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TOTAL POLYPHENOL CONTENT AND ANTIOXIDANT CAPACITY CHANGES IN DEPENDENCE ON CHOSEN GARDEN PEA VARIETIES

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ABSTRACT

The green pea is ranged between the crops with high antioxidant potential. This potential is connected with phytochemical components mainly with polyphenols. All these bioactive chemicals have disease-fighting properties. In real human diet there is no usually possibility of fresh garden pea consumption during the whole year. The total polyphenol content is significantly changed among other things by processing methods. Focus on variety, bio-fortification and other specific agricultural inputs, could be the right method of total polyphenol contents and total antioxidant capacity increasing. The main objective of the present work was to consider the changes of total polyphenols content in dependence on variety and to evaluate an antioxidant potential six garden pea varieties arranged by the ripening point of view. Variety 'Exzeleus' belongs to very early type, 'Premium' is early maturing, 'Flavora' is middle ripening variety and the last three varieties 'Utrio', 'Jumbo' and 'Ambassador' are middle late types of varieties. Every variety was grown in four replications, i.e. on 24 m² total plot in Botanical garden of Slovak University of Agriculture in Nitra during 2013. Total polyphenols were determined by the Lachman's method and expressed as mg of gallic acid equivalent per kg fresh mater. Total antioxidant capacity was measured by the Brand-Williams method using a compound DPPH (2,2-diphenyl-1-picrylhydrazyl). The highest value was reached in case of variety 'Jumbo' 1179.995 ±28.081 mg/kg, the lowest value in case of 'Premium' 674.505 ±26.541 mg/kg. When evaluating an antioxidant capacity in chosen varieties of garden pea, the interval estimated by our trail ranged from 0.523 ±0.206% ('Exzeleus') to 6.844 ±0.591% ('Flavora'). Following the both observed parameters, TPC and TAC, variety 'Flavora' (as a member of middle ripening varieties) seems to be the most optimal from the human nutrition point of view. The various varieties had significant influence on TPC and TAC according to used statistical analyzes. Within the all observed varieties, when they were arranged by ripening, there was estimated significant difference only in case of garden pea varieties 'early – middle late'. Other couples didn't show any statistical important differences in total polyphenol content.

Keywords: garden pea; total polyphenols; antioxidant capacity

INTRODUCTION

Green peas (*Pisum sativum* L.) as a good source of vegetable protein, vitamins, fiber and micronutrients belong to Pea family – Fabaceae (or Leguminosae). The legumes are rich source for lysine and tryptophan but low in sulphur-containing amino acids, methionine and cysteine. Plant proteins are cheaper than the animal proteins; therefore, the people consume legume seeds worldwide as major source of protein **Petchiammal et al., (2014)**. According to increasing scientific studies the green pea is ranged between the crops with high antioxidant potential. This potential is connected with phytochemical components mainly with polyphenols. Molecular structure of phenolic contributes to the antioxidant activity. Polyphenols are known to exhibit stronger antioxidant activity than monophenols **Troszynska et al., (2002)**. The main factor responsible for the delayed research on polyphenols is the variety and the complexity of their chemical structure **D'Archivio et al., (2007)**. Furthermore, prooxidant effects of polyphenols have been described **Elbling et al., (2005)**, having opposite effects on basic cell

physiological processes: if as antioxidants they improve cell survival, as pro-oxidant they may induce apoptosis and block cell proliferation **Lambert et al., (2005)**. Among micronutrients, peas have high contents of ascorbic acid, β-carotene, thiamine and riboflavin and, compared to other vegetables, peas are rich in iron **Nilson et al., (2004)**. Field peas are a potential dietary source of Fe, however true animal/human Fe bioavailability studies are required **Amarakoon et al., (2014)**. One of the the great aptitude of peas is ability to accumulate Se in the grain, which shows a great potential to be used as a "functional food" in Se biofortification programs **Poblaciones et al., (2013)** An increase of Se levels by fertilization translated into a much greater Se accumulation in the pea grain than in other cereals such as breadmaking wheat **Broadley et al., (2010)**.

All these bioactive chemicals have disease-fighting properties. Consumption of foods rich in antioxidant polyphenols is significantly associated with reduced risk of various non-communicable human diseases, including diabetes **(Talukdar, 2013)**. Epidemiological studies and

associated meta-analyses strongly suggest that long term consumption of diets rich in plant polyphenols offer protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Pandey and Rizvi, 2009). Important biological activities have now been suggested for these bioactive compounds like enhancement of the antioxidant, antimutagenic, anticarcinogenic and anti-hyperglycemic effects, which makes pulses an important crop for human health (Singh and Basu, 2012). Legumes in general can be considered as a therapeutic functional food due to their significant content of functional proteins and carbohydrates and their extraordinary reserve of secondary metabolites and bioactive constituents that are beneficial for managing and preventing several chronic illnesses in humans Fratianni et al. (2014). The benefit of these plants is related to the secondary metabolites that are produced by the plants even though plants produce these secondary metabolites for the benefits of the plant itself as defense against infection and injury, but it was found that the secondary metabolites have benefits to the human health and curing human diseases (Mushtaq and Wani, 2013). The antioxidant activity of plant polyphenols can retard the development of most major age-related degenerative diseases such as cancers, diabetes, cardiovascular disease, and neurodegenerative diseases (Lee, 2013; Seo et al., 2012). On the other hand, because of their sweet taste and starchy texture full of health-protective phytonutrients the pea belongs between the first vegetables, which are used for baby nourishment.

The content of mentioned bioactive compounds is significantly changed among other things by processing methods. The raw seeds of pea are the most potent antioxidant suppliers and free radical scavengers Nithiyantham et al., (2012). In real human diet there is no usually possibility of fresh garden pea consumption during the whole year. Most of time the pea is cooked, canned, dried or microwaved before it is consumed. These processes lead to changes in physical characteristics and chemical composition of pea. According to Turkmen et al., (2005) there was noticed small decrease of total antioxidant activity and total polyphenols content after boiling, steaming and microwaving of pea in comparison with fresh mass. As well as in case of (Xu and Chang, 2008) various steps of processing resulted in significant decrease in total phenolic content (TPC) and DPPH free radical scavenging content increasing. Focus on variety, bio-fortification and other specific agricultural inputs, could be the right method of total polyphenol contents increasing.

The main objective of the present work was to consider the changes of total polyphenols content in dependence on variety and to evaluate an antioxidant potential in chosen varieties of garden pea.

MATERIAL AND METHODOLOGY

For small area field trials there were chosen six garden pea varieties from the ripening point of view. Variety 'Exzeleus' belongs to very early type, 'Premium' is early maturing, 'Flavora' is middle ripening variety and the last three varieties 'Utrio', 'Jumbo' and 'Ambassador' are middle late types of varieties. Place of one trial plot was 1 m². Every variety was grown in four replications, i.e. on 24 m² total plot in Botanical garden of Slovak University of Agriculture in Nitra during 2013. Agrochemical characteristic of soil substrate are figured in table 1. Harvest of pea garden yield was made in phenological stage 'technological ripeness' and it was dried by lyophilization.

Total polyphenol content estimation (TPC)

Total polyphenols were determined by the method of Lachman et al., (2003) and expressed as mg of gallic acid equivalent per kg fresh matter. Gallic acid is usually used as a standard unit for phenolic content determination because a wide spectrum of phenolic compounds. The total polyphenol content was estimated using Folin-Ciocalteu assay. The Folin-Ciocalteu phenol reagent was added to a volumetric flask containing 100 µL of extract. The content was mixed and 5 mL of a sodium carbonate solution (20%) was added after 3 min. The volume was adjusted to 50 mL by adding of distilled water. After 2 hours, the samples were centrifuged for 10 min and the absorbance was measured at 765 nm of wavelength against blank. The concentration of polyphenols was calculated from a standard curve plotted with known concentration of gallic acid.

Total antioxidant capacity

Total antioxidant capacity was measured by the Brand-Williams et al., (1995) method using a compound DPPH (2,2-diphenyl-1-picrylhydrazyl).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was pipetted to cuvette (3.9 m³), then the value of absorbance, which corresponded to the initial concentration of DPPH solution in time A₀ was written. Then 0.1 cm³ of the followed solution was added and then 2. was immediately started to measure 1. the dependence A = f(t). The solution in the cuvette 2. was mixed and measured 1. the absorbance of 1, 5 and 10 minutes at 515.6 nm in the spectrophotometer Shimadzu UV/VIS - 1240. The percentage of inhibition reflects how antioxidant

Table 1 Agrochemical characteristic of soil substrate in mg/kg.

Agrochemical characteristic	pH (H ₂ O)	pH (KCl)	Cox (%)	Hum. (%)	
	7.55	6.36	1.39	4.01	
Nutrients	Nan	K	Ca	Mg	P
	13.3	285.0	5630.0	364.0	252.5

compound are able to remove DPPH radical at the given time.

$$\text{Inhibition (\%)} = (A_o - A_t / A_o) \times 100$$

Statistical analyzes

The analysis of variance (ANOVA), the multifactor analysis of variance (MANOVA) and the multiple Range test were done using the Statgraphic Centurion XV (StatPoint Inc. USA).

RESULTS AND DISCUSSION

On the base of reached results there were estimated changes in the total polyphenols content and also changes in total antioxidant capacity values in dependence on chosen pea varieties from the consumer point of view. The results of submitted task were compared and evaluated with scientific knowledge of foreign and home authors.

1 Evaluation of total polyphenol content and values of antioxidant capacity in chosen pea varieties in field conditions

Following the total polyphenol content parameter (as it is figured in table 2), the highest value was reached in case of 'Jumbo' variety with 1179.995 ±28.081 mg/kg. When comparing this variety from total antioxidant capacity point of view, there was estimated value 1.707 ±0.291%. This is on the lower level of observed antioxidant capacity interval. The maximum value in total antioxidant capacity was measured in case of 'Flavora' variety, with 6.844 ±0.591%. On the other hand, 'Flavora' reached 849.717 ±16.310 mg/kg in total polyphenol content, which is on the third place behind the variety 'Premium' (674.505 ±26.541 mg/kg) with the lowest polyphenol content and 'Ambassador' with the value 791.572 ±3.493 mg/kg. The results are similar as in case Han et al., (2007) where total phenolic content in case of green pea was estimated to 1200 mg/kg. According to mentioned authors, the total phenolic content (PC) was 12 mg/g in lentils, 2.2 mg/g in chickpeas, 2.3 mg/g in soybeans, 2.5 mg/g in yellow peas and 1.2 mg/g in green peas. The total phenolic content according to various sources was ranged. The differences in their TPC could be due to the ecotype, the geographical region where they grow and different extraction conditions. When the concentrations of the phenolic compounds was expressed as mg equivalents of gallic acid/g of sample (dry matter basis), as it was in our case, the values of the garden pea

occurred between the lentils (variety 'San Gerardo' with 1098 ±0.87 µg/g ±SD and 'Colliano' with 1594 ±1.97 µg/g ±SD) and chickpea ('Sassano 147 ±0.11µg/g ±SD, 'Castelcivita' 183 ±0.02 µg/g ±SD) Fratianni et al. (2014).

When comparing legumes from the TAC point of view, according to Petchiammal et al., (2014) horse gram (brown and black), cowpea (brown), common bean and masur (black) showed high protein content and also exhibited good DPPH scavenging activity, ferric reducing and reducing power activity. Comparatively, pea (white and green) and chick pea (white, green, brown) showed less antioxidant capacity.

The total polyphenols content as concentrated on the ripening according to figure 1, the highest value was estimated in case of 'Jumbo' variety, which is middle late type of variety. There is also 'Utrio' and 'Ambassador', which are middle late ripening, but variety 'Utrio' reached the total polyphenol content value 876.337 mg/kg and Ambassador 791.572 mg/kg.

From the antioxidant capacity point of view, the highest value was reached in case of middle type variety 'Flavora' with the value 6,844 %. The lowest value was found in case of very early type variety – 'Exzeleus' with the 0,523%. In comparison with results of Kavalcova et al., (2014), where the interval of statistically significant highest value of antioxidant activity was recorded in onion (from 20.22 ±0.53 to 25.76 ±0.53) and statistically significant the lowest value of antioxidant activity was recorded in garlic (from 4.05 ±0.20 to 5.07 ±0.47), our results in peas are lower, except of the variety 'Flavora', which is comparable with the values of garlic Halvorsen et al., (2002) also observed that TAC in peas was relatively low among legumes and vegetables.

2 Analyses of interactions between observed features in chosen pea varieties

2.1 Statistical evaluation of total polyphenol content differences significance within the frame of chosen varieties

When comparing all pea varieties, following the Table 3, there were significant differences according to used statistical methods on the all three types observed confidence levels between almost the all observed varieties. Only in case of couples 'Exzeleus' vs. 'Utrio', 'Exzeleus' vs. 'Flavora' and 'Utrio' vs. 'Flavora' there were not found any.

Table 2 Total phenolic content and antioxidant capacity (average and average deviation values) in chosen pea varieties after harvest in dray mass (mg/kg DM*).

Varieties (ripening)	TPC (mg/kg)*	TAC (%)*
'Exzeleus' (very early)	861.454 ±11.653	0.523 ±0.206
'Premium' (early)	674.505 ±26.541	1.797 ±0.329
'Flavora' (middle)	849.717 ±16.310	6.844 ±0.591
'Ambassador' (middle late)	791.572 ±3.493	1.908 ±0.412
'Utriuo' (middle late)	876.337 ±16.164	2.130 ±0.435
'Jumbo' (middle late)	1179.995 ±28.081	1.707 ±0.291

(TPC – total polyphenols content (mg/kg), TAC – total antioxidant capacity (%), DM – dry mass)

Within the all observed varieties, when they were arranged by ripening, there was estimated significant difference only in case of garden pea varieties 'early – middle late'. Other couples didn't show any statistical important differences in total polyphenol content.

Following to **Amarakoon et al., (2014)** significant genotypic and environmental variation was not observed ($p > 0.05$) with respect to concentrations of phenolic in field pea. Similar, according the results of **Timoracka et al., (2010)** the differences of flavonoid contents in individual pea varieties were not significant.

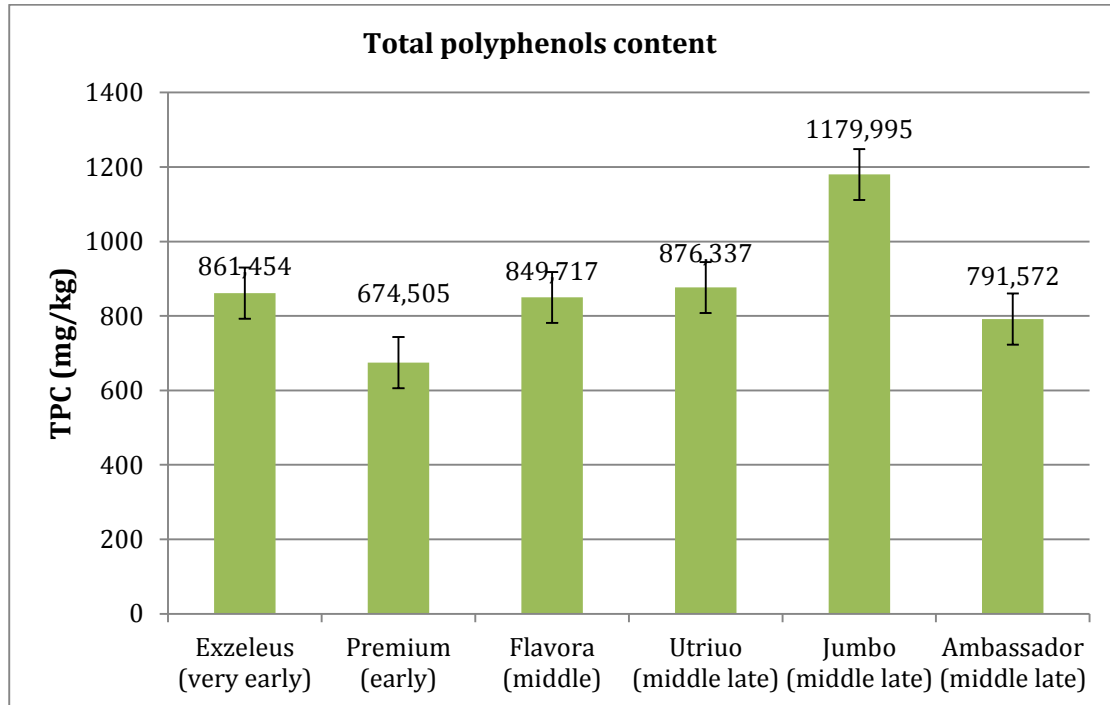


Figure 1 Average content of total polyphenols TPC (mg/kg) in chosen pea varieties arranged by ripening.

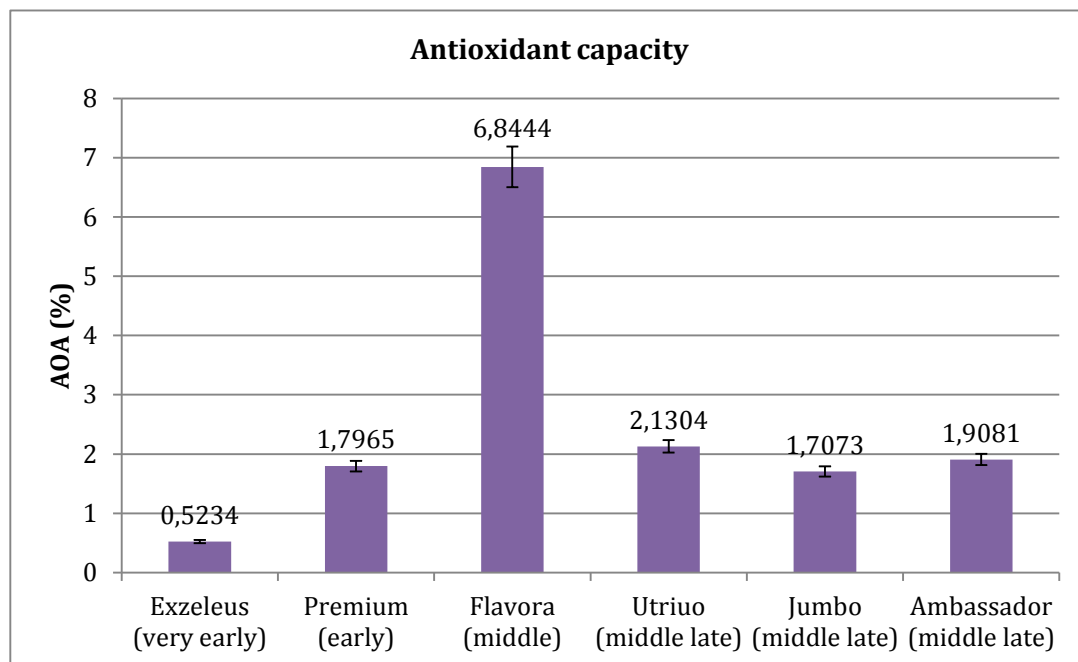


Figure 2 Average content of total antioxidant capacity TAC (%) in chosen pea varieties arranged by ripening.

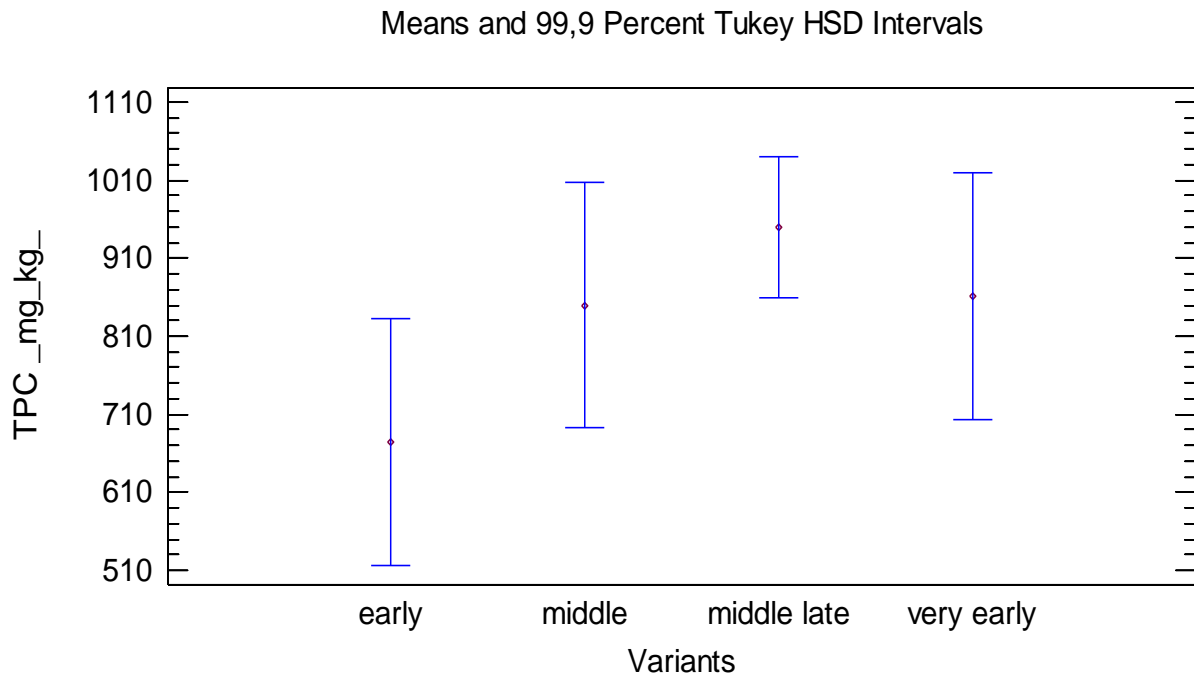


Figure 3 Dependence of total polyphenol content (TPC) on pea variety ripening.

Table 3 Statistically significant differences in total polyphenols content (TPC) of chosen pea varieties by Tukey HSD in ANOVA (Statgraphic).

Variety (mg/kg)	Premium (P)	Exzeleus (E)	Ambassador (A)	Utrio (U)	Flavora (F)	Jumbo (J)
P	–	±186.949***	±117.067***	±160.884***	±175.212***	±505.49***
E	±186.949***	–	±698816**	±148837	±117367	±318541***
A	±117.067***	±69.8816**	–	±84.7653***	±58.1448*	±388.423***
U	±160.884***	±14.8837	±84.7653***	–	±26.6204	±303.658***
F	±175.212***	±11.7367	±58.1448*	±26.6204	–	±330.278***
J	±505.49***	±318.541***	±388.423***	±303.658***	±330.278***	–

$p < 0.05$ (*): statistically significant (at 95.0 % confidence level)

$p < 0.01$ (**): high statistically significant (at 99.5 % confidence level)

$p < 0.001$ (***): very high statistically significant (at 99.9% confidence level)

2.2 Statistical evaluation of antioxidant capacity differences significance within the frame of chosen varieties

Very high statistically significant differences (at 99.9% confidence level) were found in case of variety 'Flavora' vs. all observed varieties as it is figured in table 4. Also in case of couple 'Exzeleus' vs. 'Utrio' was determined very high statistically significant differences.

'Flavora' belongs to middle type of pea variety and this group obviously reached the highest value according the figure 4, where the varieties are arranged by ripening and

statistically compared. According to Tukey test at 99.9% confidence level, there were found statistically significant differences in all compared couples except of 'early - middle late' and 'early - very early' which are not statistically different from the total antioxidant capacity point of view.

Following the both observed parameters, TPC and TAC, variety 'Flavora' (as a member of middle ripening varieties) seems to be the most optimal from the human nutrition point of view.

Table 4 Statistically significant differences at the 99.9% confidence level by Tukey HSD in ANOVA (Statgraphic) of antioxidant capacity (TAC %) in chosen varieties of peas .

Variety (%)	Premium´(P)	Exzeleus´(E)	Ambassador´ (A)	Utrio´ (U)	Flavora´ (F)	Jumbo´ (J)
P	–	±1.273*	±0.112	±0.334	±5.048 ***	±0.089
E	±1.273*	–	±1.385 **	±1.607***	±6.321 ***	±1.184 *
A	±0.112	±1.385 **	–	±0.222	±4.936 ***	±0.201
U	±0.334	±1.607***	±0.222	–	±4.714 ***	±0.423
F	±5.048 ***	±6.321 ***	±4.936 ***	±4.714 ***	–	±5.137***
J	±0.089	±1.184 *	±0.201	±0.423	±5.137***	–

$p < 0.05$ (*): statistically significant (at 95.0 % confidence level)

$p < 0.01$ (**): high statistically significant (at 99.5 % confidence level)

$p < 0.001$ (***) : very high statistically significant (at 99.9% confidence level)

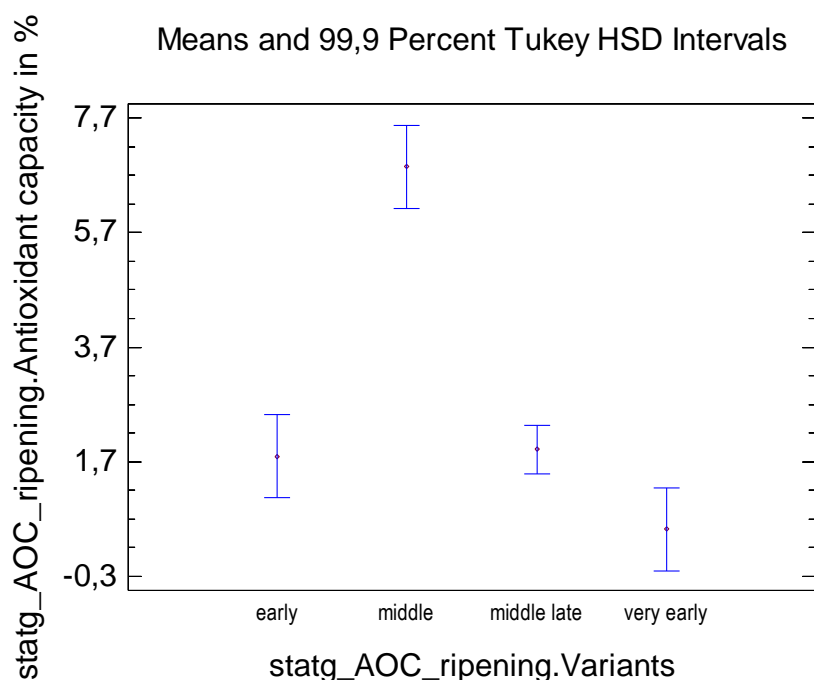


Figure 4 Dependence of total antioxidant capacity (TAC) on pea variety ripening.

CONCLUSION

The changes of total polyphenols content in dependence on chosen garden pea varieties were estimated in the article. Within the all observed varieties, when they were arranged by ripening, there was estimated significant difference in case of garden pea varieties ´early – middle late´. From dependence of total antioxidant capacity (TAC) on pea variety ripening, there were found statistically significant differences in all compared couples except of ´early – middle late´ and ´early – very early´. The various varieties had significant influence on TPC and TAC according to used statistical analyzes, that is why there is strong recommendation of multi-annual results estimation according to submitted methodology.

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INFLUENCE OF CHOSEN MICROBES AND SOME CHEMICAL SUBSTANCES ON THE PRODUCTION OF AFLATOXINS

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ABSTRACT

Aflatoxins are produced as secondary metabolites by *A. flavus*, *A. parasiticus*, *A. nomius* and *A. tamarii*. The aflatoxin biosynthetic pathway involves several enzymatic steps and genes (*apa-2*, *ver-1*) that appear to be regulated by the *afIR* gene in these fungi. The aim of this work was the detection of aflatoxins by the HPLC method and the ascertainment of factors influencing their production. *A. parasiticus* CCM F-108, *A. parasiticus* CCF 141, *A. parasiticus* CCF 3137 and two isolates *A. flavus* were used. These toxigenic isolates were recovered from spice (strain 1) and wraps (strain 2). The gene for the production of aflatoxin B1 for each species of fungi was detected using an optimized PCR method. *Rhodotorula* spp.*, *Lactococcus lactis* subsp. *lactis* CCM 1881, *Flavobacterium* spp. and fungal strain *Pythium oligandrum** were tested for inhibition of aflatoxins production and fungal growth. Having used the HPLC detection, various preservatives (propionic acid, citric acid, potassium sorbate) were tested from the viewpoint of their influence on the growth of aflatoxigenic fungi followed by the production of aflatoxins. The growth of *A. flavus* and *A. parasiticus* and aflatoxin production in Potato Dextrose Agar supplemented with propionic acid (1000-2000-3000 mg/kg), citric acid (2000-3000-4000 mg/kg) and potassium sorbate (500-800-1000 mg/kg) was tested by Agar Dilution Method. After 72 h of incubation was evaluated growth of fungi, all samples were frozen for later extraction and aflatoxins quantification by HPLC. Effect of peptone and sucrose additions were studied in yeast extract (2%) supplemented with peptone (5-10-15%) or sucrose (15%). Growth inhibition of *Aspergillus* by *Pythium oligandrum* was tested on wood surface. As shown, the highest inhibition effect on the aflatoxins production was obtained when propionic acid was applied in concentrations since 1000 mg/kg. A total inhibition of the fungi growth and aflatoxins production was observed in all samples containing peptone in the concentration range tested. Significant limitation of the growth and production of aflatoxins was also observed in the presence of other microorganisms such like *Pythium oligandrum* and *Rhodotorula* spp.

Keywords: aflatoxin; preservatives; *Pythium oligandrum*; *Rhodotorula*; inhibition; *Aspergillus flavus*; *A. parasiticus*

INTRODUCTION

Microscopic filamentous fungi present a worldwide problem. Fungal physiology refers to the nutrition, metabolism, growth and reproduction of fungal cells. It also generally relates to interaction of fungi with their biotic and abiotic environment, including cellular response to stress. The dynamics of fungal activities are central to the efficiency of forestry and agricultural operations, in all three forms: mutualistic symbionts, pathogens and saprophytes as they mobilize nutrients and affect the physical-chemical environment (Gqaleni et al., 1997; Chourasia, 1993; Paster, 1999).

Fungi are also responsible for the organic pollutants detoxification and for the bioremediation of heavy metals in the environment.

The fungal negative effect on humans, animals and plants is the main reason for monitoring the contamination of food and feed (Gunterus et al. 2007, Huang et al. 2009). Some fungi colonize plant seeds and thereby cause loss of stored harvests and reduce seed germination rates, they can also produce mycotoxins which have mutagenic properties. Some fungi are pathogens of plants or animals

(and even other fungi). Fungi can also cause diseases in humans, many of them as so-called opportunistic pathogens that attack people whose immune systems are weakened.

Aspergillus species compete with *Fusarium* and *Penicillium* species for the dominance among the world's fungal flora (Nesci et al., 2003).

Aflatoxins are secondary metabolites produced by *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* and the newly described *Aspergillus tamarii* and have the highest toxicity among mycotoxins (hepatotoxigenic, hepatocarcinogenic and immunosuppressive effects). Due to their toxicity including the carcinogenic activity, aflatoxins affect not only the health of humans and animals but also the economics of agriculture and food. For the production of aflatoxins it is particularly important where their biosynthetic pathway stops.

Aflatoxinogenic fungi are important contaminants of certain foods and animal feeds because of their ability to produce aflatoxins. When these fungi invade and grow in commodities such as peanuts, corn and cottonseed,

the resulting contamination with aflatoxins often makes the commodities unfit for consumption.

Historically, identification of filamentous fungal (mould) species has been based on morphological characteristics, both macroscopic and microscopic. These methods may often be time-consuming and inaccurate, which necessitates the development of identification protocols that are rapid, sensitive, and precise. The polymerase chain reaction (PCR) has shown a great promise in its ability to identify and quantify individual organisms from a mixed culture environment; however, the cost effectiveness of single organism PCR reactions is quickly becoming an issue (Nigam and Singh, 2000; Bennett and Papa, 1988; Weidenbörner, 2001).

The aim of this work was the determination of aflatoxins by the HPLC method and the ascertainment of factors influencing their production.

MATERIAL AND METHODS

Bacterial strains, growth medium and cultural conditions

Six fungal, two bacterial and one yeast strains were used: *A. parasiticus* CCM F-108, *A. parasiticus* CCF 141, *A. parasiticus* CCF 3137 and two isolates *A. flavus*. These toxigenic isolates were recovered from spice (strain 1) and wraps (strain 2). *Rhodotorula* spp.*, *Lactococcus lactis* subsp. *lactis* CCM 1881, *Flavobacterium* spp. and fungal strain *Pythium oligandrum** were tested for inhibition of aflatoxins production and fungal growth. Strains of CCM were provided by Czech Collection of Microorganisms (Brno, Czech Republic). Strains of CCF were provided by Department of Botany (Faculty of Science, Charles University, Praha, Czech Republic), the others (*) were provided by Department of Biological and Biochemical Sciences (Faculty of Chemical technology, University of Pardubice, Pardubice, Czech Republic). A suspension of bacterial and yeast or fungal strain was prepared from freshly grown colonies on MRS for *Lactococcus lactis*, Malt agar for yeast 24-48 h incubation at optimum temperature. Concentration of bacterial and yeast cells was adjusted to 10^8 cfu/mL using 0.5 McFarland turbidity scale. Suspensions of fungal spores were prepared from 7-days cultures grown on Malt agar's slant at 24 °C. The density of spores was adjusted to 10^6 spores/mL using Bürker counting cell. All the nutrient media used throughout the study were purchased from HiMedia, India. The growth of *A. flavus* and *A. parasiticus* and aflatoxin production was tested by Agar Dilution Method. Control Petri dishes were kept without bacteria and yeast in similar way. After 7 days of incubation was evaluated growth of fungi, all samples were frozen for later extraction and aflatoxins quantification by HPLC (Malir et al., 2006).

The growth of *A. flavus* and *A. parasiticus* and aflatoxin production in Potato Dextrose Agar (PDA, HIMEDIA, India) supplemented with propionic acid (1000-2000-3000 mg/kg), citric acid (2000-3000-4000 mg/kg) and potassium sorbate (500-800-1000 mg/kg) was tested by Agar Dilution Method. After 72 h of incubation was evaluated growth of fungi, all samples were frozen for later extraction and aflatoxins quantification by HPLC (Malir et al., 2006).

Effect of peptone and sucrose additions were also studied. Spore concentrations ranging 10^6 spores/mL were inoculated onto 10 mL of yeast extract (2%) supplemented with peptone (5-10-15%) or sucrose (15%). Each sample was examined in duplicate. Negative control (without peptone or sucrose) was also included.

After incubation (25 °C, 72 h) was evaluated growth of fungi on Malt agar, production of aflatoxins was quantification by HPLC (Malir et al., 2006).

Growth inhibition of *Aspergillus* by *Pythium oligandrum* was tested on wood surface (5x5 cm).

Extraction of aflatoxins

The sample was agitated with 50 mL of a mixture of 60% methanol with 0,8 g NaCl for 3 min at room temperature. 5 mL of solution was diluted by 10 mL deionized water. Extraction of aflatoxins was performed by Afla B affinity column (VICAM, USA). After filtration of the substrate was added 1mL to developer (8 mL deionized water, 1mL trifluoroacetic acid, 1 mL acetic acid). Then the amount of aflatoxins were detected by HPLC.

Conditions for determination of aflatoxins by HPLC

The HPLC system has been consisted of a column (OmniSpher 5 C18 (3x150, 5 µm), Varian), a precolumn (ChromSep Guard Column C18 (10x2,5 µm), Varian). A 100 µl sample was injected to HPLC system. Microfiltered methanol-acetonitrile-water (1:1:4 v/v) was used as isocratic mobile phase with a flow rate of 0,5 mL/min at room temperature. Aflatoxins detection was by fluorescence with excitation and emission wavelengths of 364 nm and 456 nm, respectively.

RESULTS AND DISCUSSION

Production of aflatoxins in potato dextrose broth

The optimized PCR reaction selectivity was tested on collection cultures and it was proven that both PCR methods are highly specific for the determination of aflatoxinogenic strains of *A. flavus* and *A. parasiticus* isolated from food and feed. When verifying the PCR selectivity to detect the regulatory gene important for the aflatoxin B₁'s production, a positive reaction was observed also in the non-aflatoxinogenic strain of *A. versicolor* CCM F-585. The aflatoxin B₁'s biosynthetic production pathway renders various intermediate products, one of which is versicolorin A, the final metabolite of the *A. versicolor* CCM F-585 fungus (Zachová et al., 2003). The production of aflatoxins was demonstrated only by *Aspergillus parasiticus* CCF 141, *Aspergillus parasiticus* CCF 3137 and *Aspergillus flavus* CCM-F 108 (Table 1).

Strain of *Aspergillus parasiticus* CCF 141 produced aflatoxin B₁ (6.6 ng/mL), aflatoxin B₂ (1.0 ng/mL), aflatoxin G₁ (6.9 ng/mL) and aflatoxin G₂ (1.0 ng/mL) (Table 1). Production of aflatoxin B₁ corresponds to the data in the literature on the production of aflatoxin B₁ found in *Aspergillus parasiticus* FVB 1981 isolated from peanut sauce, which were incubated in YES broth at 28 °C for 7 days, 10.8 ng/mL (Abarca et al., 1988). Strain of *Aspergillus parasiticus* CCF 3137 produced 18.7 ng/mL aflatoxin B₁, 0.8 ng/mL aflatoxin B₂, 6.5 ng/mL aflatoxin G₁ and 0.3 ng/mL aflatoxin G₂ (Table 1). By contrast, Tsai et al. (1984) states in YES broth at 25 °C for 72 hours difference in the production of aflatoxin B₁ *Aspergillus*

parasiticus NRRL 2999, which was only 8.2 ng/mL (Tsai et al., 1984). Strain of *Aspergillus flavus* CCM F-108 produced by aflatoxin B1, in the amount of 331.5 ng/mL and aflatoxin B2 in the amount of 2.44 ng/mL (Table 1). Authors Kady and Maraghy (1994) indicate comparable production of aflatoxin B1 to the *Aspergillus flavus* isolated from meat products cultivated in potatoes broth for 10 days at 28 °C, and that was 310 ng/mL. Production of aflatoxin B2, however, did not demonstrated in their experiment.

The production of aflatoxins by strains *A. flavus* from the samples of sunflower, barleycorn, spice *Steak afpa*, *Tandoori*, spices for chicken was not detected. This is consistent with authors Razzaghi-Abyaneh et al. (2006). Aflatoxins were produced only by isolates from wraps and spice (Table 2). Strains producing aflatoxins were used for further experiments.

Fungal growth inhibition and the production of aflatoxins by a conserving substance

Growth of selected strains of fungi was monitored in the presence of the preservative on the soil potato agar at 25 °C for 72 hours. Agar Dilution Method was used to determine the inhibition of mold growth. Size of colony was compared with the size of colonies on the medium without preservatives. Citric acid inhibited at least growth of *Aspergillus flavus* CCM F-108 and the most growth of *Aspergillus parasiticus* CCF 3137 (Table 3). Propionic acid inhibited growth of at least *Aspergillus parasiticus* CCF 3137 and the most inhibited *Aspergillus flavus* CCM F-108, whose growth was completely suppressed. Growth of *Aspergillus parasiticus* CCF-141 was inhibited completely also (Table 3). Suppression of growth of *Aspergillus flavus* detected by the agar diluted method on Sabouraud agar at 21°C for 5 days provides Čonková et al. (1993), namely even lower concentrations of propionic acid than was used for our experiment (Table 3). Potassium sorbate inhibited the growth of most *Aspergillus*

flavus CCM F-108 and a minimum growth of *Aspergillus parasiticus* CCF 3137. This strain was isolated from environment of the rainforest in Malaysia, and is therefore probably more resistant than the strains of *Aspergillus parasiticus* CCF 141 and *Aspergillus flavus* CCM F-108, obtained from the Czech Republic. Significant inhibition of growth of *Aspergillus flavus* CCM F-108 is already at a concentration of potassium sorbate and 500 mg/kg (Table 3). The concentration of potassium sorbate (higher than 1500 mg/kg) is needed for pronounced inhibition of growth for *Aspergillus parasiticus*, so there, as confirmed the authors Liewen and Marth (1984) in their study. Propionic acid inhibits growth of *Aspergillus* the most of preservatives (Table 3).

Aflatoxins production in the presence of preservatives

Conserving substance factors were tested in potato dextrose broth (30 °C, 72 h). All the preservatives inhibited the production of aflatoxins. When aflatoxins were created, aflatoxins were produced in quantities less than the detection limit determination. *A. parasiticus* CCM F-108 and *Aspergillus parasiticus* CCF 141 were the most sensitive to preservatives. Same results are featured by Chourasia (1993).

Similar to our findings also Čonková et al. (1993) feature the fungicidal efficiency of propionic acid resulting from the experiment at 88.9%. In their study, Tsai et al. (1984) determined the production of aflatoxins by strain *Aspergillus parasiticus* NRRL 2999 in the presence of 0.2 - 0.4% propionic acid.

The largest production of aflatoxins was determined in the presence of citric acid. Production of aflatoxin B1 and G1 decreased with increasing concentration of citric acid (Figure 1, Table 4 and Table 5).

In the presence of potassium sorbate were *Aspergillus parasiticus* CCF 3137 produced only aflatoxins B1 and G1, at a concentration of 500 mg/kg (B1 0,29 ng/mL, G1 0,036 ng/mL).

Table 1 Amount of aflatoxins in potato dextrose broth (25 °C, 72 hours).

Collection cultures	AFB1 (ng/mL)	AFB2 (ng/mL)	AFG1 (ng/mL)	AFG2 (ng/mL)
<i>A. parasiticus</i> CCF 141	6.6	1	6.9	1
<i>A. parasiticus</i> CCF 3137	18.7	0.8	6.5	0.3
<i>A. parasiticus</i> CCM-F 108	331.5	2.44	<0.025	<0.030
<i>A. flavus</i> CCM-F 585	<0.025	<0.035	<0.025	<0.030
<i>A. tamarii</i> CCF 3206	<0.025	<0.035	<0.025	<0.030
<i>A. parasiticus</i> CCM-F 550	<0.025	<0.035	<0.025	<0.030
<i>A. flavus</i> var <i>columnaris</i>	<0.025	<0.035	<0.025	<0.030
<i>A. flavus</i> CCM-F 171	<0.025	<0.035	<0.025	<0.030
<i>A. flavus</i> CCM-F 449	<0.025	<0.035	<0.025	<0.030

Table 2 Amount of aflatoxins in potato dextrose broth (25 °C, 72 hours).

Strains of <i>A. flavus</i> isolated from samples	AFB1 ng/mL	AFB2 ng/mL	AFG1 ng/mL	AFG2 ng/mL
Wraps (strain 2)	210	4	<0,025	<0,030
Spice (strain 1)	650	15	<0,025	<0,030

Table 3 Averages of fungal colonies on potato dextrose agar (25 °C after 72h).

Conserving substance	<i>A. parasiticus</i> CCM-F 108 (mm)	<i>A. parasiticus</i> CCM-F 141 (mm)	<i>A. parasiticus</i> CCF 3137 (mm)
	Without conserving substance	30 ±0.4	38 ±1.4
Citric acid 2000 mg/kg	29 ±0.7	27 ±1.4	27 ±0.7
Citric acid 3000 mg/kg	25 ±0.7	23 ±0.7	22 ±0.4
Citric acid 4000 mg/kg	21 ±0.4	20 ±1.8	18 ±1.4
Propionic acid 1000 mg/kg	Without growth	28 ±1.4	32 ±1.4
Propionic acid 2000 mg/kg	Without growth	Without growth	10 ±1.4
Propionic acid 3000 mg/kg	Without growth	Without growth	7 ±1.4
Potassium sorbate 500 mg/kg	18 ±0.4	36 ±1.1	45 ±2.1
Potassium sorbate 800 mg/kg	15 ±0.7	33 ±1.4	39 ±1.4
Potassium sorbate 1000 mg/kg	11 ±0.7	30 ±2.5	35 ±1.4

Information on the aflatoxin production inhibition by organic acids and potassium sorbate are rather sparse, some data can be found in **Tsai et al. (1984)**.

The reduction of aflatoxins by peptone and sucrose

The aflatoxin pathway gene expression analysis was carried out in inducing (YES medium) and non-inducing (YEP medium) conditions (**Sweeney et al., 2000; Scherm and Palomba, 2004; Scherm et al., 2005**). In our experiment, peptone and sucrose inhibited most of the aflatoxin production in all of the concentrations.

The growth of aflatoxigenic fungi was not particularly influenced by the presence of peptone and sucrose.

Fungal growth inhibition and aflatoxins production by *Pythium oligandrum*

***Pythium oligandrum* on wood block**

The growth of neither *Aspergillus* nor *Pythium oligandrum* was visible on wood block after the incubation period, therefore swabbing by sterile swab was carried out. Swab cotton was then shaken out to physiological solution and the surface of the potato dextrose agar was inoculated with 0.1 mL of the homogenate and incubation was allowed for 72 h at 25 °C.

After the incubation period, the *Pythium oligandrum* growth was quite prominent, while the growth of *A. flavus* was strongly inhibited.

Without the presence of *Pythium oligandrum* spores of *A. flavus* survive on wooden plate.

***Pythium oligandrum* in liquid medium**

This section of the experiment proved a major growth inhibition of fungi, in some strains up to four orders; hence also the aflatoxins production was reduced by *Pythium oligandrum*.

Pythium oligandrum Dresch., has been identified as one of the most numerous species observed in agricultural soils. In certain areas, it has proven its ability to induce plant disease suppression (**Floch et al., 2009**). Inoculation with *P. oligandrum* or its elicitors can sensitive plants and make them respond more quickly and efficiently to pathogen attacks and trigger systemic resistance. Additionally, *P. oligandrum* has been reported to promote plant growth according to a recent study this phenomenon is mediated by tryptamine, an auxin produced by *P. oligandrum*. Interactions between host fungi and *P. oligandrum* involve a complex set of events (**Rey et al., 2005**). In our study also showed that *P. oligandrum* not only inhibited of growth of *Aspergillus*, but inhibited production of aflatoxins as well.

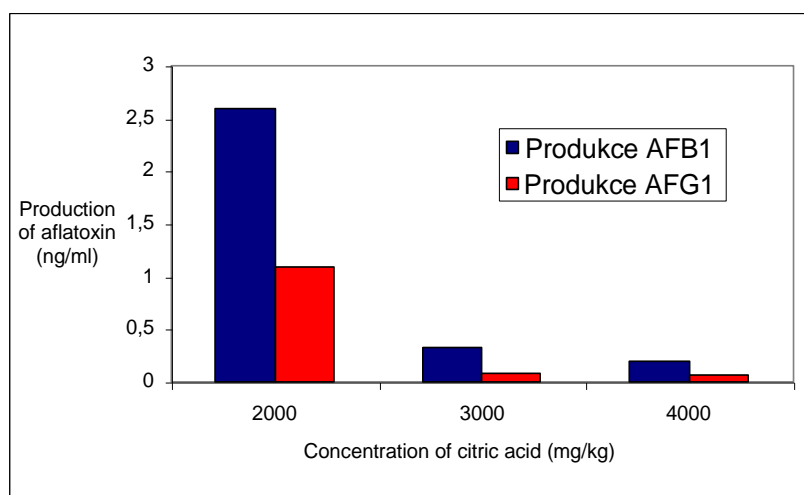


Figure 1 The dependence of the aflatoxin production on the citric acid concentration in *Aspergillus parasiticus* CCF 3137 (potato dextrose broth, 25 °C, 72 h).

Table 4 Aflatoxin production by *A. parasiticus* CCF 3137 in potato dextrose broth with preservatives (25 °C, 72 hours).

<i>Aspergillus parasiticus</i> CCF 3137		AFB1 ng/ml	AFB2 ng/ml	AFG1 ng/ml	AFG2 ng/ml
Propionic acid	1000 mg/kg	0.3	0.04	0.1	<0.030
	2000 mg/kg	0.2	0.03	<0.025	<0.030
	3000 mg/kg	0.2	0.03	<0.025	<0.030
Citric acid	2000 mg/kg	2.6	0.045	1.1	<0.030
	3000 mg/kg	0.33	<0.035	0.08	<0.030
	4000 mg/kg	0.2	<0.035	0.07	<0.030
Potassium sorbate	500 mg/kg	0.29	<0.035	0.036	<0.030
	800 mg/kg	<0.025	<0.035	<0.025	<0.030
	1000 mg/kg	<0.025	<0.035	<0.025	<0.030

Fungal growth inhibition and aflatoxins production using different microorganisms

Fungal growth inhibition using different microorganisms

The size of fungal colony was measured on a medium for selected microorganisms (Malt, MRS, BHI) (Table 6). After incubation, an analysis of aflatoxins was carried out using HPLC.

After microaerophile cultivation on MRS at 30 °C, *Aspergillus parasiticus* CCF 141 and *Aspergillus parasiticus* CCF 3137 occurred for the production of aflatoxins, which was probably caused by low pH. The fungi were stressed and resisted the production of aflatoxins in this medium, as well as his work indicates Nesci et al. (2003). Author tested the effect of pH on

aflatoxin production of *Aspergillus parasiticus* RCT 1920 and *Aspergillus parasiticus* RCD 106 on corn MALT agar at 25 °C for 5 days.

Ehrlich et al. (2005) also examined the production of aflatoxins B1 and G1 in the presence of different acidic pH. Found that gene expression for the production of aflatoxin B1 with decreasing pH increases and is the largest production of aflatoxin B1, production of aflatoxin G1 is stopped.

In our case, stress of the *A. parasiticus* probably contributed microaerophilic environment of cultivation and production of nisin by *Lactococcus*. *Lactococcus* and *Rhodotorula* inhibited growth all strains of *A. parasiticus* (Figure 2 and Figure 3).

Table 5 Aflatoxin production by *A. flavus* from spices for chicken (Strain 1) in potato dextrose broth with preservatives (25 °C, 72 hours).

<i>A. flavus</i> Strain 1		AFB1	AFB2	AFG1	AFG2
		ng/ml	ng/ml	ng/ml	ng/ml
Propionic acid	1000 mg/kg	<0.025	<0.035	<0.025	<0.030
	2000 mg/kg	<0.025	<0.035	<0.025	<0.030
	3000 mg/kg	<0.025	<0.035	<0.025	<0.030
Citric acid	2000 mg/kg	2.76	0.074	<0.025	<0.030
	3000 mg/kg	<0.025	<0.035	<0.025	<0.030
	4000 mg/kg	<0.025	<0.035	<0.025	<0.030
Potassium sorbate	500 mg/kg	0.068	<0.035	<0.025	<0.030
	800 mg/kg	0.035	<0.035	<0.025	<0.030
	1000 mg/kg	0.029	<0.035	<0.025	<0.030

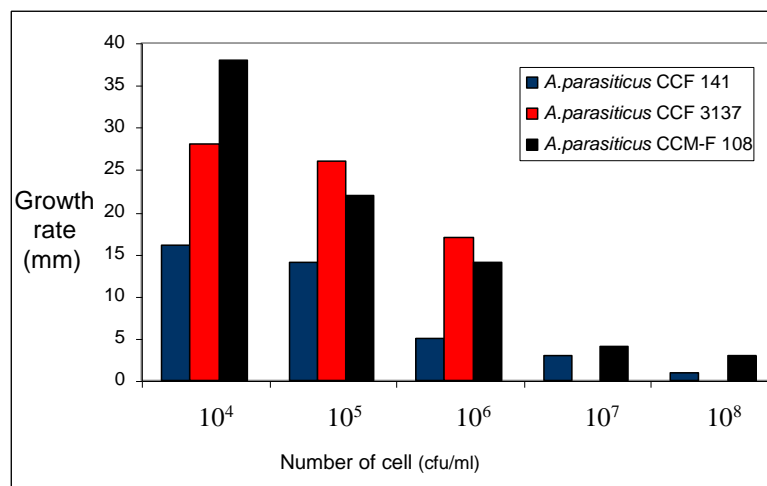


Figure 2 The dependence of the fungal growth rate on the number of *Flavobacterium* cells (BHI, 30 °C, 72 h).

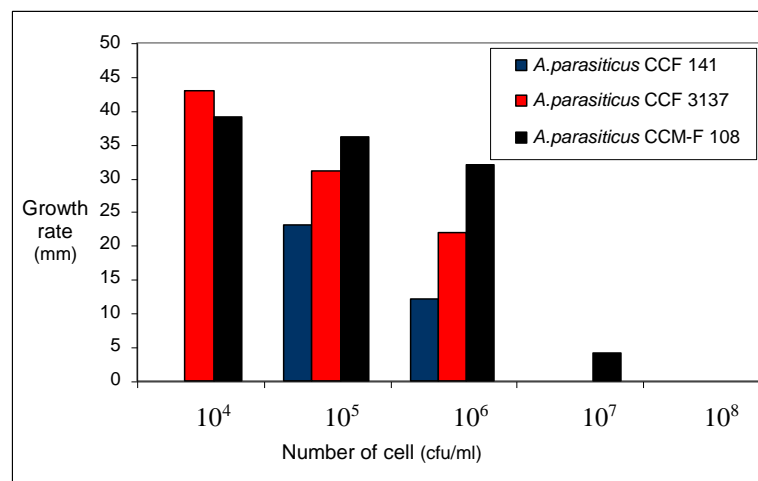


Figure 3 The dependence of the fungal growth rate on the number of *Rhodotorula* cells (MALT, 25 °C, 72 h).

Table 6 The growth rate of fungi *A. parasiticus* in the presence of different microorganisms after 72 hours.

Microorganisms [cfu/ml]	<i>A. parasiticus</i>	<i>A. parasiticus</i>	<i>A. parasiticus</i>
	CCF 141	CCF 3137	CCM F- 108
	[mm]	[mm]	[mm]
<i>Lactococcus 10⁸</i> (MRS, 30 °C)	2 ±0.7	2 ±1.4	2 ±0.7
<i>Rhodotorula 10⁸</i> .(MALT, 25 °C)	0	0	0
<i>Rhodotorula 10⁷</i> .(MALT, 25 °C)	0	0	4 ±0.7
<i>Rhodotorula 10⁶</i> .(MALT, 25 °C)	12 ±1.4	22 ±0.7	32 ±1.4
<i>Rhodotorula 10⁵</i> .(MALT, 25 °C)	23 ±1.4	31 ±2.1	36 ±0.7
<i>Rhodotorula 10⁴</i> .(MALT, 25 °C)	0	43 ±1.4	39 ±0.7

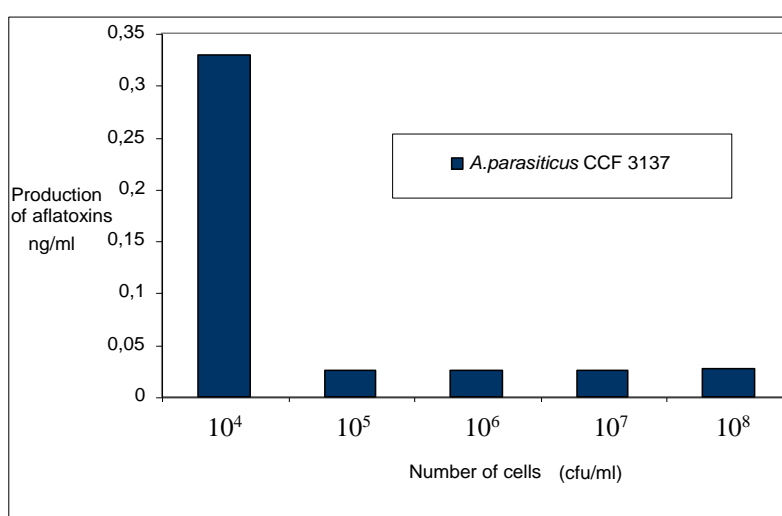


Figure 4 The dependence of aflatoxin production by *A. parasiticus* CCF 3137 on the number of *Rhodotorula* cell (MALT, 25 °C, 72 h).

Aflatoxins production inhibition by different microorganisms

In the presence of *Lactococcus*, no tested fungi produced any aflatoxins. The authors **Smiley and Draughon (2000)** tested the inhibition of aflatoxin production by protein extracts of *Flavobacterium*. *Aspergillus flavus* and *Aspergillus parasiticus* isolated from spices with protein extract of *Flavobacterium* in BHI broth at 30° C for 5 days were cultivated. Inhibition of aflatoxin production by the bacteria, according to these authors 80% (**Smiley and Draughon, 2000**).

In the presence of *Lactococcus* and *Rhodotorula*, others the strains do not produced of aflatoxins. Only *Rhodotorula* strain in density of 10⁴ cfu/mL inhibited of production of aflatoxins at least. 0,329 ng/mL of AFB1 and 0,293 ng/mL of AFG1 were produced by *A. parasiticus* CCF 3137 (Figure 4).

The aflatoxin production by *Aspergillus* sp. in the presence of peptone and sucrose was observed by the HPLC method. In all applied peptone concentrations, the strains were considerably inhibited.

Results revealed that *Pythium oligandrum* caused a total inhibition of growth and aflatoxin biosynthesis in the toxigenic *Aspergillus* sp.

A strong effect on limiting the growth of aflatoxigenic fungi and aflatoxin biosynthesis was also observed when using the *Rhodotorula*.

Also *Lactococcus* had an inhibiting effect, although only in its highest concentration (10⁸ cfu/mL).

CONCLUSION

This work also aimed to detect aflatoxins by the HPLC method and to determine factors influencing their production.

The collection cultures' aflatoxin production was quantitatively determined by the optimized HPLC method.

Chemical factors influencing growth and production of aflatoxins were tested. Tested preservatives (propionic acid, citric acid and potassium sorbate) showed different effect on different strains.

The most sensitive to preservatives was *A. flavus* CCM F-108. The most efficient growth inhibition of fungi and

aflatoxin biosynthesis was determined already for the concentration of 100 mg/kg.

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PRODUCTION OF T-2 TOXIN AND DEOXYNIVALENOL IN THE PRESENCE OF DIFFERENT DISINFECTANTS

Dana Hrubošová, Jarmila Vyřasová, Iveta Brožková

ABSTRACT

The aim of the work was to examine the effect of different disinfectants on production trichothecenes (especially of T-2 toxin and deoxynivalenol). Lipophilicity, chemical structure, the presence of bioactive groups and functional groups in their structure modifies biological activity and toxic potency of trichothecenes. For this reason, limits have been established designating maximum levels of mycotoxins in cereals while maintaining proper growing practices. Appropriate nutritive media were prepared with different concentration of tested disinfectants (Desanal A plus, ProCura spray and Guaa-Pool) and were inoculated using *Fusarium* strains. The density of *Fusarium* was 10⁵ spores per mililitre. Nutrient media was cultivated at 15 °C and 25 °C for seven days. The strains of *Fusarium graminearum* CCM F-683 and *Fusarium* species (isolated from barley) produced quantities of deoxynivalenol. *Fusarium poae* CCM F-584 and *Fusarium* species (isolated from malthouse air) produced quantities of T-2 toxin. Desanal A plus prevented *Fusarium* growth and production of T-2 toxin and deoxynivalenol at the concentration 10%. It is an alkaline disinfectant on the basis of active chlorine and the surfactant that contains <5% of NaClO. ProCura spray at the concentration 0.6% proved to be very effective. This disinfectant contains 35% of propan-1-ol and 25% of propan-2-ol. Guaa-Pool at the concentration 0.004% proved to be very effective. It is a polymeric disinfectant with anion surface-acting agent and it contains <0.9% of polyhexamethylene guanidine hydrochloride and <0.2% of alkyl (C12-C16) dimethylbenzyl ammonium chloride. Lower concentration of disinfectants that not prevented growth of *Fusarium* caused higher production of T-2 toxin and deoxynivalenol. The contents of T-2 toxin and deoxynivalenol were analyzed by enzyme-linked immunosorbent assay (ELISA) using commercially produced kits (Agra Quant® Deoxynivalenol Test kit and Agra Quant® T-2 toxin Test kit). The experiment showed that the variability in the production of T-2 toxin and deoxynivalenol depended on the *Fusarium* strain used, concentration of disinfectants and temperature of cultivation.

Keywords: disinfectant; T-2 toxin; deoxynivalenol; *Fusarium*; ELISA

INTRODUCTION

Fusarium mycotoxins cause various diseases in humans and livestock. T-2 toxin and deoxynivalenol (trichothecene mycotoxins) produced by fungi of the genus *Fusarium* (*F.*) are considered the most common contaminants in feed and food in our climatic conditions. Contamination of cereals with mycotoxins produced by *Fusarium* species is a worldwide problem (Vasatkova et al., 2009). *Fusarium* species contaminate the crops and embryonic tissue of seeds with spores during their growth and development (Thammawong et al., 2011; Schollenberger et al., 2007). *Fusarium* species occur on the ear of cereals, stalks, roots or leaves of plants. Strains of *F. poae*, *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. nivale*, *F. sporotrichioides*, *F. cerealis*, and *F. equiseti* are often isolated. They are producers of a wide range of mycotoxins (e.g., trichothecene mycotoxins, zearalenones, fumonisins, moniliformin) (Malíř a Ostrý, 2003; Gärtner et al., 2008; D'Mello et al., 1999; Malachová et al., 2010; Botalico a Perrone, 2004). They influence reproduction, prevent maturation of oocytes, disrupt of protein synthesis and affect intracellular regulators. Mycotoxins are dangerous for humans because they have mutagenic,

carcinogenic, teratogenic and immunosuppressive effects. The exposition to trichothecene mycotoxins can result in liver damage, damage to endocrine and nervous systems (Crrepy, 2002; Hussein a Brasel, 2001; Pestka et al., 2004; Bryden, 2007; Wu et al., 2012).

Trichothecene mycotoxins (trichothecenes) are the largest group of mycotoxins occurring around the world. They can be produced by taxonomically dissimilar genera of fungi, such as *Fusarium*, *Cryptomela*, *Dendrodochium*, *Trichothecium*, *Hypocrea*, *Trichoderma*, *Phomosis*, *Cylindrocarpon*, *Stachybotrys* and *Myrothecium* (Desjardins, 2006; Li et al., 2011). These are polycyclic sesquiterpenoids containing an 12,13-epoxy ring that is responsible for their toxicity. On the basis of the presence of or absence of characteristic functional groups they can be classified into four types A-D. Trichothecenes type B is different from the type A due to the presence of the carbonyl group at the C-8 position. Type A includes T-2 toxin (Figure 1) and HT-2 toxin. Type B is represented by deoxynivalenol (Figure 2) and nivalenol. In terms of toxicity trichothecene mycotoxins of type A are more dangerous than type B. Trichothecene mycotoxins of type C (crotocin, baccharin) have a second epoxy group between C-7 and C-8 or C-9 and C-10. Satratoxin and

roridin (type D) are characterized by a macrocyclic ring between C-4 and C-15 (Monaci et al., 2011; Sudakin, 2003; Li et al., 2011; Delgado et al., 2010; Krska et al., 2011). Mycotoxin production can be affected by temperature, humidity, type of substrate, and applications of fertilizer and fungicidal agents (Haidukowski et al., 2012; Hrubošová-Hrmová et al., 2011). Mold growth and production of mycotoxins can be avoided by proper choice of disinfectants. The problems in the use of disinfectants in food industry must be in the processing of food. If mycotoxins are already produced, they are usually not influenced by technological processing. Aflatoxins, ochratoxin A, deoxynivalenol, zearalenone, fumonisin and other are highly stable. Breweries worldwide had and still have problems with gushing when barley contains mycotoxins produced by *Fusarium* fungi. First step is to prevent the occurrence of *Fusarium* on barley by fungicides. Second step is preventing the occurrence using of different disinfectants in malt-houses (Běláková et al., 2012; Suchý a Herzig, 2014).

The aim of this study was to determine the effect of different disinfectants on production of T-2 toxin and deoxynivalenol.

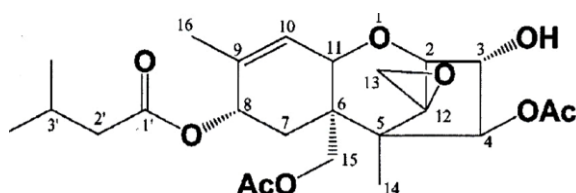


Figure 1 T-2 toxin (Li et al., 2011)

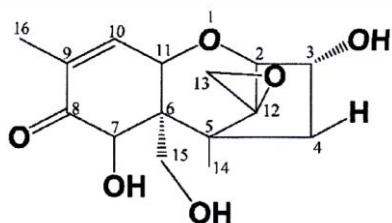


Figure 2 Deoxynivalenol (Li et al., 2011)

MATERIAL AND METHODOLOGY

Fusarium strains

Fusarium poae CCM F 584 and *Fusarium graminearum* CCM F 683 are from the Czech Collection of Microorganisms (Brno, Czech Republic). *Fusarium* species (*Fusarium* spp. 1) was isolated from malt-house air and *Fusarium* species (*Fusarium* spp. 2) was isolated from barley.

Cultivation media

MALT agar, DRBC agar, and potato-carrot agar (HiMedia, Mumbai, India) were used for isolation and identification of *Fusarium* from samples. Furthermore

MALT agar was used for isolation of T-2 toxin and deoxynivalenol in the presence of various concentrations of disinfectants.

Disinfectants

Desanal A plus (Mica, Česká Třebová, Czech Republic)

- used concentrations: 3, 5, 7 and 10%

The concentration recommended by the manufacturer is 7-10%. It is an alkaline disinfectant on the basis of active chlorine and the surfactant that contains <5% of NaClO.

ProCura spray (Agrochem, Praha, Czech Republic)

- used concentrations: 0.2, 0.3, 0.4, 0.5 and 0.6%

The concentration recommended by the manufacturer is 1-2%. This disinfectant contains 35% of propan-1-ol and 25% of propan-2-ol.

Guaa-pool (Guapex, Brno, Czech Republic)

- used concentrations: 0.0005, 0.001, 0.002, 0.003 and 0.004%

The concentration recommended by the manufacturer is 0.0004-0.0012%. It is a polymeric disinfectant with anion surface-acting agent and it contains <0.9% of polyhexamethylene guanidine hydrochloride and <0.2% of alkyl (C12-C16) dimethylbenzyl ammonium chloride.

Cultivation of *Fusarium* in the presence of disinfectants

The monitoring of the *Fusarium* mycotoxins production in the presence of disinfectants was performed as follows: tested concentrations of different disinfectants were added into the Malt agar. This prepared nutrient medium was inoculated with 0.1 mL of *Fusarium* spores at a density of 10^6 spores per millilitre (*Fusarium poae* CCM F 584, *Fusarium graminearum* CCM F 683, *Fusarium* spp. 1, *Fusarium* spp. 2). The density of spores was determined using the Bürker chamber. Cultivation was carried out at temperatures of 15 °C and 25 °C for 7 days.

Isolation of T-2 toxin and DON

The content of the respective Petri dish after cultivation (20 g of sample) was mixed with 100 mL of acetonitrile : distilled water mixture (3:1) for 30 min at room temperature at 375 g on a digital shaker (Labnet, Edison, New Jersey, USA) (Hlaváčková et al., 2012). After the filtration of the obtained volume, 1 mL was purified using on EASI-EXTRACT T-2 HT-2 immunoaffinity column (R-Biopharm, Darmstadt, Germany) for the T-2 toxin and using DONtest HPLC (Vicam, Milford, Massachusetts, USA) for DON and evaporated to dryness. Prior to the actual analysis, the residue after evaporation was dissolved in 1 mL of distilled water.

Determination of T-2 toxin and DON

T-2 toxin and DON were analysed according to the instructions in Agra Quant® Deoxynivalenol Test kit (Romer Labs, Tulln, Austria, quantification limit: $250 \mu\text{g}\cdot\text{kg}^{-1}$) and Agra Quant® T-2 toxin Test kit (Romer Labs, Tulln, Austria, quantification limit: $75 \mu\text{g}\cdot\text{kg}^{-1}$). Samples containing the toxin below the limit of quantification were measured by the method of standard addition. The standard addition of T-2 toxin was $100 \mu\text{g}\cdot\text{kg}^{-1}$ and standard addition of DON was $300 \mu\text{g}\cdot\text{kg}^{-1}$. The absorption in the micro-wells was measured with a Multiskan RC ELISA reader (Ani Labsystems Oy, Vantaa, Finland) using 450 nm of

absorbance filter. All experiments were repeated three times.

RESULTS AND DISCUSSION

In research were created suitable conditions for the germination of spores and production of T-2 toxin and deoxynivalenol. Adding of disinfectants into nutrient media had prevent *Fusarium* growth and production of T-2 toxin and deoxynivalenol.

Production of T - 2 toxin was examined in *Fusarium poae* CCM F 584 and *Fusarium* spp. 1 - isolated from malt-house air. A content of T-2 toxin is mentioned in Table 1. The highest production of T - 2 toxin at 25 °C was shown by the tested strain *Fusarium* spp. 1 in the presence of 3% of Desanal A plus. T -2 toxin at 15 °C produced mainly *Fusarium poae* CCM F 584 cultivated in the presence of 5% Desanal A plus and 0.3% Procure star namely 84.6 ±0.3 to 85.9 ±0.6 µg.kg⁻¹. When comparing two different strains was found difference production of T -2 toxin depending on the strain and temperature of the cultivation. The difference in the incidence of *Fusarium* confirms psychrophilic strains that described the Weidenbörner et al. (2001). As psychrophilic *Fusarium* can be considered strain of *Fusarium poae* CCM F 584 because the increase production of T - 2 toxin occurred

more at 15 °C in comparison to 25 °C. Kokkonen et al. (2010) found that a water activity and temperature influence the production of mycotoxins. This was observed both in the production of T-2 toxin and deoxynivalenol in our experiment.

Production of deoxynivalenol was examined in *Fusarium graminearum* CCM F 683 and *Fusarium* spp. 2 - isolated from barley. The higher production of deoxynivalenol was observed in *Fusarium graminearum* CCM F 683 cultivated in the presence of 3% Desanal A plus at 25 °C (28.9 ±0.9 µg.kg⁻¹) and *Fusarium* spp. 2 cultivated in the presence of 7% Desanal A plus at 25 °C (27.9 ±1.6 µg.kg⁻¹). The experiment showed that the use of lower concentrations of Desanal A plus disinfectants then 10% not prevented growth of *Fusarium* and production of T-2 toxin and deoxynivalenol. It was also confirmed in the study of Noske a Shearer (1985). Reynolds et al. (2012) recommended the using of low concentrations (2.4%) of NaClO for the reduction of fungi because sodium hypochlorite which is weakly alkaline (9-12 pH) can cause skin irritation (Hostynek et al., 2006). In the presence of ProCura star *Fusarium graminearum* CCM F 683 was production of deoxynivalenol in the range of 11.9 ±0.9 µg.kg⁻¹ to 35.4 ±2.2 µg.kg⁻¹ and *Fusarium* spp. 2 was production of

Table 1 Production of T-2 toxin.

Disinfectant	Concentration (%)	T-2 toxin [µg.kg ⁻¹] ±SD **			
		<i>Fusarium poae</i> CCM F 584		<i>Fusarium</i> spp. 1*	
		25 °C	15 °C	25 °C	15 °C
Desanal A plus	3	59.4 ±0.9	77.6 ±0.5	82.7 ±0.3	69.4 ±1.4
Desanal A plus	5	56.7 ±0.5	84.6 ±0.3	80.0 ±1.7	0
Desanal A plus	7	0	37.1 ±1.1	0	0
Desanal A plus	10	0	0	0	0
Guaa-Pool	0.0005	23.2 ±1.3	31.6 ±0.8	39.8 ±0.3	52.1 ±0.6
Guaa-Pool	0.001	4.5 ±0.4	2.5 ±0.6	15.2 ±0.1	44.4 ±0.3
Guaa-Pool	0.002	18.9 ±0.7	20.8 ±0.9	43.3 ±0.6	37.0 ±0.2
Guaa-Pool	0.003	12.5 ±0.7	18.3 ±0.5	46.7 ±0.8	48.5 ±1.0
Guaa-Pool	0.004	0	0	0	0
ProCura star	0,2	9.2 ±0.1	39.7 ±0.4	61.6 ±0.8	34.5 ±0.8
ProCura star	0,3	21.0 ±0.5	85.9 ±0.6	44.2 ±0.5	0.8 ±0.6
ProCura star	0,4	31.3 ±1.7	0	45.2 ±1.9	3.6 ±0.1
ProCura star	0,5	25.7 ±0.2	0	0	0
ProCura star	0,6	0	0	0	0
without disinfectants	0	13.6 ±0.9	93.7 ±1.2	71.6 ±0.9	13.0 ±2.3

* *Fusarium* spp. 1 - isolated from malt-house air

** average value ±standard deviation

deoxynivalenol in the range of $24.6 \pm 1.9 \mu\text{g.kg}^{-1}$ to $38.1 \pm 2.0 \mu\text{g.kg}^{-1}$. The 0.5% concentration of ProCura star proved to be very effective. **Suchomel et al. (2009)** found that alcoholic disinfectants containing mixtures of propan-1-ol and propan-2-ol at alcohol concentrations 70% or more fulfilled the required standard of antimicrobial efficacy. Quantity of deoxynivalenol produced by *Fusarium graminearum* CCM F 683 and *Fusarium* spp. 2 in the presence of Guaa-Pool is shown on Figure 3 and Figure 4. Guaa-Pool is a polymeric disinfectant with anion surface-acting agent. Quaternary ammonium compounds are widely used biocides with antimicrobial effect against a broad range of microorganisms. It is shown in the study of **Wessels a Ingmer (2013)**.

Our experiment showed that low concentrations of disinfectants did not prevent production of mycotoxins. It was found that using low concentrations of disinfectant

can cause increased production of toxins. This behavior of fungi has already been described when cultivated in the presence of fungicides **Havlová et al. (2006)** and **Heier et al. (2005)**. Improper application of disinfectants may act as a stress factor stimulating the production of mycotoxins, and therefore we must comply with all instructions given by the manufacturer.

CONCLUSION

The results of the study show that the most effective disinfectant for reduction of production of T-2 toxin and deoxynivalenol was Desanal A plus and ProCura star. Guaa-Pool at the concentration 0.004% proved to be very effective. It is higher concentration of disinfectant than recommended by manufacturer for full fungicidal activity. Lower concentration of disinfectants that not prevented growth of *Fusarium* caused higher production of T-2 toxin

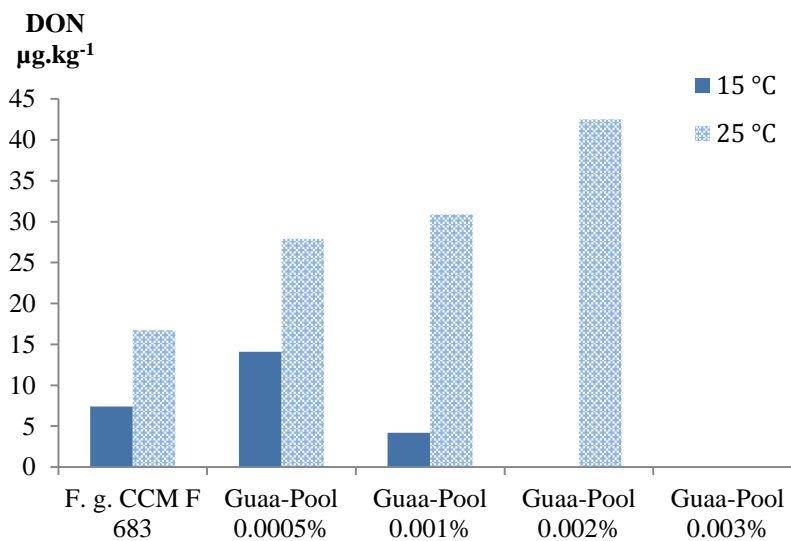


Figure 3 Production of deoxynivalenol, *Fusarium graminearum* CCM F 683, cultivation for 7 days.

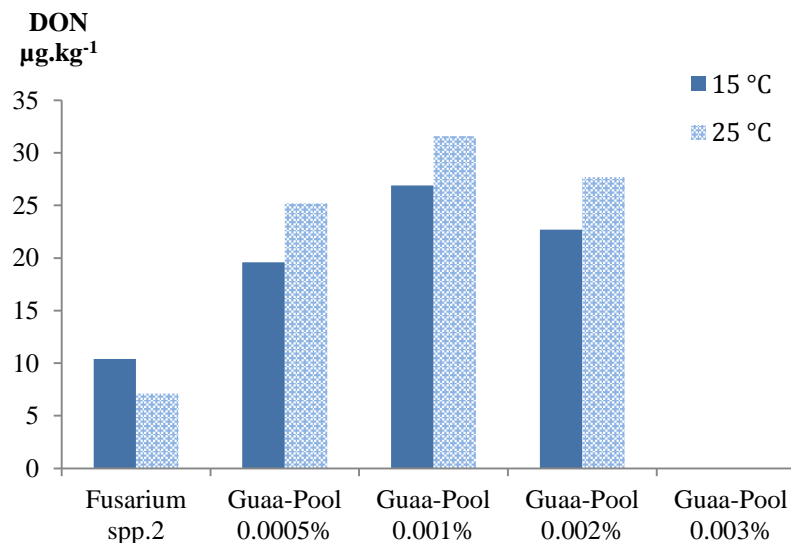


Figure 4 Production of deoxynivalenol, *Fusarium* spp. 2, cultivation for 7 days.

and deoxynivalenol. The results indicate considerable variability of individual strains in the production of T-2 toxin and deoxynivalenol. It depended on the *Fusarium* strain used, concentration of disinfectants and temperature of cultivation.

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LIQUID CHROMATOGRAPHIC DETERMINATION OF POLYPHENOLS IN CZECH BEERS DURING BREWING PROCES

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ABSTRACT

High performance liquid chromatographic (HPLC/UV) method was adapted for simultaneous determination of seven polyphenols, including derivatives of benzoic (gallic and vanillic acids) and cinnamic acids (p-coumaric, ferulic and sinapic acids), flavan-3-ols (catechin) and flavonols (rutin) in worts and beers at the various stages of the brewing process. Based on the semi-quantitative HPLC analysis, total polyphenols chromatographic index (TPCI) was in the ranges of 5.18 – 19.4 mg/L and 7.37 – 20.7 mg/L for all worts and beers, respectively. The HPLC analyses showed that relatively high levels of (+)-catechin and gallic acid were in all the worts and the beers, while the values were much lower for ferulic acid, rutin, vanillic acid, sinapic acid and p-coumaric acid. Polyphenols with relatively high concentrations, that were detected in all tested worts and beers, were gallic acid (1.29 – 4.75 mg/L resp. 2.59 – 4.97 mg/L), (+)-catechin (1.66 – 7.95 mg/L resp. 4.70 – 10.0 mg/L) and ferulic acid (0.41 – 4.53 mg/L resp. 1.05 – 2.87 mg/L). On the other side, the sinapic acid (0.72 – 1.59 mg/L resp. 0.72 – 2.5 mg/L), rutin (1.17 – 2.03 mg/L resp. 1.16 – 2.85 mg/L), p-coumaric acid (ND – 4.73 mg/L resp. ND – 1.44 mg/L) and vanillic acid (ND – 1.52 mg/L resp. 0.75 – 1.81 mg/L) were detected in lowest concentrations. In both, worts and beers investigated in this study, the changes in the contents of individual polyphenols were not uniform. In the case of some polyphenols, a decrease in the content was observed after boiling the worts with hops or after the main fermentation until maturation and filtration, but with some polyphenols, the concentrations were constant until the end of the process or even increased.

Keywords: beers; worts; brewing technology; polyphenols; HPLC; UV-VIS diode array detection

INTRODUCTION

Beer polyphenols have been mostly investigated in the light of their potential antioxidant activity claimed to enhance beer flavor and stability or even human health (Cortacero-Ramírez et al., 2003; Kondo, 2004; Nardini and Natella, 2006). The majority of polyphenols of beer are derived from malt (70–80%), whereas about 20–30% are derived from hops (Gerhauser, 2005). Further, polymerization of phenolics and formation of polyphenols, and their chemical changes can occur during wort boiling and possibly during fermentation and storage of beer. Polyphenolic constituents of beer represent a large structural variety and belong to the classes of simple phenols, benzoic and cinnamic acids derivatives, coumarins, catechins, di-, tri- and oligomeric proanthocyanidins, (prenylated) chalcones and flavonoids (Gerhauser and Becker, 2009).

In recent years, significant efforts have been made to avoid the oxygen pick-up during brewing process, the level of total packaged oxygen might be as low as 0.1 mg/L, but oxidative staling of beer is still noticeable. Minimizing the formation and reducing activity of reactive oxygen species (O_2^- , HOO^* , H_2O_2 and HO^*) in beer and wort, must be the first step for improving beer flavor stability. Antioxidants reduce the rate of oxidation

reactions. Therefore, attention is now increasingly shifting towards increasing the antioxidant activity of beer itself (Lu et al., 2007). There are many endogenous antioxidants such as polyphenols, Maillard reaction products, and sulfite present in beer. Among these antioxidants, polyphenols are of particular interest to brewers because they play a key role in the brewing process by delaying, retarding, or preventing oxidation processes (Lugasi and Hovari, 2003; Lu et al., 2007; Zhao et al., 2010).

Rapid analytical methods are necessary for the quality control department of beer producers to evaluate polyphenols that can adversely affect beer flavor and stability, what is of practical interest. Analytical methods for determining polyphenols in wort and beer are limited (Madiga et al., 1994; Montanari et al., 1999; Andersen and Skibsted, 2001; Floridi et al., 2003). Several authors determined polyphenols in beer matrices by RP-HPLC followed by ultraviolet (Hayes and Smyth, 1987), photodiode-array (PDA, Sanchez-Moreno et al., 1998; Montanari et al., 1999), fluorimetric detection (Dvorakova et al., 2008). Electrochemical detection (HPLC-ECD) has become a widely accepted and valuable technique (Rehova et al., 2004; Skeriková et al., 2004) because of its high sensitivity as well as its superior selectivity to UV absorption for analytes that are

electrochemically active, such as polyphenols (Roston and Kissinger, 1981; Wang et al., 2002). Mass (MS) and nuclear magnetic resonance (NMR) spectrometric detection can provide additional structural information and solve co-eluting compounds in complex mixtures (Whittle and Eldridge, 1999). Electrospray ionization (ESI) mass spectrometry provides the molecular masses as a soft ionization technique after chromatographic separation, while tandem mass spectrometry (MS/MS) provides extra information on the distribution of the substituents on the phenolic rings, useful for tentative identification but only rarely providing sufficient data for full structural analysis (Careri et al., 1998). An overview of recent development in HPLC determination of phenolics in beers is presented in (Chunsriimayataw et al., 2010a–c).

The general aim of this study was to detect, in a full scale industrial process, the polyphenols in all worts and beers, their fate during the main brewing steps and to compare the six kinds of “Czech brews” and their corresponding 28 worts and 17 beers from Janáček Brewery, Uherský Brod, Czech Republic from the point of view of identification and quantification of individual polyphenols by using HPLC method.

MATERIAL AND METHODOLOGY

Chemicals

Gallic acid, (+)-catechin, vanillic acid, p-coumaric acid, ferulic acid, sinapic acid, rutin, acetonitril (ACN), trifluoroacetic anhydride (TFAA) and methanol (all from Sigma–Aldrich), ferulic acid (Merck, Darmstadt, Germany), Na₂CO₃ and all other chemicals of p.a. purity were from Penta (Chrudim, Czech Republic). The stock standard solutions (ca. 1000 µg/mL) of each polyphenols were prepared in methanol by weighing approximately 0.001 g of the analyte into a 10 mL volumetric flask and diluting to volume. An intermediary mixed standard solution was prepared by dilution of the stock standard solutions in mobile phase A to give a concentration of ca. 10, 20, 30 and 50 µg/mL for each polyphenols. All standard solutions were stored in the dark at 4 °C and were stable for at least three months.

Instrumentation

A UV–VIS spectrophotometer Libra S6 (Biochrom Ltd, Cambridge, UK) and an ultrasonic bath (PSO 4000 A, Kraintek, Slovakia) were used for sample preparation. A HPLC system UltiMate 3000 system (Dionex Corporation, California, USA) consisted of a pump, an autosampler, a column compartment and a diode array detector. Chromatographic separation was carried out on a Supelcosil LC–18–DB column (250 x 4.6 mm, 5 µm, Supelco, USA) at 30°C using a gradient elution with a mobile phase consisting of solvent A (95% (v/v) acetonitrile acidified with 0.35 mL TFAA) and solvent B (50% (v/v) aqueous acetonitrile acidified with 0.25 mL TFAA). An injection volume 10 µL, flow rate of 1 mL/min, runtime 30 min were used. Phenolic compounds were identified on the basis of retention times (see Table 1) and UV spectra as compared to standard solutions of phenolic compounds. The concentrations of individual polyphenols in wort and beer samples were calculated using calibration curves constructed for all the

phenolic compounds. The analytical parameters of the calibration curves were calculated with the Excel program.

Brew samples.

Six kinds of “Czech brews” (labeled as A – F) processed by different technologies from Janáček Brewery, Uherský Brod, Czech Republic and their corresponding 28 worts and 17 beers were collected at various stages during the brewing process as follows:

1) Malt wort – front part (fresh mash) “front part” – it is the intermediate product in the process of brewing beer. It is a sweet solution without hops, containing saccharides and proteins substance that appears during the percolate. The clear fresh mash is the first part running out of a percolate bowl. It contains the highest amount of polyphenolic substances.

2) The second malt wort – after skimming of the first malt wort (extract content 16–20%), the residual spent grist flushed with hot water for last running. The goal is to get saccharides out of spent grains as much as possible. The decrease of the amount of polyphenols, which is obvious from the graph, is caused by withholding of polyphenols in the spent grains (in the filtrating layer). Temperature is important during lautern, because increasing temperature decreases viscosity and lautern is accelerated. However, temperatures above 80 °C are unfavorable. Then α-amylase is destroyed and un-dissolved starch cannot be saccharified. Wort will not be iodine normal and starch haze will result in beer.

3) Third malt wort – is the last running (extract content 0.5–1%). The main quantities of most substances have been already filtrated by previous out flowing with the previous aberration/excess. The volume of last running depends on aimed extract concentration. Extract content in spent grist fixes the end of lautern. Final extract content in spent grist has to be below 0.8%.

4) Unhoped malt wort – after lautering, brewer’s wort mixed from fronts and all low wines (usually from three of them) malt wort (front, first and third) is combined and transferred to the brewing kettle, where it is boiled during at least one hour with the addition of hops. Aims of wort boiling are wort sterilization, predication of coagulated proteins and isomerization of hop bitter substances. Next to this during hop boiling coagulate proteins with polyphenols during complex compound inception and than they come out from the solution. Coagulation has to be perfect; otherwise the rests of proteins can disturb fermentation and create later fogs.

5) Hopped wort – hop (Červeňák, Žatec hop) is added during the wort boiling. The amount of hops needed is only a fraction of the substantial quantities of malt used in the brewery. Usually, a few grams of hops are sufficient as a quantitatively minor, but qualitatively major ingredient with crucial impact on well-defined beer features. Hop dosage at the beginning of wort boiling serves for bittering and is generally carried out with bitter hop. A second dosage at the end of boiling or into the whirlpool gives a favorable hop dose.

6) Young beer – after cooling and removal of spent hops, the hopped wort is being pumped to the fermentation vessels and yeast is being added under aeration for growth. The fermentation takes about one week thereby delivering a so-called ‘young beer’ or ‘green beer’, which not

drinkable, as a number of offending (bad taste and smell) compounds are formed during fermentation. During the anaerobic phase yeast cells convert sugars to ethanol and carbon dioxide.

7) Unfiltered beer – after fermentation, beers need a maturation or lagering period of several weeks at about 0 °C, during which the unwanted components are slowly decomposed. High concentrations of diacetyl and pentane–2,3–dione are particularly obnoxious for the quality of lager beers (‘pilsner–type’) and scrupulous monitoring is required. Only after the content has decreased below critical values (ppb–ranges), beer can be bottled.

8) Filtered beer – solid and hazy particles still present in the beer (yeast, protein–tannin particles, and hop resins) are removed by filtration. Filtration also improves biological and physico–chemical stability. Filtration is carried out at low temperature (possibly at 0 to –2°C) under a counter–pressure of carbon dioxide above its saturation level, and with minimum uptake of oxygen. Beer samples were degassed in ultrasonic bath PSO 4000 A before analysis (waiver of carbon dioxide). Degassed beers and worts were filtered through 0.45 µm Nylon membrane filter (13 mm, Gronus filter, part No FFNN1345–100, SMI–LabHut Ltd., Gloucester, UK).

RESULTS AND DISCUSSION

Analysis of individual polyphenols by analytical HPLC To remedy the limitation of spectrophotometric methods (Chunsriyatav et al., 2010a–c) for total polyphenols polyphenols including derivatives of benzoic and cinnamic, flavan–3–ols and flavonols were identified and quantified by HPLC analysis in six kinds of “Czech brews” and their corresponding worts and beers from various stages during the brewing process.

The seven polyphenols standard solutions prepared by dilution of the individual stock standard solutions in mobile phase A to obtain the desired concentrations of ca. 10, 20, 30 and 50 µg/mL⁻¹ for each polyphenols. The working standard mixture was diluted 1:4, 3:7, and 1:1 (v/v) to obtain the calibration solutions. Table 2 lists the parameters of calibration curves and their calibration equations (with $c = 0$ as fixed point and omitting $c = 0$ point). The diode array detection was conducted by scanning between 205 nm, 210 nm and 275 nm (except of rutin). Comparing the absorbances at the three wavelengths, the absorbance at 210 nm showed considerable improvement in signal–to–noise ratio (better precision, sensitivity). The concentrations of seven polyphenols in worts and beers were determined using the calibration curves (with $c = 0$ as fixed point) listed in Table 2. The identification of the peaks was carried out by their retention times in comparison with standards, but also comparing the UV spectra in samples and standards by using a diode array detector. The standard polyphenols were used to examine phenol concentration in different kinds of worts and beers.

HPLC separation of standards of polyphenols

The retention times (RT) of seven standard compounds are reported in Table 1. The elution of polyphenols follows the decreasing polarity in reversed–phase HPLC, thus benzoic acid derivatives are eluted earlier than cinnamic acid derivatives. Guo et al., (1997) reported that the retention time of polyphenols increases with the number of –OCH₃ substituents. The elution order for benzoic acid is as follows: gallic acid > vanilic acid. Gallic acid is the first acid eluted (three –OH groups), whereas vanilic acid, the first –OCH₃ substituted among benzoic acids, has an RT of 11.77 min. Under the same condition, the elution order for cinnamic acids is p–coumaric acid > ferulic acid > sinapic acid. Ferulic acid eluted after p–coumaric, which indicates that the methoxy (–OCH₃) substituent is less polar, for it increases in retention.

Individual polyphenols

Table 3 reports the concentration of the seven polyphenols and the total phenolic chromatographic index (TPCI) as sum of all the polyphenolics classes calculated from the chromatogram in 28 worts and 17 beers. The standard deviation (SD) value ranges from 0.002 to 0.91 mg/L for worts and from 0.007 to 3.7 mg/L for beers. Polyphenols with relatively high concentrations, that were detected in all tested worts and beers, are gallic acid (1.29 – 4.75 mg/L resp. 2.59 – 4.97 mg/L), (+) catechin (1.66 – 7.95 mg/L resp. 4.70 – 10.0 mg/L) and ferulic acid (0.41 – 4.53 mg/L resp. 1.05 – 2.87 mg/L). On the other side, sinapic acid (0.72 – 1.59 mg/L resp. 0.72 – 2.5 mg/L), rutin (1.17 – 2.03 mg/L resp. 1.16 – 2.85 mg/L), p–coumaric acid (ND – 4.73 mg/L resp. ND – 1.44 mg/L) and vanillic acid (ND – 1.52 mg/L resp. 0.75 – 1.81 mg/L) were detected in low concentrations. Due to their low content, some individual polyphenols like p–coumaric acid could not be detected in a number of beer and wort samples.

Moreover, all the worts and beers tested in the current study exhibited relatively high levels of (+)–catechin and gallic acid, while the values were much lower for rutin, ferulic acid, sinapic acid, vanillic acid and p–coumaric acid. In both, worts and beers, the changes in the individual polyphenols were not uniform. In the case of some polyphenols, a decrease in the content was observed after boiling the worts with hops or after the main fermentation until maturation and filtration, but with some polyphenols, the concentrations were constant until the end of the technological processes or even increased (e.g., gallic acid and catechin in brew C, brew D and brew E). The concentrations of (+)–catechin and gallic acid were approximately constant or slightly decreased in most cases or increased during the brewing process (sweet wort → hopped wort → fresh beer). The results also indicated a remarkable increase of (+)–catechin contents in all beers in comparison to the corresponding worts after maturation process.

Table 1 Retention times (RT) of polyphenols.

No	IUPAC name	Current name	Abbreviation	Peak-RT ^a (min)
1	3,4,5-trihydroxybenzoic acid	Gallic acid	GA	4.16
2	trans-3,3',4',5,7-pentahydroxyflavane	Catechin	Cat	10.08
3	4-hydroxy-3-methoxybenzoic acid	Vanilic acid	VA	11.77
4	trans-4-hydroxycinnamic acid	p-Coumaric acid	pCA	18.73
5	4-hydroxy-3-methoxycinnamic acid	Ferulic acid	FA	20.64
6	3,5-dihydroxy-4-hydroxycinnamic acid	Sinapic acid	SA	20.79
7	Quercetin-3-rutinoside	Rutin	Rut	21.46

^a RT-Retention time in minutes

Table 2 Calibration curves and their calibration equations of polyphenols standards.

Compound	UV (nm)	Calibration equation ^a	R ²	Calibration equation ^b	R ²
Gallic acid	205	Y = 1552.5x - 1921	0.9907	Y = 1689.7x - 7135.2	0.9884
	210	Y = 1874.5x - 2306.8	0.9912	Y = 2039.3x - 8568.2	0.9912
	275	Y = 675.7x - 832.33	0.9925	Y = 735.15x - 3091.5	0.9925
Catechin	205	Y = 1390x - 1526.8	0.9959	Y = 1499.1x - 5671.1	0.9984
	210	Y = 946.61x - 985.34	0.9961	Y = 1017x - 3659.8	0.9982
	275	Y = 74.941x - 104.51	0.9957	Y = 82.406x - 388.18	0.9992
Vanilic acid	205	Y = 1336.1x - 385.26	0.9971	Y = 1363.6x - 1431	0.9977
	210	Y = 1337.6x - 251.56	0.9967	Y = 1355.6x - 934.37	0.9977
	275	Y = 412.52x - 33.041	0.9990	Y = 414.88x - 122.72	0.9977
p-Coumaric acid	205	Y = 654.17x + 627.88	0.9944	Y = 609.32x - 2332.1	0.9905
	210	Y = 678.88x + 644.97	0.9945	Y = 632.81x + 2395.6	0.9907
	275	Y = 823.09x + 709.47	0.9952	Y = 772.42x + 2635.2	0.9916
Ferulic acid	205	Y = 962.44 - 598.91	0.9936	Y = 1005.2x - 2224.5	0.9866
	210	Y = 1054.3x - 652.28	0.9936	Y = 1100.8x - 2422.8	0.9866
	275	Y = 746.46x - 437.55	0.9935	Y = 777.71x - 1625.2	0.9860
Sinapic acid	205	Y = 672.7x - 420.67	0.9970	Y = 702.74x - 1526.5	0.9951
	210	Y = 617.66x - 422.07	0.9969	Y = 647.81x - 1567.7	0.9955
	275	Y = 44.065x - 54.397	0.9938	Y = 254.76x - 647.51	0.9956
Rutin ^c	205	Y = 879.8x - 1396.8	0.9928	Y = 979.57x - 5188.3	1.0000
	210	Y = 734.11x - 677.09	0.9970	Y = 782.47x - 2514.9	0.9987
	275				

^a with c=0 as fixed point, ^b omitting c= 0 point, ^c rutin not detected at 275 nm

The found values agree with phenolic concentrations determined by other authors in literature. **Floridi et al., (2003)** using HPLC with coulometric array detection, described a wide range of free phenolic acids in worts. **Nardini and Ghiselli (2004)** determined free and total alkali extractable phenolic acids in three beers of Italian, Austrian and German origin. Ferulic acid was the main phenolic acid in both forms, followed by other phenolic acids present in the three beers always in considerably lower levels than ferulic acid. Phenolic acids were present

in these beers mainly in the bound form. **Vanbeneden et al., (2006)** using HPLC-ECD, determined the content of the three main phenolic acids: ferulic (main phenolic acid) followed by p-coumaric and sinapic acids, but their analytical technique was created primarily for the simultaneous detection of volatile phenols and not phenolic acids in worts or beers.

The sum of all the phenolic classes calculated from the chromatogram (total phenolics chromatographic index – TPCI) in different brews varied considerably,

Table 3 Individual polyphenols content (calculated using the corresponding calibration curves).

Brew	Comp	1	2	3	4	5	6	7	8
A	GA	4.16 ±0.08	2.89 ±0.04	4.25 ±0.26	4.75 ±0.06	3.51 ±0.05	3.85 ±0.37	3.72 ±0.48	4.16 ±0.08
B		4.15 ±0.09	2.82 ±0.004	2.95 ±0.07	3.26 ±0.09	2.87 ±0.13	3.45 ±0.48	4.15 ±0.09	2.82 ±0.004
C		3.72 ±0.05	3.79 ±0.14	2.02 ±0.01	3.22 ±0.2	1.85 ±0.07	3.64 ±0.08	2.59 ±0.01	4.85 ±0.04
D		2.31 ±0.16	1.91 ±0.06	3.17 ±0.02	2.74 ±0.32	3.76 ±0.05	4.69 ±0.01	4.97 ±0.01	2.31 ±0.16
E		3.06 ±0.04	1.95 ±0.01	1.40 ±0.003	3.28 ±0.09	3.54 ±0.91	3.36 ±0.02	4.71 ±0.02	3.06 ±0.04
F		3.27 ±0.25	3.20 ±0.01	1.29 ±0.01	3.51 ±0.02	3.37 ±0.02	4.19 ±0.02	3.70 ±0.02	4.16 ±0.01
A	Cat	4.77 ±0.07	4.06 ±0.06	5.92 ±0.02	7.53 ±0.006	7.09 ±0.007	8.44 ±0.10	4.70 ±0.55	4.77 ±0.07
B		6.98 ±0.05	5.29 ±0.06		4.05 ±0.04	4.27 ±0.01	6.56 ±0.56	5.43 ±1.2	
C		6.28 ±0.05	5.69 ±0.03	3.07 ±0.04	7.72 ±0.03	6.66 ±0.26	6.40 ±0.07	9.25 ±0.74	9.75 ±2.32
D		6.73 ±0.03	ND	4.51 ±0.46	5.46 ±0.35	8.12 ±0.02	6.27 ±0.01	5.78 ±0.03	6.73 ±0.03
E		4.94 ±0.06	2.59 ±0.29	1.66 ±0.01	5.68 ±0.06	7.95 ±0.09	10.0 ±0.16	7.45 ±0.09	8.07 ±0.06
F		3.66 ±0.3	4.02 ±0.02	ND	1.96 ±0.15	7.12 ±0.1	ND	7.91 ±1.05	6.94 ±0.59
A	pCA	- ^{a)}	- ^{a)}	0.66 ±0.06	- ^{a)}	- ^{a)}	- ^{a)}	- ^{a)}	- ^{a)}
B									
C		ND	ND	ND	ND	ND	ND	0.94 ±0.06	0.83 ±0.04
D		0.62 ±0.03	ND	ND	0.53 ±0.03	ND	1.29 ±0.09	1.23 ±0.03	0.62 ±0.03
E		ND	ND	ND	0.59 ±0.02	1.52 ±0.05	1.01 ±0.06	1.81 ±0.41	0.75 ±0.02
F		0.76 ±0.03	ND	ND	0.45 ±0.02	ND	0.89 ±0.02	1.14 ±0.05	0.55 ±0.03
A	FA	4.51 ±0.01	2.51 ±0.02	4.41 ±0.002	4.78 ±0.01	2.76 ±0.02	2.00 ±2.61	1.64 ±0.12	4.51 ±0.01
B		4.01 ±0.03	ND		4.23 ±0.007	4.45 ±0.01	2.87 ±0.15	1.60 ±0.05	
C		2.06 ±0.04	1.37 ±0.07	ND	- ^{a)}	- ^{a)}	- ^{a)}	- ^{a)}	0.31 ±0.03
D		4.73 ±0.02	ND	ND	2.40 ±0.03	- ^{a)}	- ^{a)}	- ^{a)}	4.73 ±0.02
E		4.24 ±0.005	1.42 ±0.02	1.01 ±0.004	0.97 ±0.01	0.95 ±0.28	1.12 ±0.009	1.22 ±1.21	1.12 ±0.05
F		4.26 ±0.1	0.94 ±0.09	- ^{a)}	- ^{a)}	- ^{a)}	- ^{a)}	1.44 ±0.09	1.02 ±0.05
A	SA	1.06 ±0.03	ND	0.94 ±0.06	ND	ND	ND	1.35 ±0.06	1.06 ±0.03
B		1.34 ±0.05	ND		1.23 ±0.02	1.58 ±0.03	1.18 ±0.04	1.29 ±0.07	
C		1.80 ±0.06	1.65 ±0.1	ND	1.85 ±0.06	1.59 ±0.04	1.79 ±0.06	2.50 ±0.01	1.82 ±0.65
D		3.22 ±0.06	1.61 ±0.02	4.13 ±0.06	4.23 ±0.22	1.05 ±0.02	1.44 ±1.28	1.18 ±0.03	3.22 ±0.06
E		ND	ND	ND	1.01 ±0.02	0.88 ±0.04	0.87 ±0.07	1.71 ±0.09	1.52 ±0.01
F		1.05 ±0.09	0.81 ±0.04	ND	0.94 ±0.02	0.90 ±0.02	1.07 ±0.02	1.34 ±0.05	1.10 ±0.03
A	Rut	1.19 ±0.04	ND	1.29 ±0.03	1.22 ±0.01	1.32 ±0.03	1.27	1.34 ±0.07	1.19 ±0.04
B		ND	ND		ND	1.55 ±0.05	1.33 ±0.06	1.22 ±0.05	
C		ND	ND	ND	ND	1.55 ±0.02	2.02 ±0.08	2.56 ±0.03	1.89 ±0.07
D		1.83 ±0.07	1.78 ±0.08	1.76 ±0.06	1.78 ±0.05	2.85 ±0.03	2.44 ±0.08	2.17 ±0.15	1.83 ±0.07
E		ND	ND	ND	1.65 ±0.07	2.03 ±0.05	2.13 ±0.09	2.38 ±0.04	1.24 ±0.01
F		0.87 ±0.09	1.21 ±0.02	ND	1.42 ±0.06	ND	ND	ND	1.05 ±0.02
A	TCPI ^b	15.7	9.5	17.5	18.3	14.7	15.6	12.8	
B		16.5	8.11		12.5	15.1	14.8	12.9	
C		18.4	14.8	5.18	13.2	14.4	14.6	19.2	20.7
D		19.4	6.02	13.6	18.1	16.7	17.4	16.6	19.4
E		12.2	5.96	4.07	13.2	16.9	18.5	19.3	16.8
F		15.1	11.3	1.29	9.5	12.6	7.37	16.7	17.1
		1.26 ±0.05	1.14 ±0.06	ND	1.17 ±0.02	1.19 ±0.05	1.22 ±0.06	1.16 ±0.05	2.31 ±0.41
		ND	0.72 ±0.01	ND	0.98 ±0.05	0.93 ±0.04	1.25 ±0.07	1.26 ±0.08	ND

Brew A – Patriot 11%, B – Olsavan 11%, C – in Comenius 14°, D– Extra 12°, E– Patriot PLTM 11°, F–Prima 10°, ^a each value is the mean ±standard deviation of triplicate determinations; ND – not determined, ^a – concentration below the detection limits, ^b Total Phenolic Chromatographic Index (TPCI) = sum of all the phenolic classes calculated from the chromatogram

ranging from 5.18 – 19.4 mg/L and 7.37 – 20.7 mg/L for all worts and beers, respectively. Moreover, significant differences in total polyphenols content determined by Folin–Ciocalteu and HPLC methods were found in the present study, which also verified the non–specificity of Folin–Ciocalteu method. Therefore, the measurement of phenolic profiles by HPLC method could give more information about their chemical characteristics and antioxidant activities.

CONCLUSION

HPLC analysis coupled with UV–VIS diode array detection allows separation of polyphenols in worts and beers during the brewing process. Based on the

semi–quantitative HPLC analysis, total phenolics chromatographic index (TPCI) was in the ranges of 5.18 – 19.4 mg/L and 7.37 – 20.7 mg/L for worts and beers, respectively. All the beers from different technologies contained polyphenols at concentrations that generally were similar to those detected in their corresponding worts. The HPLC analysis showed that all worts and beers tested in the current study were relatively high levels of (+)–catechin and gallic acid, while the values were much lower for ferulic acid, rutin vanillic, sinapic and p–coumaric acids, most of which changed significantly during the brewing process. This HPLC–DAD analysis set up to routinely analyze up to seven polyphenols in order to control the brewing process

and the composition of the final product. The advantage of this procedure is that reproducible results are obtained by direct injection of worts and beers without sample preparation. The influence of the brewing process on the content of free phenolic acids and other polyphenols of worts and beers can be easily evaluated. Covalently bonded polyphenols in worts and beer will be investigated in future studies. A method will be developed for the hydrolysis and extraction for determining the total concentration (free or bound) of phenolic acids, including some other polyphenols resolved with this method but not determined in this work. On the results obtained from current study, further work on optimizing brewing processes will be the improvement of beer's flavor stability through raising selectively certain polyphenols.

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CONTENT OF 4(5)-METHYLIMIDAZOLE, CAFFEINE AND CHLOROGENIC ACID IN COMMERCIAL COFFEE BRANDS

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ABSTRACT

Content of 4(5)-methylimidazole (4-MeI), caffeine and chlorogenic acid in commercial coffee brands were determined using high-performance liquid chromatography (HPLC) with UV DAD and MS detectors. Positive ion ESI mass spectra of the 4-MeI standard yielded intense signals corresponding to $[M+H]^+$ (83.0604) and $[2M+H]^+$ ions (165.1115). Also, adducts of 4-MeI with acetonitrile from mobile were detected – $[M+ACN]^+$ ions (124.0849). The LOD of 2.5 ng mL^{-1} and LOQ of 8.4 ng mL^{-1} were calculated according to the following formulas: $\text{LOD} = 3.SD/S$, and $\text{LOQ} = 10.SD/S$, where S is the slope of the calibration curve and SD is the standard deviation of the noise. The caffeine content was compared to the results of the standard addition, 1st derivative and liquid-liquid extraction spectrophotometry. 4-MeI was in tens $\mu\text{g g}^{-1}$ in the Vietnamese coffees while in units $\mu\text{g.g}^{-1}$ in all Czech and Brazilian coffees ($<2.4 \mu\text{g.g}^{-1}$ and $<4.9 \mu\text{g.g}^{-1}$, respectively). The results for caffeine were within the documented range (0.31 – 2.20%) in all coffee samples. The lower content of caffeine and chlorogenic acid was observed in Vietnamese coffees. All the methods used for determination of caffeine in the Czech and Brazilian coffees gave acceptable precision and accuracy. However, there were significant differences in the results in Vietnamese coffees. The caffeine extractability (100 °C, 3 min brewing) almost reached 100% in Czech and Brazilian coffees, while it was less than 90% in Vietnamese coffees. The Czech and Brazilian coffees tend to produce more caffeine in brews than the Vietnamese coffee because of the different composition of blends and the particle size degree.

Keywords: coffee; spectrophotometric methods; HPLC; caffeine extractability

INTRODUCTION

The study of 4(5)-methylimidazole (4-MeI) and 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) has recently become an important aspect of food chemistry. In many studies regarding the identification of the causes of carcinogenesis, 4-MeI has been a factor, which could be associated with cancer risks (NTP, 2007; OEHHA, 2010). 4-MeI can induce alveolar/bronchiolar adenoma and carcinoma in male and female mice (Chan et al., 2008). It can also inhibit the cytochrome P450 isoenzyme, which catalyzes the oxidation of many known or suspected carcinogens of low molecular mass in the human liver (Hargreaves et al., 1994). Thus, this compound has been classified as a group 2B compound “probably carcinogenic to humans” by the International Agency for Research on Cancer (IARC, 2011). 4-MeI and THI are undesired byproducts formed by heating carbohydrates (glucose, sucrose, invert sugar, etc.) in the presence of ammonia (Hodge, 1967; Kort, 1971; Tomasik et al., 1989; Gobin a Phillips, 1991; Bradbury et al., 1996). These compounds can be present in caramel color products such as soy sauce, caramel colors, carbonated soft drink, Worcestershire sauce, canned coffee, dark beer etc. (Casal et al., 2002; Cunha et al., 2011; Yamaguchi and Masuda, 2011). Because roasted coffee beans are used for brewing coffee, the levels of 4-MeI can be considered to be significant. During the

roasting process (commonly 210 – 230 °C), the coffee beans composition dramatically changes as a consequence of pyrolysis, caramelization, Maillard and Strecker reactions. The color of the beans is changing from light brown to almost black, depending on cultural and personal preferences; and the characteristic aroma of roasted coffee is formed (Nicoli et al., 1997; Paul, 2009). However, potential carcinogenic compounds may be also formed at this temperature (Tressl et al., 1998). In recent years, several researchers have focused on the determination of 4-MeI and THI contents using liquid chromatography coupled to mass spectrometry (LC-MS). Both analytes were determined in samples in canned coffee purchased from Japanese local shops (Yamaguchi and Masuda, 2011) or in caramel, dark beers and roasted coffees (Klejdus et al., 2006) after solid phase extraction (SPE). Methods of gas chromatography with flame ionization or mass spectrometric detection (GC-FID and GC-MS, respectively) after ion-pair extraction were applied for 4-MeI determination in roasted coffee beans from Brazil and Ivory Coast coffees (Casal et al., 2002) or in ground roasted coffee purchased from Czech local company using HPLC/ESI-MS via supercritical fluid (SFE) extraction (Lojková et al., 2006).

Caffeine (1,3,5-trimethylxanthine) is known as a central nervous system (CNS) stimulant. It is naturally present in leaves, seeds and/or fruits of at least 63 plant species

worldwide, such as coffee and cocoa beans, tea leaves and kola nuts (Barone and Roberts, 1996; Frary et al., 2005). Coffee beans contain between 0.8 and 2.8% caffeine, depending on species and origin. It contributes to 10 – 30% of the bitter taste of coffee brews (Burmester and Eggers, 2010). The caffeine content in *Coffea canephora* (Robusta) is about two times that of *Coffea arabica* (Arabica) (Monica, 1998). It is not significantly changed during coffee roasting (Farah, 2009). However, the amount of caffeine in coffee brewing substantially varies according to the type of product (Arabica, Robusta, or their ratio in blending), the grinding degree, brewing methods and the serving size (Bell et al., 1996; Petracco, 2001). Robusta coffee tends to produce inferior quality and aroma compared to Arabica coffee, but Robusta coffee is cheaper; as a result, more Robusta coffee are mixed into the blending products.

Raw green coffee beans are also rich in the content of polyphenols and phenolic acids. Generally, simple phenolic acids contribute to the acidity, bitterness and astringency of the coffee and act in potential biopharmacological properties (Basnet et al., 1996; Tatefuji et al., 1996). Beside the substances, an important group of polyphenolic compounds called chlorogenic acids (CQAs) is present (Clifford, 2000; Perrone et al., 2008). The levels of CQAs vary from approximately 7.88 to 14.4% dry matter for *Coffea canephora* (Robusta) and approximately 3.4 to 4.8% dry matter for *Coffea arabica* (Ky et al., 2001). Higher amount of CQAs in green coffee tends to produce an undesirable flavour because of negative notes of their products of oxidation and degradation prior to and during roasting. The methods for CQAs determination are predominantly based on HPLC (Monteiro and Farah, 2012; Gloess et al., 2013; Mills et al., 2013).

The purpose of this study was to obtain information on content of 4(5)-methylimidazole (4-MeI), caffeine and chlorogenic acid (3-caffeoylquinic acid, CQA) in selected Vietnamese, Czech and Brazilian ground roasted coffees. Chromatographic methods with UV-VIS DAD and MS detection systems were applied for identification and quantitative determination of studied analytes. Further, the caffeine content was compared to the results of the three simple and rapid UV spectrophotometric methods (standard addition, 1st order derivative and liquid-liquid extraction). Transfer efficiency of caffeine in Vietnamese, Czech and Brazilian coffee samples was also compared and discussed.

MATERIAL AND METHODOLOGY

Chemicals and reagents. Chloroform (CHCl₃; 99%), caffeine standard (>99%), chlorogenic acid (>99.5%), sodium carbonate, methanol (for HPLC, >99.9%), were obtained from Sigma Aldrich (Steinheim, Germany). Formic acid (96.5%) was from LachNer (Neratovice, Czech Republic). De-ionized water was from a Milli-Q purification system (Millipore, Bedford, MO, USA). The Vietnamese ground roasted coffee samples, including Dak Tin (Ca phe Dak Tin Ltd.), Di Linh (Cty CP Chè-Cafe Di Linh), Nam Nguyen (Công ty chế biến cà phê Nam Nguyễn), Origin (Công ty TNHH một thành viên Tín Nghĩa) and Vinacafe (Công ty cổ phần Vinacafe Biên

Hòa) were purchased at Saigon CO.OP, in HoChiMinh Town, in Vietnam. The Czech ground roasted coffee samples, including Dadák (Dadák Ltd., Valašské Meziříčí, Czech Republic), Jacobs Aroma (Kraft Foods CR Ltd., Czech Republic), Marila Standard 100% Robusta (Mokate Czech Ltd., Czech Republic), Jihlavanka (Tchibo Praha Ltd., Jihlava, Czech Republic), Grande 100% Arabica (Grande Ltd., Poland; imported by Kaufland, Czech Republic), were purchased at local market in Zlín in the Czech Republic. Coffee Caboclo Brasilia (D.E. Cafes do Brasil LTDA., Sao Paulo, Brasil) represented Brazilian coffees.

LC/MS determination of 4(5)-methylimidazole. Stock solution of coffee (2.5 g in 45 mL water of 90°C) was filtered and transferred into 50 mL volumetric flasks. Ansys SPEC 3 mL SXC SPE cartridges (30 mg, Agilent Technologies, Palo Alto, CA, USA) were used for solid phase extraction after conditioning with 1 mL of methanol and 1 mL of water. Sample solution (3 mL) containing 1000 µL of the stock solution of coffee, 1980 µL of water and 20 µL of 0.1 M HCl was transferred on the SPE cartridge, washed with 1 mL of methanol and eluted with 2 mL of methanol acidified with 5 M HCl (3:1). The extract was evaporated to dryness and dissolved in 0.5 mL of water. The solution was directly injected into the LC/MS in aliquots of 10 µL.

Sample analysis was performed under gradient elution on a Zorbax Eclipse XDB C-18 (Agilent Technologies, 4.6 x 150 mm, 5 µm) analytical column using mobile phase consisting of 5 mM NH₄OH (phase A) and acetonitrile (ACN, phase B) with following steps: 0 – 9 min: 2 - 20% ACN, 10 - 13 min: 20 – 2% ACN, 13 – 16 min: 2% ACN). Column temperature was set at 25 °C. Signal was monitored using UV detector at 215 nm. MS spectra were recorded with an Agilent 6224 Accurate-Mass TOF mass spectrometer (Agilent Technologies, Wilmington, DE, USA) calibrated in the range 30 – 1700 *m/z* using an ESI tuning solution (G1969-85000, Agilent Technologies). Dual electro-spray ionization working in a positive mode was chosen. The parameters were set as follows: nitrogen flow 6 L min⁻¹ at 350 °C, nebulizer 40 psig, capillary voltage of 4.5 kV, fragmentor voltage of 40 V and skimmer voltage of 65 V. The MS spectra were recorded from 30 to 500 *m/z* and the chromatogram for 4-MeI determination was extracted for *m/z* 83.06.

HPLC measurements. Samples (0.2 g ±0.001 g) of fine ground roasted coffee and 20 mL of boiling distilled water (100 °C) were extracted under reflux for 15 min. Clear solutions were centrifuged for 15 min at 2500 rpm after cooling to ambient temperature. Supernatant was filtered through a membrane filter (45 µm) and diluted with water (1:5) and 20 µL were injected on a chromatographic column.

A liquid chromatographic system 10 AVP consisting of two LC – 10 ADVP chromatographic pumps, a GT – 154 degasser, a CTO – 10 ASVP column thermostat, and a UV DAD detector SPD – M10AVP was controlled by a SCL – 10A unit with a Class – VP 5.02 software (all from Shimadzu, Tokyo, Japan). An analytical chromatographic column Luna C18 (250 x 3.0 mm, particle size 5 µm) with a Luna C18 Security Guard

column (4 x 2.0 mm, both Phenomenex, Torrance, CA, USA) were for caffeine and chlorogenic acid determination using mobile phase consisting of 3% aqueous formic acid (phase A, pH 2.05) and methanol (phase B) at flow rate 0.5 mL min⁻¹ and under linear gradient elution (0 – 10 min: 0 – 30% B; 10 – 20 min: 30 – 40% B; 20 – 30 min: 40 – 100% B; 35 – 38 min: 100% B; 38 – 41 min: 100 – 0% B and finally up to 50 min at 0% B). Caffeine was detected at 270 nm while chlorogenic acid was determined at 320 nm. Injection volume was 20 µL. Column temperature was set to 25 °C.

Standard addition UV spectrophotometric measurement. Working standard solutions (10.0 – 16.0 µg mL⁻¹) for the preparation of calibration curves were prepared by dilution of caffeine stock solution (100 µg mL⁻¹ in water). UV spectrum was recorded against water over the wavelength range of 190 – 300 nm. The concentration of caffeine in coffee sample was obtained from the calibration curve ($y = 0.0503x + 0.2584$, $r^2 = 0.998$) in which y is absorbance of sample and x is concentration of caffeine in the samples.

First derivative UV spectrophotometric measurement. The coffee solution (0.05 g in 50 mL of boiling water) was filtered and the cold filtrate was diluted to 100 mL by water. The first order derivative absorption spectra of caffeine solutions (6.0, 8.0, 10.0 and 12.0 µg mL⁻¹) were recorded (see Figure 1) against distilled water in range of 190 and 300 nm. Peak-to-peak measurements (Alpdogan et al., 2002) of two neighboring peaks of 260 nm (minima) and 287 nm (maxima) were used for preparation of calibration curve. The concentrations of caffeine were calculated from the regression equation ($y = 0.0483x + 0.0107$, $r^2 = 1$) in which y is peak-to-peak

amplitude of the first order spectra at extreme of each sample and x (µg mL⁻¹) is concentration of caffeine in samples. The % caffeine in coffee samples was obtained by the following formula: (caffeine content/cake mass) x 100, (cake mass: 0.05 g).

Isolation of caffeine from the coffee brews. Na₂CO₃ (0.2 g) was added to 10 ml of the filtrate (coffee solution) to remove non-caffeine solids (Heilmann, 2001). The filtrate was extracted 3 times for 10 minutes with 10 mL CHCl₃ in each run. The combined extracts were diluted by CHCl₃ to 50 mL. The absorbance of the standard solutions (10, 20, and 30 µg mL⁻¹) and the sample solutions were measured at 276.2 nm against CHCl₃ (AOAC method 979.11). The concentrations of caffeine were calculated from the regression equation ($y = 0.0489x$, $r^2 = 0.9999$) in which y is absorbance of the sample and x is concentration of caffeine in samples. The % of caffeine in coffee samples was obtained by the above-mentioned formula.

Statistical evaluation. All results were statistically evaluated using the variation statistics (ANOVA, StatSoft, Prague, Czech Republic). Correlation matrices and regression functions were calculated using the statistical package Unistat, v. 5.5 (Unistat Ltd., England).

RESULTS AND DISCUSSION

LC/MS determination of 4(5)-methylimidazole. The extracted ion chromatogram for 4-MeI (in concentration of 1 µg mL⁻¹) and MS spectrum are shown in the Figure 2. The retention time was 13.5 min. Positive ion ESI mass spectra of the 4-MeI standard yielded intense signals corresponding to [M+H]⁺ (83.0604) and [2M+H]⁺ ions (165.1115). Also, adducts of 4-MeI with acetonitrile from mobile were detected - [M+ACN]⁺ ions (124.0849).

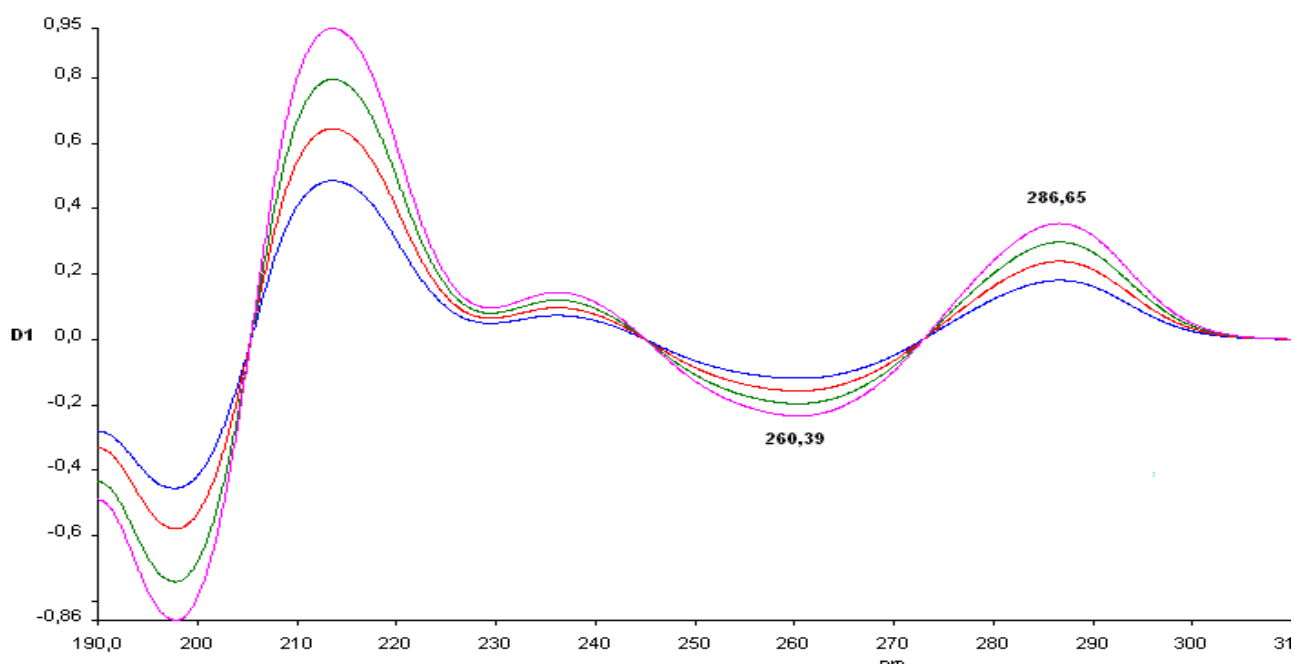


Figure 1 First derivative spectra of caffeine standards (6.0, 8.0, 10.0 and 12.0 µg mL⁻¹), reference: water.

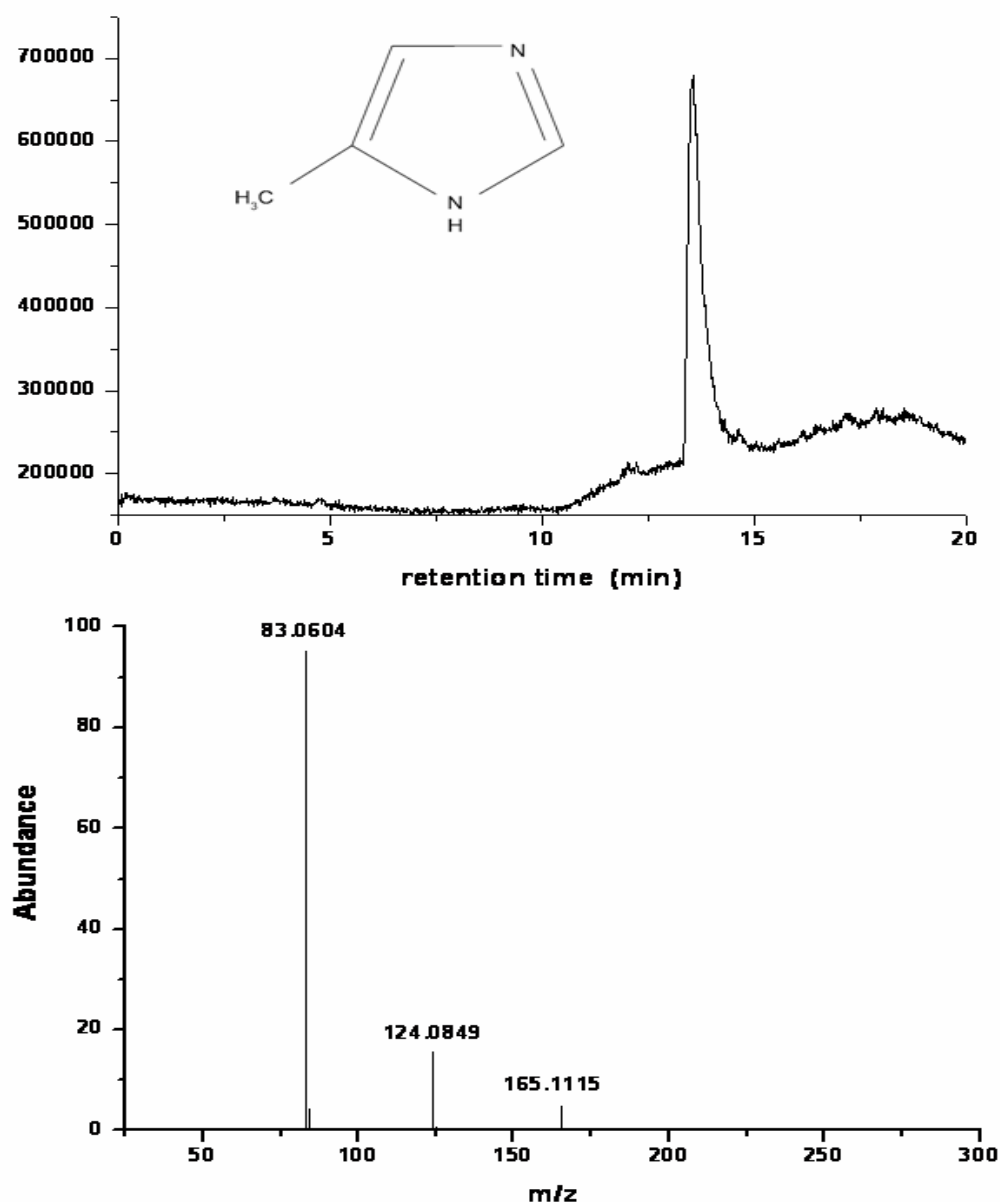


Figure 2 Extracted ion chromatogram (m/z 83.06) and ESI negative spectrum of 4-MeI.

The content of 4-MeI in the coffee samples was quantified using calibration curve ($y = 21210 \cdot 442x + 5098.331$, $r^2 = 0.998$) constructed from the peak area of the extracted ion chromatogram at m/z 83.06. The calibration was linear in the range $5 - 100 \text{ ng}\cdot\text{mL}^{-1}$. The LOD of $2.5 \text{ ng}\cdot\text{mL}^{-1}$ and LOQ of $8.4 \text{ ng}\cdot\text{mL}^{-1}$ were calculated according to the following formulas: $\text{LOD} = 3 \cdot \text{SD}/S$, and $\text{LOQ} = 10 \cdot \text{SD}/S$, where S is the slope of the calibration curve and SD is the standard deviation of the noise. The determined amount of 4-MeI varied in tens of $\mu\text{g}\cdot\text{g}^{-1}$ ($13.1 - 58.1 \mu\text{g}\cdot\text{g}^{-1}$) in the Vietnamese coffees (except of Vinacafe $5.1 \mu\text{g}\cdot\text{g}^{-1}$) while in units $\mu\text{g}\cdot\text{g}^{-1}$ in Czech coffees ($1.8 - 2.4 \mu\text{g}\cdot\text{g}^{-1}$) and Brazilian coffee

($4.9 \mu\text{g}\cdot\text{g}^{-1}$). All analyzed Vietnamese samples were characterized by intensive, suave, non-typical coffee aroma and differed from Czech and Brazilian samples in consistency. Therefore, more than ten times higher 4-MeI values observed in Vietnamese coffee samples relate with different ways of roasting or processing and with different composition of blends. Corn and soya, essential oils and even butter are also used for blending because of benefit. In some previous studies, observed values of 4-MeI in roasted coffees were also lower and ranged between $0.31 - 1.24 \mu\text{g}\cdot\text{g}^{-1}$ (Casal et al., 2002) and $0.39 - 2.05 \mu\text{g}\cdot\text{g}^{-1}$ (Klejduš et al., 2006).

Table 1 Content (mean \pm SD; in $\mu\text{g g}^{-1}$) of 4(5)-methylimidazole (4-MeI) and content (mean \pm SD; in mg 100 g^{-1}) of chlorogenic acid (CQA) in selected samples of Vietnamese, Czech and Brazilian coffees (n = 3).

Analyte	Vietnamese coffees				Czech coffees			Brazilian coffee
	Vina-cafe	Di Linh	Dak Tin	Origin	Nam Nguyen	Marila Standard	Jacobs Aroma	Caboclo Brasilia
	Content (mean \pm SD; in $\mu\text{g g}^{-1}$)							
4-MeI	5.1 \pm 0.3	58.1 \pm 1.5	38.7 \pm 1.8	13.1 \pm 0.3	29.1 \pm 0.4	1.8 \pm 0.1	2.4 \pm 0.1	4.9 \pm 0.1
	Content (mean \pm SD; in mg 100 g^{-1})							
CQA	202.4 \pm 22.1	28.6 \pm 9.0	2.2 \pm 0.8	59.9 \pm 3.7	59.6 \pm 0.1	683.4 \pm 46.7	n.d.	n.d.

n.d. - not detected

Table 2 Caffeine content (Cont.; mean \pm S.D.; in % w/w; n = 3) in coffee samples and transfer (T; in % w/w; n = 3) into coffee brewing at 100 °C and for 3 minutes.

Sample	Standard addition		Extraction using CHCl_3		1 st derivative measurement ^a		HPLC/DAD	
	Cont. (%)	T (%)	Cont. (%)	T (%)	Cont. (%)	T (%)	Cont. (%)	T (%)
Dadák	6.51 \pm 0.06	93 \pm 2	2.11 \pm 0.04	97 \pm 1	2.12 \pm 0.01	94 \pm 1	2.13 \pm 0.02	95 \pm 3
Jihlavanka	8.03 \pm 0.07	91 \pm 1	2.20 \pm 0.02	96 \pm 2	2.20 \pm 0.04	99 \pm 1	2.19 \pm 0.03	97 \pm 2
Grande	9.98 \pm 0.08	99 \pm 1	1.98 \pm 0.01	95 \pm 1	2.11 \pm 0.01	100 \pm 2	2.03 \pm 0.05	98 \pm 2
Jacobs Aroma	6.22 \pm 0.06	97 \pm 2	1.93 \pm 0.02	100 \pm 1	1.83 \pm 0.00	92 \pm 1	2.01 \pm 0.02	96 \pm 1
Marila Standard	7.04 \pm 0.10	97 \pm 2	2.10 \pm 0.1	99 \pm 2	2.11 \pm 0.02	95 \pm 1	2.13 \pm 0.01	99 \pm 1
Dak Tin	2.90 \pm 0.06	65 \pm 5	0.31 \pm 0.01	64 \pm 4	0.57 \pm 0.00	76 \pm 6	0.63 \pm 0.01	60 \pm 5
Di Linh	4.10 \pm 0.16	68 \pm 6	0.57 \pm 0.01	70 \pm 4	1.05 \pm 0.04	73 \pm 5	0.68 \pm 0.05	68 \pm 5
Nam Nguyen	4.50 \pm 0.01	64 \pm 3	1.03 \pm 0.02	77 \pm 3	1.40 \pm 0.01	85 \pm 5	1.13 \pm 0.04	75 \pm 6
Origin	5.55 \pm 0.11	66 \pm 4	1.45 \pm 0.01	90 \pm 4	1.80 \pm 0.00	86 \pm 4	1.43 \pm 0.03	89 \pm 7
Vinacafe	6.62 \pm 0.01	91 \pm 6	2.10 \pm 0.06	100 \pm 2	2.10 \pm 0.01	98 \pm 4	2.13 \pm 0.02	99 \pm 5

^a measured at 265 and 289 nm for Dadák, Grande and Vinacafe, 268 and 290 nm for Jihlavanka, 266 and 289 nm for Jacobs Aroma, 268 and 289 nm for Marila Standard, 265 and 284 nm for Dak Tin, 264 and 285 nm for Li Linh, 264 and 287 for Nam Nguyen, 263 and 284 nm for Origin

HPLC determination of caffeine and chlorogenic acid. A five points calibration curve ($y = 103858x + 170.42$, $r^2 = 0.9999$) was constructed as a peak area vs. caffeine concentration for 2.5, 5, 10, 25 and 50 $\mu\text{g.mL}^{-1}$ of caffeine with LOD = 1.56 $\mu\text{g.mL}^{-1}$ and LOQ = 5.20 $\mu\text{g.mL}^{-1}$. The calibration curve for chlorogenic acid was constructed similarly with the concentration of 0.5, 1, 2, 10 and 20 $\mu\text{g.mL}^{-1}$ ($y = 16340x + 1.478$, $r^2 = 0.9999$) with LOD = 0.08 $\mu\text{g.mL}^{-1}$ and LOQ = 0.25 $\mu\text{g.mL}^{-1}$. The contents of caffeine and chlorogenic acid (see Tables 1 and Table 2) were lower in Vietnamese coffees in comparison

with Czech coffee Marila Standard. Even in case of Dak Tin sample, the found caffeine content was less than 3 $\mu\text{g.g}^{-1}$. While the content of caffeine is not so much influenced by roasting time and relates mainly with the coffee cultivars, the level of chlorogenic acid is roasting time dependent and decrease with roasting duration (Farah, 2009). Comparison of 4-MeI, caffeine and chlorogenic acid content is illustrated in Figure 3. Our findings indicate that Vietnamese coffee samples were roasted longer than Czech samples which results in higher content of 4-MeI and lower level of chlorogenic acid.

Comparison of the spectrophotometric methods for caffeine determination. The contents of caffeine

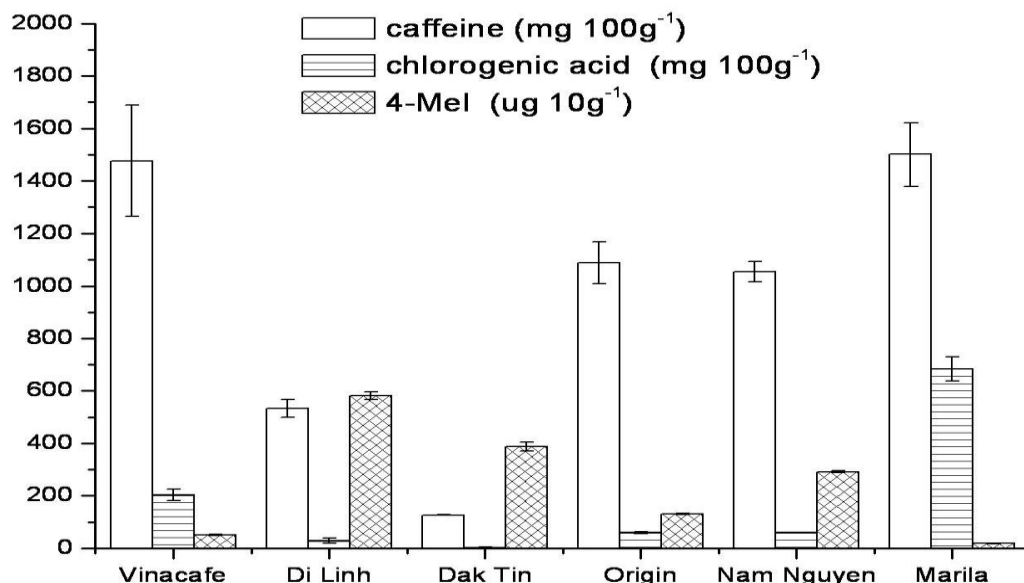


Figure 3 Comparison of caffeine, chlorogenic acid and 4(5)-methylimidazole content in Vietnamese and Czech coffee samples.

determined by liquid chromatographic, 1st derivative standard addition, and extraction spectrophotometric methods were found to be within the documented range (0.31 ± 0.01 and $2.20 \pm 0.04\%$) in all the coffee samples. There were no significant differences among the results in all the Czech coffees. However, there were significant differences in standard addition and other methods in all the Vietnamese samples (1.2 – 1.8 times), except of the Vinacafe sample ($2.10 \pm 0.06\%$ and $2.10 \pm 0.01\%$, respectively). This may be explained by a more significant interference of matrices and different composition of blends in the Vietnamese samples compared to the Czech ones (see above). The caffeine contents of the Czech coffees were higher than those of the Vietnamese coffees in all samples. The caffeine extractability at 100 °C and 3 min of brewing (commonly used time for brewing coffee) almost reached 100% in Czech coffees, while it was less than 90% in Vietnamese coffees (see Table 2). The particle size of coffee powders is probably major cause of the differences in caffeine extractability between Czech and Vietnamese coffee brands. Previous studies have also shown that caffeine content in coffee was influenced by grinding techniques (Bell et al., 1996). The results showed that the caffeine contents of Grande (100% Arabica) and Marila Standard (100% Robusta) were $1.98 \pm 0.01\%$ and $2.10 \pm 0.1\%$, respectively. However, they did not agree with the previous results published, 1.1 – 1.3% for Arabica and 2.4 – 2.5% for Robusta (Belitz et al., 2009). This may be due to deception in blending commercial ground roasted coffee.

CONCLUSION

The determination of 4(5)-methylimidazole, chlorogenic acid and caffeine content in Czech, Vietnamese and Brazilian ground roasted coffee brands illustrate significant differences in the amount of the analytes. They also indicate variant procedures in roasting and coffee processing. The contents of caffeine in five Czech and five Vietnamese coffees were also compared to the results of the 1st derivative, standard addition and extraction spectrophotometry applying CHCl_3 as an extraction solvent. The Czech coffee tends to produce more caffeine than the Vietnamese coffee because of the different composition of blends and the particle size degree. All methods can be successfully used for determination of caffeine. However, there were significant differences in the results among the standard addition and the other methods in the case of the Vietnamese coffee brands. The first derivative UV spectrophotometry gives 1.2 – 1.8 times higher results compare to the extraction spectrophotometric method.

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THE EFFECT OF STORAGE TEMPERATURE ON THE QUALITY AND FORMATION OF BLOOMING DEFECTS IN CHOCOLATE CONFECTIONERY

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ABSTRACT

The study aimed at assessing changes in the quality of certain types of chocolate products over the storage period with particular focus on the formation and development of fat and sugar bloom in chocolate products. Seven products were selected in collaboration with a chocolate factory to undergo monitoring and analysis and stored at four temperature regimens (6 °C, 12 °C, 20 °C and 30 °C). Five samplings were carried out over the storing period (18 weeks) for evaluation of the dynamics of changes in their quality. Each sampling was accompanied by sensory evaluation; selected physical attributes were also analysed: changes in colour (ΔE^*ab) within the CIE (L^*a^*b) system and changes in hardness using the TIRAtest 27025. The results showed a significant effect of storing temperature on the intensity of changes in the quality of products. The results of sensory evaluation of selected products showed that the highest quality for the majority of descriptors was achieved by products stored at temperatures of 6 °C and 12 °C. As regards samples stored under the temperature regimen of 20 °C, the products started to show visible differences, caused primarily by the formation of fat bloom while storing at 30 °C proved to be extremely unsuitable for all the tested products. Since storing temperatures of 6, 12 and 20 °C did not considerably affect hardness and colour of each product, no distinct changes occurred under such temperature regimens. From the aspect of analytical measurements of colour and hardness of each product, storing at temperatures of 20 °C can be termed appropriate. In all the analyses, the effect of the temperature regimen of 30 °C was significantly negative due to defects caused by blooms on the chocolate, meaning that such temperatures are not advisable for storing chocolate products, even over a short term.

Keywords: chocolate; storage temperature; blooming; colour; hardness

INTRODUCTION

In chocolate, durability is dependent on several parameters including storing temperature and humidity, availability of oxygen in the immediate surroundings, which is directly related to the use of packaging materials; it also relates to additions of other ingredients such as fats, nuts etc. (Nattress et al., 2004). Typically, there are two basic types of defects found on chocolate - fat bloom and sugar bloom, with the material losing its gloss and becoming covered with a fine whitish layer (Afoakwa, 2010).

Sugar bloom is a less frequent phenomenon. Often it is also confused with fat bloom. The difference can be seen under a microscope or, quite simply, when heating the chocolate to 38 °C. While fat bloom disappears at such temperature, sugar bloom remains visible. Sugar bloom occurs when the temperature over the surface of the chocolate product drops below the dew point (Čopíková, 1999). The causes for it are two: (1) storing the product at high humidity, or (2) rapid relocation of the product from a room with low temperature into one where temperature is high (Afoakwa, 2010). As a result, the formation of sugar bloom can be in particular avoided by proper storing settings and by elimination of potential

condensation of water vapour on the surface of the product (Rašper, 1963).

As regards the development of fat bloom, there are two key groups of theories: a polymorphic transformation and phase separation (McCarthy et al., 2003). The polymorphic transformation theory is based on the formation of fat bloom through the mechanism of transformation of thermodynamically unstable βV crystals of cocoa butter into polymorph modification VI (Beckett, 2008; Nöbel et al., 2009). During the durability, Form V is naturally transformed into a more stable Form VI, which leads to the formation of fat bloom, i.e., aging occur. The process is greatly influenced by storing temperature (Afoakwa et al., 2009; Beckett, 2008).

The theory of phase separation leading to the formation of fat bloom is based on triacylglycerols with different melting points found in fats used in chocolate products. Elevated temperatures may cause triacylglycerols with lower melting points to be pushed toward the surface and recrystallise (Bui and Coad, 2014). Particularly prone to this phenomenon is filled chocolate confectionery, where the filling contains a large amount of highly mobile triacylglycerols (TAGs). Fats from the filling migrate through the shell towards the surface, where they can

cause uncontrolled crystallisation, thus forming fat bloom. Other quality defects associated with the migration of fat are shell softening, filling hardening, and the overall sensory deterioration of the product (Svanberg et al., 2011).

Fat blooming occurs namely due to improper storage of chocolate. When optimally tempered products are stored at high temperatures or exposed to direct sunlight, the chocolate dissolves and during the recrystallisation, in the absence of inoculation ensuring a direct formation of the stable form V, the gradual transformation of an unstable form into a stable form leads to fat bloom to develop (Afoakwa, 2010).

Examples of fat that inhibits fat blooming include milk fat. Partial replacement of cocoa butter with milk fat has a beneficial effect on fat crystallisation in chocolate, the fact that it increases the resistance to blooming and significantly softens the texture by reducing the solid fat content, thus slowing down the rate of crystallisation, being the grounds for this (Sonwai and Rousseau, 2010). Resistance of milk chocolate to fat bloom is however only minor in the current production, as the level of milk fat and its inhibitory effect is negligible due to the use of skimmed milk powder (Bui and Coad, 2014).

The study aimed at evaluating changes in the quality of chocolate products over the period of storing them under four separate thermal regimens, thus assessing the influence and advisability of the storing temperatures for product storage.

MATERIAL AND METHODOLOGY

Materials

Seven products provided by Zora Olomouc were tested for the influence of storing temperature and the composition of chocolate mass of the product on the formation of fat/sugar bloom. The selection involved the following products:

- *Kaštany ledové* - a dark chocolate with a filling of cocoa and nuts (53%);
- *Milena* - a 50% milk chocolate with a 50% creamy filling containing a rum flavour;
- *Margot Artemis* - a double-layer soy-stick with a

37.5% coconut flavour and a 37.5% punch flavour, soaked in 25% milk chocolate;

- *Orion Krémová oříšková* - a milk chocolate with a hazel-nut filling;
- *BOCI fěkete erdö* - a dark chocolate with a cherry filling;
- *Black magic - Orange sensation* - a soft, tangy orange fondant draped in dark chocolate;
- *Orion Pistácie* - a milk chocolate with a 28% pistachio-nut and 36% hazel-nut filling, containing pieces of pistachio nuts (1.5%).

All the samples of chocolate products were produced using a standard process with the use of conventional chocolate mass tempering. *Orion Pistácie* was produced in two variants: (i) standard chocolate mass content (35%) and (ii) higher chocolate mass proportion (45%).

After the products were taken to stock, entrance analysis was carried out and a part of the samples deep frozen for later use as standards as part of sensory analysis. The remainder was split into controlled temperature regimens - warehouses cooled to 6 °C and 12 °C and a laboratory room with constant temperature of 20 °C and with the thermostat set to 30 °C; then the samples were stored under such settings without any fluctuations of temperature.

Sampling was carried out and evaluated five times. Each sampling was accompanied by sensory evaluation, determination of changes in colour using spectrophotometer in the visible region of the spectrum and hardness by texturometer. Before each analysing, a period of 24 hours was maintained to equilibrate all the samples to the lab ambient temperature.

Sensory evaluation

A sensory profile method was used for determining sensory traits of the chocolate products. Measuring perceptions for sub-descriptors used unstructured graphical scales with verbal description of the end-points with a length of 10 cm. To make mutual comparison possible as part of the respective descriptor, samples of each product from all the storing temperatures, including the standards, were administered at once. All of the sensory evaluation

Table 1 Color differentiation scale (Třešňák, 1999).

ΔE^*_{ab}	Description
0.0 – 0.2	Imperceptible
0.2 – 0.5	Minute
0.2 – 1.0	Perceptible
0.5 – 1.5	Slight
1.0 – 2.0	Recognizable
1.5 – 3.0	Clearly perceptible
2.0 – 4.0	Not yet discordant
3.0 – 6.0	Medium
4.0 – 8.0	Moderately discordant
Over 6.0	Prominent or moderately disturbing
12.0	Very prominent
16.0	Disturbing

sessions were underway in a dedicated lab under standard conditions, i.e. ISO 8586-1 (evaluators) and ISO 8589 (the premises) and the temperature of 20 °C.

The results of graphical scales were obtained by measuring the distance of the mark from the right scale end representing the lowest (0) product quality to the scale start placed to the left that represents the best (10) quality, and are graphically rendered in the form of radar charts as an average rating of all evaluators ($n = 8$). The sensory profiles of the products during the storing period clearly show the differences between each of the storing temperature regimens.

Colour measurement

Konica Minolta Spectrophotometer CM 3500d (KONICA, Japan) was used for determining the colour and its changes during the storing period. The modes selected for the colorimetric determination of colour in chocolate products using reflectance ($d/8$) were one involving gloss elimination (SCE - specular component excluded), D 65 (illumination mode: 6500 Kelvin) and 8 mm slot. Spectrophotometer measurements of colour present a welcome complement to sensory analysis. The CM-S100w program enables expression of colour in a colour space CIELAB (balls) according to the International Commission on Illumination. The values of L^* (lightness) represent the range from "0" (black) to "100" (white). The colour coordinates $+a^*$ to $-a^*$ (the axis from red to green), and $+b^*$ to $-b^*$ (the axis from yellow to blue) take positive or negative values depending on the location within the three-dimensional system (Třešňák, 1999).

Hardness measuring

Texture measurements were performed using a universal testing machine for the measurement of physical properties – TIRAtest 27025 (Germany). The penetration test was used for testing the chocolate products involving a probe of a stick shape penetrating into the sample, so obtaining a record of the force [FH] necessary to push the punch to the selected depth. The diameter of cylindrical probe was 3 mm; penetration speed was 100 mm.min⁻¹.

Statistical analysis of data

The acquired data were analysed using MS Excel. Statistical analysis was carried out for all the sourced data using STATISTICA (Edition 12) - ANOVA (analysis of variance with interactions, testing on the significance level of $p = 0.05$).

RESULTS AND DISCUSSION

Sensory evaluation

In sensory evaluation of chocolate, appearance and texture of the product are of particular importance (Neumann et al., 1990). Fat bloom on the surface of the chocolate has a negative impact on the appearance and overall acceptance of chocolate products (Bui and Coad, 2014).

The example of the sensory profile for *Milena* specifically shows a distinct deterioration of all the descriptors of the product when stored at 30 °C. The same results were achieved for *Margot Artemis*. The samples were sensorially unacceptable in terms of both appearance

and taste (Figure 1). There was overall hardening of the product, the filling dried up and manifest in loose and crumbly consistency. In *Kaštany*, on the contrary, this level of storage temperature resulted in considerable reduction of hardness and a loss of plasticity of the filling due to migration of fat components from the filling onto the product surface. In chocolate products, the typical deterioration associated with fat migration is manifest in softening, fat bloom and unacceptable textural changes within the product due to the liquid glycerides being released from the filling onto the product surface (Timms, 1984; Ziegleder, 1997).

Sensory evaluation of samples of chocolate sticks stored under the temperature regimens of both 6 °C and 12 °C showed that these appeared to be the most favourable settings for storage. Their quality almost copied the evaluation of the standards. The exception, however, was found for the *Milena* stick which was seen to develop sugar crystals in its filling. The defect was probably caused by the stiffening of incompletely dissolved sugar crystals in the water phase of the filling due to the samples being relocated into settings with a low temperature.

Storing temperature of 20 °C became improper particularly for *Margot Artemis* and *Kaštany* from the aspect of fat bloom development. The most significant blooming occurred on the surface of *Margot Artemis*, where fat bloom began to develop as early as a month of storing. Initially, the bloom formed along the sides of the product and around the cracks on the surface. By the end of the experiment, it had been present on as much as 50% of the product surface. Fat bloom also appeared inside the *Kaštany* products, more specifically on the filling where it accumulated mainly in the corners of the individual squares. According to Adenier et al. (1993), fat bloom initially accumulates at the edges of openings or along cracks on the surface layer of the chocolate. Briones and Aguilera (2005) report in their study that white spots appeared on the chocolate surface after 33 days of storing, probably due to the rapid migration of the liquid fat through defects or pores in the surface layer. Visually, the chocolate turned whitish almost throughout the surface at the end of the storing period.

In samples of chocolate sticks stored under such temperatures, deterioration generally occurred in more sensory descriptors, which namely involved gloss, scent, taste and overall acceptance. The results were also confirmed by Bui and Coad (2014) who report that as the percentage of fat bloom increases, all of the sensory attributes diminish. Increased rate of bloom therefore strongly correlates with a decrease in the visual appeal of the product. Similar conclusions were reached by Ali et al. (2001), who studied the effect of temperature (18 °C and 30 °C) during the storing period of two months in chocolates filled with dry coconut flesh. For samples stored at 18 °C no blooming was found, while as regards the second temperature regimen (30 °C) the onset of blooming was observed as early as one week of storing. The results of sensory evaluation also showed that the colour, texture and overall acceptance of samples stored at 30 °C were significantly ($p < 0.05$) lower compared with control samples and those stored at 18 °C.

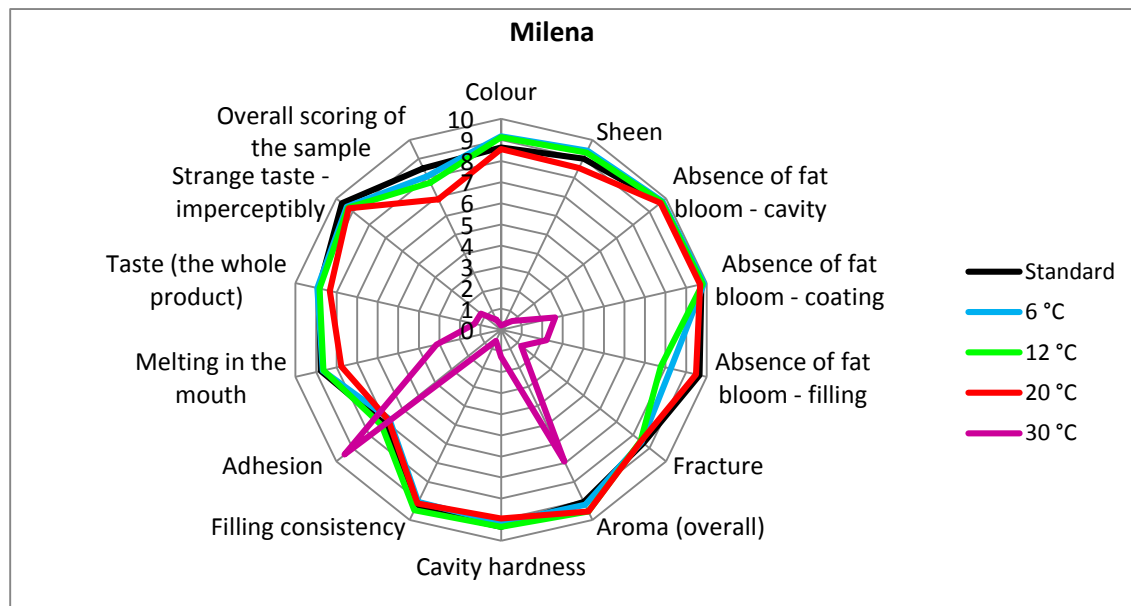


Figure 1 Sensory profile for *Milena* (sampling 5).

Evaluating the fondants showed during the storing period a significant influence of temperature regimes on changes of their sensory quality. The sensory profile produced (Figure 2) shows a considerable presence of sugar bloom for the samples stored at 30 °C. Crystals of sugar were present on both the cavity and the coating of the samples. The filling had dried up and there was overall hardening of the product due to water precipitated on the surface, which was additionally confirmed through subsequent analysis of texture.

Samples stored at temperatures up to 12 °C were found to have lost gloss, thus the overall acceptance of the product was impaired.

Conversely, the best results were reached in sensory evaluation for *Black magic - Orange sensation* fondants when stored at 20 °C. These samples were seen to have undergone minimal changes when gloss and colour was changed least of all the temperature regimens. The orange flavour and scent also unfolded in a pleasant way. Room temperature thus seems to be the most advisable way of storing these products.

For the *Krémová oříšková* chocolate bar, any greater difference or deficiency as regards the evaluated descriptors was not found between the storage regimens of 6 °C, 12 °C and 20 °C even week 18 of production date in (Figure 3). Comparable results were also observed in the remainder of chocolate bars.

The best resistance to changes even under high thermal stress in the settings with a storage temperature of 30 °C as found in the nut chocolate can be considered a positive trait. For other chocolate bars, *BOCI feketé erdő* and *Orion Pistácie*, there was overall softening, loss of fine consistency of the filling, deterioration of the look through the material becoming pale due to fat bloom, the presence of rancid off-taste, and naturally the final rating of the sample acceptance being impaired. **Nattress et al. (2004)** also reports that the storage period of dark chocolate

significantly affects bitterness, as well as the perception of sweet, sour, metallic, burnt and rancid taste. It is also a factor for hardness, viscosity and the onset of melting.

Research of **Jinap et al. (2000)** into fat bloom in chocolates stored at 18 °C, 30 °C and 35 °C for 8 weeks shows that no blooming was found during storage at 18 °C, while storing at 30 °C and 35 °C was seen to result in the development of fat bloom after week 4 and week 1, respectively.

Likewise, the results of the study by **Bui and Coad (2014)** in monitoring the sensory quality of milk chocolates during storage show that the quality of all the products stored at 35 °C significantly reduced compared with the 25 °C regimen. The quality was deteriorating exponentially with time. The highest sensitivity to deterioration was shown in the Appearance and Overall Acceptance descriptors.

Colour measurement

Analytical measurement of colour is widely used in studying surface changes of chocolate, which particularly applies to turning pale caused by fat blooming to evolve (**Kumar et al., 2003**).

Colour space CIE L*a*b* allows to identify, count, and measure objective variances between the different colours with relative ease. This difference, consisting of deviations ΔL^* , Δa^* , Δb^* , is best expressed by term ΔE^* , which is a square root of the sum of the individual deviations squared. A scale indicating the degree of disagreement between two colours was devised to facilitate the communication (Table 1).

Table 2a makes it evident that during the period of storing the *Kaštany* and *Margot Artemis* chocolate sticks there was a change in colour compared with control (K0). While the samples turned darker under the temperature regimens of 6 °C, 12 °C and 20 °C, the 30 °C mode caused them to turn paler to a significant extent.

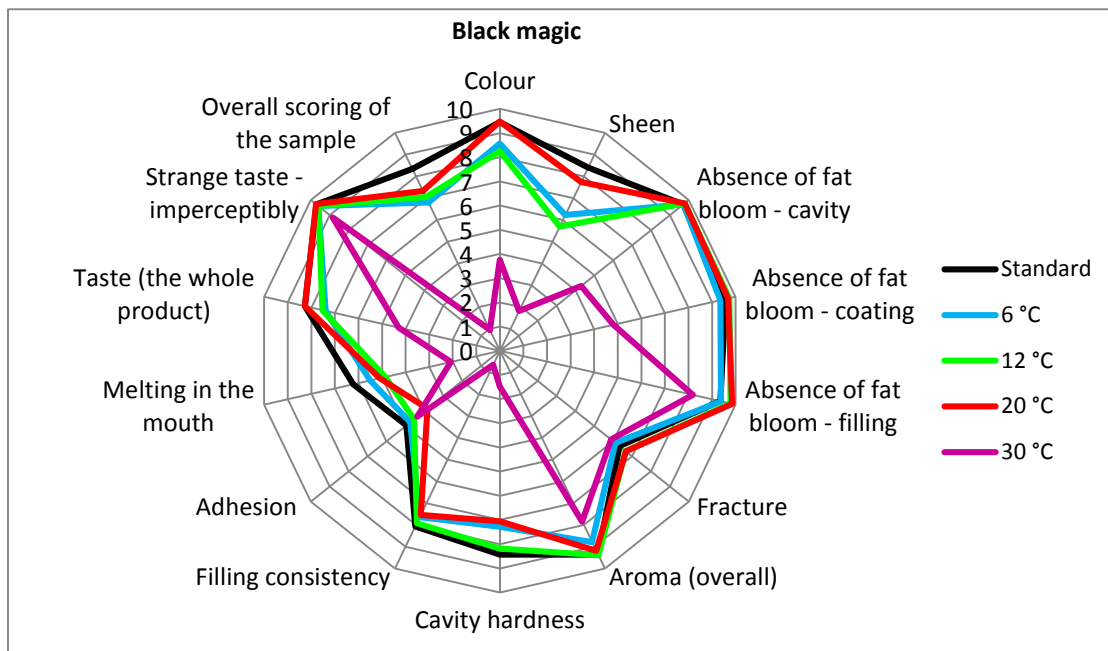


Figure 2 Sensory profile for *Black magic* - Orange sensation (sampling 5)

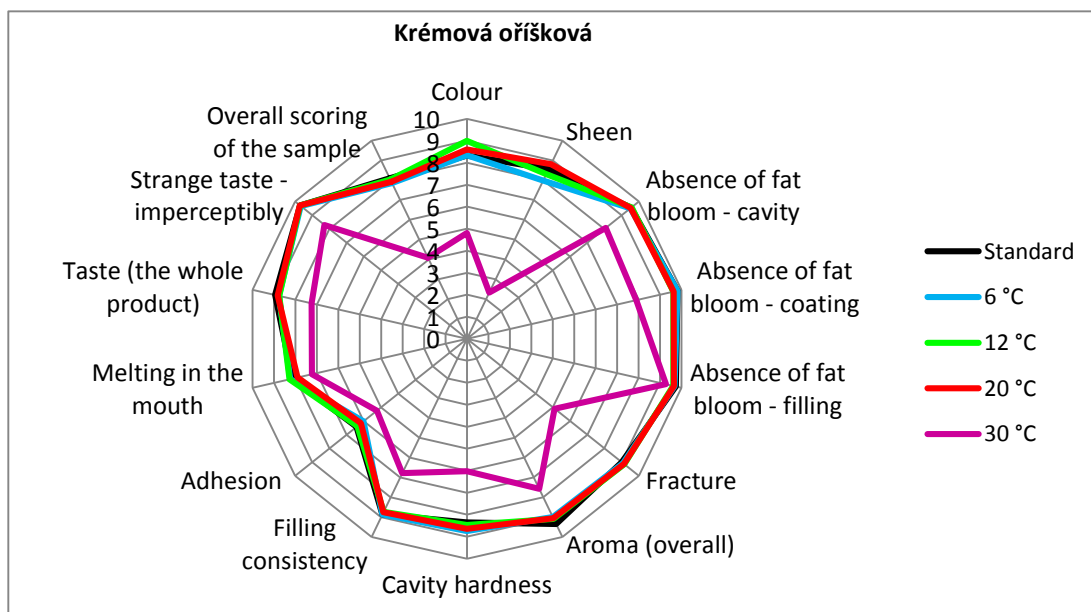


Figure 3 Sensory profile for *Krémová oříšková* (sampling 5)

The chocolate stick of *Milena* and the *Black magic* fondants were observed to significantly change colour under all of the storage regimens (Table 2b). In the event of *Milena*, similarly to the other sticks stored at temperatures of up to 20 °C, there was darkening of the samples, while they turned pale at 30 °C. Unlike control, *Black magic* fondants exhibited very visible lightening of the chocolate surface under all the storing regimens, which was probably caused by the development of sugar bloom.

Differences in colour as per temperature regimen were statistically significant ($p = 0.007$) even in chocolate bars. It results from Table 2c that *BOCI feketé erdő* samples were found to undergo the greatest extent of darkening

when stored at 12 °C. In all the cases, the storage regimen of 30 °C was significantly manifest in a negative increase in the product lightness. A similar trend was observed for pistachio chocolate. The best results were those achieved for *Krémová oříšková* čokoláda, a product which did not exhibit visible changes in colour during storage except the temperature regimen of 30 °C. The measurements confirm the results arising from sensory analysis.

Highly significant differences ($p = 0.001$) were found for all the values (L^* , a^* and b^*). The b^* values are characteristic of the coordinate on the axis from blue to yellow. The shift of values to yellow within the measurement characterises the development of fat bloom.

Table 2a Differences in colour of chocolate products.

Temperature [°C]	<i>Kaštany</i>					<i>Margot Artemis</i>				
	K0	6	12	20	30	K0	6	12	20	30
L*	29.48	28.41	28.56	28.61	31.62	36.03	34.45	34.60	34.86	40.53
a*	8.28	7.70	7.65	7.70	9.43	10.53	10.56	10.62	10.27	10.71
b*	8.17	7.13	7.08	7.16	10.39	13.06	12.44	12.55	12.21	15.18
ΔE*_{ab}	0	1.60	1.55	1.45	3.29	0	1.70	1.52	1.48	4.98

Table 2b Differences in colour of chocolate products.

Temperature [°C]	<i>Milena</i>					<i>Black magic</i>				
	K0	6	12	20	30	K0	6	12	20	30
L*	38.87	36.31	36.38	36.90	40.87	29.97	34.06	33.26	33.71	34.14
a*	10.13	10.17	10.07	9.96	10.21	8.23	8.16	8.18	8.10	8.15
b*	14.27	13.36	13.24	13.37	15.77	8.46	10.35	10.42	10.37	10.16
ΔE*_{ab}	0	2.72	2.69	2.17	2.51	0	4.51	3.83	4.21	4.51

Table 2c Differences in colour of chocolate products.

Temperature [°C]	<i>BOCI feketé erdő</i>					<i>Krémová oříšková</i>				
	K0	6	12	20	30	K0	6	12	20	30
L*	27.95	26.10	24.94	25.02	25.45	37.82	37.62	37.25	37.55	40.12
a*	6.70	6.68	6.63	6.70	7.97	10.65	10.63	10.55	10.49	10.18
b*	6.40	6.30	6.27	6.39	8.81	14.87	14.47	14.21	14.27	15.71
ΔE*_{ab}	0	1.86	3.02	2.93	3.70	0	0.45	0.88	0.68	2.49

Fat bloom induced by exposing the product to high temperatures causes chocolate to gradually change its colour change, lose gloss and appear greyish on the surface (Briones and Aguilera, 2005).

Changes in colour were noted between the experimental and control samples during storage at 30 °C even by Bui and Coad (2014) establishing that the storage period mostly influenced the L* value. For a* value no change was proved, thus no shift of values from red to green occurred. A positive change was observed for value b* in the yellow colour intensity, which made the products to become paler over time.

The same conclusion was reached in their study by Mexis et al. (2010) who report that significant changes in colour were observed as a result of fat bloom. If having a fat bloom cover, the chocolate product tends to disperse more light, so appearing to be paler and less rich-coloured (Afoakwa et al., 2008). Hartel (1999) reports that the

whitish haze in chocolate blooming is caused by light dispersion of fat crystals.

Hardness measuring

For consumers, appearance and hardness are the key attributes when choosing and deciding on the acceptability of chocolate, while taste is considered to be important when identifying the product. The final hardness of chocolate is influenced by several factors including formulas, production techniques, tempering, polymorphism (fat crystal stability) and the cooling temperature. Hardness of chocolate is a good indicator of proper temperature control and stability of the fat crystal network being formed (Afoakwa, 2010).

Differences between the storage temperature regimens were statistically significant ($p = 0.001$) for all the analysed products. Storing temperature thus significantly influences hardness of each product.

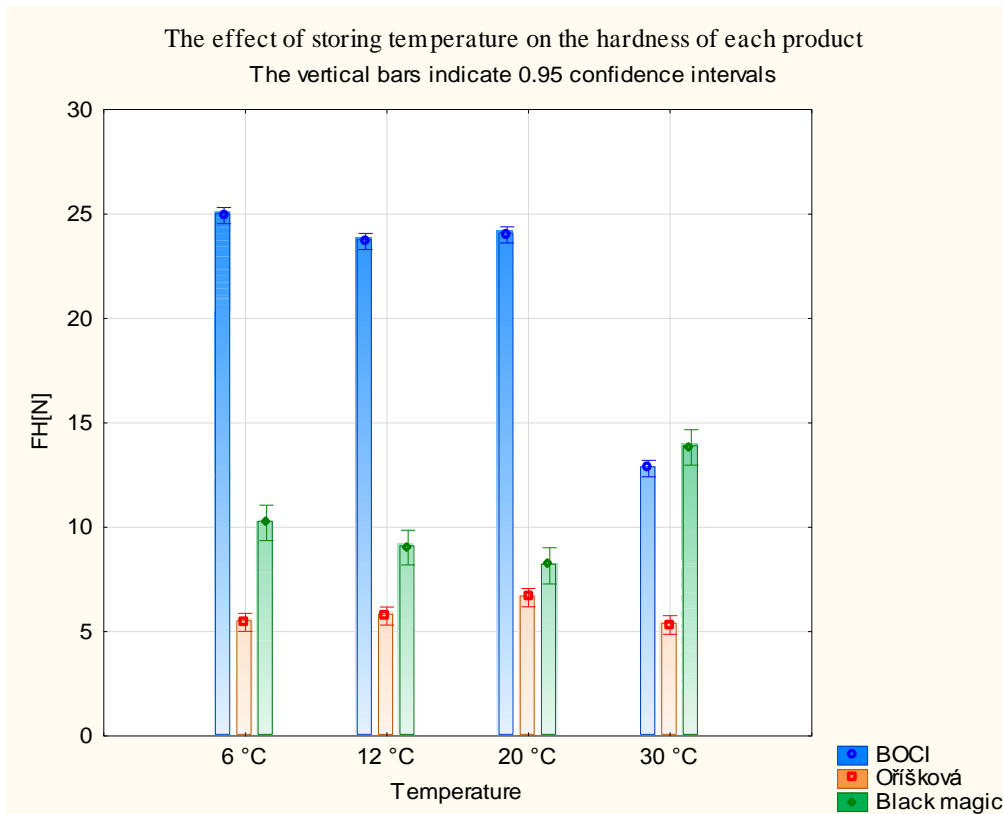


Figure 4 The effect of storing temperature on the hardness of each product.

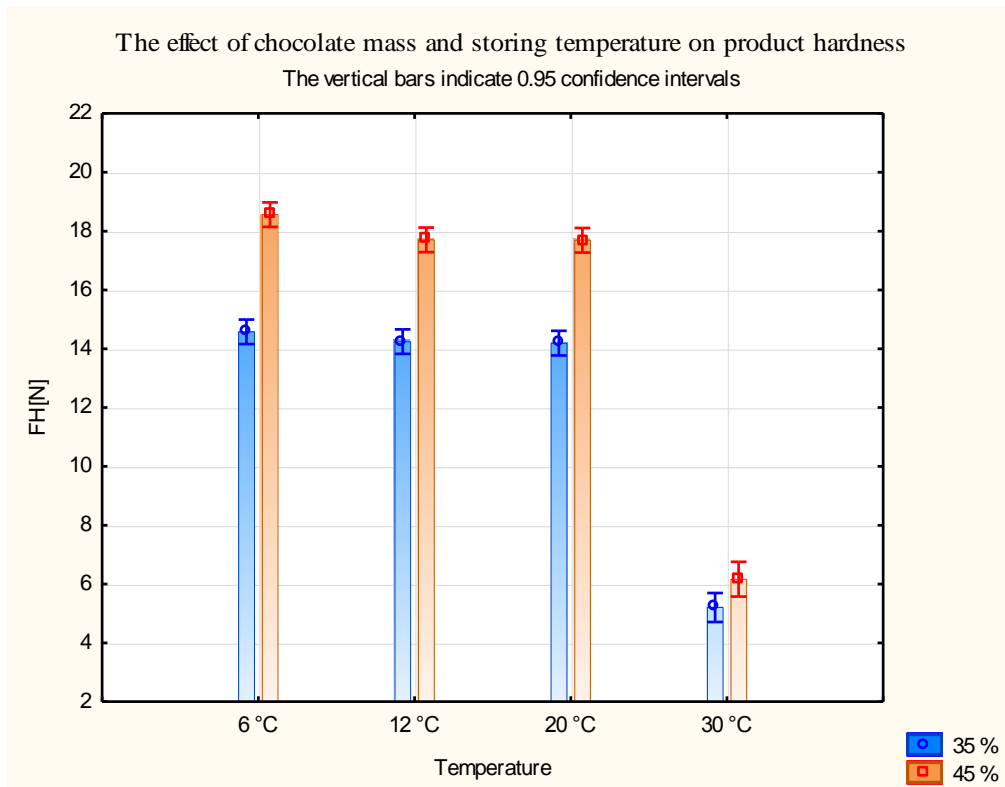


Figure 5 The effect of chocolate mass and storing temperature on product hardness.

The chart (Figure 4) makes it obvious that the highest level of hardness was found for *BOCI feketé erdő* when stored at 6 °C. This product also reflected the most the negative effect of temperature when stored at 30 °C with the samples reducing hardness by more than 10 N. We can assume that this case reflected the influence of the liquid filling on the overall texture of the product. Similar results were obtained for *Kaštany* which was probably due to a significantly different composition of the fat used in the filling where the rather high proportion of unsaturated fatty acids causes the mass melting point to decrease and the fat to migrate to the surface of the product, hence the subsequent softening.

In filled chocolate products where the filling contains lipids with low melting points, such lipids tend to migrate to the surface of the product over time, which with the highest probability initially involves lipids with the lowest melting point and the best fluidity. Such migration may cause the chocolate to become sticky and soft, while the filling becomes stiffer; the migration has even an impact on the structure of the surface (Ali et al., 2001; Ziegler, 1997). The results of Mexis et al. (2010) show as well that changes in texture accompanied by a change in colour caused by lightening through fat blooming were leading to the entire chocolate product becoming softened. All these attributes reduce the acceptability of the product for consumers. Fat migration can largely occur already at room temperature (17 °C – 23 °C) and accelerate as the temperature increases. Migration is reduced as the solid fraction of lipids increases (Wootton et al., 1970; Wacquez, 1975).

For fondant products, storing temperatures of 30 °C caused them to substantially increase in hardness compared with all other products. It is due to the fact that the filling is drying out and thereby hardening due to the composition of the filling.

The highest stability of texture was seen in the *Krémová oříšková* chocolate bar that retained its textural properties to the best extent even after 18 weeks of production under any storage regimen. It also proved the best resistance to extreme storage conditions at 30 °C. In *Milena* and *Margot Artemis*, texture was not very significantly influenced by storage temperature with differences being only units of Newtons.

Since storing temperatures of 6, 12 and 20 °C did not considerably affect hardness of each product, no distinct changes in hardness occurred under such temperature regimens. The study of Ali et al. (2001) also makes it possible to state that migration of fats with lower melting points was very slow for storage under 18 °C and the changes were minimal as regards chemical composition, hardness, gloss and polymorphic stability.

The experiment included the effect of the formula on the quality and shelf life of chocolate products being evaluated. Evaluating hardness for *Orion Pistácie* (Figure 5) makes it evident that the variants produced with use of a higher proportion of chocolate mass (45%) achieve higher hardness during storage. While softening is almost impossible to notice for the temperature regimens of up to 20 °C, for 30 °C it is significantly more intense. Yet the beneficial effect of composition of the chocolate mass used can be observed for this case as well.

CONCLUSION

The results of sensory evaluation of selected products shows that the highest quality for the majority of descriptors was achieved by products stored at temperatures of 6 °C and 12 °C. The properties of these, both visual and gustatory, were comparable with those of standards and achieving similarly high scores. As regards samples stored under the temperature regimen of 20 °C, the products started to show visible differences, caused primarily by the formation of fat bloom while storing at 30 °C proved to be extremely unsuitable for all the tested products.

From the aspect of analytical measurements of colour and hardness of each product, storing at temperatures of 20 °C can be termed appropriate. In all the analyses, the effect of the temperature regimen of 30 °C was significantly negative due to defects caused by blooms on the chocolate, meaning that such temperatures are not advisable for storing chocolate products, even over a short term.

When evaluating the effect of the composition of chocolate mass for the *Orion Pistácie* products, the higher proportion of chocolate mass was reflected mainly in the hardness. Unlike the standard production, products with the formula containing 45% chocolate mass featured a significant, 35% increase in hardness under all storage regimens.

The best results were achieved by *Krémová oříšková* in all the analysing operations, a product that was not found to have any significant shortcomings even after 18 weeks of storage. This product also received good rating even for the storage regimen with a high thermal stress.

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OXIDATIVE STABILITY OF CHICKEN MEAT AFTER PROPOLIS EXTRACT APPLICATION IN THEIR DIETS

Marek Bobko, Miroslav Kročko, Peter Haščík, Alica Bobková

ABSTRACT

In the experiment, the effect of the addition of propolis extract in a feed mixture for chicken broilers Hubbard JV on oxidative stability of breast and thigh muscles during refrigerated storage was investigated. In the experiment were included 90 pieces of one day-old chicks, which were divided into 3 groups (control, E1 and E2). Chickens were fed by *ad libitum* system until the age of 42 days. These feed mixtures were made without antibiotics preparation and coccidiostats. Propolis extract in an amount of 150 mg.kg⁻¹ (E1) and 450 mg.kg⁻¹ (E2) was added into feed mixtures for experimental groups. During whole period of refrigerated storage were higher values of MDA determined in control group compare to experimental groups. The higher average MDA value determined in breast muscles of broiler chicken hybrid combination Hubbard JV was in samples of control group (0.157 mg.kg⁻¹) compared to experimental groups E1 (0.140 mg.kg⁻¹) and E2 (0.130 mg.kg⁻¹) after 6-month of refrigerated storage. Significantly higher values of MDA were determined in control group compare to second experimental group from fourth month to the end of storage. The significantly lower value of MDA was determined in first experimental group compare to control only at 6 month of storage. Trend of thigh muscle oxidation stability of chicken hybrid combination Hubbard JV was during 6 months of refrigerated storage similar than in breast muscle. The higher average MDA value determined in thigh muscles was in samples of control group (0.170 mg.kg⁻¹) compared to experimental groups E1 (0.150 mg.kg⁻¹) and E2 (0.139 mg.kg⁻¹) after 6-month of refrigerated storage. Significantly higher values of MDA were determined in control group compare to second experimental group from fourth month to the end of storage. Higher amount of MDA in thigh muscle compare to breast muscle is due to by higher amount of fat occurred in thigh muscle.

Keywords: oxidative stability; meat; broiler chicken; propolis.

INTRODUCTION

Lipids play important role in technological, nutritional and sensory function of food. However they are liable to undergo autooxidation that leads to the formation of a number of undesirable compounds. In an effort to retard this process, various antioxidants are employed. The application of synthetic antioxidants has recently been restricted because there is suspicion that they are carcinogenic. For this reason a growing interest has been paid to the research of natural antioxidants, among which spices occupy an important position (Pokorný et al., 2001).

Lipid oxidation is a major cause of meat quality deterioration. Lipid oxidation is an important determinant of shelf life of meat and meat products. Antioxidants are natural or synthetic substances used to prevent lipid oxidation. Meat protection, primarily against lipid components, is possible by addition of antioxidants to feed mixes. This is the way to ensure oxidative stability of meat fats during the postslaughter processing of carcasses and storage of meat (Marcinčák et al., 2005). Many researchers have indicated that lipid oxidation in meat and meat products can be controlled or minimized, by the

addition of commercial synthetic or natural antioxidants (Gray et al., 1996; Kazimierczak et al., 2008; Haščík et al., 2012; Elimam et al., 2013; Kročko et al., 2014).

The *post mortem* oxidation of lipids decreases the nutritional value and the sensory quality of meat. It has been shown, in chickens, that dietary fat sources have major impact on the composition and the melting point of fat in tissues (Hrdinka et al., 1996).

Propolis is a resinous, rubbery and balsamic substance collected by honey bees from the buds of flowers, trees and other plant sources. Propolis contains resins, aromatic and ethereal oils, flavonoid pigment, vanillin, isovanillin, caffeic, benzoic and ascorbic acids as well as benzyl alcohol and cinnamic acid (Harman 1983; Greenaway et al., 1990; Said et al., 2006). These components possess antimicrobial, antifungal and antioxidant properties (Ashour, 1989; Hegazi, El-Hady, 2002; Lu et al., 2005; Trusheva et al., 2005). The composition of propolis depends on the vegetation at the site of collection; more than 180 compounds, mainly polyphenols, have been identified as constituents of propolis; the major polyphenols are flavonoids, accompanied by phenolic acid

and esters, phenolic aldehydes, ketones and others (Castaldo and Capasso, 2002).

Certainly, it is possible to state, that plant extract, propolis and the other natural supplements are considered as an alternative to the antibiotic and they have wide range of possible uses; consequently, influence of these products on human and animal health is currently evaluated and determined with regard to growth of organic farming (Tekeli et al., 2011). For this reason, the present study was aimed to investigate the effect of propolis extract Slovak multifloral addition to feed mixtures for oxidative stability of meat in the process of installation of Hubbard JV chickens.

MATERIAL AND METHODOLOGY

The experiment was realized at the test station of poultry (Slovak Agricultural University in Nitra). The experiment enrolled 90 one day old chicks of hybrid combination Hubbard JV and was formed into 3 groups: control group (C) and two experimental groups (E1, E2) of 30 pcs chickens in each group. Custom feeding insisted 42 days. Chickens were fed to 21th day of age an *ad libitum* with the same starter feed mixture HYD-01 (powdery form) and

from 22nd to 42th day of age fed with the growth feed mixture HYD-02 (powdery form). The feed mixture HYD-01 and HYD-02 have been produced without antibiotic preparations and coccidiostats. Nutritional value of feed mixtures (Table 1) given during the experiment was the same in each group, but to the experimental groups were added propolis extracts at a dose of 150 (E1) and 450 mg.kg⁻¹ (E2). Propolis extract was prepared from minced propolis (Krell, 1996). Weighed 150 g propolis was the volume of 80% ethanol, 500 cm³.

Extraction was carried out in a water bath at 80 °C under reflux for 60 minutes. After cooling was extract centrifuged. The supernatant was evaporated on a rotary vacuum evaporator at a water bath at temperature of 40 – 50 °C and then weighed. Residue in an amount of 15 and 45 g was dissolved in 1000 cm³ of ethanol concentration of 80% and applied to 100 kg of the feed mixture.

At the end of feeding (day 42th) from each group were selected 10 pieces of chicken for slaughter analysis. To determine changes in lipid degradation (determination of thiobarbiturates numbers, TBA) the samples of chickens were boned and thigh and breast muscle packed into polyethylene bags and stored for 6 months at -18 °C.

Table 1 Composition of the diets.

Ingredients (%)	Starter (1 to 21 days of age)	Grower (22 to 42 days of age)
Wheat	34.00	37.00
Maize	33.94	37.57
Soybean meal	23.00	18.00
Fish meal (71% N)	5.00	3.00
Dried blood	–	1.00
Ground limestone	1.00	0.95
Monocalcium phosphate	0.80	0.70
Fodder salt	0.10	0.10
Sodium bicarbonate	0.15	0.20
Lysin	0.13	0.08
Methionin	0.18	0.20
Palm kernel oil Bergafat	1.20	0.70
Premix Euromix BR 0.5% ¹	0.50	0.50
Analysed composition (g.kg⁻¹)		
Crude protein	212.40	191.61
Fibre	30.51	29.68
Ash	27.01	20.91
Ca	8.23	7.18
P	6.56	5.87
Mg	1.41	1.36
Linoleic acid	13.53	14.06
ME _N (MJ.kg ⁻¹)	12.07	12.16

¹active substances per kilogram of premix: vitamin A 2 500 000 IU; vitamin E 50 000 mg; vitamin D3 800 000 IU; niacin 12 000 mg; d-pantothenic acid 3 000 mg; riboflavin 1 800 mg; pyridoxine 1 200 mg; thiamine 600 mg; menadione 800 mg; ascorbic acid 50 000 mg; folic acid 400 mg; biotin 40 mg; vitamin B12 10.0 mg; choline 100 000 mg; betaine 50 000 mg; Mn 20 000 mg; Zn 16 000 mg; Fe 14 000 mg; Cu 2 400 mg; Co 80 mg; I 200 mg; Se 50 mg

TBA value expressed in number of malondialdehyde were measured in the process of first storage day of 1st, 2nd, 3rd, 4th, 5th and 6th months. TBA number was determined by **Marcinčák et al. (2004)**. Absorbance of samples was measured on UV-VIS spectrophotometer T80 (PG Limited Instruments, UK) at a wavelength of 532 nm, the translation results on the amount of malondialdehyde (MDA) in 1 kg samples.

Results of the experiment was evaluated with statistical program Statgraphics Plus version 5.1 (AV Trading Umex, Dresden, Germany), were calculated variation-statistical values (mean, standard deviation) and to determine the significant difference between groups was used variance analyze with subsequent Scheffe test.

RESULTS AND DISCUSSION

The results of the oxidation stability determined in breast and thigh muscle of chickens Hubbard JV during 6 months storage at -18 °C are shown in Table 2. Our results are in accordance with **Marcinčák et al. (2010)** who, after slaughtering and processing of poultry samples also recorded low values of MDA. During refrigerated storage of the breast and thigh muscles (6 months) were detected increased content of MDA in comparison to the first day of storage. During whole period of refrigerated storage were higher values of MDA determined in control group compare to experimental groups. The higher average MDA value determined in breast muscles of broiler chicken hybrid combination Hubbard JV was in samples of control group (0.157 mg.kg⁻¹) compared to experimental groups E1 (0.140 mg.kg⁻¹) and E2 (0.130 mg.kg⁻¹) after 6-month of refrigerated storage. Significantly higher values of MDA were determined in control group compare to second

experimental group from fourth month to the end of storage. The significantly lower value of MDA was determined in first experimental group compare to control only at 6 month of storage.

Trend of thigh muscle oxidation stability of chicken hybrid combination Hubbard JV was during 6 months of refrigerated storage similar than in breast muscle. The higher average MDA value determined in thigh muscles was in samples of control group (0.170 mg.kg⁻¹) compared to experimental groups E1 (0.150 mg.kg⁻¹) and E2 (0.139 mg.kg⁻¹) after 6-month of refrigerated storage. Significantly higher values of MDA were determined in control group compare to second experimental group from fourth month to the end of storage. Higher amount of MDA in thigh muscle compare to breast muscle is due to by higher amount of fat occurred in thigh muscle **Botsoglou et al. (2002)**.

Reached results of oxidation stability determined in chicken meat of hybrid combination Hubbard JV after propolis extract addition in their diet are in accordance with **Young et al. (2003)**, **Onibi and Osho (2007)**, **Imik et al. (2010)** and **Rahimi et al. (2011)**. The possibilities of using alternative feed supplements containing various antioxidant active substances for poultry which increase the oxidation stability of the meat during its period of freeze storage are shown in works of **Alcicek et al. (2003)**, **Šperňáková et al. (2007)**, **Mikulski et al. (2009)**, **Ahadi et al. (2010)**, **Marcinčák et al. (2010)**, **Skřivan et al. (2010)**, **Karaalp and Genc (2013)**.

Also **Samouru et al. (2007)** and **Ramos Avila et al. (2013)** state that the degradation pathways of fatty substances play one of the main causes of foods deterioration and unpleasant odors. This factor is also

Table 2 Effect of storage in freeze (-18 °C) on the concentration of malondialdehyde (mg.kg⁻¹) in breast and thigh muscle (mean ±SD).

Time of storage	Group		
	Control	E1	E2
Breast muscle			
Day - 1	0.106 ±0.014 ^a	0.096 ±0.011 ^a	0.099 ±0.014 ^a
Month - 1	0.123 ±0.012 ^a	0.117 ±0.013 ^a	0.111 ±0.005 ^a
Month - 2	0.130 ±0.013 ^a	0.120 ±0.017 ^a	0.111 ±0.014 ^a
Month - 3	0.139 ±0.020 ^a	0.126 ±0.015 ^a	0.121 ±0.016 ^a
Month - 4	0.145 ±0.007 ^a	0.130 ±0.020 ^{ab}	0.119 ±0.010 ^b
Month - 5	0.150 ±0.007 ^a	0.133 ±0.016 ^{ab}	0.126 ±0.015 ^b
Month - 6	0.157 ±0.004 ^a	0.140 ±0.011 ^b	0.130 ±0.015 ^b
Thigh muscle			
Day - 1	0.128 ±0.015 ^a	0.112 ±0.018 ^a	0.109 ±0.015 ^a
Month - 1	0.136 ±0.070 ^a	0.125 ±0.018 ^a	0.120 ±0.013 ^a
Month - 2	0.144 ±0.015 ^a	0.135 ±0.006 ^a	0.127 ±0.016 ^a
Month - 3	0.150 ±0.011 ^a	0.139 ±0.023 ^a	0.128 ±0.015 ^a
Month - 4	0.157 ±0.008 ^a	0.142 ±0.014 ^{ab}	0.134 ±0.011 ^b
Month - 5	0.163 ±0.011 ^a	0.146 ±0.010 ^{ab}	0.135 ±0.014 ^b
Month - 6	0.170 ±0.018 ^a	0.150 ±0.014 ^{ab}	0.139 ±0.013 ^b

responsible for the loss of flavor, texture, appearance, nutritional value of food, increases the drop losses, pigment, polyunsaturated fatty acids, fat-soluble vitamins, reduces the quality of meat intended for human consumption and ultimately reduces its stability, shelf life and safety.

Botsoglou et al. (2007) reported that a higher concentration of antioxidants in poultry meat has the effect of reducing lipid oxidation, ie. there is a reduction in MDA values during chilling and refrigeration storage, which was confirmed by our findings.

CONCLUSION

Results achieved in the experiment show that the addition of propolis extract in feed mixture for broiler chickens had a significantly ($p \leq 0.05$) positive impact on the reduction of oxidative processes in the breast and thigh muscles during refrigerated storage.

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THE CHEMICAL COMPOSITION OF GRAPE FIBRE

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ABSTRACT

Dietary fibres from cereals are much more used than dietary fibres from fruits; however, dietary fibres from fruits have better quality. In recent years, for economic and environmental reasons, there has been a growing pressure to recover and exploit food wastes. Grape fibre is used to fortify baked goods, because the fibre can lower blood sugar, cut cholesterol and may even prevent colon cancer. Grape pomace is a functional ingredient in bakery goods to increase total phenolic content and dietary fibre in nourishment. The aim of this study was to determine the chemical composition of commercial fibres, obtained from different Grape sources concerning their chemical properties such as moisture, ash, fat, protein, total dietary fibre. The chemical composition of Grape fibre is known to vary depending on the Grape cultivar, growth climates, and processing conditions. The obliged characteristics of the fibre product are: total dietary fibre content above 50%, moisture lower than 9%, low content of lipids, a low energy value and neutral flavour and taste. Grape pomace represents a rich source of various high-value products such as ethanol, tartrates and malates, citric acid, Grape seed oil, hydrocolloids and dietary fibre. Used commercial Grape fibres have as a main characteristic, the high content of total dietary fibre. Amount of total dietary fibre depends on the variety of Grapes. Total dietary fibre content (TDF) in our samples of Grape fibre varied from 56.8% to 83.6%. There were also determined low contents of moisture (below 9%). In the samples of Grape fibre were determined higher amount of protein (8.6 – 10.8%), mineral (1.3 – 3.8%) and fat (2.8 – 8.6%). This fact opens the possibility of using both initial by-products as ingredients in the food industry, due to the effects associated with the high total dietary fibre content.

Keywords: Grape fibre; dietary fibre; chemical composition; total dietary fibre; Grape pomace

INTRODUCTION

Consumers prefer ready to eat foods and a diet that is low in calories, low in cholesterol, and low in fat or in other words “healthy foods”. In accordance with this trend, consumers also want to eat foods with higher fibre content (Ayadi, et al., 2009). Epidemiological studies suggest that fibre consumption helps to reduce obesity, some kinds of cancer, cardiovascular diseases, and gastrointestinal diseases (Gómez et al., 2009).

Dietary fibre (DF) is a group of food components, which are resistant to hydrolysis by human digestive enzymes. Dietary fibre consists of polysaccharides, oligosaccharides and lignin (Samappito and Trachoo, 2011).

Fibre is often classified as soluble dietary fibre (SDF) and insoluble dietary fibre (IDF). Because solubility refers simply to fibres that are dispersible in water, the term is somewhat inaccurate (Figuerola et al., 2005). The physiological effects of total dietary fibre (TDF), in the forms of insoluble and soluble fractions of foods, have a significant role in human nutrition (Ramulu and Udayasekhararao 2003). Among good sources of fibre cereal grains, legumes, fruits, vegetables, nuts and seeds are of importance (Lopez et. al., 2011). Dietary fibres from cereals are more frequently used than those from fruits; however, fruit fibres have better quality (Figuerola et al., 2005). In recent decades, for economic as well as environmental reasons, there has been a continuous and

growing pressure to recover and exploit food wastes (Garau et al., 2007).

However, a large number of recent studies have suggested that fruit and vegetable by-products obtained from the processing of apples, citrus fruits, mangoes, potatoes, carrots, asparagus, and brassica vegetables among others, could be used as potential sources of DF (Centento et. al., 2010).

Dietary fibre holds all the characteristics required to be considered as an important ingredient in the formulation of functional foods, due to its beneficial health effects (Dhingra et al., 2011). Dietary fibre can also impart some functional properties to foods, e.g., increase water holding capacity, oil holding capacity, emulsification and/or gel formation. That dietary fibre incorporated into food products (bakery products, dairy, jams, meats, soups) can modify textural properties, avoid syneresis (the separation of liquid from a gel caused by contraction), stabilise high fat food and emulsions, and improve shelf-life. Fibre in foods can change their consistency, texture, rheological behavior and sensory characteristic of the end products, the emergence of novel sources of fibres, have been offering new opportunities in their use in food industry (Elleuch et al., 2011; Guillon and Champ 2000). Nowadays, there is a growing interest in the exploitation of the residues generated by the wine industry. Grape (*Vitis sp.*, Vitaceae) is one of the world's largest fruit crops, with an approximate annual production of 58 million metric

tons. The Grape belongs to the berry family as it is found attached to the stem. Grapes can be eaten raw or they can be used for making wine, jam, juice, jelly, Grape seed extract, raisins, vinegar, and Grape seed oil (Zhu et al., 2014).

The residues of Grape are composed of water, proteins, lipids, carbohydrates, vitamins, minerals, and compounds with important biological properties such as phenolic compounds (tannins, phenolic acids, anthocyanins, and resveratrol), depending on the type of waste, the cultivar and climatic and cultivation conditions (Sousa, et. al., 2014). About 80% of the total crop is used in wine making (Llobera and Cañellas, 2007; Schieber et al., 2001).

Grape pomace represents a rich source of various high-value products such as ethanol, tartrates and malates, citric acid, Grape seed oil, hydrocolloids and dietary fibre. Grape pomace consists mainly of peels (skins), seeds and stems and accounts for about 20 – 25% of the weight of the Grape crushed for wine production (Arvanitoyannis et al., 2006; Yu and Ahmedna, 2013).

Grape pomace is a rich source of polyphenols. These include catechins, namely monomeric and oligomeric flavan-3-ols (proanthocyanidins) and glycosylated flavonols. Catechins, together with other polyphenols, are potent free radical-scavengers. Thus, different epidemiological studies have demonstrated the association between a diet rich in polyphenols and the decrease in the risk of suffering cardiovascular diseases and certain types of cancer (Llobera and Cañellas, 2007).

This study is aimed to characterize commercial Grape fibres in terms of their chemical properties (moisture, ash, fat, protein, total dietary fibre) and feasibility of using in food industries improving the nutritional value of food products.

MATERIAL AND METHODOLOGY

Wheat flour was obtained from the K. K. V. – UNION, Ltd., Slovak Mills Company. Various kinds of commercial Grape fibres were purchased from market in Slovakia and their country of origin is Germany (A), Hungary (B) and Austria (C).

Chemical analysis: Moisture and ash content were

determined according to AOAC methods (1984) and Sowbhagya et al. (2007).

Lipids were determined gravimetrically by extraction with diethyl ether using a Soxhlet apparatus. Nitrogen content was estimated by Kjeldhal method and was converted to protein using a factor 6.25 (AACC methods, 2000; Ayadi et al., 2009). Total dietary fibre (TDF) content of the Grape fibre was determined with using of the Megazyme International total DF assay (adopted from AACC method 32–05 and AOAC method 985.29) (Sun-Waterhouse et al., 2010).

The measurement of pH was determined according to Kohajdová and Karovičová (2007). Caloric carbohydrates were determined by difference from the total dietary fibre, lipids, protein and ash contents (Chau and Huang, 2003). The total energy was calculated based on the energy nutrient results obtained using the conversion factors of Atwater, as described by Sousa, et al., (2014) considering 4 kcal/g for carbohydrate, 4 kcal/g for protein, and 9 kcal/g for lipids.

RESULTS AND DISCUSSION

The chemical composition of Grape fibre is known to vary depending on the Grape cultivar, growth climates, and processing conditions (Deng et al., 2011). The main characteristics of the commercialized fibre product are: total dietary fibre content above 50%, moisture lower than 9%, low content of lipids, a low caloric value and neutral flavour and taste (Larrauri, 1999). The chemical composition of Grape pomace major constituents peels and seeds, has been reported by authors, with as dietary fibre (DF) contents (Bravo and Saura-Calixto, 1998; Valiente et al., 1995; Yu and Ahmedna, 2013; Centento et. al., 2010; Deng et al., 2011).

The results of chemical composition of various kinds of commercial Grape fibres and fine wheat flour used in the study are presented in the Table 1. The commercial Grape fibre had low moisture content. Moisture content was below 9.0% pointed out by Larrauri (1999) as the upper limit for their handling and conservation. Grape fibres are not considered as an important protein source, although Grape fibre contains 9 – 13% proteins.

Table 1 Chemical composition of various kinds of commercial Grape fibres and fine wheat flour

	Fine wheat flour	Grape dietary fibre A	Grape dietary fibre B	Grape dietary fibre C
Moisture (%)	11.4 ±0.01	7.7 ±0.04	6.1 ±0.05	8.6 ±0.38
Ash (%)	0.6 ±0.00	1.3 ±0.04	2.9±0.01	3.8 ±0.04
Protein (%)	10.5 ±0.15	8.6 ±0.17	10.4 ±0.07	9.9 ±0.00
Fat (%)	1.1 ±0.02	2.8 ±0.13	5.1 ±0.03	8.6 ±0.00
pH	6.3 ±0.00	3.7 ±0.00	3.9 ±0.01	3.8 ±0.00
TDF (%)	2.3 ±0.04	83.6 ±0.67	73.2 ±0.27	56.9 ±0.08
Carbohydrate (%)	348.8	15.9	7.1	49.1
Energy value kJ/kg	3106.8	3148.6	4048.8	8135.3

TDF – Total dietary fiber

The total protein content and the amino acid composition of Grape fibre protein may vary significantly depending on the variety of Grape, location and fertilization conditions (Sousa, et al., 2014).

Protein content was also high, up to 10.5% and similar to the values reported for red Grape pomace (9%) (Rodriguez et al., 2012), white deseeded Grape pomace (11%) (Valiente et al., 1995). Higher protein values were found in the Grape skins and seeds (12%) (Bravo and Saura-Calixto, 1998). Ash content are lower (1.3 – 3.8%) than other values described for similar by-products obtained from white and red Grapes (5.7 – 9.2%) (Bravo and Saura-Calixto, 1998; Valiente et al., 1995). Similar values were found in the fresh red and white varieties (pomace and stems) (0.9 – 3.0%) (González-Centeno et al., 2010). Amount of total dietary fibre also depends on the variety of Grapes. The commercial Grape fibre B was characterized by a TDF content of 73.2%, similar contents were described with (Pérez-Jiménez et al., 2008) in the red Grape skin (73.5%) and (Llobera and Cañellas 2007) in the red Grape pomace (74.5%). Similar content total dietary fibre for sample Grape fibre C were found for red Grape skins (54%), white Grape skins (59%), white Grape seeds (56%) (Bravo and Saura-Calixto, 1998) and white deseeded Grape pomace (62%) (Valiente et al., 1995). Total dietary fibre for commercial Grape fibre A was 83.6%, this value is larger than the value determined by (Llobera and Cañellas, 2007) in the red Grape stem (77.2%). No previous studies on Grape fibre have been found in the literature to allow us to compare the high value of 83.6 of TDF obtained in our commercial Grape fibre A.

The fat content of Grape fibre depending on the variety and maturity of Grapes (Llobera and Cañellas, 2007). Fat content of the Grapes fibre were between 5.1% in Grape fibre B and 8.6% in Grape fibre C. These values are in agreement with Pérez-Jiménez et al., (2008) (7.7%), in Grape skins (6.9 – 7.8%) (Bravo and Saura-Calixto, 1998), commercial Grape fibre (6.9%) (Saura-Calixto, 1998). The lipids of the Grapes are mainly concentrated in its seeds and consist of about 90% monounsaturated fatty acids, known for their beneficial properties, particularly to the cardiovascular system (Rockenbach et al., 2011). The pH values of the Grapes fibre were (3.7 – 3.9), which led to greater stability hampering the development microorganisms because fungi generally prefer acidic pH (4.5 – 5.0) and bacteria prefer near neutral pH (6.5 -7.0) (Sousa, et al., 2014).

According to Larrauri (1999), an adequate fibre concentrate should have an energy value below than 8370 kJ/kg limit which is met by in all of various kinds of commercial Grape fibres studied.

CONCLUSION

The results obtained in this study suggest the possibility of using the most important by-products related to the winemaking process (Grape pomaces and stems) as potential sources of DF of good quality.

The commercial Grape fibres have as a main characteristic, the high content of total dietary fibre (TDF). Grape fibre is an excellent source of protein (8.6 – 10.8 %), mineral (1.3 – 3.8%), mostly fat

(2.8 – 8.6%). This fact opens the possibility of using both initial by products as ingredients in the food industry, due to the effects associated with the high TDF content.

Owing to the large quantity generated from worldwide wine and Grape juice production every year. Grape fibre has potential to serve as an important source of dietary fibre for functional food development. Dietary fibre and bioactive compounds are widely used as functional ingredients in processed foods. The market in this field is competitive and the development of new types of quality ingredients is a challenge for the food industry. In this regard, it is interesting to consider not only the nutritional quality of the ingredient, but also its distribution, cost and other additional benefits, since the use of these ingredients would give added value to the production of these materials.

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THE INFLUENCE OF *LACTOBACILLUS PARACASEI* LPC-37 ON SELECTED PROPERTIES OF FERMENTED SAUSAGES

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ABSTRACT

Fermented sausages rank among non-heat-treated meat products. Their nutritional properties are similar to the raw material, simultaneously their microbial safety and stability is ensured using additives and specific microbial cultures. The use of probiotic cultures can positively affect the processing of fermented sausages, resulting in the new technological properties and beneficial effect on human health. However, commercial application of probiotic microorganisms in fermented sausages is not common yet. *Lactobacillus paracasei* LPC-37 is a gram-positive, non-spore forming, homofermentative rod, which according to studies may modulate immune responses in human organism and survives the passage through the gastrointestinal tract. The main object of this work was to evaluate technological properties of *L. paracasei* LPC-37, which have not been fully examined. Two groups of fermented sausages were analysed in this work. The first group of fermented sausages was prepared using lyophilized starter culture (Lyocarni RHM-33). The second group of fermented sausages was prepared by the combination of lyophilized starter culture and potential probiotic culture *Lactobacillus paracasei* LPC-37. The processing and ripening of sausages were carried out in meat processing plant to simulate real conditions of production. The changes of the products (water activity, pH, concentration of organic acids and microbial growth) were evaluated during ripening (3 weeks), while sensory analysis was carried out in the final stage of the process and during storage (3 weeks). It was found that the environment of raw-fermented sausages is suitable for the growth and survival of *Lactobacillus paracasei* LPC-37 and the microbiological quality of the final product was very good (absence of *Salmonella* and *Listeria monocytogenes*). The counts of lactobacilli reached 10^7 CFU/g of the product, which meet the requirements for functional foods. The results of the sensory evaluation showed that the overall quality of raw-fermented sausages with *Lactobacillus paracasei* LPC-37 was satisfactory and differed mainly in two taste descriptors (meaty and sour).

Keywords: fermented sausage; ripening; probiotic culture; starter culture

INTRODUCTION

The quality and microbial stability of the fermented meat products depends on the raw meat quality, additives, conditions of production, and at least, on microbial cultures used in their processing. The starter cultures that are used in production of fermented sausages basically consist of staphylococci bacteria mainly in combination with lactobacilli or pediococci, which control the safety of processing and positively influence the sensory characteristics of the product. The sausage matrix, non-heat-treatment processing, and possible storage up to 24 °C represent optimal conditions for growth and another reproduction of beneficial bacteria. This fact allows to use probiotic cultures for achieving the new technological and nutritional properties of fermented sausages.

Lactobacillus paracasei LPC-37 is a gram-positive, non-spore forming, homofermentative rod (Trautvetter et al., 2012).

L. paracasei LPC-37 has been the subject of several research works focused mainly on its probiotic effects. In 2007, Roessler et al., conducted a double-blind, placebo-controlled, randomized crossover study in healthy

adults and patients with atopic dermatitis to examine the impact of a probiotic drink containing a combination of the probiotics *Lactobacillus paracasei* LPC-37 (3.9×10^8 CFU/g), *Lactobacillus acidophilus* 74-2 and *Bifidobacterium lactis* 420, on clinical and immunological parameters and their ability to survive the passage through the human GIT. *L. paracasei* LPC-37 was able to colonize transiently the intestine and was found in high numbers in faeces. No significant improvement of the skin conditions in patients with atopic dermatitis results from the study but the symptoms were described as "allayed" (Roessler et al., 2007). The study of Paineau et al. (2008), indicates that *L. paracasei* LPC-37 (1×10^{10} CFU) and several other probiotic strains may modulate immune responses of human organism (tested with oral vaccination).

The placebo controlled study of Engelbrektsen et al. (2009), showed that *Lactobacillus paracasei* LPC-37 in combination with *Bifidobacterium lactis* B1-04, *Bifidobacterium lactis* Bi-07, *Lactobacillus acidophilus* NCFM and *Bifidobacterium bifidum* Bb-02 (4×10^{10} CFU/g) minimized the disruption of faecal microbiota of healthy subjects undergoing antibiotic therapy. A similar work, which ranks among the largest,

placebo controlled studies with probiotics and antibiotic associated diarrhoea, was made by **Ouwehand et al. (2014)**. The same type of probiotic mixture as the above-mentioned was used. High doses of mixture consumed: 1.70×10^{10} CFU/g resulted in reduce of the incidence of antibiotic associated diarrhoea from 24.6 to 12.5%, significant reduce of abdominal pain and bloating.

However, there is still a need to accomplish the other relevant, randomized, double blind clinical studies to investigate the beneficial effect of *Lactobacillus paracasei* LPC-37 on human organism.

MATERIAL AND METHODOLOGY

1. Sample preparation

The first group of samples was prepared by using a standard lyophilized starter culture (Lyocarni RHM-33 produced by the Sacco company, Italy) consisting of *Staphylococcus xylosus* and *Pediococcus pentosaceus*. The second group of samples was prepared by using the combination of a standard lyophilized starter culture (Lyocarni RHM-33) and probiotic *Lactobacillus paracasei* LPC-37 (produced by Danisco). The samples were made from beef (28.7%), pork meat (33.6%), bacon (33.6%), antioxidant, sodium nitrate and flavouring substances. The processes of grinding, chopping, mixing and 3 weeks of ripening (16 – 23 °C, relative humidity: 95 – 80%) were carried out in a meat processing plant. The samples were collected over a period of one week (7 days) during the ripening process.

2. Extraction of the samples for isotachopheresis (ITP)

Sample preparation: 5 g of sample was homogenized in a grinder (3 minutes at 6000 rpm) to obtain a homogeneous mixture. Afterwards, homogeneous sample was extracted with 50 ml of hot (80 °C) deionized water, stirred for 2 minutes and tempered 1 hour at 80 °C. The supernatant was cooled to room temperature and filtered by Filtrak 388 ϕ filter paper. The filtrate was amended to volume of 50 ml by deionized water. 1 ml of the solution was transferred into 10 ml of volumetric flask and amended by deionized water. The final supernatant was transferred into reagent tubes, which have been frozen afterwards (**Pereira et al., 2012**).

2.1 The conditions of ITP analyses

The samples were slowly defrosted and filtered by 0.45 μ m microfilter prior to injection of the samples.

Leading electrolyte (LE): 10 mM HCL + ϵ -aminocaproic acid + 0.1% methylhydroxyethylcellulose

Terminating electrolyte (TE): 5×10^{-3} M caproic acid

Standards for lactic acid: solutions of lithium lactate at levels 2, 6 and 10×10^{-4} mol.dm⁻³.

Standards for acetic acid: solutions of sodium acetate at levels 2, 6 and 10×10^{-4} mol.dm⁻³.

Selectivity: The standard solutions of acids were measured and it was found that the relative step height value (RSH) for analysed acids did not change.

3. Microbial analyses

The samples with *Lactobacillus paracasei* LPC-37 were collected for microbial analyses during the fermentation process (0, 1st, 2nd and 3rd week).

3.1 Detection of *Lactobacillus*

Microbial analysis was made according to ISO Standard 15214 - horizontal method for the enumeration of mesophilic lactic acid bacteria -colony-count technique at 30 °C.

3.2 Detection of *Listeria*

Microbial analysis was made according to ISO Standard 11290-2 - horizontal method for the detection and enumeration of *Listeria monocytogenes*.

3.3 Detection of *Salmonella*

Microbial analysis was made according to ISO Standard 6579 - horizontal method for the detection of *Salmonella*, including *Salmonella typhi* and *Salmonella paratyphi*.

4. pH measurement

pH measurement was carried out according to ISO Standard 2917-using digital pH-meter OP-211/1 (Radelkis, Hungary) with a direct electrode injection into a sample.

5. a_w measurement

a_w measurement was carried out using Rotronic Hygroskop DT a_w meter after the samples were allowed to reach room temperature.

6. Sensory analysis

The sensory evaluation was performed by 9 panelists made up of males and females, selected from the Department of Food Science and Technology. The consistency, colour and appearance on cut, aroma, and taste were evaluated by hedonic test. The intensity of following taste descriptors was also described by hedonic test: meaty, salty, spicy, sour and smoked taste.

RESULTS AND DISCUSSION

ITP analysis

The results of ITP analyses are presented in Figure 1 and Figure 2. The lactic acid was found as the predominant acid created during the process of fermentation. The highest values of lactic acid in sausages was detected in the 3rd week of ripening. These values represented 16.94 g.kg⁻¹ of dry matter in sausages with starter culture Lyocarni RHM-33 (Figure 1) and 18.64 g.kg⁻¹ of dry matter (Figure 2) in sausages with addition of *Lactobacillus paracasei* LPC-37, which represented difference at 9%. Differences between samples became more apparent in the 2nd week of ripening which confirmed higher fermentative activity of *Lactobacillus paracasei* LPC-37 in fermented sausages.

Despite the homofermentative character of *Lactobacillus paracasei* LPC-37, small amounts of acetic acid were identified in sausages mainly in the 1st week of ripening which was probably due to the activity of heterofermentative, naturally presented microflora in a meat at the initial stage of ripening.

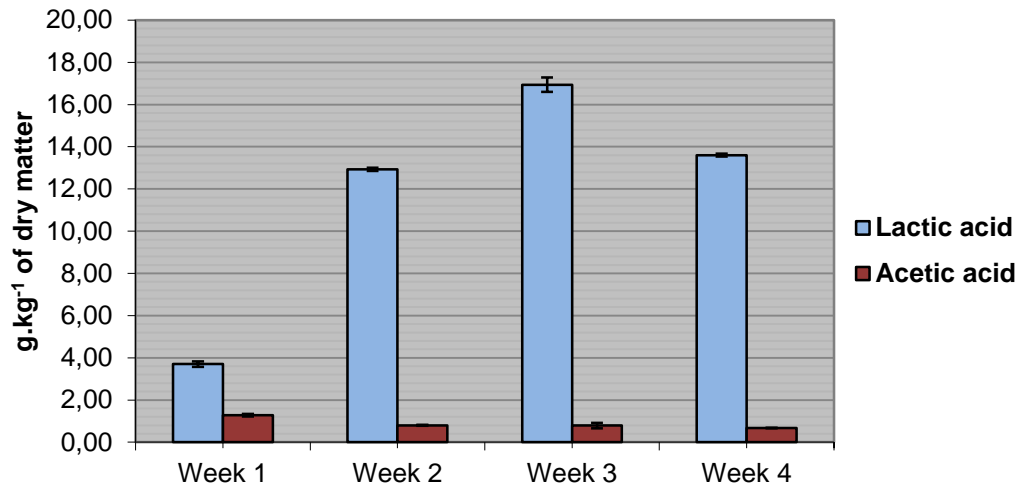


Figure 1 The concentration of the organic acids in sausages with starter culture Lyocarni RHM-33.

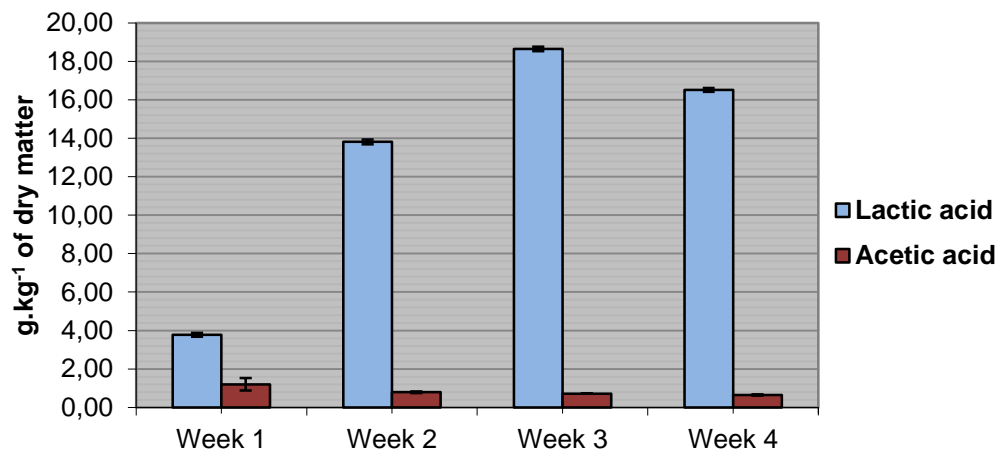


Figure 2 The concentration of the organic acids in sausages with starter culture Lyocarni RHM-33 and *Lactobacillus paracasei* LPC-37.

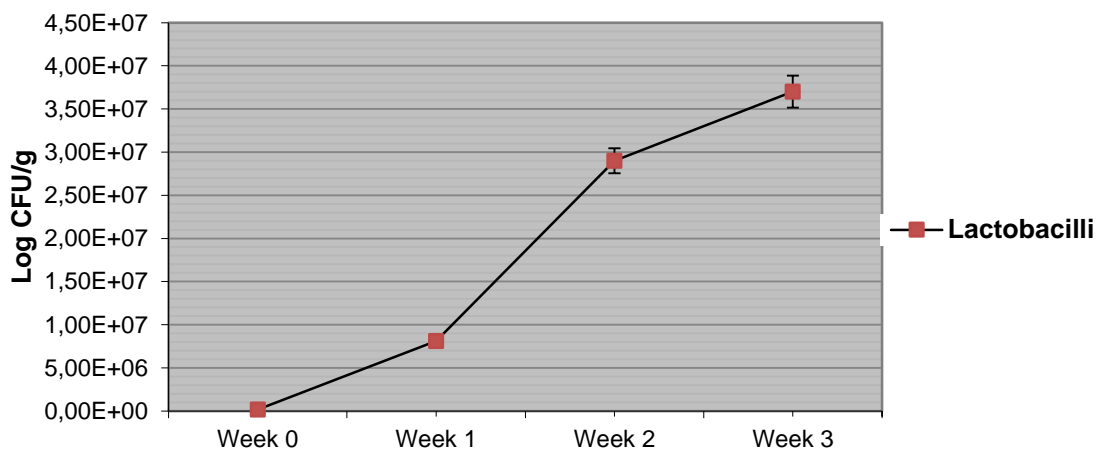


Figure 3 Evolution of the *lactobacilli* counts in sausages with Lyocarni RHM-33 and *Lactobacillus paracasei* LPC.

1st week of storage

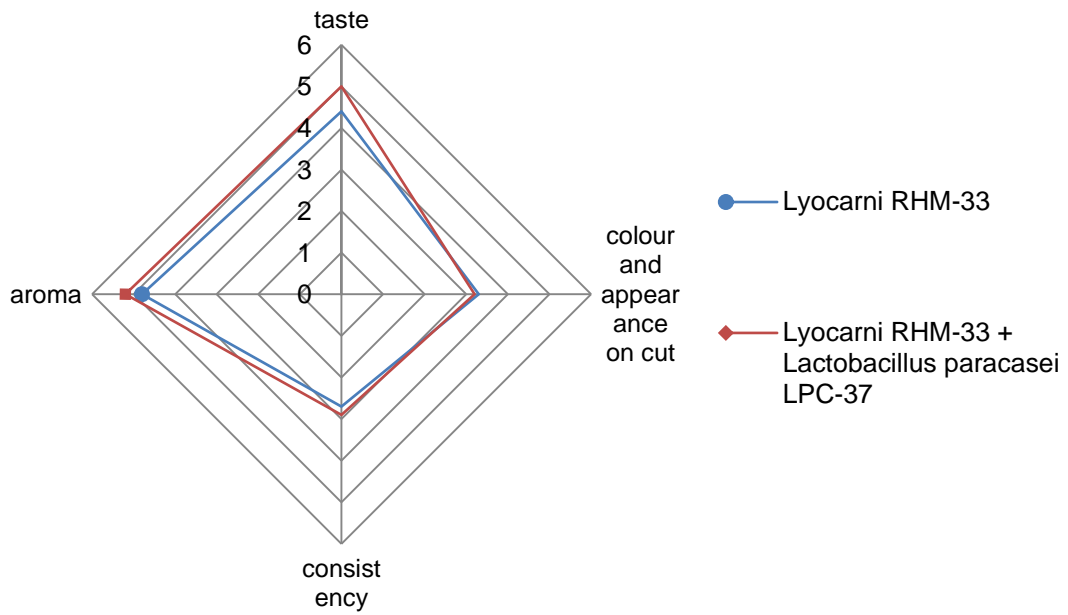


Figure 4 Sensory evaluation of sausages in the 1st week of storage.

2nd week of storage

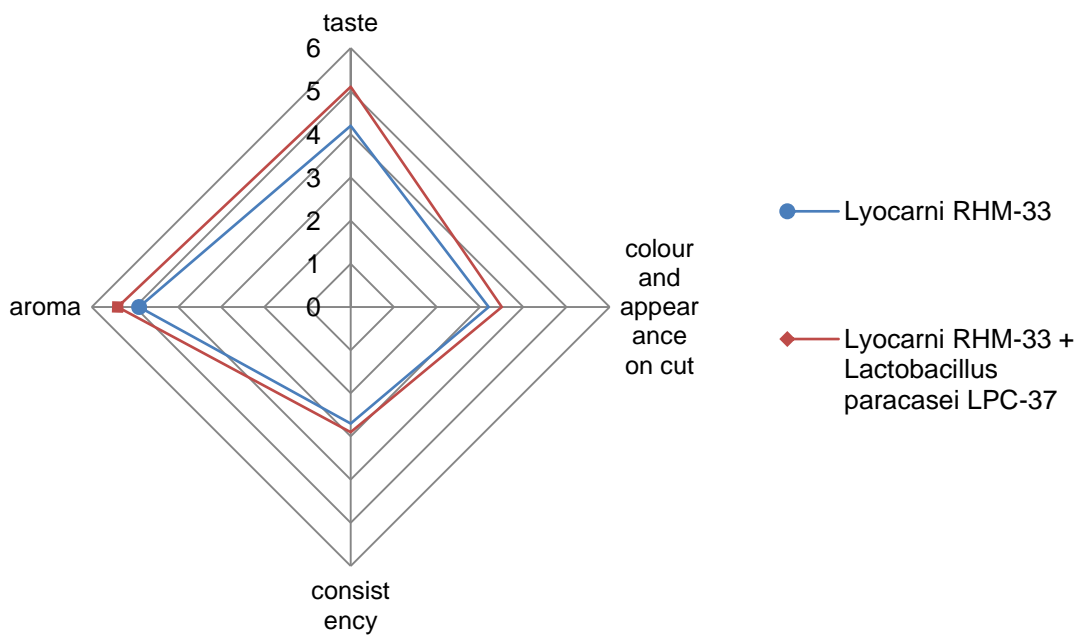


Figure 5 Sensory evaluation of sausages in the 2nd week of storage.

3rd week of storage

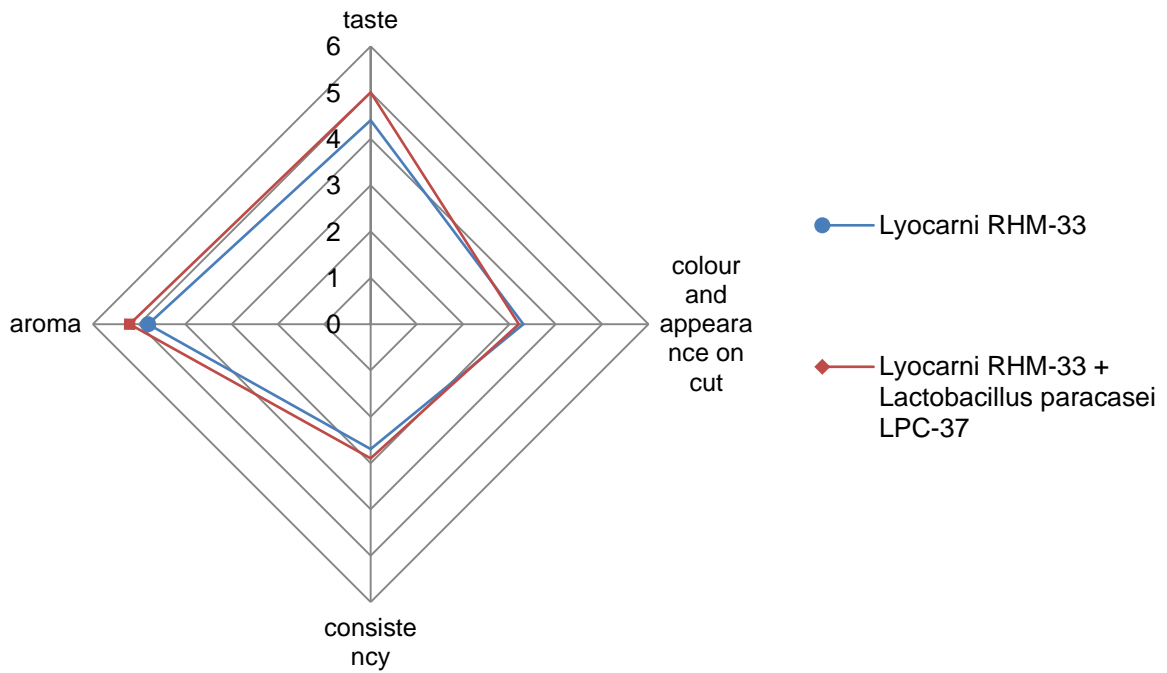


Figure 6 Sensory evaluation of sausages in the 3rd week of storage.

1st week of storage

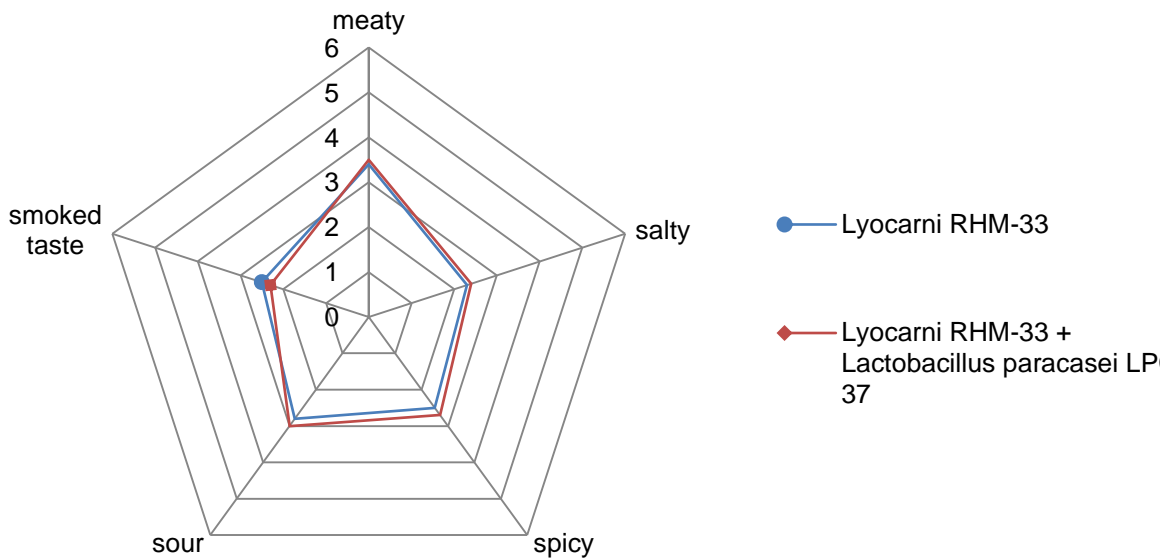


Figure 7 Sensory evaluation of sausages in the 1st week of storage - intensity of taste descriptors.

2nd week of storage

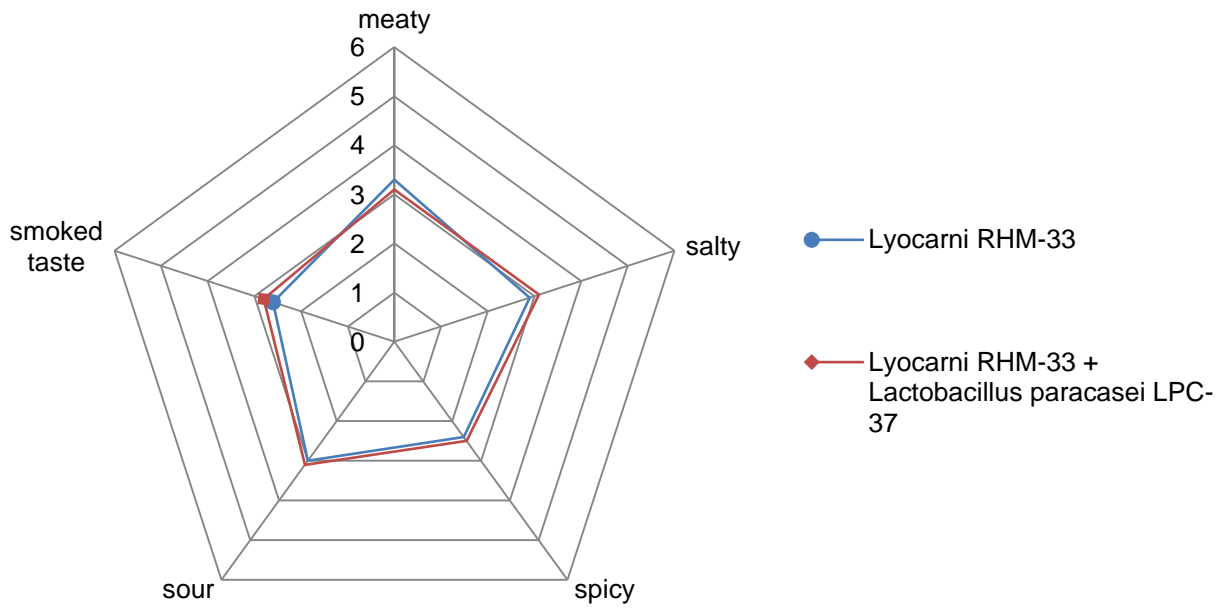


Figure 8 Sensory evaluation of sausages in the 2nd week of storage - intensity of taste descriptors.

3rd week of storage

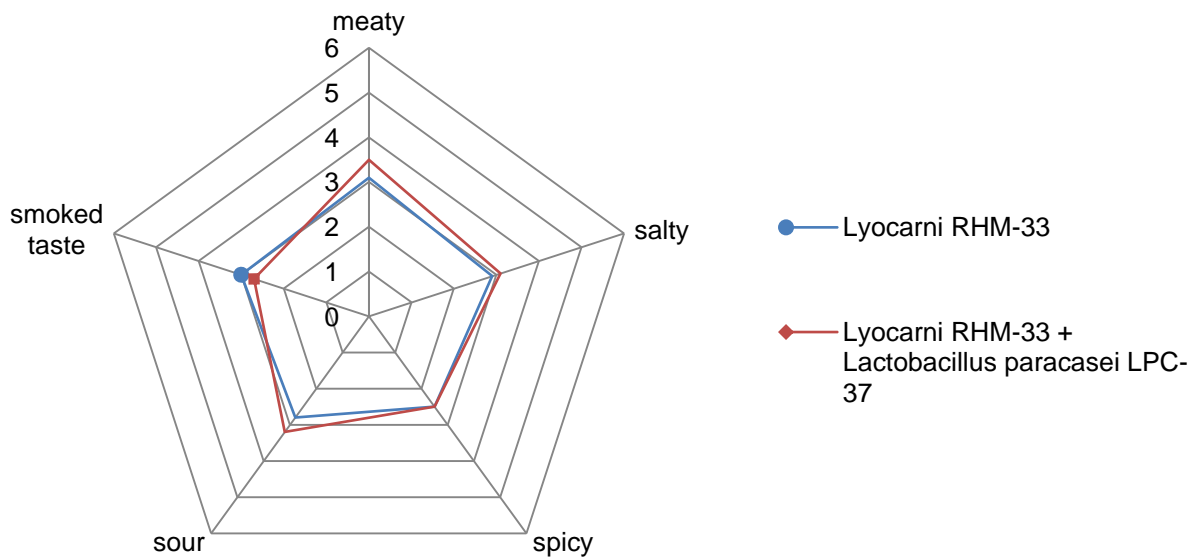


Figure 9 Sensory evaluation of sausages in the 3rd week of storage - intensity of taste descriptors.

Table 1 pH of the sausages during ripening.

	<u>Lyocarni RHM-33</u> Mean ± SD	<u>Lyocarni RHM-33 + L.paracasei LPC-37</u> Mean ± SD
Week 0	6.23 ±0.03	6,21 ±0.02
Week 1	5.82 ±0.04	5.77 ±0.01
Week 2	5.65 ±0.02	5.45 ±0.09
Week 3	4.39 ±0.01	4.30 ±0.02

Table 2 Water activity of the sausages during ripening.

	<u>Lyocarni RHM-33</u> Mean ±SD	<u>Lyocarni RHM-33 + L.paracasei LPC-37</u> Mean ±SD
Week 0	0.97 ±0.01	0.97 ±0.01
Week 1	0.96 ±0.01	0.94 ±0.01
Week 2	0.93 ±0.00	0.93 ±0.00
Week 3	0.93 ±0.01	0.93 ±0.01

Microbial analyses

The results of the microbial analyses are presented in Figure 3. The population of lactobacilli was monitored during fermentation up to the 3rd week. Week 0 represented a day in which the fermented sausages were stuffed into casings and the 3rd week represented the final week of ripening. Overall, the population of lactobacilli increased throughout fermentation from 10^5 CFU/g to more than 10^7 log CFU/g. This suggests that *Lactobacillus paracasei* LPC-37 grew rapidly and was probably dominant culture in sausages. According to **Grajek et al. (2005)**, at least 10^6 log CFU/g viable cells must reach the intestine for potential health benefits or therapeutic effect which seems to be possible in this type of culture used.

The presence of *Salmonella* and *Listeria monocytogenes* was found negative during the time of ripening which confirmed controlled and safety processing.

pH measurement

The initial pH of the sausages with starter culture was 6.23 and in samples with combination of Lyocarni RHM-33 and *Lactobacillus paracasei* LPC-37 represented 6.21 (Table 1). The lowest pH was detected in the 3rd week of ripening in sample with combination of Lyocarni RHM-33 and *Lactobacillus paracasei* LPC-37. The pH progressively increased until the end of ripening due to the proteolytic activity generated by the microorganisms. Bacterial proteases induce proteolytic degradation, generating amino acids, peptides and amines which have a buffering effect on the organic acids. The final pH of the sample with *Lactobacillus paracasei* LPC-37 was 4.35, which comply with specific requirements for this type of ready-to-eat meat products.

a_w measurement

Water activity a_w (presented in Table. 2) decrease was observed during ripening in all samples. The initial water activity in fermented sausages was 0.97. Higher water activity level (0.96) in the 1st week of ripening was determined in the samples with Lyocarni RHM-33. The samples with combination of Lyocarni RHM-33 and

Lactobacillus paracasei LPC-37 had slightly lower water activity (0.94), which is in the correlation with fact that lowering of the pH reduce the water binding properties of the meat and reduce the drying time of fermented meat products (**Holck et al., 2011**).

The values of water activity became equivalent (0.93) in the 2nd week of ripening in all samples and reached the legislative claims.

Sensory analysis

The sensory evaluation revealed no significant differences between the consistency, colour and appearance on cut, aroma, and taste during the storage of sausages. The evaluation of the intensity of taste descriptors showed some differences in meaty and sour taste during the 3rd week of storage. The fermented sausages with *Lactobacillus paracasei* LPC-37 had higher mean score (differ by 0.40 point) in both of the taste descriptors.

CONCLUSION

The objective of this study was to evaluate the water activity, pH, concentration of organic acids, microbiological quality and sensory acceptability of raw-fermented sausages with *Lactobacillus paracasei* LPC-37. The obtained data (pH, a_w and microbial quality) confirm the beneficial effects of fermentation with probiotic bacteria application in terms of the health safety of the product. Based on determined counts of lactobacilli (more than 10^7 CFU/g) represented mainly by *L. paracasei* LPC-37, product can be considered as functional food. An analysis of the sensory evaluation results showed that the overall post-fermentation quality was positively correlated with the aroma and taste of fermented sausages and the intensity of two taste descriptors (meaty and sour) was higher than in sausages with starter culture only. The results of this study showed that raw-fermented sausages with *L. paracasei* LPC-37 potential probiotic strain are of good microbiological quality. The environment of raw-fermented sausages is suitable for the growth and survival of *L. paracasei* LPC-37 potential probiotic strain.

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POTENTIAL PROBIOTIC PROPERTIES OF LACTOBACILLI ISOLATED FROM GOAT'S MILK

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ABSTRACT

The three strains of lactobacilli isolated from goat's milk – *Lactobacillus (Lbc.) casei* 21L10, *Lbc. johnsonii* KB2-1 and *Lbc. plantarum* 25/1L were selected in previous studies because they showed good processing and protective properties in production of cheeses or lactic acid beverages from heat-treated milk or in model conditions. The aim of this study was to evaluate their potential probiotic properties: resistance to lysozyme, survival in gastrointestinal tract, and hydrophobicity. Testing was carried out *in vitro* methods: comparison of growth and metabolic characteristics of the strains when cultivated in Man, Rogosa and Sharpe medium with and without the addition of lysozyme (400 µg.mL⁻¹); viability of strains after incubation in simulated environment of gastric juice (pH = 2.0, pepsin) and subsequently in simulated small intestinal juice (pH = 8.0, pancreatin, bile salts) and an ability to adhere to the non-polar solvent in the two-phase system: xylene-water. *Lbc. casei* 21L10 and *Lbc. johnsonii* KB2-1 were resistant to the effect of lysozyme, the strain *Lbc. plantarum* 25/1L was moderately susceptible. To the action of simulated gastric juice was resistant only the *Lbc. johnsonii* KB2-1, that was subsequently inhibited in simulated small intestinal juice. When using xylene as a model agent, all strains were not hydrophobic. From all the tested strains, *Lbc. johnsonii* KB2-1 showed good potential probiotic properties, particularly in relation to resistance to lysozyme and the simulated environment of gastric juice.

Keywords: *Lactobacillus*; lysozyme; simulated gastro-intestinal tract; microbial adhesion to solvent; probiotic

INTRODUCTION

FAO/WHO (2002) defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host“. It is required that these organisms are clearly described, are particularly safe and have certain features that are presumption for their beneficial effect on the host organism. Additionally, the benefits must be clearly demonstrated in clinical trials (FAO/WHO, 2002; Verdenelli et al., 2009). Selected strains of lactobacilli belong to the typical representatives of lactic acid bacteria (LAB), which may have probiotic properties. Besides human origin, they can be isolated also from the milk processing (Bao et al., 2010). They may be added to the milk products as starter adjuncts, which is one of possible methods (other than the pharmaceutical form) of their consumption (Vinderola et al., 2009).

Lysozyme is an enzyme muramidase, which is commonly found in human saliva, tears, breast milk and mucus. It can hydrolyse 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan, a common part of cell wall in Gram-positive bacteria (McKenzie and White, 1991). They are therefore more sensitive to the action of lysozyme than Gram-negative bacteria that have a different composition of the cell wall and several protective barriers, protecting of peptidoglycan layer. Lactobacilli are the Gram-positive bacteria. Thus, their resistance to lysozyme is a probiotic criterion (Kunová et al., 2011).

Some microorganisms commonly found in food, either pathogenic or beneficial, are able to survive in the low pH of the stomach, higher pH of digestive juices in the small intestine, activity of enzymes (pepsin, pancreatin), and bile salts. Thus there were created several models of simulated gastro-intestinal environment in which they are tested whether the strains of LAB are able to survive in such conditions. A number of potential probiotic strains of lactobacilli have a high survival rate (SR) in simulated gastro intestinal tract (GIT). Milk matrix itself (Guglielmottia et al., 2007), may or may not have protective effect (Mäkeläinen et al., 2009).

The ability of lactobacilli and LAB generally, to adhere to cells of GIT, is important. This feature prevents the peristaltic movements of the digestive system to remove cells immediately so that they can carry out their beneficial activity. The pathogenic microorganisms are capable of binding to GIT, too. Their ability to auto-aggregation correlates with adhesion. These are the basic conditions for colonization and subsequent infection of the GIT. If the probiotic strains are capable to co-aggregate with pathogens or to replace them, then they create a protective barrier (Collado et al., 2008). Besides, the capabilities of potential probiotics occupy binding sites in the GIT and compete for nutrients; their therapeutic effect is also based on immunomodulation (Collado et al., 2007). The rate of bacterial adhesion to human GIT depends on the physico-chemical properties of the bacterial cell surface. One of these properties is hydrophobicity (Del Re et al.,

2000). According to this *in vitro* method, it is possible to estimate whether the strains are capable of adhesion to GIT, or not.

In our previous studies the processing characteristics (Kološta et al., 2014; Slottova et al., 2014; Klapáčová et al., 2014) and protective properties (Klapáčová et al., in press) of three selected strains of lactobacilli (*Lbc. casei* 21L10, *Lbc. johnsonii* KB2-1 and *Lbc. plantarum* 25/1L), isolated from goat's milk, were described. The aim of this work was to study the susceptibility of the strains to lysozyme, model conditions of GIT (simulated gastric and small intestinal juice) and their ability to adhere to a non-polar xylene as an indicator of hydrophobicity. All these properties are important for assessment of the potential probiotic effect of lactobacilli.

MATERIAL AND METHODOLOGY

Lbc. casei 21L10, *Lbc. johnsonii* KB2-1 and *Lbc. plantarum* 25/1L were isolated from raw goat's milk and were identified with 16S rRNA PCR method (Slottova et al., 2014).

Sensitivity of the lactobacilli to lysozyme was determined by their cultivation (24.5 hours; 37 °C; statically; aerobically) in Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstadt, Germany). After one hour of cultivation, the lysozyme was added to the media – Lysozyme from chicken egg white 156733 U.mg⁻¹ (Serva Electrophoresis, Heidelberg, Germany) at a concentration of 400 µg.mL⁻¹. The growth was monitored by measuring optical density (OD) at 600 nm by spectrophotometer and their metabolic activity by pH decrease of the cultivation media by pH meter. Control samples did not contain lysozyme. From data measured, the growth lines and graphics dependence of pH on cultivation time were drawn.

The ability of lactobacilli to survive in a simulated GIT was estimated as follows: MRS medium was inoculated with an overnight culture of the strains (1%) and was cultivated for 24 hours at 37 °C, statically, aerobically. After the cultivation, the 5 mL of medium was filled into sterile centrifuge tubes. The supernatant was separated by centrifugation (Relative Centrifugal Force (RCF) = 6000; 20 min.; 30 °C) and the biomass was washed with physiological saline. After, the biomass was carefully resuspended by vortexing in 5 mL of simulated gastric juice or in 5 mL of physiological saline (control sample). Simulated gastric juice was prepared as follows: 0.5 g of NaCl (Merck) was dissolved in 100 mL of deionized water and pH was adjusted to 2.0 ± 0.1 by 36% HCl (Merck). The solution was sterilized by autoclaving. To the sterile simulated gastric juice 0.3 g of pepsin – Pepsin from porcine gastric mucosa 0.7 FIP-U.mg⁻¹ for biochemistry (Merck) per 100 ml of juice was added and the pH was checked. The strains in the simulated gastric juice were incubated statically at 37 °C for 2 hours. Subsequently, supernatant was separated by centrifugation under the same conditions. Then 5 mL of simulated small intestinal juice was added to the biomass. The simulated small intestinal juice was prepared as follows: 0.5 g NaCl and 0.3 g bile salts (Sigma-Aldrich, St. Louis, USA) were

dissolved in 100 mL of 0.1 M potassium phosphate buffer with pH 8.0 ± 0.1. The solution was sterilized by autoclaving. To the sterile simulated small intestinal juice 0.1 g of pancreatin – Pancreatin NB from porcine pancreas 5,407 U.g⁻¹ (Serva Electrophoresis, Heidelberg, Germany) was added per 100 mL of the juice and the pH was checked. The strains in simulated small intestinal juice were incubated statically at 37 °C for 4 hours. After the incubation in simulated gastric and small intestinal juice, the number of lactobacilli was determined in the samples – by cultivation on MRS agar (Merck) – 72 hours, 37 °C. SR was expressed as percentage share of logarithms of colonies of lactobacilli measured after incubation in different environments in comparison to a control sample.

Microbial adhesion to solvent of lactobacilli was assessed in a two-phase system of water-xylene. 50 mL MRS medium was inoculated with 1% of overnight culture of the strains. The medium was cultivated for 24 hours at 37 °C, statically, aerobically. After the cultivation, the medium was centrifuged (RCF = 6000; 20 min.; 30 °C), and the biomass was washed twice with buffer saline. Subsequently, the biomass was resuspended by vortexing into 50 mL of 0.1 M KNO₃ (Lachema, Brno, Czech Republic). OD of the suspension was measured at 600 nm (OD₀). From the suspension 6 mL was removed to a sterile tube and 2 mL of xylene – Xylene extra pure (Merck) was added. The mixture was pre-incubated for 10 minutes at room temperature and then was thoroughly vortexing for 2 minutes. The mixture was kept for 20 minutes at room temperature, to separate the phases. The aqueous phase was removed carefully and their OD was measured at 600 nm (OD₁). The percentage of bacterial adhesion (BA) to solvent was calculated as follows: BA = (1 - OD₁/OD₀) x 100.

Each measurement was carried out twice.

RESULTS AND DISCUSSION

From all the tested strains, only *Lbc. plantarum* 25/1L was partially sensitive to lysozyme (400 µg.mL⁻¹). Figure 1 shows that the addition of lysozyme slightly protracted lag phase and reduced specific growth rate of the strain compared to the control. Also decrease the pH of the culture medium was slightly slower (Figure 2). Growth characteristics of the strains *Lbc. casei* 21L10 and *Lbc. johnsonii* KB2-1 and the production of lactic acid (Kološta et al., 2014) were not influenced by addition of lysozyme (data not presented here).

Kunová et al. (2011) tested 13 strains of lactobacilli isolated from dairy products, or human origin for lysozyme (400 µg.mL⁻¹). The 8 strains were resistant to the effect of lysozyme, in the rest of the strains; authors reported a slight delay in the exponential phase of growth curves. The origin of the strains did not affect their susceptibility to lysozyme. On the contrary, in bifidobacteria, Kunová et al. (2012) demonstrated that the strain of *Bifidobacterium bifidum* and *Bifidobacterium longum* isolated from faeces of babies and children were more resistant to the effect of lysozyme (400 µg.mL⁻¹ and 1000 µg.mL⁻¹), than the strains of animal origin.

Besides origin of LAB, their sensitivity to lysozyme may also be influenced with the presence of other substances in the environment (e.g. nisin), cultivation temperature, growth phase or mutations of the tested strains (Neujahr et al., 1973; Guglielmotti et al., 2007; Kunová et al., 2012).

Survival of lactobacilli in a simulated environment GIT is summarized in Table 1. The only strain *Lbc. johnsonii* KB2-1 was resistant to the effect of simulated gastric juice (more than the 90% SR), but after subsequent simulation of small intestinal juice was already inhibited (less than 50% SR) – criterion according to Kejmarová et al., (2011). Strains *Lbc. casei* 21L10 and *Lbc. plantarum*

25/1L were inhibited after the effect of simulated gastric juice (SR from 32.2% to less than 23%).

Strains of lactobacilli resistant to simulated GIT have been isolated from various matrices, e.g.: from fermented olives (*Lbc. pentosus*, *Lbc. plantarum*, *Lbc. paracasei* subs. *paracasei*) (Argyri et al., 2013), cheese (*Lbc. rhamnosus*, *Lbc. paracasei*, *Lbc. casei*, *Lbc. harbinensis*, *Lbc. fermentum*) (Solieri et al., 2014), fermented meat products (*Lbc. sakei*, *Lbc. curvatus*, *Lbc. plantarum*) (Papamanoli et al., 2003). Methodology of survival varied in studies – they tested various pH of simulated gastric juice (from about 1 to 3), different additions of enzymes or bile salts, the environment, where the

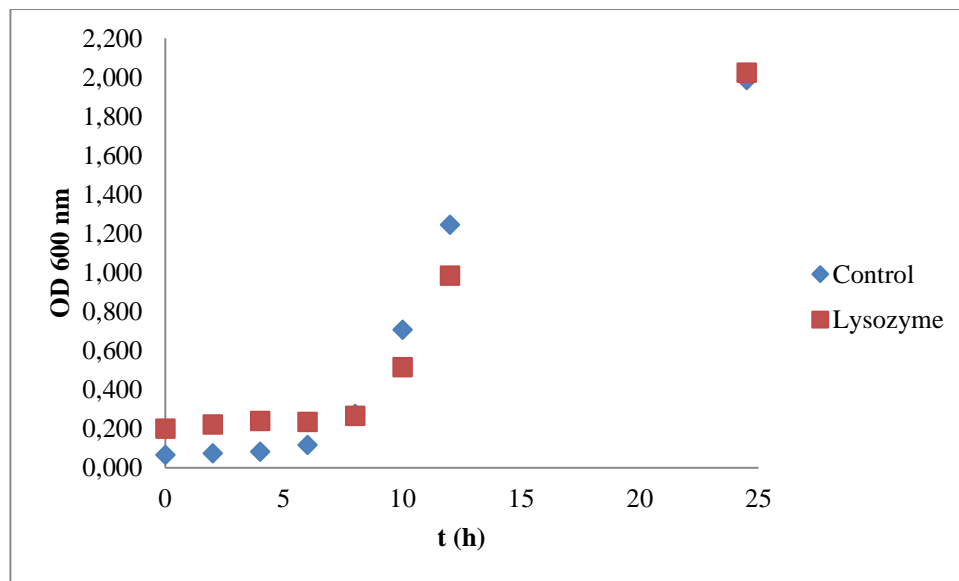


Figure 1 The growth of *Lbc. plantarum* 25/1L (37 °C, statically, aerobically) in MRS medium without and with addition of lysozyme (400 µg.mL⁻¹), detected by measuring the optical density (OD).

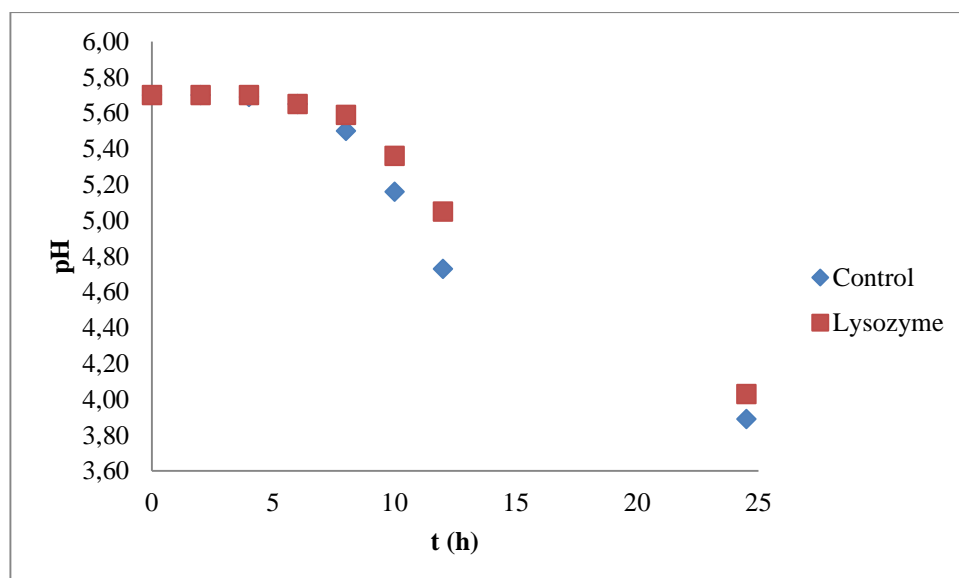


Figure 2 The decrease in pH during cultivation (37 °C, statically, aerobically) *Lbc. plantarum* 25/1L in MRS medium without and with addition of lysozyme (400 µg.mL⁻¹).

Table 1 Survival of lactobacilli in simulated environment of gastrointestinal tract at 37 °C.

Strain**	CS***		GJ****		GJ + SIJ*****	
	log ₁₀ (CFU.mL ⁻¹)	SR* (%)	log ₁₀ (CFU.mL ⁻¹)	SR (%)	log ₁₀ (CFU.mL ⁻¹)	SR (%)
21L10	9.00 – 9.08	100.0	2.91	32.2	< 1.00	< 11.1
KB2-1	6.00 – 6.20	100.0	5.49 – 5.52	90.2	2.30 – 2.32	37.9
25/1L	8.66 – 8.74	100.0	< 2.00	< 23.0	< 1.00	< 11.5

- * Survival rate
- ** *Lbc. casei* 21L10, *Lbc. johnsonii* KB2-1, *Lbc. plantarum* 25/1L
- *** Control sample
- **** Simulated gastric juice (2hours)
- ***** Simulated gastric juice (2hours) and subsequently small intestine juice (4hours)

influence was observed (MRS medium, various buffers, etc.), and the exposure time.

Tested strains did not show any adhesion to xylene – thus, they are hydrophilic. Calculated BA values were less than 1%.

Kos et al. (2003) using the same methodology, found out that the tested strain *Lbc. acidophilus* M92 was hydrophobic (71% adhesion). Whereas other probiotic strains *Lbc. plantarum* L4 and *Enterococcus faecium* L3 were hydrophilic (7% respectively 0% adhesions). The authors of this study confirmed the link between adhesion and auto aggregation (as model was used porcine ileal epithelial cells), mediated of protein components of cell membrane. **Kejmarova et al. (2011)** tested 15 strains of bifidobacteria (*Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium longum*, *Bifidobacterium species*, *Bifidobacterium dentium*) for hydrophobicity, which varied from 49% to 68%. **Bhardwaj et al. (2010)** used for testing of the hydrophobicity other agents, besides the xylene also *n*-hexadecane and *n*-octane, while changing their additional amount. *Enterococcus faecium* KH 24 showed low hydrophobicity – from 2% to 17% in case of *n*-hexadecane, but higher using xylene and *n*-octane – from 49% to 86%. It has been therefore proved that the results of such *in vitro* bacterial adhesion assay also depend on of the type and the quantity of used reagents.

Strains *Lbc. casei* 21L10 and *Lbc. johnsonii* KB2-1 were resistant to the effect of lysozyme (400 µg.mL⁻¹) and strain *Lbc. plantarum* 25/1L was affected only marginally by the tested concentration.

Strains *Lbc. casei* 21L10 and *Lbc. plantarum* 25/1L did not survive well in simulated gastric juice environment. On the contrary, strain *Lbc. johnsonii* KB2-1, this environment tolerated well. Surviving of all tested strains was inhibited by the simulated environment of the small intestine digestive juices. Excluding the effects of higher pH, pancreatin and bile salts, also autolysis of cells in the environment of usage of 0.1 M potassium phosphate buffer could occur (**El-Kholy et al., 1998**).

All the strains were not hydrophobic. Based on the results of the model (xylene), there is not an expectation of adhesion ability to the cells of the GIT. However, it is possible when another reagent is used, the adhesion will

achieve different percentage. It is also recommended to use more sophisticated models as e. g. Caco-2 cells (**Candela et al., 2008; Argyri et al., 2013**).

From all the tested strains, *Lbc. johnsonii* KB2-1 showed the best potential probiotic properties – however it was not resisted to simulated small intestine juice and was not hydrophobic.

In previous study it was shown, that the strains did not produce biogenic amines in excessive concentrations (**Klapáčová et al., in press**). The further safety criteria (resistance to antibiotics, β-haemolysis, etc.) will be evaluated too.

CONCLUSION

Strains *Lbc. casei* 21L10 and *Lbc. johnsonii* KB2-1 were resistant to the effect of lysozyme (400 µg.mL⁻¹) and strain *Lbc. plantarum* 25/1L was affected only marginally by the tested concentration.

From all the tested strains, *Lbc. johnsonii* KB2-1 showed the best potential probiotic properties – however it was not resisted to simulated small intestine juice and was not hydrophobic.

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THE BACTERIOLOGICAL QUALITY OF GOAT AND OVINE MILK

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ABSTRACT

This study concentrates on information concerning the microbiological hazards that can be present in raw milk from animal species other than cows. A total of 54 (23 of ovine and 31 of goat) bulk tank milk samples from 10 farms in the Czech Republic were collected in years 2013 – 2014. The sampling was done at regular time intervals during the whole year, with five to eight samples collected from each of the 10 dairy farms involved in the study. All milk samples were collected into sterile sampling bottles and transported in a cooler sampling case to the laboratory for immediate examination. Farms were randomly selected to cover the whole area of the Czech Republic. The prevalence and characteristic of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes* was studied. Raw cow's milk can be contaminated by *E. coli* intramammarily during clinical or subclinical mastitis and either directly through animal feces or indirectly during milk collection through farm employees or the milking equipment. *E. coli* was detected in 90.3% of the goat milk and 95.7% of the ovine milk samples. The genes encoding Shiga toxins 1 and 2– (*stx*₁, *stx*₂) were not detected and no STEC was identified. The *Eae* was detected in 3 (4.6%) isolates. *S. aureus* was detected in 9 (29.0%) samples of goat milk and 8 (34.8%) samples of ovine milk. A total 12 (57.1%) enterotoxin positive *S. aureus* were obtained; 6 (28.6%) were positive for the production of *sec* encoding enterotoxin SEC; in 4 (19.0%) isolates the gene *seh* was detected; 2 (9.5%) isolates were proven positive for *seg* (4.8%) and combination *seg* and *sei* (4.8%). The presence of MRSA was not detected in the tested samples in our study. *L. monocytogenes* was detected in 1 (3.2%) samples of goat milk and 1 (4.3%) samples of ovine milk. The serotype (1/2a, 1/2b) was detected in our study. *Campylobacter* spp. and *Salmonella* spp. were not isolated from any of the samples. These results form the basis for determining the microbiological quality standards for goat and ovine milk.

Keywords: bacteria; *Escherichia coli*; *Staphylococcus aureus*; *Salmonella* spp.; *Campylobacter* spp.; *Listeria monocytogenes*

INTRODUCTION

This study concentrates on information concerning the microbiological hazards that can be present in raw milk from animal species other than cows. Milk and dairy products are basic components of human diet. Consumption of raw milk represents a risk for the consumers, due to the possible presence of human pathogenic microorganisms in raw milk (Claeys et al., 2013). Public health problems associated with consumption of unpasteurized cow's milk and raw-milk products have been well documented (Cody et al., 1999; Kalman et al., 2000; De Buyser et al., 2001; Harrington et al., 2002). Goat and ovine production constitutes an important part of the national economy in many countries. One of the most decisive factors in the growth in the consumption of goat and ovine milk is their perceived beneficial effect on human health, which, moreover, is fully recognized by the scientific community. Goat milk has an acceptable, attractive odour and taste, and can be consumed as an alternative to cow milk because it is less allergenic. Ovine milk has a higher content of essential vitamins and minerals than cow's milk and could be used to cater to consumers' appetite for healthy and safer products (Park et al., 2007).

In general, the scientific quality of research on goat and ovine milk products is still insufficient but is continuously improving.

Milk quality can be evaluated according to hygienic, nutritional, technological and sensory parameters. One of the most important criteria that determine goat and sheep's milk quality is the control of pathogens.

The objectives of this study were: 1) to determine the microbiological status of goat and ovine milk in the Czech Republic, 2) to study the prevalence of food-borne pathogens, especially *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., and *Campylobacter* spp. and 3) typing of selected pathogens (serotyping, detection of SEs, *mecA* genes etc.).

MATERIAL AND METHODOLOGY

Samples collection. A total of 54 bulk tank milk samples from 10 farms in the Czech Republic were collected in years 2013 – 2014. The sampling was done at regular time intervals during the whole year, with five to eight samples collected from each of the 10 dairy farms involved in the study. Farms were randomly selected to cover the whole area of the Czech Republic. All milk samples were collected into sterile sampling bottles and transported in a cooler sampling case to the laboratory for immediate

examination. The milk samples were tested for the presence of: *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes* each test was performed on 25 ml of raw milk by means of qualitative methods.

Isolation and identification of *Escherichia coli* (*E. coli*). Detection of *E. coli* was carried out according to ISO 16649-1 with some slight modifications. The detection was performed after enrichment of 25 ml of milk in 225 ml of buffered peptone water (BPW, Oxoid, UK) at 37 °C for 24 hours followed by aerobic cultivation on Tryptone Bile X-glucuronide medium (TBX, Oxoid, UK) (44 °C for 24 hours). From each positive sample, one to three suspected *E. coli* isolates were used for confirmation. Confirmation of suspected colonies from TBX agar consisted of the detection of oxidase (OXItest, Pliva-Lachema, CZ) and production of indole (COLItest, Pliva-Lachema, CZ).

Isolation and identification of *Staphylococcus aureus* (*S. aureus*). Detection of *S. aureus* was carried out according to ISO 6888-1 with slight modification as follows: 25 ml of milk was diluted with 225 ml of buffered peptone water (Oxoid, UK) and homogenized. After enrichment at 37 °C overnight samples were cultivated on Baird - Parker agar (B-P, Oxoid, UK) supplemented with egg yolk-tellurite emulsion. From each plate, both the typical and atypical colonies were examined by plasmacoagulase test (Staphylo LA Seiken, DENKA SEIKEN Co. Ltd., Japan) and confirmation of suspected *S. aureus* strains was carried out by polymerase chain reaction (PCR) based on the detection of the species specific fragment SA442 (Martineau et al., 1998).

Isolation and identification of *Salmonella* spp. The detection of *Salmonella* spp. was carried out according to ISO 6579. At first, non-selective enrichment was performed in buffered peptone water (Oxoid, UK). This was followed by selective enrichment in two types of media Rappaport-Vassiliadis Soya Peptone Broth (RVS) and Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn, Oxoid, UK) simultaneously. Then isolation on media RAMBACH (MERCK, D) and XLD (Oxoid, UK) was performed.

Isolation and identification of *Campylobacter* spp. The detection of thermotolerant *Campylobacter* spp. was carried out according to ISO 10272-1. After enrichment, which was done in Bolton medium with horse blood (Oxoid, UK) and after 48 hours of cultivation at 42 °C suspension was inoculated on *Campylobacter* Selective Blood Free Agar (CCDA, Oxoid, UK) with incubation at 42 °C for 48 hours at micro-aerophilic conditions.

Isolation and identification of *Listeria monocytogenes* (*L. monocytogenes*). Detection of *L. monocytogenes* was performed according to ISO 11290-1 with a modification in the primary multiplication step which was carried out in the buffered peptone water (Oxoid, UK) at 37 °C for 24 hours. Secondary multiplication was done in Fraser broth (Oxoid, UK) at 37 °C for 24 hours and followed by aerobic cultivation on ALOA agar medium (BIO-RAD, FR) at 37 °C for 24 hours.

Typing of bacteria. More attention has been concentrated to the occurrence of methicillin-resistant *S. aureus* strains (MRSA) and *E. coli* with detection of selected genes encoding virulence factors.

For the determination of MRSA in *S. aureus* isolates polymerase chain reaction (PCR) for the detection of the *mecA* gene, which is responsible for the resistance to methicillin (Poulsen et al., 2003) was used.

For the detection of the genes encoding staphylococcal enterotoxins A–J multiplex PCR method previously published by Lovseth et al., (2004) was used.

PCR was also used for the detection of selected genes encoding virulence factors (*eae*, *hly*, *stx1*, *stx2*) in *E. coli*. The detection of the virulence genes was performed using multiplex PCR according to Fagan et al., (1999).

Typical colonies for *Listeria monocytogenes* were confirmed and serotyped by slide agglutination method using the commercially available antisera (DenkaSeiken, Japan) and verified by multiplex PCR (Doumith et al., 2004).

RESULTS AND DISCUSSION

This study was focused to map the occurrence of bacteriological risks in raw goat and ovine milk. In total, 54 (31 of goat milk and 23 of ovine milk) samples of bulk tank milk collected from 10 (5 of goat farm and 5 of ovine farm) different farms and investigated in 2013 – 2014 in the Czech Republic. The detailed results are shown in Table 1.

Differences were observed in the bacteriological quality of raw milk collected on the different dairy farms. While milk from some farms was bacteriologically safe in milk samples from other farms pathogenic microorganisms were repeatedly detected.

In our study, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* were detected.

Prevalence of *E. coli* in raw milk. Raw cow's milk can be contaminated by *E. coli* intramammarily during clinical and subclinical mastitis (Dahmen et al., 2013) and either directly through animal feces or indirectly during milk collection through farm employees or the milking equipment (Desmarchelier a Fegan, 2011). This study shows that the presence of *E. coli* in raw milk is very common. Altogether, 49 (90.7%) positive milk samples were detected. A total of 65 *E. coli* isolates were retained for further characterization. *E. coli* strains producing Shiga toxins (Stx) 1 and 2, encoded by *stx1* and *stx2* genes, respectively, are called Shiga toxin-producing *E. coli* (STEC) (Wani et al., 2009). These toxins have acytopathic effect on intestinal epithelial cells that plays a role in the development of bloody diarrhea. STEC have other, additional virulence factors, the most important of these is a protein called intimin (Robati a Gholami, 2013). Many STEC produce intimin, an adhesive protein encoded by the *eae* gene with several variants located on the pathogenicity island termed the locus of enterocyte effacement (Blanco et al., 2004). Of the adhesin coding genes, *eae* was the detected in 3 (4.6%) isolates. The genes encoding Shiga toxins 1 and 2– (*stx1*, *stx2*) were not detected and no STEC was identified.

Table 1. Results of microbiological quality of raw milk testing

Milk	Samples n	<i>Escherichia coli</i>		<i>Staphylococcus aureus</i>		<i>Listeria monocytogenes</i>	
		n	%	n	%	n	%
Goat milk	31	28	90.3	9	29.0	1	3.2
Ovine milk	23	22	95.7	8	34.8	1	4.3
Σ	54	50	92.6	17	31.5	2	3.7

Prevalence of *S. aureus* in raw milk. *Staphylococcus aureus* is one of the most important mammary gland pathogens responsible for mastitis that can cause enormous economic losses (Hata et al., 2008). When investigating the incidence of pathogenic microorganisms, we recorded the highest detection rate of *Staphylococcus aureus*. Altogether, 17 (31.5%) positive milk samples were detected. A total of 21 *S. aureus* isolates were retained for further characterization and detection enterotoxins encoding genes. Global problem of the 21st century becomes the occurrence of pathogenic microorganisms resistant to routinely used antibiotics. *S. aureus* has an impressive ability to adapt to environmental conditions and it can fast become resistant to almost all antibiotics (McCallum et al., 2010). Methicillin-resistant *S. aureus* (MRSA) were found primarily in humans, later they were detected also in animals (Lee et al., 2004). In recent years, the increase of staphylococci strains that show resistance to methicillin/oxacillin has become a serious clinical and epidemiological problem. Methicillin-resistant *S. aureus* in milk are less important as a food safety issue, since milk is almost always heat treated before consumption. However, these exceptions and raw milk consumption, which is widely practiced by farmers and their families (Oliver et al., 2009), could expose people to MRSA. Recent reports revealed that MRSA was also associated with cases of bovine and caprine mastitis (Aras et al., 2012; Vanderhaeghen et al., 2010). Occurrence of MRSA in goat milk has been observed in the Czech Republic, namely in 2008 (Štásková et al., 2009). The presence of MRSA was not detected in the tested samples in our study.

Detection of enterotoxins in *S. aureus* isolates. *Staphylococcus aureus* is an important human and animal pathogen known to produce a range of toxic substances that can cause various diseases. From the perspective of food microbiology, the most relevant characteristic of *S. aureus* is the production of heat-stable enterotoxins implicated in food-borne intoxications (Thomas et al., 2007). In terms of risk of foodborne diseases very important is the ability of approximately 50-75% of *S. aureus* strains to produce under the suitable conditions the extracellular thermostable enterotoxins (SEs) (Argudín et al., 2010).

From 54 milk samples examined, 17 (31.5%) positive

milk samples were detected. A total of 21 *S. aureus* were isolated and used for the detection of SEs. For the detection of the genes encoding enterotoxins A–J multiplex PCR was used (Lovseth et al., 2004).

A total 12 (57.1%) enterotoxin positive *S. aureus* were obtained. In our study, 6 (28.6%) of these isolates were positive for the production of classical enterotoxins SEA-SEE, which are the leading cause of foodborne diseases. In our study 6 of these isolates were positive for the production of *sec* encoding enterotoxin SEC.

It was also reported as the most frequent gene in *Staphylococcus aureus* from goat's milk from Scherrer et al., (2004) and Lyra et al., (2013), who observed the SEC in 42% and 55.6% of staphylococci. The presence of this gene was observed in 71% (Mork et al., 2010) and 86% (Silva et al., 2005) of *Staphylococcus aureus* strains isolated from healthy goats and goats with udder infections.

In our study, the 4 (19.0%) of these isolates were positive for the gene *seh* responsible for the production of SEH. Out of 21 strains investigated 2 (9.5%) were proven positive for *seg* (4.8%) and combination *seg* and *sei* (4.8%). No genes for SEs were identified in 9 (42.9%) isolates. Results are shown in Table 2.

Ikeda et al., (2005) described a mass outbreak of food poisoning caused by eating reconstituted milk contaminated by toxigenic *S. aureus* with co-productions of the SEA and SEH. The outbreak was also caused by SEH subsequently produced by *S. aureus* isolated from potato mash with raw milk (Jørgensen et al., 2005).

Prevalence of *L. monocytogenes* in raw milk. *Listeria monocytogenes* was detected in 2 (3.7%) samples. The prevalence of *L. monocytogenes* in bulk tank milk is reported to range from 1 to 12% (Oliver et al., 2005). Ninety-five percent of *L. monocytogenes* strains associated with human listeriosis and food samples belong to serotypes 1/2a, 1/2b, and 4b (Kathariou 2002). Serotype 1/2a has frequently been detected in different food matrices (Martins a Leal Germano, 2011). The same serotype (1/2a, 1/2b) was detected in our study.

Prevalence of *Salmonella* spp. and *Campylobacter* spp. in raw milk. The presence of *Salmonella* spp. and *Campylobacter* spp. was not detected in the tested samples.

Table 2. Prevalence of *S. aureus* with production staphylococcal enterotoxin genes.

Combination of toxins	Goat milk		Ovine milk	
	n	%	n	%
C	3	14.3	3	14.3
H	1	4.8	3	14.3

n- *S. aureus* with genes encoding SEs

CONCLUSION

The results of this study confirm the presence of pathogenic bacteria in goat and ovine raw milk. The study shows the fact that the consumption of raw milk is not safe for the consumers, and that heat treatment of raw milk before the consumption has a positive meaning.

Our results confirm that unpasteurized milk may be contaminated with different types of microorganisms and can be an important source of foodborne illnesses. The most effective tool for the microbiological safety of milk is pasteurization or other heat treatment. Information on health hazards associated with contaminated raw milk should be extended to the public, so that consumption of untreated raw milk could be avoided.

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THE CONTENT OF TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN RED BEETROOT

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ABSTRACT

Red beetroot (*Beta vulgaris rubra*) is an important raw material of plant origin with proven positive effects on the human body. They can be eaten raw, boiled, steamed and roasted. Red beetroot is a rich source of mineral substances (manganese, sodium, potassium, magnesium, iron, copper). Beetroot contains a lot of antioxidants, vitamins (A, C, B), fiber and natural dyes. Red beetroot is also rich in phenol compounds, which have antioxidant properties. These colorful root vegetables help protect against heart disease and certain cancers (colon cancer). In this work we evaluated content of total polyphenols and antioxidant activity in red beetroot. Samples of plant material were collected at full maturity stages from areas of Zohor, Sihelné, and Sliač. Zohor, Sihelné, and Sliač are areas without negative influences and emission sources. Samples of fresh red beetroot were homogenized and were prepared as an extract: 50 g cut beetroot extracted by 100 ml 80% ethanol for sixteen hours. These extracts were used for analyses. The content of the total polyphenols was determined by using the Folin-Ciocalteu reagent (FCR). The absorbance was measured at 765 nm of wavelength against blank. Antioxidant activity was measured using a compound DPPH' (2,2-diphenyl-1-picrylhydrazyl) at 515.6 nm in the spectrophotometer. In the present experiment it was detected, that total polyphenols content in samples ranges from 820.10 mg/kg to 1280.56 mg/kg. Statistically significant highest value of total polyphenols was recorded in beetroot in variety of *Renova* from locality of Sliač (1280.56 ±28.78 mg/kg). Statistically significant the lowest content of total polyphenols was recorded in beetroot in variety of *Renova* from the village Sihelné (820.10 ±37.57 mg/kg). In this experiment the antioxidant activity in beetroot was evaluated and compared. The values of antioxidant activity were in interval from 19.63% to 29.82%.

Keywords: red beetroot; total polyphenols; antioxidant activity; soil; locality

INTRODUCTION

Plant foods are well known sources of vitamins, such as vitamin C as well as folic acid, carotenoids, and fiber, and they are naturally free of saturated fat and cholesterol (Brat et al., 2006). Fruits and vegetables are rich sources of natural antioxidants such as water soluble vitamin C and phenolic compounds, as well as lipid soluble vitamin E and carotenoids, which contribute both to the first and second defence lines against oxidative stress (Birt et al., 2001; Harborne Williams, 2000; Halliwell et al., 2005).

Vegetable intake has long been associated with a reduced risk of chronic disease, especially cardiovascular disease and certain types of cancer (Williamson, 1996).

Red beetroot (*Beta vulgaris rubra*) is member of the *Chenopodiaceae*. This group contains important food crops, such as *Spinaciaoleracea* (spinach), which is the most consumed *Chenopodiaceae* leafy vegetable in Europe (Ruales and Nair, 1994). Red beetroot (*Beta vulgaris rubra*) seeds leaves and roots are rich in phenolic compounds, whose concentration is dependent on the stage of plant development (Ninfali and Bacchiocca, 2003; Váli et al., 2007). Red beetroot (*Beta vulgaris rubra*) contains a large amount of betalains, a group of numerous

water soluble nitrogen containing pigments derived from betalamic acid. Inside to the betalain group, there are two classes of compounds: the yellow-orange betaxanthin and the red-violet betacyanins (Stintzing and Reinhold, 2004). The principal betacyanin pigment in red beetroot (*Beta vulgaris rubra*) is betanin, which is a betanidin-5-O-β-glucoside. Betanidin is therefore the aglyconic form of the betanin (Kugler et al., 2007). To date, the food colorant extracted from red beetroot (*Beta vulgaris rubra*), known as "red beetroot", is available as E162 in USA and Europe (Castellar et al., 2012).

Red beetroot (*Beta vulgaris rubra*) have been used for a long time for their beneficial health effects, mainly consisting in stimulation of hematopoietic and immune systems as well as in the protection of kidney, liver and gut from toxic compounds. Moreover, they exhibit mineralizing, antiseptic and choleric activities as well as they contribute to the reinforcement of the gastric mucosa (Escribano et al., 1998; Kapadia et al., 2003; Winkler et al., 2005).

Oxidizing reactions occur constantly in the cells as part of aerobic life, resulting in the production of oxygen radicals. Reactive oxygen species, if produced in excess,

may damage various organic substrates, including DNA, proteins, lipids and cell membranes in living cells, and indirectly act as primary or secondary messengers to activate signaling pathways that inflict damage on living cells (Chen et al., 2011). This results in damage, diseases and disorders such as cardiovascular diseases, autoimmune diseases, cancer, rheumatoid arthritis and aging (Pham-Huy et al., 2008).

Antioxidants are substances that, at low concentrations, prevent or retard the oxidation of easily oxidisable biomolecules such as lipids, proteins and DNA. Two major groups of antioxidants are recognised, namely enzymatic and non enzymatic antioxidants (Becker et al., 2004; Ratnam et al., 2006).

Polyphenols are secondary plant metabolites of which

several thousands have been identified in higher plants and several hundred are found in edible plants. Based on their chemical structure, polyphenols are divided in e.g. phenolic acids, flavonoids, stilbenes, and lignans. Polyphenols have antioxidant properties and have several other specific biological properties Manach et al., (2004). The aim of our study was to evaluate the content of total polyphenols and antioxidant activity in red beetroot.

MATERIAL AND METHODOLOGY

Samples of plant material were collected at full maturity stages from area of Zohor, Sihelné, and Sliač. The samples of soil (Table 1, 2 and 3) and plant material were analyzed individually by selected methods, and were used in fresh material on analysis. Zohor is located in the southern

Table 1 Agrochemical characteristic of soil substrate in mg/kg (Zohor).

Agrochemical characteristic	pH	pH	C _{ox}	Humus (%)				
	7.37	6.74	2.05	3.54				
Nutrients	P	K	Ca	Mg				
	558.44	201.60	2061.8	184.50				
Heavy metals	Cd	Pb	Cu	Zn	Cr	Co	Mn	Fe
Content in aqua regia (mg/kg)	1.57	17.3	13.4	115.0	11.0	5.4	547.5	8627.7
Limit value (mg/kg)	0.4	25	30	100	50	15	-	-

Table 2 Agrochemical characteristic of soil substrate in mg/kg (Sihelné).

Agrochemical characteristic	pH	pH	C _{ox} (%)	Humus				
	6.37	5.36	3.95	6.81				
Nutrients	P	K	Ca	Mg				
	294.68	330.9	1623.4	223.0				
Heavy metals	Cd	Pb	Cu	Zn	Cr	Co	Mn	Fe
Content in aqua regia (mg/kg)	3.61	40.9	44.1	190.0	31.8	20.2	1688.2	31452.9
Limit value (mg/kg)	0.7	70	60	150	70	15	-	-

Table 3 Agrochemical characteristic of soil substrate in mg/kg (Sliač).

Agrochemical characteristic	pH	pH	C _{ox} (%)	Humus				
	8.06	6.90	2.37	4.09				
Nutrients	P	K	Ca	Mg				
	219.0	520.0	4953.2	396.2				
Heavy metals	Cd	Pb	Cu	Zn	Cr	Co	Mn	Fe
Content in aqua regia (mg/kg)	3.78	51.5	50.6	395.0	16.9	14.0	982.7	26458.2
Limit value (mg/kg)	0.7	70	60	150	70	15	-	-

part of Záhorská lowland. The altitude of the village is in the middle of 146 m.n.m. Zohor has average annual air temperature 9.5 °C, annual rainfall is 600 mm. Sihelné is located in the northern part of Orava. The altitude of the village is 731 m.n.m. Sihelné belongs to the mild cold climate zone, average annual air temperature is 4 – 7 °C, annual rainfall is 800 – 1200 mm. Sliach is located in the district of Zvolen, one of the central districts of Banská Bystrica. The altitude of the village is in the middle of 305 m.n.m. The average and annual air temperature is 8.2 °C, annual rainfall is 850 mm.

We determined the soil sample from Zohor as sandy, sandy – loam. The soil sample had a value of active soil reaction pH (H₂O) = 7.37. The soil was alkaline. Cox oxidizable carbon content was 2.05 and the humus content was 3.54. The content of potassium and magnesium was good and the content of phosphorus was very high. The total content of heavy metals (aqua regia) in soil sample was determined according to the current legislation Annex. 2 of the Law. 220/2004 Z.z. Cadmium exceeded the limit value of 3.9 times and Zinc exceeded the limit value 1.15 times.

We determined the soil sample from Sihelné as sandy – loam, loam. The soil sample had a value of active soil reaction pH (H₂O) = 6.37. The soil was weakly acidic. Cox oxidizable carbon content was 3.95 and the humus content was 6.81. The content of potassium was high and magnesium good, the content of phosphorus was very high. The total content of heavy metals (aqua regia) in soil sample was determined according to the current legislation Annex. 2 of the Law. 220/2004 Z.z. Cadmium exceeded the limit value of 5.1 times, Zinc exceeded the limit value 1.26 times and Cobalt exceeded the limit value 1.34 times.

We determined the soil sample from Sliach as sandy – loam, loam. The soil sample had a value of active soil reaction pH (H₂O) = 8.06. The soil was strong alkaline. Cox – oxidizable carbon content was determined 2.37 and the humus content was 4.09. The content of potassium, magnesium and phosphorus was very high. The total content of heavy metals (aqua regia) in soil sample was determined according to the current legislation Annex. 2 of the Law. 220/2004 Z.z. Cadmium exceeded the limit value of 5.4.

Determination of total polyphenols

Total polyphenols were determined by the method of **Lachman et al. (2003)** and expressed as mg of gallic acid equivalent per kg fresh mater. Gallic acid is usually used as a standard unit for phenolics content determination because a wide spectrum of phenolic compounds. The total polyphenol content was estimated using Folin-Ciocalteu assay. The Folin-Ciocalteu phenol reagent was added to a volumetric flask containing 100 μ L of extract. The content was mixed and 5 ml of a sodium carbonate solution (20%) was added after 3 min. The volume was adjusted to 50 mL by adding of distilled water. After 2 hours, the samples were centrifuged for 10 min. and the absorbance was measured at 765 nm of wavelength against blank. The concentration of polyphenols was calculated from a standard curve plotted with known concentration of gallic acid.

Determination of antioxidant activity

Antioxidant activity was measured by the (**Brand and Williams et al., 1995**) method-using a compound DPPH' (2,2-diphenyl-1-pikrylhydrazyl).

2,2-diphenyl-1-pikrylhydrazyl (DPPH') was pipetted to cuvette (3.9 cm³) then the value of absorbance, which corresponded to the initial concentration of DPPH' solution in time A₀ was written. Then 0.1 cm³ of the followed solution was added and then the dependence A =f(t) was immediately started to measure. The absorbance of 1, 5 and 10 minutes at 515.6 nm in the spectrophotometer Shimadzu UV/VIS – 1240 was mixed and measured. The percentage of inhibition reflects how antioxidant compound are able to remove DPPH' radical at the given time.

Inhibition (%) = (A₀ - A_t / A₀) x 100

Statistical analysis

Results were statistically evaluated by the Analysis of Variance (ANOVA – Multiple Range Tests, Method: 95.0 percent LSD) using statistical software STATGRAPHICS (Centurion XVI.I, USA).

RESULTS AND DISCUSSION

Red beetroot is ranked as one of the 10 most important vegetables. Part of red beet is the edible roots, which contain from 12 to 20% dry matter, including 4 – 12% sugar, 1.5% protein, 0.1% fat, 0.8% fibre, minerals such as sodium, potassium, phosphorus, calcium, and iron, as well as small amounts of vitamins. It also contains phenolic acids including p-coumaric, protocatechuic, ferulic, vanillic, p-hydroxybenzoic and syringic acids (**Vulić et al., 2012; Kujala et al., 2000**). In this work the content of polyphenols in red beetroot was tested and evaluated.

In the present experiment it was detected, that total polyphenols content in samples ranges from 820.10 \pm 37.57 mg/kg to 1280.56 \pm 28.78 mg/kg in varieties of red beetroot (Table 4). **Ninfali et al. (2013)** published that the content of total polyphenols was recorded in red beetroot in the interval from 720 to 1276 mg/kg. In comparison to our determined values of polyphenols their results were in similar interval. Statistically significant highest value of total polyphenols was recorded in red beetroot in variety of *Renova* from locality of Sliach (1280.56 \pm 28.78 mg/kg). **Čanadanovič- Brunet et al. (2011)** reported that the polyphenols in red beetroot was in amounts 3764 mg/kg. In comparison to our measured values their results were higher. Statistically significant the lowest content of total polyphenols was recorded in red beetroot in variety of *Renova* from the village Sihelné (820.10 \pm 37.57 mg/kg). **Wootton- Beard et al. (2011)** determined the content of polyphenols in beetroot. Their values were in similar interval 617.8 to 1450.3 mg/kg as our values.

Číž et al. (2010) referred that the content of total polyphenols was 815 mg/kg. In comparison to our measured values their results were lower. Polyphenols are thought to be particularly important in heart disease, hypertension and age-related degeneration.

From the results we can conclude that the highest content of total polyphenols in variety of *Renova* we measured in locality Sliach (1280.56 mg/kg), followed by red beetroot from Zohor (1139.82 mg/kg) (Figure 1). In this variety we recorded the lowest value of polyphenols in red beetroot

from locality of Sihelné (820 mg/kg). In the case of variety *Monorubra* we determined the highest value of total polyphenols in samples from area of Sliač (1201.6 mg/kg), followed by red beetroot from Sliač (1023.21 mg/kg) and Zohor (988.66 mg/kg). The difference between the highest and lowest parameter of polyphenol content in variety of *Renova* was 460.56 mg/kg and in variety of *Monorubra* was 212.94 mg/kg. From the measured results we conclude that the varieties (*Renova*, *Monorubra*) from Sliač had higher content of total polyphenols, which may be the result of high humus (4.0%) and potassium (520 mg/kg) content in the soil. Many authors reported that the growing area and the agrochemical composition are important environmental factors involved in the production of polyphenolic substances. The total content of polyphenolic compounds is quite variable, may be affected by postharvest climatic conditions.

Another indicator that has been evaluated and compared was the antioxidant activity of red beetroot. The

antioxidants reduce the risk for chronic diseases- cancer and heart. The main function of an antioxidant in plants is its ability to trap free radicals. Polyphenols and flavonoids scavenge free radicals (peroxide, hydroperoxides, lipidperoxyl). In the present work it was detected, that antioxidant activity in samples ranges from 19.63 ± 0.90 to 29.82 ± 0.55 % (Table 5). **Holásová et al. (2011)** said that the value of antioxidant activity was 36%. Statistically significant highest value of antioxidant activity was recorded in red beetroot in variety of *Monorubra* from Sihelné (29.82 ± 0.55). **Georgiev et al. (2010)** reported that value of antioxidant activity in beetroot was in the interval from 14.2% to 90.7%. In comparison to our measured values their results were higher. Statistically significant the lowest content of total polyphenols was recorded in red beetroot in variety of *Renova* from Sihelné (19.63 ± 0.90).

Kaur et al. (2002) referred that the value of antioxidant activity in red beetroot was in ethanol extract 73.3% and in water extract 55%. Their values of antioxidant activity in beetroot were higher.

Table 4 Average content of total polyphenols (mg/kg) in red beetroot.

Locality	Variety	TPC (mg/kg)
Sliač	<i>Renova</i>	1280.56 ±28.78 e
	<i>Monorubra</i>	1023.21 ±28.64 b
Zohor	<i>Renova</i>	1139.82 ±35.96 c
	<i>Monorubra</i>	988.66 ±45.91 b
Sihelné	<i>Renova</i>	820.10 ±37.57 a
	<i>Monorubra</i>	1201.60 ±18.62 d
HD 95%		50.0487
HD 99%		68.5709

LSD Test on the significance: α : <0.05

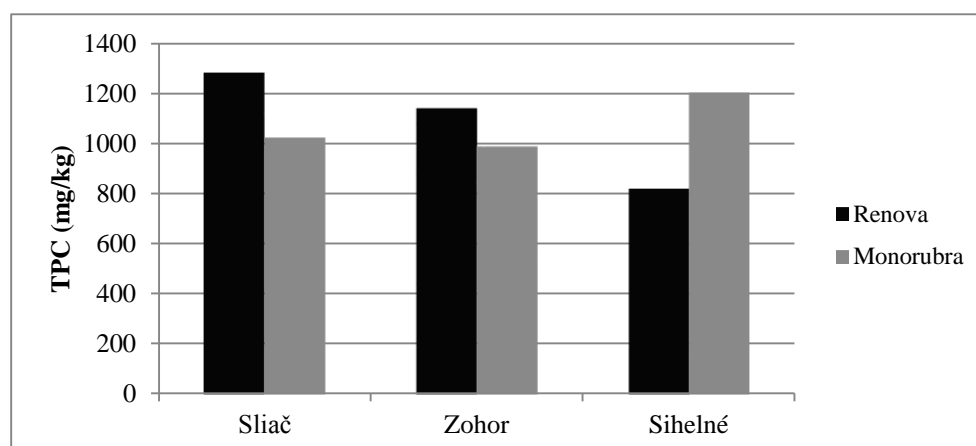


Figure 1 The content of total polyphenols (mg/kg) in red beetroot.

Table 5 Average values of antioxidant activity (% inhibition) in red beetroot.

Locality	Variety	AOA (% inhibition)
Sliach	<i>Renova</i>	28.43 ±0.90 e
	<i>Monorubra</i>	25.48 ±0.58 d
Zohor	<i>Renova</i>	24.03 ±0.77 c
	<i>Monorubra</i>	22.85 ±0.74 b
Sihelne	<i>Renova</i>	19.63 ±0.90 a
	<i>Monorubra</i>	29.82 ±0.55 f
HD 95%		1.12856
HD 99%		1.54622

LSD Test on the significance: α : <0.05

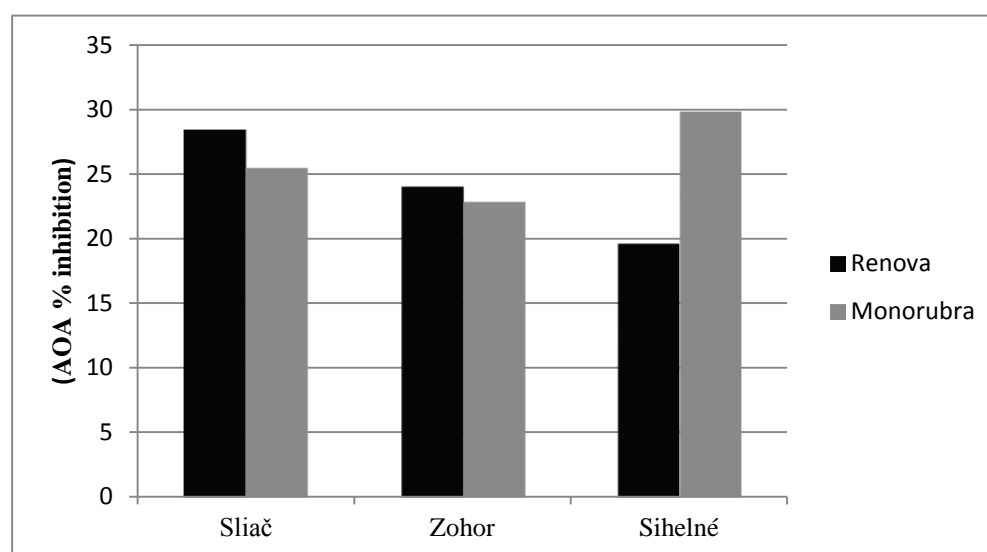


Figure 2 Values of antioxidant activity AOA (% inhibition) in red beetroot.

The above our values showed that the highest value of antioxidant activity in the case of variety *Monorubra* was in samples from Sihelne (29.82%), followed by red beetroot from Sliach (25.48%) (Figure 2). In this variety we recorded the lowest value of antioxidant activity in beetroot from locality of Zohor (22.85%). In the case of variety of *Renova* we determined the highest value of antioxidant activity in samples from area of Sliach (28.43%), followed by beetroot from Zohor (24.03%) and Sihelne (19.63%). The difference between the highest and lowest parameter of antioxidant activity in variety of *Renova* was 8.8% and in variety of *Monorubra* was 6.97%.

CONCLUSION

The present paper was focused on the content of total polyphenols and antioxidant activity in red beetroot. The results suggest that red beetroot contains higher amount of polyphenolic substances. Red beetroots are grown as vegetable rich in minerals and microelements substances. It is also a rich source of health promoting biologically active compounds (polyphenols, flavonoids, anthocyanins, dark red betaine, choline and organic acids). Values of

polyphenolic compounds contained in red beetroot are quite variable. The content of total polyphenols and antioxidant activity in red beetroot may be influenced by variety, growing and postharvest conditions. The content of chemoprotective compounds may be affected also by agrochemical composition of the soil for example content of nutrients, humus and climatic condition. The results obtained in this work provide further information about of the content of total polyphenols and antioxidant activity in red beetroot.

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THE EFFECT OF PATERNAL BULL ON MILK FAT COMPOSITION OF DAIRY COWS DIFFERENT BREEDS

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ABSTRACT

Intake of milk fat in human nutrition is important because of unsaturated and especially essential fatty acids (FAs), linoleic and α -linolenic acid, and conjugated linoleic acid (CLA), which is found only in meat and milk of ruminants. The objective of our study was to investigate the effect of paternal bulls on fatty acids composition in milk fat of dairy cows of different breeds. The milk samples were taken in total from 299 dairy cows from 11 dairy farms. In experiment Holstein (H, n = 105), Red Holstein (R, n = 120) and Pinzgau (P, n = 74) breeds originated from different bulls were used. Individual milk samples were analyzed for fatty acids in milk fat using gas chromatography (apparatus GC Varian 3800, Techtron, USA), using FID detector in capillary column Omegawax 530; 30 m. In the chromatography records there were identified 54 fatty acids inclusive of particular isomers. Their relative proportions were expressed in percent's (%). Among the studied breeds, the highest content of conjugated linoleic acid (CLA) – 0.67%, essential FAs (EFA) – 2.98%, monounsaturated FAs (MUFA) – 25.84% and the lowest atherogenic index (AI) – 3.10 was at breed P. Within this breed there was high variability and daughters of bull COS1 achieved significant above-average values of CLA content 1.07%, EFA 3.71%, MUFA 29.93% and under breed average AI = 2.40. The group of daughters of NOB3 was significant lower in CLA, 0.50% as compared with an average of P breed. . From the breed H bull MTY2 showed significantly higher value of 0.62% CLA, EFA 3.42%, 34.29% MUFA and lower value of AI, 1.9 as compared to H breed average. Statistically significantly lower levels of CLA 0.29% and 21.46% MUFA and higher AI 3.72 in milk fat of his daughters, bull STY3 may be considered as potential worsener of these properties. At the breed R bull MOR506 showed in compar to the breed average significantly higher value of the EFA 3.80% and also the higher content of CLA 0.50% and MUFA 25.09%, resulting in statistically significant lower AI = 2.91. Bull MOR506 could be considered as potential improver of milk fat composition. The above described variability in the composition of milk fat of dairy cows and the subsequent relationships between these values suggest that the selection of the bull according to the fatty acid composition of milk fat may be considered.

Keywords: cow; milk; fatty acid; paternal effect; bull

INTRODUCTION

In recent decades, the intake of milk fat in human nutrition considered a negative factor in relation to cardiovascular disease. The content of saturated acids C12:0, C14:0 and C16:0 is usually expressed in the so-calculated atherogenic index (AI). But the intake of milk fat in human nutrition is important because of unsaturated and especially essential fatty acids (EFA), linoleic and α -linolenic acid, and especially conjugated linoleic acid (CLA), which is found only in meat and milk of ruminants. Detailed examination brought knowledge of bioactive components in milk fat, which has a beneficial effect on the human body. In particular, the polyunsaturated fatty acids (PUFA) regulate the function of cell membranes and serve as precursors of bioactive mediators. Health effects of EFA are gaining prominence within the latest medical research, thus recognizing the importance of milk fat in the human diet. The latest research confirms, besides of nutrition of dairy cows, the importance of genetic effects on the composition of fatty acids in milk fat, and therefore consider it necessary to

evaluate this regard at breeds reared in Slovakia. **Capps et al. (1999)** described the Jersey breed cows that produce lower levels of conjugated linoleic acid (CLA) than Holstein when they were fed complete feed mixture with the addition of hay. By **Palmquist et al. (1993)** Holstein dairy cows produce 8 – 42% more short to medium chain fatty acid (C6:0 - C14:0), compared to the Jersey. Jersey dairy cows produced 13% more stearic acid (C18:0) and less oleic acid (C18:1). **Hanuš et al. (2008)** found insignificant differences ($p > 0.05$) between breeds Czech Pied and Holstein, where SAFA 63.97 < 65.80% and unsaturated fatty acids (USFA) 34.62 > 32.87%. In CLA content there was a clear difference 0.80 > 0.55% ($p < 0.05$). **Samková et al. (2012)** in comparison of two breeding groups of individual milk samples (Czech spotted, n = 78; Holstein, n = 86) found a difference in the representation of lauric acid (C12:0, 4.69 > 4.42%) and palmitic acid (C16:0, 32.75 < 34.1%, $p < 0.05$), and no significant differences in the C18 acids. An interesting difference (close to statistical significance) was found for the representation of health desirable CLA (0.42 > 0.38%).

The mentioned dependence shows that the use of selection for the desired change in the composition of fatty acid profile of milk fat could be possible. Higher organisms are able to incorporate double bonds in molecule by means of dehydrogenating enzyme system and reactions of chain growth (Jenkins and McGuire, 2006). Soyeurt (2010) deals with assessing the breeding value of 1993 bulls based on data corresponding to the number of their daughters (measured by a fast method FTMIR) and states that the heritability of saturated fatty acids (SAFA) and monounsaturated fatty acids (MUFA) were 44% and 22%, which means that there exists the variability needed for the development of selection in order to improve the nutritional quality of milk fat.

The objective of this paper is the study of the composition of milk fat of dairy cows of different breeds in Slovakia and to analyse the potential genetic impact of bull's sire on the content of medically important milk fatty acids in the milk of their daughters.

MATERIAL AND METHODOLOGY

The milk samples were taken from 299 dairy cows from 11 dairy farms. In the experiment primiparous Holstein

(H, n = 105), Red Holstein (R, n = 120) and Pinzgau (P, n = 74) breeds originated from different bulls were used. The dairy cows were at first lactation on different number of days in milk evenly distributed in the interval of 10 – 111 days. The milk was sampled from the whole amount of milked milk at regular milk recording performed by The Breeding Services of the Slovak Republic.

Milk fat of individual milk samples was analyzed for fatty acid composition. Milk fat was isolated from lyophilised milk samples by extraction in petroleum ether according to Röse-Gottlieb, then it was re-esterified by methanol potassium hydroxide solution, and methyl esters of fatty acids were extracted by hexane. Methyl esters of fatty acids were analysed by gas chromatography (apparatus GC Varian 3800, Techtron, USA), using FID detector in capillary column Omegawax 530; 30m. Irregular temperature gradient from 40 to 240 °C, injection and detection at 250 °C were used. Nitrogen flow rate was 6 ml.min⁻¹ (Samková et al. 2009). In the chromatography records were identified 54 fatty acids inclusive of particular isomers. Their representation was expressed relatively in percentage of peak areas (%). Groups of fatty

Table 1 The average values (%) of daughters groups of bulls Pinzgau breed

n	bulls	\bar{x}	S \bar{x}	p-value
CLA				
8	COS1	1.07	0.42	0.0298
74	\bar{x}	0.67	0.23	
8	LOZ2	0.66	0.20	0.9048
10	SBA1	0.62	0.16	0.4757
10	NOB3	0.50	0.15	0.0086
EFA				
8	COS1	3.71	1.11	0.1064
10	SBA1	2.99	0.77	0.9527
74	\bar{x}	2.98	0.84	
8	LOZ2	2.90	0.71	0.7661
10	NOB3	2.88	0.53	0.6231
MUFA				
8	COS1	29.93	4.60	0.0415
74	\bar{x}	25.84	3.44	
10	SBA1	25.77	2.29	0.9294
8	LOZ2	24.79	3.91	0.4888
10	NOB3	24.33	2.37	0.1022
AI				
8	LOZ2	3.39	0.79	0.3475
10	NOB3	3.26	0.45	0.3675
74	\bar{x}	3.10	0.56	
10	SBA1	2.96	0.46	0.4120
8	COS1	2.40	0.70	0.0246

acids and their abbreviation as well as calculated atherogenic indexes (AI) were created according to traditional structural chemical and nutrition criteria in line with works given in References. The analytical data were assessed professional statistical software SAS version 9.2 (SAS 2002) module in SAS STAT. For statistic evaluation of the bull effect only bulls with higher number of daughters were used.

RESULTS AND DISCUSSION

The evaluation of the content of medically significant fatty acids and their groups was made with respect to the bull of the monitored cows. In order to assess the possibility of genetic influence on the composition of fatty acids in milk fat of dairy cows we evaluated groups of cows according to their bull in comparison with the results of the whole group of cows of the some breed. As the healthiest important, we evaluated the content of positive CLA, EFA, MUFA and on the opposite, the AI, which relatively high value is undesirable.

Pinzgau breed (Table 1)

Essential fatty acids (EFA) were the highest in the milk of P breed (2.98%). Of these, the most nutritionally

important conjugated linoleic acid (CLA) had among studied breeds significantly highest proportion in milk of P breed (0.67%). It is believed that the establishment of heritability is related to the extension of the ruminal trans-11 C18: 1 and a lower rate of cis-9, trans-11 CLA, and to the amount and activity of the delta9-desaturase in mammary tissue (Kelsey et al., 2003). Medrano et al., (1999) revealed a difference between breeds in the enzyme activity of stearyl-CoA desaturase, which oxidizes the palmitic (C16:0) and stearic (C18: 0) acid to palmitoleic (C16:1) and oleic (C18:1) acid and acts in the production of CLA.

The highest average CLA content among the studied breeds showed P breed (0.67%). In the context of this breed as a statistically significant higher value ($p < 0.05$) can be evaluated the average value of the daughters of the bull COS1 1.07%, where there were 8 observations. Since the P breed had the highest content of CLA in milk fat among all studied breeds there were even daughters groups with values that were lower than the breed average of favorable results than in other breeds. The daughters of these bulls had relatively high levels of CLA in milk fat (LOZ2 0.66%, 0.62% SBA1). NOB3 was from an average

Table 2 The average values (%) of daughters groups of bulls Holstein black pied breed.

n	bull	\bar{x}	$S_{\bar{x}}$	p-value
CLA				
10	MTY2	0.62	0.17	0.0028
22	LU521	0.47	0.11	0.0939
106	\bar{x}	0.41	0.19	
7	STY3	0.29	0.05	0.0002
9	WEL2	0.21	0.05	0.0000
EFA				
10	MTY2	3.42	0.64	0.0069
7	STY3	2.77	0.34	0.6790
22	LU521	2.76	0.45	0.6694
106	\bar{x}	2.71	0.62	
9	WEL2	2.37	0.43	0.2376
MUFA				
10	MTY2	34.29	3.50	0.0000
106	\bar{x}	25.66	5.75	
22	LU521	25.15	3.32	0.5759
9	WEL2	24.25	6.07	0.8302
7	STY3	21.46	1.29	0.0000
AI				
7	STY3	3.72	0.32	0.0014
9	WEL2	3.39	0.99	0.9199
106	\bar{x}	3.11	0.84	
22	LU521	3.05	0.53	0.6516
10	MTY2	1.93	0.31	0.0000

Table 3 The average values (%) of daughters groups of bulls Red Holstein breed.

n	bull	\bar{x}	$S_{\bar{x}}$	p-value
CLA				
7	MOR506	0.50	0.14	0.5409
10	BS46	0.49	0.11	0.9896
7	BW8	0.47	0.11	0.1145
6	KOB505	0.46	0.12	0.3063
120	\bar{x}	0.43	0.10	
7	MES1	0.40	0.08	0.7094
10	ZAK1	0.34	0.07	0.8351
EFA				
7	MOR506	3.80	0.54	0.0015
7	BW8	2.97	0.41	0.1770
7	MES1	2.94	0.49	0.3178
10	BS46	2.82	0.29	0.3964
120	\bar{x}	2.73	0.53	
10	ZAK1	2.44	0.37	0.0425
6	KOB505	2.43	0.23	0.0203
MUFA				
7	MOR506	25.09	2.52	0.5794
10	BS46	24.80	3.55	0.8007
10	ZAK1	24.72	5.57	0.9078
120	\bar{x}	24.50	3.57	
7	BW8	24.22	1.41	0.6632
6	KOB505	22.52	1.74	0.0375
7	MES1	22.00	1.65	0.0055
AI				
7	MES1	3.72	0.30	0.0097
6	KOB505	3.64	0.38	0.0930
10	ZAK1	3.45	1.10	0.6980
10	BS46	3.36	0.65	0.8390
120	\bar{x}	3.31	0.69	
7	BW8	3.18	0.24	0.2470
7	MOR506	2.91	0.40	0.0393

of P breed statistically significant ($p < 0.01$) lower in CLA, 0.50%. Within this breed NOB3 seems to be worse of this feature, but even this value is higher than the mean values of breeds with low CLA.

The highest content of essential fatty acids (EFA) showed P breed, 2.98%. Bull COS1 showed a group of eight daughters with an average 3.71%. Bull NOB3, which had the lowest value of CLA seems also at EFA (2.88%) to be a worse within a breed, but compared breeds H and R has a higher content of EFA.

In group of daughters of the same bull COS1, the highest content of MUFA was found out, as it was mentioned above for CLA and EFA. Daughters of bull COS1 showed

statistically significant ($p < 0.05$) higher MUFA content of 29.93% than average value of the P breed 25.84%. Conversely, COS1 daughters had statistically significant ($p < 0.05$) lower AI (2.40) as the average of the P breed (3.10). NOB3 bull whose daughter had a lower content of CLA, EMC and MUFA was compared to the breed average higher ranked AI (3.26).

Holstein black pied – H breed (Table 2)

H breed showed low average CLA content of 0.41% as compared with P breed (a similar value as observed by Hanuš et al. (2008) and Samková et al. (2012)), 2.71% EFA, MUFA 25.66% and average AI = 3.11. Daughter of the bull MTY2 had significantly higher value of 0.62%

CLA, EFA 3.42%, 34.29% MUFA and lower ($p < 0.0001$) value of AI, 1.93 as compared to average value. Statistically significantly lower levels of CLA 0.29% ($p < 0.005$) and 21.46% MUFA ($p < 0.0001$) and higher AI 3.72 ($p < 0.005$) in milk fat of daughters could indicate a bull STY3 as a potential worser of these properties.

Red Holstein – R breed (Table 3)

R breed showed lower content of CLA 0.43%, EFA 2.73% and MUFA 24.50% and the highest average AI = 3.31 as compared to the average breed P. Each component about the same level with the H breed. Bull MOR506 had higher value of the EFA 3.80% ($p < 0.005$) and also the higher content of CLA 0.50% and MUFA 25.09% as compared to the breed average, resulting in statistically significant ($p < 0.05$) lower AI = 2.91. It puts bull MOR506 in to the role of potential improver. Soyeurt (2010) stated that the heritability of SAFA and MUFA were 44% and 22%, which means that there exists the variability needed for the development of selection in order to improve the nutritional quality of milk fat.

Bulls KOB505 and MES1 who had statistically significant higher AI than the breeds average (3.64, $p < 0.1$, respectively 3.72, $p < 0.01$), had statistically significant lower content of MUFA (22.52%, $p < 0.05$, respectively 22.00%, $p < 0.01$) while the CLA and EFA content was different from the mean relatively little. These results sort bull KOB505 and MES1 to potential worser of milk fatty acids composition.

CONCLUSION

Analysis of the bulls on the basis of their daughters suggests that the genetic influence on the composition of milk fat is applicable and could not be excluded. It would be appropriate to evaluate the impact of fathers using exact genetic methods. These results indicate that it was possible to find such bulls who have a positive effect on properties of the milk fat. Among the studied breeds, the highest content of conjugated linoleic acid (CLA) – 0.67%, essential FAs (EFA) – 2.98%, monounsaturated FAs (MUFA) – 25.84% and the lowest atherogenic index (AI) – 3.10 was found out at P breed. Within this breed there was high variability and daughters of bull COS1 achieved significant above-average values of CLA content 1.07%, EFA 3.71%, MUFA 29.93% and under breed average AI = 2.40. The group of daughters of NOB3 was from an average of P breed statistically significant lower in CLA, 0.50%. From the H breed bull MTY2 showed compared to H breed average significantly higher value of 0.62% CLA, EFA 3.42%, 34.29% MUFA and lower value of AI, 1.9. Statistically significantly lower levels of CLA 0.29% and 21.46% MUFA and higher AI 3.72 in milk fat of his daughters, bull STY3 may be considered as potential worser of these properties. At the R breed bull MOR506 showed significantly higher value of the EFA 3.80% and also the higher content of CLA 0.50% and MUFA 25.09%, as compared to the breed average resulting in statistically significant lower AI = 2.91. It considers bull MOR506 in to the role of potential improver of milk fat composition. The above described variability in the composition of milk fat of dairy cows and the subsequent relationships between

these values suggest that the selection of bulls according to the fatty acid composition of milk fat may be considered.

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ACRYLAMIDE CONTENT AND ANTIOXIDANT CAPACITY IN THERMALLY PROCESSED FRUIT PRODUCTS

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ABSTRACT

Acrylamide as a known processing contaminant was determined in various heat-treated plum products purchased from a local market using LC/ESI-MS-MS. The highest level of acrylamide in the range up to 60 µg/kg was detected in a plum stew known as a “povidla“, and in prunes, respectively. These products typically undergo intensive heat treatment that may take from several hours to days. Using a fruit dehydrator in home production of prunes, a low level of acrylamide under LOQ (15 µg/kg) was detected in comparison to most commercial products. Only in one of the prune samples from the market was the acrylamide content near to LOQ. The highest content of acrylamide (46 µg/kg) was detected in the Slovak sample of prune originated in Nitra region. High acrylamide content, in the range from 23 to 45 µg/kg, was observed in prunes from South America. In the rest of analysed heat-treated plum products such as plum juice, plum compote or baby food with plum puree, acrylamide was not detected due to moderate conditions during thermal processing: temperature below 120 °C and a shorter time of thermal exposure. The total phenolic content and antioxidant capacity of prunes were analysed using a UV-VIS-NIR spectrophotometer and an electron paramagnetic resonance (EPR) spectroscopy. Home-prepared prunes were characterized by the highest content of phenolics (4780 mg GAE/kg) and antioxidant capacity (14.6 mmol TEAC/kg). Commercial samples of prunes reached phenolics in the range from 1619 to 3461 mg GAE/kg, and antioxidant capacity was observed between 6.1 and 12.1 mmol TEAC/kg. Antioxidant capacity of prunes strongly correlated with total phenolic content and yellow and red colours measured in a CIELab system. However, no significant correlation between the acrylamide and antioxidative or organoleptic properties of prunes was observed. Moreover, it was noticed that bio production of plums did not demonstrate any positive impact on final acrylamide content or antioxidant capacity in comparison to conventional technology.

Keywords: acrylamide; fruit; thermal processing; antioxidant capacity

INTRODUCTION

Thermal processing is frequently used in food manufacturing to obtain safe products with prolonged shelf-life and has a strong impact on the final quality of products. One of the purposes of thermal input is to improve the sensory properties, palatability and to extend the intensity of colour, tastes, aromas and textures of food. However, undesired effects due to various chemical reactions being Maillard reaction, caramelization and lipid oxidation are the most prominent (Capuano and Fogliano, 2011). On the other hand, it is well known that some substances arising from the heating processes can play a positive role on human health. Many neo-formed compounds showing antioxidative, antimicrobial, antiallergenic effects or modulation activity in vitro have been observed (van Boekel et al., 2010). In addition to these positive effects, some detrimental consequences of thermal processes must be carefully evaluated, such as the loss of thermolabile compounds (vitamins, essential amino acids - lysine, tryptophan) or the formation of undesired tastes and off-flavours. Moreover, a major concern arising from heating processes comes from the formation of

compounds that are not naturally present in foods, but which may develop during heating or preservation processes and which reveal harmful effects such as mutagenic, carcinogenic and cytotoxic effects known as neo-formed contaminants. Well-known examples of these compounds are heterocyclic amines, nitrosamines, polycyclic aromatic hydrocarbons (Knize et al., 1999) and recently discovered acrylamide with a high toxicological potential.

Acrylamide is formed during thermal processing of many types of foods. The highest acrylamide levels have been found in fried potato products, bakery wares and coffee (Ciesarová, 2013). Acrylamide levels in food monitored between 2007 and 2010 have been compiled by the European Food Safety Authority (EFSA) in the Scientific Report (EFSA, 2010 and 2012). On the basis of investigation results obtained during 2011 and 2012, and on the basis of the monitoring results obtained pursuant to Recommendations 2007/331/EC and 2010/307/EU, it was appropriate to modify certain indicative values provided for in the Annex to the Commission Recommendation 2013/647/EU. However, acrylamide has been found in

food products other than those listed in the report of EFSA such as hazelnuts, almonds, olives or dried fruits (Amrein et al., 2007). Due to a lack of relevant information in literature and databases, the presented study focused on acrylamide analysis in thermally processed plum products available on the local market and consequently in correlation with beneficial properties of thermally processed fruit such as total phenolic content and antioxidant capacity.

MATERIAL AND METHODOLOGY

Samples of various thermally processed fruit products produced from plum (*Prunus domestica*) were purchased from a local market in the Slovak Republic with focus on dried plums (prunes) and plum stew, moreover in juice, compote and baby food.

A total of 8 samples of prunes with various origin were analysed listed in detail in Tab. 1. Three samples of traditional plum stew known as '*povidla*' produced by different companies from the Czech Republic and the Slovak Republic (represents one sample with declared 5% content of apples), plum compote (halved, pitted, sterilized) produced in Hungary, plum juice with 25% of fruit juice content (from plum puree and plum juice concentrates) originated in Austria, two samples of baby food with various content of plums (80% plums, 20% apples) from the Czech Republic, and baby food originated from Hungary with 50% plum content and with declared bio-production and starch addition.

Samples were collected for acrylamide and colour analysis and further evaluations of total phenolic content and antioxidant capacity.

Acrylamide determination

Acrylamide (ACR) was extracted from samples into water and pre-extracted into ethylacetate with an internal standard D3-acrylamide addition according to Bednáriková and Ciesarová (2012) and Ciesarová et al. (2009). Acrylamide was analysed by LC/ESI-MS-MS using an HPLC system 1200 Series (Agilent Technologies, USA) with positive electrospray ionization (ESI+) and mass spectrometer 6460 Triple Quad detection with LOQ of 15 µg/kg. The analytical separation was performed on Atlantis dC18 column (100 mm x 2.1 mm, 1.8 µm particle size; Waters, Milfor, MA, USA) using isocratic mixture of 1 % of methanol and 0.2 % of glacial acetic acid in water at flow rate 0.4 ml/min at ambient temperature.

Total phenolic content and antioxidant capacity

Prune sample was mixed with 50% ethanol (1 h, 200 rpm), centrifuged (10 000 rpm, 10 min, 20 °C) and appropriately diluted.

Total phenolic content in prune samples was determined by Folin-Ciocalteu method (Singleton et al., 1999) and expressed as an equivalent of gallic acid (GAE, mg/kg). UV-VIS-NIR spectrophotometer UV-3600 (Shimadzu, Japan) was used.

Antioxidant capacity was measured by an EPR spectroscopy and the results were expressed as Trolox equivalent using a standard solution of ABTS cation radical (TEAC_{ABTS}^{•+}) according to (Polovka et al., 2010).

Colorimetric Analysis

Measurement of product colour was carried out using a UV-3600 spectrophotometer (Shimadzu, Japan) in reflectance mode with 10° Observer, D65 Illuminant and wavelength range 380 – 780 nm. CIELAB parameters L*, a*, b* were collected and a hue angle as an attribute of a visual sensation was calculated according to the formula

$$\text{Hue} = \tan^{-1} \left(\frac{b^*}{a^*} \right)$$

Statistical Analysis

All results presented are means of three replicates along with standard deviations. Correlation coefficients were determined between colour parameters, antioxidant capacity, phenolic compounds and acrylamide content.

RESULTS AND DISCUSSION

Acrylamide content in thermally processed fruit products are not restricted till now by the law, however the European Commission have proposed an indicative value of acrylamide for baby foods of 50 µg/kg, and for products containing prunes of 80 µg/kg, respectively (EC Recommendation, 2013). In our study, acrylamide content below LOQ (<15 µg/kg) was detected in the products of plum juices, canned sterilised plum products and baby food in general. On the other hand, a significant content of acrylamide was detected in plum stew and prunes which were consequently subjected to further deeper study focused on an evaluation of beneficial properties and correlations with visual parameters measured in a CIELab system.

Acrylamide content in plum stew

The highest content of acrylamide was detected in traditional stew prepared from plums known in Czech, Slovak or Polish market as a '*povidla*'. This type of product produced by traditional technology is characterized with dark colour and a very thick consistency that is most suitable as a filling of dumplings. Traditional '*povidla*' is prepared by long term boiling without any addition of sugar or other additives from ripe fruits, harvested as late as possible, ideally after the first frosts, in order to ensure they contain enough sugar. For this moderate thermal treatment the authentic samples of plum stew were characterized by presence of acrylamide in concentration of 60 µg/kg ±5 µg/kg (data not shown). On the other hand, acrylamide content in the commercial sample of plum stew with declared 5% addition of apples was not detected.

Acrylamide content in prunes

Similarly to plum stew also prunes are typically processed by heat treatment, therefore in this type of plum products a significant acrylamide content was determined in concentrations comparable to plum stew. The drying process of prunes is intensive and slow, taking a long time to complete, up to 35 hours, depending on the drying conditions (Sabarez, 2012).

Table 1 Acrylamide, total phenolic compounds, antioxidant capacity and colour evaluation of prune samples.

Origin	Production	Acrylamide content (µg/kg)	Antioxidant activity (mmol TEAC/kg)	Total phenolic content (mg GAE/kg)	L*	a*	b*	Hue
Slovak Republic	Home	<LOQ	14.6 ±0.2	4780 ±76	-	-	-	-
Slovak Republic	Conv.	46 ±4	6.1 ±0.1	2599 ±34	69	3.6	3.2	41.5
France	Conv.	<LOQ	6.3 ±0.1	1997 ±12	72	3.0	2.4	39.1
Turkey	Conv.	19 ±3	11.5 ±0.3	3385 ±75	74	1.9	1.3	33.6
USA	Conv.	45 ±2	9.2 ±0.1	3461 ±14	71	4.2	4.7	48.2
Argentina	BIO	36 ±2	12.1 ±0.1	3426 ±21	68	4.6	4.8	46.4
USA	BIO	33 ±3	7.0 ±0.1	2503 ±34	64	2.8	1.2	23.4
Chile	Conv.	23 ±2	6.4 ±0.3	1619 ±45	73	3.4	3.0	40.9

Table 2 Correlation analysis.

Acrylamide content (µg/kg)	Antioxidant activity (mmol TEAC/kg)	Total phenolic content (mg GAE/kg)	L*	a*	b*	Hue
1						
0.1431	1					
0.4816	0.8388	1				
0.5163	-0.2392	-0.2123	1			
-0.3471	-0.7267	-0.4777	-0.2667	1		
-0.2299	-0.7458	-0.5061	0.0253	0.9442	1	
0.0148	-0.7686	-0.5378	0.4037	0.7533	0.9131	1

Results of acrylamide analysis of a sample of prunes obtained from the local market and a sample of home-prepared prunes using a kitchen dehydrator are summarized in Table 1. The lowest acrylamide content was determined in domestically prepared prunes as well as in the product of European origin (France) in concentration near to LOQ (<15 µg/kg). The rest of prune products obtained from market contained acrylamide in the range from 19 µg/kg (Turkey) to 46 µg/kg (Slovakia, obtained from marketplace in Bratislava, produced in Nitra region). Samples from South America were characterized by an acrylamide content in the range from 23 to 45 µg/kg. Higher acrylamide content led to an assumption of more intensive heat treatment of raw material or other specific technological procedure. Moreover, in prunes declared with bio origin, the acrylamide content was 33 and 36 µg/kg, respectively. It can be concluded that bioproduction declared on the packaging did not result in lower acrylamide content.

Total phenolic content and antioxidant capacity of prunes and their correlations

Prunes contain naturally high levels of fibre and have been shown to have one of the highest antioxidant levels of

the common fruits and vegetables (Stacewicz-Sapuntzakis et al., 2001; Cantu-Jungles et al., 2014; Jarvis et al., 2015). Aforementioned beneficial properties of prunes expressed as antioxidant capacity and total phenolic content are summarized in Table 1. Home-prepared prunes were distinguished from commercial samples by the highest content of phenolics (4780 mg GAE/kg) as well as antioxidant capacity (14.6 mmol TEAC/kg). Commercial samples of prunes were low in both phenolics (from 1619 to 3461 mg GAE/kg) and antioxidant capacity (from 6.1 to 12.1 mmol TEAC/kg). Phenolics, commonly found in fruits, have been reported to exhibit antioxidant activity due to the reactivity of the phenol moiety, and have the ability to scavenge free radicals (Donovan et al., 1998). A correlation analysis presented in Table 2 pointed out that antioxidant capacity of prunes strongly correlated with total phenolic content (a correlation coefficient 0.8388). However, acrylamide content as a potentially harmful compound was not correlated with health beneficial properties such as the total phenolic content or antioxidant capacity of products (correlation coefficient 0.4816 and 0.5163, respectively).

Colour of prunes and correlations with beneficial and health hazardous compounds

Colour of prune samples obtained from market varied in visual colour sensation expressed as a hue angle in the range from 23.4 to 48.2. The hue value parameter was in a good correlation with the antioxidant capacity of samples (a correlation coefficient -0.7686). On the basis of individual colour parameters measured in the CIElab system it was observed that antioxidant capacity correlated with redness (a^*) and yellowness (b^*) (correlation coefficients of -0.7267 and -0.7458, respectively), although not with colour saturation (L^*) (a correlation coefficient -0.2392).

On the other hand, statistical analysis presented in Table 2 did not show any significant correlations with neither acrylamide content nor colour of the product (a correlation of 0.0148 for hue angle), that excludes the possibility of a visual estimation of the risk of acrylamide formation in this type of product, which would be useful in practice.

CONCLUSION

The significant content of acrylamide was analysed in dried fruits from plums and plum stews in concentrations up to 60 µg/kg. Acrylamide content was not detected in thermally processed fruit products from plums, in sterilised canned plums, plum juice or baby food. Acrylamide, as a potentially harmful compound, was not correlated with health beneficial properties such as total phenolic content or antioxidant capacity of products.

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UTILIZATION OF CITRUS CROPS PROCESSING BY-PRODUCTS IN THE PREPARATION OF TARHANA

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ABSTRACT

After processing of citrus fruits (e.g. lemon, orange, grapefruit, mandarin) for juice and essential oils production, approximately 50% of the original fruit mass is left as waste material. Citrus crops processing by-products are valuable components as they contain nutrients such as pectins, saccharides, carotenoids, some vitamins, minerals, polyphenols and substances with antioxidant activity. Utilisation of these kind of side products in the recipe of various cereal product led to enhancement of final product nutritional value and better sensory attributes as well as improvement of product functional properties. In this work was studied the effect of orange and mandarin dietary fibre application at level 5 and 10% (w/w) in tarhana preparation and the influence on tarhana fermentation process. Chemical analysis showed, that dietary fibre preparations reached higher concentration of ash, fat and total dietary fibre compared to wheat flour. Wheat flour exhibited higher moisture content and protein concentration than citrus dietary fibre preparations. Orange and mandarin dietary fibre preparations showed higher values of water and oil absorption capacity, swelling capacity and least gellation concentration compared to wheat flour. Application of fruit dietary fibre preparations to tarhana recipe caused a rapid decrease in pH from 4.70 – 5.02 to values 4.31 – 4.51 during fermentation process. Reducing saccharides served as an available source of energy for fermenting microbiota and their concentration decreased from 24.5 – 32.8 to 2.2 – 0.2 g/kg after 144 h incubation. Fermentation also led to lactic acid (1.67 – 2.09 g/kg) and acetic acid (1.91 – 2.53 g/kg) production as a consequence of present microorganisms metabolic activity. Sensory evaluation of samples showed, that higher proportion of citrus dietary fibre preparations (10%) negatively affected taste, odour, consistency and sourness. Among all prepared tarhana samples with proportion of citrus dietary fibre preparation was the most acceptable tarhana with 5% of mandarin dietary fibre.

Keywords: fruit; dietary fibre; tarhana; fermentation

INTRODUCTION

Processing of citrus fruits (oranges, mandarins, etc.) in the production of juices and essential oils constitute approximately 50% of original whole fruit mass wastes. This remaining product is mainly used as feed, however, it also contains valuable nutrients such as pectins, saccharides, carotenoids, some vitamins, minerals, polyphenols and substances with antioxidant activity (Braddock, 1995; Topuz et al., 2005). Fibre-rich by-products may be incorporated into food products as inexpensive, non-caloric ingredients for partial replacement of flour, fat or saccharides, as enhancers of water and oil retention and to improve emulsion or oxidative stabilities (Elleuch et al., 2011).

Tarhana is cereal-based fermented product. It is prepared from wheat flour, yoghurt and other ingredients. After mixing of all ingredients the dough is formed, which ferment 1 – 7 days at temperature 25 – 30 °C by using lactic acid bacteria from yoghurt culture (*Lactobacillus bulgaricus*, *Streptococcus thermophilus*) and yeasts *Saccharomyces cerevisiae*. Tarhana is usually reconstituted with water and served as a hot soup generally consumed at lunch and dinner (Erbaş et al., 2006; Lar et

al. 2012; Sengun et al., 2009). Tarhana has an acidic and sour taste with a strong yeasty flavor (Kaya et al., 1999).

Tarhana is very nutritive food because of nutritional deficiency in wheat is mostly eliminated by yoghurt. Its nutritional value is increased and digestion is facilitated by fermentation (Dalgıç and Belibağh, 2008). The protein, polysaccharide and lipid components of tarhana are subjected to partial digestion and hydrolysis by lactic acid bacteria and yeasts during fermentation, resulting in a product with improved digestive properties (Tamer et al., 2007). Fermentation of tarhana also results in significant increases of riboflavin, niacin, pantothenic acid and folic acid contents (Bilgiçli, 2009). Tarhana is also a good source of calcium, iron, zinc as well as some other minerals (Daglioğlu, 2000).

The objective of this work was to prepare dietary fibre preparations obtained from citrus crops (orange and mandarin) and perform the determination of chemical and functional properties of obtained dietary fibre preparations and subsequently evaluate the suitability of fruit dietary fibre incorporation to the tarhana recipe and observe fermentation process by determination of pH, reducing saccharides, lactic and acetic acid concentrations as well as sensory evaluation of final products.

MATERIAL AND METHODOLOGY

Dietary fibre (DF) preparations were obtained from orange (cultivar *Valencia*) and mandarin (cultivar *Clemenville*) purchased from local markets. From the cleaned citrus crops were separated inner part of peels (albedo), which was subsequently dried for 6 days at 27 °C. Finally the peels were milled and sieved to obtain 400 µm particle size (Marín et al., 2007). The yoghurt was laboratory prepared from bovine UHT milk, 3.5% fat by using commercial yoghurt culture Lactoflora® and incubated at 40 °C for 6 h. Tarhana samples (control and samples with replaced a part of wheat flour with orange respectively mandarin DF preparation at level 5 and 10% w/w) were prepared according to recipe and method Ibañoğlu et al. (1995) and fermented for 144 h at 30 °C.

Within the chemical analysis of citrus DF preparations and wheat flour was performed determination of moisture, ash, fat and protein content from the total nitrogen content determined by the Kjeldhal method and converted to protein using a factor 6.25 (Kohajdová et al., 2014). Total dietary fibre (TDF) content was determined by enzymatic-gravimetric method according to Sun-Waterhouse et al. (2010). Functional properties – water absorption capacity (WAC), oil absorption capacity (OAC), swelling capacity (SWC) and least gelation concentration (LGC) were determined according to Hayta et al. (2002) and Raghavendra et al. (2004).

Fermentation process was observed by the determination of pH using inoLab pH Level 2 apparatus (WTW, Weilheim, Germany) (Ibañoğlu et al., 1999). Reducing saccharides were determined according to Schoorl. The nonreacted Cu²⁺ was determined after formation of Cu₂O. The KI was oxidized by CuSO₄ to I₂ that was determined by titration with Na₂S₂O₃ (Kohajdová and Karovičová, 2005). The concentrations of lactic and acetic acids were determined by capillary isotachopheresis method according to Kohajdová et al. (2006) by using ZKI 01 apparatus (Villa Labeco, Spišská Nová Ves, Slovakia) equipped with conductivity detector and two line recorder TZ 4200 (Laboratorní přístroje, Prague, Czech Republic). Used electrolytic system for lactic and acetic acid analysis had following composition: leading electrolyte 0.01 mol/L

HCl, counter-ion 6-aminocaproic acid, additive 0.1% methylhydroxy-ethylcellulose, pH 4.25; terminating electrolyte 0.005 mol/L capronic acid. Applied driving current was 250 µA in the pre-separation column. Lactic and acetic acids were in the samples identified according to their RSH (relative step height) values and compared with the RSH of lactic and acetic acids in standard solutions. Quantitative analysis was performed by calibration of standard solutions.

Sensory analysis of final products – tarhana soups was carried out by 11 semi-trained panellists (between 22 and 25 years of age, 9 female, 2 male, all non-smokers), informed about the preparation of samples, used raw materials and with the methods of sensory evaluation. Sensory evaluation was performed by using 7 point hedonic scale (1=extremely unacceptable to 7=extremely acceptable) and evaluate parameters were taste, odour, consistency, acidity and overall acceptability (Erbaş et al., 2005; Bilgiçli, 2009).

RESULTS AND DISCUSSION

Determination of chemical parameters (Table 1) showed, that citrus dietary fibre preparations reached higher concentration of ash, fat and total dietary fibre compared to wheat flour. Wheat flour exhibited higher moisture content and protein concentration than citrus dietary fibre preparations. Comparable concentration of proteins in fruit DF preparations also reported Yasar et al. (2007). The content of TDF in both preparations exceeded 50%, what is according to Figuerola et al. (2005) minimal criterion for TDF concentration in DF preparations. Citrus dietary fibre preparations showed higher values of water and oil absorption capacity, swelling capacity and least gelation concentration compared to wheat flour.

During tarhana fermentation was observed a decrease in pH values (Figure 1a) from 4.70 – 5.02 to 4.31 – 4.51. Ibañoğlu et al. (1999) reported a decrease in pH values of tarhana samples (control, sample without salt addition, sample with double amount of added yoghurt and tarhana prepared from whole wheat flour) from initial values of 4.7 – 5.2 to 4.3 – 4.8 after 96 h fermentation at 30 °C.

Table 1 Chemical and functional properties of wheat flour and citrus dietary fibre preparations.

Parameter	Fine wheat flour	Orange dietary fibre	Mandarin dietary fibre
Moisture (%)	11.39 ±0.25	9.19 ±0.08	9.11 ±0.70
Ash (%)	0.55 ±0.00	2.76 ±0.04	2.53 ±0.01
Protein (%)	10.48 ±0.15	3.66 ±0.11	4.35 ±0.17
Fat (%)	1.54 ±0.03	2.33 ±0.02	2.06 ±0.01
Total dietary fibre (%)	2.25 ±0.04	64.41 ±0.69	61.28 ±0.57
WAC (g.g ⁻¹)	3.20 ±0.04	5.82 ±0.05	5.16 ±0.09
OAC (g.g ⁻¹)	2.45 ±0.02	3.15 ±0.01	4.81 ±0.02
SWC (cm ³ .g ⁻¹)	2.51 ±0.07	10.87 ±0.16	7.52 ±0.10
LGC (%)	6.00 ±0.00	8.00 ±0.00	8.00 ±0.00

WAC/OAC – water/oil absorption capacity, SWC – swelling capacity, LGC – least gelation concentration

Table 2 Sensory properties of tarhana samples.

Sample	Taste	Odour	Consistency	Sourness	Overall acceptability
Control	7.0 ±0.0	6.9 ±0.1	7.0 ±0.0	6.8 ±0.1	99.2 ±0.5
ODFP 5 %	6.7 ±0.2	6.8 ±0.0	6.8 ±0.1	6.4 ±0.0	90.0 ±1.8
ODFP 10 %	6.3 ±0.3	5.7 ±0.2	5.1 ±0.3	5.4 ±0.1	87.2 ±1.1
MDFP 5 %	6.9 ±0.0	6.8 ±0.1	6.7 ±0.2	6.5 ±0.2	95.3 ±2.0
MDFP 10 %	5.9 ±0.1	5.5 ±0.1	5.3 ±0.2	5.9 ±0.2	84.5 ±0.8

ODFP – orange dietary fibre preparation, MDFP – mandarin dietary fibre preparation

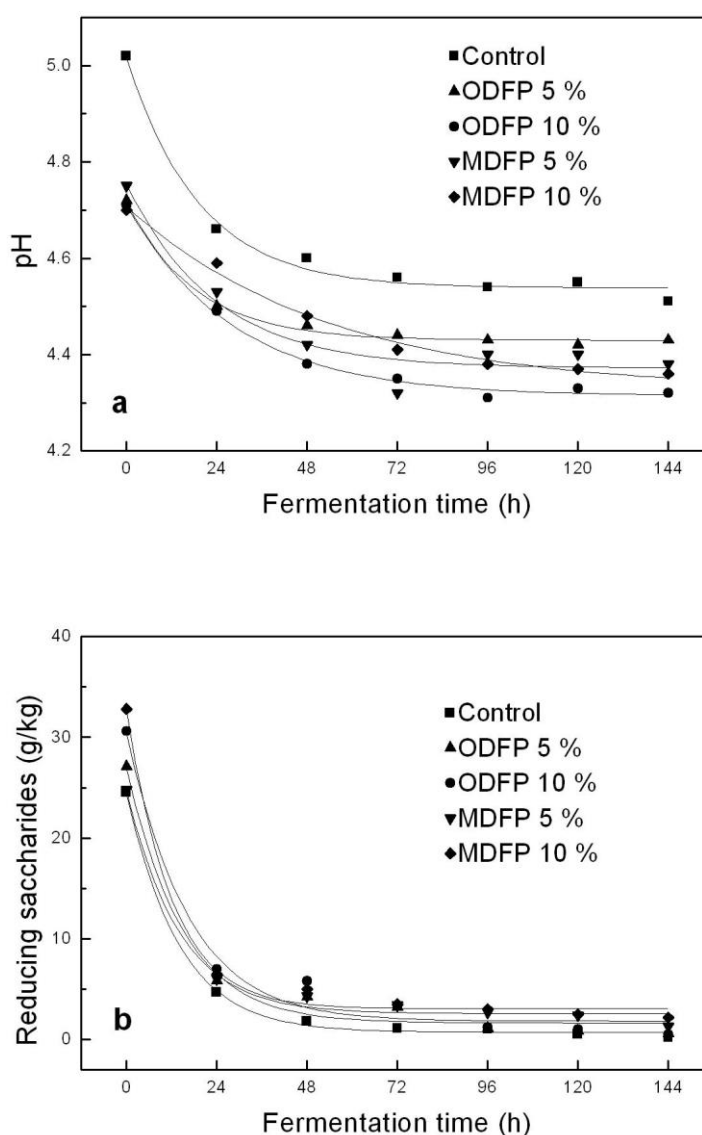


Figure 1 Changes in pH values (a) and concentration of reducing saccharides (b) during fermentation of tarhana, ODFP – orange dietary fibre preparation, MDFP – mandarin dietary fibre preparation.

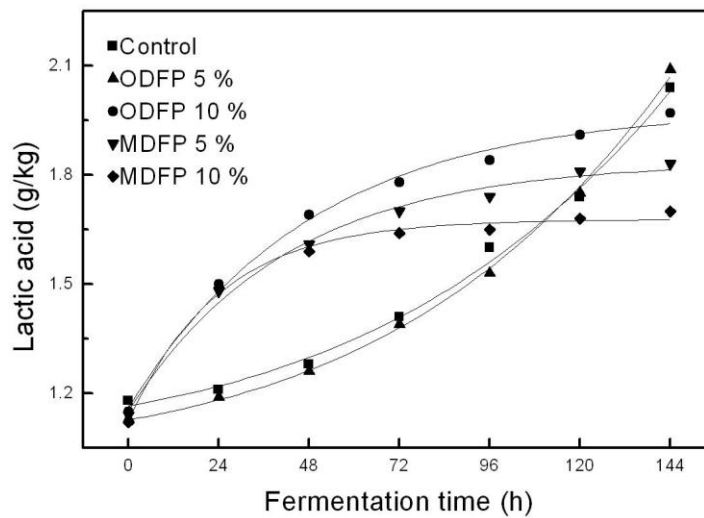


Figure 2 Changes in lactic acid concentration during fermentation of tarhana samples, ODFP – orange dietary fibre preparation, MDFP – mandarin dietary fibre preparation.

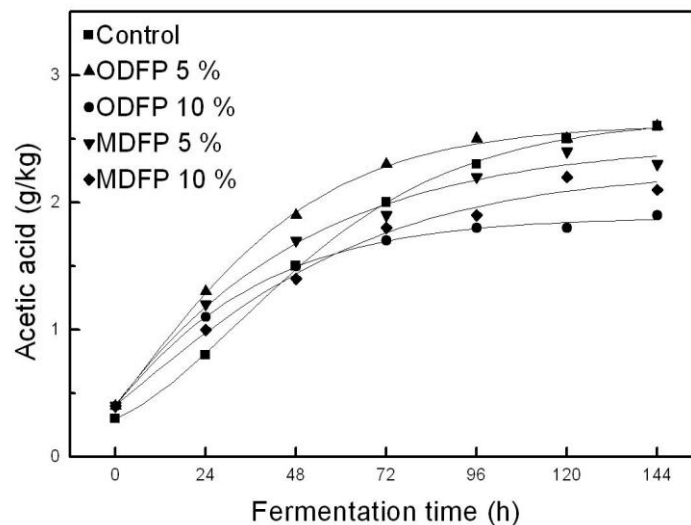


Figure 3 Determination of acetic acid during fermentation of tarhana, ODFP – orange dietary fibre preparation, MDFP – mandarin dietary fibre preparation.

Reducing saccharides are fermentable saccharides and their concentration decreased with increasing period of fermentation (Singh et al., 2013). The concentration of reducing saccharides (Figure 1b) rapidly dropped in first 24 h of fermentation and in further process decreased slightly or remains constant.

Characteristic products of lactic acid fermentation by using lactic acid bacteria are lactic acid and acetic acid, which lower pH value and thus inhibit undesirable and pathogenic bacteria (Kohajdová and Karovičová, 2008). The concentration of lactic acid (Figure 2) increased

during tarhana fermentation and at the end of the process reached values in range 1.67 – 2.09 g/kg.

Determination of acetic acid (Figure 3) showed, that its concentration gradually increased with advancing time of fermentation and after 144 h reached values in range 1.91 – 2.53 g/kg. According to Sroka and Tuszyński (2007) the production of organic acids during fermentation depends on the concentration of available saccharides and nitrogen sources as well as on the pH value.

Sensory evaluation of tarhana samples (Table 2) showed, that higher amounts (10%) of added orange and mandarin dietary fibre preparations negatively affected taste, odour,

consistency and sourness. Among all prepared tarhana samples with proportion of citrus dietary fibre preparation was the most acceptable tarhana with 5% of mandarin dietary fibre

CONCLUSION

Citrus dietary fibre preparations are valuable nutritive components of citrus crops. Prepared dietary fibre preparations obtained from orange and mandarin showed high content of total dietary fibre (64.41 and 61.28%) and higher concentration of ash and fat compared to wheat flour. Wheat flour exhibited higher moisture content and protein concentration than citrus dietary fibre preparations. Citrus dietary fibre preparations reached higher values of water and oil absorption capacity, swelling capacity and least gellation concentration compared to wheat flour.

Application of prepared orange and mandarin dietary fibre preparations to tarhana recipe led to significant decrease in pH values during fermentation. Concentration of reducing saccharides was reduced due to its utilization as an available source of energy for fermenting microorganisms. During fermentation was also observed lactic acid and acetic acid production. Sensory evaluation showed that higher amounts of added fruit preparations negatively affected taste, odour, consistency and sourness. The most acceptable among tarhana samples incorporated with citrus dietary fibre preparations was by panelists chosen tarhana with 5% proportion of mandarin dietary fibre.

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IMMUNOFLUORESCENCE DETECTION OF MILK PROTEIN IN MEAT PRODUCTS

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ABSTRACT

Nowadays there are various vegetable protein additives intended for the manufacture of meat products in the food industry. These ingredients include both, plant-origin as well as animal-origin proteins. The most common vegetable additives include various types of flour, starch, fiber and plant protein. Among animal proteins, the most commonly used are plasma, collagen or milk protein. Milk protein is added to meat products due to its functional properties, such as emulsifying fats, improving the holding capacity of meat, improving juiciness, gel-forming capacity and affecting the taste of the product. Usage of these proteins, however, is currently limited by the effective legislation, not only in order to prevent consumer deception, but also because of their potential impact on consumers' health of. Thus, this issue has received considerable attention not only in the Czech Republic, but also globally. The main risk is the impossibility of selecting a suitable foodstuff for individuals with potential allergic reactions. The only option for allergic consumers to protect themselves is to strictly exclude the given allergen from their diet. Although the number of studies dealing with the reduction or loss of allergenicity is increasing, yet these practices are not common. Most of the population suffering from food allergies is thus still dependent on strict exclusion of foodstuffs causing adverse allergic reactions from their diet. Detection of allergens in foodstuffs is unfortunately quite difficult due to the fact that they occur in trace amounts and are often masked by different parts of the foodstuff. This research dealt with the detection of milk protein in meat products purchased in the market network of the Czech Republic, whereas declaration given by the manufacturer on the packaging for the small meat products purchased from the market was used to verify the detection of milk protein by the immunofluorescence method. 20 products were examined, these were selected with regard to the presence of milk protein that was declared by the manufacturer on the packaging. Method validation was performed by comparing the positive results from the investigated method with information on the packaging of the meat product. Milk protein was detected in 84.62 per cent of samples where the manufacturer declared the presence of milk or cheese on the package and additionally in 85.71 per cent of samples where the manufacturer declared the presence of milk protein. The results show that the immunofluorescence method is suitable for the detection of milk protein in meat products.

Keywords: immunohistochemistry; allergens; milk protein; fluorescence methods; meat products

INTRODUCTION

Protein formulations are frequently used in production of meat products. From among plant-origin proteins, meat products can thus contain e.g. wheat or soy protein. Of the animal-origin proteins, they often contain plasma, collagen or milk protein (caseinate, whey, powdered skim milk, etc.) (López et al., 2006). These proteins are added due to their functional properties such as emulsification of fats or improvement of holding capacity of meat. Milk proteins are also involved in improving juiciness, gel-forming capacity and affect the delicate flavor profile of the meat product. All properties are perfectly compatible with the meat systems. On the other hand, the best known milk protein – casein – which constitutes about 80 per cent of milk protein, is relatively expensive. Conversely, proteins in whey, representing about 20 per cent of milk protein, are more economical and provide good performance in meat systems. Whey protein is primarily beta-lactoglobulin, a globular protein that can be modified (its

structure can be changed) so that it changes the functional behavior of proteins used in food industry (López et al., 2006).

On the other hand, milk protein is classified among food ingredients, which are listed in Regulation 2011/1169/EC as regards indication of ingredients present in foodstuffs. Food allergy is an abnormal immune response to foodstuffs (Bruijnzeel-Koomen et al., 1995). In this case, one's immune system responds inappropriately to the stimulus provoked by the allergen, which can be a protein or carbohydrate, for example (Ferguson, 1992). In addition, food allergens contained in foodstuffs naturally are resistant to high temperatures, low pH in one's stomach, and enzymatic digestion in the digestive tract (Hefle et al., 2007). However, it has been reported that there is no correlation between in vitro digestibility and protein allergy (Fu et al., 2002). Allergies to specific foodstuffs may in some cases exhibit also after ingestion of food of similar origin, which is known as cross-reaction.

This occurs when IgE antibodies originally produced against one allergen are produced also upon contact with a similar protein from another source (**Aalberse et al., 2001**). Food allergies have become a major health problem worldwide. Adverse health effects due to allergic reactions to food products or food ingredients occur in about 1 per cent of population and in about 4 per cent of children (including food intolerance). Food allergy is therefore more common in children than in adults. In Central Europe, typical allergies include allergies to egg, milk, temperate-zone fruits, tree nuts, poppy seed, and root vegetables; in the Asian continent critical is surprisingly not rice, but rather highly allergenic soybean with its wide range of products – at least 50 per cent of the Asian food production is soy-based, the vast majority of other foodstuffs is at least contaminated with traces of soybean (**Fuchs, 2008**). In the United States, cow's milk (2.5%), eggs (1.3%), and peanuts (0.8%) are responsible for allergic reactions in children. In contrast, in the adult population the prevailing allergies include shellfish (2%), peanuts (0.6%), nuts (0.5%), and fish (0.4%) (**Sampson, 2004; Sicherer and Sampson, 2000**). Cow's milk, eggs, soy, wheat, peanuts, tree nuts, fish, crustaceans, and molluscs cause about 90 per cent of food allergies and are also the primary foodstuffs causing anaphylaxis (**Sicherer and Sampson, 2000**). Most food allergic reactions are induced immediately after exposure to the allergenic foodstuff. Even intake of a tiny amount of foodstuffs containing allergens may cause allergic reactions in sensitive individuals. It then includes a wide range of allergic symptoms, such as digestive disorders, respiratory problems, disorders of the circulatory system, and skin irritation. In some individuals it can even lead to anaphylactic shock (**Schubert-Ullrich et al., 2009**).

In order to avoid misleading consumers and also to protect allergic consumers, analytical methods applicable to all types of foodstuffs have been developed. Among the available immunochemical methods, the Enzyme-Linked Immunosorbent Assay (ELISA) is the most frequently used method in laboratories to detect hidden allergens in foodstuffs. ELISA methods are still being improved and used in combination with other methods, as reported for example in the study by **Ben Rejeb et al., (2005)**. Polymerase chain reaction (PCR) is a method used for detection and quantification of DNA. This method is used for detection or quantification of allergens in processed foodstuffs where the DNA is generally more robust than proteins and therefore it is less likely to suffer damage or destruction during the processing of foodstuffs (**Walker et al., 2008**). There are also other immunochemical tests, for example Enzyme-Allergosorbent Test (EAST), followed by Radio-Allergosorbent Test (RAST) and Dot Immunoblotting which operate on a similar principle as ELISA.

MATERIAL AND METHODOLOGY

20 cooked meat products that, in harmony with their list of ingredients, should contain milk protein in various forms, e.g. milk protein or milk in general or that were marked: "May contain traces of milk protein", primarily sausages and pates purchased in the market network in the Czech Republic, were examined. The selected detection

method was immunofluorescence microscopy as a method more sensitive and selective than light microscopy. The samples were taken in a manner to be representative of the entire product. The samples were then processed in the accredited laboratory for investigation of foodstuffs at the Department of Vegetable Foodstuffs Hygiene and Technology, FVHE, VFU Brno. Using cryostat HM 550 (Germany, Microm) the sample was sliced into sections 10 µm thick. These sections were transferred to Thermo Superfrost slides (Germany, Thermo scientific). 9 sections were cut of each meat product. Each sample was constituted by three frozen blocks from which the microscopic sections were cut with 50 µm trimming. The selected detection method was immunofluorescence microscopy as a method more specific and selective than histochemical methods. The actual immunofluorescence procedure was launched by inserting the sections into cold acetone. After rinsing the preparations in PBS (phosphate buffer saline) for 2 x 5 min., sections were placed in humidified chamber in which blocking of nonspecific bond using Goat diluent normal serum (GB, VectorLaboratories) took place for 30 minutes. Afterwards, biotinylated primary antibody of Rabbit Anti-Beta-casein Polyclonal Antibody (USA, Bioss Antibodies) was applied to the sections, the humidified chamber was left in a refrigerator overnight. The next day, the sections were rinsed in PBS (2 x 5 min.). Thereafter, the sections were placed in the humidified chamber again and the secondary antibody (GB, VectorLaboratories) was applied to the sections for 30 min. at room temperature. Subsequently rinsing in PBS (2 x 5 min.) and application of fluorochrome followed. The fluorochrome used was Texas Red (GB, VectorLaboratories). Afterwards, the sections were mounted and examined using the fluorescence microscope of Leica DM 3000 (Germany, Leica) and further processed by Leica IM 50 software (Germany, Leica). Thus, 9 sections from each meat product was examined at a magnification of 40x and 100x.

RESULTS AND DISCUSSION

Milk protein was detected in 17 out of the total of 20 meat products samples where the manufacturer declared the presence of milk proteins or milk on the packaging. Cow's milk, wheat, eggs, soy, peanuts, tree nuts, fish, crustaceans, and molluscs cause about 90 per cent of food allergies and are also the primary foodstuffs causing anaphylaxis (**Sicherer and Sampson, 2000**). In order to protect consumers, European Commission adopted Regulation 2011/1169/EC amending Directive 2000/13/EC and Directive 2003/89/EC as regards indication of the ingredients present in foodstuffs. Annex IIIa of this guideline contains a list of food ingredients and products made from them, which are classified as potential allergens that could lead to potential intolerance, among these ingredients, is also milk (including lactose). Directive 2003/89/EC requires that each of the twelve described potentially allergenic ingredients is declared although they form less than 25 per cent of the food. The aim of the research was to verify the appropriate method for determination of milk proteins in meat products. Immunofluorescence method was selected as the examination method.

Table 1 Detection of milk protein in small meat products.

Ingredients	Milk, cheese		Milk protein	
	Declared by the manufacturer	Detected milk protein	Declared by the manufacturer	Detected milk protein
Number of samples	13	11	7	6
Percentage	100	84.62	100	85.71

Table 2 Meat products used for immunofluorescence detection.

Meat product	Declaration	Number of products	Number of detection
hamburger	milk protein content	2	2
frank	modicum of milk protein	4	3
hotdog	milk protein content	6	5
paté	Milk protein content	8	7

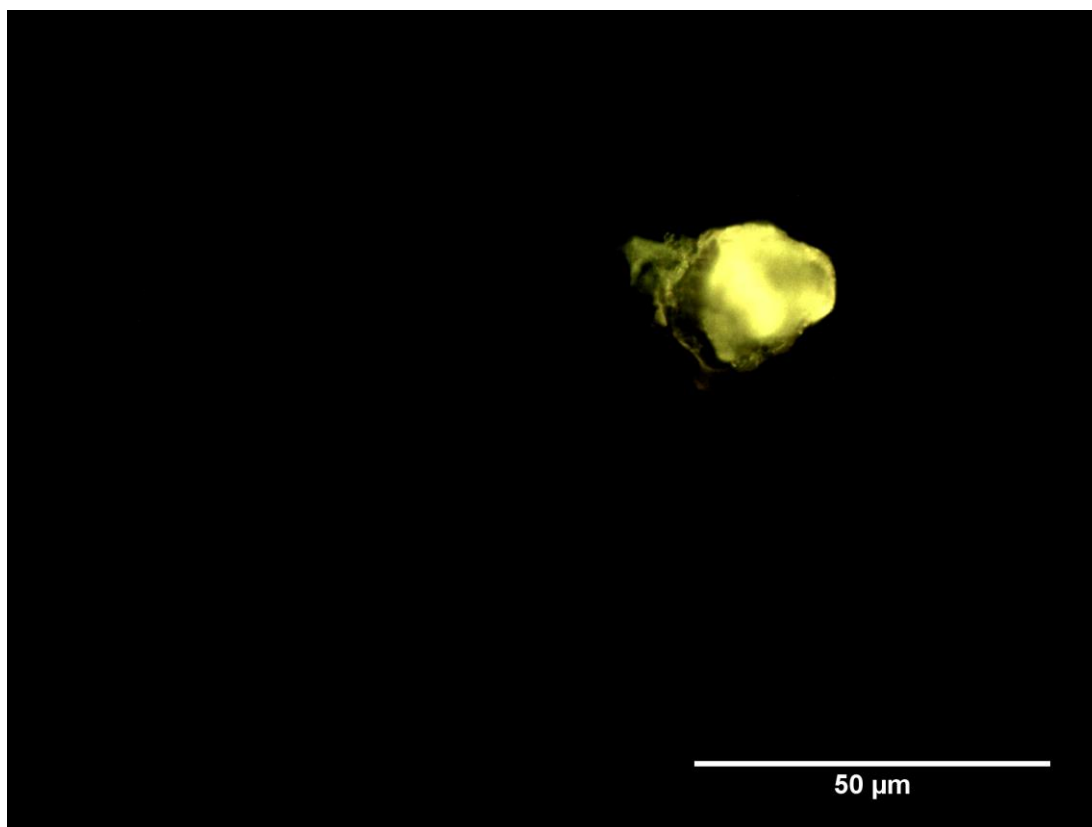


Figure 1 Milk protein green and yellow – Texas Red (magnification 400 x).

Detection was based on fluorescence which was achieved by immunohistochemical procedure and staining using fluorochromes. Immunohistochemical procedures are generally based on the reaction between the allergen and the corresponding labeled antibody (Petrašová et al., 2014; Bednářová et al., 2015). Binding of the labeled antibody was evaluated in a fluorescence microscope with a fluorescence filter I3. The examination was based on the formation of a fluorescent color, which indicates a positive reaction of the antigen with the antibody. To visualize milk protein by staining, Texas Red fluorochrome was applied. Fig. 1 shows a microphotograph of the milk protein, which differs in color from the black background that is formed by muscle, and other component of the meat product. Hereby it possible to differentiate between the milk protein and meat protein which is not fluorescent but black. We compared the results obtained in our examination with the information supplied by the manufacturer on the product packaging. The values obtained in the milk protein detection are given in Tab. 1. As apparent from this Table, the fluorescence immunohistochemical method appears suitable for determining milk protein in small meat products. Out of 13 samples where the manufacturer had declared the presence of milk or cheese, we detected milk protein in 11 products. Additionally, 7 products where the manufacturer directly declared the presence of milk protein were examined. In 6 of these products, milk protein was really detected. In one sample the presence of milk protein was not detected, which could be e.g. because of mere preventive warning on the package protecting the manufacturer for example in the production process where cross-contamination could occur, or because of deactivation of the binding sites of milk protein during the manufacturing process.

CONCLUSION

Cryosections were cut of each sample to be examined. Texas Red was used as the fluorochrome due to minimal background fluorescence. Immunofluorescence method for the detection of milk protein was verified by examination of 20 small meat products (Tab. 2) purchased from the market network. Our results obtained in this pilot study was compared with information on the packaging of the product when milk protein was declared on 7 packagings and general content of milk or cheese was stated on the packagings of 13 manufacturers. In total, milk protein was detected in 17 products. Out of that, in 11 products where milk protein was directly declared on the packaging and in 6 products where contained milk or cheese was declared in general. The results point to the possibility of using this method for the detection of milk protein in meat products. To use this method in practice, however, further validation of the method in more parameters, such as repeatability and reproducibility, is still necessary.

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OXIDATIVE STABILITY OF CHICKEN MEAT DURING STORAGE INFLUENCED BY THE FEEDING OF ALFALFA MEAL

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ABSTRACT

The aim of our experiment was to determine the effect of the alfalfa meal component in feed mixtures of Ross broiler chickens on oxidative stability of meat. Proportion of alfalfa meal in feed mixtures was 4 and 6%. The results were compared to the control group without alfalfa meal in feed mixtures. At the end of the experiment (day 38), 6 pcs of broiler chickens from each group with an average live body weight over 1 800 g were randomly selected. The samples for chemical analysis consisted of identical proportion of breast and thigh muscle, and about 1 cm² of skin with subcutaneous fat. Fat from the meat was obtained after the samples drying. A fat was determined by extraction by means of laboratory instrument Det N Gras Selecta P. The oxidative stability of meat on the basis of acid number of fat was determined by chemical analysis. Chicken meat was stored at -18 °C for 12 months and 18 months. The acid number of fat of stored meat for 12 months was 7.38 mg KOH per g in the control group, 7.42 mg KOH per g in the group with a proportion of 4% alfalfa meal, and 11.18 mg KOH per g in the group with proportion 6% alfalfa meal. An acid number of fat of stored meat for 18 months was 5.90 mg KOH per g in the control group, 4.65 mg KOH per g in the group with a proportion of 4% alfalfa meal, and 7.07 mg KOH per g in the group with a proportion of 6% alfalfa meal. Chicken meat is notably sensitive to lipid oxidation because of its high content of polyunsaturated fatty acids. Legislation in Title 5 of Part 3 of the Codex Alimentarius of the Slovak Republic and the Government Regulation No. 286/2003 Coll. in the Annex 4 in Part B provides the requirements for animal fats and meat products. Regulation of the European Parliament and Council (EC) No. 853/2004 lays down specific hygiene rules for food of animal origin. In particular, determination of free fatty acids content of rendered animal fat (tallow, lard, other animal fat). Legislative regulation does not contain requirements for the quality of chicken meat, the acid number of fat of fresh or frozen chicken meat, respectively. Chicken meat is preferred over other kinds of meat. It is characterized by certain dietary and nutritional properties that consumer prefers. A price of this kind of meat remains attractive. In terms of human health, oxidative stability of chicken meat is important, especially of stored meat. In general terms, the various food additives are currently used to maintain the food stability. Great attention is currently paid to additives of natural origin. Similar focus is presented in our study. We can state, on the basis of the oxidative stability results of chicken meat, that natural feed component has its justification. This issue requires further research.

Keywords: broiler chicken; alfalfa meal; stored meat; oxidation; acid number of fat.

INTRODUCTION

In recent years, poultry meat becomes more preferred food not only in Europe but especially in China, Brazil and India. Consumption of poultry meat increased even in Africa. If we compare global production of meat, total poultry production nearly converges to total pork production. According to forecasts, poultry production of China will increase by 37% and ten times in India by 2020. Its popularity is mainly due to the efficiency of fattening, short time of fattening and because of small area for breeding, relatively. Of course, even for dietetic properties of poultry meat. Lipids have an important role in food product quality, making them more desirable by improving the organoleptic properties of flavour, colour and texture. In addition, they confer nutritive value on the product, constituting a source of metabolic energy, essential fatty acids and fat-soluble vitamins. On the other hand, the lipid

components are susceptible to attack by molecular oxygen (Baggio, 2006). Poultry meat is in terms of dietary properties and nutritional value very interesting because of the high content of protein, minerals and vitamins and low percentage of fat. Meat poultry contains an average of 19.7 to 22.3% of protein, 1.4 to 22.16% of lipid, 57 to 75.25% of water, 1.00 to 1.07% of ashes (Benková, 2009). The composition of the meat varies depending on the type of animal, breed, gender, age and nutrition. Structure and composition of muscle depends on the method of meat processing, which affects biochemical, organoleptic and technological properties of the meat (Pipek, 1998; Brezina et al., 2001; Benková, 2009). The biological value of dietary fats is assessed as their digestibility, content in fat-soluble vitamins, essential fatty acids, cholesterol, and according to the proportion of each type of fatty acids. The quality and type of fat affects the

appearance, taste and especially energy and nutrient content of food (Jurkovičová, 2008). The lipids are presented in meat especially as fatty acid esters of glycerol. They contain lipophilic vitamins, lipids, mainly phospholipids, and essential fatty acids. The fats are rated negatively for their high energy. Fat of meat influenced a tenderness and fragility of meat (Ingr et al., 1993; Pipek 1995). Fat has an important role in the formation and in the texture of meat. Fat is a source of energy and the fat also affects tastiness properties of the meat (Pipek, 1991; Pipek, 1998). Poultry fat contains higher amounts of polyunsaturated fatty acids (PUFAs) than other fat of animals for slaughter. PUFAs are responsible for a lot of oxidative changes, for example as changes of organoleptic properties and shelf life (Korimová et al., 2000; Turk et al., 2000; Bou et al., 2001). Long chain polyunsaturated fatty acids are conditionally essential nutrients for adequate growth, development and function in humans (Gill, 2012). Chicken meat had a lower proportion of saturated ($36.4 \pm 3.6\%$; $p < 0.001$) and a higher proportion of PUFAs ($21.3 \pm 3.5\%$; $p < 0.001$) (Almeida, 2006). Because chicken meat has high content of PUFAs (Botsoglou et al., 2002), is notably sensitive to lipid oxidation. Thigh meat, as compared to breast meat, is particularly vulnerable because of its higher fat content (Jensen et al., 1998). Among them, omega-3 PUFAs (ω -3 PUFAs) have gained popularity due to their various health promoting and diseases preventing attributes. For example, ω -3 PUFAs are reported to be highly effective against cardiovascular diseases, cancer and other metabolic diseases (Wang, 2012; Gulhan, 2014). Long chain ω -3 PUFAs eicosapentaenoic and docosahexaenoic were observed only in dark chicken meat (23.0 ± 3.0 and 14.0 ± 1.0 mg per 100 g (Almeida, 2006). The oxidation of fats is one of the major problems in the meat industry due to a decrease in quality flavor and loss of nutritional value (Ladikos and Lougovois 1990; Ahn et al., 1992). Lipid oxidation causes loss of nutritional and sensory values as well as the formation of potentially toxic compounds that compromise meat quality (Cortinas, 2005). Oxidation leads to oxidative rancidity and involves oxygen attack on glycerides whereas hydrolysis leads to hydrolytic rancidity and it involves hydrothermal or enzymic (lipase) hydrolysis to free fatty acids and other products. There are some factors affecting the development of rancidity such as the degree of unsaturation of the oils, heat, prooxidants, light, certain enzymes (lipoxygenases), moisture content and availability of oxygen (Özogul et al., 2006). Secondary oxidation products such as aldehydes, ketones and esters, are responsible for the increased depreciation and deviation from the natural flavors (Ladikos and Lougovois, 1990). During storage maturation occurs in deep frozen raw meat over time (Pipek, 1992). When the meat is stored for a long time, maturing phase passes into a deep autolysis. This action is undesirable. There is degradation of the proteins on oligopeptides and amino acids. Meat acquires an unpleasant taste and undergoing to hydrolysis of fats (Kadlec et al., 2002). Oxidation might also play a role in controlling proteolytic activity of enzymes and could be linked to meat tenderness. The oxidative stability of meat depends upon the balance between anti- and pro-oxidants, including the

concentration of PUFAs (Mercier, 2004). Fatty acids are released by the hydrolysis of fats (Velíšek, 2009).

Deep autolysis of slaughtered animals is not desired, but it is not possible to completely prevent or eliminate the microbial proteolysis. Deep autolysis catalyzed by the native enzyme can take place and to continue in relatively isolated state (Steinhauser et al., 1995). The negative consequences of lipid oxidation can be overcome by the use of antioxidants in the diet (Cortinas, 2005). Meat composition of PUFAs changed at the animals, which were fed with diets with increased unsaturated fats (Lin et al., 1989; Ajuyah et al., 1993; Ahn et al., 1995; Mooney et al., 1998; Lopez Ferrer et al., 1999).

The aim of the study was to investigate the oxidative changes of stored meat at -18 °C depending on feeding of alfalfa meal in the broiler chickens.

MATERIAL AND METHODOLOGY

The feeding experiment was performed in commercial poultry farm with a final hybrid chickens Ross 308, which is used for meat production. A space for the purpose of the experiment was situated in front of the hall, with a deep bedding system of breeding. This space was divided into three equal parts to meet the requirements of the standard distribution. 100 pieces of one-day-old chickens were placed within each group. Conditions corresponding to standards of Decree of Ministry of Agriculture of the Slovak Republic no. 2136/2004-100 of 23 August 2004 were created for our feeding. The broiler chickens were fed by standard feed mixtures of soy-cereal type *ad libitum*. Standard feed mixtures, usually used in practical conditions, were used in the control group of broiler chickens. In the 1st and 2nd experimental groups were used similar feed mixtures as in control group, only feed mixtures of 1st experimental group was enriched by 4% proportion of alfalfa meal at the expense of the wheat, and feed mixtures of 2nd experimental group was enriched by 6% proportion of alfalfa meal at the expense of wheat. The experimental period was divided into 3 phases: starter with starter feed mixtures for broiler chickens at the age of 1 to 18 days, grower with grower feed mixtures for broiler chickens at the age of 19 to 31 days, finisher with finisher feed mixtures for broiler chickens at the age of 32 to 38 days. The feed mixtures were produced according to law no. 440/2006 Coll. At the end of the experiment (day 38), 6 pcs of broiler chickens from each group with an average live body weight over 1 800 g were randomly selected. A slaughtering of broiler chickens was realized by human rapid cut of the carotid artery (*Ateria carotis communis*). Subsequently, feathers as well as internal parts of broiler chickens were mechanically removed. A carcass was prepared. The slaughtering was carried out at the Department of Evaluation and Processing of Animal Products, Faculty of Biotechnology and Food Sciences, SUA in Nitra. Chicken carcasses were packaged in plastic containers and stored at -18 °C for 12 and 18 months. Chemical analysis of samples was realized after the storage period at the Department of Food Hygiene and Safety FBFS SUA in Nitra. The samples for chemical analysis consisted of identical proportion of breast and thigh muscle, and about 1 cm² of skin with subcutaneous fat. Fat from the meat was obtained after samples drying.

A fat was determined by extraction by means of laboratory instrument Det N Gras Selecta P. An acid number of fat was determined from obtained fat. Acid value of fat was determined after dissolution of fat in the extract ethanol-diethyl ether in a 1:1 alkalimetric titration against phenolphthalein. The extracted fat was slightly heated and fat was dissolved in 25.0 ml of ethanol-ether. The content in extraction flask was titrated with a few drops of the indicator with the potassium hydroxide solution until it turned to slight pink color. An acid number of fat was expressed in mg KOH per g. A fat acid value is the number of mg of potassium hydroxide required to neutralize free fatty acids per gram of fat extracted from the extracting agent. Mathematical and statistical evaluation of the results was realized by the SAS Enterprise Guide Version 1.5 system program.

RESULTS AND DISCUSSION

An acid number of fats were determined in the stored chicken meat for 12 months, which ranged from 4.72 to 10.50 mg KOH per g of fat in the control group. Due to the large margin of determined values, the coefficient of variation was 36.86%. In the experimental group with proportion of 4% alfalfa meal, the acid number of fat was determined in the range of 4.69 to 10.49 mg KOH per g with coefficient of variation 28.04%. In the group with proportion of 6% alfalfa meal, the acid number of fat was determined in the range of 5.56 to 20.61 mg KOH per g fat and the coefficient of variation was 47.99%. The differences in acid number of fat among the groups were not statistically significant ($p > 0.05$) after 12 months of meat storage. An acid number of fats determined in the stored meat for 18 months varied from 4.35 to 6.56 mg

KOH per g fat with coefficient of variation 17.60% in the control group of broiler chicken, which were fed by standard feed mixtures. The acid number of fats in the range of 4.01 to 10.69 mg KOH per g fats was obtained in the meat of broiler chickens, which were fed by feed mixtures with proportion 4% of alfalfa meal in the feed mixtures. If proportion of alfalfa meal formed 6% of feed mixtures, the acid number of fat was measured in the range of 3.29 to 10.33 mg KOH per g fat, and the coefficient of variation was 38.68%. The differences in acid number of fat among the groups were not statistically significant ($p > 0.05$) after 18 months of meat storage. Slovak legislation in Title 5 of Part 3 of the Codex Alimentarius of the Slovak Republic and the Government Regulation No. 286/2003 Coll. in the Annex 4 in Part B set out the requirements for animal fats and meat products. Regulation of the European Parliament and Council Regulation (EC) No. 853/2004 lays down specific hygiene rules for food of animal origin. In particular, determination of free fatty acids contents of rendered animal fat (tallow, lard, other animal fat). Legislative regulation does not contain requirements for the quality of poultry meat, the acid number of fats of fresh or frozen poultry meat, respectively. It was demonstrated in several studies that feeding of oxidized diets to broilers resulted in negative effects on bird performance (Cabel et al., 1988; Engberg et al., 1996), on oxidative stability of tissues and membranes (Asghar et al., 1989; Lin et al., 1989; Jensen et al., 1997; Grau et al., 2001a) and on shelf-life of meat during storage (Sheehy et al., 1994; Rhee et al., 1996; Sheldon et al., 1997; Grau et al., 2001b). Based on studies by many authors, it can be assumed that the composition of the feed mixture has a significant effect on

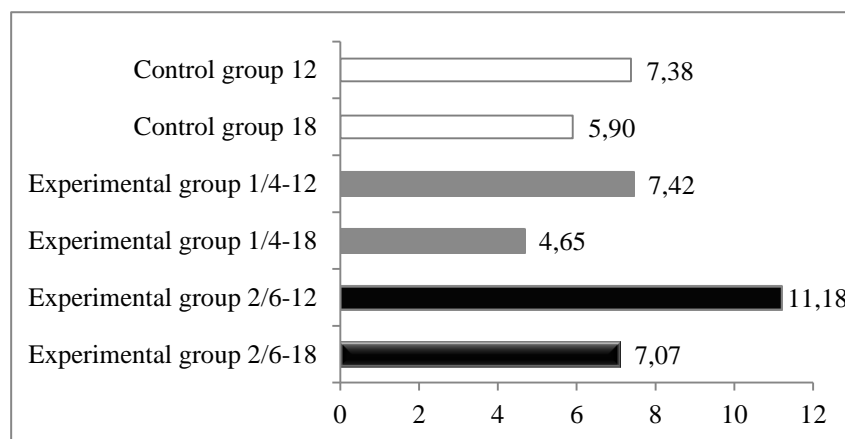


Figure 1 An acid number of fat of stored meat depending on proportion of alfalfa meal in feed mixtures of broiler chickens.

Control group 12 – stored chicken meat for 12 months at -18 °C,

Control group 18 – stored chicken meat for 18 months at -18 °C,

Experimental group 1/4-12 – stored chicken meat for 12 months at -18 °C of group with proportion 4% of alfalfa meal in feed mixtures,

Experimental group 1/4-18 – stored chicken meat for 18 months at -18 °C of group with proportion 4% of alfalfa meal in feed mixtures,

Experimental group 2/6-12 – stored chicken meat for 12 months at -18 °C of group with proportion 6% of alfalfa meal in feed mixtures,

Experimental group 2/6-18 – stored chicken meat for 18 months at -18 °C of group with proportion 6% of alfalfa meal in feed mixtures.

the chemical composition of meat broiler chickens, sensory quality and oxidative stability (Horwitt, 1986; Wood, 2004; Hugo, 2009; Bobko et al., 2012; Gibbs et al., 2013). Lipid oxidation is considered the main cause of quality damages related to flavor, color, taste, and nutritional composition of meat and meat products (Mielche and Bertelsen, 1994; Gray et al., 1996). The total amount of fat is a major factor of quality of fatty acids. The effects of fatty acid composition on meat quality are also reviewed. Fatty acid composition determines the firmness/oiliness of adipose tissue and the oxidative stability of muscle, which in turn affects flavour and muscle colour. Vitamin E is an essential nutrient, which stabilises PUFAs and has a central role in meat quality (Wood, 2008).

Free fatty acids are produced by secondary enzymatic cleavage of triglycerides (Koman et al., 1989). Higher values of acid number of fat may be according to Sopková et al. (2007) caused by the hydrolytic degradation of the fatty substance. Gutierrez (2013) studied susceptibility of unsaturated fatty acids to oxidation. It is related to the degree of unsaturation, polyunsaturated fatty acids, they are more prone to oxidation than monounsaturated fatty acids. Equally he demonstrated higher susceptibility of polar lipids to oxidation as compared with neutral lipids. Lipolysis can be responsible for a decrease of content polar lipids and releasing free fatty acids. Few authors deal with the assessment of chicken fat by acid number of fat. The authors are more concerned with assessing the quality of fats by thiobarbituric acid reactive substances (TBARS) values. Physical agents (heat, oxygen, light) and chemical factors (content of certain metals) play an important role in the development of oxidation (Ozturk and Cakmakci, 2006). Top of lipoperoxidation is derived from the free radical, such as nitrogen dioxide (Kanner et al., 1987). A speed fat oxidation of meat also depends on the presence of a prooxidants and antioxidants (Tichivangana and Morrissey, 1985; Ruiz et al., 1999).

CONCLUSION

Chicken meat is preferred over other kinds of meat. It is characterized by certain dietary and nutritional properties that consumer prefers. A price of this kind of meat remains significant. In terms of human health, oxidative stability of chicken meat is important, especially of stored meat. In general terms, the various food additives are currently used to maintain the food stability. Great attention is currently paid to additives of natural origin. Similar focus is presented in our study. We can state, on the basis of the oxidative stability results of chicken meat, that natural feed component has its justification. This issue requires further research.

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THE EFFECT OF PATULIN ON FEMORAL BONE STRUCTURE IN MALE RABBITS

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ABSTRACT

A lot of kinds of crops are susceptible to fungal attack, leading to considerable financial losses and damage the health of humans and animals. Patulin, a toxic fungal metabolite, can be found mainly in apple and apple products, with much less frequent contamination in other food products. Because of its high incidence and harmful health effects, patulin belongs to a class of mycotoxins, which are strictly monitored. However, its effect on bone structure is still unknown. This study was designed to investigate the impact of patulin on femoral bone structure in adult male rabbits. Four month-old male rabbits were randomly divided into two groups of three animals each. Rabbits from the experimental group (group A, n=3) were intramuscularly administered with patulin at dose 10 µg.kg⁻¹ body weight (b.w.) twice a week for 4 weeks. The second group without patulin administration served as a control (group B, n=3). At the end of the experiment, body weight, femoral weight and length, cortical bone thickness and histological structure of femoral bones from all rabbits were determined. The results did not show any significant differences in body weight, femoral weight and length between experimental and control groups of rabbits. On the other hand, intramuscular application of patulin induced a significant increase in cortical bone thickness ($p < 0.05$) and considerable changes in qualitative histological characteristics of compact bone in adult male rabbits. In patulin-intoxicated males, the primary vascular longitudinal bone tissue was absent near endosteal border. On the other hand, this tissue occurred near *periosteum* and also in the middle part of the femoral bone in these rabbits. The values for the primary osteons' vascular canals were significantly lower ($p < 0.05$) in males exposed to patulin as compared to the control group. Based on these findings we can conclude that intramuscular patulin administration demonstrably influences cortical bone thickness and histological structure of femoral bone in adult male rabbits.

Keywords: patulin; femoral bone; rabbit; histomorphometry

INTRODUCTION

Many species of fungi may produce secondary metabolites, known as mycotoxins (Jay, 2000; Hosseini and Bagheri, 2012; Alexa et al., 2013). Mycotoxins exert toxic effects on animals and humans (Peraica et al., 1999). They may cause mycotoxicosis, which can result in an acute or chronic disease episode (Bryden, 2007).

Mycotoxin patulin, 4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one, is produced by a number of fungi species (Bennett and Klich, 2003; Moake et al., 2005), such as *Penicillium* (mainly *Penicillium expansum*), *Aspergillus*, *Byssoschlamys* and *Paecilomyces* (Gimeno and Martins, 2006; Ionescu et al., 2010; Puel et al., 2010), which are likely natural contaminants of various food (Becci et al., 1981). Patulin has been found as a contaminant in many mouldy fruits, vegetables, cereals (Beltrán et al., 2014). According to Piqué et al. (2013), it is mainly presented in apples and apple-based products.

Although patulin had originally been considered as an antibiotic, it has adverse impacts on human health (Bennett and Klich, 2003). Several studies have found that patulin is genotoxic (Hopmans, 1997), cytotoxic (Glaser and Stopper, 2012), neurotoxic, mutagenic

(Beltrán et al., 2014), immunotoxic (Fernández-Cruz et al., 2010), teratogenic (Özsoy et al. 2008), carcinogenic (González-Osnaya et al., 2007) and embryotoxic (Piqué et al., 2013) agent.

In the study by Selmanoğlu (2006), some histopathological alternations were observed in the epididymis and prostate tissues in rats orally treated with patulin for 90 days. Also, patulin-induced degeneration and necrosis in liver tissues, as well as degeneration of glomeruli and haemorrhage between the tubules of the cortical region in kidney tissues were reported in male rats (Al-Hazmi, 2012). However, the effect of patulin on bone microstructure had not been studied prior to our experiment.

The aim of the present study was to determine the effect of patulin on selected growth characteristics (body weight, femoral weight, femoral length and cortical bone thickness) and femoral bone microstructure in male rabbits.

MATERIAL AND METHODOLOGY

Adult male rabbits (n=6) of meat line M91 (Californian broiler line) were used in the experiment. Animals (at the

age of 4 months, weighing 3.5 – 4.0 kg) were obtained from an experimental farm of the Animal Production Research Centre in Nitra, Slovak Republic. Male rabbits were used because they are less susceptible to skeletal damage than females (Riggs et al., 2004). Males were housed in individual flat-deck wire cages (area 0.3 m²) under standard conditions (temperature 20 – 22 °C, humidity 55 ±10 %, 12/12 h cycle of light and darkness) with access to food (feed mixture) and drinking water *ad libitum*.

Clinically healthy animals were randomly divided into two groups of three individuals each. In the first group (A), adult rabbits were intramuscularly injected with patulin (10 µg.kg⁻¹ b.w.) two times per week for four weeks. The dose of patulin was estimated based on literature data (FAO, 2004). The second group (B; n=3) without patulin intoxication served as a control. The Animal Experimental Committee of the Slovak Republic approved all procedures.

At the end of the experiments, all the rabbits were euthanized, weighed and their femurs were collected for macroscopical and microscopical analyses. The femurs were weighed on analytical scales with an accuracy of 0.01 g and the femoral length was measured with a caliper. For histological analysis, the right femurs were sectioned at the midshaft of the diaphysis and the segments were fixed in HistoChoice fixative (Amresco, USA). The segments were then dehydrated with a graded series (40 to 100 %) of ethanol and embedded in Biodur epoxy resin (Günter von Hagens, Heidelberg, Germany) according to the method described by Martiniaková et al. (2008). Transverse thin sections (70–80 µm) were prepared with a sawing microtome (Leitz 1600, Leica, Wetzlar, Germany) and fixed onto glass slides by Eukitt (Merck, Darmstadt, Germany) as previously described (Martiniaková et al., 2010). The qualitative histological characteristics of the compact bone tissue were determined according to the internationally accepted classification systems of Enlow and Brown (1956) and Ricqlés et al. (1991). The quantitative (histomorphometrical) variables were assessed using the software Motic Images Plus 2.0 ML (Motic China Group Co., Ltd.). We measured area, perimeter and

the minimum and maximum diameters of primary osteons' vascular canals, Haversian canals and secondary osteons in all views (i.e., anterior, posterior, medial and lateral) of the thin sections in order to minimize inter-animal differences. Diaphyseal cortical bone thickness was also measured by Motic Images Plus 2.0 ML software. Twenty random areas were selected and average thickness was calculated for each femur.

Statistical analysis was performed using SPSS 8.0 software (SPSS Inc., USA). All data were expressed as mean ± standard deviation (SD). The unpaired Student's T-test was used for establishing statistical significance ($p < 0.05$) between groups A and B.

RESULTS

Our results showed non-significant impact of patulin intramuscular administration on body weight, femoral weight and femoral length in male rabbits. On the other hand, cortical bone thickness was considerably increased ($p < 0.05$) in these animals (Table 1).

Femoral diaphysis of rabbits from the group B had a common bone microstructure. The periosteal and endosteal surfaces were formed mainly by primary vascular longitudinal bone tissue, as a basic structural pattern of all bones. The tissue was created by vascular canals, which ran in a direction essentially parallel to the long axis of the bone. Additionally, primary vascular radial bone tissue (created by branching or non-branching vascular canals radiating from the marrow cavity) was also identified in some areas near the endosteal surface. The middle part of *substantia compacta* was formed by dense Haversian (characterized by a large number of secondary osteons) or irregular Haversian (characterized by an occurrence of scattered secondary osteons) bone tissues (Figure 1).

In rabbits exposed to patulin, an absence of the primary vascular longitudinal bone tissue near the endosteal surface was found. This part of bone was created only by dense Haversian bone tissue. The periosteal surface was composed of primary vascular longitudinal bone tissue, which also occurred in some areas of the middle part of the compact bone (Figure 2).

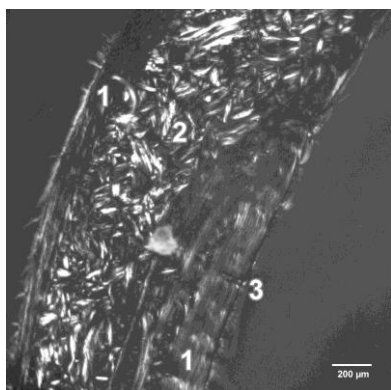


Fig. 1 Microscopical structure of compact bone in rabbits from the group B: 1 – primary vascular longitudinal bone tissue, 2 – dense Haversian bone tissue, 3 – primary vascular radial bone tissue.

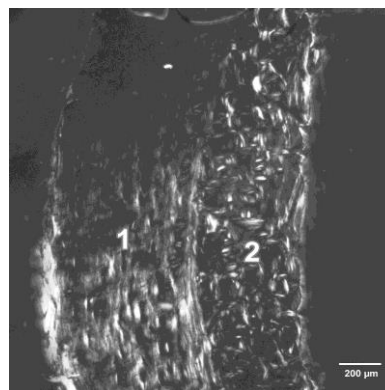


Fig. 2 Microscopical structure of compact bone in rabbits from the group A: 1 – primary vascular longitudinal bone tissue, 2 – dense Haversian bone tissue.

Table 1 Body weight, femoral weight, femoral length and cortical bone thickness in rabbits injected with patulin (10 µg.kg⁻¹ b.w.; group A) and the control rabbits (group B).

Rabbit's group	N	Body weight (g)	Femoral weight (g)	Femoral length (cm)	Cortical bone thickness (mm)
B	3	4152.9 ±212.2	14.53 ±1.39	10.51 ±0.25	997.51 ±92.95
A	3	4324.3 ±326.9	14.33 ±1.12	10.54 ±0.29	1059.31 ±118.93
T-test		NS	NS	NS	P < 0.05

N: number of rabbits, NS: non-significant changes

Table 2 Data on primary osteons' vascular canals in rabbits from A and B groups.

Rabbit's group	N	Area (µm ²)	Perimeter (µm)	Max. diameter (µm)	Min. diameter (µm)
B	124	320.76 ±48.60	64.23 ±4.87	11.13 ±1.09	9.22 ±1.02
A	125	262.05 ±58.01	58.01 ±6.32	10.03 ±1.43	8.34 ±1.12
T-test		p < 0.05	p < 0.05	p < 0.05	p < 0.05

N: number of measured structures; NS: non-significant changes

Table 3 Data on Haversian canals in rabbits from A and B groups.

Rabbit's group	N	Area (µm ²)	Perimeter (µm)	Max. diameter (µm)	Min. diameter (µm)
B	85	388.51 ±117.66	70.22 ±10.84	12.14 ±2.16	10.07 ±1.69
A	90	398.57 ±130.97	70.87 ±11.58	12.23 ±2.18	10.21 ±1.82
T-test		NS	NS	NS	NS

N: number of measured structures; NS: non-significant changes

Table 4 Data on secondary osteons in rabbits from A and B groups.

Rabbit's group	N	Area (µm ²)	Perimeter (µm)	Max. diameter (µm)	Min. diameter (µm)
B	85	8846.06 ±3950.00	332.25 ±84.17	58.98 ±16.79	45.46 ±11.05
A	90	9627.27 ±4356.66	345.41 ±77.95	60.44 ±14.31	48.54 ±11.71
T-test		NS	NS	NS	NS

N: number of measured structures; NS: non-significant changes

For the quantitative histological analysis, 249 vascular canals of the primary osteons, 175 Haversian canals and 175 secondary osteons were measured in total. The results are summarized in Tables 2, 3 and 4. We have found that all measured variables (area, perimeter, maximum and minimum diameters) of the Haversian canals and secondary osteons did not differ between rabbits from both investigated groups. On the other hand, the size of the primary osteons' vascular canals significantly decreased ($p < 0.05$) in males from the group A.

DISCUSSION

The results of our study have shown that intramuscular application of patulin had not significant effect on total body weight, femoral weight and length in adult male rabbits. Similarly, no demonstrable alterations in the body

weight gain were also reported in rats intoxicated with patulin at a dose of 0.1 mg.kg⁻¹ b.w./day in drinking water for 60 or 90 days (Selmanoğlu and Koçkaya, 2004; Selmanoğlu, 2006). Additionally, body weight and weight of various organs (e.g., liver, spleen, thymus, kidney with adrenals and lungs) were unchanged in female mice orally exposed to patulin (at the doses of 0.08, 0.16, 0.32, 0.64, 1.28 and 2.56 mg.kg⁻¹ b.w.) for 28 days (Llewellyn et al., 1998).

Skeletal growth is the result of complex interplay of nutritional, genetic and hormonal factors. Regarding the hormones, essential roles in normal skeletal development play thyroid and growth hormones. It is known that growth hormone deficiency produces severe generalized failure of osteogenesis (Braverman et al., 2005; Lieberman and Friedlaender, 2005) and decreased triiodothyronine and

thyroxine levels may reduce the basal metabolic rate, which could be associated with growth retardation (Selmanoglu and Koçkaya, 2004). The results published by above mentioned authors (Selmanoglu and Koçkaya, 2004) revealed non-significant changes in the levels of thyroid stimulating hormone and growth hormone in growing rats after application of patulin at a dose of 0.1 mg.kg⁻¹ b.w./day for a period of 60 or 90 days. This finding could signalize that patulin administration would not have an adverse impact on body weight and weight and length of femoral bone in these animals what is in accordance with our study.

Higher values for cortical bone thickness in male rabbits with patulin intoxication can be attributed to intensive formation of bone tissue within periosteal surface as an adaptive response to bone tissue against patulin toxicity. Under physiological conditions, width of bone increases with ageing due to periosteal apposition (Seeman, 2003). Periosteal bone formation can be stimulated by several agents, such as some hormones (Burr and Guillot, 2012). Parathormone is known to prevent apoptosis of periosteal osteoblasts (Jilka et al., 1999; Burr and Guillot, 2012), which could partly account for its effect on the cells in the osteogenic layer of the periosteum (Rhee et al., 2011; Burr and Guillot, 2012). The benefits of intermittent treatment with parathormone have been shown in experimental animals (Sato et al., 2002; Szulc et al., 2006). Osteoporotic postmenopausal women treated with human parathyroid hormone (1-34) had higher cortical thickness of iliac crest and higher width of distal radius (Dempster et al., 2001; Szulc et al., 2006). Sergeev et al. (1988) studied effects of aflatoxin B1 and T-2 toxin on metabolism of calcium and vitamin D. The young rats were administered daily within 7 days with these mycotoxins at the dose of 0.7 mg.kg⁻¹ and 0.54 mg.kg⁻¹ b.w. Administration of the mycotoxins caused hypocalcemia, decreased the concentration of 25(OH)D3 in blood serum and also lower activity of 25-hydroxylase D3 in liver tissue. In kidney, the activity of 24-hydroxylase tended to decrease. Likewise, the study by Glahn et al. (1991) demonstrated a decreased plasma 25-hydroxy vitamin D and 1,25-hydroxy vitamin D levels in three-week-old male broiler chickens after five days of aflatoxin treatment. In addition, total plasma calcium tended to be lower. These effects are connected with altered vitamin D and parathyroid hormone metabolism (Devegowda and Ravikiran, 2008). According to Lips (2001), deficiency in vitamin D causes secondary hyperparathyroidism, which leads to increased parathormone production. On the basis of these aspects we suppose that other mycotoxins (including patulin) should also have a similar effect on the concentrations of vitamin D and parathormone, which can be connected with evident changes in cortical bone thickness.

According to Cilotti and Falchetti (2009) androgens may prevent the loss of cancellous bone and also stimulate periosteal cortical bone apposition, resulting in a larger bone size and thicker cortical bone in males (Kung, 2003). Generally, androgen receptors are found in all bone cells, i.e., osteoblasts, osteocytes and osteoclasts (Notelovitz, 2002). The most important androgen is a testosterone (Selmanoglu and Koçkaya, 2004; Callewaert et al.,

2010). It was found that testosterone increases periosteal and endosteal apposition, bone size, trabecular (Tuck and Francis, 2009) and cortical bone thickness (Gorton et al., 2005) in adolescent males. On the other hand, a reduction in testosterone decreases the rate of periosteal bone apposition (Kung, 2003). Significant increased serum testosterone levels after patulin administration (at a dose of 0.1 mg/kg b.w./day) for a period of 60 or 90 days were documented in the study by Selmanoglu and Kockaya (2004). On the basis of these aspects we propose that enlargement of bone tissue on periosteal bone surface could also be associated with patulin-modified increased testosterone concentration.

The results of qualitative histological analysis correspond with those reported by other authors (Enlow and Brown, 1956; Martiniaková et al., 2003; Chrenek et al., 2006). The basic structural pattern of compact bone was primary vascular longitudinal in both groups of rabbits. Also, primary vascular radial and dense Haversian or irregular Haversian bone tissues were identified. In males intoxicated with patulin, an absence of the primary vascular longitudinal bone tissue near endosteal surface can be connected with intensive endosteal resorption due to patulin toxicity. On the other hand, primary vascular longitudinal bone tissue was (in some areas) present not only near the periosteal surface but also in the middle part of the compact bone. This findings point to the enhanced periosteal apposition as a compensative mechanism of bone tissue against patulin-induced bone loss from endosteal surface. Studies with liver cells or liver slices have demonstrated that patulin treatment results in glutathione depletion, which is connected with oxidative stress. Patulin-modified increased intracellular oxidative stress was reported in human embryonic kidney and human promyelocytic leukemia cells (Liu et al., 2007). Bone resorption is the unique function of the osteoclasts (Teitelbaum, 2000). Osteoclasts have shown to be activated by reactive oxygen species (ROS) to enhance bone resorption (Baek et al., 2010). Therefore, we suppose that the absence of primary vascular longitudinal tissue near the endosteal surface can be attributed to oxidative stress-induced intensive bone resorption due to patulin toxicity.

The histomorphometrical measurements showed a significant constriction of the primary osteons' vascular canals in rabbits exposed to patulin. This fact can be related to adverse effect of patulin on blood vessels, which are present in vascular canals of primary osteons (Greenlee and Dunnell, 2010). The results of Broom et al. (1944) revealed a very small, transient vasoconstriction in rabbits after patulin administration at the dose of 0.08 mg.kg⁻¹ b.w. Recent experimental studies have demonstrated the direct action of androgens on the blood vessels. Long-term administration of testosterone may elicit harmful effects, especially vasoconstriction (Akishita and Yu, 2012). Furthermore, patulin has a strong affinity for sulfhydryl groups inhibiting the activity of many enzymes (Selmanoglu and Koçkaya, 2004; Puel et al., 2010; Zbynovská et al., 2013). Magan et al. (2004) found that patulin (at the dose of 1.6 mg.kg⁻¹ b.w.) inhibited acetylcholinesterase and NaK-ATPase in the cerebral hemisphere, cerebellum and *medulla oblongata* in

rats leading to higher levels of acetylcholine in these brain segments. Martiniaková et al. (2013) noted that increased levels of acetylcholine in the endothelium of blood vessels subsequently activated muscarinic receptors in the endothelium of vascular and Haversian canals in rats. According to Yoopan et al. (2008), the alteration of muscarinic function in blood vessel may contribute to hypertension. The binding of patulin to sulfhydryl groups of acetylcholinesterase might also explain this negative impact of this mycotoxin on the size of the primary osteons' vascular canals in rabbits from the group A.

CONCLUSION

Generally, exceeded recommended level of patulin in fruit juices and other fruit products may cause potential risk in humans, particularly in children.

The current study revealed a significant effect of intramuscular application of patulin at the dose of 10 µg.kg⁻¹ b.w. twice per week for 4 weeks on cortical bone thickness and the size of primary osteons' vascular canals in adult male rabbits. In addition, patulin application induced evident changes in femoral bone microstructure of these animals.

Anyway, our results indicate considerable impact of patulin on macroscopical and microscopical structures of femoral compact bone in adult male rabbits.

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QUALITATIVE AND QUANTITATIVE CHARACTERISTICS OF SERBIAN TOMATO VARIETIES GROWN IN CONDITIONS OF SLOVAK REPUBLIC

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ABSTRACT

The aim of submitted article was to estimate qualitative and quantitative characteristic of Serbian tomato varieties grown in conditions of Slovak republic and to compare the results with control tomato variety and evaluate their potential for the Slovak market. There were included 6 Serbian tomato varieties: 'Fantom VFCTm F1', 'Marathon ASVF F1', 'Honey Heart VF F1', 'Uragan SVF F1', 'Kazanova F1 VF', 'Dinka F1' and 'Tornado F1' in control variant, which is wide spread in Slovak tomato production. As qualitative characteristic for every variety total yields (in kg) per every plant, total number of harvested fruits (in pieces for each plant) and average weight of one fruit in kg were evaluated. In Department of vegetable growing laboratory qualitative characteristics in case of all chosen tomato varieties were estimated (included firmness of fruits, total carotenoids estimation, ascorbic acid estimation). Serbian varieties reached lower total yields per plant. From the 'fruit weight' point of view they created more or less homogenous group of bigger and heavier fruits in comparison with 'Tornado F1' and statistically significant homogenous group when focusing on total number of fruits per plant in comparison with 'Tornado F1' variety. Differences between control variant and chosen Serbian varieties were even more considerable in case of qualitative characteristics, where 'Tornado F1' reached the lowest values in case of all observed characteristics - firmness of fruits, total carotenoids and ascorbic acid content. According to increasing importance of antioxidants in human diet, all tested Serbian varieties appeared to be very interesting for Slovak consumers from following both qualitative and quantitative characteristics.

Keywords: tomatoes; carotenoids; ascorbic acid; yields; varieties

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) as a one of the worldwide most grown vegetable belongs to family Solanaceae. At the present the biggest tomato producers are China, India and USA (FAOSTAT, 2014). In Slovak Republic, according to last statistical outputs from the 2013, tomatoes were the second most cultivated vegetables, grown on the total area of 2948 ha, which represented the production of 44913 t (Merava, 2014). List of registered varieties (UKSUP, 2014) refers together 85 varieties of tomatoes, which are allowed in Slovak republic, and it involves 52 plunge (indeterminate) and 33 bush (determinate) tomato varieties. Recommended dose for 1 person is 16 kg per year (Kona and Konova, 2008), whereby according to (Merava, 2014) in SR during the 2012 it was 18 kg/person, with the highest consumption of tomatoes (17. 8%) within the frame of the total vegetable consumption in Slovak republic. Tomato (*Lycopersicon esculentum*) is one of the major vegetable crops in Serbia Miličević et al., (2009). According to comparative analyses of Novaković et al., (2012) when comparing tomato indicators in Serbia, tomato were grown on area 20647 ha, with yields 8.7 t/ha and production 178823 t (values are presented as an average of the interval from 2001 till 2010).

Tomatoes are consumed raw, but also thermal processed, especially in Mediterranean, Greek, Italian, Southeast

Asian, and East European cuisine, with increased interest in its nutrient value during the last three decades. The main reason is due to the presence of different antioxidant molecules such as carotenoids, ascorbic acid, vitamin E and phenol compounds, particularly flavonoids Frusciante et al., (2007), and lycopene, the main carotenoid in tomato Yoshida et al., (2011). Tomato had all-trans-lycopene (1046-1099 µg/g Dry Weight (DW)), cis-lycopene (125-132 µg/g DW) and all-trans-beta-carotene (45-59 µg/g DW) as principal carotenoids. Tomato pulp and ketchup had all-trans-lycopene (951-999 µg/g DW and 455-476 µg/g DW), all-trans-β-carotene (76-88 DW µg/g and 20-27 DW µg/g) and cis-lycopene (71-83 µg/g DW and 14-25 µg/g DW) as the main pigments, respectively. They also contained other carotenoids in much smaller amounts (lycoxanthin, zeaxanthin, antheraxanthin, lutein, gamma-carotene, and phytofluene) Gama et al., (2006). According to various authors, the total carotenoids content as well as lycopene in fresh tomato fruits depends mainly on genotypes (Mendelová et al. 2015, Mendelová et al. 2013, and Carli et al. 2011). The big advantage of lycopene is its stability within the frame of various thermal processes. Processed tomato products like tomato juice, tomato paste, tomato puree, and tomato ketchup and tomato oleoresin have been shown to provide bioavailable sources of lycopene Basu et al. (2007). Tomatoes are the richest source of lycopene in the Western diet (Burton,

Freeman and Sesso, 2014) and they are consumed like a functional food all over the world because of health promoting compounds in its fruit. Latest research highlights the relationship between consuming tomato and its products with reduced risk of various maladies like obesity, hyperglycemic and hypercholesterolemic attributes, cardiovascular disorders and cancer insurgences (Perveen, 2015). Lycopene absorption is strongly impacted by dietary composition, especially the amount of fat. Concentrations of circulating lycopene in lipoproteins may be further influenced by a number of variations in genes related to lipid absorption and metabolism. Better understanding of the relationship between diet, genetics, and lycopene distribution will provide necessary information to interpret epidemiological findings more accurately Moran et al., (2013). The fact remains, that increasing of carotenoids, lycopene, vitamin C and other antioxidants is requested and their content is one of the important parameter estimated in case of new varieties.

The aim of submitted article was to estimate qualitative and quantitative characteristic of six Serbian tomato varieties grown in conditions of Slovak republic and to compare the results with control tomato variety 'Tornado F1' and evaluate their potential for the Slovak market.

MATERIAL AND METHODOLOGY

An experiment was founded in 2014 in Botanical Garden of Slovak University of Agriculture (below BG SUA) in tunnel conditions. Area is situated in very warm agro-climatic region, very dry sub-region. The average annual temperature is 10 °C. There were included 6 Serbian tomato varieties: 'Fantom VFCTm F1', 'Marathon ASVF F1', 'Honey Heart VF F1', 'Uragan SVF F1', 'Kazanova F1 VF', 'Dinka F1' and one Czech tomato variety: 'Tornado F1' in control variant. Variety used in control variant is extended on Slovak market, and growers have good experiences with its production in our conditions. All chosen varieties are indeterminate. For these kinds of tomatoes it is characteristic flourishing stem with indeterminate growth. The plants are usually very high and they are grown with the prop, with systematic removing of side shoots. They support lot of handmade that is the reason for their extensive growing in tunnels Uher et al., (2012). Vegetables were planted out in heatless tunnel in April 7th, 2014 in the united scale 0.7 x 0.5 m. For every variety there was planted 8 pieces of plants. After reaching phenological stage - ripening, fruits were gradually harvested from the beginning of July till middle of October, 2014.

Quantitative characteristics

For every variety total yields (in kg) per every plant, total number of harvested fruits (in pieces for each plant) and average weight of 1 fruit in kg were evaluated. Average volumes from each harvest were counted and statistically analyzed.

Qualitative characteristic

Following quantity in chosen tomato varieties, the qualitative characteristics were estimated in laboratory of Department of vegetable growing, SUA, in Nitra involving:

Firmness of fruits - Within all 7 observed varieties were measured ten replicates of firmness by penetrometer. The average volumes were evaluated.

Total carotenoids estimation - Carotenoids were estimated by spectrophotometric measurement of substances absorbance in petroleum ether extract on spectrophotometer PHARO 100 at 445 nm wavelengths. As an extraction reagent there was used acetone.

Ascorbic acid estimation - HPLC method of vitamin C content estimation was used for its quantity estimation by the help of liquid chromatograph with UV detector, for separation was used RP C18 column, mobile phase was methanol : water (5:95, v/v), UV detection was adjusted to 258 nm (HPLC fy. VARIAN).

Statistical analysis

The analysis of variance (ANOVA), the multifactor analysis of variance (MANOVA) and the multiple Range test were done using the Statgraphic Centurion XVII (StatPoint Inc. USA).

RESULTS AND DISCUSSION

Quantitative characteristic

According to submitted methods, there were evaluated total yields (kg), total number of harvested fruits (pieces) per one plant and average weight of 1 fruit in kg from each harvest (table 1).

In case of observed characteristic 'total number of fruits' all Serbian varieties was classified in homogenous group from 22.75 ± 2.92 ('Fantom VFCTm F1') to 44.00 ± 9.80 pieces per plant ('Dinka'), whereby 'Tornado F1' created much more higher amount of fruits (104.63 ± 12.39 pieces per plant). It corresponded with statistical analyzes, where it was determined statistically significant difference only between 'Tornado F1' vs. all Serbian tomato varieties at 99.9% by Tukey test. Similar parameters were evaluated as a part of research of two tomato cultivars ('Cedrico' and 'Abellus') in polytunnels in Serbia Savic et al., (2011). Their results with the values 40.45 number of fruits per plant and 5.52 kg per plant in case of 'Cedrico' and the values 35.30 number of fruits per plant and 4.10 kg per plant for 'Abellus' were comparable to Serbian varieties observed in our trial.

In opposite to high amount of fruits, following the characteristic 'average weight of one fruit' the 'Tornado F1' variety produced the most light - weighted fruits (0.07 ± 0.02 kg) in comparison with other varieties. Fruits of Serbian varieties reached the values between 0.13 ± 0.02 kg in case of 'Fantom VFCTm F1' and 0.21 ± 0.04 kg in 'Dinka F1'.

Qualitative characteristics

In Department of vegetable growing laboratory qualitative characteristics in case of all chosen tomato varieties were estimated (Table 2). The firmness of fruit was counted as an average from 10 measurements from each variety. The hardest fruits were confirmed for 'Honey Heart VF F1' (firmness of fruit = 8.36 N), followed by 'Fantom VFCTm F1' (8.07 N). In comparison with control variant represented by 'Tornado F1' with firmness 5.25 N, all Serbian varieties had harder fruits.

Followed total carotenoids content estimated and represented in Figure 1 variety 'Kazanova F1 VF' with values 13.07 mg/100g represented the upper limit of interval. Control variety 'Tornado F1' enclosed the interval on the bottom limit with 4.71 mg/100g. In case of all Serbian varieties the higher content of carotenoids was confirmed in comparison with 'Tornado F1'. **Carli et al., (2011)** monitored the content of carotenoids in 7 varieties of tomatoes and report their content from 3.63 to 17.5 mg/100g. In similar experiment which have included following tomato varieties: Bambino F1, Milica F1, (indeterminate tomato varieties) and Darina F1, Diana F1, Denar, Milica F1, Orange F1, Paulina F1, Šejk F1 (determinate tomato varieties), the carotenoid content in fresh fruits of selected varieties ranged from 2.63 to 6.55 mg/100g. The highest content of carotenoids was observed at variety Milica F1 and the lowest in Paulina F1 **Mendelova et al., (2013)**. All Serbian varieties except of 'Dinka F1' (with 4.46 mg/100g for total carotenoids) had also higher amounts of observed compounds when comparing with the experiment of Mendelova. In comparison with other experiment, led by **Mendelova et**

al. (2010), where the content of carotenoids in fruits of tomatoes for industrial processing varied in the amount of 4.41 to 7.85 mg/100 g, the Serbian varieties 'Kazanova F1 VF' and 'Fantom VFCTm F1' reached bigger content of carotenoids, and others varieties belonged to higher limits of Mendelova's interval. The content of carotenoids in the monitored varieties decreased in the order: 'Kazanova F1 VF' > 'Fantom VFCTm F1' > 'Marathon ASVF F1' > 'Honey Heart VF F1' > 'Uragan SVF F1' > 'Dinka F1' > 'Tornado F1'. Ascorbic acid content was ranged in interval from 11.51 mg/100g ('Tornado F1') to 18.72 mg/100g ('Uragan SVF F1'). Similar, as it was providing other observed qualitative characteristics, all Serbian varieties reached higher content of vitamin C. **George et al., (2004)** indicate the content of ascorbic acid in tomato pulp from 8.4 to 32.4 mg/100 g. Substantial amounts of ascorbic acid were also detected in peels (9.0–56.0 mg/100 g fw and 104–462 mg/100 g dw). The content of ascorbic acid in the monitored varieties decreased in the order: 'Uragan SVF F1' > 'Marathon ASVF F1' > 'Honey Heart VF F1' > 'Dinka F1' > 'Fantom VFCTm F1' > 'Kazanova F1 VF' > 'Tornado F1'.

Table 1 Chosen quantitative characteristic of observed tomato varieties grown in tunnel, in BG, SPU, Nitra, in 2014*.

Variety	total yields	total number of fruits	average weight of 1 fruit
	(kg/ 1 plant)	(pieces/ 1 plant)	(kg)
'Marathon ASVF F1'	7.50 ±1.40 ^{bc}	42.33 ±10.88 ^a	0.18 ±0.03 ^{bc}
'Fantom VFCTm F1'	3.05 ±0.70 ^a	22.75 ±2.92 ^a	0.13 ±0.02 ^{ab}
'Dinka F1'	8.93 ±0.56 ^c	44.00 ±9.80 ^a	0.21 ±0.04 ^c
'Tornado F1'	7.35 ±2.20 ^{bc}	104.63 ±12.386 ^b	0.07 ±0.02 ^a
'Uragan SVF F1'	5.62 ±1.52 ^{ab}	31.14 ±9.72 ^a	0.19 ±0.05 ^{bc}
'Honey Heart VF F1'	6.60 ±0.93 ^{bc}	32.33 ±9.86 ^a	0.21 ±0.07 ^{bc}
'Kazanova F1 VF'	3.21 ±0.93 ^a	20.89 ±2.86 ^a	0.14 ±0.03 ^{abc}

*Mean ±standard deviation.

Column values with different lowercase letters in superscript are significantly different at $p < 0.001$ by Tukey HSD in ANOVA (Statgraphic)

Table 2 Chosen qualitative characteristic of observed tomato varieties grown in tunnel, in BG, SPU, Nitra, in 2014*

Variety	firmness (N)	total carotenoids (mg/100g)	vitamin C (mg/100g)
'Tornado F1'	5.25 ±0.79 ^a	4.71 ±0.57 ^a	11.51 ±0.86 ^a
'Fantom VFCTm F1'	8.07 ±0.54 ^b	9.79 ±0.57 ^d	12.08 ±0.26 ^a
'Marathon ASVF F1'	6.51 ±0.66 ^a	7.61 ±0.43 ^c	15.39 ±0.56 ^b
'Honey Heart VF F1'	8.36 ±0.78 ^b	6.95 ±0.15 ^{bc}	15.03 ±0.54 ^b
'Uragan SVF F1'	6.37 ±0.81 ^a	6.62 ±0.60 ^{abc}	18.72 ±0.76 ^c
'Kazanova F1 VF'	7.87 ±0.87 ^b	13.07 ±0.23 ^e	12.03 ±0.75 ^a
'Dinka F1'	5.47 ±0.61 ^a	5.46 ±0.73 ^{ab}	13.76 ±0.25 ^{ab}

*Mean ±standard deviation.

Column values with different lowercase letters in superscript are significantly different at $p < 0.001$ by Tukey HSD in ANOVA (Statgraphic)

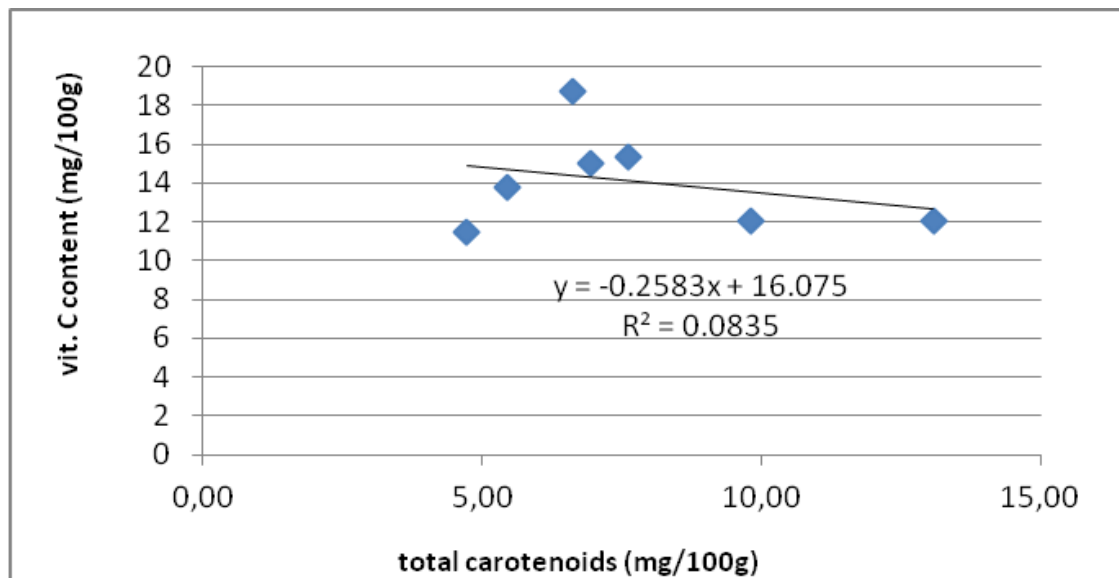


Figure 1 Ascorbic acid (vitamin C) content in chosen tomato varieties grown in tunnel, in BG, SPU, Nitra, in 2014.

According to wider spectrum of varieties, the correlation analyzes between vitamin C and carotenoid was done. Those two antioxidants are connected in some scientific studies with effort to find some relationships. It should be noted, that the usually close correlation between intake of vitamin C and carotenoids precludes any firm conclusions regarding to separate effects of dietary vitamin C and carotenoids on bladder cancer protection. In other words it is unclear if the observed dietary protective effect is due to carotenoids alone, vitamin C alone, or both (Vogelzang, 2006). No correlation was obtained with any of the antioxidant constituents such as vitamin C and carotenoids (Gil, 2002). The results corresponding with our findings figured in correlation on Figure 1.

CONCLUSION

The changes of quantitative and qualitative characteristics in dependence on chosen tomato varieties were estimated in the article. Differences between control variant and chosen Serbian varieties were founded in case of quantitative, but even more in case of qualitative characteristics, where 'Tornado F1' reached the lowest values in all observed characteristics - firmness of fruits, total carotenoids and ascorbic acid content. According to increasing importance of antioxidants in human diet, all tested Serbian varieties appeared to be very interesting for Slovak consumers. From quantitative point of view, the control variety 'Tornado F1' had the smallest fruits, whereby the values of Serbian varieties ranged in interval from 134 to 211 g. At the present on the Slovak market varieties with fruit weight in interval from 100 to 160 g are wide-spread used. Observed Serbian varieties create markedly bigger fruits in smaller amount, but they are noted for great gustative properties. On the base of complex evaluation, Serbian varieties are suitable previously to smaller growers for self-subsistent farming according to our recommendations.

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QUALITY OF MEAT OF RABBITS AFTER APPLICATION OF EPICATECHIN AND PATULIN

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ABSTRACT

The aim of the present study was to determinate the effect of epicatechin and patulin on selected parameters of meat quality of rabbits. Adult female rabbits (n=25), maternal albinotic line (crossbreed Newzealand white, Buskat rabbit, French silver) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big light silver) were used in experiment. Animals were divided into five groups: control group (C) and experimental groups E1, E2, E3, and E4. Animals from experimental groups E1, E2, E3, E4 received patulin through intramuscular injection ($10 \mu\text{g}\cdot\text{kg}^{-1}$) twice a week and animals from groups E2, E3, E4 received epicatechin three times a week through intramuscular injection. After 30 days animals were slaughtered. For analysing of meat quality the samples of *Musculus longissimus dorsi* (50 g) were used. Application of epicatechin and patulin to rabbits had slight or no effect on the pH levels in stomach, small intestine, large intestine and urinary bladder contents, however differences among the groups were insignificant ($p > 0.05$). Application of epicatechin and patulin to rabbits had slight or no effect on total water, protein, fat and differences among the groups were insignificant ($p > 0.05$). The values of amino acids concentrations were not influenced after application of epicatechin and patulin. The fatty acid profiles in animals after application of different doses of epicatechin and $10 \mu\text{g}\cdot\text{kg}^{-1}$ patulin were similar ($p > 0.05$). Concentration of cholesterol increased in experimental groups in comparison with the control group, but differences were insignificant ($p > 0.05$). pH levels of meat of rabbits in experimental group E3 was lower when compared with the control group, but differences was not significant ($p > 0.05$). Electric conductivity parameter was increased in each experimental group (in E3 the highest) against the control but without significant differences ($p > 0.05$). Colour L parameter was slightly decreased in experimental groups with comparison to the control group (in E3 the lowest). Generally we can conclude that intramuscular application of epicatechin or patulin did not affect parameters of meat quality as well as pH values of internal organs content. Further investigations are needed to prove the final answer concerning the health promoting effects of epicatechin and patulin.

Keywords: Rabbits; epicatechin; patulin; pH level; meat quality

INTRODUCTION

Flavonoids are ubiquitous in plant foods. Important dietary sources can include tea, red wine, apples, and cocoa (Renaud and de Lorgeril 1992; Hammerstone et al., 2000). Many flavonoids are potent antioxidants in *in vitro* systems (Williams et al., 2004). Epidemiologic studies have reported a reduced risk of cardiovascular disease in subjects with a high flavonoid intake (Huxley et al., 2003). Protective lipid oxidation during storage of meat is indispensable in order to preserve the quality standards and the shelf life of this product (Nieto et al., 2010). That is the reason for increasing numbers of studies examined dietary additions of natural (no synthetic) antioxidants (Nieto et al., 2010; Bodas et al., 2012; Morán et al., 2012a; Morán et al., 2012b; Capcarova et al., 2012). This strategy is especially interesting because if antioxidants are deposited in the meat during the life of the

animal no addition of exogenous products would be required after slaughter. This alternative, perceived by the consumer as a high quality standard (Sebranek and Bacus, 2007), might be especially useful to prevent meat lipid peroxidation when diets rich in polyunsaturated fatty acids (PUFAs) are administered to the animals. In this sense, attention has been paid to phenolic compounds, a group of substances present in fruits, vegetables, nuts and seeds which have shown potent antioxidant effects as metal chelators or free-radical scavenging activities (McBride et al., 2007). Most of these compounds also have shown antimicrobial properties when added directly to the meat as additives (McBride et al., 2007). However, results have been different when included in the diet of the animals. Patulin, 4-hydroxy-4H-furo(3,2c)pyran-2(6H)-one, is a mycotoxin produced by molds including *Penicillium expansum*, *Aspergillus*, and *Byssachlamys*,

also occurring world-wide in apple, apple products and sometimes in a number of foods including peaches, pears, and grain, or their products (Sommer et al., 1974; Frank et al., 1977; Scott et al., 1977; Chan et al., 2006; Morales et al., 2008; Kwon et al., 2005). Patulin level of 50 µg.kg⁻¹ in apple juice was suggested to be sufficient for protection of human health (Kwon et al., 2010). Patulin exerts its toxic effect by covalently binding to reactive sulfhydryl groups in cellular proteins, as well as by glutathione depletion, resulting in oxidative damage and generation of reactive oxygen stress (ROS) (Renaud and de Lorgeril 1992; Hammerstone et al., 2000; Wu et al., 2008). The aim of present study was to determinate the effect of epicatechin and patulin on selected parameters of meat quality (content of total water, proteins, fat, content of amino acids and fatty acids, electric conductivity, pH, colour) in muscle of rabbits and pH levels of stomach, small intestine, large intestine and urinary bladder content.

MATERIAL AND METHODOLOGY

2.1. Animals

Adult female rabbits (n = 25), maternal albinotic line (crossbreed New Zealand white, Buskat rabbit, French silver) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big light silver) were used in

experiment. Rabbits were obtained from an experimental farm of the Animal Production Research Centre Nitra, Slovak Republic. Rabbits (age 4 months, body weight 4.0 – 4.5 kg) were housed in individual flat-deck wire cages (area 0.34 m²) under a constant photoperiod of 14 h of day-light. The temperature (18 – 20 °C) and humidity (65%) of the building were recorded continually by means of a thermograph positioned at the same level as the cages. Rabbits were healthy and their condition was judged as good at the commencement of the experiment. Water was available at any time from automatic drinking troughs. Adult rabbits were fed diet of a 12.35 MJ.kg⁻¹ of metabolizable diet (Table 1) composed of a pelleted concentrate.

2.2. Experimental design and diets

Animals were divided into five groups: control group (C) and experimental groups E1, E2, E3, and E4. Animals from experimental groups E1, E2, E3, E4 received patulin through intramuscular injection (10 µg.kg⁻¹) twice a week and animals from groups E2, E3, E4 received epicatechin three times a week (Tab 2) through intramuscular injection. Experiment lasted 30 days. In this animal study, institutional and national guidelines for the care and use of animals were followed, and all experimental procedures

Table 1 Chemical composition (g.kg⁻¹) of the experimental diet.

Component	
Dry matter	926.26
Crude protein	192.06
Crude Fat	36.08
Crude Fibre	135.79
nitrogen free extract	483.56
Ash	78.78
Organic matter	847.49
Calcium	9.73
Phosphorus	6.84
Magnesium	2.77
Sodium	1.81
Potassium	10.94
Metabolizable energy	12.35 MJ.kg ⁻¹

Table 2 Application of epicatechin and patulin in injectable form (*intramuscular*).

group	n	epicatechin (µg.kg ⁻¹)	patulin (µg.kg ⁻¹)
Control C	5	0	0
Experimental E1	5	0	10
Experimental E2	5	10	10
Experimental E3	5	100	10
Experimental E4	5	1000	10

involving animals were approved by ethical committee.

2.3. Meat quality analysis

At the end of the experimental period, which lasted 30 days, 25 adult female rabbits were slaughtered in an experimental slaughterhouse. The carcasses were prepared by removing the stomach, small intestine, large intestine and urinary bladder with contents. In these organs pH levels using pH 213 Microprocessor pH meter (Hanna instruments, USA) were determined. Samples of *Musculus longissimus dorsi* (50 g) were collected from each animal. The meat samples were collected one hour after slaughter, wrapped in aluminium foil and stored at 4 °C for 24 hours. Meat quality was analysed for parameters characterizing the content of nutrients (content of total water, content of proteins, fat, content of amino acids and fatty acids) and processing technology parameters (electric conductivity, pH, colour).

The content of water, proteins, crude fat and fatty acids were analysed by method FT IR (Fourier Transform infrared Spectroscopy) using Nicolet 6700. Content of amino acids was detected using gas chromatography GC-ECD/NPD. In 1997, Oh-shin et al. [4, 5] reported a simultaneous GC-ECD/NPD method that included quantitation, for insecticides including carbaryl in drinking water. Low LOD of 0.1 ng/mL, and excellent linearity ($R_2 = 0.998-1.000$) were demonstrated.

The value of pH (24 hour post mortem) was detected by portable battery acidometer OP-109. The colour was measured on the surface of the *M. longissimus dorsi* (10 mm thickness), at 24 h post mortem. Colour data were expressed in terms of Lightness (L^*), redness (a^*), yellowness (b^*) in accordance with CIELAB colour space (CIE, 1976).

Instrumental colour measurements were recorded for L^* (lightness; 0: black and 100: white), a^* (redness/greenness; positive values: red and negative values: green), and b^* (yellowness/blueness; positive values: yellow and negative values: blue) using a spectrophotometer CM-2600d (Konica, Minolta, Japan). Due to wet surface of the sample, we evaluated the colour with shine (SCI).

2.4. Statistical analyses

The data used for statistical analyses represent means of values obtained in three blood collections performed on separate days. To compare the results, one-way ANOVA test was applied to calculate basic statistic characteristics and to determine significant differences among the experimental and control groups. Statistical software SIGMA PLOT 11.0 (Jandel, Corte Madera, CA, USA) was used. Differences were compared for statistical significance at the level $p < 0.05$.

RESULTS AND DISCUSSION

3.1. pH levels in stomach, small intestine, large intestine and urinary bladder with contents

The results of pH levels in stomach, small intestine, large intestine and urinary bladder with contents are presented in Figure 1. Application of epicatechin and patulin of rabbits had slight or no effect on the levels of pH in selected organs and differences among the groups were insignificant ($p > 0.05$). Chao- Zhi ZHu et al., (2014)

published that the antioxidant activity of peptides from Jinhua hams exhibited the strongest scavenging activity at the neutral pH, and there was no significant decrease under acidic conditions. Even when the pH was reduced to 3, it still maintained 90% of its DPPH radical scavenging activity. When the pH was increased to 9, the DPPH radical scavenging activity sharply declined and at pH 11, the activity was reduced by 40% compared with that under neutral pH condition. Deamination is promoted at higher pH values resulting in changes with structure and conformation and loss of antioxidant capacity. Different pH values will affect the actual degradation pathway used (Patel and Borchardt 1990; Bell and Labuza 1991). It is important to study the theoretical and practical aspects of the stability of the antioxidants during processing, storage, and in the gastrointestinal tract (Sannaveerappa et al., 2007).

3.2. Nutritional composition of rabbits meat

Results of total water, crude protein, crude fat are presented in Table 3. Application of epicatechin and patulin of rabbits had slight or no effect on measured parameters and differences among the groups were insignificant ($p > 0.05$). The values of amino acids (Table 4) were not influenced after application of epicatechin and patulin. The fatty acid profiles of different doses of epicatechin and 10 $\mu\text{g}\cdot\text{kg}^{-1}$ of patulin were similar and did not differ among the groups ($p > 0.05$) (Table 5). Concentration of cholesterol (Table 5) increased in experimental groups in comparison with the control group, but differences were insignificant ($p > 0.05$). Although oxidation is recognized as the main cause of deterioration of meat quality during storage and processing, it is a crucial reaction to develop typical flavor of meat products, especially for many kinds of dry-cured meat products with long-term ripening process (Chizzolini et al., 1998). It is clear that the main oxidation occurring during meat processing is auto-oxidation (Gandemer, 1999), which involves with initiation, propagation and termination steps (Frankel, 1984). It is known that polyunsaturated fatty acids undergo auto-oxidation much more readily than mono or saturated fatty acids (Chizzolini et al., 1998). Therefore, during meat products processing, the PLs, which contain greater proportion of polyunsaturated fatty acids, are more important source for volatiles compared to triacylglycerols (TGs) (Toldrá, 1998). A large number of volatiles such as alkanes, aldehydes, alcohols, esters and carboxylic acids are produced from this process, of which the volatiles with low odour threshold play important roles for meat flavour perception development. Tang et al., (2000) reported that tea catechin supplementation at levels of 200 and 300 $\text{mg}\cdot\text{kg}^{-1}$ in feed were found to be more effective in retarding lipid oxidation in all tissues. Several studies in animals have demonstrated that epicatechin has beneficial effects in chronic degenerative diseases (Al-Gayar et al., 2011; Si et al., 2011; Gómez-Guzmán et al., 2011; Mohamed et al., 2011). Animal studies have shown that patulin is carcinogenic, mutagenic, teratogenic and highly toxic (Lee and Rosenthaler 1986; Yanagisawa et al., 1987; Alves et al., 2000; Schumacher et al., 2005). There is no documented evidence of any adverse effects of patulin to man. Nevertheless, it is considered to be a contaminant in most countries. The

wide numbers of health studies done on patulin have demonstrated that it inhibits several enzymes (Ashoor, 1973). This may be attributed to its reactivity with sulfhydryl groups (Arafat, 1985). Dickens and Jones (1961) found that localized tumours developed in rats when they were repeatedly injected with sub-lethal doses of patulin. Patulin had no effect on the parameters. High doses of patulin, administered via the drinking-water, caused effects on the kidney and gastro-intestinal tract of Wistar rats. No changes in the relative weight or histological appearance of the adrenal glands were observed (Speijers et al., 1988). There are a variety of supplemental antioxidants employed in practical rabbit nutrition of which tocoferol (TOH) and vitamin C are the most widely used. TOH protects cellular membranes against oxidative damage. It reacts or functions as a chain-breaking antioxidant, thereby neutralizing free radicals and preventing oxidation of lipids within membranes (Morrissey et al., 1994; McDowell, 2000). Vitamin C can reduce the generation of ROS and might regenerate α -tocopherol from its oxidized form (Reed, 1992). Some authors suggested that phenolic compounds

could influence secondary metabolites biosynthesis (Sanzani et al., 2009), but there is no information in literature regarding the effect of these flavanones on polyacetyltrehalose (PAT) biosynthesis. Mallozzi et al., (1996) worked with quercetin and found that this flavonol reduced 55% the accumulation of aflatoxin B1 at 25 ppm, and that naringenin, the aglycone of the flavanone naringin, also reduced aflatoxin B1 in almost 41% at the same concentration.

3.3. The effect of epicatechin and patulin on processing technology parameters

The effect of epicatechin and patulin on processing technology parameters was monitored in this study (Table 6). In the experimental groups we found relatively equal levels of pH 24h, electric conductivity, colour L and a*, b* in comparison with the control group. In this study pH 24h levels in muscle samples in experimental group E3 was lower when compared with the control group, but differences were not significant ($p > 0.05$).

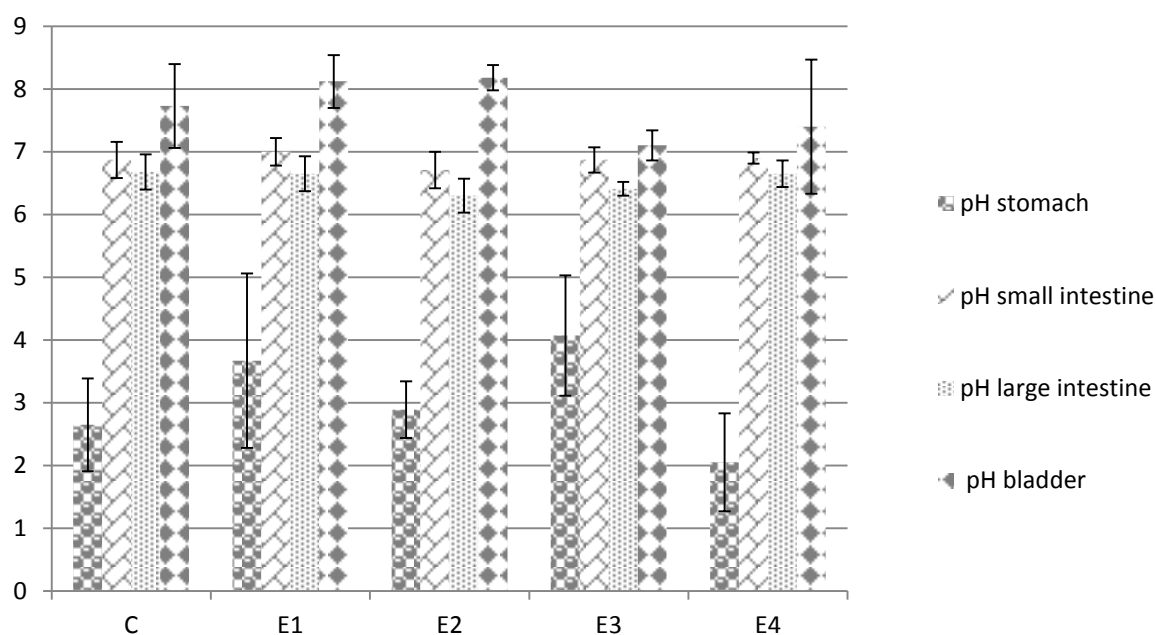


Figure 1 pH levels of stomach, small intestine, large intestine and urinary bladder with contents.

Table 3 Effect of epicatechin and patulin on selected nutrients content in samples of *Musculus longissimus dorsi* of rabbits (g.100g⁻¹).

Parameter	C	E1	E2	E3	E4
Crude protein	24.29 ± 0.46	24.67 ± 0.70	24.26 ± 0.26	23.91 ± 0.41	23.84 ± 0.07
Crude fat	1.14 ± 0.25	1.19 ± 0.17	1.06 ± 0.24	1.21 ± 0.22	1.26 ± 0.43
Total water	73.61 ± 0.38	73.58 ± 0.41	73.83 ± 0.28	73.95 ± 0.34	73.88 ± 0.41

C - control group; E1 10 $\mu\text{g.kg}^{-1}$ patulin; E2 10 $\mu\text{g.kg}^{-1}$ epicatechin and 10 $\mu\text{g.kg}^{-1}$ patulin; E2 100 $\mu\text{g.kg}^{-1}$ epicatechin and 10 $\mu\text{g.kg}^{-1}$ patulin; E3 1000 $\mu\text{g.kg}^{-1}$ epicatechin and 10 $\mu\text{g.kg}^{-1}$ patulin – experimental groups ; mean \pm SD (standard deviation).

Table 4 Effect of epicatechin and patulin on content of amino acids in samples of *Musculus longissimus dorsi* of rabbits (g.100g⁻¹).

Parameter	C	E1	E2	E3	E4
Arginine	1.47 ±0.01	1.46 ±0.13	1.52 ±0.08	1.62 ±0.02	1.54 ±0.06
Cysteine	0.34 ±0.02	0.34 ±0.03	0.35 ±0.02	0.36 ±0.01	0.35 ±0.01
Phenylalanine	0.97 ±0.07	0.96 ±0.08	1.00 ±0.05	1.06 ±0.01	1.01 ±0.04
Histidine	1.04 ±0.09	1.02 ±0.10	1.14 ±0.07	1.24 ±0.04	1.16 ±0.09
Isoleucine	0.86 ±0.08	0.86 ±0.09	0.90 ±0.05	0.98 ±0.03	0.92 ±0.05
Leucine	1.87 ±0.14	1.85 ±0.15	1.94 ±0.10	2.05 ±0.02	1.96 ±0.08
Lysine	1.96 ±0.16	1.95 ±0.17	2.04 ±0.11	2.18 ±0.03	2.06 ±0.09
Methionine	0.70 ±0.05	0.70 ±0.06	0.74 ±0.04	0.81 ±0.03	0.74 ±0.03
Threonine	1.02 ±0.08	1.02 ±0.09	1.08 ±0.05	1.13 ±0.02	1.09 ±0.06
Valine	0.96 ±0.07	0.96 ±0.07	1.03 ±0.05	1.08 ±0.02	1.04 ±0.05

C - control group; E1 10 µg.kg⁻¹patulin; E2 10 µg.kg⁻¹ epicatechin and 10 µg.kg⁻¹patulin; E3 100 µg.kg⁻¹epicatechin and 10 µg.kg⁻¹patulin; E4 1000 µg.kg⁻¹epicatechin and 10 µg.kg⁻¹patulin – experimental groups ; mean ± SD (standard deviation)

Table 5 Effect of epicatechin and patulin on content of fatty acids (g.100g⁻¹ FAME) and cholesterol (g.100g⁻¹) in samples of *Musculus longissimus dorsi* of rabbits.

Parameter	C	E1	E2	E3	E4
n-3 fatty acid	0.46 ±0.07	0.45 ±0.12	0.45 ±0.07	0.58 ±0.06	0.47 ±0.04
n-6 fatty acid	7.42 ±0.91	7.09 ±1.09	7.99 ±1.72	10.13 ±2.83	8.07 ±2.07
PUFA	8.06 ±0.74	8.52 ±0.79	8.48 ±1.22	10.59 ±2.23	8.57 ±1.51
MUFA	54.91 ±0.59	55.32 ±1.59	56.28 ±1.78	54.97 ±2.07	55.37 ±2.98
SAFA	40.12 ±0.70	40.44 ±0.69	40.47 ±2.04	38.75 ±1.72	40.47 ±2.40
Cholesterol	0.18 ±0.02	0.26 ±0.04	0.21 ±0.07	0.26 ±0.01	0.24 ±0.07

Polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), saturated fatty acids (SAFA), Fatty acids methyl ester (FAME); C - control group; E1 10 µg.kg⁻¹patulin; E2 10 µg.kg⁻¹ epicatechin and 10 µg.kg⁻¹patulin; E3 100 µg.kg⁻¹epicatechin and 10 µg.kg⁻¹patulin; E4 1000 µg.kg⁻¹epicatechin and 10 µg.kg⁻¹patulin – experimental groups; mean ± SD (standard deviation).

Table 6 Effect of epicatechin and patulin on selected processing technology parameters in samples of *Musculus longissimus dorsi* of rabbits.

item	C	E1	E2	E3	E4
pH 24 h	5.70 ±0.06	5.70 ±0.06	5.69 ±0.06	5.61 ±0.05	5.74 ±0.14
electric conductivity	0.98 ±0.21	1.35 ±0.47	1.53 ±0.32	2.17 ±0.99	1.15 ±0.10
colour L	56.83 ±1.07	56.43 ±3.14	57.40 ±0.99	56.35 ±1.02	54.50 ±1.92
a*	-2.17 ±0.87	-1.23 ±0.73	-1.35 ±0.98	-0.79 ±0.89	-1.37 ±1.17
b*	6.48 ±1.91	6.99 ±1.25	5.61 ±0.36	6.39 ±0.79	5.25 ±1.20

C - control group; E1 10 µg.kg⁻¹patulin; E2 10 µg.kg⁻¹ epicatechin and 10 µg.kg⁻¹patulin; E3 100 µg.kg⁻¹epicatechin and 10 µg.kg⁻¹patulin; E4 1000 µg.kg⁻¹epicatechin and 10 µg.kg⁻¹patulin – experimental groups; mean ± SD (standard deviation).

Meat colour is related to the energy metabolism of muscles, the processing, storage stability of meat and can be affected by many factors. **Hernández et al., (1997); Pla et al., (1998); Fushi et al., (2006)** reported a pH 24h in muscle *M. longissimus dorsi* (MLD) 5.6 and 5.7 in the thigh muscle. Similar values published **Ludewig et al., (2003)**. **Blasco and Piles (1990)** reported pH of MLD ranging from 5.66 to 5.71. In this study electric conductivity parameter was increased in each experimental groups (in E3 the highest). Colour L parameter was slightly decreased in experimental groups when compared to the control group (in E3 the lowest). The meat samples of animals from experimental groups were reddish and greenish as the control group. This was possibly due to partial cell breakdown and blood migration caused by the slow freezing process or to the oxidation of the meat pigment, which stability depends on animal species, muscle biochemical characteristics, and some external parameters (**Lyon and Lyon 2002**). The lowered pH values were observed only in rabbits received epicatechin in injectable form (100 µg.kg⁻¹), what could be a result of less intense of oxidation of myoglobin with consequent lower levels of metmyoglobin. These results are quite surprising because of the demonstrated *in vitro* antioxidant activity of epicatechin, thus the reason of the lack of the same positive effect in muscle tissue is unclear (**Wang et al., 2007**). Epicatechin protects cells from oxidative insults by modulating the cellular antioxidant defences and reducing reactive oxygen species (ROS) production in the presence of stressors (**Chen et al., 2002; Azam et al., 2004; Kinjo et al., 2006**).

CONCLUSION

In conclusion, intramuscular administration of epicatechin and patulin had no effect on the selected parameters of meat quality (*M. longissimus dorsi*) of broiler rabbits. Research on the field of interactions among epicatechin and patulin in animal bodies and the questions of safety levels can also have a positive impact on the safety of food and will be worthy of further investigation.

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HEALTH SAFETY ASPECTS OF FOODSTUFFS INTENDED FOR PHENYLKETONURICS

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ABSTRACT

Phenylketonuria is a rare metabolic disorder that occurs due to the lack of liver enzyme phenylalanine hydroxylase. Untreated it leads to mental retardation, delayed development of speech, microcephaly, epilepsy, behavioral problems etc. Its treatment consists of strict diet, very low in phenylalanine content. The aim of this study was to determine the health safety and suitability of 17 selected foods labeled as „low in protein“ respectively „foods low in phenylalanine“. Analyses were focused on their phenylalanine content and amino acid representation. The other aim was to compare phenylalanine measured with the content declared by the producers on their label. By product origin, the largest amount of foodstuffs originated from Poland (59%), followed by Germany (23%) and equal representation (6%) had Slovakia, Hungary and Sweden. Automatic analyzer AAA 400, using ion exchange chromatography, analyzed the samples. The lowest determined phenylalanine content was 1 mg.100g⁻¹ (in rubber candy) and the highest 299 mg.100g⁻¹ (in paté). Powdered egg alternative was monitored as the second highest source of phenylalanine. Phenylalanine content in the monitored foods ranged from 1 to 299.6 mg. 100 g⁻¹ respectively, and increased in the following order: Gum candies (1 mg.100g⁻¹) < Cherry jelly (1.4 mg. 100g⁻¹) < Flour (5.8 mg.100g⁻¹) < Flour (5.9 mg.100g⁻¹) < Spaghetti (8.7 mg.100g⁻¹) < Pasta (9.9 mg.100g⁻¹) < Waffle (14.2 mg.100g⁻¹) < Salty sticks (23.8 mg.100g⁻¹) < Chocolate (24.1 mg.100g⁻¹) < Bread (26 mg.100g⁻¹) < Breadcrumbs (27.1 mg.100g⁻¹) < Sticks with salt (30.3 mg.100g⁻¹) < Ice Cornets (30.8 mg.100g⁻¹) < Walnut cookie (37.2 mg.100g⁻¹) < Instant soup with noodles (46.1 mg.100g⁻¹) < Powdered egg alternative (58 mg.100g⁻¹) < Pate (299.6 mg.100g⁻¹). Only seven products of 17 observed showed low phenylalanine content, less than 20 mg.100g⁻¹.

Keywords: phenylketonuria; phenylalanine; low-protein product; acid hydrolysis; chromatography

INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive inherited disorder of phenylalanine metabolism due to a lack of the enzyme phenylalanine hydroxylase. Most forms of PKU and hyperphenylalaninemia (HPA) are caused by mutations in a gene on chromosome 12 of phenylalanine hydroxylase (PAH) (Burnett et al., 2008). Currently, more than 400 known mutations are identified in the gene for PAH. Lack of or insufficient activity of the phenylalanine hydroxylase causes accumulation of phenylalanine (Phe), and phenyl ketone excretion in the urine. Any increase in the concentration of phenylalanine in the blood results in central nervous system damage (Strnová and Úrge, 2007).

Phenylalanine (Phe) is an essential amino acid that is a precursor of melanin, dopamine, and thyroxine. It is an essential amino acid and its natural occurrence in food is usually sufficient (mean content 3.5%). In protein foods, Phe constitutes about 4-5% (Velíšek and Hajšlová, 2009). The artificial sweetener aspartame is also high source of phenylalanine. The ADI for aspartame for healthy person is 40 mg/kg body weight (EFSA, 2014).

In PKU patients, a restriction of foods rich in protein (meat, fish, eggs, bread, dairy products, nuts and seeds), as well as avoidance of drinks containing aspartame help to control blood phenylalanine levels. The developing fetus

of women suffering from PKU is particularly sensitive to their mother's phenylalanine levels (EFSA, 2014). The basic treatments of PKU are mixture preparations of amino acids. Their use ensures an adequate supply of amino acids without phenylalanine, thereby compensating reduced protein intake in low protein dietary regime. Patients with phenylketonuria usually get these foods by medical prescription as most of these foodstuffs can belong to the group of foods for special medical purposes (Strnová and Úrge, 2007), which safety is defined by the Commission Directive 1999/21/EC.

Untreated children with persistent hyperphenylalaninemia (PKU) show brain damage. Symptoms include microcephaly, epilepsy, mental disability and behavioral problems. By the use of neonatal screening and early dietary intervention, children born with PKU can live a relatively normal life (Burnett et al., 2008; Mitchell, 2000).

The application of diet at an early age of life can significantly reduce mental deficiencies associated with phenylketonuria. Dietary measures are based on the foods containing a minimum of Phe, while preserving the normal content of tyrosine and other proteins in food. This is often achieved by substitution of phenylalanine free proteins. Dietary restriction of natural proteins reduces and

maintains the level of Phe in the blood (Macdonald et al. 2009; Poustie and Wildgoose, 2010; Svačina, 2008).

The aim of this work was to analyze selected amino acids (aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine) in seventeen food samples, declared by the producer as "Low-protein foods" respectively "Foods low in phenylalanine content " by automatic analyzer AAA 400 and consequently to evaluate their safety and suitability for a particular nutritional use. To compare the content of phenylalanine declared on the label with content of phenylalanine measured.

MATERIAL AND METHODOLOGY

As material used were the foodstuffs offered for phenylketonurics obtained from internet sale and labeled as food suitable for phenylketonurics as „low in protein“ respectively „foods low in phenylalanine“. Overview of the material used is given in Table 1.

By origin of products, most of them originated from Poland (59%), followed by Germany (23%) and equal representation (6%) had Slovakia, Hungary and Sweden.

Fifteen amino acids (aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, phenylalanine, tyrosine, histidine, lysine and arginine) were determined in observed samples using ion-exchange chromatography. The total amount of amino

Table 1 Overview of the material tested.

No.	Product	Producer	Main components
1.	Pate	Producer A, Germany	bacon, pork, liver, onion, water, salt, stabilizer E 250, spice, emulsifier
2.	Breadcrumbs	Producer 1, Poland	gluten free wheat starch, corn starch, vegetable oil, glucose, sugar, yeast, salt, guar gum, E 464, fibre, raising agents, E 500, E 575
3.	Spaghetti	Slovakia	corn starch, modified corn starch, emulsifier E 471, dye curcuma (E 100)
4.	Instant soup with noodles	Producer 1, Poland	maltodextrin, gluten free pasta, corn starch, water, salt, mono and diglycerides of fatty acids, E 464, E 575, betacarotene, salt, monosodium glutamate, vegetable oil, carrot, sugar, onion, aroma, celery, leek, black pepper, parsley stalk, curcuma, citric acid
5.	Sticks with salt	Producer 1, Poland	corn starch, salt, wheat starch gluten free, vegetable oil, sugar, yeast, guar gum, E 464, salt, fibre, E 500, E 503, mono and diglycerides of fatty acids, lecithin, E 575
6.	Walnut cookie	Producer 2, Poland	corn starch, gluten free wheat starch, vegetable oil, sugar, walnuts, peanuts, oil, pectin, guar gum, pectin, glucose, butter essence, mono and diglycerides of fatty acids
7.	Powdered egg alternative	Producer B, Germany	corn starch, mono and diglycerides of fatty acids, palm oil, xanthan, rice flour, pea protein
8.	Salty sticks	Producer 2, Poland	margarine, corn starch, gluten free wheat starch, water, sugar, potato starch, yolk, guar gum, yeast, potato syrup, wheat gluten free cellulose, salt, E 503, mono and diglycerides of fatty acids, lecithin, vanillin
9.	Chocolate	Producer C, Germany	cocoa butter, sugar, butter, cocoa matter, powdered whey, vanilla
10.	Pasta	Hungary	corn starch, water, fatty acids, E 471
11.	Waffle	Producer 2, Poland	gluten free wheat starch, corn starch, guar gum, bamboo fibre, glucose, apple pectin, mono and diglycerides of fatty acids
12.	Cherry jelly	Producer 2, Poland	sugar, glucose syrup, water, agar, citric acid, cherry aroma, dye E 124
13.	Ice Cornets	Producer 2, Poland	corn starch, gluten free wheat starch, potato starch, sugar, yolk, guar gum
14.	Bread	Producer 1, Poland	gluten free wheat starch, water, vegetable fat, sugar, glucose, yeast, salt, carob, guar gum, E 464, fibre, E 500, E 575
15.	Gum candies	Producer C, Germany	potato and tapioca starch, citric acid, malic acid, galactic acid, calcium citrate, arome, fruit concentrates, vegetable extracts (blackberries, carrot, pepper, curcuma, spirulina, nettle, spinach), natural dye : chlorophyll, vegetable oil, beeswax, carnauba wax
16.	Flour	Producer 1, Poland	gluten free wheat starch, glucose, corn starch, guar gum, E 464, vegetable fibre, E 500, E 575
17.	Flour	Sweden	wheat starch (max. 0.2 g gluten/100 g), oligofructose, guar gum, vitamins, ferrum

acids and phenylalanine content of food products were monitored after 23-hour acid hydrolysis using HCl (6 mol.dm⁻³) and 115 ±2 °C. After the hydrolysis, the test tubes were cooled down to 20°C. Hydrochloric acid was evaporated and the ropy residue was diluted in loading buffer in a 25 cm³ volumetric flask. The mixture was filtered through 0.22 µm filter and loaded into an analyser. Amino Acid Analyser AAA400 (Ingos, Prague, Czech Republic) was equipped with a column (370×3.7 mm filled with a ion exchanger Ostion LG ANG – Ingos, Prague, Czech Republic), post-column ninhydrine derivatization and spectrophotometric detection (440 nm for proline and 570 nm for other amino acids). Method was performed according to the **Buňka et al. (2009)**.

Statistical analysis of results

The results were evaluated by calculating the mean, coefficient of variation and standard deviation using the statistical software Statistica 8.0.

RESULTS AND DISCUSSION

Treatment of phenylketonuria should be a combination of several diets that reduce the amount of phenylalanine: gluten-free diet, lactose-free and low proteins diet which is the most important. The principle of the diet is to reduce the amount of phenylalanine in the body and to create a balance between its income and the potential use (**Komárková and Pazdírková, 2010; Dvořák, 2009**).

Phenylalanine content in the monitored foods in our work was represented in varying amounts, its content ranged

from 1 to 299.6 mg. 100 g⁻¹ respectively, and increased in the following order:

Gum candies (1 mg.100g⁻¹) < Cherry jelly (1.4 mg.100g⁻¹) < Flour no.16 (5.8 mg.100g⁻¹) < Flour no. 17 (5.9 mg.100g⁻¹) < Spaghetti (8.7 mg.100g⁻¹) < Pasta (9.9 mg.100g⁻¹) < Waffle (14.2 mg.100g⁻¹) < Salty sticks (23.8 mg.100g⁻¹) < Chocolate (24.1 mg.100g⁻¹) < Bread (26 mg.100g⁻¹) < Breadcrumbs (27.1 mg.100g⁻¹) < Sticks with salt (30.3 mg.100g⁻¹) < Ice Cornets (30.8 mg.100g⁻¹) < Walnut cookie (37.2 mg.100g⁻¹) < Instant soup with noodles (46.1 mg.100g⁻¹) < Powdered egg alternative (58 mg.100g⁻¹) < Pate (299.6 mg.100g⁻¹).

Following legislation, it seems that national or European legislation contain no specific requirements for „low protein foods“, or foods low in phenylalanine, so in purpose to evaluate our samples we used as the standard given in the Czech legislation (**Vyhláška Ministerstva zdravotnictví České republiky č. 54/2004 Sb**), according to which food with no phenylalanine content is defined as food made by special technological process so that the phenylalanine content should not exceed 20 mg per 100 g or 100 cm³ in the food intended for consumption. Under this legislation, suitable foods for phenylketonurics could be classified as follows: Gum candies (1 mg.100g⁻¹) < Cherry jelly (1.4 mg. 100g⁻¹) < Flour no. 16 (5.8 mg.100g⁻¹) < Flour no.17 (5.9 mg.100g⁻¹) < Spaghetti (8.7 mg.100g⁻¹) < Pasta (9.9 mg.100g⁻¹) < Waffle (14.2 mg.100g⁻¹).

Table 2 Aminoacid content (mg.100g⁻¹) determined in sample of pate.

Aminoacids	Content of aminoacids [mg.100g ⁻¹]	Standard deviation	CV (%)
Aspartic acid	571.11	0.14	2
Threonine	201.60	0.04	2
Serine	156.48	0.04	3
Glutamic acid	916.62	0.55	6
Proline	546.50	0.27	5
Glycine	762.99	0.12	2
Alanine	471.35	0.02	0
Valine	371.65	0.33	9
Isoleucine	269.77	0.15	5
Leucine	522.17	0.19	4
Tyrosine	121.15	0.03	3
Phenylalanine	299.59	0.13	4
Histidine	205.64	0.03	2
Lysine	435.07	0.01	0
Arginine	387.21	0.24	6
Total	6238.90		

Table 3 Aminoacid content (mg.100g⁻¹) determined in sample of powdered egg alternative.

Aminoacids	Content of aminoacids [mg.100g ⁻¹]	Standard deviation	CV (%)
Aspartic acid	125.69	0.05	4
Threonine	33.60	0.02	7
Serín	55.26	0.03	6
Glutamic acid	163.12	0.08	5
Proline	39.72	0.01	2
Glycine	46.35	0.02	5
Alanine	58.73	0.02	3
Valine	38.43	0.00	0
Isoleucine	29.31	0.02	7
Leucine	87.81	0.04	5
Tyrosine	28.76	0.01	5
Phenylalanine	58.00	0.01	1
Histidine	32.71	0.01	4
Lysine	60.59	0.01	1
Arginine	87.63	0.02	3
Total	945.70		

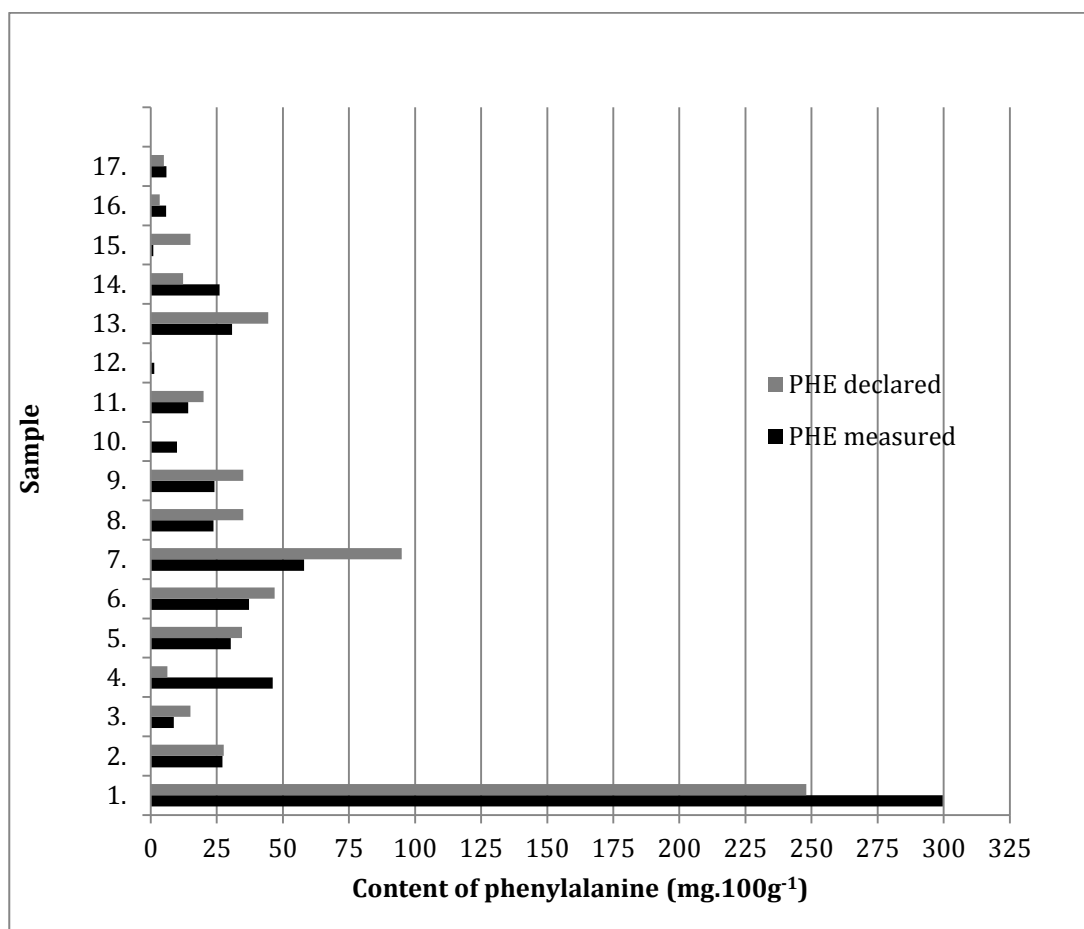


Figure 1 Differences between phenylalanine content measured and labeled by producers (mg.100g⁻¹).

Samples: 1.Pate, 2. Breadcrumbs, 3. Spaghetti, 4. Instant soup with noodles, 5. Sticks with salt, 6. Walnut cookie, 7. Powdered egg alternative, 8. Salty sticks, 9. Chocolate, 10. Pasta, 11. Waffle, 12. Cherry jelly, 13. Ice Cornets, 14. Bread, 15. Gum candies, 16. Flour, 17. Flour.

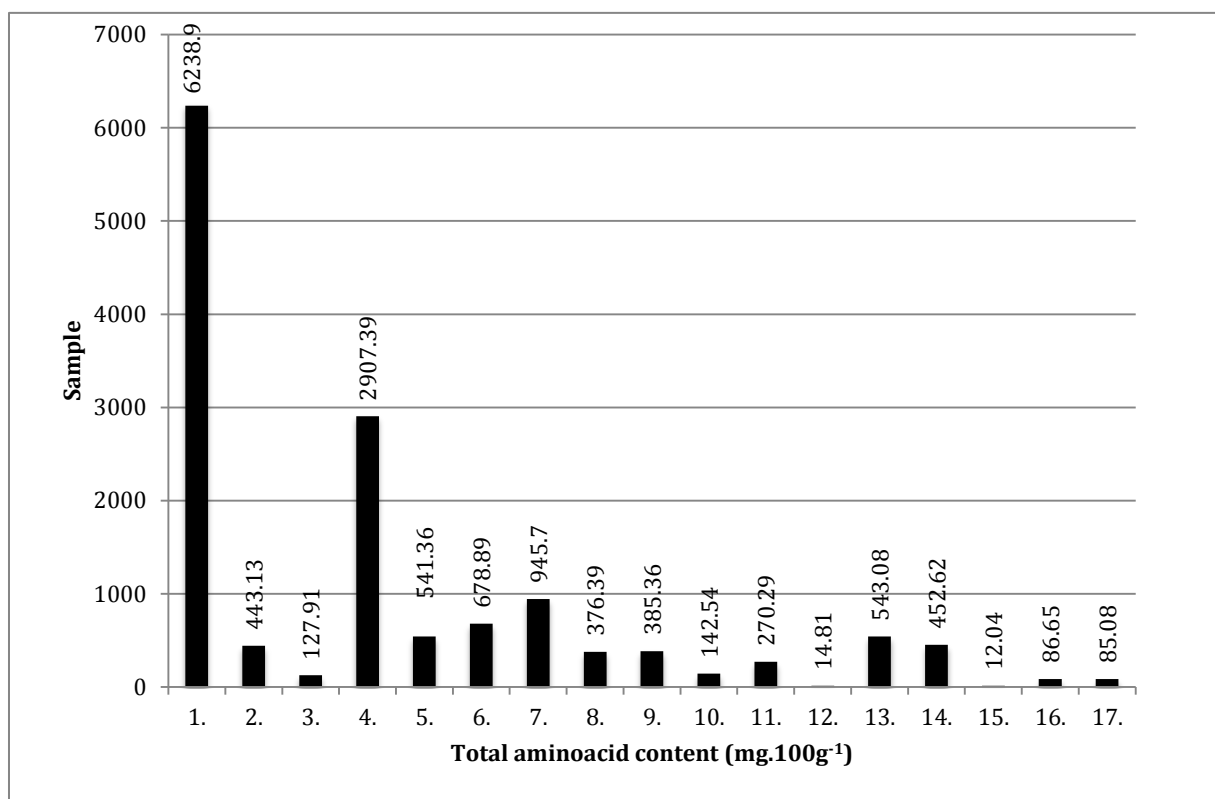


Figure 2 Total aminoacid content (mg.100g⁻¹) determined in selected products.

Samples: 1.Pate, 2. Breadcrumbs, 3. Spaghetti, 4. Instant soup with noodles, 5. Sticks with salt, 6. Walnut cookie, 7. Powdered egg alternative, 8. Salty sticks, 9. Chocolate, 10. Pasta, 11. Waffle, 12. Cherry jelly, 13. Ice Cornets, 14. Bread, 15. Gum candies, 16. Flour, 17. Flour.

Contrary, as sample with the highest content of phenylalanine (299.6 mg.100g⁻¹) was determined pate produced in Germany. The highest amino acid content was also detected (6238.90 mg.100g⁻¹) in this sample probably due to meat present, and the highest content of glutamic acid (916.62 mg.100g⁻¹) among amino acids was determined as well.

Powdered egg alternative (sample no.7) was monitored as the second highest source of phenylalanine. It consists of cornstarch, mono and diglycerides of fatty acids, palm oil, xanthan, rice flour and pea protein. Glutamic acid also prevailed in this sample (Table 3).

The only product of Slovak origin was the sample no. 3, spaghetti. Total sum of amino acids 127.91 mg.100g⁻¹ was observed, which can be assumed together with the observed phenylalanine content (8.75 mg.100g⁻¹) as low content and therefore this product can be considered as suitable for consumption by phenylketonurics.

As the most suitable for phenylketonurics with the lowest content of phenylalanine were observed gum candies and cherry jelly (less than 2 mg. 100g⁻¹). Total aminoacid content detected in these two products was also the lowest, at gum candies 12.04 mg.100g⁻¹ and cherry jelly 14.81 mg.100g⁻¹.

Sample no. 8 was the sticks of Polish origin. They are comparable with the sample no. 5, which are also sticks with salt with a similar composition but made from other producer. Phenylalanine content determined was 30.0 resp. 23.8 mg.100g⁻¹.

Comparable could be also two samples of flours originating from Poland and Sweden, with detected phenylalanine content at flour no.16 (5.8 mg.100g⁻¹), and flour no.17 (5.9 mg.100g⁻¹). Total aminoacid content were observed as similar (85.08 mg.100g⁻¹ vs. 86.65 mg.100g⁻¹) as well.

As it was stated previously, foods for phenylketonurics are often included in the category of foods for special medical purposes that means a category of foods for particular nutritional uses specially processed or formulated and intended for the dietary management of patients and to be used under medical supervision. They are intended for the exclusive or partial feeding of patients with a limited, impaired or disturbed capacity to take, digest, absorb, metabolise or excrete ordinary foodstuffs or certain nutrients contained therein or metabolites, or with other medically-determined nutrient requirements, whose dietary management cannot be achieved only by modification of the normal diet, by other foods for particular nutritional uses, or by a combination of the two (**Commission Directive 1999/21/EC**). The labelling of foodstuffs intended for particular nutritional uses shall include by the **Directive 2009/39/EC** the particular elements of the qualitative and quantitative composition or the special manufacturing process which gives the product its particular nutritional characteristics. Comparing the results in phenylalanine content measured and the content of phenylalanine labeled by producers, we observed several differences shown in the Fig. 1. Sample no. 2

represented, as breadcrumbs and sample no. 6 were the products with the lowest difference in observed and declared values of phenylalanine in the packaging. Contrary, higher differences in these two amounts were detected in samples 1, 4, 7, 8.

Total amino acid content determined in selected products is shown in the Figure 2. Its content in selected products ranged from 12.04 (gum candies) to 6238.90 mg.100g⁻¹ (in pate) and increased in the order: Gum candies (12.04 mg.100g⁻¹) < Cherry jelly (14.81 mg.100g⁻¹) < Flour (85.08 mg.100g⁻¹) < Flour (86.65 mg.100g⁻¹) < Spaghetti (127.9 mg.100g⁻¹) < Pasta (142.54 mg.100g⁻¹) < Waffle (270.29 mg.100g⁻¹) < Salty sticks (376.39 mg.100g⁻¹) < Chocolate (385.36 mg.100g⁻¹) < Breadcrumbs (443.13 mg.100g⁻¹) < Bread (452.62 mg.100g⁻¹) < Sticks with salt (541.36 mg.100g⁻¹) < Ice Cornets (543.08 mg.100g⁻¹) < Walnut cookie (678.89 mg.100g⁻¹) < Powdered egg alternative (945.70 mg.100g⁻¹) < Instant soup with noodles (2907.39 mg.100g⁻¹) < Pate (6238.90 mg.100g⁻¹).

CONCLUSION

In this work, amino acids representation, focused on phenylalanine content in selected samples intended for phenylketonurics was observed. We can conclude that may be due to the lack of legislation requirements, most of observed foods was high in content of phenylalanine. Only seven products of 17 observed showed lower phenylalanine content, less than 20 mg.100g⁻¹. The obtained results also suggest that certain foods at the content of phenylalanine differ from those listed on the product label.

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COLONIZATION OF GRAPES BERRIES AND CIDER BY POTENTIAL PRODUCERS OF PATULIN

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ABSTRACT

The aim of this study was to detect potential producers of mycotoxin patulin from grapes (berries, surface sterilized berries - endogenous mycobiota and grape juice) of Slovak origin. We analyzed 47 samples of grapes, harvested in 2011, 2012 and 2013 from various wine-growing regions. For the isolation of species we used the method of direct plating berries and surface-sterilized berries (using 1% freshly pre-prepared chlorine) berries on DRBC (Dichloran Rose Bengal Chloramphenicol agar). For the determination of fungal contamination of grape juice we used plate-dilution method and DRBC and DG18 (Dichloran 18% Glycerol agar) as media. The cultivation in all modes of inoculation was carried at 25 ± 1 °C, for 5 to 7 days. After incubation *Aspergillus* and *Penicillium* isolates were inoculated on the identification media. The potential producers of patulin were isolated from 23 samples berries, 19 samples of surface-sterilized berries and 6 samples of grape juice. Overall, the representatives of producers of patulin were detected in 32 (68.1%) samples (75 isolates). In this work we focused on the detection of potential producers of patulin, *Penicillium expansum* (the most important producer of patulin in fruits), *Penicillium griseofulvum* and *Aspergillus clavatus* were isolated. Chosen isolates of potential patulin producers were tested for the ability to produce relevant mycotoxins in *in vitro* conditions using thin layer chromatography method. The ability to produce patulin in *in vitro* condition was detected in 82% of isolates of *Penicillium expansum*, 65% of *Penicillium griseofulvum* and 100% of *Aspergillus clavatus*. Some isolates of *Penicillium expansum* were able to produce citrinin and roquefortine C, *Penicillium griseofulvum* cyclopiazonic acid, griseofulvin and roquefortin C, also.

Keywords: grapes; patulin; *Penicillium*; *Aspergillus*; mycotoxin

INTRODUCTION

Grapes and their derived products are important components of the human diet all over the world. The concern about mould in the vineyard has traditionally been linked to spoilage of grapes due to the fungal growth (Serra et al. 2006). Mycotoxins are secondary metabolites produced by filamentous fungi that have been detected in food commodities, including grapes and wine (Serra et al., 2005). Mycotoxins are abiotic hazards produced by certain fungi that can grow on a variety of crops. Consequently, their prevalence in plant raw materials may be relatively high (Marin et al., 2013). Mycotoxins are produced in plants by filamentous micro-fungi species, and naturally contaminated the food chain (Siroť et al., 2013). Some mycotoxins are acutely toxic, some are chronically toxic and some are the both. Furthermore, it is possible that mixtures of mycotoxins are acting synergistically or additively, so a mycological examination of the mycobiota, to species level, is very important, as different species produce different profiles of extrolites (Frisvad and Samson, 2002, Bürger et al., 2004). Patulin, a mycotoxin found in apples, grapes, oranges, pear and peaches, is a potent genotoxic compound (Saxena et al., 2009). Patulin is a mycotoxin produced by several species of filamentous micro-fungi, especially within *Penicillium*,

Aspergillus and *Byssoschlamys* (Weidenbörner, 2001, Bennett and Klich, 2003, Moake et al., 2005, Pitt and Hocking, 2009, Puel et al., 2010, Samson et al., 2010), from which *Penicillium expansum*, a common contaminant of damaged fruits, is the most important (Morales et al., 2007). Patulin is generally very toxic for both prokaryotes and eukaryotes, but the toxicity for humans has not been conclusively demonstrated. Several countries in Europe and the USA have now set limits on the level of patulin in apple juice (Frisvad et al., 2006, Frisvad et al., 2007b). Positive samples of grape juice, grape must be reported Rychlik and Schieberle; Altmayer et al.; grape wine Altmayer et al. (Barkai-Golan, 2008).

The aim of our study was to detect species of genera *Aspergillus*, *Byssoschlamys* and *Penicillium* from grapes of Slovak origin. The isolates of potential producers of patulin were tested for their ability to produce patulin and other mycotoxins in *in vitro* conditions.

MATERIAL AND METHODOLOGY

Samples

We analyzed 47 samples of grapes, harvested in years 2011, 2012 and 2013 from various wine-growing regions of Slovakia, from small and medium-sized vineyard. We

analyzed grape variety Alibernet (1 sample), André (2 samples), Blaufrankise (8), Cabernet Sauvignon (2), Müller Thurgau (1), Velsch Riesling (4), Grüner Veltliner (5), Pálava (1), Pinot blanc (2), Pinot gris (2), Pinot noir (2), Saint Laurent (1), Sauvignon (2), Tramin (1), Zala gyöngye (1). Samples (3 kg) were collected at the time of technological ripeness. Picked grapes were stored at 4 ± 1 °C and analyzed within 24 h after harvest.

Mycological analysis

For analysis intact berries have been used. For the isolation of species we used the method of direct plating berries, surface-sterilized berries (using 1% freshly pre-prepared chlorine) and non-sterilized berries on DRBC (Dichloran Rose Bengal Chloramphenicol agar). For each analysis were used 50 berries. The cultivation was carried at 25 ± 1 °C, for 5 to 7 days in dark. Only undamaged berries have been used for analysis. After incubation, the colonies of *Aspergillus* and *Penicillium* were transferred onto appropriate identification media.

Dilute plate technique was used for isolation of fungi from the samples of cider according to **Samson et al. (2002)**. Sample 20 ml of cider was mixed with 180 ml of saline solution (0.85% sodium chloride) with 0.05% Tween 80 on homogenizer. Then 0.1 ml of appropriate dilutions made up to 10^{-3} was applied on DRBC and DG18 (Dichloran 18% Glycerol agar). After 5 to 7 days of incubation at 25 ± 1 °C in dark, resulting colonies were transferred onto appropriate identification media.

Identification of *Aspergillus* species. Conidial suspensions were inoculated at three equidistant points both on Czapek-yeast extract agar (CYA), Czapek-yeast with 20% Sucrose (CY20S) and malt extract agar (MEA), and incubated in dark at 25 ± 1 °C, 7 days. Species identification was done according to **Klich (2002)**, **Pitt and Hocking (2009)**, **Samson et al. (2002, 2010)**, **Samson and Varga (2007)**.

Identification of *Penicillium* species.

Conidial suspensions were inoculated at three equidistant points on Czapek-yeast extract agar (CYA), malt extract agar (MEA) and Creatine Sucrose agar (CREA) and incubated in dark at 25 ± 1 °C. Sub-cultivation on CYA at 37 ± 1 °C was used as well. Species identification was done after 7 days according to (**Pitt and Hocking, 2009**; **Samson et al., 2002, 2010**; **Samson and Frisvad, 2004**).

Obtained results were evaluated and expressed in isolation frequency (Fr) at the species level. The isolation frequency (%) is defined as the percentage of samples within which the species occurred at least once (**Gautam et al., 2009**). These values were calculated according to **González et al. (1996)** as follows:

$$\text{Fr (\%)} = (\text{ns} / \text{N}) \times 100$$

where ns = number of samples with a species; N = total number of samples.

Patulin and other mycotoxins - screening by a modified agar plug method.

The abilities of selected isolates of potentially toxigenic species to produce patulin in *in vitro* conditions were screened by the means of thin layer chromatography (TLC) according to **Samson et al. (2002)** modified by **Labuda and Tančinová (2006)**.

Cultivation for screening extracellular metabolites (citrinin, griseofulvin patulin) was carried out on YES (Yeast Sucrose agar) and for intracellular (cyclopiazonic acid and roquefortin C, sterigmatocystin) on CYA (Czapek-yeast extract agar); conditions of cultivation: in dark at 25 °C, 14 days. In each tested isolate, 3 pieces of mycelium together with cultivation medium of approximately 5 x 5 mm area were cut from colonies and extracted in 1000 ml of chloroform:methanol (2:1, v/v) on vortex for 2 minutes. Then 20 µl of liquid phase of extracts along with standards (Sigma, Germany) were applied on TLC plate (Marchey-Nagel, Germany) and consequently developed in solvent system toluene:ethylacetate:formic acid (5:4:1, v/v/v). The visualisation of extrolites was carried out as follows: cyclopiazonic acid directly in daylight after spraying with the Ehrlich reagent (violet-tailed spot); patulin by spraying with 0.5% methylbenzothiazolone hydrochloride in methanol, heated at 130 °C for 8 min and then detectable as a yellow-orange spot; roquefortin C after spraying with $\text{Ce}(\text{SO}_4)_2 \times 4 \text{H}_2\text{O}$ visible as orange spot. Directly under UV light (365 nm) were visualised following mycotoxins: citrinin (yellow-green) and griseofulvin (blue spot).

RESULTS AND DISCUSSION

Mycotoxins are abiotic hazards produced by certain fungi that can grow on a variety of crops (**Marin et al., 20013**). In the current study from 68.1% of samples were isolated potential producers of patulin. We isolated species genera *Aspergillus* (*Aspergillus clavatus*) and *Penicillium* (*Penicillium expansum* and *Penicillium griseofulvum*), as potential producers of this mycotoxin. According **Dombrink-Kurtzman and Engberg (2006)** the *Byssochlamys nivea* strains produced patulin in amounts comparable to *Penicillium expansum* strains. Interest in the genus *Byssochlamys* is related to the ability of its ascospores to survive pasteurization and cause spoilage of heat-processed fruit products worldwide. Genus *Byssochlamys* was not detected in our samples. The number of isolates and isolation frequency of species recovered from the sample are listed in Table 1. According **Zouaoui et al. (2015)** patulin is a secondary metabolite, which is mainly produced by certain species of *Aspergillus* and *Penicillium* fungi.

Penicillium spp.

Penicillium species are ubiquitous, opportunistic saprophytes. A majority of the described species are soil fungi, and their occurrence in food is more of less accidental and rarely of consequence. However, quite numbers of species are closely associated with human food supplies. Some species are more specialised: several are destructive pathogen on fruit (e.g. *Penicillium digitatum*, *Penicillium expansum*, *Penicillium italicum*) (**Pitt and Hocking, 2009**). *Penicillium expansum* is one of the most common fruit pathogens; it causes soft rot known as “a blue mould rot” on a variety of fruits (**Franck et al., 2005**, **Neri et al., 2010**, **Elhariry et al., 2011**). 251 isolates from grapes berries and 180 isolates from cider (3 positive samples) have been identified as *Penicillium expansum*. **Frisvat et al. (2007b)** reported that *Penicillium expansum* is one of the most common fungal species associated with grapes. Representative isolates of *Penicillium expansum*

Table 1 Filamentous fungi – potential producers of patulin identified from grape berries and cider from 47 samples.

Species	Berries		Surface-sterilized berries		Cider	
	Number of isolates	Isolation frequency (%)	Number of isolates	Isolation frequency (%)	Number of isolates	Isolation frequency (%)
<i>Aspergillus clavatus</i>	23	27.7	7	10.6	25	4.3
<i>Penicillium expansum</i>	143	23.4	108	6.4	180	6.4
<i>Penicillium griseofulvum</i>	29	2.1	13	27.7	25	6.4

Table 2 Potential ability isolates to produce patulin and other mycotoxins in *in vitro* conditions, tested by TLC method.

Tested isolates	Mycotoxins				
	Patulin	Citrinin	Cyclopiazonic acid	Roquefortine C	Griseofulvin
<i>Aspergillus clavatus</i>	15**/15*	–	–	–	–
<i>Penicillium expansum</i>	37/45	22/45	–	42/45	–
<i>Penicillium griseofulvum</i>	11/17	–	16/17	15/17	15/17

** - number of tested isolates, * - number of isolates with ability to produce mycotoxin, TLC – thin layer chromatography

were tested for ability to produce patulin in *in vitro* condition. From 45 tested isolates 37 (82.2%) produced patulin. Postharvest diseases are the most important factors that limit commercial export of Chilean table grapes. In recent years, blue mould decay caused by *Penicillium expansum* has frequently appeared on Red Globe grapes after long period (>60 days) of cold storage, causing significant economical losses (Franck et al., 2005). *Penicillium expansum* causes significant economic losses to the fruit industry and is also one of potential public health concern because it produces toxic secondary metabolites including patulin, citrinin, and chaetoglogosins (Andersen et al., 2004). 48.9% of tested isolates *Penicillium expansum* produced citrinin and 93.3% roquefortine C *in vitro* conditions, as well. Bragulat et al. (2008) reported, that 100% isolates *Penicillium expansum*, detected from grapes, were able to produce citrinin and 60% patulin. Citrinin is a quinone methide with a powerful antibacterial effect, but toxic to humans and animals. This mycotoxin is mainly hepato-nephrotoxic (Zaied et al., 2012). Roquefortine C (another mycotoxin produced by *Penicillium expansum*) is a very widespread fungal secondary metabolite. The acute toxicity of roquefortine C is not very high (Cole and Cox, 1981). Furthermore, we have identified species *Penicillium griseofulvum* – other producer of patulin; 42 isolates from the berries and 25 from cider (2 positive samples). *Penicillium griseofulvum* is a very efficient producer of high levels of patulin in pure culture, and it may potentially produce patulin in cereals, pasta and similar products (Frisvad et al., 2006, Frisvad et al., 2007b). 11(65%) tested isolates (Table 2) were able to produce patulin. Some isolates produced cyclopiazonic acid (94%), roquefortine C (88%) and griseofulvin (88%), also. Incidence of *Penicillium griseofulvum* on grapes also described Serra et al. (2005), Bragulat et al. (2008) and other authors. As mentioned above, the *Penicillium griseofulvum* also produced cyclopiazonic acid. Persistently studies with this mycotoxin have shown that

targets correspond to muscles, liver and spleen (Burdock and Flamm, 2000). The last detected mycotoxin was griseofulvin. Griseofulvin is active against dermatophytic fungi of different species in the genera *Microsporum*, *Trichophyton* and *Epidermophyton*. Prolonged griseofulvin treatment in experimental animals provoked biochemical changes consisting mainly of disturbances of porphyrin metabolism, variation in the microsomal cytochrome levels and formation of Mallory bodies (De Carli and Larizza, 1988).

Aspergillus sp.

Species of *Aspergillus* are among the most economically important fungi, on the positive side being very widely used for synthesis of chemicals, for biosynthetic transformations and enzyme production. On the negative side, they are of great importance in food spoilage and they produce important mycotoxins (Pitt and Hocking, 2009). Lopez-Diaz and Flannigan reported that *Aspergillus clavatus*, *Aspergillus longivesica*, *Aspergillus giganteus* and other species are very efficient producers of patulin laboratory, but only *Aspergillus clavatus* may play role in human health (Frisvad et al., 2007b). 30 isolates from grapes berries and 25 isolates from cider (2 positive samples) have been identified as *Aspergillus clavatus*. All tested isolates (Table 2) were able to produce patulin.

CONCLUSION

Potential producers of patulin were isolated from 68.1% of the analysed samples. We isolated species genera *Aspergillus* and *Penicillium*. The ability to produce patulin in *in vitro* condition was detected in 82% of isolates of *Penicillium expansum*, 65% of *Penicillium griseofulvum* and 100% of *Aspergillus clavatus*. Some isolates of *Penicillium expansum* were able to produce citrinin and roquefortine C, *Penicillium griseofulvum* cyclopiazonic acid, griseofulvin and roquefortin C, also.

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EVALUATION OF MICROBIOLOGICAL QUALITY OF SELECTED CHEESES DURING STORAGE

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ABSTRACT

The aim of this article was to evaluate and compare the microbiological quality of selected types of cheeses immediately after opening and after 5 days storage in the refrigerator. Total viable counts (TVC), coliform bacteria (CB) and microscopic filamentous fungi (MFF) were determined by microbiological analysis. We analyzed 8 samples of cheese of Slovak origin. Plate dilution method was used for microbiological analysis. The Codex Alimentarius of Slovak republic (2006) just indicates number of coliforms bacteria (10^2) and microscopic fungi (5×10^2). The TVC values after opening of cheeses ranged from 1.68×10^3 CFU.g⁻¹ (3.22 log CFU.g⁻¹) in the sample no. 1 to 1.71×10^5 KTJ.g⁻¹ (5.23 log CFU.g⁻¹) in the sample no. 4 after storage in the refrigerator. All samples were negative for the presence of coliform bacteria after opening. The values of CB were 1.18×10^2 CFU.g⁻¹ (2.07 log CFU.g⁻¹) in sample no. 7 and 1.90×10^2 CFU.g⁻¹ (2.27 log CFU.g⁻¹) in the sample no. 8 after storage in refrigerator. These values are not in accordance with Codex Alimentarius of Slovak Republic (2006). Other samples were negative for presence of CB after storage at 4 °C. The values of MFF in samples ranged from 1.81×10^1 CFU.g⁻¹ (1.25 log CFU.g⁻¹) in the sample no. 1 after opening to 1.68×10^2 CFU.g⁻¹ (2.22 log CFU.g⁻¹) in sample no. 7 after storage of samples. All analysed samples were in accordance with Codex Alimentarius of Slovak republic (2006).

Keywords: cheese; microbiological quality; total viable counts; coliform bacteria; microscopic filamentous fungi

INTRODUCTION

Dairy products are characterized by reduced shelf life because they are an excellent growth medium for a wide range of microorganisms. For this reason, it is important to monitor the microbiological quality of dairy products and, in particular, the total viable count and concentration of *Escherichia coli*, as they are indicators of the hygienic state of these products (Losito et al., 2014).

Cheeses, although they have been characterized as one of the safest food products by some authors (Little et al., 2008), in 2006 the consumption of contaminated cheese accounted for the 0.4% of the total foodborne outbreaks in Europe (EFSA, 2008). Furthermore, the scientific literature has reported several food poisoning outbreaks associated with various types of cheeses.

Cheeses are ready-to-eat (RTE) food products that do not undergo any further treatment to ensure their safety before consumption. Contamination of cheese with foodborne pathogens may occur at several stages. Thus, if contamination of any type of cheese is to be prevented or controlled information on the major sources of pathogens and the mechanisms by which these contaminate the dairy chain are required. Moreover, the need for knowledge about the vectors and the routes of contamination into RTE food and quantitative data on recontamination to accurately establish Microbial Risk Assessment has been also addressed (Reij and Den Aantrekker, 2004).

Microbial contamination of cheese may originate from various sources. Such sources during cheese production

might be: starter culture, brine, floor and packaging material, cheese cloth and curd cutting knife, cold room and production room air (Temelli et al., 2006). Storage coolers have been also demonstrated to be the source of *Listeria monocytogenes* contamination of cheese made from pasteurized milk (Brito et al., 2008). Moreover, humans have been found to serve as contamination source of cheese with pathogenic bacteria like *Staphylococcus aureus* (Callon et al., 2008). However, in this section an attempt is made to review literature on two main sources of cheese contamination with pathogenic bacteria, i.e. the raw milk and routes of contamination at cheese processing plants.

The aim of the present study was to evaluate the microbiological stability of selected types of cheeses during storage.

MATERIAL AND METHODOLOGY

Microbiological analysis of 8 Slovak cheeses was performed immediately after opening and after 5 days of storage of samples in the refrigerator. Samples of evaluated cheeses:

Samples no. 1 and no. 2 spreadable processed dairy products, samples no. 3 and no. 4 melted slices with emmental, sample no. 5. Steamed cheese – smoked, sample no. 6 steamed cheese – unsmoked, samples no. 7 and no. 8 Edam cheeses.

Plate dilution method was used for the microbiological analysis of cheeses. Basic dilutions (10^{-1}) was obtained by

mixing 5 g of the sample (cheese) and 45 ml of physiological solution (0.85% NaCl), followed by homogenization of the sample for 30 minutes.

Plate dilution method was applied for quantitative cfu counts of respective groups of microorganisms in 1 g of cheese. Gelatinous nutritive substrate in petri dishes was inoculated with 1 ml of honey samples by flushing and on surface in three replications.

Determination of TVC

Plate Count Agar was used for determine of Total Viable Counts in samples. Dilutions of 10^{-3} and 10^{-4} were used to determine of TVC. Petri dishes were cultivated upside-down in a thermostat at 30 °C for 48-72 hours under aerobic conditions.

Determination of CB

Violet red bile agar was used for determine of Coliform Bacteria in samples.

Dilutions of 10^{-3} and 10^{-4} were used to determine of Coliform Bacteria. Petri dishes were cultivated upside-down in a thermostat at 37 °C for 24 – 48 hours.

Determination of MFF

Chloramfenicol yeast glucose agar was used for determine of Microscopic Filamentous Fungi.

Dilutions of 10^{-1} and 10^{-2} were used to determine of MFF. Petri dishes were cultivated upside-down in a thermostat at 25 °C for 5 – 7 days under aerobic conditions.

Calculation of microorganisms

The number of microorganisms in 1 g samples (N) were calculated using the following formula:

$$N = \Sigma C / [(n_1 + 0,1n_2) \cdot d]$$

ΣC – sum of characteristic colonies on selected plates,

n_1 – number of dishes from 1. dilutions used to calculate,

n_2 – number of dishes from 2. dilutions used to calculate,

d – dilution factor identical with 1. used dilution.

Statistics

Mathematical and statistical analyzes are processed in the tables. Arithmetic mean, standard deviation, coefficient of variation were performed using MS Excel.

RESULTS AND DISCUSSION

Microbiological quality of selected kinds of cheeses were performed. Total Viable Count (TVC), Coliform Bacteria (CB) and Microscopic Filamentous fungi (MFF) were determined immediately after opening of products and after 5 days of storage of products at 4 °C.

The factors that contribute to the safety of cheese with respect to pathogenic bacteria include milk quality, starter culture or native lactic acid bacterial growth during cheese making, pH, salt, control of aging conditions and chemical changes that occur in cheese during aging. Other technologies may provide opportunities to add additional barriers to the growth of bacterial pathogens. It is particularly important for the producers of raw milk cheeses to have a documented and systematic approach to ensure product safety (Donnelly, 2004).

Values of Total Viable Counts (TVC) in samples of Slovak cheeses after opening ranged from

1.68×10^3 CFU.g⁻¹ (3.22 log CFU.g⁻¹) in sample no. 1 to 2.91×10^3 CFU.g⁻¹ (3.46 log CFU.g⁻¹) in sample no. 7 (Figure 1). Average number of TVC after opening was 3.33 log CFU.g⁻¹ (Table 1).

Dermes-Mathieu et al. (2013) studied The efficacy of an anionic peptides-enriched extract (APEE), produced by nanofiltration of a tryptic hydrolysate from whey proteins, to inhibit the growth of *Listeria innocua* and *Listeria monocytogenes* in reconstituted Cheddar cheese was studied. The antimicrobial activity of APEE in reconstituted cheese was greater against *L. monocytogenes* than *L. innocua* and was higher in storage at 30 °C than at 4 °C. The combination of 20 mg.g⁻¹ of APEE and 1.75% salt/moisture (S/M) in cheeses incubated for 7 days at 30 °C was the most efficient condition to inhibit the growth of *Listeria*. Using these conditions, *L. monocytogenes* counts were significantly reduced by 1.1 and 1.5 log CFU.g⁻¹, compared with cheeses without APEE and prepared with lactococci at 1.75 and 3.5% S/M, respectively. These results suggest that antimicrobial anionic peptides from whey proteins can contribute to control pathogen in reduced-salt Cheddar cheeses.

Iurlina and Fritz (2004) studied the microbiological quality of Port Salut Argentino cheese during 10 days (after ripening) at two storage temperature treatments: (a) 4 °C and (b) a temperature combination of both 4 and 20 °C (4/20 °C), which implied 12 h at 4 °C and 12 h at 20 °C. Total coliforms were not higher than 10^3 CFU.g⁻¹ among samples. *E. coli* was detected at both treatments. Thirty three percent of the cheese contained *Staphylococcus aureus*. *Listeria* spp. and *Salmonella* spp. were not detected in any treatment. *Bacillus* spp. incidence was 50% of the cheese, being *B. cereus*, *B. cereus* variety mycoides and *B. pumilus*. *Bacillus cereus* and *Staphylococcus aureus* grew at 4/20 °C. Mesophilic aerobic bacteria were between 10^4 and 10^7 CFU.g⁻¹. At 4/20 °C counts decreased. At 4 °C the behaviour was variable. Moulds were lower than 10^4 CFU.g⁻¹ and yeasts were between 10^4 and 10^5 CFU.g⁻¹. pH, moisture content and titratable acidity ranges of samples were 5.5 – 6, 51 – 52.3% and 1.215 – 1.935 g.100g⁻¹ of lactic acid, respectively. Manufacturing of this cheese includes a short heat treatment and starter culture addition; consequently, our results indicate that this processing may be insufficient for achieving hygienic cheese production. The storage at refrigeration temperature will not always guarantee the cheese safety and quality.

All our samples of cheeses were negative for presence of Coliform Bacteria, all samples are in accordance with Codex Alimentarius of Slovak Republic (2006) (Figure 2).

Melanie and Siegfried (2001) determined the incidence of *Listeria* and *Listeria monocytogenes* in European red smear cheese in order to assess whether the lack of recent outbreaks of listeriosis associated with cheese is due to improved hygienic conditions in the dairies. Out of European red-smear cheese samples of various types, 15.8% contained organisms of the genus *Listeria*, 6.4% of the samples were contaminated with *L. monocytogenes*, 10.6% with *L. innocua*, and 1.2% with *L. seeligeri*. Six cheese samples contained two or more *Listeria* species, including at least one *L. monocytogenes* isolate. The incidences of *L. monocytogenes* in cheeses from various

countries were: Italy 17.4%, Germany 9.2%, Austria 10%, and France 3.3%. *Listeria* was found most frequently in soft and semi-soft cheese. Eight samples contained more than 100 *L. monocytogenes* CFU.cm⁻² cheese surface, 2 samples had counts above 10⁴ CFU.cm⁻² cheese surface. Surprisingly, a higher incidence of *L. monocytogenes* was observed in cheeses made from pasteurized milk (8.0%) than in cheeses manufactured from raw milk (4.8%).

The values of CB samples after storage in refrigerator were 1.18 × 10² CFU.g⁻¹ (2.07 log CFU.g⁻¹) in sample no. 7 and 1.90 × 10² CFU.g⁻¹ (2.27 log CFU.g⁻¹) in sample no. 8 (Figure 2). These values are not in accordance with Codex Alimentarius of Slovak Republic (2006). Other samples were negative for presence of CB after storage at 4 °C (Figure 2). Average number of CB after storage was 0.54 log CFU.g⁻¹ (Table 2).

Yucel and Ulusoy (2006) studied a total of 200 dairy (raw milk, cheese) samples obtained from Ankara, for the presence of *Yersinia* spp., total coliform and *Escherichia coli*. As expected, raw milk 55% (55/100) were significantly contaminated with *Yersinia* spp., than cheese samples 14% (14/100). *Y. enterocolitica* was the most commonly isolated species, and was recovered from 47.3% in raw milk 35.7% in cheese samples. The other *Yersinia* spp. were identified as *Y. frederiksenii* (31.0%, 21.4%), *Y. kristensenii* (12.7%), *Y. intermedia* (7.2%, 7.1%) and atypical *Yersinia* spp. (1.8%, 35.7%) in raw milk and cheese samples, respectively. All the samples of cheese examined were negative for *Y. kristensenii*. All *Y. enterocolitica* strains tested gave negative results in the autoagglutination tests and crystal violet binding test.

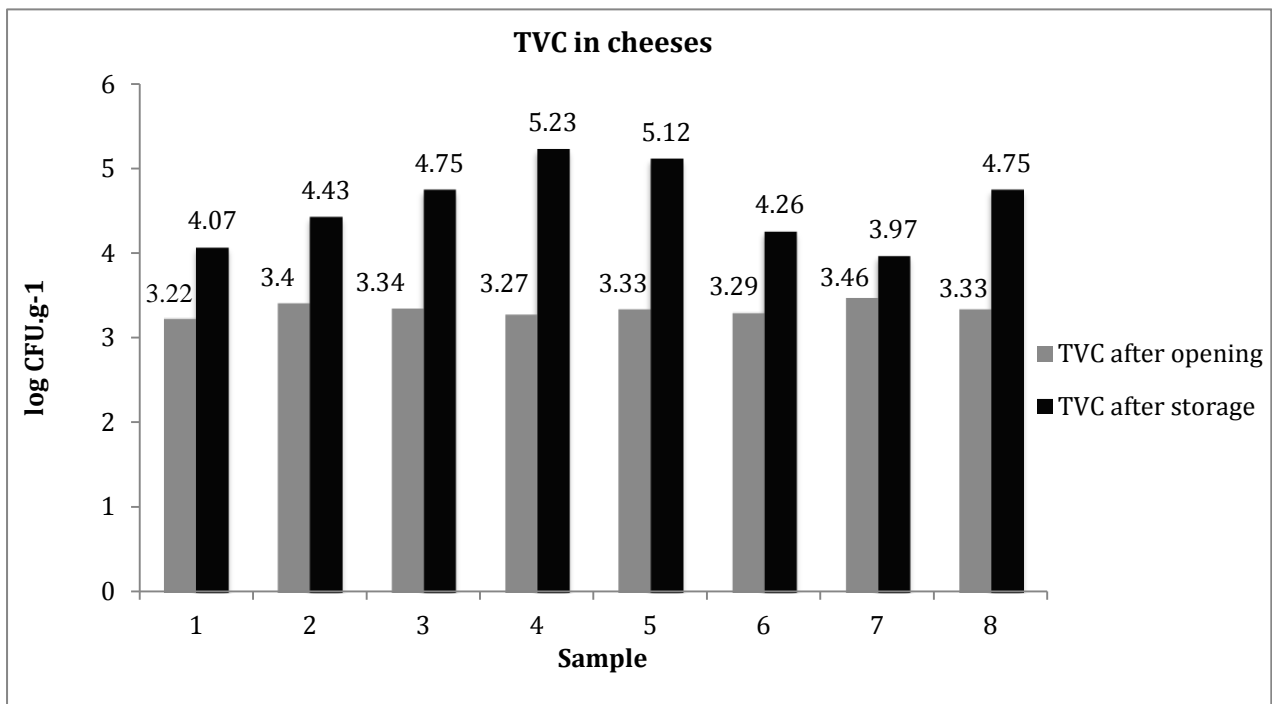


Figure 1 Values of TVC in cheeses after opening and after storage in refrigerator.

Values of TVC after 5 days of storage at temperature 4 °C were in range from 9.54 × 10³ CFU.g⁻¹ (3.97 log CFU.g⁻¹) in sample no. 7 to 1.71 × 10⁵ CFU.g⁻¹ (5.23 log CFU.g⁻¹) in sample no. 4 (figure 1). Average number of TVC after storage was 4.57 log CFU.g⁻¹ (table 1).

Table 1 Basic statistical characteristics of TVC in cheeses after opening and after storage.

	TVC after opening	TVC after storage
n	8	8
x	3.33	4.57
s	0.07	0.44
v%	2.10	9.62

n – number of samples, x – average, s - standard deviation, v% - coefficient of variation

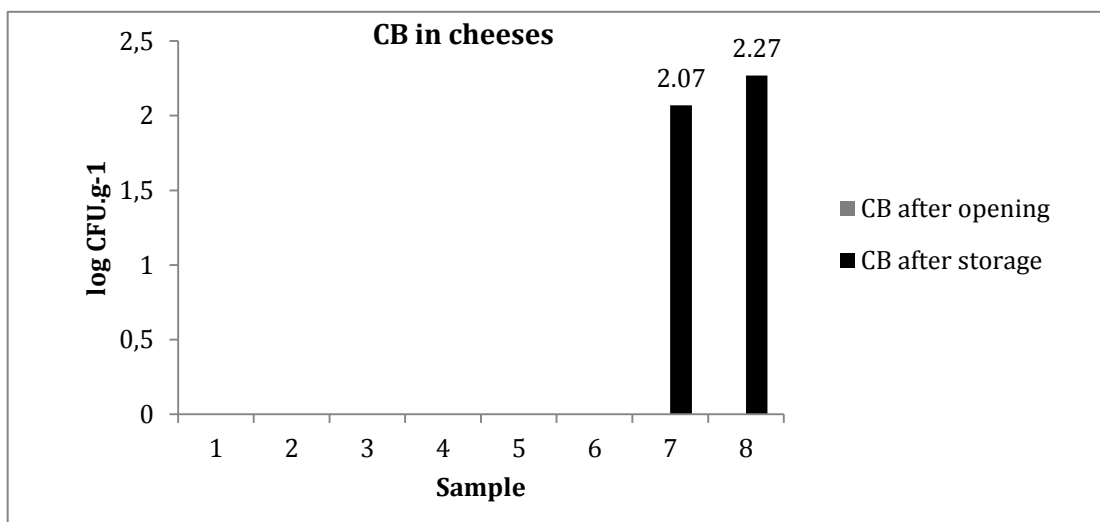


Figure 2 Values of CB in cheeses after opening and after storage in refrigerator.

The wide array of available dairy foods challenges the microbiologist, engineer, and technologist to find the best ways to prevent the entry of microorganisms, destroy those that do get in along with their enzymes, and prevent the growth and activities of those that escape processing treatments. Troublesome spoilage microorganisms include aerobic psychrotrophic Gram-negative bacteria, yeasts, molds, heterofermentative lactobacilli, and spore-forming bacteria. Psychrotrophic bacteria can produce large amounts of extracellular hydrolytic enzymes, and the extent of recontamination of pasteurized fluid milk products with these bacteria is a major determinant of their shelf life. Fungal spoilage of dairy foods is manifested by the presence of a wide variety of metabolic by-products, causing off-odors and flavors, in addition to visible

changes in color or texture (Ledenbach and Marshall, 2009). Samples no. 2, 5, 6 and 8 were negative for presence of MFF after opening. Values of MFF ranged from 1.81×10^1 CFU.g⁻¹ (1.25 log CFU.g⁻¹) in sample no. 1 to 2.72×10^1 CFU.g⁻¹ (1.43 log CFU.g⁻¹) in sample no. 7 (figure 3). Average number of MFF after storage was 0.67 log CFU.g⁻¹ (Table 3).

Values of MFF after 5 days of storage ranged from 5.4×10^1 CFU.g⁻¹ (1.73 log CFU.g⁻¹) in sample no. 4 to 1.68×10^2 CFU.g⁻¹ (2.22 log CFU.g⁻¹) in sample no. 7. Samples no. 5, 6 and 8 were negative for presence MFF after storage (figure 3). Average number of MFF after storage was 1.20 log CFU.g⁻¹ (Table 3). All analysed samples meet the requirements of Codex Alimentarius of Slovak Republic (2006).

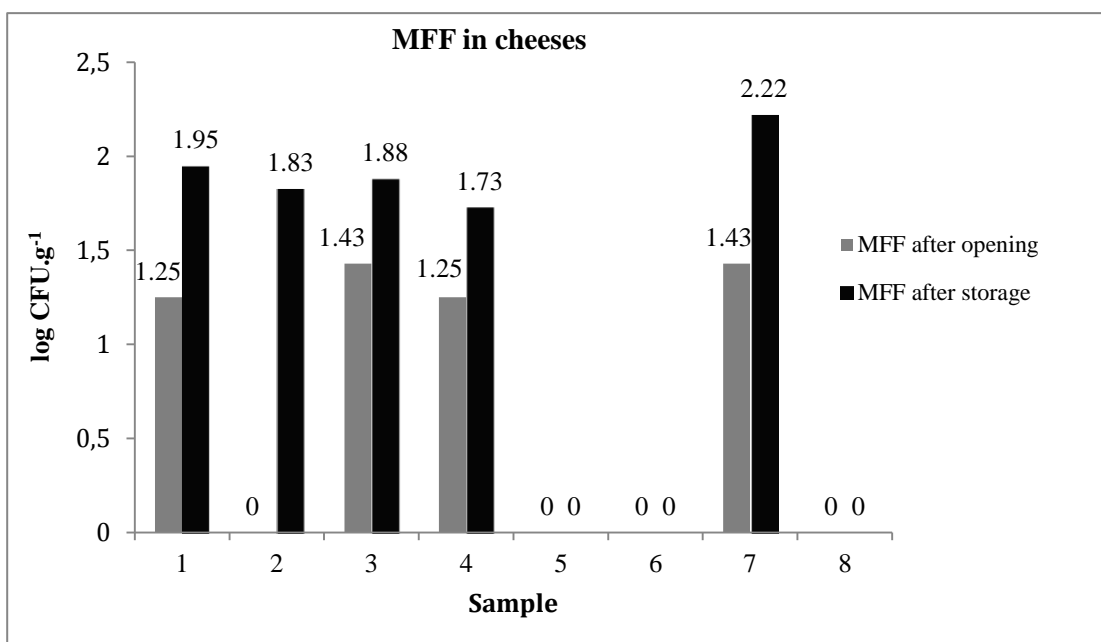


Figure 3 Values of MFF in cheeses after opening and after storage in refrigerator.

Table 2 Basic statistical characteristics of CB in cheeses after storage.

	CB after storage
n	8
x	0.54
s	0.94
v%	174.07

n – number of samples, x – average, s - standard deviation, v% - coefficient of variation

Table 3 Basic statistical characteristics of MFF in cheeses after opening and after storage.

	MFF after opening	MFF after storage
n	8	8
x	0.67	1.20
s	0.67	0.94
v%	100	78.33

n – number of samples, x – average, s - standard deviation, v% - coefficient of variation

Hayaloglu and Kirbag (2007) studied the chemical and microbial qualities, including microscopic filamentous fungi, of 30 samples of Kuflu cheese randomly purchased from different markets in Turkey. The levels of main microbial groups including total mesophilic and coliform bacteria, yeasts and moulds and the presence of some potentially pathogenic microorganisms (*E. coli*, *Salmonella* spp. and *Staphylococcus aureus*) were determined. The high numbers of all microbial groups and presence of potentially pathogenic organisms in the cheese samples suggested that the production and maturation of Kuflu cheese should be improved by better hygiene. Moulds at the cheese surface were isolated and identified. A total of 24 different mould species were detected and the genus most frequently isolated was *Penicillium* spp. which represented 70.25% of total isolates. *Penicillium commune*, *P. roqueforti* and *P. verrucosum* were the most abundant species in the cheeses sampled. The other dominant fungal groups were *Geotrichum candidum*, *Penicillium expansum* and *P. chrysogenum*. Other genera isolated from the cheese were *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Geotrichum*, *Mucor*, *Rhizopus* and *Trichoderma*. The potentially toxigenic species, including some *Penicillium* spp. and *Aspergillus flavus*, were also detected.

CONCLUSION

Microbiological quality of selected types of cheeses were evaluated after opening and after 5 days of storage in a refrigerator. The results were compared with the Codex Alimentarius of the Slovak Republic. The results show that the values of coliform bacteria in Edam cheeses after storage slightly exceeded the maximum limit established by legislation. Other samples had a good microbiological quality after opening and also during storage.

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BIOINFORMATIC APPROACH IN THE IDENTIFICATION OF *ARABIDOPSIS* GENE HOMOLOGOUS IN *AMARANTHUS*

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ABSTRACT

Bioinformatics offers an efficient tool for molecular genetics applications and sequence homology search algorithms became an inevitable part for many different research strategies. Appropriate managing of known data that are stored in public available databases can be used in many ways in the research. Here, we report the identification of RmlC-like cupins superfamily protein DNA sequence than is known in *Arabidopsis* genome for the *Amaranthus* – plant specie where this sequence was still not sequenced. A BLAST based approach was used to identify the homologous sequences in the nucleotide database and to find suitable parts of the *Arabidopsis* sequence where primers can be designed. In total, 64 hits were found in nucleotide database for *Arabidopsis* RmlC-like cupins sequence. A query cover ranged from 10% up to the 100% among RmlC-like cupins nucleotides and its homologues that are actually stored in public nucleotide databases. The most conserved region was identified for matches that possess nucleotides in the range of 1506 up to the 1925 bp of RmlC-like cupins DNA sequence stored in the database. The *in silico* approach was subsequently used in PCR analysis where the specificity of designed primers was approved. A unique, 250 bp long fragment was obtained for *Amaranthus cruentus* and a hybrid *Amaranthus hypochondriacus x hybridus* in our analysis. Bioinformatic based analysis of unknown parts of the plant genomes as showed in this study is a very good additional tool in PCR based analysis of plant variability. This approach is suitable in the case for plants, where concrete genomic data are still missing for the appropriate genes, as was demonstrated for *Amaranthus*.

Keywords: BLAST analysis; alignment; Rml-C like cupins; *Amaranthus*; PCR identification

INTRODUCTION

Bioinformatics provides an interdisciplinary tool, that is used to manage and analyse biological data and known sequences of nucleic acids (Cannataro et al., 2009). Many features of nucleic acids can be used in bioinformatic algorithms as motifs for description of their genomic variability and their better understanding. Individual sequence motifs are recognized by their order and nucleotide preference and many motif discovery algorithms have been used in different molecular or bioinformatic studies (Aravind and Koonin, 1999; Hertz and Stormo, 1999; La and Livesay, 2005; Rasouli et al., 2013; Gardner and Slezak, 2014).

Here, the bioinformatic algorithms were applied for known cupin DNA sequences. Cupin proteins are reported as to be structurally conserved and in function very divergent superfamily of proteins (Khuri et al., 2001) that are germin-related. These proteins were analysed by their EST and microbial GeneBank databases as having representatives in many prokaryotic and eukaryotic organisms, moreover, 26 residues of cupins intermotif regions were found in cereal proteins (Dunwell et al., 2000). RmlC-like cupins superfamily protein DNA sequence is available in SeedGeneDB (sgdb.cbi.pku.edu.cn/) database under the accession code AT2G18540, what makes it suitable for applying the

bioinformatic tools such as BLAST (Altschul et al. 1990) to find homology or conserved regions.

In this study, we identify the conserved nucleotides of available genomic data of *Arabidopsis* RmlC-like cupins superfamily protein suitable for bioinformatic approach based primer designation and subsequently PCR identification of the presence of this sequence in the genome of *Amaranthus*.

MATERIAL AND METHODOLOGY

Plant material of *Amaranthus cruentus* (Ficha cultivar) and a hybrid *Amaranthus hypochondriacus x hybridus* (hybrid K-433) was planted under the *in vitro* conditions. DNA was extracted following the instructions of GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific). Nanodrop Nanophotometer™ was used for quantity and quality analysis of the extracted DNA.

Bioinformatic Mega-BLAST (Zhang et al. 2000) alignment of the 2672 bp RmlC-like cupins superfamily protein DNA sequence (SeedGeneDB accession code AT2G18540) was performed. BLAST searches were used in nonredundant, nonmouse and nonhuman nucleotide databases by BLATn against plants (taxid:3193) nucleotide sequences in the NCBI database to align existing accessions. To analyse the returned alignments for the purposes of primer designations, only those nucleotide

sequences were chosen that possess the query cover more than 75% and E-value 0.0.

Primers were designed in Primer-BLAST (Ye et al, 2012) in a manner to get RmlC-like cupins amplification only based on the conservative part of this gene as predicted bioinformatically. Following primers were returned as specific and used in the study: forward 5'ccgaagtctcatccgatggc 3' and reverse 5'ctttgaaagctccccctcgg 3'. PCR amplifications were performed in a Bio-Rad C1000™ Thermocycler with the following program: an initial denaturation step at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s, 58 °C for 40 s, and 72 °C for 40 sec with a final cycle at 72 °C for 10 min. The amplified products were inspected by electrophoresis in 1.5% agarose in a 1×TBE buffer, visualized after GelRed™ staining and photographed under UV light.

RESULTS AND DISCUSSION

First, alignment of *Arabidopsis* RmlC-like cupins sequence was done using megaBLAST. Here, query cover from 10% up to the 100% was found among RmlC-like cupins nucleotides and its homologues that are actually known for taxid 3193 and stored in databases (Figure 1). In total, 64 hits were found in nucleotide database for *Arabidopsis* RmlC-like cupins sequence. Subsequently, conserved region was identified for matches that possess

the query cover more than 75% and E-value 0.0 and was returned for the nucleotides in the range of 1506 up to the 1925 bp of RmlC-like cupins DNA. Variable regions of the most conserved part of the RmlC-like cupins sequence of *Arabidopsis* are listed in the Table 1.

Comparing the obtained data for possibility to design primers for RmlC-like cupins PCR identification in plant species, candidate sites were found as displayed in figure 2 for the nucleotides 1533-1552 and 1765-1784. For primer design, Primer-BLAST (Ye et al, 2012) software was used. In literature, not only Primer-BLAST, but also similar softwares like FastPCR (Kalender et al., 2011), or csPCR (Dasu et al., 2010) are reported by (Gardner et al., 2014) as to be optimal for low throughput analyses for the purposes of manual inspection or a graphical user interface. They design the primers together with primer Tm, secondary structure and primer-dimer prediction.

Bäumlein et al. (1995) reported for cupin superfamily sharing conserved residues with vicilin and legumin storage proteins. This was confirmed by performed BLAST again, as vicilin-like and provicilin-like alignments were found in nucleotide database for *Camelina sativa*, *Morus notabilis*, *Elaeis guineensis*, *Citrus sinensis*, *Vitis vinifera*, *Brassica rapa* and *Tarenaya hassleriana*. None all of these returned alignment share the query cover more than 75% and E-value 0.0 and were not used in comparison for primer design purposes.

Table 1 Characterization of variable nucleotide motifs in the most conserved region of RmlC-like cupins superfamily protein.

Alignment	Variable nucleotides within the motif 1506-1925 nt
<i>Arabidopsis thaliana</i> cupin family protein mRNA	none
<i>Camelina sativa</i> provicilin-like (predicted)	1547, 1655, 1581, 1587, 1598, 1601, 1604, 1606, 1641, 1644, 1647, 1651, 1653, 1660, 1670, 1673-4, 1679, 1696, 1706, 1718, 1722, 1749, 1752, 1757, 1761, 1767, 1773, 1780, 1789, 1809, 1820, 1838, 1840, 1842, 1847, 1851-2, 1858-60, 1876, 1878-9, 1886, 1890, 1894-97, 1903-4, 1906
<i>Camelina sativa</i> globulin-1 S allele-like (predicted)	1547, 1556, 1565, 1580, 1586, 1601, 1604, 1606-7, 1613, 1646, 1650, 1652, 1670, 1671, 1674, 1696, 1706, 1709, 1716, 1718, 1722, 1730, 1748, 1751, 1756, 1760, 1772, 1779, 1789, 1790, 1809, 1820, 1840, 1842, 1852, 1853, 1859, 1860, 1861, 1874-88, 1885-88, 1898, 1899, 1906, 1910
<i>Camelina sativa</i> globulin-1 S allele (predicted)	1547, 1565, 1580, 1586, 1601, 1604, 1606-7, 1611, 1613, 1634, 1646, 1650, 1652, 1659, 1670, 1674, 1696, 1706, 1726, 1728, 1730, 1748, 1751, 1756, 1760, 1772, 1779, 1789, 1790, 1820, 1840, 1842, 1852, 1853, 1859, 1860, 1871, 1875-80, 1888-9, 1892, 1898, 1903, 1904, 1906, 1910
<i>Arabidopsis lyrata</i> subsp. <i>lyrata</i> hypothetical protein*	1547, 1556, 1567, 1594, 1601, 1604-5, 1607, 1613, 1628, 1637, 1640, 1646, 1650, 1652, 1666, 1670, 1647, 1694, 1700, 1706, 1709, 1714, 1716, 1718, 1730, 1772, 1776, 1779, 1786, 1801, 1840, 1845
<i>Brassica rapa</i> uncharacterized LOC103874069 (predicted)	1514, 1517, 1536, 1542, 1547, 1556, 1557, 1567, 1577, 1580-1, 1583, 1592, 1601, 1607, 1613, 1629, 1640, 1656, 1642, 1646, 1650, 1652, 1655, 1661-2, 1647, 1679-81, 1692, 1697-8, 1702, 1707, 1709-10, 1713, 1793, 1810, 1811, 1838, 1842, 1843, 1853-70, 1888, 1889, 1890, 1900-4, 1906, 1916, 1919

* alignment found here only for the nucleotides 1506-1869

In *Arabidopsis*, analysed RmlC-like cupins superfamily protein is described to have nutrient reservoir activity function (http://sgdb.cbi.pku.edu.cn/gene_info.php?id=AT2G18540). For other alignments found by BLAST in this study, beside above mentioned vicilin and provicilin like characteristics, three types of other characteristics were returned: globulin 1-S, hypothetical protein and uncharacterized one. For most of

germin-like proteins isolated from plants are in literature reported unknown function or bifunctionality (Woo et al., 2000).

After the bioinformatic analysis of *Arabidopsis* RmlC-like cupins sequence, primers were designed for the purposes of its PCR identification in those plants, for which no homologous nucleotides exist in databases. *Amaranthus cruentus* (Ficha cultivar) and a hybride

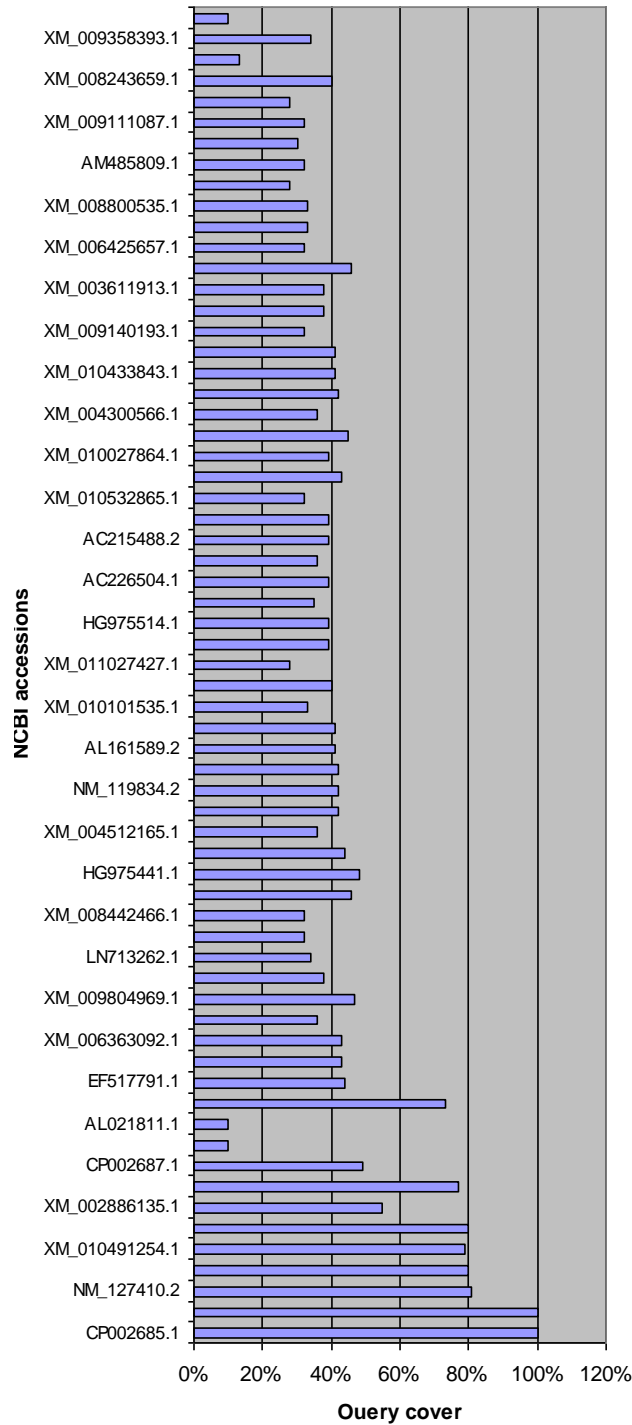


Figure 1 Differences in query covers of returned sequences in the alignment of *Arabidopsis* RmlC-like cupins sequence against taxid:3193.

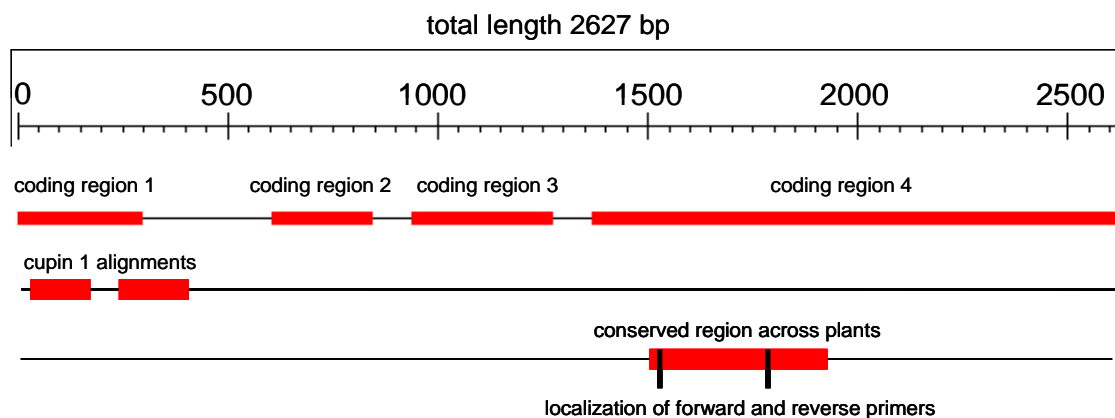


Figure 2 Characteristics of *Arabidopsis* RmlC-like cupins sequence with the position of primers for its identification designed in this study.

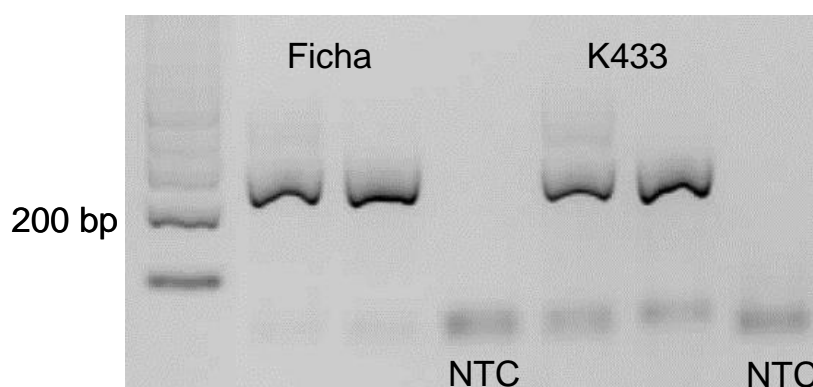


Figure 3 PCR amplification of *Arabidopsis* RmlC-like cupins sequence in *Amaranthus cruentus* and a hybrid *Amaranthus hypochondriacus* x *hybridus* based on bioinformatically predicted data.

Amaranthus hypochondriacus x *hybridus* (hybrid K-433) became the biological object for the test of the designed primer pair suitability and amplification efficiency. After optimization of PCR conditions a specific monomorphic fragment with the size of 250 bp it was obtained (Figure 3). This fragment length corresponded to the size of the conservative region that is flanked by designed primers in the bioinformatic part of the study. Amplicons were inspected for specificity on 1.5% agarose gel.

Sequence homology search algorithms became commonly used and efficient tools in molecular genetics (Karpov and Bloom, 2010). Nowadays, a number of different motifs finding algorithms are available and (Lin, <http://biochem218.stanford.edu/Projects%202012/Lin.pdf>) reported them to be impossible to provide a comprehensive report of all of them. Each algorithm has its own advantages and disadvantages. One of the aims of different patterns discovery is finding of specific motifs in nucleotide or protein sequences for the purpose of better understanding of their structure and function (Bailey, 2008) or for their identification (Khuri et al., 2001).

CONCLUSION

We presented a bioinformatic approach for identification of specific parts of the plant genomes that are known for some species, but not for all. Using nucleotide comparisons based on BLAST analysis offer a tool that

can be used for selecting the most conservative sites of known sequences. Based on comparison such as those, universal primers can be designed and used for species, where concrete genomic data are still missing for the appropriate gene.

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MICROBIAL CONTAMINATION OF SPICES USED IN PRODUCTION OF MEAT PRODUCTS

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ABSTRACT

There was investigated microbial quality of spices used in production of meat products (black pepper, allspice, coriander, juniper, cumin, cinnamon, badian, mustard, bay leaf, paprika, rosemary, garlic, ginger, thyme, cardamom). The spices were analysed on the presence of total count of mesophilic, thermoresistant and coliforming microorganisms, *Staphylococcus aureus*, methicilin resistant *S. aureus* (MRSA), *Escherichia coli*, *Salmonella* spp., *Bacillus cereus*, *Bacillus licheniformis* and moulds. For the detection of fungal contamination was used agar with glucose, yeast extract and oxytetracyclin and dichloran-glycerol agar. The cultivation was performed at $25 \pm 1^\circ\text{C}$ for 5 – 7 days. The microscopic method was used for species identification. The aflatoxin presence was confirmed by ELISA test in all of tested spices and was performed in ppb (pars per billion = $\mu\text{g}/\text{kg}$). TCM ranged from 200 to 5600000 cfu/g, TRM from 20 to 90000 cfu/g and coliforming bacteria from 30 to 3200 cfu/g. *B. cereus* was present in juniper, mustard, bay leaf, thyme and cardamom (32%), while *B. licheniformis* was confirmed in 58% of cases (allspice, pepper, ground juniper, badian, bay leaf, paprika, garlic, thyme and cardamom). *S. aureus* was detected in whole coriander, cinnamon, badian and mustard but only in law number (30, 40, 20 and 10 cfu/g respectively). No strains *S. aureus* was identified as MRSA. The presence of *Salmonella* spp. and *E. coli* was not confirmed. The fungal contamination was found in 14 spices and their count varied from 0 to 1550 cfu/g. There were confirmed the presence of *Aspergillus flavus* (allspice whole and ground, black pepper whole and ground, whole coriander, ground cumin, ground bay leaf), *Aspergillus niger* (allspice whole and ground, black pepper ground, ground juniper, cumin ground, bay leaf ground, ground rosemary, ground thyme), *Penicillium glaucum* (allspice whole and ground, whole juniper, whole cinnamon), *Penicillium claviforme* (whole black pepper, whole coriander, cardamom ground), *Alternaria alternata* (cumin ground, rosemary ground, thyme ground), *Mucor* (whole and ground coriander and thyme) and *Phoma* (ground cumin). The aflatoxin presence was confirmed in 11 of samples (57.9%) and the value ranged from 0 to 4 ppb (ground allspice, whole and ground pepper, whole juniper, cumin, cinnamon, badian, bay leaf, paprika, rosemary, thyme).

Keywords: spices; meat; bacterial species; moulds; aflatoxin

INTRODUCTION

The consumption of spices is connected with the human diet much sooner than later meat diet or the use of spices in combination with other types of food. Spices have not only the beneficial effects on the human body, but also improve and enhance the gustatory pleasures from food and promote the health. Srinivasan et al. (2004) and Srinivasan (2005a, b) confirmed the beneficial influence of turmeric/curcumin, red pepper/capsaicin, and garlic on lipid metabolism, especially anti-hypercholesterolemic effect of these spices, then anti-lithogenic effect of curcumin and capsaicin, then antioxidant effects of curcumin, capsaicin and eugenol (of clove), in his study with animals. Srinivasan (2007) also dealt with health benefits of black pepper/piperine and demonstrated in vitro studies its protective properties against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Platel and Srinivasan (2004) found the positive influences of some spices on terminal enzymes

during digestive proces. They documented that dietary curcumin, capsaicin, piperine and ginger enhanced intestinal lipase activity and also the sucrase and maltase, and dietary cumin, fenugreek, mustard and asafoetida decreased the level of phosphatases and sucrase.

Some spices can also have antibacterial properties. Arora and Kaur (1999) described the antimicrobial activity of garlic and clove to *Staphylococcus epidermidis*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*. Tajkarimi et al. (2010) found out the antimicrobial activity of herbs and spices (basil, oregano, allspice, cinnamon, clove, thyme, rosemary) containing essential oils against foodborne pathogens, such as *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*. Mandal et al. (2011) confirmed also antimicrobial effect against methicilin-resistant *Staphylococcus aureus* (MRSA) of three indian spices (cinnamon, cumin and clove). Some authors described also

antifungal activities of spices and herbs essential oils. **Omidbeygi et al. (2007)** identified antifungal activity of three essential oils (thyme, summer savory and clove) against *Aspergillus flavus*, and **Atanda et al. (2007)** described antifungal properties of sweet basil, cassia, coriander and bay leaf against *Aspergillus parasiticus* CFR 23 and aflatoxin production. **Škrinjar and Nemet (2009)** published the review of antimicrobial activity of essential oils used spices (garlic, mustard, cinnamon, cumin, clove, bay leaf, thyme, basil, oregano, pepper, ginger, sage, rosemary etc.) against common bacteria and fungi such as *Listeria* spp., *Staphylococcus* spp., *Salmonella* spp., *Escherichia* spp., *Pseudomonas* spp., *Aspergillus* spp., *Cladosporium* spp. and many others.

Although many spices show the antifungal properties, they are themselves extensively contaminated. In case of bacterial contamination, there are mostly sporeforming bacteria (genus *Bacillus*, *Clostridium*), then *Salmonella* spp. and the others such as enterobacteria, pseudomonades and aeromonades as well as lactobacilli and enterococci (**Kneifel and Berger, 1994**). *Salmonella enterica* subspecies *enterica* and *Bacillus* spp. were the most common causative agents of foodborne illness outbreaks identified in spices (**Van Doren et al., 2013**). Next to the bacterial contamination, the fungal contamination is also very common. **Mandeeel (2005)** detected 665 fungal isolates in total, representing 14 species (mostly *Aspergillus*, *Penicillium*, *Rhizopus*, *Cladosporium* and *Trichoderma*), which were recovered and identified from 17 dried and ground spice samples. The most heavily contaminated spice was observed in red chili and black pepper (1580 and 1120 cfu/g, respectively). Fungal contamination represents serious risk for human health primarily in case of *Aspergillus flavus* and *Aspergillus parasiticus* occurrence. These are able to produce aflatoxins in food and feedstuffs, which are known to be potent hepatocarcinogens in animals and humans (**Šarić and Škrinjar, 2008**).

The aim of this work was to find out the level of bacterial and fungal contamination of selected spices and to detect the aflatoxin presence in spices.

MATERIAL AND METHODOLOGY

Spices selection. There were tested spices which were used during technological process for meat products production (canned meat and pastes): black pepper, allspice, coriander, juniper, cumin, cinnamon, badian, white mustard, bay leaf, sweet paprika, rosemary, garlic, ginger, thyme, cardamom – Table 1. The mentioned spices were not treated with ionizing irradiation and originated from different countries. Their origin is included in Table 1.

Microbiological analyses. The total count of mesophilic (TCM) and thermoresistant (TRM) microorganisms and coliforms (COLI), then *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Salmonella* spp., *Bacillus cereus* and *Bacillus licheniformis* were determined in cfu in 1 g of prepared spice samples. The starting samples were prepared as follows: 5 g of spices (milled or powder) was dissolved in 45 g of sterile distilled water (the whole spices were

crushed in the bowl before). The solution was filtered and the supernatant was used for analyses.

TCM were cultivated using GTK-M Agar at 30 °C / 72 hours (according to standard ČSN EN ISO 4833) and COLI on VRLB Agar (Milcom, Tábor, CR) at 37 °C / 24 hours by standard ČSN ISO 4832. The samples for enumeration of TRM were inactivated at 85 °C during 10 minutes and then were cultivated on GTK Agar (Milcom, Tábor, CR) at 30 °C / 72 hours (ČSN EN ISO 4833).

Baird Parker Agar (HiMedia, India) was used for detection of *S. aureus* (ČSN EN ISO 6888-1). Plates were incubated at 37 ±1°C for 48 hours and colonies with zones of precipitation were submitted to the tube for free coagulase test for confirmation. STAPHYtest and the identification program TNW pro.7.5 were used for species identification (Erba Lachema, s.r.o., Brno, Czech Republic). The isolates identified as *S. aureus* were confirmed by the multiplex PCR method for the detection of the species specific fragment SA442 (**Martineau et al., 1998**). MRSA and MR-CNS (methicillin resistant coagulase-negative staphylococci) were screened for the presence of *mecA* gene, which encodes the resistance to methicillin (**Bosgelmez-Tinaz et al., 2006**).

The test for *Salmonella* spp. occurrence was performed according to norm ČSN EN ISO 6579. The samples were multiplied in buffered pepton water (Oxoid, Basingstoke, UK), cultivated at 37 ±1°C for 18 ±2 hours and then they were cultivated in selective medium RVS and MkTTn (Oxoid, Basingstoke, UK) at 37 ±1°C for 24 ±3 hours. This suspension was inoculated on the surface of Rambach medium (Merck, Germany) and XLD (Oxoid, Basingstoke, UK).

For the detection of fungal contamination was used agar with glucose, yeast extract and oxytetracyclin (GKCH, HiMedia; Indie) and dichloran-glycerol agar (DG18, HiMedia; Indie). The cultivation was performed at 25 ±1°C for 5 – 7 days according to Czech norm ČSN ISO 21257-2. The microscopic method was used for species identification.

Aflatoxin detection. The aflatoxin presence was confirmed by ELISA test in all of tested spices. Aflatoxin was determined using commercial assay kit Veratox for Aflatoxin (Neogen, USA). Veratox is a competitive direct ELISA test that provides a quantitative analysis of aflatoxin. Lower limit of detection is 2 ppb, range of quantitation is 5 – 50 ppb (parts per billion = µg/kg).

RESULTS AND DISCUSSION

Microbial contamination. The results of bacterial and fungal contamination are shown in the Table 2. From our results is evident that the count of mesophilic microorganisms ranged from 200 to 5600000 cfu/g. The highest TCM (>1 mil cfu/g) was found in cumin, bay leaf and ground coriander (5600000, 4800000 and 4000000 cfu/g, respectively). The lowest TCM was confirmed in case of whole juniper, cinnamon, badian and white mustard (TCM <1000 cfu/g). TCM <10000 cfu/g was found in whole coriander, rosemary and ginger. In the other spices, TCM varied between 20000 and 400000 cfu/g. The similar results in 55 different spices and herbs are indicated by **Kneifel and Berger (1994)**.

Table 1 Spices origin.

species	form	name	origin
allspice	whole	<i>Pimenta dioica</i>	Mexico
allspice	ground		Mexico
black pepper	whole	<i>Piper nigrum</i>	Vietnam
black pepper	ground		Vietnam
coriander	whole	<i>Coriandrum sativum</i>	Ukraine
coriander	ground		Ukraine
juniper	whole	<i>Juniperus communis</i>	Bosna-Hercegovina
juniper	ground		Macedonia
cumin	ground	<i>Carum carvi</i>	Czech Republic
cinnamon	whole	<i>Cinnamomum verum</i>	Czech Republic
badian	whole	<i>Illicium verum</i>	Vietnam
white mustard	whole	<i>Sinapis alba</i>	India
bay leaf	ground	<i>Laurus nobilis</i>	Turkey
sweet paprika	ground	<i>Capsicum annum</i>	Hungary
rosemary	ground	<i>Rosmarinus officinalis</i>	Morocco
garlic	powder	<i>Allium sativum</i>	China
ginger	ground	<i>Zingiber officinale</i>	Nigeria
thyme	ground	<i>Thymus vulgaris</i>	Poland
cardamom	ground	<i>Elettaria cardamomum</i>	Guatemala

Table 2 Results of bacterial contamination (in cfu/g).

species	TCM	TRM	COLI	<i>E.coli</i>	<i>B.cereus</i>	<i>B. licheniformis</i>	<i>S. aureus</i>	MRSA	<i>Salmonella</i> spp.
allspice whole	60000	6000	–	–	–	100	–		–
allspice ground	20000	14000	1000	–	–	150	–		–
black pepper whole	160000	15000	880	–	–	200	–		–
black pepper ground	320000	90000	3200	–	–	220	–		–
coriander whole	8000	50	600	–	–	–	30	neg.	–
coriander ground	4000000	24000	3000	–	–	–	–		–
juniper whole	200	100	–	–	10	–	–		–
juniper ground	60000	8000	100	–	120	150	–		–
cumin ground	5600000	600	2000	–	–	–	–		–
cinnamon whole	400	50	–	–	–	–	40	neg.	–
badian whole	800	140	–	–	–	10	20	neg.	–
white mustard whole	500	80	–	–	10	–	10	neg.	–
bay leaf ground	4800000	4000	480	–	80	120	–		–
sweet paprika ground	400000	9000	–	–	–	150	–		–
rosemary ground	5000	30	220	–	–	–	–		–
garlic powder	1700000	16000	–	–	–	220	–		–
ginger ground	3000	20	–	–	–	–	–		–
thyme ground	180000	6000	30	–	70	100	–		–
cardamom ground	60000	7000	–	–	80	80	–		–

cfu/g = colony forming units per gram; TCM = total count of mesophilic microorganisms; COLI = coliforming bacteria; RM = termoresistant bacteria; MRSA = methicilin resistant *S. aureus*

Table 3 Results of fungal contamination (in cfu/g).

species	total count				
allspice whole	1550	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Penicillium glaucum</i>	
allspice ground	140	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Penicillium glaucum</i>	
black pepper whole	30	<i>Aspergillus flavus</i>	<i>Penicillium claviforme</i>		
black pepper ground	20	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>		
coriander whole	140	<i>Aspergillus flavus</i>	<i>Penicillium claviforme</i>	<i>Mucor</i>	
coriander ground	40	<i>Mucor</i>			
juniper whole	180	<i>Penicillium glaucum</i>			
juniper ground	10	<i>Aspergillus niger</i>			
cumin ground	80	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Phoma</i>	<i>Alternaria alternata</i>
cinnamon whole	10	<i>Penicillium glaucum</i>			
badian whole	0				
white mustard whole	0				
bay leaf ground	340	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>		
sweet paprika ground	0				
rosemary ground	10	<i>Aspergillus niger</i>	<i>Alternaria alternata</i>		
garlic powder	0				
ginger ground	0				
thyme ground	10	<i>Aspergillus niger</i>	<i>Mucor</i>	<i>Alternaria alternata</i>	
cardamom ground	10	<i>Penicillium claviforme</i>			

Table 4 Results of aflatoxin detection (in ppb).

spice	ppb	<i>Aspergillus flavus</i>
allspice whole	0	+
allspice ground	4	+
black pepper whole	3	+
black pepper ground	4	+
coriander whole	0	+
coriander ground	0	
juniper whole	2	+
juniper ground	0	
cumin ground	2	+
cinnamon whole	4	
badian whole	4	
white mustard whole	0	
bay leaf ground	4	+
sweet paprika ground	3	
rosemary ground	3	
garlic powder	0	
ginger ground	0	
thyme ground	3	
cardamom ground	0	

More than 50% of samples contained TCM between 10^4 and 10^6 cfu/g. Some of them (chilli, black pepper and Chine spice) obtained TCM $>10^7$ cfu/g. Schwab et al.

(1982) confirmed higher count of TCM ($<100 - 3.1 \times 10^8$ cfu/g), but geometric means of cinnamon, ginger, paprika, pepper, rosemary and thyme were 39000, 21000, 100000, 820000, 7200 and 2100 cfu/g, respectively.

The count of TRM ranged from 20 to 90000 cfu/g. The highest count was confirmed in ground black pepper, ground coriander and garlic (90000, 24000 and 16000 cfu/g, respectively). The lowest count was detected in ginger, rosemary, cinnamon, whole coriander and mustard (20, 30, 50, 50 and 80 cfu/g, respectively). Compared to Kneifel and Berger (1994) that detected bacilli $> 10^5$ cfu/g in almost 40% of the samples, our results were much lower. *B. cereus* was present in juniper, mustard, bay leaf, thyme and cardamom (32%), while *B. licheniformis* was confirmed in 58% of cases (allspice, pepper, ground juniper, badian, bay leaf, paprika, garlic, thyme and cardamom). The results show that *B. licheniformis* represents the higher risk than *B. cereus* which is considered as food pathogen. This finding confirms also our previous results in case of other kind of food (Vyletřlová et al., 2001, 2002).

The count of coliforming bacteria ranged from 30 to 3200 cfu/g and their presence was found out in all of spices except for whole allspice, whole juniper, cinnamon, badian, mustard, paprika, garlic, ginger and cardamom. The similar results are described by Schwab et al. (1982). They analysed cca 1500 samples of each spices and found out that the count of coliforms ranged from <3 to 1.1×10^6 cfu/g. The geometric mean of coliform bacteria was in cinnamon, ginger, paprika, pepper, rosemary and thyme (19, 12, <3 , 9, 3.6 and 5 cfu/g, respectively).

E. coli was not detected. Sporadic occurrence of *E. coli* (geometric mean = <3 cfu/g) in some spices is confirmed also by Schwab et al. (1982). Shamsuddeen (2009) described *E. coli* presence in one mixture spice composed of ginger, cloves, black pepper, groundnut, salt and seasoning. This result confirms the finding of some authors that the spices have had antimicrobial effect against *E. coli* (Arora and Kaur, 1999, Tajkarimi et al., 2010, Sağdıç, 2003).

S. aureus was detected in whole coriander, cinnamon, badian and mustard but only in low number (30, 40, 20 and 10 cfu/g, respectively). No strains *S. aureus* was identified as MRSA. Shamsuddeen (2009) found out the high *Staphylococci* occurrence in the above mentioned mixture spice. The geometric mean was 1.73×10^9 cfu/g but they didn't specify the staphylococcal species.

The presence of *Salmonella* spp. was not confirmed. Shamsuddeen (2009) detected *Salmonella* presence only in one sample of mixture spice (0.05%), Kneifel and Berger (1994) found *Salmonella arizonae* in one sample of black pepper from all of 160 tested spices, as well as.

Fungal contamination. Results of fungal contamination are summarized in Table 3. There were confirmed the presence of *Aspergillus flavus* (allspice whole and ground, black pepper whole and ground, whole coriander, ground cumin, ground bay leaf), *Aspergillus niger* (allspice whole and ground, black pepper ground, ground juniper, cumin ground, bay leaf ground, ground rosemary, ground thyme), *Penicillium glaucum* (allspice whole and ground, whole juniper, whole cinnamon), *Penicillium claviforme* (whole black pepper, whole coriander, cardamom ground), *Alternaria alternata* (cumin ground, rosemary ground, thyme ground), *Mucor* (whole and ground coriander and thyme) and *Phoma* (ground cumin). The fungal count varied from 0 to 1550 cfu/g, where the most occurrence was in whole allspice. The count from 10 to 340 cfu/g was observed in case of ground allspice, black pepper whole and ground, coriander whole and ground, juniper whole and ground, ground cumin, cinnamon whole, ground bay leaf, ground rosemary, ground thyme and cardamom. The moulds were not detected in the rest of spices (badian, mustard, garlic and ginger). Schwab et al. (1982) stated the fungal count from <5 to 300000, while geometric mean was for cinnamon, ginger, paprika 290, 7 and 14 cfu/g respectively. They don't specify the fungal species in this work. The similar species representation (*Penicillium*, *Aspergillus*, *Mucor*) were described by Imandel and Adibnia (2000) in turmeric, black pepper and sumac. Mandeel (2005) writes that the most predominant fungal genera encountered were *Aspergillus*, *Penicillium*, *Rhizopus*, *Cladosporium* and *Trichoderma* and the most contaminated spice samples examined were observed in red chili and black pepper in order of magnitude of 1580 and 1120 cfu/g, respectively.

Aflatoxin presence. Aflatoxin detection is presented in Table 4. From obtained results is evident that the aflatoxin presence was confirmed in 11 of samples (57.9%). The presence of aflatoxin corresponds to the presence of *A. flavus* only in ground allspice, whole and ground pepper, whole juniper, ground cumin and ground bay leaf. In case of whole allspice and whole coriander there wasn't the aflatoxin occurrence detected even there were confirmed

presence of *A. flavus*. This difference can be explained by sensitivity of ELISA test or the identified *A. flavus* strain produced no aflatoxins in under this condition, which was described also by (Schindler et al., 1967, Ritter et al., 2011). In the other spices (whole cinnamon, whole badian, sweet paprika ground, ground rosemary and ground thyme) was aflatoxin determined even the *A. flavus* occurrence was negative. This difference can be explained by the used method (e.g. sample preparation - dilution) or by the presence only aflatoxins in spices.

Recorded results of aflatoxin (0 – 4 ppb) meet the requirements for limit of aflatoxin occurrence in spices according to Commission Regulation (EU) No. 165/2010 which defines the maximal limits of aflatoxin B1 (5 ppb) and total aflatoxin B1 + G1 + B2 + G2 (10 ppb) in some spices.

CONCLUSION

There are no legislative requirements for microbiological quality of spices in CR. The results can be evaluated by other regulations relating to food, which the spices are used for (e.g. Commission Regulation (ES) No. 2073/2005). According to this regulation and from the viewpoint of bacterial contamination and food pathogens (*S. aureus*, *E. coli*, *Salmonella*) it is possible to state that tested spices did not exceed allowed limits occurrence. The results fulfil also the limit value for microbiological quality according to previous Czech Regulation No. 132/2004.

The microbial contamination (TCM) in spices suggests the need for a thorough control of spices, used in food processing in general (heat treated food, semi food, food for direct consumption). Mainly food for direct consumption (including spices or herbs) represents a higher risk. The use of radiation treated with ionizing irradiation is one option to reduce or eliminate this risk. Maximum permissible overall average absorbed radiation dose which is allowed in the CR is 10.0 kGy (gray) according to Decree of Ministry of Health (133/2004).

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EFFECT OF ROSEMARY IN COMBINATION WITH YEAST EXTRACT ON MICROBIOLOGY QUALITY, OXIDATIVE STABILITY AND COLOR OF NON-FERMENTED COOKED SALAMI “INOVEC”

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ABSTRACT

The aim of this study was to determine antimicrobial and antioxidant effect of dried rosemary (1 g/kg) in combination with yeast extract 1 g/kg (1st experimental group) and 2 g/kg (2nd experimental group) in compare to control sample (without rosemary extract and treated with E316 and E621) in non-fermented heat-treated product Inovec salami. Inovec salami is a product produced all over the Slovakia. The similar product can be found in Polish (Polish salami) or in the Czech Republic (Vysocina salami). The samples were vacuum packaged and were kept at 4 °C for 30 days. Determination of psychrotrophic bacteria count (PBC), count of *Enterobacteriaceae* family (ETB), yeast and moulds, *Lactobacillus* spp. bacteria count (LAB) was done by cultivation methods. Oxidative stability was determined by TBARS value. Color spaces L*, a*, b* of Inovec salami was determined by CM 2600D spectrophotometer (Konica Minolta, Germany). It was found non-significant ($p > 0.05$) color loss (decrease in redness) over time, and the treatments had a non-significant impact ($p > 0.05$) on redness of the salami in compare to control sample. TBARS values of the rosemary treated cooked salami with different amount of yeasts extracts were significantly lower compare to control sample at the end of the shelf life. Also, lower value of TBARS was determined in salami with rosemary in combination with higher amount of yeast extract. The lowest count of PBC was determined in the second experimental group after 15 days. The rosemary extract in combination of yeast extract treatments were not able to influence the growth of the yeasts and moulds. It was found that rosemary extract in combination with yeast extract in the salami of the second experimental group after 15 days of storage significantly ($p \leq 0.05$) decrease count of *Lactobacillus* bacteria.

Keywords: rosemary; salami; oxidative stability; color; antimicrobials.

INTRODUCTION

Nowadays people are showing greater interest in foods that contain bioactive or functional components, which will give additional benefits to their health status (Cofrades et al., 2008). Many consumers believe meat and meat product consumption is unhealthy, because of their high animal fat, cholesterol, synthetic antioxidants and antimicrobials contents which may be associated with the several degenerative diseases (Serrano et al., 2007).

The extracts and essential oils of herbs and spices are widely known for their strong antioxidant, antimicrobial and antifungal activities in foods. These properties of herb and spice extracts are due to the presence of many bioactive components, including flavanoids, terpenoids, vitamins, minerals, carotenoids and phytoestrogens (Rodríguez Vaquero et al., 2010).

Addition of natural antioxidants to meat and meat products is one of the important strategies in development of healthier and novel meat products. In this regard several studies utilizing herbs, spices, fruits and vegetable extracts, and have shown that addition of these extracts to raw and cooked meat products decreased lipid oxidation, improved color stability and total antioxidant capacities which are

important characteristics for shelf stable meat products (Hygreeva et al., 2014).

Meat and meat products are good substrates for spoilage and pathogenic microorganisms because of their nutrient contents (Zhang et al., 2009). Antimicrobials are in meat products used for mainly two reasons: to preserve the food for long time (control natural spoilage process) and to increase food safety (control growth of pathogenic microorganisms). The term natural antimicrobials implies, antimicrobials derived from natural sources like plants, animals and microbes (Hygreeva et al., 2014).

Many species and herbs exert antimicrobial activity due to their essential oil fractions. Some scientists reported the antimicrobial activity of essential oils from oregano, thyme, sage, rosemary, clove, coriander, garlic, and onion against both bacteria and moulds. The composition, structure, as well as functional groups of the oils play an important role in determining their antimicrobial activity (Omidbeygi et al., 2007; Yesil Celiktas et al., 2007).

Because of concerns for the synthetic antioxidants and antimicrobials by some human health professionals and consumers, many meat processors have been seeking alternative “natural” antioxidants and antimicrobials (Karre et al., 2013). Rosemary (*Rosmarinus officinalis*)

extracts have been found to be effective in meat systems (Formanek et al., 2003; Lawrence et al., 2004).

Rosemary (*Rosmarinus officinalis*) is a plant species of the *Labiatae* family, and its major and most active extract components (e.g. carnosol, carnosic acid, carnosol, rosmarinic acid etc.) have been proved to be against cancer and inflammation diseases in experimental animals and humans (Johnson, 2011; Ngo et al., 2011). Rosemary extract also possesses antioxidant and antimicrobial properties. The diterpenes, carnosol and carnosic acid, have been shown to account for the antioxidant properties of rosemary (Offord, 2004; Rižnar et al., 2006). The phenolic compounds in rosemary extract are believed to enhance antimicrobial properties by affecting the function of the bacterial cellular membrane, the synthesis of DNA, RNA, protein and lipids, and the function of the mitochondrion (Belantine et al., 2006).

Thus, it was found that rosemary extract showed a synergistic effect with different antimicrobials in reducing the total bacterial counts fresh sausages but when it was used on its own, it was not effective (Mathenjwa et al., 2012). Seydim et al. (2006) found a similar effect when they used rosemary extract for reduction of total bacterial counts in vacuum packaged ground ostrich meat. Rosemary extract was more effective when used in a mixture or in combination with sodium lactate.

In this context the aim of this study was to determine antimicrobial and antioxidant effect of dried rosemary in non-fermented heat-treated product Inovec salami.

MATERIAL AND METHODOLOGY

Preparation of salami:

Pork (70%) and beef (30%) meat were trimmed, cured (2.0% salt and 0.01% nitrite) and together with spices extracts cooled 24 hours at 4 °C. The next day was meat minced (4 mm blade) and divided into three equal parts: meat mixture without rosemary extract and treated with E316 and E621 (C-positive control), treated with dried rosemary in amount 1 g/kg and yeast extract in amount 1 g/kg (1st experimental group), and treated with the same amount of dried rosemary, but yeast extract was in amount 2 g/kg (2nd experimental group). Each part was separately filled into 90 mm fibrous casings, dried, smoked and heat treated until the temperature in the core reached the value 70 °C for 10 min. Samples from each of the group were sliced and vacuum packaged. Samples were kept at 4 °C for 7, 15 and 30 days.

Determination of microbiological contamination:

The samples of salami (5 g) were taken after specified storage periods and homogenized in saline for 30 s by apparatus Heidolph DIAX 900 (Heidolph, Germany). The samples for enumeration of psychrotrophic bacteria count (PBC) were cultured on plate count agar at temperature 4 ±1 °C for 10 days (Himedia, India). The samples for enumeration of *Lactobacillus* spp. bacteria count (LAB) were cultured on MRS agar (Himedia, India) at temperature 37 ±1 °C for 5 days. Count of *Enterobacteriaceae* family (ETB) was determined on VRBG agar (Himedia, India) at temperature 37 ±1 °C after 24 hours of cultivation. Count of yeast and moulds were

determined on DG18 agar (Merck, Germany) at temperature 25 ±1 °C after 5 days.

Determination of antioxidant activity:

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid test (TBARS) following the recommendations of Grau et al. (2000) and measured by spectrophotometric method at 532 nm (Jenway UV/VIS – 7305, UK). TBARS values were calculated from a standard curve of malondialdehyde (MDA) and expressed as mg MDA/kg sample.

Determination of pH value:

The pH value of Inovec salami was measured using a Gryf 209 (Gryf HB, Czech Republic) apparatus during whole period of storage.

Determination of color:

Color spaces L*, a*, b* of Inovec salami was determined by CM 2600D spectrophotometer (Konica Minolta, Germany) after homogenization. Color on the surface of homogenized salami was measured with SCE (Specular Component Excluded).

RESULTS AND DISCUSSION

The pH values of salami were similar up to the seventh day of storage (6.23 – 6.26). The significant ($p \leq 0.05$) decline of pH values were recorded in experimental groups compare to control after fifteen days of storage (figure 1). Significantly ($p \leq 0.05$) lower values of pH in experimental groups were determined also at the end of storage.

The color values for lightness and redness are shown in figures 2 and 3. All samples were characterized without any significant colour discrepancies. There was non-significant ($p > 0.05$) color loss (decrease in redness) over time, and the treatments had a non-significant impact ($p > 0.05$) on redness of the salami in compare to positive control. Our results are in agreement with McCarthy et al. (2001b) and Mirshekar et al. (2009) who found that rosemary extract has been shown to reduce redness in broiler meat, fresh pork and frozen pork patties. A synergistic effect of rosemary with ascorbic acid has been observed in modified packaged fresh pork sausage, whereby the redness of the product was maintained for 12 days (Martinez et al., 2007). In contrary to our results Sebranek et al. (2005) found that rosemary extract significantly improve redness and was very effective in preserving the color of frozen pork sausage.

Yu et al. (2002) reported that rosemary extracts improved the color stability of cooked turkey rolls. Lawrence et al. (2004) also observed improved color stability as a result of rosemary extracts injected into beef loins.

However treatment of rosemary extract in our study was more effective in maintaining lightness than the control. Results of lightness are in accordance with McCarthy et al. (2001a), who also found no significant differences over the storage period in either raw or cooked patties. Fernandez-Lopez et al. (2003) apply rosemary extracts to cooked pork meat and stored for 8 days at 4 °C. They found that rosemary extract inhibited the lipid oxidation and degradation of heme pigments caused by cooking and storage.

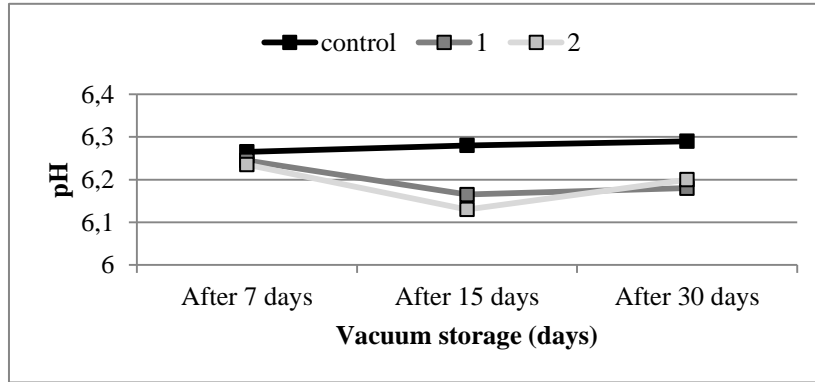


Figure 1 Effect of rosemary with combination of yeast extract on pH value of Inovec salami during vacuum storage at 4 °C.

Also, delayed metmyoglobin formation and stabilized the red meat color of the cooked meat during storage. TBARS values of the rosemary treated cooked salami with different amount of yeasts extracts were significantly lower ($p \leq 0.05$) compare to control at the end of the shelf life. Also, lower value of TBARS was determined in salami with rosemary in combination with higher amount of yeast extract.

Lipid oxidation (apart from microbial spoilage) in meat and meat products is the main cause of their quality loss. A large number of compounds are generated during the oxidation processes which adversely affect texture, color, flavor, nutritive value and safety of meat products (Lahucky et al., 2010) and this limits the shelf-life of meat and meat products (Karakaya et al., 2011).

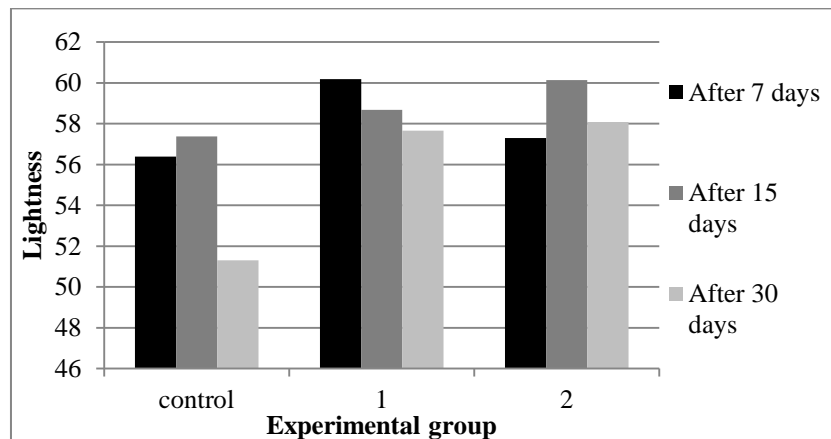


Figure 2 Effect of rosemary with combination of yeast extract on lightness of Inovec salami during vacuum storage at 4 °C.

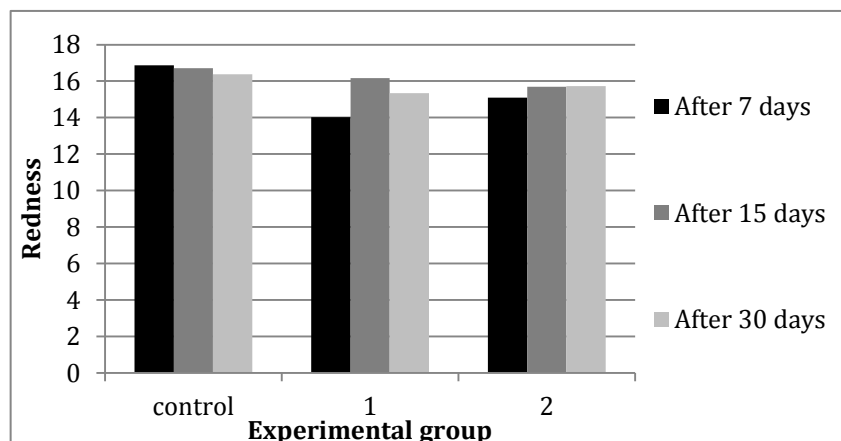
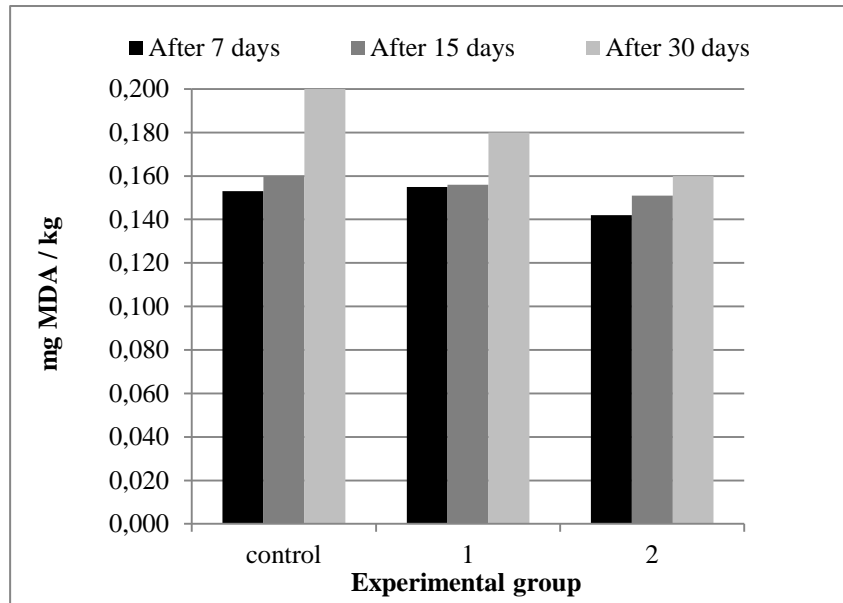


Figure 3 Effect of rosemary with combination of yeast extract on redness of Inovec salami during vacuum storage at 4 °C.



Oil-soluble carnosic acid is one of the major antioxidant compounds of rosemary extract that stabilize unsaturated fatty acids and thus retard their deterioration (Estévez et al., 2005). Martínez et al. (2006) found that rosemary extract plus ascorbic acid strongly inhibited lipid oxidation and therefore off-odour formation, delayed sausage discolouration and inhibited microbial growth, extending the shelf-life of salted fresh pork sausages by at least 4 days.

The effect of the rosemary extract, yeast extract and storage time on the total bacterial psychrotrophic count of chosen groups is indicated in figure 5. The count of PBC in control, the first and the second experimental group after seven days of storage was 3.53; 2.78 respectively 3.05 log CFU.g⁻¹. The numbers of other groups of bacteria after 7 days of storage were not detected. Also, the occurrence of bacteria *Enterobacteriaceae* family in salami during the whole period of storage was not determined. The lowest count of PBC was determined in the second experimental group

after 15 days. Jiang et al. (2011) identified that rosemary extract had strong antibacterial activity against Gram-positive and Gram-negative bacteria. Although rosemary extract in work of Mathenjwa et al. (2012) was not identified as a good antimicrobial, its antimicrobial and antioxidant properties were improved when used in combination with the SO₂. The rosemary extract in combination of yeast extract treatments were not able to influence the growth of yeasts and moulds. Also, Ibrahim and Al-Ebady (2014) found that rosemary treatments exhibited no effect in preventing fungal infection. Šošo et al. (2013) found a strong antifungal activity of rosemary oleoresin against *Aspergillus clavatus*. It was found that rosemary extract in combination with yeast extract in the salami of the second experimental group after 15 days of storage significantly ($p \leq 0.05$) decrease count of *Lactobacillus* bacteria. Especially susceptibility to the activity of rosemary extracts of the *Lactobacillus* and *Brochothrix* genus also found Fernandez-Lopez et al. (2005).

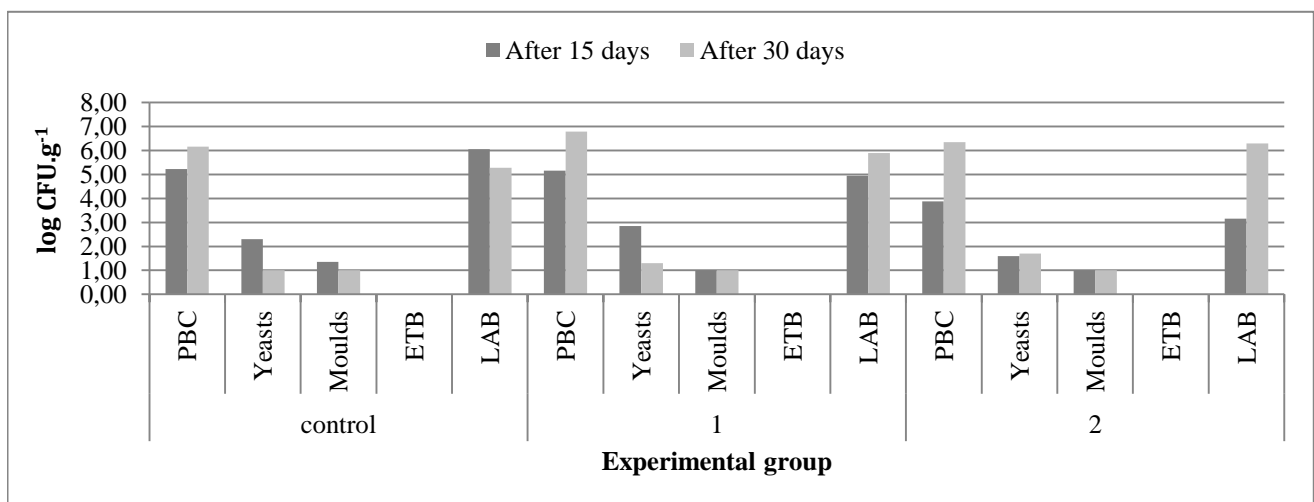


Figure 5 Effect of rosemary with combination of yeast extract on count of selected bacteria.

CONCLUSION

The negative consumer perception towards chemical antimicrobials/preservatives led to extensive search for natural antimicrobials. Numerous studies have compared plant derivatives with synthetic antimicrobials and antioxidant and reported that they are more potent and safer than synthetic compounds. While rosemary extracts have been found in this study to be not effective in preserving the color the further research has to be carried out for its natural antioxidant and antimicrobial potential in application in shelf stable non-fermented cooked salami. Also further research are required to find the appreciate doses and combinations of rosemary extract.

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REP-PCR TYPING OF STAPHYLOCOCCUS SPP. STRAINS IN MEAT PASTE PRODUCTION LINE AND IDENTIFICATION OF THEIR ORIGIN

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ABSTRACT

A meat paste production line and its microbial parameters have been evaluated in single Czech company. The raw meat paste samples before heat treatment were tested positively for the presence of three staphylococci species: *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*. Subsequent microbial analysis of meat paste components and ingredients (fresh meat, water, spices, equipment) identified only the spices used as positive for *S. aureus* (coriander, cinnamon, badian, mustard – (10 – 40 cfu/g)) and *S. haemolyticus* strains (juniper, ginger). The collection of sixteen collected strains (*S. aureus* (n = 4), *S. haemolyticus* (n = 4), *S. epidermidis* (n = 8)) has been typed with the rep-PCR method utilising (GTG)5 primer. Analysis of the fingerprints using the unweighted pair-group method using arithmetic averages (UPGMA) clustering method revealed presence of eleven strain clusters with similarity lower than 90%: two fingerprint clusters of *S. aureus*, three individual clusters characteristic for *S. haemolyticus* and six different *S. epidermidis* specific clusters. The *S. aureus* strains from different types of spice were identical, resp. very similar. Molecular tracking composed from the rep-PCR analysis of acquired isolates and comparison among all collected fingerprints confirmed the spices to be the source of both *S. aureus* and *S. haemolyticus* strains identified in raw meat paste. The additional rep-PCR analysis of the *S. epidermidis* collection confirmed usability and performance of this method. The antibiotic susceptibility to fourteen individual antibiotics has been examined among the collected staphylococci strains. The predominant erythromycin resistance (68.8%) was followed with the resistance to amoxicillin/clavulanic acid (56.2%). Other resistances observed were less frequent (clindamycin – 12.5%, oxacillin – 6.3%, tetracycline – 6.3%, sulphamethoxazole-trimethoprim – 6.3%, chloramphenicol – 6.3%, novobiocin – 6.3%). As shown by our experimental results, rep-PCR with the (GTG)5 primer is an applicable tool for typing of bacterial strains and may be used for identifying the source of contamination.

Keywords: rep-PCR; typing; meat; staphylococci; spice

INTRODUCTION

Molecular typing of bacteria is frequently used for estimation genetic relationship of the strains. Moreover, reliable identification and selection of individual bacterial strains provides a very valuable tool for further research of their dissemination and evolution. The possibility of identifying concrete pathogenic strain is very important and helpful also for effective control and monitoring of target pathogens. This can be practically utilised in protection of the food chain or for improvement of the food production technology. There are a number of methods applicable for typing bacterial strains in clinical microbiology (Gherardy et al., 2015; Collins et al., 2015). They work on different principles like PCR-based fingerprinting, pulsed-field gel electrophoresis, ribotyping, multilocus variable number tandem repeat analysis, multilocus sequence typing, whole genome sequencing with the whole genome SNPs analysis or proteomic-based mass spectrometry. However, only some of those methods are suitable for realising of powerful, time and cost-effective typing experiment. Repetitive element sequence-based PCR (rep-PCR) represents an easy-to-perform

technique utilising primers targeting repetitive sequence fragments dispersed in bacterial genomes (Versalovic et al., 1998). It may be practically utilised also for typing organism possessing genome of higher size, e.g. fungi (Abdollahzadeh and Zolfaghari, 2014). Moreover, an automated system for typing bacteria working on the rep-PCR basis has been already developed and employed by several research teams for *S. aureus* and MRSA typing (te Witt et al., 2009; Grisold et al., 2010).

In our work, we investigated microbial parameters in meat paste production line at single Czech manufacturer. The *S. aureus* and *S. haemolyticus* strains were detected in raw heat-untreated meat paste. The rep-PCR method was applied for typing acquired staphylococcal isolates with the aim to identify the source of their spreading and bring knowledge about their dissemination.

MATERIAL AND METHODOLOGY

Samples

The primary group of analysed samples consisted of raw meat paste samples acquired before heat treatment and

from final meat paste samples at selected single Czech manufacturer. Based on the positive identification of pathogenic *S. aureus* strains in the raw meat paste, we decided to analyse the main meat paste components and ingredients: fresh meat, water, spices, and equipment swab samples. The swab samples were transported to the laboratory for further procedure in a cooling box. The following spices utilised during technological process of meat paste production were tested: black pepper, allspice, coriander, juniper, cumin, cinnamon, badian, white mustard, bay leaf, sweet paprika, rosemary, garlic, ginger, thyme, cardamom. The mentioned spices were not treated with ionizing irradiation and originated from different countries.

The representative *S. aureus* and *S. haemolyticus* strains of raw meat paste origin were included in the rep-PCR analysis to confirm the source of their spreading.

Microbiological analysis

The selective cultivation on the Baird Parker Agar (HiMedia, India) was used for detection of staphylococci (ČSN EN ISO 6888-1). Plates were incubated at 37 ± 1 °C for 48 hours and colonies with zones of precipitation were submitted to the tube for free coagulase test. The suspected colonies were then inoculated onto Blood agar (Oxoid, Basingstoke, UK), cultivated at 37 °C for 24 h and subsequently identified biochemically using the STAPHYtest with the identification programs TNW Pro 7.5 (Erba Lachema, s.r.o., Brno, Czech Republic) to species level. Isolates identified as *S. aureus* were confirmed by the multiplex PCR targeting specific fragment SA442 according to **Martineau et al., (1998)**. MRSA and MR-CNS (methicillin resistant coagulase-negative staphylococci) were tested for presence of the *mecA* gene encoding the methicillin resistance phenotype as described by **Poulsen et al., (2003)**. The total count of *S. aureus* was determined in cfu per 1 g of prepared spice samples. The starting samples were prepared as follow: 5 g of spices (milled or powder) was dissolved in 45 g of sterile distilled water (the whole spices were crushed in the bowl before). The solution was filtered and the supernatant was used for analyses.

The antibiotic susceptibility testing to oxacillin, tetracycline, erythromycin, chloramphenicol, sulphamethoxazole-trimethoprim, amoxicillin-clavulanic acid, clindamycin, gentamicin, ciprofloxacin, vancomycin, teicoplanin, rifampicin, cefoxitin and novobiocin was determined by disc diffusion method following the recommendations of CLSI (**CLSI, 2012**).

Rep-PCR analysis

A loopful of individual colonies cultivated on the blood agar were transferred into clean tubes, resuspended in 180 µl solution of 20 mM Tris/HCl, 2 mM EDTA, 1% TRITON X-100 (pH 8), supplemented with 20 mg/ml lysozyme and incubated for 60 min at 37 °C. The genomic DNA was purified using the NucleoSpin Tissue kit (Macherey Nagel Inc., France); concentration of individual samples was measured on the Nanodrop 2000 instrument (Thermo Fisher Scientific Inc., DE, USA). The samples were diluted to obtain identical concentration 200 ng/µl. Rep-PCR analysis was realised with the (GTG)₅ primer (5'-GTGGTGGTGGTGGTG-3') in 25 µl reaction mixture

as follows: 1 µm primer; 1 x PPP PCR buffer containing Taq DNA polymerase (TopBio Ltd., CZE) and 1 µl of template DNA. Identical quantity of template DNA was added to each rep-PCR run to ensure maximal reproducibility of the results. The rep-PCR time and temperature profile comprised of initial denaturation 94 °C / 7 min; 30 cycles of: 94 °C / 1 min; 40 °C/1 min and 65 °C/8 min. The last cycle was followed by the final single extension step 65 °C / 16 min. Electrophoretic separation of rep-PCR products was realised in 1.5% agarose gel containing ethidium bromide ($0.5 \mu\text{g ml}^{-1}$) at 1.6 Vcm^{-1} for 12 hours. Molecular weight marker (GeneRuler 100 bp Plus, Thermo Fisher Scientific Inc., DE, USA) was positioned in lateral lines of every gel to allow later normalisation of gel images. The obtained image data with fingerprints were processed using the BioNumerics v. 6.6 software (Applied Maths NV, Belgium). The unweighted pair-group method using arithmetic averages (UPGMA) clustering method with the Dice correlation coefficient was utilized for interpretation of the results (BioNumerics, Applied Maths NV, Belgium). The fingerprints with similarity $\leq 90\%$ have been considered to represent unique strain complexes. A single control *S. epidermidis* strain was used for artificial contamination of heat-treated meat paste, to evaluate the process of cultivation and rep-PCR analysis.

RESULTS AND DISCUSSION

1 Microbial contamination

Analysis of meat paste components and ingredients revealed, only spices used were positive for *S. aureus*. It was identified in whole coriander, cinnamon, badian and mustard; no SA was identified in final meat paste product. The observed numbers were low (30, 40, 20 and 10 cfu·g⁻¹ respectively). The *S. haemolyticus* strains were cultivated also from spices (juniper, ginger) and a single *S. haemolyticus* strain was identified in final meat paste. The results indicated that the meat paste production technology involving heat treatment was able to successfully eliminate contaminants present in input ingredients. The overview of investigated spices with the results of microbial analysis is shown in Table 1. Based on the primary microbial analysis of samples in our study, we collected sixteen staphylococcal strains for further investigation (*S. aureus* (n = 4), *S. haemolyticus* (n = 4) and *S. epidermidis* (n = 8)). This was supplemented with the *S. aureus* and *S. haemolyticus* strain previously identified in raw meat paste. The overview of analysed strains and their origin is shown in Table 2.

As compared to our results, **Shamsuddeen (2009)** found out high *Staphylococci* occurrence in the mixture of spice composed from ginger, cloves, black pepper, groundnut, salt and seasoning. The geometric mean was $1.73 \cdot 10^9 \text{ cfu} \cdot \text{g}^{-1}$ but they didn't specify the staphylococcal species. **Tulu et al., (2014)** detected low numbers ($4 \pm 0.8 \text{ cfu} \cdot \text{g}^{-1}$) of *Staphylococci* in Red chilies (*Capsicum spp.*) and Turmeric (*Curcuma longa*) samples in Ethiopia. **Sospedra et al., (2010)** analysed thirty types of different spice in Spain, 7% of the samples was *S. aureus* positive. Positive detection of *S. aureus* in spice was observed also in study from Brazil (**Moreira et al., 2009**).

Table 1 Microbiological analysis investigating *Staphylococcus spp.* in spices used in meat paste production .

species	form	name	origin	species/ strain number	(cfu/g)	rep-PCR fingerprint type
allspice	whole	<i>Pimenta dioica</i>	Mexico	neg		
allspice	ground		Mexico	neg		
badian	whole	<i>Illicium verum</i>	Vietnam	SA (621)	20	B
bay leaf	ground	<i>Laurus nobilis</i>	Turkey	neg		
black pepper	whole	<i>Piper nigrum</i>	Vietnam	neg		
black pepper	ground		Vietnam	neg		
cardamon	ground	<i>Elettaria cardamomum</i>	Guatemala	neg		
cinnamon	whole	<i>Cinnamomum</i>	Czech Republic	SA (620)	40	A
coriander	whole	<i>Coriandrum sativum</i>	Ukraine	SA (619)	30	A
coriander	ground		Ukraine	neg		
cumin	ground	<i>Carum carvi</i>	Czech Republic	neg		
garlic	powder	<i>Allium</i>	China	neg		
ginger	ground	<i>Zingiber officinale</i>	Nigeria	SH (625)	/	C
juniper	whole	<i>Juniperus communis</i>	Bosna-Hercegovina	SH (626)	/	D
juniper	ground		Macedonia	SH (624)	/	C
rosemary	ground	<i>Rosmarinus officinalis</i>	Morocco	neg		
sweet paprika	ground	<i>Capsicum</i>	Hungary	neg		
thyme	ground	<i>Thymus vulgaris</i>	Poland	neg		
white mustard	whole	<i>Sinapis alba</i>	India	SA (623)	10	A

cfu·g⁻¹ = colony forming units per gram

Table 2 Overview of investigated *Staphylococcus spp.* strains and its origin.

strain number	origin	sampling	species
603	raw meat paste	Apr. 2014	SA
605	raw meat paste	Apr. 2014	SH
619	coriander whole	May 2014	SA
620	cinnamon whole	May 2014	SA
621	badian whole	May 2014	SA
623	white mustard whole	May 2014	SA
624	juniper ground	May 2014	SH
625	ginger ground	May 2014	SH
626	juniper whole	May 2014	SH
644	final liver meat paste	May 2014	SH
613	final meat paste	Dez. 2013	SE
614	final meat paste	Dez. 2013	SE
635	final roe meat paste	May 2014	SE
637	hog fresh meat	May 2014	SE
638	hog fresh meat	May 2014	SE
641	final liver meat paste	May 2014	SE
642	final liver meat paste	May 2014	SE
645	lamb liver	May 2014	SE

2 Antibiotic susceptibility

The profiles of ATB susceptibility in tested staphylococci strains bring relatively positive observation. Although

68.8%, resp. 56.2% of collected staphylococci isolates possessed resistance to erythromycin, resp. amoxicillin/clavulanic, other resistances observed were

minimally frequent: clindamycin – 12.5%, oxacillin – 6.3%, tetracycline – 6.3%, sulphamethoxazole-trimethoprim – 6.3%, chloramphenicol – 6.3%, novobiocin – 6.3% (Table 3). No *S. aureus* strain was confirmed as MRSA. Among the twelve *S. haemolyticus* and *S. epidermidis* strains, only single *S. haemolyticus* isolate was positive for the *mecA* gene and thus represented the MR-CNS (Methicillin Resistant Coagulase-Negative Staphylococci). In works of other authors and also in our previous study, the occurrence of MR-CNS among CNS of animal or human origin was more frequent (Manga and Vyletřlová, 2011; Vyletřlová et al., 2011). Huber et al., (2011) detected MR-CNS in 48.2% of samples from livestock and chicken carcasses, in 46.4% of samples from bulk tank milk and minced meat, and in 49.3% of human samples. The *S. epidermidis* together with *S. haemolyticus* and other CNS (Coagulase-Negative Staphylococci) represent a species with common occurrence on skin or mucous membranes of live animals including human. The both species are human opportunistic pathogens. However, the CNS may be also donors of genetic elements encoding antibiotic resistance to other bacterial species including *S. aureus*. A numbers of works suggested, the MR-CNS have significant impact on evolution and spreading of the MRSA strains (Berglund and Söderquist, 2008; Bloemendaal et al., 2010). The clinical CNS isolates of human origin often displays multiresistance, MR-CNS are widely frequent in hospitals and represent important carriers of methicillin resistance (Barros et al., 2012). Therefore, monitoring of antibiotic resistances in CNS of both animal and human origin has its own importance and CNS are being explored by many scientists.

3 Rep-PCR analysis

The rep-PCR analysis of *S. aureus*, *S. haemolyticus* and *S. epidermidis* isolates obtained from spices, fresh meat and meat paste gave rep-PCR fragments ranged from 180 to almost 3000 bp (Figure 1A, 1B). As regards to species

affiliation, all investigated strains have been successfully distinguished. The rep-PCR analysis of *S. aureus* (Figure 2) showed that the four *S. aureus* strains isolated from different types of spices belong to only two strain clusters (A, B). The strains in cluster type A showed 100% homology. Moreover, the high similarity (90%) between these two clusters indicates high relatedness among all four strains. With regard to different origin of spices positive for *S. aureus*, this finding is surprising. However, all spice products with positive *S. aureus* detection were derived from the same manufacturer. Therefore, we suppose the technology of final processing or packaging may be the source of contaminants. The high performance of rep-PCR method for typing bacterial strains from different species and origins was repeatedly confirmed. The *Staphylococcus spp.* rep-PCR typing experiments were successfully realised also by other authors (Reinoso et al., 2008; Nordin et al., 2011). With regard to this, we have to consider the *S. aureus* strains analysed in our work as very similar (resp. identical in one of the observed clusters). Other theoretic explanation of our findings may be a secondary contamination during sample analysis. Since we received the same results when repeated whole analysis of identical spice samples, this possibility seems to be unlikely (data not shown). Rep-PCR typing of four *S. haemolyticus* isolates coming from spices and final meat paste identified three unique strain clusters (C, D, E) (Figure 2). The two *S. haemolyticus* strains isolated from juniper and ginger (n. 624, 625) merged to the same strain cluster (C) with indicated 100% similarity. This refers to similar situation as described at *S. aureus* rep-PCR results. With other words, the presence of *S. haemolyticus* in juniper and ginger is connected in some way. In addition, the *S. haemolyticus* strain n. 644 from final meat paste representing unique fingerprint (E) featured with high homology (90%) to C type cluster. Third cluster (D type) representing with single *S. haemolyticus* strain n. 626 differed markedly from the others. Rep-PCR typing of

Table 3 Antimicrobial resistances in sixteen *Staphylococcus spp.* strains isolated in meat paste production line.

antimicrobial agents	species	SA	SA	SA	SA	SH	SH	SH	SH	SE	SE	SE	SE	SE	SE	SE	SE
	strain n.	619	620	621	623	624	625	626	644	613	614	635	637	638	641	642	645
OX		+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
TE		+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
E		-	-	+	+	-	-	+	-	-	+	-	+	-	-	-	-
C		+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
SXT		+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
AMC		-	-	-	-	+	+	+	+	-	-	+	-	-	-	+	+
DA		+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
NV		+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+

SA = *S. aureus*, SH = *S. haemolyticus*, SE = *S. epidermidis*, n = number, OX = oxacillin, TE = tetracycline, E = erythromycin, C = chloramphenicol, SXT = sulphamethoxazole-trimethoprim, AMC = amoxicillin-clavulanic acid, DA = clindamycin, NV = novobiocin; no resistance to gentamicin ciprofloxacin, vancomycin, teicoplanin, rifampicin and cefoxitin was observed

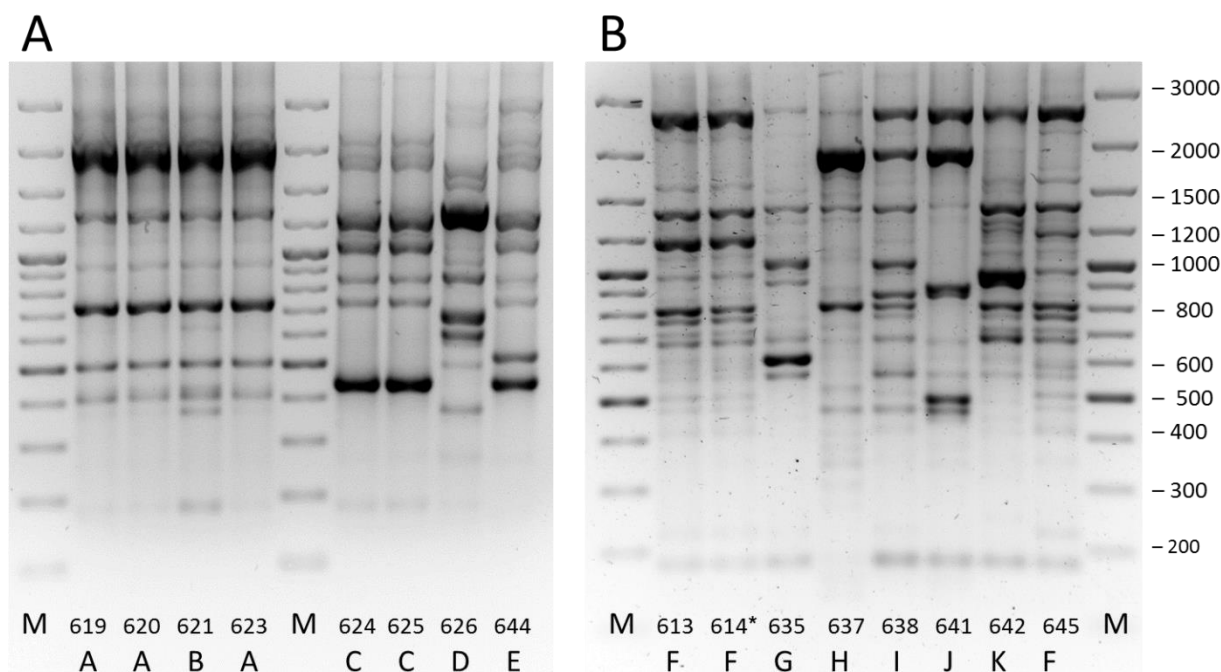
S. epidermidis isolated from meat paste and fresh meat supplemented the whole rep-PCR analysis and demonstrated discrimination power of the method for typing experiments. Among the eight *S. epidermidis* strains, six unique strain complexes were found (Figure 2). Comparison of all acquired fingerprints among sixteen isolates belonging to three staphylococcal species indicated eleven unique clusters of strains with similarity lover than 90%.

Comparative analysis of rep-PCR fingerprints among *S. aureus* strains from spices and *S. aureus* strain from raw meat paste revealed the strains were identical and belong to the same unique strain cluster (Figure 3). This provided evidence, that *S. aureus* cultivated from raw meat paste was originated in used spice. The both C and D fingerprint types characteristic for *S. haemolyticus* isolates from spices were detected also in *S. haemolyticus* isolates from raw meat paste; a representative rep-PCR analysis is indicated in Figure 3. This confirmed that the source of selected *S. haemolyticus* strains was the spice.

The control *S. epidermidis* strain (n. 614) used for artificial contamination of heat-treated meat paste was successfully cultivated and the rep-PCR analysis provided consistent result with expected fingerprint type (Figure 1 and 2). The whole process of DNA isolation and rep-PCR analysis of collected *S. aureus* and *S. haemolyticus* strains was performed twice to validate the reproducibility of the results. The acquired rep-PCR fingerprints in both independently repeated runs contained all strain-specific selective rep-PCR fragments and thus provided the

identical results (data not shown). In some cases, the individual profiles of ATB susceptibility among rep-PCR-determined strain clusters differed a little. This is most probably due to existence of horizontal gene transfer, significantly affecting the antimicrobial phenotype of individual strains at given time (Juhas, 2015).

As regards to primary strain processing and DNA isolation in rep-PCR analysis, the alkaline lysis of cultivated strains followed with direct rep-PCR analysis is an optional alternative because of rapid sample processing and DNA isolation (Švec et al., 2008). However, the unevaluated or ignored heterogeneity in template DNA quality and quantity may negatively affect the amplification efficiency as well as reproducibility of the rep-PCR analysis. In particular the rep-PCR strain typing experiments require precise laboratory work. Applying of purified DNA samples with equivalent quantity increases the reproducibility and the discrimination power of the rep-PCR (Manga and Vyleťelová, 2012). The reliable rep-PCR identification of bacterial species seems to be easier to perform (Gevers et al., 2001; Švec et al., 2010). The performance of rep-PCR method is affected also with the type of primer used for analysis. Besides the (GTG)₅ primer (Koreňová et al., 2009; Švec et al., 2010; Manga and Vyleťelová, 2012), the REP1/ REP2 (Zhong et al., 2009), ERIC (Reinoso et al., 2008), RW3A (Zee et al., 1999) or BOX primers (Begović et al., 2013) have been utilised for typing the *Staphylococcus spp.* strains. Sabat et al., (2006) compared performance of six different PCR-based methods in typing the *S. aureus* strains, the pulsed



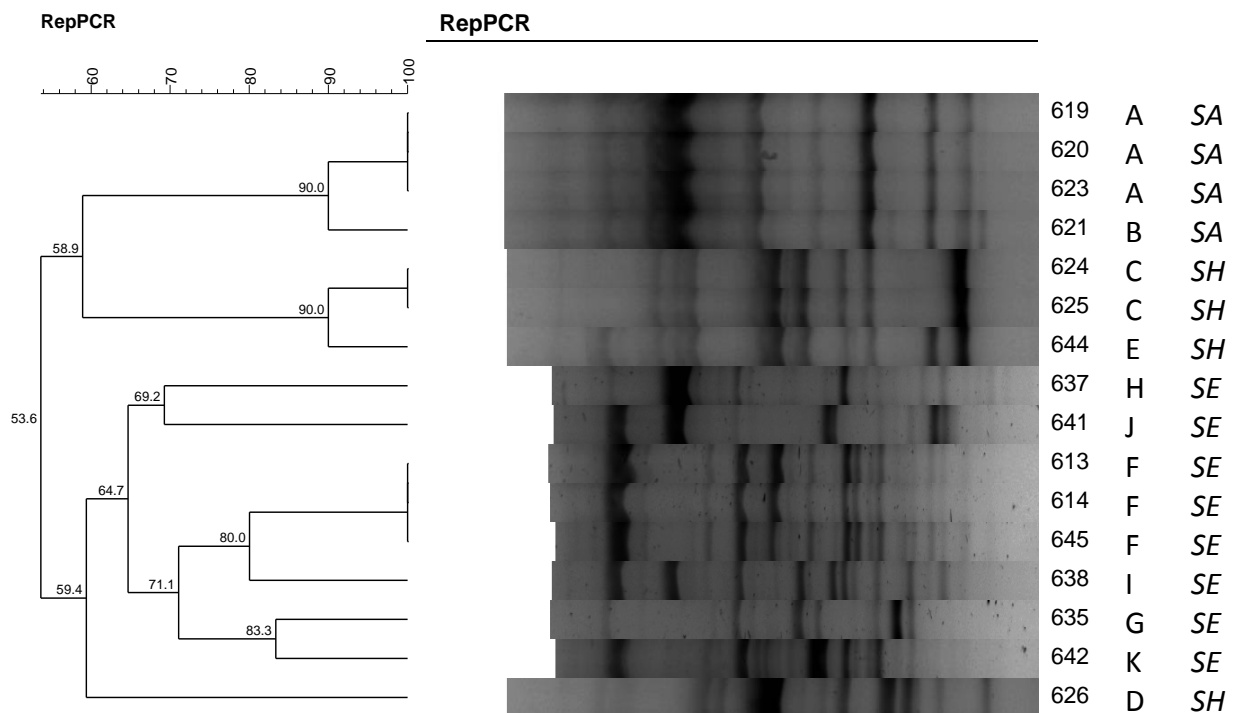
strain n. 619, 620, 621, 623 – *S. aureus*; strain n. 624, 625, 626, 644 - *S. haemolyticus*; 613, 614*, 635, 637, 638, 641, 642, 645 - *S. epidermidis*, M – ladder 100 plus (Thermo Fisher Scientific Inc., DE, USA); 614* = control strain of *S. epidermidis* (= strain 613 utilised for artificial contamination of heat-treated meat paste sample, repeatedly cultivated and analysed using the rep-PCR method)

Figure 1 Electrophoresis of rep-PCR products characteristic for *S. aureus* and *S. haemolyticus* isolates (A) and for *S. epidermidis* isolates (B).

field gel electrophoresis was utilised as a reference method. The most representative results were obtained using the VNTR typing method. As we found out in our previous work, the performance of rep-PCR method in *S. aureus* typing experiment may be full comparable even superior to pulsed field gel electrophoresis or *spa* typing (Manga et al., 2011). The other thing is that individual typing methods may provide results differing each other. With respect to evolution of current technology, application of whole genome sequencing methods may represent the most powerful and reproducible typing tool in the near future (Salipante et al., 2015). However, these methods are still high cost and high technically demanding and usually require extensive skills in bioinformatics. In

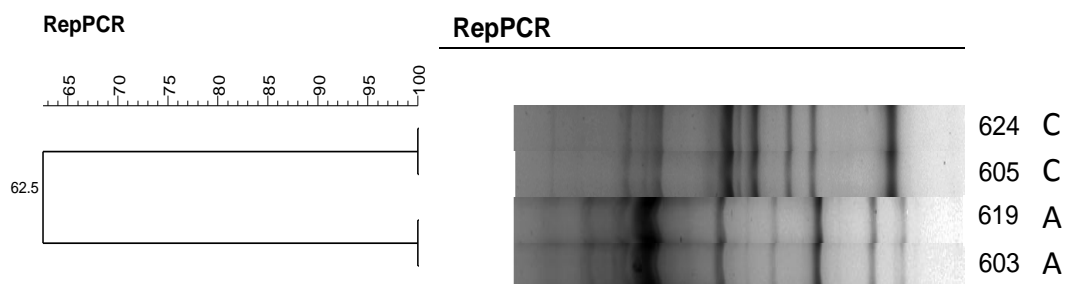
addition to this, the complex criteria and general rules for reproducible interpretation the NGS typing data have to be defined by scientific community.

As we found out, the rep-PCR typing is an alternative for low-demand and cost-effective analytic method, which may be useful in identifying the source of bacterial contamination. Besides the presented study, this may be illustrated with the work of Zhong et al., (2009) investigating spread of airborne *S. aureus* in and around chicken house using the rep-PCR. They found out that microbes in chicken feces can be aerosolized and spread indoor and outdoor, especially to downwind of the chicken houses.



SA = *S. aureus*, SH = *S. haemolyticus*, SE = *S. epidermidis*, A – K = determined strain clusters with similarity ≤ 90%

Figure 2 Clustering analysis of staphylococcal strains based on the rep-PCR data and the Applied Maths software (Applied Maths NV, Belgium); band-based UPGMA clustering (Dice correlation coefficient, Tolerance change: 1%).



cluster A type (100% similarity): 619 - *S. aureus* (coriander), 603 - *S. aureus* (raw meat paste); cluster C type (100% similarity): 624 - *S. haemolyticus* (juniper), 605 - *S. haemolyticus* (raw meat paste)

Figure 3 Confirmation the source of microbial contaminants in raw meat paste using the rep-PCR method.

CONCLUSION

We analysed meat paste production line in single Czech company to identify the source of staphylococci pathogenic strains, detected in raw meat paste. Isolates of target species *S. aureus* and *S. haemolyticus* were cultivated also from spices used in investigated manufacture.

With further rep-PCR analysis, we provided evidence that the staphylococci strains cultivated from raw meat paste were originated in spice. Moreover, molecular typing of collected isolates indicates presence of a homogeneous group of strains. This suggest on clonal spreading of the strains and their likely circulation in manufacturing or packaging link at spice manufacturer. Existence of adequate powerful diagnostic tools is one of the key prerequisite for effective monitoring of the food quality. As shown in our study, the rep-PCR method working with the (GTG)5 primer could be an effective and low cost analysis for typing microbial strains. Further, microbial contamination in spices suggests the need for regular inspection of the spices quality.

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EVALUATION OF MEAT QUALITY AFTER APPLICATION OF DIFFERENT FEED ADDITIVES IN DIET OF BROILER CHICKENS

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ABSTRACT

The present study was conducted to investigate the effect of natural feed additives, namely bee pollen extract, propolis extract and probiotic preparation, on technological properties of meat in order to evaluate the meat quality of Ross 308 broiler chickens. The feeding of chickens (180 pcs) lasted for a period of 42 days. The experiment was carried out without segregation between the genders. The chickens were randomly divided into 4 groups. The control group was fed a basal diet, whereas the other three groups were fed diets supplemented with natural additives, i.e. bee pollen extract at level of 400 mg.kg⁻¹ of feed mixture, propolis extract at level of 400 mg.kg⁻¹ of feed mixture, and probiotic preparation based on *Lactobacillus fermentum* (1.10⁹ CFU per 1 g of bearing medium) in an amount of 3.3 g added to water (for 30 pcs chickens until 21 days of age, for 20 pcs chickens from 22nd to 42nd day of age) given to group E1, group E2 and group E3, respectively. The feed mixtures were produced without any antibiotic preparations and coccidiostatics. During the whole period of experiment, the broiler chickens had *ad libitum* access to feed and water. The following technological properties were examined: cooling loss (after 24 h of storage at 4 °C), freezing loss (after 3 months of storage at -18 °C), roasting loss (performed on roasted meat that was stored at -18 °C for 3 months before thawing), colour parameters based on CIELab system (the L*, a*, b* values of raw breast and thigh muscle), and tenderness (as shear force of roasted breast and thigh muscle). We have made a finding, that the examined additives had only little impact on meat quality in most of the investigated parameters, except the significant increase ($p \leq 0.05$) in redness (a*) values and the slight decrease in roasting loss and shear force determination after propolis extract supplementation. Therefore, it may be inferred that propolis extract has been shown as the most appropriate feed additive among the applied supplements.

Keywords: chicken meat; loss; colour; shear force; bee pollen extract; propolis extract; probiotic; meat quality

INTRODUCTION

From the perspective of human nutrition, poultry meat is a valuable source of proteins, vitamins and minerals. Recent studies have affirmed that the level of those compounds, as well as meat quality, is determined not only genetically, but it is also affected by the microelement and macroelement content of feeds, the way animals are housed, their breed, sex and health, slaughter procedures, and type of muscle (Debut et al., 2003; Lombardi-Boccia et al., 2005). The ever-rising trend of poultry consumption shows the importance of controlling meat quality for the poultry industry (De Genova Gaya et al., 2011). Besides, the technological quality of poultry meat is now of major importance, since poultry meat is nowadays usually consumed as cuts or as processed products rather than as whole carcasses (Nissen and Young, 2006; Le Bihan-Duval et al., 2008).

Breast and thigh meats are the most valuable muscles of the poultry carcass (Yu et al., 2005). Water holding capacity, pH, colour and tenderness, usually determined in those parts of chicken carcass are crucial for the culinary value and technological properties of chicken meat (Musa et al., 2006; Nissen and Young, 2006).

Technological indicators such as colour and tenderness are important attributes to which consumers attach a special importance (An et al., 2013), due to the close association with factors such as freshness, flavour, desirability, storage time and food safety (Girolami et al., 2013; Wu and Sun, 2013), while variation in these indicators depends on the characteristic of muscle itself. Muscle is composed of different fiber types, on the one hand, they can be affected by sex, breed, age, etc., on the other hand, muscle fiber characteristics can influence meat quality characteristics such as colour, water-holding capacity (i.e. drip loss during storage) and the texture of meat (Lyon et al., 2004; Le Bihan-Duval et al., 2008; An et al., 2013). Producers should be concerned with environmental conditions, such as feed and housing conditions that may affect these important quality attributes (Saláková et al., 2010).

Development of pH, meat colour, and water-holding capacity (WHC) are closely connected and are associated with the energy status of the muscles at slaughter, which is highly influenced by the duration of transportation and the stress before and during slaughter (Nissen and Young, 2006). Variation of the meat colour is up to a certain point physiological, but the differentiation to pathological

alterations like pale, soft and exudative (PSE)-like meat is important because the latter is characterized by a paler colour, a heterogeneous appearance, a poorer texture and cohesiveness as well as a higher drip loss (Berri et al., 2007; Janisch et al., 2011).

The perception of colour is a very complex phenomenon that depends on the composition of the object in its illumination environment, the characteristics of perceiving eye and brain, and the angles of illumination and viewing (Wu and Sun, 2013).

The colour measurements can be conducted by visual (human) inspection, traditional instruments like colourimeter, or computer vision (Wu and Sun, 2013). Currently, meat colour is measured by colourimeter in terms of CIE L*, a*, b* values, hue angle and chroma. The L* a* b*, or CIELab is the 3-dimensional colour expression, whereby L* is the lightness component, which ranges from 0 to 100 (from black to white) and the parameters a* (from green if negative to red if positive) and b* (from blue if negative to yellow if positive) are two chromatic components which range from -120 to +120 (Leon et al., 2006; Larrain et al., 2008; Girolami et al., 2013).

Tenderness is considered the most important factor in determining the consumer-eating satisfaction of meat products (Xiong et al., 2006; Lee et al., 2009). As consumer consumption of boneless chicken meat has dramatically increased over recent years, tenderness has become increasingly important to poultry meat processors. To meet consumer expectations of tenderness, meat processors must produce tender meat products as well as understand what constitutes tender meat (Xiong et al., 2006). Meat tenderness is defined by the ease of mastication, which involves initial penetration by the teeth, the breakdown of meat into fragments and the amount of residue remaining after chewing (Kong et al., 2008). Tenderness can be determined by a trained panel (sensory analysis) or physical methods (instrumental analysis) (Cavitt et al., 2004; Li et al., 2013). Warner-Bratzler (WB) shear blade is one of the most commonly used instruments in objective estimating meat tenderness and texture quality of poultry meat, whereby the higher WB shear values are associated with less tender poultry meat (Zhuang et al., 2008). This cutting method is based on measuring the force required to shear across entire muscle fibers. The orientation of the slice needed to correspond to muscle fiber orientation so that the shearing action would be across the muscle fibers. The WB values are commonly reported in grams, kilograms, or newtons (Carranco-Jáuregui et al., 2010; Silva et al., 2015).

Water loss is directly proportional to the water holding capacity (WHC) of muscle proteins and reduced water content changes key quality parameters such as colour and texture (Ali et al., 2015). During freezing, storage and thawing, meat loses water by evaporation, sublimation and exudation, respectively. The water is also lost during the cooking. Although moisture losses make meat less attractive, they do not significantly influence its eating quality after dry-heat cooking, except in the case of very large losses, which could affect juiciness and tenderness (Pérez Chabela and Mateo-Oyague, 2006).

Chicken carcasses are chilled immediately after slaughter to reduce the temperature to 4.4 °C within 4 h (Keeton, 2001). A weight loss of 0.5% will typically occur during further processing (Sams, 2001). During the chilling, chicken carcasses usually exhibit a slight weight loss. The high relative humidity (~85%) in most coolers reduces carcass shrink and water loss (Keeton, 2001). Freezing is also responsible for weight losses of chicken meat. A slow freezing rate by the temperature zone 11.1 to 10 °C, which is the point of phase transition between intercellular crystalline ice and a combination of ice and water, results not only in large ice crystals, which generally damage the texture of meat, but also in excessive water losses when thawed (Keeton, 2001; Suzuki et al., 2006). On the contrary, a rapid freezing rate produces small ice crystals, preventing the cellular damage of meat. Furthermore, the mass transfers from cells, responsible for losses during the thawing by running water may be limited by rapid thawing (Suzuki et al., 2006). Besides the cellular and macroscopic damage, the losses also depend on the size and shape of the pieces of meat (Pérez Chabela and Mateo-Oyague, 2006).

Heating above 70 °C is often unfavourable to the meat quality due to extensive protein aggregation within the gel network, leading to water loss from the product. The gelation of the stroma protein, collagen, may also be responsible for water loss observed above 70 °C. Cooking rate can also affect the type of gel network formed and subsequent quality of heat-treated meat products. It is thought that a slower cooking rate will result in the formation of more ordered gel structures with higher water-binding abilities. Moreover, heating above 75 °C causes more fiber shrinkage, excessive moisture loss, and fat melting (Smith, 2001). Cooking loss from frozen meat depend principally on the processing of meat before freezing, especially rigor onset temperature, and on the cooking method, particularly the cooking temperature. Although cooking loss is accepted as being higher when freezing rates are slow, the effect of freezing rate on the cooking loss seem to be slight (Pérez Chabela and Mateo-Oyague, 2006).

As diet is one of the most important factors affecting meat quality (Tateo et al., 2013), various benefits in regard to meat quality characteristics can be gained by supplementing broiler diets, particularly using probiotics as feed additives (Karaoglu et al., 2004).

In the present study, probiotics, bee pollen extract and propolis extract were used in Ross 308 broiler chickens diet to investigate effects on selected technological properties of chicken meat (cooling loss, freezing loss and roasting loss) and breast and thigh muscle (colour, shear force), as the major high-value cuts of chicken meat.

MATERIAL AND METHODOLOGY

Chicks and diets

The experiment was carried out in test poultry station of Slovak University of Agriculture in Nitra. A total of 180 one day-old Ross 308 broiler chicks were randomly divided into 4 groups, namely, control (C) and experimental (E1, E2, E3) of 45 pcs chickens. The experiment lasted for 42 days and was carried out without segregation between the genders. The broiler chickens

were bred on breed litter (wood shavings), in a temperature-controlled room; the temperature began at 33 °C and was decreased gradually to 19 °C until the end of experiment. The lighting regime was steady during the feeding period. During the whole period of experiment, the broiler chickens had *ad libitum* access to feed and water.

The feeding lasted 42 days. During that period, experimental broiler chickens were fed with a starter complete feed mixture HYD-01 (until 21 days of age) and a grower feed mixture HYD-02 (from 22nd to 42nd day of age). The composition of feed mixtures is given Table 1. The feed mixtures both starter and grower were produced without any antibiotic preparations and coccidiostatics. Nutrients content and metabolizable energy in feed mixtures were balanced, in terms of broiler chickens needs (Vestník MP SR, 2005).

All the groups were fed with the same feed mixtures.

However, chickens in the control group were fed with basal diet containing no special supplement, while the diet of chickens in experimental groups contained the diet supplements as follows: bee pollen extract in amount of 400 mg.kg⁻¹ added to feed mixtures given to the group E1, propolis extract in amount of 400 mg.kg⁻¹ added to feed mixtures given to the group E2, probiotics in an amount 3.3 g added daily to the water given the group E3 (for 30 pcs chickens until 21 days of age, for 20 pcs chickens from 22nd to 42nd day of age). Besides, the groups were kept under the same conditions.

In the experiment, the probiotic preparation "Propoul" based on *Lactobacillus fermentum* (1.10⁹ CFU per 1 g of bearing medium) was used.

Bee pollen and propolis had origin in the Slovak Republic. The extracts were prepared from minced bee pollen and propolis in the conditions of the 80% ethanol in

Table 1 Composition of feed mixtures.

Ingredients (%)	Starter HYD-01 (1. – 21. day of age)	Grower HYD-02 (22. – 42. day of age)
Wheat	35.00	35.00
Maize	35.00	40.00
Soybean meal (48% N)	21.30	18.70
Fish meal (71% N)	3.80	2.00
Dried blood	1.25	1.25
Ground limestone	1.00	1.05
Monocalcium phosphate	1.00	0.70
Fodder salt	0.10	0.15
Sodium bicarbonate	0.15	0.20
Lysine	0.05	0.07
Methionine	0.15	0.22
Palm kernel oil Bergafat	0.70	0.16
Premix Euromix BR 0.5%*	0.50	0.50
Nutrient composition [g.kg⁻¹]		
Crude protein	210.76	190.42
Fibre	30.19	29.93
Ash	24.24	19.94
Ca	8.16	7.28
P	6.76	5.71
Mg	1.41	1.36
Linoleic acid	13.51	14.19
ME _N [MJ.kg ⁻¹]	12.02	12.03

* active substances per kilogram of premix: vitamin A 2 500 000 IU; vitamin E 20 000 mg; vitamin D3 800 000 IU; niacin 12 000 mg; d-pantothenic acid 3 000 mg; riboflavin 1 800 mg; pyridoxine 1 200 mg; thiamine 600 mg; menadione 800 mg; ascorbic acid 20 000 mg; folic acid 400 mg; biotin 40 mg; kobalamin 8.0 mg; choline 100 000 mg; betaine 50 000 mg; Mn 20 000 mg; Zn 16 000 mg; Fe 14 000 mg; Cu 2 400 mg; Co 80 mg; I 200 mg; Se 50 mg.

the 500 cm³ flasks, according to **Krell (1996)**. The extraction was accomplished in a water bath at 80 °C for one hour. After that, the extracts were cooled and centrifuged. The obtained supernatants were evaporated in a rotary vacuum evaporator at bath temperature 40–50 °C and weighed. Residues in an amount of 40 g were dissolved in 1000 cm³ of 80% ethanol and used for 100 kg of feed mixture.

Slaughter and measurements

The chickens were slaughtered at 42 days of age at the experimental slaughterhouse of Slovak University of Agriculture in Nitra.

After evisceration, the carcasses were kept at approximately 18 °C for 1 h *post mortem* and thereafter longitudinally divided into two parts. After that, the half-carcasses were weighed and stored at 4 °C until 24 h *post mortem*, when the first measurements were done. The left half-carcass was used in order to determine the technological properties as described below, whereas the right one was assigned to different analysis.

After 24 h, the color of breast (*Musculus pectoralis major*) and thigh muscle from the left half-carcass (n=10) was assessed using a Minolta CM 2600d spectrophotometer (Konica Minolta, Japan) and reported in the CIE system values of lightness (L*), redness (a*) and yellowness (b*). All the color readings were taken on meat without skin, in an area free of obvious color defects (over scald, bruises, and blood accumulation).

The cooling loss was determined in whole left half-carcass as the percentage of weight loss over a 24 h period, by calculating the weight differences before and after cooling.

Afterwards, the same half-carcasses were stored at -18 °C for 3 months prior to next analysis. Thereafter, the samples were thawed. After thawing was completed, the weight of the samples was obtained. To determine the freezing loss (%), the weight differences before and after freezing process were calculated (n=10). All the weight measurements were performed using the precision balance Kern 440 (Kern&Sohn, Germany) with accuracy of 0.01 g.

The heat treatment of samples was carried out in oven (Gorenje B 3300 E) at 200 °C for 60 minutes. After allowing the samples to cool at room temperature, the samples were weighed so as to calculate the percentage of roasting losses. The roasting loss was expressed as the percent weight reduction of the heat-treated sample compared to the raw sample (n=10).

The samples used to determine the roasting loss were the same used to evaluate the shear force. For this reason, tenderness of breast (*Musculus pectoralis major*) and thigh muscle was subsequently evaluated. Results have been expressed as shear force (kg.cm⁻²) (five measurements were performed on each sample to obtain an average value). First, the five cores with the same size (2.0 cm wide, 5.0 cm long and 1.5 cm high) were removed from each heat-treated sample (n=10). Then, the cores were sheared perpendicular to the muscle fibres orientation using a Warner-Bratzler shear device (Chatillon, USA), in accordance with **Goodson et al. (2002)**.

Statistical analysis

The data processing for technological attributes of raw and heat-treated samples of meat was performed using a statistical program Statgraphics Plus Version 5.1. For the determination of significant difference between the tested groups, analysis of variance (ANOVA) with Scheffé's method was used.

RESULTS AND DISCUSSION

The results of experiment with Ross 308 broiler chickens, which was aimed at selected technological properties, are presented as follows: the results of cooling loss, freezing loss and roasting loss of meat are given Table 2, the results of colour and shear force of breast and thigh muscle are given Table 3.

In the current study, the losses during the storage ranged from 3.79 to 4.04% for cooling, from 3.53 to 4.85% for freezing, from 28.50 to 30.03% for roasting. There were very similar values of cooling losses, at which 4.04% of loss, as the highest value among the tested groups, was observed in group E3. The lowest value was observed in group E2 (3.79%). The cooling losses, however, did not differ significantly ($P \geq 0.05$). It is, thus, likely, that the extracts of bee products (propolis, bee pollen), as well as probiotics, do not affect losses during the cooling of chicken meat. Nevertheless, different results were obtained for freezing and roasting losses, where significant differences ($p \leq 0.05$) were found between the groups. Among the groups, group E2 and E3 showed the highest freezing value (4.85%), whereas control group showed the lowest one (3.53%). These differences, although significant, are of little relevance as the losses in control group were lower than losses in experimental groups.

As far as roasting loss is concerned, group E3 showed the highest losses (30.03%) also in that parameter, while the lowest losses were obtained in group E2 (28.50%), i.e. the propolis extract, which was included in the feed mixture, has been shown to have the most favourable influence on losses during the roasting of chicken meat, among all the tested natural additives. These findings indicate that the probiotics supplementation (group E3) results in higher losses during the cooling, freezing and roasting than those in the other groups.

Similarly, insignificant differences between the tested groups were found in study of **Haščík et al. (2008)**, in which cooling and freezing losses of chicken meat, after probiotic supplementation (*Lactobacillus fermentum*) were investigated. Yet, both cooling ($2.74 \pm 0.34\%$) and freezing losses ($2.00 \pm 1.15\%$) were lower than those in the control ($3.14 \pm 0.57\%$ and $3.10 \pm 1.44\%$, respectively). **Bobko et al. (2009)** investigated the weight losses of chicken meat by cooling and roasting after the probiotic supplementation (*Enterococcus faecium*) besides the other feed supplements. They found out higher losses in experimental group than those in control group not only in regard to cooling ($2.49 \pm 0.57\%$ and $1.88 \pm 0.42\%$, respectively), but also to roasting ($32.27 \pm 1.75\%$ and $32.01 \pm 2.45\%$, respectively).

Table 2 Cooling loss, freezing loss and roasting loss of chicken meat (mean ±SD).

Parameter	Group				S
	C	E1	E2	E3	
Cooling loss [%]	3.97 ±0.44 ^a	3.79 ±0.36 ^a	3.94 ±0.70 ^a	4.04 ±0.51 ^a	NS
Freezing loss [%]	3.53 ±1.00 ^a	3.81 ±0.84 ^a	4.85 ±0.94 ^b	4.85 ±0.70 ^b	**
Roasting loss [%]	29.54 ±1.16 ^{abc}	29.82 ±1.12 ^{ac}	28.50 ±1.23 ^b	30.03 ±1.30 ^a	**

Legend: C – control group; E1, E2, E3 – experimental groups; mean – average, SD – standard deviation; a,b – means with different superscripts within row differ significantly; S – significance; ** $p \leq 0.05$; NS = not significant.

Table 3 Instrumental colour values and shear force value of chicken breast and thigh muscle (mean ±SD).

Parameter		Group				S
		C	E1	E2	E3	
Colour parameter						
CIE L*	breast	52.24 ±2.88 ^a	53.12 ±1.81 ^a	53.31 ±3.56 ^a	53.48 ±2.78 ^a	NS
	thigh	51.64 ±1.86 ^a	53.17 ±2.02 ^a	52.30 ±1.42 ^a	52.68 ±1.75 ^a	NS
CIE a*	breast	0.07 ±0.06 ^a	0.59 ±0.55 ^{ac}	1.33 ±0.71 ^b	0.94 ±1.04 ^{bc}	**
	thigh	1.94 ±0.64 ^a	1.33 ±0.46 ^b	1.65 ±0.55 ^a	1.84 ±1.17 ^{ab}	**
CIE b*	breast	10.08 ±1.26 ^a	10.14 ±0.98 ^a	10.69 ±1.68 ^a	10.88 ±1.38 ^a	NS
	thigh	9.60 ±1.76 ^a	10.56 ±1.33 ^a	10.22 ±0.55 ^a	10.83 ±1.13 ^a	NS
Shear force value [kg.cm ⁻²]	breast	1.97 ±0.37 ^{ab}	2.18 ±0.60 ^{ab}	1.89 ±0.33 ^a	2.28 ±0.48 ^b	**
	thigh	1.33 ±0.24 ^a	1.66 ±0.36 ^b	1.25 ±0.19 ^a	1.67 ±0.25 ^b	**

Legend: C – control group; E1, E2, E3 – experimental groups; mean – average, SD – standard deviation; a,b – means with different superscripts within row differ significantly; S – significance; ** $p \leq 0.05$; NS = not significant.

In terms of probiotics, various effects on meat quality of chickens were found in other studies. However, it was difficult to directly assess different studies using probiotics because the efficacy of a probiotic application depended on many factors (Patterson and Burkholder, 2003), such as species composition and viability, administration level, application method, frequency of application, overall diet, bird age, overall farm hygiene, and environmental stress factors (Zhou et al., 2010). Moreover, Mihok et al. (2010) suggest to examine the technological properties of other livestock species, since introducing new trends in animal nutrition can result not only in the positive effect, but also in the negative. It was clear from this study that the administration of probiotic, *Lactobacillus fermentum* via the drinking water, had not quite the effects we had expected, as regards not only the determined losses, but also the shear force.

From the data obtained by shear force measurement follows that there were significant differences between the groups. As shown in Table 3, higher value was observed in group E3 (probiotic-supplemented group), in both breast and thigh muscle (2.28 ±0.48 and 1.67 ±0.25 kg.cm⁻², respectively), as compared with the other groups. On the

contrary, as for group E2 (propolis-supplemented group), there was the lowest values observed, in both breast and thigh muscle (1.89 ±0.33 and 1.25 ±0.19 kg.cm⁻², respectively). These findings were not in agreement with the results determined by Zhang et al. (2005), who investigated the effects of *Saccharomyces cerevisiae* cell components on meat quality of male broilers. The shear forces determined in cooked breast and thigh muscle in experimental groups decreased as compared with the control. It might be explained by the different probiotic strains and culture days. In the study of Zhou et al. (2010), beneficial effects on shear force of chicken meat was observed, using different concentrations of *Bacillus coagulans* as diet supplement. In the present study, the water losses (determined as weight losses), as the important indicator of meat juiciness, have been coincided with trend of the shear force results. Thus, it may be deduced unfavourable effect of *Lactobacillus fermentum* on tenderness and juiciness of chicken meat.

According to Volpato et al. (2008), meat tenderness as a quality attribute can be negatively affected by heat-treating due to a decrease in the water content of meat during the process. Consequently, it might be appropriate the

optimising of heat-treating conditions to get better results of meat tenderness.

Since the lower shear force values indicate tenderer meat, the present study suggests that the dietary supplementation of propolis could improve meat tenderness of broilers, although the underlying mechanism is not readily understood. Anyway, propolis has been shown as the most favourable diet supplement in order to get good meat tenderness as well as getting the lowest roasting losses (mentioned above). Overall, the shear force values obtained by measurement have been appropriate owing to the fact demonstrated in study of **Lyon and Lyon (2001)**, that if the shear force value is below 3.61 kg.cm^{-2} , chicken meat can be considered as very tender. In our study, all the shear force values were below this level. The values were similar to those in study of **Alfaig et al. (2014)**, in which the shear force value of breast muscle in the probiotic-supplemented group was obtained at a level of $2.63 \pm 0.28 \text{ kg.cm}^{-2}$. What is more, the value in experimental group was higher than that in control group.

The shear force values in our study also resembled the values observed by **Rababah et al. (2005)**, who investigated chicken breast meat infused with various plant extracts. The values ranged from 1.64 to 2.28 kg.cm^{-2} . **Pelicano et al. (2005)** evaluated effects of different probiotics (*Bacillus subtilis*, *Lactobacillus acidophilus* and *casei*, *Streptococcus lactis* and *faecium*, *Bifidobacterium bifidum* and *Aspergillus oryzae*) on quality attributes of chicken meat, including the meat tenderness. They concluded that the probiotics used as diet supplements did not affect the meat quality, because of slight changes in shear force values in experimental groups as compared with the control. Moreover, the values in the experimental groups ($3.84 - 4.08 \text{ kg.cm}^{-2}$) were slightly higher than those in our study.

As far as colour parameters are concerned, only colour parameter redness (a^* value) has been shown to express the significant differences between the tested groups (Table 3). The highest a^* value of thigh muscle was observed in control group (1.94 ± 0.64). The a^* value 1.33 was, on the one hand, observed in breast muscle as the highest (E2 group), on the other hand it was observed in thigh muscle as the lowest (E1 group). The lowest a^* value in breast muscle was found in control group (0.07 ± 0.06). The redness (a^* value) of breast muscle was increased significantly ($p \leq 0.05$) after the addition of propolis in the diet, whereas the redness (a^* value) of thigh muscle was not significantly ($p \geq 0.05$) affected by addition of natural supplements. In the colour parameters lightness (L^* value) and yellowness (b^* value), the groups did not differ significantly from each other. The colour parameters ranged from 52.24 to 53.48 for L^* value, from 0.07 to 1.94 for a^* value, and from 9.60 to 10.88 for b^* value. The addition of natural supplements imparted neither darker nor lighter colour of chicken meat, since the L^* values were not as significant as the a^* component. Furthermore, the supplements did not cause changes in the yellowness (b^*) values.

In the present study, the colour of raw meat was not altered after addition of natural supplements so that was unacceptable for consumers. As mentioned **Pelicano et al. (2005)**, the different additives might be used since they did

not affect meat colour, which is an extremely important parameter that is related to the choice made by the consumer. As mentioned **Mancini and Hunt (2005)**, the instrumental measures of L^* and a^* can easily be applied to muscle colour, whereas the colours represented by b^* (blue and yellow) are not typically related to meat. Generally, as reported **Karaoglu et al. (2004)**, when a^* and b^* values increase, L^* value declines and the colour gradually darkened. With reference to study of **Lindahl et al. (2001)**, variation in a^* values is affected by pigment content and redox state in muscle, while b^* values are influenced only by redox state. In addition, L^* values are slightly correlated with haem pigment and metmyoglobin contents. In the present study, a^* values in breast muscle were rather lower due to lower pigment in the breast as compared with that in thigh.

According to study of **Bianchi and Fletcher (2002)**, comparison of absolute colour values between the different studies is difficult, because of colour difference measurements as well as differences in measurement conditions.

In the study of **Pelicano et al. (2005)**, the L^* , a^* , b^* measurements from CIELab system were evaluated, besides the above-mentioned meat tenderness. The L^* values were in the range 45.25 – 46.37, the a^* values were in the range 3.80 – 3.88, and the b^* values ranged from 2.87 to 3.36. These results were similar to findings reported by **Bianchi and Fletcher (2002)**, who investigated the effect of chicken meat thickness on colour measurement.

In another study, **Rababah et al. (2005)** observed colour parameters in the raw chicken meat, after the plant extracts supplementation, as follows: the lightness component (L^*) in the range 51.07 – 62.15, the a^* component in the range 0.95 – 3.32, and the b^* component in the range 5.21 – 7.16. In a similar manner, **Janisch et al. (2011)**, who analyzed the colour of breast muscle depending on the broiler genetic line, observed in Ross 308 line the averaged values, as follows: 51.18 ± 0.47 , 3.44 ± 0.19 and 8.73 ± 0.25 for L^* component, a^* component and b^* component, respectively. **Kilic et al. (2014)** determined the colour parameters in raw chicken meat quite similar to those above-mentioned (L^* 51.15 ± 0.13 , a^* 3.56 ± 0.19) except for b^* value, which was slightly lower as compared with those in other studies (1.50 ± 0.07).

In another study, **Ali et al. (2015)** determined the influence of multiple freeze-thaw cycles on colour of chicken meat. They obtained the lightness (L^*) value in the range 43.6 – 46.57, the redness (a^*) values in the range 2.72 – 3.92, the yellowness (b^*) values in the range 4.17 – 5.62. As the L^* component ranges from black to white and the a^* component ranges from green to red, it can be inferred that meat in the study of **Ali et al. (2015)** was observed as darker and redder as compared with that in our study.

Karaoglu et al. (2004) investigated the effect of slaughtering at different ages and the use of probiotic preparation containing *Saccharomyces cerevisiae* in chicken diet on the colour properties. In probiotic-supplemented groups, the L^* values were in the range 63.69 – 65.21, the a^* values were in the range 2.38 – 2.59, and the b^* values were in the range

10.49 – 10.64. In addition, they demonstrated that darkness of colour has increased as time progressed.

Colour parameters in breast and thigh muscle of chickens were also evaluated in study of Haščík et al. (2014), in which the bee pollen extract was included into diet of broilers. The measurement was conducted after 45 minutes *post mortem*. For this reason, some values were completely different from those in our study. The L* values were found in the range 49.38 – 52.5 and 52.31 – 53.96 for breast and thigh muscle, respectively. The a* values were found in the range -0.98 – 2.05 and 4.53 – 7.38 for breast and thigh muscle, respectively. The b* values were found in the range 7.14 – 9.52 and 5.17 – 13.56 for breast and thigh muscle, respectively.

CONCLUSION

Based on the results of present study, it may be concluded that any of applied natural additives in feed mixtures has not notable impact on losses caused by cooling, freezing and roasting, since the lowest losses were not found in experimental groups as has been expected. The results of shear force measurement, however, indicate favourable effect of propolis addition on meat tenderness, in both breast and thigh muscle. Besides, the other applied supplements did not influence the tenderness significantly. When considering the colour parameters, it can be inferred that the propolis extract addition increase the redness (a*) values in breast muscle significantly, whereas the other supplements induce rather decrease in the redness (a*), in both breast and thigh muscle. On the contrary, the lightness (L*) and the yellowness (b*) were not changed after addition of natural supplements. In addition, the results showed that probiotic administration *via* drinking water did not improve the technological properties of chicken meat, since the most of them were observed as the least convenient. On the whole, the addition of natural supplements in chicken diet requires further research to clearly understand their influence in chicken organism and the effects on technological properties of meat.

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SELECTED PARAMETERS OF QUALITY AND SAFETY OF HERBAL TEA

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ABSTRACT

The aim of this work was to assess the heavy metal presence and possible microbiological contamination in herbal teas. Evaluation of selected tea products was performed from Nitra locality during years 2009 – 2013. Microscopic filamentous fungi detection, bacteria such as *Escherichia coli* and *Salmonella* spp. were compared to requirements given in the Codex Alimentarius of Slovakia. The highest permissible limit for microscopic filamentous fungi was not exceeded (in 32 observed herbal tea samples). For incidence of *Escherichia coli*, 93 samples were investigated and for *Salmonella* spp., 91 herbal tea samples. No sample showed the presence of *Salmonella* spp., and at *E. coli* maximum permitted presence was detected below limit. Among chemical parameters, cadmium, lead and mercury content were monitored. The highest amount of lead and mercury was found in year 2012. In 2009, the highest cadmium content was found. The average content of lead in all 100 inspected herbal tea samples was 0.784 mg.kg⁻¹ so all the samples met requirements defined in the legislation. The mean content of mercury (98 investigated herbal tea samples) was 0.0161 mg.kg⁻¹ so all samples met the requirements as well. Average cadmium content was 0.1702 mg.kg⁻¹ while the highest permitted limit for cadmium is 1.0 mg.kg⁻¹. All herbal tea samples were in accordance with the legislation except one (white willow bark tea) with a very high content of cadmium (4.36 mg.kg⁻¹).

Keywords: tea; cadmium; mercury; lead; microbiological quality

INTRODUCTION

The tea is a product of plant origin, which is intended for preparing a beverage or prepared from such product. It is necessary to meet the legislative requirements for the production and import of tea, tea extracts and preparations thereof, for handling them and placing them on the market as is defined by the legislation. The composition of tea varies by variety, season, leaf age, climate and practices at harvest. Its chemical composition is a complex including carbohydrates, amino acids, proteins, alkaloids (caffeine, theophylline and theobromine), volatile compounds, polyphenols, minerals and trace elements (Bansal et al., 2013).

The results of several studies show potential health benefits of tea, due to the consumption of tea. Multiple benefits are described, such as prevention of cancer and heart disease (Sang et al., 2011).

However, known are risks associated with the microbial contamination and subsequent adverse events. The reasons of these adverse effects are common microbial contaminants that produce toxins and it is therefore important to identify them in herbal teas (Omogbai and Ikenebomeh, 2013).

Biological contamination of medicinal herbs, their preparations and products may include living organisms such as bacteria and their spores, yeasts, microscopic filamentous fungi, viruses, protozoa, insects and other organisms. There are known also chemical contaminants,

which are the products of microbial metabolism, toxic metabolites from microscopic filamentous fungi (Koslaec et al., 2010).

Microscopic filamentous fungi are prevalent contaminants of herbs; most of their microbial population survive the drying and storage (Gonzaga and Bauab, 2012).

Omogbai and Ikenebomeh (2013) at analyzing the microbiological quality of various herbal teas isolated bacteria identified as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Salmonella typhimurium*, *Pseudomonas fluorescens* and *Escherichia coli*. Among microscopic filamentous fungi they identified *Aspergillus niger*, *Aspergillus flavus*, *Penicillium expansum*, *Rhizopus stolonifer* and *Fusarium solani*.

Exceeding the threshold of microbial contamination is the most common reason to reject herbal plant material from growers (Heindl and Müller, 2006).

Keeping good manufacturing and hygiene practices with the application of HACCP at cultivation, harvesting and processing of material is essential. To reduce contamination can be used various decontamination procedures (Zweifel and Stephan, 2012). Between years 2003 and 2004 in Germany children received *Salmonella enterica* serotype Agona because of the herbal tea with aniseed from Turkey contained species.

Since it is a natural product, all parts of the plants can be contaminated with bacteria and microscopic filamentous fungi. Improper methods of cultivation and storage, improper collection and treatment, inadequate transportation, long-term drying and storage, poor hygiene at producing and natural climatic conditions cause that the raw plant materials are susceptible to microbial contaminants. Materials are often degraded by microorganisms prior to harvesting, handling, and after prolonged storage (Stevic et al., 2012).

Storage in high humidity can promote growth of microscopic filamentous fungi. Storage of the tea requires a dry, cool, dark and inert spaces (Mishra et al., 2006).

Heavy metals are a group of harmful inorganic chemical risks. Heavy metal pollution can be of anthropogenic origin, of natural origin and they can accumulate in the soil. It may be contaminated through emissions from emerging industrial areas of the landfill metal, leaded gasoline and paint, mine tailings, sewage sludge, pesticides, fertilizer use, irrigation wastewater, coal combustion, from petrochemicals, and atmospheric deposition (Nagajyoti et al., 2010; Wuana and Okieimen, 2011). Those which are most commonly found in high concentrations in the contaminated areas are chromium, copper, zinc, cadmium, mercury and lead (Cao et al., 2011).

Lead, cadmium and mercury are toxic substances of great interest for fear of danger posed. This is mainly a result of their environmental stability and potential bioaccumulation. These chemicals are widespread through the ecosystem and cause problems for all life forms. Different plants have the possibility to accumulate these contaminants when grown under natural conditions and are often used to assess environmental contamination (Storelli, 2014).

The aim of this study was to evaluate quality parameters in different herbal teas available on the market in Nitra region, focused on assessment of possible microbiological contamination of samples and analysis of selected heavy metals content such as lead, cadmium and mercury.

MATERIAL AND METHODOLOGY

Chemical and microbiological parameters of herbal teas were evaluated under the requirements of current legislation, in cooperation with the Regional Veterinary and Food Administration in Nitra, which is responsible for the safety control of these products. For the quality of selected herbal teas are responsible manufacturers, importers and sellers of teas. Sampling was performed during the period 2009 – 2013, for the control of microbiological and chemical parameters of the herbal teas were selected 100 samples of tea placed on the Slovak market by known producers (Table 1).

Investigated samples originated from different kinds of herbal teas and their mixtures (plantain, agrimony, sage, anise, tea with smartweed and ladders, tea for women, urological tea, tea for intestines, herbal tea, detox tea, tea for kidneys, virgin tea, prostate, stomach tea, herbal tea from Liquorice root, nettle leaves, elderberry, flower tea for nursing mothers, horsetail tea, cleansing tea, white willow (bark), ginkgo biloba, chicory, small-leaved lime and small-flowered, St. John's wort, Goldenrod plain,

yellow-green lady's – tops, horsetail, Stinging Nettle – leaves, tea for blood pressure, *Acorus calamus*, lemon balm, fennel, heart tea, weight loss, *Plantago lanceolata*, valerian, gentian, heather ordinary, dandelion, *Centaurium erythraea*, mallow tea, hops common, yarrow, stinging nettle, marigold, herbal blend for breathing, chamomile, herbal tea for thyroid, *Polygonum aviculare* etc.), which in some years were repeated. Total amount of analyzed samples of herbal teas was 100 kinds.

Samplings were performed within the common controls, or preventive controls in Nitra region. Investigated samples originated from different kinds of herbal teas.

Monitored parameters of herbal teas were as follows:

Microbiological indicators determined by following methods:

- *Escherichia coli* by STN ISO 16649-2. A certain amount of initial suspension was inoculated in parallel on two media with tryptone, bile salts, glucuronide (TBX). In the same way was prepared by tens dilution initial suspension spread on two agar plates for each dilution. The plates were incubated for 18 – 24 hours at 44 °C, then the presence of colonies that on the basis of their characteristics were considered as β -glucuronidasepositive *Escherichia coli*, were assessed. CFU of β -glucuronidasepositive colony forming units of *Escherichia coli* in a millilitre of sample was determined then.

- Number of microscopic filamentous fungi by STN ISO 21527-2. Microscopic filamentous fungi were assessed by horizontal method for the enumeration of viable yeasts and xerophilous microscopic filamentous fungi by Colony-count technique at 25 °C \pm 1 °C cultivation for products having a water activity of less than 0.95, or equal to 0.95.

- *Salmonella spp.* by STN EN 6579-2. To prove the presence of *Salmonella spp.* the sample was cultivated in non-selective liquid medium, followed by propagation in selective liquid media, from which we spread them on the surface of solid agar selective media. Suspected colonies were confirmed by appropriate biochemical and serological tests.

As hygiene criteria were observed *Escherichia coli* and microscopic filamentous fungi which were evaluated by the *Codex Alimentarius* of the Slovak Republic (no. 06267/2006-SL, 06267/2006-SL) as well as *Salmonella spp.* as safety indicator (no. 06267/2006).

As heavy metal content for herbal teas were determined the content of lead, cadmium, mercury. These were determined by procedures given in the Slovak Technical Standards (STN 56 0076, STN 56 0065 a STN ISO 8288). Determination of lead and cadmium was performed by atomic absorption spectrometry with graphite cuvette in herbal teas and determination of mercury by device AMA 254 (Advanced Mercury Analyzer). The method of lead and cadmium determination is based on the quantification of mineralized samples in nitric acid. Metals present in solution are getting in to the atomic state and the specific optical density at a wavelength corresponding to the resonance line is proportional to the concentration of metal in the sample to be analyzed using a spectrometer. The results were expressed as the mass fraction of each metal in milligrams per kilogram of sample ($\text{mg}\cdot\text{kg}^{-1}$). AMA 254

(Advanced Mercury Analyzer) is intended for direct determination of mercury in solid and liquid samples without the need of chemical pre-treatment.

Maximum levels for lead (10 mg.kg^{-1}), mercury (0.05 mg.kg^{-1}) and cadmium (1.0 mg.kg^{-1}) for herbal teas were evaluated by the *Codex Alimentarius* of the Slovak Republic (no. 18558/2006-SL; 608/3/2004-100).

RESULTS AND DISCUSSION

Herbal teas must meet requirements for maximum levels of microorganisms that are listed in the *Codex Alimentarius* of the Slovak Republic (no. 06267/2006-SL) and there are clearly defined contents for *Escherichia coli* as well. As safety criteria are given requirements for the absence of *Salmonella* spp. The maximum permitted levels were not exceeded in all our samples studied and thus meet the requirements and are suitable for use in the food industry.

There are some concerns about the safety of herbal preparations, a relatively high risk of contamination by pathogenic microorganisms, organic and inorganic residues, including heavy metals and non-metals, organic pollutants, mycotoxins, endotoxins and agrochemical residues (Onyambu et al., 2013).

Evaluation of microbiological indicators in herbal teas

The results achieved in controlling the microbiological quality of herbal teas within the observed period (2009 – 2013) indicate that all samples of herbal teas meet the requirements for maximum levels of microorganisms that are listed in the *Codex Alimentarius* (no. 06267/2006-SL). No presence of *Salmonella* spp. was detected in accordance with the legislation, which demands zero its presence. The maximum permitted levels

are not exceeded in all the samples studied and thus meet the requirements and are suitable for use in the food industry.

Indicator of *Escherichia coli* presence was evaluated in 93 samples, all samples complied with the legislation requirements, only three samples showed higher incidence of *E. coli* ($0.2 \times 10^2 \text{ CFU.g}^{-1}$; $0.2 \times 10^2 \text{ CFU.g}^{-1}$; $2.3 \times 10^2 \text{ CFU.g}^{-1}$). The highest amount was recorded in the “detoxification tea” ($2.3 \times 10^2 \text{ CFU.g}^{-1}$), while it also not exceeded the maximum permissible limit.

Permissible limits in the number of microscopic filamentous fungi were found, the highest amount was recorded in tea of *Equisetum arvense* ($1.2 \times 10^3 \text{ CFU.g}^{-1}$). All herbal teas also meet this requirement, and thus it can be concluded that all the samples comply with the legislation.

Similar issues were also tested by Tournas and Katsoudas (2008), who used 69 samples of various herbal tea. The teas such as peppermint, thyme, jasmine showed filamentous fungi contamination in 100% of their samples. For example, in tea of mint and papaya ranged from $1.0 \times 10^3 \text{ CFU.g}^{-1}$ to $5.6 \times 10^3 \text{ CFU.g}^{-1}$. The highest content of microscopical filamentous fungi was found in chamomile tea ($5.8 \times 10^5 \text{ CFU.g}^{-1}$) and the lowest in the tea of jasmine flowers ($1.0 \times 10^2 \text{ CFU.g}^{-1}$). The most common contaminants in herbal teas were: *Aspergillus niger*, *Penicillium* spp., *Eurotium rubrum*, *Eurotium chevalieri*, *Aspergillus flavus*, *Fusarium* spp., *Alternaria alternata* and yeasts. Contrary to these results, we did not determine any microbiological risk in observed samples from Nitra region.

Omogbai and Ikenebomeh (2013) isolated from 26 samples of herbal teas following bacteria and their percentage of occurrence in teas: *Staphylococcus aureus*

Table 1 Tea samples for microbiological and chemical examination.

Year	Number of samples	
	microbiological examination	chemical examination
2009	19	20
2010	10	17
2011	39	28
2012	13	13
2013	19	22

Table 2 The presence of microbiological indicators in samples of herbal teas for the monitored period (2009 – 2013).

year	<i>Salmonella</i> spp.		<i>Escherichia coli</i> CFU.g ⁻¹		Microscopic filamentous fungi CFU.g ⁻¹	
	max.	min.	max.	min.	max.	min.
2009	0 (no presence detected)		2.3×10^2	(< 10)	5.9×10^2	(< 10)
2010	0 (no presence detected)			(< 10)	1.2×10^2	0.5×10^2
2011	0 (no presence detected)		0.2×10^2	(< 10)	1.2×10^3	(< 10)
2012	0 (no presence detected)			(< 10)		(< 10)
2013	0 (no presence detected)		0.2×10^2	(< 10)		(< 10)

(90%), *Staphylococcus epidermidis* (60%), *Bacillus subtilis* (100%), *Pseudomonas aeruginosa* (40%), *Escherichia coli* (10%), *Klebsiella pneumoniae* (8%), *Serratia marcescens* (5%), *Salmonella typhimurium* (3%), *Pseudomonas fluorescens* (15%).

Koch et al. (2005) point out those factors such as the addition of sugar, storage temperature and time could affect the amount of *salmonella* at the time of consumption of tea.

Tournas and Katsoudas (2008) found by microbiological examination of herbal teas the number of microscopic filamentous fungi 5.8×10^5 CFU per gram of sample.

A lot of growers of medicinal plants do not have any adequate storage facilities (suitable relative humidity and circulation), and therefore there occur cross-contamination and the development of saprophytic and pathogenic microscopic filamentous fungi on medicinal herbal materials (**Stevic et al., 2012**).

Gonzaga and Bauaba (2012) deterioration of plant samples investigated that were stored for six to nine months after delivery. Some of the contaminated materials contained toxigenic strain of *A. flavus* and aflatoxin B1, which was higher than the permissible limit. In a study where the leaves were exposed to atmosphere with different relative humidity (75%, 45%, 0%), were found after 24 weeks chemical changes of important compounds. It was shown that microscopic filamentous fungi colonizing cause the largest loss of bioactive molecules of dried leaves by exposure with water.

Onyambu et al. (2013) in their study investigated unregistered herbal products for the presence of pathogenic bacterial contaminants. *E. coli*, which contaminated 75% of all samples is well known and is the most common enteropathogenic reason of childhood diarrhea of bacterial origin.

Evaluation of selected heavy metals in herbal teas

Heavy metals are a group of harmful inorganic chemical risks. Heavy metal pollution can be caused by anthropogenic origin, also of natural origin and can accumulate in the soil. It may be contaminated through emissions from emerging industrial areas, leaded gasoline and paint, mine tailings, sewage sludge, pesticides, fertilizer use, irrigation wastewater, coal combustion, from petrochemicals, or atmospheric deposition (**Nagajyoti et al., 2010, Wuana a Okieimen, 2011**). The most commonly found in high concentrations in contaminated

areas are chromium, copper, zinc, cadmium, mercury and lead (**Cao et al., 2011**).

In our work, we focused on determination of the chemical parameters, namely the determination of lead, cadmium and mercury in herbal teas and their mixtures for a period of five years. Totally 100 analyzed samples of herbal teas were observed.

The highest content of lead and mercury was measured in herbal teas in 2012 (tea for blood pressure). The highest cadmium content of herbal teas was found in 2009 (white willow - bark).

Đurža (2003) states that the subjects of global monitoring are particular elements such as arsenic, cadmium, mercury and lead. These are generally considered as the most harmful to humans and animals.

Cadmium, lead and mercury are heavy metals, which have been identified in all environmental compartments. If we do not take into account the exposure by the inhalation route, food remains a major supply of heavy metals into living organisms; hence their monitoring in animal feed and human food is very important (**Golian et al., 2004**).

Lead, cadmium and mercury are toxic substances of great interest for fear of danger posed. They are mainly a result of their environmental stability and potential for bioaccumulation. They are widespread throughout the ecosystem and cause problems for all life forms. Different plants accumulate these contaminants grown in natural conditions and are often used to assess environmental contamination (**Storelli, 2013**).

By determination of lead content by AAS in herbal teas, we can see that during the analysed period all samples meet legislative requirements. Any sample of herbal teas does not exceed the maximum quantity for lead 10 mg.kg^{-1} (*Codex Alimentarius* of the Slovak Republic no. 18558/2006-SL; 608/3/2004-100). The mean lead content in individual years ranged from 0.476 to 1.08 mg.kg^{-1} . The highest lead content was measured in 2011 in the tea of nettle (3.69 mg.kg^{-1}).

In 2012, by the Public Health Authority of the Slovak Republic the content of lead was examined at 1001 food samples and in any of the examined samples was not detected exceeding limit for lead.

At analyzing the lead content of 650 food samples state **Golian et al. (2004)**, in tea samples has not been exceeded maximum approved content, the average amount of the 49 analyzed teas focused on lead content was $0.2217 \text{ mg.kg}^{-1}$.

At herbal teas that were during the period evaluated in

Table 3 The content of lead, mercury and cadmium in all samples of herbal teas for the monitored period (mg.kg^{-1}).

		Pb			Cd			Hg		
Maximum permitted limit		10 mg.kg^{-1}			1.0 mg.kg^{-1}			0.05 mg.kg^{-1}		
year	max.	min.	mean	max.	min.	mean	max.	min.	mean	
2009	3.378	0.143	0.866	4.36	0.0055	0.31	0.0335	0.0065	0.0142	
2010	0.485	0.399	0.476	0.257	0.0052	0.056	0.049	0.0085	0.0187	
2011	3.6	0.083	0.85	0.45	0.00189	0.131	0.0181	0.0026	0.0147	
2012	2.09	0.0193	1.08	0.347	0.0022	0.121	0.0344	0.0006	0.021	
2013	2.06	0.022	0.65	0.77	0.0132	0.233	0.025	0.0006	0.012	

relation to cadmium content is shown that all the samples analyzed, except for one sample in 2009 (white willow bark tea) comply with the requirements defined in legislation (*Codex Alimentarius* no. 18558/2006-SL; 608/3/2004-100) and cadmium content in them does not exceed the maximum amount which is set 1.0 mg.kg^{-1} .

In the sample of tea from white willow, cadmium content was measured 4.36 mg.kg^{-1} , thus it can be concluded that the maximum permissible quantity has been exceeded quadrupled. This sample is the only one exceeding the maximum level of all samples of tea over monitored period. The lowest cadmium content was determined in 2009 at tea intended for nursing mothers ($0.0055 \text{ mg.kg}^{-1}$).

Bojňanská et al. (2002) investigated the cadmium content in different foods and among 61 samples exceeded the limit amount 11 of them, which is 16%. Tea showed cadmium content below the permissible limit ($1.0 \text{ mg.Cd.kg}^{-1}$).

Besides white willow, higher content of cadmium was found in chamomile tea (0.74 mg.kg^{-1}) and (0.77 mg.kg^{-1}) at agrimony. The average content of cadmium, however, was $0.1702 \text{ mg.kg}^{-1}$, which is 83% less than the maximum level.

Golian et al. (2004) measured among 48 analyzed samples of tea, the average cadmium content $0.0899 \text{ mg.kg}^{-1}$. While we analyzed mostly herbal teas, they focused on herbal, fruit, black and green teas.

The Public Health Authority of the Slovak Republic states that for the content of cadmium were examined 942 food samples in 2012, and just one sample of food supplement did not comply with legislation requirements. For the presence of mercury were examined 874 food samples, 2 samples of one food supplement did not comply with legislation requirements. The company distributing the product adopted a measure - removed product from Slovakian market (**ÚVZ SR, 2012**).

Cadmium is a common part of the plant and can be absorbed through the leaves and roots. Plants do not have excrete mechanism for cadmium. High concentrations of cadmium may contain plants growing near sources of contamination from industry. The leaves and roots of plants generally have higher cadmium content than seeds, although oil seeds are high in cadmium content. The daily dose of cadmium is usually around $10\text{--}25 \text{ } \mu\text{g.kg}^{-1}$ (**Egyúdvová and Šturdík, 2004**).

The highest mercury content was observed in tea of *Cetraria islandica* /L./ Ach. ($0.0335 \text{ mg.kg}^{-1}$). Determination of mercury in selected herbal teas showed that in 2010, the highest content of mercury was measured (0.049 mg.kg^{-1}) in one herbal tea, which was almost at the level of maximum limit (0.05 mg.kg^{-1}). It can thus be concluded that all the samples analyzed did not exceed the maximum level. The lowest mercury content was detected in 2013 in the tea named "Tea for hyperacidity" ($0.0006 \text{ mg.kg}^{-1}$).

Limmatvapirat et al. (2012) concluded that all samples of teas purchased in Thailand are not safe for human consumption, particularly in terms of heavy metals content. They determined high levels of copper, iron, lead, zinc, aluminum, manganese, nickel and only at low concentrations of arsenic, cadmium, chromium and mercury.

Of all the elements that enter the food chain and cause contamination of food, are considered as the most important arsenic, cadmium, mercury and lead. Where soils are enriched with these elements, it is usually caused by industrial, agricultural and municipal human activities. The tendency for plants to accumulate these xenobiotics depends largely on climatic factors and plant genotype. The bioavailability of contaminants in general also depends on their physico-chemical properties and composition of the diet. It is a strong link among micro-nutrients of plants, animals and humans and the absorption and action of contaminants in these organisms (**McLaughlin and Singh, 1999**).

If the heavy metals are taken in higher quantities, they show to all the living organisms adverse effects. Toxicity of heavy metals has caused various serious diseases and also led to widespread deaths (**Tasleem et al., 2013**).

Some heavy metals such as cadmium, mercury and arsenic are highly toxic to some enzymes, which may result in inhibition of growth or death of organisms (**Nagajyoti et al., 2010**).

Good manufacturing practice shall provide reducing the amount of heavy metals in the food into amounts that minimizes the estimated health risks (**Egyúdvová and Šturdík, 2004**).

Analysis of various parts of plants showed that higher content of metals generally contain roots, then the leaves and stems and the least seeds, fruits, tubers and bulbs. Monitoring of heavy metals in plants is especially important in terms of contamination of the food chain. The intensity of contamination of plants with heavy metals must be assessed according to the type of plants. Plants are able to accumulate trace elements, particularly heavy metals in their tissues due to the great ability to adapt to different chemical conditions in the environment. They become a reservoir of trace elements that can further pass to animal and human (**Đurža, 2003**). All herbal teas meet this requirement by legislation, so it can be stated that all samples meet microbiological and chemical parameters.

CONCLUSION

We can conclude that all observed herbal teas meet the requirements defined by the legislation among observed microbiological parameters. Within the monitored heavy metals only one sample of tea was not in accordance with legislation requirements, we recorded the cadmium content higher than the permissible limit (4.36 mg.kg^{-1}) while the maximum level for cadmium is 1.0 mg.kg^{-1} .

Based on our achievements, compared to the results of other authors dealing with similar issues, we can assume that the herbal teas available on the market in Nitra region during the period 2009-2013 are safe for the human health.

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MICROSCOPIC DETERMINATION OF BAMBOO FIBER IN MEAT PRODUCTS

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ABSTRACT

Fiber, a suitable additive to meat products with water-holding capacity, reduces curing losses and maintains juiciness of the meat. The risk is the use of excessive amounts of flour or other ingredients of vegetable origin, in which the fiber is contained. In some cases, sensory characteristics of products can be affected. Detection of fiber may be prevention of adulteration in some meat products. It is therefore very important to regularly detect the amount of fiber in meat products and check its contents. Fiber in meat products can be detected by various methods, applied are for example gravimetric, spectroscopic, histochemical, and microscopic methods. For this reason, a model meat product (Vysočina salami) was prepared in our experiment with the addition of bamboo fiber of selected concentrations of 0%, 2%, and 3%. Subsequently, a series of microscopic sections was made on different days of curing (day no. 7, 14 of the drying phase and 28, 42 of storage). Individual sections were examined and captured using a polarization microscope, the amounts of fiber in individual sections were analyzed by means of image analysis software and the values obtained were compared with each other. Also the influence of drying on the measured area of fiber in sections was monitored. The results indicate a noticeable reduction in the area of fiber until the seventh day of ripening, which is caused by the rapid loss of water in the product. In contrast, sections of products from the following days of drying contained mildly increased concentrations of fiber, which was caused by gradual drying of the products, while the area of fiber refrained from becoming smaller. Between the individual days of drying, a difference that was statistically significant was demonstrated from the 14th day of (storage or drying). Correlation was observed between the date of (storage or drying) and amount of added fiber. Among the tested mean values for the sample with the addition of fiber concentration of 2 % an insignificant difference was found. The difference between test values (day/fiber) in the sample with addition of 3% fiber was, however, statistically significant.

Keywords: fiber; polarization microscopy; image analysis; vysočina salami

A healthy lifestyle is currently very frequently debated issue, which is also associated with the increasing consumer demands for high quality food and thus the raw materials used. Nutritional characteristics of meat and meat products, in particular their quantity in one's diet, are also often discussed. Although their increased consumption can cause significant health problems, their popularity has not declined. In this area, an important role is played by fiber, which is a mixture of substances of plant origin and which can have a positive impact on the consumer's health. Thanks to its properties, it is a good addition to meat products. Disproportionate amount of flour and similar materials of vegetable origin affects the sensory properties of products and detection of fiber may also be an evidence of consumer deception. It is therefore important to regularly detect the amount of fiber in meat products and check its contents. There are many methods used to detect and control the composition of meat products, they are mainly used to test the quality of the product, i.e. to detect individual ingredients and assess their suitability for the product according to Czech regulation (Decree, 2001).

The aim of this paper was to verify the possibility to detect fiber in a meat product and to describe the impact of drying on the area of fiber identified by image analysis.

Fiber is a vegetable part of one's diet resistant to enzymatic degradation in the gastrointestinal tract. The individual components of fiber can be divided on the basis of their solubility/fermentability to insoluble (less fermentable) and soluble (well fermentable) substances. The former group includes cellulose, hemicellulose, and lignin. The latter group contains pectin, plant gums, and mucilages.

Dietary fiber is a complex mixture of polysaccharides with various functions in the digestive tract, where these functions are given by their physico-chemical properties, such as particle size and volume, surface characteristics and moisturizing properties (Dhingra et al., 2012).

Due to technological reasons and because of its positive impact on consumer's health, addition of fiber in meat products is on the increase. Fiber is a suitable additive to meat products because of its water-holding capacity, reduction of curing losses and maintaining juiciness of the meat.

Suitable is the use of oat bran fiber as fat replacer in ground beef and pork sausages. Other advantages include the possibility to use oat bran instead of fat in meat balls.

Dietary fiber is of great importance in food industry in terms of nutrition and technology. The popular saying “the more the better” does not apply here. Addition of 6% of fiber in the product causes a clear deterioration of sensory properties. White dots are visible in the section and consistency is too stiff and dry. Addition of fiber can reduce or even completely prevent soft center of the salami, and moreover, the time required to dry the product can be shortened. In combination with phosphate, we obtain better texture of the product, which is evident already after five days of ripening (Kameník, 2012).

Various methods are utilized to detect fiber. These can include gravimetric, spectroscopic, histochemical, and microscopic methods (Mlček et al., 2010; Mongeau, 2003; Nielsen, 2010). Gravimetric methods employ enzyme and/or a chemical to remove material, which is digestible in the small intestine; imitation of digestive processes in the colon is considered to be ideal (Mongeau, 2003). We can meet enzymatic-gravimetric methods and nonenzymatic-gravimetric methods (Davídek, 1981). NIRS (near infrared spectroscopy), ranked among the spectroscopic methods, is based on the absorption or reflection of different wavelengths of incident radiation that is affected by the chemical composition of the sample analyzed (Mlček et al., 2010). Tremlová (2000) describes histochemical methods as methods suitable for identification and localization of various chemical substances in cells and tissues. Whereas these methods are most commonly used as qualitative methods. Nielsen (2010) reports the possibility to use modern microscopy methods for a qualitative as well as quantitative examination of the products. These methods provide reliable information on the location and physical condition of all components of the sample examined. Image analysis are often used as qualitative methods for meat products (Čáslavková et al., 2014). Light microscopy is a fundamental histological method and in case of determination of fiber, it is appropriate to use one of its modifications, such as polarization microscopy. This

method is based on the ability of the optically active compounds to rotate the polarized light in the polarization microscopy (Brychtová and Hlobílková, 2008). Polarization microscopy is used for the detection of vegetable origin additions in meat products, mostly soy, starch, and spices. Another of the methods used to describe the microscopic characteristics, is fluorescence or electron microscopy (Tremlová, 2000).

MATERIAL AND METHODOLOGY

A durable cured model meat product called Vysočina was manufactured for the experiment. 3 model products were produced, where one was a control sample (Vysočina with no added fiber – control) and the remaining products contained fiber in concentrations of 2 and 3% (sample 1 and sample 2 respectively). The experiment also included monitoring changes in relation to time, therefore, sampling was performed on the 7th, 14th of the drying phase and 28th, 42nd day of storage.

Sample processing and preparation of microscopic slides included the following steps: sample fixation in 10% formaldehyde, dewatering samples, embedding them in paraffin, cutting paraffin sections, targeted staining (PAS – Calejja), and mounting the sections.

First, the most suitable microscopic method for the detection of fiber in model meat products was selected. The most suitable methods include polarization and fluorescence microscopy. After verifying both of the methods, polarization microscopy was selected for the experiment, because it gave clearer results and was less demanding in terms of sample preparation.

All the samples were photographically documented in natural as well as polarized light microscopy (Jenaval, Karl Zeiss, Jena, DDR). Images were obtained using the DSLR Remote Pro software for Microsoft Windows (USA). The very analysis of fiber in the images from polarization microscope was performed in the Adaptive Contrast Control – Image Structure and Object Analyser program, ver. 6.1 (ACC, company Sofo, Druckmüller, Štarha).

Image segmentation was preceded by decomposition of images into individual components of RGB (red, green, blue). G layer was used for the analysis since it provided

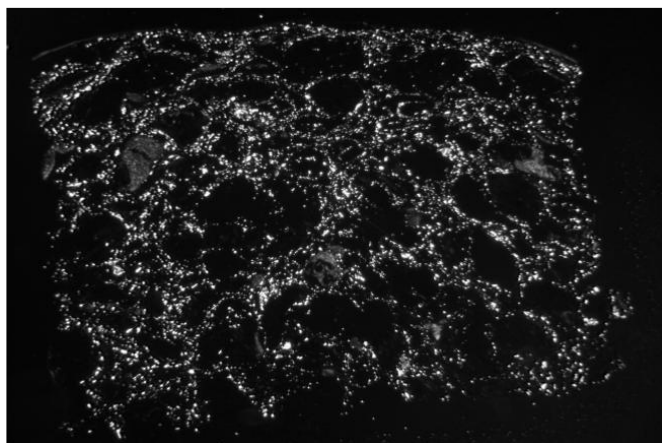


Figure 1 Decomposition into RGB – R layer.

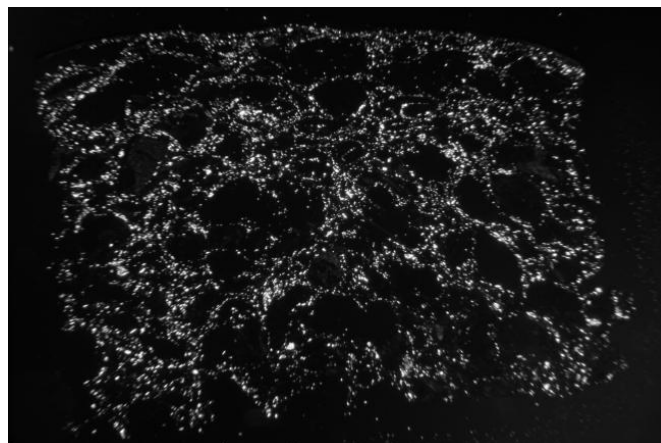


Figure 2 Decomposition into RGB – G layer.

the lowest blur and the highest contrast, thus it was selected as the most appropriate for the analysis. Fig. 1, 2, and 3 show decomposition into individual RGB layers.

Data were statistically processed by the Kruskal–Wallis one-way analysis of variance, a modification of the Tukey’s test known as the Tukey’s HSD was used (Litschmannová, 2011).

RESULTS AND DISCUSSION

The results of the experiment were focused on assessing the suitability of determination of fiber using microscopic methods. The choice to detect fiber as an anisotropic (birefringent) structure by a polarization microscope was found to be more appropriate than using a light microscope

where it is necessary to identify fiber on the basis of pink color. Vorlíčková (2010) also reports staining by hematoxylin-eosin as suitable for polarization microscopy.

The selected method was applied to examine samples with different amounts of added bamboo fiber. These results were compared with a control product in which no fiber was used. In the control sample, as well as generally in meat products, anisotropic structures were also detected. Their specific occurrence was expected with regard to the use of native spices, the source of which are in particular cell walls of plant cells. Between the control sample and samples 1 and 2 there was demonstrated a statistically significant difference ($p < 0.01$).

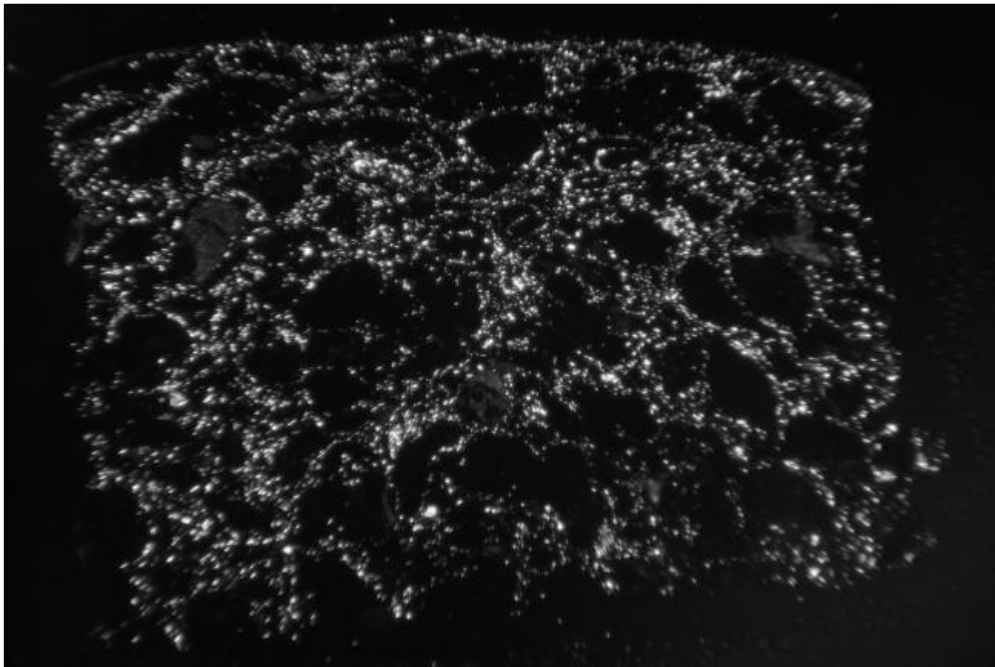


Figure 3 Decomposition into RGB – B layer.

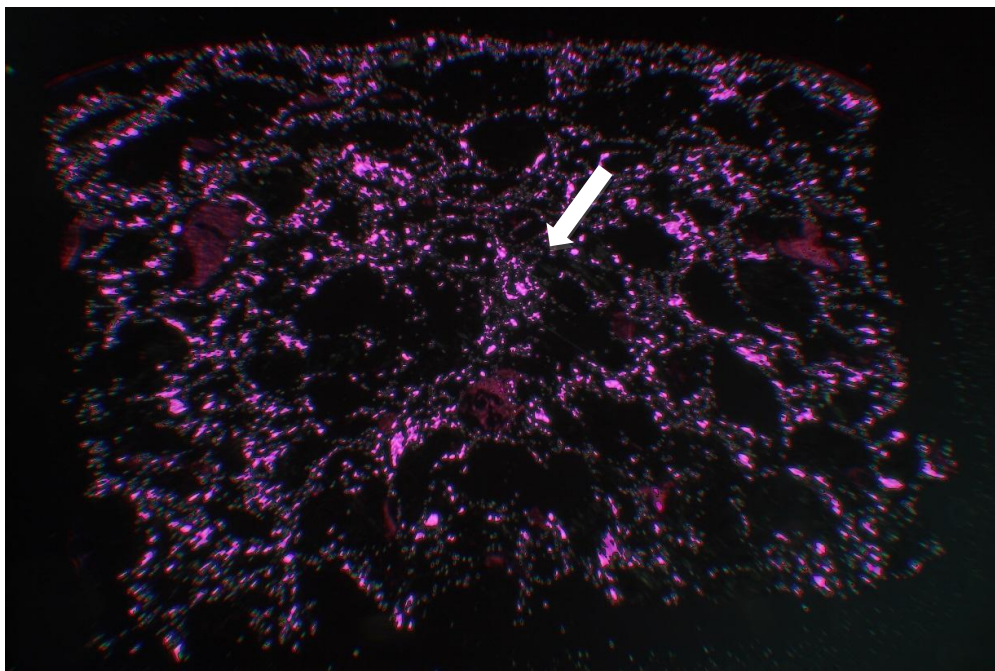


Figure 4 Analysis of fiber in the ACC Image Analyser 6.1.

Table 1 Results of measurement of fiber area [%] versus time after drying.

Day	Sample					
	Control	SD	Sample 1	SD	Sample 2	SD
1	0.086 ^{abcd}	0.04	13.99 ^{abcd}	1.66	19.90 ^{abcd}	3.70
7	0.013 ^{af}	0.01	8.63 ^a	1.30	10.21 ^{ae}	1.22
14	0.019 ^{bg}	0.01	7.10 ^b	1.45	12.49 ^b	2.45
28	0.012 ^{ce}	0.01	8.22 ^c	2.27	14.17 ^{ce}	2.38
42	0.050 ^{defg}	0.03	8.97 ^d	1.56	12.76 ^d	2.34

a, b, c, d, e, f, g – within columns, similar superscript letters indicate significant differences ($p < 0.01$)

A difference at the significance level of $p < 0.01$ was also between the samples 1 and 2. The used method can demonstrate the presence, absence, and quantity of fiber in the sample, regardless of use of spice in the product. The study further evaluated the area of fiber depending on the duration of drying.

Table 1 shows the values of fiber area for individual days of drying. On the first day of curing of the model meat product, the samples contained the highest amount of water. For this reason, the percentage of fiber measured in the section was high. This corresponds to the ability of fiber to hold water and swell, as reported by **Dhingra et al. (2012)**. From the seventh day on, however, due to release of water from the model products, rapid reduction in the area of fiber occurred, which can also be expected to be accompanied by fiber volume reduction. The fact that meat products release water and shrink in volume at the beginning of the drying phase is also confirmed by **Heinz and Hautzinger (2007)**. These authors also state that, depending on the type of meat and the size of meat pieces, the weight can drop to 45 – 35% of its original weight just after one day, and to 30 – 20% of its original weight after two days.

From the seventh day, on the contrary, a slight increase in the area of fiber began to occur. This was caused by persistent drying of the samples, however, the fiber “held” the water bound, thus the volume of fiber was not being reduced significantly any more, while the area of other ingredients in the meat product in the section was still decreasing and thus the area of fiber in the section increased in proportion to the rest. In the control sample, no significant changes in the area of anisotropic structures occurred during drying and storage. This result points out that there is no change in volume of spices and other present anisotropic structures, possibly because they are not involved in the drainage system. Between samples analyzed on the 7th day of product drying there was no statistically significant difference found between the model product 1 and 2. Up to this day area of fiber was also being reduced. The reduction in sample 2 in contrast to sample 1 could occur due to the fact that fiber creates a three-dimensional network. The network acts as a drainage system through which the water gets better from

the product to its surface where it evaporates (**Kameník, 2012**). It can be stated that the addition higher by 1% resulted in 10% higher water loss. Water bound in the fiber evaporated more easily and the total area of fiber decreased, which can be used to shorten the drying time. Between individual concentrations sampled on the 14th, 28th, and 42nd day there was a statistically significant difference detected. From the 14th day on, the sample with higher concentration achieved higher resulting values of fiber area with statistically demonstrable difference ($p < 0.01$). This points to the continuing ability of fiber to hold water in the product.

CONCLUSION

Based on the results obtained, we cannot doubt the possibility of using polarization microscopy for detection of fiber. Between the control sample and samples with added fiber, there were statistically significant differences in the content of anisotropic structures. Detection of fiber was possible already from the addition of 2%.

Drying also affected the total area of fiber. Until the seventh day after the product was cured, there was a significant loss of water, namely 38% from the sample with 2% bamboo fiber and 49% from the sample with addition of 3%, thus the reduction of area of fiber in the sample decreased as well. From the 14th day of storage, a small difference in the area of fiber was recorded. After a rapid release of water from the sample at the beginning of ripening, gradual drying occurs. During the drying phase, the total area of the meat product was decreasing and the analyzed area of fiber was slightly increasing because of the water-holding capacity of fiber. This can be used to enhance the economic profitability of meat products.

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THE INFLUENCE OF VIRAL INFECTIONS ON ANTIOXIDANT LEVELS IN THE GENETICALLY MODIFIED PLUM VARIETY “HONEY SWEET” (*PRUNUS DOMESTICA* L.)

Jiri Sochor, Boris Krska, Jaroslav Polak, Tunde Jurikova

ABSTRACT

It is well-known that polyphenolic compounds are found abundantly in fruit, but various kinds of diseases lower these levels. This work measures total polyphenolic content, antioxidant activity and the levels of specific important antioxidants in fruits of the genetically modified (GM) plum variety HoneySweet, trees which were previously inoculated with a range of different virus infections. These were the Plum Pox virus (PPV), Prune Dwarf virus (PDV) and Apple Chlorotic Leaf-Spot virus (ACLSV). Uninoculated trees were used as controls. Antioxidant activity was measured using four different photometric methods – DPPH (2,2-diphenyl-1-picrylhydrazyl), DMPD (N-dimethyl-p-phenylenediamine), ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) and FRAP (Ferric reducing antioxidant power). Total polyphenol content was measured using the Folin–Ciocalteu method. The profiles of 10 specific antioxidant constituents in the fruits of the GM plum variety HoneySweet were detected and analyzed, since these are of interest for their role in human diets and could play a role in the resistance of plants to viruses. Detection was made using HPLC with UV-VIS detection. They were: gallic acid, p-coumaric acid, 4-aminobenzoic acid, chlorogenic acid, caffeic acid, ferulic acid, vanillin, rutin and quercetin. The compound with the highest concentration was chlorogenic acid (587 mg/100 g), and that with the lowest was p-coumaric acid (0.95 mg/100 g). Of the four methods of antioxidant activity used, in three the lowest levels of antioxidant activity were seen where the PPV virus was combined with ACLSV, and in three the highest levels were seen in the un-inoculated control without any infection. The highest values of total polyphenols were seen in the control (65.3 mg/100 g), followed by infection of PPV, then treatment PPV, PDV and ACLSV, then treatment PPV and PDV and finally the lowest levels were seen in treatment PPV and ACLSV (44.2 mg/100 g), which was also that with the lowest antioxidant activity.

Keywords: *Prunus domestica* L; viral infection; antioxidants

INTRODUCTION

The levels of antioxidants in fruit is dependent on many factors, but mainly on the species and the level of ripeness (Los et al., 2000). It is also influenced by the rootstock employed (Forcada et al., 2014) and the method used for processing fruits (Miletic et al., 2014). Many scientific studies have now been published analyzing the antioxidants in plums (Gao et al., 2014, Morabbi Najafabad & Jamei, 2014, Venter et al., 2014), and it has been established that plum extracts can have an inhibiting effect on cancers (Vizzotto et al., 2014). The anti-inflammatory and antioxidant properties of plums have also been studied (Popov et al., 2014). Other studies have shown them to have anti-hyperglycemic properties (Utsunomiya et al., 2005). However, very little has been written about the changes in levels of these important constituents of tree fruits growing under the influence of viral infections. "HoneySweet" is a plum variety developed through genetic engineering to be highly resistant to plum pox potyvirus (PPV) the causal agent of sharka disease that threatens stone-fruit industries world-wide, and most specifically in Europe

(Ravelonandro et al., 2013). Polak et al., 2012 have reported that after co-infections of PPV with PDV and/or ACLSV, there was practically no effect on the quantity and quality of HoneySweet fruits, which were large, sweet, and of a high eating quality. Fruit compositional studies are continuing in the USA and Europe since quality and nutrient composition is affected by the time of harvest and environmental factors that may vary within and between years. Nevertheless, the studies to date show that "Honey – Sweet" fruit are of high quality and nutritious (Capote et al., 2008).

However, the aim of this work was to highlight the changes in the levels of polyphenols and antioxidant activity which can occur in the GM plum HoneySweet following inoculation with various viral infections.

MATERIAL AND METHODOLOGY

Biological material

Abbreviation of viruses: ACLSV – Apple chlorotic Leaf Spot Virus, PPV – plum pox virus, PDV – Prune dwarf virus.

The GM plum variety HoneySweet was first inoculated with three viruses, PPV, ACLSV and PDV, and the fruits of *Prunus domestica* – transgenic plum variety HoneySweet, were harvested at the normal level of harvest ripeness in 2011 (Prag, Ruzyne).

There were four treatments using various combinations of the viruses, plus an untreated control group: I- PPV, PDV and ACLSV, II- PPV and PDV, III- PPV and ACLSV, IV- PPV alone, and V – the controls, which were not inoculated with any virus.

Preparation of samples

Representative samples (2 g) were taken from individual fruits, transferred to three bowls and homogenized with 8 ml water. Precise volumes of the homogenized samples were placed in test tubes and automatically agitated for 30 minutes and then centrifuged for 30 minutes at 16400 rpm·min⁻¹. The supernatant fluid was then removed by pipette and used for the individual analyses.

Assesment of antioxidant components by HPLC

For the determination of the HPLC profiles of the individual cultivars, high performance liquid chromatography (HPLC) with electrochemical and UV-VIS detection was used. The system consisted of two Model 582 ESA chromatographic pumps (ESA Inc., Chelmsford, MA, USA) with a working range from 0.001 to 9.999 mL min⁻¹ and a Zorbax SB C18 (150 × 4.6; size of particles 5 µm, Agilent Technologies, USA) reverse phase chromatographic column. For UV detection, a Model 528 ESA UV detector was used. A twelve-channel CoulArray detector (ESA) was used for electrochemical detection. Samples were injected automatically by an autosampler (Model 542, ESA), which has incorporated a thermostatic space for a column. This method is described in detail in article Zitka et al., 2011.

Determination of antioxidant activity

Spectrophotometric measurements of antioxidant activity were carried out using the BS-400 automated chemical analyser (Mindray, Shenzhencity, China). Transfer of samples and reagents was provided by a robotic arm equipped with a dosing needle (error of dosage not exceeding ±5 % of volume). Cuvette contents were mixed by an automatic mixer including a stirrer immediately after addition of reagents or samples.

Determination of antioxidant activity by the DPPH test

This procedure for the determination was taken from publications by Sochor et al., 2010a. A 150 µL volume of reagent (0.095 mM 2,2-diphenyl-1-picrylhydrazyl – DPPH•) was incubated with 15 µL of the sample. Absorbance was measured at 505 nm for 10 minutes.

Determination of antioxidant activity by the ABTS test

The procedure for the determination was taken from a publication by Sochor et al., 2010a. A 150 µL volume of reagent. Seven mM 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•) and 4.95 mM potassium peroxydisulphate was mixed with 3 µL of the sample. Absorbance was measured at 660 nm for 10 minutes.

Determination of antioxidant activity by the FRAP method

The procedure for this determination was taken from a paper by Sochor et al., 2010b. A 150 µL volume of reagent was injected into a plastic cuvette with subsequent addition of a 3 µL sample. Absorbance was measured at 605 nm for 10 minutes.

Determination of antioxidant activity by the DMPD method

Procedure for the determination was taken from a publication by Pohanka et al., 2012. A 160 µl volume of reagent is injected into a plastic cuvette with subsequent addition of 4 µl sample. Absorbance is measured at 505 nm for 10 minutes.

Determination of total polyphenol content

The total level of polyphenols was determined using the Folin-Ciocalteu method, in which a 0.5 ml sample is diluted with 1.5 ml ACS water and 0.05 ml of Folin-Ciocalteu reagent (Sigma Aldrich, USA) added. Absorbance was measured after 30 minutes (at 22°C) using a double-beam spectrophotometer SPEKOL 210 (Carl Zeiss Jena, Germany) with a wavelength $\lambda = 640$ nm. The results were expressed as gallic acid equivalents, in mg·kg⁻¹.

RESULTS AND DISCUSSION

When evaluating antioxidant status, antioxidant activity was evaluated using DPPH test, methods FRAP methods DMPD and ABTS. Resulting values of antioxidant activities were converted to 1 gram of protein.

The antioxidants were studied using 1) HPLC, 2) measures of antioxidant activity and 3) measurements of total polyphenol content.

Detection of certain specific antioxidant components using HPLC

Jaiswal et al., 2013 identified forty-one comprising by *Prunus salicina* and *prunus domestica*. Were identified: caffeoylquinic acids, feruloylquinic acid, p-coumaroylquinic acids, methyl caffeoylquinates, methyl p-coumaroylquininate, caffeoylshilcemic acids, catechin, epicatechin, rutin, esculin, quercetin, quercetin-3-O-hexosides, dimeric proanthocyanidins, trimeric proanthocyanidins, caffeoyl-glucoside, feruloyl-glucoside, p-coumaroyl-glucoside, vanillic acid-glucosides, 3,4-dihydroxybenzoyl-glucoside, quercetin-3-O-pentosides, quercetin-3-O-rhamnoside, quercetin-pentoside-rhamnosides, and 3-p-methoxycinnamoylquinic acid LC-MSn method.

The profiles of 10 specific antioxidant constituents in the fruits of the transgenic plum variety HoneySweet were detected and analyzed, since these are of interest for their role in human diets and could play a role in the resistance of plants to viruses. Detection was made using HPLC with UV-VIS detection. They were: gallic acid, p-coumaric acid, 4-aminobenzoic acid, chlorogenic acid, caffeic acid, ferulic acid, vanillin, rutin and quercetin

The compound with the highest concentration was chlorogenic acid (587 mg/100 g in fruit with virus combination PPV and PDV), and that with the lowest was p-coumaric acid (0.95 mg/100 g in fruit with virus PPV).

Table 1. Concentrations of selected polyphenolic compounds (mg·100g⁻¹). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

Treatment Number	1	2	3	4	5
Virus combinations	PPV, PDV ACLSV	PPV, PDV	PPV ACLSV	PPV	Control
Gallic acid	29.8 ±2.7	34.5 ±3.4	22.16 ±2.1	33.5 ±3.4	19.9 ±3.2
p-coumaric acid	1.25 ±0.19	3.46 ±0.22	2.59 ±0.15	0.95 ±0.07	1.45 ±0.12
4-aminobenzoic acid	1.91 ±0.07	2.56 ±0.22	3.58 ±0.26	2.75 ±0.18	1.56 ±0.13
Chlorogenic acid	356 ±9.87	587 ±14.2	113 ±2.32	112 ±8.2	376 ±9.1
Caffeic acid	9.54 ±0.65	7.69 ±0.87	5.65 ±0.28	6.87 ±0.85	4.39 ±0.41
Vanillin	7.65 ±0.88	5.97 ±0.59	6.42 ±0.29	3.87 ±0.19	5.39 ±0.25
Rutin	38.8 ±1.15	42.6 ±2.4	29.9 ±1.26	26.8 ±0.21	28.6 ±0.91
Ferulic acid	2.4 ±0.13	9.8 ±0.85	5.9 ±0.58	6.5 ±0.90	3.5 ±0.12
Quercetin	11.9 ±0.12	19.7 ±1.11	8.6 ±0.54	9.6 ±0.84	7.6 ±0.65

These results are in accord with **Donovan et al., 1998** and **Rop et al., (2009)** determined chlorogenic acids and proanthocyanidins were the major phenolics present in plums. On the other hand, **Miletic et al., 2013** noticed that major phenolic compound in fresh plums and prunes (cvs. "Valjevka" and "Mildora") is neochlorogenic acid, followed by caffeic acid and chlorogenic acid. **Piga et al., 2003** noticed lower value of chlorogenic acid content 58 mg/100 g DM. In our study we determined the higher level of gallic acid (19.87 mg/L - control variant) and 28.62 rutin (28.62 mg/L - control variant) in comparison with studies of **Miletic et al., 2013** (2.56 mg/100g; 5.25 mg/100 g).

The lowest levels of the antioxidants under investigation were found in the controls (with the exception of chlorogenic acid), and the highest were found in the variant inoculated with PPV and PDV. Concretly results are in Table 1.

Assessment of antioxidant activity

The methods for measuring antioxidant activity are usually based on the reactions caused by free radicals and then their inhibition by the compounds under investigation.

The advantages are their simplicity, stability, low cost and reliability. The results of all methods are expressed in gallic acid equivalents (GAE) to make comparisons easier. Since five fundamentally different methods were used to measure antioxidant activity, the results and conclusions can be seen with some confidence (**Sochor et al., 2011**).

In our study we have used four different methods for measuring antioxidant activity. Two based on the ability of the antioxidant to destroy synthetic radicals (tests DPPH and DMPD), the method FRAP, based on the reduction of ferric iron complexes to ferrous, and the method TEAC, based on assessing sample activity in terms of its gallic acid equivalent.

Of the four methods used, in three the lowest levels of antioxidant activity were seen where the PPV virus was combined with ACLSV (treatment 3), and in three the highest levels were seen in the un-inoculated control without any infection. The results from methods ABTS, FRAP and DPPH were strongly correlated: ABTS and DPPH r = 0.96, ABTS and FRAP r = 0.95.

Assessment of total polyphenol content

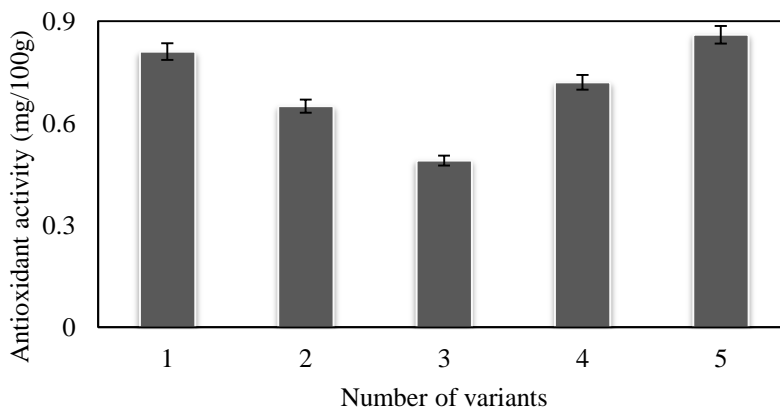


Figure 1. Antioxidant activity as measured by method DPPH and expressed in gallic acid equivalents (mg/100g). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

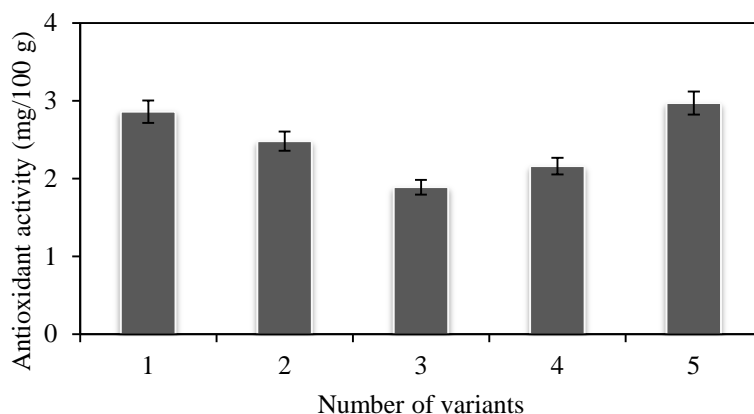


Figure 2. Antioxidant activity as measured by method ABTS and expressed in gallic acid equivalents (mg/100g). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

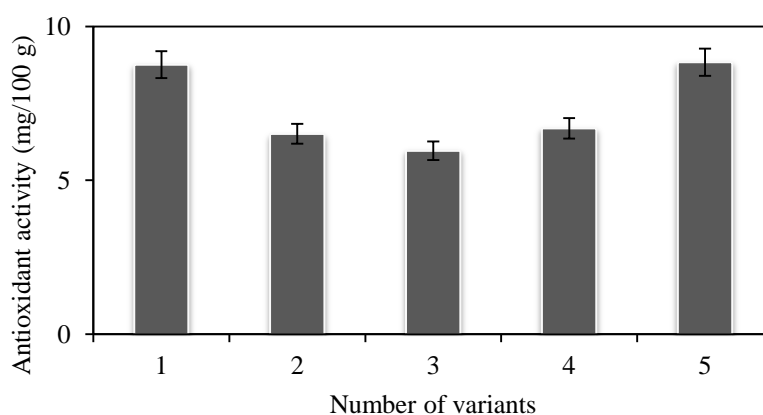


Figure 3. Antioxidant activity as measured by method FRAP and expressed in gallic acid equivalents (mg/100g). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

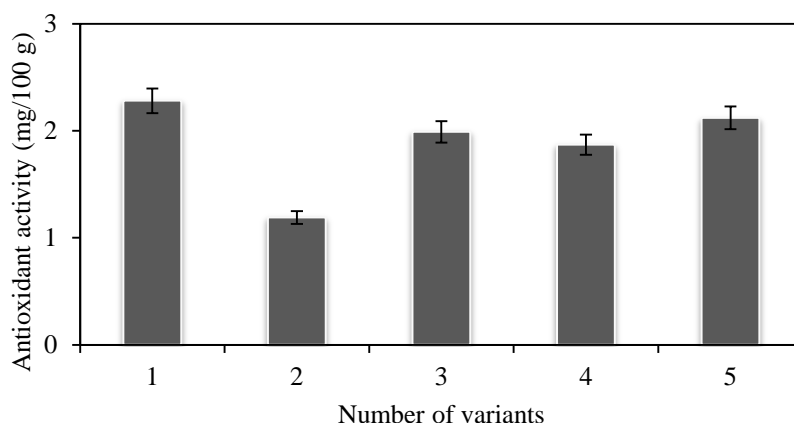


Figure 4. Antioxidant activity as measured by method DMPD and expressed in gallic acid equivalents (mg/100g). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

Polyphenols are one of the most often measured and widely discussed groups of compounds in our diet today, and at the time of writing over 8000 have been identified. The fruits of the European plum *Prunus domestica* exhibit

a great diversity in appearance including skin colors. Their skin contains many polyphenolic compounds (Treutter et al., 2012).

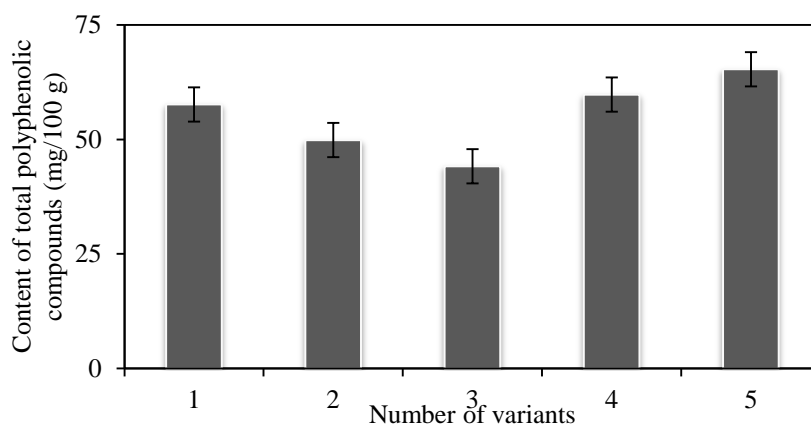


Figure 5. A comparison of total polyphenol content for each treatment.

Overall totals were measured using colorimetric methods using Folin-Ciocalteu reagent. The reagent does not only measure phenols, and will react with any reducing substance. It therefore measures the total reducing capacity of a sample, not just phenolic compounds. This method is widely used for its simplicity and reliability (Sanchez-Rangel et al., 2013).

The highest values were seen in the control (treatment 5), followed by treatment 4 (PPV), then treatment 1 with all three infections (PPV, PDV and ACLSV), then treatment 2 (PPV and PDV) and finally the lowest levels were seen in treatment 3 (PPV and ACLSV), which was also that with the lowest antioxidant activity.

In comparison, the total polyphenol content measured in plums by Luxembourg scientists showed the variety Kirk to have the highest levels, of $185 \text{ mg} \cdot 100 \text{ g}^{-1}$ (Kaulmann et al., 2014). In the USA fruit composition including study of total polyphenols content was evaluated comparing "HoneySweet" to a range of conventional plum cultivars). These analyses showed that "HoneySweet" fruit composition is generally in the range of the other plum cultivars tested (Ravelonandro et al., 2013). However, in the mentioned study HoneySweet reached up higher level of polyphenols $118 \text{ mg}/100 \text{ g}$.

CONCLUSION

It is evident from these results that the type of virus infection has an influence on the levels of antioxidants in the variety HoneySweet. The controls displayed the highest level of antioxidant activity and also had the highest total polyphenol content, from which it can be concluded that the virus infections were probably the cause of the reduced levels of antioxidants and polyphenols seen in the inoculated trees. This can only be confirmed by further studies, however.

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INTERSPECIES AND SEASONAL DIFFERENCES OF RETINOL IN DAIRY RUMINANT'S MILK

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ABSTRACT

Milk is an essential source of macronutrients and among lipophilic vitamins is significant source of retinol. The contribution of milk to the reference daily intake for retinol varies from 11% to 16%, worldwide. The most consumed dairy products are fresh, dehydrated and condensed milk in which the amounts of retinol are not modified to those of in whole milk. Retinol is essential to ensure a good functionality of the immune system and plays a critical role in vision, reproduction, cell differentiation as well as growth and development and is found only in animal tissues. The aim of our study was to evaluate the interspecies differences in the retinol concentration of whole raw bovine, caprine and ovine milk and to observe seasonal variation of retinol in bulk tank milk samples. Samples of raw milk were collected on different farms in the Czech Republic between 2013 and 2014. Retinol was measured by ultra high performance liquid chromatography with UV detection (325 nm) in isocratic mode after alkaline saponification with methanolic potassium hydroxide solution and liquid-liquid extraction into non polar organic solvent of whole raw milk. To avoid vitamin losses or degradation during the procedure, antioxidants were added to the sample extraction media. Our results indicate significant interspecies differences between bovine and ovine milk and caprine and ovine milk. Concentration of retinol is very similar in bovine and caprine milk 0.96 ± 0.11 mg/L, 0.94 ± 0.25 mg/L, respectively. The mean concentration in sheep's milk is 1.75 ± 0.24 mg/L. The seasonal variation of retinol in raw bovine milk was detected as high significant, with the highest concentration during winter. These results contribute to the nutrition evaluation of milk in the Czech Republic and indicate, that the sheep's milk is the best source of retinol among the milks of ruminants kept in the Czech Republic, however it is not used in its fluid form for human consumption.

Keywords: milk; retinol; season; small ruminant

INTRODUCTION

Milk, especially, appeared to occupy a unique position among the many foods, because it is the sole food for humans and all mammals during the first part of their lives. Therefore, milk contains everything, the young organism needs for growth and development including in particular a sufficient concentration of protein and minerals (Weber, 1997). The valuable nutritional benefits and the higher consumer demand support the production of milk of small ruminants. Consumers are requesting more and more information concerning the nutritional composition of small ruminant's milk.

The milk fat represents a good dietary source of vitamin A, which is essential for human (Väänänen et al., 2000; EFSA, 2013) and is the most quantitatively and qualitatively variable component of milk, depending on the stage of lactation, season, breed, genotype, and feeding (Raynal-Ljutovac et al., 2008). The term "vitamin A" is used for a group of several molecules with the biological activity: all-trans retinol, 11-cis retinal, retinoic acid and its esters. All-trans retinol is considered as the main form of the vitamin and simultaneously sole component of vitamin A that occurs naturally in bovine milk (Lidén and

Erisson 2006). Vitamin A is found only in animal tissues and plays a significant role in vision, immunity, cellular differentiation, and embryonic development (Maijala, 2000). In milk and meat, vitamin A occurs mainly as fatty acid retinyl esters, mainly retinyl palmitate, followed retinyl oleate and stearate (Väänänen, 2000 and EFSA, 2013). Retinoic acids are considered as the molecular species responsible for all the functions attributed to vitamin A, with the exception of vision, where only retinal is able to exert an action (EFSA, 2013). Vitamin A or precursor carotenoids with provitamin A activity are poorly soluble in water and relatively unstable during food processing and storage due to its chemical structure, which contains many double bonds susceptible of degradation. Further the stability of vitamin A is also affected by acidity and at pH below 5.0 this compound is more easily destroyed. Trace metals (especially iron and copper) and ultraviolet light accelerate the degradation of vitamin A (Andrés et al., 2014).

The vitamin A deficiency is a major public health nutrition problem (Klemm et al., 2010). Deficiencies of vitamin A caused by malnutrition include chronic illnesses, liver disease, and fat malabsorption conditions

such as celiac disease and cystic fibrosis (Majjala, 2000). Vitamin A deficiency can be severe and results in nyctalopia (night blindness), xerophthalmia (conjunctival dryness with keratinization of the epithelium), keratomalacia (ulceration and necrosis of the cornea), poor resistance to infection and abnormal epithelial morphology (Weber et al., 1997). In the year 2005 suffered by xerophthalmia 5.2 million pre school children and 9.8 million pregnant women (WHO, 2009). The recommended dietary allowances depend on life stage group. In accordance with food and nutrition board are the smallest doses for children and the highest for women in lactation. Daily reference intake according to Regulation (EU) No 1169/2011 on the provision of food information to consumers is for retinol 0.8 mg. The contribution of milk to the reference daily intake for retinol varies from 11% to 16%, worldwide. However retinol is removed with the fat during the separation process thus making it a requirement to replenish the lost vitamin in the form of a vitamin premix in skim and partially skimmed milks. In some countries (i.e. Canada) vitamin A is added into skim and partially skimmed milks is mandatory in level 0.7/100 ml (CFIA, 2013). Fresh, dehydrated and condensed milk represent approximately 46% of world dairy products and the amounts of vitamin A are not modified compared to those in whole milk (Sauvant et al., 2003).

A number of spectroscopic and separation methods are used for the determination of vitamin A in food. At present, the technique of choice is high performance liquid chromatography (HPLC). The normal-phase and reversed-phase HPLC conditions coupled to UV and fluorescence detection has been widely used, as well as the mass spectrometry detection system. Recently ultra-high performance liquid chromatography (UHPLC) has emerged as a liquid chromatographic technique offering much rapid and efficient separation (López-Cervantes J. et al., 2006).

In our study we investigated how is the retinol concentration influenced by the season and observed the differences between the bovine, caprine and ovine milk. These results are needed for nutritional evaluation of milk from different ruminant's species.

MATERIAL AND METHODOLOGY

Samples of farm milk in the number of 155 were collected and analysed during 2013-2014. The breed representation on the dairy farms was Holstein Friesian cattle and their cross-breeds, Czech Fleckvieh cattle and their cross-breeds, Jersey and Montbeliarde. From the goats were included white short-haired goats and from sheep different breeds – Lacaune, Cigaja and East-Frisien sheep. The feeding management on the farm was balanced during whole year.

Whole raw milk samples were kept frozen prior to the analysis. The samples were homogenized before processing and analysed in duplicate.

Chemicals used for extraction and HPLC analyses were

methanol, acetonitril and n-hexane, all HPLC gradient grade (Merck, Germany), sodium-1-heptane sulfonate (Sigma-Aldrich, Germany), potassium hydroxide, hydroquinone, ascorbic acid, sodium sulphate anhydrous, hydrochloric acid all in p.a. purity (Penta, Czech Republic). Vitamin A-acetate concentrate 50 % (w/w) in peanut oil was purchased from Fluka (Buchs, Switzerland). The stock standard solutions of vitamin A for analyses were prepared to the concentration of approximately 500 mg/L of retinol and in methanol. Working standard solutions at concentrations 0.1 – 20 mg/L were prepared by dilution of the stock solution in methanol.

The sample preparation procedure consisted of direct saponification with methanolic potassium hydroxide and liquid-liquid extraction in n-hexane. Antioxidants hydroquinone and ascorbic acid were added to the sample. The separation of retinol was provided by the analytical reverse phase UPLC system Acquity with dual wavelength absorbance detector (Waters, USA). The chromatographic column Acquity BEH C8 (Waters, USA) was used. The analysis was performed in isocratic mode and the mobile phase consisted of methanol and water (93:7) at a flow rate of 0.4 mL/min, injection volume was 4 µL, run time 2.7 minutes. The UV detection was performed at the wavelength of 325 nm.

For statistical analysis was used Microsoft Office Excel 2010 and Unistat 6.0. The statistical significance was set at $p=0.01$ and $p=0.05$.

RESULTS AND DISCUSSION

The characteristic of small ruminant's milk fat is its globule size. The caprine milk has higher proportion of small globules compared to cow milk. This property should support the hypothesis, that the small ruminant's milk has higher content of retinol than bovine milk (Raynal Ljutovac et al., 2008). The Table 1 shows the mean retinol concentration in milk of different ruminant's species held in the Czech Republic. The determined results from our study are higher in comparison with the available literature (Debier, 2005; Raynal-Ljutovac et al., 2008; Park and Haenlein 2013; Hulshof 2006). This difference should depend on the method of extraction and the type of breed held in different countries. Analysis of retinol is paved with difficulties due to the labile nature of the compound, low concentration, and the need for saponification in order to remove fat and to hydrolyse retinyl esters (Hulshof et al., 2006). There are various endogenous and exogenous factors, which can influence the concentration of biological active compounds, among other seasons of the year, stage of lactation, environmental factors, feed fortification and the diet during the stage of lactation and the difference in retinol precursor concentration in the animal diet and/or a difference in bioavailability of the precursors in the animal body (Raynal-Ljutovac et al., 2008; Fedele et al., 2004).

Table 1 Concentration of retinol in milk of different ruminant's species

	Type of milk		
	Cow	Goat	Ewe
Retinol concentration (mg/L)	0.96 ±0.11*	0.94 ±0.25	1.75 ±0.24
Retinol in 1 g of milk fat (µg)	26.25	24.12	29.56
Retinol (mg/L) **	0.3 – 0.52	0.4 – 0.62	0.44 – 0.83

*mean±SD

** Data from Park and Haenlein (2013); Raynal-Ljutovac (2008); Hulshof (2006)

The type of breed is significant endogenous factor, which influences the milk fat content and subsequently the retinol concentration. The mean retinol concentrations in caprine and bovine milk are very similar ($p > 0.05$). In bovine milk were the values in the range from 0.28 mg/L to 1.61 mg/L with median 0.66 mg/L and in caprine milk from 0.46 mg/L to 1.75 mg/L with median 0.85 mg/L. The comparison of the results, however, shows greater variability retinol concentration in caprine milk (0.94 ±0,25 mg/L versus 0.96 ±0,11 mg/L). The same results, significant higher concentration of retinol in ewe milk compared to goat's milk were found by **Kondyli et al. (2012)**.

The significant difference was observed in comparison with ovine milk ($p < 0.01$). The concentration in ewe milk is 0.72 – 1.96 mg/L with median 1.39 mg/L. According to **Hulshof et al., (2006)** raw milk contains on average 0.40 mg retinol per 1000g of milk. It should be hypothesised, that goat's and ewe milk contains higher concentration of retinol in contrast to that of cow's, because both goat and sheep milk are lacking β-carotene, which is entirely converted into retinol (**Raynal-Ljutovac et al., 2008**). This fact is not practically observed, because the concentration of retinol expressed for 1 gram of fat is not significant between ruminant's species. This reason

should be in retention of retinol in the liquid and semi/liquid dairy products, which is above 80% (**Hulshof et al., 2006**) and small proportion of the retinol is associated with whey proteins and/or concentrated in the milk fat globule membrane (**Revilla et al., 2014**), which is not cleaved into the fat extract. Very important factor is the farming system. Two major systems of small ruminant farming are pasture and indoor systems. Between these two farming systems is a wide scope of mixed systems such as summer pasture/winter indoors or alternatively indoors/outdoors subject to climatic differences (**Morand-Ferhr et al., 2007**). According to the study **Fedele et al., (2004)**, is found the higher concentration (often significantly) in goat's milk from goats reared on pasture system than on indoors farming system. The concentration on pasture was observed 650.5 ±133.9 µg/100 DM and in indoor system, the concentration was 498.6 ±49.9 µg/100 DM. The green herbage increased the total retinol content by 20% (**Fedele et al., 2004**).

The seasonal variation in cow's farm milk samples (Figure 1) was observed at significance $p < 0.01$. However, the statistical difference was found between winter and the other time of year. The mean concentration in winter was 1.01 mg/L and the lowest was observed in autumn 0.77 mg/L. **Hulshof et al. (2006)** investigated the seasonal

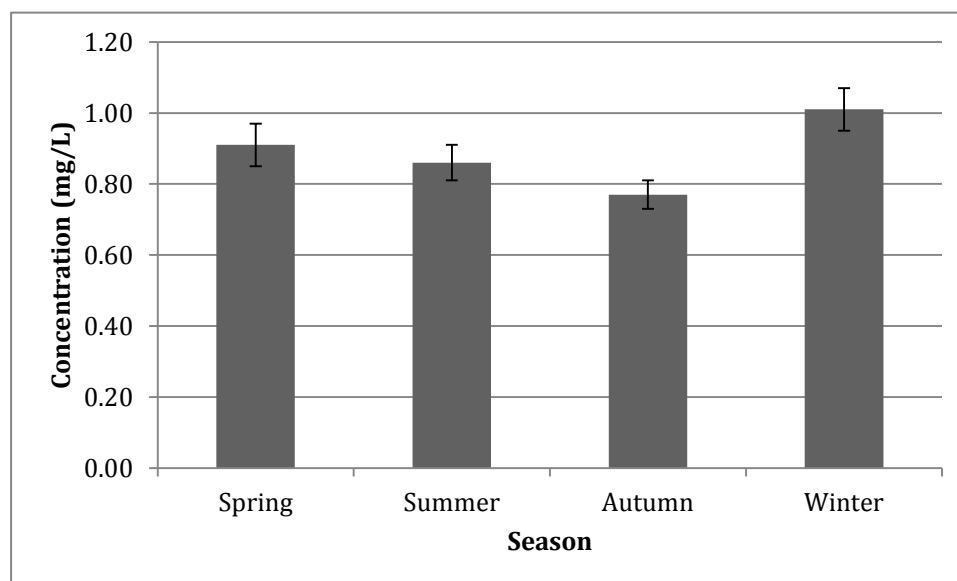


Figure 1. The seasonal variation of retinol in cow's farm milk (mean ±SD).

variation in retinol content in Dutch milk. They observed opposite results than in our study. Winter milk contained 20% less retinol compared to summer milk. The mean concentration per g fat was 9.2 µg and was dependent of the fat content. Seasonal differences in animal feeding practices may be the main cause for these differences in nutrient content. During winter and spring cows stay in stables and are mainly fed in silage. The highest levels are normally found during spring and summer, when the cows are fed on fresh vitamin rich pasture (Sauvant et al., 2002). This feeding management is not so used in the Czech Republic. The breeders are trying to compile the diet balanced during the year, but the dairy cows are often in the stable whole year and on the pasture are approximately half of the farms from the May to September.

CONCLUSION

Based on our results we can conclude that milk is good source of vitamin A and the highest concentration was observed in the ewe milk, respectively sheep's cheeses, where its content is increasing during the processing. From the perspective of the consumer our research shows an important finding that the concentration of vitamin A in farm cow's milk, which the consumer buys in milk vending machine, is high throughout the year and against expectations is rising during winter. This finding also applies to milk purchased for processing dairies. From the nutrition point of view the whole bovine milk contains approximately 192 µg of vitamin A per glass (200 mL), which provides about 24% of the daily vitamin A requirement.

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EVALUATION OF DAILY MILK PRODUCTION IN TSIGAI EWES BY SOMATIC CELL COUNT

Martina Vršková, Vladimír Tančin, Katarína Kirchnerová, Petr Sláma

ABSTRACT

The objective of our research was to study daily milk production which was affected by somatic cell count (SCC). The study was performed on a selected flock of purebred Tsigai ewes (326 animals). Regular milk yield recording was performed during the evening milking in around the middle of April, May and June. Milk samples were analyzed for basic milk composition (fat, protein and lactose) and somatic cells count. SCC were evaluated using decadic logarithm (logSCC). According to animals, the dairy ewes were divided into the four groups on the basis of individual SCC ($G_1 = \text{SCC} < 100 \times 10^3 \text{ cells.mL}^{-1}$, $G_2 = \text{SCC}$ between $100 - 300 \times 10^3 \text{ cells.mL}^{-1}$, $G_3 = \text{SCC}$ between $300 - 600 \times 10^3 \text{ cells.mL}^{-1}$, $G_4 = \text{SCC} > 600 \times 10^3 \text{ cells.mL}^{-1}$ to study the frequency of distribution of animals in selected group of ewes throughout experimental period. The average daily milk production in selected flock of Tsigai was 421.02 mL. We reached the highest daily milk production in April 476.40 mL and the highest content of fat and protein in June, while milk production was the lowest. From this flock of purebred Tsigai 76% of ewes were below $\text{SCC} 300 \times 10^3 \text{ cells.mL}^{-1}$. This SCC indicated a good health status of experimental ewes, at which 61% sheep were at the first lactation. We found a tendency to lower milk production by a higher SCC. With the increasing SCC decreased lactose content from 4.78% (G_1) to 4.32% (G_4). Reduced lactose content refers to the occurrence of mastitis and there is a need for performing bacteriological examination in milk.

Keywords: sheep; milk yield; milk composition; SCC

INTRODUCTION

The small ruminants rearing have a rich history in Slovakia. Nevertheless, this sector has always been a marginal part of agricultural. Except the production function the sheep and goat rearing has a very big importance in non-productive meaning (environmental and rural development, [Margetín et al., 2013a](#)). Ewe's milk is mainly used for making cheese in Slovakia. [Bianchi et al. \(2004\)](#) presented in their work that SCC was associated with various udder health statutes, and lactational phases were evaluated to verify their role in milk quality with regards to its cheese-making properties. In particular, udder inflammation (indirectly diagnosed using SCC) and late lactation were associated with high plasmin activities in milk that, in turn, were responsible for marked proteolytic phenomena. Casein index (ratio of casein to crude protein) was reduced in ewes with infected udders compared to milk from healthy ones. The most evident consequence of protein degradation in milk from infected udders was a significant decrease in the frequency of samples reactive to rennet. Secondly, milk from infected glands was associated negatively with curd characteristics. Although the SCC is not considered as factor influencing the price of milk, it is also an important factor determining the yield and quality of the final product ([Oravcová et al., 2007](#); [Margetín et al., 2013b](#)). Selection for milk yield, milk components and health of an ewe's udder may have an impact on the further improvement of Slovakian breeds

of local origin traditionally, i.e. Tsigai and Improved Valachian ([Margetín et al., 1995](#), [Margetín, et al., 1996](#)). In dairy cattle, detection of subclinical mastitis by milk somatic cell counts (SCC) is standard practice. In the past the suitable methods for the detection of subclinical mastitis (NK test, California Mastitis Test – CMT, Whiteside Test – WST) were rarely used in milking of sheep, although their use is generally recommended ([Bergonier et al., 2003](#); [Špánik et al., 1996](#)). In dairy ewe's instantaneous physiological and pathological thresholds of SCC ranging from $(0.25 \text{ to } 1.0) \times 10^6 \text{ cells.mL}^{-1}$, have been available since the early 1990s ([Ariznabarreta et al., 2002](#)). In sheep and goats, mastitis episodes are the main reason for culling because of sanitary problems, which occur mainly during the first 2 – 3 months of lactation ([Bergonier et al., 2003](#); [Leitner et al., 2008](#)).

[Berthelot et al. \(2006\)](#) recommends that a decision rule proposes to consider an udder as healthy if every SCC are lower than $0.500 \times 10^6 \text{ cells.mL}^{-1}$ and infected if at least two individual SCC are higher than 1 or 1.2 million cells.mL^{-1} . [Arias et al. \(2012\)](#) found in manchega sheep that milk yield was always higher for ewe with $\text{SCC} \leq 300 \times 10^3 \text{ cells.mL}^{-1}$ than for those with $\text{SCC} > 300 \times 10^3 \text{ cells.mL}^{-1}$. Subclinical mastitis should be always suspected as one of the primary causes in cases of decreased milk production in dairy flocks ([Fragkou et al., 2014](#)). In fact, coagulase-negative *staphylococci*, which are the most common aetiological agents of subclinical

mastitis (Contreras et al., 2007), are also frequent inhabitants of the skin of the udder. Most sheep mastitis occurs before the end of lactation (at the beginning of dry period) and also during the rearing lambs (Albenzio et al., 2003; Bergonier et al., 2003; Contreras et al., 2007).

Although none of the definitions of udder halves infection status would reflect perfectly the Dynamics of infections observed (one-monthly sample throughout lactation), the third one (healthy, brief and durable infections) could represent an acceptable compromise describing the diversity observed under field conditions. Thus, many halves were culture-positive only during the suckling-milking period (mainly during the first week post-partum) and never later; a strict application of the first definition could lead to consider these halves infected. Likewise, the interpretation of the bacteriological results of samples collected at the end of lactation (close to drying-off) is difficult (Fthenakis, 1994).

The objective of our research was to study the effect of parity, season and udder health on daily milk yield, milk composition and somatic cell count of Tsigai ewes in the year 2014.

MATERIAL AND METHODOLOGY

The study was performed in the pedigree breeding of purebred Tsigai ewes (326 animals) in the year 2014. We have evaluated ewes at first and second lactation. The ewes were on pasture with addition 0.5 kg grain by milking. The lambing of the ewes mostly performed in February. The ewes were machine milked twice a day after weaning of their lambs at the beginning of April. Regular milk yield recording was performed during the evening milking in around the middle of April, May and June. Individual milk samples were obtained from whole milk collection as an average sample. Milk samples from each udder were transported to the certificated Central laboratory of the Breeding Services of the Slovak Republic (Plemenárske služby š.p. Bratislava) for milk analysis. Milk samples were analyzed for basic milk composition (fat, protein and lactose) and somatic cells count (SCC). Basic milk composition was done by MilkoScan FT120 (Foss, Hillerød, Denmark) and somatic cells count were determined using a Fossomatic 90 instrument (Foss Electric, Hillerød, Denmark) after heat treatment at 40 °C for 15 min. SCC were evaluated using decadic logarithm (logSCC).

According to animals, the dairy ewes were divided into four groups on the basis of individual SCC ($G_1 = \text{SCC} < 100 \times 10^3 \text{ cells.mL}^{-1}$, $G_2 = \text{SCC}$ between $100 - 300 \times 10^3 \text{ cells.mL}^{-1}$, $G_3 = \text{SCC}$ between $300 - 600 \times 10^3 \text{ cells.mL}^{-1}$, $G_4 = \text{SCC} > 600 \times 10^3 \text{ cells.mL}^{-1}$). According the number of lactation animals were divided into two groups: first and second lactation. The sampling period was divided into three periods related to April, May, Jun, which also partially corespond to lactation stage.

The effect of above mentioned factors on milk yield, composition (fat, protein, lactose content) and on SCC was analysed by SAS/STAT 9.2 (PROC Mixed, 2009).

RESULTS AND DISCUSSION

The average daily milk production (DMP) per milking was 421.02 ml in selected flock of Tsigai. We found the average fat content of 7.72% and 6.41% protein per lactation. The DMP and milk component contents are meeting the criteria for the breed standard specifies for Tsigai breed (ZCHOK, 2014). Dairy ewe's at 1st lactation achieved 395.87 ml of daily milk production and the second one 446.17 ml. Although, the ewe's in the 2nd lactation had higher milk yield ($p < 0.05$), they reached a similar fat and protein content (Tab. 1). Nevertheless ewes on both lactations reached negligible differences in fat and protein content in addition to lactose. The significantly lower lactose content between the first and second lactation may indicate problems with mastitis in ewes while SCC is the same.

Asimilar fat content, but higher protein content was observed as compared to results of Oravcová et al. (2007). These ewes reached higher milk yield and similar components in milk in the year 2014 by comparison with 2013 (Vršková et al., 2015). From the available publications Špánik et al. (1996), Margetín et al., 1995, Margetín, et al., 1996, Margetín et al. (1998) and Oravcová et al. (2005), which researched the composition of Tsigai milk rearing in Slovakia, a positive trend of increasing milk production was observed.

The fat content differences have been observed, but the protein content has positively grown by 0.30%. Similarly, we found a positive trend in the SCC, which came to its decline. Consider a change in the technique of preparation for milking ewes (Vršková et al., 2015).

Sheep Breeder machine milked already during rearing lambs after their suckling and did not begin machine milking until weaning of lambs. Antonič et al. (2013) have found that immediately after weaning there was relatively high percentage of ewes with high SCC indicating health problem of the udder during suckling period. Increase in SCC during next two milkings could be caused by stress from weaning and starting of machine milking.

When monitoring the impact of the season, we found the highest milk yield was in April (Tab. 2). The highest content of fat and protein was observed in the month of June, when the average milk production was lower ($p < 0.0001$) compared to the April. Milk yield had decreasing trend by season ($p < 0.0001$). Components in milk had increasing trend ($p < 0.0001$). In June we observed the lowest content of lactose. These changes of the milk yield and milk composition are mainly related and could be also explained by the stage of lactation.

We found that the protein content of milk is more than 6%, in each season. Compared with a team of Antonič et al. (2013), we found a higher daily milk yield and content of components excluding lactose (0.5% less) for a similar logSCC (Tab. 2).

It was found that the protein content of milk was over 7% within each season. Last year had lower content of protein by lower milk yield (Vršková et al., 2015). Compared to Antonič et al. (2013) in April we reached increased daily milk yield and containing components, excluding lactose 0.5% less and lower SCC.

Table 1 The investigated parameters depending on the order of lactation.

	Lactation			
	First (n=198)		Second (n=128)	
	mean	S. E.	mean	S. E.
Daily Milk Production (ml)	395.87 ^a	16.71	446.17 ^b	20.19
Fat (%)	7.65	0.11	7.78	0.14
Protein (%)	6.41	0.05	6.40	0.06
Lactose (%)	4.65 ^a	0.03	4.56 ^b	0.03
logSCC	5.30	0.04	5.30	0.05

Legend: Daily Milk Production – DMP per milking

Table 2 The investigated parameters depending on the season.

	Season (months)						
	April		May		June		Significant
	mean	S. E.	mean	S. E.	mean	S. E.	
DMP (ml)	476.40	17.31	440.92	16.99	345.74	17.57	1:3, 2:3
Fat (%)	6.99	0.14	7.24	0.13	8.86	0.14	1:3, 2:3
Protein (%)	6.23	0.05	6.17	0.05	6.81	0.05	1:3, 2:3
Lactose (%)	4.55	0.03	4.46	0.03	4.81	0.03	1:2, 1:3, 2:3
logSCC	5.27	0.05	5.31	0.05	5.31	0.05	–

Legend: significant $p < 0.0001$

SCC is used to assess the health status of the cows, sheep or goats udder. The strong relationship observed between the annual geometric mean of bulk SCC and the estimated prevalence of intramammary infections can be considered as an indirect validation of our decision rule and thresholds (Lagriffoul et al., 1999). SCC was stable for each season at 250×10^3 cells.mL⁻¹ (Tab. 1). Depending on the season, there were no differences by logSCC (Tab. 2). LogSCC was balanced throughout lactation, indicating good health udder of experimental ewes. In this breeding was milked ewes during rearing lambs and thus to eliminate the stress of weaning lambs and transition to only machine milking. SCC was lower values by comparison with last year (Vrškova et al., 2015).

In Table 3, the animals were divided into groups by SCC. We found a trend toward lower milk production with higher SCC. With increasing SCC the lactose content of

4.78% (G₁) was reduced to 4.32% (G₄). This phenomenon was also statistically significant. Reduced lactose content is related to the incidence of mastitis in which microorganisms utilize lactose as an energy source. This is best observed in the groups G₃ and G₄, which have the same daily milk production, but the difference in lactose content of 0.28%.

For individual animals, the best approach has been provided by Berthelot et al. (2006). The mentioned author suggested that values $< 0.5 \times 10^6$ cells mL⁻¹ indicate a healthy mammary gland and values $> 1.0 \times 10^6$ cells.mL⁻¹ indicate a mammary gland with clinical or subclinical mastitis. Furthermore, there is no need to perform a simultaneous bacteriological examination of milk samples to confirm the problem. Values between 0.5×10^6 and 1.0×10^6 cells.mL⁻¹, according to those authors, indicate ‘suspected disease’.

Table 3 The milk yield and milk composition by SCC.

	SCC group								
	G ₁ (1), n=86		G ₂ (2), n=163		G ₃ (3), n=35		G ₄ (4), n=42		Significant
	mean	S. E.	mean	S. E.	mean	S. E.	mean	S. E.	
DMP (ml)	463.37	19.11	431.41	14.93	394.56	27.27	394.74	25.67	
Fat (%)	7.20	0.15	7.56	0.11	7.99	0.22	8.04	0.21	1:3, 4
Protein (%)	6.17	0.06	6.35	0.04	6.48	0.08	6.61	0.08	1:2, 3, 4; 2:4
Lactose (%)	4.78	0.03	4.73	0.02	4.60	0.05	4.32	0.05	1:3, 4; 2:4, 3:4

Legend: G₁ = Group1 of (SCC $< 100 \times 10^3$ cells.mL⁻¹), G₂ = (SCC between $100 - 300 \times 10^3$ cells.mL⁻¹), G₃ = (SCC between $300 - 600 \times 10^3$ cells.mL⁻¹ and G₄ = (SCC $> 600 \times 10^3$ cells.mL⁻¹), significant $p < 0.05$.

hence there is a need for performing bacteriological examination in milk. From a practical point of view, individual milk SCC are used, for subclinical mastitis control; “doubtful” ewes are grouped either with “healthy” (when farmers decide to cull “infected” females) or “infected” ewes (in order to implement a selective drying-off therapy). The lambs' mouths and milkers' hands are the sources of milk contamination (Albenzio et al., 2003). These authors have found that within 4 weeks lasting experiment there was higher SCC at machine milking of ewes when compared to suckled ones, as a consequence of higher bacterial positive samples at machine milking.

CONCLUSION

From this flock of purebred Tsigai 76% of ewes were below SCC 300×10^3 cells.mL⁻¹. This SCC indicated good health status of experimental ewes, at which 61% sheep were at the first lactation. We found a tendency to lower milk production by a higher SCC. With the increasing SCC decreased the lactose content from 4.78% (G₁) to 4.32% (G₄). Reduced lactose content refers to the occurrence of mastitis and there is a need for performing bacteriological examination in milk. However more detail study is needed to see relationship between high SCC and presence of microorganisms to better understanding thereasons the physiological and pathological SCC in the udder. Individual milk SCC represents a useful tool for the detection of subclinical mastitis in dairy ewes. It is recommended to evaluate a series of SCC, take into account the stage of lactation and use two thresholds allowing to distinguish three classes of ewes: healthy, doubtful (or briefly infected) and infected (or persistently infected).

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Comparison of selected sensory properties of wholemeal breads

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ABSTRACT

The aim of this study was to compare textural (firmness) and sensory properties (surface colour, crumb colour, crumb texture, appearance, appearance and colour preferences) between whole breads made from conventional wheat (*Triticum aestivum*, L.) and whole breads made from unconventional wheat (*Triticum aestivum*, L., winter variety Skorpion, grains with blue aleuron). Wholemeal breads (control samples with marks C1, C2 and C3) were prepared of 100, 90 and 80 % amount of conventional wholemeal wheat flour to 0, 10 and 20% amount of smooth white flour made from conventional wheat using baker's experiment. Wholemeal breads (experimental samples with marks S1, S2 and S3) were prepared of unconventional wholemeal wheat flour to smooth white wheat flour in same ratio as control samples of breads. Our results showed, that negative evaluation of some sensory attributes of breads made from unconventional wheat correlated with increasing addition of wholemeal flour of this wheat. It was found that breads made from conventional wheat (100, 90 and 80% amount of conventional wholemeal flour) were more acceptable in surface colour, colour and appearance preferences than these sensory attributes of breads made from unconventional wheat (same amount of wholemeal flour). Appearance and crumb colour of breads made from conventional wheat (100 and 90% amount of wholemeal flour) were evaluated better than these attributes of breads made from blue coloured wheat (same amount of wholemeal flour). Appearance and crumb colour of both groups of whole wheat breads (80:20) was similarly evaluated. Crumb texture of breads made from conventional wheat (90% and 80% amount of wholemeal flour) was worse than crumb texture of breads produced of same ratio of flour, but made from unconventional wheat. Breads with addition of 80% of wholemeal flour milled of blue coloured wheat were most acceptable in all of sensory attributes for evaluators. Significant difference ($p < 0.05$) of crumb firmness of breads were demonstrated between samples of breads (80:20) made from conventional or unconventional wheat. Crumbs of breads (100:0 and 80:20) made from blue coloured wheat had higher firmness than crumbs of breads (100:0 and 80:20) made from conventional wheat. But crumbs of breads (90:10) made from conventional wheat were firmer than crumbs of breads (90:10) made from unconventional wheat. Breads produced of blue coloured wheat were also more friable than breads made from conventional wheat. Although evaluation of wholemeal breads made from unconventional wheat had not better scores than evaluation of wholemeal breads made from conventional wheat, but evaluation of breads produced of unconventional wheat wholemeal flour were not below average and its market position could be very high in the future, due to the content of health benefit substances, which will be the subject of further research.

Keywords: wholemeal wheat flour; blue aleuron; firmness; sensory evaluation

INTRODUCTION

Whole grain foods are rich source of fiber, antioxidants and other nutrients, which have positive impact on human health. Fiber, mineral substances, vitamins (especially vitamin E), phytoestrogens and phenol acid are located in bran and germ of cereal grains, therefore, whole-grains products have greater benefits for human health than products made from the inner parts of cereal grain (Chaturvedi et al., 2011; Shepherd et al., 2012).

Wheat varieties with unconventional coloured grains (blue aleuron, purple pericarp and yellow endosperm) contain anthocyanins, which also protect the human body against free radicals. It is estimated that the long-term and regular consumption of products made from wheat with coloured grains would be beneficial to human health, and

thus it would be possible to define these grains as functional foods (Martinek et al., 2013).

Recent scientific studies have shown reduced risk of cardiovascular diseases, carcinogenesis, type 2 diabetes and obesity as a consequence of consumption of whole grains and foods of whole grains (Yu et al., 2013). But consumption of whole grain breads versus white breads is limited by consumers, because these products achieve lower volume, they have a rougher texture and faster staling as reported Rosell et al. (2009).

The relationship between foods and health has an increasing influence on the development of foods with functional properties (Peressini and Sensidoni, 2009). Balestra et al. (2011) reported that development of functional food is important, particularly in development of bread which will have not only physiological efficiency

for the consumer, but it will be also organoleptically acceptable for them.

The aim of present study was to assess textural (firmness) and sensory properties (surface colour, crumb colour, crumb texture, appearance, appearance and colour preferences) between whole wheat breads produced of conventional wheat (*Triticum aestivum* L.) or unconventional wheat (*Triticum aestivum* L., winter variety Skorpion, grains with blue aleuron).

MATERIAL AND METHODOLOGY

Preparation of whole wheat breads and baking (baker's experiment)

All samples of whole wheat breads were prepared by direct way using baker's experiment in laboratory conditions of Department of Vegetable Foodstuffs Hygiene and Technology, FVHE UVPS Brno, Czech Republic. The preparation of whole wheat breads and baking were performed in three repeating. Wholemeal flour of wheat with blue kernels (winter wheat variety Skorpion; provided by Mendel University Brno) was milled on laboratory mill LM 3100 (Perten Instruments AB, Sweden). Wholemeal flour (Voženílek Mills Inc., CZ) of conventional wheat were bought in supermarket. Breads dough were prepared of 100, 90 and 80 % amount of wholemeal flour (breads of unconventional flour: control samples with marks S1, S2 and S3; breads of conventional flour: experimental samples with marks C1, C2 and C3) to 0, 10 and 20 % amount of smooth white flour (Voženílek Mills Inc., CZ) using yeast (UNIFERM GmbH&Co.KG, Germany), sunflower oil (Usti Oils Inc., CZ), salt (Salt Mills PLC, CZ) and lukewarm drinking water. The amounts of ingredients for making breads are represented in Table 1. Recipe we gain from Penam a.s. Breads dough were prepared by mixing ingredients in a kneading machine Vorwerk (Vorwerk & Co. KG, Germany) for 8.5 minutes. After that, doughs matured at 31 °C in humid conditions for 15 minutes (temperature and time according recipe). Matured doughs was divided into 40 g pieces, they were hand-moulded and after that, they matured at 31 °C in humid conditions for 30 minutes. Risen pieces of doughs were placed on baking sheets, sprayed by water and they were baked at 250 °C for 15 minutes in the oven. Pieces were also sprayed by water in half of baking time and 5 minutes before ending of baking. After baking, each loaves of breads were placed on plates and they cooled in room temperature for 1 hour. Subsequently, the

determination of textural properties and sensory evaluation of breads were performed.

Texture properties of breads

The texture profile analysis (TPA) was performed with TA-XT Plus Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK). The samples were examined using a Stable Micro Systems Type (version 5.0, 9.0). The firmness of bread crumbs was determined on loaves of breads samples according to modification of AACC Method 74-09 (AACC, 1996). Modification of this method was in usage a cylindrical-shaped piston, 38 mm in diameter, for compression of all samples to 40 % of their original height. For each compression test, three loaves of bread (each sample) were used. Whole loaf of breads sample were placed centrally under the cylinder probe, avoiding any irregular or non-representative areas of crumb. A three-inch diameter compression plate was installed to the 25 kg load cell of the analyzer. A 5-kg weight was used to calibrate the 25 kg load cell prior to analysis and the setting was adjusted at a pretest speed of 1.0 mm/s, a test speed of 1.7 mm/s and a posttest speed of 10.0 mm/s.

Sensory analysis

The finished whole wheat breads were evaluated by 15 evaluators (6 males and 9 females – staffs and students of doctoral study programme of two Departments of FVHE UVPS Brno: Department of Meat Hygiene and Technology and Department of Vegetable Foodstuffs Hygiene and Technology), 24 hours after baking, using an organoleptic questionnaire. All the instructions were given to evaluators before evaluation. Prior to the sensory test, each loaf of whole wheat breads was cut into slices of 10.0 mm thick and immediately placed in plastic bags. Each bag was codified arbitrarily with three digits numbers. The bread samples were evaluated for attributes such as surface colour, crumb colour, crumb texture, appearance, appearance and colour preferences by using a five-point scale ranging from like extremely (1) to dislike extremely (5).

Statistical analysis

Data from three different experiments are reported as the means \pm standard deviation (SD) of three measurements, which were performed on three wholemeal breads (each sample). The data of texture profile analyses were subjected to Student's t-test by using Unistat software version 6.0 (Unistat Ltd., England). Differences were

Table 1 Ingredients for making breads using conventional or unconventional wholemeal flour.

Component	Samples of breads					
	C1	S1	C2	S2	C3	S3
Wholemeal flour * [g]	200	200	180	180	160	160
Smooth white flour [g]	0	0	20	20	40	40
Water [mL]	120	120	120	120	120	120
Yeast [g]	8	8	8	8	8	8
Sunflower oil [g]	8	8	8	8	8	8
Salt [g]	4	4	4	4	4	4

* wholemeal flour of conventional wheat or wheat grains with blue aleuron

considered significant at $p < 0.05$. Kruskal-Wallis one-way analysis of variance (Unistat software version 6.0, Unistat Ltd., England) were used to compare of each sensory attributes of sensory evaluations of breads made from conventional or unconventional wheat.

RESULTS AND DISCUSSION

Sensory analysis

The results of the sensory evaluation of whole wheat breads stored for 24 h at room temperature are shown in Figures 1 – 3.

Colour of surface

The surface colour of bakery products made from wholemeal flour of conventional wheat was evaluated better than surface colour of same products made from unconventional wheat. Specifically, average values for whole wheat breads made from conventional wheat were 1.9 for 100%, 1.8 for 90% and 1.7 for 80% amount of wholemeal flour. The surface colour of breads made from

unconventional wheat flour were evaluated: 2.7 for 100%; 2.6 for 90% and 2.1 for 80% of flour amount. Breads made from 80% of wholemeal flour of both conventional and unconventional wheat was evaluated as samples with the best surface colour. The worst surface colour had whole breads made from 100% of both conventional and unconventional whole wheat flour.

Appearance of breads

The appearance of breads made from 100% amount of wholemeal flour of conventional wheat was evaluated better (average points: 2.2) than the appearance of breads made from 100% amount of wholemeal flour of unconventional wheat (average points: 2.73). Whole breads made from 90% amount of conventional wholemeal flour was evaluated 1.75 points, ie. more acceptable than breads made from 90% amount of wholemeal flour of unconventional wheat (3.0 points). The appearance of bread made from 80% amount of both wholemeal flour of conventional and unconventional wheat was evaluated similarly: 2.4 for breads produced of unconventional

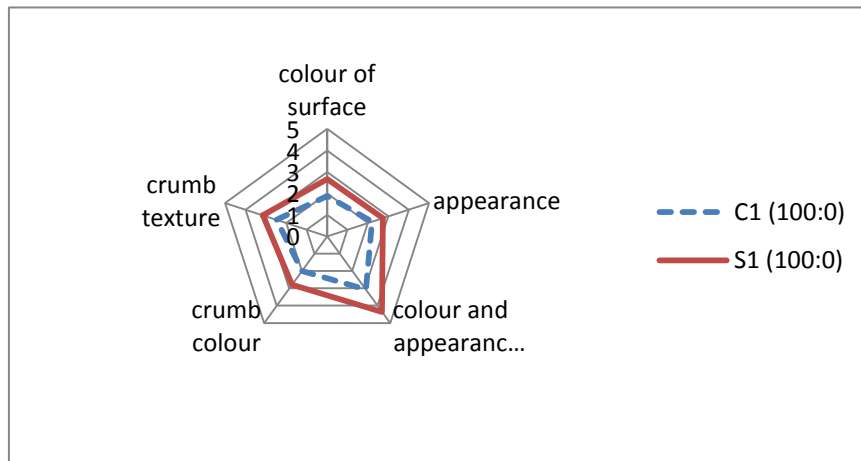


Figure 1 Comparison of sensory properties between breads made from conventional (C1) or unconventional (S1) wholemeal flour.

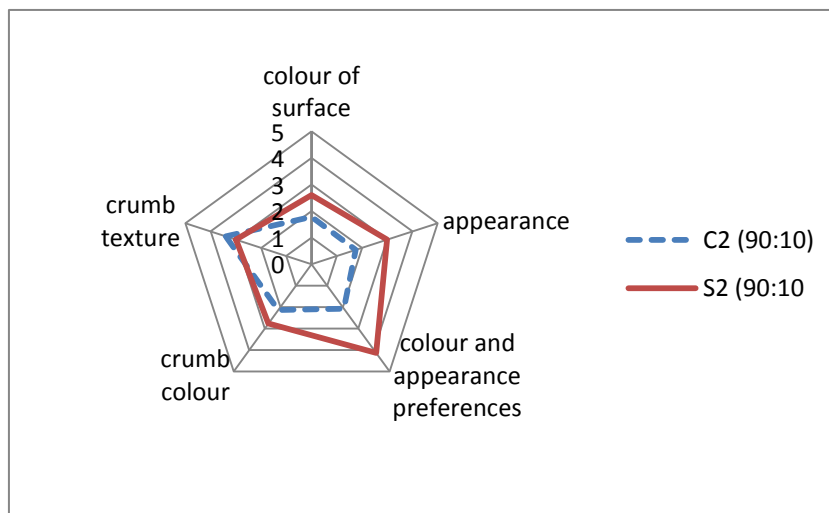


Figure 2 Comparison of sensory properties between breads made from conventional (C2) or unconventional (S2) wholemeal flour.

wheat, 2.46 for breads made from conventional wheat. The best design properties according to sensory evaluation had samples produced of conventional wheat (90% amount of flour) and samples made from unconventional wheat (80 % amount of flour). The worst appearance of breads had breads made from 80% amount of wholemeal flour of conventional wheat and samples made from 90% amount of wholemeal flour of unconventional wheat. The appearance of baked products made from unconventional wheat (samples S1, S2 and S3) with decreasing addition of wholemeal flour were evaluated more positively (data not shown), because the majority of evaluators were probably people who white wheat breads most frequently consumed. It is not accordance with work of **Challacombe et al. (2011)** who produced crackers and breads using flour of red wheat. They reported that the appearance of products (crackers and breads) made from red wheat were more acceptable than same products produced of white wheat. In their work, the majority of consumers were people who whole wheat bread most frequently consumed. They said, that it could be a contributing factor, why products of red wheat had greater acceptability for appearance.

Colour of crumb

The crumb colour of breads made from unconventional wheat was evaluated more negatively: 2.8 (100% amount of flour), respectively 2.8 (90% amount of flour) than crumb colour of breads made from conventional wheat: 2 (100% amount of flour), respectively 2.13 (90 % amount of flour). Breads made from 80 % amount of wholemeal flour of conventional wheat was rated lower (2.3) than breads made from same amount of wholemeal flour, but flour milled of unconventional wheat (2.2). The best crumb colour had breads made from 100% amount of flour (conventional wheat) and breads made from 80% amount of flour (unconventional wheat). Breads made from 90% amount of flour of conventional wheat and breads made from 100% amount of flour of unconventional wheat had the worst crumb colour. **Duchoňová et al. (2012)** evaluated crumb colour of breads made from coloured

wheat varieties (purple wheat variety: Konini and blue wheat variety: RU 440-6) and with and without the addition of β -glucans. The pleasantest crumb colour had a loaf of bread made from purple wheat variety (Konini) without the addition of β -glucans. A little less pleasant evaluation of colour of crumb (compared to the previous evaluation) had bread made from purple wheat variety with addition of β -glucans. Crumb colour of breads made from blue wheat variety with and without the addition of β -glucan was less acceptable than crumb colour of breads made from purple wheat variety.

Texture of crumb

Better evaluation of crumb texture showed breads made from 100% amount of wholemeal flour of conventional wheat (2.5 points) than crumb texture of breads made from 100% amount of wholemeal flour of unconventional wheat (3.2 points). The crumb texture of breads made from wholemeal flour of conventional wheat were evaluated worse (3.4 points for breads made from 90% amount of flour and 3.1 points for breads made from 80% amount of flour) than crumb texture of breads made from wholemeal flour of unconventional wheat (3 points for breads made from 90% amount of flour and 2.9 points for breads made from 80% amount of flour). The best evaluation of crumb texture had breads made from 100% amount of flour of conventional wheat and breads made from 80% amount of flour milled of unconventional wheat. On the other hand, breads made from 80% amount of flour of conventional wheat and breads produced of 90% amount of flour of unconventional wheat had the worst evaluation for this sensory attribute. **Duchoňová et al. (2012)** reported that more flexibility of bread crumb had bread made from purple variety of wheat (Konini) without addition of β -glucans. A little less pleasant evaluation of texture of crumb (compared to the previous evaluation) had bread made from purple wheat variety with addition of β -glucans. Crumb texture of breads made from blue wheat variety (both types of breads - with and without the addition of β -glucans) had less flexible crumb in

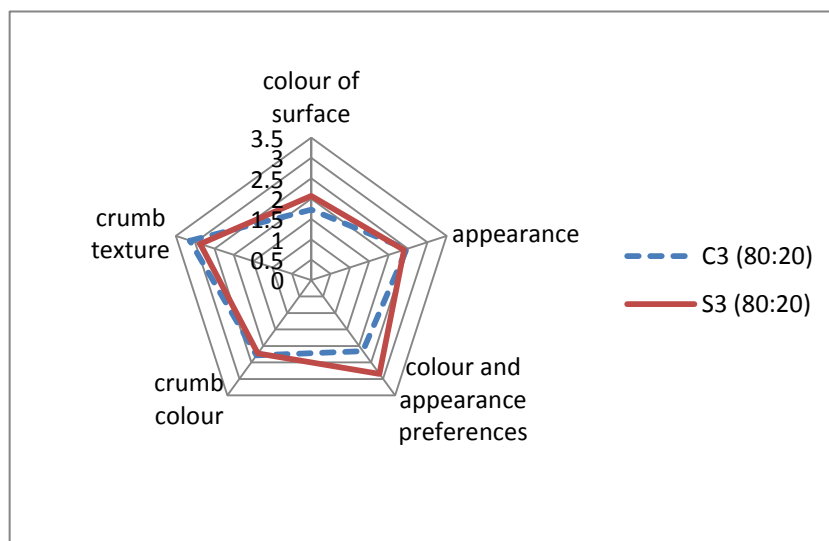


Figure 3 Comparison of sensory properties between breads made from conventional (C3) or unconventional (S3) wholemeal flour.

comparison of crumb of breads made from purple wheat variety.

Preferences of colour and appearance

Colour and appearance preferences of whole breads made from conventional wheat had better evaluation than evaluation of same attributes of whole breads made from unconventional wheat. Specifically, mean points of evaluation of breads made from conventional wheat were 3.1 (100% amount of flour), 2.1 (90% amount of flour) and 2.2 (80% amount of flour). Colour and appearance preferences of breads made from unconventional wheat were evaluated by these points: 4.4 (100% amount of flour), 4.1 (90% amount of flour) a 2.8 (80% amount of flour). Evaluators most preferred colour and appearance of breads made from conventional wheat (90% amount of flour) and same attributes of breads made from unconventional wheat (80% amount of flour). The worst acceptability of colour and appearance had whole breads made from 100 % amount of both conventional or unconventional wheat flour.

Because products made from unconventional wheat are not available in the market network in the Czech Republic, we expected that the results of scores point of preferences colour and appearance of bread made from wheat with blue aleurone will be worse. According to **Baik et Ullrich (2008)**, who said that colour and appearance of products are the indicators of food safety and quality and first factors of selection before buying and consumption of foods. Consumers prefer a specific colour for each food product. If a food product has not colour, which is expected or has a deviation in colour, product losing popularity regardless of other quality characteristics.

Texture properties of breads

Texture is an important qualitative property for assessing the quality and acceptability of fresh and processed products of food industry (Chen and Opara, 2013). The results of the texture profile analysis (TPA) of whole wheat breads stored for 1 hour at room temperature are shown in Table 2. The firmness is an important factor in bakery products since it is strongly correlated with consumers' perception of bread freshness (Onyango et al.

2010). Breads made from flour of conventional wheat (90% amount of flour) and breads made from flour of unconventional wheat (90% amount of flour) had the firmest structure (41.56 N for breads made from conventional wheat and 40.26 N for breads made from unconventional wheat). A statistically significant difference (p 0.002) was proved between mean value of firmness of breads made from 80% flour amount (conventional wheat) and mean value of firmness of breads made from 80% flour amount (unconventional wheat). The value of firmness of breads made from 80% amount of flour of conventional wheat was 24.88 N and value of firmness of breads made from 80% amount of flour of unconventional wheat was 34.79 N. Crumbs of breads (100:0 and 80:20) made from unconventional wheat had higher firmness than crumbs of breads (100:0 and 80:20) made from conventional wheat. But crumbs of breads (90:10) made from conventional wheat were firmer than crumbs of breads (90:10) made from unconventional wheat.

Whole wheat breads produced of wheat with blue aleuron showed higher friability than whole wheat breads produced of conventional wheat (data not shown). It is not accordance with results of work by **Pasqualone et al. (2015)** who baked biscuits using flour of purple wheat. They reported that biscuits produced of purple wheat had lower friability than conventional biscuits, due to higher gluten index of starting meal.

CONCLUSION

Our results showed, that negative evaluation of surface colour, appearance, crumb colour, crumb texture, colour and appearance preferences of breads made from unconventional wheat correlated with increasing addition of wholemeal flour of this wheat, in comparison to breads made from conventional wheat. Nevertheless, breads with addition of 80% amount of wholemeal flour milled of unconventional wheat were most acceptable for evaluators in all of sensory attributes. Breads produced of 100 and 80% amount of flour (unconventional wheat) caused higher firmness of crumbs than breads produced of same amount of wholemeal flour made from conventional

Table 2 Results of firmness of bread crumbs and their comparison between control samples of breads (C1 – C3) and samples (S1 – S3) of breads from wheat kernels with blue aleuron on $p < 0.05$.

Samples	Firmness of samples ($\bar{x} \pm SD$) [N]	p value
C1	30.73 \pm 1.25	0.384
S1	32.77 \pm 3.15	
C2	41.56 \pm 2.94	0.578
S2	40.26 \pm 2.25	
C3	24.88 \pm 1.21	0.002
S3	34.79 \pm 0.47	

C – control samples of breads, S – samples of breads from wheat grains with blue aleuron, C1 – 100:0, C2 – 90:10, C3 – 80:20, S1 – 100:0, S2 – 90:10, S3 – 80:20, highlighted value – significantly difference between samples, SD – standard deviation.

wheat. But crumbs of breads (90:10) made from conventional wheat were firmer than crumbs of breads (90:10) made from unconventional wheat. However, statistically significant differences between values of firmness showed breads made of 80% amount of wholemeal flours. Breads made from unconventional wheat were also more friable than breads made from conventional wheat.

Although the evaluation of whole breads made from unconventional wheat had not better scores than evaluation of whole breads made from conventional wheat, but evaluation of breads produced of wholemeal flour made from unconventional wheat were not below average and its market position in the future could be very high, due to the content of health benefit substances, which will be the subject of further research.

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THE TESTING OF SANITIZERS EFFICACY TO ENTEROCOCCI ADHERED ON GLASS SURFACES

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ABSTRACT

The aim of this work was to test the ability of 6 strains of enterococci to adhere on glass surfaces in environment with different content of milk residues and then to evaluate efficacy of 2 commercial sanitizers (alkaline and acidic) used in milk production. Tested enterococci were isolated from milk, dairy products and from rinse water after sanitation milking machine. Suspension of enterococci ($8 \log \text{CFU} \cdot \text{mL}^{-1}$) was prepared in phosphate buffered saline (PBS), PBS with content 0.1% and 1% of skimmed reconstituted milk. Glass plates were immersed into bacterial suspension for 1 h at 37 °C. The number of enterococci adhered on glass surface in PBS achieved an average value $3.47 \log \text{CFU} \cdot \text{mm}^{-2}$, in PBS with 0.1% of milk $2.90 \text{CFU} \cdot \text{mm}^{-2}$, in PBS with 1% of milk $2.63 \text{CFU} \cdot \text{mm}^{-2}$. Differences between the tested files were not statistically significant ($p > 0.05$). In the second part of work the glass plates with adhered enterococci were exposed to the effect of alkaline sanitizer (on basis of NaOH and NaClO), respectively acidic sanitizer (on basis of H_3PO_4). Sanitation solutions were prepared and tested according to manufacturer recommendations (concentration 0.25%, contact time 20 min, temperature 20 °C). Alkaline sanitation solution was 100% effective against all tested enterococci regardless to content of milk residues in environment. Acidic sanitation solution was 100% effective only against *E. faecalis*_D (isolated from rinse water after sanitation). Average value of reduction of enterococci with acidic sanitation solution, which were on glass plates in environment PBS was $2.84 \text{CFU} \cdot \text{mm}^{-2}$, in PBS with 0.1% of milk was $2.45 \text{CFU} \cdot \text{mm}^{-2}$ and in PBS with 1% of milk was $2.16 \text{CFU} \cdot \text{mm}^{-2}$. It can be concluded, that increase of milk residues in environment decrease the adhesion of enterococci on glass surface, but also effectiveness of acidic sanitation solution.

Keywords: enterococci; sanitation; glass; biofilm

INTRODUCTION

Traditionally enterococci are considered as part of the lactic acid bacteria. Like most other lactic acid bacteria, some enterococcal strains are used as starter or protection cultures or feed supplements as well as probiotics (Klein, 2003). Enterococci are present in the microbial association of a variety of fermented foods such as cheeses (Koluman et al., 2009) or meat products (Barbosa et al., 2010).

The positive influence of enterococci on cheeses, respectively on other fermented foods, seems due to specific biochemical traits such as proteolytic, lipolytic activity, citrate utilisation, and production of aromatic volatile compounds (Giraffa, 2003; Foulquie Moreno et al., 2006). Some strains of enterococci are used in “food technology” because of their ability to produce bacteriocins and to act as a starter in fermented product (Settanni and Moschetti, 2010).

Another important characteristic of genus *Enterococcus* is that enterococci are not considered “generally recognised as safe” due to its use as an indicator of faecal contamination (Foulquie Moreno et al., 2006; Cassenego et al., 2011), because they are part of humans and animals intestinal microbiota. For enterococci is typical also intrinsic resistance to some antimicrobial agents

commonly prescribed for Gram-positive cocci such as cephalosporin, lincomycin, cotrimoxazole, and low levels of penicillin and aminoglycosides (Marinho et al., 2013; Medeiros et al., 2014). Several investigations showed the occurrence of vancomycin resistant enterococci also in food of animal origin (Klein, 2003; Kročko et al., 2011; Ducková et al., 2014a). Enterococci also exhibit resistance to a wide variety of other antimicrobials, by acquisition of resistance genes via transposons or plasmids (Marinho et al., 2013; Medeiros et al., 2014).

Several studies have also shown that enterococci posse virulence determinants. Although enterococcal virulence factors are found more frequently among clinical strains, they are also detected in food isolates. Over the years several virulence factors have been identified in food enterococci which include: aggregation substances (agg), cytolysin (cyt), gelatinase (gelE), enterococcal surface protein gene (esp), cell wall adhesions (efaAfm and efaAfs) (Valenzuela et al., 2009; Barbosa et al., 2010; Jahan and Holley, 2014). One of the main factors of enterococci virulence is also the biofilm formation (Necidová et al., 2009). Biofilm production can promote increase resistance to antibiotic and other antimicrobials (Tsikrikonis et al., 2012).

The formation of biofilm creates major problem in the food industry since it may represent an important source of contamination for materials or foodstuffs coming into contact with them, so leading to food spoilage or transmission of diseases (Hamadi et al., 2013). Biofilm can be defined as matrix-embedded bacterial population adhered to a surface or to each other (Jahan and Holley, 2014). The process of bacterial biofilm formation is occurring in four depended stages. The adhesion of bacteria to surface is the first and essential stage in the formation of biofilm. The bacterial adhesion stage is associated with the production of exopolysaccharides, DNA and proteins. The initial stage of bacterial adhesion was reported to be a reversible because of the weakness of the interactions between bacteria and surfaces however this stage becomes irreversible as a result of anchoring by appendages and/or production of extracellular polymers mainly exopolysaccharides. This adhesion depends on both physicochemical properties of cell surface, and also on characteristics of the surrounding medium (Hamadi et al., 2013; Ouali et al., 2014).

Milk, the main raw material dealt with at dairies, is very good growth medium for bacteria. According to the literature, biofilm problems in the dairy process have been found in air-handling systems, cooling systems, milk transfer lines, on conveyors, in packaging machines, in heat exchangers, on ultra-filtration surfaces, in mixers, tanks and other equipment, on floor and in drains (Salo et al., 2006). It has also been found that biofilm cells of bacteria were more resistant than planctonic cells to disinfectants containing e.g. chlorine, iodine, quarternary ammonium and anionic acid compounds (Wirtanen and Salo, 2004; Salo et al., 2006).

The aim of this study was therefore to test the ability of enterococci to adhere on glass surface in environment with different content of milk residues and then to evaluate efficacy of 2 commercial sanitizers (alkaline and acidic) used in milk production.

MATERIAL AND METHODOLOGY

Tested strains of enterococci were isolated from different sources – *E. faecalis*_A and *E. faecalis*_B from traditional Slovak bryndza cheese, *E. faecalis*_C and *E. faecalis*_D from rinse water after milking machine sanitation, *E. faecium*_I from sheep milk and *E. faecium*_{II} from rinse water after milking machine sanitation.

The adhesion of enterococci to glass was determined modified method described by Carballo and Araújo (2012). Overnight cultures of enterococci (37 °C) in Tryptone Soy Broth (TSB) (HiMedia, India) were pelleted by centrifugation (4000 rpm, 20 min). Separated bacteria were washed three times with phosphate buffered saline (PBS) and then were suspended in PBS. Bacterial cell density was adjusted with PBS to 8 log CFU.mL⁻¹ by spectrophotometer. Except enterococcal suspension in PBS, were also prepared enterococcal suspensions in PBS with content of 0.1% and 1% of skimmed reconstituted milk. Glass was cut in plates 10 x 25 x 1 mm, washed and sterilized (160 °C, 4 h).

Glass plates were immersed into bacterial suspensions (4 mL) for 1 h at 37 °C. After incubation, plates were rinsed twice with 4 mL of PBS and immersed in 4 mL

TSB. Adhered enterococci were immediately released from glass plates with ultrasonic probe UP 100 H (Hielscher ultrasound technology, Germany) (30 W, 20 s). Ten-fold serial dilutions of TSB in saline were made. After 48 h incubation at 37 °C on Slanetz-Bartley agar (HiMedia, India) the number of enterococci was counted. Each experiment was performed three times.

In the second part of this work, efficacy of two commercial sanitizers was tested on enterococci adhered on glass plates. The sanitation solutions were prepared and tested according to manufacturer recommendations (concentrations 0.25%, contact time 20 min, temperature 20 °C). Both tested sanitizers are commonly used for sanitation of machine and equipments processing milk. Alkaline sanitizer contained NaOH and NaClO, acidic sanitizer contained H₃PO₄.

The glass plates with adhered enterococci, obtained as explained previously, were immersed into 4 mL of each sanitation solution for 20 min. Then the plates were washed with PBS (4 mL, twice) and the number of surviving enterococci was determined as already explained.

RESULTS AND DISCUSSION

Numbers of enterococci adhered on glass plates in environment with different content of milk residues are in Table 1.

Carballo and Araújo (2012) found out similar results with ours. They determined numbers of *Salmonella* strains attached on stainless steel after one hour incubation in TSB at room temperature in the range from 3.9 to 4.7 log CFU.mm⁻² and numbers of *Listeria monocytogenes* strains were higher in the range from 5.1 to 5.5 log CFU.mm⁻².

The biofilm forming capability of *Staphylococcus aureus* on stainless steel and glass surface verify Marques et al. (2007). Their results obtained after 15-day incubation showed biofilm formation on both surfaces with bacterial count in the order of 10⁷ CFU.mm⁻² and 10⁸ CFU.mm⁻² on stainless steel and glass surfaces, respectively.

Necidová et al. (2009) monitored the capability of enterococci to form biofilm. These authors determined biofilm formation potential in glass tubes containing suspension of tested stains (35 °C, 2 days) and after staining glass tubes by safranin solutions. The capability of forming biofilm was detected in 28% of *Enterococcus* spp. strains. Higher number of biofilm forming strains of the *Enterococcus faecium* (33%) than *Enterococcus faecalis* (28%) has been registered.

Table 1 shows effect of different concentrations of milk residues in environment to adhesion of enterococci on glass surface. It can be concluded that increase of milk residues in environment, paradoxically decreased the adhesion of enterococci on glass surface. The differences between compared numbers of enterococci were not statistically significant ($p > 0.05$).

Comparable results (average values 3.36, 2.73 and 2.52 log CFU.mm⁻² in PBS, in PBS with 0.1% of milk and in PBS with 1% of milk respectively) for the same strains enterococci adhered on stainless steel plates have been previously published by Ducková et al. (2014b).

The role of milk or milk components in inhibiting bacterial adhesion was reported previously by several works.

Barnes et al. (1999) reported that adhesion to the milk treated stainless steel varied with the organism used. With *Staphylococcus aureus*, *Listeria monocytogenes*, *Serratia marcescens* cells, attachment was reduced to levels $\leq 20\%$ of clean surface values. In contrast, *Escherichia coli* and *Pseudomonas fragi* cells adhered in small numbers to the clean stainless steel surface, with less than 1 organisms per held of view, making any effect of protein film difficult to assess.

Also **Hamadi et al. (2013)** reported that milk reduces *Staphylococcus aureus* adhesion and the level of this reduction depends on contact time. The adhesion results were interpreted in terms of hydrophobicity and electron donor/electron acceptor properties of both surfaces (cell surface, stainless steel surface).

Dat et al. (2010) and **Srey et al. (2013)** explain lower bacterial adhesion on surfaces with milk residues with repulsion between negatively charged milk proteins and equally charged surfaces of bacterial cells. Another explanation of mentioned results is the lack of nutritional substances in the environment, because according **Mah and O'Toole (2001)**, initiation of biofilm formation is the natural behavior of bacteria in nutrient deficient environment.

Tables 2 and 3 show the effect of alkaline and acidic sanitation solutions, respectively, on enterococci adhered on glass plates.

Alkaline sanitation solution containing NaOH and NaClO was 100% effective against all tested strains enterococci, which were adhered on glass plates regardless to content of milk residues in environment.

Acidic sanitation solution containing H_3PO_4 was 100% effective only against *E. faecalis_D* (isolated from rinse water after sanitation). Average value of reduction of enterococci with acidic sanitation solution, which were on

glass plates in environment PBS was 2.84 CFU.mm^{-2} , in PBS with 0.1% of milk was 2.45 CFU.mm^{-2} and in PBS with 1% of milk was 2.16 CFU.mm^{-2} . The values of reduction of enterococci adhered on glass after application of acidic sanitation solution decrease with increase of milk residues content in environment which glass plates were in contact. Effectiveness of acidic sanitation solution obviously decreased presence of milk residues.

Ducková et al. (2014b) found out similar results for the same strains of enterococci adhered on stainless steel plates. Alkaline sanitation solution containing NaOH and NaClO was 100% effective against all tested strains enterococci adhered on stainless steel. Average value of reduction of enterococci with acidic sanitation solution, which were on stainless steel plates in environment PBS was 2.76 CFU.mm^{-2} , in PBS with 0.1% of milk was 2.37 CFU.mm^{-2} and in PBS with 1% of milk was 1.97 CFU.mm^{-2} .

Trachoo and Frank (2002) reported also similar results. They found out that sanitizers containing chlorine are more effective than acidic sanitizers based on peracetic acid or mixture of peracetic and peroxyoctanoic acid against *Campylobacter jejuni* in biofilms.

The efficiency of sanitizers: hydrogen peroxide, sodium dichloroisocyanurate and peracetic acid on formation of biofilm by *Staphylococcus aureus* on stainless steel and glass surfaces tested **Marques et al. (2007)**. Peracetic acid was the most efficient in removing adhered cells, presenting 5.26 and 4.5 decimal reduction for adhered cells on stainless steel and glass surfaces, respectively.

Carballo and Araújo (2012) reported that by the manufacturer recommended concentrations of sanitation solutions (quaternary ammonium compounds, alquyldiethylenediamineglycine and di-alquyldiamineethylglycine) were not effective to kill *Listeria monocytogenes* and *Salmonella* spp., especially they were adhered to surfaces.

Krebs-Artimová et al. (2010) tested effectiveness of

Table 1 Numbers ($\log \text{CFU.mm}^{-2}$) of adhered enterococci released from glass plates after 1 h cultivation at 37 °C in different environments.

Strains of enterococci	Initial numbers of enterococci in suspension ($\log \text{CFU.mL}^{-1}$)	Adhered enterococci ($\log \text{CFU.mm}^{-2}$)		
		in PBS	in PBS with 0.1% of milk	in PBS with 1% of milk
<i>E. faecalis_A</i>	8.74	4.31	3.48	3.14
<i>E. faecalis_B</i>	8.71	3.63	3.35	3.34
<i>E. faecalis_C</i>	7.61	2.44	1.79	1.55
<i>E. faecalis_D</i>	8.71	3.46	2.90	2.65
<i>E. faecium_I</i>	8.69	3.51	3.06	2.74
<i>E. faecium_{II}</i>	8.66	3.49	2.84	2.37
<i>x</i>	8.52	3.47	2.90	2.63
x_{\min}	7.61	2.44	1.79	1.55
x_{\max}	8.74	4.31	3.48	3.34

Table 2 Effectiveness of alkaline sanitation solution (concentration 0.25%, temperature 20 °C, 20 min) against enterococci adhered on glass surface in environment with different content of milk residues

Strains of enterococci	Adhered enterococci (log CFU.mm ⁻²) on glass surface					
	in PBS		in PBS with 0.1% of milk		in PBS with 1% of milk	
	before sanitation	after sanitation	before sanitation	after sanitation	before sanitation	after sanitation
<i>E. faecalis</i> _A	4.31	-	3.48	-	3.14	-
<i>E. faecalis</i> _B	3.63	-	3.35	-	3.34	-
<i>E. faecalis</i> _C	2.44	-	1.79	-	1.55	-
<i>E. faecalis</i> _D	3.46	-	2.90	-	2.65	-
<i>E. faecium</i> _I	3.51	-	3.06	-	2.74	-
<i>E. faecium</i> _{II}	3.49	-	2.84	-	2.37	-

Table 3 Effectiveness of acidic sanitation solution (concentration 0.25%, temperature 20 °C, 20 min) against enterococci adhered on glass surface in environment with different content of milk residues.

Strains of enterococci	Adhered enterococci (log CFU.mm ⁻²) on glass surface					
	in PBS		in PBS with 0.1% of milk		in PBS with 1% of milk	
	numbers before sanitation	reduction after sanitation	numbers before sanitation	reduction after sanitation	numbers before sanitation	reduction after sanitation
<i>E. faecalis</i> _A	4.31	2.40	3.48	1.95	3.14	0.80
<i>E. faecalis</i> _B	3.63	2.45	3.35	3.30	3.34	3.32
<i>E. faecalis</i> _C	2.44	2.42	1.79	1.78	1.55	1.54
<i>E. faecalis</i> _D	3.46	3.46*	2.90	2.90*	2.65	2.65*
<i>E. faecium</i> _I	3.51	3.50	3.06	2.62	2.74	2.52
<i>E. faecium</i> _{II}	3.49	2.83	2.84	2.17	2.37	2.15
<i>x</i>	3.47	2.84	2.90	2.45	2.63	2.16
<i>x</i> _{min}	2.44	2.40	1.79	1.78	1.55	0,80
<i>x</i> _{max}	4.31	3.50	3.48	3.30	3.34	3.32

* total elimination

sanitary detergents against enterococci in planktonic form. They found out that alkaline sanitary detergent on chlorine base applied as 0.75% was sufficiently effective on enterococci damage, also at conditions with reduced temperature (40°C), in presence of organic matters (0.1% of milk) and also with water hardness 45°. Acidic sanitary detergent on base of phosphoric acid applied as 0.75% solution in combination with 40 °C temperature had 100% of effectiveness on enterococci damage only in the environment without organic matters regardless of water hardness.

Also Lavová et al. (2011) found out that presence of organic loads (1% of milk) and lower temperature decreased the sanitation effect of the sanitary detergents on

the base of NaClO or H₃PO₄ against enterococci in planktonic form. They found also a weaker powerful of acidic sanitation solution in comparing with alkaline.

CONCLUSION

It may be concluded that obtained results contribute to the better understanding of enterococci adhesion as initial phase of forming biofilm. Results also indicate that adhered enterococci can survive sanitation process, especially by using acidic sanitation solutions and in environment with residues of milk. In food and especially in dairy industry it is necessary to prevent biofilm formation and the contamination of food undesirable microorganisms by thorough cleaning and sanitation

process. The risks of enterococci biofilm formation not consist only in food contamination but also in possibility of antibiotic resistance genes transfer or other virulence factors.

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THE EFFECT OF FOOD WITH DIFFERENT GLYCAEMIC INDEX ON THE BLOOD GLUCOSE LEVEL

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ABSTRACT

Blood glucose levels are affected by many factors including the type of foods consumed, processing technology and cooking method. Hormone insulin lowers blood glucose to its constant level, while glucagon, growth hormone, adrenalin and glucocorticoids have the opposite effect. High steepness of the blood glucose level rise after meals may be unfavourable for the organism. Sugars are transferred into the blood at different speeds according to the type of food. Therefore the aim of this study was to confirm experimentally the effect of food on blood glucose levels in men and women of different ages. Two types of low, medium and high-glycaemic index (GI) foods were given to 4 men and 4 women of different age (from 35 to 65 years). All volunteers were healthy, slightly overweight, and without any regular sporting activity. None of them had any idea about their daily carbohydrates consumption and what the term glycaemic index meant. The volunteers came to the GI determination fasted in the morning. Their rise in blood glucose level was monitored by glucometer before the meal and after 1 and 2 hours of the consumption of baked potatoes (GI 85), white bread bun (GI 70), boiled potatoes (GI 64), rye bread (GI 62), potato dumplings (GI 52) and white cooked spaghetti (GI 41). Fasting blood sugar levels of volunteers highly depended on their age ($p < 0.0001$) and gender ($p < 0.0001$). The blood glucose values increased with age and were higher in men than in women. Significant influence of food GI on blood glucose levels in both men and women in all the age categories was observed ($p < 0.0001$). An interaction between age and gender was also statistically highly significant ($p < 0.0001$). One hour after consuming food the blood glucose values were significantly different from the values of fasting ($p = 0.0035$). The differences of these values did not depend on the age ($p = 0.0574$) and sex ($p = 0.8256$) of volunteers, but there was a significant difference on the GI value of food ($p < 0.0001$). Significant interactions were also found in case of sex*age ($p = 0.0002$), age*GI ($p < 0.0001$) and age*sex*GI ($p < 0.0001$). Medium correlation was found between the GI values of food and the rise of blood glucose levels after 1 h ($r = 0.6468$). After the consumption of high-GI foods the values of glycaemia did not returned to their fasting levels even after 2 hours. There was still significant difference ($p = 0.0032$), but the values after 2 h were also statistically different from those after 1 hour ($p < 0.0001$). The response to a particular type of consumed food depended on age ($p = 0.0018$) and especially the GI of foods.

Keywords: glycaemia; glycaemic index; age; gender; time after consumption

INTRODUCTION

Blood sugar (blood glucose) levels in the human body are normally maintained by regulatory mechanisms within a constant range. In a healthy fasting person its values lie between 3.5 to 5.5 mmol.L⁻¹ (Gaerth, 2003). Many factors affect these levels including physical activity, psychological stress, drugs, cyclical changes in metabolites during the day, week, month and year, pregnancy, age, gender and race. Blood glucose level increases several times during the day after food intake. Insulin acts by means of homeostatic mechanism and lowers blood glucose to its constant level, while glucagon, growth hormone, adrenalin and glucocorticoids have the opposite effect. They increase the level of sugar in the blood when it is in shortage. Blood sugar level usually reaches a maximum within 30 to 60 minutes after meal consumption to approximately 6.8 mmol.L⁻¹. It then

gradually decreases about two hours after a meal to its normal fasting levels.

Blood glucose levels are affected by the types of foods consumed (Rovner et al., 2009). The composition, content and type of carbohydrates, and also its processing technology, cooking method and the length of storage can influence blood glucose levels. Glycaemic index (GI) of food is defined as the incremental area under the two-hour blood glucose response curve following a 12-hour fast and ingestion of 50 g of reference food (either glucose giving GI units or white bread giving BE units) and multiplied by 100 (Svačina and Bretšnajdrová, 2008). The GI value of glucose is 100 by definition.

Food may be classified into three groups according to their GI. Firstly low-GI foods (GI 55 or less) releases glucose more slowly and steadily, which leads to more suitable postprandial blood glucose readings. Secondly

medium-GI foods (GI 56 – 69) rise blood glucose levels moderately. Thirdly high-GI foods (GI 70 and above) causes a rapid rise in blood glucose level and is suitable for recovery after exercise. Values of glycemic indexes for common foods may be found in **Foster-Powell et al. (2002)** or on <http://www.glycemicindex.com>.

Carbohydrates are transferred into the blood at different speeds according to the type of food and the type of carbohydrate present. High steepness of the blood glucose level rise after meals may be unfavourable for the organism. If the blood sugar raises slowly it can be gradually transferred into the cells and therefore does not contribute to the development of undesirable complications (**Frost and Dornhorst, 2005**).

Selection and consumption of carbohydrate-containing foods according to their GI can greatly influence the metabolism and physiology of the human body. This can be used for the prevention and treatment of some chronic diseases such as diabetes mellitus or obesity. Physical and mental performance can also be affected by the level of food GI (**Jenkins et al., 2004**). The low-GI foods can help to keep the blood glucose level in the desired range and thereby prevent complications of diabetes (**Frost and Dornhorst, 2000**). The low GI of food has a positive effect on HDL cholesterol and insulin sensitivity, which is related to the prevention of cardiovascular disease (**Dickinson and Brand-Miller, 2005**).

A high-GI food is a risk factor for coronary heart disease (**Denova-Gutiérrez et al., 2010**). Results of **Randi et al. (2008)** with 5830 volunteers aged 20 – 70 years also indicate a link between high-GI diet and a higher risk of thyroid cancer. Postprandial rise of glucose also corresponds to the decrease of antioxidants in blood serum. Low-GI diet may therefore have a positive impact on reduction of oxidative cell damage (**Jenkins, 2002; Leeds, 2002**). The consumption of foods with high GI also has an impact on the reduction of attention, the ability to remember and ability to concentrate (**Dickinson and Brand-Miller, 2005**). According to **Mondazzi and Arcelli (2009)** and **Little et al. (2010)**, the GI can also be an important factor in sports nutrition and weight-reduction diets.

Results of **Kong et al. (2014)** showed that overweight girls and boys aged 15 to 18 years on the diet with a low glycemic index exhibited a significant reduction in body weight and BMI. In addition waist circumference (WC) was significantly reduced. **Papadaki et al. (2010)** found that a high-protein diet with low glycemic index was effective for reduction of obesity in children aged 5 to 15 years. Similar conclusions were reported by **Gogebakan et al. (2011)** who focused on adults. The results of their study showed that a high-GI diet coupled with a sedentary lifestyle leads to an increase in body weight, body fat and WC especially in women. This was not seen in the case of women with higher physical activity. These changes were also not observed in men having a high-GI diet. This indicates some differences between men and women in relation to a diet with a high glycemic index and the development of obesity.

The aim of this study was to confirm experimentally the effect of food with different GI on blood glucose levels in men and women of different ages.

MATERIAL AND METHODOLOGY

Tested volunteers

Eight volunteers (4 men and 4 women) were involved in the experiment. They have no special education in health and nutrition sciences. One men and one woman of the same age were allocated into one of four groups (35, 45, 55 and 65 years). All of them were healthy; their Body Mass Indexes were 30-34.9 (class I obesity) (**WHO, 2000**). No individual participated in any sport activity. Prior to the experiment, the volunteers filled out a short questionnaire. The results showed that none of them had any idea about their daily carbohydrates consumption and what the term glycaemic index meant. The volunteers came to the GI determination fasted in the morning. Sampling was carried out using a finger glucometer (Accu-Chek Performa Combo, Roche Ltd.). The glycemic index was determined in the blood before the meal consumption, 1 h and 2 h after the consumption of 50 g of selected food.

Tested food samples

Two kinds of food with low, medium and high GI were chosen for the experiment. Baked potatoes (GI 85) and white bread bun (GI 70) were selected from the category of high-GI food. Boiled potatoes (GI 64) and rye bread (GI 62) were the representatives of the medium-GI category. Potato dumplings (GI 52) and white cooked spaghetti (GI 41) were chosen as low-GI meal. Each person repeated the experiment three times with each food. Altogether there were obtained 432 results (from 8 persons, 6 different foods measured three times in 3 intervals: 0, 1 and 2 h).

Statistical analysis

The data obtained were analysed using statistical software Statistica 12.0 (StatSoft Inc.). Analysis of variance (a multi-dimensional ANOVA with interactions) was performed and the significant differences in the means were separated using the Tukey's test. The data were expressed as an average of triplicates ± 0.95 confidence interval. For all statistical tests, a 5% level of significance was used.

RESULTS AND DISCUSSION

Fasting blood sugar levels of volunteers depended on their age ($p < 0.0001$) and gender ($p < 0.0001$). The blood glucose values increased with age (Fig. 1) and were higher in men (6.3 ± 0.5) than in women (5.2 ± 0.3). An interaction between age and gender was also statistically highly significant ($p < 0.0001$). The blood glucose level was above the limit values for the average healthy person in case of older volunteers, which may indicate a greater likelihood of health complications including diabetes (**Frost and Dornhorst, 2000**). According to their BMI all volunteers were slightly obese. Consumption of high-GI food could contribute to this status (**Kong et al., 2014**).

One hour after consuming food the blood glucose values were significantly different from the values of fasting ($p = 0.0035$). The differences of these values did not depend on either age ($p = 0.0574$) and sex ($p = 0.8256$). There was however, a significant difference depending on the GI value of food ($p < 0.0001$). Significant interactions were also found in case of sex*age ($p = 0.0002$), age*GI ($p < 0.0001$) and age*sex*GI ($p < 0.0001$). Differences in blood glucose levels corresponded with the GI values of

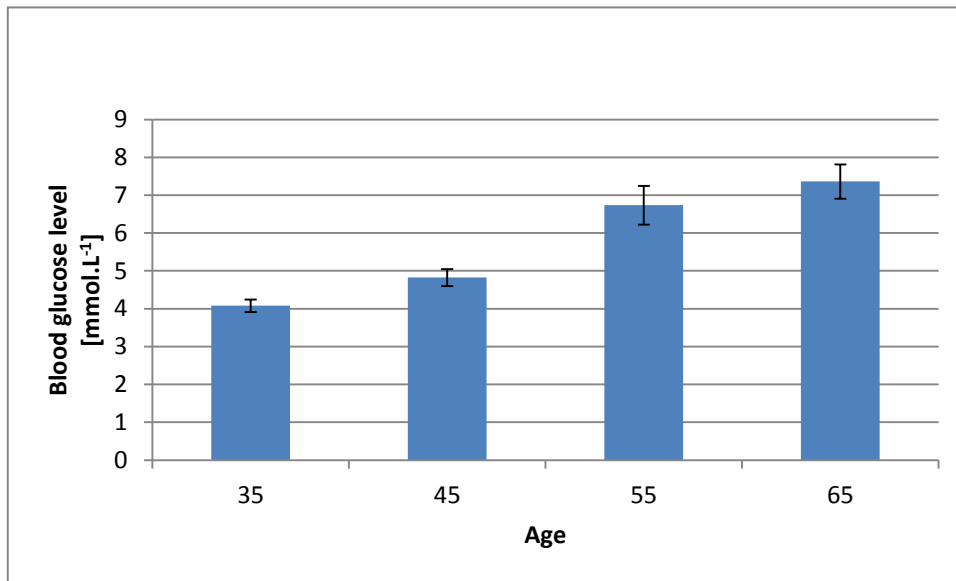


Figure 1 The blood glucose levels of fasting volunteers from different age groups.

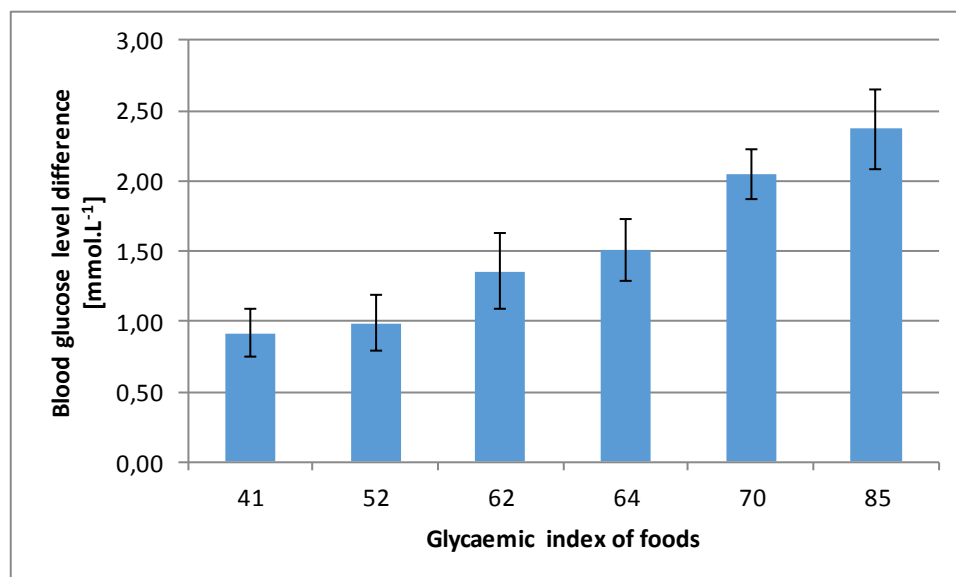


Figure 2 The blood glucose levels differences (1 h after meal – fasting values) in relation with the GI values of food.

foods (Fig. 2). The correlation coefficient between GI and blood glucose level was $r = 0.6468$. These results are consistent with the findings of **Taki et al. (2010)**. It was confirmed that a high-GI food increases blood sugar to higher levels.

According to the literature, blood glucose levels return to the fasting baseline usually within 2 hours after food consumption. In this study, however, there was still significant difference between the values after 2 h and fasting level ($p = 0.0032$). The values after 2 h were also statistically different from those after 1 hour ($p < 0.0001$).

The response to a particular type of consumed food depended on age ($p = 0.0018$) and especially the GI of foods (Fig. 3). It was noted that after consuming of high-GI food, the glucose levels return to its original state much slower than after medium and low-GI meals. This is in line with the conclusion of **Frost and Dornhorst (2000)**, who observed that foods with a low glycemic index value help maintain blood glucose in the desired range of values and thus contribute to reducing the risk of health problems including obesity and diabetes (**Jenkins, 2002; Leeds, 2002; Denova-Gutiérrez et al., 2010**).

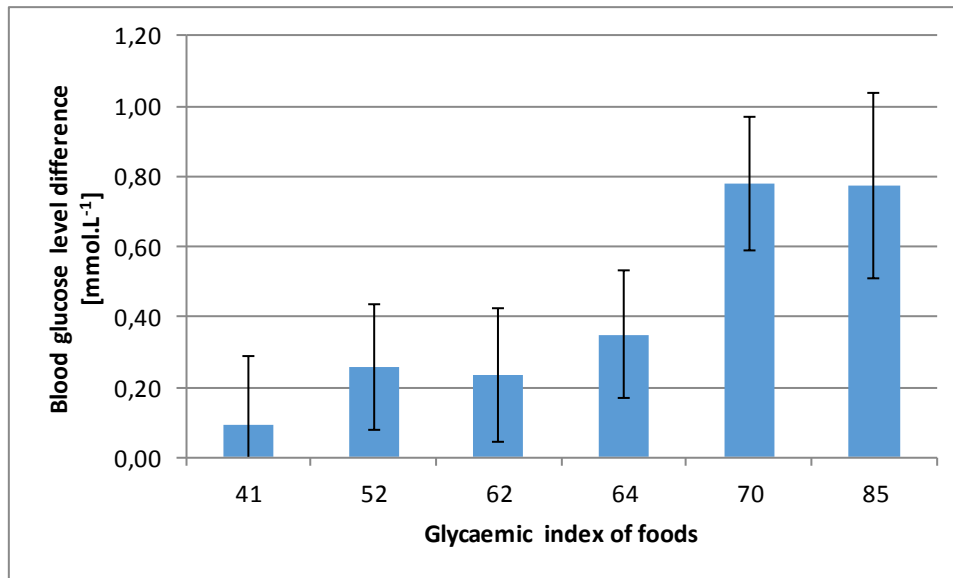


Figure 3 The blood glucose levels differences (2 h after meal – fasting values) in relation with the GI values of food.

CONCLUSION

A significant influence of food GI on blood glucose levels in both men and women in all the age categories was confirmed in this study. Sex was observed to be significant in fasting glycaemia levels only. Different responses of men and women after consumption of food were not demonstrated. Medium correlation was found between the GI values of food and the rise of blood glucose levels after 2 h. The values of glycaemia after eating foods with high GI did not return to their original values after 2 hours.

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THE PREVALENCE OF *SALMONELLA* INFECTIONS IN LAYING HEN FLOCKS PRODUCING EGGS AND THEIR IMPACT ON THE PUBLIC HEALTH

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ABSTRACT

Since 2008, Slovakia has implemented the National control program of *Salmonella* infections in laying hen flocks. This program requires the farm operators to monitor and investigate the invasive types of *Salmonella* (*S. Enteritidis* and *S. Typhimurium*) according to STN ISO 6579. The aim of this study was to perform a microbiological examination of dust and chicken droppings samples of laying hens in the Trenčín region for the presence of *Salmonella* by Horizontal method according to STN ISO 6579: 2002, to compare results with the statistics across Slovakia and selected EU countries and to evaluate the impact of official controls of salmonellosis in animals and humans. In the years 2009 – 2013 in the Trenčín region, 730 samples of dust from the conveyor belts and droppings of laying hens were taken to determine the prevalence of *Salmonella* in individual rearings. In these years, the incidence of positive samples was found from 0% to 29.17%. For the period from 2009 till 2013 was reported 22833 salmonellosis cases in human population of Slovakia, while in the Trenčín region it was 2636. Five-year EU-trend (2009 – 2013) showed a statistically significant decrease of salmonellosis occurrence (with a mean reduction of 12% per year). The Wilcoxon signed-rank test was performed in order to provide in-depth epidemiological assessment of salmonellosis cases in Trenčín region in relation to selected characters: etiological agents, transmission mechanism, age, location as well as seasonality of infection.

Keywords: *Salmonella* spp.; laying hens; eggs; salmonellosis; legislation

INTRODUCTION

The safety of eggs and egg products is at present time one of the priorities of the institutions that deal with health risks and foodborne diseases. Angelovičová et al. (2012, 2013 a,b) and Medved' and Angelovičová (2010) examined hygienic, nutritional and health quality aspects of broiler chickens, laying hens as well as eggs.

Microbial contamination on the shell eggs is one of the major factors to indicate egg quality, affecting the level of exogenous microbial contamination in the egg contents (Englmaierová and Tůmová, 2007). Cwiková and Nedomová (2014) assessed the microbiological quality of egg liquid products. Statistical analysis showed ($p > 0.05$) no correlation between seasons and microbial counts for any of the egg substances investigated.

It poses a fundamental problem in the production of eggs intended for consumers (Dev et al., 2013), particularly regarding the total aerobic count and contamination with Gram-negative bacteria from the family *Enterobacteriaceae* (Németh et al., 2011). *Salmonella* has long been recognised as an important zoonotic pathogen of economic significance in animals and humans. More than 2500 serovars of zoonotic *Salmonella* exist and the prevalence of the different serovars changes over time (e.g. *S. Typhimurium*). Non-typhoidal *Salmonella enterica* infection is one of the leading causes of gastrointestinal illness, responsible for several million human cases and

thousands of deaths worldwide each year (Clarkson et al., 2010).

Today, reliable identification of many serotypes is performing by different analytical methods, from microbiological and biochemical ones (Mirmomeni et al., 2009) up to the DNA based (Žiarovská et al., 2013).

The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals which result in a variety of foodstuffs covering both food of animal and plant origin as sources of infections (Latimer et al., 2008).

When specific control programs are performed, variations in the progress of these programs might follow a geographic pattern, leading to spatial heterogeneity. However, climate may affect maintenance and transmission of *Salmonella*, and wildlife may act as a source of infection. Under these circumstances and considering that information on geographical location is available, spatial analysis can be considered as a key step in epidemiology. The objectives include generating hypotheses on the risk factors and on processes underlying the transmission of infections (Nabila et al., 2010).

In this regard, since 1 January 2008 Slovakia has implemented the National control program of *Salmonella* infections in laying hen flocks (*Gallus gallus*) producing eggs for human consumption. It monitors the incidence of *Salmonella* in rearings and their possible transfer to table eggs. This program is mandatory throughout the territory

of Slovakia at all commercial rearings of laying hens of domestic fowl (*Gallus gallus*) producing eggs for human consumption and those eggs placed on the markets. The aim is to reduce the incidence of *Salmonella Enteritidis* and *Salmonella Typhimurium* (including monophasic *Salmonella Typhimurium* strains with the antigenic formula 1,4, [5], 12: i:-) in adult laying hens of *Gallus gallus*, which is determined as an annual reduction of positive flocks of adult laying hens by at least 10%, if the prevalence in the preceding year was less than 10%. Eradication program is evaluated annually.

The legal basis of the national control program are: **Act. 39/2007 Coll. on veterinary care, Regulation (EC) No 2160/2003, Government Regulation No 626/2004, Commission Regulation (EU) No. 517/2011, Commission Regulation (EC) No. 1177/2006.**

Slovakia is actively involved in the Early Warning Response System (EWRS) in the case of epidemiological situation emergency in the EU. The aim of the system is a rapid exchange of information on the incidence of infectious diseases and epidemics that have the potential to spread beyond countries where they start respectively, or may be a threat to the population of the EU or are rare and interesting in professional view. Slovakia cooperates in international activities in the area of foodborne diseases and zoonoses with the WHO, EFSA, and also in particular with the European Centre for Disease Prevention (ECDC) in Stockholm at the European level. In addition to sending data to the TESS (The European Surveillance System) is managed as the task under a special program of the European Food and Waterborne Diseases (FWD). Based on the FWD program, European network of special Epidemiological Information System (EPIS, since 2006) for the FWD was established in Slovakia. EPIS network is involved in the solutions of so-called "urgent inquires," what is a signal of possible threats of international epidemics. ECDC teams distribute the data to all member states including Slovakia. FWD monitors the surveillance of 6 priority diseases (salmonellosis, campylobacteriosis, VTEC-*E. coli*, yersiniosis, listeriosis and shigellosis) and partially 10 other diseases.

MATERIAL AND METHODOLOGY

The aim of the study was to perform the microbiological examination of dust and chicken droppings samples of laying hens in the Trenčín region for the presence of *Salmonella* by Horizontal method according to STN ISO 6579: 2002, to compare results with the statistics of Slovakia and selected EU countries and to evaluate the impact of official controls of salmonellosis in animals and humans. In the Trenčín region during the years 2009 – 2013, totally 730 samples of dust from the conveyor belts and droppings of laying hens were taken to determine the prevalence of *Salmonella* in individual rearings.

Epidemiological analysis of reported cases of salmonellosis was based on factual material that was obtained from the following sources:

- The Epidemiological Information System (EPIS) in Slovakia

- The Community Summary Report on Trends and Sources of Zoonoses and Zoonotic Agents in the European Union by EFSA.

We performed epidemiological analysis of reported cases of salmonellosis in the Trenčín region (Slovakia) for the period from 2009 till 2013, resp. 2000 till 2014 in relation to the selected characters: etiological agent, transmission mechanism, age, location and seasonality of infection.

Statistical evaluation of the results were performed in the program *Tanagara 1.4.43*. (Rakotomalala, Lyon, France). Based on the parameters arised from our results we chosed the Wilcoxon signed-rank test which is a non-parametric statistical hypothesis test.

RESULTS AND DISCUSSION

Since 2008, Slovakia has implemented the National control program of *Salmonella* infections (*S. Enteritidis* a *S. Typhimurium*) in laying hen flocks (*Gallus gallus*) producing eggs for human consumption by the **Regulation (EC) No. 2160/2003**. Control programs consist of effective measures to prevent, detect and control *Salmonella* on whole production line in processing of eggs mainly at the level of primary production to reduce the prevalence of *Salmonella* and risk to public health.

Table 1 summarizes the prevalence of *Salmonella* in laying hen flocks in selected EU countries in the period 2009 – 2013. The study of *Salmonella* prevalence focuses mainly on the serotypes of *S. Enteritidis* and *S. Typhimurium*. Since during the period 2008 – 2010 went three-year period, after which the results were evaluated, we present only the results of 2010.

In the following section is described a detailed overview of the samples examined in the context of the National control program for *Salmonella* infections in laying hen flocks of domestic fowl (*Gallus gallus*) producing eggs for human consumption in the Trenčín region and Slovakia during the years 2009 – 2013.

In 2009, the prevalence of *Salmonella* at laying hens in the Trenčín region was 29.17%, due to the low number of samples taken (24), of which 7 samples were observed positive. In Slovakia, 263 samples were taken and positive prevalence was found at 7.60%. In 2010 in the Trenčín region was taken 623 samples and only 5 of them were positive (0,80%). A similar prevalence was detected in Slovakia, among collected 1491 samples, in 14 positive cases the prevalence was determined 0.94%.

In 2011, in the Trenčín region under self-control of farmers and official controls of hens producing eggs for human consumption among 13 samples observed, no positive case of *Salmonella* occurred. In this year a slight decrease in the number of human infections in zhe Trenčín region was shown. Slovakia confirmed the occurrence of *Salmonella* at two rearings, being 1.14% increase compared to 2010. The number of samples taken was up to five times lower than in the previous year, which could affect the results. The situation in the EU in 2011 improved, the prevalence of *Salmonella* in laying flocks was 4.2%. Prevalence of serotypes *S. Enteritidis* and *S. Typhimurium* was 1.5%. Compared to 2010, it represents a decrease of 1.7% incidence of all serotypes of *Salmonella* spp. and decrease of 0.4% of two most common serotypes of flocks (**EFSA Journal, 2012**).

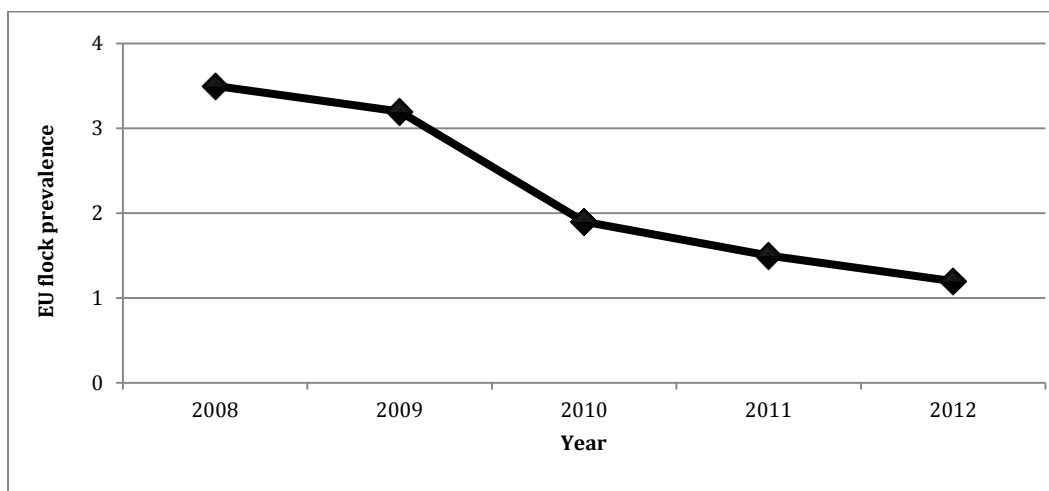


Figure 1 Prevalence of *S. Enteritidis* and *S. Typhimurium*-positive laying hen flocks of *Gallus gallus* during the production period in the EU (2008 – 2012) (EFSA Journal, 2014)

The overall prevalence of positive samples at laying hen flocks in Slovakia in 2012 was 4.68%. Compared to previous year, incidence increased of 2.6% depending on the number of samples. According to the EFSA report on zoonoses in 2012 the prevalence of *Salmonella Enteritidis* and *Salmonella Typhimurium* was 1.8%. Compared to previous year in Slovakia by the EFSA report on zoonoses in 2011, it's an increase.

The overall prevalence of positive samples at laying hen flocks in Slovakia in 2013 was 1.06%. Compared to previous year, this represents a decrease of 3.62% depending on the number of samples.

In the Czech Republic, according to the EFSA, prevalence of *Salmonella* in rearings of laying hens was detected in 2012 at around 2%, of which 1.5% were *S. Enteritidis* and *S. Typhimurium* and 0.5% were other unspecified serotypes. This means a total decrease since 2005, when the study was done based on the prevalence of *Salmonella* in laying hen flocks in the European Union and the Czech Republic ranked the fourth with the percentage of *Salmonella* in laying hen flocks with 65.6% of prevalence and 62.5% of serotypes *S. Enteritidis* and *S. Typhimurium*. Larger percentage of positive occurrence of *Salmonella* spp. in laying hen flocks was in this study recorded in Portugal (79.5%), Poland (77.2%), Estonia (73.2%) (EFSA Journal, 2013).

In Poland, from where the most often eggs are imported to Slovakia (Jamborová, 2013), was in 2012 the situation in the breeding of layer hen similar to the trend of the EU.

The incidence of *Salmonella* spp. was 4.3%, and of monitored two serotypes was 2.8%, that means a decrease in the incidence of 0.9% compared to 2011 (EFSA Journal, 2013).

As it is shown, in recent years in the Trenčín region has been observed the overall decrease in the incidence of *Salmonella* infections in laying hens flocks, but in Slovakia as well. The most important role showed the adoption of eradication programs and good cooperation of the veterinarians, medical doctors and food producers.

In terms with the above described trend of decreasing incidence of *Salmonella* in rearings of laying hens and by the implementing of national control programs for *Salmonella* infection in broiler chickens and laying hens, the next section will be focused on the epidemiological analysis of reported human salmonellosis cases in the Trenčín region and Slovakia.

Salmonellosis (A020) belongs to the diseases with the highest morbidity in Slovakia. In the Trenčín region from 2009, situation with the incidence of human salmonellosis is relatively stable and has a decreasing tendency. However, when comparing the period before the introduction of control programs, such as 2004 – 2007 and the current situation, we can see a large decrease in occurrence of these infections. While in 2004 there were found about 260 cases of salmonellosis per 100000 inhabitants, in 2013 only 75 cases of salmonellosis per 100100 inhabitants were confirmed. Total number of salmonellosis cases in the Trenčín region, but also in

Table 1 Overview of *Salmonella* prevalence (%) in laying hen flocks in selected EU countries (EFSA Journal, 2014)

	2009	2010	2011	2012	2013
Trenčín region: <i>Salmonella</i> spp.	29.17	0.80	0	2.63	6.25
Slovakia: <i>Salmonella</i> spp.	7.60	0.94	2.08	4.68	1.06
Czech republic: <i>Salmonella</i> spp.	12.80	3.20	3.20	2.00	
Poland: <i>Salmonella</i> spp.	12.90	7.00	5.50	4.30	
EU: <i>Salmonella</i> spp.	6.70	5.90	4.20	3.20	

Table 2 Overview of the samples examined in the context of the National control program for *Salmonella* infection in laying hen flocks of domestic fowl (*Gallus gallus*) producing eggs for human consumption in the Trenčín region and Slovakia in the years 2009 – 2013 (own processing).

Year	Trenčín region			Slovakia		
	Samples	Positive samples / %		Samples	Positive samples / %	
2009	24	7	29.17	263	20	7.60
2010	623	5	0.80	1491	14	0.94
2011	13	0	0	289	6	2.08
2012	38	1	2.63	534	25	4.68
2013	32	2	6.25	282	3	1.06
Total	730	15	7.77	2 859	68	3.27

Slovakia is shown in Figure 2.

The latest findings published by the EFSA regarding zoonoses in the European Union (EU) corroborate the fact that salmonellosis remains the most frequently reported zoonoses in the EU (Pires et al., 2009; Lahuerta et al., 2011). Within Europe in 2012 it was reported 91034 human salmonellosis cases, it is decrease of 4.7% compared to 2011. A statistically significant decreasing trend in the European Union was seen in the period of 2008 – 2012 as well.

Zeleňáková et al. (2012) analyzed the changes in the epidemiology of salmonellosis and campylobacteriosis diseases in Slovakia over the past 10 years and evaluated them in the context of epidemiological changes comparing to the EU. For the period from 2001 till 2010 was reported 109304 salmonellosis cases in human and 3 327 cases of *Salmonella* carriage in Slovakia. They focused on more in-depth epidemiological analysis of salmonellosis cases in Slovakia in relation to the infection agent and the outbreak of disease transmission mechanism, age and gender, location and seasonality of disease.

The epidemiologic report mapping the year 2014 is still not reported and published in the annual Community Summary Report, which covers 15 diseases. It can be

assumed that the number of human salmonellosis cases in the EU reported via BSN (Basic Surveillance Network) has decreased since 2004. However, there were member states with specific variations in trend. Although ten countries showed a significant decreasing trend (the greatest average annual decline of 28% was observed in the Czech Republic), there was still one MS, Malta that showed a significant increasing trend. Trends were not significant in the rest of 14 countries that reported data on *Salmonella* for the five consecutive years.

The incidence of salmonellosis in the Trenčín region is not different from those reported by other regions within Slovakia. The current situation is due to a strong activity of *S. Enteritidis*, which dominates in the etiology of salmonellosis and the number of cases has increased since the beginning of the last decade. The second most frequently occurring serotype of *S. Typhimurium* accounted for 3% and *S. Infantis* was 1%. Other serotypes occurred only sporadically and usually represent only a fraction of a percentage of a total (8%). As it was already mentioned, the number of salmonellosis illnesses since 2001 is gradually declining, but new serotypes of *Salmonella* are still isolated.

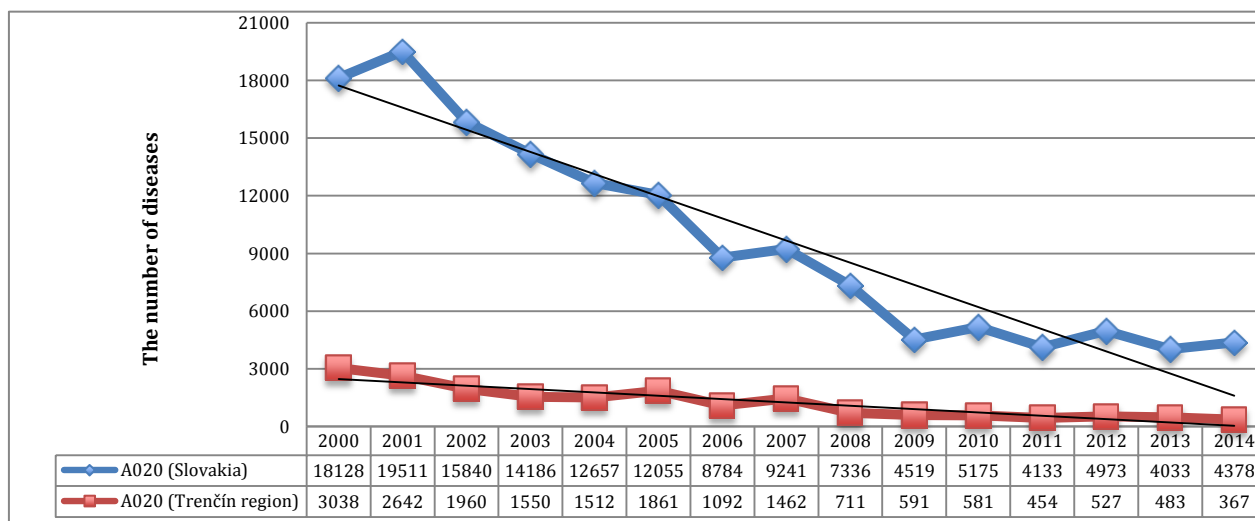


Figure 2 The trend of the human salmonellosis incidence in Slovakia as well as in the Trenčín region (2000 – 2014) (own processing)

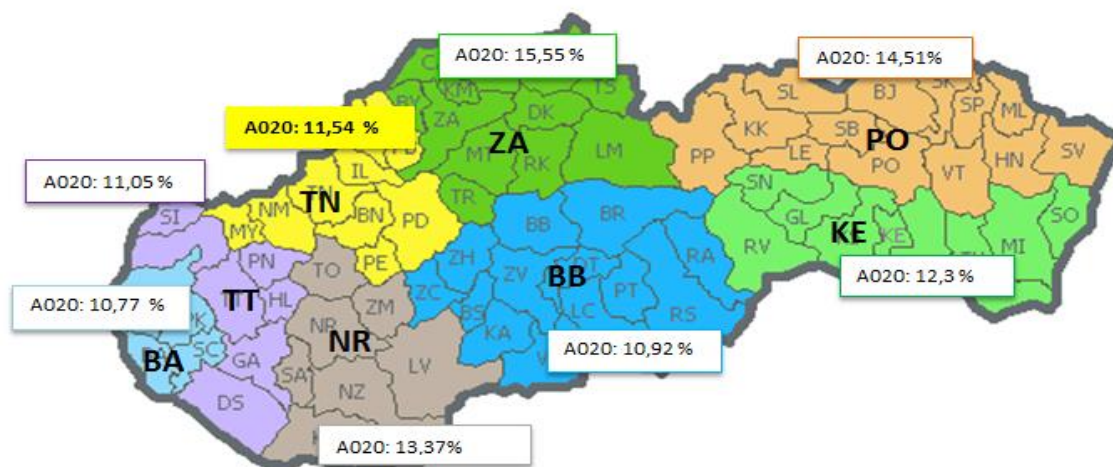


Figure 3 Regional expansion of human salmonellosis in Slovakia (2009 – 2013) (own processing)

Note. The percentages show the proportion of the number of diseases in various regions to the total number observed in Slovakia. Regions: BA – Bratislava; TT – Trnava; NR – Nitra; TN – Trenčín; BB – Banská Bystrica; KE – Košice; PO – Prešov; ZA – Žilina

The common reservoir of *Salmonella* spp. is the intestinal tract of a wide range of domestic and wild animals which result in a variety of foodstuffs covering both food of animal and plant origin as sources of infections (Montserrat and Yuste, 2010).

Salmonella spp. is an enteric pathogen associated with animal and slaughter hygiene. In the EU, eggs and egg products are the most frequently implicated sources of human salmonellosis. Meat is also an important source, with poultry and pork implicated more often than beef and lamb (EFSA Journal, 2008). *S. Enteritidis* is associated primarily with poultry and eggs. It has been observed that *Salmonella* spp. usually persists during chilling (D’Aoust-Maurer, 2007; Voetsch et al., 2004).

The analysis of single epidemics in the Trenčín region according to the place of occurrence shows that the most salmonellosis cases was detected in private (usually within the family celebrations) but also at firms, schools and open forms of catering (inadequate storage temperatures, inadequate cooking or cross contamination

of ready-to-eat food). The most common factors of *Salmonella* transmission were eggs, home and business networks, egg products inadequately treated, unpasteurised milk, water from individual sources, blended food, confectionery, cheese and poultry. Transfer factor has been proven by laboratories, but also by epidemiological investigation.

Overall, in the EU, *S. Enteritidis* and *S. Typhimurium* are the serovars most frequently associated with human illness. Human *S. Enteritidis* cases are the most commonly associated with the consumption of contaminated eggs and poultry meat, while *S. Typhimurium* cases are mostly associated with the consumption of contaminated pig, poultry and bovine meat (EFSA Journal, 2011).

Salmonella was rarely detected in other foodstuffs, such as dairy products, fruit and vegetables. Products non-compliant with EU *Salmonella* criteria were mainly observed in minced meat and meat preparations as well as in live molluscs (EFSA Journal, 2014).

Table 3 The most frequent factors of *Salmonella* transmission in the Trenčín region (2009 – 2013) (own processing).

Age	Agens - <i>Salmonella</i>	Factors			
		1	2	3	4
2009	<i>Enteritidis, Typhimurium</i>	Domestic eggs	Poultry, meat products	Fast food	Confectionery
2010	<i>Enteritidis, Typhimurium</i>	Eggs from the supermarket	Meat products	Mixed food	Ice cream
2011	<i>Enteritidis</i>	Egg products	Poultry	Fast food	Ice cream
2012	<i>Enteritidis, Typhimurium</i>	Domestic eggs	Meat products	Fast food	Confectionery
2013	<i>Enteritidis, Bovismorbificans</i>	Domestic eggs	Milk products	Milk products	Fast food

Table 4 Verification of differences in the incidence of salmonellosis in the Trenčín region (2000 – 2014) for the factor „Age“ with the Wilcoxon signed-rank test (own processing).

Age	65+	55-64	45-54	35-44	25-34	20-24	15-19	10-14	5-9
65+	-	-	-	-	-	-	-	-	-
55-64	**	-	-	-	-	-	-	-	-
45-54	NS	**	-	-	-	-	-	-	-
35-44	*	**	***	-	-	-	-	-	-
25-34	**	**	***	**	-	-	-	-	-
20-24	*	NS	***	***	***	-	-	-	-
15-19	NS	*	**	***	***	**	-	-	-
10-14	NS	*	NS	**	***	**	*	-	-
5-9	***	***	**	*	*	***	**	***	-
0-4	***	***	***	***	***	***	***	***	***

NS – non significant different, * $p < 0.05$ - significant different, ** $p < 0.01$ - high significant different, *** $p < 0.001$ - very high significant different

Investigations of geographical localization of infections show that salmonellosis was reported in all regions of Slovakia (Fig. 3). However it should be noted that slightly higher incidence of salmonellosis has been reported in the Eastern Slovakia. It can be assumed that it is due to more traditional rural way of life regarding the preference of eggs produced in the local farms. **Zeleňáková et al. (2012)** analyzed occurrence of human salmonellosis in Slovakia (2001 – 2010) from geographical aspect and by the Sheffe’s analysis observed homogeneous groups with statistically significant differences.

Salmonellosis infections in the Trenčín region (2009 – 2013) were reported in each age group (Table 4), while the highest number of 662 was recorded for 0 – 4 year old (age-specific morbidity 477/100000). For more in-depth statistical analysis was used the Wilcoxon signed-

rank test (Table 4). At the age range of 0 – 4 years, it was found very high statistically significantly difference compared to other categories. The results confirmed that the formation of salmonellosis is higher in risk groups - newborns, infants and people with lower immunity, respectively people with decreased gastric acidity, whose infection is sufficient due to substantially lower amount of micro-organisms (the infection dose is $10^6 - 10^9$ of live microorganisms in 1 g of contaminated food).

Considering the year occurrence, salmonellosis occurred with the highest incidence from May to September over the period of 2003 – 2009 in the Trenčín region. We focused on more in-depth epidemiological analysis of salmonellosis cases in Trenčín region during years 2000 – 2014 in relation to seasonality. The existence of differences in the incidence of salmonellosis in different

Table 5 Verification of differences in the incidence of salmonellosis in the Trenčín region (2000 – 2014) for the factor Seasonality with the Wilcoxon signed-rank test (own processing).

Month	January	February	March	April	May	June	July	August	September	October	November
January	-	-	-	-	-	-	-	-	-	-	-
February	**	-	-	-	-	-	-	-	-	-	-
March	NS	*	-	-	-	-	-	-	-	-	-
April	NS	***	*	-	-	-	-	-	-	-	-
May	***	***	**	**	-	-	-	-	-	-	-
June	***	***	**	***	NS	-	-	-	-	-	-
July	***	***	**	***	NS	NS	-	-	-	-	-
August	***	***	***	***	*	*	*	-	-	-	-
September	***	***	**	***	*	*	*	NS	-	-	-
October	***	***	**	**	NS	NS	NS	*	***	-	-
November	**	**	**	*	NS	*	*	*	**	*	-
December	NS	NS	NS	*	**	***	***	***	***	***	**

NS – non significant different, * $P < 0.05$ - significant different,

months of the year we performed using the the Wilcoxon signed-rank test (Table 5). We can state that the months of May, June, July and August were very high statistically significantly different compared to others.

By Zelenáková et al. (2012) during the years 2001 – 2010 in summer months, the incidence of *Salmonella* spp. ranged from 54% (2009) to 65.07% (2010) in Slovakia. Very interesting was the year of 2008, when the most infected cases were detected in February and March that was associated with rapid warming and favorable conditions for multiplication of microorganisms.

Regarding the EU situation, the number of human salmonellosis was stable over the five-year period, but the incidence was always higher during the summer months. This could be due to a seasonal effect that has not been addressed through traditional *Salmonella* control programs for food and animals (Lahuerta et al., 2011). The incidence of salmonellosis especially in the warmer months is conditional upon the most food-borne zoonotic diseases benefiting from the higher temperatures. Their optimum growth temperature is around 37 °C. In the summer season comes to faster food spoilage, especially in non-compliance with time and temperature during transportation and storage. When supplying raw materials to processing services the time plays an important role. Food contamination can be caused by humans too when a poor personal hygiene is observed (Kangas et al., 2007).

Ambient temperature may influence people's behaviour, which in turn may affect the chance of a foodborne illness occurring. For example, increased temperature may lead to elevated consumption of raw foods such as fruit and salad (at risk of cross-contamination), and higher temperatures may encourage riskier cooking practices such as barbecuing. Finally, warmer temperatures may lead to increased outdoor recreational activity which may make it more likely that people will be exposed to environmental sources of the relevant gastrointestinal pathogens. Although these illnesses are not strictly 'foodborne', routine surveillance data cannot readily distinguish between these illnesses and those which are foodborne (Tam et al., 2003).

CONCLUSION

Over the past 20 years, there has been a major change in the epidemiology of salmonellosis. A lot of factors have contributed to the change, including genetic factors, host susceptibility, antimicrobial resistance and a substantial increase in international travel and in globalization of food trade. Common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals which result in a variety of foodstuffs covering both food of animal and plant origin as sources of infections. Transmission often occurs when organisms are introduced in food preparation areas and are allowed to multiply in food, e.g. due to inadequate storage temperatures, inadequate cooking or cross contamination of ready-to-eat food. In the EU, eggs and egg products are the most frequently implicated sources of human salmonellosis.

Although in all EU countries, since 2008, health programs have been established to reduce the number of *Salmonella* positive laying flocks, eggs and poultry meat are the most common sources of human salmonellosis. In

recent years in the Trenčín region the overall decrease in the incidence of *Salmonella* infections in laying hen flocks has been observed, and in Slovakia as well. The most important role showed the adoption of eradication programs and good cooperation of the veterinarians, medical doctors and food producers. In Slovakia with 5424925 inhabitants (as reported in 31.12.2009), the epidemiological situation in the incidence of human salmonellosis is relatively favorable, but still is important very close collaboration between human and veterinary health authorities. There is a need to maintain effective surveillance and control programs, including reliable and sufficiently discriminative methods with rapid turn-around times, for providing epidemiological information on foodborne illness outbreaks and so reducing the prevalence of pathogens. This requires a collective effort by public health authorities.

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QUALITATIVE PARAMETERS OF NON-TRADITIONAL TYPES OF VEGETABLES - DETERMINATION OF NITRATES AND ASCORBIC ACID CONTENT

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ABSTRACT

The main aim of this study was to determine selected quality indicators of non-traditional types of leafy vegetables. Mizuna (*Brassica rapa japonica*), Chinese mustard (*Brassica juncea*), edible chrysanthemum (*Chrysanthemum coronarium*) and arugula (*Eruca sativa*) belonged among the selected species of vegetables. During the one-year experiment, spring and autumn sowing was carried out for these species of vegetables. The measured quality parameters were the content of nitrates and ascorbic acid. Sampling was done in the morning and in the laboratory, the samples were further processed according to the type of determination. To determine the content of nitrates and ascorbic acid, leaves were removed from plants. The filtrate from the leaves was then prepared. Determination of nitrates and ascorbic acid was carried out using a special test strip and device Rqflex plus 10. The results of measurement of both sowing varieties were compared. Total nitrate content was higher up to 22% in plants sown in the autumn except edible chrysanthemum (*Chrysanthemum coronarium*). The highest content was recorded in arugula (*Eruca sativa*), which was recently implemented to the studies of the European Union and for which there were set the limits of nitrates. Overall, the nitrate content ranged from 221 to 334 ppm in spring varieties and from 249 to 384 mg/kg in autumn varieties. Ascorbic acid content was very high in Chinese mustard (*Brassica juncea*), edible chrysanthemum (*Chrysanthemum coronarium*) and arugula (*Eruca sativa*) in both spring and autumn varieties. Values of ascorbic acid ranged from 839 in autumn sowing up to 2909 mg/kg in spring sowing. These non-traditional types of leafy vegetables could be included among the other important sources of vitamin C in the future.

Keywords: ascorbic acid; nitrate; mizuna; Chinese mustard; edible chrysanthemum; rocket

INTRODUCTION

Leafy vegetables contain many nutritionally valuable substances but also compounds that negatively affect human metabolism. Leafy vegetables are crops in which the whole leaves or their parts are eaten (Richardson, 2012), mostly fresh, heat unprocessed. Many species of leafy vegetables are grown throughout the world and thrive in different climatic regions. Generically, leafy vegetables varied greatly and the most of types can be grown during the whole year. The main parts are leaves which contain large amount of various substances. Highly valued is particularly vitamin C and A as well as E, H, K and the B group. Significant is also the mineral content, primarily K, Fe, Ca, and Zn (Pokluda, 2006; Açıkgoz 2012; Fujime, 2012). New, non-traditional kinds of vegetables come to the Czech Republic mainly from Western Europe and in recent years also from the Orient.

Mizuna (*Brassica rapa japonica*) is an annual plant, native to Asia which has at least 16 known varieties. However it thrives well even in temperate climates and tolerates frost down to -10 °C. The leaves have high content of vitamin C, potassium and calcium and are used particularly for preparing of fresh salads, cooking or pickling. Chinese mustard (*Brassica juncea*) is an annual, the longest known and most widely used of oriental

vegetables in Europe. The leaves are rich in vitamin C, they have dietetic attributes and to a certain extent they are germicidal. For its pungent flavour it is most commonly used for preparing of fresh salads. Chinese mustard can withstand winter temperatures up to -10 °C, as well as mizuna (Pekárková, 2002; Bhandari and Kwak, 2015). Edible chrysanthemum (*Chrysanthemum coronarium*) is an annual plant which is used especially in Asian cuisine. Young stems and leaves which contain a large amount of vitamins, fiber, flavonoids, calcium and potassium are used for the culinary processing. Unlike the mizuna and Chinese mustard, this vegetable is sensitive to low temperatures and thus at temperatures around zero its above-ground parts get frosted and lose their palatability (Kopeck, 2010). Arugula (*Eruca sativa*) is an annual, originally weedy plant, which has a place of origin in the Mediterranean and Asia. Thanks to its pungent taste it is used for preparing of salads. It contains lots of vitamin C and at higher doses of nitrogen fertilization also nitrate content. This vegetable is very resistant to drought and in winter can withstand temperatures up to -4 °C (Miyazawa et al., 2002; Warwick, 2011).

Nitrates are natural metabolites of plants (Pekárková, 2002) which accumulate mainly in the leaves, stems and roots, and at higher concentrations they adversely affect

the human organism. Therefore it is important to eliminate their content by providing of sufficient amount of light and moisture to the plants and reduce fertilization with high doses of nitrogenous fertilizers (Hlušek, 2004). Intake of ascorbic acid, pectins, fiber and certain minerals, which significantly suppress the conversion of nitrate to N-nitroso compounds, is also favourable (Šrot, 2005). Nitrates which are received in the usual amount together with food are not dangerous for humans (Velíšek and Hajšlová, 2009). Crops harvested in cooler conditions and grown in northern areas have higher nitrate content than summer varieties grown in southern areas (Weighman et al., 2006; Prugar et al., 2008). The highest nitrate content have leafy vegetables (EFSA, 2008; Prugar et al., 2008), where amount can be up to 6000 mg NO₃/kg (EUR-Lex, 2015). The determination of nitrates is performed by colorimetry, using ion-selective electrode (Prugar and Prugarová, 1985), HPLC/IC, enzymatically catalyzed reaction of the aqueous extract of the sample, continuous flow (ČSN, 1998) or by reflectometry (Merck, 2013).

Ascorbic acid known as vitamin C is synthesized by all autotrophic plants (Velíšek and Cejpek, 2008). It helps the absorption of iron, preventing LDL oxidation, supports the immune system of humans and inhibits the formation of nitrosamines (Brambilla and Martelli, 2007; Begum et al., 2009). The main source are citrus fruits, another important source are then a leafy and brassicaceous vegetables (Lee and Kader, 2000; Fořt, 2011), which cover about 30 – 40% of the daily intake of vitamin C (Mindell and Mundisová, 2010). There are considerable losses of vitamin C during post-harvest handling, storage and heat treatment (Lee and Kader, 2000; Toledo et al., 2003), which may be reduced by using of crops in the fresh state, by limiting the contact of vegetables with air and by freezing. The determination is performed by colorimetry (Velíšek and Cejpek, 2008), spectrophotometrically (Valášek and Rop, 2007), plarographically (Arya et al., 2000), chromatographically (Škrovánková et al., 2007) or by reflectometry.

Given the increasing popularity and occurrence of lesser-known varieties of leafy vegetables in our market was the main aim of this study assessment of selected quality indicators (content of nitrates and ascorbic acid) in mizuna (*Brassica rapa japonica*), Chinese mustard (*Brassica juncea*), edible chrysanthemum (*Chrysanthemum coronarium*) and arugula (*Eruca sativa*).

MATERIAL AND METHODOLOGY

The seeds of mizuna (*Brassica rapa japonica*), Chinese mustard (*Brassica juncea*), edible chrysanthemum (*Chrysanthemum coronarium*) and arugula (*Eruca sativa*) originated from the SEMO company were used for this research. Half of the seeds were used for spring sowing (April) and the other half of seeds was kept in dry place for the autumn sowing (August). Plants were seeded at 40 m line in 5 cm spacing between the individual seeds and 40 cm between rows. All plants were maintained by weeding, they were watered three times a week and they were not fertilized during the whole growing.

Four plants from each species and always from every quarter row were taken in the process of sampling. The plants were further processed according to the type of

determination in the laboratory. Morning sampling for nitrate determination is very important because their content in plants change significantly during the day. Especially in the warm season, nitrate reductase activity grow in plants, thereby the content of accumulated nitrates is on the decrease (Weightman et al., 2006). Plants were harvested in the morning for the determination of nitrate and ascorbic acid content.

The leaves from all plant species were removed for the determination of nitrates and a representative sample was created from these leaves. There were took 4 x 10 g of material. The sample was weighed into a 200 ml volumetric flask with a widened neck, supplemented with distilled water to 2/3 of the content of the flask and heated in a water bath at approximately 100 °C. After removing the sample was allowed to cool down, the flask was filled with distilled water to 200 ml and filtered. The filtrate was poured into a 50 mL beaker, to which was subsequently inserted a special test strip for the determination of nitrate on RQflex plus 10, which was set to Nitrate Test with a measuring range of 5 – 225 mg.L⁻¹. The strip was left in the beaker for 10 seconds and then lightly wiped dry and inserted into the apparatus, which assessed the nitrate content in mg.L⁻¹. According to the scope of the results there was made calibration solution of NaNO₃, which was measured using test strips on the device RQflex plus 10. There was created calibration curve from the measured values.

For the determination of ascorbic acid content was prepared 3% solution of 1 L metaphosphoric acid for acid extraction. The leaves from freshly harvested plants were collected, and a mixed sample was made from them. For determination was used 4 x 5 g of sample. The sample was picked and weighed into a 200 mL beaker, by pipette was added 50 mL of metaphosphoric acid, and the mixture was homogenized by mixer for 1 minute. The extract was filtered through paper and the filtrate was poured into a 50 ml beaker. Into a beaker were then inserted a special test strip for the determination of ascorbic acid on the RQflex plus 10. The device was set to the Test for the determination of ascorbic acid content with a measuring range of 25 – 450 mg.L⁻¹. The strip was left in the beaker for 5 seconds, then wiped dry and placed into a meter, which provided the ascorbic acid content in mg/l. According to extent, there were created calibration solutions of ascorbic acid of known concentration. From the measured values was made calibration curve.

Each sample was measured four times and the results were expressed as the mean ± standard deviation. The results were then analyzed using Microsoft Excel and Statistica 12 (t-test), and compared. The obtained values of nitrate content on the device RQflex plus 10 were calculated according to calibration curve. Nitrate content determined by reflectometer in mg.L⁻¹ was converted to nitrate content in mg/200 mL, which took place in determining the sample and finally through the sample size was calculated nitrate content in mg/kg (ppm). The values of ascorbic acid generated by the RQflex plus 10 were calculated using the calibration curve to a more accurate value indicating the ascorbic acid content in mg.L⁻¹. From the found values was converted ascorbic acid content in 50 mL used in determination and through a sample weight

was calculated content in mg/kg (ppm). The method is based on reflectometry, and its simplicity enables quantitative determination in the field.

RESULTS AND DISCUSSION

According to measurement results (Table 1) was found that the spring varieties contain minor amount of nitrates. This is corresponding with research of **Weightman et al. (2006)** about the favourable effect of light and heat on the reduction of nitrate content in plants. Surprisingly, the ascertained values for all crop species ranging between medium to low nitrate content, although the leafy vegetables belong to a group with a high content of nitrates. The resulting values may be influenced by the way of cultivation without fertilization using nitrogenous or other fertilizers.

Chrysanthemum, mustard and mizuna do not have yet so widespread use in Europe to make their nitrate content deal with EU legislator. It is different e.g. in wild rocket which is becoming more popular and where the nitrate level may be up to 9300 mg/kg, such as in the study of **Santamaria (2006)**. Plants measured in this work did not exceed the limit for rucola (7000 mg/kg), nor remotely came close to this limit. It is possible to affect and significantly reduce the amount of nitrates in leafy

vegetables due to climatic conditions and cultivation methods, which describe **Fontana and Nicola (2009)** in their work.

The measurement results confirm that the given types of vegetables are a good source of vitamin C (Table 2), especially arugula (*Eruca sativa*), which included nearly 3000 mg/kg. This finding not correlates with the results of **Kim and Ishii (2007)** who recorded the amount of 1420 mg of ascorbic acid per kg in leaves of arugula, and found that values are reduced by the influence of storage conditions. From the nutritional perspective it is therefore more advantageous consumption of fresh vegetables. The content of ascorbic acid in chrysanthemum, mustard and arugula was not affected by sowing period and despite the diverse values of differences in ascorbic acid content between spring and autumn sowing, both varieties seem to be a suitable source of vitamin C for human consumption. Only autumnal variation of mizuna (*Brassica rapa japonica*) showed up to two-thirds smaller amounts of vitamin C than the spring variant. These results are coincidental with measurements of **Kalisz et al. (2012)** who found that variants of mizuna sown in July had higher ascorbic acid content than those that were seeded in August. Also **Aires et al. (2011)** point out to a higher content of ascorbic acid in spring varieties for the genus

Table 1 Content of nitrates.

Plant species	Content of nitrates in mg/kg		
	Spring sowing	Autumn sowing	Difference
Edible chrysanthemum (<i>Chrysanthemum coronarium</i>)	309 ±43	249 ±41	60
Chinese mustard (<i>Brassica juncea</i>)	221 ±37	283 ±91	-62
Mizuna (<i>Brassica rapa japonica</i>)	221 ±38	277 ±56	-56
Arugula (<i>Eruca sativa</i>)	334 ±43	384 ±48	-50

Table 2 Content of ascorbic acid.

Plant species	Content of ascorbic acid in mg/kg		
	Spring sowing	Autumn sowing	Difference
Edible chrysanthemum (<i>Chrysanthemum coronarium</i>)	1890 ±59	1889 ±75	1
Chinese mustard (<i>Brassica juncea</i>)	2068 ±21	2047 ±29	21
Mizuna (<i>Brassica rapa japonica</i>)	2479 ±50	839 ±21	1640
Arugula (<i>Eruca sativa</i>)	2909 ±85	2717 ±66	192

Brassica in general. On the contrary Açıkgöz (2012) states that the vitamin C in vegetables is influenced by the seasons. He indicates in his work that the detected level of vitamin C in spring variant of mizuna (*Brassica rapa japonica*) was 702 mg/kg and in autumn variant 576 mg/kg, which correspond to our results, when ascorbic acid content was also higher in spring variant.

Ascorbic acid content detected in spring varieties ranged from 1890 to almost 3000 mg/kg which is much more than in the study of Kopta and Pokluda (2006), where the measured values ranged from 590 to 720 mg/kg. Also Bhandari and Kwak (2015) found lower values in *Brassica* vegetables. Recorded values in both experiments may vary due to the use of different methods for the determination of ascorbic acid. In this experiment, the values were found by reflectometry, while Kopta and Pokluda (2006) used HPLC for determination.

Based on the one-year experiment, there was not found correlation between the observed qualitative parameters, $r = 0.361$. Except ascorbic acid content of mizuna ($p < 0.0001$) and arugula ($p = 0.0119$), there were not found any statistical significant differences between spring and autumn sowing in volumes of nitrates and ascorbic acid.

CONCLUSION

The total content of nitrates in plants was not high, neither in spring nor in autumn varieties. In both cases of sowing the greatest amount of nitrates was found in rocket (*Eruca sativa*) but even in this case the measured values were very low. Arugula is the most widely culinary used of all determined kinds and as one of the crops mentioned above, there were set the limits for nitrates by the European Union for this plant. All types of observed vegetables showed high ascorbic acid content and thus proved to be another important source of vitamin C.

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THE INFLUENCE OF ADDITIONAL FLOURS ON THE RETENTION ABILITY OF DOUGH AND THE TECHNOLOGICAL QUALITY OF BAKERY PRODUCTS

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ABSTRACT

The work monitored rheofermentation properties of dough prepared from composite flours formed by 70% of wheat flour T650 and the addition of 30%. Three kinds of additions were used, namely spelt flour, amaranth flour and buckwheat flour. To determine rheofermentation properties Rheofermentometer Rhea F4 was used, by means of which the dough development, the production of fermentation gases, retention ability of dough and the activity of used baking yeast were analysed. The best ability to retain formed fermentation gas had wheat flour (control) and composite flour with the addition of spelt flour. The composite flour with addition of amaranth flour showed a retention coefficient compared to the control lower by 13%, and the composite flour with addition of buckwheat flour showed a retention coefficient compared to the control reduced by 20%. Control flour and composite flours were then processed in the baking experiment. Based on its results it was possible to evaluate the effect of the addition and retention capacity of dough on the quality of the final products (experimental loaves). The biggest loaf volume (200 cm³) and the optimal vaulting (0.65) were found in the control and a loaf made of composite flour with addition of spelt. Loaf volume, produced from composite flours with the addition of amaranth, and buckwheat was compared to control lower by 18.7%, and 16.3% respectively. The value of vaulting of these products (0.40) can be evaluated as unsatisfactory. Based on the evaluation of results observed by measuring on the rheofermentometer and baking experiment results it can be concluded that a better ability to retain the formed fermentation gas, thus ensuring high volume, had loaves made from wheat flour T650 and composite flour with addition of spelt flour. Based on the findings, it is possible to state that the results of rheofermentometric measurements predict the volume and vaulting of bakery products. By means of Rheofermentometer valuable information has been gained concerning the quality of bakery ingredients, especially flour and yeast.

Keywords: Rheofermentometer; dough properties; retention coefficient; baking experiment

INTRODUCTION

Cereals belong to the most important raw materials of plant origin. They are a source of carbohydrate, proteins, vitamins and minerals. Wheat and rye are bread baking basic raw materials, and the quality of bread and bakery products whose consumption varies in Slovakia at around 66 kg per person per year (the Statistical Office SR, 2014), is significantly influenced by the quality of raw materials, mainly flour and yeast.

The term pseudocereals commonly refers to seed crops used in the same way as cereals, but they do not belong to the family *Poaceae*. Compared to grains they tend to have higher levels of nutritionally relevant components, such as essential amino acids, minerals and fibre. Their application to products is reflected by a change in sensory properties, and such products have usually more interesting taste and are more attractive for consumers. Currently we can see higher interest of consumers in pseudocereals, especially in relation to the nutritional composition that is able to enrich ordinary bakery products (Ikeda, 2002; Kohajdová and Karovičová, 2008; Prugar, 2008; Kocková and Valík, 2011; Arendt et al., 2013; Bojňanská et al., 2013).

Protein of wheat flour and spelt flour, however, differ from the protein of pseudocereals flour by ability to form gluten. Gluten is a water-insoluble portion of wheat proteins (gliadins and glutenins) (Hampl, 1981; Dodok and Szemes, 1998; Rosell et al., 2007), whose peptide chains are linked together by hydrogen, disulphide and methylene bridges, and which are capable of forming in dough viscoelastic three-dimensional network. The unique properties of wheat flour to form viscoelastic dough which is able to retain gas are due to protein characteristics of wheat gluten when it is mixed with water (Roccia et al., 2009). High quality and well matured flour with high gluten content after processing produces non-sticky dough that has high water absorption ability and the ability to retain gases. Such dough provides for quality bakery products with good volume (Lazaridou et al., 2007).

Yeast bakery product cannot be prepared without bakery yeast to ferment sugar to carbon dioxide, ethanol and secondary metabolites. By that a sufficient amount of leavening gases that contribute to the desired dough structure is ensured. It is also an important contribution to the formation of aroma and taste of fermented bakery products (Szemes and Mainitz, 1999; Rezaei et al. 2014).

Yeast in addition to these characteristics, has the ability to plastify leavened wheat dough (Hampl et al., 1981; Verheyen et al., 2014), reflecting the fact that they affect the rheological properties of the dough, which have a decisive influence on the final technological quality of baked bakery products. By measuring of these characteristics the properties of baked loaves might be predicted (Campos et al., 1997). Rheofermentometer is used to estimate dough properties during fermentation, by measuring the released CO₂ or produced pressure, knowing the fact that produced CO₂ serves to expand dough and achieve the final bread loaf volume. Rheofermentometer can be used in baking industry for punctual determination of time when fermentation is finished (Torbica et al., 2008).

The aim of the research was to study the rheofermentation properties of dough prepared from composite flours made up of wheat flour T650 (70%), and the addition of 30%, and evaluate changes caused by addition in the ability of dough to retain fermentation gases.

MATERIAL AND METHODOLOGY

The work studied the impact of composite flours on the retention properties of flour dough comparing the results with objective baking properties of the baked bakery products. For the production of dough the wheat flour T650 (Mill Pohronsky Ruskov, Inc.) was used, which was a control sample. Composite flours were created by mixing wheat flour T650 (70%) with addition of flour (from spelt – A, amaranth – B and buckwheat – C) in an amount of 30%. Spelt semi-coarse wholemeal flour and buckwheat flour were from the producer J. Vince Ltd., amaranth wholemeal flour from J. Kroner Ltd.

In flours content of minerals,% (ICC Standard No. 104/1 (1990) and crude protein content (Nx5.7),% (ICC Standard No. 105/2 (1995) was determined). An important ingredient – fresh pressed bakery yeast came from the production of Old Herold Hefe, s.r.o., Trenčín, Slovakia. All of flours and yeast were purchased commercially.

Additionally, rheofermentometer studies were used to analyse dough development according to fermentation time and gas production and release of gas from the dough. The recipe of dough tested in rheofermentometer comprised of the portion of flour, or composite flour in an amount of 250 g and of fresh yeast in an amount of 2.8%. The dough was kneaded in the farinograph. After the first

minute of dough making the salt was added in an amount of 2%, and mixing continued for six more minutes. The volume of water added depended on the water absorption of flour (to create dough with optimal consistency). Subsequently was the test dough (315 g) inserted in Rheofermentometer Rhea F4 (Trippetta & Renaud Chopin, Villeneuve-la-Garenne, France) (AACC Method 89-01.01), in which following properties were established during the three-hour test: *the total volume of gas formed* (cm³) *the volume of gas leaked to environment* (cm³), *the volume of gas retained in dough* (cm³) and *the retention coefficient R* (%) which is the ratio of the volume actually detained in dough to the total volume of formed CO₂.

Baking experiment was conducted by the standard method used in the workplace: to flour or composite flour, weighing 500 g yeast (4%), sucrose (1%), salt (1.8%) were added and water according to water absorption of flours determined by farinograph. The dough was processed in a laboratory mixer Diosna SP 12. Then the dough was shaped into loaves, which were rising in the rising room for 20 minutes at 30 °C and then baked at 240 °C for 20 minutes in a furnace Miwe Condo. The baked loaves were evaluated by objective methods, establishing *volume of products* (cm³), a *specific product volume* (cm³/100g⁻¹), *baking loss* (%) and *cambering* (the ratio between height and width).

RESULTS AND DISCUSSION

Bread and pastries are the source of the energy from polysaccharides and proteins, and their consumption is an important part of the Central European eating habits. Therefore, the demands for the technological quality of these products are high. The practical significance of gluten for bakery technology lies in the fact that during the dough development it retains fermentation gas, allows dough rising, its thorough baking and the pore structure of a bakery product (Bojňanská, 2004; Gajdošová and Šturdík, 2004). Flours other than of wheat raw materials cannot be used alone for the production of bread, pastries, and other fermented products. As these flours do not contain gluten forming protein, they can be used only in certain amounts as additional flours (Palenčarová and Gálová, 2010) to the wheat flour. The reason for their addition is mainly nutritional enrichment of bakery products. This was confirmed and selected results are shown in Table 1.

All additional flours (A, B, C) had higher ash content as compared to the wheat flour; the maximum level was in

Table 1 Content of crude protein and ash content in used flours

Flour sample	T650 control	A	B	C
Crude protein, %	13.4	12.3	15.9	7.6
Ash content, %	0.48	1.3	2.3	0.7

Legend:

- A – spelt flour
- B – amaranth flour
- C – buckwheat flour

amaranth flour and spelt flour. As whole grain flours were used, they contained the coating grain layers rich in minerals. For amaranthus high ash content is characteristic and phosphorus, potassium, magnesium and calcium dominant minerals in the raw seeds (Gamei et al., 2006). In spelt wheat the content of zinc, iron and selenium is important (Gomez-Becerra et al., 2010; Zhao et al., 2009).

Amaranth is a raw material with high content of crude protein. The advantage of amaranth grains compared to conventional cereals is a relatively high content of proteins and more balanced composition of essential amino acids. Amaranth grain is rich in lysine, methionine, arginine and tryptophan (Michalová, 1999; Písaříková et al., 2005; Mota et al., 2014). The flour of spelt wheat, and buckwheat contain in comparison to wheat flour lower amount of crude protein. From a nutritional point of view proteins in pseudocereals can be classified as very valuable, because the portion of albumins and globulins in their seeds is about twice that of wheat (Mota et al., 2014). Písaříková et al. (2005) indicate that the index of essential amino acids EAAI has in amaranth value of 90.4% while in wheat values EAAI are around 50 – 55%. Mixing of wheat flour, in which the limiting amino acid is lysine with flour from pseudocereals in which the proportion of lysine is approximately twice as much, might improve amino acid profile of bakery products (Fornal, 1999; Ikeda, 2002; Arendt et al., 2013). Bread made with the addition of pseudocereal flours has a fuller flavour and

is nutritionally more varied (Mashayekh et al., 2008; Dhinda et al., 2012; Ma, 2013; Sanz-Penella et al., 2013; Biney et al. 2014).

By the means of rheofermentometer the retention capacity of formed composite mixtures (70% flour T650 + 30% additional flour) was compared with the control flour T650 (Table 2).

The data in Table 2 show that the control sample produced during the test the smallest volume of the fermentation gas, but was able to retain it in a very large quantity (84.4%). The volume of gas formed in dough of the composite flour with the addition of spelt wheat (A) was higher by 12.5%, with the addition of amaranth (B) by 21%, and with the addition of buckwheat (C) as much as by 27.3% compared to the control.

The volume of gas leaks was lowest in control and then in the composite flour with the addition of spelt, which resulted in the highest retention coefficients. The amount of gas leaks in a composite flour with the addition of amaranth was higher by 105% compared to the control, and in the flour containing buckwheat flour the volume of gas leaks was higher by 165% compared to the control. Doughs with addition of pseudocereals had significantly lower retention coefficient, mainly due to a lower proportion of gluten in the flour composition. The retention coefficient of the finest flours obtained from the endosperm, which are low-milled, has a value close to 100%, in high-milled flours with a higher content of outer grain layers reaches this value about 50% (Chopin

Table 2 Rheofermentometric properties of composite flours

	control	A	B	C
Total volume, cm ³	1408.0	1584.0	1705.0	1792.0
Volume of CO ₂ lost, cm ³	219.0	318.0	451.0	580.0
Retention volume, cm ³	1189.0	1267.0	1254.0	1212.0
Retention coefficient, %	84.4	80.0	73.5	67.6

Legend:

- control – 100 % wheat flour T650
- A – 70 % T650 + 30 % spelt wheat flour
- B – 70 % T650 + 30 % amaranth flour
- C – 70 % T650 + 30 % buckwheat flour

Table 3 Selected results of baking experiment

	control	A	B	C
Product volume, cm ³	200.0	200.0	162.5	166.3
Specific product volume, cm ³ .100g	231.4	226.7	192.0	196.5
Baking loss, %	14.6	14.6	15.8	17.9
Cambering	0.65	0.65	0.4	0.4

Legend:

- control – 100 % wheat flour T650
- A – 70 % T650 + 30 % spelt wheat flour
- B – 70 % T650 + 30 % amaranth flour
- C – 70 % T650 + 30 % buckwheat flour

Technologies: Methods and Equipments, 2013). Lower amount of gluten forming proteins in composite flours has a negative impact directly on a quality of final products, but also on the rheological properties of doughs and their ability to retain fermentation gases (Wiesner, 2007).

The best ability to retain formed fermentation gas was found in a control and in a sample A (with the addition of spelt wheat), despite the fact that in those doughs the smallest volume of gas was formed during the test. Gas forming ability of flours is significantly affected by the amount of originally present (pre-existing) sugars in them (Muchová, 2005; Bojňanská et al., 2013), and precisely composite flours with the addition of amaranth and buckwheat have a lower gluten content and higher originally present sugar content (Michalová, 1999; Steadman et al., 2001, Bojňanská, 2004, Muchová 2005; Steertegem et al., 2014).

Poor retention is an indication of an insufficient ability of dough to retain gas produced during fermentation, and it manifests itself by less volume of baked products. Table 3 shows the results of baking test, in which the same composite flours were used as in the rheofermentometric evaluation.

Results of baking experiment, which are shown in Table 3 indicate that the best breadmaking quality of the baked products was observed in the control and the sample A (spelt wheat), mainly thanks to the largest volume and specific volume of products. The volume of the loaves prepared from composite flours (B and C) was by 18.7%, and by 16.8% respectively lower than in control loaves. Best cambering was found in the control and in the sample with spelt wheat, unlike the loaves with amaranth and buckwheat, in which cambering can be identified according Muchová (2005) and Hampl et al. (1981) as unsatisfactory. Losses during baking are a natural part of making bread and pastries, but values above 15% are not desirable. The control and the sample with the spelt wheat the losses were adequate, and both samples had optimal retention coefficient. In samples with the addition of amaranth and buckwheat their lower retention capacity manifested itself by higher losses during baking. Overall, the best breadmaking quality was observed in control and in a sample of composite flour with an addition of spelt wheat.

CONCLUSION

Based on the evaluation of results obtained by measuring on rheofermentometer and baking experiment results it can be concluded that a better ability to retain the formed fermentation gas, thus ensuring high volume, had loaves made from wheat flour T650 and composite flour with the addition of spelt wheat at 30%.

Based on the findings, it can be stated that the results of rheofermentometric measurements predict the volume and vaulting of bakery products. By means of rheofermentometer valuable information about the quality of baking raw materials were obtained.

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THE QUALITY OF PROCESSED CHEESES AND CHEESE ANALOGUES THE SAME BRAND DOMESTIC AND FOREIGN PRODUCTION

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ABSTRACT

Processed cheeses belong to Slovakia favorite dairy products. Processed cheeses are made from natural cheeses. In recent years the trend is to replace natural cheeses with other raw materials of non-dairy nature. The composition of the processed cheese analogues is not in many countries defined by legislation. The objective of this study was to determine and compare chemical properties (fat, dry matter, fat in dry matter, NaCl) two samples of processed cheeses (C, D – Veselá krava) and two samples cheese analogues (A, B - Kiri) the same brand domestic and foreign production. The evaluated was taste of processed cheeses and cheese analogues, too. Chemical analysis and sensory analysis were repeated four times. The results of chemical analysis shows that all rated samples processed cheeses made on Slovakia fulfilled demands declared (dry matter and fat in dry matter) as producers provided on the label. The most commonly fluctuate content of NaCl from 1 to 1.24 g.100g⁻¹. The higher coefficient of variation in the determination of NaCl (3.88%) was found in processed cheeses made in France. Processed cheese and cheese analogues made in France had not specified parameters for dry matter and fat in dry matter on the label. For production cheese analogues Kiri made in Slovakia was used different raw material than Kiri made in France. The taste of products was determined by descriptors – salty, slightly sweet, milky, buttery-creamy, fatty, sour, bitter, and unknown. The interesting that Kiri made in Slovakia had stronger milky and buttery-creamy taste than cheese analogue Kiri made in France. Significant differences were found in the slightly sweet taste of processed cheeses, the most points won processed cheese Veselá krava made in Slovakia.

Keywords: processed cheese; cheese analogues

INTRODUCTION

The term “processed cheese” describes a dairy product made by heating a mixture of various cheese types of different degrees of maturity in the presence of appropriate emulsifying salts (mostly sodium phosphate, polyphosphates, citrates and/or their combinations), usually under reduced pressure (vacuum) with constant stirring, commonly in the temperature range of 90 – 100 °C, until a smooth and homogenous compact mass is formed with desired textural properties (Kapoor and Metzger, 2008; Sołowiej et al., 2014; Salek et al., 2015).

Key components for the production of processed cheeses are emulsifying salts (ES). Application of ES has several functions in the creation of a stable emulsion (homogenous and not phase separated), until spray drying for cheese powder, or during cooling and storage for processed cheese, e.g., binding of Ca²⁺, pH adjustment, casein dispersion and fat emulsification (Nagyová et al. 2014; Hougaard et al., 2015).

The products from natural cheeses in that they are not made directly from milk (or dehydrated milk), but rather from various ingredients such as skim milk, natural cheese, water, butter oil, casein, caseinates, other dairy ingredients, vegetable oils, vegetable proteins and/or minor ingredients. The two main categories, namely pasteurized processed

cheese products (PCPs) and analogue cheese products (ACPs) (Guinee et al., 2004).

There are various types of PCPs (e.g., processed cheese, cheese spread, and cheese foods) defined by national legislation. Such legislation defines the composition, natural cheese content (ranging from 51 to about 96% of the final dry matter), and permitted ingredients for the different types. Optional ingredients may include dairy ingredients, condiments, flavors, colors, and preservatives (Guinee, 2011). Cheese analogues are usually defined as products made by blending individual constituents, including non-dairy fats or proteins, to produce a cheese-like product to meet specific requirements. They are being used increasingly due to their cost-effectiveness, attributable to the simplicity of their manufacture and the replacement of selected milk ingredients by cheaper vegetable products (Cunha et al., 2010).

Sales of cheese analogues are closely linked to developments in the convenience food sector, where they extend the supply and lower the cost. Moreover, there is an ever-increasing interest among consumers in food products which contain less total fat, saturated fat, cholesterol, and calories. Development of cheese analogues involves the use of fat and/or protein sources other than those native to milk, together with a flavour system simulating as closely as possible that of the natural product. It is also necessary to develop a suitable processing regime capable of

combining these elements to provide the required textural and functional properties (Bachmann, 2001).

Regarding processed cheese, several studies have been carried out on sensory characterization in relation to processing factors and chemical composition. Their effects on structure, texture and rheological properties have been studied in order to improve our understanding and obtain acceptable products (Hanaei et al., 2015).

The objective of this study was to determine and compare chemical properties (fat, dry matter, fat in dry matter, NaCl) two samples of processed cheeses (C, D – Veselá krava) and two samples cheese analogues (A, B - Kiri) the same brand domestic and foreign production.

MATERIAL AND METHODOLOGY

Material

For evaluation were used 2 samples of processed cheeses – C, D and 2 samples of cheese analogues – A, B the same brand. Composition of cheeses showed Table 1.

Chemical analysis

Dry matter was determined by drying at 102 ± 2 °C according to ISO 5534:2004.

Fat content was measured by the method of van Gulik according to ISO 3433:2008.

Fat in dry matter was determined by mathematical calculation.

NaCl content in cheeses was determined by methodology described by Cvak et al. (1992).

Sensory evaluation

Sensory analysis was attended by five lay tasters. The taste of products was determined by descriptors – salty, slightly sweet, milky, buttery-creamy, fatty, sour, bitter and unknown. The rating was done by assigning points of 0 to 5. The descriptors were described by Horčín (2002).

Chemical analysis and sensory analysis were repeated four times.

RESULTS AND DISCUSSION

Chemical analysis

The results of the selected chemical parameters of cheeses shown Tables 2.

As shown in Table 2 the composition of cheese analogue Kiri produced in Slovakia was almost unchanged. The evaluated product has declared on the package label the fat content in the dry matter of 65% and 41.5% dry matter. Our results confirm (given the accuracy of the methods) declare content of the fat in dry matter. The dry matter of the analyzed products was even higher than the government requirements. Unchanging salt content in these products probably related with the fact, that the base formula of the product was formed by a curd and not by the sweet natural cheeses. Especially for sweet natural cheeses contain higher amount of salt, which is influenced by more factors. From that reason content of salt in sweet cheeses fluctuates, which of course must also be reflected in various salt content in processed cheeses.

On the package label of the Kiri product (made in France) were not declared content of dry matter and content of fat in dry matter, except content of salt. Consequently, it was not possible to determine whether it satisfies the quality. Since it is the same brand name of one company (sample A was produced in Slovakia and sample B in France), so the values could be compare with each other. In the comparison of these two products was detected the same content of NaCl, while in the products from the French production, salt content fluctuated significantly. Non significantly higher content of fat and fat in dry matter were determined in the Kiri product, which was made in France. The interesting point of this comparison was that both products were manufactured from different raw materials – Table 1.

In the product Veselá krava (made in Slovakia) often fluctuated fat in dry matter and NaCl content. The evaluated product has declared on the package label fat content in the dry matter 45% and dry matter content 40%. Our results confirm these declared parameters.

Table 1 Composition samples of processed cheeses and cheese analogues declared on the label

Sample	Materials	Composition
A	curd 76% (50% cream), water, butter, milk proteins, emulsifying salts: E452, E341, E331, E330, salt, milk protein concentrate, thickener: E407	fat in dry matter 65%, dry matter 41.5%
B	cream cheese (50%), cream (27%), water, milk protein, emulsifying salts: E341, E452, E339, E331, sodium chloride, mineral substances, milk, stabilizers: E407. The salt content of at least: 0.7%.	not specified
C	skimmed milk, cheese, butter, emulsifying salts: E452, E341, E450, E330, milk protein, salt	fat in dry matter 45%, dry matter 40%
D	skimmed milk, cheese, butter, emulsifying salts: E452, E341, E450, E330, milk protein, salt	not specified

A – Kiri, Slovakia; B – Kiri, France; C – Veselá krava, Slovakia; D – Veselá krava, France;

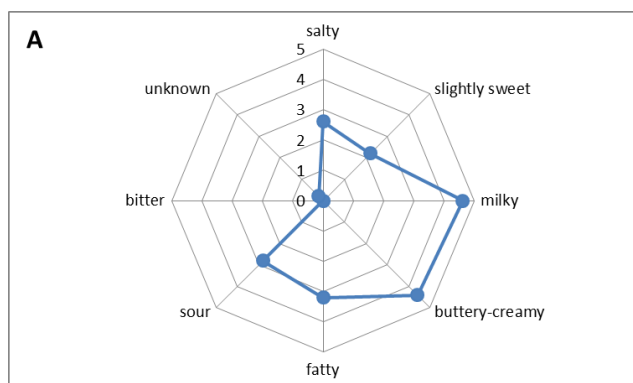


Figure 1 The taste of „Kiri“ samples manufactured in Slovakia.

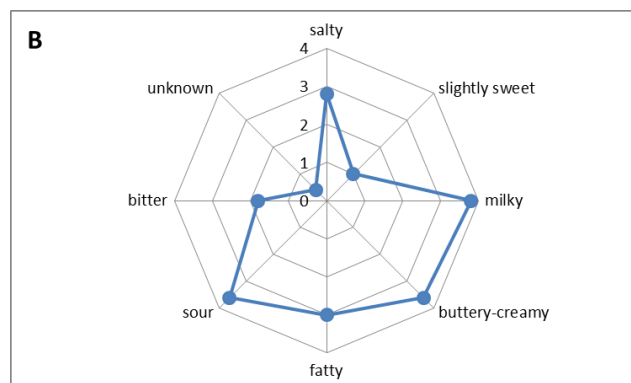


Figure 2 The taste of „Kiri“ samples manufactured in France.

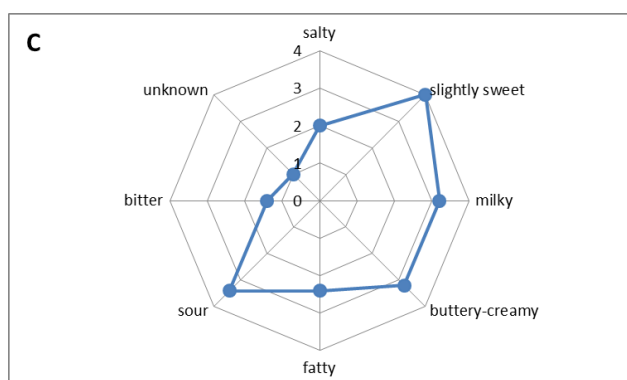


Figure 3 The taste of „Veselá krava“ samples manufactured in Slovakia.

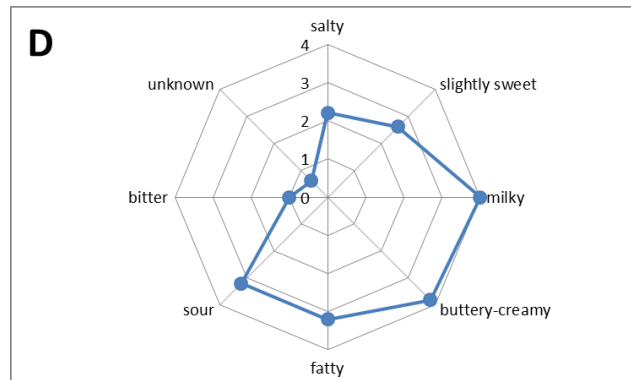


Figure 4 The taste of „Veselá krava“ samples manufactured in France.

The dry matter content and content of NaCl most commonly fluctuated in the products of Veselá krava manufactured in France (D). On the package label of this product were not declared contents of these parameters. Since it is the same brand name of one company (sample C was produced in Slovakia and sample D in France), so the values could be compare with each other. The processed cheese Veselá krava manufactured in Slovakia has higher salt content and the value of fat in dry matter. However, on the package label of both products were declared the same raw materials.

From the results can be concluded that most frequently fluctuated content of salt. The determination of salt content is not assessed by statutes. The average sodium content in the processed cheese reported by **Vojtaššáková et al. (2000)** is 751 – 1347 mg.100 g⁻¹. According to **Dostálová (2005)** many products contains a higher amount of sodium than 1000 mg.100g⁻¹, which is in accordance with our results.

For healthier population is necessary to ensure consumption of safety foods. However, permits of these foods on the market are often not possible, because the foods contain higher amount of salt which is responsible for various health problems such as the cardiovascular disease (**Zachar, 2008**).

Among cheeses made in Slovakia and France were not determined significant differences.

Sensory analysis

Results of the sensory evaluation shown Fig. 1 – 4.

Panelists most often detected milky flavor and buttery-creamy taste after evaluation of Kiri samples manufactured in Slovakia. The bitter taste was not detected.

The milky flavor and creamy-buttery flavor were most intensive in Kiri samples manufactured in France. Interested is that Kiri produced in Slovakia has strong milky and buttery-creamy taste than the cheese of same brand manufactured in France.

Panovská et al. (2001) reported that the cheeses with low fat contents are perceive as more salty but less creamy and

In the product „Veselá krava“ manufactured in Slovakia, panelist most often detected slightly sweet taste and sour taste. Salty taste of processed cheese Veselá krava manufactured by Slovakia to obtain less points than Veselá krava manufactured by France.

The same milky flavor and creamy-buttery flavor which were detected in Kiri samples manufactured in France were also most intensive in Veselá krava samples manufactured in same country. From the evaluation of taste Panelists found that among samples manufactured in Slovakia and France were significant differences. It is probably due to with cheeses used for production of these products.

Table 2 Chemical composition of processed cheeses and cheese analogues.

Sample	Parameter	Fat (g.100g ⁻¹)	Dry matter (g.100g ⁻¹)	Fat in dry matter (%)	NaCl (g.100g ⁻¹)
A	\bar{x}	28.13	43.66	64.42	1.03
	X _{min}	27.75	4.64	63.59	1.03
	X _{max}	28.25	43.70	64.73	1.03
	S _x	0.25	0.03	0.55	0
	v (%)	0.89	0.06	0.85	0
B	\bar{x}	29.68	44.50	66.71	1.03
	X _{min}	28.75	44.42	64.56	1.00
	X _{max}	30.25	44.63	68.08	1.07
	S _x	0.66	0.10	1.58	0.04
	v (%)	2.22	0.20	2.26	3.88
C	\bar{x}	19.13	41.36	46.24	1.20
	X _{min}	18.75	41.26	45.13	1.16
	X _{max}	19.50	41.55	47.26	1.24
	S _x	0.32	0.13	0.91	0.03
	v (%)	1.67	0.31	1.97	2.49
D	\bar{x}	18.75	41.73	43.59	1.03
	X _{min}	18.00	41.28	42.57	1.00
	X _{max}	18.25	42.87	44.14	1.06
	S _x	0.13	0.77	0.73	0.03
	v (%)	0.66	1.82	1.65	2.91

A – Kiri, Slovakia; B – Kiri, France; C – Veselá krava, Slovakia; D – Veselá krava, France;
s_x – standard deviation, v – coefficient of variation

CONCLUSION

Based on these results it can be concluded that the products from Slovakia contains declared composition, which is labeled on package and in conformity with legislative requirements. The quality of evaluated processed cheeses produced in Slovakia and abroad is very good. Efforts of manufacturers should be a tendency of going back to the original recipe and not the substitution by cheeses and milk commodities, various stabilizers and emulsifiers, which also leads to the use of a wider range of melting salts. From the observed results can be concluded that it should be aware to consumption of processed cheese and processed cheese products with relatively high content of salt in high amount.

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THE EFFECT OF DRYING ON ANTIOXIDANT ACTIVITY OF SELECTED LAMIACEAE HERBS

Anna Adámková, Lenka Kouřimská, Barbora Kadlecová

ABSTRACT

Antioxidant activity and total phenolics content of selected fresh and dried herbs from the *Lamiaceae* family were compared. The analysed herbs included *Thymus vulgaris*, *Origanum vulgare*, *Satureja hortensis*, *Origanum majorana*, and *Origanum heracleoticum* from the 1st and the 2nd harvests. The antioxidant activity was determined using DPPH method and the total content of phenols was analysed using the Folin-Ciocalteu reagent. Ascorbic and gallic acids were used as reference standards. All the analysed herbs had the reasonable potential to reduce the DPPH radical. The dried herbs from the 2nd harvest had the highest antioxidant activity. Oregano exhibited the highest antioxidant activity from the analyzed samples of both harvests together. The descending order of the samples was oregano > Greek oregano > marjoram > summer savory > thyme. Marjoram from the 2nd harvest had the highest antioxidant activity from the fresh samples. The lowest activity was observed in thyme from the 2nd harvest. In case of dried samples, the highest antioxidant activity was measured in sample of Greek oregano from the 2nd harvest. The lowest activity was observed in thyme from the 1st and 2nd harvest again. The descending order of total phenolics content for both harvests together was oregano > Greek oregano > marjoram > summer savory > thyme. In case of fresh herbs the highest total phenolics content was measured in oregano from the 1st harvest, the lowest content was measured in summer savory from the 2nd harvest. Greek oregano from the 2nd harvest had the highest values from dried herbs. Dried thyme from the second harvest had the lowest total phenolics content. The correlation between the DPPH values and the total content of phenols was determined (for fresh herbs: 0.4917; for dried herbs: 0.8698). According to the total content of phenols a statistically significant difference between the fresh and dried herbs from the 2nd harvest ($p = 0.0185$) was found.

Keywords: Herb; *Lamiaceae*; antioxidant activity; DPPH method; total phenolics contents

INTRODUCTION

People's interest in health and healthy lifestyle is increasing at present. People are mainly focused on sport and a healthy diet. For proper nutrition it is important to know not only the composition of food, but also its quantity and technological processing. This processing must be chosen so as to preserve the food nutritional value.

The use of herbs and their processing have a long tradition. Medicinal, aromatic and culinary plants, which synthesize many useful chemical compounds, are traditionally eaten in fresh and dried forms. Antioxidants belong to the biologically important compounds in herbs. They represent chemical compounds, which are able to inhibit oxidation reactions caused by free radicals. Free radicals can cause damage of biologically important molecules, cells and tissues. This process can be one of the main factors of various pathological lifestyle diseases such as cardiovascular diseases (atherosclerosis, ischemia, hypertension, etc.) (Jacob, 1995). Therefore it is important to have a sufficient amount of antioxidants in our nutrition. Moreover, antioxidants in foods prolong their shelf life and protect them against undesirable oxidation (rancidity changes of lipids and other easily oxidizing agents) (Cao et al., 1997).

Antioxidants can be classified according to the various aspects – their source and origin (natural and synthetic), chemical structure, etc. The most important antioxidants in food are vitamin C and E, carotenoids, flavonoids, and selenium. Herbs of the *Lamiaceae* family are an important source of antioxidants and other biologically active substances. Summer savory (*Satureja hortensis* L.), marjoram (*Origanum majorana* L.), Greek oregano (*Origanum heracleoticum* L.), oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.) have been commonly used in households for culinary food processing for many years. They have also an irreplaceable role in the food industry. Furthermore, they are used in pharmacy and cosmetics.

The antioxidant activity of the material produced from plants of the family *Lamiaceae* depends on many factors, including the plant cultivation conditions (conventional and organic farming methods, soil composition, irrigation and plant protection, harvest period, place of growing), collection method, plant processing (drying method and conditions - natural drying, oven drying, lyophilisation, exposure and intensity of light, temperature, humidity, etc.), the way of sample processing and extraction, and the selection of the antioxidant activity method.

Phenolics are a heterogeneous group of compounds with antioxidant activity found in many food resources (Halliwell, 1995). They are often part of plant essential oils and are responsible for characteristic aroma of individual foods.

MATERIAL

Herbs

Lamiaceae family herbs: thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*), summer savory (*Satureja hortensis*), marjoram (*Origanum majorana*) and Greek oregano (*Origanum heracleoticum*) were planted and analyzed. The seeds were pre-grown in flowerpots, sown on 7th April 2013 and replanted to bed on 21st May 2013. All herbs were planted on the sunny, unfertilized plot of sandy loam medium soil in the locality Malá Hraštica (49° 48' N, 14° 16' E, district Přeborn).

The seeds purchased from different companies were sowed in the following depths:

- thyme, oregano and marjoram (NOHEL GARDEN): 0.5 cm,
- summer savory (MORAVO SEED): 0.5 cm,
- Greek oregano (SEMO) 0.4 cm.

Plant parts were harvested on 18th July 2013 (1st harvest) and on 16th September 2013 (2nd harvest). The proportion of herbs was then dried.

Herb samples were taken before flowering when they should have the highest content of bioactive compounds. Fresh and dried leaves from herbs were used for the analysis.

Chemicals

- Methanol, p. a., CH₄O, M = 32.04 g/mol, Lachner, PP/2011/08626
- Sodium carbonate anhydrous, Na₂CO₃ p. a., M = 105.99 g/mol, Lachner, PP/2012/08988
- DPPH 2,2-difeny-1-pikrylhydrazyl, Sigma Aldrich, USA
- Folin & Ciocalteu's phenol reagent, Merck, UN 3264
- Ascorbic acid p. a., Penta, batch 1507080710, M = 176.13 g/mol
- Gallic acid p. a., Sigma Aldrich.

Equipment

- Spectrophotometer UV- 2900 PC, Tsingtao Unicom-Optics Instruments Co., Ltd., China
- Analytical balances AND, ER- 180A, max. 180 g, d = 0.01 mg
- Ultrasonic bath Tesla

- Balances with infrared dryer, Precisa HA 300, Precisa Instruments, Switzerland
- Thermostat Memmert 54853, Germany

METHODOLOGY

Drying

Herbs were dried in the open air at 25 °C for one week.

Determination of total dry matter

Infrared balances Precisa HA 300 were used for dry matter content determination. Fresh and dried herbs (0.5 g) from both harvests were ground and spread on aluminium foil. Program for vegetable drying was applied: maximum temperature 105 °C, constant value if the weight difference was less than 2 mg for 30 s. Samples were measured in triplicate and the average was calculated.

Herb extraction

Fresh herbs (6 g) or the equivalent amount of dried herbs (calculated from total dry matter of individual herbs, **Table 1**) were taken for the preparation of water extracts. Herb samples were extracted twice by 50 mL of hot demineralized water in the ultrasonic bath for 10 min. Samples were then filtered into 100mL volumetric flasks and filled up to the mark after cooling. The extracts were analysed on the same day.

Determination of antioxidant activity by the DPPH method

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method is one of the commonly used methods for antioxidant activity assessment. The principle of this method is based on the reduction of stable DPPH radical to DPPH-H by compounds with antioxidant activity and the measurement of the intensity of the violet DPPH radical solution at 522 nm. The method was calibrated with ascorbic acid and the results were expressed as equivalents of ascorbic acid per unit mass of sample. This method was taken from **Chrpová et al. (2010)** and **Buřičová et al. (2011)**. Samples were kept in dark and measured after 1, 2 and 3 hours to reach the reaction maximum.

Determination of total phenolic compounds (TPC)

The content of total phenolic was determined spectrophotometrically at 760 nm by using Folin-Ciocalteu reagent. The results were expressed as the content of gallic acid per unit mass of the sample. The method was taken from **Dorman et al. (2003)** and **Stratil et al. (2008)**.

Statistical evaluation

Table 1 Weight of dried herbs for extraction.

Herb	1 st harvest	2 nd harvest
Oregano	1.2265 g	1.8887 g
Greek oregano	1.2039 g	1.1279 g
Marjoram	1.7091 g	0.6223 g
Thyme	1.1047 g	1.9311 g
Summer savory	1.2467 g	1.2104 g

Table 2 Herbs dry matter content [%]

Harvest	Fresh herbs		Dried herbs	
	1 st	2 nd	1 st	2 nd
Oregano	20.2 ±0.6	28.0 ±0.3	98.6 ±0.2	88.5 ±0.1
Greek oregano	18.1 ±0.4	16.5 ±0.2	90.2 ±0.2	87.9 ±0.3
Marjoram	25.8 ±0.5	9.2 ±0.5	90.5 ±0.1	89.2 ±0.1
Thyme	16.8 ±0.2	28.5 ±0.4	91.3 ±0.3	88.6 ±0.2
Summer savory	19.7 ±0.3	17.4 ±0.5	94.8 ±0.1	86.3 ±0.2

Using the software STATISTICA 12 (StatSoft Inc.), statistical evaluation of values recalculated to dry matter content was performed. Statistical evaluation of the difference between the fresh and the dried samples was done by paired t-test at a probability level of $p < 0.05$.

RESULTS AND DISCUSSION

Dry matter content

Dry matter content results of analysed herbs are presented in Table 2.

Antioxidant activity

Results of antioxidant activity of herbs per 100 g of sample or dry matter are shown in Tables 3 and 4. It is seen that the herbs in the dried state have a higher antioxidant activity than fresh herbs.

Total phenolics content

Each measurement was repeated four times. Total phenolics content was then recalculated to 100 g of the extracted sample as well as to 100 g of dry matter of herbs. From the results shown in Table 5 and 6 is clear that the herbs have a reasonable content of phenolics. Higher

values can be observed in samples of dried plants. The only exception was the Greek oregano from the first harvest in Table 6.

Statistical evaluation

There was a statistically significant difference between fresh and dried samples, in terms of the potential to quench free radical DPPH (1st and 2nd harvest) and total phenols (second harvest). A statistically significant difference was not detected in TPC between fresh and dried herbs from the first harvest (Table 7).

Furthermore, the correlation between TPC and DPPH results was analysed (Table 8). It was found that there is a strong correlation between the values from dried herbs and a medium correlation in case of fresh herbs.

DISCUSSION

Dry matter content of herbs

Dry matter content results of dried and fresh herbs (Table 2) are consistent with **Vidovic et al. (2014)** and **Sabolová (2012)**. Some variability of dry matter content in fresh herbs can be observed, which can be affected by

Table 3 Antioxidant activity of herbs in grams of AA per 100 g of sample.

Harvest	Fresh herbs		Dried herbs	
	1 st	2 nd	1 st	2 nd
Oregano	1.80 ±0.16	1.84 ±0.01	11.53 ±0.06	12.03 ±0.01
Greek oregano	1.35 ±0.17	1.85 ±0.07	6.54 ±0.01	12.46 ±0.05
Marjoram	0.86 ±0.07	1.07 ±0.01	5.63 ±0.05	11.69 ±0.04
Thyme	0.64 ±0.08	0.74 ±0.02	5.38 ±0.01	5.31 ±0.07
Summer savory	0.63 ±0.08	0.92 ±0.14	6.30 ±0.02	8.20 ±0.06

Table 4 Antioxidant activity of herbs in grams of AA per 100 g of dry matter.

Harvest	Fresh herbs		Dried herbs	
	1 st	2 nd	1 st	2 nd
Oregano	8.92 ±0.78	6.56 ±0.03	11.70 ±0.06	13.59 ±0.01
Greek oregano	7.47 ±0.92	11.16 ±0.40	7.25 ±0.01	14.18 ±0.05
Marjoram	3.32 ±0.26	11.59 ±0.07	6.22 ±0.05	13.11 ±0.05
Thyme	3.80 ±0.50	2.61 ±0.60	5.89 ±0.01	6.00 ±0.08
Summer savory	3.20 ±0.40	5.26 ±0.80	6.65 ±0.02	9.50 ±0.07

Table 5 Total phenolics content of herbs in grams of GA per 100 g of sample.

Harvest	Fresh herbs		Dried herbs	
	1 st	2 nd	1 st	2 nd
Oregano	0.84 ±0.10	0.61 ±0.01	4.66 ±0.04	4.28 ±0.01
Greek oregano	0.64 ±0.03	0.58 ±0.01	2.71 ±0.01	4.26 ±0.01
Marjoram	0.62 ±0.10	0.48 ±0.01	3.24 ±0.05	3.57 ±0.01
Thyme	0.40 ±0.01	0.19 ±0.01	2.21 ±0.02	2.09 ±0.01
Summer savory	0.45 ±0.10	0.23 ±0.02	3.06 ±0.02	2.41 ±0.03

Table 6 Total phenolics content of herbs in grams of GA per 100 g of dry matter.

Harvest	Fresh herbs		Dried herbs	
	1 st	2 nd	1 st	2 nd
Oregano	4.15 ±0.48	2.18 ±0.01	4.73 ±0.04	4.83 ±0.01
Greek oregano	3.51 ±0.11	3.49 ±0.06	3.00 ±0.01	4.84 ±0.02
Marjoram	3.15 ±0.38	2.77 ±0.05	3.42 ±0.06	4.13 ±0.01
Thyme	1.44 ±0.02	2.09 ±0.03	2.44 ±0.02	2.35 ±0.01
Summer savory	2.68 ±0.49	0.81 ±0.09	3.36 ±0.02	2.72 ±0.04

many factors, including watering, temperature and humidity. Dried herbs results are not so variable because majority of the water was lost during the drying process.

Antioxidant activity

There are many studies describing significant antioxidant activity of plants of the Lamiaceae family (Srinivasan, 2014). Herbs and spices are therefore important sources of natural antioxidants. Chrpová et al. (2010) presented significant activity of selected Lamiaceae herbs. Another important activity and content of phenolic compounds were observed by Katalinic et al. (2006) in lemon balm *Melissa officinalis* L. Significant antioxidant activity was demonstrated also in the analysed samples. From Table 4 it is clear that all herbs from the first and the second harvests have the potential to quench free DPPH radical on a level comparable with presented references.

Drying process may cause some changes in antioxidant activity, total phenolics content as well as composition of

essential oils. There is higher concentration of stable phenolic compounds involved in the antioxidant activity in dried herbs. Our results by DPPH method are in line with these findings (Hossain et al., 2010). Antioxidant activities of analysed samples of thyme (T), oregano (D), summer savory (S), marjoram (M) and Greek oregano (G) from the first (1), the second and (2) both harvests were in the following descending order:

- a) fresh: M2>G2>D1>G1>D2>S2>T1>M1>S1>T2
- b) dried: G2>D2>M2>D1>S2>G1>S1>M1>T2>T1
- c) both harvests together: D>G>M>S>T

From this comparison it is clear that among fresh herbs the highest antioxidant activity was showed by marjoram from the second harvest. The important antioxidant activity of marjoram, which has potential to be used as a natural antioxidant, was also mentioned by Roby et al. (2013). In case of dried herbs, the highest values were detected in Greek oregano from the second harvest. This herb also showed the highest antioxidant activity by the

Table 7 Statistical comparison of fresh and dried herbs samples.

Method	Compared samples	<i>p</i>
DPPH	1 st harvest - dry x fresh herbs	0.0264
	2 nd harvest - dry x fresh herbs	0.0135
TCP	1 st harvest - dry x fresh herbs	0.1934
	2 nd harvest - dry x fresh herbs	0.0185

Table 8 Correlations between TPC and DPPH methods.

Material	Correlation coefficient
Fresh herbs	0.4917
Dry herbs	0.8698

DPPH method when both harvests were calculated together. These results are comparable with **Chrpová et al. (2010)** who found significantly higher antioxidant activity in mint and oregano comparable to that of green tea.

A statistically significant difference between fresh and dried herbs from the 1st and the 2nd harvests was detected. Dried herbs from the second harvest had the highest antioxidant activity.

Total phenolics content

Higher TPC values (Table 5) were observed in dried herbs. The only exception was the Greek oregano from the first harvest which shows slightly higher value in fresh state. TPC of analysed samples were in the following descending order:

- a) fresh: D1>G1>G2>M1>M2>S1>D2>T2>T1>S2
 b) dried: G2>D2>D1>M2>M1>S2>G2>S2>T1>T2
 c) both harvests together: D>G>M>S>T

Among the fresh herbs, the highest total phenolics content was detected in oregano from the first harvest, the lowest content was detected in summer savory. Among the dried herbs, the highest total phenolics content was detected in Greek oregano from the second harvest, which also showed the highest antioxidant activity for DPPH. Dried thyme from the second harvest had the lowest total phenolics content. Comparing both harvests together, the highest values were detected in oregano sample.

As far as the TPC is concerned, statistically significant difference was found between fresh and dried herbs from the second harvest. Dried oregano, Greek oregano and marjoram showed the highest values in case of the 2nd harvest, thyme and summer savory in the first harvest.

Comparison of fresh and dried herbs

Our results confirmed higher antioxidant activity and TPC in dried herbs. There are many different enzymatic and non-enzymatic reactions taking place during the drying process as well as tissue decomposition. As a result of these processes, different substances (including phenolics) are created.

A statistically significant difference between dried and fresh herbs was also confirmed by **Sefidkon et al. (2006)**, and **Pirbalouti et al. (2013)**, who analysed two varieties of basil. **Rhim et al., (2009)** investigated the influence of drying on the antioxidant activity. Their results were significantly higher for dried samples too. Results of **Hossain et al. (2010)** again showed significantly higher content of TPC, rosmarinic acid and higher antioxidant capacity of the air dried samples versus fresh samples.

There was a strong correlation between DPPH and TPC (Table 8) which is in line with the study of **Mechergui et al. (2010)**. **Yesiloglu et al. (2013)** also presented strong antioxidant activity correlating with the content of phenols and flavonoids.

CONCLUSION

Selected herbs of the Lamiaceae family exhibited a reasonable antioxidant potential and phenolics content in both fresh and dried form. According to the DPPH method results a statistically significant difference between fresh and dried herbs from the 1st and the 2nd harvests was

determined. High correlation between TPC and DPPH values was found. Dried herbs from the 2nd harvest had the highest antioxidant activity.

TPC results showed a statistically significant difference between fresh and dried herbs from the 2nd harvest. Dried *Origanum vulgare*, *Origanum heracleoticum*, and *Origanum majorana* had the highest results from the 2nd harvest, *Thymus vulgaris* and *Satureja hortensis* had the highest results from the 1st harvest.

Both antioxidant activity and the total content of phenols increased after the drying process.

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OCCURRENCE OF *ENTEROCOCCUS* SPP. ISOLATED FROM THE MILK AND MILK PRODUCTS

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ABSTRACT

Enterococcus spp. is the most controversial group of lactic acid bacteria that have been for years ascribed with beneficial or detrimental role in food and feed. The aim of our study was to monitor the occurrence of *Enterococcus* spp. as the indicator of the contamination from collected samples of raw cow's milk, goat's colostrum and whey (n = 186). Cultures of enterococci were cultivated and purified and identified by the genus-specific and species-specific PCR method (n = 230). Among suspected isolates in total 222 isolates (96.5%) were identified as *Enterococcus* spp. The results were the same in all samples separately, more than 90% each of them were positive to *Enterococcus* spp. The results of counting the number of cultivated colonies showed that the largest number of enterococci is found in the samples of whey taken after the process of electro dialysis and the smallest in the native whey sample. From collected whey samples, 64 samples (90%) were PCR positive for enterococci species. Afterwards within the identification of several selected isolates that were identified, as *Enterococcus* spp. by the species-specific PCR method the most frequently presented in all of isolates was *Enterococcus faecalis*. Apparently the presence of enterococci was detected in all samples, but in amounts that aren't hazardous for human health. Although enterococci are opportunistic pathogens, it seems that they occur frequently in foods (especially fermented) in large numbers.

Keywords: enterococci; PCR; raw cow's milk; colostrum; whey

INTRODUCTION

Lactic acid bacteria (LAB) is a group of Gram-positive bacteria united by a constellation of morphological, metabolic and physiological characteristics. The group consists of around 20 genera producing lactic acid as the end product of carbohydrates fermentation (Salmien et al., 2004).

The genus *Enterococcus* is the most controversial and one of the largest group that belongs to LAB group. They are Gram-positive homofermentative cocci, occurring singly or in pairs and can be found in variety of habitats including humans and animals. From the taxonomic point of view enterococci have been reviewed several times and today genus consists of at least 53 species of which *Enterococcus faecalis* and *Enterococcus faecium* are the most important (Giraffa, 1999; Giraffa, 2003; Khan et al., 2010). These bacteria play important role in the food and feed fermentation and nowadays these strains are frequently used as probiotics. They are considered as a potential cholesterol-lowering agents, in treatment of gastrointestinal diseases and for immune regulation (Franz et al., 2011). With their ability to produce enterocins (class II of bacteriocins) *Enterococcus* strains can provide natural preservation of dairy products and hurdle in the growth of microorganisms (antimicrobial activity against spoilage or pathogenic bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium* spp.). Also on the other hand enterococci have their negative influence – opportune pathogens and can cause diseases

(like endocarditis, bacteremia, infection of urinary tract, central nervous system, intra-abdominal and pelvic infections) and also carrying enzymes involved in biogenic amines production (Fouquié Moreno et al., 2006; Franz et al., 2011).

During the process of lactation colostrum is the first milk that comes from the mammary glands of mammals and after that raw milk. The consumption of colostrum for ruminant species (such as cow, goat and sheep), but also humans and other mammals has a fundamental role in passive immune transfer and in the survival rate of newborns. For that reason it is necessary that the newborns obtain adequate amount of colostrum during their first days of life to obtain adequate passive immune transfer and increase its future productivity. The amount and composition of produced colostrum can be affected by several factors such as nutrition or litter size (Uruakpa et al., 2002; Hernández-Castellano et al., 2015).

Milk is the most completed natural fluid, one of the most important basic and healthiest raw materials, which plays important role in the dairy nutrition of all population (necessary for human health and normal function of the human body). With its consumption we start pretty early (in early childhood, when we are born) and continue in all stages of life till the late elderly (Zulueta et al., 2009; Tratnik and Božanić, 2012). Goat's milk with its ameliorative composition of its components plays an important role in the specific diet and the economy of many developing countries (Guo, 2003).

During the manufacturing of milk after precipitation of casein the greenish translucent liquid that is obtained is whey. For a long time it has been viewed as one of the major disposal problems of the dairy industry, but not anymore. Today there are several types of whey, which depending mainly on the processing sequence resulting in casein removal from fluid milk, but the most often encountered types (sweet or acid whey) originates from the manufacture of cheese. With its nutritional aspects and enriched with some other additions like fruits is one of the healthy products that can be found on the market (Jelen, 2002, Pescuma et al., 2008; Tratnik and Božanić, 2012).

With its very complex and changeable composition, milk and dairy products are favorable environment for growing initial and subsequent microflora involved in fermentation or food spoilage.

The aim of this study was to monitor the occurrence of *Enterococcus* spp. in collected samples of raw cow's milk, goat's colostrum and whey as the indicators of contamination.

MATERIAL AND METHODOLOGY

All together 186 samples of raw cow's milk, goat's colostrum and whey were collected in the different areas of South Moravia Region in the Czech Republic in a period of 2013 and 2014. The samples of raw cow's milk were collected from milk vending machines available and goat's colostrum samples were collected from several dairy farms. Whey samples were only periodically collected in different manufacturing stages, from one dairy plant in South Moravia in the Czech Republic, in the period of middle of February to the middle of March 2014 (one month). The collection of samples and their origin are described in the Table 1.

Basic processing of the samples was carried out immediately according to the ISO 7218 and ISO 6887-1 standards. The amount of 25 mL of each sample was diluted in 225 mL of Buffered Peptone Water (Oxoid, England) and made decimal dilutions. From diluted sample

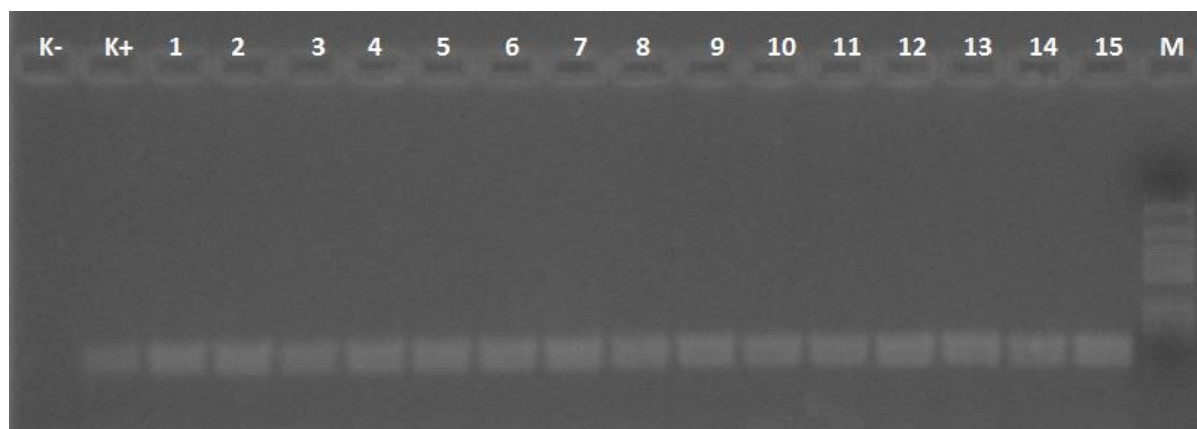
200 µL was aseptically spread on Slanetz Bartley agar (Oxoid) and cultivated at 37°C for 24h aerobically. All colonies from each sample that showed different morphological characteristics and pigment production were selected and purified for further characterization on Bile Esculin agar (Oxoid) and Blood agar (Oxoid).

Isolation of bacterial DNA was performed by 20% Chelex 100 (Bio-Rad Laboratories, USA). Isolates were identified by the genus-specific polymerase chain reaction (PCR) method based on the detection of *sodA* genes encoding enzyme mangan-dependent superoxide dismutase for the rapid identification of enterococci (Jackson et al., 2004). The PCR amplification was carried out in the PTC-200 thermocycler (MJ Research, USA). Isolates were also identified by the species-specific PCR method also according to the Jackson et al. (2004).

RESULTS AND DISCUSSION

A total amount of grown colonies, that after cultivation showed typical growth as enterococci was 230 suspected isolates (Table 1). Nowadays several molecular methods are available for identification of microorganisms. The commonly used molecular method for identification of enterococci is PCR method (Figure 1). Based on the detection of *sodA* genes encoding enzyme mangan-dependent superoxide dismutase for the rapid identification of enterococci, only 8 strains (3.5%) of suspected isolates didn't show the specific PCR product.

Enterococci occur as nonstarter LAB in variety of cheese production and ripening (e.g. the development of flavor) probably due to their thermal resistance during pasteurization of milk. High level of contaminating cheese with enterococci, except as starter cultures, can result in the poor hygienic practices during manufacturing (Pieniz et al., 2014). During all phases of whey manufacturing enterococci were presented (Table 2). This could be due to their ability of biofilm formation (electrodialysis) or maybe due to their resistance so they continually remain during the process. Whey is a by-product of the cheese



K-: negative control (reaction admixture with PCR H₂O), K+: positive control (*Enterococcus* spp., 112 bp), M: marker (155-970 bp), 1-15: *Enterococcus* spp. (112 bp)

Figure 1 Agarose gel electrophoresis of PCR products with genus-specific primers obtained by DNA amplification of 15 bacterial strains of the genus *Enterococcus*.

industry, which was often disposed as the waste. During the cheese production some compounds present in milk and / or cheese end up in the composition of whey. The major factors to determine the survival of this bacteria include particular characteristics of the strains, composition of food ingested and competition of microbiota (Pieniz et al., 2014). Whey and its protein content are ingredients used in dairy industry mainly due to their foaming and emulsifying properties and increasing nutritional quality (cheese, dairy desserts etc.). LAB in whey can decrease the high content of lactose and increase the digestibility of BLG (β -lactoglobulin) and ALA (α -lactalbumin) proteins. This can contribute to the change and increase the flavor aroma and texture of the end product and provide extra nutritional value (Pescuma et al., 2008). Enterococci were present in all phases of whey processing, except in the native way sample, but in the amount that is not hazardous for human health. Besides of maybe bad hygienic conditions, handling with and cleaning the equipment during whey manufacturing, the high prevalence of enterococci in whey may be also attributed to their resistance to heat, acid, salt and harsh conditions during food processing. To conclude, the application of electro dialysis, in the process of whey manufacturing, proved to be good.

Today, the consumers are offered a wide range of commercial types of milk including raw milk sold by milk vending machines or on the farm. According to the Food and Agriculture Organization of the United Nations (FAO)

that number is daily increasing. Not like before, within the consumption of raw milk consumers are today very concern about their health and what kind of food they are consuming.

The difference in microbiological quality of raw milk collected out of vending machines at different places was noted. Within the microbiology analysis form collected samples of raw milk ($n = 30$) in total more than 90% of the collected samples ($n = 28$) contained *Enterococcus* spp. A total of 38 suspected enterococci isolates were obtained from collected samples of raw milk. During the cultivation period from each sample was selected one or more colonies, that showed different morphological characteristics. Analyzing the isolated DNA with the PCR method for the identification of *Enterococcus* spp. more than 90% of isolates showed positive results ($n = 36$) and only 2 of suspected isolates were identified as non-enterococci. The presence and detection of enterococci in the samples of raw milk during the collection period time has been showed repeatedly. Comparing to the other works made on the occurrence of enterococci in raw milk, results were similar (Kagli et al. 2007; Franciosi et al., 2009).

The major enterococci in raw milk originates from plants (silage), udder skin, storage tanks, where they come from environments contaminated by human and animal fecal material (water, soil, urban sewage etc.) cause they are already naturally presented in the intestinal microflora (Giraffa, 2003; Kagli et al. 2007).

Table 1 The origin and frequency of *Enterococcus* isolates in all samples.

Commodity	Number of samples	Number of positive samples	Number of suspected isolates	Number of positive isolates
raw cow's milk	30	28 (93.3%)	38	36 (94.7%)
goat's colostrum	85	82 (96.5%)	106	103 (97.2%)
whey	71	64 (90%)	86	83 (96.5%)
Total	186	174	230	222

Table 2 The origin and frequency of *Enterococcus* isolates in the samples of whey.

Commodity*	Number of samples	Number of positive samples	Number of suspected isolates	Number of positive isolates
native whey sample	6	0 (0%)	2	0 (0%)
w in drying station	6	5 (83%)	7	6 (86%)
w from the tank	9	9 (100%)	11	10 (91%)
w before the electro dialysis	13	13 (100%)	18	17 (95%)
w after the electro dialysis	13	13 (100%)	17	17 (100%)
desalted w before concentration	7	7 (100%)	13	13 (100%)
desalted w behind the pasteur	5	5 (100%)	8	8 (100%)
concentrated, desalted w	4	4 (100%)	5	5 (100%)
sample of dried powder	5	5 (100%)	5	5 (100%)
Total	71	64 (90%)	86	83 (96.5%)

*w = the sample of whey

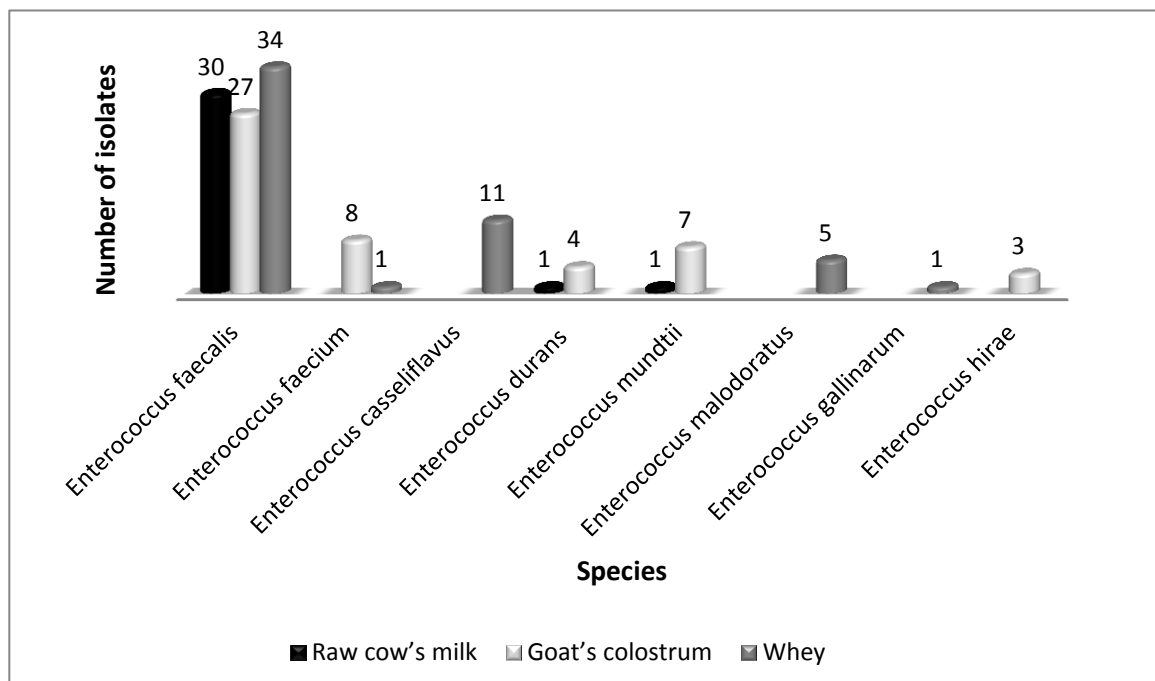


Figure 2 The frequency of several selected positive *Enterococcus* spp. Isolates.

For years it has been considered the milk is directly contaminated with fecal, but it seems that maybe due to the uncleaned milk equipment, milk vending machines and the lack of maintenance of hygienic conditions milk is becoming its source. Besides of that because of their psychrotrophic nature, their heat resistance and growth conditions, they can also increase during the milk refrigeration period (Giraffa, 2003).

Several of suspected isolates, which showed positive specific PCR product, were selected and subjected to further analysis. From each sample were taken several positive isolates and identified by the species-specific PCR method (Figure 2). The most of tested isolates were identified as *Enterococcus faecalis*, which is one of the most common species presented in the milk. According to the work of Citak et al. (2006) *Enterococcus faecalis* was detected in more than 50% of the isolated strains. Besides the *Ent. faecalis* and *Ent. faecium*, afterwards the major presence of *Enterococcus durans* was also identified as the most frequently presented (Kagli et al. 2007; Franciosi et al., 2009) and in our case especially in the samples of goat's colostrum.

CONCLUSION

This study confirmed the presence of *Enterococcus* spp. in all of collected samples: raw cow's milk, goat's colostrum and during the whole whey manufacturing process. Apparently the presence of enterococci in raw milk trails all the way from the colostrum since it is the first milk or from unkeeping the safty hygienic conditions. In whey manufacturing process the presented amount of enterococci is amout that is not hazardous for human health. Although enterococci are not pathogenic microorganisms, it seems that they occur frequently in foods (especially fermented) in large numbers. On the other hand besides the small share with their ability to

have beneficial effects on human health, these bacteria are accompanied with the great future opened for the long and more detailed exploration. Isolation of bacteria may have the advantage of giving the choice of safety for further operation in making dairy products.

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THE EFFECT OF FEEDING WHEAT WITH PURPLE PERICARP ON THE GROWTH OF CARP

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ABSTRACT

This study assessed and compared the influence of feeding wheat with purple pericarp (variety Konini) and standard coloured wheat (red variety Bohemia) on the growth characteristics of fingerling carp (*Cyprinus carpio* L.) of the “Amurský lysec” line. The total content of anthocyanins converted to cyanidin 3-glucoside in the control Bohemia wheat was 24.95 mg.kg⁻¹ and in the Konini purple wheat 41.70 mg.kg⁻¹. Two experimental variants for feed were evaluated: dipped wheat grain and crushed wheat grain. The feed dose for wheat was 1.5% of the fish stock weight and for natural food (frozen Chironomid larvae) was 0.2% of fish stock weight to all variants. Growth parameters (body length, body weight, Fulton’s condition factor and feed conversion ratio) of the fish were evaluated after one month of administration. The feed consumption and physico-chemical parameters (temperature, oxygen saturation, pH, N-NH₄⁺, N-NO₂⁻, N-NO₃⁻ and Cl⁻) of the environment were observed. During the feeding test, no major differences in food consumption among variations feeding on either wheat and on Chironomid larvae were noted. Satisfying results for mass and length gain were achieved in V2 wheat with purple pericarp (Konini variety – dipped grain), where the average total body length was 156.56 mm and the average unit mass was 60.81 g. In this variant, higher values of the parameters were achieved compared to the control group (100.6%, resp. 104.2%). A positive impact of wheat with purple pericarp on the evaluated parameter of fish condition factor was demonstrated. This trend was confirmed in all variants. No effect was demonstrated for mechanical disruption of kernels on the level of utilization of nutrients. In further experiments on growth characteristics we would like to determine antioxidant parameters in the blood and liver of fry.

Keywords: purple pericarp of wheat; carp; growth; feeding test

INTRODUCTION

Wheat grain is considered a good source of fibre, phenols, tocopherols and carotenoids. Anthocyanins are another group of bioactive agents contained in blue and purple wheat grains. It is well known that herbal anthocyanins play a role as antioxidants and they also have antibacterial and anti-carcinogenic effects (Varga et al., 2013). In addition to white and red grains, wheat with a purple colour can occur. The purple colour of the grain is caused by anthocyanins in the pericarp. Purple grains occur mainly in tetraploid wheats from Ethiopia, and one kind of bread wheat is known from China (Zeven, 1991).

The first testing of the feeding value of some cultivars of purple wheat with broilers was carried on in 1976 by Gregoire et al. (1976). However, the cultivation process has advanced for wheat since then. The higher antioxidation effect could lead to a reduction in the oxidation of fats in food derived from animals fed on coloured wheat. Effect of feeding purple wheat on quality in hens was studied by Ruckschloss et al. (2010). The result was a higher average body weight of hens fed with purple wheat (up to 6.22% comparing to the control group). On the contrary, Štastník et al. (2014) found no statistically significant effect on the weight of broilers’ performance and carcass parameters when fed with purple

wheat. Increased antioxidant capacity of the liver in rats fed with purple wheat was described by Karásek et al. (2014). No similar study of the effect of feeding coloured wheat to fish has yet been realized.

The common carp *Cyprinus carpio* (L.), is the most frequently raised fish species in Central and Eastern Europe, with production levels reaching more than 80% of total fish production in some countries, including the Czech Republic (Adámek et al. 2012). The species is commonly reared in earth ponds using extensive and semi-extensive management regimes, thereby allowing the use of natural resources for growth and development (Adámek et al. 2012). Fish production in the Czech Republic is based on the principle of maximum utilisation of the full nutrient source in the form of natural food to promote growth but on the other hand to minimize energy conversion losses. For this reason cereals play an important role in carp production, as they are inexpensive and a rich source of energy. Nutritionally, cereals cannot be considered a fully-fledged food, because they contain a low amount of protein which is poor in essential amino acids (lysine and methionine) without which the synthesis of body tissues cannot occur. Therefore, increasing carp production is limited due to the availability of natural food (Hůda, 2009; Przybyl and Mazurkiewicz, 2004). For

additional carp feeding all sorts of cereals can be used, but which is the most valuable is still in question (Hůda, 2009). According to Przybyl and Mazurkiewicz, (2004) wheat has the highest nutritional values of cereals for carp protein, followed by rye – triticale – barley. Wheat is the primary source of energy due to the high starch content of about 70% of the dry mass (Heuzé et al. 2013). The starch digestibility of the whole grains is around 70% in carp (Čirković et al. 2002). Technological treatment of cereals can increase the production efficiency of the feed. Heat treatment of the kernels can increase the starch digestibility to 90% (Przybyl and Mazurkiewicz 2004). Additional feeding with cereals in the absence of natural food for the carp causes an increase in visceral fat content and fat in muscle tissue. The feed intake and metabolism of carp is affected by many biotic and abiotic factors. Aside from age and pedigree the content of dissolved oxygen and water temperature also have an influence. Water temperature affects the activity of digestive enzymes in carp fry. At water temperature of 22 – 28 °C activity of α -amylase is higher than above that range.

The aim of this study was to evaluate the growth characteristics of carp fingerlings fed on purple wheat (Konini variety).

MATERIAL AND METHODOLOGY

Common carp (*Cyprinus carpio L.*) fingerlings of the “Amurský lysec” line (Figure 1) from Rybníkářství Pohelice Inc., in the Czech Republic, were used for the feeding test. Fish were placed in 200 L glass tanks, 50 fish per tank (100 fish per variant) (Figure 2). The rearing tanks were connected to a recirculation system with

mechanical and biological phases of water treatment. Tanks were sorted into four groups, each with two replicates: V1 dipped standard wheat – control, V2 dipped wheat with purple pericarp, V3 crushed standard wheat – control, V4 crushed wheat with purple pericarp. The Feed dose for wheat was 1.5% of fish stock weight and for natural food (frozen Chironomid larvae) was 0.2% of fish stock weight for all variants. The whole grain wheat was weighed out dry and subsequently dipped for 14-16 hours. A table grinder was used to grind the wheat. The grinding process was set to maximum graininess for mechanical disruption of each grain. Such prepared wheat was not further dipped. The feeding strategy was set at three feeds per day during the light period at 8am, 1pm and 6pm. Both forms of prepared wheat were divided into two sub-parts and administered to fish at 8am and 1pm. Natural food was administered at 6pm. The light regime was set at 13 hours light and 11 hours dark. Fish were weighed in groups once a week for modification of the feeding dose. The total content of anthocyanins converted to cyanidin 3-glucoside in the control Bohemia red wheat was 24.95 mg.kg⁻¹ and in Konini purple wheat 41.70 mg.kg⁻¹ (Figure 3). The Konini variety (pedigree: Fortuna/Arawa//Kopara/Purple-Hilgendorf) was grown for research purposes in Brno-Tuřany. Seeds of genetic resources were obtained from Agrotest fyto Ltd. Kroměříž (Ing. Petr Martinek, CSc).

The basic physicochemical parameters of the water were measured twice per day during the test. Samples of water for chemical analysis were taken once per day. The range of values is given in Table 1.

Parameters such as Fulton’s condition factor (FC) and the



Figure 1 Common carp – the “Amurský lysec” line.



Figure 2 Glass tanks.



Figure 3 Comparison of Bohemia (left) and Konini (right) grain varieties.

feed conversion ratio (FCR) were calculated as follows: $FC = (w/TL^3)*100$, where w is the body weight (g) and TL is the total body length (cm); $FCR = F/(w_t - w_0)$, where w_t is the final body weight (g), w_0 is the initial weight (g), and F is the feed consumption (g). The values obtained were compared using ANOVA at $p < 0.05$.

RESULTS AND DISCUSSION

During the feeding test, no major differences in food consumption among variations fed each of the wheats and the Chironomid larvae were noted (Table 2).

Conversely, differences in growth parameters, i. e. length and weight of individual fish between the feeding variants were found (Table 3).

Differences in basic growth characteristics in both variants can be explained by the method applied to kernels. Satisfactory results in the growth characteristics of carp were achieved in V2 (Table 3) at a FCR (Food Conversion Ratio) value of 11.15. The bioavailability of nutrients from kernels the structure of which was treated with water before administration increased in comparison with mechanical disruption of the grain. This is demonstrated by the relatively high value of FCR in the V4 variant (70.54).

Table 1 The range of values of basic physico-chemical parameters during the feeding test.

Parameter	Average	Min.	Max.
Temperature (°C)	24.34	23.4	25.2
Oxygen saturation (%)	81.74	64.4	90.0
pH	7.13	5.71	7.82
N-NH ₄ ⁺ (mg.L ⁻¹)	1.31	0.0	7.5
N-NO ₂ ⁻ (mg.L ⁻¹)	0.19	0.06	0.61
N-NO ₃ ⁻ (mg.L ⁻¹)	61.01	44.37	89.80
Cl ⁻ (mg.L ⁻¹)	55.28	44.86	64.64

Table 2 Feed consumption during the feeding test.

	Wheat (g)	Chironomids larvae (g)
V1	2828.3	433.0
V2	2904.7	436.1
V3	2827.0	428.3
V4	2786.8	421.3

V1 – Bohemia (dipped), V2 – Konini (dipped), V3 – Bohemia (crushed), V4 – Konini (crushed)

Table 3 The average values of the basic growth characteristics of fish.

	Total body length (mm) ±SD	Weight (g) ±SD	Fulton's condition factor ±SD
Beginning of the test	151.80 ±11.30	56.75 ±13.36	3.15 ±0.19
v_x	7.44%	23.54%	6.15%
End of the test			
V1	155.64 ±25.23	58.34 ±22.72	3.05 ±0.47
v_x	16.21%	38.95%	15.98%
V2	156.56 ±12.78	60.81 ±13.58	3.17 ±0.30
v_x	8.16%	22.33%	9.57%
V3	155.87 ±10.27	58.81 ±10.93	3.17 ±0.32
v_x	6.59%	18.58%	10.24%
V4	153.47 ±13.07	57.91 ±11.52	3.23 ±0.35
v_x	8.52%	19.89%	10.72%

V1 – Bohemia (dipped), V2 – Konini (dipped), V3 – Bohemia (crushed), V4 – Konini (crushed)

Table 4 Composition of whole fish (carp given in (%)).

	Dry matter ±SD	Fat ±SD	Protein ±SD
V1	31 ±0.74	13.03 ±0.96	14.77 ±0.51
Vx	0.02%	0.07%	0.03%
V2	31.53 ±1.31	13.32 ±1.04	15.18 ±0.54
Vx	4.15%	7.83%	3.58%
V3	34.43 ±2.71	14.73 ±2.25	15.93 ±1.31
Vx	7.88%	15.27%	8.24%
V4	30.54 ±1.77	11.96 ±1.94	15.27 ±0.59
Vx	5.81%	16.22%	3.88%

Values are given as a fresh matter. SD – standard deviation, v_x – coefficient of variation, V1 – Bohemia (dipped), V2 – Konini (dipped), V3 – Bohemia (crushed), V4 – Konini (crushed)

The values of production parameters are influenced by the quality of diet. Under controlled conditions without the presence of natural food we used a complete feed mixture for the type and age category of the farmed fish. When testing the cereal in recirculating systems used to treat water and natural food in the form of frozen Chironomids at a dose of 0.2 to 0.5%. A higher proportion negatively affects the intake of the tested cereals. To improve digestibility of the feed given different methods are used to improve the availability of nutrients. One is crushing (squeezing) the kernels or dipping. As is apparent from the results the values of the monitored parameters are influenced by the treatment of the kernels (Tables 3 and 4). Relative feeding coefficient values of cereals (in pond conditions with natural food available) generally range between 2 to 5. The value is influenced in part by environmental conditions feed quality and proportion of natural food. **Másilko et al. (2014)** gave FCR test values ranging from 2.08 to 2.50 with depending on the cereals used and method of treatment. A decline in the share of natural food leads to an increase in FCR values to greater than 10.

In Table 4 the chemical composition of whole fish are presented. Technological dipping treatment did not affect the chemical composition of whole fish in variants V1 and V2. Differences in composition were observed in technological treatment with homogenization, when in variant V3 (control) higher values were achieved in all monitored indicators in comparison with V4 and throughout the test. **Hůda (2009)** obtained in pond conditions similar results for fat content 13.27 ±0.36% in the muscle of carp fed with corn supplements to our variants V1 and V2, where in wheat values the fat content was 11.21 ±0.31% and so nearest to variant V4. The food (cereals) used significantly affects the composition of tissues produced in carp, especially the fat content and its composition (**Urbánek et al. 2010**).

Adult carp are an omnivorous species utilizing a relatively high proportion of animal prey in its diet, mainly chironomids and other benthic invertebrates (**Anton-Pardo et al. 2014**). Carp as an omnivorous species has a high level of amylase activity in the digestive tract in

comparison with piscivores. The production efficiency of wheat feed can be improved by increasing the bioavailability of nutrients from the kernels. The production efficiency of cereal feed processed in different ways was studied by **Másilko et al. (2014)** who observed the growth of carp fed with different kinds of cereal in fishponds. The highest individual weight was discovered in carp fed technologically unprepared rye, while the lowest individual weight was discovered in the pressed wheat and oilseed variant. Not only the means of technological modification of kernels but also the kind or a variety can possibly affect the utilization of nutrients in carp. For the purposes of the elimination of differences between group trends, further observations are necessary. The positive impact of coloured wheat on the value of the evaluation parameter of fish condition factor (FC) was demonstrated (Table 3). The method of treating wheat (crushing x dipping) may affect the achieved output parameters, as is evident from the data in Table 3 and the composition of the tissues (Table 4). However, the difference observed was not statistically significant.

CONCLUSION

In this work it was found that inclusion of wheat with purple pericarp (Konini) in feed can influence the growth parameters of the fish. These were mainly the weight of the fish, and this change was reflected in changes in Fulton's condition factor. Within further experiments with growth characteristics we would like to establish the determination of antioxidant parameters in the blood and liver of fry, but these experiments are more financially demanding.

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GENOME-WIDE SELECTION SIGNATURES IN PINZGAU CATTLE

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ABSTRACT

The aim of this study was to identify the evidence of recent selection based on estimation of the integrated Haplotype Score (iHS), population differentiation index (F_{ST}) and characterize affected regions near QTL associated with traits under strong selection in Pinzgau cattle. In total 21 Austrian and 19 Slovak purebred bulls genotyped with Illumina bovineHD and bovineSNP50 BeadChip were used to identify genomic regions under selection. Only autosomal loci with call rate higher than 90%, minor allele frequency higher than 0.01 and Hardy-Weinberg equilibrium limit of 0.001 were included in the subsequent analyses of selection sweeps presence. The final dataset was consisted from 30538 SNPs with 81.86 kb average adjacent SNPs spacing. The iHS score were averaged into non-overlapping 500 kb segments across the genome. The F_{ST} values were also plotted against genome position based on sliding windows approach and averaged over 8 consecutive SNPs. Based on integrated Haplotype Score evaluation only 7 regions with iHS score higher than 1.7 was found. The average iHS score observed for each adjacent syntenic regions indicated slight effect of recent selection in analysed group of Pinzgau bulls. The level of genetic differentiation between Austrian and Slovak bulls estimated based on F_{ST} index was low. Only 24% of F_{ST} values calculated for each SNP was greater than 0.01. By using sliding windows approach was found that 5% of analysed windows had higher value than 0.01. Our results indicated use of similar selection scheme in breeding programs of Slovak and Austrian Pinzgau bulls. The evidence for genome-wide association between signatures of selection and regions affecting complex traits such as milk production was insignificant, because the loci in segments identified as affected by selection were very distant from each other. Identification of genomic regions that may be under pressure of selection for phenotypic traits to better understanding of the relationship between genotype and phenotype is one of the challenges for livestock genetics.

Keywords: bovine SNP50 BeadChip; F_{ST} ; iHS score; Pinzgau cattle; selection sweep

INTRODUCTION

Genome-wide screening of single nucleotide polymorphisms (SNPs) can improve the understanding of the connection between genotype and phenotype changes resulting from the formation of modern livestock breeds. The analysis of a large number of SNPs across the genome will reveal aspects of the population genetic structure, including evidence of adaptive selection across the genome (Barendse et al., 2009). Variations identified within the genome of cattle breeds have been primarily caused by human selection during the processes of domestication and subsequent breed formation. Domestication greatly changed the morphological and behavioral characteristics of cattle and with breed formation and selection programmes for improving the production traits allowed the formation of very diverse breeds (De Simoni Gouveia et al., 2014).

The explanation and identification of selection signatures can provide not only basic knowledge about evolutionary changes which shaped the genome but also can be very perspective for identifying domestication-related loci that ultimately may help to further genetically improve of economically important traits (De Simoni Gouveia et al., 2014; Qanbari et al., 2014). Much of the variation across the genetically diverse ancestral population was either lost

due to limited numbers of animals within the areas of domestication or was divided into the subpopulations that were later recognized as distinct breeds. The strong selection to fix favourable mutations underlying domestication and formation of each breed created selective sweeps in which the variation was also lost (Ramey et al., 2013). If the mutation was recent and the selection is strong all alleles under positive selection will increase in frequency by producing selective sweep or selection signature. For neutral mutation, this will take many generations until the mutated allele has reached a high population frequency through drift. Where the loci selection is slight or the mutation is old little evidence of this selection may be left in the genome (Qanbari et al., 2010a; Kemper et al., 2014).

The evaluation of genes underlying phenotypic variation can be prepared based on two approaches: firstly from phenotype to genome that is carried out by linkage disequilibrium based association mapping and may involve positional cloning of QTL or by targeting particular candidate genes identified based on homology to known genes and secondly from genome to phenotype that includes the statistical estimation of genomic data to identify likely targets of past selection. The elimination of standing variation in regions linked to a recently fixed

beneficial mutation is known as a “selective sweep” (Moradi et al., 2012; Qanbari et al., 2010b, Utsunomiya et al., 2013). Several methods were used to assess the evidence of positive selection including the analysis of population differences using F_{ST} , the analysis of ancestral states in connection with extended haplotype homozygosity (EHH) of derived alleles (iHS) and the modeling of distribution of allele frequencies along chromosome under expectation of a selective sweep analyses by composite likelihood ratio (CLR) (Barendse et al., 2009). For detection of recent selection via selective sweeps identification Sabeti et al. (2002) suggested basic concept resulting from the extended haplotype homozygosity statistic. Sabeti et al. (2002) proposed the use of the contrast of EHH statistic of one core haplotype vs. other haplotypes in the same position if the selection carries an allele on a specific haplotype to higher frequency faster than the rate at which it is broken down by recombination when the high frequency will be longer than expected under neutrality (Qanbari et al., 2010b; Simianer et al., 2010). The integrated Haplotype Score as an extension of EHH statistic was proposed by Voight et al. (2006). This method is based on the comparison of EHH between derived alleles and ancestral alleles present in the wild ancestor or outgroups within population (Simianer et al., 2010). The allele frequency differences between breeds or populations in a segment of genome caused by different histories between them can be analysed by F_{ST} . Fixation index F_{ST} can be interpreted also as a measure of gene frequencies dispersion among groups relative to the variation expected in the population from which such groups was derived (Kemper et al., 2014).

The aim of our study was to identify the signatures of strong and recent selection based on estimation of the integrated Haplotype Score and population differentiation index and characterise genomic regions which have been subjected to selective sweeps.

MATERIAL AND METHODOLOGY

In this study three data sets of animals were used to detect signatures of recent selection in Pinzgau cattle. In total 21 Austrian and 19 Slovak purebred bulls registered by their breed association were genotyped using Illumina BovineHD and Illumina BovineSNP50 BeadChip. Dataset of 21 Austrian sires was builded with the aim of having common ancestors or being related to Slovak ones. The detailed description of sample size and data source for each set can be found in Table 1. The 36393 SNPs

common to applied Illumina genotyping arrays were retained in reduced panel of SNPs. Markers assigned to unmaped regions or with unknown chromosomal position according to the latest bovine genome assembly (Btau 4.0) and SNPs positioned to sex chromosomes were removed (634). Quality control of data was carried out according to Purcell et al. (2007). Autosomal loci with call rate <90%, minor allele frequency <0.01 and Hardy-Weinberg equilibrium limit of 0.001 were excluded from subsequent analyses (5221). The evidence of positive selection was evaluated based on two approaches: integrated Haplotype Score (iHS) statistic and Wright’s fixation index (F_{ST}) measure.

The analysis of selection sweeps using iHS statistic is based on haplotype frequencies as specified Voight et al. (2006). The haplotypes were reconstructed for each autosome using default parameters according to Scheet and Stephens (2006). The iHS statistic evaluate the extent of local linkage disequilibrium which is partitioned into haplotypes positioned upon and loci that carry the ancestral versus the derived allele. The iHS score reflects the structure of haplotype and essentially indicates unusually long haplotypes carrying the ancestrall and derived allele (Qanbari et al., 2011). The set of ancestrall alleles resulting from research of Matukumalli et al. (2009) was used in our study. In iHS statistic each loci is treated as core SNP and the test begins with calculation of extended Haplotype Homozygosity for each core SNP. If SNPs are biallelic loci, then each core SNP can be ancestral or derived. This integrated EHH (iHH) (summed over both directions away from the core SNP) is denoted iHH_A or iHH_D , depending on whether it is computed for the ancestral or derived core allele (Qanbari et al., 2011; De Simoni Gouveia et al., 2014). According Voight et al. (2006) the iHS score is described as within population score for the ratio between iHH_A and iHH_D :

$$iHS = \ln \frac{(iHH_A)}{(iHH_D)}$$

The negative iHS values indicate greater homozygosity outlying the ancestral allele and positive values denote greater homozygosity outlying the derived allele. The iHS within analysed population was evaluated using the *rehh* package that is incorporated in R software (Gautier and Vitalis, 2012). Subsequently, the iHS values were averaged in genome-wide non-overlapping 500 kb windows.

Table 1 Description of used sample and genotyping array.

Breed	Country	Sample (n)	SNPs on array	Production type
Pinzgau	Austria	17	700k	
Pinzgau	Austria	4	54001	Dual-purpose
Pinzgau	Slovakia	19	54609	

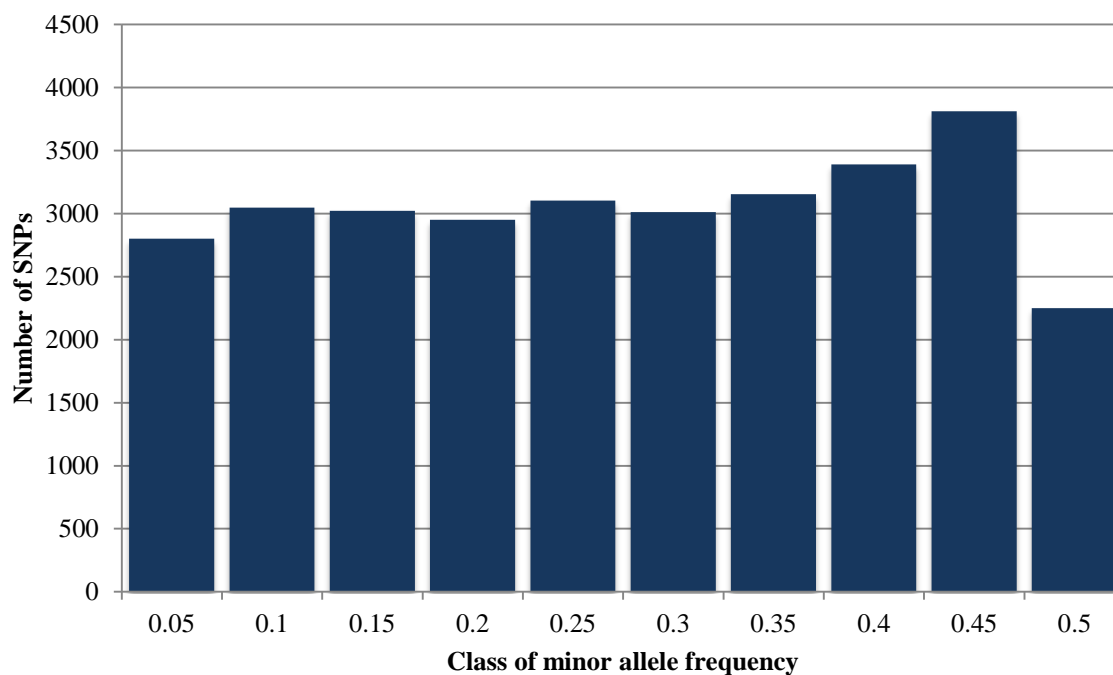


Figure 1 Distribution of MAF across genome.

The genome-wide pattern of selection signatures were estimated also by calculating the basic form of Wright’s F_{ST} fixation index corrected by Weir and Cockerham (1984) at each syntenic locus and visualised using SNP & Variation Suite v8.x (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com). The F_{ST} index describing the degree of genetic differentiation between subpopulations can theoretically range from 0 to 1, but it is also possible to assume negative values (Akey et al., 2002). Selection signatures can be recognized when adjacent SNPs all show high F_{ST} (Weir et al., 2005), due to the hitch-hiking effect (Maynard-Smith et al., 1974), implying divergent selection between breeds, or where adjacent SNPs all show low F_{ST} , implying balancing selection between breeds. Smoothing, where a moving average of a certain number of markers is taken, is a method of looking for regions where selection is apparent over multiple markers, rather than one-off high values (Barendse et al., 2009; Moradi et al., 2012). The F_{ST} values were also evaluated against genome position based on sliding windows approach and averaged over 8 consecutive SNPs.

RESULTS

The dataset consisting of the total 30538 autosomal SNPs that passed the filtering criteria have been used to identify genomic regions in Pinzgau bulls that may be influenced by recent selection. This subset of loci covered 25084.85 Mbp of the genome with 81.86 kb average adjacent SNPs spacing. The distribution of minor allele frequency (MAF) across the panel of loci was not uniform (Figure 1). The average value of MAFs (0.24 ± 0.14) was comparable with other dual-purpose cattle breeds (Edel et al., 2011; Su et al., 2012; Mancini et al., 2014). Across all analysed individuals the average heterozygosity at level 0.34 ± 0.01 was observed.

Two approaches were used for evidence of recent selection. Firstly, the iHS statistic was applied on dataset to detect selection sweeps. The iHS score was calculated for each SNPs and then averaged into non-overlapping 500 kb segments across the genome. The size of sliding windows was chosen based on sufficient number of SNPs for each segment. Genomic regions were considered as recently selected when the iHS score of multiple loci located within 0.5 Mb was greater than 1.7. In total

Table 2 Detected autosome segments identified as regions under selection.

Region	BTA	Position (Mb)	Number of SNPs	iHS value	Closest gene to max
1	1	65.66 – 65.99	3	1.70	HGD
2	5	55.58	1	1.73	LOC101904412
3	7	12.83	1	2.24	DCAF15
4	9	72.11 – 72.31	2	1.70	EYA4
5	15	52.03 – 52.47	7	1.86	NUMA1
6	18	22.26 – 22.38	2	1.73	FTO
7	19	0.47	1	1.90	CA10

Table 3 Summary of autosome regions identified as affected by positive selection.

Region	BTA	Position (Mb)	Number of SNPs	F _{ST} value
1	1	3.40 – 23.73	3	0.23
2	2	1.32 – 13.28	2	0.25
3	4	57.33 – 72.54	5	0.24
4	5	73.45 – 85.22	3	0.22
5	10	12.71 – 26.17	2	0.23
6	28	5.17 – 11.15	3	0.22

4889 windows involving 23677 iHS values were tested. Table 2 showed summaries of the autosomal regions that displaying significant iHS values. Using iHS statistic was found only few SNPs that can be evaluated as loci under selection. Across genome only 7 windows exceeded the iHS value greater than 1.7. Figure 2 displays the genome-wide plot of iHS values against the genomic position. The observed segments were localized close to the different genes. The average value of iHS score was 0.05 and the highest score (2.24) was identified for region on chromosome 7 with only one observed locus. In this genome location was found the presence of gene encoding DDB1 and CUL4 associated factor 15. Most of SNPs that showed significant iHS values were located on chromosome 15 in genomic region ranged from 52.03 to 52.47 Mb. In this bovine autosomal region is located the nuclear mitotic apparatus protein 1 (NUMA1) gene which is gene conserved across different species including human. The NUMA1 gene was tested for evidence of its role in proliferative activity and meiotic cell division (Taimen et al., 2004). The region on chromosome 18 consisting of only 2 loci was near to the FTO (the fat mass and obesity-associated gene) gene which was significantly associated with carcass traits and meat quality in cattle and pigs (Zhang et al., 2011; Dvořáková et al., 2012). However, the distribution of segments with clustered loci was across autosome non-uniform. The values of iHS

score across autosome segments indicated that the analysed regions which can be affected by recent selection showed no major overlap. The slight signals of recent selection can be caused by the smaller sample size or mainly by the fact that the analysed individuals were genetically related.

Secondly, to estimate genome-wide pattern of positive selection within evaluated population of Pinzgau bulls the F_{ST} index for each SNP was calculated. The level of genomic differentiation was evaluated between two groups based on their origin which can lead to increase of allele frequencies in loci that were potentially affected by positive selection. The higher allele frequencies of these loci can be representative to the differentiation in balancing or directional selection, neutrality or other processes that were used in breeding programs of Austrian and Slovak Pinzgau cattle populations.

Theoretically, the F_{ST} values varied from 0 to 1, when both extremes means the total identity (F_{ST}=0) or differentiation (F_{ST}=1) within analysed populations. The selection signatures could be recognized when adjacent loci all show high F_{ST} due to hitch-hiking effect resulting from divergent selection or when adjacent SNPs all show low F_{ST} resulting from the balancing selection between populations (Qanbari et al., 2011). In our study the autosomal regions were recognized as affected by positive selection when the adjacent SNPs showed F_{ST} values

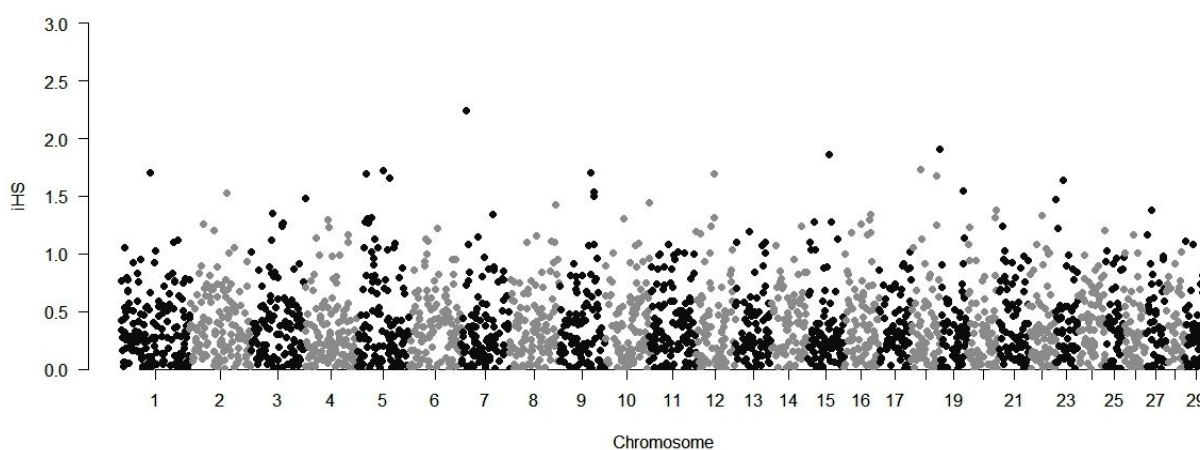


Figure 2 Genome-wide plot of the iHS score averaged for 500 kb.

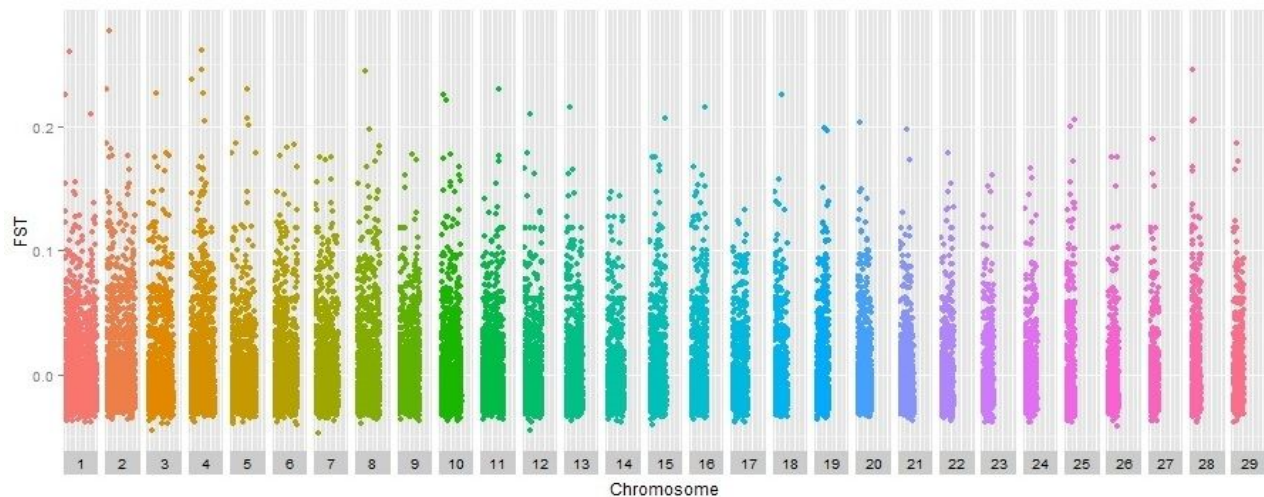


Figure 3 The distribution of F_{ST} values within the autosomes.

higher than 0.20. The observed F_{ST} values ranged from -0.05 to 0.28, with an average values of 0.0005. In total 76.23% of F_{ST} values were lower than 0.01. The highest average F_{ST} was found for BTA 4 (0.004). As indicated in figure 3 only few loci had a tendency to cluster into similar region. The slight signals of positive selection was found in 6 regions localized on chromosomes 1, 2, 4, 5, 10 and 28 (Table 3).

In the next step of F_{ST} estimation the values were averaged over 8-wide SNPs windows within each autosome to determine global pattern of F_{ST} across genome. More than 95% of clusters across all autosomes showed F_{ST} values lower than 0.01. The results indicated unimodal distribution and pretty much uniform scheme of selection in all loci included in analysis. The low level of genetic differentiation between Austrian and Pinzgau bulls detected based on F_{ST} index is also apparent from the similar origin and common ancestors in pedigree data of the analysed Pinzgau bulls.

DISCUSSION

The level of genetic variation among cattle population is a result of both neutral demographic processes, weak but sustained natural selection and strong short-term artificial selection for divergent breeding goals (Qanbari et al., 2011). In our study we used the genome-wide SNP data to detect the evidence of positive selection signals based on the iHS and F_{ST} scan in Pinzgau bulls originating from Austria and Slovakia. The results from both analyses indicated low level of genomic differentiation between analysed groups. Based on the iHS statistic seven regions that can be potentially affected by recent selection were detected. The total number of SNPs for each region was low. Only one of identified segments was found in genome region associated in previous studies with bovine quantitative trait locus (Table 2). The F_{ST} values averaged in 8-wide windows indicated that the selection programs of Slovak and Austrian bulls are similar and therefore the genomic regions showed only very small differences.

Both of the applied statistics were used successfully in the evaluation of selection signatures in different cattle

populations. Qanbari et al. (2010b) found in population of German Holstein-Friesian cattle segment with an outlier value on chromosome 18 that contains the Sialic acid binding Ig-like lectin 5 gene and the Zink finger protein 577 gene. These genes are considered as candidate for calving ease, longevity and total merit index in Holstein cattle (Cole et al., 2009). Gu et al. (2009) reported the positive selection signatures in the genomic region surrounding muscle related genes. The evidence of strong selection in the region near to the growth hormone gene located on chromosome 20 was found by Flori et al. (2009) in Angus and by Hayes et al. (2009) in Holstein breeds. Simianer et al. (2010) reported the outlier F_{ST} windows for the two regions on chromosome 2 and 5 in the vicinity of ZRANB3, R3HDM1 and WIF1 genes which are known as genes affecting feed efficiency and mammalian mesoderm segregation. In studies of three French dairy cattle breeds published by Van Tassell et al. (2008) and Karim et al. (2011) was found the differentiations in the region located on chromosome 18 which was associated with coat color (MC1R gene) and in the segment on chromosome 14 harbouring the PLAG1 gene that is important for the cattle growth. The platelet-derived growth factor alpha polypeptide (PDGFA) was identified as a potential candidate gene underlying the selective sweep on BTA25 in Simmental cattle and the receptor for this growth factor (PDGFRA) was identified as differentiated among the French dairy breeds (Ramey et al., 2013).

The genome-wide scan for evidence of selection signatures in livestock is one of the many approaches that are available for estimation of genomic diversity due to development of high throughput SNP genotyping arrays. The large observed datasets with high SNPs density provide a much better insight into the biological processes underlying natural and artificial selection of animals. The results of these studies can provide the valuable data for increase of animal selection strategies efficiency and also may help to understanding of biological limits and signals resulting from the high selection pressure of the achievement of breeding goals.

CONCLUSION

The genome-wide scan based on estimation of positive selection signatures using iHS and F_{ST} statistics led to the detection of few regions that were affected by recent selection in Pinzgau cattle. Our results indicated use of similar selection programs in Slovak and Austrian Pinzgau populations. The conditions that would result in a clear evidence of selection signatures were rare. The response to the selection resulted from the small allele frequency changes in many loci that were polymorphic before start of selection in population. The results show low level of genetic differentiation or high genetic relatedness between analysed Austrian and Slovak Pinzgau bulls what is due to the fact that bulls from both populations had common ancestors. Pinzgau cattle are recognized as producer of food resources of specific quality due to its mountaineous origin. Observed results confirmed previously stated assumption of common genetic pool of Slovak and Austrian populations and indicated importance of both populations in preservation and utilization of Pinzgau cattle.

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PHTHALATE MIGRATION FROM PACKAGING MATERIALS INTO FOOD

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ABSTRACT

The content of dibutylphthalate (DBP) and di- (2-ethylhexyl) phthalate (DEHP) in samples of packages used for packaging meat products and the phthalate migration from packaging materials to meat products were studied. Five samples of textile packaging intended for cooked meat production were analysed as well as the final product which was filled into packages. Subsequently an analysis was carried out (after 1, 7, 14, 21, and 28 days of storage) of the finished meat products stored over the course of their intended shelf life at ambient temperature of 4 °C. Determination of phthalates was conducted by high performance liquid chromatography (HPLC) method with UV detection at a wavelength of 224 nm on the Zorbax Eclipse C8 column. The content of phthalates in the final product was below the limit of quantification. According to the Regulation of the Commission (EU) No. 10/2011, the specific migration limit of products intended for food contact is 1.5 mg.kg⁻¹ of food simulant for DEHP and 0.3 mg.kg⁻¹ for DBP. After filling and the first day of storage of the meat product, four package samples release the said phthalates to an extent that it exceeded the limits of the Commission Regulation (EU) No. 10/2011. Already after the seventh day of storage, all samples (with the exception of sample 2 for DBP) exceeded SMLs. Monitoring of each phthalate migration in individual samples during storage (for 28 days) produced a rising tendency. In sample 1, DBP increased from 0.40 to 3.37 mg.kg⁻¹, while DEHP from 0.58 to 14.66 mg.kg⁻¹. In sample 2, DBP increased from ≤0.2 mg.kg⁻¹ to 4.34 mg.kg⁻¹, while DEHP from 1.46 to 28.20 mg.kg⁻¹. In sample 3, DBP increased from ≤0.2 mg.kg⁻¹ to 8.27 mg.kg⁻¹, while DEHP from 1.67 to 14.84 mg.kg⁻¹. In sample 4, DBP increased from 0.27 to 6.12 mg.kg⁻¹, while DEHP from 2.37 to 13.22 mg.kg⁻¹. In sample 5, DBP increased from 0.32 to 11.11 mg.kg⁻¹, while DEHP from 1.91 to 15.42 mg.kg⁻¹.

Keywords: package; Gothaer salami; phthalic acid esters; thermal treatment; migration limit

INTRODUCTION

Phthalates are a group of dialkyl- or alkyl aryl esters of 1,2-benzenedicarboxylic acid derived from the trivial name for this acid, phthalic acid. Phthalates have become ubiquitous contaminants (Net et al., 2015) because of their volatilization and leaching (Wormuth et al., 2006). High molecular weight phthalates, such as di- (2-ethylhexyl) phthalate (DEHP), are primarily used as plasticizers (PVC) (Rahman et al., 2004) and low molecular weight phthalates, such as di-n-butyl phthalate (DBP), are added e.g. in colours to increase their adhesion to the surface (Xue et al., 2010).

Negative effects of phthalates on living organisms have been demonstrated. They pose teratogenic, carcinogenic, mutagenic, and reproductive hazards. Contamination of the body by phthalates may take different routes, e.g. through inhalation, skin absorption and food (YIN et al., 2003; Borchers et al., 2010; Witassek et al., 2011).

The highest human exposure to phthalates comes from food. One of the sources of food contamination is the packaging material, from which phthalates migrate to food (Cao, 2010). Bradley et al., (2013) conducted an analysis of the phthalate content in 261 food samples, 20 UK Total Diet Study food groups. Phthalate diesters were confirmed to be present in 77 samples and DEHP was detected in 66 samples. Other studies confirmed that packaging materials

contribute to the concentration of phthalate diesters in some food.

Zhang et al. (2008) determined the 2,6-diisopropyl-naphthalene (DIPN) and dibutylphthalate (DBP) in 110 domestic and foreign packaging and food in marketplaces in the United States. Concentrations of DIPN and DBP in packaging ranged from 0.09 to 20.0 and from 0.14 to 55 mg.kg⁻¹; mostly they were less than 20 mg.kg⁻¹. DIPN was not detected (<0.01 mg.kg⁻¹) in 41 samples of foods and DBP was only detected in two domestic and four imported food samples with a concentration from 0.01 to 0.81 mg.kg⁻¹. Phthalates were part of the packaging printing inks.

For the period of 14 days Cirillo et al. (2013) have analysed the diet of patients eating in the hospital for the contents of DEHP and DBP. Packaging contained the polyethylene terephthalate (PET) and the food was covered with a polypropylene (PP) foil. The study found that the highest concentration was in bread (0.307 ± 0.138 µg.g⁻¹) of the original sample for DEHP and 0.174 ± 0.091 µg.g⁻¹ of the original sample for DBP. Although the main route of exposure for hospital patients may be the hospital facilities, even the diet containing phthalates may contribute to increasing the Tolerable Daily Intake (TDI) of phthalates.

In many cases the source of phthalates may not be the material used for packaging food, but the technological equipment. Such was the finding of **Bach et al. (2012)** in case of packaged water. Mineral water bottled in PET (polyethylene terephthalate) bottles, where phthalates as plasticisers are not used, was contaminated by equipment from bottling lines.

Phthalates are used in many industries and are produced in extremely large quantities worldwide. Since phthalate-based plasticisers are not firmly bonded through covalent bonding in the material, they slowly release into the surrounding environment by volatilisation, leaching or migration. Large amounts of phthalates are released not only during use, but also when handling plastic waste (storing at landfills, incineration). Improper disposal of such products helps to release PAE into the environment where they contaminate air, soil or water resources, which results in the leakage of about 63%. Soil is the environmental component contaminated to the major extent (77%), followed by water (21%). These factors increase the risk of contamination of raw foods and human food chain. Foods can be contaminated either primarily during primary production, as a result of contaminated water, soil and air, or secondarily during subsequent processing and all kinds of handling (**Wormuth et al., 2006**).

The aim of the study was to monitor the migration of phthalates (DEHP and DBP) from packaging to meat products during storage.

MATERIAL AND METHODOLOGY

Meat products packages, samples of final meat product and samples of the finished meat product were analysed. Packages of meat products ($n = 5$) were purchased from the company that supplies packages for the processing industry. These were coloured textile packages designed for the production of cooked meat products. From each type of package a sample was taken in size of 10 dm², when the samples were analysed in duplicate.

Final meat product intended for further heat treatment was produced in pilot conditions of the Department of Food Technology at Mendel University in Brno. Six samples of final meat product were collected, and then the final meat product was filled into packages. Thirty samples were produced for each package. After being filled into packages, the samples were stored at 4°C. Samples were taken after 1, 7, 14, 21 and 28 days of storage.

A total of 20 samples of packaging, 6 samples of final meat product before being filled into packages and 150 samples of the meat product after heat treatment and storage were analysed. All samples were analysed in duplicate, i.e. 352 analyses were performed.

Packaging samples were leached in *n*-hexane:dichloromethane (1:1) solvent mixture for 72 hours and then extracted three times (60, 30, and 30 minutes). The combined extraction shares were filtered, evaporated on a rotary vacuum evaporator and finally dried under nitrogen. The extract was then transferred into vials using hexane (5 mL) and centrifuged. The upper portion of the extract (1.5 mL) was collected into vials for HPCL (high-performance liquid chromatography) determination and dried under nitrogen. Again, the vials

were centrifuged; the upper layer of the extract (1.5 mL) was taken away and dried under nitrogen. Subsequently, the vials were supplemented with acetonitrile to make a volume of 1 mL. If the extracts were coloured or turbid, they were purified with sulphuric acid.

In order to analyse PAE in samples of final meat product and finished meat products proven methods for determining DBP and DEHP in food have been used (**Jarošová et al., 1998, 1999**).

Samples were homogenized, weighted into metal plates and frozen. Gradually, the frozen samples were lyophilized and subsequently the PAE residues were extracted with *n*-hexane. PAEs were separated from co-extracts by gel permeation chromatography on Bio Beads S-X3 gel. Eluates were purified by a cleaning process using the concentrated sulphuric acid.

Phthalates were determined by HPLC method with UV detection at a wavelength on 224 nm on the Zorbax Eclipse C8 column. The quantity of the samples sprayed on the column was 10 µL. Final concentrations were calculated based on the calibration curve in the Agilent Chemstation software for LC and LC/MS systems. The range of calibration curve was between 1.06 µg.mL⁻¹ and 106.00 µg.mL⁻¹ for DBP and between 1.01 µg.mL⁻¹ and 100.50 µg.mL⁻¹ for DEHP. The correlation coefficient was 0.9999 both for DBP and DEHP. The limit of detection was 0.05 µg.mL⁻¹ for DBP and 0.11 µg.mL⁻¹ for DEHP. In the final phase, the results were statistically processed.

All the laboratory glass was flushed with hexane during sample preparation. Simultaneously dry matter and fat content were determined for each sample. Samples of the meat product contained about 30 % of fat. All samples were analysed in duplicate. Concentrations of DEHP and DBP are related to the original sample.

RESULTS AND DISCUSSION

Concentrations of phthalates in the analysed packages are expressed in µg.dm² and are given in Table 1. Each value represents the average of two parallel determinations.

The suitability of packaging for food is defined by a migration limit (ML), which is the maximum amount of packaging components, which can be released from the packaging per unit area. According to the Commission Regulation (EU) No. 10/2011, products intended to come into contact with food and meals must not release their ingredients into the food in amounts greater than 10 mg.dm⁻² or 60 mg.kg⁻¹ of the food or food simulant. The said regulation also includes a specific migration limit, which is max 1.5 mg.kg⁻¹ of food simulant for DEHP and max 0.3 mg.kg⁻¹ of food simulant for DBP. As far as the samples of packaging given in Table 1 are concerned, four samples (1, 3, 4, and 5) would not comply with the said regulation with regard to the specific migration limit (Table 2) after 1 day of storage of a meat product packaged in these packages.

Concentrations of monitored phthalates (DEHP and DBP) in the meat product before thermal processing and in finished meat products are expressed in mg.kg⁻¹ of the original sample and they are given in Table 2. Each value represents the mean of 12 values (six parallel samples and each sample analysed in duplicate).

The phthalate content in the final meat product and migration of phthalates after heat treatment and storage were monitored. In samples of final meat product taken immediately after mixing of the final meat product, concentrations of both phthalates were below the limit of quantification ($\leq 0.2 \text{ mg.kg}^{-1}$).

After 1 day of storage DBP content moved from ≤ 0.2 to 0.40 mg.kg^{-1} and DEHP content from 0.58 to 2.37 mg.kg^{-1} . Migration limit was not exceeded in sample 2 (DBP and DEHP), sample 1 for DEHP and samples 3 and 4 for DBP. After 7 days of storage the DBP content was between 0.22 and 1.32 mg.kg^{-1} and DEHP content between 7.12 and 11.93 mg.kg^{-1} . Migration limit was exceeded in all analysed samples, except for sample 3, where DBP value was measured 0.22 mg.kg^{-1} below the migration limit. After 14 days of storage DBP content was between 1.78 and 6.84 mg.kg^{-1} and DEHP content between 10.55 and 19.10 mg.kg^{-1} . Migration limit was exceeded in all analysed samples. After 21 days of storage DBP content was between 2.93 and 8.00 mg.kg^{-1} and DEHP content between 10.80 to 21.56 mg.kg^{-1} . Migration limit was exceeded in all analysed samples. After 28 days of storage DBP content was between 3.37 and 11.11 mg.kg^{-1} and DEHP content between 13.22 to 28.20 mg.kg^{-1} . Migration limit was exceeded in all analysed samples.

Monitoring of each phthalate migration in individual samples during storage produced a rising tendency.

According to the Commission Regulation (EU) No. 10/2011, under which the specific migration limit is 1.5

mg.kg^{-1} for DEHP and 0.3 mg.kg^{-1} for DBP, the DBP migration limit was exceeded after the first day of storage in two samples (1 and 5) and DEHP migration limit in three samples (3, 4, and 5). After the seventh day of storage all samples exceeded the migration limits set for the said phthalates, except for sample 3 for DBP.

Our experiments have shown that the content of plasticizers leached from packages increases with temperature (heat treating temperature of $70 \text{ }^\circ\text{C}$ for 10 minutes) and time of storage and our finding is consistent with results of other authors.

Condyle et al. (1992) examined the migration of dioctylphthalate (DOP) and dioctyladipate (DOA) from PVC into ground meat with a different fat content stored at $4 \text{ }^\circ\text{C}$ and $-20 \text{ }^\circ\text{C}$. After 8 days of storage at $4 \text{ }^\circ\text{C}$ from 2 to 80 mg.kg^{-1} (0.12 to 4.8 mg.dm^{-2}) plasticizers migrated to meat and from 2 to 60 mg.kg^{-2} (0.13 - 4 mg.dm^{-2}) migrated after 212 days of storage at $-20 \text{ }^\circ\text{C}$. Their experiments demonstrated that the migration in samples with a lower fat content was proportionally lower.

Also Moreira et al. (2015) concluded that phthalates are released from packaging materials. They investigated the content of eight plasticizers in samples of spices and baked chicken meat stored in plastic packages. They found diisobutyl phthalate and dibutyl phthalate, which migrated from the package. Higher concentrations of plasticizers were detected in spices.

Migration of dioctyladipate (DOA) and acetyltributylcitrate (ATBC) plasticizers in ground meat

Table 1 Mean concentrations of DBP and DEHP ($\mu\text{g.dm}^{-2}$) in samples of packages used for packaging meat products (standard deviation for DBP = 7.33 and for DEHP = 36.1)

Sample	DBP	DEHP
$\mu\text{g.dm}^{-2}$		
1	4.35	19.1
2	8.26	16.79
3	23.95	103.33
4	15.09	26.54
5	5.26	0.3

Table 2 Mean concentrations of DBP and DEHP (mg.kg^{-1}) in the samples in the meat product before thermal processing and in finished meat products (n = 5) after 1, 7, 14, 21, and 28 days of storage at $4 \text{ }^\circ\text{C}$ (standard deviation for DBP = 3.1 and for DEHP = 7.2)

Sample	Meat product before thermal processing		Day 1		Day 7		Day 14		Day 21		Day 28	
	DBP	DEHP	DBP	DEHP	DBP	DEHP	DBP	DEHP	DBP	DEHP	DBP	DEHP
mg.kg^{-1}												
1	LOQ	LOQ	0.40	0.58	1.32	8.79	2.08	11.28	3.14	14.43	3.37	14.66
2	LOQ	LOQ	LOQ	1.46	0.70	11.93	1.78	19.10	2.93	21.56	4.34	28.20
3	LOQ	LOQ	LOQ	1.67	0.22	7.12	4.38	12.05	6.59	13.76	8.27	14.84
4	LOQ	LOQ	0.27	2.37	0.43	7.90	4.68	13.09	5.17	13.16	6.12	13.22
5	LOQ	LOQ	0.32	1.91	0.64	9.32	6.84	10.55	8.00	10.80	11.11	15.42

due to the effect of microwave heating was studied by **Badeka et al. (1998)**. The samples varied in fat, and all were packed into foil containing PVC and polyvinylidenechloride (PVDC/PVC). They were heated in a microwave oven for the period from 0.5 to 4 minutes. The values of DOA and ATBC migration in samples with 55 % fat content and 4 minutes of heating were 846.0 mg.kg⁻¹ (14.7 mg.dm⁻²) and 95.1 mg.kg⁻¹ (2.5 mg.dm⁻²).

Mei-Lien Chen (2008) conducted a study in Taiwan, where they monitored the level of migration of phthalates (DEHP and DBP) from PVC foil. Food was covered with PVC foil and heated in a microwave oven. Results showed that the DEHP level in food increased significantly during 3 minutes of heating.

CONCLUSION

The aim of our study was to monitor the content of phthalates (DEHP and DBP) in packaging intended for meat products and track the possible migration of phthalates from packaging into the product after heat processing and storage over the product shelf life. Given the overall migration values under the Commission Regulation (EU) No. 10/2011, the monitored packages did not exceed the migration limits. In comparison with the specific migration limits for DBP (0.3 mg.kg⁻¹) and DEHP (1.5 mg.kg⁻¹) all analysed packages exceeded the limits imposed by the said regulation already after the seventh day of storage of the finished meat products. The DBP values ranged from ≤0.2 mg.kg⁻¹ to 11.11 mg.kg⁻¹ and DEHP values from 0.58 mg.kg⁻¹ to 28.20 mg.kg⁻¹ in all the analysed samples during storage lasting 28 days.

Currently, there is sufficient information about contamination of food by phthalates and their content in food packaging materials. Considering the presence of phthalates in the environment and food chain as well as due to the migration of PAE from packages into the food as was proved in this study, it is desirable to accept all of the necessary legal measures to reduce migration of PAE into the environment and foodstuffs.

One of the ways of the progressive reduction of risks of phthalates is promoting the substitution of toxic phthalates by other health-safe substances, e.g. citrates, phenol alkylsulphonate or benzoates, particularly in the production of materials used in agriculture, food and health care industries.

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THE USE OF FRUIT EXTRACTS FOR PRODUCTION OF BEVERAGES WITH HIGH ANTIOXIDATIVE ACTIVITY

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ABSTRACT

Free radicals and reactive oxygen species can cause many diseases of the circulatory and nervous system as well as tumors. There are many ways of preventing and treating these diseases including the consumption of products that contain significant amounts of antioxidant compounds, such as polyphenols and antioxidative vitamins. However, currently food stores offer mainly convenient food, ready-to-eat foodstuffs or highly processed products. During numerous technological treatments they have been deprived of many valuable compounds occurring in fresh products. Therefore, an important element of the food production technology is to ensure a proper composition of valuable human health-promoting compounds, mostly vitamins, minerals and polyphenols in final food product. Consumers often and willingly drink beverages. They are also a good starting base for supplementation. Drinks can be enriched with polyphenols, which may reduce the risk of lifestyle diseases, owing to their antiradical potential. The aim of this study was to use the fruit extracts for beverages enrichment in order to increase their antioxidative potential and polyphenol content. For the experiment the fruits of Cornelian cherry, lingonberry, elderberry, hawthorn and Japanese quince were used. Fruit was extracted with 80% ethanol, and then thickened by distillation under reduced pressure. Extracts were used to enrich the apple, orange and grapefruit beverages. Antioxidative activity and total polyphenols content in final beverages were determined. Also, sensory analysis was carried out. The fortification of tested beverages resulted in an increased antioxidative activity and total polyphenol content in case of all applied fruit extracts. Among the beverages composed, the best antioxidative properties were found in a beverage of red grapefruit, whereas the best organoleptically evaluated was the orange beverage. The scores of on the sensory evaluation revealed that the addition of extracts from Japanese quince fruit (in the case of apple and orange beverages) and lingonberry extract (grapefruit beverage) were preferred than the other samples.

Keywords: beverage; enrichment; antioxidative potential; polyphenol compound

INTRODUCTION

Current lifestyles and unhealthy eating habits contribute to the development of many diseases, known as lifestyle diseases, including heart disease, cardiovascular and nervous systems diseases and tumors. One of the main reason of mentioned diseases is the too high concentration of free radicals and reactive oxygen species. One of the method of treatment is introducing of properly balanced diet, which should be rich in compounds with antiradical potential, such as polyphenols (Robards et al., 1999; Knekt et al., 2002). Exogenous antioxidants include, among others, vitamin E and C, carotenoids, and polyphenols. The antioxidant properties of polyphenolic compounds result, inter alia, from their ability to metal chelating, quenching of free radicals and preventing the Fenton and Haber-Weiss reactions, preventing for example a lipid peroxidation. They also react with intermediate products, generated in the course of peroxidation, leading to the termination of free radical reaction (Manach et al., 2004). Antioxidative properties have mainly phenolic acids and flavonoids. These abilities largely depend on the structure of the molecule, mainly on the amount and localization of hydroxyl groups (Gawlik-Dziki, 2004). The antioxidative activity increases with increasing

number of hydroxyl groups in the molecule. Moreover, antioxidative potential of molecules is higher when -OH groups are attached in position ortho- or para- than in meta-. It has been also demonstrated that glycosylation of flavonoids reduces their antioxidative activity comparing with the corresponding aglycones. Flavonoids exert also protective impact on vitamin C and E, and may decrease the activity of enzymes that are involved in peroxidation of membrane lipid. Antioxidative activity of phenolic acids could be increased by attaching additional methoxy groups to the ring (Heimet et al., 2002). Very good source of phenolic compounds in our diet are fruits and vegetables.

Fruit beverages, juices and nectars are one of the most commonly consumed groups of food products. They increasingly attract attention with new flavor compositions and interesting, tempting packaging. Physicians and nutritionists encourage people to make the consumption of vegetables and fruits more frequent, including tasty and readily available juices, which are an excellent source of vitamins and polyphenols in the human diet. However, not every fruit drink or juice has a positive effect on health. The final beverages often irretrievably had lost their health properties, mainly due to the technology used by the manufacturer or because of low quality of raw materials

(Oszmiański et al., 2007; Nosecka, 2008; Sokół-Łętkowska and Kucharska, 2008). The most valuable health benefits have cloudy fruit and vegetable juices. They contain significant amounts of polyphenolic compounds, vitamins, minerals and fiber. However, the majority of the commercially available juices are clarified juices. Unfortunately, as a result of the clarification many valuable components are lost, which is detrimental to consumers. When clarified juice is produced, the compounds with the highest antioxidative activity that originates from fruits are largely lost. Low molecular weight polyphenols are oxidized, while the high molecular polyphenols are removed during the clarification. However, the clarified juice can be enriched only in sugars and acids, and there is no possibility to add polyphenolic compounds in any form. On the other side, beverages are the best group of products to use with functional additives, which in other products could not be found (Oszmiański and Wojdyło, 2006; Sokół-Łętkowska and Kucharska, 2008).

Food fortification has been defined as the addition of one or more essential nutrients to a food, whether or not it is normally present in that food, for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population group (FAO/WHO, 1994). Food fortification is one of the possibilities of enrichment food products with ingredients that have been removed or inactivated during the production process. In addition, producers may provide new properties for foodstuff through addition of the ingredients, which are not naturally occurring in the product (Gębczyński and Jaworska, 2009).

The aim of the study was to evaluate the possibility of applying the fruit extracts for enrichment fruit beverages in polyphenol compounds and hence increasing their antioxidative activity.

MATERIAL AND METHODOLOGY

Apple, orange and grapefruit juice concentrates (Tymbark, Poland) were used for preparing the beverages. Apple beverage (20% concentrated apple juice, 80% distilled water), orange drink (18% concentrated orange juice, 2% sugar syrup, 80% distilled water) and grapefruit drink (18% concentrated grapefruit juice, 14% sugar syrup, 68% distilled water) were prepared. Then the beverages were fortified with fruit extracts.

The extracts were obtained from fruits purchased from the pomological orchard of University of Agriculture in Krakow, located in Garlica Murowana near Krakow or from the organic farms in Malopolska and Podkarpacie areas. As the raw material for extracts elderberry fruits (*Sambucus nigra* L.), Japanese quince (*Chaenomeles japonica* (Thunb.)), Cornelian cherry fruits (*Cornus mas* L.), lingonberry (*Vaccinium vitis-idea* L.), and hawthorn (*Crataegus oxyacantha* L.) were used.

Preparation of extracts

10 g of seedless fruit was soaked with 90 mL of ethyl alcohol (80% v/v) and extracted using a high speed homogenizer (19 000 rpm; 5 min; UltraTurrax T25 Basic, IKA). The extracts were filtered, adjusted to 100 mL by adding a solvent and stored at -20°C. Before introducing to

the fruit beverages, extracts were concentrated four-fold by distillation under reduced pressure (60°C) under an inert atmosphere (N₂).

Evaluation of antioxidative activity (Tarko et al., 2009)

The antioxidative activity in final fruit beverages was determined by using the active radical cation ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), Sigma). ABTS radical was generated by chemical reaction between 7 mM aqueous solution of diammonium salt of 2,2'-azynobis (3-ethylbenzthiazoline-6-sulfonate) and 2.45 mM potassium persulfate solution (K₂S₂O₈). The solution was kept overnight in the dark at ambient temperature, to terminate the reaction and to stabilize ABTS cation. During analysis, the concentrated solution of ABTS was diluted with phosphate buffer saline (PBS) at pH 7.4, to obtain solution with absorbance value of $A = 0.70 \pm 0.02$ (ABTS_{0,7}) measured by a spectrophotometer (Beckman DU 650) at a wavelength of 734 nm. 100 µL of the appropriate diluted samples were added to 1 mL of ABTS_{0,7} and the absorbance was measured at 6 minutes after mixing. The antioxidative capacity of the samples was calculated using a standard curve performed with solutions of synthetic vitamin E (Trolox; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma) and expressed in mg of Trolox/100 mL.

Determination of total polyphenol contents (Tarko et al., 2009)

Total polyphenol content was determined by the modified Folin-Ciocalteu method. 45 mL of redistilled water was added to 5 mL of diluted beverages. Then, 0.25 mL of Folin-Ciocalteu reagent (water dissolved at 1:1 v/v, Sigma) and 0.5 mL 7% Na₂CO₃ (POCh) were added. Samples were incubated for 30 minutes in the dark, before measuring the absorbance by a spectrophotometer at the 760 nm (against PBS as a blank). The results of total polyphenols content were obtained based on the calibration curve and were expressed as mg of catechin/100 mL of beverage.

Sensory evaluation (PN-64/A-04022)

Evaluation was carried out by the panel comprising 20 qualified and tested for their sensory sensitivity people. They assessed three basic quality factors. For the sensory evaluation the 5-point scale with following weights: flavor (0.5), odor (0.3) and color (0.2) was used.

Statistical analysis

There were a minimum of three repetitions of the analysis and the results are shown as the arithmetic mean with standard deviation (\pm SD). Statistical analysis was performed using InStat v. 3.01 (GraphPad Software Inc., USA). A single-factor analysis of variance (ANOVA) with post hoc Tukey's test was applied to determine the significance of differences between means. The Kolmogorov-Smirnov test was carried out to assess the normality of distribution.

RESULTS AND DISCUSSION

In the first stage of the study, beverages were prepared with addition of various extracts at doses from 1 to 10%. The sensory evaluation was performed and the obtained results showed that extracts of Japanese quince (a final

concentration 2 and 5%), Cornelian cherry (2%) or lingonberry (2%) were suitable for the enrichment of apple beverages; Japanese quince extracts (2 and 5%) and lingonberry (2%) for orange beverages and Japanese quince extract (2%), Cornelian cherry (2 and 10%) and lingonberry (2%) for grapefruit beverages. Extracts from the elderberry and hawthorn fruits were rated as not acceptable by the sensory panel.

Apple beverages were characterized by the lowest antioxidative activity and the total polyphenols content in comparison with other samples. For this reason their fortification raised antiradical capacity much more than in case of other beverages (Table 1). The increased antioxidative capacity of fortified beverages (by 10 to 32% in comparison with control beverage) has been shown. However, fortification with fruit extracts first of all affected the amount of polyphenolic compounds in apple beverages (from 48 to 138% more than in the beverage without additives). The highest differences were observed for the 5% addition of Japanese quince extract, which is a raw material rich in organic acids (malic and citric acids), catechin (140 mg/100 g) and leucoanthocyanins (270 mg/100 g) (Domański et al., 1994; Fronc and Oszmiański, 1994). It could be also noted that only the addition of 2% of the Japanese quince extract had a positive effect on the score of sensory evaluation. Fortification of apple beverages with lingonberry and Cornelian cherry extracts was negatively evaluated by the sensory panel. A strong correlation between the content of total polyphenols and antioxidative activity of beverages ($R_2 = 0.94$) was also shown.

In the case of enrichment of orange drink with fruit extracts a lower impact on the total polyphenol content was demonstrated than in the corresponding apple beverages. Enrichment of orange drink in Japanese quince extract and lingonberry extract contributed to an increase of their antioxidative capacity, as well as the total polyphenol content (Table 1). It has been shown that the additives in a similar way increased the quality parameters of analyzed orange drink. Lingonberry, like Japanese

quince, is characterized by significant catechin and organic acids content (Matuszkiewicz, 2006). The 5% addition of Japanese quince extract was associated with increased antioxidative activity and polyphenol content in orange beverage (by 24 and 56%, respectively). It is worth noting that the supplementation of orange beverages with extracts from Japanese quince, both at concentration of 2 and 5%, resulted in the higher scores of sensory evaluation. Japanese quince extracts are characterized by high acidity (3.5–4.5%) (Lopez, 2006), which matched well with a relatively high content of organic acids in orange drink and for this reason, they could be attractive for evaluators. High sensory evaluation score is also related to the content of sugars in the fruit. Japanese quince contains improper sugar to acid ratio (three times more acids than sugars, while fruits intended for direct consumption should contain at least 10 times more sugar than acids) (Lesińska, 1988). The proportions of acids and sugars in the fruits of Cornelian cherry disqualify these fruits for direct consumption, but the orange beverage has been much sweeter than apple beverage and therefore it better masked the sour taste sensation of added extracts.

Grapefruit drinks are a good raw material for enrichment due to high acidity, high sugar content and a dark color. These features well mask the undesirable sensory traits of introduced additives (eg. dark color of extracts). The enrichment of grapefruit beverages in fruit extracts slightly contributed to the change of their antioxidative capacity and total polyphenol content (Table 1). These values, especially the amount of polyphenols, did not differ significantly. Only 10% addition of Cornelian cherry extract caused an increase of the analyzed parameters values. However, such high content of the extract in the grapefruit beverage was negatively rated by the sensory panel, and scores were significantly lower than in the case of beverage without additives. Worth mentioning is the fact, that only in the case of this beverage, addition of Japanese quince extract did not increase the scores of sensory evaluation. It was also shown that fortification of grapefruit beverages with extracts of lingonberry increased

Table 1 Antioxidative activity, total polyphenols content and sensory evaluation of beverages enriched with plant extracts (mean ± SD).

Parameter	Beverages	Control	Japanese quince 2%	Japanese quince 5%	Lingonberry 2%	Cornelian cherry 2%	Cornelian cherry 10%
Antioxidative activity [mg Trolox/100 mL]	Apple	177 ± 1.6 ^a	206 ± 2.2 ^b	234 ± 3.4 ^c	194 ± 1.1 ^d	207 ± 1.3 ^b	–
	Orange	329 ± 5.7 ^a	357 ± 3.4 ^b	407 ± 5.2 ^c	364 ± 1.5 ^b	–	–
	Grapefruit	274 ± 3.1 ^a	300 ± 3.9 ^b	–	306 ± 0.9 ^{b,c}	323 ± 2.3 ^c	396 ± 3.3 ^d
Total polyphenols content [mg catechin/100 mL]	Apple	34 ± 1.7 ^a	57 ± 2.3 ^b	81 ± 0.8 ^c	51 ± 1.1 ^d	51 ± 1.0 ^d	–
	Orange	66 ± 5.5 ^a	91 ± 2.0 ^b	103 ± 1.9 ^c	92 ± 2.8 ^d	–	–
	Grapefruit	62 ± 5.9 ^a	72 ± 0.6 ^b	–	67 ± 0.7 ^a	66 ± 0.3 ^a	105 ± 3.7 ^c
Sensory evaluation score [points]	Apple	3.89 ± 0.3 ^a	4.01 ± 0.4 ^b	3.88 ± 0.4 ^a	3.79 ± 0.5 ^c	3.70 ± 0.2 ^c	–
	Orange	3.99 ± 0.4 ^a	4.32 ± 0.4 ^b	4.10 ± 0.2 ^c	3.88 ± 0.3 ^a	–	–
	Grapefruit	4.01 ± 0.5 ^a	3.99 ± 0.2 ^a	–	4.15 ± 0.4 ^b	3.69 ± 0.3 ^c	3.47 ± 0.3 ^c

Note: means marked with different letter (within the analyzed parameter and beverage - lines) differ statistically at $p < 0.05$

sensory evaluation scores. In case of this beverage a very strong influence of the content of polyphenolic compounds on antioxidative activity was also shown ($R^2 = 0.96$).

CONCLUSION

This study has shown that addition of fruit extracts positively affected antioxidative activity and total polyphenolic content of beverages. Based on the sensory evaluation, the samples with addition of extract from Japanese quince fruit (in the case of apple and orange beverages) and lingonberry (grapefruit beverage) were preferred before the others. It can be assumed that beverages fortified with ingredients that may provide a health benefits will be very popular in the future, mainly due to increasing consumer awareness.

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THE INFLUENCE OF *PICHIA* KILLER TOXINS ON THE WINE SPOILAGE YEASTS

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ABSTRACT

Killer yeasts are able to produce toxins that antagonize the growth of susceptible yeasts cells of the same species or the ones that are related to them. Killer strains are resistant to their own toxins but can be sensitive to killer proteins of other yeasts. The killer proteins of *Pichia* spp. are known for its broad spectrum of antifungal activity including pathogens such as *Candida albicans*. The aim of the study was to investigate the potential of the partly purified killer toxins to inhibit the growth of selected yeast strains which can contribute to wine spoilage. Three *Pichia* killer yeast strains (CBS 1982, CBS 5759, CBS 7373) were used in the study. The killer protein secreted by *Pichia anomala* CBS 1982 was characterized by the highest antifungal activity. The most pronounced effect of the reduction of cell proliferation by killer toxin preparations was found after 2 and 20 h cultivation. Among the 13 tested strains, all *Pichia* killer toxin preparations inhibited the growth of *Rhodotorula graminis* Rg, *Rhodotorula mucilaginosa* Rm and *Schizosaccharomyces pombe* DSM 70576. Killer toxins produced by *Pichia anomala* CBS 1982 (K8) and CBS 5759 (K4) limited the growth of *Candida pulcherrima* K5 and *Hanseniaspora guillermondii* DSM 3432 after 2, 20 and 168 h of incubation. A significant reduction of *Debaryomyces hansenii* DSM 3428 biomass was observed in medium with the addition of one toxin preparation (*Pichia anomala* CBS 1982). The growth limitation of *Candida glabrata* DSM 6425, *Hanseniaspora uvarum* DSM 2768, *Metchnikowia pulcherrima* DSM 70321 and *Cryptococcus laurentii* DSM 70766 was noticed only after 2 hours cultivation in presence of killer protein preparations. The killer toxins could be used in the food industry as selective tools to control infections during the fermentation of wine and improve the quality of the final product.

Keywords: *Pichia*; killer yeast; killer toxin; wine spoilage

INTRODUCTION

The killer phenomenon was first described by Bevan and Makower in 1963 in a *Saccharomyces cerevisiae* strain which was isolated as a brewery contaminant. Since then, killer systems have been reported in other yeast genera such as *Ustilago*, *Kluyveromyces*, *Pichia*, *Candida*, *Debaryomyces*, *Torulopsis*, *Cryptococcus*, *Metchnikowia*, *Williopsis* and *Zygosaccharomyces* (Schmitt and Breinig 2002; Pfeiffer et al., 2004; Izgü et al., 2006; Santos et al., 2009). Killer activity is one of the most important mechanisms of competition between strains and plays a significant role in the ecology of yeasts especially at low nutrient availability in the environment. The killer effect may represent a model of biological competition similar to that of bacteriocins among bacteria (Magliani et al., 1997).

Killer yeasts secrete toxins (usually proteins or glycoproteins) which kill cells of sensitive strains of yeasts belonging to the same or related species without direct cell–cell contact (Selitrennikoff, 2001; Wang et al., 2007). Killer strains are immune to their own toxins but can be sensitive to killer proteins of other yeasts. Each toxin has unique properties which differ considerably depending on the strain which it produces. The killer proteins of *Pichia* spp. have broad spectrum of antifungal activity including pathogenic *Candida albicans* (Santos

and Marquina, 2004; Izgü et al., 2006). They are relatively stable in comparison to toxins of *Saccharomyces* spp. (Sawant et al., 1989).

Several applications for the killer yeasts and their toxins have been considered. Starter cultures with killer activity could be used to eliminate undesirable yeasts and filamentous fungi during the production of wine or beer. Killer strains are regarded useful in biological control of spoilage yeasts and the preservation of food (Izgü et al., 2006). Killer toxins could be also considered as novel antimicrobial agents in the treatment of human fungal infections (Schmitt and Breinig, 2002).

The aim of this study was to determine the ability of *Pichia* killer toxins to inhibit the growth of 13 selected yeast strains that are associated with fermentation of grape must and infection during winemaking.

MATERIAL AND METHODOLOGY

Yeast strains

The killer strains employed in this study (*Pichia anomala* CBS 1982 producing K8 toxin, *Pichia anomala* CBS 5759 secreting K4 toxin, *Pichia membranifaciens* CBS 7373 producing K7 toxin) were provided from the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands. The sensitive yeast strains such as *Rhodotorula graminis* Rg,

Rhodotorula mucilaginosa Rm, *Candida pulcherrima* K5, *Kloeckera apiculata* 66 were sourced from the Culture Collection of the Department of Fermentation Technology and Technical Microbiology, University of Agriculture in Krakow, Krakow, Poland. These yeasts were isolated from Wegierka Zwykła plums (Satora and Tuszyński, 2005). Other sensitive yeast strains used in this study (*Schizosaccharomyces pombe* DSM 70576, *Candida glabrata* DSM 6425, *Candida sake* DSM 70763, *Debaryomyces hansenii* DSM 3428, *Hanseniaspora guillermondii* DSM 3432, *Hanseniaspora uvarum* DSM 2768, *Metschnikowia pulcherrima* DSM 70321, *Cryptococcus laurentii* DSM 70766, *Pichia anomala* DSM 6766) were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. All pure yeast cultures were stored on agar slants with YEPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar at 4 °C.

Killer yeast cultivation

Killer yeast strains were cultured on agar slants with YEPD medium for 24 h at 28 °C. Next they were transferred into 50 mL of liquid YEPD medium and incubated at 28 °C for 24 h. At next stage 125 mL of liquid medium was inoculated with killer strain (the final dry substance of yeast cells 2g/L). Killer cultures were cultivated at 20 – 22 °C with shaking at 120 RPM on a gyratory shaker for 96 h. When the cells reached to stationary phase they were removed by centrifugation (4000 rpm for 10 min) and the culture supernatant was filtered through and 0.2 µm cellulose acetate membranes. Next the filtrate was adjusted to a final glycerol content of 15% (v/v) and concentrated 40-fold by using a centrifugal concentrator devices with a 10 kDa cut-off membrane.

Killer activity against wine spoilage microorganisms

Pure wine spoilage strains were cultivated on agar slants with YEPD medium at 28 °C for 24 h. At next stage each starter culture was prepared by inoculating four loops of slant culture into 125 mL and incubated at 28 °C for 24 h. Next yeast suspension was diluted 10-fold with sterile Ringer's solution. 1 mL of diluted yeast suspension was added to 8 mL of liquid medium containing 0.8% (w/v) nutrient broth, 1% (w/v) glucose and 0.01% (w/v) chloramphenicol, and then 1 mL of killer toxin preparations in 0.1 M citrate-phosphate buffer (pH 4.4) were added. A toxin-free control sample contained 8 mL of liquid medium, 1 mL of diluted yeast suspension and 1 mL of 0.1 M citrate-phosphate buffer pH 4.4. Measurements of the turbidity at 600 nm were done after 2, 20 and 168 h, using a standard curve of absorbance against dry cell mass concentration. All experiments were performed four times.

Statistical analyses

Statistical analyses were performed using InStat software, version 3.01 (GraphPad Software Inc., San Diego, USA). A single-factor analysis of variance (ANOVA) with a post hoc Tukey-Kramer's test was used to find means that are significantly different from each

other. The means for the experimental groups were compared at 5% probability level.

RESULTS AND DISCUSSION

The influence of killer toxins on wine fermentation was investigated in several research studies (Shimizu, 1993; Medina et al., 1997; Gutiérrez et al., 2001; Pérez et al., 2001; Satora et al., 2014). In some cases the presence of killer yeasts may decrease wine quality or even cause stuck or sluggish wine fermentation. On the other hand, must inoculation with killer yeast may reduce undesirable wild yeast strains, thus protect wine quality (Maqueda et al., 2012). In the case of food preservation biological control with yeasts has been considered as a desirable alternative to the application of chemicals (Santos et al., 2009).

To examine the potential of *Pichia* killer toxins as biocontrol agents 13 cultures of yeast which often cause the diseases of wine were selected. The results of *Pichia* killer activity against spoilage wine strains are presented in Table 1. Toxin activity was expressed as the percentage reduction in growth of the sensitive strain yeast with respect to a toxin-free control.

The pronounced inhibition of *Rhodotorula graminis* Rg growth by *Pichia* killer proteins was noted. The major effect was found when K8 killer toxin was added to medium. A considerable reduction of the biomass growth was also observed when killer proteins secreted by *Pichia* strains CBS 5759 and CBS 7373 were used. A smaller increase in a biomass production relative to control sample was noticed during *Rhodotorula mucilaginosa* Rm cultivation with addition of all three killer preparations. In the case of *Schizosaccharomyces pombe* DSM 70576 the effect of the growth limitation was observed after 2 h and 20 h incubation. During following days more intensive growth in relation to control sample was found. It could be explained by assimilation of nitrogen compounds as proteins of toxin preparation by studied yeasts.

It was also found that killer toxins produced by strains CBS 1982 (K8) and 5759 (K4) inhibited the growth of *Candida pulcherrima* K5 and *Hanseniaspora guillermondii* DSM 3432 after 2, 20 and 168 h of incubation. A significant reduction of *Debaryomyces hansenii* DSM 3428 biomass was observed in medium with the addition of only one toxin preparation (*Pichia anomala* CBS 1982). In the case of *Candida glabrata* DSM 6425, *Hanseniaspora uvarum* DSM 2768, *Metschnikowia pulcherrima* DSM 70321 and *Cryptococcus laurentii* DSM 70766 strains the greatest inhibition of growth was evident after 2 hours. The limitation of cell proliferation was noted only in the case of usage of toxins produced by *Pichia anomala* CBS 1982 and CBS 5759. After two hours of cultivation, the inhibition of *Pichia anomala* DSM 6766 and *Kloeckera apiculata* 66 growth was observed in the presence of K8 toxin secreted by the strain CBS 1982. In another study on the *Pichia* killer activity, the action of PMKT2, a toxin from *Pichia membranifaciens* CYC 1086 which is active against *Brettanomyces bruxellensis*, was reduced significantly in the first hour, then killer activity was constant for 10 hours (Santos et al., 2009).

Table 1 The impact of *Pichia* killer toxins on the growth of yeast which can contribute to the spoilage of wine. Results are expressed as the percentage of biomass of yeast strains cultivated after treatment with killer toxins in relation to the growth without addition of killer proteins.

Sensitive yeast strain	Killer strain	Cultivation time		
		2 h	20 h	168 h
<i>Rhodotorula graminis</i> Rg	1982	9 ^a ±4	65 ±11	82 ^a ±3
	5759	20 ^b ±4	78 ±4	97 ^b ±2
	7373	37 ^b ±18	68 ±8	86 ^a ±3
<i>Rhodotorula mucilaginosa</i> Rm	1982	34 ±20	83 ^a ±2	106 ^a ±6
	5759	33 ±17	85 ^a ±1	90 ^b ±5
	7373	52 ±27	70 ^b ±6	76 ^c ±1
<i>Schizosaccharomyces pombe</i> DSM 70576	1982	52 ±33	74 ±6	116 ±7
	5759	33 ±2	74 ±1	118 ±2
	7373	55 ±38	80 ±4	132 ±10
<i>Candida pulcherrima</i> K5	1982	42 ±22	66 ±15	59 ±28
	5759	67 ±37	75 ±10	61 ±20
	7373	115 ±24	82 ±14	77 ±15
<i>Candida glabrata</i> DSM 6425	1982	27 ±10	99 ±2	72 ±20
	5759	44 ±27	103 ±3	99 ±2
	7373	70 ±35	108 ±2	102 ±1
<i>Candida sake</i> DSM 70763	1982	152 ±77	103 ±9	92 ±2
	5759	61 ±47	102 ±3	94 ±2
	7373	119 ±17	96 ±1	86 ±1
<i>Debaryomyces hansenii</i> DSM 3428	1982	18 ^a ±10	62 ±6	53 ^a ±2
	5759	80 ^b ±27	88 ±5	55 ^a ±11
	7373	102 ^b ±18	75 ±10	91 ^b ±3
<i>Hanseniaspora guillermondii</i> DSM 3432	1982	46 ^a ±31	58 ±17	62 ^a ±2
	5759	62 ^a ±17	73 ±13	60 ^a ±2
	7373	121 ^b ±23	83 ±14	92 ^b ±6
<i>Hanseniaspora uvarum</i> DSM 2768	1982	36 ^a ±18	95 ±6	98 ^a ±2
	5759	43 ^a ±22	102 ±3	96 ^{ab} ±4
	7373	99 ^b ±1	92 ±1	84 ^b ±2
<i>Kloeckera apiculata</i> 66	1982	40 ^a ±9	83 ±15	79 ^a ±6
	5759	124 ^b ±21	88 ±10	76 ^a ±4
	7373	110 ^{ab} ±14	95 ±5	98 ^b ±2
<i>Metschnikowia pulcherrima</i> DSM 70321	1982	25 ^a ±16	90 ±2	100 ±2
	5759	55 ^a ±25	91 ±2	102 ±4
	7373	120 ^b ±19	81 ±10	89 ±12
<i>Cryptococcus laurentii</i> DSM 70766	1982	36 ^a ±13	71 ±8	93 ±2
	5759	19 ^a ±10	86 ±6	91 ±3
	7373	125 ^b ±14	89 ±13	95 ±3
<i>Pichia anomala</i> DSM 6766	1982	42 ^a ±13	82 ±5	82 ±6
	5759	70 ^{ab} ±17	86 ±6	82 ±4
	7373	110 ^b ±20	81 ±3	81 ±5

Note: The values with different superscript letters mean statistically significant differences at 5% levels of probability

The results showed relatively weak influence of killer toxin preparations on the certain tested yeasts. Among the *Pichia* strains used in the study, the killer toxin preparation of *Pichia anomala* CBS 1982 was characterized by the

highest antifungal activity, whereas killer toxin K7 secreted by *Pichia membranifaciens* CBS 7373 inhibited less effectively the growth of selected strains.

Killer toxins as proteinaceous compounds have a limited stability in solution. In many cases, killer proteins are characterized by a high susceptibility to various factors such as an elevated temperature or the presence of proteases. It would be important to conduct further research on the action of *Pichia* killer toxins against tested spoilage yeasts in winemaking conditions. It is necessary to determine how the presence of killer toxins could influence the metabolites production by tested yeasts and in consequence change the quality of the wine. It would also be important to increase the stability of studied killer toxins or consider the application of different killer proteins which are more stable or more efficient in their inhibitory action.

CONCLUSION

The killer toxin secreted by *Pichia anomala* CBS 1982 was distinguished by the highest antifungal activity. Of the 13 wine spoilage strains, all *Pichia* killer toxin preparations inhibited the growth of *Rhodotorula graminis* Rg, *Rhodotorula mucilaginosa* Rm and *Schizosaccharomyces pombe* DSM 70576. The most pronounced effect of the reduction of cell proliferation by killer toxin preparations was found after 2 and 20 h cultivation. Further research should be done to determine the activity of *Pichia* killer toxin preparations under winemaking conditions. The obtained results may find the application in the food industry. The killer toxins could be used as selective tools to control infections during the fermentation of wine.

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TOXOPLASMA GONDII IN WILD RUMINANTS BRED IN GAME PRESERVES AND FARMS WITH PRODUCTION DESTINED FOR HUMAN CONSUMPTION IN THE CZECH REPUBLIC

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ABSTRACT

Toxoplasma gondii is the causative agent of the most common parasitic infection in humans. Almost all warm-blooded animals, as well as humans, can act as intermediate hosts that harbour infective cysts in their tissues. Felids act as definitive hosts excreting oocysts in faeces. In humans, *T. gondii* can cause subclinical infection but also severe clinical disease with a wide range of symptoms, especially in immunocompromised individuals. The infection is usually asymptomatic in animals and is not recognized at either *ante-* or *post-mortem* inspection. The consumption of undercooked meat from infected animals is one of the most important routes by which the infection can be transmitted to humans. Handling of the organs and other tissues of game animals and eating their undercooked meat have been described as a risk of *T. gondii* infection. For diagnosis of toxoplasmosis, the combination of serological and molecular methods has been described as a suitable approach. Antibodies against *T. gondii* were detected in 20.8%, 50.0%, 23.1%, and 24.4% of red deer, sika deer, fallow deer and mouflons, respectively, coming from game preserves and farms in the Czech Republic. *T. gondii* DNA was found in the muscle tissue of red deer (8.3%) and mouflons (14.6%). The lower prevalence rates based on molecular screening could be due to the random distribution and low density of cysts in tissues of infected animals. Bearing in mind the increase in the number of hunted animals and the growing trend in game consumption, it is important to educate hunters and game meat consumers about the risk of exposure to this zoonotic infection during handling and consumption of the meat.

Keywords: zoonosis; food safety; meat; tissue cyst; antibodies

INTRODUCTION

Toxoplasma gondii is a ubiquitous zoonotic parasite of significant concern to human health (EFSA, 2007). *Toxoplasma* only rarely causes severe clinical symptoms, the infection is mostly asymptomatic or only mild symptoms occur (self-limiting lymphadenopathy, fever or intraocular inflammation). However, it can cause life-threatening infections in immunocompromised individuals (disseminated disease with encephalitis, meningoencephalitis, myocarditis or hepatitis). Parasitaemia in a primarily infected pregnant woman may result in congenital toxoplasmosis with abortion, neonatal death, or fetal damage as encephalomyelitis, retinochoroiditis, intracranial calcifications, hydrocephalus or mental retardation in survivors (Tenter et al., 2000; EFSA, 2007; Kijlstra and Jongert, 2008).

Almost all warm-blooded animals, as well as humans, can serve as intermediate hosts of *Toxoplasma* with the formation of infective tissue cysts having a high affinity for neural and muscular tissues. Tissue cysts may persist for the life of the hosts. Wild and domestic cats and other felids act as definitive hosts excreting oocysts in their faeces. Infection in hosts, including humans, can be acquired by the consumption of raw or undercooked meat containing tissue cysts, from soil, water or food contaminated by oocysts, or congenitally. Also, contact

with infected carcasses during evisceration, dressing and processing presents a risk of infection to humans (Cook et al., 2000; Tenter et al., 2000; EFSA, 2007; Jones et al., 2009; EFSA 2013b).

The infection usually does not cause clinical signs in animals or visible lesions in carcasses and is not recognized at either *ante-* or *post-mortem* inspection. Although toxoplasmosis is the most reported parasitic zoonosis in humans in EU, there is inadequate system for routine monitoring and therefore the incidence of toxoplasmosis in humans and animals and the presence of *T. gondii* in food is underestimated (EFSA, 2007). According to the European Food Safety Authority (EFSA), *T. gondii* was identified as a relevant biological hazard to be addressed in revised meat inspection regulations for pigs, sheep, goats, farmed deer and farmed wild boar (EFSA, 2011; 2013a; 2013b). Infected game can serve as a source of infection for other animals, especially carnivores, and humans (Ross et al., 2001; EFSA, 2013b). Wildlife may also be a good indicator of environmental contamination with *T. gondii* oocysts (Olamendi-Portugal et al., 2012; Ferroglio et al., 2014).

One of the suitable diagnostic methods is the detection of specific antibodies against *T. gondii* in serum or meat juice and seropositivity has been correlated with the presence of cysts in muscles and other animal tissues (Dubey, 1995).

Molecular methods, which can detect the genome of the parasite in tissues but without confirmation of its viability, are less sensitive because the density of these parasites is low in meat (Dubey, 1988; Dubey et al., 2014).

According to EFSA, there are limited data on the prevalence of toxoplasmosis predominantly in farmed cervids (EFSA, 2013b). By means of both serological and PCR testing, the aim of this study was to determine the prevalence of *T. gondii* in wild ruminants from game preserves and farms in the Czech Republic, destined for human consumption.

MATERIAL AND METHODOLOGY

During the period 2012-2014, muscle samples (musculus gluteus, diaphragma and/or masseter) were collected from 82 carcasses of wild ruminants (red deer, sika deer, fallow deer, and mouflons) immediately following hunting and killing. Animals came from game preserves (n=5) and farms (n=6) in the Czech Republic (regions Kralovehradecky, Pardubicky, Stredocesky and Vysocina) and their meat was intended for human consumption. The ages of the majority of the animals were not available for the purposes of this study.

Meat juices were obtained by subsequent freezing and thawing of the muscle tissue and stored at -20 °C until tested for antibodies to *T. gondii* by the ID Screen® Toxoplasmosis Indirect Multi-species ELISA kit (IDVET, Montpellier, France) according to the manufacturer's instructions. This ELISA kit was evaluated as a screening test for toxoplasmosis in sera or meat juice of ruminants, cats, dogs and pigs and was also used in serological surveys in wild ruminants (Roqueplo et al., 2011).

In addition, 25 g of muscle tissue were analyzed with real time PCR specific for *T. gondii*. Tissue samples were processed according to Opsteeght et al. (2010) prior to DNA isolation based on the manufacturer's protocol (QIAGEN) slightly modified to include mechanical homogenization with zirconia/silica beads (0.2 mm) in a MagNALyser instrument (Roche, Mannheim, Germany). The isolated DNA was used as a template for the triplex

real time PCR assays. The detection of *T. gondii* via primers and probes specific for *BI* and *529rep* was adopted from Lass et al. (2012) and Opsteeght et al. (2010). The previously published internal amplification control was introduced to eliminate false negative samples (Slana et al., 2008).

RESULTS AND DISCUSSION

It has been suggested that the handling of the organs and other tissues of game animals and eating their undercooked meat carries a risk to humans of potential *T. gondii* infection (Cook et al., 2000; EFSA, 2007). Ross et al. (2001) described acquired ocular toxoplasmosis with flu-like symptoms in deer hunters after ingestion of venison. Three cases of acute toxoplasmosis in deer hunters were reported by Sacks et al. (1983). Ingestion of raw or rare venison was considered to be the most likely route of infection.

The consumption of game in the Czech Republic is relatively low and traditionally confined to the hunting fraternity. However, there is an increasing trend towards a wider appreciation amongst those who seek occasional dietary diversification (0.5 kg of game per capita per year in 2006 in comparison with 0.9 kg in 2013; Anonymous, 2015a). This increase is linked to the growth of wild populations and number of hunted animals in recent years (Anonymous, 2015b).

We collected muscle samples from a total of 82 wild ruminants of four species whose meat was intended for human consumption. *T. gondii* antibodies were detected in 24.4% of animals (Table 1). Using real time PCR, the presence of *T. gondii* DNA was detected in tissues of eight (9.8%) animals. The lower prevalence rates, based on molecular screening as compared with serological testing, could be due to the random distribution and low density of cysts in tissues of chronically infected and asymptomatic animals (one cyst per 50-100 g of tissue; Dubey et al., 1988; Dubey et al., 2014). Nevertheless, real time PCR is a useful method for demonstrating the presence of parasites in muscle samples of food-producing animals. *T. gondii* DNA was detected in four out of eight cases in

Table 1 Prevalence of *Toxoplasma gondii* infection in wild ruminants from game preserves and farms in the Czech Republic.

Species	No. examined	Prevalence of <i>T. gondii</i> antibodies		Detection of <i>T. gondii</i> DNA in muscles	
		No. positive	Prevalence (%)	No. positive	Prevalence (%)
Red deer (<i>Cervus elaphus</i>)	24	5	20.8	2	8.3
Sika deer (<i>Cervus nippon dybowskii</i>)	2	1	50.0	0	0
Fallow deer (<i>Dama dama</i>)	13	3	23.1	0	0
Mouflon (<i>Ovis musimon</i>)	41	10	24.4	6	14.6
Total	82	20	24.4	8	9.8

seronegative animals (two red deer and two mouflons). It indicates either recent infection without the development of antibodies or that the antibody titers had declined to undetectable levels (Dubey et al., 1995). The combination of serological and molecular methods should be used for accurate diagnosis of toxoplasmosis (EFSA, 2007; Halová et al., 2012).

We did not find significant differences in seroprevalence amongst the investigated species. The highest prevalence of *T. gondii* antibodies was observed in sika deer (50.0%), but the number of available samples was too low (only two animals, Table 1). *T. gondii* DNA was found in tissues of red deer and a higher prevalence in mouflons (8.3% and 14.6%, respectively). Feeding habits are different in these species - deer feed mainly on grass, leaves and berries, but also on young shoots and twigs, whereas mouflons graze on short grasses and thus may be more frequently infected by oocysts shed by felids. However, the higher prevalence of infection in mouflons than in deer in our study was not confirmed by other authors (Hejlíček et al., 1997; Gauss et al., 2006; Bartova et al., 2007).

In the study of Hejlíček et al. (1997) carried out in the Czech Republic (south Bohemia) during the period 1981-1990 which targeted mainly free-ranging wild ruminants, antibodies were detected in 15% (46/303), 100% (3/3) and 10% (2/20) of red deer, fallow deer and mouflons, respectively, but tissue cysts were not isolated from these animals. However, we found a higher occurrence of antibodies, especially in red deer (20.8%) and mouflons (24.4%). It could be influenced by the origin of the investigated animals in our study coming from game preserves and farms. In these smaller, fenced areas with more cats having access and with higher animal densities, the risk of infection increases. The association between positive ELISA results for *T. gondii* and high animal density with the presence of domestic cats being the main sources of toxoplasmosis in wild animals, has already been described (Hejlíček et al., 1997; Gauss et al., 2006; Olamendi-Portugal et al., 2012). Gauss et al. (2006), however, did not find a statistically significant difference in seroprevalence in red deer from open versus fenced areas in Spain (20.9% versus 14.0%).

Bartova et al. (2007) surveyed the prevalence of *T. gondii* antibodies in wild ruminants from the countryside and captivity in the Czech Republic. They detected a higher seroprevalence in red deer (45%) which also came primarily from farms and game preserves than in our study (20.8%). On the other hand, we found *T. gondii* antibodies more frequently in fallow deer (23.1% vs. 17%) and mouflons (24.4% vs. 9%) in comparison with the aforementioned study carried out in 1998-2006.

Previous European studies reported seroprevalence of 26.7% amongst white-tailed deer in Finland (Jokelainen et al., 2010), 14.8% of mouflons and 22.8% of fallow deer in Spain (Gauss et al., 2006), 7.7% of red deer in Norway (Vikøren et al., 2004), 6.6% of deer in Ireland (Halová et al., 2013) and 0% of red deer in Northwestern Italy (Ferroglio et al., 2014). In France, similarly as in our study, Aubert et al. (2010) detected *T. gondii* antibodies in 23%, 17% and 25% of mouflons, red deer and fallow deer, respectively, and viable parasites were recovered by bioassay from one mouflon and one red deer. The

identified prevalence of toxoplasmosis in deer hunted in Poland was 8.8% using direct agglutination and 11.6% using PCR, according to the report of EFSA and ECDC (EFSA, 2014).

However, it is difficult to compare results from various studies because different diagnostic methods (serological and molecular) were used and the animal's age, geographical location and climatic conditions can also influence the prevalence of infection (Vikøren et al., 2004; Gauss et al., 2006; Jokelainen et al., 2010; Olamendi-Portugal et al., 2012; Dubey et al., 2014).

CONCLUSION

Although toxoplasmosis is the most commonly reported parasitic zoonosis in humans, the pathogen is not routinely monitored in food-producing animals. It is obvious from our study, and previous ones, that wild cervids and mouflons are commonly exposed to *T. gondii* and may serve as a reservoir of infection for humans. When game animals are killed and butchered in the field, discarded viscera left unattended can infect other animals, especially carnivores and omnivores.

To prevent the transmission of infection by ingestion, meat should be cooked well at a minimum temperature of 70 °C. It is also common to freeze game meat for later culinary use. Freezing at a temperature of at least -12 °C for 3 days should be sufficient to inactivate the parasites (Dubey et al., 1988; Tenter et al., 2000; Kijlstra and Jongert, 2008). Hunters and consumers of game should be educated about the risk of exposure to this zoonotic disease during evisceration, handling and consumption.

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COMPARISON OF THE YEAST MICROBIOTA OF DIFFERENT VARIETIES OF COOL-CLIMATE GRAPES BY PCR-RAPD

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ABSTRACT

The yeast microbiota occurring on different varieties of grapes grown in cool-climate is not completely researched. Therefore, its identification is important to research. On the other hand, yeasts occurring in these fruits can be potentially used as starter cultures to obtain particularly demanded features in the production of wine. In addition, rapid methods for yeast identification allow to eliminate the contamination with pathogenic yeasts, which could cause the loss of wine production. The aim of the study was to isolate and identify the yeasts occurring on the surface of the different varieties of white and red grapes, grown in cool-climate of Poland. Also, the aim was to compare the qualitative and quantitative composition of yeasts on the tested grapes. The 84 cultures of yeasts were isolated, that were initially macroscopic and microscopic analyzed and the purity of cultures was rated on the WL medium. Identification of yeasts by PCR-RAPD was carried using the M13 primer. In the PCR-RFLP method ITS1 and ITS4 primers, as well as restriction enzymes *HhaI*, *HinfI*, *HaeIII*, were used. Preliminary identification of yeasts by standard methods produced results very different from the results obtained by molecular methods. Among the isolated microorganisms yeasts were dominating, but bacteria and molds were also present. Using the PCR-RAPD method most strains of yeasts were identified. Yeast microflora of different varieties of white and red grapes was very similar as the same species of yeasts were identified. Yeasts of the genus *Saccharomyces* were present in all varieties of grapes. The *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Rhodotorula minuta*, *Pichia kluyveri*, *Hanseniaspora uvarum* and *Rhodotorula mucilaginosa* were identified by PCR-RAPD. 4 of the 33 tested strains of yeasts were identified by PCR-RFLP. By PCR-RAPD only *Hanseniaspora uvarum* was identified. The quantity and quality of microorganisms living on the surface of grape fruits is very important for the process of winemaking. Yeasts influence the course of alcoholic fermentation, the flavor, aroma, and thus the quality of the produced wine. To a large extent their presence depends on the condition of the surface of the fruit. Many researchers reported significant differences between yeast microflora in grapes of Mediterranean and cool-climate vineyards. As they are expected to affect the final wine properties precise researching of the microflora of cool-climate grapes may lead to the isolation of new species of yeasts and thus the wines with unique characteristics can be obtained.

Keywords: grape; yeast; PCR-RAPD

INTRODUCTION

The grapes on the surface have a very rich microflora, including yeasts, bacteria and molds. Yeasts, on the surface of matured grapes are from 10^3 to 10^6 cfu/g. Yeasts of the genus *Hanseniaspora/Kloeckera* constitute 50-75% of the total yeast population. Other types of yeasts occurring in grapes are: *Aureobasidium*, *Brettanomyces*, *Bulleromyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Issatchenka*, *Kluyveromyces*, *Lipomyces*, *Metschnikowia*, *Pichia*, *Rhodospiridium*, *Rhodotorula*, *Saccharomyces*, *Sporidiobolus*, *Sporobolomyces*, *Torulaspora*, *Yarrowia*, *Zygoascus*, *Zygosaccharomyces* (Fleet et al., 2002; Renouf et al., 2007).

Many genera and species of yeasts during the wine production were found. The low pH and high sugar content quickly produce anaerobic conditions while the presence of phenolic compounds creates an ideal environment for yeast growth. Metabolic activity of microorganisms has a great influence on the composition of wine, and therefore

its taste and aroma properties (Gil et al., 1996; Lema et al., 1996; Romano et al., 2003; Fleet, 2003). In fact, the types of wine could depend on the specific metabolites of yeasts. Microorganisms that affect the taste of the wine can be derived from a vineyard or be carried by insect vectors of fruit flies, bees and wasps (Fleet et al., 2002). The presence of different types of yeasts depends on regional and climate factors, grape varieties, atmospheric pressure, damage to grapes and vineyard practices (Prakitchaiwattana et al., 2004).

Wine yeasts have specific metabolic characteristics that make it possible to produce wines of exceptional bouquet and interesting organoleptic properties. Small vineyards choose the production of wines with a unique taste, corresponding to the region and consumers. In such vineyards the spontaneous fermentation is used to produce wine. Yeast strains used for the production of wine should be characterized by a period of rapid adaptation to the environment of the must, which leads to rapid

fermentation. During fermentation, the yeasts should be stable and reproduce. In addition, the by-products of fermentation produced by yeasts should affect the aroma and taste of the wine. Moreover, the yeasts should be characterized: production of a specific quantity of ethanol, the ability to the spontaneous flocculation and sedimentation after the fermentation process, and the ability to metabolize malic acid, which is the main cause of the excessive acidity of grapes. On the other hand, the yeasts may contaminate the wine and cause their diseases. Yeasts of *Candida* and *Pichia* could lead to a significant reduction of alcoholic fermentation. This could result the organoleptic change of wines (Ribereau-Gayon et al., 2006).

Microorganisms living at the surface of the grapes are an important element affecting the taste, aroma, and thus the quality of the finished wine. Their presence depends to a large extent on the condition of the fruit surface. Previous studies show that are differences between the species of microorganisms occurring on grapes in cool-climate and Mediterranean. Through evolution, new types of microorganisms were created that are dependent on the climatic conditions in which they live. Nowadays, the new test methods for the identification and analysis of the microflora have been established which are based on molecular biology techniques. Particularly noteworthy is PCR analysis, which has given rise to other methods. Identification methods are continually improved, which makes the obtained results reliable and fast. This is very important because different types and species of yeasts on the grapes of cool climate can identify. Such yeasts can be used to production of wines, as starter cultures about a particularly important characteristics. On the other hand, rapid methods of yeast identification allow to eliminate harmful or pathogenic microflora. The aim of this study was to isolate yeast microflora present on the surface of the different varieties of white and red grapes grown in Poland, as well as identification of yeasts by PCR-RAPD and RFLP-PCR methods.

MATERIAL AND METHODOLOGY

The experiments were performed on white grape varieties, i.e.: Seyval blanc, Hibernál, Johanniter, Jutrzenka, Solaris, as well as red grapes: Rondo, Regent, Cabernet cortis and Dornfelder, from vineyard Srebrna Góra (Krakow). All the grapes were mature, without mechanical and microbiological damages.

Grape samples from each variety (10 g), were placed in the sterile stomacher bags. For each of bags the 90 mL of sterile 0.85% NaCl were added. Samples were homogenized using a stomacher (Bad Mixer). From each sample decimal dilutions to 10^{-2} were performed. For the isolation of microorganisms the nutrient agar for total bacteria count (incubation at 32 °C for 24 h), DRBC agar for yeast (28 °C for 2 – 3 days), and Czapek-Dox agar for molds (28 °C for 2 – 3 days) were used. After incubation, the colonies were counted by a colony counter.

Pure cultures of yeasts were grown at 28 °C and stored at 4 °C for further analysis. Analysis of yeasts was carried out macro- and microscopically. The ability to sporulation (acetate agar, 28 °C, 72 h), as well as the growth on WL medium were assessed.

The isolation of the genomic DNA of yeasts was started from the propagation of yeasts on the Sabouraud Agar medium (28 °C, 48 h) and Sabouraud Broth (10 mL, 28 °C, 24 h). Cultures were centrifuged four-times (5000 rpm for 5 min). The density of the yeasts was determined by the McFarland densitometer (DEN-1B BIOSAN).

From each yeast strain DNA was isolated using kit for isolation and purification of yeasts genomic DNA (Genomie Mini AX Yeast Spin, A&A Biotechnology) according to the protocol. The reaction mixture (50 µL) for PCR containing: One Taq Standard Buffer (BioLabs), dNTPs (Gene DireX), M13 (5'GAGGGTGGCGTTCT3'; Genomed) primer, One Taq Polymerase (BioLabs), DNA-ase free water (Sigma) and DNA template. DNA amplification was carried out in a thermocycler (MultiGene Mini, Labnet). PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 95 °C for 1 min, annealing at 36 °C for 1 min and extension at 68 °C for 2 min; and a final extension at 68 °C for 7 min. PCR products was stored at -20 °C for further analysis. The PCR products were separated on 1,5% agarose gels stained (Lab Empire) with ethidium bromide (Sigma), with 1xTAE buffer. Separation was performed at a flow of 100 V for 70 min in the apparatus for electrophoresis (Labnet). After electrophoresis, gels were visualized under UV light and photographed (InGenius, IG-LHR). Sizes were estimated by comparison against a DNA length standard 100-1000 bp ladder (A&A Biotechnology).

The reaction mixture for PCR-RFLP containing: One Taq Standard Buffer, One Taq Polymerase, dNTPs, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer (Genomed), DNA template and DNA-ase free water. PCR conditions were as follows: initial denaturation at 94 °C for 1 min; 30 cycles of denaturing at 94 °C for 30 s, annealing at 55.5 °C for 1 min and extension at 68 °C for 3 min; and a final extension at 68 °C for 5 min. PCR products ($1 \mu\text{g}\cdot\text{mL}^{-1}$) were digested with the restriction endonucleases *HhaI*, *HinfI*, *HaeIII* (EURx) according to the supplier's instructions. Electrophoresis and its analysis was carried out as for PCR-RAPD.

Data obtained were expressed as the mean \pm standard deviation (SD). A single-factor Analysis of Variance test (ANOVA) with a *post hoc* Tukey's test were applied to perform a statistical analysis. Distribution of normality was determined by Kolmogorov-Smirnov test using the program InStat3.

RESULTS AND DISCUSSION

Determination of the amount of microorganisms is the primary method of microbiological analysis (Walczak et al., 2013). Table 1 shows the amount of bacteria, yeasts and molds occurring in the surface of the grapes. The least number of microorganisms was found in Seyval blanc and Rondo grapes, the average for the Regent and Solaris grapes, and the largest for Hibernál and Dornfelder grapes. Numerical range varied between 345 cfu/g and 845 cfu/g. A significant impact on the populations of microorganisms have weather, climate, habitat and the varieties of grapes (Ribereau-Gayon et al., 2006).

Table 1 Number of bacteria, yeasts and molds occurring in the tested varieties of grapes.

Vine varieties	Bacteria cfu/g grape	Molds cfu/g grape	Yeasts cfu/g grape
Seyval blanc	2 ±1 ^a	6 ±2 ^a	385 ±5 ^a
Solaris	16 ±2 ^{b,c}	43 ±3 ^{b,c}	615 ±6 ^{b,c}
Hibernal	31 ±4 ^{d,e}	112 ±8 ^{d,e}	729 ±9 ^{b,d,e}
Johanniter	9 ±1 ^{b,d,f,g}	69 ±3 ^{b,d,f,g}	440 ±6 ^{b,d,f,g}
Jutrzenka	10 ±2 ^{b,f,h}	92 ±3 ^{b,d,f,h}	412 ±4 ^{b,d,f,h}
Rondo	8,5 ±2 ^a	100 ±3 ^a	345 ±4 ^a
Regent	28 ±2 ^{b,c}	52 ±2 ^{b,c}	610 ±7 ^{b,c}
Cabernet cortis	36 ±4 ^{b,d,e}	29 ±2 ^{b,d,e}	625 ±6 ^{b,d,e}
Dornfelder	19 ±3 ^{b,d,f}	10 ±1 ^{b,d,f}	845 ±4 ^{b,d,f}

Note: Mean value ±standard deviation; n = 3; a - h – values in columns and denoted by different letters differ statistically significantly at $p < 0.05$.

The amounts of yeasts in the varieties of white and red grapes were very similar. On the varieties of white grapes were from 385 to 729 cfu/g, and varieties of red grapes were from 345 to 606 cfu/g of yeasts. On the white and red grapes were much less bacteria and molds. Thus, for white grapes 14 cfu/g of bacteria and 64 cfu/g of molds were observed, whereas varieties of red grapes contained 23 cfu/g of bacteria and 48 cfu/g of molds. The quantitative composition of yeasts depends on many factors. The number of microorganisms is influenced by the variety of grapes, maturity and chemical composition of grapes, the climatic conditions and the treatments used. The grapes were harvested at full maturity, where there has been modification of the composition of organic compounds in berries. These changes take place at the final stage of grape ripening (Ribereau-Gayon et al.,

2006). The microflora of the vineyard has a large impact on the production of wines. Biofilm is readily formed on different surfaces. It may be on slippery stainless steel tanks and wooden barrels of rough, difficult to disinfect (Joseph et al., 2007). The results of the quantitative analysis showed that the yeasts were the largest group of microorganisms present on the fruit surface.

Before identification of yeasts by molecular methods, it was important to obtain pure cultures of these microorganisms. The pure cultures allow to investigate the morphological, physiological and genetic characteristics of microorganisms. Using the classical methods were tested the purity of 84 isolated strains of yeasts. Such evaluation allows to select cultures before molecular studies.

By PCR-RAPD 84 strains of yeasts and by PCR-RFLP

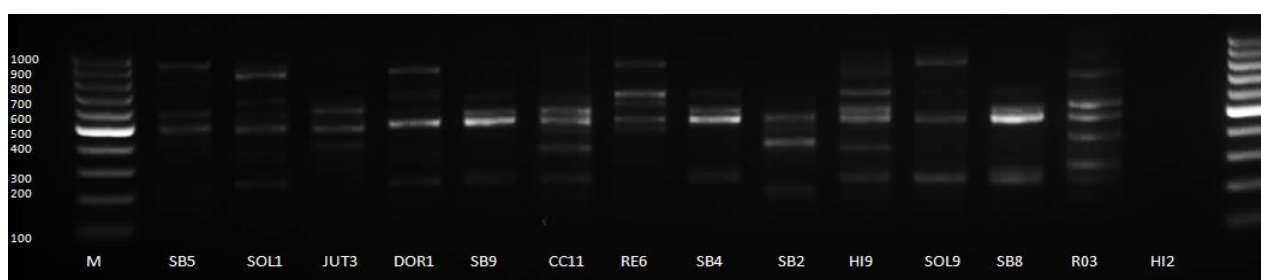


Figure 1 The separation of genetic material of yeasts in a 1.5% agarose gel by PCR-RAPD, M – is a marker, abbreviations of yeast strains described in table 2.

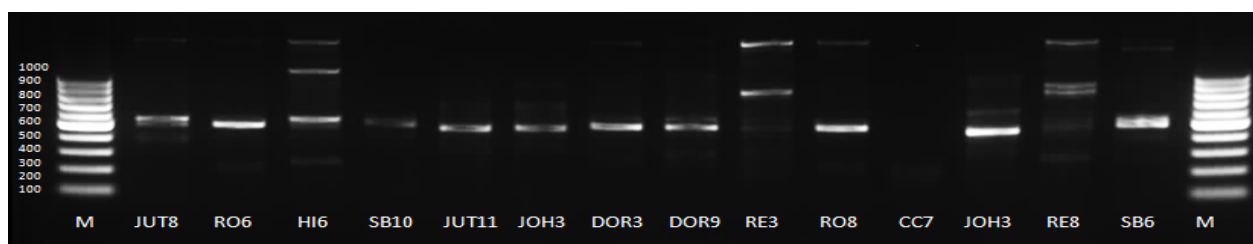


Figure 2 The separation of genetic material of yeasts in a 1.5% agarose gel by PCR-RAPD, M – is a marker, abbreviations of yeast strains described in Table 3.

33 strains of yeasts were identified. We were unable to replicate 7 samples that did not contain enough DNA. The RFLP technique allows to detect the restriction sites, the presence of which makes possible to distinguish the different strains of a single species. In both methods size of the PCR-amplified DNA region of the yeast strains was obtained which was compared with the image reference strains (Łojkowska and Śledź, 2012). Figures 1 and 2 show examples of electrophoretic pattern of the genetic material of yeasts isolated from grapes after PCR-RAPD. Tables 2 and 3 show the size in bp of the PCR products the selected yeast species and their name, identified on the basis of literature (Hierro et al., 2004; Cordero-Bueso et al., 2010).

The PCR-RFLP method allows to distinguish the yeast strains after treatment with the restriction enzymes. Because the genetic material of yeasts were degraded, by PCR-RFLP method only one strain of the yeast: *Hanseniaspora uvarum* was identified. Thus, 29 of strains of yeasts were not identified. Although the PCR-RFLP method gives reliable and precise results, it is more laborious and needs more genetic material than a PCR-RAPD.

The studied varieties of grapes grown in cool-climate, did not exhibit a significant diversity in the yeast population. Six species of yeasts were isolated, which belong to five genera, i.e.: *Rhodotorula*, *Saccharomyces*, *Pichia*, *Metschnikowia* and *Hanseniaspora*. Among the *Rhodotorula* genus two different species were determined: *Rh. mucilaginosa* and *Rh. minuta*. The grapes of Jutrzenka variety were characterized by the highest yeast species diversity as following species were identified: *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Rhodotorula mucilaginosa* and *Rhodotorula sp.* On the each varieties of grape has

been identified an average of 3-4 types of yeasts.

As already stated, the surfaces of the grape berries were inhabited by a variety of microorganisms. Microorganisms have specific physiological characteristics which affect the final wine properties. The presence of these microorganisms is dependent on the stage of ripening of the grapes and availability of nutrients. A significant impact on the occurrence of microorganisms has the condition of fruit surface. Untouched skin of grapes may have traces of microcracks and dents, thereby changing the availability of nutrients. In this case, the population of yeasts with a low of fermentation activity is decreased. Such yeasts include *Candida sp.*, *Hanseniaspora sp.*, *Metschnikowia sp.* and *Pichia sp.* When the grape skin is significantly damaged, automatically the availability of sugars at high concentrations increases. This favors the growth of yeasts having a higher fermentation activity such as *Pichia sp.* and *Zygoascus hellenicus*. Well-fermenting sugars yeasts, such as *Saccharomyces cerevisiae* are rarely found on the undamaged grapes. Condition of fruit is a major factor influencing on ecology of microorganisms, their numbers and variety (Barata et al., 2012).

Identified species of *Hanseniaspora*, *Metschnikowia* and *Pichia* were found on the undamaged surface of the grape (Barata et al., 2012). The studied grapes did not have mechanical and microbiological defects. The *Saccharomyces* yeasts are rare in undamaged fruit. Number of the identified *Saccharomyces* could indicate the specific conditions in the vineyards (Milanović et al., 2013). The *Saccharomyces* are highly desirable in the wine technology, because of the good ability to ferment sugars (Ribereau-Gayon et al., 2006). This yeast can be used as a starter culture in the fermentation of Polish wines. Yeasts isolated from the vineyards of southern Europe could be

Table 2 Size in bp of the PCR product and identified yeasts.

Symbol of sample	Size of PCR product (bp)	Identified yeast species
SB5	500, 600, 950	<i>Rhodotorula mucilaginosa</i>
SOL1	250, 500, 600, 700, 900	<i>Saccharomyces cerevisiae</i>
JUT3	400, 500, 600	-
DOR1	250, 500, 900	<i>Metschnikowia pulcherrima</i>
SB9	250, 500, 600	<i>Saccharomyces cerevisiae</i>
CC11	250, 350, 500, 600	-
RE6	500, 700, 900	<i>Rhodotorula minuta</i>
SB4	250, 500, 600, 700	<i>Saccharomyces cerevisiae</i>
SB2	200, 400, 500	<i>Metschnikowia pulcherrima</i>
HI9	250, 350, 500, 600, 700	<i>Saccharomyces cerevisiae</i>
SOL9	250, 500, 850	<i>Saccharomyces cerevisiae</i>
SB8	600, 500, 250	<i>Saccharomyces cerevisiae</i>
RO3	300, 400, 500, 600, 800	-
HI2	-	absence of DNA

Table 3 Size in bp of the PCR product and identified yeasts.

Symbol of sample	Size of PCR product (bp)	Identified yeast species
JUT8	400, 500, 600	<i>Rhodotorula sp.</i>
RO6	300, 500	-
HI6	300, 600, 1000	<i>Pichia kluyveri</i>
SB10	600	<i>Metschnikowia pulcherrima</i>
JUT11	500, 700	<i>Hanseniaspora uvarum</i>
JOH3	500, 700, 800	<i>Rhodotorula minuta</i>
DOR3	500, 600, 900	<i>Saccharomyces cerevisiae</i>
DOR9	250, 500, 600, 900	<i>Saccharomyces cerevisiae</i>
RE3	500, 800	<i>Hanseniaspora uvarum</i>
RO8	150, 500, 1000	-
CC7	150	-
speciesJOH3	500, 650, 900	<i>Saccharomyces cerevisiae</i>
RE8	250, 500, 800, 900	<i>Saccharomyces cerevisiae</i>
SB6	500	<i>Metschnikowia pulcherrima</i>

different from those isolated in Poland. These differences are primarily affected by the total annual precipitation in each month, the average daily temperature, solar radiation and atmospheric fronts (Gawęcki and Libudzisz, 2011).

Knowledge about the microflora of fruits from the Polish vineyards is still too small, so it is necessary to carry out series of studies using modern research methods. In the case of PCR-RAPD the identification of the DNA fragment length is problematic. This is associated with lack of complete database for analysis of microorganisms by PCR-RAPD. It is important to perform as much research on these organisms to be able to make up database, which could be widely available for all interested in the subject. The computer analysis allows to nominate fragments, which are located in the database (Łojkowska and Śledź 2012). The second technique, PCR-RFLP analyzes the restriction fragment length polymorphism. It is used to identify closely related species of bacteria and fungi. Both methods are useful in the study of the identification and differentiation within a species of yeast. The RAPD-PCR method is faster and cheaper. Furthermore, the analysis requires a smaller amount of DNA template than for PCR-RFLP.

CONCLUSION

On different cool-climate varieties of grapes similar yeast species were identified. The *Saccharomyces* species on red and white grapes were identified. *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Rhodotorula minuta*, *Pichia kluyveri*, *Hanseniaspora uvarum* and *Rhodotorula mucilaginosa* were identified by PCR-RAPD methods, whereas PCR-RFLP allowed to identify *Hanseniaspora uvarum*. Among the isolated microorganisms yeasts were dominant, but bacteria and molds were also present. The quantity and

quality of the yeasts on the surface of grapes are very important parameters for the production of wines. Therefore the obtained results require further investigation. Yeasts from different varieties of grapes, in different vineyards and within a few years need to be identified. Moreover, isolated yeasts shall be tested for use in the production of wine.

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ADAPTABILITY OF CULTIVARS AND HYBRIDS OF TANGERINE IN A SUBTROPICAL ZONE OF RUSSIA

Julia Abilphazova, Oksana Belous

ABSTRACT

Results of researches of various cultivars and hybrids of tangerine growing in the conditions of the humid subtropics of Russia and possessing valuable physiological and biochemical markers are presented in article. The stress factors limiting cultivation of tangerine culture in Krasnodar region are defined. The assessment of the water saving and enzymes activity in tangerine leaves is given. Changes of physiological parameters at influence of a stressful factor are shown. The analysis of water deficiency is revealed existence of essential distinctions between cultivars and hybrids. Change of thickness of a leaf blade of tangerine is the important diagnostic characteristic testifying the ability of leaf tissues to accumulate and keep water. Researches are showing that difference between catalase action of cultivars and hybrids are insignificant. Strengthening of enzyme activity can testify to bigger violation of a functional state of plant cultivars in comparison with the hybrids. Water deficiency and drought resistance coefficient can be used for diagnostics of stability of tangerine to hydrothermal factors. Correlation coefficients between the studied markers were calculated and regression models of their interrelation were given. So, there was a high correlation between thickness of a leaf blade and water deficiency index; close correlation between water deficiency and thickness of a leaf blade, as well as dependence of average degree on water deficiency and catalase activity were noted. There were revealed tangerine cultivars and hybrids which are stable in the changing environmental conditions and have high biochemical value of fruits.

Keywords: tangerine; water deficit; turgor; catalase; resistance

INTRODUCTION

Humid subtropics of Krasnodar region – the only place in Russia where citrus plants, and first of all, tangerines can be grown up in field conditions. However, in recent years field cultivation of a citrus in this zone was reduced to a minimum, first of all, unprofitability of their cultivation due to high costs of watering during a drought and acquisition modern and reliable materials for frost protection. The abnormal phenomena existing in subtropics of Russia (severe droughts, frosts, etc.) have an adverse effect on course of physiology-biochemical processes that is expressed in change not only exchange processes in cages, but also growth, development, duration of fructification of plants. So, one of the major stressful factors in a subtropical zone of Krasnodar region is irregularity of rainfall distribution; the necessary quantity during all vegetative period are 500 – 600 mm, but in some years was no more than 150 – 200 mm, and often they have storm character. Besides, the summer period in subtropics of Russia is characterized by high air temperature, with not sufficient available soil moisture. It creates here on the coast conditions for annually repeating droughty periods in which the inhibition of plants growth, their wilting and even drying are observed. The stressor's influence leads to considerable cropping losses of citrus which have a strengthened ovary abscission. In response to instability of abiotic factors a citrus becomes more

susceptible to various diseases that conduct to decrease in efficiency and deterioration of fruits. Regarding to this there is a need for studying of adaptive potential of citrus plants using the methods of express diagnostics for an assessment of their physiological state and development of new cultivars and hybrids with adaptability to this region.

In a number of scientific institutions of the country were developed various methods of diagnostics of plants stability recommended for practical using containing various receptions of an assessment of resistance to extreme factors (Udovenko, 1995; Goncharova, 2007; Reutsky and Radionov, 1992; Technique of researches, 1985). The offered methods differ on labor input, level of differentiation of the estimated objects on stability and except of their practical application. Besides, methods of diagnostics are depending on the studied species and specifics of stressful factors. However at the heart of plants including tangerine stability diagnostics are the general principles of their assessment which are based on mechanisms of adaptation of plants to stresses (Udovenko, 1973; Goncharova, 2007; Radchenko, 2005).

The purpose of these long-term researches was to study of citrus plants biological features, their reactions to adverse factors of the environment for establishment of diagnostic criteria of stability, selection of assortment of tangerines which are steady to humid subtropics of Russia.

MATERIAL AND METHODOLOGY

The present researches were conducted using followed tangerine genotypes: Sentyabrsky; Kodorsky; Unshiu; hybrid 16939 (Miagava-Vase x Natsu); hybrid 16954 (Miagava-Vase x Yuka). Plants were grown up in a field conditions in the Institute collection in 3-fold frequency since 1986, the total area of a site makes 400 m², the schema are 4 x 2 m. Soils are brown forest and low unsaturated. Physiologically uniform leaves of tangerine which finished their growth were selected for analyses. Selection of tangerine leaves was made from June to August depending on a cultivar, terms of leaves increase and approach of stressful conditions (a drought, high or low air temperatures, etc.). The agriculture is standard for tangerine planting.

Researches were conducted in field and laboratory conditions on the basis of Russian Research Institute of Floriculture and Subtropical Crops with use of classical methods: determination of water deficiency by Pochinok (Pochinok, 1976); activity of enzyme of a catalase – by method of gasometry (Gunar, 1972); thickness of a leaf was determined by a field turgor meter, coefficient of heat resistance – by express method of diagnosing (Kushnirenko et al., 1986).

The program STATGRAPHICS Centurion XV and the mathematical software package of MS Excel 7.0 were applied an assessment of experiment results.

RESULTS AND DISCUSSION

On the Black Sea coast of Krasnodar region the limiting factor in summertime is the drought which can proceed two and more months. And, the most critical and droughty months for tangerine was June – August when average temperature rose above +25.0 °C at high relative humidity of air (to 80%).

Use of physiological methods in periods of low humidity of the soil, air and high temperatures allowed to establish influence of a drought on a condition of plants and to reveal features of formation of drought resistance by them

(Abilfazova, 2002; Belous, 2009). At diagnostics of stability of tangerine cutivars we used an assessment in a complex of parameters of the water mode and enzyme’s activity (Goncharova, 2007; Radchenko, 2005; Romanova, 2008). These parameters are characterized by variability and high response on many abiotic factors therefore it was necessary to observe special care in selection of plant material for the analysis and taking note of stressful factors on plants. Water deficiency during the spring period leads to delay of a shoots gain, to blooming of buds and budding, later blossoming of tangerine (in average for 2 weeks later, than usually) was established in our research. During this period the content of water in leaves is an important diagnostic indicator of a physiological condition of citrus plants for which is necessary optimal moisture, especially in the spring and at the beginning of summer, when active growth of vegetative and generative organs is going on.

At the beginning of the stressful period was revealed that water deficiency of tangerine is ranging from 9.5% (grade Yubilejny) to 16.3% (grade Slava Vaviliv). At this time the high values of parameter at plants (more than 11%) are connected with lack of necessary watering. We were showing that further a strengthening of water deficiency on average for 1 – 5% (Figure 1).

Variability of an indicator on cultivars was from 26% (cultivar Miagava-Vase) to 31.8% (cultivar Yubilejny). High values of variability coefficient at a cultivar Yubilejny testify to its plasticity that is important at cultivation of the cultivar in unstable climatic conditions. The analysis of an indicator «water deficiency» revealed existence of essential distinctions between cultivars and hybrids (least significant difference at 95% level = 4.4). In general, cultivars Yubilejny and Kodorsky which water deficiency is significantly lower both in optimum and during the stressful period are the steadiest. At the same time, the cultivar Slava Vaviliv has a higher water deficiency throughout the entire period of supervision.

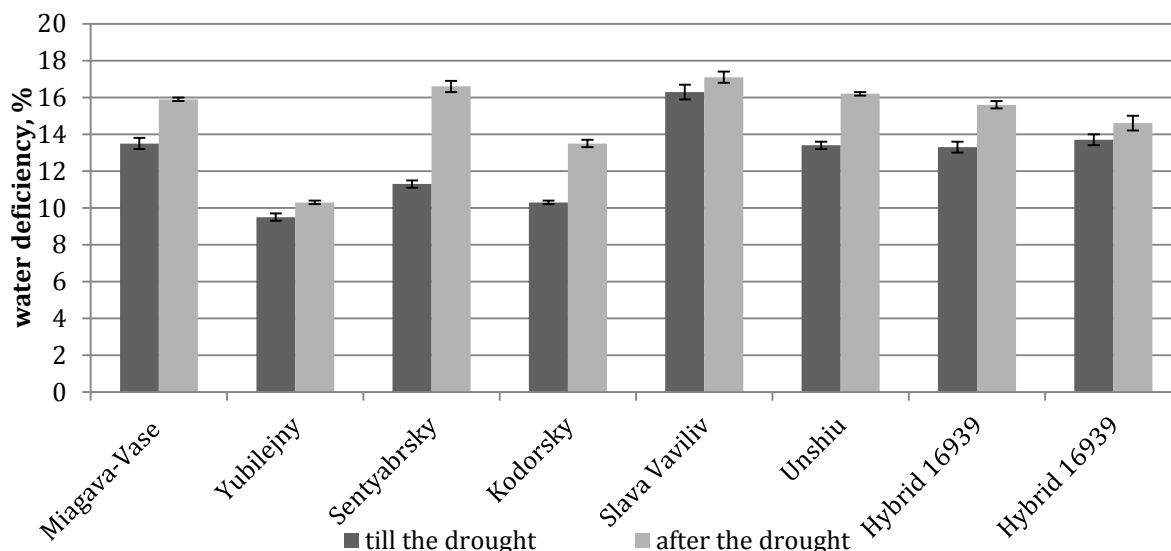


Figure 1 Water deficiency of tangerine.

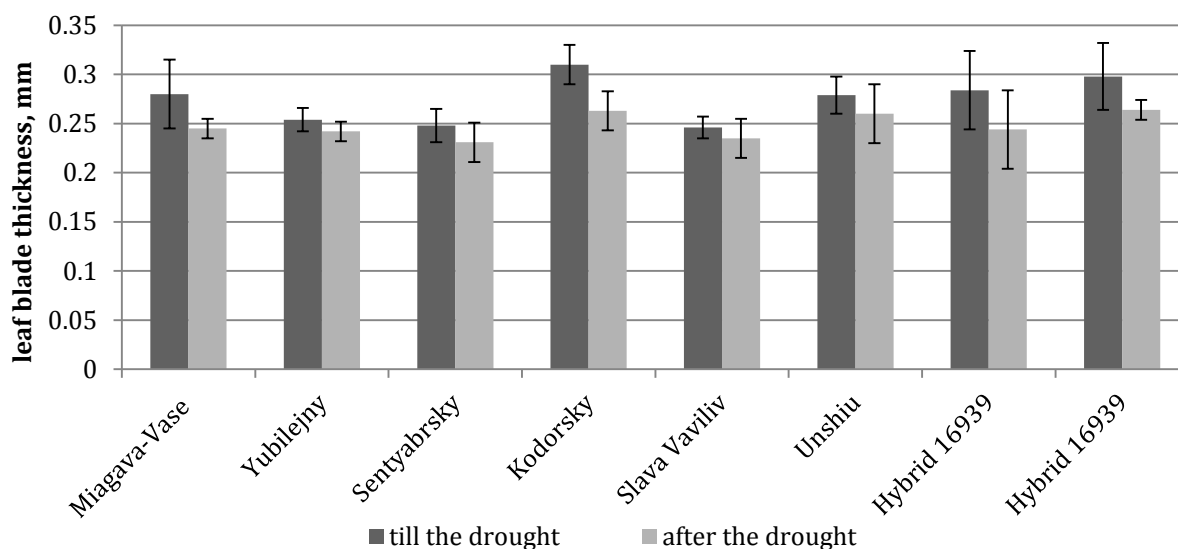


Figure 2 Change of tangerine leaf blade thickness.

There is a considerable loss of plant cells turgor during the drought that is a consequence of water deficiency. Change of thickness of a tangerine's leaf blade is the important diagnostic characteristic testifying the ability of leaf tissues to accumulate and keep water. As was shown in our researches, there is a close correlation ($r = 0.72$) between thickness of a leaf blade and the value of water deficiency. The analysis of biometric parameters of leaf blade of tangerine at till the drought period showed that the greatest thickness of a leaf characterizes a cultivar Kodorsky, by the smallest – a cultivar Slava Vaviliv, distinctions between cultivars are essential: least significant difference at 95% level = 0.020 (Figure 2). Increase of water deficiency leads to decrease in leaf turgor that is connected with deterioration of hydrothermal factors; changing the relation of T1/T2 which is stability coefficient changes (the coefficient equal 1.0 testifies to

high resistance of plants to a drought). Stability of this indicator during action of stressful factors is a sign of plants stability. In the cultivar Yubilejny high resistance to action stress factor was observed as it was shown in our researches.

The mechanisms which function at the level of enzymes systems and provide the adaptation of plants to adverse conditions of environment are significant for a plant. These mechanisms were shown in the form of change of concentration of enzymes and/or activity of their multiple molecular forms (Romanova, 2008). Enzymatic processes characterize features of a metabolism at different plants. Various physiological properties, including biological stability are connected with their action. One of the main oxidation-reduction enzymes of plants is the catalase. Change of enzymatic activity testifies to change of adaptability of plants to an adverse effect of environment;

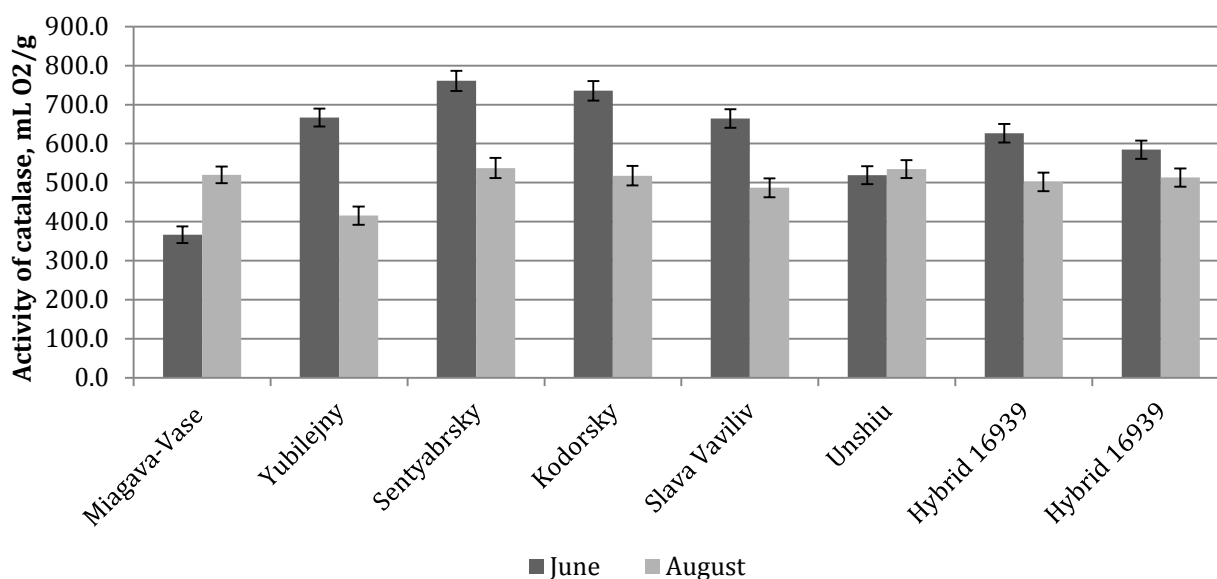


Figure 3 Enzymatic activate of tangerine.

Table 1 Characterize of physiology parameters of cultivars and hybrids of tangerine.

Cultivars	Water deficit %	V %	Enzymatic activity, mL O ₂ /g	V %	Leaf blade mm	V %
Miagava-Vase St.	14.7 ±1.7	26	443.4 ±21.1	21	0.263 ±0.025	20
Yubilejny	9.0 ±0.6	32	541.2 ±23.3	23	0.248 ±0.008	20
Sentyabrsky	14.0 ±3.7	27	649.2 ±25.5	25	0.240 ±0.012	20
Kodorsky	11.9 ±2.3	29	626.7 ±25.0	25	0.287 ±0.033	19
Slava Vaviliv	16.7 ±0.6	24	575.7 ±24.0	24	0.241 ±0.008	20
Unshiu	14.8 ±2.0	26	526.9 ±23.0	23	0.270 ±0.013	19
Hybrid 16939	14.5 ±1.6	26	564.4 ±23.8	24	0.264 ±0.028	19
Hybrid 16954	14.2 ±0.6	27	548.8 ±23.4	23	0.281 ±0.024	19
Least significant difference (95% confidence level)	4.4	-	283.5	-	0.020	-

therefore the task to study dynamics of enzymatic activity of various grades and hybrids of tangerine was set.

In June activity of a catalase in tangerine leaves was ranging from 366.7 mL O₂/g (grade Miagava-Vase) to 760.8 mL O₂/g (grade Yubilejny) by us was established. Strengthening of stressful influence led to minor change of enzymatic activity (V = 3.9 – 4.7) that points to stability of the parameter (Figure 3).

The cultivar Miagava-Vase had a strengthening of activity of a catalase against its falling at other cultivars, however, distinctions between cultivars and hybrids are insignificant (least significant difference at 95% level = 283.5). Strengthening of enzyme activity can testify to bigger violation of a functional state at plants of this cultivar in comparison with the others that characterizes this cultivar as unstable (Table 1).

CONCLUSION

Adaptability of the most perspective cultivars and hybrids of citrus cultures that revealed difficult mechanisms of interaction between the studied indicators was analyzed. Close correlation between water deficiency and leaf blade thickness, dependence of average degree between water deficiency and activity of a catalase are noted. We was defined that on two diagnosed indicators (size of water deficiency and coefficient of drought resistance) the cultivar Yubilejny is steadier than other cultivars. These characteristics can be used for diagnostics of stability of tangerine to hydrothermal factors. And also, can be used for a fast assessment of the cultivars and hybrids introduced and created developed at institute.

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PROPERTIES AND NUTRITIONAL VALUE OF WHEAT BREAD ENRICHED BY HEMP PRODUCTS

Ivan Švec, Marie Hrušková

ABSTRACT

Hemp (*Cannabis sativa*) is annual plant that is native to China and remained as important material for food, industrial and medical purposes. As source of cannabinoids belongs to controversial, but due to its excellent nutritional profile, non-gluten protein, fat and fibre it has potential in bakery products. Addition of 5% – 20% of hemp press cake fine flour and fine wholemeal significantly increased dietary fibre content, but their influence on volume of laboratory baked bread was different. Reflecting actual dosage, both types of hemp press cake flour diminished bun sizes about 6% – 33%; volumes of bread containing hulled wholemeal were comparable to standard (mean 310 mL/100 g vs. 333 mL/100 g, respectively). Only dehulled wholemeal hemp form increased the bread specific volume (6% – 30%), especially as 10% fortification (434 mL/100 g). Six Canadian hemp products were added as 10% and 20% on wheat flour base, comprising fine hemp flour and coarse hemp powder, dehulled whole seeds, hulled hemp seeds with sea salt as well as 50% and 43% hemp protein concentrates (KP1-KP6, respectively). The higher level of KP1, KP2, KP5 and KP6, the lower bread specific volumes were determined (decrease about 9% – 48%). Soft increase in buns size caused by 10% and 20% KP3 (323 and 319 mL/100 g vs. 296 mL/100 g) was insignificant. The effect of KP4 was reversely verifiable, magnifying the parameter about 25% and 17%, respectively. In terms of protein content in bread, a level 11.75% in wheat bread has risen to approx. 14.5% and 18.0% when 10% and 20% of KP3 and KP5, respectively, was included into bread recipe. All six Canadian hemp products increased dietary fibre content in bread, mainly owing to KP4 and both protein concentrates (up to 4 and 3 times, respectively). Incorporation of hemp flour up to the level of 10% positively affected bread sensorial properties.

Keywords: hemp flour; protein concentrates; food usage; dietary fibre; bread

INTRODUCTION

Nowadays, the Czech society has knowledge about hemp due to its procedure of marihuana legalization for medicine purposes. In general, hemp is perceived mostly as dangerous drug threatening human health. Similarly to the fact, that poppy is not opium, hemp is not marihuana. It belongs to utility plant, known and used by mankind for thousand years. Within the Czech Republic, end of hemp planting and hemp seeds usage is dated at 1936 – consecutively to the Marihuana tax law adoption, hemp breeding was forbidden.

Within temperate latitudes, hemp specie *Cannabis sativa* *culta* is the most spread and bred botanical variant, and all parts of plant could be economically utilized. From year 1999, hemp varieties Juso and Beniko are permitted, and these sorts conform to the Law 167/1998 Sb. about addictive substances. Both varieties are planted for production of hemp thread, dry organic matter used for technic or energetic purposes as well as for seeds production to gain technic or food oil.

Fibre is the part of plant-based foods (grains, fruits, vegetables, nuts, and beans) that the body cannot break down. It passes through the body undigested, keeping digestive system clean and healthy, easing bowel

movements, and flushing cholesterol and harmful carcinogens out of the body.

Insoluble fibre does not dissolve in water and helps to prevent constipation, and is found in whole grains, wheat cereals, and vegetables. Soluble fibre dissolves in water and helps control blood sugar levels and reduce cholesterol.

In general, the more natural and unprocessed the food, the higher it is in fibre. Refined or “white” foods, such as white flour and bread, have had all or most of their fibre removed. Good sources include barley, oatmeal, beans, nuts, and fruits. Many seeds as hemp, chia, quinoa and teff contain both soluble and insoluble fibre and can be good source for fortification of cereal foods.

Usage of hemp seed in food industry

In past times, food prepared from hemp belonged to diet of low and middle society. By seeds roasting or milling, thick porridge was prepared, and later hemp oil was pressed. Hemp seed proteins and dietary fibre support sensation of satiation, so hemp food was served to hardly working people. Hemp flour is suitable for celiatics, because here's a lack of gluten fractions of protein. Whole seeds can be used as component of cereal sticks, biscuits and purees, or they could be consumed after roasting

or dehulling treatment. Seed skin softens after heating, thus hulled seeds may season cooked dishes as pasta, rice or sausages. Dehulled seeds could also be included into non-cooked dishes. Mixed with water, 'hemp milk' is prepared suitable for taste emphasizing of chips, pasta and tortillas (Heroudková, 2013). In amount between 10% and 15%, hemp flour could be added into bakery products (Ruman, 2014). Incorporated into cut off cookies, pleasant coffee brown colour and specific by-taste could be reached (Hrušková et al., 2011). Pasta containing hemp products from hulled and dehulled seeds were characterised by up to four times higher fibre content compared to wheat ones (Hrušková and Švec, 2012).

For bakery usage, different commercial product could be applied, to which fine or wholemeal flour gained by disintegration of hulled or dehulled seeds could be counted, or protein concentrates of domestic or foreign origin. From technological point of view, chemical composition limits their potential usage as their presence in recipe affects bread dough machinability in terms of dilution of wheat gluten proteins.

The goal of the pilot study, supported by the grant NEW FOOD, was to compare different hemp products and their effect on consumer and nutritional value of wheat bread manufactured in laboratory scale.

MATERIAL AND METHODOLOGY

For all rheological and laboratory baking proofs, commercial type of bright fine wheat flour was used as a base (mill Delta Prague). Flour composites were blended from wheat flour and 5% – 20% of the Czech samples of fine hemp press cake flour (K1, K2, respectively) and fine wholemeals K4 and K5 (from dehulled and hulled seeds; Hemp Production, Chraštica). Hulled seeds represent a hemp seed in its nature form, while dehulled ones are soft hearts of the seed (i.e. peeled with hard cover layers removed). Within further experimental set, six composite flours contained 10% and 20% of Canadian hemp products of the Hemp Oil Canada Inc. Specimens of fine hemp flour and coarse hemp

powder (KP1, KP2, respectively), dehulled whole seeds (KP3), hulled hemp seeds with sea salt (KP4) as well as 50% and 43% hemp protein concentrates (KP5 and KP6, respectively). According to inner method of the Cereal laboratory of the UCT Prague, baking trial concerned on leavened bread evaluation was performed. For standard dough preparation of consistency 600 units, the Farinograph Brabender was employed. Manufactured buns quality as volume and shape was described objectively after two hours cooling at room conditions. Also their sensorial profiles were evaluated by three skilled panellists, using intense sensorial model of the Cereal laboratory. The profiling comprises bread shape, crust and crumb properties and represents overall quality depicted by 9 attributes of sensorial acceptability. Following the standards ČSN 56 05 12-11 and AOAC 985.29, nutritional score was quantified in terms of protein and dietary fibre contents, respectively.

RESULTS AND DISCUSSION

Compared to wheat flour, flour composites involving hemp products of the Czech origin used for laboratory bread preparation were characterised by higher content of all dietary fibre forms. Contrasted hemp products K1, K2, K4 and K5 together, any significant difference was not determined (Figure 1).

Considering dough machinability, hemp products addition did not led to verifiable increase of water absorption, but they softly affected stability after optimal dough development during the farinograph test. According to mixolab test results, impact of non-gluten proteins of prolamin groups was revealed out (Hrušková and Švec, 2012). Regardless to tested hemp type, specific volumes and shapes of breads enhanced by four domestic hemp products (K1 - K2, K4 - K5) could be considered as comparable to standard (Figure 2). Products containing 5% addition were characteristic by pleasant coffee brown colour, which intensified to dark brown correspondingly to actual dosage. Sensorial scores confirmed acceptability of such fortified bread recipes for common consumers

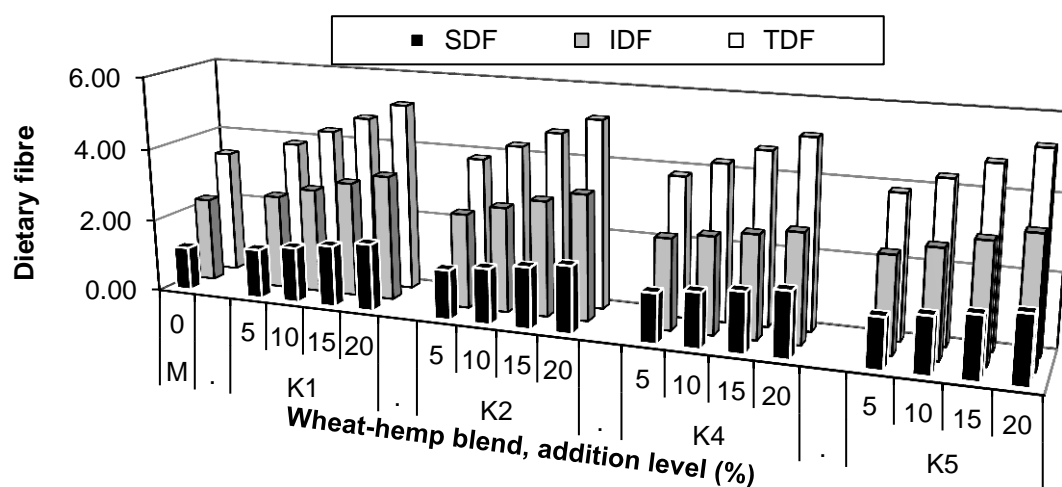


Figure 1 Dietary fibre content in blends of wheat and hemp flour (IDF, SDF, TDF: insoluble, soluble and total dietary fibre contents, respectively).

up to 10% of hemp added. Higher hemp product dosages led to less acceptable scores, mainly owing to taste difference (partially bitter taste). Disagreeable sensory perception of weaker extent was noticed in case of dehulled hemp wholemeal K4 testing (Figure 3) and it can be suppressed by e.g. higher sugar content like in case of biscuits. Worse sensorial profile was observed for product containing K2 addition, depending on tested dosage.

Canadian hemp products influenced bread volumes dependently on used type and addition level. Compared to non-fortified wheat flour, 10% of fine hemp flour KP1

did not change bread volume, whilst 20% did (a decrease about 100 mL/100 g; Figure 4). Similar trend was recorded during coarse hemp powder KP2 examination; however, ca 15% diminishing was determined already by 10% KP2 in recipe. Lower bread volumes resulted also from protein concentrates KP5 and KP6 testing, when the lowest bun size corresponds to 20% enhancement. Positive effect in terms of volumes increase was measured as a result of whole seed hemp products (KP3 and KP4) addition. During sensorial scoring, a worse point rating obtained samples with 20% of hemp products in recipe in relation to ones containing lower dosage (10%). Bread items richer

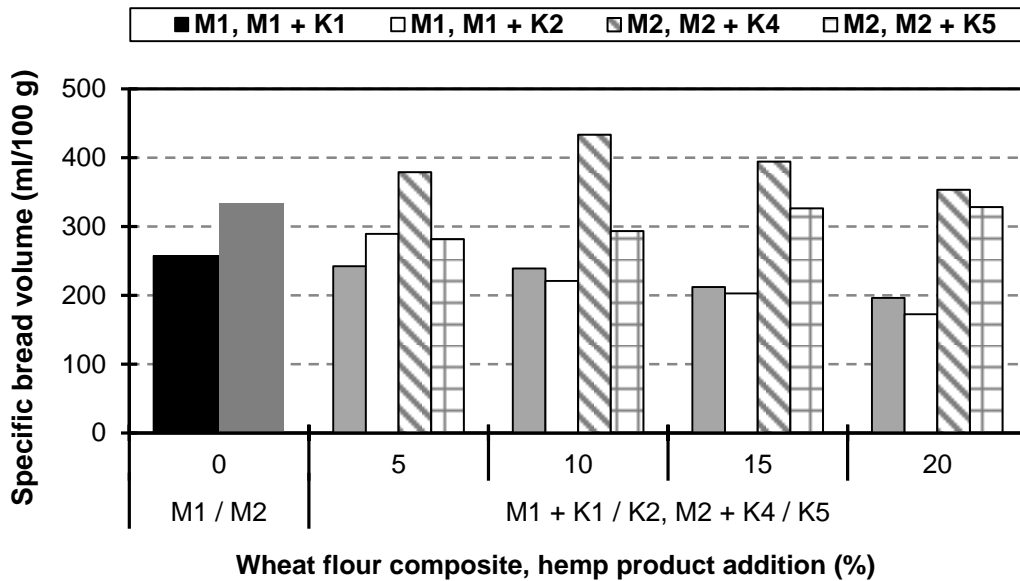


Figure 2 Specific volumes of bread with hemp products K1, K2, K4 and K5 (5% – 20%).

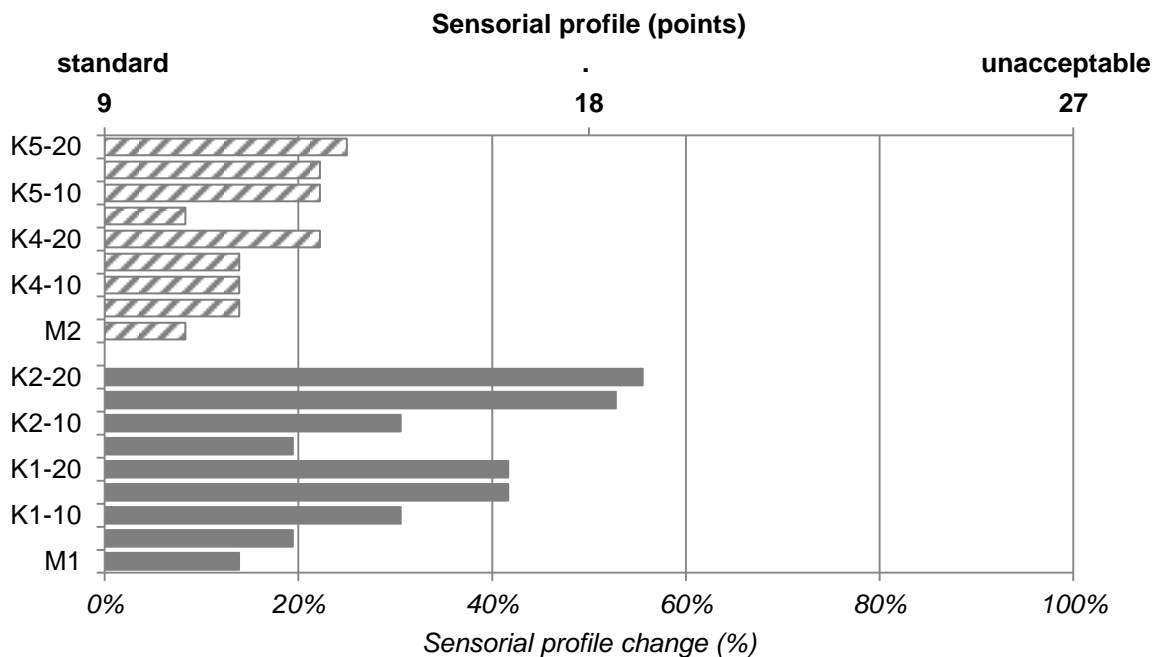


Figure 3 Sensorial profile of bread containing hemp products K1, K2, K4 and K5 (5% – 20%).

in KP were appraised as worse mainly due to lower vaulting of buns and non-typical taste. Bread enhancement by dehulled seeds (KP3) on both levels tested end in satisfying consumer's properties similarly to application of domestic dehulled hemp wholemeal K4. Mentioned components have also positive effect on taste perceptions masking in case of e.g. barley flour supplied in bread recipe (Hrušková and Švec, 2014).

In case of Canadian hemp products, bread nutritional value is higher compared to non-fortified one. Protein content, determined according to Kjeldahl's method (ČSN 56 0116) as well as proportions of single dietary fibre forms (AOAC 985.29) are illustrated on Figures 5 and 6.

The greatest protein rate was analytically confirmed in bread enhanced by 50% hemp protein concentrate,

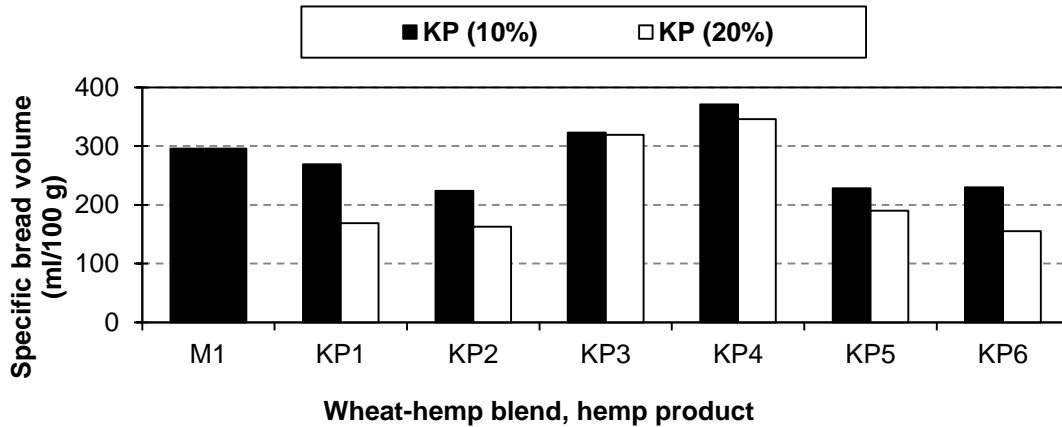


Figure 4 Specific volumes of bread with the Canadian hemp products in recipe.

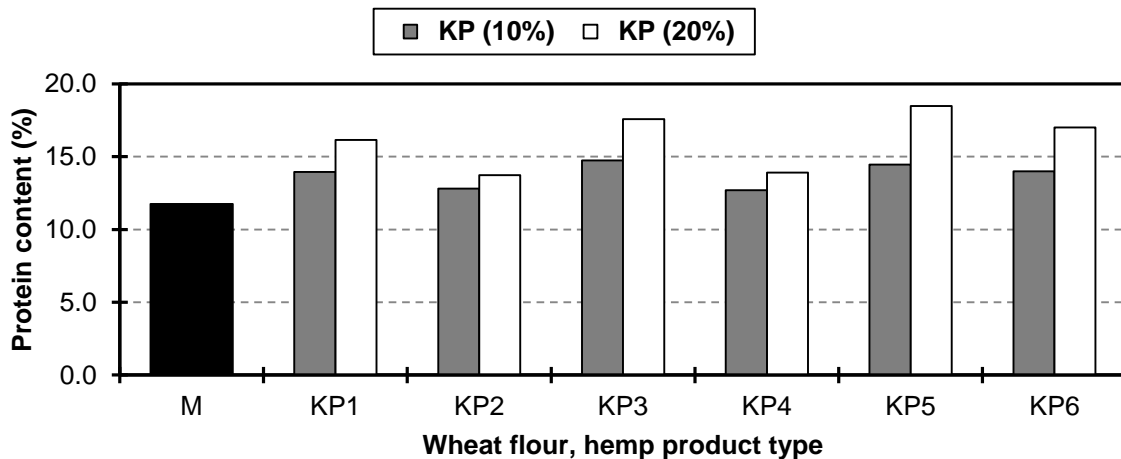


Figure 5 Protein content in bread with the Canadian hemp products in recipe.

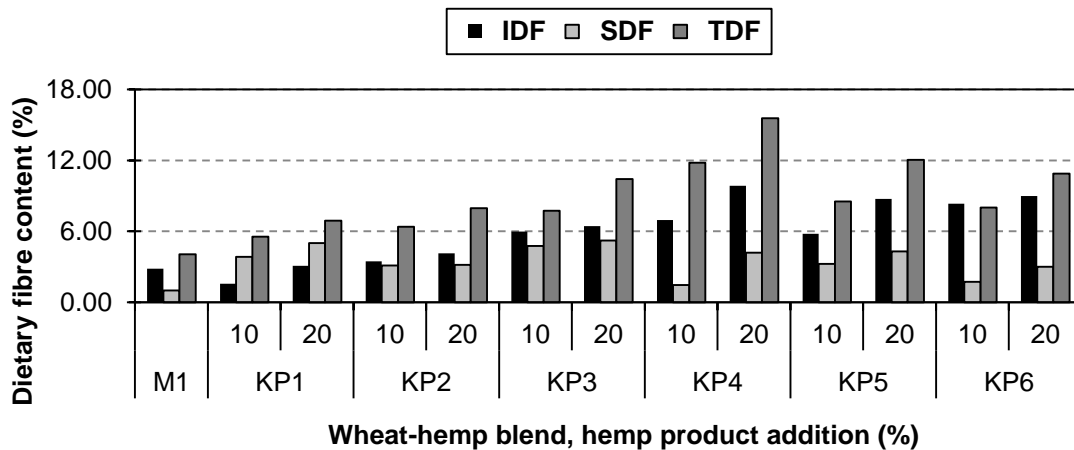


Figure 6 Dietary fibre content in bread with the Canadian hemp products in recipe (IDF, SDF, TDF: insoluble, soluble and total dietary fibre contents, respectively).

and in relation to wheat bread, that increase represents ca 45% of value in standard. Magnified content of total as well as insoluble fibre (TDF and SDF respectively) was measured in bread involving hulled hemp seeds with sea salt. In case of its 20% dosage, TDF level was high (15.55%) and protein content overcomes an average (13.91%). Added at lower level, bread reached better sensorial score due to the greatest volume, optimal shape and high crumb elasticity. Supplement of KP3 (dehulled hemp seed) could be recommended also on base satisfying SDF portion (5.23%).

CONCLUSION

Hemp is categorised among utility plants and it has a nutritional potential for usage in food industry. Common hemp seeds render higher protein and dietary fibre content to leavened bread, and also supply indispensable essential fatty acids presented in hemp oil. For bakery products, different forms of hemp constitute a source of fortification and innovation of offered assortment. Results of the pilot study could be concluded by statement, that higher dosage of hemp components into bakery products affects differently specific volume, shape and sensorial score of leavened bread. Also they verifiably contribute to higher contents of proteins and dietary fibre. Commercial hemp products but vary in their effect on consumer's quality and according to type, a supplement between 5% and 10% could be recommended.

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CHANGES IN THE MICROFLORA COMPOSITION OF GOAT AND SHEEP MILK DURING LACTATION

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ABSTRACT

The aim of this work was to determine extend of microbial contamination of raw milk in individual seasons. Raw goat milk (3 farms) and sheep milk (2 farms) were analyzed. Milk was produced on farms of different way of farming and with a different number of milked animals. Samples were taken during lactation three terms in the beginning, middle and end of lactation. In milk, following groups of microorganisms were determined by standard methods: total count of microorganisms (TCM), psychrotrophic microorganisms, *Enterobacteriaceae*, lactic acid bacteria (lactobacilli), enterococci, aerobic and anaerobic thermoresistant microorganisms (TMRae, TMRan), micromycetes (yeast and moulds). In goat milk, the following numbers of microorganisms were detected: total count of microorganisms (TCM) from 10^5 to 10^9 CFU x mL⁻¹, lactobacilli from 10^2 to 10^5 CFU x mL⁻¹, bacteria fam. *Enterobacteriaceae* from 10^1 to 10^5 CFU x mL⁻¹, enterococci from 10^1 to 10^5 CFU x mL⁻¹, thermoresistant aerobic and anaerobic microorganisms (TMRae and TMRan) from units to 10^3 resp. 10^5 CFU x mL⁻¹, psychrotrophic microorganisms from 10^1 to 10^6 CFU x mL⁻¹, micromycets from 10^1 to 10^4 CFU x mL⁻¹. In the sheep milk, the following numbers of microorganisms were determined: TCM from 10^5 to 10^6 CFU x mL⁻¹, lactobacilli from 10^3 to 10^6 CFU x mL⁻¹, bacteria fam. *Enterobacteriaceae* from 10^1 to 10^5 CFU x mL⁻¹, enterococci from 10^1 to 10^4 CFU x mL⁻¹, TMRae and TMRan from units to 10^5 CFU x mL⁻¹, psychrotrophic microorganisms from 10^4 to 10^6 CFU x mL⁻¹, micromycets from 10^2 to 10^4 CFU x mL⁻¹. From the above mentioned results, the following conclusions can be suggested. The bacterial counts of raw goat and sheep milk are highly variable and influenced by a number of important factors in the course of lactation and year (temperature, health, secondary contamination etc.). The bacterial numbers are not affected by the stage of lactation. High numbers of microorganisms in goat and sheep milk may be primarily caused by the insufficient cleaning and sanitizing of milking equipment or low hygiene of hand milking. An important role may also act the cooling rate of milk and purity of cooling equipment.

Keywords: goat milk; sheep milk; microflora of raw milk; total count of microorganisms

INTRODUCTION

Milk due to its beneficial composition and properties is suitable environment for the development of contaminating microorganisms. A relatively low number of microorganisms is contained in the milk of healthy animals mainly tending to settle in the teat canal and passes into the milk tank of udder. The number of microorganisms in milk ranges from 10^1 to 10^3 respectively 10^4 CFU x mL⁻¹ in the process of the leaving udder. A 10^5 CFU x mL⁻¹ could be obtained in the milk from the animal with the textured flaccid sphincter. The bacteria of the genus *Micrococcus*, *Enterococcus*, coryneform bacteria are represented, and infrequently e.g. staphylococci. In the diseased animals, e.g. *Streptococcus agalactiae*, *S. dysgalactiae*, *Staphylococcus aureus*, *E. coli*, and *Klebsiella* etc. can also occur (Hejlová, 1997; Görner and Valík, 2004). During milking, the milk is exposed to secondary contamination from the environment of the milking equipment, hands of the milker, and udder etc. (Hejlová, 1997; Malá et al., 2010). The quality of milk hygiene is microbiologically significant, which varies in terms of the types and numbers according to climate,

weather, grazing, lactation, housing, health, herd management etc. According to Švejarová et al. (2010), inferior microbiological parameters may prove a negative effect on the final product. When the bacteria content is too low, not only pathogenic microflora can be disturbed but also the natural non-pathogenic one, which can significantly affect the properties of dairy products made from raw milk (Kalantzopoulos, 2003).

The criteria for hygienic quality of raw milk are listed in the Regulation of the European Parliament and Council Regulation (EC) no. 853/2004 as amended. The microorganism content at 30 °C (per mL) should be ≤ 1500000 in goat and sheep milk (rolling geometric mean over a two-month period, at least two samples per month). However, if the milk is intended for the production of dairy products from raw milk by a process without heat treatment, the milk should contain $\leq 500\ 000$ microorganisms per mL. These regulations are the only applicable microbiological criteria in the Czech Republic for raw goat and sheep milk.

The aim of this study was to find out the dynamics of the microflora composition of raw goat and sheep milk from different farm breeds during lactation.

MATERIAL AND METHODOLOGY

Raw goat milk (3 farms) and sheep milk (2 farms) were analyzed. Milk was produced on farms of different way of farming and with a different number of milked animals (Tab. 1 and 2). Samples were taken during lactation in the beginning (spring), middle (summer) and end of lactation (autumn). In milk, following groups of microorganisms were determined by standard methods: total count of microorganisms (TCM) on PCA-AB medium (MILCOM – Tábor, Czech Rep.) at 30 °C for 72 h, psychrotrophic microorganisms on PCA medium (MILCOM – Tábor, Czech Rep.) at 6.5 °C for 10 days, fam. *Enterobacteriaceae* on VRBG medium (MILCOM – Tábor, Czech Rep.) at 37 °C for 24 h, lactobacilli on MRS medium (MILCOM – Tábor, Czech Rep.) anaerobic cultivation at 37 °C for 48 h, enterococci on Slanetz-Bartley Agar (Merck, Germany) at 37 °C 48 h, aerobic and anaerobic thermoresistant microorganisms (TMRae, TMRan) after thermoinactivation (85 °C 10 min.) on PCA at 37 °C for 48 h, micromycetes (yeast and moulds) on YGC medium (MILCOM - Tábor, Czech Rep.) at 25 °C for 120 h. After the cultivation of particular Petri dishes accrued colonies were counted and the result was expressed in CFU x mL⁻¹.

RESULTS AND DISCUSSION

The results of microbiological analyzes of goat and sheep milk samples are shown in Table 3. In goat milk, the

following numbers of microorganisms were detected: total count of microorganisms (TCM) from 10⁵ to 10⁹ CFU x mL⁻¹, lactobacilli from 10² to 10⁵ CFU x mL⁻¹, bacteria fam. *Enterobacteriaceae* from 10¹ to 10⁵ CFU x mL⁻¹, enterococci from 10¹ to 10⁵ CFU/mL, thermoresistant aerobic and anaerobic microorganisms (TMRae and TMRan) from units to 10³ resp. 10⁵ CFU x mL⁻¹, psychrotrophic microorganisms from 10¹ to 10⁶ CFU x mL⁻¹, micromycets from 10¹ to 10⁴ CFU x mL⁻¹. In the sheep's milk, the following numbers of microorganisms were determined: TCM from 10⁵ to 10⁶ CFU x mL⁻¹, lactobacilli from 10³ to 10⁶ CFU x mL⁻¹, bacteria fam. *Enterobacteriaceae* from 10¹ to 10⁵ CFU x mL⁻¹, enterococci from 10¹ to 10⁴ CFU x mL⁻¹, and TMRae and TMRan from units to 10⁵ CFU x mL⁻¹, psychrotrophic microorganisms from 10⁴ to 10⁶ CFU x mL⁻¹ micromycets from 10² to 10⁴ CFU x mL⁻¹.

TCM is only one legislative covered group of microorganisms in milk of small ruminants. The numbers are regulated by the European Parliament and Council Regulation (EC) no. 853/2004 and the maximum of ≤1.5 million cells should be contained in 1 mL. The average values of two-year monitoring for individual farms and lactation period are shown in Fig. 1. The results show that the limit value was multiply exceeded in the monitoring period for most of the farms. The exception is the farm no. 1, for which the limit value is not exceeded in any single case and TCM values ranged from 10⁴ to 10⁵ CFU x mL⁻¹. This state of farm no. 1 is held in the long term (Kalhotka et al., 2013). The observed values of TCM of farm no. 1 prove to be very close to the average values of 1.1 x 10⁵ CFU x mL⁻¹ as indicated Kouřimská

Table 1 Chacterization of farms.

Farm	Typ of milk	Breed	Type of farming	Milking animals	Region
1	goat	White Shorthaired Goat	conventional	131	dist. Blansko (SMR)
2	goat	White Shorthaired Goat	conventional	18	dist. Prostějov (OR)
3	goat	Crossbreed of Anglo-Nubian goat	organic	20	dist. Vyškov (SMR)
4	sheep	Lacaune	conventional	83	dist. Šumperk (OR)
5	sheep	Lacaune	organic	100	dist. Vsetín (ZR)

dist. – district, SMR – South Moravian Region, OR – Olomouc Region, ZR – Zlín Region

Table 2 Selected parameters relating to milking on farms.

Farm	Milking per day	Toilet m. gland	Parlor	Milking post-treatment	Cooling equipment
1	2x	wet	parallel	no	cooling equipment
2	2x	wet	parallel	barrier	spec. glasses in the refrigerator
3	2x	wet	spec. box	no	spec. glasses in the refrigerator
4	2x	wet	parallel	barrier	cooling equipment
5	2x	dry	circular	no	cooling equipment

and Dvořáková (2008). TCM fluctuated during lactation between the farms and it can not be clearly said that TCM rose with the increasing duration of lactation. At the same time, significant annual differences were also found out up to two logarithmic orders. Nevertheless, the individual

milk samples obtained from healthy animals contained small numbers of microorganisms, as for instance mentioned Králíčková et al. (2011), TCM can achieve high numbers in mixed samples. The average values of TCM for the whole period are shown in Fig. 2. Even here,

Table 3 Counts of microorganisms in goat and sheep milk in CFU x mL⁻¹.

Farm	Seasons	Year	TCM	Lbc.	Entero.	Ent.	TMRae	TMRan	Psych.	Mikromyc.
Farm 1	spring	2013	2.8×10^5	3.7×10^3	2.0×10^3	3.5×10^2	11	1	2.0×10^4	2.1×10^3
		2014	2.1×10^5	4.6×10^3	7.1×10^3	22	28	23	1.5×10^5	1.9×10^3
	summer	2013	2.3×10^5	1.2×10^4	1.6×10^3	4.4×10^2	23	28	8.4×10^4	2.9×10^3
		2014	9.5×10^5	3.2×10^5	1.6×10^4	1.7×10^2	24	21	2.6×10^5	2.5×10^3
	autumn	2013	7.0×10^4	8.6×10^3	30	2.5×10^2	9.8×10^3	4.2×10^3	3.7×10^3	9.1×10^2
		2014	7.7×10^5	3.9×10^2	2.7×10^4	6.6×10^2	2	1	2.2×10^5	1.6×10^3
Farm 2	spring	2013	2.3×10^7	9.5×10^5	5.6×10^3	1.2×10^5	2.9×10^3	9.8×10^2	4.7×10^6	1.9×10^4
		2014	9.5×10^5	2.4×10^3	3.2×10^2	8.7×10^2	1.4×10^5	20	2.3×10^5	1.2×10^3
	summer	2013	1.1×10^5	2.7×10^2	11	2.0×10^2	8	1	3.5×10^3	1.6×10^2
		2014	1.7×10^6	1.0×10^5	3.8×10^4	1.7×10^4	23	16	4.3×10^5	6.9×10^3
	autumn	2013	1.1×10^7	8.4×10^5	7.2×10^4	1.6×10^5	7.8×10^3	82	1.1×10^6	3.2×10^3
		2014	3.0×10^6	1.4×10^3	6.9×10^3	7.7×10^2	5.6×10^2	3.8×10^2	2.1×10^5	1.0×10^4
Farm 3	spring	2013	7.1×10^5	6.0×10^3	3.6×10^3	61	5	0	1.2×10^6	1.6×10^3
		2014	1.4×10^5	5.3×10^3	3.9×10^2	94	20	2.2×10^2	5.1×10^3	65
	summer	2013	2.3×10^5	3.0×10^2	2.6×10^2	32	3	1	<100	23
		2014	5.2×10^6	5.4×10^4	9.2×10^5	3.9×10^3	1.1×10^2	90	1.4×10^4	1.5×10^3
	autumn	2013	1.2×10^9	2.1×10^3	7.9×10^2	5.4×10^2	10	15	7.0×10^3	2.7×10^3
		2014	3.7×10^7	1.3×10^4	1.6×10^4	6.0×10^4	3.3×10^2	92	1.5×10^6	7.8×10^2
Farm 4	spring	2013	3.8×10^6	9.2×10^4	4.5×10^4	3.8×10^2	58	1.2×10^2	1.3×10^6	1.7×10^4
		2014	5.3×10^6	1.1×10^5	6.6×10^3	1.6×10^3	5.1×10^3	3.6×10^3	1.6×10^6	5.7×10^3
	summer	2013	3.8×10^5	1.4×10^5	4.7×10^3	2.2×10^3	15	9	1.6×10^4	1.1×10^4
		2014	8.2×10^5	1.3×10^5	4.6×10^4	1.8×10^4	32	20	6.7×10^5	3.5×10^4
	autumn	2013	5.9×10^6	2.2×10^3	2.1×10^5	5.4×10^2	1.1×10^5	1.5×10^5	1.2×10^6	5.0×10^4
		2014	2.5×10^6	6.5×10^3	6.8×10^3	1.4×10^4	90	7.1×10^2	6.6×10^5	4.8×10^4
Farm 5	spring	2013	4.5×10^5	1.8×10^3	4.0×10^3	95	48	5	1.3×10^4	1.6×10^2
		2014	3.7×10^6	1.9×10^4	<100	9.5×10^3	8.0×10^2	13	3.5×10^4	3.4×10^2
	summer	2013	1.1×10^6	1.8×10^4	1.4×10^3	2.2×10^4	8	11	4.5×10^4	4.9×10^2
		2014	4.0×10^6	2.3×10^6	3.8×10^4	9.9×10^4	2.1×10^3	57	8.1×10^5	1.7×10^3
	autumn	2013	5.2×10^6	1.1×10^6	2.3×10^3	1.1×10^4	5.3×10^2	4.0×10^2	2.2×10^6	2.5×10^4
		2014	4.5×10^6	1.8×10^3	5.5×10^2	6.1×10^2	3.7×10^2	2.7×10^2	2.5×10^5	1.5×10^3

TCM – Total count of microorganisms, Lbc. – lactobacilli, Entero. – fam. *Enterobacteriaceae*, Ent. – enterococci, TMRae – aerobic thermoresistant microorganisms, TMRan – anaerobic thermoresistant microorganisms, Psychro. m. – psychrophilic microorganisms, mikromyc. – micromycetes (yeasts and moulds)

it can be easy to see that the best results were achieved at the farm no. 1.

In goat milk, the highest average values of TCM were detected at the farm no. 3 using an environmentally friendly method. For sheep's milk, no significant difference was found out between the farms using

conventional or organic method.

The recommended values based on CSN 570529 related to cow's milk can be used for the evaluation of other monitored microorganism groups also proving technological importance. The number of psychrotrophic microorganisms should be up to 50000 in 1 mL,

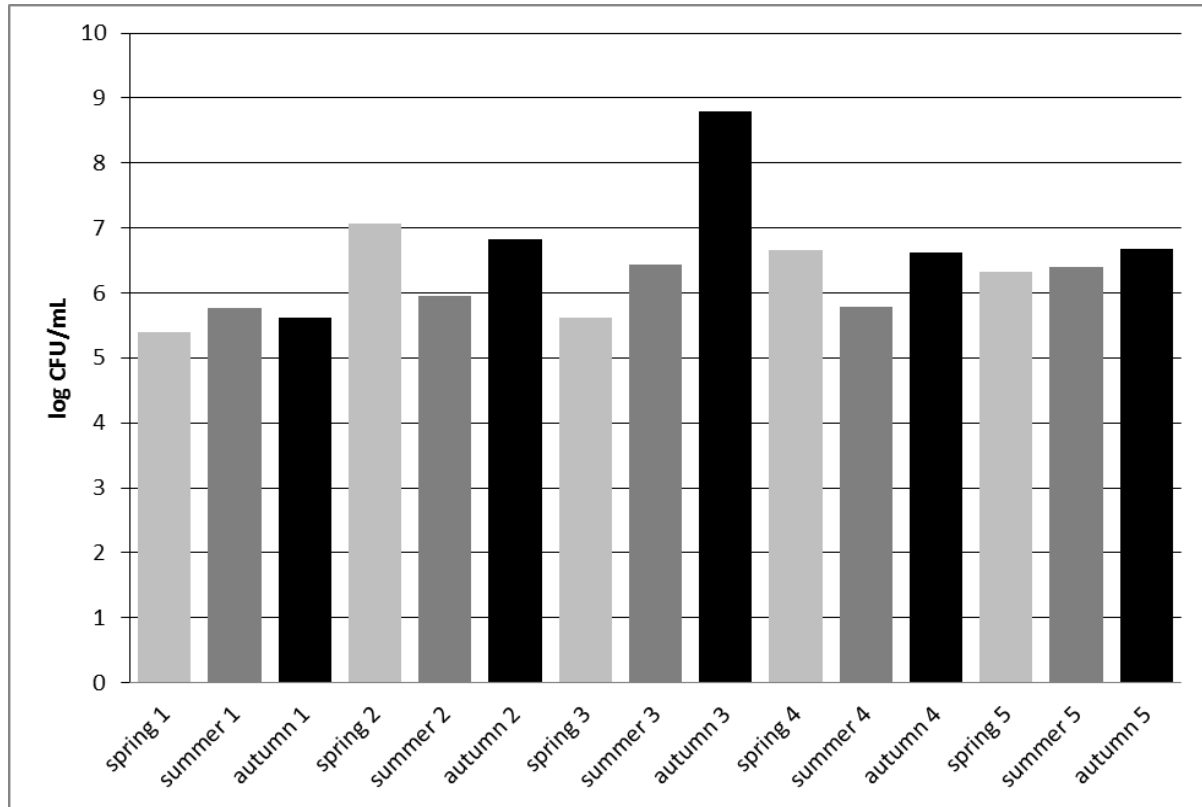


Figure 1 The average value of TCM in each seasons (2013 - 2014) for farms 1-5.

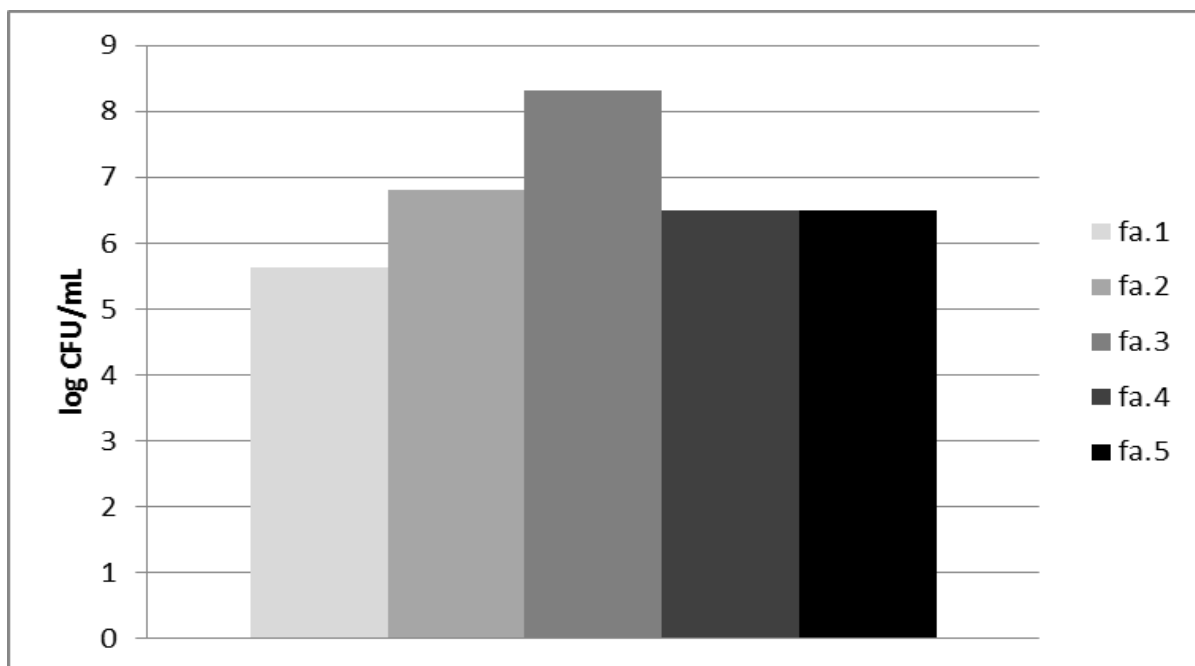


Figure 2 Average TCM the whole monitored period (2013 - 2014) for farms 1-5.

heat-resistant microorganisms up to 2000 in 1 mL, the highest number of coliforms up to 1000 in 1 mL, spore anaerobic bacteria should be negative in 0.1 mL. These microbiological limits are not determined for milk of small ruminants. In goat and sheep milk, the numbers are expected proportionately higher. Our identified microbial counts are corresponding with the findings (Tab. 3). High numbers TCM and coliform bacteria also mentioned **Oliveira et al. (2011)** and **Morgan et al. (2003)**.

The numbers of enterococci, lactobacilli but also coliform bacteria, yeasts and TCM samples of raw goat milk are lower up to several orders which are presented in the study by **Foschino et al. (2002)**, where significant differences are indicated between the farms.

High numbers of microorganisms in milk can cause spoilage unless the milk is quickly and efficiently cooled and subsequently pasteurized before further processing. In pasteurized milk, where the majority of contaminating microflora is destroyed, present microbial enzymes remaining active can cause the spoilage except to surviving microorganisms. The surviving microorganisms or microbial enzymes are also active and can spoil dairy products, which are made from such milk.

The results of microbiological analyzes show that the number of microorganisms in milk has been extensively individual and changeable. The final microbiological quality of the milk is caused by a number of factors (**Hejlová, 1997; Kalantzopoulos, 2003; Morgan et al., 2003; Malá et al., 2010; Cempírková et al., 2012**). The stage of lactation (Tab. 3, Fig. 1) did not prove a significant effect on the number of microorganisms in goat and sheep milk opposite to the number of somatic cells corresponding to the data of **Foschino et al. (2002)** and **Švejcárová et al. (2010)**. High numbers of microorganisms in goat and sheep milk may be primarily caused by the insufficient cleaning and sanitizing of milking equipment or low hygiene of hand milking. An important role may also act the cooling rate of milk and purity of cooling equipment. The method for cooling milk (cooling equipment versus the use of special glasses, see Tab. 2) may play an important role, especially in the farms from 1 to 3 producing goat milk.

CONCLUSION

From the above mentioned results, the following conclusions can be suggested. The bacterial counts of raw goat and sheep milk are highly variable and influenced by a number of important factors in the course of lactation and year. The bacterial numbers are not affected by the stage of lactation. Due to the small sample size and short term of monitoring, it can not be clearly said which farming method is preferable in the terms of microbial milk contamination. More important role acts the access, care and responsibility of the farmer apart from the mentioned factors. Not only for this reason, the microbiological analysis should be repeated for the following periods.

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DIETARY PATTERNS AND LIFESTYLE OF PATIENTS AFTER MYOCARDIAL INFARCTION

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ABSTRACT

Civilization diseases, including cardiovascular, are major health problems in current modern society. Numerous studies provided sufficient evidence that variety of risk factors are involved in cardiovascular diseases formation. Of the most important is the lifestyle that largely contributes to our health, up to 50 – 60%. Lifestyle includes all modifiable risk factors that together affect the development of these diseases. In our study we searched and evaluated the nutritional parameters and lifestyle of patients hospitalized in Cardiocentre Nitra. In order to obtain the necessary information we chose the questionnaire method. In our survey 194 patients were included, of which 155 were men (79.89%) and 39 (20.11%) women. These patients were hospitalized with acute myocardial infarction diagnosis. The vast majority of patients had overweight and obesity caused by improper eating habits. The high prevalence of overweight, BMI in the range 25 – 30 kg/m², was also confirmed as statistically significant, $p < 0.05$ ($P=0.02$). BMI over 25 kg/m² was present in 85% of men and 80% of women. The consumption of selected food commodities with evidence of a positive or negative impact on the emergence of acute myocardial infarction was not statistically significant ($p > 0.05$) when evaluating dietary habits. But we can confirm too frequent consumption of chicken at the expense of other types of meat and relatively frequent consumption of fish (once a week). We recorded the preference of semi-skimmed dairy products, but also high and inappropriate consumption of full-fat dairy products in men and women (37% and 17.8%, respectively). Daily intake of fruits and vegetables was in 85.9% of women and 64.7% of men. Everyday intake of bread and pastries was confirmed by 100% of respondents. Lifestyle was evaluated according to the presence of smoking, excessive alcohol consumption, and physical activity. Up to 60.5% of men and 26.4% of women admitted smoking, while 34.6% of men and 7.5% women ended up with the habit after myocardial infarction. The excessive alcohol consumption was not detected in the study group. Approximately 67% of men and 56% of women carry out an easier walking and moderate physical activity while taking into account their health status.

Keywords: dietary habits; lifestyle; nutrition.

INTRODUCTION

Cardiovascular diseases are one of the major causes of death and sick leave of inhabitants all around the world despite the ever increasing medical expertise and care. The World Health Organization (WHO) defines cardiovascular diseases (CVD) as a group of disorders of the heart and blood vessels that includes coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, and deep vein thrombosis and pulmonary embolism. The most common acute clinical manifestations of CVD are myocardial infarction and stroke (Michas, Micha, Zampelas, 2014). In the European Union, in 2000, 115 people per 100000 died due to coronary heart disease. By 2010, this figure had dropped to 76 per 100000. However, despite this progress, CVD still represents the major cause of adult morbidity and mortality in most developed and many developing countries (Murray et al., 2012). At current death trend in the world, the number of cardiovascular diseases in 2020 may increase up to 25 million (Riečanský, 2009). Cardiovascular diseases are

disproportionately high price that mankind pays for an unhealthy lifestyle. It includes unbalanced caloric intake and expenditure. This is connected with the increase in obesity during childhood (Riečanský, 2009). Childhood obesity has been identified as one of the most important risk factors of developing cardiovascular diseases. Many of overweight or obese children will become obese adults with enhanced risk for cardiovascular diseases. Childhood obesity is often accompanied by serious consequences such as dyslipidemia, hypertension, diabetes, pro-inflammatory state and non-alcoholic fatty liver disease (Sypniewska, 2015). Since a large share on these disorders development have modifiable risk factors, mainly related to lifestyle, the effort of all professionals in the medical circles as well as among nutritionists about their reversal is therefore understandable. The aim is to show and teach the general public a healthier lifestyle. Hypertension, high LDL cholesterol and triglyceride concentrations, insulin resistance, inflammation and disturbances in adipocytokines secretion are associated with endothelial dysfunction which precedes the

development of atherosclerosis (Sypniewska, 2015). Atherosclerosis is a chronic inflammation of the arteries, which develops over decades in response to the biologic effects of underlying risk factors (Nabel and Beaunwald, 2012), a multi-factorial disease with both genetic and environmental etiology. The primary modifiable risk factors are dyslipidemia, hypertension and smoking. Nutritional habits, especially dietary fat are implicated in the process of atherosclerosis (Perk et al., 2012). Atherosclerosis's deceit lies in the fact that etiopathogenetic process occurs in the blood vessels for years confidently and without pain, and the first manifestation of the disease may mean the death of man. Today it is clear that atherosclerosis etiology is multifactorial. It is considered to be an inflammatory and immunomodulatory response taking place in the vessel wall (Balagopal, 2011). Risk factors of CVD are influenced by both genetic and environmental factors. Although it is difficult to alter genetic factors, modifiable environmental factors such as smoking or dietary patterns could be targeted in preventive interventions aimed at lowering these risk factors (Mirmiran et al., 2009).

MATERIAL AND METHODOLOGY

We chose the questionnaire method to obtain information on dietary habits and lifestyle of people after myocardial infarction. Of the 238 hospitalized patients, 194 patients were diagnosed acute myocardial infarction, of which 155 were men and 39 women. The collection of data was carried out by a questionnaire method through guided conversation. The survey covered lying patients who were hospitalized in Cardiocentre Nitra. The questionnaire was anonymous, its completion was voluntary with only one response to be circled for each question. Data collection was carried out simultaneously with somatometric and biochemical examination of the respondents. Recorded eating habits and lifestyle of respondents were compared with the current recommendations of nutritional criteria for patients with cardiovascular disease.

RESULTS AND DISCUSSION

The number of hospitalized patients with acute myocardial infarction was 194, of which 155 were men and 39 women. Average age of patients was 59.2 for men and 64.4 years for women. From the social factors we positively consider the fact that up to 86% of patients lived with their family in the same household. More than half of the patients were retired, while their highest level of education was mainly secondary education with graduation. In the context of a positive family history it is an interesting fact that up to 51.5% of men indicated cardiovascular diseases among first-degree relatives. This finding is even more important, whereas up to 88.9% of men reported that the disease occurred among male relatives. Among women, the positive family history was confirmed in 22%.

We calculated BMI from data on height and weight of respondents. The results are shown in Figure 1. Our survey confirms high prevalence of overweight and obesity among people with cardiovascular disease. We also confirmed a statistically significant overweight occurrence (BMI in the range 25 – 30 kg/m²), *p* <0.05 (*p* =0.02), in patients with acute myocardial infarction. Overweight and obesity are considered to be a significant risk factor influencing creation of these diseases and their presence often worsens their course. Obese patients develop more CVD risk factors than normal weight controls. BMI ≥40 kg/m² exhibits increased prevalence of type 2 diabetes mellitus and hypertension. Dyslipidaemia peaked around BMI 35 to <37.5 kg/m² and CVD at BMI 37.5 to <40 kg/m² in men and BMI ≥40 kg/m² in women. A 10% weight loss reduced the OR for type 2 DM by 30% and CVD by 20%, while 10% weight gain increased type 2 DM risk by more than 35% and CVD by 20% (McQuigg et al., 2008).

High BMI is associated with the development of cardiovascular risk factors such as hypertension, dyslipidemia, insulin resistance, and diabetes mellitus (Wormser et al., 2011). BMI alone seems to present a U- or a J-shaped association with clinical outcomes and

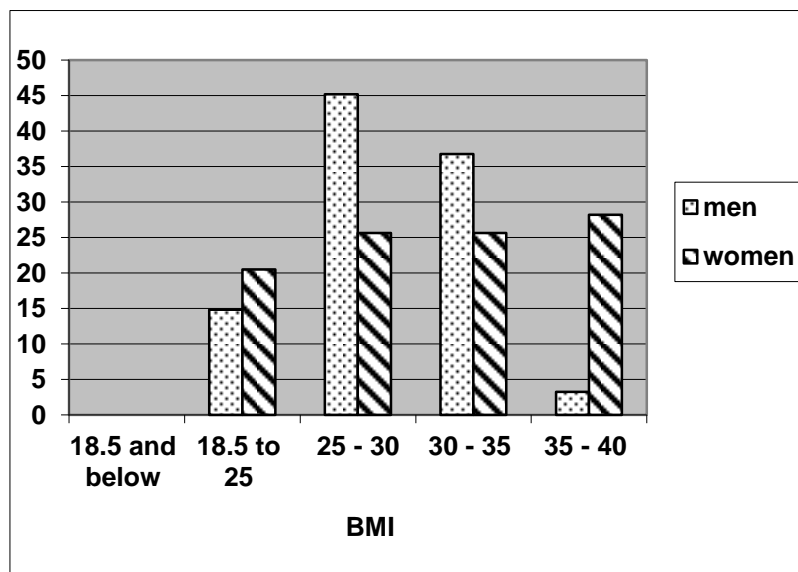


Figure 1 BMI of patients with myocardial infarction in %.

mortality (Calle et al., 1999). Such an inverse relationship fuels a controversy in the literature, named the ‘obesity paradox’, which associates better survival and fewer CVD events in patients with mildly elevated BMI afflicted with chronic diseases (Romero-Corral et al., 2007).

Dietary habits

Meat and meat products

Meat and meat products are often associated with an increased risk of cardiovascular diseases because they increase the total intake of fat, especially saturated. We consume 30% of total fat daily intake in form of the meat and meat products. The fat content depends on the type of meat (Sergyi et al., 2011). Polyunsaturated fatty acids are mostly represented in the lean meat. All previous findings confirm that the saturated fatty acids increase the levels of total cholesterol and LDL cholesterol levels in the blood and vice versa, unsaturated fatty acids reduce them. Atherogenic potential of saturated fatty acids is significantly higher (about twice that high) than anti-atherogenic effect of unsaturated fatty acids (Bada, 2001). The notion that meat consumption is associated with CVD risk has been reflected in the dietary guidelines that emphasize selecting lean meats. The association between meat consumption and the incidence of chronic disease and mortality has been evaluated in hundreds of observational epidemiologic studies over the past few decades. Despite this wealth of data, it is unclear whether higher intakes of specific meat groups (e.g., total, unprocessed or processed red meat) or individual meats (e.g., beef or pork) independently contribute to disease risk, or whether they are part of a broader diet and lifestyle pattern that is ultimately responsible for the disease (Micha et al., 2013). Several individual studies and meta-analysis focusing on the relationship between dietary meat and CVD and total mortality have been carried out. These analyses support an association between red meat consumption and total mortality and CVD-related deaths, as well as the risk for CVD, ischemic stroke and type 2 DM. However, this association was driven in many cases by the consumption of processed meats rather than by that of fresh red meat (Brown and Hazen, 2014). Therefore, some investigators propose that the preservatives used in food processing may be driving the deleterious effects. In

fact, it has been suggested that the deleterious effects may relate to other ingredients, such as sodium, nitrites, heme iron, or L-carnitine (Brown and Hazen, 2014). Pan et al. (2012) by combining data from the Health Professionals Follow-up Study and from the Nurses’ Health Study concluded that both red and processed meat consumption is associated with an increased risk of CVD mortality. However, a recent investigation in the EPIC cohort, including 448,568 participants in 10 European countries, provided more evidence that unprocessed red meat intake was not significantly associated with CVD mortality; conversely, processed meat was associated with 30% higher CVD (Rohrmann et al., 2013). Preferred types of meats between hospitalized patients are shown in Figure 2.

The consumption of poultry meat was the most common among the patients, however the consumption was not statistically significant, $p > 0.05$ ($p = 0.358$).

Fish

There is strong scientific evidence in many studies that n-3 fatty acids derived from fish or fish oil significantly reduce the effect of risk factors of heart diseases. Omega-3 polyunsaturated fatty acids have been observed to decrease the production of pro-inflammatory eicosanoids and cytokines and thus, fish consumption is believed to protect from diseases involving inflammatory processes (Wall et al., 2010). Fish consumption and omega-3 polyunsaturated fatty acids intake have also been suggested to slow the progression of atherosclerosis (Massaro et al., 2008) and to reduce arterial stiffness (Hall, 2009). Optimum preventative dose for the average consumer is considered to be 40 – 60 g of fish meat twice a week (Kerestés et al., 2011). However, this fact has not been confirmed by our research, whereas 87% of women and 77.5% of men indicate the frequency of fish consumption at least once a week, mainly in a canned form. It can also be affected by smaller portions of fish, despite the fact that they are consumed relatively quite frequently. Fish consumption in the frequency of once a week is not statistically significant in both men and women, $p > 0.05$ ($p = 0.17$).

Milk and milk products

Dairy products, in their natural form, contain relatively high fat and high saturated fat and cholesterol. Therefore, after occupying a prominent position among recommended

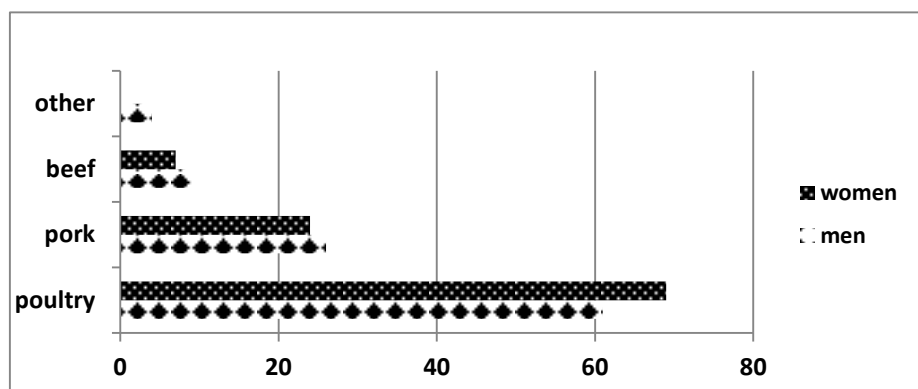


Figure 2 The preference of each types of meat in %.

foods for decades, especially for children and adolescents, this food group also suffered from the consequences of the fight against saturated fat and cholesterol. Some of the fats previously deemed as good might not be so healthy (ie, omega-6 polyunsaturated fats) and, conversely, that some of the bad fats might be healthy (ie, saturated fats from dairy foods) (Lawrence, 2013). Milk and dairy products are not only a source of energy and high quality proteins, but also an important source of trace elements in our diet. It has been also proven that they have beneficial effect on lowering blood pressure and increasing HDL cholesterol, which is associated with a reduced risk of cardiovascular diseases (German, 2009). Milk fat has a high content of saturated fatty acids (60%) and it is known that some of the saturated fatty acids may increase serum cholesterol (Riečanský, 2009). A systematic literature review of observational studies on the relationship between dairy fat and high-fat dairy foods, obesity, and cardiometabolic disease was conducted by Kratzet et al. (2013). Results suggest that dairy fat or high-fat dairy foods do not contribute to obesity or cardiometabolic risk, and imply that high-fat dairy consumption within typical dietary patterns is inversely associated with obesity risk. O'Sullivan et al. (2013) found that high intakes of dairy products were not associated with a significantly increased risk of mortality compared with low intakes. Some authors use the current evidence to recommend even an increase of dairy products in order to achieve a more complete and balanced nutrition. Fulfilling the recommended amounts, ie, 3 servings daily for individuals ≥ 9 years, helps to accomplish current overall nutrient intakes and recommendations. Moreover, consuming more than 3 servings of dairy per day leads to better nutrient status and improved bone health and is associated with lower blood pressure and a reduced risk of CVD and type 2 DM (Rice, Quann, Miller, 2013). It can be concluded that consumption of dairy products is either protective against CVD or has no adverse effects. In our research, two thirds of patients reported that they consume milk and dairy

products every day and the remaining one-third of them indulges that at least two to four times a week (Figure 3).

However, only 16.2% of men and 26.1% of women choose low fat dairy products deliberately. It is highly inappropriate that there is up to 37% preference of full fat dairy products among men and 17.8% among women. Milk and milk products consumption was not statistically significant ($p > 0.05$). The most popular dairy products are yoghurt and cheese.

Fruits and vegetables

Fruits and vegetables have always been considered health-promoting foods. This is due to the association of a higher intake of these products with a reduced risk of developing chronic illnesses, including CVD (Berciano and Ordovás, 2014). Therefore, current dietary guidelines and WHO recommend increasing fruit and vegetables intake to ≥ 5 servings per day. This recommendation is based on the belief that eating fruit and vegetables may reduce cardiovascular risk through a combination of beneficial micronutrients, antioxidants, phytochemicals and fiber in these foods. It has been found that those with higher carotene intake had about 46% lower risk of dying from cardiovascular diseases than those who had low or no intake of these active substances (Liu et al., 2000). A recent report based on the Health Survey for England studied the eating habits of 65226 people. The report found that eating ≥ 7 portions of fruit and vegetables daily reduced the specific risks of death by cancer and heart disease by 25% and 31% respectively. This report also showed that vegetables have significantly higher health benefits than fruit (Oyebode et al., 2014). Mirmiran et al. (2009) found that consumption of fruit and vegetables was inversely related to total cholesterol and LDL cholesterol concentrations, independent of age, sex, smoking status, exercise, and educational attainment. According to the Italian study, a diet rich in bulb vegetables can have a beneficial influence on the risk of acute myocardial infarction occurrence. This is explained by the effect of

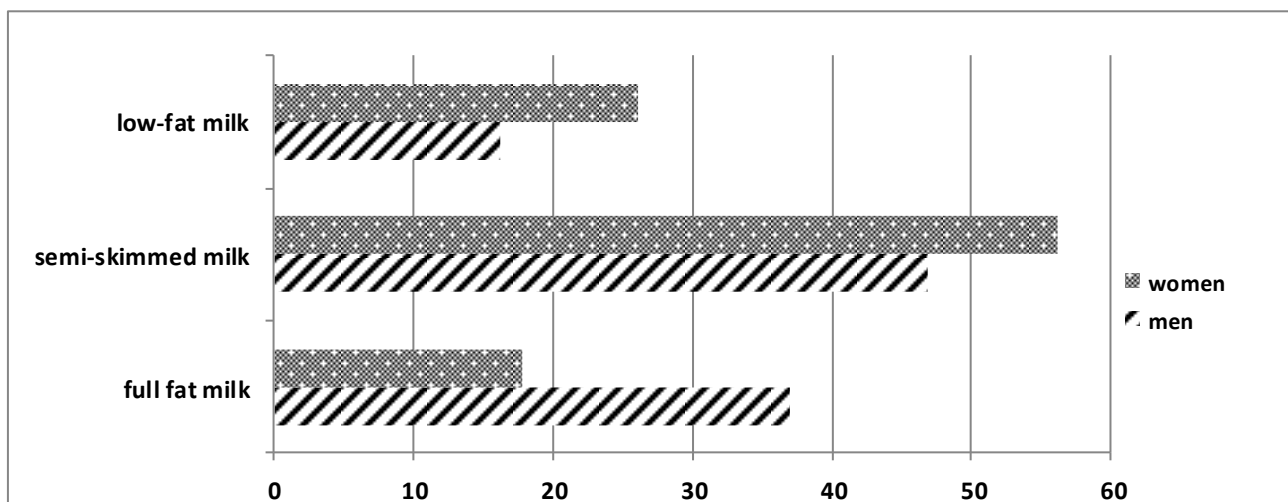


Figure 3 The preference of each types of milk and milk products in %.

bioactive ingredients such as phenol and organosulphate compounds which have antithrombotic and protective effects on the endothelium (Galeone et al., 2009). Despite daily intake of fruits and vegetables, which was declared by 85.9% of women and 64.7% of men among hospitalized patients in our survey, these people were diagnosed by myocardial infarction. This may be caused by an insufficient amount of eaten fruit and vegetables, despite their introduction into the daily diet. The consumption of fruit and vegetables was statistically insignificant in the study group ($p > 0.05$). According to the recent findings it is recommended to eat up to 8 servings of fruit and vegetables to the people with cardiovascular risk, while one serving is considered to be 80 g. This corresponds to a small banana, medium sized apple, pear, orange or medium-sized carrot (Marmot, 2011). Among the most preferred fruit in our survey were

apples, pears and bananas. Citrus and exotic fruits were less consumed. The most preferred kinds of vegetables were leafy and root vegetables.

Bread and pastry

Researchers from the University of Barcelona found out that eating bread on every day basis is a good way how to prevent the development of cardiovascular diseases. Its consumption is associated with better lipid profile. This means that the person has lower levels of bad LDL cholesterol and higher levels of good HDL cholesterol. In a sample of older volunteers with high risk of cardiovascular diseases occurrence, the results showed that those who ate bread daily had healthier lipid profile and lower insulin levels than those who did not consume it daily. Metabolite which caused change in the lipid profile was also determined. The content of this metabolite was higher in those who ate wheat bread (Nordqvist, 2012).

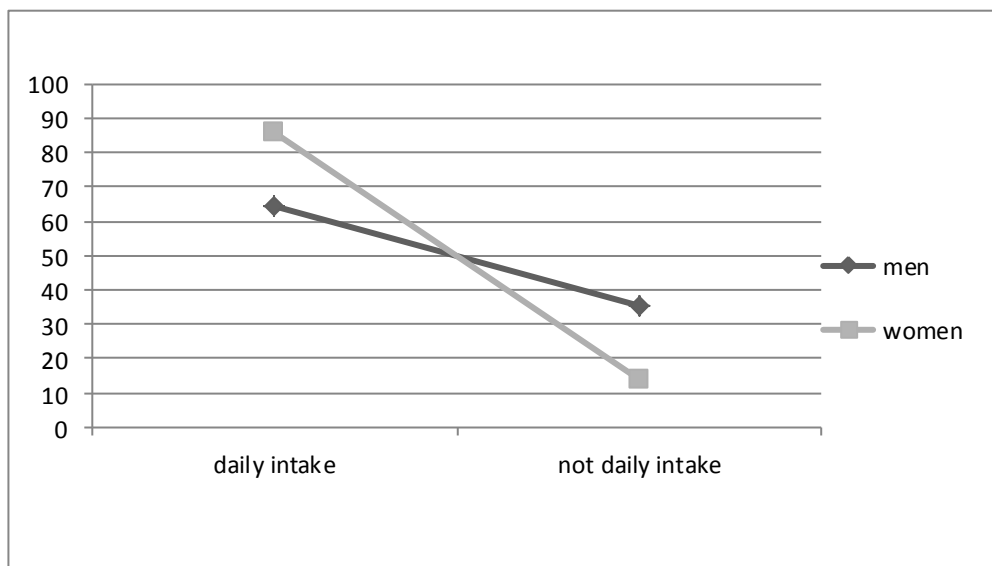


Figure 4 The consumption of fruit and vegetables in %.

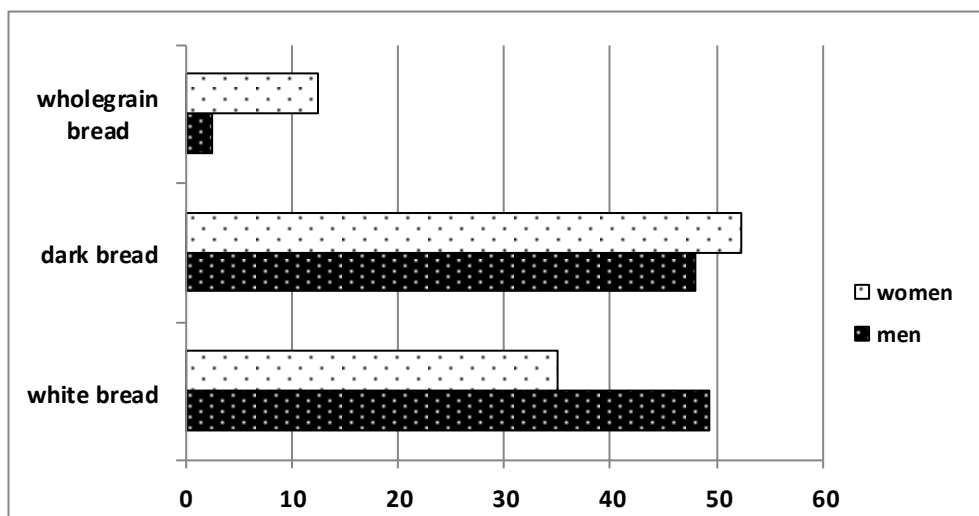


Figure 5 The preference of each types of bread and pastry in %.

American scientists highlight the high proportion of salt, which is found in some types of bread. Too much sodium in the diet increases the risk of high blood pressure and it is one of the major risk factors of heart diseases (**Paddock, 2012**). From the answers of our respondents we found out that bread and pastries are daily consumed by 100% of the respondents, while white bread is preferred by 49.4% of men and 35.1% of women. Dark breads are preferred by 48.1% of men and 52.45% of women. The rest of the respondents are consuming cereal kinds of bread (2.5% of men, 12.45% of women).

Lifestyle

Smoking

Smoking, both active and passive, is an established vascular risk factor and one of the most serious global health problems and its harm to human health is of no doubt. It clearly leads to the progression of atherosclerosis. This risk increases with the number of cigarettes smoked per day (**Jurkovičová, 2005**). Severity of disease and mortality sharply increases from 20 cigarettes per day. The average life-shortening of smokers regardless of the type and method of smoking in men is more than 13 years and in women it represents 14.5 years (**Rosamond et al., 2007**). The results of our research show that up to 60.5% of men smoked in the past and 34.6% of men stopped smoking due to the disease. Smoking among women in the past was confirmed by 26.4%, while 7.5% gave up this habit due to hospitalization. In the number of cigarettes smoked per day, up to 58.7% of all smokers reported under the category of 10 to 20 cigarettes per day. More than 20 cigarettes per day are lighted by 24% of smokers (mostly men).

Cigarette smoking exaggerates the link between dyslipidaemia and CVD; total cholesterol, triglycerides and low-density lipoprotein levels are elevated, whereas HDL cholesterol levels are decreased in smokers, possibly due to changes in lipid transport enzymes (**Chelland et al., 2008**). Of note, abstinence from smoking was associated with increases in HDL cholesterol, total HDL and large HDL particles despite weight gain, especially in women (**Gepner et al., 2011**).

Alcohol consumption

A lot of attention is given to the relation between alcohol and cardiovascular diseases. Epidemiological studies have shown that persons consuming small amounts of alcohol have a lower death rate from cardiovascular diseases, in particular acute myocardial infarction, compared with those who drink much or do not drink at all. Overall, the evidence suggests a J-shaped association between alcohol consumption and coronary heart diseases risks, in which a moderate alcohol intake (eg, 2 glasses of wine per day) causes a significant increase in HDL levels and a reduction of coronary heart diseases risk, while the disease risk for a heavy drinker would be twice as high (**Eilat-Adar et al., 2013**). Recent reviews suggest that beer and especially red wine (**O'Keefe et al., 2014; Chiva-Blanch et al., 2013**) are associated with greater reductions in CVD risk due to their high polyphenol content. A recent meta-analysis found out a reduction of the overall mortality risk in men of 17% and women of 18% (**Di Castelnuovo, 2006**). Described effects are not attributable solely to the alcohol

but to the other non-alcoholic substances in wine (especially red wine) and some types of beer (mainly black beer). These include polyphenols (catechin, quercetin, resveratrol) which improve endothelial function and increase HDL cholesterol (**Xin et al., 2010**). In our survey, the most preferred was the consumption of beer and wine among men and wine and liqueurs among women. The regular use of alcohol (at least once a week) was reported by 55.35% of men, while drinking at least 1 litre of alcoholic beverage per week and 33.4% of women consume 0.2 to 0.5 litres of alcoholic beverage per week.

Physical activity and inactivity

Concomitant with the rise in sedentarism has been an epidemic of chronic disease and mortality. Epidemiological data strongly suggest that the lack of physical activity significantly contributes to the increasing incidence of chronic diseases, especially cardiovascular. Physical inactivity increases the risk of disease to 1.5 times (**World Health Report, 2002**). The best type of exercise is endurance physical activity (walking, running, swimming) with alternating by resistance training. Regular physical activity has wide beneficial effects (**Perk et al., 2006**). In relation to the physical activity, we did not focus only on the current physical activity, but especially on sport activities in the past that could have had an impact on cardiovascular disease formation. Current physical activity of our respondents is very limited due to the bad health condition. In the past, 53% of men and 3% of women practised active sports, 37% of men and 70% women practised recreational sports. 27% of women and 10% of men said they had had no physical activity. Currently 67% of men and 56% of women practise easier walking and moderate physical activity. The health benefits and cardioprotective effects of physical activity are irrefutable. Reduced mortality and improvements in metabolic function, body composition, hemodynamics, musculoskeletal, and psychologic functioning are a few of the myriad benefits of increased physical activity. Even small increments in physical activity via reductions in sedentary behavior are beneficial, given that each is an independent risk factor for CVD (**Archer, Blair, 2011**).

CONCLUSION

Based on our research, we confirmed that the accumulation of various risk factors, particularly overweight and obesity, smoking, or poor composition of dietary habits, including physical inactivity has resulted in the development of cardiovascular diseases, which resulted in myocardial infarction. Based on research results we suggest to modify the diet of hospitalized patients to such an extent that approaches the most nutritious food. That means the regulation of energy intake according to the physical activity (control of body weight and its maintaining in a range of BMI between 18.5 to 25 kg/m²). It is necessary to increase the consumption of fish and partially limit the meat consumption, and to incorporate other types of meats (beef and veal, turkey) into the diet more often, instead of chicken meat. When consuming dairy products it is necessary to draw attention to the low-fat products. Increased consumption of fresh fruit and vegetables according to the proposed standards is also

desirable. Within lifestyle it is indispensable to appeal to stop smoking and to introduce physical activity, of course, to the extent that is permitted by the health of the patient. We can leave moderate red wine consumption within the recommended values, but certainly not higher. In order to supplement information on healthy lifestyle, regardless of the incomplexity of our research it is recommended to choose the vegetable oils and margarines enriched by mono- and polyunsaturated fatty acids instead of hydrogenated margarines and butter, while limiting foods with high salt content, preparing meals low in salt, or without salt and limiting sugary drinks and foods.

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EFFECT OF SELENIUM ON ITS CONTENT IN MILK AND PERFORMANCE OF DAIRY COWS IN ECOLOGICAL FARMING

Pavel Horký

ABSTRACT

Currently, the ecological farming is increasingly spread in the European Union. The aim of this relatively young farming method is a friendly approach to agricultural production with an emphasis to deliver healthy raw materials and food to final consumer. Selenium is included in an essential trace micronutrients which are necessary for the proper process of physiological reactions. It is a part of glutathione peroxidase, which is a powerful antioxidant. At present, selenium-deficiency can occur in feed and food in central Europe. Selenium deficiency is one cause of the higher occurrence of cardiovascular diseases. The aim of the experiment was to study whether the addition of selenium to the diet of dairy cows in ecological farming can increase its concentration in milk and affect quantitative (milk yield) and quality (content of protein, fat, lactose, somatic cells and urea) milk indicators. The experiment included twenty cows of Holstein breed. The first experimental group of cows ($n = 10$) was fed with selenium in an amount of $0.3 \text{ mg} \cdot \text{kg}^{-1}$ (as selenomethionine) in the feed dose. The control group ($n = 10$) was not fed with the increased selenium in the feed dose. The basic feed dose contained 0.17 mg of Se/kg in the diet. For dairy cows, daily intake was of 20.5 kg of dry matter feed. The duration of the experiment was set at 45 days. The selenium concentration in milk was measured from 0.13 to $0.15 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ in the experimental group of cows during the evaluation. The control group of cows without the addition of selenium to the diet showed a selenium concentration below the detection limit. During the experiment, milk yield, lactose, fat and protein were not affected. A significant decrease ($p < 0.05$) of somatic cells by 58% occurred in milk in the experimental group. The amount of urea was significantly lower in both groups in the experimental (by 52%; $p < 0.05$) and control (50%; $p < 0.05$). These results show that the addition of selenium may increase the selenium concentration in milk (the production of functional food). The addition of selenium reduces the amount of somatic cells, which are the indicators of inflammatory diseases of the mammary gland.

Keywords: selenium; milk; cow; ecological farming

INTRODUCTION

Ecological farming has recently developed in the European Union. The food from this production is increasingly popular for final consumer (Gambelli et al., 2014; Horký, 2014). Selenium is important as the essential microelement in animal and human areas. The receiving adequate level of selenium in the diet is essential for the maintaining of good health and reproduction parameters. Selenium is a part of the enzyme glutathione peroxidase (GPx) transforming hydrogen peroxide to water and molecular oxygen (Horký et al., 2012a; Horký et al., 2013). The food is low on selenium content and the total amount of antioxidants, which are associated with civilization diseases in many cases (Hadas et al., 2015; Navia et al., 2014). The amount of selenium varies significantly in the soil by the region. Selenium proves the ability to activate cells of the defense system of the organism and thus prevents serious diseases e.g. cancerous tumors (Mehdi et al., 2013). The application of selenium in feed doses is one of the ways how to increase selenium in animal food and products (Horký, 2014; Van Metre and Callan, 2001). The addition of minerals (especially selenium) to the diet of ruminants can also improve the

reproductive performance and animal health (Horký et al., 2012b; Nevrkla et al., 2014; Pechova et al., 2012). Selenoproteins support the metabolism of hormones because of improving activity of the thyroid gland (Horký and Cerkal, 2014; Wichtel, 1998).

The aim of the experiment was to study the effect of addition of organically bound selenium on the quality of the produced milk in terms of functional food into the diet of dairy cows in an ecological farming.

MATERIAL AND METHODOLOGY

The experiment was carried out on an ecological farm (the farm was registered in accordance with the Czech Republic's Act No. 242/2000 Coll. under registration number 42318335) keeping dairy cattle in Lesoňovice, the Czech Republic. The experiment included 20 Holstein dairy cows divided into two equal groups by weight (the experimental group had an average weight of $622 \pm 15 \text{ kg}$; the average weight of the control group was $630 \pm 11 \text{ kg}$) and at the same stage of lactation. Dairy cows in the experimental group had completed an average of 3.4 ± 0.1 lactations prior to the experiment while cows in the control group had completed an average of 3.6 ± 0.1 lactations.

Table 1 Composition of the feed ration and dairy cows' average daily intake per head (Dry matter/kg).

Feedstuff	Control group	Experimental group
Maize silage	6.38	6.38
Clover–grass haylage (first cutting)	5.95	5.95
Grass haylage (first cutting)	4.00	4.00
Winter wheat	2.64	2.64
Spring barley	1.76	1.76
Detamin GA Spezial ^b	0.13	0.13
Total intake	20.86	21.86

Note: ^bDetamin GA Spezial is a mineral supplement for ruminants intended to be used in organic farming systems (H. Wilhelm Schaumann GmbH, Pinneberg, Germany).

The experimental and control group of cows received up to 20.5 kg of dry matter of feed /piece/day. The basic diet contained 0.17 mg of selenium/kg. The animals had ad-libitum access to water. The first group of cows (n = 10) was added the selenium in the diet at a dose of 0.3 mg.kg⁻¹ (as selenomethionine). The second group of cows (n = 10) served as a control group without the addition of selenium (this group of animals received only selenium from native sources). The premix of selenium was mixed to the basic ration (TMR) and fed in the morning dose. The average milk yield of the animals was 7,600 ±50 kg/lactation. All animals were fed a basic feed ration in the form of a total mixed ration (Table 1 and Table 2) and were allowed *ad libitum* access to water. The quantity of feed provided was recorded by the mixer-wagon (Luclar, the Czech Republic). Uneaten amounts were estimated and not analysed in any way.

In the control group of cows, the average duration of lactation lasted for 103 days at the beginning of the experiment (stage of lactation varied from 86 to 110 days). The experiment began on average 107th day of lactation (lactation phase ranged from 85 to 114 days) in the experimental group of cows. The cows were in free housing. The feeding was carried out twice a day (morning and evening). The duration of the experiment was set at 45 days.

Before starting the experiment and then at 15 day intervals (0, 15, 30 and 45th day), milk samples were taken for selenium determination. Milk samples for the determination of yield and milk components (protein, fat, lactose, somatic cells, urea) were taken at the beginning and end of the experiment, ie. 0th and 45th day. The samples were taken before feeding in each morning and

subsequently subjected to an appropriate analysis.

Determination of Selenium Concentration in the Silage

The determination of selenium concentration in the silage was carried out using the atomic absorption spectrometry.

Determination of Selenium Concentration in Milk

Selenium has been set on the 290Z Agilent device using method of atomic absorption spectrophotometry (Agilent, USA) with the electrochemical atomization. The ultrasensitive lamp with hollow cathode (Agilent) was used for selenium. The lamp of power of 10 mA was utilized as a radiation source. The spectrometer operated at 196 nm with a spectral bandwidth of 1.0 nm. The volume of sample was of 20 mL, which was injected into the graphite tube. The flow rate of argon as the inert gas was of 300 mL/min. The repetition was applied in the field strength of 0.8 Tesla. Selenium was determined in the presence of palladium as a chemical modifier. The samples were always measured in two repetitions.

Microwave Decomposition for the AAS determination

A 40 mL of milk was pipetted into tubes, in which took place the decomposition. Nitric acid (65%) and hydrogen peroxide (30%) were used for the decomposition of the samples. Overall, a 500 ml of folding mixture was used. The ratio between the nitric acid and hydrogen peroxide was 7:3. The samples were determined using a 3000 microwave (Anton Paar GmbH, Austria), MG - 65 rotor. The program is repeated at regular ten minute intervals with power ranging from 50 W to 0 W (cooling). The microwave power was 100 W in the main portion of the program (duration 30 minutes) at 140 °C.

Assessment of milk components

Table 2 Composition of Detamin GA Spezial mineral supplement for dairy cows (the content is presented in 1 kg of premix).

Parameter	Unit	Quantity
Zinc (as zinc oxide)	mg	8000
Manganese (as manganese oxide)	mg	6000
Copper (as copper sulfate pentahydrate)	mg	1200
Iodine (as calcium iodate)	mg	100
Cobalt (as carbonate cobaltous hydroxide)	mg	18

The milk was preserved using 2-bromo-2-nitropropane-1,3-diol and cooled to 4 – 6 °C until the analysis, which was carried out within hours of the sampling. The milk components were analysed in a commercial laboratory using a MilkoScan FT2 (Foss Electric, Hillerød, Denmark). Fat was acidobutyrometrically established according to standard CSN ISO 2446. Crude protein was established by the Kjeldahl method according to CSN 57 0530. The urea in milk was enzymatically determined using a commercially available urea/ammonia assay kit (Megazyme, Wicklow, Ireland) according to CSN 57 0533. Somatic cells were analyzed using FTIR technology (Fourier Transform InfraRed) - (MilkoScan, FT 6000) according to ČSN EN ISO 13366-2.

Statistica

The data were processed statistically using STATISTICA.CZ, version 10.0 (the Czech Republic). The results were expressed as mean ± standard deviation (SD).

RESULTS

In the experiment, the effect of selenium on selenium concentration in milk and performance of dairy cows was

studied. During cow feeding of selenium in an ecological farming, the selenium content was monitored in the milk of animals before the beginning of the experiment under the detection limit. In the experimental group, a detectable amount of selenium was monitored after 15 days of the application of selenium to the diet, which was in the constant level throughout the duration of the experiment. The experimental group with selenium level ranged from 15th to 45th day in the interval from 0.13 to 0.15 g.mL⁻¹. In the control group, the amount of selenium was under the detection limit for the entire duration of the experiment (Table 3).

Within the experimental observation, the qualitative and quantitative parameters of the produced milk were also evaluated. Milk yield was similar in both groups with no significant changes. At the end of the experiment, an increase of 22 resp. 18 % was observed in the experimental and control groups. For lactose, no differences were also observed. The lactose value were within the physiological range (from 4.6 to 4.8%). The values of milk yield and lactose are shown in Figure 1A and Figure 1B.

Table 3 Effect of selenium feeding on its content in milk and antioxidant potential.

Index	Experimental group				Control group			
	Day of experiment				Day of experiment			
	0	15	30	45	0	15	30	45
Selenium content (µg/ml)	UDL	0.15 ±0.01	0.13 ±0.05	0.14 ±0.03	UDL	UDL	UDL	UDL

UDL - under the detection limit

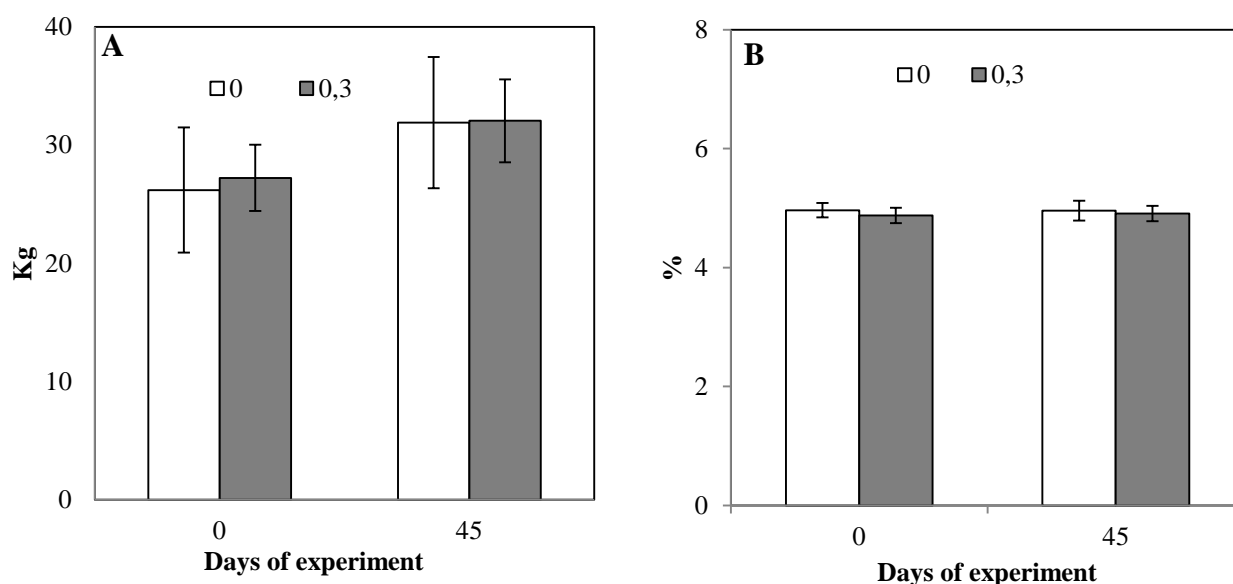


Figure 1 Effect of selenium feeding on milk yield (A) and lactose content (B) in dairy cows in ecological farming.

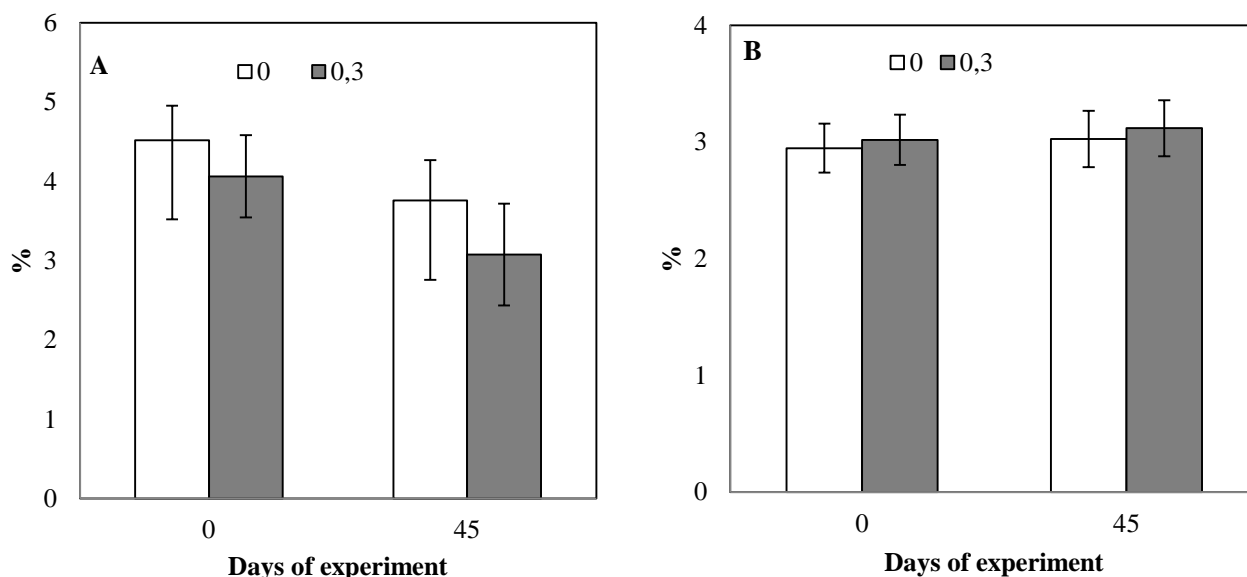


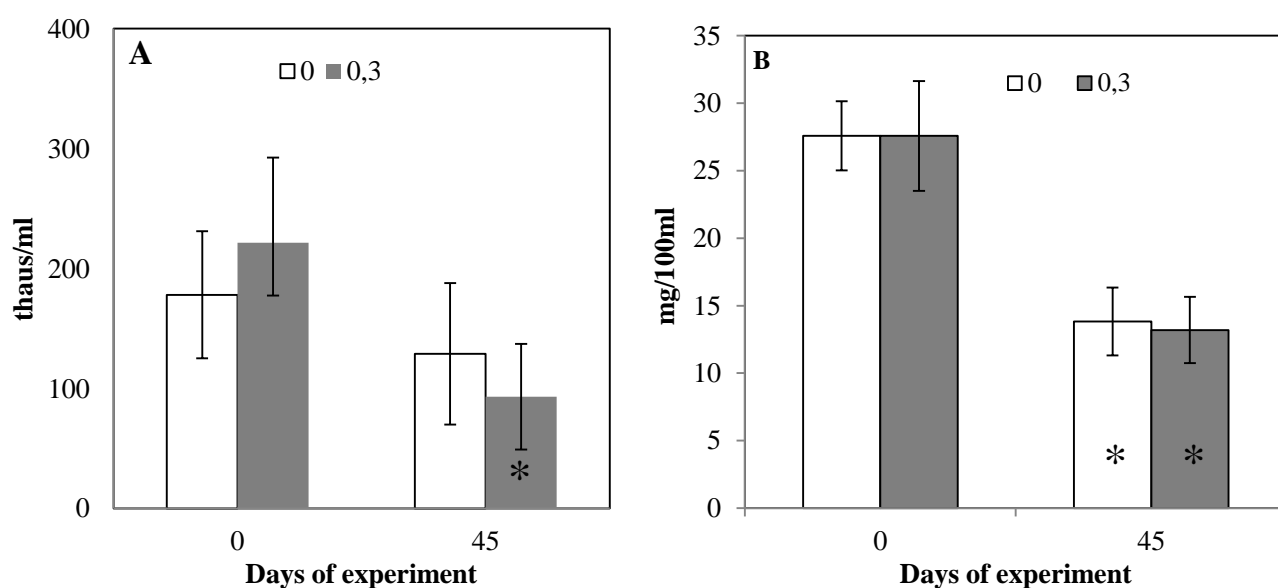
Figure 2 Effect of selenium feeding on the content of fat (A), protein (B) in dairy cows in ecological farming.

The fat decrease occurred in both groups in the experimental (by 17%) and control (by 24%) during the observation. The protein in the milk of the experimental and control groups of cows was practically in the identical level throughout the experiment. The fat and protein values in the milk of dairy cows can be seen from the Figures 2A, 2B.

The amount of somatic cells was significantly reduced in the test group of dairy cows by 58% ($p < 0.05$). The decrease of the number of somatic cells was also observed in the control group (by 28%). The urea concentration was significantly decreased in the experimental and control groups by 52% ($p < 0.05$) respectively 50% ($p < 0.05$). The values of somatic cells and urea in milk are shown in Figures 3A, 3B.

DISCUSSION

The experiment was focused on the feeding of organic selenium ($0.3 \text{ mg} \cdot \text{kg}^{-1}$ of diet) in the ecological farming of dairy cows in order to increase the element in cow milk (the production of functional food with higher selenium level). We also studied the quantitative and qualitative indicators of the produced milk. In the experiment, in which the selenium of the same amount ($0.3 \text{ mg} \cdot \text{kg}^{-1}$ of diet) was fed by dairy cows similarly to our observation, was higher selenium level of the serum (Hall et al., 2014). The dose of $0.278 \text{ mg Se} \cdot \text{kg}^{-1}$ of diet affected neither the performance of dairy cows nor selenium concentration in milk. No significant differences were observed in the content of lactose and protein. Conversely, the increased milk fat, the reduction of somatic cells in the milk and the



* = statistical significance $p \leq 0.05$ between Day 0 and Day 45 of the experiment

Figure 3: Effect of selenium feeding on the amount of somatic cells (A), urea (B) in dairy cows in ecological farming.

occurrence of mastitis were occurred (Oltramari et al., 2014). In our observation, no significant increase was detected in milk fat content. However, the decrease of somatic cells in cow group treated with selenium was found out as in the previous study. It may suggest that selenium may prove the anti-inflammatory effect from this point of view associated with a lower occurrence of mastitis. The concentration of selenium was effected in this case contrary to the results according to Oltramari et al. (2014). Conversely, the milk yield and protein content are in accordance with the above mentioned authors. Selenium with vitamin E was fed to other groups of cows. A higher synergistic effect was expected because of shared action. Selenium and vitamin E were applied in the injection (three weeks before the birth). In terms of selenium concentration in the animal blood, no difference was found out between oral and injection application (Kafilzadeh et al., 2014). From this viewpoint, it is possible to take the selenium injection as a possible alternative to its application. In another experiment, the selenium was given in a single injection (48.4 mg of selenium for 21 days before birth). After the birth, the decrease of mastitis (by 13%) and somatic cell count in milk was observed in the experimental group (Zigo et al., 2014). As stated in other studies, selenium-enriched milk can be an alternative source of selenium in human nutrition (as a functional food that has a positive effect on the consumer health). We tested two forms of selenium (inorganic and organic). The use of inorganic form may cause an increase in milk content from 13.3 to 17.6 to 19.7 mg.L⁻¹. and the application of organic selenium (selenomethionine) was increased from 13.9 to 24.6 to 54.8 mg.L⁻¹. In both cases, the dose was of 0.4 mg selenium /kg per diet (Meyer et al., 2014). In our case, selenium increase was observed from 13 to 15 µg.L⁻¹ after the feeding of organic form. Because of taking lower selenium dose by 0.1 mg than Mayer et al (2014) did, the selenium increase was not so significant. The control group of cows without the selenium addition showed the levels below the limit detection. It is not identified whether the selenium addition in the diet of cows can increase the milk production and quality. In the experimental observation lasted for 60 days (in this case for 30 days), the dose of 0.3 mg selenium /kg of diet was given to dairy cows in the organic or inorganic form. The performance of the animals was not affected. During evaluation of dairy ingredients (percentage of fat, protein and lactose) no significant differences were also observed. In the assessment, the antioxidant parameters in blood of dairy cows were observed in a higher activity of antioxidant enzymes (catalase and GPx) in a group of cows feeding an organic source of selenium. Selenium was also significantly higher in milk during using of selenomethionine (Gong et al., 2014). In our experiment, we have managed to confirm the results. On the other hand, it must be said that our aim was not to compare the two forms of selenium (organic or inorganic). Our objective was to focus on selenomethionu applications. During the use of organic selenium, neither the milk production nor affecting of milk components were observed. The antioxidant capacity of milk was not measured because of low quantitation of the antioxidant

enzymes. Selenium is still rare element in cattle feed doses. Its deficiency may cause not only a reduction of antioxidant potential but mainly getting lower the reproductive performance. This trend is widespread throughout central Europe (Balicka-Ramisz and Jastrzębski, 2014). The study (Pilarczyk et al., 2011) was devoted to the monitoring of the concentration of selenium in dairy cows from the ecological and conventional farming. In both farming methods, high variability was found out. In ecological farming, the average level of selenium was up to 0.016 µg.mL⁻¹ in milk. In conventional farming, the average level of selenium was at the level of 0.005 µg.mL⁻¹ in milk. As the authors stated, the deciding factor of selenium content is the nutrition in cow milk. Our study only monitors the different methods of cow farming.

CONCLUSION

In an experimental monitoring, the effect of organic selenium addition on its concentration, quantitative and qualitative parameters of milk in the diet of dairy cows in an ecological farming was studied. The selenium addition at a dose of change to 0.3 mg.kg⁻¹ of the diet increased the concentration of selenium in the milk and reduced the number of somatic cells ($p < 0.05$). Other components of milk (protein, fat, lactose, and urea) and milk yield were not affected. From these results, it is obvious that the selenium addition to the diet of cows increases the concentration of essential micronutrients in milk (production of functional food) and reduces the production of somatic cells, which are indicators for the inflammation of the mammary gland.

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THE OCCURENCE OF RISK FACTORS OF CARDIOVASCULAR DISEASES AND THE EFFECT OF SELECTED DIETARY HABITS ON THE LIPID PROFILE AND BODY MASS INDEX

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ABSTRACT

In a group of 204 randomly selected patients hospitalized in the Cardiocentre Nitra, of which 63 were women (30.88%) and 141 men (69.12%), we evaluated the prevalence of modifiable risk factors for cardiovascular diseases and the impact of dietary habits on the lipid profile and body mass index (BMI). We have recorded a high prevalence of risk factors, especially overweight and obesity, where 87.3% of women and 92.91% of men had BMI ≥ 25 . Normal weight was observed only in 12.70% of women and in 7.09% of men. In the study group up to 60.32% of women and 57.45% of men had blood pressure higher than $\geq 130/85$ mmHg. More than half of the respondents were simultaneously overweighted or obese together with high blood pressure occurrence. The total cholesterol level higher than 5.2 mmol/L was recorded in 41.24% of women and 34.75% of men. There was statistically significant difference between men and women ($p < 0.05$) in the prevalence of low HDL cholesterol to the detriment of men while the value below 1.3 mmol/L was recorded in 31.75% of women and the value lower than 1.1 mmol/L in 52.48 % of men. Values of triglycerides (TG) ≥ 1.7 mmol/L were recorded in 28.57% of women and in 35.42% of men. Fasting blood glucose levels ≥ 5.6 mmol/L were recorded in up to 68.25% of women and 71.63% of men. There was not statistically significant difference ($P > 0.05$) in the occurrence of increased levels of cholesterol, triglycerides, blood pressure and glycemia according to gender. We found out that most of the respondents consumed food 3-4 times per day, i.e. 53.97% of women and 60.99% of men. Food intake for five to six times a day was reported only by 28.57% of women and 19.15% of men. The number of daily meals was significantly reflected in the BMI values in men who consumed food 1-2 times a day compared to the men who ate 3-4 meals daily ($p < 0.001$). We detected lower BMI values in women with more frequent food consumption, however the difference was not statistically significant. While assessing the nutritional history, we have recorded frequent consumption of meat and meat products which are consumed by 49.21% of women and 60.28% of men ($p < 0.001$) more than four times a week, while more than 30% of women and men consume them daily. On the contrary, women consume fish more often, while 53.97% of women and 48.23% of men consume it 1 to 2 times a week. Although fruit and vegetable are part of daily diet of almost all patients, it is insufficient in portions of one or two pieces a day compared with dietary recommendations. We noticed significantly higher BMI ($p < 0.05$) in men who consume sweetened beverages, than men who consume mostly non-sweetened beverages.

Keywords: cardiovascular disease; risk factor; dietary habits; lipid profile; body mass index

INTRODUCTION

Cardiovascular diseases (CVD), including coronary heart disease (CHD) and stroke, currently represent the major causes of mortality and morbidity all over the world. In Europe, CVD are responsible for 43% of deaths in men and 55% in women and for 30% of all deaths before the age of 65 years. Eighty percent of cardiovascular accidents could probably be avoided by lifestyle adjustment (weight control, smoking abstinence, physical activity, and a healthy diet), together with proper management of clinical and biological risk factors (Carpentier and Komsa-Penkova, 2011).

The major typical feature of CVD is the atherosclerotic plaque, an inflammatory lesion which develops insidiously around cholesterol depots in the intima of the arterial wall

over many (20-30) years (Peters et al., 2011). Cholesterol deposition and development of inflammatory lesions are prevented by high-density lipoproteins (HDL) which ensure a reverse cholesterol transport to the liver (and glands producing steroid-derived hormones) and reduce inflammatory and peroxidative reactions. It is important to understand that endothelial dysfunction and inflammatory reactions may be corrected by appropriate lifestyle and therapeutic measures, causing the atherosclerotic lesion to be reversed, modified or stabilised. As a corollary, the vast majority of CVD accidents can and should be prevented (Carpentier and Komsa-Penkova, 2011). For a long time plasma cholesterol concentration has been considered as the major (if not the only one) risk factor for CVD (Ferrieres, 2004). The major responsible factors for CVD

are the higher levels of low density lipoprotein cholesterol (LDL) and lower levels of the high density lipoprotein cholesterol (HDL) present in blood plasma (Fruchart and Duriez, 2002). Since cholesterol deposition in the arterial wall is a key and early step in the initiation of the atherosclerotic process, decreasing the number of LDL particles (and/or avoiding their retention in the intimal space) has been a primary target (Brunzell et al., 2008). Attention is currently paid not only to decreasing LDL cholesterol, but also to increasing the number of HDL particles; this can be achieved by lifestyle adjustments: weight control, physical activity, no-smoking and proper nutrition. A high concentration of plasma triglycerides is recognised as a direct risk factor (increased coagulation, impaired endothelial function, but may also have indirect effects by decreasing HDL level and increasing LDL atherogenicity via formation of atherogenic small dense LDL.

The American Heart Association (AHA) recently defined a construct of "ideal cardiovascular health" comprising 7 health metrics: no smoking, engaging in sufficient physical activity, consuming a healthy diet, maintaining a normal body mass index (BMI), and having optimal levels of total cholesterol, blood pressure, and fasting blood glucose (Lloyd-Jones et al., 2010). The more of these risk factors a person has, the more it runs the risk of development of CVD. The risk, of course, also increases with increasing intensity of a specific risk factor. Specific treatment for individual risk factors of atherosclerosis is different, but the principles of treatment are the most common of them. The basic treatment of most risk factors is suitable diet regime and compliance measure (Whitney-Rolfes 2002).

It is widely recognized that diet plays a critical role in the development of CVD. While foods from animal sources contain important nutrients that may not be readily available from plant sources, high consumption of red meats has been shown in association with greater risk of morbidity and mortality from CVD. Such diets may contain high levels of total and saturated fats and cholesterol. In addition, accumulated evidence supports that fish or sea food consumption may have cardioprotective effects. Long-chain omega-3 polyunsaturated fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are suggested to be the key nutrients responsible for the observed benefits of fish consumption on CVD. Numerous studies have demonstrated the benefits of diets high in fruits, vegetables, whole grains, and fish, and low in red meat, high-fat dairy products, and trans and saturated fats (Davíglus and Pirzada, 2008). High consumption of fruit and vegetables is likely to reduce hypertension, coronary heart disease and cancer. Furthermore a high consumption of milk and dairy products, is negatively associated with all-cause deaths, ischaemic heart disease, stroke and incidence of diabetes (Boeing et al., 2012; Sayon-Orea et al., 2013). Whereas, the increased consumption of pre-prepared, fatty, and salty food is known for promotion cardiovascular risk factors and therefore should be avoided (Perk et al., 2012). Healthy lifestyle, including healthy diet, is the best strategy for prevention of CVD and other

diseases (Davíglus and Pirzada, 2008; Osler, 2002).

MATERIAL AND METHODOLOGY

We evaluated the prevalence of modifiable risk factors for cardiovascular disease (CVD) and the relationship between dietary habits and lipid profile and BMI in a group of 204 randomly selected patients hospitalized in the Cardiocentre Nitra, of who 63 were women (30.88%) and 141 men (69.12%). Respondents were 25 – 89 years old, where the average age of women was 65.40 ± 10.59 years, and the average age of men was 61.04 ± 11.04 years. Most of the respondents (65.08% of women and 62.41% of men) were 56 – 75 years old. Selected respondents have either overcome the myocardial infarction or were diagnosed with angina pectoris and hospitalized after a procedure so called catheterization. The largest proportion of respondents was hospitalized due to the myocardial infarction, i.e. 50.79% of women and 56.74% of men. The data necessary for detection of the presence of modifiable risk factors for CVD and dietary habits were obtained by a questionnaire method through guided conversation. The questionnaire was anonymous, its completion was voluntary with only one response to be circled for each question. Data collection was carried out simultaneously with somatometric and biochemical examination of the respondents ensured by the Cardiocentre Nitra. The following parameters were evaluated: total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides and blood glucose, because these parameters are considered to be one of the major risk factors for cardiovascular diseases. We used the diagnostic criteria of the metabolic syndrome (MS) according to ATP III criteria for the evaluation of the risk factors, as the MS brings together components that are associated with the risk of CVD, in particular, with the risk of coronary heart disease. Given the close correlation of parameters of total and central obesity in our population, the positive criteria besides the waist perimeter are considered to be also the values of $BMI \geq 25 \text{ kg.m}^{-2}$. The presence of risk determinants was set as follows: $BMI \geq 25 \text{ kg.m}^{-2}$, triglycerides $\geq 1.7 \text{ mmol/L}$, HDL cholesterol $< 1.0 \text{ mmol/L}$ in men and $< 1.3 \text{ mmol/L}$ in women, blood pressure $\geq 130/85$, fasting blood glucose $\geq 5.6 \text{ mmol/L}$. We also followed a total cholesterol level $\geq 5.2 \text{ mmol/L}$. The results were evaluated with appropriate standard mathematical-statistical methods and were listed in the tables. We used the program STATISTICA Cz version 10 belonging to the available statistical programs and MS Excel 2007 and the following tests: Tukey test and χ^2 test.

RESULTS AND DISCUSSION

Table 1 shows the average values of biochemical markers and BMI. The average values were higher in men than in women with the exception of triglycerides and BMI.

There are more than 300 known risk factors associated with coronary heart disease and the risk of stroke. Risk factors for CVD are important in all population groups, while one third of all CVD is connected with five risk factors in developed countries.

Table 1 Average values of selected biochemical markers and BMI.

parameter	women	men
Glucose (mmol/L)		
average ±SD	7.51 ±3.13	7.44 ±3.10
min	4.36	4.26
max	19.88	24.12
Total cholesterol (mmol/L)		
average ±SD	5.19 ±1.48	4.78 ±1.39
min	2.05	1.99
max	8.97	9.21
HDL cholesterol (mmol/L)		
average ±SD	1.47 ±0.42	1.12 ±0.40
min	0.57	0.54
max	2.6	4.12
LDL cholesterol (mmol/L)		
average ±SD	2.96 ±1.10	2.96 ±1.08
min	0.93	1.03
max	5.49	7.19
Triglycerides (mmol/L)		
average ±SD	1.34 ±0.61	1.62 ±1.06
min	0.35	0.45
max	2.79	7.39
BMI (kg.m⁻²)		
average ±SD	28.48 ±3.32	30.13 ±4.57
min	20.96	21.95
max	34.13	50.50

According to the numerous studies, it was determined that the most important risk factors contributing to the higher frequency of coronary heart disease could be elevated cholesterol level, hypertension, smoking, alcohol and stress (Kamenský, 2007).

Table 2 presents the prevalence of selected risk factors of cardiovascular disease in the study group.

The most frequent risk determinants among the respondents were overweight and obesity, when BMI ≥25 was recorded in 87.30% of women and 92.91% of men. Normal weight was observed in only 12.70% of women

and 7.09% of men. High BMI values are associated with the development of cardiovascular risk factors such as hypertension, dyslipidemia, insulin resistance and diabetes mellitus which resulting in cardiovascular diseases such as coronary heart disease and ischemic stroke (Wilkins et al., 2010; Wormser et al., 2011). The development of these co-morbidities in proportion to BMI and obesity is considered as an independent risk factor for cardiovascular diseases (Poirier et al., 2006). Several studies demonstrated that high BMI was demonstrably associated in men and women with the manifestation of

Table 2 The occurrence of selected risk factors of cardiovascular diseases (%).

Risk factor	women	men	p
BMI ≥25 kg.m ⁻²	87.30	92.91	0.1909
Blood pressure ≥130/85 mmHg	60.32	57.45	0.7008
Cholesterol ≥5.2 mmol/L	41.24	34.75	0.3723
HDL cholesterol F <1,3 mmol/L, M <1.0 mmol/L	31.75	52.48	0.0060
Triglycerides ≥1,7 mmol/L	28.57	35.42	0.3348
Fasting blood glucose ≥5.6 mmol/L	68.25	71.63	0.7005

cardiovascular diseases such as angina pectoris, myocardial infarction, heart failure and sudden death (Bastien et al., 2014). People with BMI of 25 to 28.9 have two times higher relative risk of cardiovascular diseases than people with BMI <21, while those with BMI >29 have a three-fold risk (Peeters et al., 2003). Hypertension is a clinical consequence of obesity, with all its well-known consequences (especially left ventricular hypertrophy, development of heart failure and coronary heart disease) (Huang, 1998), which was also confirmed by our results. Up to 60.32% of women and 57.45% of men in the study group had blood pressure higher than $\geq 130/85$ mmHg. Blood pressure of 120/80 mmHg was recorded in 25.40% of women and 28.37% of men. More than half of the respondents was simultaneously overweighted or obese with high blood pressure (53.19% of women and 53.97% of men). The relationship between obesity and hypertension is clear: BMI increase of 3 kg.m^{-2} increases systolic blood pressure for 2 mmHg. The incidence of hypertension is four times higher in individuals with overweight, and in obese ones even six to eight times higher than in individuals with normal weight (Dixon and Brian, 2002). Several studies have confirmed that the reduction of the weight in an average of 3.8 kg lowered systolic blood pressure by 2.9 mmHg and diastolic blood pressure by 2.3 mmHg in 18 months (Jones et al., 1999).

The cholesterol level higher than 5.2 mmol/L was recorded in 41.24% of women and 34.75% of men. There was statistically significant difference in the prevalence of low HDL cholesterol between men and women ($P < 0.05$) to the detriment of men, with a value lower than 1.3 mmol/L recorded in 31.75% of women and the value lower than 1.1 mmol/L recorded in 52.48% of men. The triglycerides ≥ 1.7 mmol/L were recorded in 28.57% of women and 35.42% of men and fasting blood sugar levels ≥ 5.6 mmol/L were recorded in 68.25% of women and 71.63% of men. There was no statistically significant difference ($p > 0.05$) in the occurrence of elevated levels of cholesterol, triglycerides, blood pressure and blood glucose between both genders.

The combination of several factors, high cholesterol, hypertension and smoking markedly enhances the risk of cardiovascular accidents (Carpentier and Komsa-Penkova, 2011). Simultaneous occurrence of two of risk factors such as BMI ≥ 25 with impaired fasting glucose levels ≥ 5.6 mmol/L was present in 47.62% of women and 68.08% of men. Up to 36.68% of women and 39.72% of men had three risk factors present (BMI ≥ 25 , fasting blood glucose ≥ 5.6 mmol/L, blood pressure $\geq 130/85$ mmHg). Almost 20% of the respondents (19.05% of women and 17.02% of men) had even four risk factors present (BMI ≥ 25 , fasting blood glucose ≥ 5.6 mmol/L, blood pressure $\geq 130/85$ mmHg, cholesterol ≥ 5.2 mmol/L).

Eating habits and their impact on lipid profile and BMI

During the twentieth century, modern technologies had increased food production (through industrialization of agriculture and food processing) and shelf life (through preservation and transport) in developed countries. These changes led to the oversupply of food, as well as to the decline in food prices, which consequently led to a change

in the traditional healthy eating habits (Golzarand et al., 2012). The resulting increase in consumption of meat, dairy products, and industrially prepared meals caused a high-energy nutrition rich in protein, sugar and fat, which enhances the occurrence of cardiovascular diseases, as well as the diet-dependent risk factors such as obesity, hypercholesterolemia and diabetes (Popkin, 2014).

Distribution of all-day energy intake into at least five meals is an appropriate measure in the prevention of lipid metabolism disorders and obesity, since it has been recorded that people with low quantity of food doses per day had increased levels of total blood cholesterol, higher prevalence of obesity and CVD (Jurkovičová et al., 2010), which was also confirmed by our results. We have recorded higher mean total cholesterol levels in men and women who consumed less daily meals, however the difference was not statistically significant. We found out that most respondents consumed food 3-4 times a day, i.e. 53.97% of women and 60.99% of men. Five to six daily food intakes were reported only by 28.57% of women and 19.15% of men. We detected statistically insignificant differences in the influence of the number of daily meals on HDL cholesterol, which was higher in women as well as men who consumed 3 – 4 meals compared to 1 – 2 daily meals. The number of daily meals is significantly reflected in the values of BMI in men who consumed 1 – 2 times a day (BMI = 33.36 kg.m^{-2}) compared with men who ate 5-6 meals daily (BMI = 30.19 kg.m^{-2}) ($p < 0.05$), we have recorded lower BMI values in women with more frequent food consumption, but the difference was not statistically significant. Neither Keller et al. (2014) have recorded the impact of dietary habits on BMI. Table 3 shows the consumption of selected food types and Table 4 shows the effect of the frequency of consumption of selected foods on lipid profile and BMI.

With the exemption of the impact of vegetables consumption on LDL cholesterol level in women, the different frequency of consumption of monitored food did not show to be statistically significant for the levels of lipid profile and BMI both in women and men. Consumption of meat more than four times per week was recorded in 49.21% of women and 60.28% of men, while more than 30% of women and men eat meat daily. Respondents indicated poultry and pork as the most commonly consumed meat. Both men and women had insignificantly higher values of HDL for the less frequent consumption, while the values of triglycerides and LDL cholesterol were higher for more frequent consumption. BMI values in men were insignificantly higher for daily consumption and higher for women with less frequent consumption. The interesting fact is that women consume meat products more often than men, about 66.67% of women and only 55.32% of men consume them several times per week ($p < 0.001$). In our group of respondents the consumption of fish for 1-2 times a week was recorded in only 53.97% of women and 48.23% of men. Alarming finding is that up to 34.92% of women and 48.23% of men consume it only one to two times per month. Different frequency of fish consumption in men nor women did not show to be statistically significant for the lipid profile, neither for the BMI values.

Table 3 Consumption of selected food types (%).

	women	men	<i>p</i>
Meat ¹	49.21	60.28	0.0000
Meat products ²	66.67	55.32	0.0005
Fish ³	53.97	48.23	0.0006
Milk and dairy products ⁴	46.03	51.77	0.0908
Fruit ⁴	61.90	70.21	0.0010
Vegetable ⁴	57.14	62.59	0.0057
Sweets ⁴	28.81	35.25	0.0046

¹- consumption more than four times a week, ² – consumption several times a week, ³-consumption one to two times a week, ⁴ – daily consumption

Our research showed, that almost half of women (46.03%) and 36.14% of men consume semi-skimmed milk and, surprisingly, up to 32% of men do not consume it at all. The daily consumption of milk and milk products was recorded in 46.03% of women and 51.77% of men (*p* >0.05). Milk type neither different frequency of its consumption did not affect the lipid parameters nor the BMI values. The levels of total and LDL cholesterol were higher at the daily consumption of milk and dairy products, while the TG and BMI values were higher for the less frequent consumption.

Fruit and vegetables consumption is associated with a reduction in the incidence and mortality of various chronic diseases: cardiovascular diseases, stroke, hypertension, diabetes, obesity, and certain types of cancer (Bazzano,

2006). An interesting finding is that more men than women consume fruit and vegetables daily (*p* <0.05), which is inconsistent with the results of Pérez (2002), who states higher consumption among women. Regarding the amount of fruit consumed per day, the amount was comparable between the sexes. More than a half of the respondents consumes 1 – 2 pieces of fruit per day (55.56% of women and 57.45% of men). Similarly, the consumption of 3 – 4 pieces a day was about the same in men and women (15.87% of women and 19.14% of men).

Several studies (Serdula et al., 1996; Subar et al., 1990; Bazzano, 2002) state more frequent consumption of fruit and vegetables in people with normal weight compared to the obese ones. Women who eat fruit daily had statistically

Table 4 Effect of the frequency of consumption of selected foods on lipid profile and BMI.

Frequency of consumption	Total cholesterol mmol/L		LDL cholesterol mmol/L		HDL mmol/L		TG mmol/L		BMI kg/m ²	
	women	men	women	men	women	men	women	men	women	men
Meat and meat products										
daily	5.35	4.92	3.07	3.10	1.28	1.06	1.47	1.98	27.03	30.26
3x/week	5.16	4.78	3.04	3.08	1.53	1.09	1.27	1.51	29.17	30.59
Fish										
1-2x/week	5.03	4.80	3.09	3.04	1.46	1.15	1.38	1.62	28.90	29.54
1-2x/month	5.09	4.85	3.05	3.14	1.47	1.09	1.26	1.63	28.04	31.02
Milk and dairy products										
daily	5.27	4.91	3.07	2.89	1.45	1.09	1.41	1.61	28.12	29.70
not daily	5.11	4.67	2.89	2.91	1.50	1.08	1.42	1.66	28.92	30.77
Fruit										
daily	5.11	4.80	2.89	2.88	1.46	1.09	1.46	1.51	28.32	30.32
not daily	5.31	4.74	3.17	2.98	1.49	1.10	1.32	1.90	28.93	29.69
Vegetable										
daily	4.87	4.78	2.64	2.86	1.42	1.07	1.47	1.51	28.17	30.50
not daily	5.57	4.85	3.47	3.04	1.53	1.12	1.31	1.85	29.02	29.56
Sweets										
daily	5.46	5.07	3.27	3.05	1.45	1.11	1.16	1.76	27.14	30.07
not daily	5.12	4.67	2.93	2.86	1.46	1.08	1.53	1.57	29.04	30.20

insignificantly lower BMI values compared with women who consumed it less often. The difference between men and women was also recorded in the consumption of vegetables, which is consumed in the amount of 1 – 2 pieces a day by 44.44% of women and 58.28% of men. Consumption of vegetables in a higher amount (3 – 4 pieces a day) was observed in 23.81% of women and 12.95% of men. Negative finding is that up to 30.16% of women and 28.78% of men do not consume vegetables every day. We have detected a significant effect of the consumption of vegetables on LDL cholesterol in favor of daily consumption ($p < 0.05$) in women. Women and men who consumed fruits and vegetables daily had statistically significantly lower levels of HDL. Considering the number of fruits and vegetables consumed daily, men and women who consumed 3 – 4 pieces per day had statistically insignificantly higher level of HDL than those who consumed only 1 – 2 pieces per day.

The excessive consumption of simple sugars - particularly in form of sweetened beverages has become an enormous problem of the past decades. Numerous meta-analyses have shown that a decreased consumption of added sugar significantly reduces body weight, while the increased intake of sugar leads to the increase in weight (Hu, 2013). We found significantly higher BMI ($p < 0.05$) in men who consume sweetened beverages compared with those who consume the unsweetened ones. The consumption of sweets did not have significant effect on BMI values in men neither in women, which were lower for less frequent consumption in both cases.

CONCLUSION

Through the research carried out, we recorded high incidence of risk factors, in particular overweight and obesity, high blood pressure and high prevalence of hypercholesterolemia and hypertriglyceridemia. More than a half of the respondents was simultaneously overweighted or obese with high blood pressure.

Nutritional factors which have a correlation with the occurrence of cardiovascular diseases include, in particular, the consumption of inappropriately selected fats with over-representation of saturated fatty acids, a lack of the protective agents, e.g. unsaturated fatty acids, fiber, vitamins and antioxidants. When assessing the nutritional history we have recorded frequent consumption of meat and meat products, insufficient consumption of fish and milk. Although fruits and vegetables are a part of daily diet of almost all patients, it is insufficient in amount of one or two pieces per day compared with the dietary recommendations.

We detected statistically non-significant differences in the effect of number of daily meals on HDL cholesterol value, which was higher in women and men who consumed 3-4 meals compared to 1 – 2 meals per day. The number of daily meals was statistically significantly reflected in the higher BMI in men who ate 1-2 times a day (BMI = 33.36 kg.m⁻²) compared with men who ate 5-6 meals per day (BMI = 30.19 kg.m⁻²) ($p < 0.05$). We found lower BMI values in women with more frequent consumption of food. With the exception of the impact of vegetables consumption on lower LDL cholesterol levels in women, different frequency of monitored food

consumption did not show to be statistically significant for the level of lipid profile and BMI in women neither in men.

Cardiovascular prevention should be a lifelong endeavour and more emphasis should be given to the behavioral aspects of prevention and life-long healthy life style adherence. It is therefore necessary to emphasize that without the lifestyle changes and without the improvements in control of hypertension, hyperlipidemia and diabetes, the reduction of mortality from cardiovascular diseases can not be expected

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THE IMPACT OF CARROT JUICES ON INTESTINAL AND PROBIOTIC BACTERIA

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ABSTRACT

There is a growing interest in non-dairy probiotic products. The main aim of the study was to evaluate the impact of juice prepared from 15 various cultivars of carrot on the growth of representatives of human intestinal microbiota (*Bifidobacterium catenulatum*, *Escherichia coli*) and probiotic strains (*Lactobacillus acidophilus* LA-5, *Lactobacillus casei* 01). Carrot juice was added to liquid medium at a final concentration of 5.0% and their impact on the bacteria number was assessed by measurement of the turbidity after 24 h of culture. The number of cells was expressed as % of positive control (medium without juice addition). Juices prepared from all tested cultivars of carrot inhibited the growth of *Bifidobacterium catenulatum*, and the strongest inhibitory effect was observed for juices obtained from the 'Kongo F1' cultivar (3.40 ± 2.85% of positive control), 'Rumba F1' (4.17 ± 2.27%) and 'Broker F1' (5.35 ± 2.14%). The majority of tested juices also inhibited the growth of *E. coli*, but those prepared from the 'Niland F1', 'Napa F1', 'Afro F1' and 'Samba F1' cultivars stimulated the growth of this bacterium. The probiotic strains were less sensitive to carrot juice impact than intestinal species, however both stimulation and inhibition could be observed. Juices made from the cultivars 'Kongo F1' and 'Deep Purple F1' acted negatively on the growth of both probiotic strains, while juice from 'Bangor F1' cultivar inhibited *L. casei* 01 growth, but stimulated the growth of LA-5. The obtained results suggest that 'Kongo F1' and 'Deep Purple F1' cultivars are not suitable as an additive or raw material for the production of probiotic products, because of their inhibitory properties against probiotic strains. Concluding, carrots can be used as raw material for the production of probiotic beverages, however both the cultivar of carrot and the strains of probiotic bacteria used for the production should be selected carefully. The most suitable for production of probiotic beverages seems to be the 'Rumba F1', followed by the 'Polka F1' and 'Yellowstone F1' cultivars. They inhibited the growth of intestinal bacteria, which are undesirable in the final product, without negative effect on the probiotic species.

Keywords: carrot juice; antibacterial activity; intestinal microbiota; probiotic

INTRODUCTION

Generally, there are two basic groups of carrots: with orange roots (*Daucus carota* ssp. *Sativus* var. *Sativus*) and purple-violet (*Daucus carota* ssp. *Sativus* var. *Atrorubens*); but varieties of yellow and white root are also known. Various cultivars differ in chemical composition, shape, and size of root, the length of the growing season, the storage stability and application. The most visible difference is associated with the root color and it is caused by the presence of high content of carotenoids in orange varieties, while in purple ones xanthophylls dominate (Alasalvar et al., 2001; Gajewski et al., 2007; Majkowska-Gadomska and Wierzbicka 2010; Mech-Nowak et al., 2012).

The energy value of 100 g edible parts of carrots is only 27 kcal; moreover, a carrot is easy to digest, which makes it a valuable component of many diets, including for infants, convalescents, and people wanting to lose weight. Additionally, carrots are rich in antioxidants, both of a lipophilic fraction (carotenoids) as well as hydrophilic

(phenolic compounds) (Gajewski et al., 2007; Leja et al., 2010; Mech-Nowak et al., 2012).

Poland is the second European producer of carrots. In 2011, the domestic production has been estimated at about 900 thousands of tons. Carrot is a vegetable available almost all year round and can be eaten raw in the form of salads, cremogenes or juices. It is also suitable for cooking and freezing.

Owing to its taste, color and nutritional value is increasingly fashionable to use different cultivars of carrots for production of fresh unpasteurized carrot juices or as an ingredient in juice multi-vegetable juices. The more that the diversity of cultivars with different root color (from white through orange to the almost black) allows for a wide selection and enables to create interesting compositions. Some producers try also to introduce vegetable juices with added probiotic bacteria, into the market (Nazzaro et al., 2008). The rich composition of carrots is certainly valuable to the consumer organism, but it can have negative effects on microorganisms added. It was proved that some polyphenols can inhibit the growth

of intestinal bacteria (Duda-Chodak, 2012) and probiotics, such as *Lactobacillus casei* (Duda-Chodak et al., 2008). The effect of carrot juice on the probiotic strains of bacteria or bacteria that reside in the human intestine has not been studied yet.

The aim of the study was to evaluate the impact of juices prepared from 15 cultivars of carrot on the growth of selected species of intestinal and probiotic bacteria.

MATERIAL AND METHODOLOGY

Material

Fifteen varieties of carrot (*Daucus carota* L.) with different colors of their edible root were selected for the study: 'Samba F1', 'Salsa F1', 'Polka F1', 'Rumba F1', 'Afro F1', 'Korund F1', 'Broker F1', 'Kongo F1', 'Niland F1', 'Nerac F1', 'Bangor F1', 'Napa F1' (all have orange root), 'Deep Purple F1' (purple root), 'Yellowstone F1' (yellow root), 'White Satine F1' (white root). All cultivars were obtained from the Experimental Station of the University of Agriculture in Krakow, located in Mydlniki, Poland.

Bacterial culture

Bifidobacterium catenulatum (DSM 16992) /BF/ and *Escherichia coli* (DSM 1116) /EC/ were used as the representatives of intestinal microbiota, and were purchased from Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ Germany). The probiotic strains *Lactobacillus acidophilus* LA-5 /LA5/ and *Lactobacillus casei* 01 /LC01/ were obtained from Christian Hansen (Denmark).

Bacteria were grown anaerobically at 37°C in media specially designed for particular microorganisms. The medium for *Bifidobacterium* contained (per 1 L): peptone from casein, pancreatic digest (10.00 g), yeast extract (5.00 g), beef extract (5.00 g), soy peptone (5.00 g), glucose (10.00 g), K₂HPO₄ (2.00 g), MgSO₄ × 7 H₂O (0.20 g), MnSO₄ × H₂O (0.05 g), Tween 80 (1.00 mL), NaCl (5.00 g), cysteine-HCl × H₂O (0.50 g), resazurin (1 mg), and 40 mL salt solution. Composition of salt solution (per 1 L): 0.25 g CaCl₂ × 2 H₂O, 0.50 g MgSO₄ × 7 H₂O, 1.00 g K₂HPO₄, 1.00 g KH₂PO₄, 10.00 g NaHCO₃, 2.00 g NaCl. For lactic acid bacteria MRS Broth was used, while a Nutrient Broth was used for *E. coli* (both from Biocorp, Poland).

Preparation of carrot juice

The raw material for the preparation of the juice were thoroughly washed roots of appropriate carrot cultivar from which inedible parts were removed. The juice (ap. 1 L from each cultivar) was prepared with the use of a juicer. Then the juice was centrifuged at 14 000 rev./min. and the clear supernatant obtained after centrifugation was separated, frozen and stored (at -20°C) as such until the experiments.

The impact of carrot juice on the growth of bacteria

The impact of the carrot juices on bacteria was assessed in liquid medium inoculated with the tested bacteria (3×10^6 /mL) with or without 5% of carrot juice. Biomass

of bacterial cultures after 24 h of incubation at 37°C was assessed nephelometrically by measuring the turbidity in McFarland scale (densitometer DEN-1B, Biosan), which then enabled to calculate cells number.

As controls the number of bacteria in medium without carrot juice (positive control), and turbidity of empty medium without bacteria (double blank) was determined. As the addition of carrot juices (especially those from dark cultivars) to different media changed the medium color and turbidity, blank samples (medium with appropriate concentration of juice without bacteria) were also prepared. The value obtained for the blank was each time subtracted from the value obtained for the sample turbidity, taking into account the kind of medium.

The obtained results were expressed as % of positive control in order to facilitate the comparison between different bacteria species.

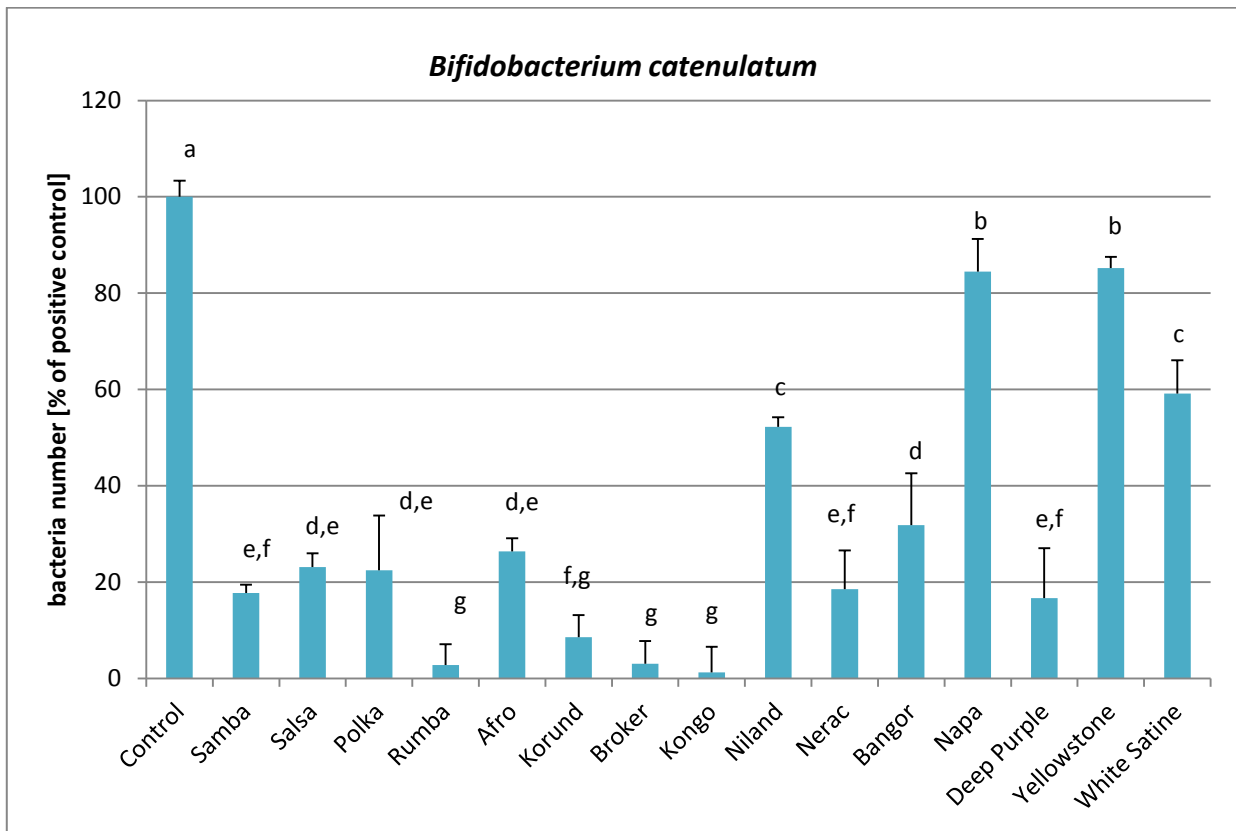
All determinations were performed at minimum three replications, and the results were presented as arithmetic mean ± SD. The ANOVA with a *post hoc* Tukey test was applied to perform statistical analysis (GraphPad InStat version 3.01 for Windows, GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

Analysis of the impact of carrot juice to *Bifidobacterium catenulatum* showed that all 15 cultivars inhibited the growth of the bacterium under conditions of the experiment (Fig. 1). The strongest inhibitory effect was observed for juices obtained from the 'Kongo F1' cultivar (3.40 ± 2.85% of positive control), 'Rumba F1' (4.17 ± 2.27%) and 'Broker F1' (5.35 ± 2.14%).

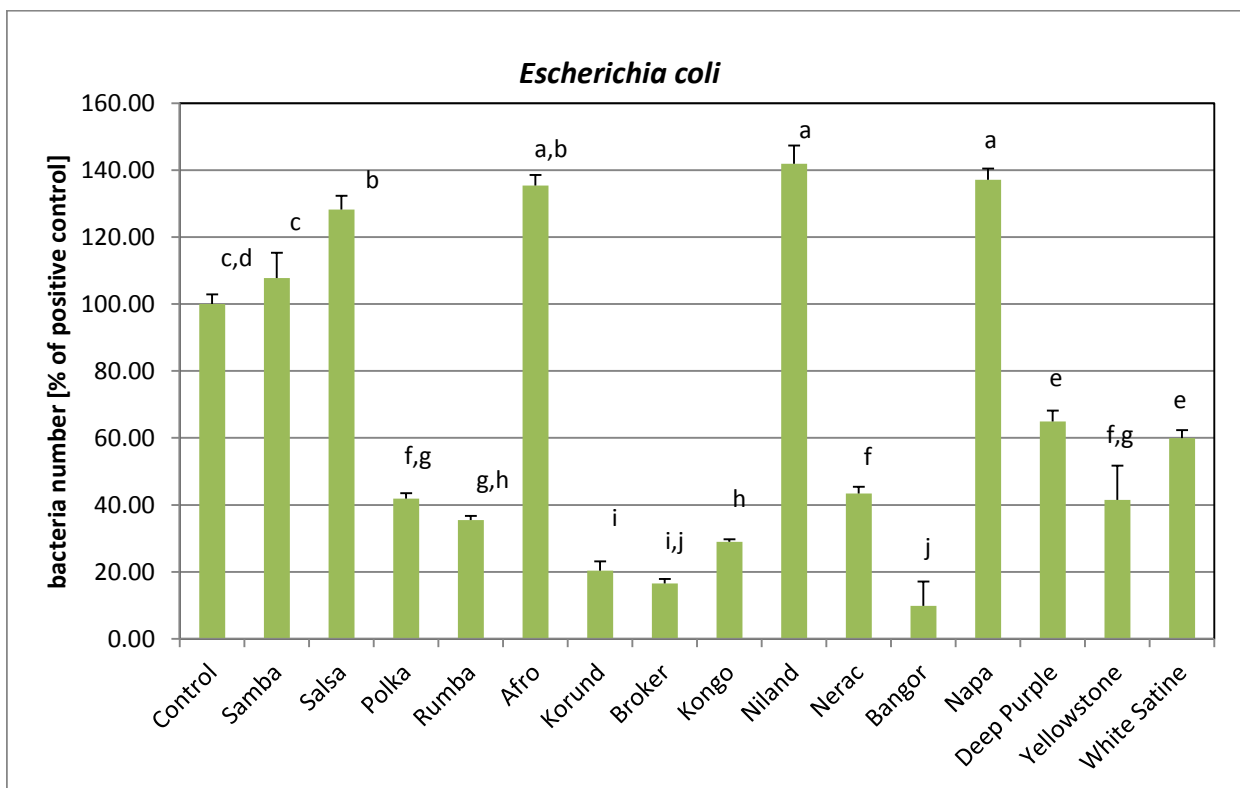
In the case of *E. coli* some cultivars of carrot positively influenced growth of the bacterium (Fig. 2). The highest stimulation of *E. coli* growth was observed in medium with the addition of 5% of juice prepared from the 'Niland F1' cultivar (141.91 ± 5.45% of positive control), 'Napa F1' (137.09 ± 3.39%), 'Afro F1' (135.43 ± 3.14%), 'Salsa F1' (128.25 ± 4.07%) and 'Samba F1' (107.78 ± 7.51%). Juices made from other cultivars of carrots caused inhibition of the bacterium, and the stronger inhibition was characteristic of the 'Bangor F1' (14.67 ± 1.49%) and 'Broker F1' (16.58 ± 1.30%).

Generally, probiotics were less sensitive to the presence of carrot juices. The growth of *Lactobacillus acidophilus* LA-5 was inhibited by juices obtained from the cultivars 'Napa F1' (55.10 ± 1.84% of positive control), 'Kongo F1' (58.30 ± 2.08%), 'Deep Purple F1' (72.20 ± 0.00%), and to a lesser extent by the 'Salsa F1', 'Broker F1', 'Niland F1' and 'Nerac F1' cultivars (Fig. 3). In contrary, the juices prepared from cultivars 'Bangor F1' and 'White Satine F1' slightly stimulated growth of this probiotic strain (105.29 ± 2.06% and 104.83 ± 2.62%, respectively). Juices from the 'Deep Purple F1' and 'Kongo F1' carrots inhibited also *Lactobacillus casei* 01 (52.25 ± 28.62% and 62.51 ± 3.71%, respectively), but the stronger inhibition of LC01 was observed for the 'Bangor F1' cultivar (37.71 ± 3.06%) (Fig. 4).



a,b,c – different letters indicate that differences between the number of bacteria are statistically significant at $p < 0.05$

Figure 1 The impact of carrot juices on the growth of *Bifidobacterium catenulatum*.

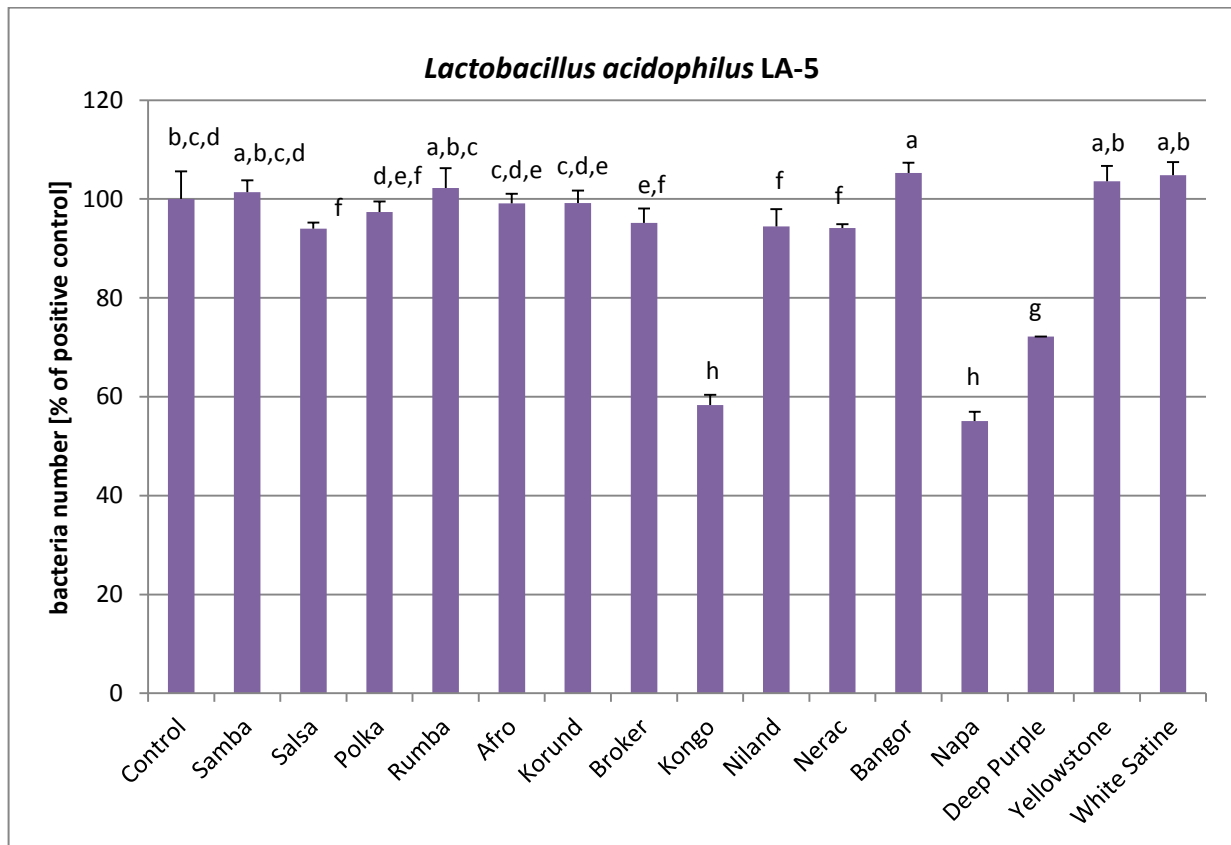


a,b,c – different letters indicate that differences between the number of bacteria are statistically significant at $p < 0.05$

Figure 2 The impact of carrot juices on the growth of *Escherichia coli*.

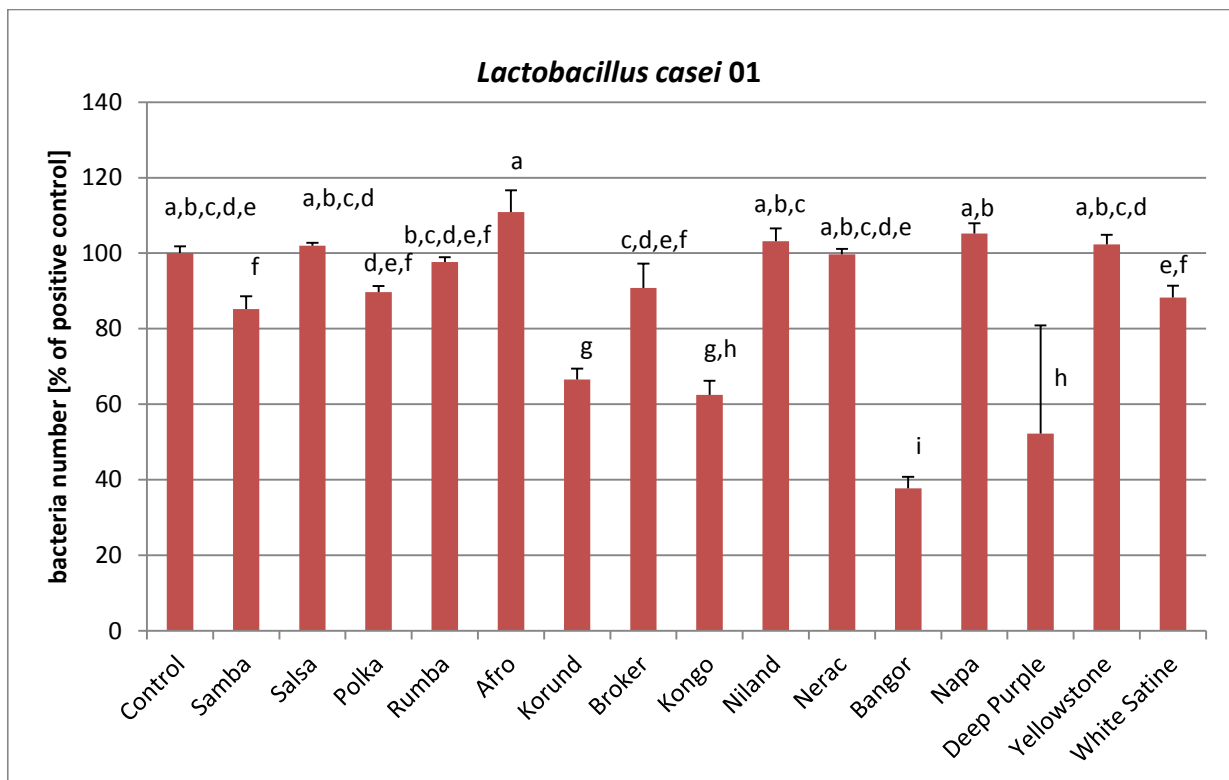
There is a growing interest in non-dairy probiotic products because of the rising frequency of lactose

intolerance, elevated blood cholesterol, as well as due to economic aspect, traditions, and dietary modifications



a,b,c – different letters indicate that differences between the number of bacteria are statistically significant at $p < 0.05$

Figure 3 The impact of carrot juices on the growth of *Lactobacillus acidophilus* LA-5.



a,b,c – different letters indicate that differences between the number of bacteria are statistically significant at $p < 0.05$

Figure 4 The impact of carrot juices on the growth of *Lactobacillus casei* 01.

caused by lifestyle (eg. a vegan diet) (Prado et al., 2008). The obtained results suggest that some carrot cultivars are

not suitable as an additive or raw material for the production of probiotic products. The cultivars ‘Deep

Purple F1' as well as 'Kongo F1' inhibited growth not only of intestinal bacteria, which is a desirable effect, but also of probiotic strains. Likewise, the 'Korund F1', 'Bangor F1' and 'White Satine F1' cultivars should not be used for the production of probiotics products. Our results revealed that the most suitable cultivar for this purpose was 'Rumba F1'; it inhibited growth of intestinal bacteria but had no impact on probiotic strains. The 'Polka F1' and 'Yellowstone F1' cultivars also could be used. The 'Bangor F1' cultivar of carrot can be considered as additive for probiotic products, but the proper selection of probiotic strains should be made. Juice prepared from the 'Bangor F1' inhibited strongly the growth of intestinal bacteria but also of the *L. casei* 01, whereas the growth of LA5 was stimulated. The juice obtained from 'Afro F1' cultivar stimulated growth of probiotic strain LC01, but stimulated also *Escherichia coli* growth, so it should not rather be used for probiotic juices production.

CONCLUSION

Carrot juice can be used as raw material for the production of probiotic beverages, however both the cultivar of carrot and the strains of probiotic bacteria used for the production should be selected carefully. The most suitable for production of probiotic beverages seems to be the 'Rumba F1' cultivar, followed by 'Polka F1' and 'Yellowstone F1'. Their valuable property was the inhibition of the growth of intestinal bacteria without negative effect on the tested probiotic species.

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LIPID OXIDATION IN CHICKEN MEAT AFTER APPLICATION OF BEE POLLEN EXTRACT, PROPOLIS EXTRACT AND PROBIOTIC IN THEIR DIETS

Marek Bobko, Peter Haščík, Alica Bobková, Adriana Pavelková, Jana Tkáčová, Lenka Trembecká

ABSTRACT

In the experiment, the effect of the addition of bee pollen, propolis extract and probiotic in a feed mixture for chicken broilers Ross 308 on oxidative stability of breast and thigh muscles during chilled storage was investigated. In the experiment were included 180 pieces of one day-old chicks, which were divided into 4 groups (control, E1, E2 and E3). Chickens were fed by *ad libitum* system until the age of 42 days. These feed mixtures were made without antibiotics preparation and coccidiostats. Bee pollen extract in amount of 400 mg.kg⁻¹ (E1), propolis extract in an amount of 400 mg.kg⁻¹ (E2) was added into feed mixtures and probiotic (*Lactobacillus fermentum*) (E3) in an amount 3.3 g added daily to the water given the experimental group. During whole period of chilled storage were higher values of MDA determined in control group compare to experimental groups. The higher average MDA value determined in breast muscles of broiler chicken hybrid combination Ross 308 was in samples of control group (0.129 mg.kg⁻¹) compared to experimental groups E1, E3 (0.125 mg.kg⁻¹) and E2 (0.115 mg.kg⁻¹) after 7-day of chilled storage. Significantly higher values of MDA were determined in control group compare to second experimental group on the end of storage. Trend of thigh muscle oxidation stability of chicken hybrid combination Ross 308 was during 7 days of chilled storage similar than in breast muscle. The higher average MDA value determined in thigh muscles was in samples of control group (0.142 mg.kg⁻¹) compared to experimental groups E1 (0.137 mg.kg⁻¹), E2 (0.125 mg.kg⁻¹) and E3 (0.138 mg.kg⁻¹) after 7-day of chilled storage. We have not determined statistically significant differences between testing groups on the end of storage. Higher amount of MDA in thigh muscle compare to breast muscle is due to by higher amount of fat occurred in thigh muscle.

Keywords: oxidative stability; meat; broiler chicken; propolis; bee pollen; probiotic

INTRODUCTION

Consumer concerns on the quality of meat and meat products have greatly increased during past decades. „Quality“ and „healthfulness“ were reported to be one of the most important factors for influencing consumers choice for food (Lennernas et al., 1997).

Lipid oxidation is one of the primary causes of quality damages related to flavour, colour, taste and nutritional composition of meat and meat products (Kanner, 1994; Gray et al., 1996; Marcinčák et al., 2005; Min et al., 2008).

Many factors can contribute to the initiation and development of lipid oxidation process in meat, such as fat content and fatty acid profile, degree of processing, storage conditions, and the balance between tissue pro and antioxidants content (Jensen, et al., 1997). Poultry meat is notably sensitive to lipid oxidation because of its high content of polyunsaturated fatty acids (Botsgolou, et al., 2002) and thigh meat, as compared to breast meat, is particularly vulnerable because of its higher fat content (Jensen, et al., 1998).

Lipid oxidation products have harmful biological effects and some have been related to the etiology of various neurodegenerative and cardiovascular diseases as well as different types of cancer (Cohn, 2002; Schroepfer, 2002).

Thus, it is important to not only improve the nutritional value of foods but also to minimize lipid oxidation to provide healthy food products. Consumers reject some antioxidants that are very effective in controlling lipid oxidation whereas they accept natural products with antioxidant activity since they are often perceived as safer and more nutritious than food containing additives or food coming from animal feed ingredients of a non-natural origin. While natural products are desired by many consumers, these products can be difficult to define since some man-made food additives and feed ingredients can be completely identical to those present in nature, slightly different, or modified for a better use (Bou et al., 2009). The negative consequences of lipid oxidation of meat and meat products can be overcome by the use of antioxidants in the diets (Kazimierczak et al., 2008; Haščík et al., 2012; Elimam et al., 2013). In recent period, after ban of antibiotics and coccidiostats in poultry nutrition in EU,

different alternative supplements e.g. probiotics, plant essential oils and their extract, enzymatic preparations and bee pollen products (pollen, propolis or their extracts), have begun to use for their positive influence on health state, feed utilization, nutritional and sensory quality of product as well as economics of poultry industry production (Wang et al., 2004; Shalmany and Shivazed, 2006; Seven et al., 2008).

The aim of the experiment was to determine the oxidative stability in the most valuable parts of chicken carcasses (Ross 308 hybrid combination) during the cold store (7 days) after application of bee pollen extract and propolis extract added to feed mixtures and probiotic added into drinking water.

MATERIAL AND METHODOLOGY

The experiment was carried out in test poultry station of Slovak University of Agriculture in Nitra. A total of 180 one day-old Ross 308 broiler chicks were randomly divided into 4 groups, namely, control (C) and experimental (E1, E2, E3) of 45 pcs chickens. During the whole period of experiment, the broiler chickens had *ad libitum* access to feed and water.

The feeding lasted 42 days. During that period, experimental broiler chickens were fed with a starter complete feed mixture HYD-01 (until 21 days of age) and a grower feed mixture HYD-02 (from 22nd to 42nd day of age). The composition of feed mixtures is given in Table 1. The feed mixtures both starter and grower were produced without any antibiotic preparations and coccidiostatics.

All the groups were fed with the same feed mixtures. However, chickens in the control group were fed with basal diet containing no special supplement, while the diet of chickens in experimental groups contained the diet supplements as follows: bee pollen extract in amount of 400 mg.kg⁻¹ added to feed mixtures given to the group E1, propolis extract in amount of 400 mg.kg⁻¹ added to feed mixtures given to the group E2, probiotic in an amount 3.3 g added daily to the water given the group E3. The groups were kept under the same conditions.

In the experiment, the probiotic preparation "Propoul" based on *Lactobacillus fermentum* (1.10⁹ CFU per 1 g of bearing medium) was used.

Bee pollen and propolis had origin in the Slovak Republic. The extracts were prepared from minced bee pollen and propolis in the conditions of the 80% ethanol in

Table 1 Composition of the diets.

Ingredients (%)	Starter (1 to 21 days of age)	Grower (22 to 42 days of age)
Wheat	35.00	35.00
Maize	35.00	40.00
Soybean meal	21.30	18.70
Fish meal (71% N)	3.80	2.00
Dried blood	1.25	1.25
Ground limestone	1.00	1.05
Monocalcium phosphate	1.00	0.70
Fodder salt	0.10	0.1.50
Sodium bicarbonate	0.15	0.20
Lysin	0.05	0.07
Methionin	0.15	0.22
Palm kernel oil Bergafat	0.70	0.16
Premix Euromix BR 0.5% ¹	0.50	0.50
Analysed composition (g.kg⁻¹)		
Crude protein	210.76	190.42
Fibre	30.19	29.93
Ash	24.24	19.94
Ca	8.16	7.28
P	6.76	5.71
Mg	1.41	1.36
Linoleic acid	13.51	14.19
ME _N (MJ.kg ⁻¹)	12.02	12.03

¹active substances per kilogram of premix: vitamin A 2 500 000 IU; vitamin E 50 000 mg; vitamin D3 800 000 IU; niacin 12 000 mg; d-pantothenic acid 3 000 mg; riboflavin 1 800 mg; pyridoxine 1 200 mg; thiamine 600 mg; menadione 800 mg; ascorbic acid 50 000 mg; folic acid 400 mg; biotin 40 mg; vitamin B12 10.0 mg; choline 100 000 mg; betaine 50 000 mg; Mn 20 000 mg; Zn 16 000 mg; Fe 14 000 mg; Cu 2 400 mg; Co 80 mg; I 200 mg; Se 50 mg.

the 500 cm³ flasks, according to **Krell (1996)**. The extraction was accomplished in a water bath at 80 °C for one hour. After that, the extracts were cooled and centrifuged. The obtained supernatants were evaporated in a rotary vacuum evaporator at bath temperature 40 – 50 °C and weighed. Residues in an amount of 40 g were dissolved in 1000 cm³ of 80% ethanol and used for 100 kg of feed mixture.

At the end of feeding (day 42th) from each group were selected 10 pieces of chicken for slaughter analysis. To determine changes in lipid degradation (determination of thiobarbiturates numbers, TBA) the samples of chickens were boned and thigh and breast muscle packed into polyethylene bags and stored for 7 days at 4 °C.

TBA value expressed in number of malondialdehyde were measured in the process of first storage day of 1st, 3rd, 5th and 7th day. TBA number was determined by **Marcinčák et al. (2004)**. Absorbance of samples was measured on UV-VIS spectrophotometer T80 (PG Limited Instruments, UK) at a wavelength of 532 nm, the translation results on the amount of malondialdehyde (MDA) in 1 kg samples.

Results of the experiment was evaluated with statistical program Statgraphics Plus version 5.1 (AV Trading Umex, Dresden, Germany), were calculated variation-statistical values (mean, standard deviation) and to determine the significant difference between groups was used variance analyse with subsequent Scheffe test.

RESULTS AND DISCUSSION

The results of the oxidation stability measured in breast and thigh muscle of chickens Ross 308 during 7 days storage at 4 °C are shown in Table 2. Our results are in accordance with **Marcinčák et al. (2010)** who, after slaughtering and processing of poultry samples also show low values of MDA. During chilled storage of the breast and thigh muscles (7 days) were detected increased content of MDA in comparison to the first day of storage. During testing period of chilled storage were higher values of MDA measured in control group compare to experimental groups. The higher average value of MDA measured in

breast muscle of broiler chickens Ross 308 was in samples of control group (0.129 mg.kg⁻¹) compared to experimental groups E1, E3 (0.125 mg.kg⁻¹) and E2 (0.115 mg.kg⁻¹) after 7-day of chilled storage. Significantly higher values of MDA on the end of storage were determined in control group compare to second experimental group.

Trend of oxidation stability in thigh muscle of chicken hybrid combination Ross 308 was during 7 days of chilled storage similar than in breast muscle. The higher average value of MDA measured in thigh muscle was in samples of control group (0.142 mg.kg⁻¹) compared to experimental groups E1 (0.137 mg.kg⁻¹), E2 (0.125 mg.kg⁻¹) and E3 (0.138 mg.kg⁻¹) after 7-day of chilled storage. We have not found statistically significant differences between testing groups. Higher concentration of MDA in thigh muscle compare to breast muscle is due to by higher amount of fat passes into thigh muscle **Botsoglou et al. (2002)**.

Reached results of oxidation stability determined in chicken meat of hybrid combination Ross 308 after propolis extract addition in their diet are in accordance with **Betti et al. (2009)** and **Yasin et al. (2012)**. The possibilities of using alternative feed supplements containing various antioxidant active substances for poultry which increase the oxidation stability of the meat during its period of freeze storage are presented in works of **Mikulski et al. (2009)**, **Ahadi et al. (2010)**, **Marcinčák et al. (2010)**, **Karaalp and Genc (2013)**.

Ramos Avila et al. (2013) stated that the degradation pathways of fatty substances play one of the main causes of foods deterioration and unpleasant odours. This factor is also responsible for the loss of sensory properties such as flavour, texture, appearance, nutritional value of food, increases the drop losses, pigment, polyunsaturated fatty acids, fat-soluble vitamins, reduces the quality of meat intended for human consumption and ultimately reduces its stability, shelf life and safety.

Botsoglou et al. (2007) reported that a higher concentration of antioxidants in poultry meat has the effect of reducing lipid oxidation, ie there is a reduction in MDA values during chilling storage, which was confirmed by our results.

Table 2 Effect of storage (4 °C) on the concentration of malondialdehyde (mg.kg⁻¹) in breast and thigh muscle (mean ±SD).

Time of storage	Group			
	Control	E1	E2	E3
Breast muscle				
Day - 1	0.034 ±0.008 ^a	0.021 ±0.002 ^b	0.022 ±0.009 ^b	0.025 ±0.005 ^b
Day - 3	0.049 ±0.006 ^a	0.044 ±0.009 ^{ab}	0.042 ±0.011 ^{ab}	0.046 ±0.005 ^b
Day - 5	0.084 ±0.004	0.079 ±0.006	0.076 ±0.009	0.830 ±0.002
Day - 7	0.129 ±0.003 ^a	0.125 ±0.012 ^{ab}	0.115 ±0.006 ^b	0.125 ±0.010 ^{ab}
Thigh muscle				
Day - 1	0.043 ±0.002 ^a	0.030 ±0.008 ^b	0.028 ±0.002 ^b	0.033 ±0.011 ^{ab}
Day - 3	0.059 ±0.006 ^a	0.052 ±0.009 ^{ab}	0.047 ±0.006 ^b	0.050 ±0.004 ^b
Day - 5	0.097 ±0.012	0.092 ±0.014	0.087 ±0.007	0.095 ±0.011
Day - 7	0.142 ±0.019	0.137 ±0.020	0.125 ±0.008	0.138 ±0.022

Legend: Mean values in the same lines with different superscripts (a, b) are significantly different at $p < 0.05$ level.

CONCLUSION

Results achieved in the experiment show that the addition of propolis extract in feed mixture for broiler chickens had positive impact on the reduction of oxidative processes in the breast and thigh muscles during chilling storage, but with the addition of bee pollen extract and probiotic has been recorded significant effect on the oxidation of fat in the breast and thigh muscles meat broiler chickens Ross308.

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MODIFIED CARBON PASTE ELECTRODE AS A TOOL FOR THE EVALUATION OF OXIDATIVE STABILITY OF RAPESEED OIL

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ABSTRACT

Carbon paste electrode was used for evaluation of oxidative stability of rapeseed oil samples using cyclic voltammetry in 0.1 mol.L⁻¹ HCl as a supporting electrolyte. Rapeseed oil samples were exposed to daylight and oxygen in open glass baker at the laboratory condition in order to obtain oils with accelerated primary and secondary products of oxidation. The oxidation status was determined by peroxide value and p-anisidine value. Total oxidative stability was expressed as TOTOX index. The edible oils were used for preparation (modification) of the carbon paste composite material followed by the cyclic voltammetric measurement. Peroxide values significantly increased whereas p-anisidine value rather fluctuated during 40 days of storage in all the samples. Cyclic voltammograms showed anodic current peaks at 575 – 600 mV and cathodic current peaks at 400 – 425 mV. The oxidation and reduction waves diminished at pH \geq 3.0 suggesting not only phenolic compounds contributed to the electrochemical characteristic of oil samples. The peroxide value or p-anisidine value did not correlate with oxidation or reduction peak currents at the potential 575 – 600 mV and 400 – 425 mV, respectively. Both cathodic and anodic currents increased with increasing TOTOX index exhibiting positive correlation with high Spearman correlation coefficient ($r = 0.894$ and $r = 0.914$ for anodic and cathodic current, respectively). Linear relationship was found for each sample individually. A caution has to be done when interpreting results since the correlation seems to be of oil sample specific. Nevertheless, the modified carbon paste electrode with rapeseed oil represents a suitable and alternative tool for determination of the oxidative state of edible oils without use of organic solvents.

Keywords: edible oil; peroxide value; cyclic voltammetry; carbon paste electrode

INTRODUCTION

Plant seed oils represent the significant source of fatty acids, sterols and other biologically active substances for human nutrition (McKevith, 2005). Rapeseed oil belongs to the most frequently used vegetable oil in European Union countries. Global rapeseed production gradually increased over the past 20 years with the EU being the principal consumer (including biodiesel production) (Carré and Pouzet, 2014).

Rapeseed oil composition varies among the cultivars of *Brassica* sp. but it is well known that oleic, linoleic and α -linolenic acids dominated together with other health-promoting compounds such as glucosinolates (progoitrin, gluconapin), tocoferols and phenolics (Pawłowicz et al., 2013; Zheng et al., 2014; Szterk et al., 2010). Phenolic substances also have protective effect against oxidative changes in the edible oils. The oxidation mechanism of the edible oils is very complex, and is influenced by the fatty acid composition, oil processing, energy of heat or light, the type and the concentration of oxygen and transition metals, pigments and antioxidants. The oxidation of the edible oils decreases their nutritional quality and sensory acceptance by the formation of aldehydes, carboxylic acids, alcohols and hydrocarbons as the secondary oxidation products (Choe and Min 2006). Therefore, the

knowledge of the oxidative status is crucial during manufacturing and storage of plant seed oils.

The analytical methods are divided in those determining the primary oxidation products (hydroperoxides) and the secondary ones. The versatile methods for determination of hydroperoxides and secondary oxidation products are peroxide value (PV) and p-anisidine value (p-AV), respectively. A comprehensive reviews upon the other methods for measurement of oxidation rancidity have been recently published (Gromadzka and Wardencki, 2011; Pignitter and Somoza, 2012) including UV/VIS, infrared and Raman spectroscopy, chromatography, nuclear magnetic resonance, scanning calorimetric techniques or luminescent method. Compared with those methods above, electrochemical techniques have some advantages such as high sensitivity, accuracy, simplicity, low expense and the possibility of miniaturization. The changes of electrical conductivity during the reaction of KI and hydroperoxides (Yang et al., 2014) or an optical transmission/capacitance bridge based on the reaction of moisture during heating of the edible oils (Shelke and More, 2013) represent interesting approaches to the evaluation of rancidity. Potentiometric determination of PV and p-AV in the edible oils with triiodide-selective membrane has been proposed for flow injection technique (Saad et al., 2006; Saad et

al., 2007) and Adhoumad and Monser (2008) described direct measurement of hydroperoxides using Prussian-blue modified glassy carbon electrode. Electrochemical characteristics of the edible oils were also used for the determination of the origin or adulteration of the seed oils using chemometric treatment of the data (Gambarra-Neto et al., 2009; Oliveri et al., 2009, Apetrei et al., 2005).

Gambarra-Neto et al., (2009) classified various kinds of edible oils and unsuccessfully attempted to distinguish expired and non-expired oil samples using square wave voltammetry and platinum- and gold-disk electrodes. Oliveri et al., (2009) used Pt-microelectrode in mixture of the edible oil with ionic liquid and evaluated the whole shape of cyclic voltammograms. Our research was inspired by Apetrei et al., (2005) who used different olive oils as a binder material for preparation of carbon paste electrode for their subsequent discrimination. Among all the carbon electrodes, carbon paste electrode (CPE) is an appealing and widely used electrode material in the fields of electrochemistry, electroanalysis, etc. due to its attractive advantages, such as simple preparation, low-cost implementation, renewability, low background current, and wide potential window (Švancara et al., 2012). The aim of this study was to evaluate the rapeseed oils oxidation status measuring their electrochemical characteristics by cyclic voltammetry using the carbon paste electrode.

MATERIAL AND METHODOLOGY

Reagents and equipment

All the reagents were purchased in Sigma-Aldrich spol. s r. o. (Prague, Czech Republic). Deionized water was used in this study ($G \leq 0.055 \mu\text{S}$).

A three-electrode system consisting of CPEs (working), Ag/AgCl/3.0 M KCl (reference) and platinum wire (counter electrode) connected to PalmSens (Ivium Technologies, Eindhoven, The Netherlands) was used for electrochemical measurement. The surfaces of CPE were regenerated by renewing and polishing them on wet filter paper before each measurement.

Preparation of CPE

The carbon paste composite material was prepared by mixing of 0.5 g graphite powder $5.5 \mu\text{m} - 7.0 \mu\text{m}$ (CR-5, Maziva Týn n. L. s. r. o., Czech Republic) with 140 μL of rapeseed oil sample in a ceramic mortar. The resulting paste was packed into the teflon piston holder (3.0 mm inner diameter) (Švancara and Metelka, 2000). The resistance of the composite material was always $\leq 15.0 \Omega$. The cyclic voltammetry (CV) was performed with CPE, where RO1-RO3 was used as a binder liquid in 0.1 mol.L⁻¹ HCl at a scan rate 100 mV.s⁻¹ (potential range from -200 to +1000 mV, potential step 25.0 mV) or in 0.4 mol.L⁻¹ Britton-Robinson buffer at the pH 3.0, 7.05 and 10.05 if necessary.

Sample preparation

Two refined rapeseed (RO1, RO2) oils under private label were obtained from local market (Billa spol. s r. o., Prague and Lidl Holding s.r.o., Prague, Czech Republic), RO3 (*Brassica rapa*) sample was purchased in Sigma

Aldrich spol. s.r.o. (Prague, Czech Republic). The oil samples were stored in refrigerator until use.

After opening the original package of the oil, 75 mL of each sample was put into the 150 mL glass baker and was allowed to expose to daylight and oxygen at laboratory condition ($23.0 \pm 2.0 \text{ }^\circ\text{C}$) for 40 days. The geometry of the liquid in baker was 65 mm \times 35 mm (diameter \times height). A portion of each oil sample was taken off at 0, 7th, 14th, 28th and 40th day of storage for determination of PV (AOAC 2007; Method 965.33), p-AV (IUPAC 1992; Method 2.504) and CPE preparation. Total oxidation value (TOTOX) was calculated as follows (Cao et al., 2014):

$$\text{TOTOX} = 2 \times \text{PV} + \text{p-AV}$$

Statistical analysis

Statistical analysis was performed by Origin Pro software (OriginLab Corp., MA, USA) on the probability level $p < 0.05$. Relationship among electrochemical characteristics of oil samples and oxidation values was evaluated using Spearman correlation coefficient (r). Comparison of the dataset was performed using analysis of variance. The chemical and electrochemical analysis was performed at six replicates.

RESULTS AND DISCUSSION

Peroxide value and p-anisidine value were determined for three rapeseed oil samples during storage in open baker at ambient temperature exposing to daylight for 40 days. As can be seen from Table 1, the initial PVs for RO1 and RO2 samples are below the required level ($< 10.0 \text{ mmol.kg}^{-1}$) for refined vegetable oils recommended in International Food Standard by Codex Alimentarius (IFS 1999).

The sample RO3 is not primarily designed for human consumption but for analytical purposes. The exposition of rapeseed oil samples to daylight and oxygen resulted in increase of PV during 40 days of storage. It was previously published that the light is more important in ¹O₂ oxidation than temperature (Choe and Min, 2006) forming the hydroperoxides, which are slowly decomposed to the secondary oxidation products. It is in agreement with our results where PVs gradually increased until the end of experiments whereas p-AV showed fluctuations in all the samples.

A typical cyclic voltammogram of the CPE modified with RO1 sample is depicted in Figure 1. An anodic wave appeared at the potential range from +575 to +600 mV followed by the cathodic wave from +400 to +425 mV at the pH 1.0 in the reverse scan. Apetrei et al., (2005) stated that some phenolic compounds were oxidized and reduced when they examined olive oils modified CPE. However, electrochemical studies of various phenolic compounds using CPEs described the shifting of the redox potentials to negative region with increasing pH (Švancara et al., 2012).

In our study, the redox waves disappeared even at pH 3.0. We may imply that phenolics naturally occurred in rapeseed oils did not act electrochemically at the experimental conditions described above.

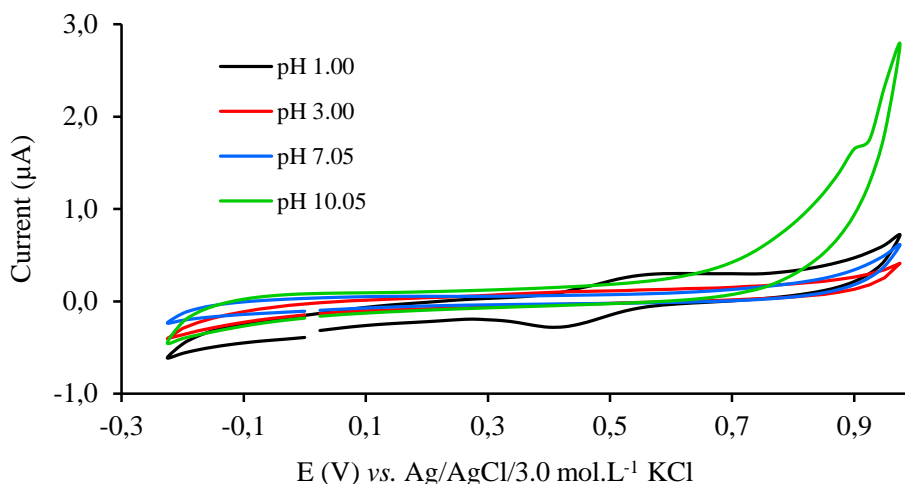


Figure 1 Cyclic voltammogram of carbon paste electrode modified with rapeseed oil (RO1) at different pH. Scan rate 100 mV.s⁻¹ and potential step 25.0 mV.

Table 1 Changes in peroxide value and p-anisidine value during the autooxidation of rapeseed oils (RO1-RO3)** at 23 °C exposed to sunlight (n = 6).

Storage time Days	Peroxide value mmol.kg ⁻¹ ±SD*			p-Anisidine value ±SD*		
	RO1	RO2	RO3	RO1	RO2	RO3
0	^a 4.35 ±0.72	^a 1.82 ±0.23	^a 25.50 ±1.31	^b 2.41 ±0.43	^{cd} 3.11 ±0.12	^c 6.33 ±0.25
7	^a 5.42 ±0.63	^b 9.21 ±0.20	^b 35.62 ±1.45	^a 1.30 ±0.21	^{ad} 2.70 ±0.11	^b 5.81 ±0.36
14	^b 9.53 ±0.54	^c 11.55 ±0.19	^b 42.11 ±1.26	^c 3.51 ±0.09	^{bc} 3.34 ±0.16	^b 5.23 ±0.21
28	^c 15.91 ±1.00	^d 16.50 ±0.91	^d 68.90 ±0.36	^e 5.34 ±0.15	^b 3.62 ±0.23	^a 2.64 ±0.30
40	^d 41.90 ±1.75	^f 47.22 ±0.16	^f 105.71 ±0.45	^c 3.85 ±0.35	^b 3.71 ±0.30	^c 6.84 ±0.42

Different small letters (a-f) in superscript indicate statistical differences in columns ($p < 0.05$).

* Values are presented as mean ±SD of duplicate samples obtained from three individual trials.

** RO1 (Rapeseedoil 1, Billa spol. s r. o., Prague, Czech Republic), RO2 (Rapeseed oil 2, Lidl Holding s.r.o., Prague, Czech Republic), RO3 (Sigma Aldrich spol. s r. o., Prague, Czech Republic).

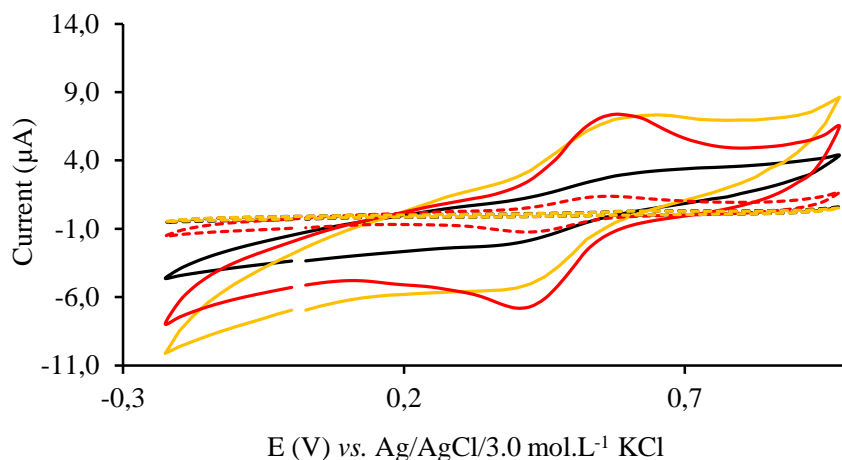


Figure 2 Cyclic voltammogram of carbon paste electrode modified with rapeseed oil samples RO1 (black line), RO2 (yellow line) and RO3 (red line) in initial oxidation state (dotted lines) and after 40 days (solid lines) of storage exposed to daylight and oxygen at 23 °C. Scan rate 100 mV.s⁻¹, potential step 25.0 mV, pH 1.0.

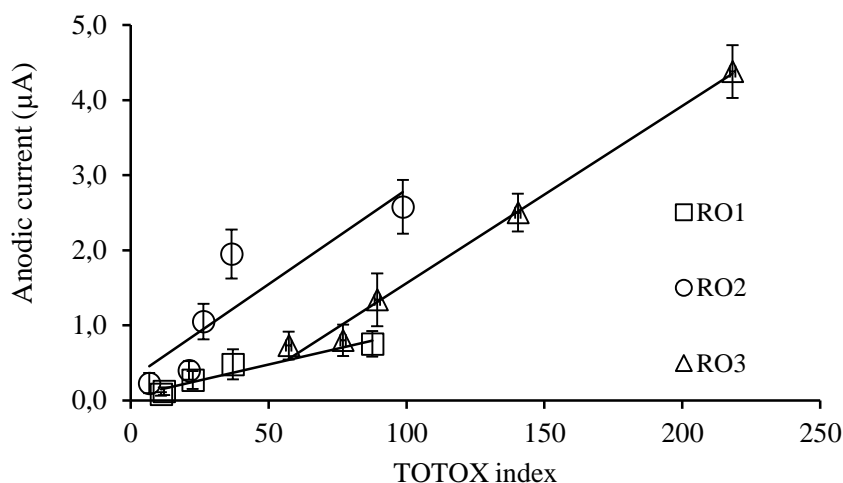


Figure 3 Linear regression of oxidation current of CPE modified with rapeseed oil samples upon TOTOX index. Mean \pm SD ($n=6$). RO1 (Rapeseed oil 1, Billa spol. s r. o., Prague, Czech Republic), RO2 (Rapeseed oil 2, Lidl Holding s.r.o., Prague, Czech Republic), RO3 (Sigma Aldrich spol. s r. o., Prague, Czech Republic).

The same type of working electrode may give different results at similar conditions. For instance, **Oliveri et al., (2009)** analyzed the whole CV curves using Pt-microelectrode for discrimination of edible oils and found no specific oxidation and reduction wave. On the other hand, **Garbarra-Neto et al., (2009)** used Pt-discelectrode for the same purpose, and used currents appearing in cathodic potential region specific for fatty acids detection for chemometric analysis. When performing cyclic voltammetry with CPEs prepared with rapeseed oils in different oxidation state, an increase of the both oxidation and reduction currents appeared (Figure 2). An attempt to find correlation between peroxide value and oxidation (I_a) and reduction (I_c) current was performed resulting in Spearman correlation coefficients $r = 0.321$ ($p = 0.508$) and $r = 0.412$ ($p = 0.516$) for I_a and I_c , respectively. Correlations of some edible oil quality parameters have been studied in some research papers.

Zheng et al., (2014) found negative linear correlation between the canolol content in rapeseed oils and PV and p-anisidine values. The concentration of nonpolar carbonyl compounds correlated with the TOTOX index in vegetable oils (**Cao et al., 2014**). Statistically significant positive correlation of the current and TOTOX index for all the samples was found in our study giving $r = 0.894$ ($p = 7.125 \cdot 10^{-6}$) and $r = 0.914$ ($p = 1.841 \cdot 10^{-6}$) for I_a and I_c , respectively. No correlation was found between the current and p-AV in this study. The TOTOX index is used to estimate total oxidative deterioration of oils combining the amounts of primary oxidation products (hydroperoxides) with secondary oxidation products (principally alkenals and alkadienals) (**Cao et al., 2014**). In addition, **Apetrei et al., (2005)** found strong correlation between anodic potential of the virgin olive oil and specific absorbance at 232 nm and 270 nm (showing the amount of polar compounds with conjugated multiply bonds), free fatty acid content, and peroxide value. In our study, the shift of both positive and negative potential values was observed but correlation was not confirmed.

Despite the strong correlation between TOTOX index and anodic or cathodic current, it was unable to mathematically describe such relationship for prediction purposes for all the samples. Therefore, we tried to evaluate each sample independently. The results presented in Figure 3 show the linear relationship between anodic peak current and TOTOX value for each of the rapeseed oil sample with r^2 to be 0.915 ($p = 0.007$), 0.730 ($p = 0.041$) and 0.989 ($p = 3.394 \cdot 10^{-4}$) for RO1, RO2 and RO3, respectively. The same linear correlation was found between TOTOX value and cathodic current with the calculated values r^2 to be 0.971 ($p = 0.014$), 0.819 ($p = 0.022$) and 0.979 ($p = 8.510 \cdot 10^{-4}$) for RO1, RO2 and RO3, respectively.

CONCLUSION

Rapeseed oil used as a binder liquid instead of mineral oils for the preparation of CPE is an alternative tool for determination of its oxidative stability. Despite the positive correlation between electrochemical characteristics of the binder liquid and the TOTOX index, one should be cautious interpreting results. We proved that each oil sample has different linear relationship of both anodic or cathodic currents, and oxidation quality parameters (TOTOX, p-AV). This behavior is probably rapeseed oil specific and should not be generalized. Nevertheless, the results of our research introduced a novel and a different approach for determination of oxidation stability of edible oils.

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IDENTIFICATION OF SWEET CHESNUT POLLEN IN BEE POLLEN PELLET USING MOLECULAR ANALYSIS

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ABSTRACT

Castanea sativa possesses many characteristics that are used by humans for different purposes, not only as a part of the food. One of them is the utilization of the sweet chesnut pollen for its pharmacological benefits. Actually, no information about the DNA based identification of the sweet chesnut exists. Here, an identification of *Castanea sativa* based on the specific DNA fragment amplification is described for the first time. Sweet chesnut identification was performed in the very complex sample of bee pollen pellets that were identified as to contain sweet chesnut pollen grains by morphological analysis. First, bioinformatic analysis was performed to find a *Castanea sativa* conservative part of galactol synthase gene. BLAST alignment of the CDS of Gols1 gene was performed by BLASTn against plants nucleotide sequences in the NCBI database to ensure for the specificity or existing nucleotide differences. Then, specific primers were subsequently designed and PCR amplification was performed. All the PCRs have run in duplicates for pollen pellet sample and two independent samples of *Castanea sativa* pure pollen. Restriction cleavage of the PCR amplified fragment was performed to confirm the specificity of the obtained PCR product with the positive confirmation as the predicted three restriction fragments were obtained that fully correspond by the length to those from virtual cleavage. Restriction endonuclease Hpy166II was used in restriction cleavage analysis. *Castanea sativa* pollen grains were confirmed reliable in multifloral pollen pellet by PCR and this approach has the potential to be used effectively for the authentication purposes of sweet chesnut.

Keywords: *Castanea sativa*; pollen; pollen pellet; identification; PCR

INTRODUCTION

Castanea sativa is reported as to have ecological and economical value (Beyhan and Serdar, 2008; Lusini et al., 2014). It is cultivated throughout the whole Europe and historically, it is used for different purposes – it produces large amount of nectar, its fruits are edible and its pollen is valuable for the medicinal properties (Hrga et al., 2010). *Castanea* pollen was described by Peeters and Zoller (1988) as small (18,3 x 12 µm), prolate and tricolporate with a smooth exine. Maurizio and Grafl (1969) reported the liberation time of *Castanea* pollen to be situated between 7-19 h, but to have two maxima at 10 h and 16 h. The pollination is entomophilous and anemophilous (Hrga et al., 2010).

In spite of good knowledge about the genetic diversity and population structure of sweet chesnuts that was analysed using different biochemical and molecular markers, no information about the effective identification of the species within a complex samples – like bee pollen pellets, can be found. Different DNA markers were utilized for describing the variability of *Castanea sativa* – cpDNA (Fineschi et al., 2000), RAPD (Galderisi et al., 1998) or microsatellites (Martín et al., 2012; Mattioni et al., 2013; Lusini et al., 2014). Hasegawa et al., (2009) analyzed pollen genetic structure using microsatellite genotyping of pollen grains, seeds, and potential paternal trees in the self-incompatible monoecious tree species *Castanea crenata*.

Bee pollen is used in medicine as a part of supplementary nutrition and in alternative diets, because of its reported nutritional properties and health benefits. The components of bee pollen are: carbohydrates, crude fibers, proteins, lipids, minerals, trace elements, vitamins, carotenoids, phenolic compounds, flavonoids, sterols and terpenes (Bogdanov, 2014).

Bee pollen is described as the perfectly complete food, because it contains all essential amino acids that humans need for their nutrition. The biochemical composition of bee pollen is affected by many factors such as plant source, geographic origin, climatic conditions, soil type or beekeeper activities (Feás et al., 2012).

Single pollen identification was reported for the first time by Suyama et al., (1996) for the pollen grain from Pleistocene peat. Matsuki et al., (2007) modified this method and have used it for the purpose of multilocus genotyping of a single pollen grain by PCR. Using the PCR for pollen identification is now utilized in parentage analysis, discrimination of self-pollen, or evaluation of the genetic diversity of pollen grains (Isagi, 2011).

The aim of the study was the identification of sweet chesnut pollen as to be a part of bee pollen pellet using molecular approach.

MATERIAL AND METHODOLOGY

Biological material

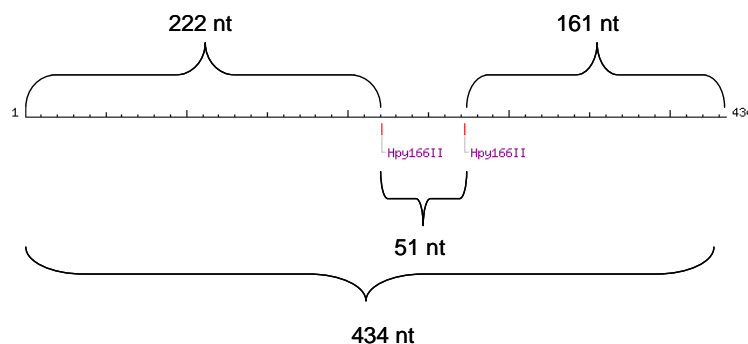


Figure 1 Restriction scheme for the obtained PCR fragment of the Go1 S1 gene of the *Castanea sativa*, L.

A pure pollen of *Castanea sativa*, Mill was collected through the chesnut flowering season during 2013 and 2014 in the form of biological duplicate. Samples were desiccated and frozen in plastic aseptic container until further analysis. Pollen was obtained in the Radošina (Slovak republic), where a group of old trees of sweet chesnut can be found. Pollen pellets were collected by bee keepers in the same locality.

DNA extraction

Total genomic DNA from pollen and bee pollen pellet was extracted using the GeneJET™ Plant Genomic DNA Purification Kit (ThermoScientific) according to the manufacturer's recommendations. DNA quality and the concentration were quantified by spectrophotometer.

Bioinformatic alignment of *Castanea sativa* galactinol synthase (Go1S1) gene and primer design

BLAST (Zhang et al., 2000) alignment of the CDS of Go1S1 gene, (NCBI accession code JX512438) was done by BLASTn against plants (taxid:3193) nucleotide sequences in the NCBI database to ensure for the specificity or existing nucleotide differences. Primer design was performed in Primer-BLAST (Ye et al., 2012) in a manner to get *Castanea sativa* galactinol synthase specific amplification. Following primers were returned and used in the study:

RESULTS AND DISCUSSION

Molecular markers are reported by Allendorf et al. (2010) as an efficient tool for characterization of long-life species, mainly for its facilitation of conservation decisions. DNA based markers that were applied in sweet chesnut analysis have aimed in past mainly on mapping studies for disease resistance genes or population studies. Different DNA markers were utilized for this purpose, such as RAPDs, RFLPs or ISSRs (Fineschi et al., 2000; Casasoli et al., 2001; Goulão et al., 2001). In nucleotide databases, a total of 55 different microsatellite loci are stored for *Castanea sativa*, Mill. Martín et al. (2012) used 7 microsatellite loci for genetic structure analysis of sweet chesnut in Spain and Lusini et al., (2014) used 6 of the same plus 1 different locus for genetic structure analysis of sweet chesnut in Bulgaria. Both of the analysis showed a high level of genetic diversity. That is why finding of reliable conservative sequences is important for identification studies of *Castanea sativa*, Mill.

First, bioinformatics alignment was performed for Go1S1 gene of *Castanea sativa*, Mill.. Any of returned

forward 5' AAGCCCCTCCCTCTGGTATT 3' (matching the nucleotides 4693 - 4712 of JX512438) and reverse 5' GCTCAGGCATGGAAGCCATA 3' (matching the nucleotides 5107 - 5126 of JX512438) with the amplification product length of 434 bp.

PCR conditions

PCR runs were performed in duplicates, each containing KAPA2G™ Robust HotStart Ready Mix (2x) (Kapa Biosystems); 300 nmol of forward and reverse primer 50 ng of template DNA, for 40 cycles using a BioRad C1000. Cycle conditions were as follows; 3 min. at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C and 15 s at 72 °C. The resulting reaction was run on a 2% agarose gel to confirm the product was unique and of the correct size.

Product specificity verification

Nucleotide sequence that was obtained by bioinformatic analysis was uploaded into the NEBcutter v 2.0 (Vincez et al., 2003) and the appropriate restriction enzyme was selected for the verification of the results of PCR. The amplified PCR product was inspected for the specificity using the Hpy166II restriction endonuclease that possess in total two restriction sites (figure 1) within the amplified fragment of the Go1S1 gene of the *Castanea sativa*, L.

alignments don't possess the relevant similarity with the Go1S1 gene (table 1).

Based on the obtained data, *Castanea sativa* galactinol synthase specific amplification primers were designed and used in subsequent PCR analysis. PCR amplification was performed in duplicates for pollen pellet sample and two independent samples of *Castanea sativa* pure pollen. Optimization of annealing temperature and primer concentration was performed, were 150, 300 and 450 nmol primers were tested. A combination of 300 nmol primers and 60 °C annealing temperatures resulted in fully reproducible PCR product for both of pure pollen samples. Optimized PCR conditions were used for the analysis of pure *Castanea sativa* pollen, pure *Betula verrucosa* pollen and tested pollen pellet sample.

A single 434 bp long PCR product was obtained (figure 2) for all the samples except the pure pollen sample of *Betula verrucosa* that was used as negative control in the study. The analysis was repeated one more time with the same results.

The analytical methods used for species identification and authenticity rely mainly on protein and DNA analysis.

Table 1 Alightment characteristics for similar sequences for GolS gene.

Sequence	Max score	Total score	Query cover	E value	Ident	NCBI Accession
<i>Theobroma cacao</i> Galactinol synthase 4	231	231	5%	9,00E-57	81%	XM_007044611.1
<i>Ricinus communis</i> conserved hypothetical protein	228	336	7%	1,00E-55	82%	XM_002525318.1
Predicted: <i>Nelumbo nucifera</i> galactinol synthase 1-like	220	428	9%	2,00E-53	83%	XM_010252922.1
<i>Manihot esculenta</i> galactinol synthase (GolS3)	211	211	4%	1,00E-50	84%	KJ722606.1
Predicted: <i>Sesamum indicum</i> galactinol synthase 1-like	209	209	4%	4,00E-50	82%	XM_011072789.1
Predicted: <i>Nelumbo nucifera</i> galactinol synthase 1-like	200	371	9%	2,00E-47	80%	XM_010244766.1
Predicted: <i>Nelumbo nucifera</i> galactinol synthase 1-like	200	371	9%	2,00E-47	80%	XM_010244765.1
<i>Camellia sinensis</i> galactinol synthase	196	196	5%	3,00E-46	80%	JX624168.1
<i>Camellia sinensis</i> cultivar Longjin43 galactinol synthase 1	195	195	4%	1,00E-45	81%	KF472133.1
<i>Pop. alba</i> x <i>Pou. grandidentata</i> galactinol synthase I	171	171	3%	2,00E-38	81%	JF499886.1
<i>Amborella trichopoda</i> hypothetical protein	169	169	4%	7,00E-38	78%	XM_006840664.1
<i>Solanum tuberosum</i> cultivar Umatilla putative galactinol synthase (GolS)	150	150	4%	3,00E-32	77%	GU596453.1
<i>Solanum commersonii</i> cultivar PI243503 putative galactinol synthase (GolS)	148	148	4%	9,00E-32	78%	GU596447.1
<i>Solanum commersonii</i> cultivar PI243503 putative galactinol synthase (GolS) gene	148	148	4%	9,00E-32	78%	GU596446.1
Predicted: <i>Solanum tuberosum</i> galactinol synthase 2-like	145	145	4%	1,00E-30	77%	XM_006346903.1
<i>Arabidopsis thaliana</i> chromosome 1 sequence	141	141	3%	2,00E-29	80%	CP002684.1
<i>Arabidopsis thaliana</i> chromosome I BAC F25P12 genomic sequence	141	141	3%	2,00E-29	80%	AC009323.4
<i>Arabidopsis thaliana</i> glycosyltransferase (AT1G56600)	135	135	3%	7,00E-28	79%	KJ138960.1
<i>Arabidopsis thaliana</i> galactinol synthase 2	135	135	3%	7,00E-28	79%	NM_104537.2
<i>Arabidopsis thaliana</i> At1g56600/F25P12_16 mRNA, Arabidopsis thaliana Full-length cDNA Complete sequence from clone GSLTSIL64ZG05	135	135	3%	7,00E-28	79%	BX818170.1
<i>Arabidopsis thaliana</i> At1g56600/F25P12_16 mRNA, complete cds	135	135	3%	7,00E-28	79%	AY050410.1
<i>Arabidopsis thaliana</i> AtGolS2 mRNA for galactinol synthase	135	135	3%	7,00E-28	79%	AB062849.1
<i>Populus trichocarpa</i> galactinol synthase family protein	128	128	4%	1,00E-25	77%	XM_002319437.2
<i>Populus trichocarpa</i> galactinol synthase 2-2	128	128	4%	1,00E-25	77%	HQ727694.1
PREDICTED: <i>Populus euphratica</i> galactinol synthase 2-like	122	122	4%	5,00E-24	76%	XM_011025526.1
<i>Populus trichocarpa</i> putative galactinol synthase family protein (POPTR_0002s19230g)	121	121	4%	2,00E-23	75%	XM_002301495.2
<i>Populus trichocarpa</i> galactinol synthase 8 (GolS8)	121	121	4%	2,00E-23	75%	KF496091.1
<i>Populus trichocarpa</i> x <i>Populus deltoides</i> galactinol synthase 4 (GOLS4)	121	121	4%	2,00E-23	75%	EU305724.1
<i>Arabidopsis thaliana</i> At1g56600/F25P12_16 mRNA	115	115	3%	9,00E-22	79%	AF412094.1
<i>Solanum lycopersicum</i> chromosome ch02, complete genome	111	111	4%	1,00E-20	75%	HG975514.1
<i>Populus alba</i> x <i>Populus grandidentata</i> galactinol synthase II (GolSII)	111	111	4%	1,00E-20	75%	JF499887.1
<i>Populus trichocarpa</i> galactinol synthase 2-1 mRNA	111	111	4%	1,00E-20	75%	HQ727693.1
Predicted: <i>Solanum lycopersicum</i> galactinol synthase 2-like (LOC101244339)	106	106	4%	5,00E-19	74%	XM_004233483.2
<i>Populus trichocarpa</i> galactinol synthase family protein (POPTR_0013s00720g)	106	106	4%	5,00E-19	75%	XM_002319436.2
<i>Ajuga reptans</i> mRNA for galactinol synthase, isoform GolS-2	73.1	73.1	1%	6,00E-09	79%	AJ237694.1
Predicted: <i>Camelina sativa</i> galactinol synthase 5-like	62.1	62.1	0%	1,00E-05	88%	XM_010437916.1

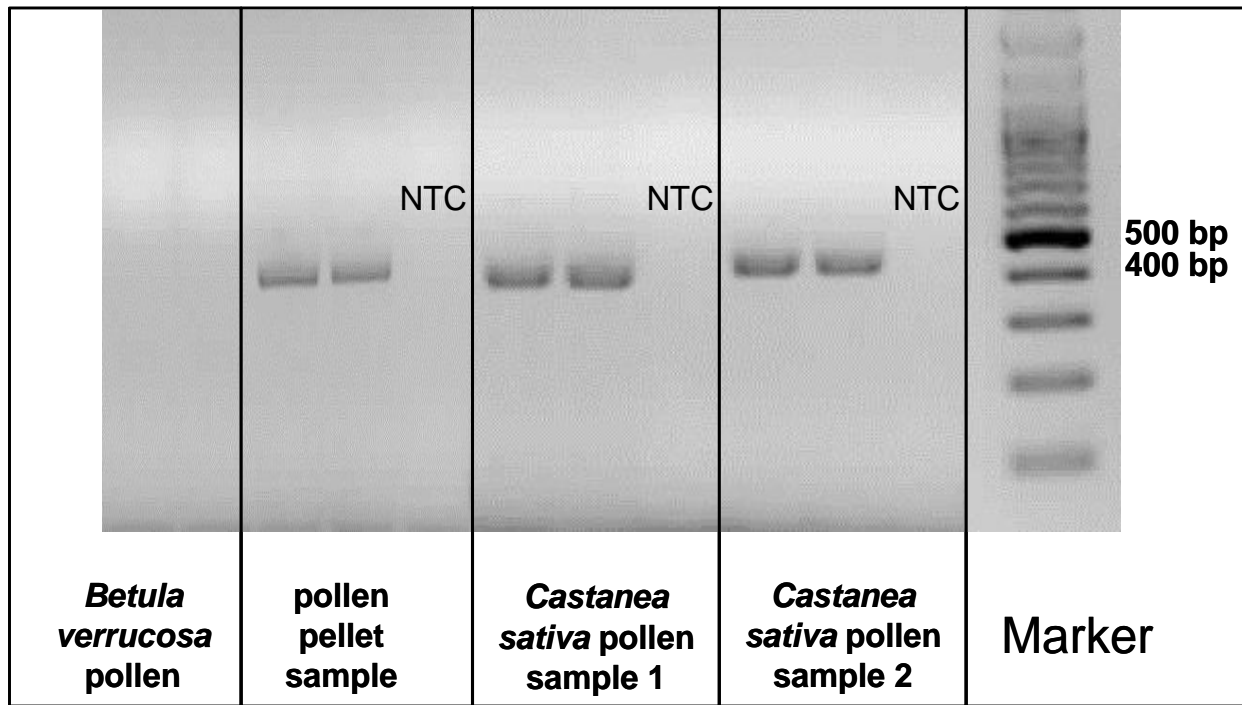


Figure 2 PCR amplification of conservative part of the Gos1S1 gene.

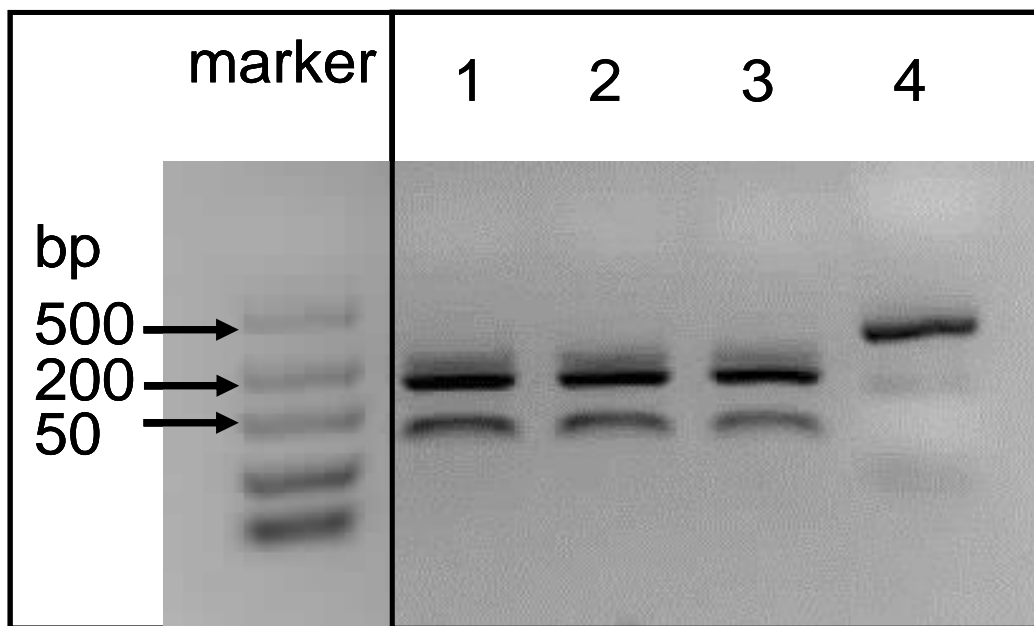


Figure 3 Restriction analysis of amplified fragment of Gos1S1 gene. 1 – pollen pellet sample; 2 – *Castanea sativa* pollen sample 1; 3 – *Castanea sativa* pollen sample 2; 4 – unrestricted fragment of pollen pellet sample.

The specific amplification of one fragment by PCR followed by gel electrophoresis for fragment size verification is the simplest strategy to evaluate the presence of a species (Mafra et al., 2000). In the standard PCR a single internal primer mismatch does not affect the PCR results seriously when other PCR conditions are optimized (Kwok et al., 1990; Christopherson et al., 1997). But chosen of the perfectly matched primers is one of the crucial steps in pollen DNA analysis, as the amount of template DNA can be extremely low and even a small mismatch can cause failing of the PCR (Ito et al., 2008).

For *Chenopodiace* Zhou et al., (2007) used internal transcribed spacer sequence (ITS) for DNA based identification of the pollen in the soil samples on the level of species. Here, using a bioinformatic alignment, specific primers were possible to be designed for the purposes of *Castanea sativa* identification in the DNA targeted sequences extracted from pollen samples.

The specificity of amplified fragments of the galactinol synthase (Gos1) gene of *Castanea sativa* was performed using the Hpy 166II restriction endonuclease. In total, three restriction fragments were obtained with the length

that corresponded to those of predicted by NEBcutter software (figure 3).

Nowadays, molecular and DNA based identification of different, not only plant species is a well-established method (Kántor et al., 2014; Židek et al., 2012). Molecular analysis that utilized the knowledge based on molecular genetics became an inevitable part of the research in many areas. Plant genome analysis are performed in a wide range of different approaches ranged from DNA markers based analysis (Petrovičová et al., 2015; Oslovičová et al., 2014; Trebichalský et al., 2013; Milella et al., 2011) up to the specific analysis of plant allergens detection or their expression (Revák et al., 2014; Ražná et al., 2014).

Pollen and pollen pellet samples are in the centre of scientific research in a wide range of different analysis – from antioxidant (Fatrovová-Šramková et al., 2013) through protein (Longhi et al., 2009; Bryce et al., 2010) and animal food supplements (Haščík et al., 2014) up to the allergenic ones (D'Amato et al., 2007; Alche', 2012; Ražná et al., 2014). Genotyping methods for pollen and single pollen are utilized in many areas including ecology, evolutionary and genomic research (Isagi et al., 2011). In plant population analyses, this technique will allow the estimation of pollen flow by directly tracking the actual movement of individual pollen grains (Paffetti et al., 2007). In genomic studies this technique will enable direct analysis of the haploid DNA sequence of single pollen grains (Zhou et al., 2007).

Here, a PCR based identification of *Castanea sativa*, Mill. was proved as effective when used in multifloral pollen pellets.

CONCLUSION

Bioinformatic approach can be used well in pollen DNA based species identification. It provides an efficient method for rapid species specific primers designing that work in PCR reliable. *Castanea sativa* pollen can be detected in complex pollen pellets samples by specific PCR amplification of galactinol synthase. Such as identification of *Castanea sativa* galactinol synthase is efficient in multifloral pollen pellet for *Castanea sativa* authentication purposes.

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TOTAL POLYPHENOL CONTENT AND ANTIOXIDANT CAPACITY OF COWPEA EFFECT OF VARIET AND LOCALITY

Ismael S. Dalaram

ABSTRACT

Leguminous seeds belong to plant foods which are generally rich in phenolic compounds. Cowpea seeds are a major source of plant proteins and vitamins for man, feed for animals. Polyphenolic compounds are secondary metabolites of amino acids present in many plant species, including legume. Their content depends on various factors, such as cultivar, pedoclimatic and cultivation conditions. The influence of cultivar, locality on the total polyphenols (TPC) and antioxidant activity (TAC) of Cowpea seeds was studied. Cowpea cultivars were cultivated under different climatic conditions in Iraq Republic. The main objective of the present work was to consider the changes of total polyphenols content in dependence on variety and to evaluate an antioxidant potential of three Cowpea varieties (white, light brown and red color) in different localities of Erbil City in Kurdistan Region Iraq and to evaluate the content of bioactive compounds (polyphenolics) in legumes commonly utilized in the human diet in Iraq, to compare their antioxidant capacity and to evaluate the influence of grown locality on observed parameters. Total polyphenols were determined by the Lachman's method and expressed as mg of Gallic acid equivalent per kg dry matter. Total antioxidant capacity was measured by the Brand-Williams method using a compound DPPH (2,2-diphenyl-1-picrylhydrazyl). Analysis of variance indicated significant differences ($p < 0.05$) among locality and color for phenolic contents and antioxidant capacity. The various varieties of white color cowpea had significant influence on TPC and TAC and affected by locality too. From tested seeds the highest polyphenol content was measured in red color ($802.323 \pm 15.937 - 825.700 \pm 8.494 \text{ mg.kg}^{-1} \text{ GAE}$). The lowest value was in white color ($480.195 \pm 15.286 - 721.952 \pm 25.004 \text{ mg.kg}^{-1} \text{ GAE}$). The similar trend was observed at values of TAC. The highest TAC value was determined in red color ($28.709 \pm 15.937 - 34.777 \pm 8.494\% \text{ DPPH}$). The lowest value was in white color ($6.065 \pm 0.836\% - 9.578 \pm 0.884\% \text{ DPPH}$). The various varieties had significant influence on TPC and TAC according to used statistical analyses. Correlation between the phenolic contents and antioxidant activity was significantly positive ($r = 0.783645$). Our results confirmed that legumes can be a good source of bioactive compounds in the human nutrition.

Keywords: Cowpea; total polyphenols; antioxidant capacity; locality

INTRODUCTION

Cowpeas (*Vigna unguiculata* L. Walp), are an important part of the staple diet in many developing countries since the earliest practice of agriculture. The increasing agricultural production became an urgent issue since projections suggest that the global population will reach 9 billion people by the middle of this century (Godfray et al., 2010). Cowpea is a major staple food crop in sub Saharan Africa, especially in the dry savanna regions of West Africa. Cowpea has found utilization in various ways in traditional and modern food processing in the world. Traditionally in Africa, cowpeas are consumed as boiled vegetables using fresh and rehydrated seeds or processed into flour to make other food products (Odedeji and Oyeleke, 2011) noted that flour produced from whole seeds presents better functional properties compared to the dehulled seed flour which is common practice in processing of cowpea. Cowpeas provide a rich source of proteins and calories, as well as minerals and vitamins. A cowpea seed can consist of 25% protein and is low in anti-nutritional factors (Angel et al., 2003). This diet

complements the mainly cereal diet in countries that grow cowpeas as a major food crop. The seeds are a major source of plant proteins and vitamins for man, feed for animals, and also a source of cash income. The young leaves and immature pods are eaten as vegetables. Cowpea can be grown under rainfed conditions as well as by using irrigation or residual moisture along river or lake flood plains during the dry season, provided that the range of minimum and maximum temperatures is between 28 and 30°C (night and day) during the growing season. Cowpea performs well in agroecological zones where the rainfall range is between 500 and 1200 mm/year. Cowpea and horse gram are low in fat and are excellent sources of protein, dietary fibre, a variety of micronutrients and phytochemicals with potential health benefits (Kadam and Salunkhe, 1985; Siddhuraju and Becker, 2007). The nutritional and functional properties of their flours are comparable to chickpea flour (Sreerama et al., 2012). Due to their favourable flour functionality and their phytochemical-associated health benefits, these flours offer an enormous potential for the production of legume

composite flours. Pulses have shown numerous health benefits, e.g. lower glycemic index for people with diabetes, increased satiation and cancer prevention as well as protection against cardiovascular diseases due to their dietary fiber content (Chillo et al., 2008). There is a dearth of information on the specific health beneficial components in these lesser known legumes with specific reference to their phenolic compounds on the regulation of oxidative stress and their influence on enzyme activities associated with hyperglycemia and hypertension. These insights may help to exploit the use of under-utilized legumes as ingredients in composite legume flours and functional foods to promote their use in disease risk reduction and overall health. The health-promoting effects of dehulled cowpea flours derived from phenolic compounds and other antioxidants make this legume a potential source of functional food ingredients. Phenolic compounds (tannins, flavonoids and phenolic acids) are secondary metabolites in plants and as such are present in some plant foods (Manach et al., 2004; Wu et al., 2006). Several studies have reported on antioxidant and antiradical activity of tannins (Amarowicz, 2007). Cowpea is known also as containing a low amount of fat and high level of fiber which can prevent heart disease by reducing the low-density lipoprotein (Phillips et al., 2003). In addition, cowpea consumption increases glucose blood more slowly because of the slowly digestibility of the legume starch promoting its usage for diabetics (Phillips et al., 2003). Knowledge of the genetic basis and heritability of these health beneficiary phytochemical profiles is essential for efficient development of new cultivars for food processing industries and breeders. Phenolic extracts have been reported to retard lipid oxidation in oils and fatty foods (Rodriguez et al., 2007) vascular disorders including diabetes and hypertension (Van der Zwan et al., 2010). Therefore, it is important to control both blood glucose level and cellular redox status for managing these diabetic complications. Alpha-Amylase and alpha-glucosidase are key enzymes involved in starch break down and intestinal glucose absorption (McDougall and Stewart, 2005). Phenolic compounds constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites and are responsible for various beneficial effects in a multitude of diseases (Soobrattee et al., 2005). These are believed to work synergistically to promote human health through their antioxidant properties and their ability to modulate the activity of various enzymes. These phenolics are also potent inhibitors of α -amylase and α -glucosidase, the two important enzymes involved in the regulation of glucose homeostasis (McDougall and Stewart, 2005).

MATERIAL AND METHODOLOGY

Material. Cowpeas (*Vigna unguiculata* L. Walp). Samples at full maturity were obtained from different localities in Erbil city, four varieties (C1, C2, C3, C4) were white color, two varieties (C5, C6) light brown and two varieties (C7, C8) red color were collected in Iraq.

Extraction. For 12 hours extraction, dry material (5 g) was used and continuously extracted by a Twisselmann extractor with methanol (80%, v/v).

Total polyphenols determination. Total polyphenols were determined by the method of Lachman et al. (2003) and expressed in mg eq. Gallic acid per kg dry matter. The total polyphenol content was estimated using Folin-Ciocalteu assay on the spectrophotometer Shimadzu 710 (Japan).

Total antioxidant capacity determination. The free radical scavenging activity of the extracts was measured using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method of Brand-Williams et al., (1995).

Statistical analysis. Processing of the results was carried out by software STATGRAPHICS. Procedure compares the data in 8 varieties. The F-test in the ANOVA test whether there are any significant differences amongst the means. If the P-value of the F-test is less than 0.05, there is a statistically significant difference between the means at the 95% confidence level; the Multiple Range Tests will tell which means are significantly different from which others. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure.

RESULTS AND DISCUSSION

On the base of reached results there were estimated changes in the total polyphenols content and also changes in total antioxidant capacity values in dependence on Cowpea varieties in different localities.

1 Evaluation of total polyphenol content and values of antioxidant capacity in white color Cowpea.

Following the total polyphenol content parameter in Cowpea white color (Figure 1, Table 1). According to the obtained results, the polyphenols content (TPC) in the tested was significantly different and was influenced by locality the highest value was reached in C1 (Shaqlawia) variety with $721.952 \pm 25.004 \text{ mg.kg}^{-1}$. When comparing this variety from total antioxidant capacity point of view, there was estimated value $9.578 \pm 0.884\%$. This is on the highest level of observed antioxidant capacity interval white color. For growing cowpeas are the best warm and dry areas. Therefore the C2 and C3 seeds have shown significantly lower TP content (these cultivars were grown in area suitable for growing cowpea). While the C1 variety, grown in mountain areas with higher rainfall, recorded higher amount of polyphenols. Lachman et al., (2006) investigated the effect of weather conditions on the TPC contents of potatoes and their results showed that an upland cooler site with higher rainfall provide the tubers with a higher content of TPC. C2 and C3 varieties were grown in a similar area condition, contain similar values of (TPC) 685.386 ± 40.956 , $678.751 \pm 40.348 \text{ mg.kg}^{-1}$ respectively and the lowest value Of (TPC) was found in C4 variety Koysinjak with $480.195 \pm 15.286 \text{ mg.kg}^{-1}$. Manach et al., (2004) noted that environmental and genetic factors have a major effect on polyphenols content. Our results suggest that severe climatic conditions have caused a slight increase in the total content of polyphenolic substances in white color by locality.

Table 1 Total phenolic content and antioxidant capacity (average and average deviation values) In chosen white Cowpea varieties after harvest in dray mass (mg.kg⁻¹ DM).

Varieties	Locality	Color	TPC (mg.kg ⁻¹)	TAC (%)
C1	Shaklawa	White	721.952 ±25.004	9.578 ±0.884
C2	Kalak	White	685.386 ±40.956	6.325 ±0.429
C3	Qushtapa	White	678.751 ±40.348	7.332 ±0.404
C4	Koysinjak	White	480.195 ±15.286	6.065 ±0.836

Data expressed as means of six replications ± standard deviation. Values in the same column with the different letters present significant differences *p* <0.05 using F-test for independent samples.

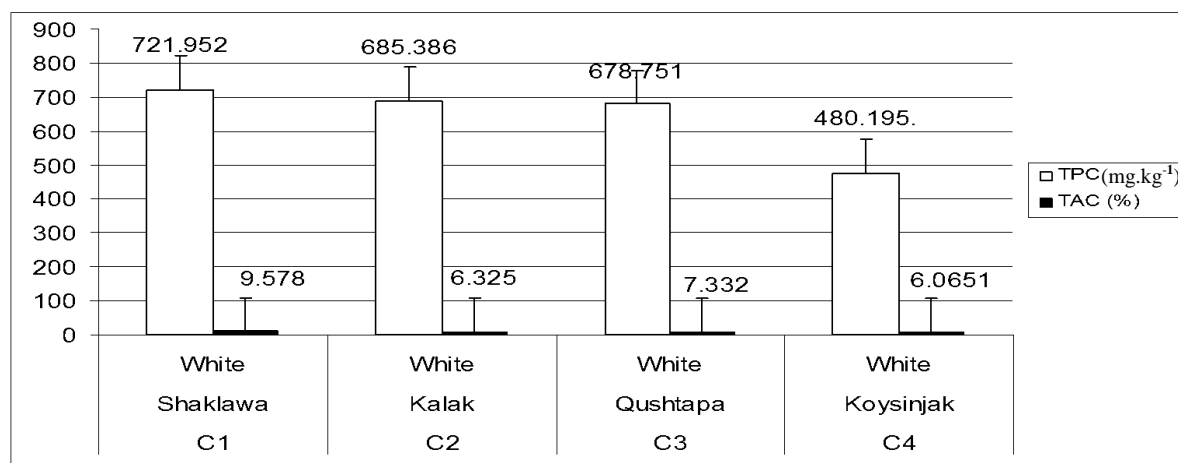


Figure 1 Average content of total polyphenols TPC (mg.kg⁻¹) and Average content of total antioxidant capacity TAC (%) in chosen white cowpea varieties arranged by locality.

When comparing legumes from the antioxidant capacity (TAC%) point of view (Figure 1 and 3; Table 3). *p*-value <0.05 there is a statistically significant difference between the means of the 4 variables at the 95.0% confidence level. The highest value was reached in case of C1 (shaqlawa) variety with the value 9.578 ±0.884% c. The lowest value was found in case of C4 (koysinjak) variety with the value 6.065 ±0.836% a, C2 and C3 varieties with the value 6.325 ±0.429% a, 7.332 ±0.404% b respectively. Our results suggest that antioxidant capacity was affected by location too.

2.1 Statistical evaluation of total polyphenol (TPC) content differences significance within of chosen varieties.

When comparing all cowpea varieties, following in Table 2, there were significant differences according to used statistical methods on the all three types (white, light brown, red) observed confidence levels between almost the all observed varieties. Effect of varieties analysis (Table 2, figure 2) for the phenolic compounds contents (TPC) of seeds flours of tested showed the presence of significant variety differences (*p* <0.05).

Table 2 Total phenolic content (average and average deviation values) in chosen Cowpea varieties after harvest in dray mass (mg.kg⁻¹DM).

Varieties	Locality	Color	TPC (mg.kg ⁻¹)		
			M ± SD	LSD I.	LSD II.
C1	Shaklawa	White	722.952 ±25.004	c	C
C2	Kalak	White	685.386 ±40.956	a	B
C3	Qushtapa	White	678.751 ±40.349	b	B
C4	Koysinjak	White	480.195 ±15.286	a	A
C5	Shaklawa	Red	825.703 ±8.493	c	E
C6	Kalak	Red	802.323 ±15.938	b	E
C7	Shaklawa	Light Brown	811.223 ±7.200	b	E
C8	Kalak	Light Brown	771.890 ±7.440	a	D

Notes: Data expressed as means of eight varieties ± standard deviation. Since the P-value of the F-test is less than 0,05, there is a statistically significant difference between the means of the 8 variables at the 95,0% confidence level. Values in the LSD I. column present significant differences using LSD tests among white varieties (3 homogenous groups) and among red and light brown varieties (3 homogenous groups). Values in the LSD II. column present significant differences using LSD tests among all varieties, 5 homogenous groups are identified.

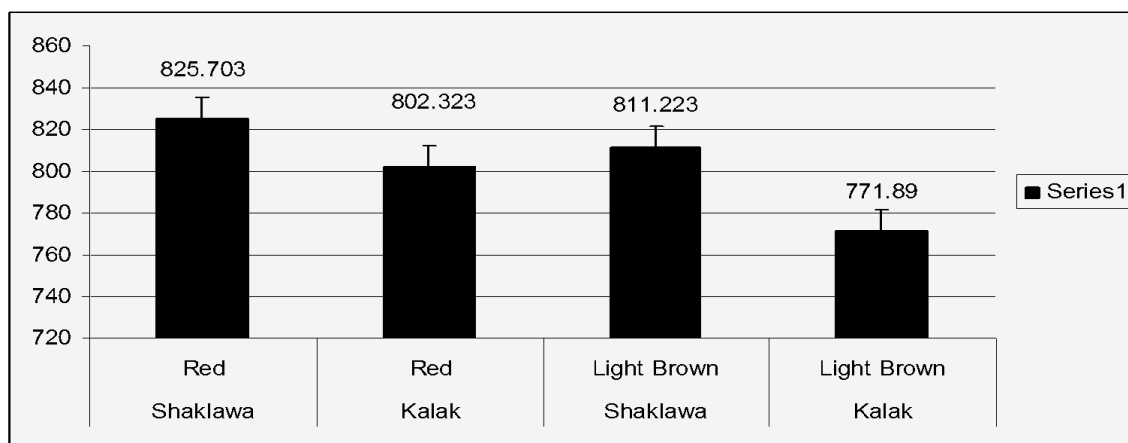


Figure 2 Average content of total polyphenols TPC (mg.kg⁻¹) in red and light brown color.

The TPC varied from 480.195 mg.kg⁻¹ (white color Koysinjak) to 825.700 mg.kg⁻¹ (red color shaklawa), our result similar, according to the results of Dalaram et al., (2013) recorded a range of (555.12 ±4.13 – 969.30 ±6.39) mg.kg⁻¹ for TPC in chickpea seeds. As shown in (Table 2 and 3) cultivar significantly affected phenolic accumulation and antioxidant capacity of flours from cowpea seeds. The cultivar shaklawa with a red seed coat, possessed the highest AOA and TPC levels followed by light brown seeds then in white color seeds. As shown in (Table 2, 3) According to Warington et al., (2002), Siddhuraju and Becker (2007), pigmented cowpea varieties had favorable factors that enhance AOA of seeds. The total polyphenolic compounds contents (TPC) in red color with the range (802.323 ±15.938 to 825.703 ±8.493) mg.kg⁻¹ higher than content (TPC) in light brown color with the range (771.890 ±7.440 to 811.223 ±7.200) mg.kg⁻¹ and the lowest contents (TPC) was found in white color with the range (480.195 ±15.286 to 722.952 ±25.004) mg.kg⁻¹. Papoulias et al., (2009) pointed out that polyphenols accumulation in plants is affected by genetic factors, environmental and cultural conditions and also various stresses (Krvacic et al., 2009), storage and cooking (Manach et al., 2004).

2.2 Statistical evaluation of antioxidant capacity (TAC) differences significance within the frame of chosen Varieties

The highest value was reached in case of red color (shaklawa red) varieties as it is (Figure 3, Table 3) in Table with the range (28.709 ±0.425 to 34.777 ±1.827 c) %. The lowest value was found in case of white color variety with the range (6.065 ±0.836 to 9.578 ±0.884) %, and for light brown color with the range (20.615 ±0.665 to 23.288 ±0.678) %. In comparison with results of Kavalcova et al., (2014), where the interval of statistically significant highest value of antioxidant activity was recorded in onion from (20.22 ±0.53 to 25.76 ±0.53) % and statistically significant the lowest value of antioxidant activity was recorded in garlic (from 4.05 ±0.20 to 5.07 ±0.47) %, our results in cowpeas light brown color with the range (20.616 ±0.665 to 23.288 ±7.199) % are similar. In comparison with results in variety in onion from (20.22 ±0.53 to 25.76 ±0.53) % of Kavalcova et al., (2014), our results in white color cowpeas with value range (6.065 ±0.836 to 9.578 ±0.884) % are higher than garlic of Kavalcova et al., (2014).

Table 3 Average content of total antioxidant capacity TAC (%) in chosen cowpea varieties (average and average deviation values).

Varieties	Locality	Color	TAC (%)		
			M ±SD	LSD I.	LSD II.
C1	Shaklawa	White	9.578 ±0.884	c	C
C2	Kalak	White	6.326 ±0.429	a	a, b
C3	Qushtapa	White	7.333 ±0.404	b	B
C4	Koysinjak	White	6.065 ±0.836	a	A
C5	Shaklawa	Red	34.778 ±1.828	c	F
C6	Kalak	Red	28.696 ±0.401	b	E
C7	Shaklawa	Light Brown	23.288 ±7.199	b	D
C8	Kalak	Light Brown	20.616 ±0.665	a	D

Notes: Data expressed as means of eight varieties ± standard deviation. Since the *p*-value of the F-test is less than 0,05, there is a statistically significant difference between the means of the 8 variables at the 95,0% confidence level. Values in the LSD I. column present significant differences using LSD tests among white varieties (3 homogenous groups) and among red and light brown varieties (3 homogenous groups). Values in the LSD II column present significant differences.

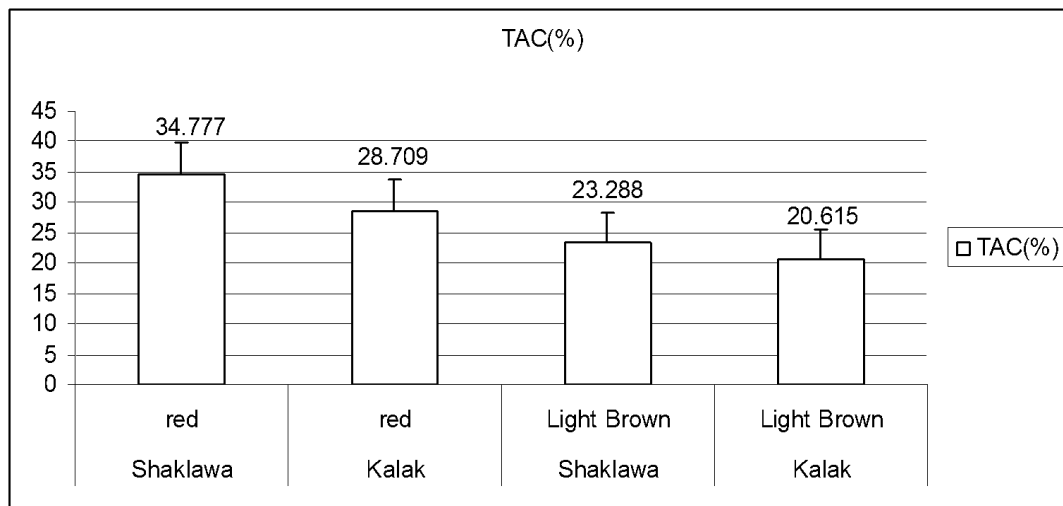


Figure 3 Average content of total antioxidant capacity TAC (%) in red and light brown varieties using LSD tests among all varieties, 6 homogenous groups are identified.

Significant variation of the AOA among varieties was also pointed out for many grains (Adom and Liu, 2002), for wheat flour (Akond et al., 2010) and for common bean (Golam et al., 2011). For fababean (Dalaram et al., 2013), with the range 16.62 ± 0.81 to $24.54 \pm 0.64\%$. Mokgope (2007) noted that in general, the efficacy of phenolic constituents as antioxidants depends on factors such as the number of hydroxyl groups bonded the aromatic ring, the site of bonding, mutual position of hydroxyls in aromatic ring and their ability to act as hydrogen or electron donating agents and free radical scavengers.

2.3 Correlation between the total antioxidant activity values and total phenolics contents:

ANOVA linear correlation coefficients were used to assess the relationships between TPC and TAC. Correlation: Our result confirmed a strong statically

correlations between total polyphenol content and total antioxidant capacity values. A statistically strongly significant correlation ($R = 0.783645$; $p < 0.05$) was found (Figure 5). Amarowicz et al., (2005) analyzed the extracts of fababean, broad bean, adzuki bean, red bean, pea, red lentil and green lentil seeds using 80% (v/v) acetone and confirmed a statistically significant correlation between the total antioxidant activity values and total phenolics ($p = 0.01$). A strong correlation between total polyphenol content and antioxidant activity ($R = 0.86$; $p < 0.05$) was observed also by Akond et al., (2011) in common bean and a statistically strongly significant correlation (P -value $2.391E-06$; $R = 0.802$) was found between total polyphenol content and total antioxidant capacity values by Dalaram et al., (2013) in lentil. According these authors this finding suggests that total polyphenol content is a good predictor of in vitro antioxidant activity.

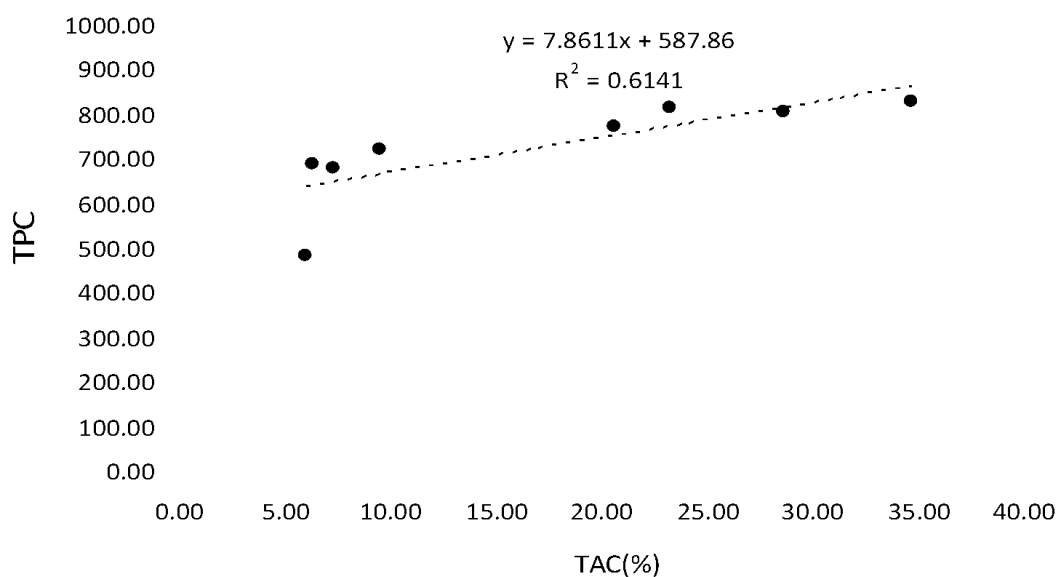


Figure 4 Correlation between TP and TAC.

CONCLUSION

Cultivar significantly affected phenolic accumulation and antioxidant capacity of flours from cowpea seeds. The present study showed that there are also differences in these traits among red, brown and white cowpea cultivar. The Shaqlawa cultivar with red seed coat possessed the higher AOA and TPC levels than light brown color seed coat and white color seeds cowpea. The positive interrelationship between these two parameters demonstrates that the antioxidant activity depends mainly on polyphenols contents. Based on our results the followed order of total polyphenol content as well as total antioxidant capacity in investigated cowpea obtained from Iraq can be created: red color cowpea >light brown color cowpea >white color cowpea. The present study of cowpea is also affected by locality. Our results confirmed that legumes can be a good source of bioactive compounds in the human nutrition. The consumption of cowpea provides potential nutraceuticals for human health. Prevention of degenerative diseases associated with free radical damage, in addition to their traditional role of preventing protein malnutrition.

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ASSESSMENT OF WELFARE AND EGG PRODUCTION OF LAYING HENS MORAVIA SSL IN SMALL-SCALE BREEDING

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ABSTRACT

The purpose of this study was to investigate the welfare of laying hens Moravia SSL housed in small-scale hen house with free range, behavior, egg production and selected physical indicators of eggs and chemical indicators of egg mass. The laying hens were kept in a hen house on deep litter. Breeding facility of hen house was within the meaning of recommendation for applying the principles of welfare, i.e. the space and breeding facility within the meaning of enriched breeding environment. Stocking density of the laying hens corresponded with recommendations for unrestricted movement and implementing natural activities. The hen house was equipped with the perch, nest, feeder and drinker. The commercial feed mixture was used for feeding, which is intended for laying hens. The kitchen remains were added to feed mixture, as are wet bread, the non-edible remains of foodstuffs. A feed mixture was served to laying hens 825 g per day. The laying hens had free access to drinking water, grazing, ground pecking, ground scratching and dust-bathing and in the free range. We focused investigation of on the egg laying intensity, selected parameters of physical egg quality and chemical egg contents. Time to relax of laying hens was adjusted according to the summer and winter breeding seasons. The main activities of free-range hens are grazing, ground pecking, ground scratching and dust-bathing. The main activities of free-range hens are grazing, ground pecking, ground scratching and dust-bathing. These activities were investigated in laying hens too in dependent of year period, more in the summer. Housing of the hens was equipped with the perch. The laying hens regularly used a perch. A beginning of occupation the perch was at the time of time growing dark, at the end of the light day. A nesting material was selected regular, monthly exchange. It was meadow hay of excellent quality for the collection of high quality and safe eggs from nests in the hen house. The laying hens had unlimited access to feed and water. The commercial feed mixture was available *ad libitum* and fresh kitchen remnants were added in a small amount, provided that they fed in the short term, so that not subject to harmful degradation process. The management of our experiment was scheduled at age of laying hens 30 to 90 weeks. The egg laying intensity was observed 50% in a moment of the experimental 1st week (age of laying hens 30 weeks). High egg laying intensity of laying hens was at their age between 39 to 63 weeks. At the end of the experiment, at age of laying hens 90 weeks was decreased egg laying intensity at 26.19%, which represents almost half of egg laying intensity recorded at the beginning of the experiment. An indicator of egg production in the our experiment was studied under defined conditions for small-scale breeding, i.e. in alternative production system with free range, under defined conditions of nutrition and timing of investigation more than one year, from the end of October to the end of December of the following year. The results of our experiment can be related to the season, months of the year. The laying hens laid the eggs by individual weeks 33-90 weeks of age about average weight from 57.5 to 75.0 g. The fat content of an egg mass was 11.3 g.100⁻¹ and protein 12.39.g.100⁻¹. The table eggs from conditions of small-scale breeding are an important source of foodstuffs for the population, especially in rural areas. It must be given to this source of table eggs for human nutrition the highest quality and health safety too.

Keywords: Moravia SSL; small-scale breeding; welfare; production; table egg

INTRODUCTION

The European Commission has issued the **Council Directive 1999/74/EC**, which prohibits conventional the battery cages for laying hens from January 1, 2012 onward (**European Communities, 1999**). This Directive relates to welfare of the laying hens. From then on, the furnished cages and the alternative non-cage systems, such as aviaries and the floor systems, the conventional cages housing was replaced for the purpose of to improve the

welfare of layers (**Tauson, 2005**). Yet, limited information is available about the consequences of alternative housing systems on transmission of zoonotic pathogens, such as *Salmonella enterica* serovar *Enteritidis*, in hens housed in these systems. Concerns were raised about the decreased hygienic status found in alternative housing systems, which could result in an easier spread of infectious agents (**Duncan, 2001; EFSA, 2005**). Most epidemiological data showed a higher prevalence of *Salmonella* in layer flocks

housed in conventional cages compared with flocks housed in alternative systems (Methner et al., 2006; EFSA, 2007; Snow et al., 2007, 2010; Namata et al., 2008; Huneau-Salaün et al., 2009; Van Hoorebeke et al., 2010), with some exceptions (Schaar et al., 1997; Mollenhorst et al., 2005; Pieskus et al., 2008).

The purpose of this study was to investigate the welfare of laying hens Moravia SSL housed in small-scale hen house with free range, behavior, egg production and selected physical indicators of eggs and chemical indicators of egg mass.

MATERIAL AND METHODOLOGY

Object investigation

As a material were used the laying hens Moravia SSL to investigation. The laying hens were housed in alternative production system with free range in the small-scale conditions. Laying hen Moravia SSL is interlineal hybrid Rodajlenky with low proportion of Leghorn.



Picture 1 Laying hens Moravia SSL in hen house (Polačková, 2012).

Housing conditions

The laying hens Moravia SSL were kept in a hen house on deep litter. Breeding facility of hen house was within the meaning of recommendation for applying the principles of welfare, i.e. the space and breeding facility within the meaning of enriched breeding environment. Stocking density of the laying hens corresponded with recommendations for unrestricted movement and implementing natural activities. The hen house was equipped with the perch, nest, feeder and drinker. As litter material was used a wheat straw. The commercial feed mixture was used for feeding, which is intended for laying hens. The kitchen remains were added to feed mixture, as are wet bread, the non-edible remains of foodstuffs (fresh: potato peel, peel of carrots etc.). Water was exchanged in the summer three times daily and in the winter once daily.

The experiment lasted from October 29, 2011 to December 31, 2012. There were six birds in the experiment. We served a feed mixture to laying hens 825 g per day. The laying hens had free access to drinking water, grazing, ground pecking, ground scratching and dust-bathing and in the free range. We focused investigation of on the egg laying intensity, selected parameters of physical egg quality and chemical egg contents.



Picture 2 Free range next to hen house (Polačková, 2012).



Picture 3 Natural activity of laying hens – dust-bathing (Polačková, 2012).



Picture 4 Laid eggs in the nest box (Polačková, 2012).

Egg collection was carried each day at 9.00 am, to determine the egg weight, regular one day per week (Saturday) and to chemical analysis of the egg contents, once (n = 10 pcs of eggs) at age of laying hens 60 weeks.

Indicators of production

The following indicators were observed in the production of laying hens type Moravia:

- the number of laid eggs every day, at age of laying hens 30 to 90 weeks, which was the basis for the computation of the intensity of egg production (the number of laid eggs, the number of laying hens and the number of days of investigation),

- selected physical characteristics of the eggs: egg weight, egg albumen weight, egg yolk weight and egg shell weight of the egg samples collected weekly (Saturday) and weighted on the instrument type Kern 440-49N ECB with an accuracy of $\pm 0.1 \text{ d} = \text{g}$, the weight of the albumen was calculated based on the weight of the egg, reduced by the sum of the weight of yolk and shell,

- selected chemical parameters egg content:

- dry matter content in the JR Selecta oven, at 105 °C,
- protein content by the Kjeldahl method on a laboratory instrument Kjeltec™ 8200,
- fat extraction method for an agent petroleum ether to a laboratory instrument type DET-GRAS N,
- the average body weight of laying hens aged between 30 and 90 weeks on the instrument type Kern ECB 20K20 with an accuracy of $\pm d = 0.1 \text{ g}$.

Raw data were evaluated according to the elementary of statistical characteristics.

RESULTS AND DISCUSSION

Behaviour of laying hens

Welfare of the laying hens can be threatened by many external breeding factors. It is important to know the cause of their possible occurrence and action to their prevention. Laying hens can develop several kinds of foot injuries related to the surfaces on which they stand and walk (Lay et al., 2011). Ulcerative pododermatitis is seen most often in hens housed in litter based systems because of the presence of wet litter and feces. Environments can further compromise animal welfare if they promote the multiplication and spread of pathogens and parasites. Pathogens can be in litter, feed and soil of range area (Fraser et al., 2013). Production efficiency is often compromised when air quality is poor; for example, feed efficiency of chickens decreases at ammonia levels of 25-60 parts per million (ppm) (Quarles and Kling, 1974; Beker et al., 2004). Parasites were not observed in laying hens Moravia SSL during the experimental period. There was not a disease and mortality there. The hen house was opened daily at 7:30 am and closed in the summer at 9:00 pm, in winter after dusk, i.e. 5:00 pm. Time to relax of laying hens was adjusted according to the summer and winter breeding seasons. The main activities of free-range hens are grazing, ground pecking, ground scratching and dust-bathing. The extent of these is weather-dependent (Hughes and Dun, 1983). These activities were investigated in laying hens Moravia SSL too in dependent of year period, more in the summer. Domestic fowl kept in non-cage systems prefer to roost on high perches at night if these are provided (Blokhuis, 1983, 1984), but standard cages prevent this behaviour. Housing of the hens Moravia SSL was equipped with the perch. The laying hens regularly used a perch. A beginning of occupation the perch was at the time of time growing dark, at the end of the light day. The laying hens show strong motivation to gain access to perches for roosting, and the hens accustomed to roosting show signs of frustration when they are denied access to perches (Olsson and Keeling, 2002). Hence, prevention of roosting is likely to be another cause of frustration. On the basis of such research, there is a growing trend for animal welfare standards to require that hens be allowed to perch and nest (Fraser et al.,

2013). According to Peter et al. (1986), light regime is an important factor that significantly affects the development, growth and production, as well as indirectly viability of laying hens. This information was important for us to investigation of the behaviour of laying hens Moravia SSL in the hen house during the night and during the day especially in the free range. The laying hens rested in the hen house during the night, and during day had unlimited space in free range for move not only to feed and water, but also to dust-bath and scratching. The laying hens well acclimatized to temperature of external environment, which manifested to the viability and health of these laying hens. Hrnčár and Civaň (2005) underlined in their study that at high ambient temperatures fail in the laying hen thermoregulatory system, it means that failed heat release. A body overheats, i.e. hyperthermia. In this case, there is water intake increased and feed intake decreased in the laying hens. This negative effect is reflected in the reduction to stop egg production. In our experiment, the laying hens Moravia SSL was not affected by these variables feed intake, or egg production. A frequent source of infection is the dustiness of environment and non-compliance with hygiene of the drinkers and feeders (Peter et al., 1986; Tittl, 2010). We addressed more attention to investigation of breeding environment hygiene on the basis of literary knowledge, in the hen house as well as in the free range. Housing space was regularly was regularly cleaned and litter exchanged. For non-compliance of hygiene and the welfare principles in the breeding of laying hens in relation to the reduction in egg production also reminded O'Connor et al., (2011). Even, Musgrove et al., (2012) state that failure to comply with environmental hygiene breeding of hens, namely the nest, is a reservoir for Salmonella. Red mite (*Dermanyssus gallinae*), a nest-dwelling parasite of chickens that can cause anemia and deaths, resides in cracks and crevices in the bird's environment is especially common in non-cage systems (Chauve, 1998; Lay et al., 2011). We selected regular, monthly exchange nesting material, meadow hay of excellent quality for the collection of high quality and safe eggs from nests in the hen house. The hay corresponded with organoleptic indicators of compliance with the terms of excellent hay, i.e. a smell, a color and the impurities. In domestic fowl, a strong motivation to perform 'nest-building' behaviour is triggered by hormonal events at ovulation, 24 h earlier (Wood-Gush and Gilbert, 1973). Under natural conditions, the hen separates from the flock 60-90 min before an egg is laid, seeks and enters a nesting site, and then performs nest-building activity (Duncan and Kite, 1989). The laying hens Moravia SSL had unlimited access to feed and water. Zelenka et al., (2006) in their study indicated that the animal receives enough food to satisfy their need for energy content. The feed mixture of laying hens Moravia SSL was available *ad libitum* and fresh kitchen remnants were added in a small amount, provided that they fed in the short term, so that not subject to harmful degradation process. Even Hrnčár (2006) notes if egg production is intensifying, egg protein production is increased proportionally. The author explains the important of protein need in feed mixture of laying hens. Therefore, the crude protein content 160 g.kg⁻¹ is important in feed

mixture of laying hens. The feed mixture in our experiment was obtained from the official commercial feed companies. The feed mixture was in accordance with the requirements of with the Code of feed. The feed mixture was composed of suitable feed raw material and fulfils the conditions to ensure saturation with essential nutrients and energy of laying hens Moravia SSL. Nutrition is an important factor that influences not only the egg production of laying hens, but also their health. Similarly, as feed and water quality, the role of mineral element supply should not be overlooked (Anyanwu et al., 2008). Calcium and phosphorus are essential macro minerals. They are forming a significant component of the egg shell and phosphorus playing an important role in skeletal calcium deposition (Frost and Roland, 1991) and subsequent availability of calcium for egg shell formation (Boorman et al., 1989). According to many literary knowledge is known that to feed of laying hens are used biologically active materials such as the enzymes (Hůrka, 2010), to prevent the growth depression in terms of additional fat oxidation, or the antioxidants (Kočí and Kočiová, 1999). Even Mazzuca et al., (2011) recommend adding to a feed mixture an alternative feed containing soybean hulls to increase egg production. With this in mind, our experiment was carried out in conditions of small scale breeding and available fresh kitchen remains were available, these were used in addition to the feed mixture.

Egg production

Sexual maturity means the age of the poultry in laying the first egg or the average age of the population in the achievement of 50% lay eggs. Already in 1981, was published study (Ledeč, 1981), according to which sexual maturity of laying hens can be affected especially nutrition, but also by other factors.

Light breeds of hens begin egg laying at age 150 to 170 days, combined breeds aged 160 to 180 days. Halaj and Chmelničná (1983) reported that after reaching sexual maturity, the egg laying weekly doubles and the laying hens reach a peak (90 to 92%) between weeks 27 to 34 their age. Then the egg laying decreases to 55 to 65%, at age of the laying hens 72 to 82 weeks. The management of our experiment was scheduled at age of laying hens Moravia SSL 30 to 90 weeks. The egg laying intensity was observed 50% in a moment of the experimental 1st week (age of laying hens 30 weeks). Egg laying intensity 90 to 92% was not reached in the small-scale breeding conditions. In contrast, there are more data on egg laying hens with a statement Halaj and Chmelničná (1983). High egg laying intensity of laying hens Moravia SSL was at their age between 39 to 63 weeks. At the end of the experiment, at age of laying hens Moravia SSL 90 weeks was decreased egg laying intensity at 26.19%, which represents almost half of egg laying intensity recorded at the beginning of the experiment. Roubalová (2011) characterized an egg laying intensity as very variable

Table 1 Egg laying intensity % according to weeks and for total experimental period.

Age of laying hens, weeks	Egg laying intensity, %	Age of laying hens, weeks	Egg laying intensity, %	Age of laying hens, weeks	Egg laying intensity, %	Age of laying hens, weeks	Egg laying intensity, %
30	50.00	46	54.76	62	45.24	78	23.81
31	66.67	47	59.52	63	73.81	79	21.43
32	61.90	48	61.90	64	59.52	80	23.81
33	59.52	49	64.28	65	50.00	81	23.81
34	71.43	50	73.81	66	50.00	82	21.43
35	59.52	51	64.28	67	54.76	83	21.43
36	64.28	52	66.67	68	54.76	84	23.81
37	64.28	53	61.90	69	50.00	85	23.81
38	61.90	54	71.43	70	33.33	86	19.05
39	73.81	55	69.04	71	23.81	87	26.19
40	54.76	56	69.04	72	26.19	88	19.05
41	59.52	57	61.90	73	30.95	89	19.05
42	69.04	58	64.28	74	26.19	90	26.19
43	57.14	59	64.28	75	26.19		
44	50.00	60	61.90	76	30.95		
45	45.24	61	50.00	77	26.19		

\bar{x} 48.24

\bar{x} - mean

indicator that can be influenced by various factors. An indicator of egg production in the our experiment was studied under defined conditions for small-scale breeding, i.e. in alternative production system with free range, under defined conditions of nutrition and timing of investigation more than one year, from the end of October to the end of December of the following year. The beginning of the experiment was scheduled from age of laying hens Moravia SSL 30 weeks and winter feeding period. Thereby that has been extended investigation of egg laying intensity at laying hens Moravia SSL, our interest was concentrated to quality of this indicator in relation to breeding of the laying hens in the small-scale conditions. Typically, the egg production in the large-scale farms by **Halaj et al., (2002)** is described as the laying cycle 12 months for effective hybrids. The egg laying cycle by **Verhoef-Verhallen and Rijs (2003)** is finished with molting and after this period follows a phase of natural physiological rest. After molting, i.e. exchange of the feathers, the laying hen begins to lay the eggs and started the second a laying cycle. This laying cycle is characterized by lower egg production but egg weight is higher. In our experiment, we observed only the first laying cycle, nevertheless that investigation continued until the age of laying hens 90 weeks. There are the molting at these hybrids Moravia SSL was not observed. There was not molting process carried out. What was interesting in the investigation of egg production, sharply

their number was decreased in the 70th week of age laying hens Moravia SSL. This age of laying hens falls into the autumn period. The results of our experiment can be related to the season, months of the year. Because the laying hens were daily in the free range, the influence of the season could also manifests in the egg production. And of course, a hybrid of hens and nutrition are important. **Pospíšilová (2011)** reported that poultry has several specifications of the digestive tract. One of these characteristics is the crop, which is used for the collection of feed. Specificity is also the gizzard, which mechanically processed feed by small stones. Respecting of specifics of laying hens Moravia SSL and enabling of free movement in the free range, there was created the conditions for the implementation of natural activities, such as scratching, pecking and dust-bathing. **Verhoef-Verhallen and Rijs (2003)** emphasize a need of grit addition or egg shell. They must be separately in a dish. These additions to feed facilitate of mechanical processing of the feed and subsequent enzymatic utilization of nutrients. The authors also recommend the use of green fodder to feed mixture for laying hens. They also state that the laying hens can intake the green feed in the free range. The laying hens can rake and search small insects, such as earthworms, snails, larvae, and etc. Commercial brown-egg laying hens have the genetic potential to produce 26.5 kg egg mass per hen (405 eggs with average weight of 65.5 g) in a prolonged laying period of 16 months, with a feed conversion ratio of

Table 2 Average egg weight according to weeks of laying hens during experimental period.

Age of laying hens, weeks	\bar{x} , g	Age of laying hens, weeks	\bar{x} , g	Age of laying hens, weeks	\bar{x} , g	Age of laying hens, weeks	\bar{x} , g
33	70.00	49	65.00	65	65.00	81	75.00
34	72.00	50	65.00	66	66.66	82	-
35	61.25	51	68.00	67	71.25	83	70.00
36	63.33	52	71.25	68	67.50	84	65.00
37	66.25	53	75.00	69	65.00	85	72.50
38	71.25	54	65.00	70	72.50	86	70.00
39	71.25	55	69.00	71	70.00	87	70.00
40	60.00	56	62.50	72	72.50	88	70.00
41	63.75	57	72.50	73	75.00	89	70.00
42	65.00	58	65.00	74	67.50	90	70.00
43	63.33	59	66.25	75	70.00		
44	57.50	60	67.00	76	72.50		
45	62.50	61	61.66	77	75.00		
46	65.00	62	71.25	78	67.50		
47	60.00	63	68.33	79	-		
48	60.00	64	70.00	80	70.00		

\bar{x} 65.44

\bar{x} - mean

2.1 kg feed per kg egg mass (Lohmann Tierzucht, 2011). The genetic potential of birds to efficiently convert feed nutrients into eggs and poultry meat for human consumption can be fully exploited when birds are well managed, remain healthy and receive highly digestible, concentrated and well-balanced feed rations (Jeroch et al., 2013). The challenges for commercial poultry diets formulation are to optimize feed composition based on limited choice of raw materials and limited inclusion rates, and to eliminate antinutritional factors. In our experiment, fresh kitchen remnants were added to feed mixture on the basis of literary knowledge by Malík (1995). Such regulation of feed ration increases a tastiness and utilization of the nutrients from feed. The laying hens take feed from two longitudinal, wooden feeders on the floor in the hen house. Width feeders allowed simultaneously to intake of feed from feeders for all laying hens.

Selected physical indicators of egg quality

Average egg weight according to experimental weeks

In the production of table eggs attention is paid to physical variables in relation to their quality (Halaj et al., 2002), which include the weight of the eggs. The laying hens Moravia SSL, according to the results of our experiment, laid the eggs by individual weeks 33-90 weeks of age about average weight from 57.5 to 75.0 g. At present, the farms are used types of hybrid combinations, such as the

Isa Brown, in contrast to the SSL Moravia laying hens, which were used in our experiment. Isa Brown is hybrid combination of color sexing type with a lower live weight. In experiment of Angelovičová et al. (2013) these laying hens reached an average egg weight during whole cycle 62.30 g. A slight increase of eggs can be achieved adjusting the diet. The feed additives may be. Arpašová et al. (2012), Angelovičová et al. (2013), found a tendency to increase egg weight after application of probiotics in the feed of laying hens compared to the control group. Differences between groups were not statistically significant ($p < 0.05$).

Average body weight of laying hens Moravia SSL

At the beginning of our experiment reached laying hens Moravia SSL aged 30 weeks on average body weight 1.92 kg and at the end of the experiment at age 90 weeks 3.08 kg. In hens were not observed in the incidence of parasites in the lips, disease and death.

Selected physical and chemical parameters of egg quality at age of laying hens 60 weeks

When laying hens Moravia SSL achieved 60 weeks of age, it was selected 10 pcs of eggs to determine the weight of the individual egg components and protein and fat of egg mass.

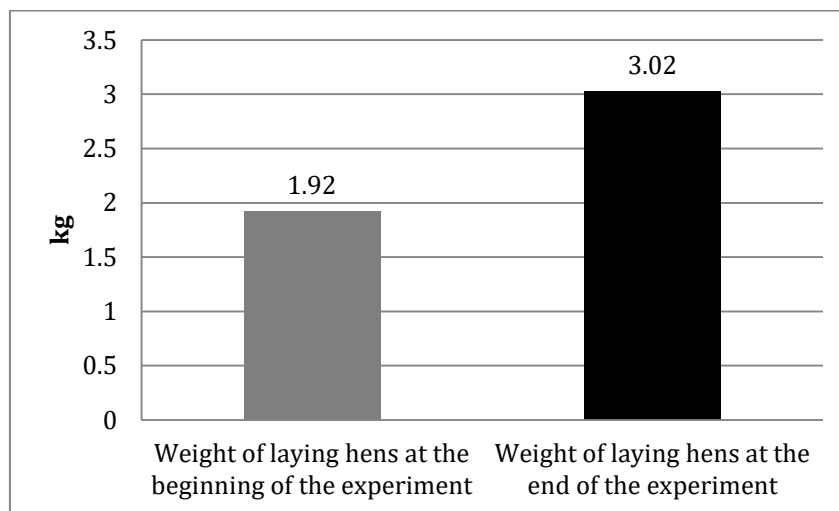


Figure 1 Average body weight of laying hens Moravia SSL.

Table 3 egg weight, albumin weight, yellow weight and eggshell weight of laying hens Moravia SSL at age 60 weeks.

Indicators	\bar{x}	SD	c_v , %
Egg weight, g	65.46	3.26	4.98
Albumin weight, g	41.21	2.12	5.14
Yellow weight, g	17.82	0.75	4.21
Eggshell weight, g	6.27	0.31	5.01

\bar{x} - mean, SD - standard deviation, c_v - coefficient of variation

Table 4 Content of crude protein and fat in egg mass.

Indicators	\bar{x} , g.100 ⁻¹	SD, g.100 ⁻¹	c _v , %
Dry matter content	25.08	0.21	0.86
Fat content	11.30	0.31	2.75
Crude protein content	12.39	0.26	2.13

\bar{x} - mean, SD - standard deviation, c_v - coefficient of variation

Age of laying hens 60 weeks **Halaj et al. (2002)** indicate as the second phase of the laying cycle, namely, it is the second half of the second phase of the laying cycle. Eggs are classified as the “food protein group”. Eggs contain high quality protein, with 100% of chemical score (essential amino acid level in a food protein divided by the level found in an “ideal” food protein), 97% of egg protein being digestible and 94% of biologic score (a measure of how efficiently dietary protein is turned into body tissue) (**McNamara and Thesmar 2005; WHO/FAO/UNU, 2007**). The eggs are an important source of nutrients due to their high quality protein (**Samooel et al., 2011**). The eggs are low in fat (5.3 g fat per egg). Overall, 50 g edible portion of a boiled egg has an energy value of 324 kJ (77.39 kcal) and the consumption of one egg daily would contribute only around 5% of the average energy requirement e.g. of a child aged 6 years in a 5.86-6.70MJ (1400 to 1600 kcal) diet (**Kliegman et al., 2011; USDA 2012**). In terms of preservation of hygienically safe content egg, an egg mass, it is increasing attention paid to the egg shell quality as to natural packaging. Many factors have been found to affect eggshell quality, such as disease, nutritional status of the flock, heat stress and age (**Roberts, 2004**). A decrease in eggshell quality of older hens has been reported by **Elaroussi et al., (1994)**. Egg production rate decreases and egg weight increases as age advances, also egg composition change and shell thickness decrease with production level and age of laying hens (**Summers and Leeson, 1983; Seeland et al., 1995; Machal and Simeonovova, 2002**). An egg shell quality is affected by many factors. **Halaj et al., (2002)** divided into several groups, such as physiological, pathological, nutritive factors, and breeding environment. The quality of the egg shell plays an important role in the production of eggs in small scale conditions for the application of the principles of welfare. Laying hens Moravia SSL old 60 weeks in our experiment achieved an average weight of egg shells 6.27 g, with variation of the values expressed as a coefficient of variation 5.01%. **Halaj et al., (2002)** points out that the biggest effect on the egg shell quality has from environmental factors mainly mineral nutrition of laying hens (calcium, phosphorus), especially at the end of egg laying. Egg shell formation by these authors is formed 19 to 21 hours. Because the egg shell is a natural packaging of an egg contents, it plays an important role in maintaining its hygiene and health safety. An interesting knowledge of an egg whites indicate **Velíšek and Hajšlová (2009)**, that the fraction ovotransferin exhibits antimicrobial effects. The egg proteins consist, essentially, 40 different proteins, among which a significant antimicrobial activity has lysozyme (**Rao et al., 2012**).

The average weight of egg white is 41.21 g according to our measurements for whole experimental period. Egg yolk is an important source of highly nutritional and functional ingredients in a wide variety of food products (**Jaekel et al., 2008**). The content of individual nutrients in egg yolk can be influenced by various factors Nutrition (**Kočí and Kočiová; 1999 Kotrbáček, 2010**). When laying hens Moravia SSL achieved an age 60 weeks, a weight yolk their eggs was 17.82 g.

The fat content of an egg mass was 11.3 g.100⁻¹ and protein 12.39.g.100⁻¹. These two indicators of the chemical composition of an egg masses are significant in terms of nutritional value of the egg. The aim of poultry nutrition is to convert feed protein to egg and poultry meat proteins. According to calculations, the conversion ratios of dietary protein to edible protein are 33% in eggs (opposite 26% in broiler meat) (**Jeroch et al., 2013**). The ten ‘General Principles for the Welfare of Animals in Livestock Production Systems’ adopted by the World Organization for Animal Health provide a framework to guide the development of specific animal welfare standards for various animal species. The principles are based on decades of multi-disciplinary research relevant to animal welfare. Research on animal welfare complements work in traditional fields to provide a more comprehensive scientific basis for the care and management of animals (**Fraser et al., 2013**). The table eggs from conditions of small-scale breeding are an important source of foodstuffs for the population, especially in rural areas. It must be given to this source of table eggs for human nutrition the highest quality and health safety too.

CONCLUSION

The table eggs from conditions of small-scale breeding are an important source of foodstuffs for the population, especially in rural areas. It must be given to this source of table eggs for human nutrition the highest quality and health safety too. The present study was a contribution of investigation the welfare of laying hens Moravia SSL housed in small-scale hen house with free range, behavior, egg production and selected physical indicators of eggs and chemical indicators of egg mass. The welfare principles were applied in the breeding conditions. An egg laying has been affected by the season of year. An egg weight, fat and protein content of egg mass were in accordance with the general requirements of the quality of table eggs.

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EFFECTS OF STORAGE ON THE MAJOR CONSTITUENTS OF RAW MILK

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ABSTRACT

Milk testing and quality control should be carried out at all stages of the dairy chain. Milk can be tested for quantity, organoleptic characteristic, compositional characteristic, physical and chemical characteristics, hygienic characteristics, adulteration or drug residues. The content of the major constituents of raw milk is important for milk payment system. Enzymes naturally present in the milk can change the chemical composition of raw milk. Also, enzymes secreted by bacteria or enzymes from somatic cells can degrade the raw milk composition. Products of these degradation reactions can have undesirable effects on milk structure, smell and taste. It is very important that farm-fresh raw milk be cooled immediately to not more than 8 °C in the case of daily collection, or not more than 6 °C if collection is not daily. During transport the cold chain must be maintained. An authorized person, properly trained in the appropriate technique, shall perform sampling of bulk milk in farm. Laboratory samples should be dispatched immediately after sampling to the dairy company and consequently to the testing laboratory. The time for dispatch of the samples to the testing laboratory should be as short as possible, preferably within 24 h. Laboratory samples shall be transported and stored at temperature 1 to 5 °C. Higher temperatures may adversely affect the composition of the laboratory sample and may cause disputes between the farmer, the dairy company and the laboratory. The effect of refrigerated storage at temperature 4 °C during 24 h on the composition of raw milk were investigated in this work, because we wanted to know how the milk composition will be changed and how the laboratory results will be affected. In many cases, the samples are not preserved with chemical preservatives like azidiol, bronopol, potassium dichromate or Microtabs. We found, that the composition of raw cows' milk after 24 h was changed significantly ($p > 0.005$). We found an average decrease in the fat content of -0.04 g/100g, increase in the protein content of +0.02 g/100g, increase in the lactose content of +0.02 g/100g, increase in the solid-not-fat content of +0.02 g/100g and decrease in the total solid content of -0.02 g/100g. It is necessary to cool the raw cows' milk after the milking to decrease the changes in milk composition caused mainly due to the lipolytic activity of lipase.

Keywords: raw milk; milk composition; fat content; protein content

INTRODUCTION

The aim of this work was to investigate how composition of raw milk changes after 24 hours of storage at temperature 4 °C. According to the international standard **ISO 707 (2008)** the raw milk should be immediately transported to the laboratory at temperature 1 – 5 °C and analysed within 24 hours after collection. When sample refrigeration is not possible, sample must be preserved by appropriate means (**Kroger, 1985**).

There are several studies, which were focused on the relationship between quality of dairy products and quality of raw milk. Very important factor is temperature during the storage (**Bachman and Wilcox, 1990; Valík et al., 2011**). Also, contamination of raw milk before processing is an important factor (**Forsbäck et al., 2010**).

According to the **Celestino, Iyer and Roginski, (1996)** storage of bulk raw milk resulted in increased numbers of lipolytic and proteolytic bacteria. On average, the number of psychrotrophs as a proportion of the total plate count increased from 47 to 80% after two days storage. The different trends in bacterial growth in bulk milk samples collected in three seasons suggested the importance of not only the initial load of bacteria but also of the type and activity of microflora present. Significant effects of raw

milk storage on lipolysis and proteolysis were observed. The bacterial and enzyme action in the stored raw milk was greater than that in fresh raw milk and subsequently resulted in increased free fatty acids content and lower pH.

According to the **Ralyea et al., (1988)** enclosed pipeline milk systems, better sanitary design of equipment, cleaner cows, and more effective “clean in place” systems have provided the opportunity for farms to produce raw milk with less microbial contamination. Rapid cooling of raw milk before the bulk tank with inline plate coolers has reduced the growth of contaminating bacteria. Rapid cooling and refrigerated storage of raw milk has favored the growth of psychrotrophic bacteria in raw milk.

If the raw milk bacterial count is <25,000 cfu/mL, then the raw milk somatic cells count will be the most important determinant of shelf life. The influence of raw milk somatic cells count on pasteurized fluid milk quality is caused by increasing levels of heat-stable proteases and lipases originating from the cow with increasing milk somatic cells count (**Barbano, Ma and Santos, 2006**).

Numerous organisms commonly found in raw milk produce degradative enzymes. Once these enzymes have been secreted, they have the potential to degrade both raw and processed milk components. Furthermore,

refrigeration conditions under which raw milk is stored selects for growth of psychrotrophs, many of which produce heat-stable enzymes. These psychrotrophs can grow and secrete heat-stable enzymes while milk waits processing (Mottar, 1989). Pathogenic bacteria like *Staphylococcus aureus* can pose an elevated health hazard and have to be eliminated (Pukáčová, Pořáková and Dudriková, 2010; Vasil' et al., 2012; Bogdanovičová et al., 2014).

Ideally, microbial contamination of raw milk should be addressed primarily through preventive measures on the farm and throughout processing. However, too many contamination sources exist to prevent entry of all bacteria. Therefore, milk handling and processing strategies are designed to reduce and control bacterial numbers in processed products to protect milk quality and milk safety. The first of these measures involves efficient cooling of milk to 4 °C immediately following milking (Marth and Steele, 2001).

Milk must be cooled immediately to not more than 8 °C in the case of daily collection, or not more than 6 °C if collection is not daily. During transport the cold chain must be maintained and, on arrival at the establishment of destination, the temperature of the milk must not be more than 10 °C (Commission regulation (EC) Regulation No 1662, 2006).

Reduced temperatures inhibit growth of mesophils and thermophils and reduce the activity of degradative enzymes. Modern dairy farms use refrigerated bulk storage tanks which maintain milk at 4 °C or below. As bulk tank milk pick-up typically occurs daily or every other day, product from multiple milkings is frequently mixed and stored in the same tank. To prevent fresh, warm milk from the most recent milking from raising the temperature of milk already present in the bulk tank, many farms employ pretank cooling systems to reduce product temperature before addition to the tank (Marth and Steele, 2001).

The presence and growth of bacteria in milk affects milk quality. Chemical components of milk can be degraded by bacterial metabolism and various enzymes secreted by bacteria. Products of these degradation reactions can have undesirable effects on milk structure, smell and taste. Fermentative metabolisms of lactose by a variety of lactic acid bacteria can occur in milk (Cousin, 1982; Baylund, 1995; Jay, Loessner and Golden, 2005; Bezeková et al., 2012). *Enterococcus spp.* is the group of lactic acid bacteria, which can enter the milk from environment through milking machines (Fabianová et al., 2010; Krebs-Artiová, Ducková and Kročko, 2013; Lačanin et al., 2015). Proteins can be digested by extracellular proteases. Lipase will cause break down of triglycerides. Phospholipases hydrolyze phospholipids present in fat globule membranes making interior lipids more susceptible to lipase attack (Baylund, 1995; Cousin, 1982 and Jay, Loessner and Golden 2005).

MATERIAL AND METHODOLOGY

Milk samples

Raw cows' milk from morning milking was sampled from the bulk tank in farm into sterile bottles according to

the standard ISO 707 (2008) and immediately transported to the laboratory at temperature 1 – 5 °C.

Instruments

To perform this research we used MilkoScan FT 120 infrared absorption analyser (FOSS, Hillerød, Denmark; distributor: Milcom servis a.s., Prague, Czech Republic). It was calibrated quarterly with calibration samples (Actalia - Cecalait, Poligny, France) preserved with 0.02 % Bronopol.

Infrared milk analysis

Samples of fresh raw cows' milk were analysed 2 and 24 hours after milking. Each sample was analysed 10 times and the average result was calculated. Milk composition was determined in compliance with ISO 9622 (2013) and the FOSS (1998) working manual for the Milkoscan FT 120. The samples were analysed at the State Veterinary and Food Institute in Bratislava, Slovakia, at the National Reference Laboratory for Milk and Milk Products, which is accredited in accordance to the international standard ISO 17025 (2005). The experiment was replicated 10 times.

Deviation calculation

Deviations between the results of laboratory determination of milk composition were calculated following this equation:

$$\text{Deviation of result of analyte (g/100g)} = (A) - (B)$$

Where:

(A) is the result of analyte of raw cows' milk after 24 hours storage at temperature 4 °C and

(B) is the result of analyte of fresh raw cows' milk.

Statistical analysis

The statistical analysis was performed using statistical program Tanagra 1.4 (Lumière University, Lyon, France) according to Rakotomalala (2005). To evaluate the results, data was classified into two groups representing the composition of raw cows' milk and the composition of raw cows' milk after 24 hours. Subsequently, the Principal Components Analysis (PCA) was performed with the Hierarchical Clustering Procedure (HAC). To evaluate the difference between the results with paired samples of fresh raw cows' milk and raw cows' milk after 24 hours storage at temperature 4 °C, the Student's t-test was used and the *p*-value was calculated.

RESULTS AND DISCUSSION

The composition of the fresh raw cow's milk used in experiments is presented in Table 1. The effect of 24 h storage at temperature 4 °C on milk composition is presented in Figure 1. Figure 2 represents the Principal Component Analysis of data of fresh (▲) and stored (●) raw cow's milk. The data do not overlap. It means the composition of raw cow's milk after 24 h was changed significantly (*p* > 0.005). We found an average decrease in the fat content of -0.04 g/100g, increase in the protein content of +0.02 g/100g, increase in the lactose content of +0.02 g/100g, decrease in the total solid content of -0.02 g/100g and increase in the solids-not-fat content of +0.02 g/100g.

Table 1 The composition of fresh raw cows' milk.

Experiment No.		Composition of raw cows' milk (g/100g) ^a					Composition of raw cows' milk after 24 hours stored at 4 °C (g/100g)				
		Fat	Protein	Lactose	Total solid	Solids-not-fat	Fat	Protein	Lactose	Total solid	Solids-not-fat
1	Average (g/100g)	3.82	3.22	4.65	12.37	8.54	3.80	3.23	4.66	12.36	8.56
	Cv (%)	0.39	0.35	0.21	0.17	0.20	0.17	0.33	0.10	0.12	0.12
	SD (± g/100g)	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.00	0.01	0.01
2	Average (g/100g)	3.40	3.25	4.68	12.01	8.60	3.35	3.27	4.70	12.00	8.65
	Cv (%)	0.19	0.13	0.15	0.08	0.10	0.14	0.16	0.10	0.08	0.07
	SD (± g/100g)	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.01
3	Average (g/100g)	4.26	3.36	4.80	13.10	8.84	4.20	3.38	4.85	13.04	8.84
	Cv (%)	0.19	0.42	0.52	0.18	0.34	0.19	0.00	0.22	0.14	0.12
	SD (± g/100g)	0.01	0.01	0.03	0.02	0.03	0.01	0.00	0.01	0.02	0.01
4	Average (g/100g)	4.00	3.30	4.71	12.68	8.69	3.93	3.33	4.73	12.65	8.72
	Cv (%)	0.13	0.20	0.51	0.12	0.27	0.12	0.15	0.53	0.13	0.19
	SD (± g/100g)	0.01	0.01	0.02	0.01	0.02	0.00	0.00	0.03	0.02	0.02
5	Average (g/100g)	3.43	3.24	4.73	12.07	8.63	3.41	3.27	4.75	12.04	8.63
	Cv (%)	0.24	0.57	0.18	0.17	0.30	0.09	0.15	0.18	0.07	0.10
	SD (± g/100g)	0.01	0.02	0.01	0.02	0.03	0.00	0.00	0.01	0.01	0.01
6	Average (g/100g)	4.00	3.28	4.78	12.69	8.73	3.96	3.31	4.79	12.67	8.72
	Cv (%)	0.32	0.10	0.37	0.44	0.30	0.18	0.13	0.10	0.07	0.06
	SD (± g/100g)	0.01	0.00	0.02	0.06	0.03	0.01	0.00	0.00	0.01	0.01
7	Average (g/100g)	3.50	3.27	4.78	12.20	8.70	3.48	3.29	4.81	12.20	8.72
	Cv (%)	0.09	0.17	0.24	0.06	0.14	0.20	0.20	0.15	0.11	0.13
	SD (± g/100g)	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
8	Average (g/100g)	3.72	3.29	4.74	12.41	8.69	3.68	3.29	4.76	12.40	8.72
	Cv (%)	0.29	0.24	0.42	0.13	0.23	0.21	0.22	0.33	0.15	0.21
	SD (± g/100g)	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02
9	Average (g/100g)	3.44	3.25	4.73	12.10	8.66	3.40	3.28	4.74	12.06	8.66
	Cv (%)	2.87	0.22	0.12	0.80	0.12	0.14	0.13	0.17	0.08	0.08
	SD (± g/100g)	0.10	0.01	0.01	0.10	0.01	0.00	0.00	0.01	0.01	0.01
10	Average (g/100g)	4.10	3.38	4.80	12.91	8.83	4.01	3.37	4.81	12.87	8.85
	Cv (%)	0.13	0.28	0.28	0.10	0.18	0.21	0.12	0.15	0.07	0.07
	SD (± g/100g)	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01

^a Raw cows' milk from morning milking was sampled from the bulk tank immediately after the end of milking. Composition of raw cows' milk was analysed two hours after sampling.

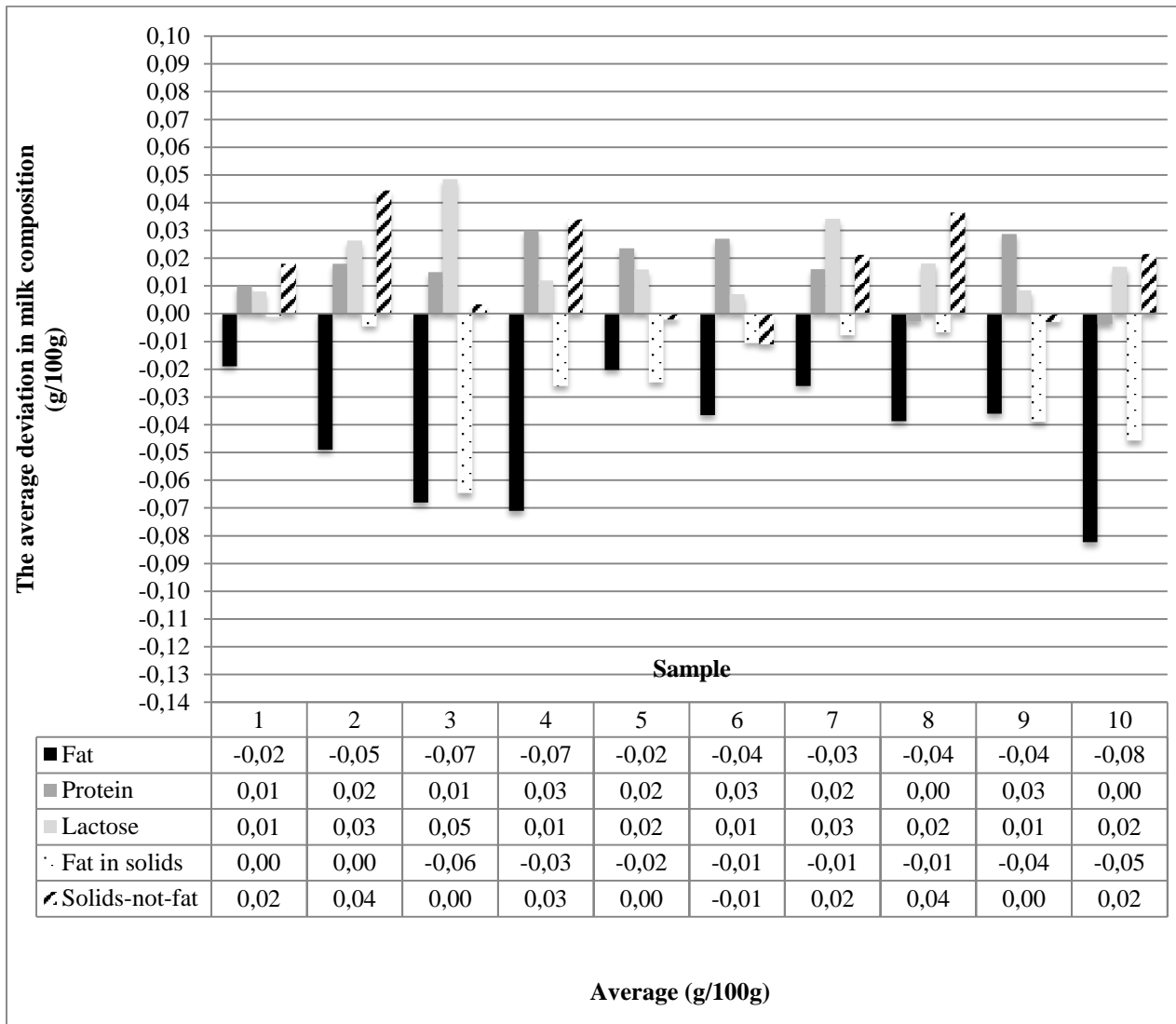


Figure 1 The effect of 24 h storage at temperature 4 °C on milk composition. Each sample (n = 30).

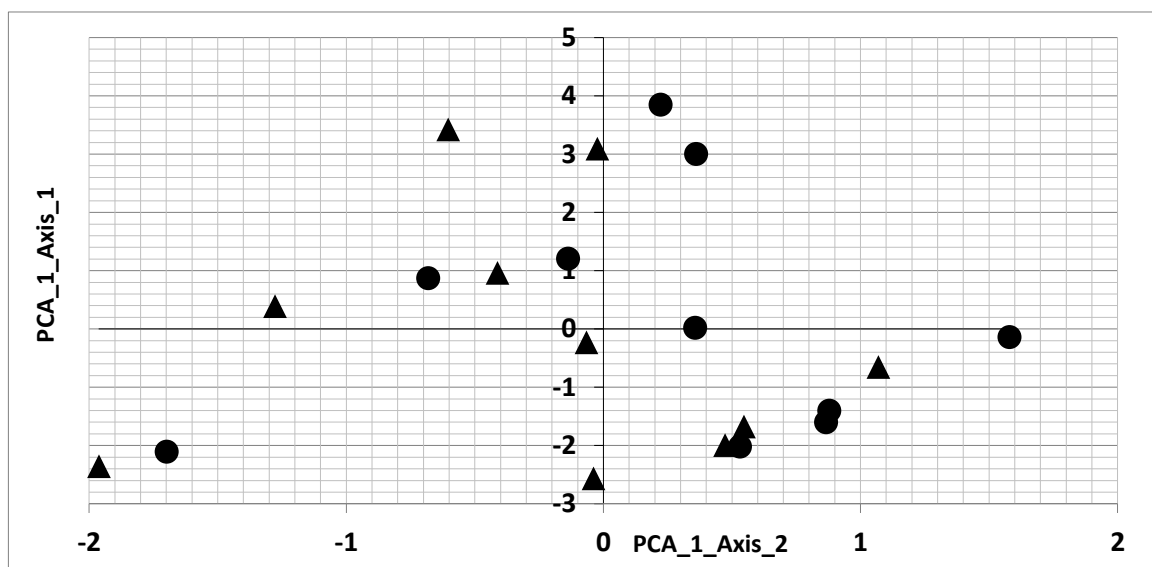


Figure 2 The Principal Component Analysis of the composition of (▲) fresh raw cows' milk versus (●) raw cows' milk stored 24 hours at temperature 4 °C. The PCA_1_Axis_1 and PCA_1_Axis_2 represent the results of fat, protein, lactose, total solids and solids-not-fat content.

In our opinion, lipolysis of milk can be initiated both by indigenous milk lipases and by microbial lipases, which could change the milk composition.

The liberation of fatty acids by the action of lipases can change the instrument's readings. Increasing the lipolysis index by 1 milliequivalent per 100 g of fat changes the instrument's signal for fat by -0.022% and signal for protein by +0.013% (ISO 9622, 2013). Bovine milk contains a lipoprotein lipase that accounts for most, if not all, of its lipolytic activity. The total lipase activity in raw milk is sufficient to cause rapid hydrolysis of a large proportion of the fat. Physical damage to milk fat globule membrane in raw milk initiates lipolysis. Furthermore, simply cooling milks soon after secretion can initiate the so-called spontaneous lipolysis (Deeth, 2006). Raw milk stored at 4 °C enables the growth of lipolytic psychrotrophic bacteria (Fonseca et al., 2013). Extracellular microbial lipolytic and proteolytic enzymes may cause spoilage problems (Baur et al., 2015). Also, Leitner et al., (2011) described the negative effect of bacterial infection on milk composition. Barbano, Ma & Santos (2006) expect the activity of various enzymes in milk. The microbial count and somatic cell count determine the load of heat-resistant enzymes in milk and these enzymes reducing the shelf life of the milk. Proteolysis can occur during 4 °C storage of preserved milk samples (Santos et al., 2003). Proteolysis in milk during storage at 4 °C for six days points to the greater importance of microbial proteinases than plasmin activity. Plasmin activities decreased during the six days of storage at 4 °C (Guinot-Thomas et al., 1995). Temperature during cold storage can have a significant influence on plasmin levels and thus contribute to the subsequent proteolysis rate in milk (Schroeder, Nielsen & Hayes, 2008). Marino et al. (2005) stated that the proteolytic activity associated with somatic cells in milk could affect milk composition. Verdi & Barbano (1991) were observed casein proteolysis of milk by enzymes isolated from somatic cells. The higher protease activity may be present due to the higher concentrations of activated macrophages. Different somatic cells counts and milk composition during the lactation, activities of cathepsin D, cysteine proteases and another unidentified milk proteinase in milk were fluctuate during lactation (Larsen et al., 2006). Fifteen per-cent of 19,830 samples analysed for total bacterial count and twenty-six per-cent of 13,037 samples analysed for somatic cells count didn't meet the legal requirements. It means the enzymatic activity due to the presence of microorganisms and somatic cells in bulk tank milk have to be expected (Zajác et al., 2012). The activity of these enzymes can lead to the laboratory results deviations when unpreserved laboratory samples or improper lower concentrations of preservatives are used. As shown in Figure 2, only the lipolysis of fat content occurred after 24 hours storage at temperature 4 °C. In contrast, the protein and lactose content was slightly increased. Because, the fat content was changed, the calculation of the other milk components by instrument was affected. According to Kaylegian et al., (2007), the proteolytic activity in milk increased only about 1% after 8 days storage at temperature 4 °C. The activity of lipases can decrease the fat content during infrared readings and

increase other milk components (ISO 9622, 2013). It is then necessary to analyse the milk samples as soon as possible after collection; otherwise, they must be preserved by appropriate means and stored in the temperature 5 °C (ISO 707, 2008) to eliminate changes in milk composition. The application of milk preservatives can extend the shelf life of the sample as well (Chalermnan et al., 2004).

CONCLUSION

The composition of raw cows' milk after 24 was changed significantly ($p > 0.005$). We found an average decrease in the fat content of -0.04 g/100g due to the lipolytic activity of lipase. We found increase in the protein content of +0.02 g/100g, increase in the lactose content of +0.02 g/100g, increase in the solid-not-fat content of +0.02 and decrease in the total solid content of -0.02 g/100g. Increased content of these milk components was caused due to the instrument's readings, because the fat content was decreased, subsequent calculation of other components was affected. It is necessary to cool the raw cows' milk after the milking to decrease the changes in milk composition. Also, it is necessary to analyse the milk samples and process the milk as soon as possible, preferably within 24 hours.

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STUDY OF ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF GRAPEVINE SEEDS, GRAPE AND ROSEHIP PRESSINGS

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ABSTRACT

In our experiment, we studied the antimicrobial and antioxidative effect of phytogetic additives. Three additives (grapevine seeds, grape and rosehip pressings) were selected to be monitored. The extracts about concentrations of 1:3 and 1:5 were prepared from them. The monitoring of antimicrobial properties was focused on the pathogenic bacteria *Clostridium perfringens* and *Escherichia coli* causing a serious disease in avian species. The bacteria were prepared in the dilutions of 10^2 , 10^4 and 10^6 . The antimicrobial effect was observed in the inhibition zones. The antioxidant activity was determined using DPPH method within the antioxidant analysis. Furthermore, the content of flavanols, hydroxycinnamic acids and the total content of polyphenolic compounds was also determined. In the monitoring of the antimicrobial effect of grapevine seeds, grape and rosehip pressings at *E. coli*, a reduced growth of KTJ (colony forming units) was observed in the disk area during the dilution of 10^6 and 10^4 . Reduced growth of *C. perfringens* at a dilution of 10^6 was noticed using the extracts of grapevine seeds and grape pressings. Low reduced growth of *C. perfringens* at a dilution of 10^6 was found out using rosehip pressings. In a dilution of 10^2 and 10^4 in *C. perfringens* and 10^2 in *E. Coli*, a very low increase of KTJ was observed therefore the zones of inhibition were not possible to measure. In all monitored additives, the antimicrobial effect was proved. The additives reduced the growth of pathogenic *E. coli* and *C. perfringens*. Within the antioxidant analysis, the highest antioxidant activity was found out in grapevine seeds (7.021 g.L^{-1} GAE), which also contained the highest content of flavanols (3000 times higher than the rosehip pressings and 300 times higher than grapevine seeds pressings), hydroxycinnamic acids (1000 times higher than in grape pressings and 7600 times higher than in rosehip pressings) and the total content of polyphenolic compounds (580 times higher than grape pressings and 2000 times higher than the rosehip pressings) of the monitored additives.

Keywords: *Clostridium perfringens*; *Escherichia coli*; grapevine seeds; pressings; rosehip

INTRODUCTION

Natural products are an important source of phenolic compounds. The interest in the compounds is in their ability to bond the important free radicals causing lipid oxidation, which is the main factor of lower quality of foods during the processing and storage. Furthermore, they have an important role in the progression of a wide variety of diseases such as cancer, atherosclerosis, inflammation and aging depending on the formation of free radicals. Catechin and resveratrol are the two most frequent phenols present in natural products. Catechin is found out in significant quantities, for example grapes, apples and tea. Phenolic compounds are extracted from grape extracts commonly used as active ingredients in the manufacture of pharmaceutical products. These compositions are used in skin preparations for the treatment of hemorrhoids, or to reduce platelet aggregation and oxidation abilities. Their effectiveness as preservatives was demonstrated in peeling fruit and vegetables, juices and other natural products (Pinelo et al., 2005). Phenolic compounds may affect the growth and metabolism of bacteria. They cause the activating or inhibitory effect on the growth of microorganisms according to their composition and concentration (Vaquero et al., 2007a). Some studies have shown that phenolic compounds, present in the wine, can

influence bacterial growth and metabolism but the antimicrobial effect depends on the particular compound (Ganan et al., 2009). The content of phenolic compounds in natural materials is quite variable. It depends on the particular crop species but also their varieties. Their content is conditioned genetically and influenced by climatic and agronomic conditions. Changes in the content of phenolic compounds largely also indicates germination, degree of ripeness as well as technical processing and storage of plant products (Boncikova et al., 2012). After making wine, about 20 % of grapes remain in the form of skin pressings, seeds and stalks. The pressings contain significant quantities of phenolic compounds, which are not extracted into the wine. Resveratrol, present in wine in small quantities, was probably the most studied flavonoid. The phenolic compound inhibits the growth of microbial species (*S. aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*) and several pathogenic fungi as dermatophytes causing skin infections and also inhibits the biofilm formation of *E. coli* and *P. aeruginosa*, *V. cholerae*. It appears that resveratrol has antimicrobial, antiparasitic and anti-inflammatory effects showing the potential to be used for microbial food safety against the infections endangering people (Friedman, 2014). The applications of grape pressings in food technology

demonstrated the potential use of oil with an effective antioxidant capacity as an inhibitor of lipid oxidation in fish, frozen fish muscle and cooked, chilled turkey meat during storage (Goni et al., 2007, Horky et al., 2013). The antioxidants, occurring in grapevines, are phenolic acids (benzoic acid and hydroxycinnamic), stilbene derivatives, flavan-3-ols (catechin and epicatechin), flavonols (quercetin and myricetin), and anthocyanidins. The antioxidant potential of grape seed is twenty times higher than E vitamin and fifty times higher than C vitamin which is obvious from higher levels of polyphenols, proanthocyanidins and units of flavan-3-ol oligomers particularly catechin and epicatechin occurring in the extract of grapevine seeds. However, the use of natural antioxidants in the diet of animals could be limited by the low bioavailability of polyphenols (Brenes et al., 2010; Horky, 2014; Horky et al., 2012). Another interesting source may be rosehip fruits (Loetscher et al., 2013). Currently, rosehip is widely used as aromatic and medicinal plant with high antioxidant activity (Yesilbag et al., 2011) In vitro tests showed high antioxidant capacity of the rosehip especially in the lipophilic fraction extract, which is probably caused by the content of phenolic compounds (Loetscher et al., 2013).

Rosehip has long been used in many European countries such as herbal teas, vitamin supplements or food products as it contains large amounts of vitamin C. Except to ascorbic acid, rosehips contain also carotenoids and phenolics, which are also the important antioxidants (Gao et al., 2000).

MATERIAL AND METHODOLOGY

Experimental design

Three phytogetic additives were selected to be monitored - grapevine seed (Marlen variety) grape and rosehip pressings. The effect of selected additives was determined using laboratory methods. Their antimicrobial and antioxidant effects were also found out.

Microbiological analysis

Extract preparation

The extracts were prepared from grape seeds, grape and rosehip pressings. The samples of the plant additives were first dried at 45 °C to a constant weight and then milled. The extracts were prepared at two concentrations of 1:3 (30 g sample +90 mL water) and 1:5 (20 g +100 mL water). Weighed samples were filled with boiled water and then placed in a water bath at 95 °C for 1 hour. After removal from the bath, the samples were centrifuged on a centrifuge (Biosan, Latvia) at 1500 rpm for 20 minutes.

Microbe preparation

For *Escherichia coli* bacterium, a pure culture of the Czech Collection of Microorganisms was used. *Clostridium perfringens* was grown on a selective medium - Tryptone neomycin sulfite agar (TSN) for the microorganism at 46 °C for 24 hours. For each bacteria, the suspension was prepared in the application of a microbe in a saline solution with a density of 1 McFarland (108). Subsequently, three dilutions (10^2 , 10^4 , 10^6) were

prepared in decimal dilutions. Petri dishes with Violet Red Bile agar (VRBL) - *E. coli* and sulfite neomycin Tryptone agar (TSN) - *C. perfringens* was inoculated with 0.1 mL of suspension with sterile pipette and spread using sterile stick. Violet red bile agar (VRBL) contains in 1 liter of medium: 7.0 g peptic digest of meat, 3.0 g yeast extract, 10 g lactose, 1.5 g bile salts, 5.0 g sodium chloride, 30 mg neutral red, 2 mg crystal violet, 12.0 g bacteriological agar. Tryptone sulfite neomycin agar (TSN) contains in 1 liter of medium: 15 g, 10 g tryptone yeast extract, 1 g sodium sulfite, 0.5 g ferric ammonium citrate, 50 mg neomycin sulfate, 20 mg polymixin B sulfate, 13.5 g bacteriological agar. The extracts were pipetted in quantities of 30 µL onto sterile paper disks of 9 mm in diameter, which were then placed on a petri dish. Petri dish with *C. perfringens* was then inserted into the anaerostat (Merck, Germany) with the generator of anaerobic environment - Anaerocult A (Merck, Germany) and placed in a thermostat of 46 °C for 24 hours. Petri dishes with *E. coli* were put into a thermostat at 37 °C for 24 hours. Two repetitions were prepared in each dilution. After the incubation, the inhibition zones were measured using ruler (weakened growth zones of KTJ - colony forming units of bacteria) around the disks.

Antioxidant parameters

Determination of antioxidant activity by DPPH

The radical solution of DPPH (2,2-diphenyl-β-picrylhydrazyl radical) of 2000 µL was dosed in 3 mL cuvette using pipette and $m = 9.35$ mg of DPPH radical was weighed. The measured amount was put into a volumetric flask of 250 mL and supplemented with methanol. Then 40 ml of the sample was added. In this case, it was the sample of wine and left at 22 °C for 25 minutes. After a given time, the absorbance was measured at 505 nm. The calculation of the antioxidant activity was performed from the calibration curve, as a standard was used gallic acid ($10 - 200$ mg.L⁻¹). The results were expressed in mg.L⁻¹ of the antioxidant equivalents of gallic acid.

Determination of total flavanols

The preparation of reagents - 40 µL volume of sample was dispensed into 3 mL cuvette and then diluted in 1960 µL of reagent [(0.1 % DMCA = p-dymethylaminocinnamaldehyd) and 300 mL of MHCl in MeOH (methanol)]. The mixture was shaken and incubated for 12 minutes at the room temperature (about 22 °C). After 12 minutes, the absorbance was measured at the machine, called double beam spectrophotometer of SPECORD 210 brand, Carl-Zeis Jena, Germany, at $\lambda = 640$ nm against the empty cuvette. The results were expressed as catechin equivalents.

Determination of total hydroxycinnamic acids

The measurement was performed using SO₂ method. In a 2 ml vial, 200 µL sample with 1.8 mL and 1.1 mol HCl were shaken. Blank test to each sample was prepared in the same way. HCl solution was replaced with fresh 0.22 mol solution of K₂S₂O₅ (SO₂). After 180 minutes, the

absorbance of samples with HCl was measured in the cuvette at 280 nm. The results were calculated for values at mg.L⁻¹.

The calculation of the total hydroxycinnamic acids: OD280 = 10 * dilution * A (HCl) 280

Determination of total polyphenolic compounds

Wine sample of volume (50 mL) was pipetted into a cuvette and diluted with 1.5 mL ACS water. Subsequently, 0.05 mL of Folin-Ciocalt reagent (Sigma-Aldrich, US) was added. After 30 minutes at 22 °C, the absorbance was measured at dual beam spectrophotometer called SPEKOL 2000 at wavelength λ = 640 nm and λ = 670 nm against a blank sample (gallic acid). The results were expressed as gallic acid equivalents in mg/100 g.

RESULTS AND DISCUSSION

The experiment was aimed to find out the antioxidant and antimicrobial activity of grapevine seeds, grape and rosehip pressings. The task was also to determine the relationship between the antioxidant and antimicrobial activity. As markers of antioxidant potential were selected the contents of flavones, hydroxycinnamic acids, polyphenol compounds, DPPH. The main observed parameters from a microbiological point of view were *C. perfringens* and *E. coli*, which are directly tied to the grapevine seeds, grape and rosehip pressings.

Determination of antimicrobial activity

The basic characteristics of plant additives is their wide antimicrobial activity (Jakubcova et al., 2014a). Many studies proved a potential of use of various phytochemical additives into feed rations of poultry as alternative to antibiotics (Jakubcova et al., 2014b).

The attention was focused on the study of two important pathogens such as *C. perfringens* and *E. coli* in the digestive tract of chickens. These microorganisms are responsible for serious poultry diseases. The antimicrobial activity was determined in the inhibition zones. Reduced growth of *C. perfringens* was found out very low using the extract of rosehip pressings at a dilution of 10⁶. Reduced growth of *C. perfringens* was recorded using the extract of

grapevine seeds and grape pressings at a dilution of 10⁶. During the monitoring of the antimicrobial effect of the extracts of grapevine seeds and grape pressings for *E. coli*, a reduced growth of KTJ (colony forming units) was detected in the disc area at a dilution of 10⁶ and 10⁴. In the extracts of rosehip, a strong reduced growth of KTJ was recorded around the disc areas at a dilution of 10⁴ and 10⁶. For the dilutions of 10² and 10⁴ in *C. perfringens* and 10² in *E. coli*, a low increase was observed in KTJ. The antimicrobial effect of the extracts was not possible to be evaluated. During the dilution of 10⁴ in *C. perfringens* and 10² in *C. perfringens* and *E. coli*, a very low increase was found out in KTJ. The inhibition zones around the discs were impossible to be measured.

Vaquero et al., (2007b) demonstrated the antimicrobial effects of various concentrations of phenolic compounds contrary to *Listeria monocytogenes* in three types of wine. In this study, all samples of wine proved the inhibition zone contrary to bacteria, which was higher with increasing concentration of polyphenols. Further, it was demonstrated the antibacterial effect of three types of wine to the bacterium such as *Escherichia coli*, *Preteus mirabilis*, *Serratia marcescens*, *Flavobacterium sp.* and *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* (Vaquero et al., 2007a). The extract of grapevine seed pressings showed a high antibacterial activity *in vitro* contrary to bacteria such as *S. aureus* and *E. coli*, and a low activity contrary to *Salmonella sp* (Rotava et al., 2009). In our monitoring, we managed to prove that the pressings have the antibacterial effect on bacteria such as *E. coli* and *C.perfringens*. Based on the results of his study (Viveros et al., 2010) states that the products of grapes rich in polyphenols grapes affected the increase of beneficial bacteria in the ileum, as well as on increasing the villus height and crypt depth in the jejunum. These factors can have a significant impact on the physiology and biochemistry of cancer. The advances in the understanding of interactions between bioactive compounds in feed mixtures and specific intestine bacteria could contribute to a better understanding of positive and negative interactions *in vivo* and to identify new functional intestinal microorganisms. The study of (Gadang et al., 2008) demonstrated the antimicrobial effect in a combination of

Table 1 Averages of inhibition zones.

MO	Concentration MO [McF]	Dilution	Averages of inhibition zones [mm]					
			Grapevine seeds		Grape pressings		Rosehip pressings	
			1:3	1:5	1:3	1:5	1:3	1:5
<i>C.perfringens</i>	1	10 ⁶	14	12	9	10	9	11
		10 ⁴	-	-	-	-	-	-
		10 ²	-	-	-	-	-	-
<i>E.coli</i>	1	10 ⁶	15	9	13	10	12	14
		10 ⁴	16	11	12	16	11	12
		10 ²	-	-	-	-	-	-

n=72

MO – microorganisms

McF – McFarland

nisin, malic acid and extract grapevine seeds to *L. monocytogenes*. The results of the study of **Vaz et al., (2012)** showed that wine proves a strong inactivation effect against vegetative cells of two strains of *B. cereus*. Wine also affects the viability of *C. jejuni*, while red and rose wines are more effective than white wines. However, alcohol reduces the survival rate of *C. jejuni*, phenolics also significantly affect their viability, especially p-hydroxybenzoic acid and gallic acid. Wine thus creates a hostile environment for the survival of this pathogen. It would be interesting to study the possible use of wine phenolic compounds as alternatives to the use of the antimicrobial growth promoters contrary to these bacteria in broilers (**Ganan et al., 2009**).

Determination of antioxidant properties

Three major groups of substances - polyphenolic compounds, flavanols and hydroxycinnamic acids were selected to study the antioxidant properties. Further, the antioxidant activity represents the antioxidant power of antioxidant components.

Determination of antioxidant activity

DPPH method was used to determine the antioxidant activity. It is one of the most basic and most commonly used methods for the determination of the antioxidant activity. It is based on the reaction of a biological matrix (grape seeds and pressings in our case) with free radical DPPH. The results are then expressed in the equivalent amount of the antioxidant substances gallic acid – GAE in our experiment) which is capable of putting out the amount of the radical. In Table 2, the results of the antioxidant activity of observed samples are showed. The highest antioxidant activity was observed in the grapevine seeds. The measurement for the grapevine seeds was 500 times higher than for grape pressings and 575 times higher than for the rosehip pressings.

Determination of flavonols content

Flavonols are primarily synthesized in grape skin. In the red grapes, they are found out in smaller amounts than anthocyanins. Particularly, flavonols are important during co-pigmentation with anthocyanins (**Hilbert et al., 2015**). For the flavanol determination, the method, based on a reagent reaction with DMAC, was used. The highest content of flavanols was measured with grapevine seeds, and almost 3000 times higher than the rosehip pressings and 300 times higher than grapevine seeds pressings. The content of flavanols in the monitored additives is shown in Table 3.

Determination of the hydroxycinnamic acid content

Hydroxycinnamic acids are the principal group of phenolic compounds located in the pulp of grapes. They occur in the form of esters of tartaric acid in the skin and

pulp. The highest content of hydroxycinnamic acids was determined in the grapevine seeds. The measured values of grapevine seeds were 1000 times higher than in grape pressings and 7600 times higher than in rosehip pressings. The results of measurement of hydroxycinnamic acids content are given in Table 4.

Determination of the total polyphenolic compounds

Polyphenols create one of the most abundant and widely distributed group of natural products of the plant kingdom. They include a sufficient amount of molecules with polyphenolic structure but also molecules with one phenolic circle. Polyphenols contained in grapes and wine can generally be divided into two main groups: non flavonoids (hydroxybenzoic and hydroxycinnamic acids and their derivatives, and stilbene phenol alcohols) and flavonoids (anthocyanins, flavanols, flavonols and dihydroflavonol). Many of polyphenols have been identified in grape pressings, in which are the most abundant anthocyanins, flavanols, flavonols, hydroxybenzoic and hydroxycinnamic acids and stilbenes (**Antonioli et al., 2015**).

The highest content of total polyphenolic compounds has been measured in grapevine seeds. This value was almost 580 times higher than grape pressings and 2000 times higher than the rosehip pressings. For content of pressings coming from grapevine seeds, the value was measured 10 times higher than the rosehip pressings. The measured values are given in Table 5.

Spranger et al., (2008) evaluated the antioxidant activity of test compounds determining the trapping capacity of different types of radicals in his study. All test methods showed that polymeric procyanidins on an equimolar basis had the highest antioxidant activity followed by oligomeric procyanidins, while catechins reached a lower antioxidant activity than oligomers and polymers. The antioxidant activity of procyanidins of grape seeds positively related to their level of polymerization. Additionally, procyanidins showed greater antioxidant activity than other antioxidants as e.g. vitamin C.

According to (**Rotava et al., 2009**) the antioxidant activity of extracts from grapevine seed pressings is comparable to ascorbic acid. Due to the high content of antioxidants in the rosehip extracts, they proved high antioxidant activity in all measurements using different methods. The total antioxidant activity significantly contributed to the phenolic fraction. The comparison of the results based on the ratio of the content of the antioxidant capacity of antioxidants proved that lipophile component has been effective (**Gao et al., 2000**). In the study, in which was evaluated the antioxidant capacity using DPPH, was found out in the red wine polyphenol content of 456 mg.L⁻¹ GAE (**Wang et al., 2015**). In the comparison with our results, we can state that only a part of antioxidants passes to the wine compared to the native seeds. In our experiment, the GAE concentration (grapevine seeds) was measured by method of DPPH 7.021 g.L⁻¹.

Table 2 Results of determination of antioxidant activity using DPPH method. Results are expressed in mg.L⁻¹ GAE.

	DPPH	
Grapevine seeds	7 021 ±217,7	mg.L ⁻¹ GAE
Grapevine seed pressings	14 ±0,462	mg.L ⁻¹ GAE
Rosehip pressings	12 ±0,35	mg.L ⁻¹ GAE

n=6

Table 3 Results of determination of total flavonols content. Results are expressed in mg.L⁻¹.

Values of total flavonols content		
Grapevine seeds	3070.0 ±116,7	mg.L ⁻¹
Grapevine seed pressings	10.3 ±0,361	mg.L ⁻¹
Rosehip pressings	1.1 ±0,034	mg.L ⁻¹

n=6

Table 4 Results of determination of total hydroxycinnamic acids. Results are expressed in mg.L⁻¹.

Values of hydroxycinnamic acids content		
Grapevine seeds	6 870 ±254,2	g.L ⁻¹
Grapevine seed pressings	6.1 ±0,207	mg.L ⁻¹
Rosehip pressings	0.9 ±0,028	mg.L ⁻¹

n=6

Tabulka 5 Results of determination of total content of polyphenolic compounds. Results are expressed in mg/L.

Values of polyphenolic compound content		
Grapevine seeds	7 540 ±271,4	mg.L ⁻¹
Grapevine seed pressings	37.6 ±1,25	mg.L ⁻¹
Rosehip pressings	3.7 ±0,13	mg.L ⁻¹

n=6

CONCLUSION

This work deals with the study of the antimicrobial and antioxidant properties of grapevine seeds, grape pressings and rosehip pressings. The presented results highlighted the impact of grapevine seeds, grape and rosehip pressings on bacteria *C. perfringens* and *E. coli*. The highest antimicrobial effect was observed in the variants with grapevine seeds. This fact also corresponded with the content of the antioxidant components that were the highest in grapevine seeds. The values of the antioxidant activity in the grapevine seeds were much more higher than in grape pressings (500 times) and rosehip pressings (575 times higher). The grapevine seeds also contained the highest content of flavanols, hydroxycinnamic acids and the total content of polyphenolic compounds from the monitored additives.

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DETECTION OF HONEY ADULTERATION USING HPLC METHOD

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ABSTRACT

This work deals with the determination of undeclared or illicitly added sugar content in honey samples evaluated using the High-Performance Liquid Chromatography or HPLC with refractive index detection. Labelling of samples was also evaluated in accordance with current legislation. In a total of 21 samples of honey purchased in the fall of 2013, 13 samples were obtained from the regular shopping network, 2 samples were purchase in Health Food stores and 6 samples came directly from local beekeepers and were purchased at the Christmas Markets in Brno.

We have determined the contents of fructose, glucose, sucrose, and oligosaccharides using the HPLC method. We have calculated the basic statistics such as the mean and standard deviation for each sample. Samples have been evaluated according to the Council Directive 2001/110/EC, which lays down limit values for the parameters of honey.

Only four out of 21 honey samples complied with the requirements of Council Directive 2001/110/EC. These were three samples obtained from the regular shopping network and one obtained directly from the local beekeeper. Six samples did not meet the requirements for the sum of fructose and glucose, two samples could not be determined due to the failure to specify the honey type, and fourteen samples failed the requirement of sucrose content.

We have further assessed whether honey samples comply with legislative requirements relating to this product or consumer misleading practices take place. Our analysed samples often lacked indication whether it is a floral honey or honeydew honey; this information was missing in eight out of 21 samples. Samples 5 and 9 did not mention the name of manufacturer. Sample 10 did not mention the country of origin.

Keywords: honey; HPLC; labelling; fructose; glucose; sucrose

INTRODUCTION

Honey is a natural product produced by bee workers from the nectar or honeydew, without any human interference (Roman and Popiela, 2011). It is an easily digestible, energetically valuable food of natural carbohydrate character. It is composed mainly of sugar and water, and also contains other ingredients such as vitamins and minerals (Vallianou, 2014). Honey as a natural food of carbohydrate nature composed mainly of glucose, fructose, organic acids, enzymes (Kňazovická et al., 2011) and solid particles captured by bees while collecting sweet flower juice (nectar), excretions of insects on plant surfaces (honeydew) or on living parts of plants. Bees (*Apis mellifera*) collect the components, transform them, combine them with their own specific substances, store them and let them dehydrate and mature in combs (Decree no. 76/2003 Coll.). "Codex Alimentarius" (2001) defines honey as a non-fermenting sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, and leave in the honey comb to ripen and mature. According to Council Directive 2001/110/EC (2001), honey is 100% bee product, to which nothing can be added and from which nothing can be removed. Therefore in order to maintain its therapeutic values, it is necessary to deliver it to the consumer in its natural form without any additives and major technological modifications.

Honey is among the most adulterated food products, as it is a natural product with limited production and relatively high cost (Megherbi et al., 2009). Honey adulterations can take place by substitution of botanical and geographical origin, confusion of honeydew honey with floral honey, selling of artificial honey (flavoured sugar solutions), and failure to comply with quality and hygiene requirements (unauthorized quantities of residues of antibiotics and sulphonamides). Honey adulterating may include even heating or storage under unsatisfactory conditions (Čížková et al., 2010). Freshly bottled honey contains virtually no hydroxymethylfurfural (HMF), but its content may increase during storage (Kalábová et al., 2003; Frank, 2010; Bogdanov, 2014). The presence of HMF thus becomes an indicator of food quality deterioration caused by excessive heating during thermal treatment as well as improper and long-term storage, and is also an indicator of possible adulteration (Borkovcová, 2011). Also honey made by feeding bee colonies sugar syrup in the summer and declared as a pure honey, can be judged as adulterated (Titěra, 2006). Honey flavour can be modelled by heating a solution of a monosaccharide with phenylalanine, since almost all phenyl acetic esters are known for possessing honey flavour (Kolínek, 2007).

The natural content of sucrose in honey is (with some exceptions) to 5% (Kameník, 2013). Sugar content of honey depends on its botanical and geographical origin, weather, storage conditions and processing technology (Dobre et al., 2012). Honeydew honey is lower in sugar

than the nectar (floral) honey (Bentabol et al., 2011; Escuredo et al., 2013). Harvesting of honey with high moisture content, or subsequent addition of water to honey can result in honey fermentation and spoilage (Šroll, 2012). Sometimes honey is artificially coloured, because darker honey can give consumers the impression that it is forest honey (Přidal, 2005).

The aim of this study was to detect illegal or undeclared addition of sugar in honey using high performance liquid chromatography (HPLC) and determine whether the honey vendors comply with legal requirements applicable to their product and whether consumers are not deceived.

MATERIAL AND METHODOLOGY

We have analysed a total of 21 honey samples. Thirteen honey samples came from the regular shopping network, two samples were purchase in Health Food stores and six samples were obtained directly from local beekeepers (Table 1). We have prepared 10% solution from the relevant sample of honey. After mixing, two parallel samples were prepared, centrifuged at 18000 rpm for 5 min and analysed by HPLC. Conditions of analysis: column: steel 7.8 x 300 mm, packing: Rezex RCM-Monosaccharide Ca²⁺ (8%), temperature: 80 °C, mobile phase: deionized water, flow rate: 0.8 mL / min, injection volume: 5 µL, pressure: 2.6 MPa, detection: refractometric, detector sensitivity: 0.32. Equipment:

double piston pump LCP 4000, dispense valve D, column oven LCO 101, columns supplied by Phenomenex, differential refractive index detector, laboratory instruments Praha RIDK-102nd. Further, deionized water was used for HPLC, standards used were of HPLC grade, and laboratory centrifuge was Hobbolab 2110 (France). Evaluation software was Clarity.

Calibration: 0 - 1 - 2 - 5 g/100 mL of maltose, sucrose, glucose, fructose, glycerol, methanol, ethanol (Merci, Germany).

Samples were evaluated according to the Council Directive 2001/110/EC, which lays down limit values for the parameters of honey.

We have calculated the basic statistics such as the mean and standard deviation for each sample (n = 3).

RESULTS AND DISCUSSION

We have determined the amounts of fructose, glucose, sucrose, and oligosaccharides using the HPLC method. Besides floral honeys, samples contained also honeydew honey, for which different values apply than those listed in Council Directive 2001/110/EC. Table 2 shows that only four out of 21 samples met the requirements of **Council Directive 2001/110/EC of 20 December 2001 relating to honey**. These were three samples from regular shopping network and one obtained directly from the beekeeper.

Table 1 List of honey samples.

Sample no.	Honey type	Purchased from	Country of origin	Additional information
1	Not stated	Beekeeper	Czech Republic	Honey from the Moravian Karst
2	Floral	Beekeeper	Czech Republic	Czech forest honey, KLASA
3	Floral	Shopping network	Blend of EU and non-EU honeys	Meadow
4	Not stated	Shopping network	Blend of EU and non-EU honeys	Mixture of floral and honeydew honey in certain proportions
5	Not stated	Shopping network	Blend of EU and non-EU honeys	Forest honey, Mixture of floral and honeydew honey in certain proportions
6	Floral	Shopping network	Blend of EU and non-EU honeys	Meadow
7	Not stated	Health Food	Czech Republic	Bio buckwheat honey
8	Floral	Shopping network	Blend of EU and non-EU honeys	Meadow
9	Floral	Shopping network	Blend of EU and non-EU honeys	-
10	Floral	Shopping network	Not stated	Bio
11	Floral	Shopping network	Cuba, Mexico, Nicaragua	-
12	Honeydew	Shopping network	Blend of EU and non-EU honeys	Forest
13	Floral	Shopping network	Blend of EU and non-EU honeys	„Honey bear“- honey in bear-shaped bottle
14	Not stated	Beekeeper	Czech Republic	Acacia
15	Floral	Beekeeper	Czech Republic	-
16	Not stated	Beekeeper	Czech Republic	Blended honey
17	Floral	Beekeeper	Slovak Republic	Slovak honey
18	Not stated	Shopping network	Czech Republic	Blend of honeydew and floral honeys
19	Floral	Shopping network	Czech Republic	Bio meadow honey
20	Not stated	Health Food	Greece	-
21	Floral	Shopping network	Czech Republic	-

Table 2 The average content of carbohydrates (g/100 g) provided by HPLC method (n = 3); Oligosaccharides I (maltosa), Oligosaccharides II (maltotriosa).

Sample no.	Fructose [g/100 g]	Glucose [g/100 g]	Sucrose [g/100 g]	Oligosaccharides I [g/100 g]	Oligosaccharides II [g/100 g]
1	31.2 ±0.62	28.4 ±0.57	7.9 ±0.16	2.1 ±0.04	10.8 ±0.21
2	38.2 ±0.76	37.9 ±0.76	6.7 ±0.13	0.5 ±0.01	0.7 ±0.01
3	38.4 ±0.62	41.6 ±0.89	5.4 ±0.35	0.2 ±0.25	0.40 ±0.21
4	39.9 ±0.68	39.6 ±0.79	5.4 ±0.68	0.3 ±0.56	0.4 ±0.58
5	38.1 ±0.46	39.2 ±0.82	5.9 ±0.59	0.3 ±0.35	0.4 ±0.36
6	41.4 ±0.32	32.0 ±0.45	3.8 ±0.55	0.2 ±0.37	0.3 ±0.78
7	36.9 ±0.55	33.5 ±0.52	8.6 ±0.79	1.2 ±0.56	5.1 ±0.88
8	42.1 ±0.76	32.2 ±0.78	4.5 ±0.46	0.3 ±0.51	0.6 ±0.49
9	34.7 ±0.45	31.2 ±0.57	8.8 ±0.32	0.6 ±0.32	1.2 ±0.78
10	26.6 ±0.51	29.9 ±0.61	2.3 ±0.58	0.2 ±0.11	0.5 ±0.68
11	34.2 ±0.48	29.0 ±0.85	8.5 ±0.87	0	1.1 ±0.23
12	36.6 ±0.73	30.1 ±0.55	12.7 ±0.87	0.7 ±0.58	2.0 ±0.46
13	45.0 ±0.67	33.7 ±0.32	3.9 ±0.98	0.2 ±0.69	0.4 ±0.28
14	43.9 ±0.69	27.9 ±0.66	7.9 ±0.58	0.1 ±0.89	2.3 ±0.25
15	32.8 ±0.85	33.9 ±0.67	6.4 ±0.69	0.9 ±0.25	1.2 ±0.89
16	24.7 ±0.88	22.4 ±0.42	8.0 ±0.85	0.5 ±0.39	1.1 ±0.82
17	27.3 ±0.69	26.0 ±0.51	5.6 ±0.25	0.1 ±0.54	1.3 ±0.78
18	30.7 ±0.89	23.1 ±0.78	4.3 ±0.34	0.9 ±0.65	3.6 ±0.71
19	28.0 ±0.76	27.7 ±0.45	5.7 ±0.54	0.4 ±0.23	1.4 ±0.59
20	23.5 ±0.47	18.1 ±0.58	3.9 ±0.48	1.0 ±0.66	1.2 ±0.53
21	24.4 ±0.65	22.6 ±0.36	3.9 ±0.35	0	0.2 ±0.48

Six samples did not meet the requirements for the sum of fructose and glucose, two could not be included because failing to specify the type of honey and fourteen samples failed the requirement of sucrose content.

The above mentioned directive states that honey if placed on the market or used in any product intended for human consumption must meet the following criteria: the sum of fructose and glucose in floral honey should be no less than 60 g / 100 g and no less than 45 g / 100g for honeydew honey and blends of honeydew honey with floral honey.

Sucrose content: generally, not more than 5 g / 100g; not more than 10 g / 100 g for acacia honey (*Robinia pseudoacacia*), alfalfa honey (*Medicago sativa*), banksia honey (*Banksia menziesii*), sulla honey (*Hedysarum*), eucalyptus honey (*Eucalyptus camadulensis*) leatherwood honey (*Eucryphia lucida*, *Eucryphia milliganii*) and citrus honey (*Citrus spp.*), and not more than 15 g / 100 g for lavender honey (*Lavandula spp.*) and starflower honey (*Borago officinalis*).

Limits for the oligosaccharides are not determined by legislation, but their value should be around 10 % in honeydew honeys and between 2 and 3 % in floral honeys.

Honey samples have been further assessed for their compliance with legislative requirements relating to the product or whether the consumers are not deceived. As reported by Titěra and Vořechovská (2010), the compulsory indications on the label include manufacturer's business name and address, quantity, date of minimum durability or 'best before' date (just the month and year),

the country of origin (CR, EC or non-EC). According to Horňáčková (2009), the most frequently reported minimum durability of honey is two years. Yet we can see much longer minimum durability indicated on honey labels. Horňáčková (2009) further states that each beekeeper can determine the minimum durability of his/her own honey based on laboratory tests, when honey even after his/her determined period of minimum durability meets all requirements for wholesomeness and quality. For products intended to be supplied into stores outside the local district, it is important to indicate the registration number assigned by the competent State Veterinary Administration. Other important information, which must be included on the label, is the type of honey, i.e. whether it is floral (nectar) or honeydew honey. According to Decree no. 113/2005 Coll., the label indications must not include words like true, fresh, pure, home-made, high-quality, natural or healing. It addition, it must not give any information about the preventive effects or healing power of honey. The compulsory indications on the label of honey from the beekeeper include the name and address of the beekeeper, quantity, date of minimum durability or 'best before' date (just the month and year) and information referring to its floral or honeydew origin.

Such label is not necessary, if honey is sold e.g. at 'yard sale'. Our analysed samples often had no indication about the honey origin (floral or honeydew). Specifically, this information was missing on eight out of 21 samples. Samples 5 and 9 lacked any indication about the manufacturer. The country of origin was absent only on

sample 10. The study by **Vlkovič, Vorlová and Přidal (2011)**, which dealt with the issue of proper honey labelling, reported that the most common deficiency in the labelling of honey in stores is the absence of honey type indication.

Přidal (2012) therefore suggests that the use of the word 'forest' and the like be restricted by law, thereby to ensure that the label is not misleading and ambiguous. Another possible solution **Přidal (2013)** sees in the absence of any identification of the honey type on the label. Honey type could be indicated only if specific properties prevail which are characteristic of a given generic honey (e.g. sunflower honey, acacia honey, honeydew honey, etc.).

CONCLUSION

The HPLC method was used to determine the amounts of fructose, glucose, sucrose and oligosaccharides in samples of honey purchased in the Czech Republic. Only four out of 21 samples complied with the requirements set out in Council Directive 2001/110/EC relating to honey. These were three samples from the regular shopping network and one purchased directly from the beekeeper.

The most common deficiency in evaluating the compliance with labelling requirements was the absence of honey type identification. In total, this information was missing in 8 samples. Often, the type of honey was replaced by the word 'forest', which is optional and can mislead the consumer into believing that the product is honeydew honey. This absence of type identification caused also difficulties in assessing the content of sugar, as it was not clear into which category the honey should be classified. Two samples do not identify the manufacturer.

Manufacturers often inundate labels of their products with optional information, which can create a feeling of product exceptionality among the customers and mislead them. While honey adulteration usually does not endanger consumers' health, in any event such practice deceives consumers because instead of natural honey with many favourable properties they consume factory product based on sucrose and starch.

Existing legislation provides for the evaluation of honey rather inaccurately as it does not define the generic status of honey, does not distinguish between floral and honeydew honeys and does not limit the use of the word 'forest' in labelling of honey. The results show that the consumer cannot entirely rely on always buying properly identified honey of the highest quality in specialized stores or regular shopping network. Possible improvements can be brought about by the Regulation no. 1169/2011 of the European Parliament and of the Council, which specifies the mandatory particulars that must be included on packaging.

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TESTING OF DNA ISOLATION FOR THE IDENTIFICATION OF HEMP

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ABSTRACT

Hemp is diploid organism ($2n = 2x = 20$, genome size 534 Mb) with nine pairs of autosomes plus XX (♀) or XY (♂) chromosomes. *Cannabis sativa* L. is an important economic plant for the production of food, fibre, oils, and intoxicants. Genotypes (varieties or chemovar) of hemp with low Δ^9 -tetrahydrocannabinol content are used for industrial applications. Varieties with high Δ^9 -tetrahydrocannabinol or high cannabidiol content are used for medicinal applications. Biochemical and molecular methods can be used for identification and classification. An important step for molecular biology methods is to obtain the matrix of the native and sufficiently pure DNA. We tested two different experimental variants of samples (20 mg and 100 mg) of seeds, oilcake and dried flowers for analysis of the Italian variety Carmagnola for analysis (harvested in 2014, Hempoint Ltd., Czech Republic). The DNeasy® Plant Mini Kit (Qiagen, GE) was used to isolate the DNA. The DNA concentration and purity was assessed by agarose electrophoresis and via a spectrophotometer. Samples of lower weight yielded lower values of DNA concentration (average $16.30 - 38.90 \text{ ng} \cdot \mu\text{L}^{-1}$), but with better purity than samples of higher weight (ratio $A_{260\text{nm}}/A_{280\text{nm}}$ for low-weight samples was near 1.80). To test the applicability of DNA analysis, we used two SSR markers (*CAN1347* and *CAN2913*). PCR products were separated on 1% agarose and on 8% polyacrylamide electrophoresis. DNA samples obtained from samples of higher weight exhibited less PCR amplification than samples of lower weight. We found no effect of sample weight on the formation of non-specific amplification products during the PCR reaction. Based on our results we can be recommended for practical isolation procedure using DNeasy® Plant Mini Kit with lower of sample weight (20 mg). In future work the procedure for DNA isolating from wheat-cannabis products, e. g. breads, rolls or pasta, will be optimized.

Keywords: *Cannabis*; seed; oilcake; dry flower; DNA isolation

INTRODUCTION

Hemp (*Cannabis* L.) is one of the oldest cultivated plants. It is both cultivated and grows wild around the world, and is used in diverse applications (Gilmore and Peakall, 2003). *Cannabis sativa* L. has been distributed as a source of fibre, feed, oils, medicine and intoxicants (Small and Cronquist, 1976). However, *Cannabis* is the botanical genus of the plant and marijuana describes *Cannabis* plants that contain high Δ^9 -tetrahydrocannabinol (THC) content and are used for their psychoactive potency (Alghanim and Almirall, 2003). Hemp is used to describe *Cannabis* plants that have low THC content and are cultivated for industrial applications. Therefore, there are two distinctive strains; one is generally cultivated for fibre (hemp) and the other for drug use (marijuana) (Mechoulam, 1970). Historically, there were three recognized varieties of *Cannabis*: *C. sativa*, *C. indica*, and *C. ruderalis*. For many years, botanists considered each of them to be a distinct species. However, most botanists now generally agree that *Cannabis* is a genus with a single highly variable species (*C. sativa*) that has diversified into a wide variety of ecotypes and cultivated races (Siniscalco Gigliano, 2001). Identification of *Cannabis* is also important for farmers and industry.

There are two main methods in most classification schemes that can be applied to hemp identification.

For marijuana, both biochemical (Debruyne et al., 1994) and DNA tests (Siniscalco Gigliano, 1999) are available to identify a substance as *Cannabis*. Biochemical methods to establish geographic origin of a plant have met with variable success (Pitts et al., 1992). Biochemical profiling has also successfully differentiated between resinous and textile *Cannabis* (Debruyne et al., 1981). One of the most useful and widely used DNA markers is SSR, otherwise known as microsatellite, or short tandem repeat (STR) (Alghanim and Almirall, 2003). Microsatellites have become well suited for a fingerprint and genotype identification (Gregáňová et al., 2005; Musilová et al., 2013), seed purity evaluation and germplasm conservation (Brown et al., 1996), and marker assisted selection (Röder et al., 1998).

The first step for the application of DNA markers in hemp is DNA isolation. In our study we tested sources of DNA and subsequent application of the DNA for DNA fingerprinting in *Cannabis*.

MATERIAL AND METHODOLOGY

Genomic DNA was isolated from seeds, oilcake and dry flowers (Figure 1) using the isolation kit DNeasy® Plant Mini Kit (Qiagen, GE). Italian variety Carmagnola from Hempoint, Ltd. (Czech Republic), harvested in 2014, was used. Two experimental sample variants were used for

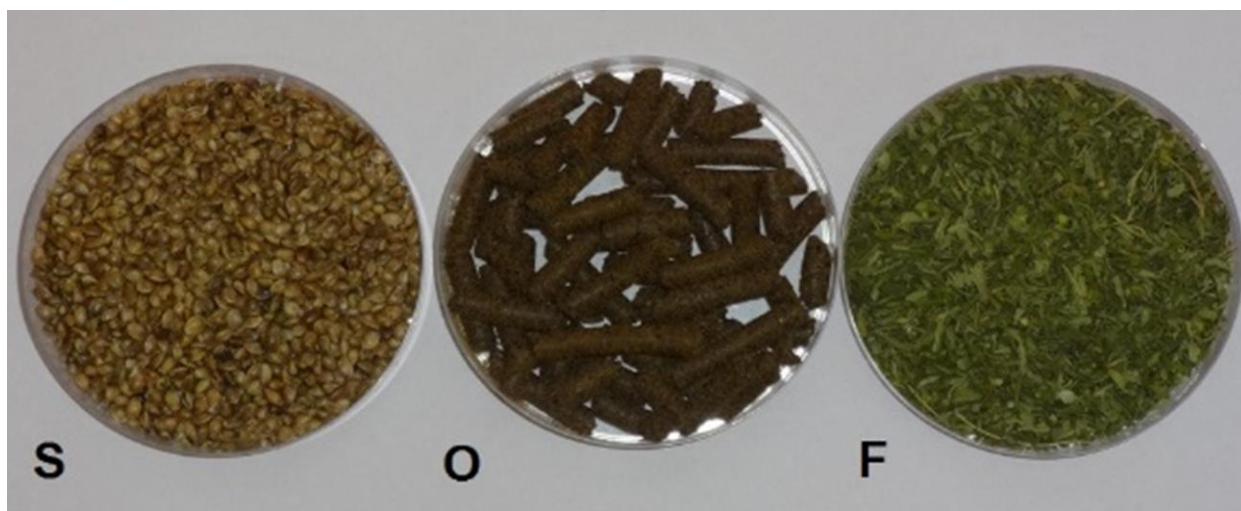


Figure 1 Matrix used for analysis. S – seeds, O – oilcake, F – dry flower.

analysis: 20 mg and 100 mg (in triplicate). The DNA concentration and purity was assessed by 1% agarose electrophoresis and a spectrophotometrically by Picopet 1.0 (Picodrop, UK). The values obtained were compared using ANOVA at $p < 0.05$.

To test the applicability of DNA analysis for identification, two SSR markers (*CAN1347* and *CAN2913*) were used, as described by Gao et al., (2014). PCR analyses were repeated twice. The reaction mixture for PCR of a total volume 25 μL contained 0.5 U *Taq* polymerase (Promega), 1 \times aliquot buffer, 0.1 mM of each dNTP (Promega), 0.3 M of each primer and 20 ng of

RESULTS AND DISCUSSION

In our experiment we tested two experimental variants of cannabis weighed in three different matrices, which are commonly used in agriculture and food industry. Sample weights were used according to the manufacturer's protocol (Qiagen, 2012). The obtained results show that in samples weighing 100 mg, the average DNA yield was about 2 times higher ($56.80 - 68.80 \text{ ng}\cdot\mu\text{L}^{-1}$) than that of samples weighing 20 mg (Table 1). The observed variability in the values was not dependent on the weight of the material or on the biological matrix. The observed variability could be due to human factors in the course of

Table 1 Parameters of obtained DNA.

Sample	Concentration ($\text{ng}\cdot\mu\text{L}^{-1}$)	v_x (%)	Purity	v_x
			($A_{260\text{nm}}/A_{280\text{nm}}$)	(%)
average \pm SD			average \pm SD	
S100	63.13 \pm 17.92	28.38	1.64 \pm 0.04	2.31
O100	68.80 \pm 6.94	10.09	1.42 \pm 0.04	2.67
F100	56.80 \pm 4.30	7.58	1.67 \pm 0.06	3.51
S20	19.03 \pm 4.82	25.31	1.77 \pm 0.18	10.25
O20	16.30 \pm 6.16	37.78	1.83 \pm 0.28	15.42
F20	38.90 \pm 16.48	42.36	1.60 \pm 0.06	3.82

S – seeds, O – oilcake, F – dry flower, 100 – sample weight 100 mg, 20 – sample weight 20 mg, SD – standard deviation, v_x – coefficient of variation.

template DNA; the reaction conditions of PCR in T3 cyclor (Biometra) by Gao et al., (2014). The PCR reaction profile comprised a 10 min incubation at 94 $^{\circ}\text{C}$, then a cycle of 94 $^{\circ}\text{C}$ for 30 s, 57 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 40 s, repeated 35 times. Following cycling, the reaction was held at 72 $^{\circ}\text{C}$ for 10 min, before a final 10 $^{\circ}\text{C}$ hold. Useful step seems to be control electrophoresis on 1% agarose gel (stained with ethidium bromide) and on polyacrylamide gels. The amplification of SSR products was then visualized on 8% non-denaturing polyacrylamide (PAA) gels in TBE (Tris-borate-EDTA) buffer followed by staining with silver (0.2% AgNO_3).

DNA isolation, especially during homogenisation of the sample using a mortar in the presence of liquid nitrogen. Homogenization of the matrix is one of the most critical steps of the entire DNA isolation and significantly affects the yield of the applied protocol (Blim and Stafford, 1976).

For subsequent use of the DNA for molecular biology methods, however, DNA purity is a more important factor. Sufficiently pure samples satisfy the condition $A_{260\text{nm}}/A_{280\text{nm}} \geq 1.8$ (Moeller et al., 2014).

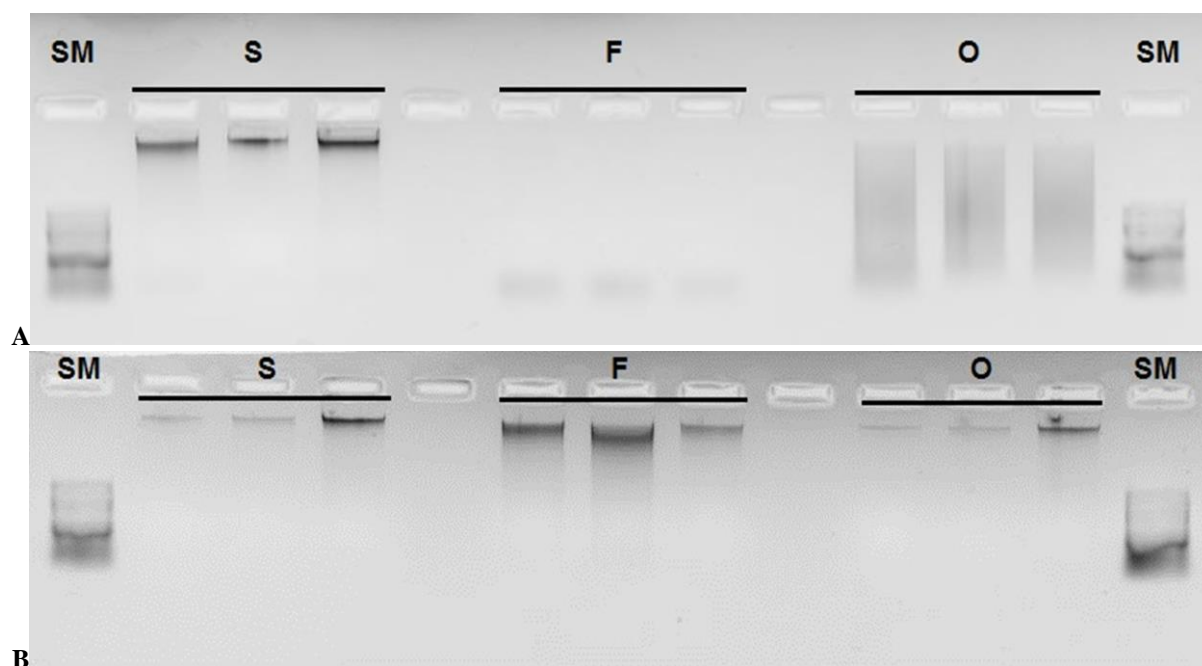


Figure 2 Control electrophoresis of DNA (1% agarose). A – sample weight = 100 mg, B – sample weight = 20 mg, SM – size marker, S – seeds, O – oilcake, F – dry flower.

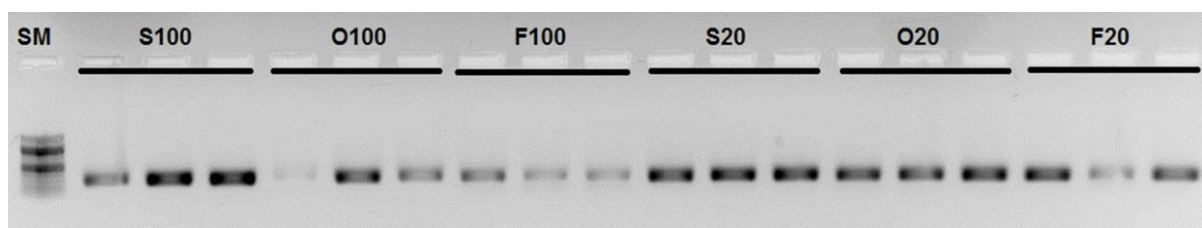


Figure 3 Agarose electroforetogram of SSR marker (*CAN2913*). SM – size marker (100 bp), S – seeds, O – oilcake, F – dry flower, 100 – sample weight 100 mg, 200 – sample weight 20 mg.

When comparing samples for analysis to achieve better parameters for weighing 20 mg with an average purity of 1.60 to 1.83 (Table 1). This may be influenced by the absorption capacity of the purification columns provided in the kit and by removing large quantities of impurities from the final DNA sample (Qiagen, 2012). The visible difference in the quantity and quality of DNA obtained from 20 mg samples indicates that the lower weight samples are preferable over higher weight samples (Figure 2). During electrophoresis, the 100 mg oilcake sample exhibited distinct smears indicating a possible degradation of DNA. Although these are the products (oilcake) from cold pressing, but in the high pressure process that heats (Small and Marcus, 2002), which may negatively affect the DNA and lead to its degradation. Increasing the purity of the obtained DNA is possible via purification procedures, but purification processes can reduce the final concentration of DNA in the sample (Demeke and Jenkins, 2010).

Dirt and degraded DNA may negatively affect the progress of the PCR reaction (Collard et al., 2007). Therefore, we decided to use two SSR for testing the effect of concentration and purity of DNA on the progress of the PCR reaction. Our results confirm the known fact (Ning et al., 2009), that the level of purity has a much greater

influence on the course of the PCR reaction than DNA concentration. Especially during electrophoresis with agarose gels (Figure 3), compared to polyacrylamide gels (Figure 4), were observed greater PCR amplification from 20 mg samples for analysis with a lower concentration, but higher purity in comparison with samples weighing 100 mg, where the values were reversed. The most significant negative influence on the formation of the PCR product was demonstrated at 100 mg variants - dried flowers, which could adversely exhibit high essential oil content in the flowers of hemp (Hazekamp and Fishedick, 2012). Simultaneously, it was not shown to affect the formation of non-specific amplification in the PCR reaction, which is visible on a polyacrylamide gel (Figure 4).

Previous reports indicated that the ideal concentration of DNA for analysis using SSR markers is 20 - 30 ng.μL⁻¹ (Gregáňová et al., 2005; Musilová et al., 2013; Ovesná et al., 2014). Within the isolation of DNA from the portion of the lower concentration of DNA was achieved on the border of the reference value. Given this fact and the results of the analysis of SSR markers of cannabis, the DNeasy® Plant Mini Kit (f. Qiagen) can be recommended for DNA isolation of samples weighing 20 mg.

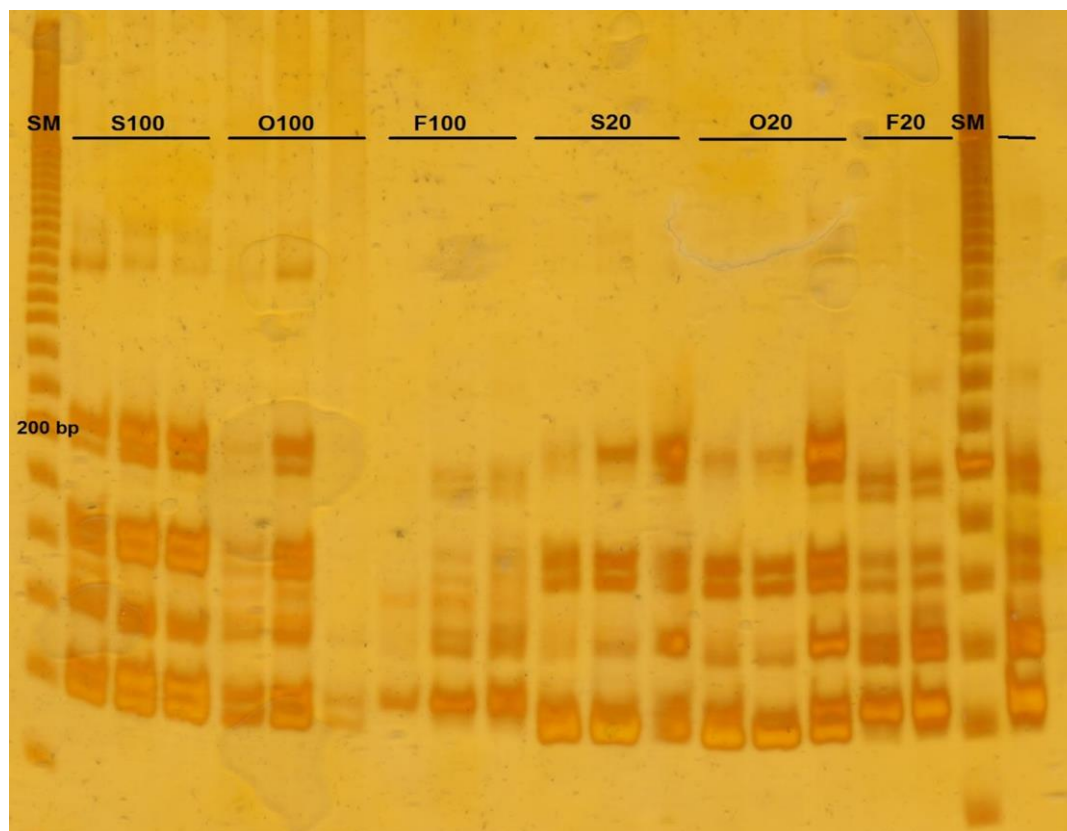


Figure 4 Polyacrylamide electroforetogram of SSR marker (*CAN2913*). SM – size marker (20 bp), S – seeds, O – oilcake, F – dry flower, 100 – sample weight 100 mg, 200 – sample weight 20 mg.

CONCLUSION

Based on our results we can recommend practical DNA isolation procedure using the DNeasy® Plant Mini Kit with sample weights of 20 mg. From this size sample, we obtained the best results for DNA quality and purity, and there was no effect on subsequent analysis of DNA variation using microsatellite markers. In future work we will optimize the procedure for DNA isolation from cannabis products and we will look for a combination of SSR markers to identify varieties of industrial hemp.

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DETERMINATION OF MERCURY, CADMIUM AND LEAD CONTENTS IN DIFFERENT TEA AND TEAS INFUSIONS (*CAMELLIA SINENSIS*, L.)

Július Árvay, Martin Hauptvogel, Ján Tomáš, Ľuboš Harangozo

ABSTRACT

The present paper deals with assessing the level of contamination of green ($n = 14$) and black – fermented ($n = 10$) teas of different origins (country of origin) (China, India, Japan, Nepal and Taiwan), which are normally available in Slovakia. The contents of the studied contaminants (mercury, cadmium and lead) were observed in samples of dried teas and their infusions. The contaminant contents were investigated by atomic absorption spectrometry with Zeeman background correction and a graphite furnace GF-AAS (Cd, Pb). The total mercury content was analyzed by CV-AAS method. Concentrations of the studied contaminants in the dried tea samples were as follows: green tea: Hg: $0.0027 \pm 0.0010 \text{ mg.kg}^{-1}$ (median \pm standard deviation); Cd: $0.161 \pm 0.084 \text{ mg.kg}^{-1}$, Pb: $0.875 \pm 0.591 \text{ mg.kg}^{-1}$, black tea: Hg: $0.0022 \pm 0.0014 \text{ mg.kg}^{-1}$, Cd: $0.397 \pm 0.077 \text{ mg.kg}^{-1}$, Pb: $1.387 \pm 0.545 \text{ mg.kg}^{-1}$. The contents of the contaminants in the tea infusions were as follows: green tea: Hg: $0.03 \pm 0.04 \text{ } \mu\text{g.L}^{-1}$, Cd: $0.278 \pm 0.068 \text{ } \mu\text{g.L}^{-1}$, Pb: $1.975 \pm 0.503 \text{ } \mu\text{g.L}^{-1}$, black tea: Hg: $0.050 \pm 0.080 \text{ } \mu\text{g.L}^{-1}$, Cd: $0.291 \pm 0.054 \text{ } \mu\text{g.L}^{-1}$, Pb: $1.955 \pm 1.264 \text{ } \mu\text{g.L}^{-1}$. According to the currently valid maximum limits for a particular contaminant in Slovakia, it can be stated that the health standards were not exceeded in any of the tea samples. The limit value of the lead content ($2.0 \text{ mg.kg}^{-1} \text{ DM}$) was exceeded (by 12.4%) only in one sample of the dried black tea from China (Yunnan – Golden Snow), however it is the limit value valid in China. The results of the analysis of 24 tea samples show that even regular consumption does not pose a health risk to consumers.

Keywords: mercury; cadmium; lead; teas; tea infusions; *Camellia sinensis*, L.

INTRODUCTION

Tea (*Camellia sinensis*, L.) is one of the most popular non-alcoholic beverages consumed by over two-third of the world's population due to its medicinal, refreshing and mild stimulant effects. Tea plays a major role in terms of the intake of a number of nutritional trace elements and biologically active compounds in humans (Sharangi, 2009). The Tea plant is evergreen plant with three races (*C. sinensis*, *C. assamica* and *C. cambodiensis*). Tea plant grows in more than 36 countries spread over all continents (Jeszka-Skowron et al., 2015). Tea plant grows best in tropical and subtropical areas with adequate rainfall, good drainage and acid soils. Tea leaf comprises of two leaves and the terminal apical bud of a growing shoot of a tea bush. Made teas are classified into six main types such as white, green, oolong, black, compressed and flavored based on their respective manufacturing techniques (The United Kingdom Tea Council Ltd., 2014).

Green teas (compared with other tea types) are characterized by the highest biological effect on consumers. They contain a broad spectrum of catechins, and/or flavan-3-ols and caffeine (theine) (Naldi et al., 2014; Zimmermann and Gleichenhagen, 2011). Substances from the group of catechins and flavan-3-ols have high antioxidant ability (Nováková et al., 2010).

Regular consumption of tea beverage has a positive effect on various aspects of human health. Tea drinking is associated with the reduction of blood serum cholesterol

(Dong et al., 2007), prevention of low density lipoprotein oxidation (Zhang et al., 2009), decreased risk of cardiovascular disease and cancer (Chung et al., 2003). Polyphenols are the most biologically active group of tea components, which have antioxidative, antimutagenic, and anticarcinogenic effects (Yao et al., 2004). Tea contains also other compounds beneficial to human health like fluoride, caffeine and essential minerals (Cabrera et al., 2003).

Regular consumption of tea (and/or herbal tea) also brings negative aspects on human health and it is therefore necessary to know the contents of some harmful elements in teas and tea infusions (Bobková et al., 2015). Some heavy metals, particularly Hg, Cd and Pb are characterized by high persistence in different environmental compartments or different agricultural (plant and/or animal) products, especially legumes (Timoracká et al., 2011), potatoes (Musilová and Bystrická, 2010), bee honey (Roman and Popiela, 2011) and high bioaccumulate ability in the consumer's body, causing various diseases such as cancer and/or Alzheimer disease (De Sole et al., 2013). According to the findings of Chen et al., (2011), contents of some hazardous metals (especially aluminum and mercury) are positively correlated with the levels of catechins or total phenolic contents.

Due to the fact that beverages prepared with hot or cold extraction of teas belong to the most popular non-alcoholic

beverages, it is necessary to pay attention to the monitoring their phytochemical composition, especially in terms of the content of harmful ingredients, often naturally present in them. The paper focuses on determination of the content of monitored contaminants (Hg, Cd and Pb) in teas and their subsequent transfer to a tea beverage prepared by hot extraction.

MATERIAL AND METHODOLOGY

For the purposes of this paper, 24 samples of tea (*Camellia sinensis* L.) that represent two basic groups of teas: green tea ($n = 10$) and black – fermented tea ($n = 14$) were chosen. The samples were obtained from Tea House of Good People, Nitra, Slovakia in 2014. The samples come from different countries of East Asia (China, India, Japan, Nepal and Taiwan). The main characteristics of the tea samples are shown in Table 1.

Pre-analytical and analytical procedure

Before their mineralization, the samples of dried tea were homogenized in a ceramic mortar. The homogenized tea samples (1.0 g) were mineralized in a closed system of microwave digestion using Mars X-Press 5 (CEM Corp., Matthews, NC, USA) in a mixture of 5 mL HNO_3 (Suprapur, Merck, Darmstadt, Germany) and 5 mL deionized water ($0.054 \mu\text{S}\cdot\text{cm}^{-1}$) from Simplicity185 (Millipore SAS, Molsheim, France). Digestion conditions for the applied microwave system comprised of the heat which ran up to 160°C for 15 minutes, keeping it constant for 10 minutes. A blank sample was carried out in the same way. The digest were subsequently filtered through a quantitative filter paper Filtrak 390 (Munktell & Filtrak GmbH, Bärenstein, Germany) and filled up with deionized water to a volume of 50 mL (Árvay et al., 2014).

To assess the degree of possible intoxication of a consumer resulting from the consumption of a tea beverage, 2 g of the sample was extracted with 200 mL of boiling water for 10 minutes and afterwards the extract was filtered through filter paper Filtrak 390 (Munktell & Filtrak GmbH, Bärenstein, Germany).

The determination of metals were performed in a Varian spectrAA240Z (Varian Inc., Mulgrave, VIC, Australia) atomic absorption spectrometer with Zeeman background correction. The graphite furnace technique was used for the determination of Cd and Pb (detection limits for GF-AAS: 10.0 and 10.0 $\text{ng}\cdot\text{kg}^{-1}$ for Cd and Pb, respectively). The total mercury content was determined in the homogenized dried samples of teas (0.005 – 0.01 g) and tea infusions using a cold-vapour AAS analyzer AMA 254 (Altec, Prague, Czech Republic) with a detection limit of $1.5 \text{ ng}\cdot\text{kg}^{-1}$ DM (Svoboda et al., 2006). Calibration standard was used for the calibration of GF-AAS, CertiPUR® (Merck, Darmstadt, Germany).

Statistical analysis and risks assessment

All statistical analyses were carried out using the statistical software Statistica 10.0 (Statsoft, USA).

Descriptive data analysis included minimum value, maximum value, median, mean and standard deviation. The limit of statistical significance was set up at $p < 0.05$ for all descriptive statistical analysis. In order to assess the level of contamination of the dried green and black teas, the results of the studied contaminant contents were compared with maximum allowable levels (MAL) of the contaminants that are listed in relevant legislative regulations valid in China and Slovakia.

RESULTS AND DISCUSSION

Heavy metals in the tea samples (dried)

The contents of the studied elements in the dried tea samples are shown in Table 1. The contents varied in different intervals, depending mainly on the type of tea. Significantly higher concentrations of mercury were recorded in green teas compared with black – fermented teas, which is caused by technological processes (higher temperature during the tea fermentation). Hg concentration was $0.0027 \pm 0.0010 \text{ mg}\cdot\text{kg}^{-1}$ (median \pm standard deviation) in the green teas and $0.0022 \pm 0.0014 \text{ mg}\cdot\text{kg}^{-1}$ in the black teas. Paradoxically, the highest concentration of Hg was recorded in one sample of black tea (Darjeeling Singbulli: $0.00511 \text{ mg}\cdot\text{kg}^{-1}$). Almost identical results of mercury content in Pu-Erh teas (fermented teas) recorded Cao et al., (2010), who found in 17 samples concentration interval of $0.0002 - 0.0390 \text{ mg}\cdot\text{kg}^{-1}$ with an average of $0.0030 \text{ mg}\cdot\text{kg}^{-1}$. The maximum allowable levels of the monitored contaminants defined by the legislative standards of China, and/or Codex Alimentarius of Slovakia were not exceeded in the results of the Hg content in the tea samples.

In the case of cadmium and lead, the situation was different. Higher concentration of Cd and Pb was recorded in black teas compared with green teas. The cadmium content ranged from $0.161 \pm 0.084 \text{ mg}\cdot\text{kg}^{-1}$ in the green teas and $0.397 \pm 0.077 \text{ mg}\cdot\text{kg}^{-1}$ in the black teas. Similar conclusions were published by many authors who analyzed samples of green and black teas from different countries. For example Salahinejad and Aflaki, (2010) analyzed samples of black tea from India and recorded a cadmium content ranging from ND- $0.770 \text{ mg}\cdot\text{kg}^{-1}$. Moreda-Pinero et al., (2003) found Cd content ranging from 2.22 to $2.39 \text{ mg}\cdot\text{kg}^{-1}$ in different kinds of teas from India, Japan, Kenya, Malaysia, etc. Lower Cd content was found in samples of green tea from China and Japan, ranging from 0.051 to $0.114 \text{ mg}\cdot\text{kg}^{-1}$ (Marcos et al., 1998). Approximately 3 – 4 fold higher concentrations were found in lead concentration compared with cadmium. The Pb content was $0.875 \pm 0.591 \text{ mg}\cdot\text{kg}^{-1}$ in the green teas and $1.387 \pm 0.545 \text{ mg}\cdot\text{kg}^{-1}$ in the black teas. The relatively high value of the standard deviation indicates high variability of the set of values (Table 1). Similar results reported Lv et al., (2013), who recorded Pb concentration ranging from 0.66 – $4.66 \text{ mg}\cdot\text{kg}^{-1}$ with an average of $2.32 \text{ mg}\cdot\text{kg}^{-1}$ in fermented Pu-Erh teas. It confirms the findings of Cao et al., (2010).

Relatively large concentration interval of lead in individual tea samples can be caused by harvest period and age of the tea leaves. Han et al., (2006a) reported 2 – 2.5 times higher Pb content (as well as other elements) in older leaves compared with younger ones. Other major factors

affecting the different concentrations of lead in teas are local environmental conditions (Qin and Chen, 2007), use of fertilizers (Frankl et al., 2005) and technological processing. Han et al., (2006b) state that technological processes are major source of the product contamination

Table 1 Basic characteristics of tea samples and heavy metals content in tea and tea infusion samples.

Name	Sub-name	Country of origin	Heavy metals in teas (mg.kg ⁻¹ DM)			Heavy metals in tea infusions (µg.L ⁻¹)			
			Hg	Cd	Pb	Hg	Cd	Pb	
Black teas									
Darjeeling	Jungpana 2013 SF	India	0.00193	0.269	1.036	0.00030	0.440	4.530	
Darjeeling	Muscatel	India	0.00082	0.338	1.392	0.00004	0.275	0.340	
Darjeeling	Singbulli	India	0.00511	0.382	1.382	0.00009	0.261	2.580	
Nepal	Guranse 2013 FF	Nepal	0.00177	0.491	1.456	0.00004	0.282	2.940	
Aiko	--	Japan	0.00247	0.324	0.370	0.00005	0.277	1.770	
Assam	Bherjan Bio	India	0.00101	0.425	1.624	0.00001	0.336	1.900	
Ruby	--	Japan	0.00132	0.447	1.901	ND	0.257	1.360	
Yunnan	Golden Snow	China	0.00365	0.411	2.248^b	0.00009	0.314	2.850	
Yunnan	Black Needles	China	0.00369	0.431	0.767	0.00005	0.318	0.315	
Gaba	Black	Taiwan	0.00279	0.260	1.078	0.00006	0.299	2.010	
Descriptive statistics			Minimum	0.00082	0.260	0.370	ND	0.257	0.315
			Maximum	0.00511	0.491	2.248	0.00030	0.440	4.530
			Median	0.00220	0.396	1.387	0.00005	0.291	1.955
			St. dev.	0.00137	0.077	0.545	0.00008	0.054	1.264
Green teas									
Lung Ching	Superior	China	0.00416	0.222	1.832	0.08600	0.276	1.830	
Pi Lo Chum	Extra	China	0.00226	0.230	1.475	0.03000	0.338	2.090	
Mao Feng	Fu Tien	China	0.00365	0.090	0.448	ND	0.304	2.360	
Tai Mu	Long Zhu	Chinan	0.00278	0.117	0.880	0.05300	0.238	3.190	
Gaba	Imperial	Taiwan	0.00237	0.189	0.996	0.02300	0.222	2.090	
Kate	Benituki	Japan	0.00339	0.272	1.315	0.03900	0.319	1.790	
Gaba	Katsura	Japan	0.00202	0.134	0.157	0.01800	0.382	1.770	
Gyokuro	Asahina	Japan	0.00435	0.049	0.196	0.07100	0.255	1.240	
Genmaicha	Extra	Japan	0.00268	0.050	0.299	0.04500	0.132	1.890	
Sakura	Kukicha	Japan	0.00119	0.245	1.048	ND	0.360	2.460	
Sencha	Shizouka	Japan	0.00227	0.245	0.870	0.13300	0.276	2.270	
Matcha	Iri Genmaicha	Japan	0.00351	0.094	0.315	0.11300	0.279	1.750	
Sencha	Ashikubo	Japan	0.00445	0.072	0.717	0.01900	0.210	1.300	
Shambala	Nepal Exclusive	Nepal	0.00226	0.262	1.968	ND	0.358	2.440	
Descriptive statistics			Minimum	0.00119	0.049	0.157	ND	0.132	1.240
			Maximum	0.00445	0.272	1.968	0,13300	0.382	3.190
			Median	0.00273	0.161	0.875	0,03450	0.278	1.975
			St. dev.	0.00098	0.084	0.591	0,04208	0.068	0.503
Maximum Allowable Levels			0.300^a 0.500^c	1.000^{a, c}	2.000^b 10.0^c	--	--	--	

ND – not detected.

DW – dry weight.

^aMaximum allowable levels of Cr, Cd, Hg, As and F in tea (NY659-2003) (MOAC, 2002).

^bMaximum allowable levels of contaminants in green foods – tea (NY/T 288-2002) (MOAC, 2003).

^cMaximum allowable levels of contaminants – Codex alimentarius of Slovakia (PKSR, 2006).

by lead, especially spreading out. MAL of the cadmium and lead content defined by relevant legislative standards was not exceeded almost in all samples. An exception was one sample of Chinese black tea (Yunnan – Golden Snow), where MAL was exceeded by 12.4% (Table 1), which was reflected also in the Pb content of the tea infusion. However, this concentration does not possess any health risk arising from a long-term and regular consumption of the tea beverage. According to MAL valid in the Slovak Republic the Pb content was not exceeded.

Heavy metals in the tea infusion samples

The contents of the monitored elements in the tea infusions are listed in Table 1. The concentration of mercury in the infusions of green tea were $0.03 \pm 0.04 \mu\text{g.L}^{-1}$ and $0.05 \pm 0.08 \mu\text{g.L}^{-1}$ in the black tea infusions. The highest concentration of Hg was recorded in the black tea Darjeeling Jungpana 2013 SF infusion ($0.30 \mu\text{g.L}^{-1}$). The cadmium content ranged from $0.278 \pm 0.068 \mu\text{g.L}^{-1}$ in the green tea infusions and $0.291 \pm 0.054 \mu\text{g.L}^{-1}$ in the black tea infusions. Similarly to mercury, the highest concentration of cadmium was recorded in the sample of Darjeeling Jungpana 2013 SF ($0.44 \mu\text{g.L}^{-1}$). Similar results obtained Nookabkaew et al., (2006) and Sofuoglu and Kavcar, (2008), who recorded, cadmium contents at intervals from 0.04 to $0.24 \mu\text{g.L}^{-1}$ and $0.02 - 0.79 \mu\text{g.L}^{-1}$, respectively in samples of green and black tea from China, India, Japan and Turkey.

The lead content was in the range of $1.975 \pm 0.503 \mu\text{g.L}^{-1}$ in the green tea infusions and $1.955 \pm 1.264 \mu\text{g.L}^{-1}$ in the black tea infusions. The relatively high value of the standard deviation in the black teas is caused by high levels of lead in the sample of Darjeeling Jungpana 2013 SF ($4.53 \mu\text{g.L}^{-1}$). This value is by almost 132% higher in comparison with other values (median). Our results are considerably lower than results of green and black tea from Iran and China recorded by Nookabkaew et al., (2006) and Karimi et al., (2008).

CONCLUSION

The present paper aims at determining of the studied xenobiotics – heavy metals (Hg, Cd and Pb) in samples of green ($n = 14$) and black – fermented ($n = 10$) teas originating from different countries and beverages prepared from them. Our results show that MAL values defined by appropriate legislative norms were not exceeded in the majority of the dried tea samples. An exception was only one sample of Chinese fermented tea (Yunnan – Golden Snow), in which the allowable amount of lead was exceeded by 12.4%. The contents of the monitored contaminants in the tea infusions were not compared with the limit values. It can be concluded that even regular consumption of the tea beverages prepared from the tea samples does not pose a health risk to consumers.

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MICROBIOLOGICAL QUALITY OF SMEAR-RIPENED CHEESES STORED IN DIFFERENT TEMPERATURE REGIMES

Olga Cwiková

ABSTRACT

The purpose of this work was to study smear-ripened cheese, especially its microbiological quality. Samples were stored in different temperature conditions. The first group (A) of samples was stored in a refrigerator at 6 °C. The second group (B) of samples was stored at 6 °C for 21 days, next at -18 °C for 7 days and 7 days at 6 °C. The third group (C) of samples was stored at 6 °C before the date of minimum durability, next 7 weeks at -18 °C and after that at 6 °C for 7 days. I have observed lactic acid bacteria, *Brevibacterium linens*, coliforms, psychrotrophic organisms, *Escherichia coli*, moulds, and yeast. The number of *Brevibacterium linens* was higher ($p < 0.05$) at cooling/freezing for 1 week ($\log \text{CFU.g}^{-1}$) than cooling ($\log \text{CFU.g}^{-1}$) and cooling/freezing for 7 weeks after 35 days. A higher ($p < 0.05$) number of psychrotrophic microorganisms was recorded at the end of the monitoring in samples stored in a refrigerator (A/49) in comparison with cheeses stored using cooling/freezing regime for one week (B/49). Among the samples stored at these temperature regimes, there were no statistically significant differences ($p > 0.05$) in the numbers of LAB, coliforms, *E. coli*, moulds and yeast, neither at the end of the DMD nor at storage for 49 or 91 days, respectively.

Keywords: smear-ripened cheese; lactic acid bacteria; coliforms; moulds; yeast; *Brevibacterium linens*

INTRODUCTION

Cheese production is based on the use of both defined starter cultures and the presence of undefined indigenous microbial populations, including diverse yeasts and moulds, Gram-positive and Gram-negative bacteria (Monnet et al., 2015; Adouard et al., 2015; Cotona et al., 2012). Smear-ripened cheeses belong to the group of acidic cheeses, characterized by distinctive to spicy fragrance and flavour typified by orange or golden yellow smear (Teubner, 1998; Galaup et al., 2015). They gain these properties during aging especially through the action of proteolytic bacteria *Brevibacterium linens* (Fox, 2004). This sebaceous micro flora facilitates typical ripening predominantly from the surface toward the centre (Kadlec et al., 2012) and contributes (apart from the influence of physical and chemical properties of milk, starter culture, and non-starter bacteria) to the development of the cheese characteristics (Smit, 2003). In the cheese core, the dominant micro flora usually corresponds to lactic acid bacteria (LAB) species, while Gram-positive and catalase-positive bacteria, as well as yeasts, moulds and diverse Gram-negative bacteria (*Citrobacter spp.*, *Enterobacter spp.*, *Pseudomonas spp.*) constitute the subdominant micro flora (Cotona et al., 2012). The presence of some Gram-negative bacteria is often used as a marker for hygiene conditions, as coliforms are indicative of faecal contamination, and they are also considered to be undesirable cheese contaminants (Bockelmann et al., 2005). Contamination can occur not only during the processing of raw materials or food, including slicing, washing or cooling with contaminated water, contaminated tools, surface, or production worker, but also in packaging, transporting and distribution or through reservoir

organisms, insects, soil and air (Capozzi et al., 2009). The aim of this study was to perform microbiological analysis of smear-ripened cheese stored in three different temperature regimes and to monitor the development of cultural and contaminating microflora.

MATERIAL AND METHODOLOGY

We have analysed samples of smear-ripened cheeses, which were supplied by the manufacturer in consumer packaging weighing 100 g in cold boxes always on the day of production. Then we have stored the samples at the selected temperature conditions. For monitoring of microbiological indicators, we have chosen three types of storage regimes in accordance requirements of producer:

Type A: Storage in a refrigerator at 6 °C up to the date of minimum durability (DMD) and 14 days beyond this date. We have collected samples on the day of production (A/0), at the end of the DMD (A/35), and two weeks after the end of the DMD (A/49).

Type B: Storage of cheese after manufacture at cold storage temperature of 6 °C for 21 days. Cheeses were then frozen to a temperature of -18 °C for 7 days. For the following 7 days, they were again kept at cold storage temperature of 6 °C. We have collected samples on the day of production (B/0), at the end of the DMD (B/35), and two weeks after the end of the DMD (B/49).

Type C: Storage of cheese at cold storage temperature of 6 °C throughout the DMD (35 days). The samples were then kept frozen at -18 °C for 7 weeks. Subsequently the samples were unfrozen and stored at cold storage temperature of 6 °C for 7 days. We have collected samples at the end of the DMD (C/35) and 8 weeks after the end of the DMD (C/91).

We have monitored the following microbiological indicators: lactic acid bacteria (according to **ISO 15214:1998**), cultivation on MRS agar (Noack, France) at 30 °C, the counting of colonies was carried out after 72 hours. We have determined psychrotrophic microorganisms according to **ISO 17410:2001** on PCA growth medium (Noack, France); cultivation was carried out at 6.5 °C for 10 days. We have determined moulds and yeasts according to **ISO 21527-1:2008** on the DRBC growth medium (Noack, France); cultivation was carried out at 25 °C for 3 to 5 days. We have determined coliform microorganisms according to **ISO 4832:2010** on VRBL growth medium (Noack, France), at 37 °C. We have carried out counting of colonies after 48 hours. We have cultivated *Escherichia coli* on COLIFORM agar (Merck, Germany) for 48 hours at 37 °C. We have performed determination of *Brevibacterium linens* on agar M17 (Noack, France), cultivation was at 30 °C for 48 hours. During the experiment, we have successively analysed 5 batches of cheese. For one analysis we have always used 3 consumer packages. We have carried out analyses in the microbiology laboratory of the Department of Food Technology at the Mendel University in Brno from March to July 2011, according to **ISO 7218: 2007**.

From each 100 g package we have cut off 10 g of sample using a sterile scalpel. In order to get a representative sample, the sector passed through all of the segments. The sample included both the centre and edge of the cheese. After adding of 90 mL saline, we have homogenized the sample and diluted it to the desired decimal dilution.

We have performed statistical analysis using Statistica CZ, version 10. The program calculated basic statistical characteristics, such as mean and standard error of the mean. For comparison of groups, it has used simple analysis of variance. We have also used regression analysis.

RESULTS AND DISCUSSION

The aim of the experiment was to monitor the number of different groups of microorganisms during storage under different temperature conditions and to compare these numbers at the end of minimum durability and at the end of storage. The microorganisms included coliforms, psychrotrophs, moulds, yeast, *Escherichia coli*, *Brevibacterium linens*, and lactic acid bacteria.

Lactic acid bacteria (LAB)

At the beginning of the monitoring (A, B, C/0), the number of LAB was 8.6 log CFU.g⁻¹. Number of LAB (Figure 1) at the end of the minimum durability after 35 days of storage in regime A was 8.3 log CFU.g⁻¹. In the storage regime B, the number of LAB was 8.7 log CFU.g⁻¹ and in regime C it was 8.3 log CFU.g⁻¹. At the end of storage, the number of LAB for cheeses stored in regime A after 49 days was 8.9 log CFU.g⁻¹. In regime B, it was 8.7 log CFU.g⁻¹ and in storage regime C after 91 days, it was 8.8 log CFU.g⁻¹. Among the samples stored at these temperature regimes, there were no statistically significant differences ($p > 0.05$) among the number of LAB both at the end of the DMD and the storage for 49 and 91 days respectively. Lactic acid bacteria used in manufacturing of

smear-ripened cheese belong to the group of mesophilic bacteria, whose temperature minimum is between 5 °C and 15 °C. It is interesting, that the difference between the appropriate minimum temperature and the reasonable optimum temperature is about 22 °C. On the other hand, the difference between the optimum temperature and the maximum temperature is about 6.5 °C. This indicates that microorganisms tolerate suboptimal temperatures better than hyperoptimal ones. As stated by **Görner and Valík (2004)**, freezing is not the cause of death of all cells and this may be the cause for permanently higher numbers of LAB in samples stored in various temperature regimes. **Komprda et al., (2012)** also found the same number of lactic acid bacteria (8.2 log CFU.g⁻¹) at the end of the DMD in the smear-ripened cheeses stored at 5 °C. Likewise, according to **Tan et al., (2008)**, the numbers of LAB in smear-ripened cheeses range between 8 and 9 log CFU.g⁻¹.

Brevibacterium linens

The determined quantity of *Brevibacterium linens* at the beginning of storage (A, B, C/0) was 8.6 log CFU.g⁻¹. At the end of the DMD after 35 days of storage (Figure 2) in storage regime A, the detected value was 7.8 log CFU.g⁻¹. In the storage regime B, the value was 8.3 log CFU.g⁻¹ and in the storage regime C, the value was 7.8 log CFU.g⁻¹ of *Brevibacterium linens*. At the end of storage in the regime A after 49 days, the recorded value was 8.4 log CFU.g⁻¹. In the storage regime B, the value was 8.1 log CFU.g⁻¹ and under the conditions of regime C after 91 days, it was 7.8 log CFU.g⁻¹ of the bacteria *Brevibacterium linens*. Statistically significant differences ($p < 0.05$) were found in the numbers of *Brevibacterium linens* at the end of the DMD (after 35 days) and at the end of monitoring after 49 and 91 days, respectively. Higher ($p < 0.05$) counts of *Brevibacterium linens* have occurred at the end of the DMD in the storage regimen B (B/35), when compared to other storage methods. At the end of monitoring after 49 and 91 days, respectively, I have found higher number ($p < 0.05$) of bacteria in samples stored in the refrigerator (A/49), compared to the samples stored in the regime C (C/91). *Brevibacterium linens* may assert itself on the surface of the cheese after the present lactic acid is metabolized and neutralized by yeast and cocci and the surface pH rises to values of 5.7 to 6 (**Kadlec et al., 2012**, **Mounier et al., 2008**). **Eliskases-Lechner and Ginzinger (1995)** found after 6 weeks of aging in refrigerated conditions *Brevibacterium linens* at the surface of the cheese to be 9 log CFU.cm⁻². During freezing, the concentration of water useful for microorganisms decreases and their growth is partly or completely inhibited (**Görner and Valík, 2004**). However, freezing only brings the activity of microorganisms to a standstill, rather than killing them (**Šilhánková, 2008**). This fact may cause a lower number of *Brevibacterium linens* at the end of storage after long-term freezing. Storing in a refrigerator leads to multiplication of bacteria due to a suboptimal temperature, but in the other temperature regimes, it leads to stabilization of the number of *Brevibacterium linens*. Therefore, a short-term freezing should not influence the number *Brevibacterium linens*.

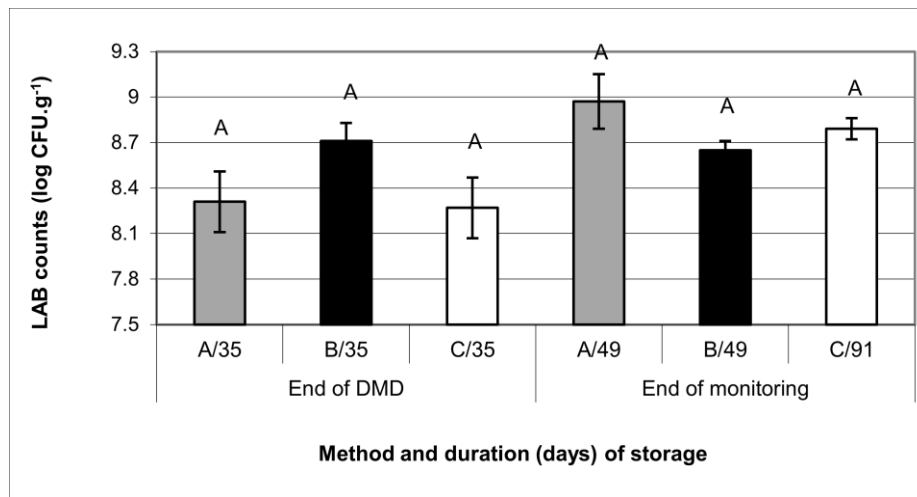


Figure 1 Number of LAB (log CFU.g⁻¹) in the smear-ripened cheese stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

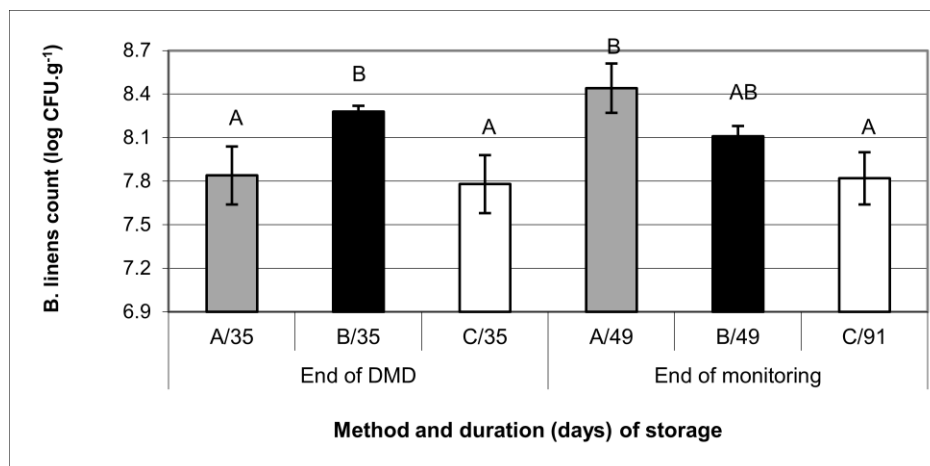


Figure 2 Number of *Brevibacterium linens* (log CFU.g⁻¹) in the smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C). Averages labelled with different letters were statistically different ($p < 0.05$) within a given factor, n = 15.

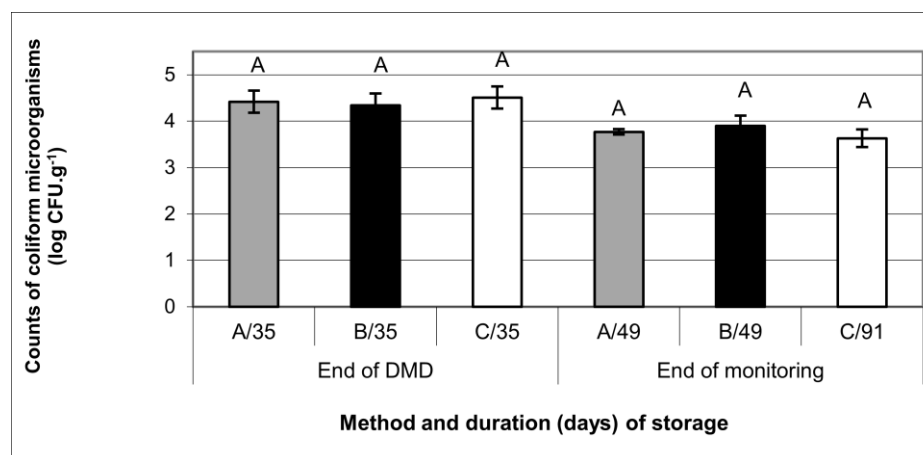


Figure 3 Number of coliform microorganisms (log CFU.g⁻¹) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

Coliform microorganisms

In the analysed cheeses stored at different temperature conditions, the counts of coliform microorganisms were comparable ($p > 0.05$) both at the end of the DMD and at the end of monitoring (Figure 3). At the beginning of storage (A, B, C/0), the count of coliforms detected in cheeses was $4.7 \log \text{CFU.g}^{-1}$. When stored in the regime A after 35 days, I have found $4.4 \log \text{CFU.g}^{-1}$ of coliform microorganisms. In the storage regime B, it was $4.3 \log \text{CFU.g}^{-1}$ and under the temperature conditions of regime C, it was $4.5 \log \text{CFU.g}^{-1}$. At the end of the monitoring, the number of coliform microorganisms in the storage regime A, after 49 days, was $3.8 \log \text{CFU.g}^{-1}$.

In the storage regime B, it was $3.9 \log \text{CFU.g}^{-1}$ and in the regime C, it was $3.6 \log \text{CFU.g}^{-1}$. **Dolci et al., (2009)** found on the surface of smear-ripened cheeses $5\text{-}6 \log \text{CFU.cm}^{-2}$ of coliform microorganisms. **Maher et al., (2000)** stated an increase in the number of coliforms within 42 days of ripening of these cheeses. **Doležalová et al., (2013)** have found lower counts of coliform bacteria in smear-ripened cheeses stored at temperatures up to $8 \text{ }^\circ\text{C}$, namely $4.6 \log \text{CFU.g}^{-1}$, which corresponds with our results.

Escherichia coli

I have not found any statistically significant differences ($p > 0.05$) among the numbers of *E. coli* in cheeses stored in various temperature regimes at the end of the DMD or at the end of monitoring after 49 and 91 days respectively (Figure 4). At the start of storage (A, B, C/0), the number of *E. coli* was $1.0 \log \text{CFU.g}^{-1}$. The number of colonies in storage regime A after 35 days was $1.2 \log \text{CFU.g}^{-1}$. When stored at the temperature regime B, the count was $1.7 \log \text{CFU.g}^{-1}$ and under the conditions of regime C, it was $1.0 \log \text{CFU.g}^{-1}$. At the end of the monitoring in the regime A after 49 days, the count of *E. coli* was $1.8 \log \text{CFU.g}^{-1}$. In the storage regime B, I found it to be $0.9 \log \text{CFU.g}^{-1}$ and under the conditions of regime C, after 91 days, it was $1.8 \log \text{CFU.g}^{-1}$. In comparison to our data, **Maher et al., (2000)** detected much larger numbers of *E. coli*. After 42 days of curing, in smear-ripened cheeses they found $4.0 \log \text{CFU.g}^{-1}$ of these bacteria. According to **Görner and Valík (2004)**, breeding of *Escherichia coli* does not take place at temperatures below $5 \text{ }^\circ\text{C}$. This is consistent with our results as well as with those from many other studies. According to **Frazier and Westhoff (1988)**, the growth rate of microorganisms decreases after reaching this minimum temperature. However, a slow metabolic activity can proceed, which may cause a tendency of slow growth of bacteria in the storage conditions of the regime A. It also explains the tendency to increase the number of bacteria in the regime C that occurred after defrosting the sample and its subsequent storage at $6 \text{ }^\circ\text{C}$. **Heredia et al., (2009)** have also confirmed that *Escherichia coli* can reliably survive temperature of $-20 \text{ }^\circ\text{C}$ in a freezer. **O'Brien et al., (2009)** state the minimum temperature for the growth of these pathogens to be approximately $7 \text{ }^\circ\text{C}$. In cheese, *E. coli* is used as an indicator for assessing post-pasteurisation contamination. Its presence may indicate inadequate pasteurisation, poor hygiene conditions during processing, or post-processing contamination (**O'Brien et al., 2009**).

Psychrotrophic microorganisms

Their number was at the beginning of monitoring (A, B, C/0) $7.9 \log \text{CFU.g}^{-1}$. At the end of the DMD after 35 days, the number of psychrotrophic microorganisms in regime A was $7.9 \log \text{CFU.g}^{-1}$. In the storage regime B, it was $8.3 \log \text{CFU.g}^{-1}$. For the samples stored under the regime C $7.9 \log \text{CFU.g}^{-1}$ (Figure 5). There were no statistically significant differences ($p > 0.05$) among the samples in the number of psychrotrophic microorganisms. At the end of monitoring after 49 days in the regime A, I have detected $8.8 \log \text{CFU.g}^{-1}$ of them. In the storage regime B, the count was $8.4 \log \text{CFU.g}^{-1}$. In the storage regime C after 91 days, I have detected $8.5 \log \text{CFU.g}^{-1}$ of psychrotrophic microorganisms. I have recorded a higher ($p < 0.05$) number of psychrotrophic microorganisms at the end of the monitoring, in samples stored in the refrigerator (A/49) in comparison with cheeses stored by using cooling/freezing method for one week (B /49). Decreasing temperature of the environment significantly extends the generation time and the lag phase of psychrotrophic microorganisms. According to **Görner and Valík (2004)** these microorganisms grow in acidic cheeses at temperatures higher than $5 \text{ }^\circ\text{C}$, which explains the increase in the number of microorganisms after 49 days at conditions in a cooler. Microbial cells do not tolerate repeated freezing and thawing, but devitalization in this manner is not reliable. If thawing follows a short time after the freezing, there is an increase in the number of these bacteria and under these conditions they can survive in the long run (**Žiška and Martinková, 1990**).

Moulds

At the beginning of storage (A, B, C/0), the number of moulds was $7.5 \log \text{CFU.g}^{-1}$. After 35 days (Figure 6), in the storage regime A, I have found $7.6 \log \text{CFU.g}^{-1}$ of moulds. In storage regime B, I have found $7.1 \log \text{CFU.g}^{-1}$ of moulds and in the storage regime C their number was $7.4 \log \text{CFU.g}^{-1}$. I have found no statistically significant differences ($p > 0.05$) in the number of moulds among the samples stored in different storage regimes. At the end of monitoring of samples stored at the regime A after 35 days, we have detected $7.9 \log \text{CFU.g}^{-1}$ of moulds. In the storage regime B, the count was $7.8 \log \text{CFU.g}^{-1}$ and in samples stored in the regime C after 91 days it was $7.6 \log \text{CFU.g}^{-1}$. At the end of monitoring, after 49 and 91 days respectively, I could not demonstrate any statistically significant differences ($p > 0.05$) in the numbers of moulds among samples stored at different temperature regimes. The reason for the high numbers of moulds, even in frozen specimens, may be the fact that some species can be extremely psychrotrophic. They stop their growth at temperatures as low as $-20 \text{ }^\circ\text{C}$ to $-30 \text{ }^\circ\text{C}$ when all water freezes out in the food (**Görner and Valík, 2004**). According to **Sørhaug (2011)**, moulds are commonly present not only in the air, but also occur on the production equipment and can degrade various types of cheeses. Using the cooler temperatures, however, reduces the risk of mycotoxins, which could adversely affect the health of consumers.

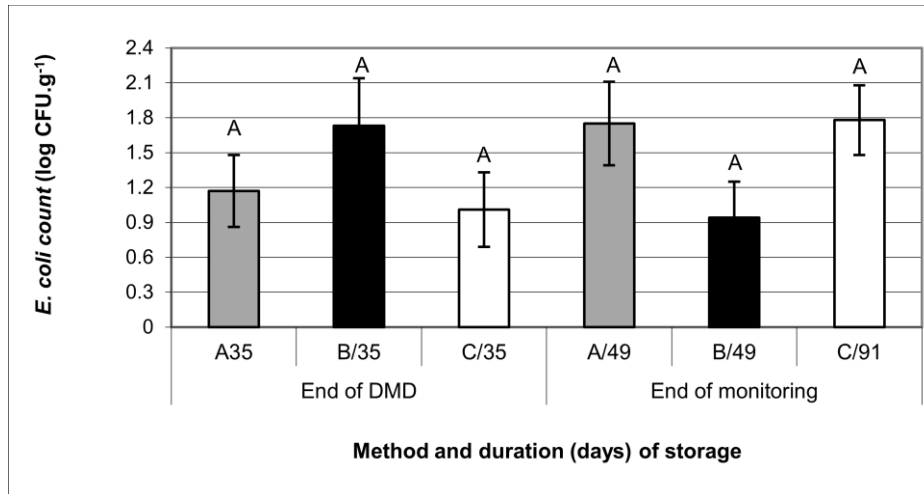


Figure 4 Number of *E. coli* (log CFU.g⁻¹) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

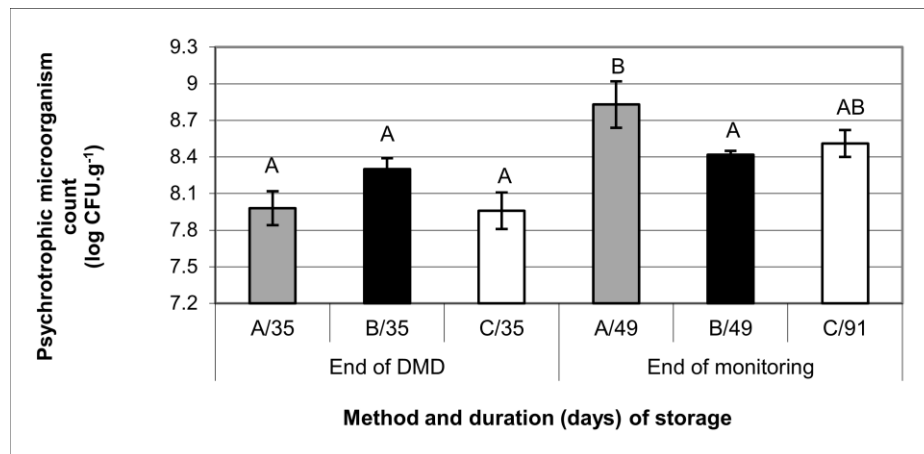


Figure 5 Number of psychrotrophic microorganisms (log CFU.g⁻¹) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C). Averages labelled with different letters were statistically different ($p < 0.05$) within a given factor, n = 15.

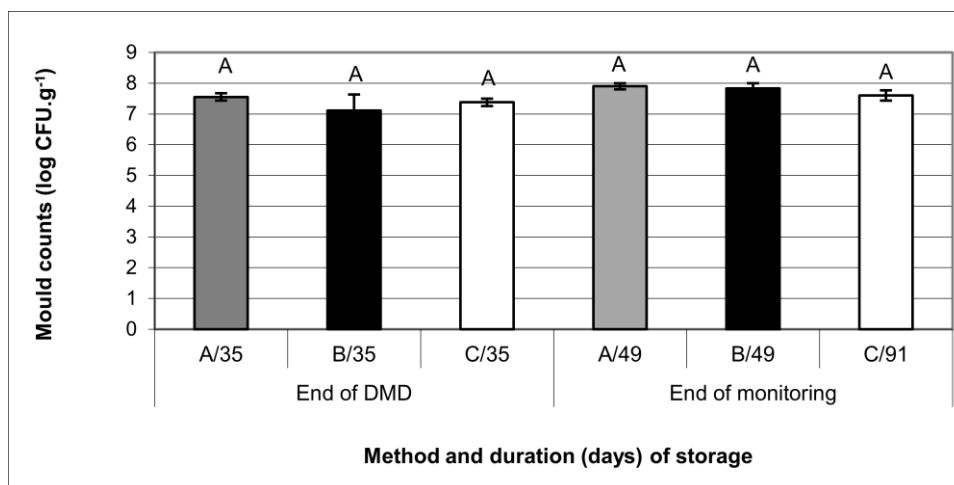


Figure 6 Number of moulds (log CFU.g⁻¹) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

Moulds can easily contaminate the cheese production site and the product itself. They grow very easily on cheese because they can overcome conditions unfavourable to other microorganisms, such as low temperatures, reduced water activity, high salt concentrations, low pH, and lack of carbohydrates (Bachmann et al., 2005).

Yeast

At the beginning of monitoring (A, B, C/0), their count was $7.9 \log \text{CFU.g}^{-1}$. Both at the end of the DMD and at the end of storage, we could not detect any statistical difference in the number of yeasts ($p > 0.05$). In samples stored in the temperature regime A after 35 days, I have detected $7.9 \log \text{CFU.g}^{-1}$ of yeast. In the samples of the temperature regime B, the yeast count was $8.1 \log \text{CFU.g}^{-1}$. In the samples in the temperature regime C, the yeast count was $8.0 \log \text{CFU.g}^{-1}$. In samples analysed at the end of the monitoring, after 49 days in the regime A, I have detected $8.1 \log \text{CFU.g}^{-1}$ of yeast. In the regime B, the count was $8.3 \log \text{CFU.g}^{-1}$ and after 91 days in regime C, I found $8.1 \log \text{CFU.g}^{-1}$ of yeast (Figure 7). The number of yeasts in the samples examined at the end of the monitoring after 49 and 91 days respectively, I have not found any statistical difference ($p > 0.05$) among the storage regimes temperatures. Higher yeast counts are caused by their addition to the sebaceous cultures. This also agrees with the findings by Eliskases-Lechner and Ginzinger (1995), who investigated the occurrence of yeasts in soft, smear-ripened cheeses. They found that after 21 days, the yeast count was $7 \log \text{CFU.g}^{-1}$. Dolci et al., (2009) reported the surface yeast counts to be $6 \log \text{CFU.cm}^{-2}$. Likewise Doležalová et al., (2013) reported the number of yeasts and moulds up to $7 \log \text{CFU.g}^{-1}$. According to Görner and Valík (2004) the yeast counts during maturation range between 4 and $9 \log \text{CFU.cm}^{-2}$. In an experiment by Wyder and Puhán (1999) yeast counts during maturation reached $8 \log \text{CFU.g}^{-1}$. Carminati (1999) reported $7.7 \log \text{CFU.g}^{-1}$ after 40 days of storage. Irlinger and Mounier (2009) recorded similar yeast counts. At the beginning of ripening, the yeast count in their experiment was $7 \log \text{CFU.cm}^{-2}$, while in the middle

of the ripening time it increased to $7.7 \log \text{CFU.cm}^{-2}$.

The results show that at the end of the experiment the number of LAB or *Brevibacterium linens* did not decrease, which means that after thawing the process of ripening continued, while maintaining the sensory quality of the product (Jarošová and Cwíková, 2014). In connection with it the number of coliform microorganisms decreased. *B. linens* namely produce antimicrobial substances that inhibit the growth of many food-poisoning bacteria as well as several yeasts and moulds. Regarding *E. coli*, the Commission Regulation (EC) No 2073 (2005) gives as a criterion for processing cheese made from heat-treated milk in 5 samples number up to $2 \log \text{CFU.g}^{-1}$, wherein two of the five samples may contain $3 \log \text{CFU.g}^{-1}$. These values were not achieved even after 91 days of storage. Although *E. coli* survives cooling plant temperatures, subsequent low temperature storage ($6 \text{ }^\circ\text{C}$) and high number of cultural micro flora prevented the increase of the number of the bacteria. *Escherichia coli* are used as an indicator of direct or indirect faecal contamination of food, and therefore the possible presence of enteric pathogens. Our figures therefore point to a strict adherence to hygiene throughout the production process. Yeast counts were comparable in all types of storage regimes, both at the end of the DMD and at the end of monitoring and matched the values reported in other studies. Relatively high number of yeast is due to the fact that they are added as culture microorganisms. Coexisting yeasts and bacteria often reach high population densities, typically between 8 and $10 \log \text{CFU.g}^{-1}$ of cheese at the time of consumption. The high number of moulds is related to the fact that *G. candidum* although taxonomically a yeast, is usually automatically assigned to moulds because of its appearance. However, white moulds, which were found during our experiment on smear-ripened cheeses, are still generally considered to be undesirable contaminants. The issue of moulds in smear-ripened cheeses is still little explored. One possible way to minimize contamination by moulds (excluding high-quality raw materials and adherence to good manufacturing and hygienic practices in the production) may be filtering the air in the indoor

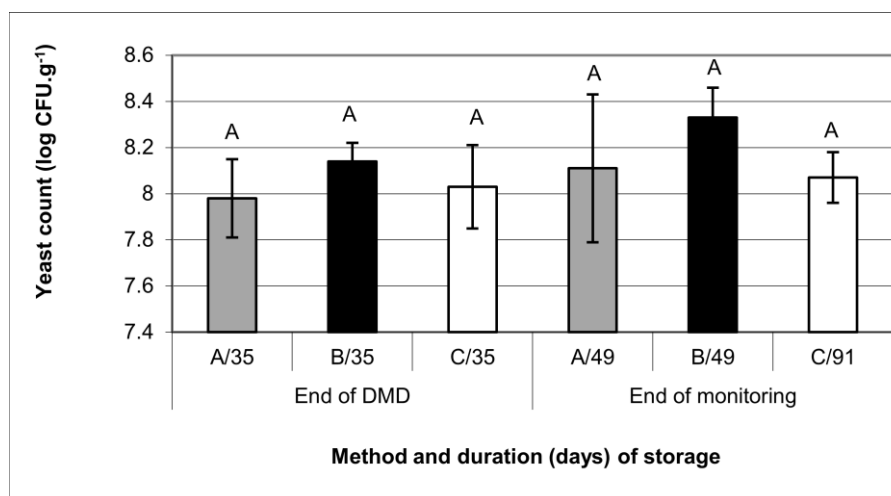


Figure 7 Number of yeasts ($\log \text{CFU.g}^{-1}$) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), $n = 15$.

facility.

The gained results will be used by the manufacturers of smear-ripened cheeses to find possible ways to extend the DMD of cheeses without compromising their quality or breaching of safety.

CONCLUSION

Smear-ripened cheeses in addition to cultural microflora also contain undesirable microorganisms. They contribute not only to spoilage, but may also include dangerous pathogens that are able to cause foodborne illness. One of the easiest ways to reduce potential risk to a minimum and maintain the required quality is to keep these products at the appropriate temperature. This reduces the growth of undesirable microorganisms, while allowing for the maturation and development of the sensory properties of cheese.

The aim of our study was to assess whether the different temperature regimes will maintain the development of cultural micro flora and will continue maturing and how the selected temperature regime will affect the number of contaminating microorganisms.

During the storage (49 days), the number of LAB, *Brevibacterium linens*, *E. coli*, and yeasts remained unchanged ($p > 0.05$). The number of coliform microorganisms decreased ($p < 0.001$), while the number of psychrotrophic microorganisms and moulds increased ($p < 0.001$).

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SEED PROTEIN ELECTROPHORESIS FOR IDENTIFICATION OF OAT REGISTERED CULTIVARS

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ABSTRACT

The protein diversity of 15 registered oat genotypes (*Avena sativa* L.) was examined. Acid-PAGE (acid polyacrylamide gel electrophoresis) of avenins and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) of the glutelins were used for seed analyses of oat varieties. The result of this study indicated that the genotypes of oat cultivars could effectively be differentiated on the basis of polymorphism, detected between protein patterns. SDS-PAGE result revealed that glutelins presented a higher differentiation power than avenins and could be used as a rapid method for the identification of oat varieties in breeding programmes. Avenin protein band numbers and molecular weight ranged from 5 to 11, and 8 to 45 kDa, respectively. Genetic similarity analysis based on avenin protein ranged from 0,071 to 1. The tree-cluster analysis illustrates the distribution of varieties in two major groups. The first major group was large with eight varieties and following varieties were determined as identical: Vendelín-Zvolen and Prokop-Václav. The second group consisted of six varieties and Detvan-Hronec and Izak-Vážec were identical pairs. The number of glutelin protein markers was 22. Genetic similarity coefficients resulted from comparisons by glutelin pattern and they ranged from 0,125 to 0,929 all oat varieties were different from each other. Two Slovakian varieties Izak and Vážec from Breeding Station Viglaš-Ptruša with similarity value 92,9% were grouped with variety Vendelín. Two varieties Kanton and Viliam with similarity value 92% were grouped with Prokop and Valentin. All analysed cultivars could be distinguished by their glutelin pattern and will be useful in oat breeding process.

Keywords: *Avena sativa* L.; genetic diversity; avenins; glutelins; electrophoresis

INTRODUCTION

The oat belongs to the grass family Gramineae (Poaceae), to the tribe Aveneae, and to the genus *Avena*. It contains 30 different species which form distinct polyploidy series ranging from diploid through tetraploid to hexaploid with base chromosome number of seven. Centre of origin of genus *Avena* L. lies in the western part of the Mediterranean region where species *A. byzantina* C. Koch originated too. The secondary centre of formation of *Avena* L. species and origin of cultivated oat (*Avena sativa* L.) is situated within the Asia Minor centre of crop origin (Loskutov, 2007). Proteomic techniques have been applied successfully to the identification of cereal seeds, differentiation of proteins from the wheat kernel (Šramková et al., 2011), identification of hordein from barley (Bradová et al., 2001) and verification of zeins from corn (Gregová et al., 2013). Oats (*Avena sativa* L.) have relatively high protein content (12.4 – 24.5%) and an excellent nutritional score of amino acids can be a good source for protein composition for food application. (Lásztity, 1996). Cultivated oats are hexaploid cereals belonging to the genus *Avena* L., which is found worldwide in almost all agricultural environments. Oat grains are mainly used as animal feed and only a few percent are used for human nutrition. Recent results showing the suitability of oats for celiac patient moreover

raises the status of oats healthy part of their diet. Oat provides well balanced food for human and has been used for feeding for long time. Oat protein fractions are albumins (10 – 20 %), globulins (12 – 55%), prolamins (12 – 14%) and glutelins (23 – 54%) (Haard, 1999). Globulins and avenins are located in vacuolar protein bodies. Protein belonging to the albumin group are mostly enzymes and functional proteins. In oat globulin and glutelin fraction were presented as a major components. Shotwell et al. (1988) presented the oat 12S globulin is 70 % homologous to rice storage globulins and 30% – 40 % homologous to storage globulins present in legumes. Generally the glutelin fraction is defined as the protein fraction extracted with basic or acidic solution after the removal of water-soluble albumins, salt-soluble globulins and alcohol-soluble prolamins (Anderson, 2014). German workers reported that glutelins are the major fraction and concentration of globulin is only 21 – 27% of total protein (Weiser et al. 1980). Other authors reported that globulins are the major fraction and Peterson (1976) found 54 – 56% globulin concentration of the total protein. Storage globulins are the major endosperm proteins in oats and rice, although technically the rice globulins were earlier classified as glutelins due to their solubility properties (Shewry 1995). Polymorphism in the prolamins is more heterogeneous than in the globulin pattern (Dvoráček et al., 2005; Gregová et al., 1996, Polišínská

et al., 2011) and has been investigated as a tool for cultivar identification (Šliková et al., 2015). Alcohol soluble prolamins with molecular weights ranging from 22 kD to 33 kD and there are deficient in the essential amino acid lysine. The avenins are located in the protein bodies (Pernollet et al 1982) and have been divided to alpha-avenins (lower molecular weight) and beta-avenins (higher molecular weight) which cannot readily be extracted separately, both types are present in Finnish oat cultivars (Jussila et al 1992). In comparison with wheat gliadins, the avenins have been little studied, and the number of full avenin genes present at the moment in the databases is limited and from few genes. The variability of avenin genes in oats is not well represented. In developing oat endosperms, globulins and avenins are located in vacuolar protein bodies. The globulins aggregate within the protein bodies whereas the avenins aggregate in the rough endoplasmic reticulum (Lending et al 1989). Several workers used reversed-phase high-performance liquid-chromatography (Lookhart, 1985, Lookhart and Bean, 1995) or two-dimensional polyacrylamide gel electrophoresis for the specification of phylogenetic relationships among *Avena* species and variations (Nalecz et al., 2009). Western blot analysis with gluten polyclonal antibody is useful method for qualitative detection of prolamin complex. (Socha et al., 2011). Soluble protein and aggregates in oat was studied using asymmetric flow field-flow fractionation (AF4) coupled with online UV vis spectroscopy and multiangle light-scattering detection (MALS) and AF4 fraction is useful for characterization by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). AF4 fraction revealed three peaks, which were determined to be monomeric forms of soluble proteins, globulin aggregates, and B-glucan, respectively (Runyon et al., 2013). In this study, 15 registered oat cultivars were examined based on avenin and glutelin pattern using acid-PAGE and SDS-PAGE techniques for identification and verification.

MATERIAL AND METHODOLOGY

From List of Registered Varieties 12 oat varieties are originated from research and Breeding Station Viglas-Pstruša, two varieties originated from Germany and one originated from Czech Republic. Five varieties (Detvan, Hronec, Izak, Tatron and Važec) were analysed of naked oats (*Avena nuda*) and ten varieties (Flamingsgold, Atego, Kanton, Prokop, Václav, Valentín, Vendelín, Viliam, Vojtech and Zvolen) of husked oats (*Avena sativa*). Acid-PAGE was carried out according to the standard reference ISTA method (Draper, 1987). Proteins were extracted from seeds of 20 mg finely ground powdered seeds with 240 µl extracting solution (0.05 g Methylgreen, 25% 2-chloroethanol), stained overnight at room temperature, and centrifuged for 5 minutes at 5 000xg. From gel solution of 10% acid-PAGE (pH: 3.1) was prepared 0.75 mm thick gel, using vertical Hoefer 800 unit. Electrophoresis was carried out at 200 V for 20 minutes, 600 v for 3 hours at 4 °C. Staining of gels was performed in a solution of Coomassie Brilliant Blue R250 dissolved in acetic acid and methanol solution. Gel was scanned with densitometer GS 800 (Bio-Rad) and evaluated with Quantity One-1D Analysis Software.

SDS-PAGE was carried out according to UPOV. Stock extraction buffer was used according to ISTA method for wheat. Extracting solution for oat seed was prepared fresh from 2.8 ml stock extraction buffer, 0.79 ml mercaptoethanol and 1.5 ml DMF (dimethylformamide) with distilled water. Crushed seed (45 mg) was extracted with 1 ml of extracting solution. Extraction was performed at room temperature overnight and heating in boiled water for 5 minutes, centrifugation at 5000 xg for 5 min. 10 µl of extracts were applied to the sample wells. The gel (1.0 mm thick) consists of two parts: stacking gel (3.5% acrylamide, pH = 6.8 acrylamide) and resolution gel (10% acrylamide, pH = 6.8). Vertical unit Bio-Rad protean II xi Cell was used for gel electrophoresis. Protein separation was carried overnight (2 hours at 20 mA, 16 hours at 30 mA at 4 °C). Gels were fixed with fixing solution (water/methanol/acetic acid/700ml:200ml:100ml for 20 minutes and then stained in a solution of Coomassie Brilliant Blue R250, dissolved in acetic acid and methanol solution. Gel was scanned with densitometer GS 800 and evaluated with Quantity One-1D Analysis Software.

RESULTS AND DISCUSSION

Alcohol – soluble seed proteins (avenins) present a typical spectrum of this plant species (Dumlupinar et al., 2011; Dvoráček et al., 2003; Hansen et al., 1988). Avenin proteins have structural homology to the S-rich subgroup α -gliadins and γ -gliadins of wheat, the B-hordeins of barley, and γ -secalins of rye. Prolamin is more variable in structure and sequence than globulin, because the major groups of prolamins in the Triticeae (wheat, barley, rye) maize and millet had a separate evolutionary origins (Shewry and Halford, 2002). In addition rye, wheat and barley contain a high concentration of prolamin and a comparatively small amount of globulins, whereas the situation is reversed in oat. The relatively low final concentration of prolamins in oat may partly be due to the fact that major avenin synthesis begins approximately one week after that of the globulins (Robert et al., 1983).

Avenin protein band numbers ranged from 5 to 11. In previous studies 7 to 11 bands were found (Portyanko et al., 1998), 7 to 14 (Jussila et al., 1992), 4 to 16 (Dumlupinar et al., 2011), in *Avena sativa* and 24 to 34 in *Avena fatua* (Mirza et al., 2007) with a different method. On the basis of component mobility we evaluated all bands which were used for clustering of oat varieties. Jaccard's similarity coefficient was calculated from the data generated by 2-state scoring of the band patterns obtained in Acid-PAGE system. From the similarity matrix a dendrogram was constructed using statistical analysis by means of SPSS software 22 (IBM SPSS, Chicago, Illinois, USA). The tree-cluster analysis illustrates the distribution of varieties in two major groups. The first major group was large with nine varieties from *Avena sativa* L. (Vendelín, Zvolen, Prokop, Václav, Vojtech, Valentín, Viliam, Kanton, and Atego) and following varieties were determined as identical: Vendelín-Zvolen and Prokop-Václav. The second group consisted of five varieties of *Avena nuda* L. (Detvan, Hronec, Izak, Važec and Tatron) and one from *Avena sativa* L. genotype Flamingsgold. The two identical pairs with identical composition of protein pattern were found Detvan-Hronec and Izak-Važec.

Table 1 Genetic similarity between oat varieties estimated by A-PAGE of avenins.

Proximity Matrix

Case	Jaccard Measure														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1:Detvan	1.000														
2:Hronec	1.000	1.000													
3:Flamingsgold	.778	.778	1.000												
4:Izak	.333	.333	.333	1.000											
5:Važec	.333	.333	.333	1.000	1.000										
6:Tatran	.385	.385	.385	.500	.500	1.000									
7:Atego	.071	.071	.071	.154	.154	.308	1.000								
8:Kanton	.273	.273	.273	.167	.167	.333	.300	1.000							
9:Prokop	.182	.182	.182	.083	.083	.364	.333	.375	1.000						
10:Václav	.182	.182	.182	.083	.083	.364	.333	.375	1.000	1.000					
11:Valentín	.273	.273	.273	.077	.077	.333	.300	.714	.571	.571	1.000				
12:Vendelín	.077	.077	.077	.000	.000	.231	.182	.200	.571	.571	.333	1.000			
13:Viliam	.273	.273	.273	.077	.077	.231	.182	.714	.375	.375	.714	.333	1.000		
14:Vojtech	.273	.273	.273	.167	.167	.455	.300	.200	.571	.571	.333	.333	.200	1.000	
15:Zvolen	.077	.077	.077	.000	.000	.231	.182	.200	.571	.571	.333	1.000	.333	.333	1.000

This is a similarity matrix

Table 2 Genetic similarity between oat varieties estimated by SDS-PAGE of glutelins.

Proximity Matrix

Case	Jaccard Measure														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1:Vendelin	1.000														
2:Zvolen	.600	1.000													
3:Detvan	.294	.571	1.000												
4:Hronec	.467	.571	.667	1.000											
5:Izak	.786	.786	.438	.643	1.000										
6:Važec	.733	.733	.412	.600	.929	1.000									
7:Prokop	.533	.438	.400	.400	.412	.389	1.000								
8:Václav	.200	.125	.143	.143	.188	.176	.308	1.000							
9:Tatran	.333	.333	.385	.385	.400	.375	.357	.077	1.000						
10:Atego	.125	.125	.231	.231	.188	.176	.133	.333	.273	1.000					
11:Flamings	.438	.438	.615	.615	.500	.471	.467	.308	.462	.417	1.000				
12:Kanton	.375	.375	.429	.333	.278	.333	.615	.143	.385	.067	.400	1.000			
13:Valentin	.375	.375	.538	.538	.353	.333	.750	.231	.385	.231	.615	.667	1.000		
14:Viliam	.400	.400	.462	.357	.294	.278	.667	.154	.417	.071	.429	.900	.727	1.000	
15:Vojtech	.250	.333	.385	.286	.313	.294	.267	.273	.231	.273	.357	.286	.385	.308	1.000

This is a similarity matrix

Glutelins from oat varieties reveal a higher distinguishing power than acid-PAGE does. The number of protein markers was 22. Jaccard's similarity coefficient was calculated from the data generated by 2-state scoring of the band patterns obtained in SDS-PAGE system. The dendrogram tree (Fig.1) demonstrated the relationship among the fifteen registered oat varieties according to the similarity index detected by glutelin protein patterns, using UPGMA cluster analysis. The dendrogram was divided into two main clusters. The first one contained only two varieties from *Avena sativa* L. (Václav and Atego), while the second cluster contained the rest varieties. The second cluster was divided into 4 subclusters. One group consisted of four varieties from *Avena nuda* L. (Izak, Važec, Vendelín and Zvolen). The second group consisted of one variety from *Avena nuda* L (Detvan) and two varieties of *Avena sativa* L. (Hronec and Flamingsgold). Two Slovakian varieties Izak and Važec from Breeding Station Viglaš-Ptruša with similarity value 92.9% were grouped

with variety Vendelin. Two varieties Kanton and Viliam with similarity value 92% were grouped with Prokop and Valentin. They indicated that most of registered varieties of oat were readily differentiated, some very closely related varieties gave identical glutelin pattern and these could be used as genetic markers.

CONCLUSION

Both techniques may provide useful information on the level of polymorphism and diversity in oat cultivars. 13 registered cultivars originated from Breeding Station Viglaš-Pstruša were very closely related. Glutelins from oat varieties reveal a higher distinguishing power than avenins do. Result from this study show that protein markers are powerful and efficient in characterising and identifying of oat varieties in addition to their usefulness in phylogenetic studies.

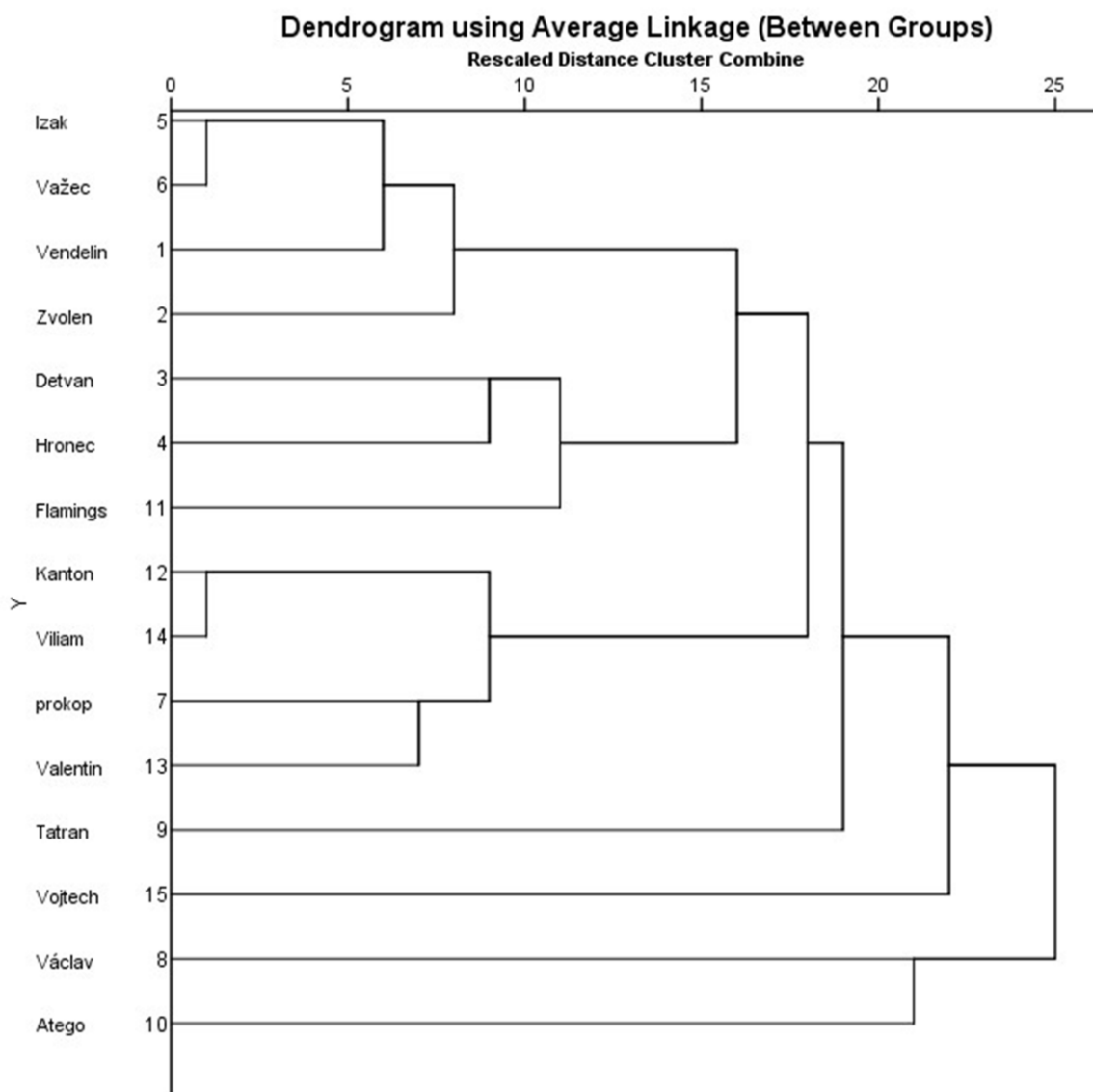


Figure 1 Dendrogram of genetic relationships between 15 oat cultivars, constructed by UPGMA based Jaccard coefficient, based on glutelin patterns.

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OXIDATIVE STABILITY OF FATTY ACID ALKYL ESTERS: A REVIEW

Michal Angelovič, Juraj Jablonický, Zdenko Tkáč, Marek Angelovič

ABSTRACT

The purpose of this study was to investigate and to process the current literary knowledge of the physico-chemical properties of vegetable oil raw used for biodiesel production in terms of its qualitative stability. An object of investigation was oxidative stability of biodiesel. In the study, we focused on the qualitative physico-chemical properties of vegetable oils used for biodiesel production, oxidative degradation and its mechanisms, oxidation of lipids, mechanisms of autooxidation, effectiveness of different synthetic antioxidants in relation to oxidative stability of biodiesel and methods of oxidative stability determination. Knowledge of the physical and chemical properties of vegetable oil as raw material and the factors affecting these properties is critical for the production of quality biodiesel and its sustainability. According to the source of oilseed, variations in the chemical composition of the vegetable oil are expressed by variations in the molar ratio among different fatty acids in the structure. The relative ratio of fatty acids present in the raw material is kept relatively constant after the transesterification reaction. The quality of biodiesel physico-chemical properties is influenced by the chain length and the level of unsaturation of the produced fatty acid alkyl esters. A biodiesel is thermodynamically stable. Its instability primarily occurs from contact of oxygen present in the ambient air that is referred to as oxidative instability. For biodiesel is oxidation stability a general term. It is necessary to distinguish 'storage stability' and 'thermal stability', in relation to oxidative degradation, which may occur during extended periods of storage, transportation and end use. Fuel instability problems can be of two related types, short-term oxidative instability and long-term storage instability. Storage instability is defined in terms of solid formation, which can plug nozzles, filters, and degrade engine performance. Biodiesels are more susceptible to degradation compared to fossil diesel because of the presence of unsaturated fatty acid chain in it. The mechanisms of oxidative degradation are autoxidation in presence of atmospheric oxygen; thermal or thermal-oxidative degradation from excess heat; hydrolysis in presence of moisture or water during storage and in fuel lines; and microbial contamination from contact with dust particles or water droplets containing fungi or bacteria into the fuel. The oxidation of lipids is a complex process in which unsaturated fatty acids are reacted with molecular oxygen by means of free radicals. The radicals react with lipids, and cause oxidative destruction of unsaturated, polyunsaturated fatty acids, therefore, known as lipid peroxidation. The factors such as heat, oxygen, light, and some metal ions, especially iron and copper, also play a significant role in creating oxidation. Oxidative products formed in biodiesel affect fuel storage life, contribute to deposit formation in tanks, and they may cause clogging of fuel filters and injection systems. The volatile organic acids formed as secondary by products of the oxidative degradation, may stimulate corrosion in the fuel system. Poor stability can lead to increasing acid numbers, increasing fuel viscosity, and the formation of gums and sediments. In general, antioxidants can prevent oxidation. Biodiesel, because it contains large numbers of molecules with double bonds, is much less oxidatively stable than petroleum-based diesel fuel. Oxidation stability is the important parameter to determine the storage of biodiesel for longer period of time. Biodiesel samples were evaluated according to methods on the base of kept in contact with pure oxygen at elevated temperatures and pressures. The results show that the performance antioxidants variation is observed for biodiesel. The most commonly used primary synthetic antioxidants.

Key words: biodiesel; vegetable oil; fatty acid; oxidation; stability

INTRODUCTION

Traditionally, oil and fat consumption was shared between food, feed, and industrial use in the ratio 80:6:14. But with growing production of biodiesel this ratio is probably now closer to 74:6:20 (Gunstone, 2008).

Palm and rapeseed oils contribute most to the growing industrial use, palm oil mainly because of the development of the oleochemical industry in southeast Asia and

rapeseed oil mainly because of the biodiesel industry in Europe (Gunstone, 2009).

In 2008 biodiesel production and capacity amounted globally to 11.1 and 32.6 million tonnes, respectively (Biodiesel 2020: A global market survey, 2008). The huge gap between capacity and production is most likely due the fluctuation of subsidies. This situation could offer an opportunity for the chemical industry, since biodiesel (a mixture of C₁₆ and C₁₈ fatty acid methyl esters) should be

considered as a potential chemical feedstock. For instance, applications of biodiesel as a polymerization solvent have already been studied (Salehpour and Dube, 2008; Salehpour et al., 2009).

The production of fatty acids is the highest volume oleochemical process and accounts for about 52% of industrially used oils and fats (Gunstone, 2001).

Biodiesel is chemically fatty acid alkyl esters (FAAEs) of long-chain fatty acids derived from renewable sources such as vegetable oils, animal fats, waste greases, recycled cooking oils etc., through the esterification and transesterification reactions of free fatty acids (FFAs) and triglycerides, respectively (Borugadda and Goud, 2012; Atabani et al., 2013; Ong et al., 2013; Sanjid et al., 2013; Silitonga et al., 2013).

Biodiesel also has a much lower oxidative stability than mineral diesel (Bouaid et al., 2007; Knothe, 2007; Santos et al., 2007; Araújo et al., 2009; Jain and Sharma, 2011; Karavalakis et al., 2011).

Biodiesel also has a much lower oxidative stability than mineral diesel (Bouaid et al., 2007; Knothe, 2007; Santos et al., 2007; Araújo et al., 2009; Jain and Sharma, 2011; Karavalakis et al., 2011).

Previous literature (Dunn, 2000; Litwinienko et al., 2000; Litwinienko and Kasprzycka-Guttman, 2000; Haas et al., 2001; Abreu et al., 2004) produced by transesterification reaction of vegetable oils with short (Litwinienko, 2001; Dunn, 2002; Dunn, 2005a) on the oxidative stability of oleochemicals utilizing DSC studied a variety of vegetable oils and biodiesel, along with selected fatty acids and their corresponding ethyl esters. However, individual fatty acid alkyl ester was not investigated. Other reports (Dunn, 2000; Knothe and Dunn, 2003a; Dunn, 2005b; Dunn, 2008b) on the oil stability index of various fatty acid alkyl esters (including biodiesel) and vegetable oils were accomplished following

the American Oil Chemists' Society (AOCS) official method Cd 12b-92 at a variety of block temperatures (50–110 °C). These studies did not include a number of fatty acid alkyl ester commonly found in biodiesel, nor were EN 14112 or differential scanning calorimetry (DSC) methods utilized. A more recent report (Knote, 2008) on the oil stability index of selected fatty acid alkyl ester employed both EN 14112 and the American Oil Chemists' Society official method Cd 12b-92 at 110 °C, but once again did not include a number of fatty acid alkyl ester commonly found in biodiesel, nor were the effects of chain length, double bond location or orientation investigated.

The purpose of this study was to investigate and to process the current literary knowledge of the physico-chemical properties of vegetable oil raw used for biodiesel production in terms of its qualitative stability.

An object of investigation was oxidative stability of biodiesel. In the study, we focused on the qualitative physico-chemical properties of vegetable oils used for biodiesel production, oxidative degradation and its mechanisms, oxidation of lipids, mechanisms of autooxidation, effectiveness of different synthetic antioxidants in relation to oxidative stability of biodiesel and methods of oxidative stability determination.

Vegetable oils and biodiesel

The main constituents of vegetable oils are triglyceride molecules. If the vegetable oil allowed to be reacted with methanol in the presence of a catalyst, the result is biodiesel and glycerol. Triglyceride molecules are transesterified to form fatty acid alkyl esters (Marchetti et al., 2007; Gui, 2008; Atadashi et al., 2012; Motasemi and Ani, 2012; Atabani et al., 2013; Yaakob et al., 2013).

Transesterification reaction can be catalyzed by both homogeneous (alkalies and acids) and heterogeneous

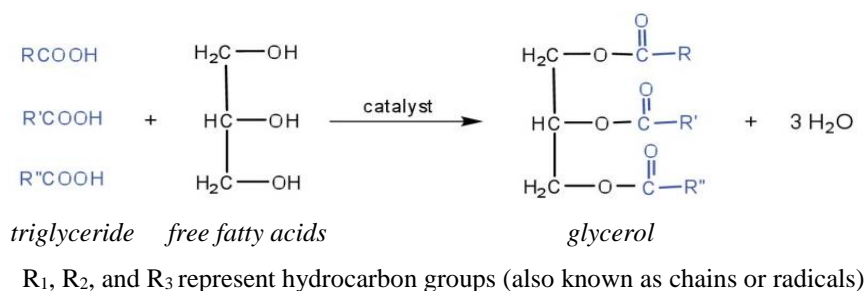


Figure 1 A triglyceride is the condensation product of one molecule of glycerol with three molecules of fatty acids (Blagdon, 2007).

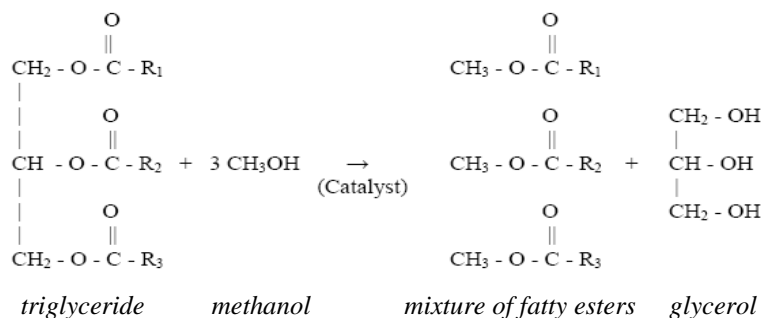


Figure 2 Transesterification reaction (Van Gerpen et al., 2004).

catalysts. The most commonly used alkali catalysts are sodium hydroxide (NaOH), sodium methoxide (CH₃ONa), and potassium hydroxide (KOH) (Encinar et al., 2010).

Thus the biodiesel consists of long-chain fatty acid esters (Haas et al., 2001; Abreu et al., 2004) produced by transesterification reaction of vegetable oils with short chain alcohols (Nouredini et al., 1998; Encinar et al., 2002).

Biodiesel typically comprises alkyl fatty acid (chain length C₁₄-C₂₂) esters of short-chain alcohols, primarily, methanol or ethanol (Ma and Hanna, 1999; Demirbas, 2009).

The transesterification reaction consists in the conversion of the triglyceride molecules, by means of the action of short chain alcohol, i.e., ethanol, into the corresponding fatty acids esters. According to the source of oilseed, variations in the chemical composition of the vegetable oil are expressed by variations in the molar ratio among different fatty acids in the structure. The relative ratio of fatty acids present in the raw material is kept relatively constant after the transesterification reaction (Costa Neto et al., 2000).

Currently, more than 95% of the world biodiesel is produced from edible oils which are easily available on large scale from the agricultural industry (Gui, 2008).

However, continuous and large-scale production of biodiesel from edible oils has recently been of great concern because they compete with food materials, the food versus fuel dispute (Refaat, 2010).

Biodiesel physico-chemical properties

The quality of biodiesel physico-chemical properties is influenced by the chain length and the level of unsaturation of the produced fatty acid alkyl esters. According to Schuchardt et al. (1998) the chain length and the level of unsaturation of the produced fatty acid alkyl ester correspond to that of parent oil. Therefore, in general the fatty acids ethyl esters profile obtained by transesterification is reflected by the composition in fatty acids of the employed raw material. This fact can be proven by comparing the fatty acid compositions in employed raw materials, with the fatty acids composition in esters of the produced biodiesel (Ferrari et al., 2005).

Knothe and Dunn (2003a) reported that biodiesel is thermodynamically stable. Its instability primarily occurs from contact of oxygen present in the ambient air that is referred to as oxidative instability. The term 'oxidation stability' is a general term. It is necessary to distinguish 'storage stability' and 'thermal stability', in relation to oxidative degradation, which may occur during extended periods of storage, transportation and end use. Monyem et al. (2001) reported also that some chemical and physical properties of biodiesel can be affected by oxidation of the fuel during storage.

The rates of reactions in autoxidation schemes are dependent on hydrocarbon structure, heteroatom concentration, heteroatom speciation, oxygen concentration, and temperature. Fuel instability problems can be of two related types, short-term oxidative instability and long-term storage instability (Mushrush et al., 2000). Storage instability, is defined in terms of solid formation, which can plug nozzles, filters, and degrade engine performance (Mushrush et al., 2001).

Oxidative degradation

Oxidative degradation during transport and storage causes deterioration of the physical properties of the biodiesel making it unstable and unusable (Bouaid et al., 2007; Hoshino et al., 2007).

Biodiesels are more susceptible to degradation compared to fossil diesel because of the presence of unsaturated fatty acid chain in it (carbon double binds C=C) (Prankl and Schindlbauer, 1998; Pullen and Saeed, 2012).

The mechanisms of degradation are: autoxidation in presence of atmospheric oxygen; thermal or thermaloxidative degradation from excess heat; hydrolysis in presence of moisture or water during storage and in fuel lines; and microbial contamination from contact with dust particles or water droplets containing fungi or bacteria into the fuel (Dunn, 2008; Pullen and Saeed, 2012; Obadiah et al., 2012).

This degradation is exasperated if there is at least two or higher number of carbon double bonds (polyunsaturation) are extant in their fatty acid chains (Graboski and McCormick, 1998).

More than half of a century has been elapsed after the establishment of autoxidation mechanism of polyunsaturated fatty acids as a radical chain reaction (Bolland, 1949; Bergström et al., 1950). This was followed by interpretation on role of antioxidants as inhibiting agent (Ingold, 1961).

Oxidation

The oxidation of lipids is a complex process in which unsaturated fatty acids are reacted with molecular oxygen by means of free radicals (Gray, 1978). The radicals react with lipids, and cause oxidative destruction of unsaturated, polyunsaturated fatty acids, therefore, known as lipid peroxidation. Lipid peroxidation is chain reaction (Sakac and Sakac, 2002).

The factors such as heat, oxygen, light, and some metal ions, especially iron and copper, also play a significant role in creating oxidation (Ozturk and Cakmakcib, 2006).

The oxidation of biodiesel is due to the unsaturation in fatty acid chain and presence of double bond in the molecule which offers high level of reactivity with O₂, especially, when it is placed in contact with air/water. The primary oxidation products of double bonds are unstable allylic hydroperoxides which are unstable and easily form a variety of secondary oxidation products. This includes the rearrangement of product of similar molecular weights to give short chain aldehydes, acids compounds and high molecular weight materials. The oxidation reactivity is related to the degree of C=C bonds in the fuel, increased content of the C=C bonds correlates to decreased oxidative stability of the fuel. The increase in instability of a given diesel fuel molecule is generally directly proportional to the number of C=C bonds in the molecule (i.e., a molecule containing two C=C bonds has half the stability of a molecule containing one C=C bond) (Berman et al., 2011). The methylene groups adjacent to double bonds have turned out to be particularly susceptible to radical attack as the first step of fuel oxidation (Knothe and Dunn, 2003b). The rate of oxidation of fatty compounds depends on the number of double bonds and their position (Knothe, 2005). The oxidation chain reaction is usually

initiated at the positions allylic to double bonds (Hoshino et al., 2007). The oxidation can be described in terms of initiation reactions as a three phases (Formo et al., 1979; Kemin and Industries Inc., 2010).

In the start-up phase are highly active free radicals formed due to temperature, light or metal ions. The second step in the process is called phase oxidation promotion, in each reaction, because the new, reactive radicals are formed, followed by the two radicals may be combined and mutually neutralized, resulting in the formation of stable oxidation formed, followed by a chain reaction. During the final products stages of the two radicals may be combined and mutually neutralized, resulting in the formation of stable oxidation products (Jain and Sharma, 2011).

The mechanism of autooxidation of lipids describes Kanner and Rosenthal (1992), Choe and Min (2006) as a free radical chain reaction that involves three stages: initiation, propagation and termination.

Autoxidation of oils requires fatty acids or acylglycerols to be in radical forms. Fatty acids or acylglycerols are in nonradical singlet states, and the reaction of fatty acids with radical state atmospheric ³O₂ is thermodynamically unfavorable due to electronic spin conservation (Min and Bradley, 1992).

The oxidation products formed in biodiesel affect fuel storage life, contribute to deposit formation in tanks, and they may cause clogging of fuel filters and injection systems (McCormick et al., 2007). The volatile organic acids formed as secondary by products of the oxidative degradation, may stimulate corrosion in the fuel system (Araújo et al., 2009).

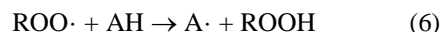
Antioxidants

The antioxidants can be generally categorized into primary and secondary types (Dubey, 2015). Stability of the biodiesel is an important attribute if the biodiesel is to be stored for a prolonged period. Poor stability can lead to increasing acid numbers, increasing fuel viscosity, and the formation of gums and sediments. Information about the stability of the stored biodiesel can be achieved by monitoring the acid number and viscosity. Storage stability

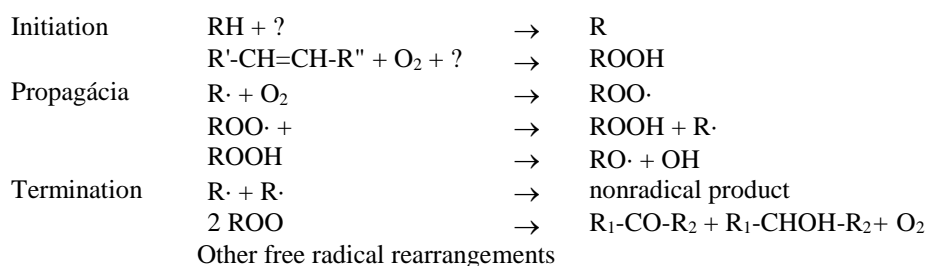
of biodiesel has not been extensively examined relative to its composition. Therefore, the current best practice involves not storing biodiesel or biodiesel blends for more than six months. The stability of stored diesel fuels can be enhanced by using antioxidants (Van Gerpen et al., 2004).

In general, antioxidants can prevent oxidation (Ozturk and Cakmakcib, 2006). Tkačová et al. (2015) reported that they could be on a natural basis. Oxidation cannot be entirely prevented but can be significantly slowed down by the use of antioxidants which are chemicals that inhibit the oxidation process (Pospisil and Klemchuk, 1990). One area where the biodiesel producer needs to give serious consideration is oxidative stability. Biodiesel, because it contains large numbers of molecules with double bonds, is much less oxidatively stable than petroleum-based diesel fuel. Fortunately, stability-enhancing additive technology is well-developed in the food industry and many of these additives can be carried over to stabilizing biodiesel (Van Gerpen et al., 2004). Naturally occurring antioxidants were shown to provide relatively poor oxidation stabilization of biodiesels compared to synthetic antioxidants (Ball et al., 2009). Two types of antioxidants are generally known: chain breakers and hydroperoxide decomposers (Pospisil and Klemchuk, 1990). Literature related to hydroperoxide decomposers is very scarce. The two most common types of chain breaking antioxidants are phenolic and amine-types. Almost all the work related to stability of fatty oil and ester applications is limited to the phenolic type of antioxidant (Natarajan, 2012).

According to Dubay (2015), primary, or chain-breaking antioxidant, are free radical scavengers that delay or inhibit the initiation step of autooxidation or interrupt the propagation step.



Where: *ROO*·=peroxyl radical, *AH*=primary antioxidant, *ROOH*=hydroperoxide, *A*·=antioxidant radical.



where:

RH=hydrogen containing compound

R·=alkyl radical

R'-CH=CH-R"=reactive alkene

O₂=air oxygen, triplet

ROOH=hydroperoxide

ROO·=peroxyl radical

RO·=alkoxyl radical

OH·=hydroxyl radical.

Figure 3 Mechanism of lipid autoxidation as a free radical chain reaction involving initiation, propagation and termination (Min and Bradley, 1992).

Antioxidants significantly slow down the biodiesel degradation process. According to their mode of action, antioxidants could be classified in to various groups: free radical terminators, metal ion chelators capable of catalyzing lipid oxidation, or as oxygen scavengers that react with oxygen in closed systems (Shahidi et al., 1992). The antioxidants sú described in papers by Manura (1994), Imahara et al. (2008), Jain and Sharma (2013), Atabani et al. (2013), Dwivedi and Sharma (2014a).

The study by Dwivedi and Sharma (2014b) shows that oxidation stability is the important parameter to determine the storage of biodiesel for longer period of time. The study also shows that out of various natural and synthetic antioxidants Pyrogallol is best for storage of biodiesel. There are various type of metal and alloys containers are used for storage of biodiesel. Out of various metal and alloys iron and aluminium alloy is best for storage of biodiesel. The future scope of study includes the thermal stability of various biodiesel and the long term effect of various antioxidant, metal and alloys on biodiesel.

Determination of lipid oxidative stability

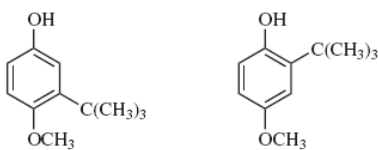
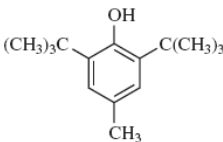
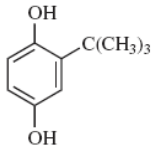
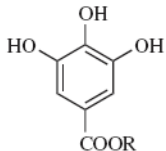
It is believed according to Wąsowicz et al. (2004) that for the determination of oxidative stability and shelf life, methods for the determination of lipid oxidation can be ranked in the following order: sensory analysis > headspace analysis of volatiles > oxygen absorption > peroxide value > thiobarbituric acid reactive substances (TBARS) > carotene bleaching by cooxidation with linoleic acid > Rancimat test (Frankel, 1993). The diversity and abundance of methods used to monitor lipid

oxidation reflect the complexity of this issue and confirm the fact that multiple methods should be applied to get the maximum information available.

Method for determine of the biodiesel oxidative stability

Method for determine of the biodiesel oxidative stability according to Ferrari et al. (2005) can be evaluated by means of the Rancimat® equipment model 617, under temperatures of 100 and 105 °C and air flow of 20 L.h⁻¹. Samples of 5.0 g are utilized, weighed in the Rancimat® flask. The oxidation is then induced by the passage of the air flow through the sample, kept under constant temperature. The volatile products of the reaction, which are blown with the air, are collected in distilled water and measured by the change in electric conductivity of this water. They are expressed through a curve from which the induction period can be calculated by the interception of two lines: a tangent to the inclination and the another tangent to the curve level part. The method used is adapted from the American Oil Chemist's Society (1997) recommended method Cd 12b-92. The cleanliness of the containers used in the Rancimat® apparatus is essential to get truthful and save results, therefore traces of oxidized fats or metals may have a harmful effect on the induction period. In order to avoid problems the cleaning practical procedures of the used material were performed according to Pacheco's (1991) specifically. Moreover, Angelovič et al. (2013) reported that no comprehensive risk assessment for diesel engine emissions from biodiesel and its blends is possible in regard to a comprehensive hazard characterization it is urged to develop a panel

Table 1 The most commonly used primary synthetic antioxidants (Dubay, 2015).

Name	Chemical structure	
Butylated hydroxyanisole (BHA) Molecular formule: C ₁₁ H ₁₆ O ₂		(Shahidi and Zhong, 2005)
Butylated hydroxytoluene (BHT) Molecular formule: C ₁₅ H ₂₄ O		(Shahidi and Zhong, 2005)
tert-Butylhydroquinone (TBHQ) Molecular formule: C ₁₀ H ₁₄ O ₂		(Shahidi and Zhong, 2005)
Different alkyl gallate: Propyl gallate (PG) C ₁₀ H ₁₂ O ₅ Octyl gallate (OG) C ₁₅ H ₂₂ O ₅ Dodecyl gallate (DG) C ₁₉ H ₃₀ O ₅ , most of them propyl gallate (PG)		(Shahidi and Zhong, 2005) (Dubay, 2015)

of standardized and internationally accepted protocols which allow a reliable assessment also of possible health hazards which may arise from the combustion of new fuels compared to conventional diesel fuel.

The effectiveness of various antioxidants and biodiesel oxidative stability

Biodiesel samples were evaluated according to methods on the base of kept in contact with pure oxygen at elevated temperatures and pressures. The results show that the performance antioxidants variation is observed for biodiesel. **Karavalakis et al. (2011)** reported that butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) displays the lowest effectiveness in neat biodiesel. Propyl gallate (PG) and pyrogallol (PY) additives showed the strongest effectiveness in both the neat biodiesel and the biodiesel blends.

Stability was enhanced in canola biodiesel with antioxidant 2,5-di-tert-butylhydroquinone (DTBHQ) or poly(1,2-dihydro-2,2,4-trimethylquinoline) (Orox PK) (**Focke et al., 2012**).

Accordingly, the review by **Jain and Sharma (2010)** focussed on data for eight representatives of the class phenolic antioxidants. Overall, propyl gallate (PG), pyrogallol (PA) and tert-butyl hydroquinone (TBHQ) were considered the most effective additives in both the neat biodiesel and the biodiesel blends. The superior performance of propyl gallate (PG) and pyrogallol (PA) was confirmed by **Karavalakis et al. (2011)**. These authors reported that butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) displays the lowest effectiveness in neat biodiesel. Propyl gallate (PG) and pyrogallol (PY) additives showed the strongest effectiveness in both the neat biodiesel and the biodiesel blends. **Schober and Mittelbach (2004)** used a Rancimat instrument at 110 °C and investigated the potential of 11 synthetic phenolic antioxidants to improve the oxidation stability of four biodiesel samples derived from different feedstocks. 2,5-di-tert-butylhydroquinone (DTBHQ) was found to be a very effective antioxidant even at low concentrations. **Focke et al. (2012)** prepared biodiesel samples using base-catalyzed methanolysis of sunflower, soybean and canola oils. Fatty acid methyl ester analysis results, in conjunction with Fourier Transform Infrared (FTIR) spectroscopy (FTIR spectra), confirmed the absence of water and methanol but indicated that the fuels contained unreacted vegetable oils. The stability of the neat oils correlated with the degree of unsaturation of the fatty acid methyl esters. The Rancimat induction periods were 0.61 h, 3.3 h and 7.1 h for the neat (and fresh) sunflower, soybean and canola fuels respectively. The measured Rancimat induction periods decreased by about 25% when the neat oils were exposed to air for 12 days. The neat canola biodiesel already conformed to the European Rancimat specification **EN 14214** or biodiesel stability of > 6 h. Its stability was further improved by the addition of the synthetic antioxidants di-tert-butylhydroquinone (DTBHQ) or poly(1,2-dihydro-2,2,4-trimethylquinoline) (Orox PK). Surprisingly it was found that low additions of the synthetic antioxidant tris(nonylphenyl) phosphite (Naugard P) to canola oil actually resulted in an apparent decrease in the oxidative stability as quantified by Rancimat. Such an effect was

also observed, albeit to a lesser extent, with DTBHQ as inhibitor in sunflower and canola biodiesels.

CONCLUSION

Biodiesel is chemically fatty acid alkyl esters of long-chain fatty acids derived from renewable sources, which include also the vegetable oils. The quality of biodiesel physico-chemical properties is influenced by the chain length and the level of unsaturation of the produced fatty acid alkyl esters. A biodiesel is thermodynamically stable. Its instability primarily occurs from contact of oxygen present in the ambient air that is referred to as oxidative instability.

The great deal of knowledge accumulated on the general chemistry of oxidation of lipids suggests that further efforts should be directed to a detailed understanding of the effects of this process in biodiesel.

Potential targets for future research would be based on the following:

- For biodiesel is oxidation stability a general term. It is necessary to distinguish 'storage stability' and 'thermal stability', in relation to oxidative degradation, which may occur during extended periods of storage, transportation and end use.

- Fuel instability problems can be of two related types, short-term oxidative instability and long-term storage instability. Storage instability is defined in terms of solid formation, which can plug nozzles, filters, and degrade engine performance. Biodiesels are more susceptible to degradation compared to fossil diesel because of the presence of unsaturated fatty acid chain in it.

- The mechanisms of oxidative degradation are autooxidation in presence of atmospheric oxygen; thermal or thermal-oxidative degradation from excess heat; hydrolysis in presence of moisture or water during storage and in fuel lines; and microbial contamination from contact with dust particles or water droplets containing fungi or bacteria into the fuel.

- The factors such as heat, oxygen, light, and some metal ions, especially iron and copper, also play a significant role in creating oxidation.

- Oxidative products formed in biodiesel affect fuel

- Storagelife, contribute to deposit formation in tanks, and they may cause clogging of fuel filters and injection systems.

- The volatile organic acids formed as secondary by products of the oxidative degradation, may stimulate corrosion in the fuel system.

- Poor stability can lead to increasing acid numbers, increasing fuel viscosity, and the formation of gums and sediments.

- Oxidation stability of biodiesel is the important parameter to determine the storage of biodiesel for longer period of time. Biodiesel samples were evaluated according to methods on the base of kept in contact with pure oxygen at elevated temperatures and pressures. Antioxidants showed preventive effects on oxidative stability of biodiesel, especially primary synthetic.

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EFFECT OF DIFFERENT DIETARY SUPPLEMENTS ON SELECTED QUALITY INDICATORS OF CHICKEN MEAT

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ABSTRACT

The aim of the study was to evaluate the effect of different feed additives (bee pollen extract, propolis extract, and probiotic) on meat quality of broiler chickens. A total of 180 one day-old broiler chicks of mixed sex (Ross 308) were randomly divided into 3 groups. Dietary treatments were as follows: basal diet, free of supplements (control group; C); basal diet plus 400 mg bee pollen extract per 1 kg of feed mixtures and 3.3 g probiotic preparation added to drinking water (group E1); basal diet plus 400 mg propolis extract per 1 kg of feed mixtures and 3.3 g probiotic preparation added to drinking water (group E2). In the experiment, the probiotic preparation based on *Lactobacillus fermentum* (1.10^9 CFU.g⁻¹ of bearing medium) was used. Fattening period lasted for 42 days. Feed mixtures were produced without any antibiotic preparations and coccidiostatics. Meat quality was evaluated by following technological properties: cooling, freezing and roasting loss; colour parameters based on CIELab system; and shear force. Both dietary supplementations led to decrease in cooling ($p \leq 0.05$) and freezing ($p \geq 0.05$) losses compared with control. On the contrary, the supplemented diet tended to increase roasting losses ($p \leq 0.05$) and shear force values in thigh muscle ($p \leq 0.05$). Significantly higher L* values ($p \leq 0.05$) in breast and thigh muscles, as well as the b* values in thigh muscle, were found when broiler chickens were fed the supplements, especially bee pollen extract and probiotics. In addition, the supplements improve redness (a*) of meat. The redness of breast muscle appeared to be the most affected ($p \geq 0.05$) by propolis extract plus probiotics supplementation, while thigh muscle had the highest value ($p \leq 0.05$) in bee pollen extract plus probiotics supplemented group. These findings suggested that the supplements have a beneficial effect on quality of chicken meat due to positive changes in most of quality indicators investigated in the study.

Keywords: chicken meat; loss; colour; shear force; dietary supplement

INTRODUCTION

Worldwide, chicken meat continues to be the most popular poultry meat, representing about 85% of total poultry meat output (Soriano-Santos, 2010). The poultry market has grown substantially due to various marketing practices, such as selling individual cuts. Another reason for the increased popularity of poultry is its low fat and cholesterol contents. Poultry products are especially lean compared to other animal products, such as pork or beef. Consumer interest in natural or organic products is increasing at a fast rate and has contributed to the increase in poultry consumption. Many poultry producers have met consumer needs by producing antibiotic- and hormone-free meat (Padilla, 2010; Lázaro et al., 2015).

There are many criteria that drive a consumer decision to purchase certain products, including appearance, taste, aroma, and texture (Akiba et al., 2001; Padilla, 2010; An et al., 2013). Water-holding capacity, colour, pH, tenderness, and sensory acceptability are commonly used in order to evaluate chicken meat quality because consumers prefer meat that is juicy, tender, and not pale (Schilling et al., 2010; Miezieliene et al., 2011).

Colour and appearance of fresh meat are presumed to be indicators of meat freshness and quality. Chicken muscle colour is affected by a variety of factors, including age, environment, diet, and feed withdrawal. The colour of raw muscle is due to the light-scattering properties (Brewer, 2010) and ranges from pink to red due to hemoglobin and myoglobin within the muscle (Padilla, 2010). One of the important factors affecting meat colour is the pH of the meat. Broilers produced by organic methods had a lower pH and a lower water-holding capacity, which may have been responsible for producing meat that appeared more yellow as well as less red than broilers produced by a traditional system (Castellini et al., 2002).

Tenderness involves all the mouth feel characteristics perceived kinesthetically: those perceived prior to mastication (particle size, oiliness), during mastication (tenderness, juiciness), and after mastication (fibrous residue, mouth coating) (Brewer, 2010). In general, consumers rate tenderness as the major factor that determines the eating quality of meat (Brewer and Novakofski, 2008).

Carcass chilling time is important processing procedure that influences the quality of meat. Slow, inadequate

chilling decreases the pH of the meat from lactic acid build up and begins to denature proteins within the muscle. This defect, known as pale, soft, and exudative (PSE) meat, is a growing problem in the poultry industry (**Padilla, 2010; Bowker et al., 2014**).

The defect PSE affects colour negatively, as well as meat texture and integrity. Meat quality as well as water-holding capacity begins to decline, which can make meat tough. To minimize the occurrence of PSE, the temperature of the carcass should be less than 25 °C by 60 min *post mortem* (**Alvarado and Sams, 2002**).

In order to eliminate the use of antibiotics as growth promoters, search of effective alternatives is a very important task in poultry industry (**Fasina and Olowo, 2013; Cai et al., 2015**). Plant-derived substances received considerable interest because of their antioxidant and antimicrobial effects reported in many studies. Bee pollen is a natural product, which is collected from plants by honey bees (**Attia et al., 2014**). The pollen is rich in proteins, essential amino acids, oils, fatty acids, minerals, enzymes and co-enzymes, carbohydrates and flavonoids, carotenoids and phytosterols (**El-Asely et al., 2014**). Propolis is a natural resinous product produced by honey bees from the gum of various plants and trees, and is used in the beehive as a protective barrier against their enemies (**Duman and Özpolat, 2015**). It contains amino acids, minerals, ethanol, vitamin A and E, B complex vitamins, and flavonoids and has strong antimicrobial properties (**Aygun et al., 2012; Da Silva Frozza et al., 2013**).

Among the possible alternatives, probiotics are considered a promising alternative to antibiotics, as well. Probiotic is defined as a live microbial feed supplement that beneficially affects the host animal by improving the intestinal microbial balance (**Daneshmand et al., 2015**). Application of probiotics can prevent the occurrence of diseases, replace or reduce the use of antibiotics, stimulate the immune system, inhibit the inflammatory processes (**Vidová et al., 2013**). Various studies have reported a wide variety of health-promoting properties influencing the host intestinal balance (**Shim et al., 2012; Blajman et al., 2015**), as well as quality of chicken eggs (**Angelovičová et al., 2013**) and chicken meat (**Bobko et al., 2015**).

In the previous study (**Haščík et al., 2015**), we reported debatable effects of bee pollen, propolis and probiotics on technological properties of chicken meat, since the results observed in the study were not satisfactory. For this reason, we have decided to investigate whether the effect of the natural feed supplements will be more obvious when administered in combination, namely the bee pollen extract with probiotic preparation and the propolis extract with probiotic preparation.

Thus, the objective of the present study includes assessment of influence of the natural supplements in the combination on quality of chicken meat by determination of selected technological properties of chicken meat, namely cooling, freezing and roasting loss, colour and shear force.

MATERIAL AND METHODOLOGY

Chickens and dietary treatments

The experiment was carried out in test poultry station of Slovak University of Agriculture in Nitra. A total of 180 one day-old broiler chicks of mixed sex (Ross 308) were randomly divided into 3 groups, namely, control (C) and experimental (E1, E2) of 60 pcs chickens. The experiment lasted for 42 days. The chickens were bred on breed litter (wood shavings), in a temperature-controlled room; ambient temperature in test poultry station was maintained at 33 °C during the first week and gradually decreased by 2 °C, and finally fixed at 19 °C thereafter. Throughout the entire experimental period, the broilers were provided with *ad libitum* access to feed and water and were kept under constant light regime.

Table 1 lists the basal diet formulated according to nutrient requirements of broilers. The broiler chickens were fed a starter diet (HYD-01) from the 1st to the 21st day and grower diet (HYD-02) from the 22nd to the 42nd day. The feed mixtures both starter and grower were produced without any antibiotic preparations and coccidiostatics.

The dietary treatments were as follows: basal diet without any supplementation (C; control group), basal diet plus 400 mg bee pollen extract per 1 kg of feed mixtures and 3.3 g probiotic preparation added to drinking water (group E1), basal diet plus 400 mg propolis extract per 1 kg of feed mixtures and 3.3 g probiotic preparation added to drinking water (group E2). Besides, the groups were kept under the same conditions.

In the experiment, the probiotic preparation based on *Lactobacillus fermentum* (1.10⁹ CFU.g⁻¹ of bearing medium) was used.

Bee pollen and propolis had origin in the Slovak Republic. The extracts were prepared from minced bee pollen and propolis in the conditions of the 80% ethanol in the 500 cm³ flasks, according to **Krell (1996)**. The extraction was accomplished in a water bath at 80 °C for one hour. After that, the extracts were cooled and centrifuged. The obtained supernatants were evaporated in a rotary vacuum evaporator at bath temperature 40 – 50 °C and weighed. Residues in an amount of 40 g were dissolved in 1000 cm³ of 80% ethanol and used for 100 kg of the feed mixtures.

Slaughter and measurements

At the end of the experiment (42 days of age), 120 broiler chickens, randomly selected from each group (n = 40), were slaughtered at the experimental slaughterhouse of Slovak University of Agriculture in Nitra.

After evisceration, the carcasses were kept at approximately 18 °C for 1 h *post mortem* and thereafter longitudinally divided into two parts. After that, the half-carcasses were weighed and stored at 4 °C until 24 h *post mortem*, when the first measurements were done. The left half-carcass was used in order to determine the technological properties as described below, whereas the right one was assigned to different analysis.

Table 1 Composition of basal diet and nutrient content.

Ingredients (%)	Starter (HYD-01) (day of age 1 – 21)	Grower (HYD-02) (day of age 22 – 42)
Wheat	34.00	37.00
Maize	33.92	37.52
Soybean meal (48% N)	23.00	18.00
Fish meal (71% N)	5.00	3.00
Dried blood	-	1.00
Fodder lime	1.00	0.95
Monocalcium phosphate	0.80	0.70
Fodder salt	0.10	0.10
Sodium bicarbonate	0.15	0.20
Lysine	0.13	0.08
Methionine	0.18	0.20
Clinacox 0.5% ¹	0.02	-
Sacox 12% ²	-	0.05
Bergafat (palm kernel oil)	1.20	0.70
Euromix BR 0.5% ³	0.50	0.50
Nutrient content [g.kg⁻¹]		
Linoleic acid	13.53	14.05
ME _N [MJ.kg ⁻¹]	12.07	12.16
Fibre	30.50	29.67
Crude protein	212.40	191.61
Ash	27.00	20.90
Ca	8.22	7.18
P	6.55	5.86
Na	1.77	1.70

¹ Active ingredient: each kg contains 5 g of diclazuril; ² Active ingredient: each kg contains 120 g of salinomycin;

³ Active substances per kilogram of premix: vitamin A 2,500,000 IU; vitamin E 20,000 mg; vitamin D₃ 800,000 IU; niacin 12,000 mg; D-pantothenic acid 3,000 mg; riboflavin 1,800 mg; pyridoxine 1,200 mg; thiamine 600 mg; menadione 800 mg; ascorbic acid 20,000 mg; folic acid 400 mg; biotin 40 mg; kobalamin 8.0 mg; choline 100,000 mg; betaine 50,000 mg; Mn 20,000 mg; Zn 16,000 mg; Fe 14,000 mg; Cu 2,400 mg; Co 80 mg; I 200 mg; Se 50 mg.

After 24 h, the colour of breast (*Musculus pectoralis major*) and thigh muscle (*Musculus biceps femoris*) from the left half-carcass (n = 10) was assessed using a Minolta CM 2600d spectrophotometer (Konica Minolta, Japan) and reported in the CIE system values of lightness (L*), redness (a*) and yellowness (b*). Lightness (L*) is the amount of incident light that a surface reflects; positive a* values represent the red colour and negative a* values represent the green one; positive b* values represent yellow and negative b* values represent blue. Colour measurements were taken at three locations on each sample and averaged. All the colour readings were taken on skinless meat, in an area free of obvious colour defects (over scald, bruises, and blood accumulation).

The cooling loss was determined in whole left half-carcass as the percentage of weight loss over a 24 h period, by calculating the weight differences before and after cooling.

Afterwards, the same half-carcasses were stored at -18 °C for 3 months prior to next analysis. Thereafter, the samples were thawed. After thawing was completed, the weight of the samples was obtained. To determine the freezing loss (%), the weight differences before and after freezing process were calculated (n = 10). All the weight measurements were performed using the precision balance

Kern 440 (Kern and Sohn, Germany) with accuracy of 0.01 g.

The heat treatment of samples was carried out in oven (Gorenje B 3300 E) at 200 °C for 60 minutes. After allowing the samples to cool at room temperature, the samples were weighed so as to calculate the percentage of roasting losses. The roasting loss was expressed as the percent weight reduction of the heat-treated sample compared to the raw sample (n = 10).

The samples that were used for roasting loss determination were also used for shear force determination. Results for tenderness of breast (*Musculus pectoralis major*) and thigh muscle (*Musculus biceps femoris*) have been expressed as shear force (kg.cm⁻²) (five measurements were performed on each sample to obtain an average value). First, the five cores with the same size (2.0 cm wide, 5.0 cm long and 1.5 cm high) were removed from each heat-treated sample (n = 10). Then, the cores were sheared perpendicular to the muscle fibres orientation using a Warner-Bratzler shear device (Chatillon, U.S.A.), in accordance with Goodson et al. (2002).

Statistical analysis

The data processing for technological attributes of raw and heat-treated samples of meat was performed using a statistical program Statgraphics Plus Version 5.1 (AV Trading Umex, Dresden, Germany). For the

determination of significant difference among the tested groups, analysis of variance (ANOVA) with Scheffé's method was used.

RESULTS AND DISCUSSION

The effects of the feed supplements administration on selected technological properties of chicken meat are shown in Table 2 and Table 3. Regarding cooling losses of chicken meat, there was positive effect ($p \leq 0.05$) of feed additives, with the lowest losses being observed in bee pollen plus probiotic supplemented group (3.35%), followed by propolis plus probiotic supplemented group (3.58%). Also, there was found the positive effect for freezing losses of chicken meat, with, however, no statistical significance. The lowest value was observed in propolis plus probiotic supplemented group (2.78%), followed by bee pollen plus probiotic supplemented group (3.38%). The effect of the supplements was rather inappropriate for roasting losses owing to the higher values

in experimental groups than that in control. Results indicated that supplementation of chicken diet with the feed additives was more effective in lowering of losses caused by cooling and freezing than those caused by roasting. In addition, the values of cooling and freezing losses were lower than those achieved in the study of **Haščík et al. (2015)** when the supplements were administered singly.

As far as colour measurement is concerned, more obvious results were achieved in thigh muscle. Lightness (L^*) of thigh muscle was significantly ($p \leq 0.05$) improved by feed supplements. The improvement was shown by higher values in both bee pollen plus probiotic (54.32) and propolis plus probiotic supplemented group (54.44) when compared with control (51.64). In breast muscle, the significantly ($p \leq 0.05$) higher L^* value was observed in bee pollen plus probiotic supplemented group (55.58) when compared with the other two groups.

Chicken breast muscle can be classified according to the

Table 2 Cooling loss, freezing loss and roasting loss of chicken meat (mean \pm SD).

Parameter	Group			S
	C	E1	E2	
Cooling loss [%]	3.97 \pm 0.44 ^a	3.35 \pm 0.43 ^b	3.58 \pm 0.45 ^{ab}	**
Freezing loss [%]	3.53 \pm 1.00 ^a	3.38 \pm 0.68 ^a	2.78 \pm 0.97 ^a	NS
Roasting loss [%]	29.54 \pm 1.16 ^a	31.18 \pm 1.26 ^b	30.01 \pm 1.06 ^a	**

Legend: C – control group; E1 – experimental group with basal diet plus 400 mg bee pollen extract per 1 kg of feed mixtures and 3.3 g probiotic preparation added to drinking water; E2 – experimental group with basal diet plus 400 mg propolis extract per 1 kg of feed mixtures and 3.3 g probiotic preparation added to drinking water; mean – average, SD – standard deviation; a, b – means with different superscripts within row differ significantly; S – significance; ** $p \leq 0.05$; NS = not significant.

Table 3 Instrumental colour values and shear force value of chicken breast and thigh muscle (mean \pm SD).

Parameter		Group			S
		C	E1	E2	
Colour parameter					
CIE L*	breast	52.24 \pm 2.88 ^a	55.58 \pm 3.37 ^b	52.65 \pm 3.60 ^{ab}	**
	thigh	51.64 \pm 1.86 ^a	54.32 \pm 2.18 ^b	54.44 \pm 2.90 ^b	**
CIE a*	breast	0.07 \pm 0.06 ^a	0.13 \pm 0.43 ^a	0.49 \pm 0.78 ^a	NS
	thigh	1.94 \pm 0.64 ^a	4.17 \pm 1.58 ^b	2.30 \pm 1.39 ^a	**
CIE b*	breast	10.08 \pm 1.26 ^a	10.73 \pm 1.58 ^a	10.46 \pm 2.08 ^a	NS
	thigh	9.60 \pm 1.76 ^a	12.56 \pm 1.60 ^b	11.42 \pm 2.17 ^{ab}	**
Shear force value [kg.cm ⁻²]	breast	1.97 \pm 0.37 ^a	2.00 \pm 0.42 ^a	1.88 \pm 0.51 ^a	NS
	thigh	1.33 \pm 0.24 ^a	1.67 \pm 0.33 ^b	1.62 \pm 0.45 ^{ab}	**

Legend: C – control group; E1 – experimental group with basal diet plus 400 mg bee pollen extract per 1 kg of feed mixtures and 3.3 g probiotic preparation added to drinking water; E2 – experimental group with basal diet plus 400 mg propolis extract per 1 kg of feed mixtures and 3.3 g probiotic preparation added to drinking water; mean – average, SD – standard deviation; a, b – means with different superscripts within row differ significantly; S – significance; ** $p \leq 0.05$; NS = not significant.

colour as: lighter than normal ($L^* > 53$), normal ($48 < L^* < 53$) and darker than normal ($L^* < 48$), as mentioned in the study of **Qiao et al. (2001)**. Since the L^* value in bee pollen plus probiotic supplemented group (E1) exceeded the value of 53, the meat can be classified as lighter than normal while that in other groups (52.24 – group C, 52.65 – group E2) can be classified as normal.

Numerically higher redness (a^*) of breast muscle, though not confirmed statistically ($p \geq 0.05$), was observed in group of chickens receiving the supplements (0.13 – group E1, 0.49 – group E2) in comparison with control (0.07). Redness (a^*) of thigh muscle was significantly improved ($p \leq 0.05$) when bee pollen extract and probiotics were used (4.17 – group E1) in comparison with other groups (2.30 – group E2, 1.94 – C).

The values of yellowness (b^*) of breast muscle were similar among the groups. However, the yellowness (b^*) of thigh muscle increased by the dietary supplementation of bee pollen extract and probiotics (12.56 – group E1), as well as propolis extract and probiotics (11.42 – group E2) compared with control diet (9.60).

Jiang et al. (2014) have noticed that a^* value (redness) is the most favoured by consumers and lower b^* value (yellowness) indicates less pale meat. For this reason, we assume that low a^* values found in our study are not quite convenient for consumers.

Results for the colour measurements are in agreement with **Lei and Kim (2013)** who investigated the effect of whole egg powder on meat quality in broiler chickens (Ross 308). In the study, L^* values of breast muscle ranged from 55.1 to 57.8, a^* values ranged from 14.0 to 14.9 and b^* values ranged from 12.4 to 13.5. It is obvious that b^* values observed in the study were much more high than those in the present study, that is why the meat has appeared as more red. However, there was no effect of added egg powder on colour parameters of chicken meat found.

Similar finding were reported by **Jiang et al. (2014)** using isoflavone as dietary supplement for Lingnan yellow male broilers. On the one hand, the supplementation resulted in a significant ($p \leq 0.05$) decrease in L^* colour parameter (55.17 – 57.49), but on the other hand in a significant ($p \leq 0.05$) increase in a^* colour parameter (12.55 – 14.00). Yellowness (b^*) (17.59 – 20.92) was not affected by isoflavone supplementation.

Ros-Polski et al. (2015) reported significant influence ($p \leq 0.05$) of lightness (L^*) (46.47 – 56.43) and redness (a^*) (3.21 – 3.92), but not yellowness (b^*) (12.63 – 12.80) adding sodium chloride (NaCl) to the chicken meat, which results are similar to this study.

Results of the present study, however, are not consistent with those obtained by **Dotas et al. (2014)**, who found much higher L^* values (74.5 – 77.3 in breast muscle, 73.6 – 77.3 in thigh muscle) after partial replacement of soybean meal and corn with raw field peas, perhaps also because male broiler chickens (Ross 308) were used. Redness (a^*) ranged from 4.3 – 6.4 in breast and 4.2 – 5.5 in thigh muscle, yellowness (b^*) ranged from 17.4 – 23.3 in breast and 13.3 – 19.3 in thigh muscle.

Min et al. (2012) observed lower L^* values in breast fillets of male broilers receiving distillers dried grains with

solubles (DDGS) (54.94 – 58.6) compared with those fed a basal diet (59.02) while a^* values were in range of 12.61 – 13.48 (11.90 in control). Rather different values were found in b^* colour parameter (31.99 – 38.95 vs. 30.29 in control).

In another study, **Schilling et al. (2010)** also investigated DDGS as feed supplement in diet of broiler chickens and evaluated the effect on meat quality. They found no differences ($p \geq 0.05$) among breast meat from the different groups with respect to colour parameters. Yet, the results achieved in the study were similar to those in our study (except for b^* values), the L^* (52.9 – 53.8), a^* (2.2 – 2.7) and b^* values (2.4 – 3.2) for all groups were characteristic of normal broiler breast meat ($L^* < 55$) at 24 h *post mortem*.

Also, **Cai et al. (2015)** observed similar values of colour parameters of breast muscle after supplementation of rare earth elements-enriched yeast in diet of broilers (Ross 308), however, with significant differences only in redness (b^*). L^* values ranged from 57.73 – 57.81, a^* values ranged from 14.05 – 15.57, and b^* values ranged from 15.34 – 17.17.

Akiba et al. (2001) evaluated effect of diet supplemented with *Phaffia rhodozyma*, yeast containing high levels of astaxanthin, on meat colour in broiler chickens. They found following values in breast muscle (*Pectoralis major*): L^* values in range of 39.6 – 41.7, a^* values in range of -0.4 – 2.4 and b^* values in range of 8.2 – 8.8. The values in thigh muscle were as follows: 41.2 – 42.6 for lightness (L^*), -1.2 – 3.8 for redness, and 15.8 – 16.0 for yellowness (b^*). The effect was considered as very positive, owing to visible redness of meat from broilers receiving the supplement.

Miezeliene et al. (2011) reported that diet containing the addition of selenium in broilers diet had a significant effect on meat colour. Lightness of chicken breast significantly decreased ($p \leq 0.05$) (73.38 vs. 56.51), but redness (3.05 vs. 6.75) and yellowness (3.71 vs. 5.20) significantly increased ($p \leq 0.05$) with the addition of selenium in diet.

Our previous study (**Haščík et al., 2015**) showed that diet containing bee pollen, propolis and probiotics did not affect lightness and yellowness of chicken meat, but increased ($p \leq 0.05$) redness of breast muscle (0.59 – 1.33) and decreased ($p \leq 0.05$) redness of thigh muscle (1.33 – 1.84), which was not in accordance with this study, where all the colour parameters were increased.

Regarding shear force measurement (Table 3), none of supplements caused significant changes ($p \geq 0.05$) in tenderness of breast muscle, whereas both bee pollen plus probiotic and propolis plus probiotic supplementations (1.67 and 1.62 kg.cm⁻², respectively) increased ($p \leq 0.05$) the shear force in thigh muscle compared with control (1.33 kg.cm⁻²). When comparing shear force values among the groups, bee pollen plus probiotic supplemented group showed higher values than the other two groups. Thus, the bee pollen extract in combination with probiotics has been considered as the least appropriate supplement.

These findings are consistent with the previous study (**Haščík et al., 2015**), in which very similar shear force values were observed. In addition, the lowest values were found in both breast (1.89 kg.cm⁻²) and thigh

(1.25 kg.cm⁻²) muscle of broilers receiving propolis extract. In the present study, the best results were observed also in group receiving propolis extract (in combination with probiotics). It is, thus, likely that the propolis is the most effective in improving the tenderness of chicken meat among the supplements.

According to **Chen et al. (2007)**, tenderness is the most important factor in consumer perception of palatability and quality of meat products. Therefore, this attribute has drawn much attention from researchers.

Castellini et al. (2002) assessed effect of organic production on meat quality of male broiler chickens (56 days of age). In the organic group, there were higher shear force values of roasted samples found in both breast and thigh muscle (2.25 and 3.08 kg.cm⁻², respectively) compared with control (1.98 and 2.39 kg.cm⁻², respectively). When comparing with our study, the higher values observed in the study of **Castellini et al. (2002)** can be correlated to the higher age of chickens.

Since the most of researches have used various devices and cooked samples for the shear force measurement, it is difficult to directly compare the shear force values among the different studies.

In the study of **Min et al. (2012)**, significant difference was observed ($p \leq 0.05$) between broilers fed the distillers dried grains with solubles (DDGS) and control group with respect to shear force. With the addition of DDGS, shear force almost doubled (24.29 – 42.32 N), compared with that of control (12.79 N). Since the tender meat is more acceptable to consumers, dietary DDGS were evaluated as less suitable supplement.

Schilling et al. (2010) found relatively low shear force values (15.1 – 16.3 N) which indicate very tender meat that would be highly acceptable to consumers.

Similarly, **Chen et al. (2007)** reported shear force values of breast muscle for Arbor Acres broiler, Jingxing 100 crossbred chicken and Beijing fatty chicken at levels 17.36, 17.06 and 11.90 N, respectively.

CONCLUSION

In conclusion, the supplements evaluated in the study (bee pollen extract plus probiotics and propolis extract plus probiotics) reduced cooling ($p \leq 0.05$) and freezing ($p \geq 0.05$) losses. However, they slightly increased ($p \leq 0.05$) the losses during roasting. As regards colour measurement, the supplements elevated the values of L* colour parameter (lightness) in both breast and thigh muscle compared with group containing no supplements. The most noticeable effect of the supplements was, however, observed in redness (a*), because of presenting higher values. The b* colour parameter (yellowness) did not appear to be positively affected by the supplementation, as well as shear force values, which did not differ from each other except lower ($p \leq 0.05$) shear force value of thigh muscle in control. The present study demonstrated that bee pollen and propolis extract in combination with probiotics could be considered as suitable additives without negative indications in broiler chickens, because of apparent synergistic effect of mixture of the supplements.

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SELENIUM CONTENT INCREASING IN THE SEEDS OF GARDEN PEA AFTER FOLIAR BIOFORTIFICATION

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ABSTRACT

Selenium plays an important role as an antioxidant in the prevention of cardiovascular disease. Content of selenium in the crops is constantly in the spotlight of professional public. Vegetables, as an important source of chemo protective substances, have irreplaceable position within the food of plant character. The aim of research work was to solve the Se content increasing in the seeds of garden pea (varieties Premium and Ambassador) through the foliar biofortification of the plants (50 g Se / ha and 100 g Se / ha) and to monitor its effect on production of photosynthetic pigments. In the seeds of fresh garden pea, the chlorophyll *a* and chlorophyll *b* content was determined by spectrophotometer depending on a variety and the doses of selenium. In lyophilized seeds there was measured content of selenium by ET-AAS methods. The statistically significant increase of selenium was confirmed with its increasing concentrations in case of both varieties. In the var. Ambassador there was increasing from 0.083 ± 0.009 mg.kg⁻¹ DM to 4.935 ± 0.598 mg.kg⁻¹ DM (60-fold) and in a var. Premium the values increase from 0.067 ± 0.007 mg.kg⁻¹ DM to 3.248 ± 0.289 mg.kg⁻¹ DM (48-fold) after application of 100 g Se / ha. After application of 50 g Se / ha in both varieties of peas there was reported 25-fold increasing in the selenium content in comparison with control. The content of photosynthetic pigments was also increased, or possibly left at level of un-fortificated variant (chl_a – Ambassador – 50 g Se / ha; chl_b – Premium – 100 g Se / ha) by foliar biofortification. Chlorophyll *a* content was high significantly increased according to used statistical methods in varieties Premium, from the content of 24.527 ± 5.156 mg.kg⁻¹ FM to 66.953 ± 6.454 mg.kg⁻¹ FM, likewise the content of chlorophyll *b* from the value of 19.708 ± 5.977 mg.kg⁻¹ FM to 37.488 ± 6.146 mg.kg⁻¹ FM (after 50 g / ha application). Foliar biofortification of different vegetable species can provide large-scale intake of minerals with antioxidant properties for human as well as an increase of certain biologically active substances as a result of their synergies.

Keywords: garden pea; foliar biofortification; selenium; chlorophyll *a*; chlorophyll *b*

INTRODUCTION

At present, there is increasing attention to the impact of nutrition on human health, mainly due to a significant increase of the number of cancer and cardiovascular diseases. Increasing of antioxidants intake has an important position in the prevention from this kind of diseases. The selenium belongs to such an elements and that's the reason of its intensive studying in the recent years. Potential positive effects of selenium on the human health are reflected especially in the fight against cardiovascular disease, since endothelial cells need enough of selenium to maximize selenium protective activities. Maintaining of optimum concentrations and activity of selenoproteins appears to be important in the prevention of the so-called lifestyle diseases (Holben and Smith, 1999; Arthur, 2003; Zeng and Combs, 2008). Moreover, a low selenium intake is associated with health disorders, including oxidative stress, epilepsy, impaired fertility, immune deficiency, etc. (Rayman, 2012; Whanger, 2004; Zeng and Combs, 2008). Selenium is part of glutathione peroxidase; it protects the arterial endothelium against damage of lipid peroxides. In the absence of selenium lipoperoxides accumulate in the heart, they damage the

cell membrane and they are harmful in transport of the calcium, which is accumulated in a cell (Hegedús et al. 2007; Li et al. 2008; Rayman, 2012).

The level of selenium in the body depends on its concentration in the food. In to the food chain the selenium gets primarily from the soils and drinking water. Its content in plants is a function of the conditions of soil – plant system (Arthur, 2003). Slovak soils are generally poor in selenium, which is related to its insufficient quantity in agricultural products. Content of selenium in the crops is constantly in the spotlight of professional public. The biological value of grown food raw materials depends on qualitative state of growing mediums - soils. Biogenic elements presented in the soils are taken by plants and thereby entering into the food chain. Plants are able to receive the inorganic selenium added to the soil (in the form of selenate and selenite) and to convert its part or all of it to the organic components. Agronomic biofortification through the application of fertilizers enriched with selenium is one of the possible ways of its content increasing in the soil. On the other hand, there is potential danger of soil contamination. Because of selenium content increasing in the edible parts

of plant there is promoted its combination with other biofortification approaches, such as the foliar biofortification, i.e. selenium application directly to the plant (Graham et al. 2007; White and Broadley, 2009). Foliar application was shown to be several times more efficient than application in fertilizers (Aspila, 2005; Hegedúsová et al. 2015).

Selenium content in different parts of plants depends on the plant species. The concentration of selenium is two or three times higher in grain and roots as in the stems and leaves. The level of selenium achieves the values in the range from 0.001 to 0.034 mg.kg⁻¹ in vegetables. Crops containing the average amount of sulphur such as e.g. cabbage tend to higher Se accumulation than plants with low sulphur content (Baghour et al. 2002). Selenium is arguably the naturally occurring trace element of greatest concern worldwide. In excessive amounts it can lead to toxicosis and teratogenesis in animals, while the impact of selenium deficiency can be even more significant (Bañuelos et al. 2013; Timoracká and Vollmannová, 2010).

The aim of the work was to solve the selenium content increasing in the seeds of garden pea plants through the foliar biofortification and to monitor its impact on the content of photosynthetic pigments.

MATERIAL AND METHODOLOGY

The small – area field experiment of two garden pea varieties (Premium and Ambassador) was established on 22nd April, 2014 on the Botanical Garden of Slovak University of Agriculture in Nitra. There were applied doses of selenium on two concentration levels (50 g Se / ha a 100 g Se / ha) and without selenium application (in case of control), every variant in four replications. Total area of field trials was 24 m². Agrochemical characteristic of soil substrate are figured in Table 1.

Before planting the soil was prepared according to the technological demands of garden peas. During vegetation of the pea the field trial was treated by hand hoeing, loosening and irrigation in the absence of moisture. Foliar application of an aqueous solution of sodium selenate was carried out on garden pea plants in the flowering stage by using of a hand sprayer. The harvest was done at the stage of consumer maturity for every garden pea variety in suitable time. In fresh garden pea seeds the chlorophyll

content was determined for chlorophyll *a* as well as for chlorophyll *b*, in lyophilized seeds it was estimated the content of selenium.

Variants:

K – control without foliar biofortification

SeI – application of 50 g Se / ha in the form of an aqueous solution of sodium selenate in the stage of flowering

SeII – application of 100 g Se / ha in the form of an aqueous solution of sodium selenate in the stage of flowering.

Characterisation of garden pea varieties

Var. Premium - is an early variety, which needs 680 thermal units for its maturing. Plant height is 60 – 65 cm. The first pods are on 9. – 10. node and the number of pods on the node is 1 – 2. The length of the pods is 8 – 9 cm; the pods are straight and blunt-ended. Number of seeds in the pod is 7 – 8, seed colour is dark green and calibration of grain is moderate. Weight of 1000 seeds is about 220 g. It belongs between the suitable varieties of garden peas for industrial processing, as well as for gardens. It has high resistance to *Fusarium oxysporum*.

Var. Ambassador - is a medium late variety, for the maturing it needs 845 thermal units. Plant height is 75 – 80 cm. The first pods are formed at 15. – 16. node, their number is 2. The length of the pods is 8 – 9 cm, are straight and blunt-ended. Number of grains in the pod is 8 – 9 and grain colour is dark green. Weight of 1000 seeds is about 200 g. He has medium resistance to mosaic virus pea and mildew, high resistance to the bean yellow mosaic virus, and *Fusarium oxysporum*. This variety of garden pea is suitable for industrial processing and even in gardens.

Chlorophyll *a* and chlorophyll *b* content

Chlorophyll *a* and chlorophyll *b* were determined spectrophotometrically (Spektralquant PHARO 200) laterally in the acetone extract on the wavelengths $\lambda_{\alpha} = 649 \text{ nm}$ a $\lambda_{\beta} = 665 \text{ nm}$ (Hegedúsová et al. 2007). Fresh garden pea seeds (150 – 200 g) were homogenized and 1 g of sample weight was wiped in a mortar with sea sand by the addition of 3 – 4 mL of acetone. After perfect homogenization, the acetone extract was filtered through the glass filter S4 device according to Morton. The extraction was repeated until the acetone stayed discoloured. Clear filtrate was decanted into a volumetric flask and it was filled up with 80% acetone to 50 mL. By

Table 1 Agrochemical characteristic of soil substrate.

Agrochemical characteristic	pH (H ₂ O)	pH (KCl)	Cox (%)	Hum. (%)	
		7.55	6.36	1.39	4.01
Nutrients	N _{an} [mg.kg ⁻¹]	K [mg.kg ⁻¹]	Ca [mg.kg ⁻¹]	Mg [mg.kg ⁻¹]	P [mg.kg ⁻¹]
	13.3	285.0	5630.0	364.0	252.5

the help of the measured absorbance values chlorophyll *a* and chlorophyll *b* were calculated.

Selenium content estimation

The total content of selenium was determined in a digestion of plant material patterns. Quantitative determination of selenium was done by using of ET-AAS method with Zeeman background correction. Atomic absorption spectrometer SpectrAA240FS (Varian, Mulgrave Virginia, Australia) was used to measure the total selenium content. Conditions for selenium measurement were set in the equipment according to the recommendations of the manufacturer (Rothery, 1988) for ET-AAS technique.

Statistical analyzes

The analysis of variance (ANOVA), the multifactor analysis of variance (MANOVA) and the multiple Range test were done using the Statgraphic Centurion XV (StatPoint Inc. USA).

RESULTS AND DISCUSSION

Evaluation of selenium, chlorophyll *a* and chlorophyll *b* content

The selenium content in the seeds of the garden pea var. Ambassador without foliar application of selenium was on

the low level 0.083 ± 0.009 mg.kg⁻¹ of DM (Table 2). As the dry matter of garden pea is 20%, the selenium content in the fresh seeds in the control treatment is the 0.017 mg.kg⁻¹ of FM. High statistically significant changes were found after foliar biofortification of the peas with selenium. The dose of 50 g Se / ha caused an increasing of the selenium content in the seeds 25-times (2.031 ± 0.339 mg.kg⁻¹ DM = **0.406 mg.kg⁻¹ FM**), and 100 g Se / ha about 60-times (4.935 ± 0.598 mg.kg⁻¹ DM = **0.987 mg.kg⁻¹ FM**) (var. Ambassador, Table 2).

The level of selenium content in the seeds of the garden pea var. Premium without the foliar selenium application was low (0.067 ± 0.007 mg.kg⁻¹ of DM) (Table 3). Highly statistically significant changes were demonstrated after foliar biofortification of the peas with selenium. The dose of 50 g Se / ha increased the selenium content in pea seeds of the var. Premium also 25-fold as well as in case of var. Ambassador (1.648 ± 0.228 mg.kg⁻¹ DM = **0.330 mg.kg⁻¹ FM**) and the dose of 100 g Se / ha about 48 multiply (3.248 ± 0.289 mg.kg⁻¹ DM = **0.650 mg.kg⁻¹ FM**) (Table 3). The results correspond with research of (Kreft et al. 2013), where Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) plants were tested. The Se was effectively assimilated by the plants and preserve into the seeds, where its concentration was more than double that in

Table 2 Selenium content, chlorophyll *a* and chlorophyll *b* content in garden pea seeds (*Pisum sativum*) – variety ‘Ambassador’.

Ambassador	selenium	Chla	Chlb	Chla / Chlb
	[mg.kg ⁻¹ DM]	[mg.kg ⁻¹ FM]	[mg.kg ⁻¹ FM]	
K	0.083 ±0.009 ^a	25.960 ±1.419 ^a	19.130 ±2.090 ^a	1.36
Se I	2.031 ±0.339 ^b	25.653 ±2.340 ^a	22.698 ±2.030 ^a	1.13
Se II	4.935 ±0.598 ^c	31.913 ±1.304 ^b	21.745 ±2.860 ^a	1.47

Note: DM = dry matter, FM = fresh matter, Means ± standard deviation. Column values with different lowercase letters in superscript are significantly different at P < 0.05 by Tukey HSD in ANOVA (Statgraphic).

Table 3 Selenium content, chlorophyll *a* and chlorophyll *b* content in garden pea seeds (*Pisum sativum*) – variety ‘Premium’.

Premium	selenium	Chla	Chlb	Chla / Chlb
	[mg.kg ⁻¹ DM]	[mg.kg ⁻¹ FM]	[mg.kg ⁻¹ FM]	
K	0.067 ±0.007 ^a	24.527 ±5.156 ^a	19.708 ±5.977 ^a	1.24
Se I	1.648 ±0.228 ^b	66.953 ±6.454 ^c	37.488 ±6.146 ^b	1.79
Se II	3.248 ±0.289 ^c	41.005 ±3.305 ^b	19.603 ±4.328 ^a	2.09

Note: DM = dry matter, FM = fresh matter, Means ± standard deviation. Column values with different lowercase letters in superscript are significantly different at P < 0.05 by Tukey HSD in ANOVA (Statgraphic).

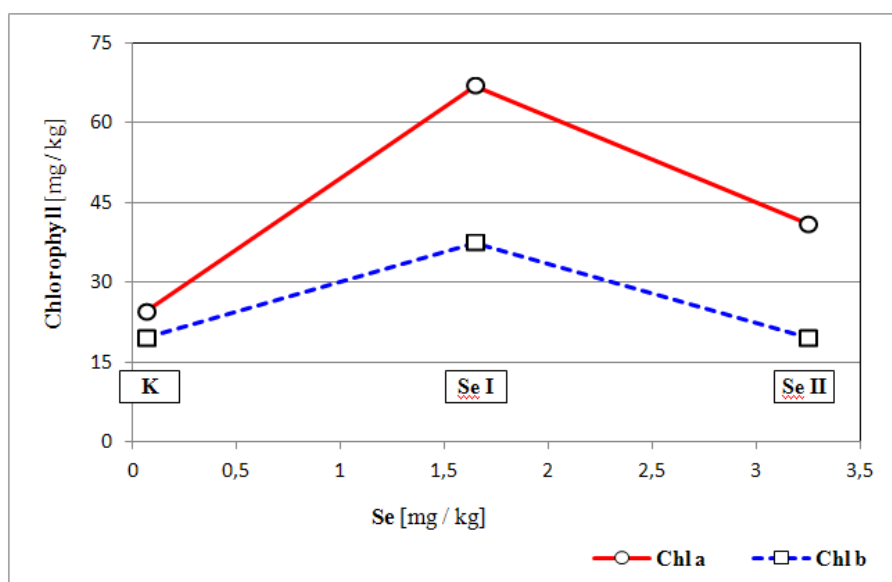


Figure 1 Dependence between of chlorophyll *a*, chlorophyll *b* and selenium content in seeds of garden pea, var. Premium.

untreated plants. Distribution of selenium and its increasing content was tested in *Fagopyrum esculentum* Moench by (Vogrincic et al. 2009). The total Se content in plant parts in the untreated group was low, whereas in the Se-sprayed group it was approximately 50- to 500-fold higher, depending on the plant part. We observed a similar distribution of Se in plant parts in both control and treated groups, with the highest difference in Se content being in ripe seeds. Concerning to preserving and increasing of the selenium in to pea grains, according to (Smrkolj et al. 2006), the Se content of pea seeds obtained from the untreated (UT group), once (OT) and twice (TT) foliarly treated plants, was, in each case, directly proportional to the number of spraying applications. Selenium-enriched pea seeds are a potential source of dietary selenium, on

account of their ability to accumulate Se, and that this Se is present mainly as SeMet, known to be favourable for human consumption. Increasing of selenium content in peas after biofortification with inorganic form was confirmed by (Poblacionset al. 2013). For each gram of Se fertilization as sodium selenate or sodium selenite, the increase of total Se concentration in the seed was 148 and 19 µg Se / kg dry weight, respectively.

There was found the difference of selenium preserving in pea seeds within the frame of observed varieties. Seeds of var. Ambassador contain more selenium in comparison with the var. Premium, about 19% more after application of 50 g Se / ha and 34% more after application of 100 g Se / ha. This difference is probably related to a shorter growing season of var. Premium (early variety), while var.

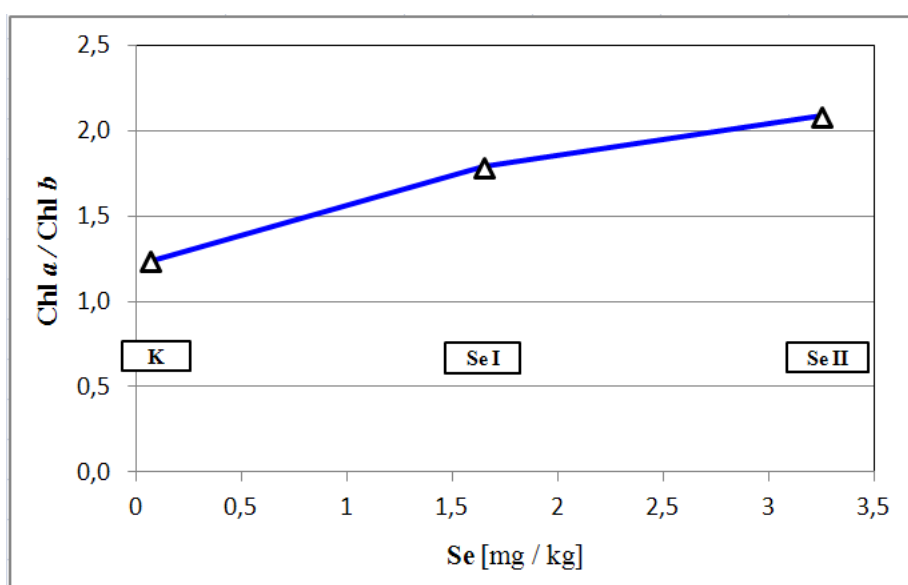


Figure 2 Dependence of chlorophyll *a* - chlorophyll *b* ratio on selenium content in seeds of garden pea, var. Premium.

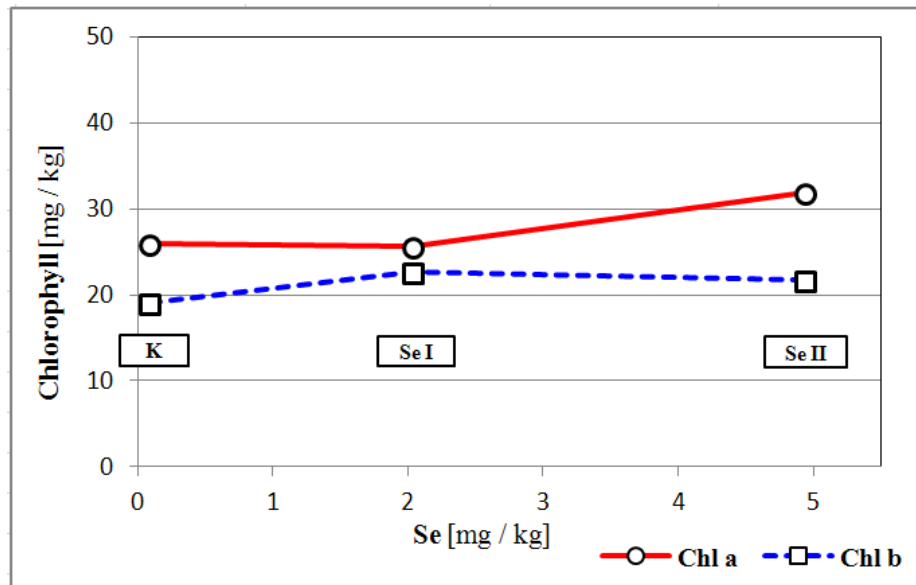


Figure 3 Dependence between contents of chlorophyll *a*, chlorophyll *b* and selenium in seeds of garden pea, var. Ambassador.

Ambassador is medium late variety.

Based on data, where average intake of selenium in Slovakia is 40 µg / person / day (0.04 mg), then after consuming of 100 g of fresh garden peas (fortified at least 50 g Se / ha) the average daily intake of selenium could be covered. Recommended daily intake of selenium for adults is 50 to 70 µg / person / day (0.05 to 0.07 mg) (Vestník MZ SR, 1997). According to analysis results, after the foliar selenium application even in 50 g Se / ha, there is achieved a very significant increase of its total content in seeds, which is very important in term of biological value increasing.

Considering the mentioned deficit of daily intake, there can be said that by the addition of inorganic forms of

selenium in the soil, in the form of selenate, the foodstuffs with high content of selenium can be obtained, in which selenium is bound in organic form in a significant proportion. Inside plants, inorganic selenium is converted to low molecular weight amino acids and finally into selenoproteins. These proteins are responsible for most of the physiological functions mediated by selenium such as antioxidative action, redox regulation, immune function etc. (Priyadarsini et al. 2013).

Protective effects of chlorophyll are related to their ability to modulate the activation of endogenous extraneous detoxification systems and caspase of polymerase pathway, as well as with their antioxidant and antimutagenic properties (Ferruzzi and Blakeslee, 2007).

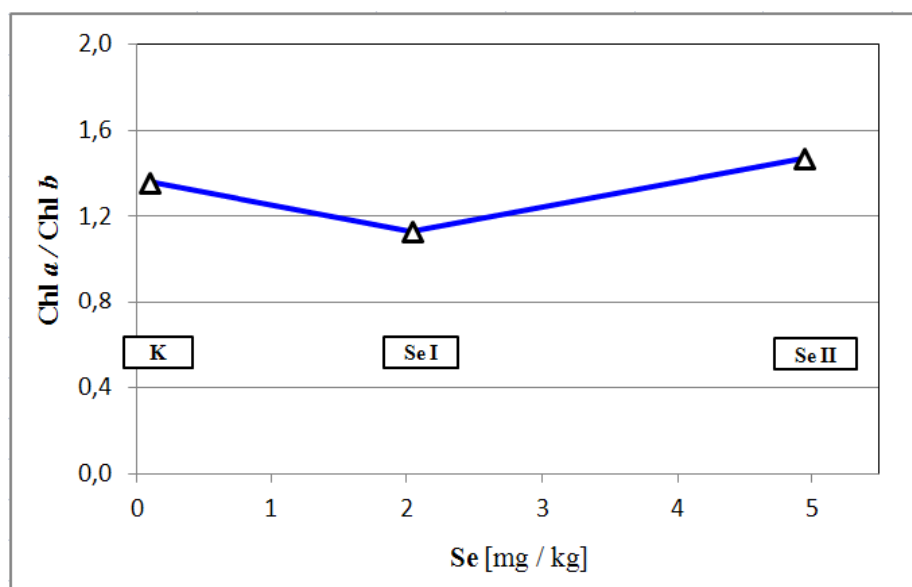


Figure 4 Dependence of chlorophyll *a* - chlorophyll *b* ratio on selenium content in seeds of garden pea.

Chlorophyll content – *chl_a*, *chl_b* was increasing with increased concentration of selenium, or it remained at the level of un-fortificated variants (*chl_a* – Ambassador SeI, *chl_b* – Premium SeII). Statistically significant changes were found after foliar application of selenium in case of var. Premium, where it occurred two - fold (100 g Se / ha) and even three - fold (50 g Se / ha) increase of *chl_a* and two - fold increase of *chl_b* (50 g Se / ha in comparison with control (Table 3). Increasing of the concentration of applied selenium dose from 50 g Se / ha to 100 g Se/ha caused reduction of *chl_a* and *chl_b* content (Figure 1) in case of var. Premium and increasing of the *chl_a/chl_b* ratio (Figure 2). For var. Ambassador it wasn't confirmed the statistical significance in the *chl_a* and *chl_b* content increasing, except of chlorophyll *a* content increasing after the application of 100 g Se / ha *chl_a* in comparison with control (Table 2). Increasing of the concentration of applied Se dose from 50 g Se/ha to 100 g Se/ha caused increasing of *chl_a* content and moderate decreasing of *chl_b* content (Figure 3) and increasing of the *chl_a/chl_b* ratio (Figure 4) in var. Ambassador.

Selenium significantly increased the content of chlorogenic acid, chlorophyll *a*, chlorophyll *b* and carotenoids in leaves of *Lycium chinense* (Dong, 2013).

Increasing of the *chl_a/chl_b* ratio is caused by deceleration or by blocking of the chlorophyll production. Due to stress and aging the chlorophyll content is decreasing and their ratio reaches a value 3, and in the end decomposition of chlorophyll is occurring. From the ratio of *chl_a/chl_b* in our experiments for var. Ambassador there can be said that its value was almost always close to value of the control, which indicates insignificant differences in the reduction of both types of chlorophyll. In case of var. Premium the *chl_a/chl_b* ratio was the most different in case of variant with application of 100 g Se / ha comparing to control, which means that in case of this concentration it may be happened partial reduction of the chlorophylls.

CONCLUSION

Based on the evaluation of the total selenium content in the seeds of two garden pea varieties after the foliar fortification by the solution of sodium selenate there was shown statistically significant increase connected with concentration increasing of applied selenium. Results show that after consuming of 100 g seeds of fresh garden pea (fortified with at least 50 g Se / ha), the average daily intake of selenium may be covered, although there were some differences between the varieties.

The increasing concentrations of selenium resulted in an increase, in some cases statistically significant increase in the content of photosynthetic pigments (*chl_a*, *chl_b*), except of chlorophyll *b* content in case of var. Ambassador. From the *chl_a/chl_b* ratio results that partial reduction of the chlorophylls could be occurred only in var. Premium after 100 g Se / ha application. Foliar biofortification of different vegetable species can provide large-scale intake of minerals with antioxidant properties for human as well as an increase of certain biologically active substances as a result of their synergies.

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THE TABLE EGGS AND THEIR QUALITY IN SMALL-SCALE BREEDING

Mária Angelovičová, Lucia Ševčíková, Marek Angelovič

ABSTRACT

The purpose this study was to investigate quality of the table eggs, their damage and soiling in various age of the laying hens (47-62 weeks) during the second phase of the laying cycle. The object of the research was table eggs, egg white, egg shell, damage and soiling of the egg shell. The eggs were of the final laying hybrid ISA Brown reared in the non-cage system with deep litter and free range at small-scale breeding. In the breeding system with free range at small-scale conditions were secured requirements for laying hens in accordance with welfare principles. The eggs were collected each day at 4:00 pm. Weights of egg and egg shell samples of were measured on scales type KERN 440-35N. A white weight was calculated. Damage and soiling of eggs were investigated under the desk lamp lighting up to 100 W bulb. Statistical evaluation of the results was carried out in the program system SAS. From the existing conclusions of the various scientific and professional published works, it is known that both quality and safety are interrelated. In many works are the risk factors referred together as the quality standards. We found an important fact in assessing the trend of values of the egg shell weight, depending on the age of laying hens. Based on this fact, it can be assumed that the values of egg shell weight were not directly related to egg weight and egg white weight. It follows that the egg shell weight must be assessed comprehensively, and account must be taken of other factors. For the characteristics of the deformed egg shape has been one pc, representing 3.33% of the samples taken for analysis of eggs at 53 weeks of age of the total sampled eggs. In the following 56 weeks of age hens laying eggs there was not a deformed shape. The next subsequent sampling 59 weeks of age laying hens were recorded two pcs of eggs with deformed shape, i.e. 6.67%. At the last sampling of eggs at age 62 weeks, the number of eggs with deformed shape increased to three pcs, representing 10.0% of the sampled eggs for analysis. The sediments on the shell eggs were observed as variable ridge shapes of the shell matter. At the first sampling of eggs at age 47 weeks were found 2 pcs of this defect, representing 6.67% of the sampled eggs. The ridges on the egg shell belong to deviations of the natural shell associated with its texture. These defects of egg shell frequently occur depending on the age of laying hens but can also be caused by other factors. In our experiment was studied mainly the age factor of laying hens. It was interested from aspect of clarifying the relation between ridges on the egg shell and weight of the egg, white, egg, respectively. The results of investigated ridges on the egg shell showed that there occurred in every age of laying hens. Their highest number was recorded at age 59 and 62 weeks (5 pcs, i.e. 16.67% of the 30 egg samples). After 59 weeks of age was found also reduce the weight of the white and shell and at age 62 weeks of age was reduced egg shell weight. Egg production with calcareous sediments on the shell is probably hereditary. Currently, the consumer prefers the consumption of eggs with brown shell. Egg shell color is determined primarily by genetics of laying hens. The results of our experiments have shown that the occurrence of the dots on egg shell had a shade darker brown colour or bright dots, especially on the ends of the egg. The occurrence of dotted eggs was recorded in each assessed age of laying hens (5 – 7 pcs, i.e. 16.67 to 23.33%), while in the last, evaluated 62 weeks of age even up to 9 pcs. These dots and their occurrence are related to the intensity of pigment deposition in the shell cuticle. The process of distribution of pigment in egg shell is the same as is formed by packing egg shell and therefore the cuticle. With the increasing age of laying hens reduces the intensity of pigment deposition in the egg shell and increases of egg surface area, of the egg shell area, to which the pigment is stored. This fact is confirmed by our results especially at week 62 of age, when occurrence of pigment dots was the highest on the shell and also these eggs had the highest average weight.

Keywords: table egg; egg shell; demange; soiling; age of laying hen

INTRODUCTION

Raising awareness of food safety among consumers of the table eggs was observed a change in perception of egg quality through high quality and health-safe, clean egg shell. The shell is a natural food packaging material of egg

contents. Quality and safe egg shell are generally assessed based on physical properties and microbiological integrity. The microorganisms can contaminate the eggs at different stages of their production, processing until preparation and consumption. A brief overview of requirements for table

eggs distributed worldwide is clearly illustrated. According to European regulations eggs, which are intended for the EU market are classified as the eggs of class A or B (**Commission Regulation (EC) No 2295/2003; Council Regulation (EC) No. 1028/2006**) and only graded eggs can be sold for the purpose of direct human consumption or sold at retail (**Council of the European Union, 2006**). Similarly, the US Department of Agriculture (USDA) has approved three levels of these eggs in the basic of internal egg quality, appearance and condition of the shell (**USDA Food Safety and Inspection Service, 2013**). AA grade eggs and A grade eggs are generally intended for sale to the consumer and B grade eggs are usually destined for further processing.

Conventional cages are banned for rearing of laying hens in the European Union since 2012, according to the EU directive 1999/74 (**Council Directive 1999/74/EC on 19 July 1999**).

Since 2012, the laying hens can be kept in the enriched cage systems or non-cage systems which can be applied aviary, litter, respectively. The alternatives to conventional cages were till now more evaluated from a business

perspective, in terms of productivity and welfare of laying hens (**Abrahamsson and Tauson, 1995; Tauson et al., 1999; Tauson, 2002; Wall and Tauson, 2002; Wall et al., 2002; Rodenburg et al., 2005, 2008**).

Furthermore, attention was paid to influence of breeding system of the laying hens on the egg hygiene. A development towards the of farming in the enriched cages and non-cage-system may have consequences for the deterioration of egg hygiene and increase the percentage of the cracked and dirty eggs (**Wall and Tauson, 2002**), bacterial contamination of egg shell, respectively (**De Reu et al., 2005; Mallet et al., 2006**).

The high number of cracked eggs have a negative impact on the efficiency of production of table eggs and breeding economy of the laying hens on the farm. One of the most obvious reasons for egg shell cracking (including thick cracks, hairline cracks and stellar cracks) is mechanical damage (**Awosanya et al., 1998**).

Mechanical damage of the egg shell caused either alone or hens is caused due to poor management practices, such as frequent collection of eggs, excessive handling of eggs, bad shape cages or cage maintenance. Egg shell strength

Table 1 Causes of shell quality problems (**Jacob et al., 2000**).

Condition of egg shell	Possible causes
Odd shaped	Inherited Disease: Newcastle disease, infectious bronchitis, laryngotracheitis, egg drop syndrome 76 Age of hens: incidence is higher in older hens
Thin, porous or shell-less	Inheritance influences porosity and ability to produce strong shells Lack of sufficient calcium, phosphorus, manganese or vitamin D ₃ Vitamin D ₂ mistakenly substituted for D ₃ Excess phosphorus consumption, especially, by older hens Ingestion of sulphanilamide (sulpha drugs) Disease: Newcastle disease, infectious bronchitis, avian influenza, egg drop syndrome 76 Hens exposed to temperature over 85-90°F Age of hens: incidence higher with older hens Premature laying of the egg
Rough or abnormal shell texture	Inherited Newcastle disease, infectious bronchitis Excessive use of antibiotics Excess calcium consumption by the hens Copper deficiency
Mottled shells	Primarily caused by high or low extremes in humidity Inherited Manganese deficiency Artificially induced White strain layers producing tinted
White strain layers producing tinted eggs	Primarily inherited
Yellow shells	Extended use of high levels of certain antibiotics
Tremulous or loose air cells	Newcastle disease Infectious bronchitis Rough handling of eggs Eggs stored large end down
Depigmented brown shell	Infectious bronchitis High stress in the flock Egg Drop Syndrome 76

affects health safety egg packing mass. Eggs with weak shells are more prone to rupture, cracking and subsequent microbial contamination (Yörük et al., 2004).

The purpose this study was to investigate and statistically to evaluate quality of the table eggs, their damage and soiling in various age of the laying hens during the second phase of the laying cycle.

MATERIAL AND METHODOLOGY

The object of research

The objects of the research were table eggs, egg white, egg shell, damage and soiling of the eggs. The eggs were of the final laying hybrid ISA Brown reared in the non-cage system with deep litter and free range at small-scale breeding.

Characteristic of experimental conditions

The laying hens were chosen for the experiment in the non-cage system of free range at small-scale conditions. The experiment was carried out with 36 pcs of laying hens. Sampling of eggs was carried out in the laying hens aged 47 – 62 weeks, every three weeks. The number of samples for the measurement and assessment was 30 pcs of eggs (Table 2).

In the breeding system with free range at small-scale conditions were secured requirements for laying hens in accordance with the Regulation of the Slovak Republic dated 11. 12. 2002 laying down minimum standards for the protection of laying hens and its complement 9. 7. 2003 to low no. 736/2002, Regulation of the Slovak Government of 9. 7. 2003 on the protection of animals kept for farming purposes, as amended low 368/2007 coll. lows and welfare principles applying the five freedoms.

Breeding facilities stall was windowless hall with deep litter (wheat straw). Right from the door on the right side of the hall were six nests. Hen house was constructed from wood Two-storey structures. In each floor there were three nests. In one nest can lay eggs six laying hens. In the middle of the hall are the whole length perches, which are constructed as to Prevent the movement of the hens below. Therefore, under the perches were placed containers. A height of 30 perches was the direction from the top container. The laying hens without restriction went out in the range from hen house. The door of hen house was opened daily at 6:00 am, and closed at 7:00 pm. Feeding and watering of the laying hens were carried out in the hen house and in the free range. The laying hens had unlimited access to feed in tubular feeder and to water in bucket watering place. The laying hens were fed with complete feed mixture, which was complemented in feeders daily. A drinking water was complemented in watering place daily. Feeding and watering equipment was handmade. Eggs were collected by hand and each day at 4:00 pm.

Investigated indicators

- . egg weight,
- . egg white weight,
- . egg shell weight,
- . damage to the eggs by deformation of shape,
- . damage to the eggs by ridges on the shell,
- . damage to the eggs by stained dots on the shell,
- . damage to the by rupture of the shell eggs and cracks
- . soiling of eggs with blood,
- . soiling of eggs with dung.

Working procedures

An egg weight was measured on scales type KERN 440-35N, with an accuracy of 0.01 g and a maximum weight of 400 g.

Sample preparation: The egg was broken, separated the yolk and the white. The yolk placed in pre-weighed watch glass and the egg shell with membranes were washed with tap water and dried in a drying cabinet preheated to 55 °C. Yolk and shell were weighed on scales of type KERN 440-35N, with an accuracy of 0.01 g and a maximum weight of 400 g.

White weight was calculated using the formula:

$$x = \text{egg weight, g} - (\text{weight of egg yolk, g} + \text{weight of egg shell, g})$$

Damage and soiling of eggs were assessed in the laboratory of the Department of Food Hygiene and Safety. Each sample of egg was laid on white paper under the desk lamp lighting up to 100 W bulb. Investigation damage and soiling of eggs was carried out by rotating of egg at first in the equatorial plane and followed assessment of blunt and sharp egg end. There was recorded a number of damaged eggs with deformed shape, the shell ridges, dotted with different shade of colour (deviation) as staining of shell area, and with ruptured shell and the shell cracks. It was recorded also the number of eggs soiled with blood or dung (stains).

Statistical methods

The obtained data were assessed according to basic statistical characteristics \bar{x} = mean, SD = standard deviation and c_v = coefficient of variation). Scheffe's test at the significance level of $\alpha = 0.05$ was used to compare a difference between indicator values in the program system SAS, version 8.2.

RESULTS AND DISCUSSION

A procedure for assessment of quality of table eggs is carried out according to certain methods, which are described and characterized in many scientific and professional works and legislative measures. By making was altered breeding system of laying hens in the European Union according to the principles of welfare, including the Slovak Republic, January 1, 2012, there is

Table 2 Sampling schemes to measure.

Age of birds, week	47	50	53	56	59	62
Number of egg samples, pcs	30	30	30	30	30	30
Number of birds, pcs	36	36	36	36	36	36

not enough unambiguous conclusions of literary knowledge founded on the experimental results. From literary knowledge it is not clearly determined, based on which indicators may be method of assessment of table eggs safety. From the existing conclusions of the various scientific and professional published works, it is known that both quality and safety are interrelated and in many works are the risk factors referred together as the quality standards. Overview of quality standards for table eggs of laying hens already in 2000 published **Jacob et al. (2000)**. These authors included among the qualitative indicators dirty stains on the shell, stuck dirt or contaminants on the shell, egg shape, and shell texture, ridges on the shell and egg shell thickness.

Weight of eggs, white, yolk and shell

Egg weight

European Cmmission (2003), as well as **Council of the European Union (2006)** determined the classification of table eggs intended for marketing in the European Union by legislative measure. These are the eggs of class A or class B. In these legislative measures it is laid down that only graded eggs can be sold for the purpose of direct human consumption or

sold at retail-trade.

In our experiment, we focused on the weight of the eggs, because according to **Butcher and Miles (2003)**, **Rajkumar et al. (2009)** smaller eggs generally have a stronger shell compared to larger eggs. This fact is explained by the fact that the laying hens have a limited capacity to store calcium in shells. The result is the same amount of calcium spread over a smaller area of the smaller eggs and a larger area at a larger egg. Weight of table eggs in the experiment was 63.52 g of laying hens aged 47 weeks, which had an increasing tendency to 53 weeks of age of laying hens. After 56 weeks of age of laying hens decreased an average egg weight of 0.21 g and the end of the reporting period entered into an upward trend. Average egg weight for the whole examined period in our experiment was 64.69 g. A similar egg weight 65.46 g, **Angelovičová and Polačková (2015)** state at Moravia SSL laying hens aged 60 weeks or more 68.98 g is published in work by **Angelovičová et al. (2013)** at ISA Brown laying hens aged 61 weeks. We found an interesting fact about the average egg weight when assessing of trend according to individual weeks of age of laying hens. With increasing age of laying hens was also mild increasing of egg weight, besides the average egg weight at 56 weeks of age, when the trend of increasing values interrupted.

Table 3 Average egg weight according to age of laying hens.

Age of laying hens weeks	n	\bar{x} (g)	SD (g)	c_v (%)
47	30	63.52	±4.41	6.94
50	30	64.14 ^a	±5.59	8.71
53	30	64.71	±3.89	6.01
56	30	64.50 ^b	±4.33	6.71
59	30	65.49	±5.81	8.87
62	30	65.80 ^a	±5.78	8.78
Average egg weight for the entire period of investigation	180	64.69		

Note: n – number of samples, \bar{x} – mean, SD – standard deviation, c_v – coefficient of variation, a, b – value within a column with different superscript letter is significantly different ($p < 0.05$).

Table 4 Average egg white weight according to age of laying hens.

Age of laying hens weeks	n	\bar{x} (g)	SD (g)	c_v (%)
47	30	38.21	±3.42	8.95
50	30	39.15	±4.19	10.70
53	30	39.47	±3.98	10.08
56	30	39.72	±2.93	7.,38
59	30	39.53 ^a	±3.73	9.44
62	30	40.70 ^b	±4.08	10.02
Average egg weight for the entire period of investigation	180	39.46		

Note: n – **number** of samples, \bar{x} – mean, SD – standard deviation, c_v – coefficient of variation, a, b – value within a column with different. superscript letter is significantly different ($p < 0.05$).

The lowest values of variation in egg weight according to the results of the standard deviation was at the laying hens aged 53 weeks (SD = ±3.89 g), which represents a variation of values according to the results of the coefficients of variation $c_v = 6.01\%$. The highest variation of values according to the results of the standard deviation was at the laying hens aged 59 weeks (SD = ±5.81 g), which represents a variation of values according to the results of the coefficients of variation $c_v = 8.87\%$. Statistically significant difference of egg weight ($p < 0.05$) according to specified age of laying hens was found between 50 to 56 weeks and 56 to 62 weeks.

Egg white weight

Abanikannda et al. (2007) state, the egg shell quality directly depends on the egg white quality. This fact explicates that the egg white is situated under egg membranes providing a structure for storing the egg shell. We found a trend of average weight egg white by individual weeks of age of laying hens, which does not correspond to the trend of increasing values of average egg weight. The trend of white weight was interrupted at the laying hens aged 59 weeks. The average weight of the egg white over the entire follow-up period was 39.46 g.

The lowest values of variation in egg white according to the results of the standard deviation was at the laying hens aged 56 weeks (SD = ±2.93 g), which represents a variation of values according to the results of the coefficients of variation $c_v = 7.38\%$. The highest variation of values according to the results of the standard deviation was at the laying hens aged 50 weeks (SD = ±4.19 g), which represents a variation of values according to the results of the coefficients of variation $c_v = 10.70\%$. Statistically significant difference of egg weight ($p < 0.05$) according to specified age of laying hens was found between 59 to 62 weeks.

Egg shell weight

We found an important fact in assessing the trend of values of the egg shell weight, depending on the age of laying hens.

Based on this fact, it can be assumed that the values of egg shell weight were not directly related to egg weight and

egg white weight. It follows that the egg shell weight must be assessed comprehensively, and account must be taken of other factors. The average egg shell weight for the whole period of follow-up was 5.99 g. The lowest values of variation in egg white according to the results of the standard deviation was at the laying hens aged 47 weeks (SD = ±0.47 g), which represents a variation of values according to the results of the coefficients of variation $c_v = 7.64\%$. The highest variation of values according to the results of the standard deviation was at the laying hens aged 62 weeks (SD = ±0.71 g), which represents a variation of values according to the results of the coefficients of variation $c_v = 12.47\%$. Statistically significant difference of egg weight ($p < 0.05$) according to specified age of laying hens was found between 47 to 50 and 59 to 62 weeks.

Damage and soiling of egg shell

In terms of known knowledge about the safety of food of animal origin which published Chukwuka et al. (2011), it is determined specification of products intended for human consumption, which is characterized by criteria that must be fulfilled. According to these authors, great emphasis is placed on the egg shell, which must be for eggs intended for trading visibly clean and must not contain any defects that are visible under lighting. The table eggs must not show signs of embryonic development or decay, and no blood clots. They must not be incubated. They must be processed and stored under conditions which prevent condensation of water on the surface of the egg. From above mentioned conclusions and literary knowledge is evident that a methods and indicators for assessing of the safety of table eggs are different in the published works. In our view, it is difficult to assess the safety of table eggs by risk factors of chemical, biological and physical origin, as these factors capture the essence of assessment also the quality of table eggs, whether it is chemical composition of the egg contents or shell eggs indicators. The table eggs are specific and different from other foods of animal origin. Their edible part is protected by natural packing, egg shell. Based on the known information on the prohibition of using the conventional breeding of laying hens from 2012, many authors researched alternative to conventional cages (Abrahamsson and Tauson, 1995;

Table 5 Average egg shell weight according to age of laying hens.

Age of laying hens weeks	n	\bar{x} (g)	SD (g)	c_v (%)
47	30	6.15 ^a	±0.47	7.64
50	30	6.19 ^b	±0.66	10.66
53	30	5.97	±0.50	8.37
56	30	6.01	±0.56	9.32
59	30	5.91 ^a	±0.69	11.67
62	30	5.72 ^b	±0.71	12.41
Average egg weight for the entire period of investigation	180	5.99		

Note: n – number of samples, \bar{x} – mean, SD – standard deviation, c_v – coefficient of variation, a, b – value within a column compared between 47 to 50 and 59 to 62 weeks with different superscript letter is significantly different ($p < 0.05$).

Tauson et al., 1999; Tauson, 2002; Wall and Tauson, 2002; Wall et al., 2002; Rodenburg et al., 2005, 2008). The essence of research of alternative to conventional breeding had more commercial standpoint in respect of the production and the welfare of laying hens and not the quality or safety of table egg. In the same time horizon, it was published also experimental works. A research bol oriented to comparison of breeding system for laying hens in enriched cages and non-cage system in relation to hygiene, egg safety, respectively. Wall and Tauson (2002), in the same time horizon, it was published also experimental works. A research bol oriented to comparison of breeding system for laying hens in enriched cages and non-cage system in relation to hygiene, egg safety, respectively. Similarly, De Reu et al. (2005), Mallet et al. (2006) warned of possible bacterial contamination egg shells. On the base of conclusions of published work, it can be stated that a research and risk assessment methods for table eggs remain open.

Following the above issue, we focused on research to address the safety of table eggs. We chosed the selection of individual indicators under legislation by the European Union and some published works. Already in 1998 Awosanya et al. reported that the damaged egg shell has a negative effect on the efficiency of the production of table eggs and overall economy of breeding of laying hens on the farm.

From this point of view it is very important attending to damage but also clean egg shell. For example, egg producers Federation of New Zealand assessed table eggs on the adopted Code (EPF and NZFSA, 2002) by 14 potential flaws of egg shell, which are divided into five major categories: flaws of eggs associated with the

integrity of the shell, texture of shell, shape, color and cleanliness.

For the characteristics of the deformed egg shape has been one pc, representing 3.33% of the samples taken for analysis eggs at age 53 weeks of total sampled eggs for analysis. In the following 56 weeks of age there was not a deformed egg shape. The next subsequent sampling 59 weeks of age were recorded two pcs of eggs with deformed shape, i.e. 6.67%. At the last sampling egg at 62 weeks, the number of eggs with deformed shape increased to three pcs, representing 10.0% of total sampled eggs for analysis.

The sediments on the shell eggs were observed as variable ridge shapes of the shell matter. At the first sampling of eggs at age 47 weeks of laying hens were found 2 pcs of this defect, representing 6.67% of total sampled eggs. The ridges on the egg shell belong to deviations of the natural shell associated with its texture. These defects of egg shell frequently occur depending on the age of laying hens but can also be caused by other factors (Coutts and Wilson, 1990).

We focused in our experiment mainly on the age factor of laying hens that was interested from aspect of clarifying the relation between ridges on the egg shell and weight of the egg, white, egg, respectively. The results of investigated ridges on the egg shell showed that there occurred in every age of laying hens. Their highest number was recorded in the age of laying hens 59 and 62 weeks (5 pcs, i.e. 16.67% of the 30 egg samples). After 59 weeks of age of laying hens was found also reduce the weight of the white and shell and in the age 62 weeks of age was reduced egg shell weight.

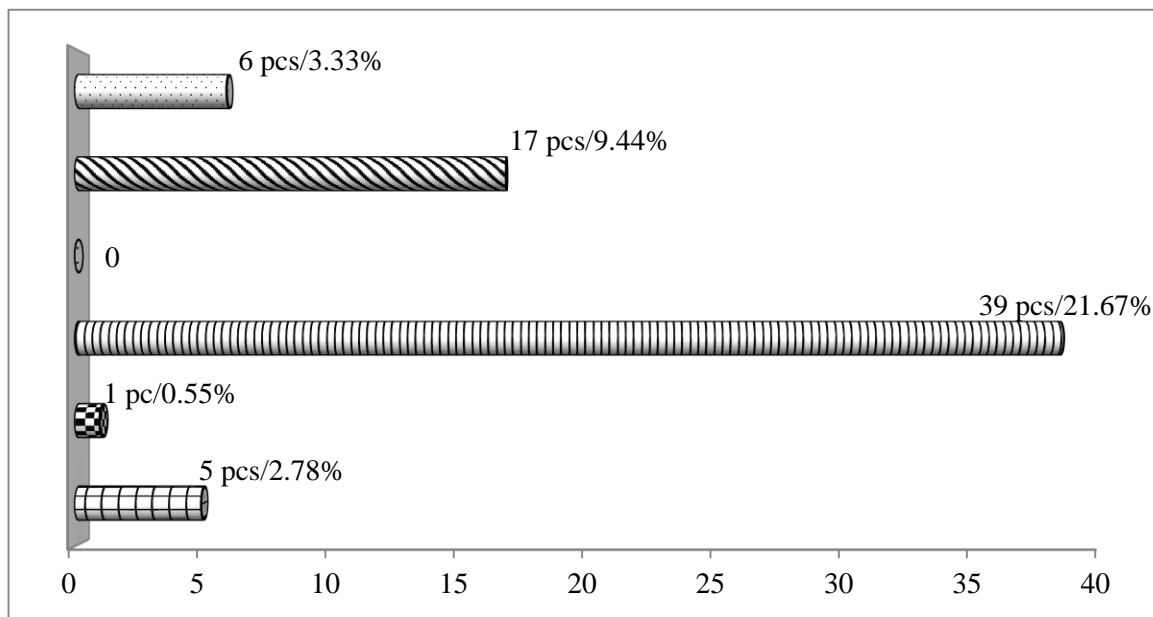


Figure 1 The number and proportion of eggs with dirty and damaged shell for the whole period of the experiment (n = 180). Note: 6 pcs/3.33% – the number and proportion of deformed eggs, 17 pcs/9.44% – the number and proportion of eggs with ridges on the shell, 0 – the number and proportion of eggs with ruptured shell, cracks, 39 pcs/21.67% the number and proportion of eggs with dots of pigments, 1 pc/0.55% – the number and proportion of eggs with blood stains, 5 pcs/2.78% – the number and proportion of eggs with dung stains.

Table 6 The number of eggs with dirty and damaged shell and their proportion of the number of eggs investigated according to age of laying hens (n = 30).

Age of laying hens, weeks	47		50		53		56		59		62	
	pc	%	pc	%	pc	%	pc	%	pc	%	pc	%
Deformed egg shape	0	0	0	0	1	3.33	0	0	2	6.67	3	10.00
Ridges on the egg shell	2	6.67	1	3.33	1	3.33	3	10	5	16.67	5	16.67
Dots of pigments on the egg shell	6	20.00	7	23.33	5	16.67	6	20.00	6	20.00	9	30.00
Ruptured egg shell, cracks	0	0	0	0	0	0	0	0	0	0	0	0
Blood stains	0	0	0	0	0	0	0	0	1	3.33	0	0
Dung stains	3	10.00	0	0	0	0	1	3.33	0	0	1	3.33
Total	11	36.67	8	26.67	7	23.33	10	33.33	14	46.67	18	60.00

Khan et al. (2004) state that egg production with calcareous sediments on the shell is probably hereditary.

Currently, the consumer prefers the consumption of eggs with brown shell. Egg shell color is determined primarily by genetics of laying hens (**Fairfull and Gowe, 1990**). The results of our experiments have shown that the occurrence of the dots on egg shell had a shade darker brown color or bright dots, especially on the ends of the egg. The occurrence of dotted eggs was recorded in each assessed age of laying hens (5 – 7 pcs, i.e. 16.67 to 23.33%), while in the last, evaluated 62 weeks of age laying hens even up to 9 pcs. These dots and their occurrence are related to the intensity of pigment deposition in the shell cuticle (**Fairfull and Gowe, 1990**). The process of distribution of pigment in egg shell is the same as is formed by packing egg shell and therefore the cuticle. **Khan et al. (2004)** state the pigment distribution may not achieve a coherent area of the cuticle, of the entire package surface. Any factor that causes disruption to the process of synthesis and deposition of pigment in the cuticle or the ability of the epithelial cells of the pigment synthesis affects to color of the egg shell. These factors include stress according to **Gerber (2009)**. In our investigations of relations, we eliminated this factor, because for laying hens were secured all the rearing conditions under principles of welfare.

An important factor was in our experiment an age of laying hens, which **Abdullah et al. (2003)** marked also as an important factor. According to them, with the increasing age of laying hens reduces the intensity of pigment deposition in the egg shell. They reason this state that with the age of laying hens is reduced pigment synthesis. But they do not exclude even increasing of egg surface area, of the egg shell area, to which the pigment is stored. This fact is confirmed by our results especially in week 62 of age of laying hens, which was the highest occurrence of pigment dots on the shell and also these eggs had the highest average weight.

The eggs with ruptured shell or with cracks not included for direct consumption. The occurrence of such eggs with damaged shell did not occur between samples of eggs in our experiment.

Blood stains on the shell of assessed eggs which have been the object of our investigation, were rare. We found only one egg sample with soiled shell at laying hens aged 59 weeks. In this age was also found reduced weight of the egg shell and egg white. **Chukwuka et al. (2011)** note the table eggs should be visibly clean. Similarly, **Jacob et al. (2000)** reported that on the shell of table eggs may not be glued extraneous soilings or elements.

An observance of cleanliness and hygiene in rearing of laying hens is the basis of egg production management. An occurrence of egg shell with dung stains in our experiment confirms that this soiling is not connected with age of laying hens. Number 3 eggs instantly at the first sampling of eggs in hens laying hens aged 47 weeks was associated with dung dirty floors. The floor was cleaned once per day. One egg with the dirty egg shell occurred in samples taken from laying hens aged 56 and 62 weeks. According to current knowledge, it is known that hens lay eggs clean. After laying, may be dirty with dung or other impurities (**Sauter and Petersen, 1974; Nascimento and Solomon, 1991**).

The accumulation of persistent toxic substances in the environment by human activity negatively affects the quality and safety of the egg shell. Many researchers focus their tasks of investigation to this aspect. Egg shell a good indicator for research of soiling (**Falk et al., 2006; Castilla et al., 2009a**). Therefore, the egg shell is the center of attention of research teams primarily in terms of its characteristics (**Hunton, 1995; Carnarius et al., 1996; Massaro and Davis, 2004, 2005; Castilla et al., 2007, 2009b**).

On the base of literary knowledge and results of our experiment, we can state that the shell is an important factor of the table egg quality and safety. With regard to the literary sources supported by experiments are poor in unambiguous conclusions, this area remains opened for further research.

CONCLUSION

From the existing conclusions of the various scientific and professional published works, it is known that both quality and safety are interrelated and in many works are

the risk factors referred together as the quality standards. We found an important fact in assessing the trend of values of the egg shell weight, depending on the age of laying hens. Based on this fact, it can be assumed that the values of egg shell weight were not directly related to egg weight and egg white weight. Egg white creating a direct contact with the egg shell, its membranes. In connection with the new welfare conditions of rearing laying hens need to be experimentally verified:

- which indicators should form the basis for assessing the health safety of eggs,
- which should form the basis for relations between the evaluation indicators of technological quality indicators and health safety of eggs,
- or qualitative indicators eggs must be distinguished from safety indicators eggs in relation to consumer health protection.

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OXIDATIVE STABILITY OF CHICKEN THIGH MEAT AFTER TREATMENT OF *ABIES ALBA* ESSENTIAL OIL

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ABSTRACT

In the present work, the effect of the *Abies alba* essential oil in two different concentrations on oxidative stability of chicken thigh muscles during chilled storage was investigated. In the experiment were chickens of hybrid combination Cobb 500 after 42 days of the fattening period slaughtered. All the broiler chickens were fed with the same feed mixtures and were kept under the same conditions. The feed mixtures were produced without any antibiotic preparations and coccidiostatics. After slaughtering was dissection obtained fresh chicken thigh with skin from left half-carcass which were divided into five groups (n = 5): C - control air-packaged group; A1 - vacuum-packaged experimental group; A2 - vacuum-packaged experimental group with ethylenediaminetetraacetic acid (EDTA) solution 1.50% w/w; A3 - vacuum-packaged experimental group with *Abies alba* oil 0.10% v/w and A4 - vacuum-packaged experimental group with *Abies alba* oil 0.20% v/w. The *Abies alba* essential oil was applicate on ground chicken things and immediately after dipping, each sample was packaged using a vacuum packaging machine and storage in refrigerate at 4 ± 0.5 °C. Thiobarbituric acid (TBA) value expressed in number of malondialdehyde was measured in the process of first storage day of 1st, 4th, 8th, 12th and 16th day after slaughtering and expressed on the amount of malondialdehyde (MDA) in 1 kg sample. The treatments of chicken things with *Abies alba* essential oil show statistically significant differences between all testing groups and control group, where higher average value of MDA measured in thigh muscle of broiler chickens was in samples of control group (0.4380 mg.kg⁻¹) compared to experimental groups A1 (0.124 mg.kg⁻¹), A2 (0.086 mg.kg⁻¹), A3 (0.082 mg.kg⁻¹) and A4 (0.077 mg.kg⁻¹) after 16-day of chilled storage. Experiment results show that the treatment of chicken thigh with *Abies alba* essential oil positively influenced on the reduction of oxidative processes in thigh muscles during chilling storage and use of essential oil is one of the options increase shelf life of fresh chicken meat.

Keywords: oxidative stability; chicken meat; essential oil; *Abies alba*

INTRODUCTION

For chicken meat products, freshness, as one of the most important quality attributes, has attracted attention from producers and consumers and has a strong relationship with product sales and consumption (Rzepka et al., 2013).

The production and consumption of chicken meat has become very popular worldwide owing to its desirable nutritional characteristics, such as high protein, low fat and relatively high concentrations of polyunsaturated fatty acids (PUFAs) compared to beef or pork (Brenes and Roura, 2010). However, the higher level of PUFAs in muscle membranes increases the susceptibility of oxidative deterioration of lipid (Engberg et al., 1996), which impairs the organoleptic characteristics and shortens the shelf-life of meat and meat products. Lipid oxidation is a major cause of meat quality deterioration, resulting in rancidity and the formation of undesirable odours and flavours, which lowers the functional, sensory and nutritive values of meat products; and therefore, consumer acceptability (Bou et al., 2004). In meat, lipid peroxidation is initiated by the abstraction of hydrogen radicals from unsaturated fatty acids, induced by light (Boselli et al., 2005), heat, metal ions (Kanner et al., 1988), or other

oxidizing agents. The reaction of oxygen with preformed free radicals results in accelerated lipid peroxidation (Frankel, 1984), which leads to the formation of secondary by-products from PUFA such as MDA and the potential appearance of lower sensory scores.

The antioxidants can be of synthetic or natural origin. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) have been widely used in meat and poultry products (Biswas et al., 2004; Jayathilakan et al., 2007). But the demand for natural antioxidants, especially of plant origin has increased in the recent years due to the growing concern among consumers about these synthetic antioxidants because of their potential toxicological effects (Juntachote et al., 2006; Naveena et al., 2008; Nunez de Gonzalez et al., 2008).

One option for reducing lipid oxidation is the use of various natural plant antioxidants presented in essential oils.

Essential oils (EOs) are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). They can be obtained

by expression, fermentation, enfleurage or extraction but the method of steam distillation is most commonly used for commercial production of EOs (Van de Braak and Leijten, 1999). EOs obtained from various herbs are widely used in cosmetics and food manufacturing and can be used for prolonging the shelf-life of food for their antimicrobial (Skandamis et al., 2002; Mihajilov-Krstev et al., 2009), and antioxidant activities (Burt, 2004; Bobko et al., 2015a, b).

Studies have shown wide effective in spices to retard lipid oxidation in meat products (Juntachote et al., 2006, 2007a, b; Chouliara et al., 2007; Mariutti et al., 2008; Sasse et al., 2009; Lee et al., 2010; Marcinčák et al., 2010; Viuda-Martos et al., 2011; Tkáčová et al., 2015).

Antioxidants can slow the oxidative reactions of both lipids and pigments in meat, and much interest has been focused on natural antioxidants due to negative public perception of synthetic additives (Cuppert et al., 1997). Frankel (1996) commented that the use of natural antioxidants has increased according to a presumption of their safety. Natural plant-derived antioxidants are primarily composed of polyphenolic compounds (Shahidi et al., 1992), and antioxidants containing 2 or more phenolic hydroxyl groups have been found to be effective antioxidants (Dziedzic and Hudson, 1984). Plant phenolics are multi-functional antioxidants that can act as reducing agents (free radical terminators), metal chelators, and singlet O₂ quenchers. Namiki (1990) listed several common natural antioxidants used commercially in food, for example, tocopherols, ascorbic acid, soybean products, oat products, components of crude vegetable oils, amino acids, peptides and proteins, guaiac gum, flavonoids, spices, and herbs.

One possible use EOs from another source e.g. trees from the family *Pinaceae*, which includes many of the well-known conifers of commercial importance such as cedars, firs, hemlocks, larches, pines and spruces. Trees are rich in polyphenolic compounds, and might serve as a source of potentially useful substances, if their antimicrobial properties and safety can be established (Välilmaa et al., 2007). The essential oil showed antioxidative and antibacterial activities (Yang et al., 2009). However, many other fir species have been recognized as rich sources of lignans, flavonoids and other phenols with antioxidant activity (Yang et al., 2008; Li et al., 2011).

The essential oils isolated from *Abies alba* needles are used commercially in the cosmetic and fragrance industries; for example, silver fir needle oil is a component in air fresheners, perfumes, and household products (Góra and Lis, 2012).

Wajs-Bonikowska et al. (2015) stated that in samples *Abies alba* and *Abies koreana* essential oils obtained by hydrodistillation 135 compounds were identified, constituting 98.3 – 99.9% of the total oil compositions.

Zeneli et al. (2001) have been reported that α -pinene, camphene, β -pinene, limonene and bornyl acetate were the major component in the needle oleoresin, and α -pinene, β -pinene, limonene, β -caryophyllene and germacrene D comprised the majority of cortical oleoresin of silver fir in Albania.

Data from recent scientific literature suggest an increased interest in studying the composition of the essential oil isolated from different pine species as well as biological activity. Generally, monoterpenes and sesquiterpenes are dominant components of pine needle essential oils (Menkovic et al., 1993; Roussis et al., 1994; Dormont et al., 1998; Barnola and Cedeno, 2000; Yong-Suk and Dong-Hwa, 2005; Dob et al., 2006; Dob et al., 2007; Nikolic et al., 2007; Oluwadayo et al., 2008). It is important to notice that these essential oils have antimicrobial (Sacchetti et al., 2005; Yong-Suk and Dong-Hwa, 2005; Oluwadayo et al., 2008), antifungal and antioxidant activity (Gülçin et al., 2003; Pinelo et al., 2004; Sacchetti et al., 2005; Guri et al., 2006; Jerez et al., 2007a, b; Limei et al., 2008).

In this study we aimed to investigate the combined effect of ethylenediaminetetraacetate acid (EDTA) and plant essential oil (*Abies alba*) on the oxidative stability of fresh chicken thighs stored under vacuum packaging (VP), at 4 ± 0.5 °C for a period of 16 days.

MATERIAL AND METHODOLOGY

The experiment was implemented in the local poultry station (Hydinaren a.s., Zamostie). The tested were broiler chickens of hybrid combination Cobb 500 both sexes. All the broiler chickens were fed with the same feed mixtures and were kept under the same conditions. The feed mixtures were produced without any antibiotic preparations and coccidiostatics. At the end of the fattening period (42. day) were chickens slaughtered for analysis in laboratory of Slovak University of Agriculture in Nitra. After slaughtering was dissection obtained fresh chicken thighs with skin from left half-carcass, which were divided into five groups (n = 5):

- Air-packaged (C, control group): chicken thigh fresh meat was packaging to polyethylene backs and stored aerobically in refrigerator at 4 ± 0.5 °C for a period of 16 days;
- Vacuum-packaged (VP) (A1, experimental group): chicken thigh fresh meat was packaging to polyethylene backs and stored anaerobically in vacuum and in refrigerator at 4 ± 0.5 °C for a period of 16 days;
- VP with EDTA solution 1.50% w/w (A2, experimental group): chicken thigh fresh meat was treated with EDTA for 1 min and packaging to polyethylene backs and stored anaerobically in vacuum and in refrigerator at 4 ± 0.5 °C for a period of 16 days;
- VP with *Abies alba* oil 0.10% v/w (A3, experimental group): chicken thigh fresh meat was treated with *Abies alba* oil for 1 min and packaging to polyethylene backs and stored anaerobically in vacuum and in refrigerator at 4 ± 0.5 °C for a period of 16 days;
- VP with *Abies alba* oil 0.20% v/w, (A4, experimental group): chicken thigh fresh meat was treated with *Abies alba* oil for 1 min and packaging to polyethylene backs and stored anaerobically in vacuum and in refrigerator at 4 ± 0.5 °C for a period of 16 days.

Immediately after dipping, each sample was packaged using a vacuum packaging machine type VB-6 (RM Gastro, Czech Republic).

Ethylenediaminetetraacetic acid (EDTA) (C₁₀H₁₄N₂O₈.Na₂.2H₂O) was 99.5% purity, analytical

grade, (Invitrogen, USA). A stock solution of 500 mM concentration was prepared by diluting 186.15 g.L⁻¹ distilled water. A final concentration of 50 mM EDTA solution was prepared from the stock solution. The pH of the solution was adjusted to 8.0 with the addition of the appropriate quantity of NaOH solution. The amount of EDTA added to the treat chicken thighs was 0.28 g.kg⁻¹. Essential oil (Calendula, Nova Lubovna, Slovakia) were added to the coated chicken thigh surface (both sides) of each sample using a micropipette so as to achieve a 0.1% and 0.2% v/w final concentration of essential oils.

TBA value expressed in number of malondialdehyde (MDA) was measured in the process of first storage day of 1st, 4th, 8th, 12th and 16th day. TBA number was determined by **Marcinčák et al. (2004)**. Absorbance of samples was measured on UV-VIS spectrophotometer T80 (PG Limeted Instruments, UK) at a wavelength of 532 nm, the translation results on the amount of MDA in 1 kg samples.

Results of the experiment was evaluated with statistical program Statgraphics Plus version 5.1 (AV Trading Umex, Dresden, Germany), were calculated variation-statistical values (mean, standard deviation) and to determine the significant difference between groups was used variance analyse with subsequent Scheffe test.

RESULTS AND DISCUSSION

The results of the oxidation stability of fresh chicken thigh muscles of chicken Cobb 500 after application EDTA and plant essential oil (*Abies alba*) during 16 days storage at 4 ±0.5 °C are shown in Table 1 and Figure 1. Oxidation of lipids can occur in both fresh and cooked meats (**Min and Ahn, 2005; Jo et al., 2006**), and can have significant impact to meat industry.

The higher average value of MDA measured in thigh muscle in 0 day of experiment was in samples of vacuum-packaged chicken thighs group with *Abies alba* oil 0.10% v/w group A3 (0.027 mg.kg⁻¹) compared to experimental

groups A1 (0.021 mg.kg⁻¹), A2 (0.024 mg.kg⁻¹), A4 (0.026 mg.kg⁻¹) and air-packaged control group (0.023 mg.kg⁻¹). We have not found statistically significant differences between testing groups chicken thighs. During chilled storage of the thigh muscles were detected increased content of malondialdehyde in comparison to the first day of storage.

On the fourth day of storage were measured below the values of malondialdehyde in all experimental groups (0.042 mg.kg⁻¹ – groups A3, A4 and 0.055 mg.kg⁻¹ – group A1) opposite control group C (0.071 mg.kg⁻¹). We have found statistically significant differences ($p \leq 0.05$) between control group C and tested groups A2, A3 and A4.

A similar trend of improving the oxidation stability after eight days of refrigerate storage in the thigh muscle of hybrid combination Cobb 500 we found in the experimental groups (0.053 mg.kg⁻¹ – A4 to 0.069 mg.kg⁻¹ – A1) compared with control group C (0.172 mg.kg⁻¹).

After 12 days of thigh muscle storage was statistic significantly ($p \leq 0.05$) improved the oxidative stability of all test groups chicken things (0.066 mg.kg⁻¹ – A4 to 0.081 mg.kg⁻¹ – A1) compared to the control group C (0.246 mg.kg⁻¹). We have found statistically significant differences ($p \leq 0.05$) between control group C and tested groups and between tested group A1 and groups A2, A3 and A4.

During testing period of chilled storage were higher values of malondialdehyde measured in control group C compare to experimental groups. The higher average value of MDA measured in thigh muscle of broiler chickens Cobb 500 was in samples of control group C (0.438 mg.kg⁻¹) compared to experimental groups A1 (0.124 mg.kg⁻¹), A2 (0.086 mg.kg⁻¹), A3 (0.082 mg.kg⁻¹) and A4 (0.077 mg.kg⁻¹) after 16-day of chilled storage. At the end of the test period we have found statistically significant differences between all testing groups and control group of chicken thighs.

Table 1 Effect of *Abies alba* essential oil on the concentration of MDA (mg.kg⁻¹) in thigh muscle (mean ±SD) (n = 5).

Day	C	A1	A2	A3	A4
0	0.023 ±0.007	0.021 ±0.008	0.024 ±0.006	0.027 ±0.005	0.026 ±0.008
4	0.071 ±0.012 ^a	0.055 ±0.008 ^{ac}	0.049 ±0.006 ^{bc}	0.042 ±0.005 ^b	0.042 ±0.011 ^{bc}
8	0.172 ±0.017 ^a	0.069 ±0.005 ^b	0.058 ±0.006 ^c	0.055 ±0.007 ^c	0.053 ±0.010 ^c
12	0.247 ±0.024 ^a	0.081 ±0.008 ^b	0.072 ±0.013 ^b	0.075 ±0.007 ^b	0.066 ±0.016 ^b
16	0.438 ±0.052 ^a	0.124 ±0.020 ^b	0.086 ±0.013 ^c	0.082 ±0.008 ^c	0.077 ±0.008 ^c

Legend: C – air-packaged control group; A1 – vacuum-packaged control group; A2 – vacuum-packaged control samples with EDTA solution 1.50% w/w; A3 – vacuum-packaged experimental group with *Abies alba* oil 0.10% v/w; A4 – vacuum-packaged experimental group with *Abies alba* oil 0.20% v/w. Mean values in the same lines with different superscripts (a, b, c) are significantly different at $p \leq 0.05$ level

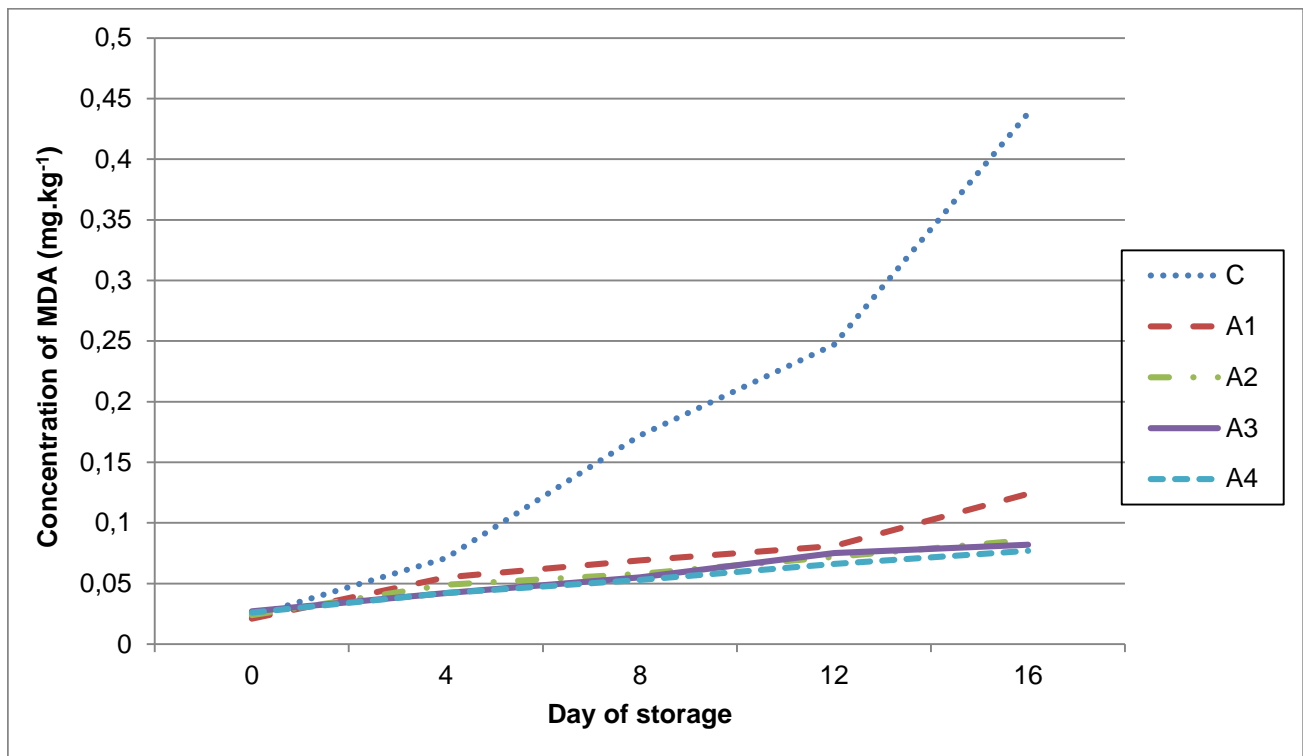


Figure 1 Concentration of MDA (mg.kg⁻¹) in thigh muscle.

Note: C – air-packaged control group; A1 – vacuum-packaged control group; A2 – vacuum-packaged control samples with EDTA solution 1.50% w/w; A3 – vacuum-packaged experimental group with *Abies alba* oil 0.10% v/w; A4 – vacuum-packaged experimental group with *Abies alba* oil 0.20% v/w.

Like plant essential oils such as oregano, thyme, sage etc. (Economou et al., 1991; Yanishlieva and Marinova, 1995; Man and Jaswir, 2000), also *Abies alba* essential oil exhibits substantiating positive effect on oxidation stability of lipids in meat. Botsoglou et al. (2007) reported that a higher concentration of antioxidants in poultry meat has the effect of reducing lipid oxidation, i.e. there is a reduction in malondialdehyde values during chilling storage. Sampaio et al. (2012) examined the effect of combinations of sage, oregano and honey on lipid oxidation in cooked chicken meat (thigh and breast) during refrigeration at 4 ± 0.5 °C for 96 h as measured by TBARS numbers. The analysis of variance on the TBARS data indicated that the TBARS values were significantly affected by natural antioxidants throughout refrigeration ($p < 0.05$). Analysis their data showed that all of the three combinations of natural antioxidants tested would be beneficial for reducing the velocity of lipid oxidation in both chicken meats during storage, what are corroborated by other authors who have added honey and herbs and thereby inhibited the development of lipid oxidation in cooked meats during refrigeration time (McKibben and Engeseth, 2002; Juntachote et al., 2007a).

Overall, we can state that such addition *Abies alba* essential oil as well as the packaging method (system of vacuum packing) improved the oxidative stability of chicken thigh stored cooling at 4 ± 0.5 °C for a period of 16 days.

Due to consumer preferences, chicken dark meat is typically in an overabundance; thus, finding value-added

options for dark chicken meat is of interest to the poultry industry. One reason ground chicken meat shelf life is limited is the rapid loss of fresh appearance.

Meat containing unsaturated fatty acids is very sensitive to lipid oxidation especially during storage, because polyunsaturated fatty acid esters are easily oxidized by molecular oxygen. This kind of oxidation is called autoxidation and proceeds by a free radical chain mechanism (Brewer, 2011).

Gong et al. (2008) used TBARS values as an indicator of secondary lipid oxidation products, which were determined in minced breast and thigh muscles from chicken, turkey and duck during -4 °C storage. TBARS formation was slowest in minced chicken thigh, intermediate in duck thigh and fastest in turkey thigh ($p < 0.01$).

Ramos Avila et al. (2013) stated that the degradation pathways of fatty substances play one of the main causes of foods deterioration and unpleasant odours. This factor is also responsible for the loss of sensory properties.

Rhee et al. (1996) observed that raw poultry meat is less prone to lipid oxidation than beef or pork meat because of its lower iron content.

CONCLUSION

The essential oil from natural sources can be used as alternatives to chemical additives which could extend the meat and meat products shelf life. Results achieved in the experiment show that the treatment of chicken thigh with *Abies alba* essential oil in concentration 0.10% v/w and

0.20% v/w with combination vacuum packaging had positive influence on the reduction of oxidative processes in thigh muscles during chilling storage at 4 ± 0.5 °C in comparative with tested groups - control air-packaged group, vacuum-packaged experimental group and vacuum-packaged experimental group with EDTA solution 1.50% w/w. The use of essential oil is one of the options increase shelf life of meat.

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ANALYSIS OF TEXTUROMETRIC PROPERTIES OF SELECTED TRADITIONAL AND COMMERCIAL SAUSAGES

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ABSTRACT

Food texture is one of the main features that affect the consumer's judgment. Instrumental texture analysis is suitable method for objective assessment of the texturometric characteristic of food. In this experimental work we have analysed textural properties of different traditional and commercial sausages originating from Slovakia. Twenty sausages were classified in four groups. Group 1 (traditional home-made sausages purchased directly from a producer), Group 2 (traditional sausages purchased from butchery), Group 3 (non-traditional sausages purchased from a supermarket) and Group 4 (non-traditional sausages purchased from a hypermarket). Once taken, samples were immediately transported to the laboratory. Samples were analysed immediately and after the storage 72 h at 25 °C and 80% relative humidity. Samples were analysed with texturometer TA-XT2 plus and we have used the Warner-Bratzler probe. The main reason of this experiment was to find differences for two selected textural parameters, firmness and toughness of the fresh and stored sausages. The average firmness and toughness of fresh sausages before storage were 1.83 kg and 12.86 kg.s⁻¹ respectively. These values were increased after the storage. The average firmness and toughness of stored sausages were 2.74 kg and 19.23 kg.s⁻¹ respectively. It means, storage affects the textural properties of sausages ($p < 0.05$). We were observed decrease of the water activity after the storage. The loss of free water was 5.1% higher in the case of commercial sausages. Also, the protein content, fat content and minerals elements content were analysed. The content of overall protein was 5.8% higher in the traditional sausages. The fat content in commercial sausages was 3.36% higher in comparison to traditional sausages. The sensory quality of traditional sausages was better than commercial sausages.

Keywords: traditional sausage; commercial sausage; firmness, toughness; work of shear; texturometer

INTRODUCTION

There are different kinds of meat products all over the world, among which fresh sausages represent an important part (Feiner, 2006). In general, meat products are made from various meat and non-meat components (from different origins and suppliers), which are combined at the formulation stage with respect to criteria of composition, technological factors, sensory characteristics, legal regulations, functionality and production cost (Jiménez-Colmenero et al., 2010). The quality of meat products depends on the raw meat quality, additives, conditions of production, storage temperature and handling conditions (Čurlej et al., 2011; Kunová et al., 2014; Kročko et al., 2014; Sedghi et al., 2014; Mati et al. 2015). Kozelová et al. (2011) investigated consumer's opinion about quality of meat and meat products. Authors found 30% of respondents highlighted the quality as lower and 19% as very low. Consumers identified in many cases as a reason for dissatisfaction textural properties. According to Feiner (2008) fresh sausages (and eventually the raw-semidry ones) are produced from diverse kinds of meat such as beef, pork, mutton, chicken, turkey, etc. and usually pork fat or fatty tissues. Furthermore, various non-meat ingredients (salt, herbs, spices, juices, vinegar, etc.) and additives (nitrites, phosphates, sorbates, etc.) can be added

according to the type of sausage, geographical traditions or manufacturing practices. According to (Lee, 1999) actual making process of fresh sausages includes both traditional and non-traditional methods. Apart from flavour, smell and colour, food must have appropriate textural parameters. Texture is not only a basic objective food property but to some extent it also depends on a person that examines or consumes food. Texture is an important attribute of food quality and it extensively influences an impression from food (Brenner, 2012). In food production process, there are several technological steps like mixing, pumping, kneading and many others. This process may affect the technological and final product quality (Pollar, 2003). Instruments designed for texture analysis can help meat producers with quality of product (Nollet and Toldra, 2008).

In this experiment we aimed at the determination of firmness and toughness of meat sausages originating from Slovakia. Main aim of this experimental study was to compare the traditional and commercial sausages in order to identify the textural differences. We were analysed fat content, protein content and minerals elements content and water activity. Also, we were analysed the changes in water activity in relation to sausages storage in regulated conditions.

MATERIAL AND METHODOLOGY

Samples

Twenty samples of sausages of different origin were divided into four groups and used for comparison of selected textural parameters. Each sample consisted with 5 pieces of sausages. Description of tested groups: Group 1 (traditional sausages purchased directly from producer), Group 2 (traditional sausages purchased from butchery), Group 3 (non-traditional sausages purchased from supermarket) and Group 4 (non-traditional sausages purchased from hypermarket).

Samples preparation

1. Samples were tempered to a room temperature (25 °C),
2. Samples were stored under controlled environmental conditions (stored for 72 h at 25 °C and 80% relative humidity).

Samples analysis

Samples were analysed:

- Immediately and
- After storage under controlled conditions.

Instruments

Determination of the selected textural parameters was performed with the TA XT2 plus texturometer (Stable Micro Systems, Surrey, UK) using the Heavy duty platform / Warner Blatzer set.

Determination of water activity a_w was performed with FA-st lab, (GBX, Lyon, France).

Determination of protein content was performed with Kjeltec 8200 (Foss, Eden Prairie MN, USA).

Determination of fat content was performed with Soxhlet Selecta DET-GRAS N (JP Selecta S.A. Barcelona, Spain).

Determination of Cu, P, Mg, Fe, K, Na, Cu and Zn was performed with AES-ICP (Agilent 5100 ICP-OES, Santa Clara USA).

Instrument setup

Setting of texturometer parameters in the Exponent software 6.1.9.1 (Stable Micro Systems, Surrey, UK) were as follows:

- load capacity 5 kg,
- texturometer arm movement before test 7 mm.s⁻¹,
- probe penetration into a sample 6 mm.s⁻¹,
- probe speed after measurement 10.0 mm.s⁻¹,
- penetration depth of the probe into the sample 30 mm.

Measurement

Analysis of samples was performed: each sausage was sliced into 1 cm wide rings (6 rings per one piece of sausage, total number of pieces per sample was 30), which were placed into the water activity meter and water activity was measured. Consequently, rings of sample were placed into the central position of texturometer base table. Each sample was measured and the mean value was calculated for each selected textural parameters: firmness (maximum peak force in kg) and toughness (peak area - work of shear in kg.s⁻¹).

Protein content was measured according to the STN ISO 937:2001 – Kjeldahl method.

Fat content was measured according to the ČSN ISO 1443: 2002).

The Ca, P, Mg, Fe, K and Na content was measured according to the STN EN ISO 11885, 2009; STN EN 13805, 2015.

The Cu and Zn content was measured according to the STN EN 14082, 2003.

Statistical analysis

Obtained results were evaluated by the Exponent software 6.1.9.1 (Stable Micro Systems, Surrey, UK), its macro function for the obtainment of mean values, standard deviation and coefficient of variation. We used the statistical program Tanagra 1.4 (Lumière University, Lyon, France) according to **Rakotomalala (2005)**. Shapiro-Wilks test was used to test the normality of data. Statistical differences between two groups of sausages (traditional and non-traditional) and two groups of sausages (fresh and stored) in relation to firmness and toughness was evaluated with one-way MANOVA. We were testing the null hypothesis (H_0) for main effects of factor A (traditional sausage) and factor B (non-traditional sausage) and the same for main effects of factor A (fresh sausage) and factor B (stored sausage). Furthermore, tested H_0 for interaction between variables of firmness and toughness ($p < 0.05$). Consequently, we have used the paired Student's t-test for evaluation of differences among obtained values of individual products. Differences between samples were considered as statistically significant at $p < 0.05$. Subsequently, the Principal Components Analysis (PCA) was performed to reducing the original data and show position of products according to the textural parameters firmness and toughness. Also, the Principal Components Analysis (PCA) was performed with the Hierarchical Clustering Procedure (HAC) to show differences between the results of paired samples of fresh and stored sausages in relation to firmness and toughness. Evaluation of the organoleptic characteristics of sausage samples was performed using the Kramer and Friedman test.

RESULTS AND DISCUSSION

In this experiment we were focused on the determination of firmness and toughness of different kind of sausages. Samples were analysed by the TA XT2 plus texturometer and Warner-Bratzler stainless-steel probe.

The PCA analysis of the products according to the firmness and toughness is presented in the Figure 1.

Statistically significant differences were found between ten samples of traditional and ten samples of commercial sausages ($p < 0.05$) and also between commercial and traditional sausages ($p < 0.05$) in measured texture parameters firmness and toughness (according to MANOVA test and paired t-test).

Results of water activity determination are presented in Table 1. The water activity of stored sausages was significantly ($p < 0.05$) lower in comparison with fresh sausages. The lost of water during the storage is affecting the firmness and toughness of both, traditional and commercial sausages.

Firmness and toughness of fresh and stored sausages are shown in Table 2. The PCA analysis of the fresh and stored sausages according to the firmness and toughness is presented in the Figure 2.

When evaluating toughness in within both groups, the highest values were observed in samples of traditional sausages. Specifically, we recorded the highest average value 4.46 kg for the product no. 1. The main reason for the expected higher levels was higher percentage of meat in the analysed samples. The lowest average value was recorded in samples from a group of commercial sausages for the product no. 20 where the average toughness reached 0.69 kg. It can be concluded that the strength of untreated samples of sausages was influenced by their composition and the ratios of various kinds of meat. For soft processed meat products, made by industrial production, in which nearly always other than a relatively small portion of meat, will contain the skin, mechanically separated meat, often soya or other protein substitutes, wheat flour, potato etc. (Pipek et al., 2002). The increase in the strength of meat products is specified by Benito et al. (2005), who detected an increase in strength during the ripening of sausages and found that at the end of maturation, the strength parameter of the sample in comparison to control one increased two times, similar results were observed in our study. From the analyse of

samples stored under modified environmental conditions in thermal chambers (72 hours at 25 °C and 80% RH) is clear, that all treated samples exhibited higher values in comparison to untreated sample. These conditions caused partial dehydration of the samples and thereby increasing their toughness. The highest average value of toughness in has sausage product no. 1 of the group of traditional sausages presented by the value 5.73 kg, generally the highest total values were recorded with traditional sausages. The lowest average value was recorded in samples from a group of commercial sausages for product no. 13 where the value was 1.02 kg. Sausages have been issued to specific conditions in order to develop a model situation that can occur in the case of incorrect storage of the product. The highest average value which determines toughness parameter had fresh product no. 1 with the value 34.89 kg.s⁻¹, after storage under modified terms the product no. 4 was characterized by the value 45.04 kg.s⁻¹. Both samples were from the group of traditional sausages. The lowest average value of toughness parameter had fresh product no. 8 presented by the value 3.91 kg.s⁻¹ from the group of traditional sausages, after treatment by modified conditions measured value was 8.14 kg.s⁻¹ for the product no. 13 from commercial sausages. The values of toughness before and after storage, varied depending on the composition of the sample.

Table 1 Water activity of fresh and stored sausages.

Product no.	Product identification name	a_w Wate activity before storage	a_w Water activity after storage
Product 1	Home produced sausage	0.97	0.78
Product 2	Home produced sausage	0.93	0.79
Product 3	Home produced sausage	0.92	0.76
Product 4	Home produced sausage	0.93	0.69
Product 5	Home produced sausage	0.88	0.72
Product 6	Home produced sausage	1.00	0.85
Product 7	Home sausage	0.95	0.85
Product 8	Hlohovecká sausage	0.99	0.90
Product 9	Mojmírovská sausage	1.00	0.88
Product 10	Trampská sausage	0.99	0.83
Product 11	Vysočánska sausage	0.99	0.75
Product 12	Spišská sausage	1.00	0.78
Product 13	Ipeľská sausage	1.00	0.79
Product 14	Laborecká sausage	0.98	0.78
Product 15	Tesco Gazdovská sausage	1.00	0.86
Product 16	Gazdovská sausage	0.98	0.83
Product 17	Dargovská sausage	0.99	0.79
Product 18	Prešovský kabanos	1.00	0.81
Product 19	Smoked sausage from Berto	0.99	0.78
Product 20	Zipser sausage	1.00	0.81

Note: n = 5 sausages (6 rings per one piece of sausage, total number of analysed pieces per sample was 30).

Table 2 Firmness and toughness of sausages (Part 1).

Product no.	Product group	Product identification name		Firmness before storage (kg)	Toughness before storage (kg.s ⁻¹)	Firmness after storage (kg)	Toughness after storage (kg.s ⁻¹)
Product 1	Group 1	Home produced sausage	Mean	4.395	36.374	5.676	36.544
			SD	0.55	3.76	0.33	6.22
			CV (%)	12.46	10.34	5.85	17.02
Product 2	Group 1	Home produced sausage	Mean	2.377	14.933	3.658	19.638
			SD	0.46	2.81	1.51	6.60
			CV (%)	19.15	18.83	41.16	33.63
Product 3	Group 1	Home produced sausage	Mean	2.632	22.884	5.460	40.072
			SD	0.31	2.35	0.43	2.73
			CV (%)	11.95	10.29	7.96	6.82
Product 4	Group 1	Home produced sausage	Mean	3.049	23.954	5.378	43.188
			SD	0.31	1.78	0.48	3.84
			CV (%)	10.27	7.44	9.00	8.90
Product 5	Group 1	Home produced sausage	Mean	3.337	24.119	4.475	35.701
			SD	0.75	1.58	0.86	5.21
			CV (%)	22.44	6.54	19.18	14.58
Product 6	Group 2	Home produced sausage	Mean	3.143	20.359	2.664	19.264
			SD	0.44	2.13	0.29	2.85
			CV (%)	14.07	10.48	10.97	14.78
Product 7	Group 2	Home sausage	Mean	0.694	4.569	1.478	10.191
			SD	0.24	1.71	0.16	0.84
			CV (%)	35.20	37.50	10.62	8.24
Product 8	Group 2	Hlohovecká sausage	Mean	1.987	9.862	3.127	18.308
			SD	0.66	2.57	0.82	2.39
			CV (%)	33.27	26.04	26.17	13.05
Product 9	Group 2	Mojmírovská sausage	Mean	1.260	6.484	2.776	18.138
			SD	0.21	1.00	0.33	4.64
			CV (%)	16.66	15.49	11.73	25.58
Product 10	Group 2	Trampská sausage	Mean	0.734	5.256	1.247	8.821
			SD	0.15	0.76	0.14	1.38
			CV (%)	20.44	14.42	11.58	15.67
Product 11	Group 3	Vysočánska sausage	Mean	0.887	7.130	1.272	11.295
			SD	0.08	0.78	0.28	1.84
			CV (%)	9.41	10.97	21.81	16.25
Product 12	Group 3	Spišská sausage	Mean	0.991	8.102	1.537	13.620
			SD	0.12	1.23	0.32	3.72
			CV (%)	12.37	15.15	21.06	27.30

Table 2 Firmness and toughness of sausages (Part 2).

Product no.	Product category	Product identification name		Firmness before storage (kg)	Toughness before storage (kg.s ⁻¹)	Firmness after storage (kg)	Toughness after storage (kg.s ⁻¹)
Product 13	Group 3	Ipeľská sausage	Mean	0.995	8.059	1.584	10.529
			SD	0.13	0.78	0.37	1.34
			CV (%)	13.21	9.62	23.08	12.71
Product 14	Group 3	Laborecká sausage	Mean	0.710	5.354	1.401	9.607
			SD	0.12	1.02	0.42	2.17
			CV (%)	17.34	18.98	30.28	22.55
Product 15	Group 3	Tesco Gazdovská sausage	Mean	1.669	12.210	2.667	17.033
			SD	0.16	0.82	0.54	1.58
			CV (%)	9.74	6.70	20.44	9.29
Product 16	Group 4	Gazdovská sausage	Mean	2.798	17.565	3.175	18.322
			SD	0.43	2.14	1.00	5.06
			CV (%)	15.44	12.20	31.56	27.60
Product 17	Group 4	Dargovská sausage	Mean	0.760	6.875	0.983	8.396
			SD	0.10	1.06	0.13	1.17
			CV (%)	13.38	15.38	13.71	13.97
Product 18	Group 4	Prešovský kabanos	Mean	0.898	7.664	1.202	10.496
			SD	0.20	0.91	0.22	2.30
			CV (%)	22.50	11.84	18.05	21.94
Product 19	Group 4	Smoked sausage from Berto	Mean	1.101	7.849	1.770	16.330
			SD	0.23	1.14	0.52	4.80
			CV (%)	21.34	14.50	29.37	29.38
Product 20	Group 4	Zipser sausage	Mean	2.231	7.584	3.228	19.176
			SD	0.74	0.79	0.75	2.81
			CV (%)	33.14	10.47	23.38	14.63

Note: n = 5 sausages (6 rings per one piece of sausage, total number of analysed pieces per sample was 30).

It can be assumed that the value of the toughness can be affected by natural packaging materials used for various sausages. Assignment of water activity (a_w) of each sample was used in order to verify the objectivity of the results obtained by measuring the texture. Water activity was assessed by use of the apparatus FA-st lab. The suitability of measurement of water activity was clearly demonstrated by **Mati et al. (2014)**, who assessed the water activity of dried meat purchased in a commercial network and from the manufacturer immediately after opening during the 24 hrs., 48 hrs., 96 hrs., 168 hrs., during storage in a dark room for 168 hrs. and after storage at a hermetically sealed package in the same time period.

In our study, we assessed the water activity of commercial and traditional sausages at room temperature. Under the same conditions, assessment of water activity

realized for commercial and industrially produced sausages points to a higher free water loss in comparison to traditionally produced sausages.

The percentage decline was in commercial products purchased in the supermarket as follows: product no. 11 → 25%, no. 12 → 22%, n. 13 → 21%, no. 14 → 21%, no. 15 → 14%. For commercial products bought in hypermarket: no. 18 → 20%, no. 20 → 19%, no. 19 → 21%, no. 17 → 20%, no. 16 → 15%. Traditional sausages directly from the producer: product no.1 → 16%, no. 2 → 15%, no. 3 → 18%, no. 4 → 16%, no. 5 → 19%. For products of traditional sausages purchased from the butcher: product no. 6 → 15%, no. 7 → 11%, no. 8 → 10%, n. 9 → 13%, no. 10 → 16%.

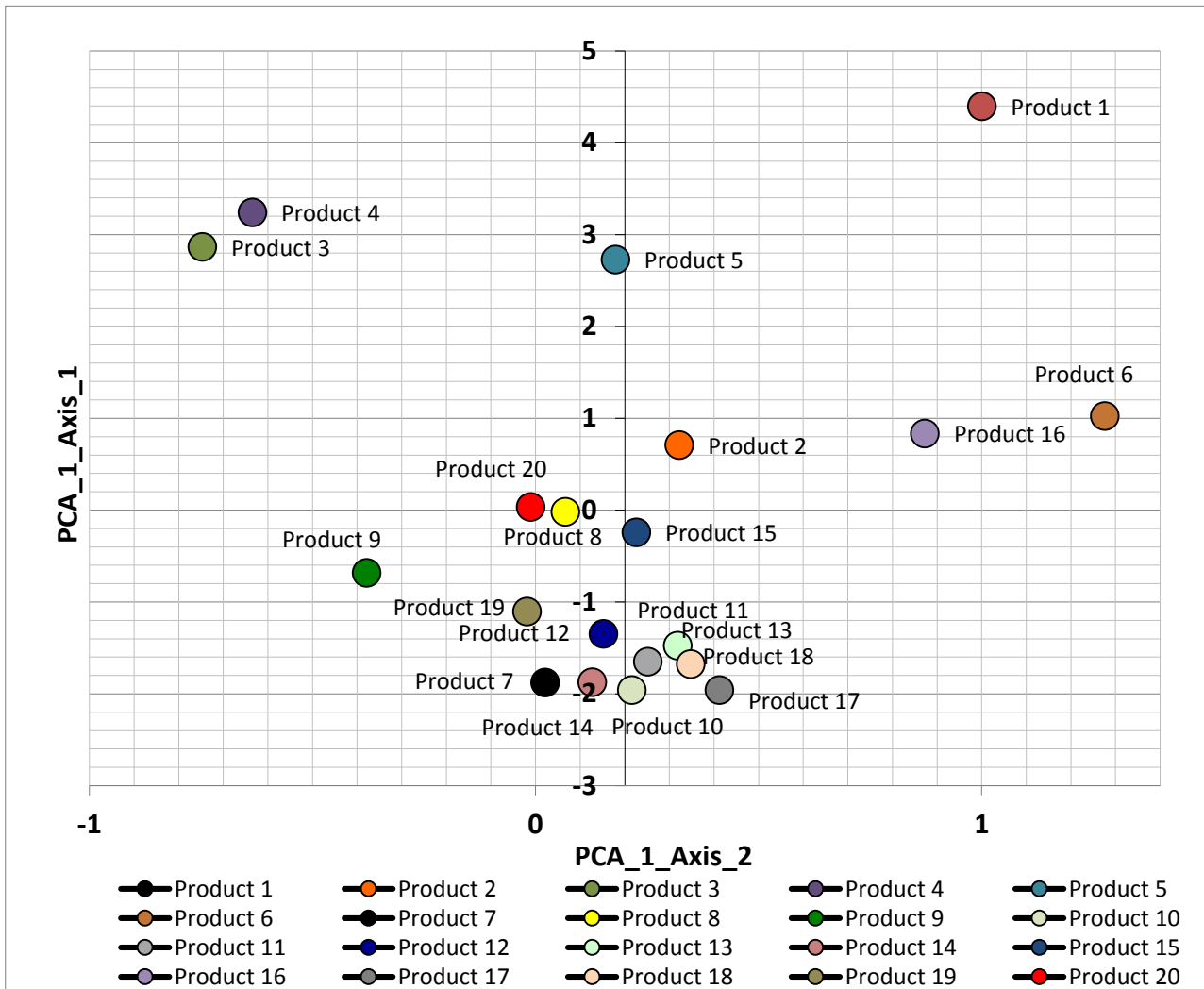


Figure 1 PCA analysis of the products according to the firmness and toughness (PCA_1_Axis_1 and PCA_1_Axis_2 represents the data of firmness and toughness before and after storage).

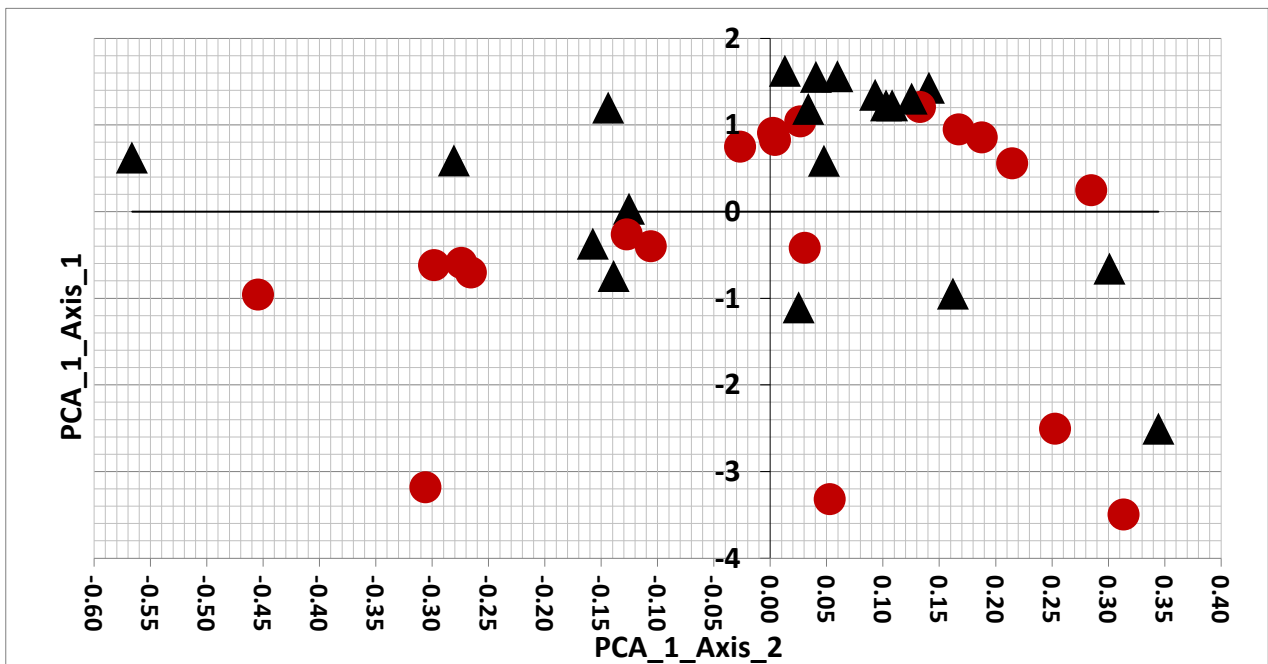


Figure 2 PCA analysis of the fresh (▲) and stored (●) sausages (PCA_1_Axis_1 and PCA_1_Axis_2 represents the data firmness and toughness).

Table 3 The composition of sausages.

Product	Ca (mg.kg ⁻¹)	Fe (mg.kg ⁻¹)	P (mg.kg ⁻¹)	K (mg.kg ⁻¹)	Mg (mg.kg ⁻¹)	Na (mg.kg ⁻¹)	Zn (mg.kg ⁻¹)	Total protein content (%)	Total fat content after hydrolysis (%)
Product 1	132	13.9	2580	4700	221	11160	36.3	29.19	26.30
Product 2	165	11.2	2610	4690	219	11390	38.0	26.30	27.51
Product 3	136	9.7	3030	5400	269	9290	41.6	32.19	23.97
Product 4	160	6.7	2110	3970	203	10550	32.3	24.36	41.22
Product 5	158	10.1	2190	4520	233	10510	22.6	22.55	32.02
Product 6	192	6.4	2340	3100	174	8470	17.2	16.74	22.55
Product 7	212	6.6	2200	3480	193	10020	21.8	17.69	29.66
Product 8	147	11.3	1920	3370	214	9110	27.3	18.07	28.45
Product 9	189	8.5	2420	3030	186	9490	26.4	16.82	21.50
Product 10	207	6.3	2480	2680	163	8220	18.4	14.60	30.33
Product 11	173	3.2	2130	2330	130	9150	17.5	14.72	31.99
Product 12	205	4.6	1840	1800	126	8650	10.7	0.77	31.49
Product 13	149	3.9	1920	3460	188	9800	19.2	19.06	30.49
Product 14	202	6.1	1360	1630	101	8820	11.4	11.94	33.59
Product 15	138	8.4	2020	3380	209	10110	17.0	18.41	31.88
Product 16	264	6.4	2010	1790	109	10030	10.5	11.90	18.12
Product 17	169	6.4	1620	2510	155	8180	15.3	15.30	28.48
Product 18	273	6.2	2090	993	125	10690	11.3	13.08	18.62
Product 19	152	5.1	2180	1330	145	8270	17.6	16.19	30.26
Product 20	430	11.4	2160	1180	145	9450	13.3	14.01	29.12

Note: n = 6

According to **Mati et al. (2014)** samples of commercially produced sausages and purchased in outlets are more stable in comparison with traditional sausages. In contrast, we found the commercially purchased samples of sausages had significantly greater decrease in free water in comparison with traditional sausages.

According to this we can conclude that during the production of sausages are used in small proportion other meat and additives of non-meat origin which ultimately may extend the shelf life of the product but on the other hand may adversely affect the nutritional value of the final product, compared with traditionally-made sausages.

Water activity can vary widely respectively it may be affected by the presence of various soluble substances and their level, such as sugar and salt (**USDA-FSIS 2007**).

The use of different ways for the production of sausages may significantly affect the nutritional composition of the final product. In order to prevent negative impacts on customers, these changes are regulated in many countries by the legislation. For example, in most countries maximum fat content and minimal proportion of lean meat is established. Furthermore, it is generally required minimum content of proteins, but they may be derived from meat or cheaper sources such as wheat gluten and soy

protein (**Freiner, 2008**). In our study it was not possible, to rely on the legal requirements during the analysis of the product due to the fact that the sausages are classified as other meat products for which there are no specified limits of protein and fat. This is also one of the findings of the project confirming observations of the practice that such legislation is not a sufficient protective tool that may control and block the trend of decreasing quality of Slovak soft meat products and is not an effective tool to control it. As **Pipek (1999)** shown in his paper focused to analytics of the meat content of meat products, should always be based on the fact that meat is presented as a muscle consist of approximately 20% protein content, about 70% water content with varying fat content and about 1 – 2% content of extractive substances and minerals. If is during the production of meat characterized by these properties incorporated only technologically requirement water amount (about 10 – 20%) can be expected in meat products 10 – 20% protein content. Collagen and other proteins as a pure meat protein are not considered, so the final value of protein is in that case affected by about 1%. This finding demonstrated the results of the work where the observed variability within each group of sausages (assessed by standard deviation) was high. This could be

attributed mainly to variations in the amount of fats used in the production process of sausages and their degree of drying. Commercial sausages bought at the supermarket reporting higher percentages of fat compared to sausages produced in the traditional way. The commercially purchased sausage exhibited 28.68% average fat content. For traditional sausages an average value was 25.32% for the fat content. On the basis of these findings we can conclude that commercial sausages reached higher fat content (about plus 3.4% more) in comparison to traditional ones. When evaluating the total protein for commercial sausages, the average value reached the level of 14.60%, for traditional sausages it was 20.40%. The proportion of total protein in traditional sausages was increased by 5.8% value. Results coming from comparison of the mineral content, expressed in mg.kg^{-1} based on the total weight of fresh samples shown significant differences between traditional and commercial sausage. As **González-Tenorio et al. (2012)** reported in their study, these differences could be attributed to different ingredients, additives and also dryness of sausage samples. Comparing the results with the above mentioned study carried out by **González-Tenorio et al., (2012)**, who were focused to comparing the content of fat, protein and minerals between home-made and commercial sausages marked as Chorizo from Mexico, similarly than in our study they reached higher level of protein and lower level of fat in traditional sausages and higher values of the fat and lower protein levels in commercial sausage. On the base of these findings they concluded that a higher proportion of the protein in traditional type sausage is related to higher proportion of lean meat in comparison to non-traditional sausages. Lean meat is the main ingredient in the composition of the sausages and it has relatively high protein content, about 65% of dry matter (**USDA, 2010**).

In terms of nutritional value minerals are essential nutrients and our results provides useful information on what customers consume. The content of minerals (Ca, Fe, P, K, Mg, Na, Zn) in sausages is presented in Table 3. We have found different concentration of mineral elements between both categories of sausages. The content of minerals in traditional sausages was: Na 9821 mg.kg^{-1} , K 3894 mg.kg^{-1} , P 2388 mg.kg^{-1} , Ca 169.8 mg.kg^{-1} , Fe 9.07 mg.kg^{-1} , Zn 28.19 mg.kg^{-1} , Mg 143.3 mg.kg^{-1} . The content of minerals in commercial sausages was: Na 9315 mg.kg^{-1} , K $2040.3 \text{ mg.kg}^{-1}$, P 1933 mg.kg^{-1} , Ca 215.5 mg.kg^{-1} , Fe 6.17 mg.kg^{-1} , Zn 14.38 mg.kg^{-1} , Mg 207.5 mg.kg^{-1} .

Traditional sausages contained higher amounts of iron and zinc. In the consumption of meat brings these mineral micronutrients health benefits most significantly (**McAfee et al., 2010**). Higher values of zinc and iron in traditional sausage rather than in non-traditional ones could be explained by the use of higher proportion of lean meat originated from older animals (with higher iron content). **González – Tenorio et al., (2012)** reported high levels of iron in sausages from rural markets. This finding does not only relate to the age of the animals, but also with the possibility of iron ions migration to meat and sausage mixtures from surfaces of cast iron tools, that means from dishes, grinders (**Quitaes et al., 2004**), which are commonly used in domestic production. Despite the small

amount of iron in non-traditional sausage was its concentration in sausages purchased from urban wholesale markets comparable to traditional ones. This may be an indicator of the application of mechanically separated meat containing higher amounts of iron, about two-times higher iron content than handmade deboned meat. The differences between both groups of sausages regarding the content of sodium may be explained by typically used higher amounts of salt under the non-traditional production processes. Similarly, higher sodium content in the traditional type of sausages was also reported by **González-Tenorio et al., (2012)**. A similar trend was observed at concentrations of phosphor. Physiological phosphor is a component of protein structures in animal tissues and its concentration in meat products can be estimated from the protein concentration, this could be the reason of higher values of this substance in traditional sausages. **González-Tenorio et al., (2012)** reported higher phosphor concentration in non-traditional sausages what can be caused by controlled addition of phosphates and soya granulate (non-meat protein ingredients with high phosphor content) commonly used under commercial sausage production. To notice, the maximum phosphates content regulated by the EU is set to 5 g.kg^{-1} (expressed as P_2O_5) in respect to soya granules is the ratio to the protein content higher than those in meat. Calcium concentrations were higher in non-traditional sausages (made for the lowest possible costs). As reported by **González – Tenorio et al., (2012)** elevated concentrations may be associated with the use of mechanically separated meat (mechanically separated meat from the bones) and soy granules. Mechanically separated meat is cheaper than conventional meat and in sausage production is used to reduce the costs. It has higher calcium content than meat deboned by hands, 40 – 500 mg depending on the raw material and used devices (**Newman, 1981; USDA, 2010**). Higher values of potassium in traditional sausages could be due to different feeding of pigs, as it was confirmed by the study of **González – Tenorio et al., (2012)**, as well as authors we also confirmed lower levels of magnesium in traditional sausages, probably due the use of pure muscle without the use of mechanically separated meat. In our study evaluating the organoleptic characteristics of sausage samples using Kramer and Friedman test did not found statistically significant differences between the versions at significance level $\alpha = 0.05$. On the base of the results we can conclude, that different samples are similar from the quality determination. During the first and second measurement of sausages best fits the groups B and C. Group A has lost more points in spot test, compared to other ones. During the first and second measurement of sausages we did not revealed statistically significant differences by the use of non-parametric tests such as Kramer and Friedman test. In Kramer test interval calculated amount is completely covered by tabular interval. As reported **Zajác et al. (2013)**, the main objective of food safety policy of the European Union is to achieve the highest possible level of human health protection and consumers' interests in this field. Therefore, is focused on food safety and appropriate labelling, taking into account the diversity of traditional products while trying to ensure the efficient functioning of the market.

Each handling of food from the producer to the final consumer must be conducted in a hygienic manner to protect the quality and safety.

CONCLUSION

Texture is one of the most important sensory properties of sausages. Consumer rejects product even if it is safe, unless it has got desirable sensory attributes. Assessment of textural properties gives space for their optimization. Obtained knowledge from the texture assessment of traditional and commercial sausages can be useful for producers. Due to the continuous increase in consumers' requirements for food quality, they can more effectively improve the textural properties quality of their products in comparison with traditional homemade sausages. The average firmness and toughness of fresh sausages before storage were 1.83 kg and 12.86 kg.s⁻¹ respectively. These values were increased after the storage. The average firmness and toughness of stored sausages were 2.74 kg and 19.23 kg.s⁻¹ respectively. It means, storage affects the textural properties of sausages ($p < 0,05$). The loss of free water was 5.1 % higher in the case of commercial sausages. The protein content, fat content and minerals elements content was analysed. The content of overall protein was 5.8 % higher in the traditional sausages. The fat content in commercial sausages was 3.36 % higher in comparison to traditional sausages. The sensory quality of traditional sausages was better than commercial sausages. The content of minerals in traditional sausages was: Na 9821 mg.kg⁻¹, K 3894 mg.kg⁻¹, P 2388 mg.kg⁻¹, Ca 169.8 mg.kg⁻¹, Fe 9.07 mg.kg⁻¹, Zn 28.19 mg.kg⁻¹, Mg 143.3 mg.kg⁻¹. The content of minerals in commercial sausages was: Na 9315 mg.kg⁻¹, K 2040.3 mg.kg⁻¹, P 1933 mg.kg⁻¹, Ca 215.5 mg.kg⁻¹, Fe 6.17 mg.kg⁻¹, Zn 14.38 mg.kg⁻¹, Mg 207.5 mg.kg⁻¹.

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VARIETAL DEPENDENCE OF CHEMOPROTECTIVE SUBSTANCES IN FRESH AND FROZEN SPINACH (*SPINACIA OLERACEA* L.)

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ABSTRACT

Spinach (*Spinacia oleracea* L.) is an important source of bioactive compounds. It is commonly consumed fresh or frozen products. Spinach is rich sources of polyphenols, it is a good source of vitamin C and has potential beneficial properties for human health. This study provides some knowledge about content of total polyphenols, and antioxidant activity in selected varieties of fresh and frozen spinach samples. Four spinach cultivars ('Boa', 'Hudson', 'Chica', 'Trombone') were analysed. The content of the total polyphenols (TPC) was determined by the Folin-Ciocalteu reagent (FCR). Antioxidant activity (AA) was measured using a compound DPPH (2,2-diphenyl-1-picrylhydrazyl). The content of total polyphenols in fresh samples of spinach ranged from 975 ± 97.15 mg.kg⁻¹ to 1493 ± 50.42 mg.kg⁻¹ and values of antioxidant activity were in interval from $77.55 \pm 0.34\%$ to $82.57 \pm 0.83\%$. The highest level of TP content in fresh spinach was recorded in variety 'Hudson' (1493 mg.kg⁻¹) and the lowest in variety 'Chica' (975 mg.kg⁻¹). Between these varieties statistically significant difference in the content of total polyphenols was found. The highest value of antioxidant activity in fresh spinach was recorded in variety 'Trombone' (82.57%) and the lowest in variety 'Boa' (78.59%). This difference was also statistically significant. The highest level of TP content in frozen spinach samples was found in variety 'Hudson' (1749 mg.kg⁻¹) and the lowest in variety 'Chica' (855 mg.kg⁻¹). The values of antioxidant activity in frozen spinach samples were in range from $45.86 \pm 7.84\%$ to $79.67 \pm 0.88\%$. The highest value of antioxidant activity in frozen spinach was found in variety 'Hudson' (79.67%) and the lowest in variety 'Chica' (45.86%).

Keywords: spinach; total polyphenols; antioxidant activity; freezing

INTRODUCTION

Spinach (*Spinacia oleracea* L.) belongs to the *Amaranthaceae* family with relatively short growing season. It is native to South-West Asia and cultivated through the world as vegetables (Rao et al., 2015), it is an important dietary vegetable and a common raw material in the food processing industry.

Spinach is not a popular plants in Europe, despite its high content of health-promoting substances. It is the most important leafy vegetable commonly consumed fresh or as canned or frozen products (Koh et al., 2012). It is widely known for their nutraceutical value, but there is relatively little information about its polyphenols content. Polyphenols are divided into two main groups, flavonoids and phenolic acid (Tapiero et al., 2002). Phenolic acids and their derivatives exhibit effects of primary antioxidants. Their activity depends on the number of hydroxyl groups in the molecule (Musilová et al., 2013). It is known that phenolic compounds are involved in maintenance of redox status of cell and its response to cold, UV radiation and the effect of pathogens (Lukaszewicz et al., 2004). Spinach is rich source of polyphenols often associated with beneficial health effects. Their content depends on various factors, such as cultivar or cultivation conditions. These compounds possess an

aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer (Balasundram et al., 2005). Spinach is a good source of carotenoids, flavonoids, vitamin C, vitamin A, vitamin B-6, vitamin K, and minerals such as magnesium, calcium, manganese, potassium (Bacchetti et al., 2015; Rao et al., 2015) and a lot of water. The major carotenoids identified in spinach were lutein, β -carotene, violaxanthin and neoxanthin (Bunea et al., 2008). In green vegetables such as spinach only the green chlorophylls are seen by the consumer because they mask the bright colours of carotenoids (Altemimi et al., 2015). It also contains very good amount of polyphenols. Polyphenols (flavonols and flavones) present in spinach are important components, and their consumption is associated with reduced incidence of civilization diseases (Ergene et al., 2006; Musilová et al., 2013; Altemimi et al., 2015; Kavalcová et al., 2015; Rao et al., 2015). Epidemiological studies suggest that long term consumption of diets rich in plant polyphenols offer protection against development of cancers, osteoporosis, cardiovascular diseases, neurodegenerative diseases and diabetes (Pandey and Rizvi, 2009). The major three flavonols (quercetin, myricetin, kempferol) and flavones (luteolin, apigenin)

were investigated in the fresh spinach leaves (Dehkharghanian et al., 2010). Spinach is ranged between the crops with high antioxidant potential. Fresh spinach leaves contain 1 g.kg⁻¹ of total flavonoids and other phenolic constituents that act as antioxidants due to the free radical scavenging properties of their hydroxyl groups (Lomintski et al., 2003). Spinach it also good source of *p*-coumaric acid contribute significantly to its antioxidant activity (Edenharder et al., 2001). Molecular structure of phenolic substances contributes to the antioxidant activity, but not all polyphenolic compounds are characterized as antioxidants. Antioxidants are important components, which protect against free radicals.

Raw vegetables are subjected to some form of preservation in order to make them available for later consumption. Freezing is considered an effective technique for preserving of total polyphenol content, ascorbic acid and antioxidant activity in all vegetables.

Freezing of fruits and vegetables is generally regarded as superior to other food preservation techniques such as canning and dehydration, with respect to retention in sensory attributes and nutritive properties. Freezing is often employed to maintain fresh-like characteristics with minimal loss of nutrients such as vitamins and antioxidant contents (Prochaska et al., 2000).

The aim of this study was to evaluate the content of total polyphenols and antioxidant activity of chosen varieties of fresh and frozen spinach.

MATERIAL AND METHODOLOGY

Characteristic of varieties

'Boa' is excellent as early spring and also late autumn cultivation. It has a large dark green leaves, suitable for industrial processing but also for fresh market. 'Hudson' is early variety of spinach. The variety has characteristic smooth leaves. 'Hudson' variety tends to run out into flower. 'Chica' is a slow-growing variety. The variety has a bright green smooth leaves. 'Trombone' is characterized by smooth leaves, very profitable for industrial processing.

Chemical and instruments

Total polyphenol content (TPC) and antioxidant activity (AA) were analysed by colorimetric methods (Shimadzu UV/VIS-1240 spectrophotometer; Shimadzu, Kyoto, Japan).

The chemicals used for all analyses were as follows: Folin-Ciocalteu reagent, monohydrate of gallic acid, p.a., anhydrous sodium carbonate, p.a., ethanol, p.a., methanol, p.a., 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, p.a., Trolox (97%).

Plant samples

Four spinach cultivars ('Boa', 'Hudson', 'Chica', 'Trombone') were obtained from a local producer in area Vinica, Slovak Republic. All cultivars were cultivated conventionally under the same condition. Altitude of this area is in the range from 164 to 400 meter above the sea level. Climatic conditions: this area belongs to the warm climatic area, regional middle dry. The annual mean temperature is 9.1 °C, during growing season is 15 °C. Annual mean rainfalls is 640 mm.

Samples of selected varieties of spinach were washed with distilled water several times, gently dried and part immediately stored in plastic bags at -18 °C. Samples of spinach were homogenized (50 g) in 100 mL 80% ethanol 12 h at 250 rpm. Extracts were then filtered through filter paper (130 g.m⁻²; Filtrak Brandt GmbH, Thermalbad Wiesenbad, Germany) and kept at 8 °C for further analysis. The experiment was based on four replications.

Total polyphenol content (TPC) determination

Total polyphenol content was determined by the method of Lachman et al., (2003) and expressed as mg of gallic acid equivalent per kg fresh mater. Gallic acid is usually used as a standard unit for phenolic content because a wide spectrum of phenolic compounds. The Folin-Ciocalteu phenol reagent was added to a volumetric flask containing 100 mL of extracts. The content was mixed and 5 mL of a sodium carbonate solution (20%) was added after 3 min. The volume was adjusted to 50 mL by adding of distilled water. After 2 hours, the samples were centrifuged for 10 min. and the absorbance was measured at 765 nm of wave length against blank. The concentration of polyphenols was calculated from a standard curve plotted with known concentration of gallic acid.

Antioxidant activity (AA) determination

Antioxidant activity was measured by the Brand-Williams et al., (1995) method-using a compound DPPH' (2,2-diphenyl-1-picrylhydrazyl). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was pipetted to cuvette (3.9 mL) then the value of absorbance which corresponded to the initial concentration of DPPH' solution in time A₀ was written. Then 0.1 mL of the followed solution was added and then the dependence A = f (t) was immediately started to measure. The absorbance of 1, 5 and 10 minutes at 515.6 nm in the spectrophotometer Shimadzu UV/VIS – 1240 was mixed and measured. The percentage of inhibition reflects how antioxidant compound are able to remove DPPH' radical at the given time.

Table 1 Total polyphenol content (TPC) in mg GAE.kg⁻¹ and antioxidant activity (AA) in % inhibition.

Variety	Spinach (fresh)		Spinach (frozen)	
	TPC	AA	TPC	AA
Chica	975 ±97.15 ^a	82.30 ±0.66 ^a	855 ±41.66 ^a	45.86 ±7.84 ^a
Trombone	1459 ±51.22 ^b	82.57 ±0.83 ^a	1523 ±82.66 ^b	50.97 ±3.78 ^a
Hudson	1493 ±50.42 ^b	82.02 ±1.16 ^a	1749 ±95.16 ^b	79.67 ±0.88 ^b
Boa	1481 ±20.28 ^b	78.59 ±1.68 ^b	1533 ±98.72 ^c	79.34 ±1.43 ^b

^{a-c} values with different letters mean significant differences (*p* <0.05) among selected varieties, values TPC and AA are expressed as arithmetic mean

Formula: Inhibition (%) = $(A_o - A_t / A_o) \times 100$

Statistical analysis

Results were statistically evaluated by the Analysis of Variance (ANOVA – Multiple Range Tests, Method: 95.0 percent LSD) using statistical software STATGRAPHICS (Centurion XVII, USA).

RESULTS AND DISCUSSION

Spinach (*Spinacia oleracea* L.) contains a range of phytochemicals such as polyphenols (flavonols, flavones), vitamins and minerals, which significantly contribute to their antioxidant activity. Freezing of spinach influenced the content of total polyphenols and antioxidant activity in spinach, however freezing helps to preserve fruit and vegetables through the slowing of senescence. Both studied parameters – the content of total polyphenols and antioxidant activity in selected varieties of fresh and frozen spinach are described in Table 1.

The content of total polyphenols in fresh spinach samples

ranged from $975 \pm 97.15 \text{ mg.kg}^{-1}$ to $1493 \pm 50.42 \text{ mg.kg}^{-1}$. The highest level of total polyphenol content was found in variety of Hudson and statistically significant lowest content of total polyphenols was recorded in variety of Chica. Based on the measured values of total polyphenols content varieties of spinach can be classified as follows: 'Hudson' (1493 mg.kg^{-1}) > 'Boa' (1481 mg.kg^{-1}) > 'Trombone' (1459 mg.kg^{-1}) > 'Chica' (975 mg.kg^{-1}). **Bunea et al., (2008)** determined total polyphenol content in fresh spinach to be $2088 \text{ mg GAE.kg}^{-1}$. **Turkmen et al., (2005)** determined a lower content of polyphenols in fresh spinach, in comparison with our results. Their value was $589 \text{ mg GAE.kg}^{-1}$. **Yosefi et al., (2010)** referred that total polyphenol content in fresh spinach was in interval from 3.19 to $11.03 \text{ mg GAE.g}^{-1}$. **Ko et al., (2014)** determined total polyphenol content in fresh spinach to be $500 \text{ mg GAE.kg}^{-1}$. According **Musilová et al., (2013)** cultivar is one of the most important internal factors affecting polyphenol concentration in the plants.

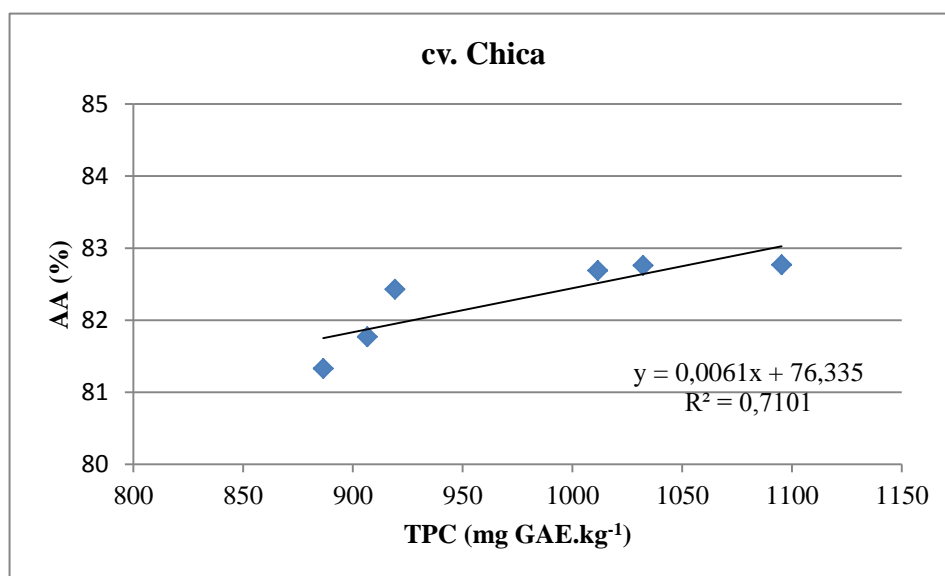


Figure 1 Relationship between TPC and AA in fresh spinach in cv. 'Chica'.

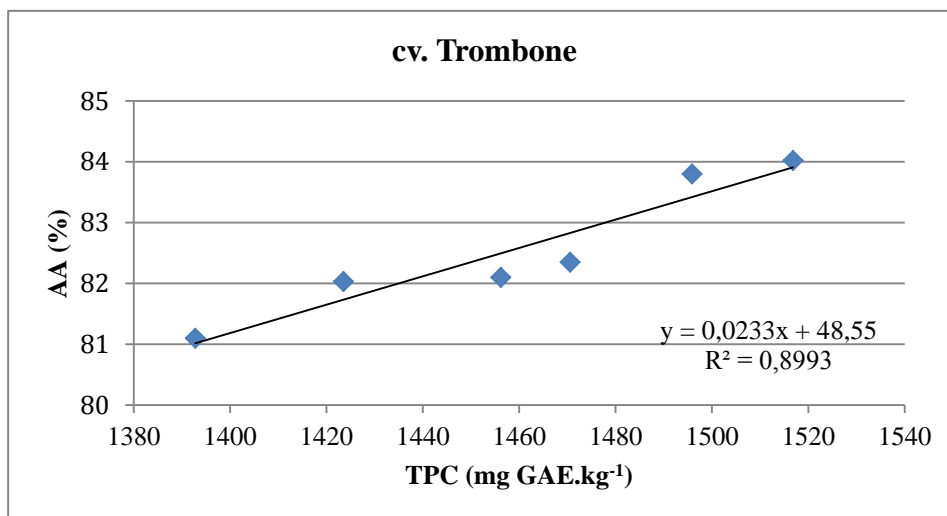


Figure 2 Relationship between TPC and AA in fresh spinach in cv. 'Trombone'.

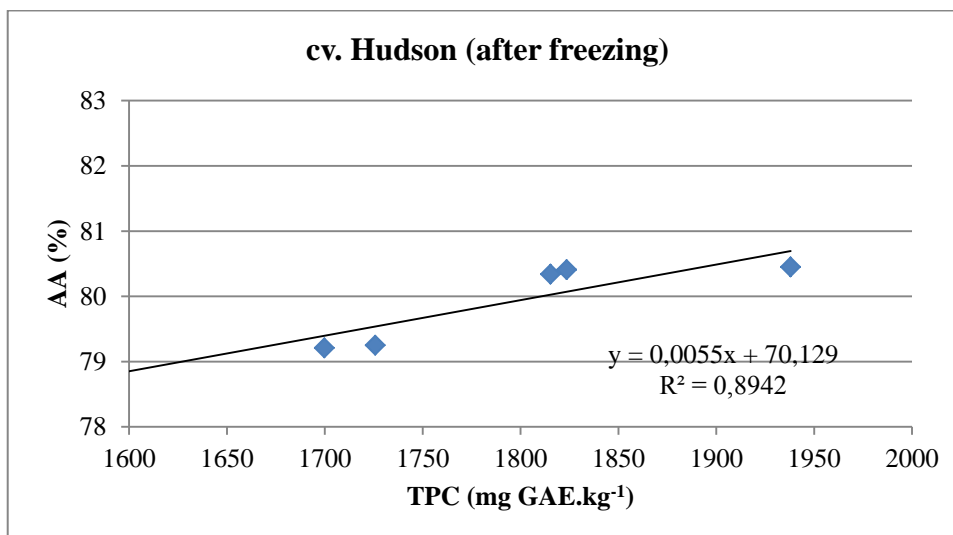


Figure 3 Relationship between TPC and AA in frozen spinach in cv. 'Hudson'.

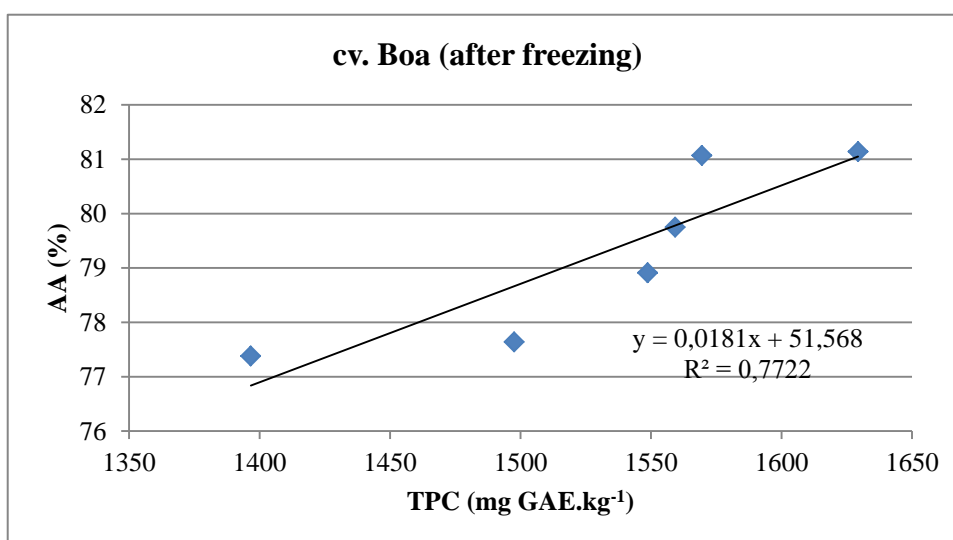


Figure 4 Relationship between TPC and AA in frozen spinach in cv. 'Boa'.

Another indicator that has been evaluated and compared was the value of antioxidant activity in varieties of spinach. The values of antioxidant activity in fresh spinach samples were similar and ranged from $78.59 \pm 1.68\%$ to $82.57 \pm 0.83\%$. Based on the measured values of antioxidant activity in fresh spinach samples cultivars can be classified as follows: 'Trombone' >'Chica' >'Hudson' >'Boa'. Turkmen et al., (2005) found the value of antioxidant activity in spinach in range from 67 to 85%. Statistically significant lowest level of antioxidant activity was recorded in variety 'Boa' (78.59%), on the other hand there was measured the highest level of total polyphenol content. These gained results indicate a fact that phenolic compounds may not be necessarily able to reflect antioxidant activity. Mainly structure of present phenolic substances is very important for antioxidant properties. Zhu et al., (2001) reported that the phenolic compounds with *hydroxy* groups in the position *ortho* (1, 2), have a stronger antioxidant properties than phenolic compounds which have a *hydroxy* groups at other position. Wojdylo et al., (2007) also referred that the differences in the structure

and substitution influenced the phenoxyl radical stability and thereby the antioxidant properties of the phenolic compounds.

In the present work the content of total polyphenols and antioxidant activity in frozen spinach samples after 3 months of freezing was detected. In the frozen spinach samples (except a variety 'Chica') slight increase of total polyphenol content was recorded. The highest level of total polyphenol content in frozen spinach samples was found in variety 'Hudson' (1749 mg.kg^{-1}) and the lowest in variety 'Chica' (855 mg.kg^{-1}). The greatest increase of total polyphenol content after 3 months of freezing was found in variety 'Hudson' (17.15%). The total polyphenol content is changed among other things by processing methods (Hegedúsová et al., 2015). Ganbaatar et al., (2015) also reported a decline of some polyphenols after boiling or after the fermentation. Some researchers reported an increase of total polyphenol content after freezing the spinach (Ligor et al., 2013; Song et al., 2013), whilst others observed a decrease (Bajčan et al., 2013). The antioxidant activity of vegetables is often associated with a

content of β -carotene, L-ascorbic acid and present polyphenolic compounds such as quercetin and rutin (Kavalcová et al., 2014).

The values of antioxidant activity in frozen spinach samples were in range from 45.86 \pm 7.84% to 79.67 \pm 0.88%. Patras et al., (2011) referred that frozen treatments caused a significant decrease in the values of antioxidant activity and ascorbic acid content of selected vegetable samples. In our study in the frozen spinach samples (except a variety 'Boa') decrease of the antioxidant activity level was recorded. The greatest decrease of the antioxidant activity level after 3 months of freezing was found in variety 'Chica' (55.70%). This finding agrees with several results (Hunter and Flechter, 2002; Bajčan et al., 2013), where also a decline in antioxidant activity in frozen spinach was recorded. Gil et al., (1999) also recorded a decrease of the antioxidant activity level in spinach during storage. Ligor et al., (2013) also investigated the antioxidant activity of fresh and frozen spinach and they obtained, besides one sample, higher level in antioxidant activity in frozen spinach than in fresh spinach. On the contrary Ismail et al., (2004) found a significantly higher level of antioxidant activity in fresh spinach (66%).

Between the content of total polyphenols and antioxidant activity in fresh and frozen spinach statistically significant (p -value = 3.10^{-2} , p -value = $3.9.10^{-3}$, p -value = $4.3.10^{-3}$, p -value = 2.10^{-2}) positive correlation was observed (Figure 1 – 4). Several studies have reported a good correlation between TP content of plants extracts and their antioxidant activity (Kiselova et al., 2006; Burin et al., 2010; Saeed et al., 2012).

CONCLUSION

The study indicated that spinach (fresh and frozen) is a potential source of nutritionally valuable components and its consumption improves antioxidant status. The high level of the total polyphenol content in spinach influences the high antioxidant activity. Freezing had a different influence on the levels of total polyphenol content and antioxidant activity in individual spinach samples. Based on the finding it was concluded that total polyphenol content in spinach samples slightly increased (except a variety 'Chica') and the level of antioxidant activity decreased (except a variety 'Boa') after 3 months of storage. As it is well known the level of total polyphenol content and antioxidant activity can be affected by factors as growth conditions, cultivar, the conditions of storage (e.g. temperature, time) and the preparation of the samples. Therefore in future research several more studies should be performed for more exact conclusions about the effect of freezing on the total polyphenol content and antioxidant activity in spinach samples.

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B-CAROTENE CONTENT OF *M. LONGIFOLIA* SEED OIL IN DIFFERENT AGRO-CLIMATIC ZONES IN SRI LANKA, THE EFFECT OF HEAT ON ITS STABILITY AND THE COMPOSITION OF SEED CAKE

Mihiri Munasinghe, Jagath Wansapala

ABSTRACT

M. longifolia is a plant with a seed rich of edible oil (more than 50%), but is still under-utilized for edible purposes in Sri Lankan context. It shows a wide distribution throughout the country representing several agro-climatic zones. No studies have done yet to discover variations of *M. longifolia* seed oil with respect to its different geographical locations. In this study, the content of β -carotene in *M. longifolia* seed oil samples obtained from four different agro-climatic zones in Sri Lanka was evaluated. The effect of heat on the stability of β -carotene in *M. longifolia* seed oil was also studied. Dried, fallen seeds were collected from randomly selected trees in four agro-climatic zones in Sri Lanka named low country dry zone (LD), low country wet zone (LW), low country intermediate zone (LI) and mid country intermediate zone (MI). Oil was extracted with a small scale, mechanical oil expeller (cold pressed method). β -carotene content in four samples was assessed with MPOB test method using Ultraviolet-Visible (UV-VIS) spectrophotometer and with High Performance Liquid Chromatography (HPLC) using Chase et al., (1994) method. A series of heat treatment (50 °C – 300 °C) was given and the content of β -carotene was determined at each temperature with the above mentioned two methods. There were some differences in the content of β -carotene for two types of methods. β -carotene content varies from 17.69 to 13.51 ppm in four agro-climatic zones for HPLC method and 20.46 – 27.69 ppm for spectrophotometric method. The reduction of β -carotene content up to 150 °C from the room temperature (30 °C) was not prominent. But after 150 °C, a sudden, sharp decrease was reported. Nutritional composition of seed cake varied significantly ($p < 0.05$) among the different agro-climatic zones. Protein content, similar to Palm kernel was reported which ranged from 15.44 – 17.76%.

Keywords: *Madhuca longifolia*; β -carotene; heat stability

INTRODUCTION

M. longifolia is a large grown, woody tree of family Sapotaceae and reported to have its origin in Southeast Asia. This plant plays a significant role in both Indian and Sri Lankan traditional ayurvedic medicines. Almost all the parts of the plant including bark, flowers, seeds and leaves are used for that. The strong, hard and durable heartwood of the tree is used for house construction like activities. Flowers are edible and used as a food item of tribal either in raw or cooked forms. They are fermented to produce an alcoholic drink called as Mahua, country liquor. This liquor is used to produce vinegar also.

M. longifolia plant produces fruit which is economically valued for its seed which yield high quantity of fat (ca. 50%), commercially known as mahua butter or mowrah butter, and has many edible and medicinal applications (Singh and Singh, 1991). It is one of the single largest sources of natural hard fat (Bringi, 1987). This fat is used as a substitute for cocoa butter and ghee. Other than that, it has applications in cooking, frying and manufacturing chocolates. The seed fat has emulscent property. Besides edible and medicinal uses, mahua has industrial application as it can be utilized in the manufacture of laundry soaps and lubricants (Parrota, 2001).

The oil yield from the crops is always the key factor to decide its suitability for nutritional and industrial purposes from economic point of view (Yadav et al., 2011). In that

case, *M. longifolia* yields a considerable amount of fat as mentioned previously as well as it has a beneficial fatty acid profile with less saturated fatty acid content. In *M. longifolia* seed fat, 46% of the fatty acids present are saturated, 37.4% are monounsaturated, and 16.5% are polyunsaturated (Ramadan et al., 2006). But the use of this oil for the food industry has been carried out in a limited scale. In Sri Lanka, almost all these edible purposes have limited only for cooking in traditional culture. This also is in very small scale and considers that seeds as an under-utilized seed type for the production of oil. According to the past reports, the under-utility of this fat may probably due to the lack of technical information regarding to the properties and potential uses.

Even though few reports in previous literature highlight several compositional characteristics and thermal properties of *M. longifolia* seed fat, no details can be found with the available pigments in it. The bright yellow color of *M. longifolia* seed fat gives evidences for that it contains considerable amount of pigments, especially β -carotene. Carotenoids are the phytonutrients that impart a distinctive yellow, orange, and red color to various plant parts. Among the carotenoids, β -carotene is important for its associated health benefits more than its color imparts for food stuffs. It is the most potent precursor of vitamin A and is present naturally as a mixture of various isomers (cis and trans) of β -carotene molecule. It has a potent

antioxidant capacity and offers an array of health benefit such as lowering the risk of heart diseases and certain types of cancers, enhancing the immune system, and protection from age-related macular degeneration; the leading cause of irreversible blindness among adults. Consumer attitude towards bioactive compounds, including β -carotene, as natural colorants and for health benefits is promising (Gul et al., 2015). Therefore, identifying novel sources of β -carotene which acts as primary or only source of vitamin A in several countries is important.

After the extraction of oil from oil bearing seeds, a major portion of the raw materials is left over as the oil seed cake. Oil cakes are of two types, edible and non-edible (Ramachandran et al., 2007). Edible oil cakes have a high nutritional value; especially have protein content ranging from 15% to 50% (www.seaofindia.com). Due to their rich protein content, they are used as animal feed, especially for ruminants and fish. Their composition varies depending on their variety, growing condition and extraction methods (Ramachandran et al., 2007). The market value of the cakes is governed by its protein contents and quality of its proteins (Kureel et al., 2009). Therefore, the determination of proximate composition of *M. longifolia* seed cake will be beneficial to identify and enhance its potential uses.

The objective of this study was to determine the β -carotene content in *M. longifolia* seed oil grown in different agro-climatic zones in Sri Lanka and to assess the stability of β -carotene prior to any heat treatment and after a series of heat treatments. Other than that, the proximate composition of seed cake was also analyzed based on their different growing locations.

MATERIAL AND METHODOLOGY

Seeds Sample collection

The dried, fallen fruit seeds of *M. longifolia* were collected from four different agro-climatic zones in Sri Lanka during August-December 2014 using the random sampling method. Even though there are seven agro-climatic zones in Sri Lanka, these four were selected for the study based on the availability of plant. The four agro-climatic zones were low country dry zone (LD), low country wet zone (LW), low country intermediate zone (LI) and mid country intermediate zone (MI). The collected samples were named with symbols as LD, LW, LI and MI with respect to their agro-climatic zone. The species and the variety of the plant were confirmed by comparing the morphological characteristics.

Oil extraction

The seed coat was broken by hand and the kernels were removed out. Thereafter, kernels were ground to obtain a power using a domestic grinder. That powdered kernel was put into a small scale mechanical oil expeller and the oil was extracted (cold pressed method). The extracted oil was covered with Black papers and stored in a refrigerator (4 °C) for the determination of β -carotene content.

Heat treatment

The oil stored at refrigerator was taken out and allowed to come to the room temperature (30 °C). Then about

2 – 3 g of oil at room temperature was kept as it is to determine the β -carotene content at room temperature. All four samples at room temperature obtained from different agro-climatic zones were used to determine and compare the β -carotene content. After that, only the oil sample obtained from low country dry zone (LD) was heated to 50 °C, 70 °C, 100 °C, 150 °C, 200 °C, 250 °C and 300 °C to determine the stability of β -carotene. At each temperature, 2 – 3 g of oil was taken and stored for the determination of β -carotene content at those temperatures.

Determination of β -carotene content

Spectrophotometric determination was done by Ultraviolet-Visible (UV-VIS) spectrophotometer (UV mini 1240, SHIMADZU, Japan) at 450 nm using MPOB test method.

The sample was homogenized and weighed to the nearest ± 0.0001 g into a 25 mL volumetric flask. The sample was dissolved with *n*-hexane and diluted to the mark. The solution was transferred into a 1 cm quartz cuvette and the absorbance was measured at 450 nm against *n*-Hexane. The carotene content was calculated as β -carotene in parts per million (ppm).

The calculation was as follows:

$$\beta\text{-carotene content (ppm)} = [V \times 383 \times (A_s - A_b)] / (100 \times W)$$

Where:

V = the volume used for analysis

383 = the extinction coefficient for carotenoids

A_s = the absorbance of the sample

A_b = the cuvette error

W = the weight of the sample in g

HPLC analysis was performed according to the method of Chase et al., (1994).

About 1 g of sample was measured to a 25 mL volumetric flask with the 0.0001g accuracy. The content of the flask was filled with *n*-Hexane, mixed until dissolved and left for 12 hours at dark. Thereafter, it was centrifuged at 500 rpm in a laboratory centrifuge. A standard β -carotene sample was purchased from Merck and solution of β -carotene standard (5 mg/100 mL) was freshly prepared in *n*-Hexane and stored in the dark. This solution was further diluted with *n*-Hexane to the final concentration of 1 μ g/mL. HPLC determination was performed with HPLC, Agilent technologies, Column = 4.6 x 250 mm Eclipse Plus C₁₈ (5 μ m), injector volume = 50 μ L, Mobile phase = Acetonitrile: Ethyl Acetate: Methanol, 70:20:10, Flow rate = 1 mL/min, open temperature = 30 °C, UV-VIS detector, λ = 450 nm. Peaks were identified by comparing the retention times for the samples of oil and standard solution of known concentration of β -carotene. The quantity of β -carotene was expressed as parts per million (ppm) by comparing the area of peaks after separation of the β -carotene standard and oil samples. All samples were prepared in duplicate and the analysis at each temperature was done twice.

Determination of proximate composition

Powdred *M. Longifolia* seed kernel was obtained by grinding the kernel. Moisture content was determined according to A. O. A. C. method 925.13. Ash content was determined with A. O. A. C. method 923.03 - direct method. Protein and Fibre contents were analyzed with A. O. A. C. official method 984.13 and A. O. A. C. method 1985.

RESULTS AND DISCUSSION

β-carotene content and its stability

Table 1 indicates the β-carotene content (ppm) of *M. longifolia* seed oil (determined by two methods) obtained from four different agro-climatic zones in Sri Lanka. According to the results, β-carotene content varies from 13.51 to 17.69 ppm in four agro-climatic zones for HPLC method. The highest β-carotene content has reported in the sample obtained from low country dry zone (LD). The second highest content was there in the mid country intermediate zone (MI) sample. Under the spectrophotometric method, the results were somewhat higher than the HPLC. Here also, the sequence of β-carotene content from highest to least is similar to the

results of HPLC method. The β-carotene content was ranged from 20.46 – 27.69 ppm by spectrophotometric method. Fig. 1 compares the value determined by two different methods. The differences in the sensitivity of the two methods may be the reason for the variations obtained under two methods in determining β-carotene content.

According to the previous studies, the quantity of β-carotene in other vegetable oils determined by spectrophotometric method is 542.09 ppm for Red palm olein, 0.00 ppm for palm olein, 0.91 ppm for corn oil and 0.00 ppm for coconut oil (Daugan *et al.*, 2011). The literature regarding the other vegetable oil types is difficult to find. Above results shows that except Red palm olein, other oil types are lack of β-carotene. However since these oils were commercially available oils, it is difficult to get an idea about the naturally available content of β-carotene. During processing, a significant amount can be lost because the stability of β-carotene depends on light, heat etc. like external factors. When compared with above oils, β-carotene content in *M. longifolia* seed oil is considerable (20.46 – 27.69 ppm, determined by spectrophotometric method).

Table 1 β-carotene content (ppm) determined by two different methods at room temperature (30 °C)

Sample code	β-carotene content (ppm, HPLC method)	β-carotene content (ppm, spectrophotometric method)
LD	17.69 ±0.23	27.69 ±0.00
LW	13.51 ±0.31	20.46 ±0.32
LI	14.32 ±0.05	22.67 ±0.10
MI	17.52 ±0.11	27.42 ±0.00

Note: Results are Mean ±Standard Deviation (n=3).

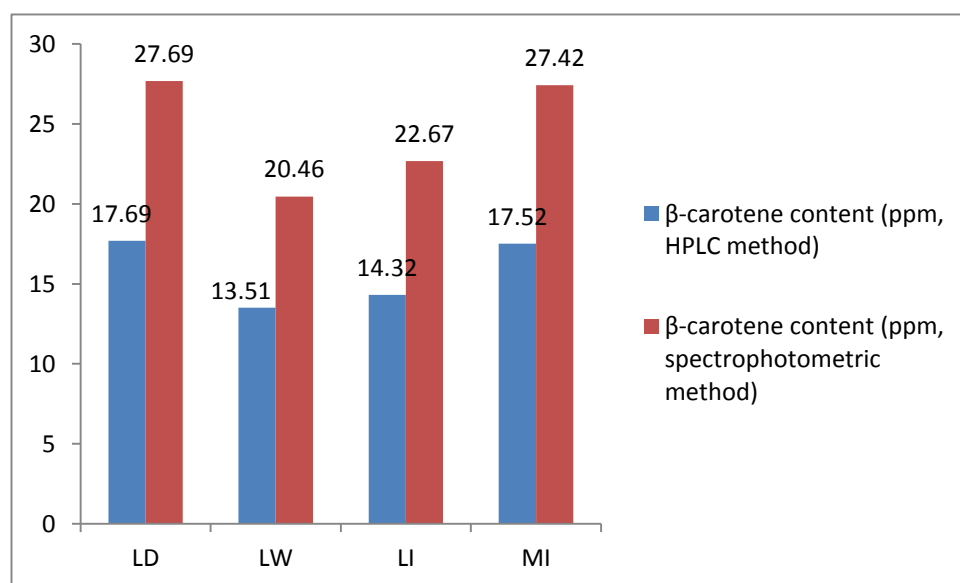


Figure 1 β-carotene content (ppm) of *M. longifolia* seed oil in four agro-climatic zones.

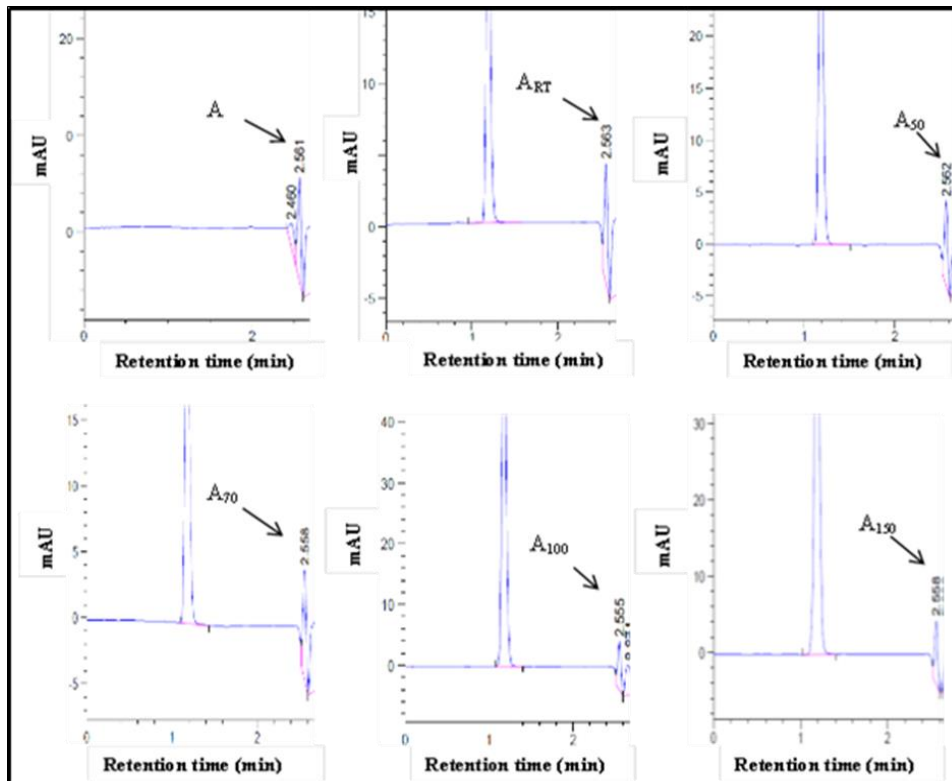


Figure 2 Peaks for β -carotene in HPLC chromatogram for standard (A), at room Temperature (A_{RT}), at 50 °C (A_{50}), at 70 °C (A_{70}), at 100 °C (A_{100}) and at 150 °C (A_{150}).

Table 2 β -carotene content (ppm) determined by two different methods at different temperatures.

Temperature (° C)	β -carotene content (ppm, HPLC method)	β -carotene content (ppm, spectrophotometric method)
50	17.70 \pm 0.00	26.53 \pm 0.00
70	17.48 \pm 0.20	25.64 \pm 0.00
100	17.47 \pm 0.14	22.82 \pm 0.01
150	17.25 \pm 0.27	21.31 \pm 0.32
200	0.00	9.41 \pm 0.19
250	0.00	4.13 \pm 0.00
300	0.00	1.45 \pm 0.00

Note: Results are Mean \pm Standard Deviation (n=3).

The oil sample obtained from low country dry zone (LD) was used to study the effect of heat treatment on β -carotene content. Here, a prolonged heat treatment was not given and only the stability of β -carotene at higher temperatures was evaluated. Fig. 2 illustrates how the peaks in HPLC chromatogram were obtained for the standard sample of β -carotene and for the other samples of *M. longifolia* seed oil which were subjected to the heat treatment. According to the results of Table 2 for HPLC method, the reduction of β -carotene content up to 150° C from the room temperature was not prominent as it has decreased from 17.70 ppm to 17.25 \pm 0.27 ppm only. But after 150 °C, a sudden, sharp decrease (almost zero value) was observed as shown in Fig.3. This pattern of behavior was common for the spectrophotometric method also. By the way, even after 150° C also, small quantity of β -carotene was detected by spectrophotometric method in contrast to HPLC method.

In many parts of the less industrialized world, vitamin A deficiency is a problem that affects nutritional status and health. In several countries, provitamin A carotenoids are

the primary, if not the only, source of vitamin A (Oliveira *et al.*, 1998). For humans, β -carotene of vegetables is considered as an important source of vitamin A. Among the vegetable sources, vegetable oils is a suitable carrier of β -carotene since it is a daily fat source, high in energy, polyunsaturated fatty acids, and naturally occurring antioxidant vitamin E. Therefore, vegetable oils like *M. longifolia* seed oil which is rich with β -carotene would be beneficial in human nutrition since it is stable even at higher temperatures like 150 °C.

Proximate composition of seed cake

When consider about the parameters mentioned in Table 3, significant differences ($p < 0.05$) were there in all the analyzed parameters for different agro-climatic zones. Moisture content was ranged from 9.5 – 10.86%, protein from 15.44 – 17.76%, ash from 1.62 – 2.44% and fibre from 28.03 – 30.59%. In oil seeds, protein content is the most valuable parameter which determines its potential applications. The content of protein of widely available oil seeds are as follows.

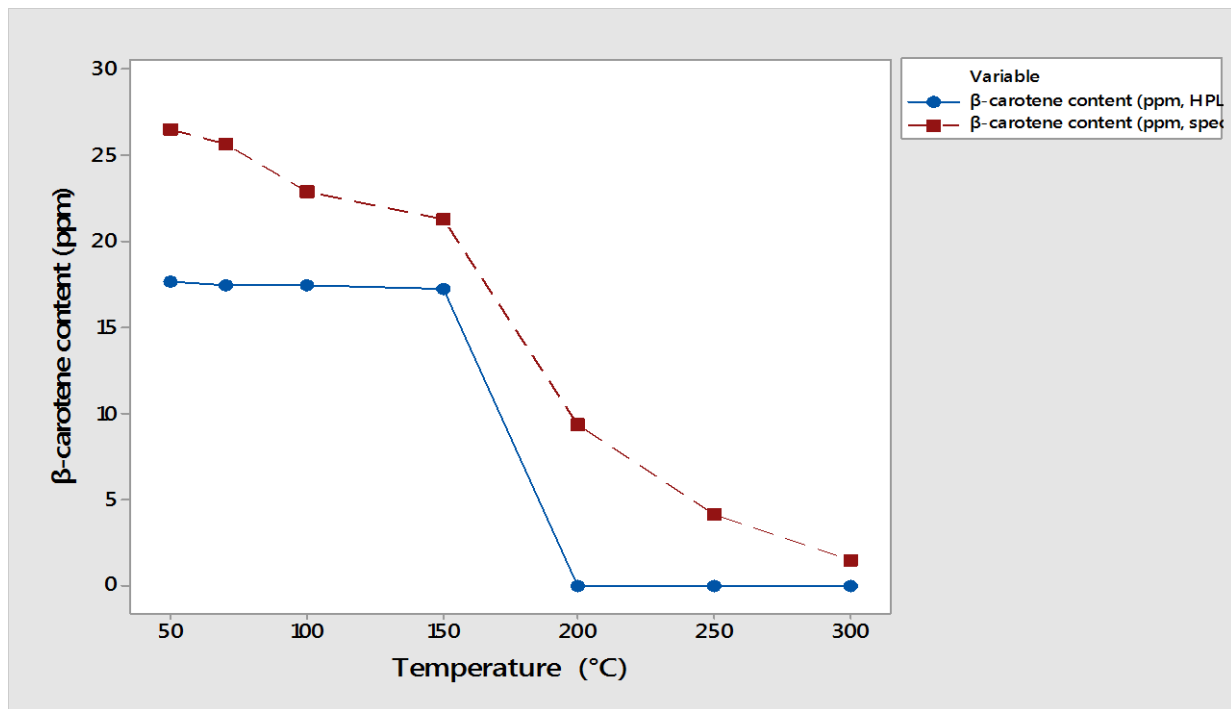


Figure 3 Stability of β-carotene at higher temperatures in *M. longifolia* seed oil.

Table 3 Proximate composition of seed cake in different agro-climatic zones

Parameter	LD	LW	LI	MI
Moisture (%)	9.52 ±0.46 ^c	10.09 ±0.21 ^b	10.29 ±0.25 ^b	10.86 ±0.32 ^a
Protein (%)	15.44 ±0.43 ^b	15.72 ±0.34 ^b	15.87 ±0.60 ^b	17.76 ±0.46 ^a
Ash (%)	2.06 ±0.10 ^b	2.44 ±0.22 ^a	1.67 ±0.05 ^c	1.62 ±0.03 ^c
Fibre (%)	30.09 ±0.75 ^a	28.03 ±0.51 ^b	28.09 ±0.33 ^b	30.59 ±0.38 ^a

Note: Results are Mean ±Standard Deviation (n=3),

Means that do not share a letter are significantly different at significant level 0.05 (Fisher LSD test).

For Coconut, Cotton seed, Ground nut, Olive, Palm kernel, Soy bean and Sunflower, values are 25.2%, 40.3%, 49.5%, 6.3%, 18.6%, 47.5% and 34.1%. With them, *M. longifolia* seed cake has a value for protein similar to Palm kernel (18.6%). Location wise differences showed that, samples from mid country intermediate zone has the highest protein content (17.76%) compared to other three zones. Fibre values for above mentioned seed cakes are as 10.8%, 15.7%, 5.3%, 40%, 37%, 5.1%, and 13.1% for Coconut, Cotton seed, Ground nut, Olive, Palm kernel, Soy bean and Sunflower respectively. Fibre value of *M. longifolia* seed cake is also (28.03 – 30.59) somewhat closer to that of Palm kernel (37%).

CONCLUSION

β-carotene content in four agro-climatic zones varies from 13.51 to 17.69 ppm by HPLC method and 20.46 – 27.69 ppm by spectrophotometric method. The loss of β-carotene is not prominent up to 150° C and thereafter, a sharp decrease can be seen. When compared with the commercially available other vegetable oils, *M. longifolia* seed oil is rich with β-carotene. *M. longifolia* seed cake is composed of a considerable amount of protein comparable to other commonly available oil seed cakes and therefore it can be utilized as a source of protein in food applications.

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TOTAL POLYPHENOLS CONTENT IN FRUITS OF SELECTED CULTIVARS OF STRAWBERRIES IN RELATION TO CONCENTRATIONS OF CADMIUM AND LEAD IN SOIL

Pavol Trebichalský, Juliana Molnárová, Daniel Bajčan, Mária Timoracká, Janette Musilová, Ľuboš Harangozo

ABSTRACT

The key aim of our study was to define the correlation of total polyphenols contents in seven cultivars of strawberries (Clery, Sonata, Alba, Korona, Ázia juh, Anthea, Joly) grown in the parcels with exceeded levels of pseudo-total Cd and mobile Pb in soil. Strawberries were grown in rows long 200 m, the distance of the plants was 0.35 m and inter-row distance was 1 m. Following fertilizing was applied - at the beginning of vegetation period the doses of elements (in kg.ha⁻¹) were: N_{48.2} +P_{78.3} +K_{66.3} +Mg_{7.2}, afterwards 23 days of elements were applied in liquid irrigation in amount: N_{46.5} +P_{34.8} +K_{118.7}. Heavy metals in contamination soil were analysed by the method of atomic absorption spectroscopy and total contents of polyphenols spectrophotometrically. The highest content of all monitored parameters (pseudo-total and mobile Cd and Pb, as well as the amount of total polyphenols) was found in Ázia juh cultivar. There was statistically significant positive relation of total polyphenols contents in Anthea cultivar to levels of all monitored forms of metals in soil and contrary, in Clery cultivar there was found non-correlation of total polyphenols contents on levels of monitored heavy metals in soil (mainly of mobile contents of Cd and Pb contents). Contents of mobile forms of individual heavy metals positively correlated with total polyphenols (except of Clery and Ázia juh cultivars). Varietal relation on levels of total polyphenols was confirmed in experiment prior to relation of these organic compounds to the presence of monitored heavy metals in soil.

Keywords: strawberries; cadmium; lead; polyphenols

INTRODUCTION

Several epidemiological studies verify that the consumption of fresh fruits, vegetables and beverages of plant origin (such as herbal teas) can play a preventive role in free radical mediated disorders (e.g. cancer, heart disease, stroke), which is due to a variety of constituents, including vitamins, minerals, fiber and numerous phytochemicals, including polyphenols (Eastwood, 1999; Hertog et al. 1995; Bončíková et al. 2012, Hegedúsova et al. 2015, Kvalcová, et al. 2015, Timoracká, et al. 2010).

The health effects of polyphenols depend on the amount that is consumed and on their bioavailability (Manach et al., 2004). Several factors may affect the polyphenol content of plants, such as ripeness at the time of harvest, genotype (Picchi et al., 2012), environmental factors, processing and storage. The degree of ripeness considerably affects the concentrations and proportions of the different polyphenols. In general, the phenolic acid concentrations decrease during ripening, whereas the anthocyanin concentrations increase (Crecente-Campo et al., 2012).

Furthermore, environmental factors have a major effect on the polyphenol content, and these factors may be pedoclimatic or agronomic (e.g., culture in greenhouses or

fields, biological culture, hydroponic culture, and fruits yield per tree) (Manach et al., 2004). Several studies have shown that the content of phenolic compounds is higher in organic products, whereas other studies have found similar or lower contents of phenolic compounds in organic products (Vinkovic et al., 2011; Huber et al., 2011).

The strawberry is a relevant source of bioactive compounds because of its high levels of vitamin C, folate, and phenolic constituents (Proteggente et al., 2002), most of which express relevant antioxidant capacities *in vitro* and *in vivo* (Scalzo et al., 2005a; Scalzo et al., 2005b; Tulipani et al., 2009; Wang et al., 2000). These properties are mainly attributed to high fruit polyphenolic content, especially anthocyanins – the type of polyphenols quantitatively most important in strawberry fruits – as well as flavonoids, phenolic acids and vitamin C (Roussos et al. 2009; Meyers et al., 2003; Olsson et al., 2004; Cordenunsi et al., 2005).

Moreover, strawberries are economically and commercially important and widely consumed fresh or in processed forms, such as jams, juices, and jellies. That is why they are among the most studied berries from the agronomic, genomic, and nutritional points of view (Giampieri et al., 2012).

The aim of this work was to determine the effects of the abiotic stress caused by cadmium and lead on the polyphenol levels in strawberries fruits.

MATERIAL AND METHODS

The cultivars of strawberries were planted 20th August 2011 and were grown in 200 m long rows in area Dolná Malanta in Nitra (Table 1). The space between the plants was 0.35 m and the inter-row space was 1m. 200 m long cultivation line of strawberries was divided in half and one average sample was collected from each part (samples were signed as 1 and 2). The following fertilizers were applied 5th March 2012 (at the beginning of the vegetation period) and the doses of elements were (in kg/ha): N_{48.2} + P_{78.3} + K_{66.3} + Mg_{7.2} - in form of granulated fertilizer, after 23 days the elements were applied in liquid irrigation (48 days) in this quantity: N_{46.5} + P_{34.8} + K_{118.7} (Table 1). The strawberries were planted in contaminated soil of anthropogenic action- from air pollutants of vehicles in transportation and emissions from industrial enterprises. Soil samples were taken in soil horizons (0 – 0.2 m) in accordance with the exact method (pedological instrument GeoSampler). Agrochemical characteristics of the soil were assessed in soil samples (changeable soil reaction (pH/KCl), contents of nutrients (K, Ca, Mg, P) were assessed by Mehlich II method (Table 2). Heavy metals in soil were analysed in individual rows as following: the first 100 meters of rows – marked by sequential number 1 and the remaining 100 meters – marked by number 2 (Table 1, 3). Pseudototal contents of risk metals in extract of aqua regia and the contents of mobile forms of risk metals (Cd and Pb) in soil extract NH₄NO₃ (c = 1 mol.dm⁻³) were assayed by the flame atomic spectrometry (AAS Varian AA Spectr DUO 240FS/240Z/UltrAA, manufacturer Varian Australia Pty Ltd, A.C.N. 004 559 540, Mulgrave, Australia) (Koppova, 1955).

Four samples of fresh fruit (50 g) from the strawberries was extracted using 250 mL of methanol: water (80 : 20 v / v) for 18 hours on 125 rpm rotary shaker. After filtering, the crude extracts were obtained. The amount of total phenolics in extract acts was determined with the Folin-Ciocalteu reagent. 100 µL of crude extract (20 mg.mL⁻¹) was mixed with 0.2 mL of Folin-Ciocalteu reagent, 2 mL of purified water and 1 mL of 15% Na₂CO₃. The absorbance of sample was measured at 765 nm (UV-1800 spectrophotometer, Shimadzu, Japan) after 2 hours at room temperature. Gallic acid was used as a standard and the total phenolics were expressed as mg.k⁻¹ g gallic acid equivalents (Slinkard and Singleton, 1977).

Results were evaluated by statistical program Statgraphics 4.0 (Statpoint Technologies, Inc., Czech republic), the data were analyzed by means of one-way analysis of variance (ANOVA) and also mutually by regression and correlation analysis (by Microsoft Excel, version 2010).

RESULTS AND DISCUSSION

Values of total polyphenols in fresh matter of seven tested strawberry cultivars are shown in Table 3. According to the average contents of total polyphenols in fresh matter of strawberries there is the following line of varieties from both sampling sites in our work: Ázia juh > Sonata > Joly > Clery > Anthea > Korona > Alba. Contents of total polyphenols in tested fruits were from 1262.91 to 2343.63 mg.kg⁻¹. Slightly higher values of total polyphenols in Fragaria cultivar were also achieved by Crecente-Cambo et al., (2012), who presented values of total polyphenols of certain cultivar in interval 2600 – 2880 mg.kg⁻¹ of fresh matter, overleaf, Pinely et al., (2011), found the dynamics of total polyphenols contents in two ripening cultivars of strawberries (Osogrande and Camino real) in range of 1743.5 – 2169.4 mg.kg⁻¹. Wang and Lin (2000) reported total polyphenols values of

Table 1 Methodology of planting and fertilization of selected varieties of strawberries.

Length of rows	200 m		
1. variant	the first 100 m of rows		
2. variant	the remaining 100 m of rows		
The inter-row space	1 m		
The space between the plants	0.35 m		
Date of planting	20.August 2011		
Fertilization of granulated fertilizer	5.March 2012	The dose of macronutrients	N 48.2 kg.ha ⁻¹ P 78.3 kg.ha ⁻¹ K 66.3 kg.ha ⁻¹ Mg 7.2 kg.ha ⁻¹
Fertilization of irrigation solution	28.March 2012 – 15.May 2012	The dose of macronutrients	N 46.5 kg.ha ⁻¹ P 34.8 kg.ha ⁻¹ K 118.7 kg.ha ⁻¹

Table 2 Agrochemical characteristics of soil (horizons 0 – 0.2 m).

Soil reaction	Humus content	Nutrients			
		K	Ca	Mg	P
(pH/KCl)	(%)	(mg.kg ⁻¹)	(mg.kg ⁻¹)	(mg.kg ⁻¹)	(mg.kg ⁻¹)
4.7 – 5.8	1.57	374.3	1,523.8	219.9	46.3

Table 3 The contents of polyphenols (mg.kg⁻¹ fresh matter) in strawberries and contents of two analysed heavy metals (mg.kg⁻¹) in soil of samples (numbers 1 and 2 mean the average sample from each of the half of 200 m cultivation row). Above limit contents of both heavy metals in soil are marked by bold letters.

Cultivars	Total polyphenols (mg.kg ⁻¹)	Cd (mg.kg ⁻¹) (extract NH ₄ NO ₃)	Cd (mg.kg ⁻¹) (extract of aqua regia)	Pb (mg.kg ⁻¹) (extract NH ₄ NO ₃)	Pb (mg.kg ⁻¹) (extract of aqua regia)
Clery 1	1960.21 ^{Aac}	0.07 ^{ABab}	1.50 ^{ABbc}	0.22 ^{ABa}	19.60 ^{Bac}
Clery 2	2154.56 ^{Abcd}	0.07 ^{Aabc}	1.10 ^{Aa}	0.16 ^{Aab}	16.60 ^{Aac}
Sonata 1	1991.09 ^{ABabcd}	0.06 ^{ABabc}	1.54 ^{Bcb}	0.17 ^{ABab}	17.20 ^{A^Cabc}
Sonata 2	2245.17 ^{Aacd}	0.07 ^{Ac}	1.56 ^{Acb}	0.20 ^{Aab}	17.20 ^{Aabc}
Alba 1	1262.91 ^{ABabd}	0.07 ^{Cab}	1.56 ^{ABDcb}	0.19 ^{Dab}	17.40 ^{ADabc}
Alba 2	1492.04 ^{ABabd}	0.06 ^{Bbc}	1.70 ^{ABabc}	0.11 ^{ABa}	16.60 ^{ABbc}
Korona 1	1648.49 ^{ABCad}	0.05 ^{ABa}	1.80 ^{ACbc}	0.10 ^{ABCab}	20.60 ^{ABb}
Korona 2	1348.59 ^{Aacd}	0.06 ^{Aabc}	1.46 ^{Abc}	0.17 ^{Aab}	18.60 ^{Aabc}
Ázia Juh 1	2343.63 ^{Abc}	0.07 ^{ABab}	1.78 ^{Bab}	0.20 ^{ABa}	20.40 ^{Bbc}
Ázia Juh 2	2305.01 ^{ABCac}	0.08 ^{ABab}	1.60 ^{Bab}	0.22 ^{ACa}	20.40 ^{Cabc}
Anthea 1	1734.76 ^{ABbcd}	0.08 ^{Aabc}	1.50 ^{Cb}	0.17 ^{Cab}	20.00 ^{Cabc}
Anthea 2	1344.00 ^{Aabcd}	0.06 ^{Bab}	1.28 ^{Cab}	0.12 ^{ABCb}	18.20 ^{ACbc}
Joly 1	1758.52 ^{Abc}	0.08 ^{ABa}	1.50 ^{ABabc}	0.16 ^{ABa}	19.20 ^{Ba}
Joly 2	2310.20 ^{Acd}	0.07 ^{Aabc}	1.68 ^{Aabc}	0.14 ^{Aab}	17.40 ^{Aabc}
Critical values		0.10	0.70	0.10	70.00

Note: Capital letters in table stand for statistical significance in rows ($p < 0.01$) and small letters stand for statistical significance in columns ($p < 0.01$). Their conformity means that the values are statistically non-significant and different letters characterize statistical significance ($n = 4$).

Table 4 Percentages of variability between contents of two heavy metals in soil and contents of total polyphenols in fresh weight of strawberries (values above 70% mean highly significant correlation, the interval 30 – 70% means significant correlation and below 30% non-significant correlation).

cultivars	Cd (mg.kg ⁻¹) (extract NH ₄ NO ₃) (%)	Cd (mg.kg ⁻¹) (extract of aqua regia) (%)	Pb (mg.kg ⁻¹) (extract NH ₄ NO ₃) (%)	Pb (mg.kg ⁻¹) (extract of aqua regia) (%)
Clery	48.50	28.98	28.01	40.50
Sonata	11.49	28.94	19.74	6.16
Alba	69.94	76.29	93.50	63.03
Korona	16.49	75.73	74.82	65.21
Ázia Juh	60.37	29.44	42.43	34.04
Anthea	93.75	96.30	94.80	86.98
Joly	54.20	93.97	85.97	93.24

strawberry juice (cv. Allstar) at different stages of maturity: 2560 and 1030 mg.kg⁻¹ for green and ripe fruit, respectively. The total polyphenol content also varies when the stem is attached.

Our results indicate (as well as the results from above mentioned studies) that there is substantial relation of cultivar to content of these secondary organic metabolites in strawberry fruit. However, it is obvious that the influence of cultivar on total polyphenols content is not the main factor, as the content of organic compounds in plants is more influenced by other factors, such as: soil type, nutrient supply, or, to the contrary – the presence of contaminants and climate. According to **Fernandes et al., (2012)**, strawberries of the same tested cultivars grown in two different conditions (organic farming and integrated pest management) had in average different contents of polyphenols: 108 μM.g⁻¹ and 81 μM.g⁻¹ of fresh matter

(after conversion via molecular weight of gallic acid, the results are 1837.1 mg.kg⁻¹ and 1377.8 mg.kg⁻¹ of fresh matter). For comparison, the total polyphenolic content in cranberries 3-fold higher than in strawberries (**Rop et al., 2010**).

In our work, there were exceeded mobile contents of Pb and pseudototal contents of Cd in all analysed samples of soil (with the exception of one value of Pb mobile content, from the first part of sampling site from Korona cultivar). The statistical non-significant (significance level 99%) highest mobile and pseudototal contents of both tested heavy metals were in the Ázia juh cultivar, which also had the highest values of total polyphenols – also statistically non-significant on mentioned level in comparison to other variants. It could be presumed that the increased amount of total polyphenols in plants of strawberries is a response to this stress, because in **Márquez-García et al., (2012)**

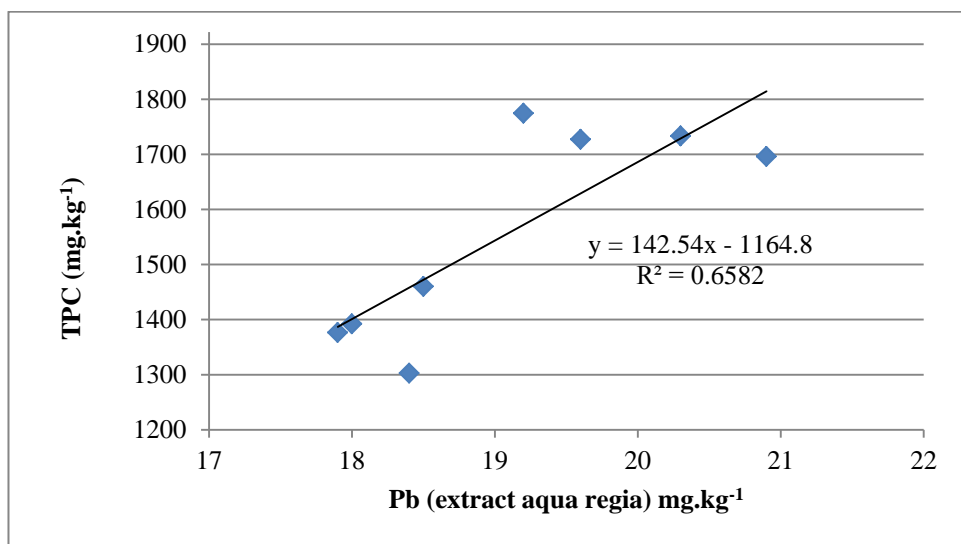


Figure 1 Linear regression between total polyphenols (TPC) and pseudototal Pb contents in soil (ANTHEA cultivar). The collection points from specific experimental area were realized and obtained samples were analyzed.

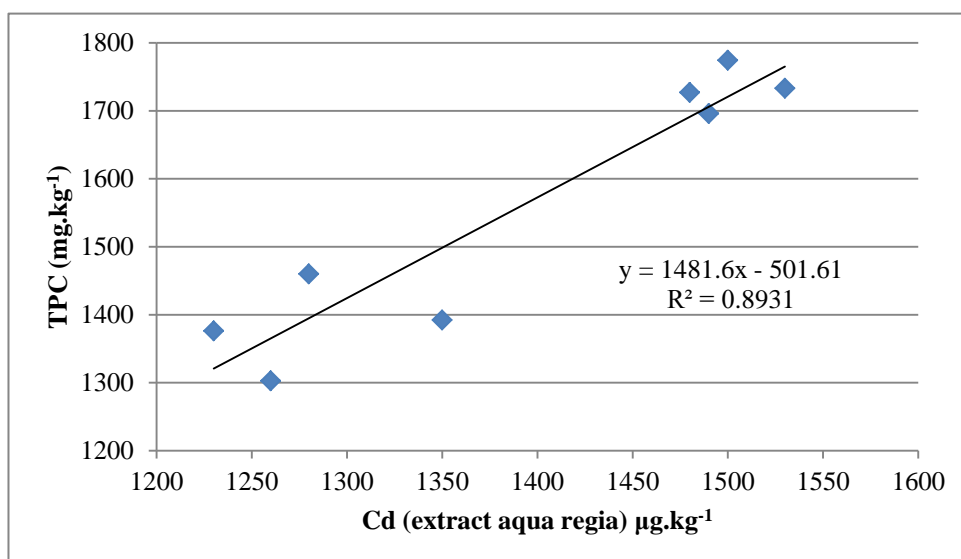


Figure 2 Linear regression between total polyphenols (TPC) and pseudototal Cd contents in soil (ANTHEA cultivar). The collection points from specific experimental area were realized and obtained samples were analyzed.

work, there is also an evidence that added Cd (5 mg.kg⁻¹ in soil) had an influence on the highest content of phenol compounds in tissues of *Erica andevalensis* flower. Moreover, the authors found increased amount of derivatives of cinnamic acid in comparison with control in variants with applied Cd in soil. Also in hydroponic experiment by **Dudjak et al., (2004)**, the application of Cd into nutrient solution with concentration 10⁻⁶ mol.dm⁻³ had the consequence in higher total polyphenols in plants of barley in comparison with control variant without applied Cd as follows: leaves by 95.2%, stems 16.7% and roots 20.3%. The authors also found out that the relationship between the total polyphenol content and Cd content in all investigated barley organs was linear.

This condition is similar to stress caused by external factors: UV, γ-radiation, drought, or higher temperature (**Lachman et al., 2001; Orsák et al., 2001; Hakala et al., 2002; Hideg et al., 2002**).

Not only cadmium, as a stressor, has great effect on increased total polyphenols content, another heavy metal – lead, also affects the content of observed secondary metabolites in plants. The content of total polyphenols increased (**Musilová et al., 2011**) with the increased content of Pb in soil, as well as in potato tubers according to positive correlation R = 0.961 (p < 0.01). In our study the line of average values of both sampling sites, according to measurements of all analysed heavy metals in soil where the plants of strawberries were grown, were not consistent with the line of cultivars in content of total polyphenols in the fruit and this result was also confirmed by their statistical non-significant relation (Table 3). Very low approximate conformity (as well as statistical non-significant relation) was found in pseudototal contents of Pb in soil on amount of total polyphenols in strawberries (with exceptions from cultivars Sonata and Korona).

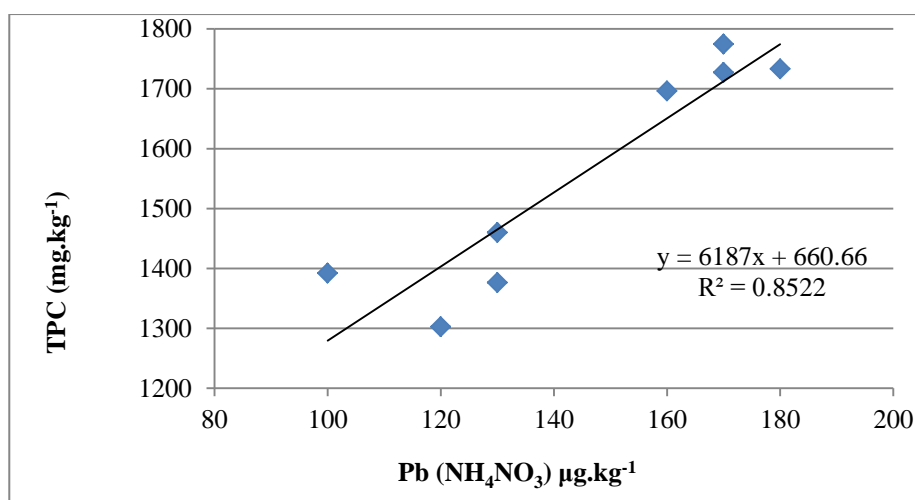


Figure 3 Linear regression between total polyphenols (TPC) and mobile Pb contents in soil (ANTHEA cultivar). The collection points from specific experimental area were realized and obtained samples were analyzed.

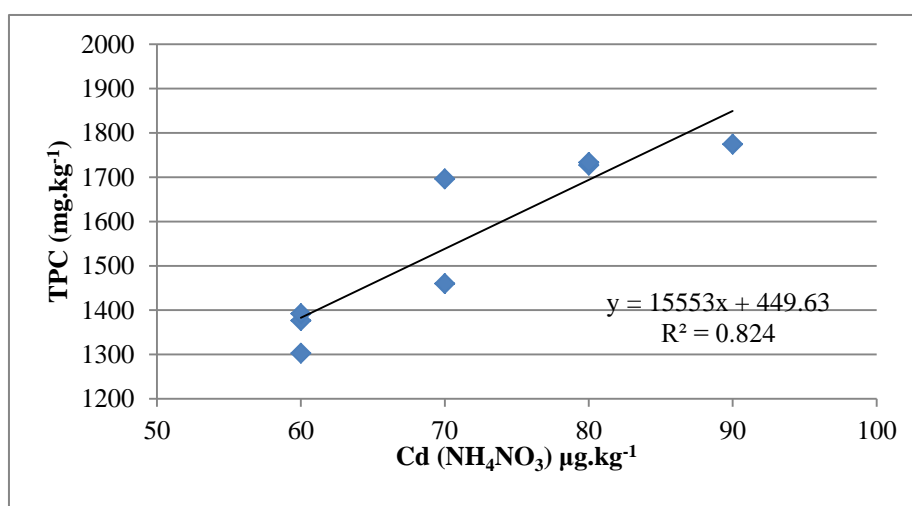


Figure 4 Linear regression between total polyphenols (TPC) and mobile Cd contents in soil (ANTHEA cultivar). The collection points from specific experimental area were realized and obtained samples were analyzed.

The least significant effect on content of total polyphenols in strawberry cultivars (with exception of above mentioned cultivar Ázia juh) had the content of exceeded pseudototal Cd, only by cultivar Anthea there was statistical high significant relationship of these parameters. Non-exceeded limit of mobile content of Pb, as well as the lowest content of mobile Cd level in soil, where the plants of Korona cultivars were grown, had relatively low statistical non-significant effect on the value of total polyphenols in fruit of this cultivar; this is the borderline after which there is stressing action of heavy metals on plants.

When mentioning correlation relations (Table 4), the contents of total polyphenols in fruit are statistically high positively correlated with amounts of all tested heavy metals in soil (Figure 1 – 4) only in Anthea cultivar (variability in all cases was always above 86.98%) (Table 4), in Joly cultivar (with exception of mobile content of Cd) there was also high positive variability (above 85.97%) on amounts of total polyphenols in strawberries. In Alba and Korona cultivars there was only significant positive relation of all heavy metals in soil to amount of polyphenols in fruits. Only in Clery and Ázia

juh cultivars there is absolute non-correlation of tested parameters.

CONCLUSION

The amount of total polyphenols in fruits of strawberries was statistically non-significantly ($p > 0.01$) affected by two factors: type of cultivar and stress caused by of heavy metals (cadmium and lead) present in soil. In our work, the stronger influence of stress was invoked by heavy metals, while the plants were grown in soils with higher metallic burden by cadmium and lead. These limits in which more significant influence of stress occurred were evaluated as: mobile content in soil above 0.07 and 0.1 mg.kg⁻¹ for Cd and Pb, respectively, and pseudototal content in soil above 1.1 and 17.0 mg.kg⁻¹ for Cd and Pb, respectively. Beside this limit the type of grown cultivar of strawberry is manifested in higher range as a factor on content of total polyphenols in their fruits that were in our study in interval of values 1262.91 to 2343.63 mg.kg⁻¹ – only by Anthea cultivar there was high statistical relation of total polyphenols contents, but only with pseudototal content of cadmium in soil.

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QUANTITATIVE INDICATORS OF FRUIT AND VEGETABLE CONSUMPTION

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ABSTRACT

The quantitative research of the market is often based on surveys and questionnaires which are finding out the behavior of customers in observed areas. Before purchasing process consumers consider where they will buy fruit and vegetables, what kind to choose and in what quantity of goods. Consumers' behavior is affected by the factors as: regional gastronomic traditions, price, product appearance, aroma, place of buying, own experience and knowledge, taste preferences as well as specific health issues of consumers and others. The consumption of fruit and vegetables brings into the human body biological active substances that favorably affect the health of consumers. In the presented research study we were interested in differences of consumers' behavior in the consumption of fruit and vegetables according to the place of residence and gender. In the survey 200 respondents has participated; their place of residence was city or village. The existence of dependences and statistical significance were examined by selected statistical testing methods. Firstly we analyzed the responses via statistical F-test whether observed random samples have the same variance. Then we applied two-sample unpaired t-test with equal variance and χ^2 -test of statistical independence. The statistical significance was tested by corresponding p values. Correlations were proved by the Cramer's V coefficient. We found that place of residence has no impact on the respondents' consumption of fruit. The gender of respondents does not affect their consumption of fruit. Equally, the gender does not affect the respondents' consumption of vegetables. Only in one observed case the significant differences proved that the place of respondent residence has impact on the consumption of vegetables. Higher consumption of vegetables is due to the fact that the majority of citizens, who live in villages, have a possibility to grow their own vegetables and, thus, the demand for it in village shops is low.

Keywords: fruit; vegetable; consumption; consumer; behavior; respondent

INTRODUCTION

In the food market the customers' behavior is affected by different factors. The most important factors, which have an impact on the customers' behavior, are the quality of the products and price of products. Other important factors are: information about the goods, the offer and access to goods, discount, eating habits and rules, national or regional gastronomic traditions, taste preferences as well as specific health issues of consumers. The differences in consumers' behavior in the food market were analyzed by **Rousseau and Vranken (2013)**, **Kutnohorská and Tomšík (2013)**; new trends in eating and impacts on purchasing habits of consumers were examined by **Horská et al., (2012)**, economic and management aspects of the production, sales and demand for food by **Bielik et al., (2014)**, **Kozáková et al., (2014)**, **Tóthová and Prčík (2011)**. Purchasing habits of consumers are also changing by the effects of new information of global character in relation to climate changes. Interesting results were obtained by **Chuanmin et al., (2014)** scenario experimental methodology of carbon labeling on agri-food products, coupled with 873 questionnaires collected from six cities in China; then they made a statistical analysis of different types of consumers' behavior on the low-carbon agri-food purchase.

In regard to the existence of substantial regional disparities at district level, a place of residence has a major impact on customers' behavior when purchasing food. These differences are related to wage levels, purchasing power of people, employment rate, vacancies and job offers as well as land use. The above stated facts demonstrate works of **Fáziková (2012)**, **Chreneková and Jíříček (2013)**, **Kadlečíková et al., (2013)**. Consumers' behavior, when purchasing organic food, dependent on the place of the residence, was examined also by **Müller and Gaus (2015)**. Whereas eating habits are established mainly in the family, in kindergartens and primary schools, the importance of introducing healthy eating habits for children and adolescents are pointed at by **Lesschen et al., (2011)** and others.

The aim of this paper is to analyze consumers' eating habits focusing on fruit and vegetables in relation to the two criteria: the place of residence and the gender of respondent.

MATERIAL AND METHODOLOGY

The partial goal of this paper was to carry out the monitoring of consuming of fruit and vegetable and then to evaluate results in relation to the place of residence and the gender of respondent. The survey was conducted by the method of the questionnaire in February 2015 and 200

respondents were included in the statistical sample. The questionnaire consisted of 16 questions about consumption of fruit and vegetables and 4 classificatory issues (in this paper we present 2 of them).

In the survey participated 46% of women and 54% were men. In terms of age the structure was following: 18% were young people from 14 till 19 years; group of 18% of respondents belonged to the age from 20 till 25 years; age group 26 – 35 years accounted for 19% of respondents; age group 36 – 45 years accounted for 15% of respondents; age group 46 – 55 years 19%, and respondents aged 56 years and over were represented in the 13% share. The structure of the respondents according to their economic activity was as follows: 35% students, 30% were employed, 23% unemployed and 13% of retirees. In terms of the place of residence 31% of respondents live in a village and in the city 70% of respondents.

Using a research questionnaire we investigated whether there are significant differences in the level of daily consumption of fruit (resp. of vegetables) in relation to the place of respondent residence and in relation to the gender of the respondent. The term "1 serving" was explained to respondents as the amount of approximately 100 – 150 g of fruit or vegetables. We will analyze the responses by selected methods of mathematical statistics; more precisely we will use two-sample unpaired t -test with equal variance and χ^2 -test of independence. Before running t -test and χ^2 -test we will verify using the F -test whether observed random samples have the same variance. Calculations and graphical interpretation of the results will be realized by MS Excel 2010.

We will describe the main characteristics of the F -test. We assume that there are given two samples with ranges n_1, n_2 with variances s_1^2, s_2^2 that are selected from basic files with normal distribution $N(\mu_1, \sigma_1^2)$ and $N(\mu_2, \sigma_2^2)$. We test the null hypothesis $H_0: \sigma_1^2 = \sigma_2^2$ versus the alternative hypothesis $H_1: \sigma_1^2 \neq \sigma_2^2$. The test criterion has the Fisher-Snedecor distribution $F(n_1 - 1, n_2 - 1)$. If $F > F_{\frac{p}{2}}(n_1 - 1, n_2 - 1)$, we reject the hypothesis H_0

and accept the alternative hypothesis H_1 .

In implementing the unpaired t -test, we assume that the selected samples are independent and justified assumption of equal variances σ_x^2, σ_y^2 . We test the null hypothesis

$H_0: \mu_1 = \mu_2$ versus the alternative hypothesis

$H_1: \mu_1 \neq \mu_2$. As a test criterion we apply

$$t = \frac{\bar{X} - \bar{Y}}{\sqrt{S_p^2 \cdot \left(\frac{1}{n} + \frac{1}{m}\right)}}$$

Critical region is the set $W_\alpha = (-\infty, -t_\alpha(n+m-2)) \cup (t_\alpha(n+m-2), \infty)$

where $t_\alpha(n+m-2)$ is the critical value of Student's t -distribution with $n+m-2$ degrees of freedom. If $t \in W_\alpha$ (i.e. if $|t| > t_\alpha(n+m-2)$), we reject hypothesis

H_0 at the significance level α . Otherwise if $t \notin W_\alpha$ we cannot reject null hypothesis H_0 and accept the alternative hypothesis H_1 .

Measurement of dependencies through qualitative characters, i.e. measurement of associations, we will use for verification of the existence of dependence between own questions and classification questions using χ^2 -test of independence. The statistical evidence supporting relationships will be assessed on the basis of the significance of test characteristics (p -value). Dependence of two variables can be either symmetrical (mutual) or asymmetrical (one-sided). The basic test used for the detection of dependence of two categorical characters (without taking into account the direction of statistical dependence) is χ^2 -test of independence. That is based on the consideration that if two characters are independent, then the distribution of frequencies in the pivot table is proportional to the row's and column's marginal frequency (Řezanková, 2011). Using χ^2 -test test we verify the existence of dependence between responses to questions in relation to the classification criteria (in general it can be economic activity, gender, age, place of residence and others). The relationship is considered statistically significant if the p -value is less than the significance level $\alpha = 0.05$.

In research organizing we were in line with formulated targets and suggested following assumptions:

1. We assume that women consume more portions of fruit than men.
2. We assume that people living in the villages consume more fruit than city residents.
3. We assume that women consume more portions of vegetable than men.
4. We assume that people living in the villages consume more vegetable than city residents.

RESULTS AND DISCUSSION

In this part we will verify formulated preconditions through testing of hypotheses. We will apply the F -test to find if observed random samples have the same variance. Then we use the unpaired t -test and χ^2 -test. The aim of the testing is to determine whether:

- There are significant differences in the consumption of fruit in relation to the gender of a respondent,
- There are significant differences in the consumption of fruit in relation to the place of residence of a respondent,
- There are significant differences in the consumption of vegetable in relation to the gender of a respondent,
- There are significant differences in the consumption of vegetable in relation to the place of residence of a respondent.

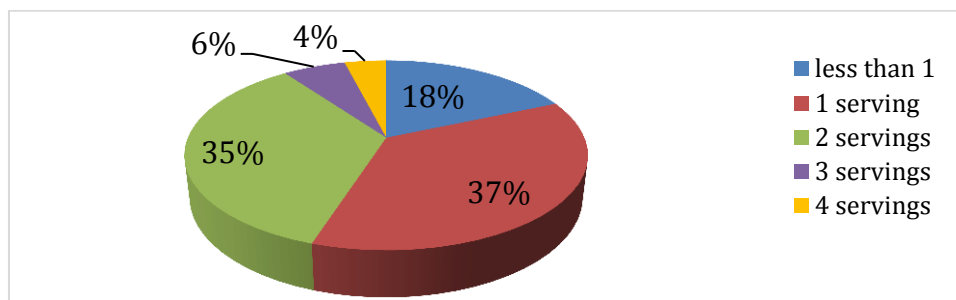


Figure 1 Consumption of fruit per day.

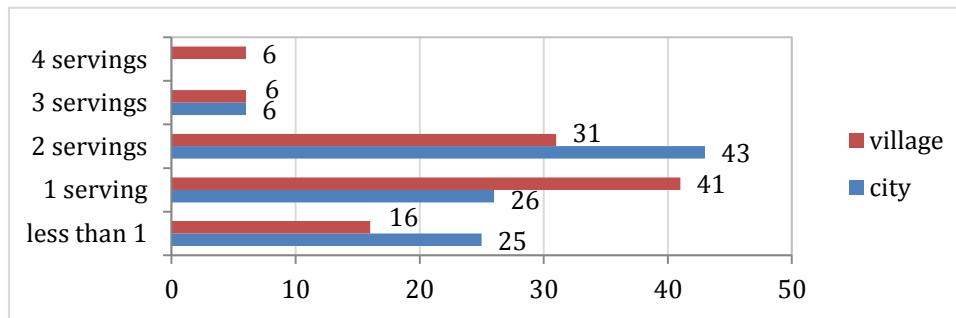


Figure 2 Consumption of fruit in relation to the place of residence (in %).

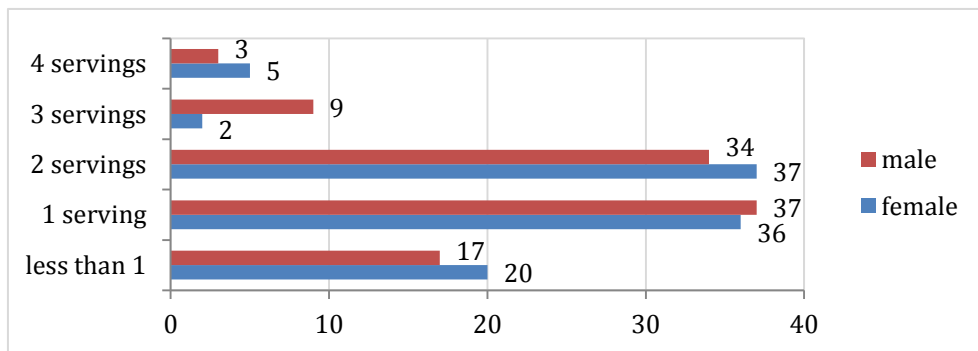


Figure 3 Consumption of fruit in relation to the gender (in %).

Table 1 Consuming fruits by respondents according to the residence place.

Number of servings	City	Village	Testing method	Outcome	Result
Less than 1	15	22	<i>F</i> -test	0.174	$p > 0.05$
1 serving	16	57	<i>t</i> -test	0.189	$p > 0.05$
2 servings	26	44	χ^2 -test	0.409	$p > 0.05$
3 servings	4	8	Cramer's V	0.216	Weak relationship
4 servings	0	8	coefficient		

Source: authors

Table 2 Consuming fruits by respondents according to the gender.

Number of servings	Female	Male	Testing method	Outcome	Result
Less than 1	19	18	<i>F</i> -test	0.897	$p > 0.05$
1 serving	33	40	<i>t</i> -test	0.784	$p > 0.05$
2 servings	34	36	χ^2 -test	0.776	$p > 0.05$
3 servings	2	10	Cramer's V	0.168	Weak relationship
4 servings	5	3	coefficient		

Source: authors

The fruit consumption and habits of respondents

The fruits can be consumed fresh as well as frozen, sterilized or otherwise modified. We were interested how many servings of fruit respondents of the survey eat daily. Majority of respondents (37%) declare that they eat 1 serving of fruit daily. Frequency of daily consumption of fruit is as follows: 18% of respondents eat less than 1 serving; 35% of respondents eat 2 servings; 6% of respondents eat 3 servings daily; 4% of respondents eat 4 servings of fruit. Not one respondent declares that eat 5 servings of fruit per day (Figure 1).

We assumed that people living in the villages consume more servings of fruit than city residents. We formulated and tested the following null hypothesis H_1 : Differences in the consumption of fruit in relation to the place of residence of a respondent are not significant.

In the Table 1 there are results of hypothesis testing to the question about the daily consumption of fruit in relation to the place of residence. From summarized results follows: Based on F -test we can conclude that differences in the variances of the samples are not statistically significant, $p = 0.174 > 0.05$. It is evident from results of two sample un-paired t -test and χ^2 -test that differences in the consumption of fruit in relation to the respondent's place of residence are not statistically significant. At a significance level of $\alpha = 0.05$ we accept the null hypothesis. And further, the value of Cramer's V coefficient proves that the relationship between the city and village in the consumption of fruit is weak. Place of residence has no impact on the consumption of fruit of respondents.

Next we assumed that women consume more servings of fruit than men. We formulated and tested the following null hypothesis H_2 : Differences in the consumption of fruit in relation to the gender of a respondent are not significant.

In the Table 2 there are results of hypothesis testing to the question about the daily consumption of fruit in relation to the gender of a respondent. From results follows: based on F -test we can conclude that differences in the variances of the samples are not statistically significant. From results of two sample un-paired t -test and χ^2 -test is evident that differences in the consumption of fruit in relation to the respondents' gender are not statistically significant. At a significance level of $\alpha = 0.05$ we cannot reject the null hypothesis. The value of Cramer's V coefficient proves that the relationship between the women's and men's consumption of fruit is weak. Gender of respondents has no impact on the consumption of fruit.

Many epidemiological studies examine the health benefits of a diet rich in fruits and vegetables. Increasing individual fruit and vegetable consumption by up to 600 g per day (the baseline of choice) could reduce the total worldwide burden of disease by 1.8% (Lock et al., 2005). During the transition from adolescence to young adulthood, the intake of fruit and vegetables tends to decline (Larson et al., 2008). Studies among young adults, university students, seem to have found a high prevalence of low fruit and vegetable consumption (<5 servings/day), e.g. Brazil 85.2% (Ramalho et al., 2012), Chile 94.8% (Mardones et al., 2009), Germany 95% (Keller et al., 2008), Saudi Arabia 73.6% (Elsoadaa et al., 2013), and UK 70% (Dodd et al., 2010).

Via consumption of fruit and fruit products, the body can receive biologically active substances, such as: total polyphenols, anthocyanins, quercetin, chlorogen acid, pterostilbene and antioxidant activity in bilberry and blueberry analyzed Habánová et al., (2013). In the Slovak Republic in year 2013 the consumption of fruits from temperate zone increased per capita per year, reaching 53.1 kg; fresh fruit consumption was 41.1 kg. Tropical fruit consumption per capita was 25.8 kg (Meravá, 2014a).

The vegetables consumption and habits of respondents

We were interested how many servings of vegetables respondents eat daily. Majority of respondents (29%) declare that they eat two servings of vegetables daily. Frequency of daily consumption of vegetables is as follows: 20% of respondents eat less than 1 serving; 26% of respondents eat 1 serving; 14% of respondents eat 3 servings daily; 11% of respondents eat 4 servings of vegetables. Not one respondent declares that eat 5 servings of vegetables per day (Figure 4).

We were interested whether the gender of the respondents demonstrates the impact on the consumption of vegetables. We assumed that women consume more servings of vegetable than men. We tested the following null hypothesis H_3 : Differences in the consumption of vegetable in relation to the gender of a respondent are not significant.

In the Table 3 there are results of hypothesis testing to the question about the daily consumption of vegetables in relation to the gender of a respondent. From results we can conclude: From the F -test follows that differences in the variances of the samples are not statistically significant. From results of two sample un-paired t -test and χ^2 -test is evident that differences in the consumption of vegetables in relation to the respondents' gender are not statistically significant. At a significance level of $\alpha = 0.05$ we accept the null hypothesis. The value of Cramer's V coefficient proves that the relationship between the women's and men's consumption of vegetables is weak. Gender of respondents has no impact on the consumption of vegetables.

In the last research question we assumed that people living in the villages consume more vegetable than city residents. We tested the null hypothesis H_4 : Differences in the consumption of vegetable in relation to the place of residence of a respondent are not significant.

In the Table 4 there are summarized results of hypothesis testing to the question about the daily consumption of vegetables in relation to the place of residence of a respondent. From results we can formulate conclusions:

From the F -test follows that differences in the variances of the samples are not statistically significant. From results of two sample un-paired t -test and χ^2 -test follow that differences in the consumption of vegetables in relation to the respondents' gender are statistically significant. At a significance level of $\alpha = 0.05$ we reject the null hypothesis and accept the alternative hypothesis that differences in the consumption of vegetable in relation to the residence place of a respondent are significant. The value of Cramer's V coefficient proves that the relationship in the consumption of vegetables is moderately strong between the respondents from the city and village.

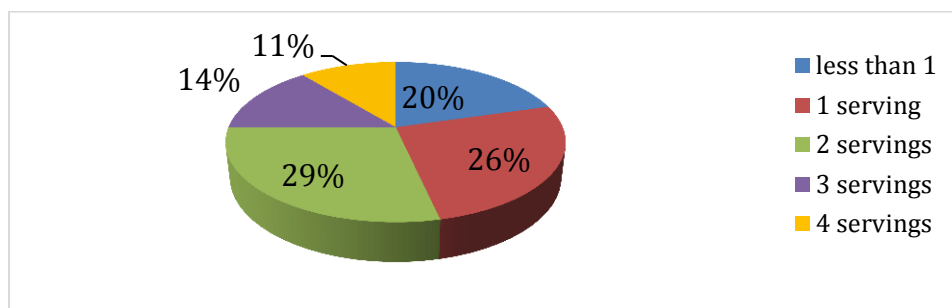


Figure 4 Consumption of vegetable per day.

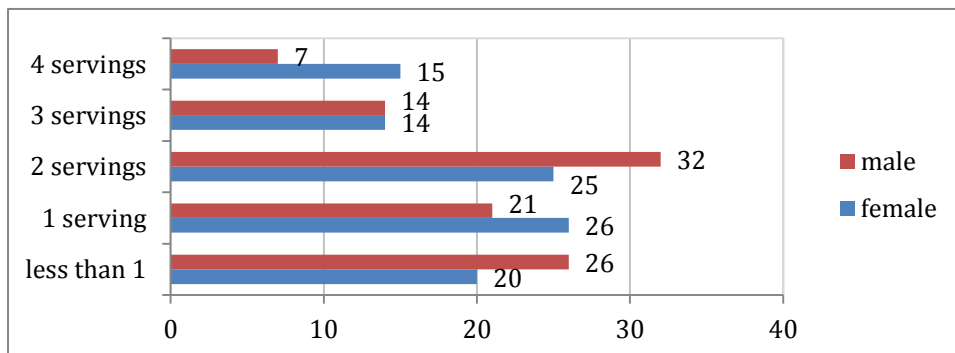


Figure 5 Consumption of vegetable in relation to the gender (in %).

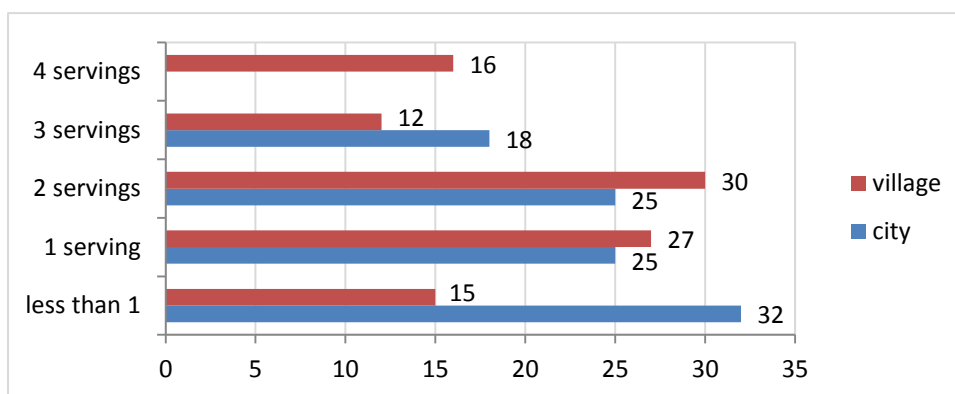


Figure 6 Consumption of vegetable in relation to the place of residence (in %).

Table 3 Consuming vegetables by respondents according to the gender.

Number of servings	Female	Male	Testing method	Outcome	Result
Less than 1	19	28	F-test	0.195	$p > 0.05$
1 serving	24	22	t-test	0.599	$p > 0.05$
2 servings	23	34	χ^2 -test	0.855	$p > 0.05$
3 servings	13	15	Cramer's V		Weak
4 servings	14	8	coefficient	0.154	relationship

Source: authors

Table 4 Consuming vegetables by respondents according to their residence place.

Number of servings	City	Village	Testing method	Outcome	Result
Less than 1	20	21	F-test	0.482	$p > 0.05$
1 serving	15	37	t-test	0.031	* $p < 0.05$
2 servings	15	42	χ^2 -test	0.039	* $p < 0.05$
3 servings	11	17	Cramer's V		Moderately strong
4 servings	0	22	coefficient	0.297	relationship

Source: authors

In this case we found that the place of residence of respondents has impact on the consumption of vegetables. We assume that increased consumption of vegetables is due to the fact that the majority of village citizens have the possibility of growing their own vegetables.

The presence of biological active substances in vegetables is examined by several authors, e.g. in spinach by Mendelová et al., (2014), in garden pea varieties Hegedüsová et al., (2015). It is widely known that healthy diet is a key factor in the prevention of chronic diseases and in maintaining the health throughout one's life. The low consumption of fruit and vegetables was classified as the 5th cause of disability. The high consumption of fruit and vegetables is linked to reduced risk of chronic diseases such as heart disorders, diabetes or cancer. The positive association between the consumption of fruit and vegetables and health improvement was demonstrated also by studies of Takaoka and Kawakami (2013).

The consumption of vegetables and vegetable products (in the case of fresh vegetables) in 2012 rose over the year by 2 408 t (+0.4 %) to 545 454 t, but the consumption of fresh vegetables decreased by 9 026 t less (-2.3%) to 375 156 t, what means that vegetable products had a higher consumption than fresh vegetables. The increase in consumption of vegetables and vegetable products per capita in the year 2013 represented 5.2 kg and consumption of fresh vegetables 5.5 kg. The highest consumption had tomatoes (17.8%), cabbage (15.1%), carrot (11.1%) and onion (8.4%). The lowest consumption had spinach (0.4%), garlic and peas (each 0.8%) and beans (0.7%) (Meravá, 2014b).

CONCLUSION

We assumed that citizens who live in villages consume more portions of fruit and vegetables than those who live in towns. In this case we accept null hypothesis $p = 0.409 > 0.05$, place of residence does not affect the respondents' consumption of fruit. Demand for fruit in the village shops is low, since local citizens grow fruit in their gardens, or they buy it directly from a producer as a sale from a farm yard (Hypothesis 1). We assumed that women consume more fruit than men. This hypothesis was not confirmed because $p = 0.776 > 0.05$. Gender does not affect the respondents' consumption of fruit (Hypothesis 2). We assumed that gender of the respondents has impact on the consumption of vegetables. This hypothesis was not confirmed, $p = 0.855 > 0.05$ and gender does not affect the consumption of vegetables (Hypothesis 3). We assumed that citizens, who live in villages, consume more vegetables than those who live in towns. This hypothesis was confirmed, testing result is $p = 0.039 < 0.05$. Higher consumption is due to the fact that the majority of citizens, who live in villages, have a possibility to grow their own vegetables and, thus, the demand for it in village shops is low (Hypothesis 4).

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CONTENT OF TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN SELECTED VARIETIES OF ONION (*ALLIUM CEPA* L.)

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ABSTRACT

Onion (*Allium cepa* L.) is one of the most important vegetable crops widely consumed in the world. The bulb onion is grown as fresh shoots and as bulbs for consuming uncooked, cooked, and pickled or production of seed and sets. They can be eaten raw, boiled, steamed and roasted. Onion has high nutritional value. Onions are a good source of vitamins, minerals and major component like polyphenols, flavonoids, fructooligosaccharides, thiosulfates and other sulfur compounds. Polyphenols belong to significant antioxidants in human diet. Antioxidant compounds (polyphenols, flavonoids) scavenge free radicals, inhibit the oxidative mechanisms that lead to degenerative diseases. Antioxidant compounds in food play an important role as a health protecting factor. Onions have a wide range of beneficial properties for human health, such as anti-cholesterolaemic, anti-mutagenic and antioxidant capacity. In this work we evaluated content of total polyphenols and antioxidant activity in selected varieties of onion. Samples of plant material we collected at the stage of full maturity in the locality of Pružina. Pružina is locality without negative influences and emission sources. Total polyphenols content and antioxidant activity were measured in six varieties of onion, namely in red variety (Red matté), in yellow varieties (Boston, Bingo, Sherpa) and white varieties of onion (Diamond, White dry). The content of the total polyphenols was determined by using the Folin-Ciocalteu reagent (FCR). Antioxidant activity was measured by using a compound DPPH'. In the present experiment it was detected, that total polyphenols content in samples ranges from 142.01 mg.kg⁻¹GAE (white variety of onion- White dry) to 1083.04 mg.kg⁻¹GAE (red variety of onion- Red matté). Statistically significant highest value of total polyphenols (1083.04 mg.kg⁻¹GAE) was recorded in variety Red matté. Another indicator that has been evaluated and compared was the antioxidant activity. The values of antioxidant activity were in interval from 7.74% to 41.67%. Based on the measured values of antioxidant activity in onion the samples can be classified as follows: Red matté (41.67%) > Boston (25.77%) > Sherpa (24.34%) > Bingo (19.54%) > White dry (8.10%) > Diamond (7.74%).

Keywords: onion; variety; color; polyphenols; antioxidant activity

INTRODUCTION

Onions are one of the oldest vegetables in continuous cultivation dating back to at least 4,000 BC. Onion (*Allium cepa* L.) is botanically included in the *Liliaceae* and species are found across a wide range of latitudes and altitudes in Europe, Asia, North America and Africa. World onion production has increased by at least 25% over the past 10 years with current production being around 44 million tonnes making it the second most important horticultural crop after tomatoes **Griffiths et al., (2002)**.

Onions are grown mainly as food materials. They are highly valued for their flavour and for their nutritional value. Onion bulb (red, white or yellow in colour) is consumed in its tender state, raw, ripe, pickled or in form of powder. Onion can grow on most soil types. However, well-drained medium textured soils with pH 6-7 are particularly good for the crop. Flat land enhances good yield. Varieties of onion can differ for pungency, sugar content, disease resistance, seed stem formation, double centers, bulb shape, and bulb size.

Onions have a high content of hydrophilic vitamins (vitamin C, B, A) and a lipophilic vitamin (vitamin D and E). Onion is a source of minerals such as iron, selenium, iodine, potassium, calcium, sulfur, and many others. Onion is characterized by not only rich in vitamins and minerals, but is characterized by a strong content of biologically active substances, especially polyphenolic compounds (flavonoids, quercetin, rutin) and phenolic acids (cinnamic acid derivatives and benzoic acid), fructooligosaccharides (FOS), thiosulfates and other sulfur compounds.

Allium species are referred to possess anti-bacterial and anti-fungal activities, and they contain the powerful antioxidants, sulphur and other numerous phenolic compounds which have aroused great interests for food industries **Benkeblia (2005)**.

Polyphenols are compounds possessing one or more aromatic rings with one or more hydroxyl groups. Polyphenols are secondary metabolites of plants, currently known more than 8000 phenolic structures ranging from simple molecules (phenolic acids) to highly polymerized

substances (tannins). Phenolics are generally involved in protection against ultraviolet radiation, by pathogens, parasites and predators **D'Archivio et al., (2007)**. Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes and lignans **Spencer et al., (2008)**.

Polyphenols are directly involved in the response of plants to different types of stress: they contribute to healing of damaged areas possess antimicrobial properties, and their concentrations may increase after infection (**Parr and Bolwell, 2000**).

Another factor that directly affects the polyphenol content of the foods is storage. Studies have proved that polyphenolic content of the foods change on storage, the reason is easy oxidation of these polyphenols **Manach et al., (2004)**.

Epidemiological studies suggest that long term consumption of diets rich in plant polyphenols offer protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (**Pandey and Rizvi, 2009**). Polyphenols are characteristic in an antioxidant and anti-inflammatory effect.

Antioxidants are vital substances, which possess the ability to protect the body from damages caused by free radical induced oxidative stress. A variety of free radical scavenging antioxidants is found in a number of dietary sources **Qusti et al., (2010)**. The main characteristic of antioxidant is its ability to trap free radicals. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxy and inhibit the oxidative mechanisms that lead to degenerative diseases **Prakash et al., (2007)**. The aim of our study was to evaluate content of total polyphenols and antioxidant activity in selected varieties of onion (*Allium cepa* L.).

MATERIAL AND METHODOLOGY

The experiment was established in the year 2014 in the area of Pružina block methods (seeds of varieties of onion

was planted by hand to 4 lines, size of the experimental flat- 1 m²). Samples of plant material were collected at full maturity stages from area of Pružina. The samples of soil (Table 1) and plant material were analyzed individually by selected methodologies, and we used fresh material for analysis. Pružina is located under the Strážovské hills – Strážov. The attitude of the village is in the middle of 381 m.a.s.l. Pružina belongs to the mild cold climate zone, average annual temperature is 7 °C, annual rainfall is 800-1000 mm. Pružina is area without negative influences, emission sources (carbon), relatively pure from point of view of content permissible forms of risk elements.

We determined the soil samples from Pružina as sandy – loam, loam. The soil samples had a value of active soil reaction pH (H₂O) = 7.95. The soil was strongly alkaline. Cox oxidizable carbon content was 1.46 and the humus content was 2.51. The content of potassium and magnesium was high; the content of phosphorus was very high. The total content of heavy metals (aqua regia) in soil sample was determined according to the current legislation Slovak republic (Slovak decree no. 220/2004 Coll., Annex. 2) Cadmium exceeded the limit value of 1.91-times, Zinc exceeded the limit value 2.03-times.

Sample preparation

Samples of fresh onion were homogenized and we prepared an extract: 25 g cut onion extracted by 50 mL 80% ethanol for sixteen hours. These extracts were used for analysis.

Characteristic varieties of onion:

Red matté is red variety of onion. Red matté can be grown from seed or seedlings. It is suitable for consumption and storage.

Diamond is white variety of onion. It is suitable for consumption, industrial processing and short-term storage.

White dry is white variety of onion, suitable for consumption and short-term storage.

Boston is yellow variety of onion. Boston is suitable for consumption and longer storage.

Sherpa is yellow variety of onion. This variety is suitable for consumption and storage. Sherpa can be grown from seed and seedlings.

Bingo is yellow variety of onion, suitable for consumption and longer storage. Onion is medium, firm and round. The yield of this variety in optimal conditions is large.

Table 1 Agrochemical characteristic of soil substrate in mg.kg⁻¹ (Pružina).

Agrochemical characteristic	pH(H ₂ O)	pH(KCl)	C _{ox} (%)	Humus				
	7.95	7.18	1.46	2.51				
Nutrients	P	K	Ca	Mg				
	259.78	354.40	8049.30	439.60				
Heavy metals	Cd	Pb	Cu	Zn	Cr	Co	Mn	Fe
Content in aqua regia (mg.kg ⁻¹)	1.34	29.40	36.90	305.30	21.00	13.3	636.50	25696.4
Limit value (mg.kg⁻¹)	0.7	70	60	150	70	15	-	-

Note: *Limit value for Aqua regia – Slovak decree no. 220/2004 Coll.

Determination of total polyphenols

Total polyphenols were determined by the method of **Lachman et al. (2003)** and expressed as mg of gallic acid equivalent per kg fresh mater. Gallic acid is usually used as a standard unit for phenolics content determination because a wide spectrum of phenolic compounds. The total polyphenol content was estimated using Folin-Ciocalteau assay (Merck). The Folin-Ciocalteau phenol reagent was added to a volumetric flask containing 100 mL of extract. The content was mixed and 5 mL of sodium carbonate solution (20%) (Sigma Aldrich) was added after 3 min. The volume was adjusted to 50 mL by adding of distilled water. After 2 hours, the samples were centrifuged for 10 min. and the absorbance was measured at 765 nm of wave length against blank (Shimadzu UV/VIS-1240, Japan). The concentration of polyphenols was calculated from a standard curve plotted with known concentration of gallic acid (Sigma Aldrich).

Determination of antioxidant activity

Antioxidant activity was measured by the **Brand and Williams et al., (1995)** method-using a compound DPPH' (2,2-diphenyl-1-pikrylhydrazyl). 2,2-diphenyl-1-pikrylhydrazyl (DPPH') was pipetted to cuvette (3.9 m³) then the value of absorbance, which corresponded to the initial concentration of DPPH' solution in time Ao was written. Then 0.1 cm³ of the followed solution was added

and then the dependence A = f(t) was immediately started to measure. The absorbance of 1, 5 and 10 minutes at 515.6 nm in the spectrophotometer Shimadzu UV/VIS-1240 was mixed and measured. The percentage of inhibition reflects how antioxidant compound are able to remove DPPH' radical at the given time.

$$\text{Inhibition (\%)} = (A_o - A_t / A_o) \times 100$$

Antioxidant activity was measured by the **Brand and Williams et al., (1995)** method-using a radical DPPH' (2,2-diphenyl-1-pikrylhydrazyl) (Merck). 2,2- diphenyl-1- pikrylhydrazyl (DPPH') was pipetted to cuvette (3.9 m³), then the value of absorbance was written, which corresponded to the initial concentration of DPPH' solution in time Ao. Then 0.1 cm³ of onion extract was added. Solution in the cuvette was mixed and then was immediately started to measure the dependence A = f (t). The absorbance after 1, 5 and 10 minutes was measured at 515.6 nm in the spectrophotometer (Shimadzu UV/VIS -1240). The percentage of inhibition reflects how antioxidant compound are able to remove DPPH' radical at the given time. Inhibition (%) = (Ao - At / Ao) x 100

Statistical analysis

Results were statistically evaluated by the Analysis of Variance (ANOVA – Multiple Range Tests, Method: 95.0% LSD) using statistical software STATGRAPHICS (Centurion XVII.I, USA).

Table 2 Average content of total polyphenols (mg.kg⁻¹) in selected varieties of onion.

vegetable	variety	TPC (mg.kg ⁻¹)
onion	Boston	441.32 ±26.29 ^b
	Sherpa	455.22 ±44.86 ^b
	Bingo	451.71 ±38.21 ^b
	Red matté	1083.04 ±56.03 ^c
	Diamond	160.49 ±7.30 ^a
	White dry	142.01 ±15.91 ^a
HD 95%	HD _{0,05}	52.9127
HD 99%	HD _{0,01}	72.4949

Legend: *Multiple Range Tests, Method: 95,0 percent LSD, Different letters (a, b, c,) between the factors show statistically significant differences (p <0.05) – LSD test, TPC- total polyphenols content.

Table 3 Average values of antioxidant activity (% inhibition) in selected varieties of onion

vegetable	variety	AOA (% inhibition)
onion	Boston	25.77 ±0.29 ^d
	Sherpa	24.34 ±0.39 ^c
	Bingo	19.54 ±0.98 ^b
	Red matté	41.67 ±0.70 ^e
	Diamond	7.74 ±0.69 ^a
	White dry	8.10 ±0.31 ^a
HD 95%	HD _{0,05}	0.949236
HD 99%	HD _{0,01}	1.30053

Legend: *Multiple Range Tests, Method: 95.0% LSD, Different letters (a, b, c, d, and e) between the factors show statistically significant differences (p <0.05) – LSD test AOA- antioxidant activity.

RESULTS AND DISCUSSION

Onion is recognized as one of the most important onion vegetable in our diet for its content of polyphenols compounds, antioxidants with beneficial effect on the human body.

Onions are very low in calories and fats. 100 grams carry just 40 calories. However, onions are rich in soluble dietary fiber. Onion is rich source of chromium and mineral manganese, vitamin C, B- complex group of vitamins. It is also good source of antioxidant polyphenols, which has anti-carcinogenic, anti-inflammatory, and anti-diabetic functions.

In this work the content of total polyphenols in selected varieties of onion was evaluated. Total polyphenols content was measured in six varieties of onion, namely in red variety (Red matté), in yellow varieties (Boston, Bingo, Sherpa) and white varieties of onion (Diamond and White dry). Onions are grown and distributed in three colors- white onion (5%), red varieties of onion (8%) and yellow onions (88%).

In the present experiment it was detected, that total polyphenols content in samples ranged from 142.01 mg.kg⁻¹GAE (in white variety of onion- White dry) to 1083.04 mg.kg⁻¹GAE (in red variety of onion- Red matté) (Table 2). Statistically the highest value of total polyphenols (1083.04 mg.kg⁻¹ GAE) was recorded in variety of Red matté. The lowest content of total polyphenols was recorded in white variety of White dry (142.01 mg.kg⁻¹ GAE) and in white variety of Diamond (160.49 mg.kg⁻¹ GAE). In the case of yellow varieties of onion Sherpa (455.22 mg.kg⁻¹ GAE), Bingo (451.71 mg.kg⁻¹GAE) and Boston (441.32 mg.kg⁻¹ GAE) lower levels of polyphenols than in red variety of onion (Red matté) were measured. Our results correspond to the results of **Armand et al., (2012)**, which reported the highest values of total polyphenols in variety of red onion (982.03 mg.kg⁻¹) yellow variety (mg.kg⁻¹) and white variety (280 mg.kg⁻¹). **Andrejiová et al., (2011)** also reported the content of total polyphenols in red onion 1088.51 mg.kg⁻¹, yellow onion 652.15 mg.kg⁻¹ and in white onion 105.19 mg.kg⁻¹. **Karadeniz et al., (2005)** reported that the polyphenols in onion was in the amount 536 mg.kg⁻¹. **Amin et al., (2013)** referred that the content of polyphenols was 132.2 mg.kg⁻¹. **Brat et al., (2006)** published that the content of total polyphenols in onion was 761 mg.kg⁻¹. In comparison to our determined values of polyphenols their results were in similar interval. **Benkeblia et al., (2005)** reported that the highest content of polyphenols was in red onion (473 mg.kg⁻¹), followed by yellow variety (347 mg.kg⁻¹). **Apak et al., (2007)** referred that the content of total polyphenols in yellow onion was 880 mg.kg⁻¹. In comparison to our measured values in yellow onion (455.22 mg.kg⁻¹ GAE – 441.32 mg.kg⁻¹ GAE) their results were higher. **Bystrická et al., (2014)** published that the content of polyphenols in yellow onion was 508.16 – 638.2 mg.kg⁻¹. In comparison to our determined values of polyphenols in yellow onion, their results were in similar interval.

Lu et al., (2011) found similar results in the evaluation of onions of different colors. From the results we can

conclude that the highest content of total polyphenols we measured in variety of Red matté (1083 mg.kg⁻¹ GAE), followed by variety of Sherpa 455.22 mg.kg⁻¹ GAE. In variety of Red matté is the average content of 6.74-times higher than that of the variety Diamond. The lowest content of total polyphenols was recorded in variety of White dry (142.01 mg.kg⁻¹ GAE). Based on the measured values of total polyphenols in onion the samples can be classified as follows: Red matté (1083 mg.kg⁻¹GAE) > Sherpa (455.22 mg.kg⁻¹ GAE) > Bingo (451.71 mg.kg⁻¹ GAE) > Boston (441.32 mg.kg⁻¹ GAE) > Diamond (160.49 mg.kg⁻¹ GAE) > White dry (142.01 mg.kg⁻¹ GAE). The content of polyphenolic compounds in onion also can be affected by the type of variety and color of bulb of onion.

Another indicator that has been evaluated and compared was the antioxidant activity in selected varieties of onion. Antioxidant activity of onion is often associated with L-ascorbic acid and polyphenolic compound such as anthocyanins, quercetin, and rutin. Antioxidant activity was also determined in six varieties of onion (Red matté, Boston, Bingo, Sherpa, Diamond and White dry).

In the present work we found that, antioxidant activity in samples ranges from 7.74% to 41.67%. The DPPH method is frequently used to determine the antioxidant activity. DPPH assay is a primary antioxidant activity test that determines the free radical scavenging activity of the respective samples. Statistically the highest value of antioxidant activity (41.67%) was recorded in red variety- Red matté. The lowest value of antioxidant activity (7.74%) was recorded in white variety- Diamond. In the case of yellow varieties of onion Sherpa (24.37%), Bingo (19.54%) and Boston (25.77%) lower levels of antioxidant activity than in red variety of onion (Red matté) but higher levels antioxidant activity than in white varieties of onion (Diamond, White dry) were measured. **Škerget et al., (2009)** published that the value of antioxidant activity in yellow onion was 35%. In comparison to our measured values in yellow varieties their results were higher. **Prakash et al., (2007)** published that the value of antioxidant activity in red onion was 50.6% and in white onion 13.6%. In comparison to our measured values of antioxidant activity their results were in similar interval. **Nuutila et al., (2003)** reported that the value of antioxidant activity was in interval from 32.9% (yellow onion) to 44.5% (red onion). **Cheng et al., (2013)** determined that red onion extracts showed good antioxidant activity varying from 53.36% to 85.53% and better than in the yellow variety ranging from 52.32% to 72.25%. In comparison to our measured values their results were higher. **Kavalcová et al., (2014)** published that the value of antioxidant activity in yellow onion was 25.7%.

Based on the measured values of AOA in onion can be samples classified as follows: Red matte > Boston > Sherpa > Bingo > White dry > Diamond.

Polyphenols are natural substances in plants that are antioxidants with the potential to protect the human body from diseases.

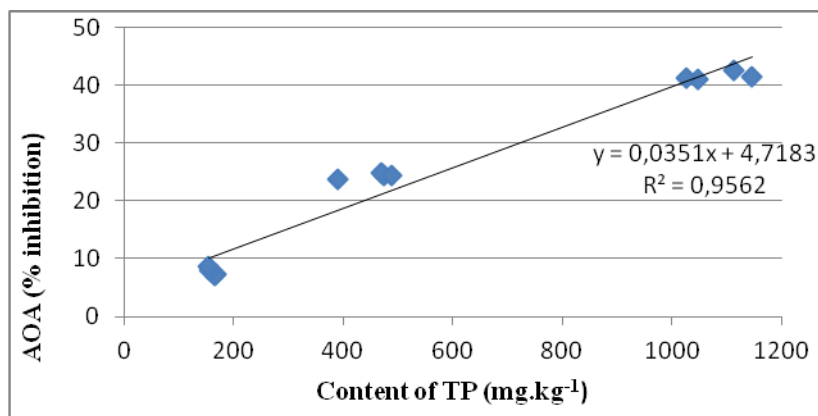


Figure 1 Relationship between TP (total polyphenols) and AOA (antioxidant activity) (red, yellow and white onion).

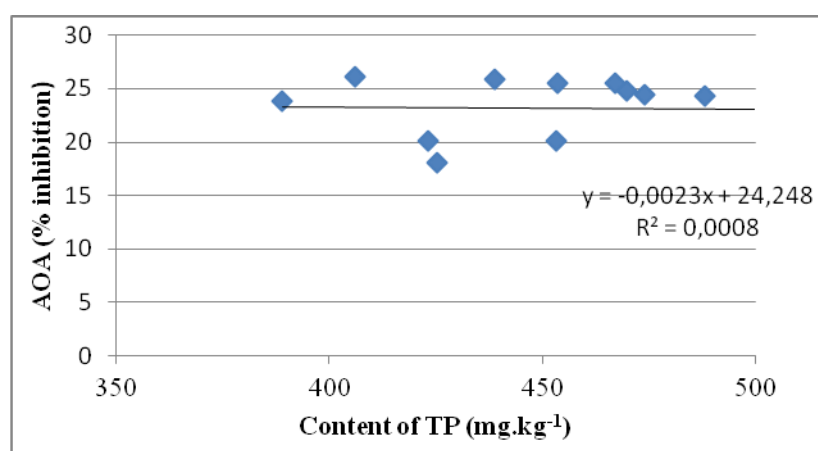


Figure 2 Relationship between TP (total polyphenols) and AOA (antioxidant activity) yellow onions (Sherpa, Bingo, Boston).

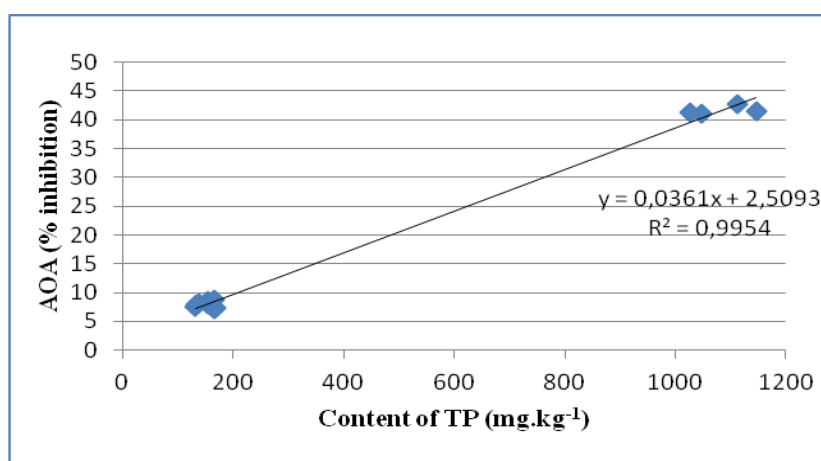


Figure 3 Relationship between TP (total polyphenols) and AOA (antioxidant activity) (Red matte, Diamond and White dry).

Between the content of total polyphenols and antioxidant activity in red, yellow and white onions we have found positive correlation. We can conclude that with increasing the content of total polyphenols also increased antioxidant activity (Figure 1). Several studies have reported a good correlation between the TP content of plant extracts and antioxidant activity. **Lu et al., (2011)** found the positive

relationship between the content of total polyphenols and antioxidant activity. Most of the researches have mentioned that high phenolic content will lead to high radical scavenging activity (**Silva et al., 2007; Tawaha et al., 2007**).

In the case of yellow varieties of onion, we have not found relationship between the content of total

polyphenols and antioxidant activity (Figure 2). Mamamury (2002) also not found positive relationship between TPC and AOA. Antioxidant activity depends not only on the total phenolic content but is significantly influenced by the structure of these phenolic compounds, in particular the position and the number of hydroxyl groups. The antioxidant activity of onion is often associated with a L-ascorbic acid, vitamin E and present polyphenolic compounds such as quercetin, rutin.

Red onion is rich in anthocyanins and yellow onion has high concentration of flavonoids (quercetin, kaempferol). We recorded the highest content of polyphenols and antioxidant activity was in red variety of onion (Red matté) (Figure 1 and Figure 3). Anthocyanins are known to have the most potent antioxidant effects. Kong et al. (2003) reported that for high antioxidant activity in red varieties of onion are responsible anthocyanins.

CONCLUSION

Onions are characterized by a high content of nutritionally valuable components, which are essential for the management of biochemical processes that positively affect the cardiovascular, cancer and other civilization diseases. The contribution focused on the total of polyphenol content and antioxidant activity in selected varieties of onion. The results suggest that statistically the highest value of total polyphenols and antioxidant activity was in red onion. In the case of white varieties of onion, we determined significantly the lowest values of total polyphenols and antioxidant activity. From the results we can conclude that more colorful varieties of onions have a higher content of polyphenols and a higher antioxidant activity. This statement is not always true, because not all polyphenols have antioxidant effects. Polyphenolic compounds in onion are quite variable, may be affected by type of variety, post-harvest, climatic condition, agrochemical composition of soil.

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MYCOBIOTA OF SLOVAK WINE GRAPES WITH EMPHASIS ON *ASPERGILLUS* AND *PENICILLIUM* SPECIES IN THE SMALL CARPATHIAN AREA

Soňa Felšöciová, Dana Tančinová, Ľubomír Rybárik, Zuzana Mašková, Miroslava Kačániová

ABSTRACT

The Slovak wine-growing region is divided into six viticulture areas. The largest in size and the most important over the centuries has been the Small Carpathian area (around 5800 ha of vineyards) spreads in the western of Slovakia. The objectives of this study were: to gain more knowledge about mycobiota on grapes originating from Slovakia, with a focus on genera *Aspergillus* and *Penicillium* and their ability to produce mycotoxins in *in vitro* conditions by thin layer chromatography method. From the twelve vineyards were collected 14 samples of wine grapes (white 6, blue 8) during harvesting 2011, 2012 and 2013. Fifty wine grapes per bunch (approximately 7-8 berries per plate) that showed no symptoms were randomly selected on Dichloran Rose Bengal Chloramphenicol agar medium. The plates were then incubated aerobically at 25 ±1 °C for 5 to 7 days in the dark. Of these samples were identified 22 genera. Ninety-three percent of samples were colonies by the genus *Penicillium* and 79% by the genus *Aspergillus*. During the survey, 251 isolates belonging to 14 *Penicillium* species (*P. aurantiogriseum*, *P. citrinum*, *P. coprophylum*, *P. crustosum*, *P. expansum*, *P. funiculosum*, *P. glabrum*, *P. griseofulvum*, *P. chrysogenum*, *P. oxalicum*, *P. polonicum*, *P. purpurogenum*, *P. roqueforti* and *P. thomii*) and 37 isolates belonging to 7 *Aspergillus* species (*A. clavatus*, *A. flavus*, *A. section Nigri*, *A. ostianus*, *A. parasiticus*, *A. versicolor* and *A. westerdijkiae*) were isolated and identified from exogenous contamination. The main occurring penicillium species of the samples were *P. chrysogenum* (36% Fr), followed *P. crustosum* (29% Fr), *P. griseofulvum* (21% Fr) and *P. expansum* (21% Fr). The main occurring aspergillus species of the samples were *A. section Nigri* (64%). Thirteen potentially toxigenic species were tested for their toxigenic ability. It was confirmed the production of various mycotoxins such as aflatoxin B₁, G₁, citrinin, griseofulvin, patulin, cyclopiazonic acid, penitrem A, roquefortin C and sterigmatocystin. Out of 124 strains, 84% produced at least one mycotoxin.

Keywords: wine grapes; Slovak Republik; fungi; mycotoxin

INTRODUCTION

Grapevine can be attacked by a number of fungi and fungus-like organisms which affect the berries and cause loss of quality and influence the taste of the wine (Pitt and Hocking, 2009). Several fungi are pathogenic to grapevines, infecting the roots, trunk, canes, leaves and berries (Pearson and Goheen, 1988). Fungi which commonly infect berries include the mildew pathogens *Erysiphe necator* and *Plasmopara viticola*, as well as *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Cladosporium* spp., *Penicillium* spp., *Epicoccum* spp. and *Rhizopus* spp. (Bellí et al., 2004; Sage et al., 2002). During maturation, the spoilage agents, *Aspergillus*, *Botrytis*, *Penicillium* and *Rhizopus*, increase their incidence. When the temperature is higher than 37 °C, species in *Aspergillus* section *Nigri*, usually called "black aspergilli", predominate (Valero et al., 2005). At harvest time the conditions are optimal for fungal invasion, especially if physical damage has occurred on berries. From single infected berries the whole cluster may be affected causing mummified clusters covered with green mould *Penicillium expansum*. Green mould produce mycotoxins (Abrunhosa et al., 2001; Serra et al., 2006)

for example patuline which is however degraded during fermentation and by sulphurization. Berries affected by green mould have an off-flavor and even a small amount of infected berries add a mouldy taste to the wine (Kassemeyer and Berkelmann-Löhnertz, 2009). Samson et al., (2004) considered 15 species provisionally accepted in *Aspergillus* section *Nigri*, four of those producing ochratoxin A (OTA) and only two occurring on grapes, raisins and in wine – *Aspergillus carbonarius* and to a lesser extent *A. niger*. Ochratoxin A is the main mycotoxin of concern in grape products. OTA is produced primarily when *Aspergillus carbonarius* infects berries before harvest. The relatively few toxigenic strains of the related species, *Aspergillus niger* is by far the most common species of *Aspergillus* present on grapes (Leong et al., 2007). The aflatoxigenic species, *Aspergillus flavus* and *Aspergillus parasiticus*, have occasionally been isolated from grapes (Sáez et al., 2004). Toxigenic isolates of *Aspergillus ochraceus* have also only occasionally been isolated from grapes. Generally, the colonisation of grape bunches by black aspergilli and other fungi occurs when berry skin damage allows the entry into fruit tissues, where the low pH and high sugar content under aerobic

conditions provide a competitive advantage for moulds. However, fungal invasion may occur without visible symptoms (Bellí et al., 2007).

The aim of our study was to detect mycobiota on grapes and species of genera *Aspergillus* and *Penicillium* and potentially toxigenic producing species tested by thin layer chromatography for the ability to produce selected mycotoxins in *in vitro* conditions.

MATERIAL AND METHODOLOGY

Study area

Twelve vineyards were studied (Modra, Zeleneč, Svätý Martin, Doľany, Dolné Orešany, Dvorníky, Pezinok, Moravany n. Váhom, Gajary, Skalica, Bratislava – Rača) during a 3-year period (2011 – 2013) in Small Carpathian wine-growing region. Slovak republic has 6 distinct wine-growing zones (the Small Carpathians, the Southern Slovak, the Nitra, the Central Slovak, the Eastern Slovak and the Tokaj wine regions). They spread from the west to the east of the country along its southern and south-western borders. The largest in size and the most important over the centuries has been the Small Carpathian area (around 5800 ha of vineyards) spreads in the western of Slovakia. The Small Carpathian wine region is divided to 12 subregions. The subregion is the area with the same soil and climate conditions. Wine-growing zones are defined as geographic regions with distinct climatic conditions for grape cultivation. The Small Carpathian wine-growing region has medium climates and abundant moisture.

Samples

Samples were collected from early September to late October, in the maturation stage harvest. Fourteen samples: 6 of white grape varieties (Sauvignon, 2 x Pinot Blanc, 2 x Green Veltliner, Riesling) and 8 of blue grape varieties (2 x Cabernet Sauvignon, 2 x André, 4 x Blue Frankish) were mycologically analyzed. Three kilograms of samples were collected at the time of technological ripeness. Picked grapes were stored at 4 ± 1 °C and analyzed within 24 h after harvest.

Mycological analysis of grapes

A total of 50 berries (7 – 8 berries per bunch) from each sample were plated in Dichloran Rose Bengal Chloramphenicol agar medium (DRBC) and incubated at 25 ± 1 °C in the dark for one week. The spore-producing filamentous fungi detected were identified to genus level based on morphological characters according to the manual of Pitt and Hocking (2009). Different media were used for the taxonomic identification of obtained fungi according to that used for standard strains. Specifically, *Penicillium* and *Aspergillus* strains were identified down to the species level first using Malt extract agar (MEA) (Pitt and Hocking, 2009), Czapek yeast extract agar (CYA) (Samson et al., 2002a), Czapek yeast extract with 20% sucrose agar (CY20S) (Pitt and Hocking, 2009), Yeast extract agar (YES) (Samson et al., 2010), Creatine-Sucrose agar (CREA) (Samson et al., 2010) and identified to species level according to the manuals of Samson et al., (2002a), Samson and Frisvad (2004), Pitt and Hocking

(2009). The berries from the vineyards sampled were generally in good condition without visible damage.

The obtained results were evaluated and expressed according to isolation frequency (Fr) and relative density (RD). The isolation frequency (%) is defined as the percentage of samples within which the species or genus occurred at least once. The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample (Guatam et al., 2009). These values were calculated according to González et al. (1999) as follows:

$$\text{Fr (\%)} = (\text{ns} / \text{N}) \times 100 ; \text{RD (\%)} = (\text{ni} / \text{Ni}) \times 100$$

Where: ns – number of samples with a species or genus; N – total number of samples; ni – number of isolates of a species or genus; Ni – total number of isolated fungi.

Toxinogenity analysis

Toxinogenity of selected isolates was screened in *in vitro* conditions by means of thin layer chromatography (TLC) according to Samson et al., (2002b), modified by Labuda and Tančinová (2006). Extracellular metabolites – citrinin, patulin, griseofulvin, ochratoxin A, aflatoxin B₁, G₁ were carried out on YES agar and intracellular roquefortin C, penitrem A, cyclopiazonic acid and sterigmatocystin on CYA agar. A few pieces of mycelium with approximate size 5 x 5 mm were cut from colonies and placed in an Eppendorf tube with 500 µL of chloroform:methanol – 2:1 (Reachem, Slovak Republic). The content of the tubes was stirred for 5 min by Vortex Genie® 2 (MO BIO Laboratories, Inc. – Carlsbad, CA, USA). The volume 30 µL of liquid phase of extracts along with 10 µL standards (Sigma, Germany) was applied on TLC plate (Alugram® SIL G, Macherey – Nagel, Germany). The plate was put into TEF solvent (toluene:ethyl acetate:formic acid – 5:4:1, toluene – Mikrochem, Slovak Republic; ethyl acetate and formic acid – Slavus, Slovak Republic). After elution the plate was air-dried. Identification of the metabolites was done by comparison with metabolite standards. Roquefortin C was visible after spraying with Ce(SO₄)₂ x 4 H₂O as an orange spot. Cyclopiazonic acid was visible directly in daylight after spraying with the Ehrlich reagent as a violet-tailed spot. Penitrem A after spraying with 20% AlCl₃ in 60% ethanol and heating at 130 °C for 8 min as a dark blue spot. Patulin by spraying with 0.5% methylbenzothiazolone hydrochloride (MBTH), (Merck, Germany) in methanol and heating at 130 °C for 8 min and then detectable as a yellow-orange spot. Directly under UV light with a wavelength of 365 nm was visualized citrinin as a yellow-green-tailed spot, griseofulvin as a blue spot, ochratoxin A as a blue-green spot, aflatoxin B₁ as a blue spot, aflatoxin G₁ as a green-blue spot and sterigmatocystin as a reddish spot.

The filamentous fungi identified from samples by the direct plating method are indicated in Table 1. Without surface disinfection, a total of 2774 strains belonging to 20 genera were identified. The three most abundant genera found by descending order were *Alternaria* (42%), *Cladosporium* (33%) and *Penicillium* (9%). *Epicoccum*, *Botrytis*, *Fusarium*, *Rhizopus*, *Trichoderma* were detected in more than 1% of the berries analyzed. The remaining 12 genera were detected in less than or equal to 1% of the berries. The genus *Alternaria*, *Cladosporium*, *Fusarium*,

Rhizopus, *Trichoderma* colonised 100% of samples, followed *Epicoccum*, *Penicillium* (93%, each) and *Aspergillus* (79%). In our study was also unidentified *Mycelia sterilia* without creation fruiting bodies.

RESULTS

The *Aspergillus* and *Penicillium* strains were isolated and identified to species level. The isolation rates for *Aspergillus* from the berries were 79%. The relative densities were low (Table 1). Table 2 shows the number of isolates and isolation frequency (%) of *Aspergillus* spp. The species of *Aspergillus* section *Nigri* were the predominant in mycobiota. The species of *A. clavatus* and *A. flavus* were the other most important species recorded with high isolation frequency.

The incidence of *Penicillium* species on agar DRBC revealed the occurrence of 13 different *Penicillium* species (Table 3) with high frequency – 93% (Table 1). The relative density was 9%. From the 251 *Penicillium* strains identified, the most frequent were *Penicillium chrysogenum* (64%), *P. crustosum* (12%) and *P. griseofulvum* 8% of the isolates. Isolation frequency among species was maximum for *P. chrysogenum* (36%), *P. crustosum* (29%), *P. expansum* and *P. griseofulvum* (21%, each).

In total 124 isolates representing 13 potentially toxigenic species were tested for their toxigenic ability (Table 4). Out of 124 strains, 84 % produced at least one mycotoxin as revealed by the method used here. Positive toxigenity

was detected in *A. clavatus*, *A. parasiticus*, *P. crustosum* and *P. chrysogenum*. *Aspergillus flavus* produced aflatoxin B₁ and cyclopiazonic acid (CPA, 2 out of 5 strains screened, each) but did not produce aflatoxin G₁. *Aspergillus ostianus* produced sterigmatocystin and did not produce OTA and citrinin. Ochratoxin A production was tested also in 7 strains belonging to *Aspergillus* section *Nigri*. Among them, the production of ochratoxin A was not confirmed. *Penicillium citrinum* produced citrinin (1 out of 2), *Penicillium expansum* produced roquefortin C (RC), patulin (3 out of 5) and citrinin (two out of 5), *P. griseofulvum* produced CPA, RC, griseofulvin (12 out of 13) and patulin (10 out of 13). Negative toxigenity was detected in *A. versicolor*, *P. coprophilum* and *P. roqueforti*.

DISCUSSION

The native mycobiota of seven grape varieties (14 samples) grown in Small Carpathian wine-growing region (Slovak Republic) has been studied. We used plating methods without surface disinfection to detect the sporulating fungi colonizing the grape surface. Most of the fungi found are ubiquitously distributed, such as the field fungi *Alternaria* and *Cladosporium*, which occur commonly in the air, plant surfaces, debris and soil. *Alternaria*, *Cladosporium* and *Penicillium* occurred with high isolation frequency and relative density. **Magnoli et al., (2003)** showed that *Alternaria* (80% of the samples), *Aspergillus* (70%), *Cladosporium* (40%) and *Penicillium*

Table 1 Fungi identified in Slovak wine grapes from 2011 to 2013 by the direct plating method.

Fungal taxa	No.	Fr (%)	RD (%)
<i>Absidia</i>	1	7	<1
<i>Acremonium</i>	4	7	<1
<i>Alternaria</i>	1180	100	42
<i>Arthrinium</i>	3	21	<1
<i>Aspergillus</i>	37	79	1
<i>Botrytis</i>	59	71	2
<i>Cladosporium</i>	901	100	33
<i>Epicoccum</i>	90	93	3
<i>Fusarium</i>	59	100	2
<i>Gibberella</i>	3	7	<1
<i>Geotrichum</i>	1	7	<1
<i>Chaetomium</i>	1	7	<1
<i>Mucor</i>	25	43	1
<i>Nigrospora</i>	1	7	<1
<i>Penicillium</i>	251	93	9
<i>Phoma</i>	2	14	<1
<i>Rhizopus</i>	43	100	2
<i>Trichoderma</i>	54	100	2
<i>Ulocladium</i>	1	7	<1
<i>Mycelia sterilia</i>	58	79	2
Total isolates	2774		

Note: No. – number of isolates, Fr – isolation frequency, RD – relative density.

(30%) species were the predominant microfungi in harvested grapes from Argentina. In Spanish wine grapes the most prevalent reported genera were *Alternaria*, yeasts, *Aspergillus*, *Cladosporium*, *Rhizopus* and *Penicillium* (Belli et al., 2006).

Also, in some other research from Tunisia, *Aspergillus* (33%), *Botrytis* (23%), *Alternaria* (13%), *Cladosporium* (11%) and *Penicillium* (8%) have been isolated as the most frequent fungi genus (Fredj et al., 2007). In three-year a study, Serra et al., (2006) investigated the fungal species present on the surface of grape berries from Portuguese vineyards in four winemaking regions. According to their results, in more humid climates, *Botrytis* was the main pathogen and spoiling agent, and the incidence of black *Aspergillus* was minimal. *Alternaria*, *Botrytis*, *Cladosporium* and *Penicillium* were four of the most frequent genera in all the regions. *Botrytis*, *Cladosporium* and *Penicillium* were also reported as the predominant mycobiota by Abrunhosa et al., (2001) in Portugal. In 41

samples of grape fruits grown in Eastern Spain the most infected samples were *Cladosporium*, *Alternaria* and *Aspergillus* section *Nigri* (Sáez et al., 2004).

Certainly the *Aspergillus* species are present worldwide, in all the grape products and under all environmental conditions, most frequent in warmer regions and heat-generating substrates (Somma et al., 2012). Our results agree with this because this genus represented 1% of all the fungi found in the region so the occurrence of *Aspergillus* spp. in our samples was generally low. *Aspergillus* section *Nigri* were the most prevalent, followed by *A. clavatus* and *A. flavus*. The isolation frequency of *Aspergillus* section *Nigri* in our contaminated samples was 64% and relative density 40% in non-disinfected grapes. From the thirteen samples of wine grapes in Czech Republic, a Slovak neighbouring country, Ostrý et al., (2007) were not found ochratoxigenic microfungi, e. g. *Aspergillus carbonarius*, and other species of section *Nigri*, *A. ochraceus*, *Penicillium*

Table 2 *Aspergillus* species identified in Slovak wine grapes from 2011 to 2013 by the direct plating method.

<i>Aspergillus</i> species	No.	Fr. (%)
<i>A. clavatus</i>	7	21
<i>A. flavus</i>	6	21
<i>A. section Nigri</i>	15	64
<i>A. ostianus</i>	1	7
<i>A. parasiticus</i>	1	7
<i>A. versicolor</i>	1	7
<i>A. sp.</i>	6	43
Total isolates	37	

Note: No. – number of isolates, Fr – isolation frequency.

Table 3 *Penicillium* species identified in Slovak wine grapes from 2011 to 2013 by the direct plating method.

<i>Penicillium</i> species	No.	Fr. (%)
<i>P. aurantiogriseum</i>	2	7
<i>P. citrinum</i>	3	14
<i>P. coprophylum</i>	1	7
<i>P. crustosum</i>	30	29
<i>P. expansum</i>	17	21
<i>P. funiculosum</i>	1	7
<i>P. glabrum</i>	1	7
<i>P. griseofulvum</i>	21	21
<i>P. chrysogenum</i>	160	36
<i>P. oxalicum</i>	3	7
<i>P. purpurogenum</i>	2	14
<i>P. roqueforti</i>	1	7
<i>P. thomii</i>	3	14
<i>P. sp.</i>	6	29
Total isolates	251	

Note: No. – number of isolates, Fr – isolation frequency.

Table 4 Toxinogenicity of selected strains, isolated from exogenous mycobiota of wine grapes.

Species	AFB ₁	AFG ₁	OTA	C	G	P	CPA	PA	RC	STER
<i>A. clavatus</i>						5/5				
<i>A. flavus</i>	2*/5**	0/5					2/5			
<i>A. ostianus</i>			0/1	0/1						1/1
<i>A. parasiticus</i>	1/1	1/1								
<i>A. section Nigri</i>			0/7							
<i>A. versicolor</i>										0/1
<i>P. citrinum</i>				1/2						
<i>P. coprophilum</i>					0/1				0/1	
<i>P. crustosum</i>								14/14	14/14	
<i>P. expansum</i>				2/5		3/5			5/5	
<i>P. griseofulvum</i>					12/13	10/13	13/13		13/13	
<i>P. chrysogenum</i>									68/68	
<i>P. roqueforti</i>						0/1			0/1	

Note: * - number of isolates with ability to produce mycotoxin, ** - number of tested isolates, AFB₁,G₁ – aflatoxin B₁,G₁, OTA – ochratoxin A, C – citrinin, G – griseofulvin, P – patulin, CPA – cyclopiazonic acid, PA – penitrem A, RC – roquefortin C, STER – sterigmatocystin.

verrucosum and *P. nordicum*. Occurrence of *Aspergillus* spp. in grapes from Slovakia was surveyed during 2 years 2008 and 2009 by Mikušová et al., (2012). A large number of *Aspergillus* spp., including *A. flavus*, *A. japonicus*, *A. niger*, *A. carbonarius* and *A. ibericus* were identified.

Penicillium is a common component of the grapes mycobiota. This genus is ubiquitous saprophyte whose conidia are easily distributed in the atmosphere (Serra et al., 2006). *Penicillium* is described as being frequent in soils and temperate regions. *Penicillium* was more frequent than *Aspergillus* in all our samples. Isolation frequency among *Penicillium* species was maximum for *P. chrysogenum* (36%), followed by *P. crustosum* (29%), *P. expansum* and *P. griseofulvum* (21%, each). Cabañes et al., (2002) found *Penicillium purpurogenum* in all samples of the white Garnacha grape variety that they studied. Their samples were from Tarragona, Spain. *Penicillium purpurogenum* was isolated in our samples, however in low relative density (1%). Magnoli et al., (2003) found among *Penicillium* spp. *P. chrysogenum*, as the most frequent species isolated in 22% of the samples what correspond with our results. The most frequent *Penicillium* species in grape berries from Portuguese vineyards in four winemaking regions were *P. brevicompactum*, *P. thomii* and *P. glabrum/spinulosum* which together accounted for approximately 71% of the strains identified in the genus (Serra et al., 2006). In our study we isolated them by only in a low density. *Penicillium thomii* represented 1% of the isolates and *P. glabrum* 0,4%. The genera *Penicillium* (present in the range 27 – 54%) was predominant in harvest time in grapes from Slovakia, and it was represented by *P. brevicompactum*, *P. chrysogenum*, *P. crustosum*, *P. expansum*, *P. palitans*, *P. polonicum*, *P. verrucosum*, *P. citrinum* and *P. glabrum* (Mikušová et al., 2012). Most of them also were isolated from our samples. It should be noted that despite the differences in geographic location, the varieties studied by the different

authors were different as well, which could explain the disagreement of the results found among the samples.

Grapes that are heavily infected with moulds alter in chemical composition and secondary metabolites such as mycotoxins. These mycotoxins of greatest significance in grapes and grape products produced by *Aspergillus* and *Penicillium* spp., include ochratoxin A, aflatoxins, patulin and citrinin (Magnoli et al., 2003). Mycotoxins such as aflatoxin, patulin and citrinin are less common than ochratoxin A in grape and grape products. Ochratoxin A is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties, which has received growing interest from the scientific community and food committees in the last few years (Chulze et al., 2006). Ochratoxin A is a kidney toxin and probable carcinogen (Varga and Kozakiewicz, 2006). According to studies ochratoxin A producing strains from the group *Aspergillus* section *Nigri* (*A. carbonarius* and *A. niger* aggregate) are the source of ochratoxin A in wines, grapes and dried vine fruits (Hocking et al., 2007). Ochratoxin A is produced primarily when *A. carbonarius* infects berries before harvest. *Aspergillus niger* may also contribute to ochratoxin A contamination. It is by far the most common species of *Aspergillus* present on grapes (Chulze et al., 2006). Toxigenic isolates of *A. ochraceus* have only occasionally been isolated from grapes (Abrunhosa et al., 2001). *Penicillium verrucosum* and *P. nordicum*, the only confirmed *Penicillium* species that are able to produce OTA, were not isolated. Ochratoxin A producers in grapes were isolated in 24.74% in the year 2008 and only one species *Aspergillus niger* (7.01%) in the year 2009 in South Slovak region by Mikušová et al., (2012). The higher presence of *Aspergillus carbonarius* (9.68%) was observed only in year 2008. However, their results confirmed a low production of the OTA, what indicates that there is low risk of OTA contamination of Slovak wine. This conclusion agrees with reported survey data, where OTA has been detected in less than 50% of analysed

wine samples, however wines produced and sold in Slovakia had lower level of OTA than imported wines and OTA concentrations found were far below the proposed European limit of 2 µg.L⁻¹ (Belajová and Rauová, 2007).

Aflatoxins are potent carcinogens, produced by *Aspergillus flavus* and *A. parasiticus* (Pitt, 2000). Aflatoxins and aflatoxin producing strains (Fredj et al., 2007) have been detected in wine and must occasionally, as reported in Lebanon and Turkey (El Khoury et al., 2008). So far, aflatoxin contamination in the grape and wine product chains does not seem to be a real risk for human and animal health. Aflatoxins may occur as common contaminants of dried vine fruits in some countries, i.e. Iran (Feizy et al., 2012), Egypt (Youssef et al., 2000) and Greece (Kollia et al., 2013) at very high levels. The strain of *A. parasiticus* isolate from our study was able to produce *in vitro* aflatoxins B₁ and G₁. Other toxigenic species *A. flavus* produced AFG₁ (2 out of 5) but not produced AFB₁.

Citrinin, a hepato-nephrotoxic compound, also has been detected in grapes before storage (Bragulat et al., 2008). It is produced by different species of *Penicillium*, *Aspergillus* and *Monascus*. Citrinin producing strains *A. ostianus*, *P. citrinum* and *P. expansum* were isolated from our Slovak samples.

Patulin can occur in many moldy fruits including grapes. Patulin causes gastrointestinal problems, skin rashes, and is known to be mutagenic (Abrunhosa et al., 2001). Patulin has been demonstrated to be acutely toxic (Dailey et al., 1977), genotoxic (Alves et al., 2000), teratogenic (Dailey et al., 1977), and possibly immunotoxic (Escuela et al., 1988) to animals. Patulin production was confirmed by *P. expansum* and *P. griseofulvum*. The production of patulin by *P. roqueforti* was not confirmed. Tančinová et al., (2015) analyzed 47 samples of grapes, harvested in 2011, 2012 and 2013 from various wine-growing regions. The potential producers of patulin were isolated from 23 samples berries, 19 samples of surface-sterilized berries and 6 samples of grape juice. Overall, the representatives of producers of patulin were detected in 32 (68.1%) samples (75 isolates). The ability to produce patulin in *in vitro* condition was detected in 82% of isolates of *Penicillium expansum*, 65% of *Penicillium griseofulvum* and 100% of *Aspergillus clavatus*.

Penicillium chrysogenum may produce a very wide range of toxic compounds – roquefortine C, meleagrins and penicillins. These metabolites could be considered as a potential hazard to human health (Samson et al., 2002a). We tested 68 strains on roquefortine C from exogenous mycobiota which all were positive.

CONCLUSION

Grapes were analyzed by plating methods from Small Carpathian wine-growing region at the harvest time between 2011 and 2013. From the 4463 strains detected and identified from exogenous mycobiota, the most frequent genera were *Alternaria*, *Cladosporium* and *Penicillium*. Potentially toxigenic *Aspergillus* and *Penicillium* species were tested for their toxigenic ability by thin layer chromatography. Out of 124 exogenous strains representing 7 potentially toxigenic species, 84% produced at least one mycotoxin. Potential producers of

ochratoxin A *Aspergillus* section *Nigri* and roquefortin C *Penicillium chrysogenum* were the most frequent mycotoxigenic species isolated from grapes. In line with the results on OTA content of Slovak grapes, it appears that the mycotoxin does not present a significant hazard to consumers.

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EVALUATION OF DRIED SALTED PORK HAM AND NECK QUALITY

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ABSTRACT

The aim of the present study was analysed chemical and physical parameters of dried salted pork ham and neck. Dry-cured meat is a traditional dry-cured product obtained after 12 – 24 months of ripening under controlled environmental conditions. Ham and neck was salted by nitrite salt mixture during 1 week. Salted meat products were dried at 4 °C and relative humidity 85% 1 week after salting. The quality of dry-cured meat is influenced by the processing technology, for example length of drying and ripening period. The average moisture of dried salted pork ham was 63.77% and dried salted pork neck was 59.26%. The protein content was 24.87% in dried salted pork ham and significantly lower (20.51%) in dried salted pork neck. The value of intramuscular fat in dried salted pork ham was 4.97% and 14.40% in dried salted pork neck. The salt content was 5.39% in dried salted pork ham and 4.83% in dried salted pork neck. The cholesterol content was 1.36 g.kg⁻¹ in dried salted pork ham and significant lower in dried salted pork neck (0.60 g.kg⁻¹). The value of lightness was 44.36 CIE L* in dried salted pork ham and significantly lower in dried salted pork neck (40.74 CIE L*). The pH value was 5.84 in dried salted pork ham and 5.80 in dried salted pork neck. The shear work was 9.99 kg.s⁻¹ in dried salted pork ham and 6.34 in dried salted pork neck. The value of water activity (a_w) was 0.929 in dried salted pork ham and similar 0.921 in dried salted pork neck.

Keywords: chemical and physical parameters; cholesterol content; color; water activity; dried meat

INTRODUCTION

Variation in technological properties of meat largely depends on the productive conditions of pig (including genetics and feeding) and processes of muscle conversion into meat (Čandek-Potokar and Skrlep, 2011) and it is an important drawback in the commercial setting.

The pork meat quality is defined as a combination of different characteristics of raw and processed meat (Joo et al., 2013). These characteristics relate to acceptability for technological and consumers' aspects, such as color, water-holding capacity (WHC) and texture. Biochemical processes take place in the muscle *post mortem* affect all of these characteristics. The result of these biochemical changes is influenced by pH value, which is considered as one of the most important factors determining the meat quality (Van der Wal et al., 1997).

The demand for livestock products has rapidly increased in recent times as a result of the income and population growths, as well as along with changing food preferences (FAO, 2013). However, consumers' demands meat products with high quality, safety, health benefits, palatability and convenience.

However, processing of the muscle into final products for instance, ready-to-eat products with high nutritional value, typical palatability and convenience to use is necessary to add value to this muscle far beyond its usual profitability. So far, dry-cured meat is one of the most popular meat products processed mainly from pork muscle, and has been

received considerable attention by consumers due its palatability and typical flavour (Muriel et al., 2004a; Ventanas et al., 2007). The dry-cured meat is produced by salting a mixture of curing agents (salt and nitrite) and other additives, drying and ripening. The ripening process product becomes partially dried and develops its typical aroma and taste characteristics due to the biochemical changes. The quality characteristics of dry-cured meat products as a whole are greatly affected by a number of factors related to the raw material and processing technology (Andres et al., 2004; Jurado et al., 2007).

The ripening process of dry-cured meat products involves complex chemical and biochemical changes in the main components of raw meat (proteins and lipids) which lead to the generation of volatile compounds with olfaction thresholds and distinct aromatic notes (Ruiz et al., 2002).

The acceptance of dry-cured meat by consumers is mainly determined by their sensory properties. The flavour is probably the most important quality parameter and it is affected by raw material, processing techniques, and ripening time (Sánchez-Peña et al., 2005). Processing has an important influence on the final flavour of dry-cured meat. The ripening and drying phases involve complex chemical and biochemical changes in the main components of the raw meat (proteins and lipids) leading to the generation of volatile compounds, which are mainly esters and sulphide compounds (Lorenzo, 2014a; Muriel et al., 2004b). These compounds are responsible of the

characteristic flavour of these products and they have influence on the consumer acceptance (Ruiz et al., 2002).

Sodium chloride (NaCl) is the most important ingredient in the manufacturing process of dry-cured meat for its contribution to the water-holding capacity, prevention of microbial growth, reduction of water activity, facilitating the solubilisation of certain proteins and conferring a typical salty taste (Lorenzo, 2014b). Moreover, salt affects some chemical and biochemical reactions such as proteolysis, lipolysis and lipid oxidation which contribute to the development of texture and typical flavour of dry-cured meat (Lorenzo, 2014b).

In *m. longissimus dorsi* the protein content ranged from 21.23 until 22.34 g.100 g⁻¹ (Haščik et al., 2011).

Intramuscular fat of dry-cured hams contributes to flavour and odour perception through different mechanisms (lipid oxidation, Maillard reactions etc.) involved in volatile compounds formation (Ruiz-Carrascal et al., 2002). Intramuscular fat also plays an important role in the perception of the texture of dry-cured hams, particularly in juiciness, since these products are strongly dehydrated and the contribution of moisture to the perception of this attribute is limited (Ventanas et al., 2005). Fat stimulates the saliva secretion and contributes directly to juiciness by coating the tongue, teeth and other parts of the mouth acting as a lubricant agent (Lynch et al., 1993).

The quality of dry-cured meat is also influenced by the processing technology (for example length of drying and ripening period). Practically, the product, after draining ripen for several months and is decreased the water activity (a_w) below 0.90 (Soto et al., 2008). The ripening time may cause an increase in weight loss which affects the quality characteristics and nutritional value as well as production cost of dry-cured meat.

The color is a significant parameter of the meat quality. It is one of the most important parameter which influence evaluation of meat (Valous et al., 2010). Measurement and evaluation of color can be done with determining the L^* , a^* , b^* values in CIELAB color parameter and computer image analysis (Du and Sun, 2004). The most often used methods of detection of ripening meat process are pH measurement in combination with measurement of the color of meat in the CIEL*a*b* system (Lesiów and Xiong, 2013). By Scheier et al., (2013) the color (L^* - value) influences the consumers purchasing decision more than any other quality factor.

The aim of this article was to determine chemical and physical parameters of dried salted pork ham and neck.

MATERIAL AND METHODOLOGY

Ham (14 samples) and neck (14 samples) was salted by nitrite salt mixture during 1 week (dry salting). Salt mixture contains salt, dextrose, stabilizer E316, maltodextrin, taste enhancer E621, flavourings, nitrite mixture. The weight of samples was approximately 1 kg and they were dried at 4 °C and relative humidity 85% 1 week after salting.

Chemical composition analysis

The chemical composition of the neck and ham was measured (50 g) by the FT IR method using the device Nicolet 6700 (Thermo Scientific, USA). The total proteins in g.100g⁻¹, the intramuscular fat in g.100g⁻¹, total water in

g.100g⁻¹ and cholesterol (g.kg⁻¹) were analysed. The infrared spectrum of the muscular homogenate analysis was carried out by the molecular spectroscopy method. The principle of this method is the absorption of the infra-red spectrum during the sample transition. There is a change of the rotary vibrating energetic conditions of the molecule depending on the changes of the dipole momentum molecule.

Determination of salt (NaCl)

Samples of approximately 2 g with 2 mL of indicator potassium chromate were titrated by solution of silver nitrate until a light orange color. The amount of silver nitrate was divided by weight of sample.

Water activity determination (a_w)

Water activity (a_w) of the dried ham and neck was determined at 25°C with a measuring device FA-st lab (GBX advanced technology, Switzerland). Calibration was done by using several saturated solutions of known a_w .

Colour determination

Color parameters of the salted and dried ham and neck were measured using the spectrophotometer CM-2600d Minolta Chroma Meter CR-400 (Minolta Camera Co., Ltd., Osaka, Japan). Color was expressed according to the Commission International de l'Eclairage (CIE) system and reported as CIE L^* (lightness), CIE a^* (redness), CIE b^* (yellowness).

Shear work measurements (W – B)

Shear force was measured by device TA XT2 plus (Stable Micro Systems, United Kingdom).

Measurement of pH value

The pH value of dried ham and neck at different ripening periods was measured using a pH meter Gryf 209L (Sigma-Aldrich, Czech Republic).

Statistical analyse

The data were subjected to statistical analysis using the Statistic Analysis System (SAS) package (SAS 9.3 using of application Enterprise Guide 4.2). Differences between groups were analysed by t-test.

RESULTS AND DISCUSSION

Dry-cured ham is a traditional dry-cured meat product obtained after 12 – 24 months of ripening under controlled environmental conditions (Dall'asta et al., 2010), at present time is the tendency of shortening the aging time.

Chemical and physical parameters of dried salted pork ham and neck were analysed in this article.

The average moisture of dried salted pork ham was 63.77% and ranged from 61.06 to 67.32%. The average moisture of dried salted pork neck was 59.26% and ranged from 53.75 to 65.68% (Table 1 and Table 2). The moisture was significantly lower in neck in comparison with ham.

Benedini et al., (2012) found out opposite our results in ham lower moisture 61.2% in dried salted *biceps femoris*. The average protein content was 24.87% in dried salted pork ham and significantly lower (20.51%) in dried salted pork neck. The protein content ranged from 23.86% to

25.63% in dried salted pork ham and from 18.92 to 21.76% in dried salted pork neck.

Benedini et al., (2012) found out opposite our results protein content 27.00% in dried salted *biceps femoris*.

Lorido et al., (2015) found out opposite our results higher content of proteins (39.26%) in *semimembranosus*, but in lower moisture content (40.84%).

The average value of intramuscular fat in dried salted pork ham was 4.97% and 14.40% in dried salted pork neck. The differences between values in intramuscular fat were significant and they related to the different content of fat in the raw meat. **Lorido et al., (2015)** found out higher content of intramuscular fat (10.62%) in *semimembranosus*.

The average salt content was 5.39% in dried salted pork ham and 4.83% in dried salted pork neck. The salt content ranged from 4.50 to 5.94% in dried salted pork neck. The salt content in the dried salted pork ham was more

variable (3.56 – 7.35%). **Lorido et al., (2015)** found out similar content of salt (4.38%) in *semimembranosus*. The average cholesterol content was 1.36 g.kg⁻¹ in dried salted pork ham and significant lower in dried salted pork neck (0.60 g.kg⁻¹).

The cholesterol content ranged from 0.73 to 1.93 g.kg⁻¹ in dried salted pork ham and from 0.38 to 1.23 g.kg⁻¹ in dried salted pork neck.

The average value of lightness was 44.36 CIE L* in dried salted pork ham and significantly lower in dried salted pork neck (40.74 CIE L*). The lightness in the dried salted pork ham ranged from 34.68 to 55.27 CIE L* and in dried salted pork neck ranged from 36.06 to 45.49 CIE L* (Table 3).

The average value of redness was 10.02 CIE a* in the dried salted pork ham and significantly higher in dried salted pork neck (14.45 CIE a*). The redness in the dried salted pork ham ranged from 8.08 to 12.49 CIE a* and in

Table 1 The content of moisture, dry mater and proteins in pork ham and neck.

Parameters		Moisture (%)	Dry mater (%)	Proteins (%)
ham	x	63.77	36.23	24.87
	s	2.12	2.12	0.71
	S _x	0.80	0.80	0.27
	min.	61.06	32.68	23.86
	max.	67.32	38.94	25.63
	v%	3.32	5.85	2.83
neck	x	59.26	40.74	20.51
	s	4.55	4.55	0.91
	S _x	1.72	1.72	0.34
	min.	53.75	34.32	18.92
	max.	65.68	46.25	21.76
	v%	7.67	11.16	4.43
	t-test	+	+	+++

Table 2 The content of intramuscular fat, salt and cholesterol in pork ham and neck.

Parameters		Intramuscular fat (%)	Salt (%)	Cholesterol (g.kg ⁻¹)
ham	x	4.97	5.39	1.36
	s	2.50	1.23	0.44
	S _x	0.77	0.46	0.17
	min.	3.20	3.56	0.73
	max.	9.15	7.35	1.93
	v%	41.18	22.73	32.69
neck	x	14.40	4.83	0.60
	s	4.79	0.69	0.29
	S _x	1.81	0.26	0.11
	min.	7.89	4.50	0.38
	max.	20.92	5.94	1.23
	v%	33.29	14.29	48.09
t-test	+++	-	++	

dried salted pork neck ranged from 11.71 to 16.32 CIE a*.

Compared with our results, those of Alino et al., (2010) found lower CIE a* values (5.7 to 6.3) in dry-cured meat processed with different ripening at 60 days. In general, redness is considered as one of the most attractive color parameters of cured meat products (Honikel, 2008), however, the extended ripening time up to 90 days did not improve the redness, suggesting that the ripening beyond 60 days may not be effective in improving redness of dry-cured loins.

The average value of yellowness was 9.22 CIE b* in the dried salted pork ham and 9.75 CIE b* in dried salted pork neck. The yellowness in the dried salted pork ham ranged from 7.44 to 11.90 CIE b* and in dried salted pork neck

ranged from 8.05 to 10.49 CIE b*.

The average pH value was 5.84 in dried salted pork ham and 5.80 in dried salted pork neck (Table 4). The pH value ranged from 5.80 to 5.93 in dried salted pork ham and from 5.67 to 5.98 in dried salted pork neck. The pH values of both products showed that the meat has not been ripened.

Several studies have shown that pH is a good predictor of the colour and drip loss of meat. Bednářová et al., (2014) measured pH of *semimembranosus*. They found out pH values in range from 5.56 to 5.63. There is a high relationship between pH and moisture diffusivity and mechanical and sensory textural properties in salted or dry-cured meat (Gou et al., 2002, Guerrero et al., 1999 and Ruiz-Ramirez et al., 2006).

Table 3 Color results of dried salted pork ham and neck.

Parameters		CIE L*	CIE a*	CIE b*
ham	x	44.36	10.02	9.22
	s	6.71	1.59	1.80
	S _x	2.54	0.61	0.68
	min.	34.68	8.08	7.44
	max	55.27	12.49	11.90
	v%	15.12	15.89	19.51
	neck	x	40.74	14.45
s		3.18	1.41	1.02
S _x		1.20	0.54	0.39
min.		36.06	11.71	8.05
max		45.49	16.32	11.30
v%		7.82	9.80	10.49
t-test		+	+	-

(Note: $p > 0.05$; + $p \leq 0.05$).

Table 4 Physical parameters of dried salted pork ham and neck.

Parameters		pH	Shear work (kg.s ⁻¹)	Water activity (a _w)
ham	x	5.4	9.99	0.929
	s	0.05	6.48	0.011
	S _x	0.02	2.45	0.004
	min.	5.80	3.81	0.910
	max	5.93	20.88	0.939
	v%	0.89	64.83	1.226
	neck	x	5.80	6.34
s		0.10	1.86	0.021
sx		0.04	0.70	0.008
min.		5.67	4.19	0.897
max		5.98	8.76	0.947
v%		1.79	29.33	2.379
t-test		-	-	-

(Note: $p > 0.05$; + $p \leq 0.05$; ++ $p \leq 0.01$; +++ $p \leq 0.001$).

The average shear work was 9.99 kg.s⁻¹ in dried salted pork ham and 6.34 in dried salted pork neck. The shear work ranged from 3.81 to 20.88 kg.s⁻¹ in dried salted pork ham and from 4.19 to 8.76 kg.s⁻¹ in dried salted pork neck. **Franci et al., (2007)** found out higher shear work opposite our results (ranged from 13.69 to 13.71 kg.cm⁻²).

The average value of water activity (a_w) was 0.929 in dried salted pork ham and similar 0.921 in dried salted pork neck. The water activity value ranged from 0.91 to 0.939 in dried salted pork ham and from 0.897 to 0.947 in dried salted pork neck. **Bjarnadottir et al., (2015)** found out opposite our results lower water activity in dried ham (0.809 to 0.906 a_w).

CONCLUSION

The aim of this article was to determine chemical and physical parameters of dried salted pork ham and neck. The moisture was significantly lower in neck in comparison with ham. The protein content in dried salted pork ham was significantly higher in comparison with dried salted pork neck. The value of intramuscular fat in dried salted pork ham was significantly lower in comparison with dried salted pork neck. The content of salt was higher in dried salted pork ham compared to dried salted pork neck. The cholesterol content in dried salted pork ham was significant lower in comparison with dried salted pork neck. The lightness was significantly higher in dried salted pork ham in comparison with dried salted pork neck. The pH value was similar in dried salted pork ham as in dried salted pork neck. The shear work in dried salted pork ham was higher compared to dried salted pork neck. The value of water activity (a_w) was similar in dried salted pork ham as in dried salted pork neck.

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DEPENDENCE AMONG TOTAL POLYPHENOLS CONTENT, TOTAL ANTIOXIDANT CAPACITY AND HEAVY METALS CONTENT IN POTATOES

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ABSTRACT

Polyphenols belong to the most significant compounds with antioxidant effects in potatoes. Their content depends on several factors. The most important factor is the variety of potatoes and the conditions of their growing such as temperature, rainfall, altitude, agronomic and chemical characteristics of the soil. We have compared two potato cultivars in the study which have been grown in the Slovak localities Dolné Obdokovce and Vrbová nad Váhom (cv. Agria) and Nitra, Radošina and Vrbová nad Váhom (cv. Impala). In lyophilized samples of potatoes total polyphenols content in ethanolic extracts using Folin-Ciocalteu agents and in methanolic extracts total antioxidant capacity using DPPH· were spectrophotometrically determined. The average total polyphenols content in the potato variety Impala was 604.26 ± 120.70 mg.kg⁻¹ dry matter (DM) and 529.37 ± 59.35 mg.kg⁻¹ DM in the variety Agria. The average value of total antioxidant capacity, expressed in % inhibition, was $8.44 \pm 1.68\%$ in the potato variety Impala and $8.09 \pm 1.14\%$ in Agria. The results obtained were evaluated by One-way analysis of variance ANOVA (LSD-test), using Statistical Analysis Software Statgraphics. Mutual correlations among the total polyphenols content, total antioxidant capacity and heavy metals content (Pb, Ni, Cd - determined using atomic absorption spectrometry (AAS) method: Cd, Pb: GF-AAS and Ni F-AAS) were evaluated using the correlation and regression analysis (Microsoft Excel). Statistically significant interdependence ($p < 0.05$) was only confirmed between the Cd content and total antioxidant capacity in both cultivars, or between the Ni content and total antioxidant capacity in the variety Impala.

Keywords: potatoes; polyphenols; cultivar; antioxidant capacity; heavy metals

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the world's most important crops, ranking fifth in terms of human consumption and sixth in worldwide production with 324 million tonne production. The main producing countries are China, India and Russia (FAOSTAT, 2012).

Potatoes are promoted as healthy food items due to the presence of essential amino acids, vitamins, minerals and antioxidants. They also contain important amounts of polyphenols, a class of secondary plant metabolites (Deußer et al., 2012). All these bioactive chemicals have disease-fighting properties. Consumption of foods rich in antioxidant polyphenols is significantly associated with reduced risk of various non-communicable human diseases, including diabetes (Talukdar, 2013). Brat et al. (2006) classified potatoes as the important sources of polyphenols for their relatively high consumption (compared to 24 fruits, only the contribution of apples was higher than that of potatoes).

Polyphenols constitute a very heterogeneous group of compounds, with over 500 different molecules that have different properties and bioavailabilities, there are secondary plant metabolites and are important determinants of the sensory and nutritional qualities of fruits, vegetables and other plants (Tresserra-Rimbau et al., 2013; Xi et al., 2015).

Plant phenolic compounds represent a very diverse group of organic compounds, of a very heterogeneous in terms of chemical structure. They are characterized by the presence of an aromatic ring bearing one or more hydroxyl substituent. Based on the structure, they could be divided into several groups, for example, flavonoids, phenolic acids, hydroxycinnamic acids as well as flavonols (Bončíková et al., 2012; Liu et al., 2015).

The contamination by hazardous elements can influence the quality of potatoes to a larger degree, including their hygienic safety. Intake of heavy metal-contaminated vegetables may pose a hazard to the human health. Heavy metal contamination of the food items is one of the most important aspects of food quality assurance (Ali and Al-Qahtani, 2012).

Cadmium (Cd) is a toxic heavy metal that can accumulate in the human body and the environment for lengthy periods (Gonçalves et al., 2012; Zhang et al., 2014). Thus, prolonged exposure to it has been linked to toxic effects since it gets accumulated over time in a variety of structures including kidneys, liver and central nervous system and may cause proteinuria, glucosuria, and aminoaciduria with final renal dysfunction (Minh et al., 2012; Xu et al., 2013).

Lead (Pb) with its long history of use in the industry and its high persistence in the environment belong to most

serious environmental contaminants (Chen et al., 2005). Food is the major source of exposure to Pb and a possible hazard for the population. It has been shown that Pb can disturb hemoglobin synthesis and influence behaviour and the neurological system in children and can cause a number of cardiovascular dysfunctions in adults. Some studies have also indicated that Pb can influence kidney function. The bone is the main organ in which Pb is accumulated, and more than 90% of Pb is found in the bone (Zheljazkov et al., 2008; Chen et al., 2014).

Nickel is the essential element for plants and some animals. When nickel is present in high concentration in soil, then it is toxic for plants. Also toxic effect on the human health is known at high Ni levels. Nickel exceeding its critical level might bring about serious lung and kidney problems aside from gastrointestinal distress, pulmonary fibrosis and skin dermatitis. Nickel is also known as a respiratory tract carcinogen that is deposited in the lungs (Fu and Wang, 2011; Ogunbileje et al. 2013).

The aim of this work was to investigate the cultivar influence on the accumulation of Cd, Pb and Ni and compare dependence among total polyphenol content, total antioxidant capacity and contents of heavy metals in the two potato cultivars grown in several localities.

MATERIAL AND METHODOLOGY

Plant samples – potatoes (*Solanum tuberosum* L.)

Cultivars: maturity – shape of tubers – colour skin/colour flesh – cooking type:

- Impala: very early – oval – yellow/light yellow – AB;
- Agria: mid-early – oval – yellow/yellow – BC.

Locality: region, altitude, annual mean temperature, annual mean rainfalls:

- Dolné Obdokovce: Podzoborský region, 180-227 m a.s.l., 8.5 °C, 600-700 mm;
- Vrbová nad Váhom: Nitriansky region, 111-114 m a.s.l., 9.9 °C, 400-500 mm;
- Nitra: Nitriansky region, 130-558 m a.s.l., 9.5 °C, 500-800 mm;
- Radošina: Tribečsko-Inovecký region, 180-561 m a.s.l., 9.0 °C, 600-700 mm.

Standard technology of potato cultivation was used in all localities. Potatoes were harvested in their physiological maturity. Samples from each cultivar were collected in four repetitions in an amount of about 2 kg from each sample site. About 150 g potatoes were randomly selected from undamaged tubers which were homogenized and subsequently (after removal of about 30 g for the determination of dry matter) lyophilized after washing, peeling and chopping.

Determination of total polyphenols content (TPC) was realized spectrophotometrically by Spectrophotometer UV-VIS 1601, Shimadzu, Japan. Total polyphenols content was determined in ethanolic extracts using Folin-Ciocalteu agents. Analysis conditions were as follows: extraction of samples using Twisselman Extractor 80% EtOH (Sigma - Aldrich, Germany), duration of extraction 12 h, preparation of samples for spectrophotometric determination according to Lachman et al. (2006), measurement of absorbance (against blank) at wavelength

$\lambda = 765$ nm. TPC was expressed as mg gallic acid eqv. to kg of dry matter.

For determination of total antioxidant capacity (TAC) method based on radical reaction of 2,2-diphenyl-1-picrylhydrazyl (DPPH \cdot) according Brand-Williams et al., (1995) was used. To obtain a stock solution 0.025 g of DPPH (Sigma-Aldrich, USA) was diluted to 100 mL with methanol and kept in a cool and dark place. Immediately before the analysis, a 1:10 dilution of the stock was made with methanol. For the analysis, 3.9 mL of the DPPH working solution was added to a cuvette and the absorbance at 515 nm was measured (A_0) with a Shimadzu 710 spectrophotometer (Shimadzu, Japan). Subsequently, 0.1 mL of the extract was added to the cuvette with DPPH \cdot , and the absorbance was measured after 10 min (A_{10}). An increasing amount of antioxidants present in the methanol extract of the sample reduced DPPH \cdot and faded the colour of the solution in a correlation proportional to the antioxidant concentration. The percentage of DPPH \cdot inhibition was calculated according to the following equation:

$$\% \text{ inh. DPPH}\cdot = [(A_0 - A_{10})/A_0] * 100;$$

Where: A_0 - absorbance at time $t = 0$ min

A_{10} - absorbance at time $t = 10$ min.

All analyses were run in quadruplicate.

Determination of heavy metals as the contents of Cd, Pb and Ni was done in potatoes in extracts of freeze-dried samples. Mineralization of the samples was performed by microwave digestion (MARS X-press, CEM, USA). The contents of heavy metals were determined using AAS (atomic absorption spectrometry) method: Cd, Pb: GF-AAS and Ni F-AAS. The measured results were compared with multielemental standard for GF AAS (CertiPUR®, Merck, Germany) and subsequently expressed in mg.kg $^{-1}$ of fresh matter (FM).

Contents of heavy metals determined in plant samples were evaluated according to maximal allowed amounts given by Foodstuffs Codex of Slovak Republic and EC No. 1881/2006.

Soil samples

Immediately with the plant material also soil samples in horizon 0 – 0.2 m were collected (into pedological probe GeoSampler fy. Fisher). In all samples contents of Cd, Pb and Ni and also agrochemical characteristics after previous preparing at the Department of Chemistry SUA in Nitra were determined.

Determination of agrochemical indicators, contents of nutrients and heavy metals:

- exchange soil reaction (pH/KCl), c (KCl) = 1 mol/L, KCl: CentralChem, Slovakia; 691 pH Meter Metrohm, Swiss),
- content of oxidizable carbon (C_{OX} , %) volumetric method (H_2SO_4 , $K_2Cr_2O_7$, $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$: Merck, Germany) and content of humus (Hum, %) calculated from value of C_{OX} content,
- contents of nutrients (method by Mehlich III: P – spectrophotometrically ($\lambda = 666$ nm, spectrophotometer UV-VIS 1800, Shimadzu); K, Ca, Mg – using AAS method; NH_4NO_3 , NH_4F , EDTA,

HNO₃, H₂SO₄, (NH₄)₂MoO₄, C₈H₄K₂O₁₂Sb₂•3H₂O, ascorbic acid: Merck, Germany),

- content of mobile forms of Cd, Pb and Ni, was determined in soil extract by NH₄NO₃ (c = 1 mol/L, NH₄NO₃: Merck, Germany);

The contents of Cd, Pb and Ni in soil were determined using F-AAS method and GF-AAS method, or. (VARIAN AASpectr DUO 240FS/240Z/UltrAA equipped with a D2 lamp background correction system, using an air-acetylene flame, Varian, Ltd., Mulgrave, Australia) and compared with limit and critical values according to Act No. 220/2004.

Statistical analysis.

Results were statistically evaluated by the Analysis of Variance (ANOVA - Multiple Range Tests, Method: 95.0 percent LSD) using statistical software STATGRAPHICS (Centurion XVII, USA) and the regression and correlation analysis (Microsoft Excel) was used.

RESULTS AND DISCUSSION

The results of chemical analysis aimed at agrochemical characteristics of soil (values of exchangeable soil reaction, content of humus, oxidizable carbon and contents of available nutrients: P, K, Ca, Mg) are presented in Table 1.

The above results show that there are soils with weak acid to alkaline soil reaction, little to moderate high humus supply, high to very high in phosphorus, convenient to high in potassium and very high in magnesium. Agrochemical properties of soil were evaluated according the Code of Good Agricultural Practise in Slovak Republic (Bielek 1996). Supply of nutrients in the soils observed for the growing of potatoes was sufficient, or in some cases higher than the conforming content (100 – 125 mg P, 140 – 220 mg K and 110 – 180 mg Mg per kilogram of soil). Satisfactory soil reaction shall be in the range

pH 5.5 – 6.5 and optimum humus content should be higher than 2% (Vokál et al., 2003). This value has been exceeded only in soils from the locality Dolné Obdokovce.

The evaluation of hazardous heavy metals content was used as hygienic criterion for assessing the suitability of the soil for the growing of potatoes as follows: Cd, Pb a Ni. The main reason for the determination of risk elements content in soils is followed from their toxicity to plants and through subsequent entry into the food chain for other organisms (Harangozo et al., 2012). Their contents determined by the AAS method were compared to critical values according to legislation valid in the Slovak Republic (Law 220/2004) (Table 2).

We determined higher content of permissible levels of lead which was also reflected in its accumulation in the crop grown in all soil samples (except for that of the locality Vrbová nad Váhom). In the samples of potatoes, the lead content exceeded the maximum allowable quantity laid down in EU as well as Slovak applicable legislation (EU No. 1881/2006; FC SR).

Statistically significant differences in accumulation of hazardous metals have not only been found between the potatoes of the same variety grown in different localities, but also between those in the same locality. Many authors (Galdón et al., 2012; Lachman et al., 2012; Ezekiel et al., 2013; Marchettini et al., 2013) indicated that variety significantly influences the nutritional content in potatoes, chemical composition of tubers and also the appropriateness of harvest time of potatoes, pest resistance, suitability for processing in the kitchen and the quantity of potatoes harvested. The dependence on variety was also confirmed in the statistical evaluation of TPC and TAC determined in potato tubers (Table 3).

The TPC in potatoes of the variety Impala was on average 14% higher than in Agria, and we also found statistically significant differences between the TPC values within one variety grown in different locations.

Table 1 Basic agrochemical indicators and contents of nutrients (mg.kg⁻¹).

Cultivar	Locality	pH/KCl	Humus (%)	C _{ox} (%)	P (mg.kg ⁻¹)	K (mg.kg ⁻¹)	Ca (mg.kg ⁻¹)	Mg (mg.kg ⁻¹)
Agria	DO1	6.26	2.60	1.51	221.2	308.5	3130	284.0
	DO2	6.53	2.24	1.30	246.6	351.0	3444	376.0
	DO3	6.27	3.15	1.83	204.4	292.5	2798	338.0
	DO4	6.35	2.24	1.30	172.1	312.0	2886	400.0
	VnV1	7.24	1.57	0.91	157.2	207.1	5904	314.6
Impala	N1	7.18	1.75	1.02	192.3	286.4	6105	601.3
	N2	7.26	1.83	1.06	184.7	297.3	5982	586.3
	N3	7.14	1.69	0.98	196.4	391.4	7355	631.7
	R1	7.57	1.33	0.77	145.9	250.1	6417	311.4
	R2	7.34	1.39	0.81	139.4	215.5	6334	263.4
	VnV2	7.16	1.42	0.82	153.2	163.0	5807	325.6

Note: DO – Dolné Obdokovce, VnV – Vrbová nad Váhom, N – Nitra, R – Radošina.

Table 2 Contents of heavy metals in soil and potato tubers (mg.kg⁻¹ FM).

Cultivar	Locality	soil			potato		
		Cd (mg.kg ⁻¹)	Pb (mg.kg ⁻¹)	Ni (mg.kg ⁻¹)	Cd (mg.kg ⁻¹ FM)	Pb (mg.kg ⁻¹ FM)	Ni (mg.kg ⁻¹ FM)
Agria	DO1	0.06	0.27	0.17	0.050 ^b ±0.002	0.724 ^b ±0.029	0.175 ^a ±0.007
	DO2	0.07	0.32	0.19	0.075 ^c ±0.003	0.987 ^d ±0.039	0.269 ^b ±0.011
	DO3	0.06	0.27	0.17	0.087 ^d ±0.003	0.749 ^b ±0.029	0.350 ^c ±0.014
	DO4	0.06	0.29	0.17	0.113 ^e ±0.004	0.832 ^c ±0.033	0.440 ^d ±0.017
	VnV1	0.03	0.02	0.02	0.033 ^{a,A} ±0.001	0.250 ^{a,A} ±0.010	0.526 ^{e,A} ±0.021
Impala	N1	0.06	0.17	0.04	0.054 ^c ±0.002	0.232 ^b ±0.009	0.542 ^c ±0.021
	N2	0.08	0.21	0.06	0.052 ^c ±0.002	0.233 ^b ±0.009	0.491 ^b ±0.019
	N3	0.01	0.23	0.10	0.072 ^d ±0.003	0.284 ^c ±0.011	0.698 ^e ±0.027
	R1	0.07	0.14	0.07	0.049 ^b ±0.002	0.325 ^d ±0.013	0.596 ^d ±0.023
	R2	0.06	0.13	0.05	0.051 ^{b,c} ±0.002	0.206 ^a ±0.008	0.462 ^{a,b} ±0.018
	VnV2	0.03	0.03	0.02	0.042 ^{a,B} ±0.002	0.245 ^{b,A} ±0.010	0.440 ^{a,B} ±0.017
<i>Critical value</i>		0.1	0.1	1.5			
<i>EU No. 1881/2006 (FC SR)</i>					0.1 (0.1)	0.1 (0.1)	- (0.5)

Note: DO – Dolné Obdokovce, VnV – Vrbová nad Váhom, N – Nitra, R – Radošina,

^{a, b, c, d} – statistically significant differences between content of heavy metals in potato tubers from different locality (Multiple Range Tests for HMs by locality; Method: 95.0 percent LSD).

^{A, B} – statistically significant differences between content of heavy metals in different cultivars from one locality (Multiple Range Tests for HMs by cultivar; Method: 95.0 percent LSD).

Table 3 Total polyphenols content (TPC) (mg.kg⁻¹ DM) and total antioxidant capacity (TAC) (%) in potato tubers.

Cultivar	Locality	TPC (all sampling sites)			TAC (all sampling sites)			
		Min.	Max.	Average	Min.	Max.	Average	
Agria	DO1	545.39 ^b ±21.669			7.87 ^b ±0.159			
	DO2	480.33 ^a ±9.567			7.27 ^{a,b} ±0.300			
	DO3	459.38 ^a ±25.171	430.6	659.6	8.77 ^c ±0.781			
	DO4	614.42 ^c ±30.635			9.72 ^d ±0.134			
	VnV1	547.35 ^{b,A} ±4.770			6.82 ^{a,A} ±0.492			
Impala	N1	649.89 ^c ±7.171			9.92 ^c ±0.327			
	N2	387.64 ^a ±61.23			8.43 ^b ±0.586			
	N3	603.05 ^b ±5.950	334.22	788.24	10.75 ^c ±0.562			
	R1	589.14 ^b ±2.923			7.57 ^b ±1.422			
	R2	614.00 ^{b,c} ±3.777	604.26 ±120.70			7.81 ^b ±0.340		
	VnV2	781.81 ^{d,B} ±5.757			6.15 ^{a,A} ±0.470			

Notes: DO – Dolné Obdokovce, VnV – Vrbová nad Váhom, N – Nitra, R – Radošina,

^{a, b, c, d} – statistically significant differences between content of TPC and TAC in potato tubers from different locality (Multiple Range Tests for TPC and TAC by locality; Method: 95.0 percent LSD).

^{A, B} – statistically significant differences between content of TPC and TAC in potato tubers from one locality (Multiple Range Tests for HMs by cultivar; Method: 95.0 percent LSD).

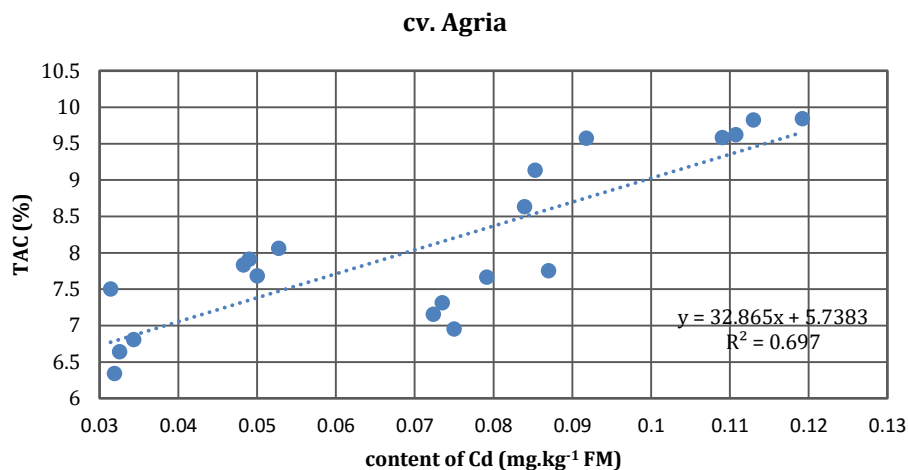


Figure 1 Total antioxidant activity (TAC, %) in relationship to the Cd content (mg.kg⁻¹ FM) in potato tubers (cv. Agria).

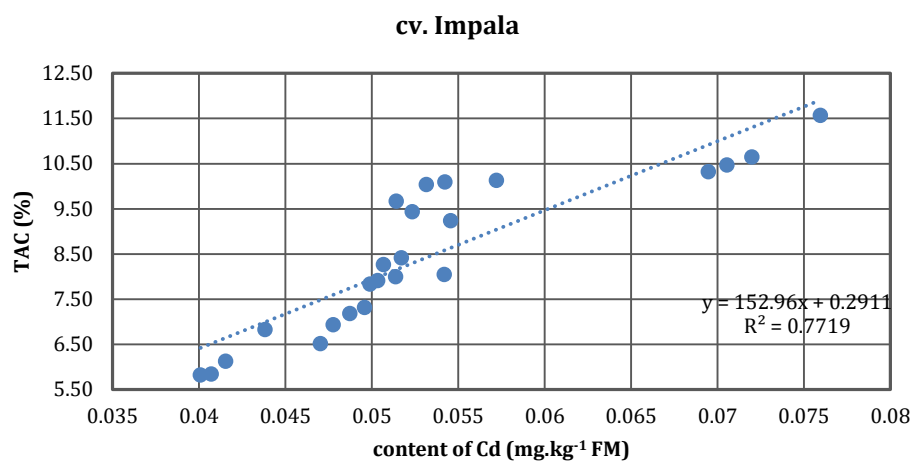


Figure 2 Total antioxidant activity (TAC, %) in relationship to the Cd content (mg.kg⁻¹ FM) in potato tubers (cv. Impala).

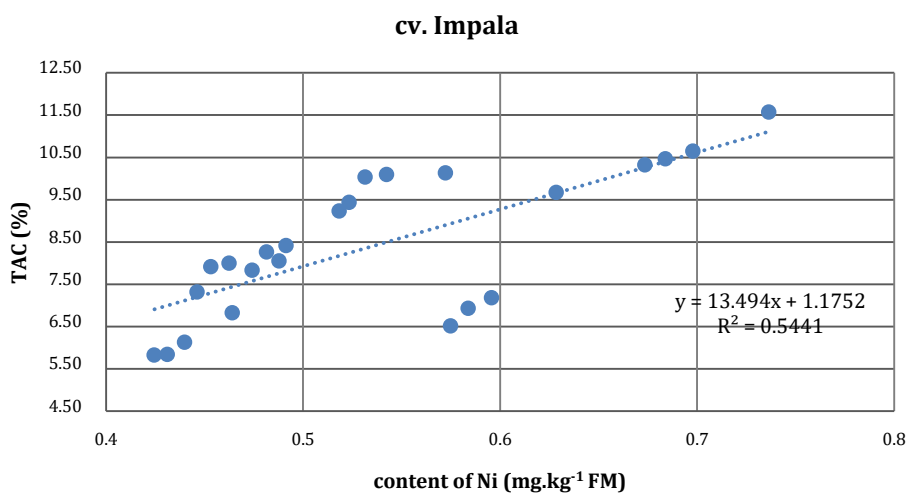


Figure 3 Total antioxidant activity (TAC, %) in relationship to the Ni content (mg.kg⁻¹ FM) in potato tubers (cv. Impala).

The effect of locality was confirmed in both observed cultivars. Conditions of potato cultivation are the second factor influencing polyphenolic levels in potatoes (Reddivari et al., 2007). We have determined the following the TPC values which were higher than those in potatoes grown *in vitro* on peat substrate in a greenhouse: from 256.44 to 425.37 mg.kg⁻¹ FM (Musilova et al., 2015).

Lachman et al. (2006) determined 2.46 – 3.44 g CP.kg⁻¹ in yellow cultivars of potatoes, the levels of polyphenols in purple cultivars were 58.1% higher on average. The significant difference can not only be caused by the variety, but also the methodology of sample preparation. The above mentioned authors have used undivided tubers, and we used peeled potatoes for our analysis. Rytel et al. (2014) also indicated approximately three and a half times higher amounts of the polyphenols in potato skins than the potato internal tissue.

The results shown by other authors correspond with our findings. Ezekiel et al. (2013) determined total polyphenols content in the potato's flesh ranging between 30 – 900 mg.kg⁻¹ DM and in the peel it was 1000 – 4000 mg.kg⁻¹ DM. Burgos et al. (2013) evaluated the range of 596 – 4196 mg TPC.kg⁻¹ DM. In addition to variety, the content of polyphenols is also influenced by year, storage conditions and kitchen processing to a considerable extent (Faller, Fialho, 2009; Galdón et al., 2012).

TAC as well as TPC was higher in the variety Impala. There were also statistically significant differences in the TAC values found between the localities within one variety (Table 3). Plant polyphenols are antioxidants because they scavenge free radicals. The antioxidant capacity of polyphenols depends on the number and position of their hydroxyl substituent and structure of aromatic nucleus (Bassama et al., 2010). However, the relevance between antioxidant capacity and inhibitory effects is still unclear (Liu et al., 2015) and not all phenolics present in potatoes have an antioxidant activity (Burgos et al., 2013). In our case, the correlation between TPC and TAC has not been confirmed in either variety (cv. Agria: *p*-value = 0.0955, cv. Impala: *p*-value = 0.2296). Rumbaoa et al., (2009) even confirmed a negative correlation between the TPC and TAC in four Philippine potato cultivars. However, Albishi et al., (2013) confirmed that samples which had the highest phenolic content were most effective as free radical scavengers. The positive correlation indicates that the higher phenolic content resulted in a higher antioxidant activity; the strongest correlation existed between bound phenolics and different antioxidant activity assays employed. However, no significant linear or weak relationship existed between total free or esterified phenolic content and DPPH radical scavenging capacity. Lachman et al., (2008) referred about a strong positive correlation between TAC and TPC in potatoes (yellow cultivars Karin, Impala, Dita, Saturna; purple cultivars Valfi, Violette). Also Reyes et al., (2005); André et al., (2009); Al-Weshahy et al., (2013) confirmed a high positive correlation between TAC and TPC.

In our work both cultivars, however, showed a positive correlation between the Cd content in potato tubers and

their TAC (Figure 1 and Figure 2), there was also a positive correlation between the Ni content in potatoes and their TAC in the cultivar Impala (Figure 3).

The relationship between the Pb content and TAC in both potato cultivars, between the Ni content and TAC in the variety Agria was not statistically significant (Pb – cv. Agria: *p*-value = 0.0573, cv. Impala: *p*-value = 0.5349; Ni – cv. Agria: *p*-value = 0.8387). The formation of polyphenols in potatoes was not significantly influenced by the accumulation of cadmium, lead or presence of nickel (Cd – cv. Agria: *p*-value = 0.4734, cv. Impala: *p*-value = 0.2784, Pb – cv. Agria: *p*-value = 0.4637, cv. Impala: *p*-value = 0.8866; Ni – cv. Agria: *p*-value = 0.1276, cv. Impala: *p*-value = 0.5760).

CONCLUSION

The influence of the variety on the Cd, Pb and Ni accumulation ratio, polyphenols content and antioxidant capacity were confirmed in the potato cultivars Agria and Impala grown in the localities Dolné Obdokovce, Nitra, Radošina and Vrbová nad Váhom. The influence of locality was confirmed in the case of the Pb accumulation, there were not any significant differences between TAC in the potato cultivars Agria and Impala from the locality Vrbová nad Váhom. The correlation between TPC and TAC, or the content of hazardous metals and polyphenols were not confirmed in none of the cultivars. A positive significant correlation was found between the Cd content in potato tubers and their TAC in both cultivars and between the Ni content in potatoes and their TAC in the variety Impala.

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QUALITY EVALUATION OF KORBAČIK CHEESE

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ABSTRACT

The aim of the present study was analysed the physical and chemical parameters in lump cheese and Korbáčik cheese. Sensory evaluation was performed only in Korbáčik cheese. There was compared quality of Korbáčik cheese made from lump cheese ripened one and three weeks. The statistical analysis of the moisture showed significant differences ($p < 0.001$) among Korbáčik cheese made from raw material ripened one and three weeks. Average moisture of the Korbáčik made from cheese ripened one week was 44.73% and of the Korbáčik made from cheese ripened three weeks was 53.73%. The statistical analysis of the dry mater value showed significant differences ($p < 0.001$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average value of dry matter of the Korbáčik made from cheese ripened one week was 55.27% and of the Korbáčik made from cheese ripened three weeks was 46.27%. The statistical analysis of the fat content showed significant differences ($p < 0.01$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average fat content of the Korbáčik made from cheese ripened one week was 22.67% and of the Korbáčik made from cheese ripened three weeks was 20.20%. The statistical analysis of the fat content in dry matter showed significant differences ($p < 0.001$) among Korbáčik cheese made from raw material ripened one and three weeks. Average NaCl content in the Korbáčik made from cheese ripened one week was 3.78% and in the Korbáčik made from cheese ripened three weeks was 2.93%. The statistical analysis of MDA content showed significant differences ($p < 0.05$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average MDA content in the Korbáčik made from cheese ripened one week was 0.29 mg.kg⁻¹ and in the Korbáčik made from cheese ripened three weeks was 0.36 mg.kg⁻¹. Korbáčik cheese made from cheese aged 3 weeks was practically in all sensory parameters better evaluated than the Korbáčik cheese made from cheese aged one week.

Keywords: physico-chemical parameters; sensory parameters; malondialdehyde; lump cheese

INTRODUCTION

Korbáčik cheese is traditional Slovak products and is listed in Council Regulation (EC) No. 510/2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs. Korbáčik cheese belongs to the steamed cheese. Steamed cheese production has a long tradition in Slovakia, although originally made from sheep's milk. Steamed cheeses form a special group of cheeses whose production is especially typical for countries in southern Europe, countries located on the Balkan peninsula, Greece and Italy, which are called general Pasta filata. This term has been extended and is now used internationally as a term for a group of steamed cheeses.

Several studies have been carried out to describe the influence of pasteurization on the cheese proteolysis, but mainly in cow milk cheeses (Rosenberg et al., 1995; Beuviel et al., 1997; Skeie and Ardö, 2000). These studies have shown that there is little consistency among different varieties of cheeses, in relation to the influence of milk pasteurization on the primary and secondary proteolysis of cheeses.

Raw milk cheeses represent a significant proportion of matured cheeses in most Mediterranean countries, mainly those made from goat and ewe milk. To knowledge, few studies have been made to compare cheeses made from raw or pasteurized milk.

Cheese ripening represents an important technological process, during which cheese occurs microbiological and biochemical changes. It is influenced by intensity of proteolysis, contents of dry matter, NaCl and fats and by pH (Everard et al., 2006; Saint-Eve et al., 2009). Cheese functional, texture and sensory properties develop during the process of maturing; nevertheless, proper maturation is a costly process (Forde and Fitzgerald, 2000). The length of cheese ripening depends on the type of cheese.

Manufacturers of cheese try to decrease time of ripening (due to reduction of production costs). Therefore, unripe cheeses can be supplied to distribution networks. Their organoleptic properties are different in the comparison with the qualities of the cheeses matured under standard conditions (Pachlová et al., 2011). Milk quality, chemical composition (e.g. moisture, fat or NaCl contents), using of proper starter lactic acid bacteria (SLAB) and presence of

non-starter lactic acid bacteria (NSLAB) may also influence processes occurring in ripening cheeses (Al-Otaibi and Wilbey, 2004; Fenelon and Guinee, 2000; Flourey et al., 2009; Pachlová et al., 2011; Shakeel-Ur-Rehman et al., 2000).

Acceleration of the maturing process is possible to solve the aforementioned problems of deteriorated cheese quality. It can compress the maturing period and, at the same time, ripeness of the products can reach a sufficient level. Fox et al., (1996) reported that the following factors can accelerate cheese ripening: elevated ripening temperatures, exogenous enzymes, chemically or physically modified cells, genetically modified starters, adjunct cultures, cheese slurries.

Increasing of temperature is regarded as the simplest method for acceleration of the ripening process from the technical point of view (Sihufe et al., 2010a). Besides the quick growth of both SLAB and NSLAB, propagation of other undesirable contaminating microorganisms can occur and they can cause decreasing of quality of cheese and also cause alimentary intoxication or infection (Iurlina and Fritz, 2004). Sihufe et al., (2010b) report possible double or triple decrease of ripening period by increase of ripening temperature by 6 °C.

Cheese ripening represents a very important technological process, during which cheese undergoes significant microbiological and biochemical changes. For consumers, texture plays a key role in cheese purchase. It is mainly influenced by intensity of proteolysis, contents of NaCl, dry matter and fats and by pH (Everard et al., 2006 and Saint-Eve et al., 2009). Cheese texture and functional and sensory properties develop during the process of ripening; nevertheless, proper maturation is a costly process (Forde and Fitzgerald, 2000). The length of cheese ripening depends on the type of cheese. Cheese manufacturers try to decrease ripening time (due to reduction of production costs). Therefore, insufficiently ripened cheeses can be supplied to distribution networks. Their organoleptic properties differ substantially from the qualities of the cheeses matured under standard conditions (Pachlová et al., 2011). Acceleration of the ripening process is suggested to solve the aforementioned problems of deteriorated cheese quality. It can compress the ripening period and, at the same time, ripeness of the products can reach a satisfactory level. As reported by Fox et al., (1996), the following factors can accelerate cheese ripening: elevated ripening temperatures, exogenous enzymes, chemically or physically modified cells, genetically modified starters, adjunct cultures, cheese slurries.

As reported by Buňková et al., (2010); Komprda et al., (2007); Pachlová et al., (2011), the extent of microbiological and biochemical processes in individual parts of cheese might differ, that is why differences in development of texture parameters in individual cheese segments can be expected.

Salt had a significant effect on moisture, uptake of salt, pH, and hardness. Results indicated that higher percentages of salt in brine developed a harder cheese with higher salt content and higher pH values but lower moisture content. High rennet concentration had a significant effect on the uptake of salt. Increasing the

concentration of rennet gave a softer cheese with higher salt content. Rennet level had no significant effect on the moisture or protein contents or pH of the cheese (Prasad and Alvarez, 1999).

Prepared cheese is processed to mash by hot water. The recommended lower limit water temperature is 70 °C. The cheese is mixed with a paddle until the not achieve the correct structure of matter - suppleness, smoothness and elasticity. Recommended acidity of raw materials for steamed cheese is pH 5.1 to 5.0.

Cheese which is poorly fermented are difficult kneaded, small, and there is elastic enough - or at textured yarn cheese strip is torn and formed large differences in the thickness of the thread. In this case, the raw material is suitable and only after maturing may be produced from it. It can be done manually or with the using of machines. In the production Korbáčik from the steamed cheese are forms to a thread (a diameter of 2-3 mm) that falls straight into cold water (Konečná and Šustová, 2012). Kunová et al., (2015) found out values of TVC after 5 days of storage at temperature 4 °C were in range from 3.29 to 5.12×10^3 CFU.g⁻¹, but MFF were no found.

Oravas Korbáčik is a steamed cheese made from lump cheese contains especially thermoresistant lactic acid microflora of the genera: *Lactococcus*, *Streptococcus* and *Lactobacillus*. Chemical requirements: dry matter at least 40%. Fat in dry matter at least 25%, salt content of more than 4.5% unsmoked and 5.5% smoked. Microbiological characteristics: raw material for korbáčik the lump cheese, which contains mainly genera *Lactococcus*, *Lactobacillus* and *Streptococcus* - thermoresistant lactic acid microflora (Council Regulation 510/2006).

The aim of the present study was analysed the quality of the Korbáčik cheese made of cheese aged 1 and 3 weeks.

MATERIAL AND METHODOLOGY

There were analysed samples of Korbáčik cheese quality, which were made from lump cheese aged 1 and 3 weeks. There were analysed 10 samples of Korbáčik cheese made from lump cheese aged 1 week and 10 samples aged 3 weeks.

Chemical composition analysis

The chemical composition of the raw material (cheese) and Korbáčik cheese was measured (50 g) by the FT IR method using the device Nicolet 6700. The total proteins in g/100g, the intramuscular fat in g/100g and total water in g/100g were analysed. The infra-red spectrum was carried out by the molecular spectroscopy method. The principle of this method is the absorption of the infra-red spectrum during the sample transition. There is a change of the rotary vibrating energetic conditions of the molecule depending on the changes of the dipole momentum molecule.

Determination of salt (NaCl)

Samples of approximately 2 g with 2 ml of indicator potassium chromate were titrated by solution of silver nitrate until a light orange color. The amount of silver nitrate was divided by weight of sample.

Determination of titratable acidity

The samples were homogenised, total 50 g of sample were used for analysis. Titratable acidity was determined by titration with 0.25 mol.L⁻¹ NaOH and phenolphthalein was added before titration. Acidity was determined as 2 times the volume of NaOH used in titration. The results were expressed as titration activity in °SH (Soxhlet-Henkel).

Determination of malondialdehyde

The degradation products of Korbáčik cheeses were analysed. Malondialdehyde (MDA) was measured in the Korbáčik cheese. MDA number was determined according to **Marcinčák et al. (2006)**. Absorbance of samples was measured at a wavelength of 532 nm on UV-VIS spectrophotometer Jenway 7305 (United Kingdom - JENWAY). Results will be calculated as the mg of MDA in 1 kg of sample.

Sensory analysis

These organoleptic characteristics were evaluated in sensory analysis: aroma and taste, consistency, color and appearance. Sensory analysis was performed using the sensory evaluation using scale. A five-point scale was used to the characteristics of each point. Assessment system was chosen, the highest number of points (5) was evaluated as, excellent "and 1 point as "unacceptable".

Statistical analyse

The data were subjected to statistical analysis using the Statistic Analysis System (SAS) package (SAS 9.3 using of application Enterprise Guide 4.2). Differences between groups were analysed by t-test.

RESULTS AND DISCUSSION

Korbáčik cheese is traditional Slovak products and it belongs to the steamed cheese. Physical and chemical

parameters were evaluated in lump cheese and Korbáčik cheese. Sensory evaluation was performed only in Korbáčik cheese.

Average moisture of lump cheese was 44.77% and ranged from 44.20 to 45.10% after first week of ripening. Average moisture of lump cheese was 45.17% and ranged from 44.60 to 45.40% after third week of ripening (Table 1). **Okpala et al., (2010)** found out moisture in the fresh cheese 63.10% on the 1st day and 59.9% on the 8th day after processing.

Average value of dry matter was 55.23% in the lump cheese after first week of ripening and 54.88% after third week of ripening.

Average fat content was 25.33% in the lump cheese after first week of ripening and 22.07% after third week of ripening. Fat content ranged from 25.20 to 25.50% after first week and from 21.90 to 22.30% after third week of ripening in the lump cheese. **Okpala et al., (2010)** found out fat content 16.02% in the fresh cheese.

The statistical analysis of the fat content in dry matter showed significant differences ($p < 0.05$) among the lump cheese after first week of ripening and after third week of ripening. Average content of fat in dry matter was 45.87 after first week of ripening and 40.21% after third week after ripening in the lump cheese. Content of fat in dry matter was in range from 45.34 to 46.36% after first week and from 39.53 to 40.81% after third week of ripening in the lump cheese. Fresh cheese is classified as semi-soft cheeses (**Bozoudi et al., 2015**).

Average content of NaCl was 0.39 after first week of ripening and 0.38% after third week of ripening in the lump cheese. Content of NaCl in the lump cheese ranged from 0.31 to 0.43% after first week and from 0.30 to 0.45% after third week of ripening.

Table 1 Physical and chemical parameters of raw materials (lump cheese) after the first and third week of ripening.

Parameters	Moisture (%)	Dry matter (%)	Fat (%)	Fat in dry matter (%)	NaCl (%)	Acidity °SH	MDA (mg/kg)
The raw materials (lump cheese) after the first week of ripening							
mean	44.77	55.23	25.33	45.87	0.39	98.27	0.37
S. D.	0.49	0.49	0.15	0.51	0.07	0.70	0.02
S. E.	0.28	0.28	0.09	0.29	0.04	0.41	0.01
Min.	44.20	54.90	25.20	45.34	0.31	97.60	0.36
Max.	45.10	55.80	25.50	46.36	0.43	99.00	0.40
CV%	1.10	0.89	0.60	1.11	17.22	0.71	6.18
The raw materials (cheese) after the third week of cheese ripening							
mean	45.17	54.88	22.07	40.21	0.38	108.33	0.59
S. D.	0.45	0.45	0.21	0.64	0.08	2.08	0.13
S. E.	0.26	0.26	0.12	0.37	0.04	1.20	0.07
Min.	44.60	45.60	21.90	39.53	0.30	106.00	0.51
Max.	45.40	55.40	22.30	40.81	0.45	110.00	0.73
CV%	0.99	0.82	0.94	1.60	19.92	1.92	21.49
t - test	-	-	-	+	-	+	+

Note: °SH – Soxhlet-Henkel, MDA – malondialdehyde, $p > 0.05$; $+ p \leq 0.05$.

Table 2 Physical and chemical parameters of a Korbáčik cheese made from raw materials after the 1st and 3rd week of cheese ripening.

Parameters	Moisture (%)	Dry matter (%)	Fat (%)	Fat in dry matter (%)	NaCl (%)	°SH	MDA (mg/kg)
The Korbáčik cheese made after the first week of cheese ripening							
mean	44.73	55.27	22.67	40.35	3.78	99.00	0.29
S. D.	0.55	0.55	0.76	0.34	0.33	2.00	0.03
S. E.	0.32	0.32	0.44	0.19	0.19	1.15	0.02
Min.	44.10	54.90	22.00	40.07	3.40	97.00	0.27
Max.	45.10	55.90	23.50	40.72	4.00	101.00	0.32
CV%	1.23	1.00	3.37	0.83	8.80	2.02	10.51
The Korbáčik cheese made after the third week of cheese ripening							
mean	53.73	46.27	20.20	43.66	2.93	109.73	0.36
S. D.	0.68	0.68	0.26	0.57	0.76	4.06	0.07
S. E.	0.39	0.39	0.15	0.33	0.45	2.34	0.04
Min.	52.95	45.80	19.90	43.31	2.15	107.00	0.31
Max.	54.20	47.05	20.4	44.32	3.70	114.4	0.43
CV%	1.27	1.48	1.31	1.30	26.43	3.70	18.5
t - test	+++	+++	++	+++	-	+	+

Note: $p > 0.05$; $+ p \leq 0.05$; $++ p \leq 0.01$; $+++ p \leq 0.001$.

Table 3 Sensory evaluation of Korbáčik cheese made from raw materials after 1st week and 3rd week of cheese ripening.

Parameters	Smell	Taste	Color	Consistency	Appearance
The Korbáčik cheese made after the first week of cheese ripening					
mean	4.17	4.33	4.67	5.00	3.83
S.D.	0.29	0.29	0.29	0.00	0.29
S.E.	0.17	0.17	0.17	0.00	0.17
min	4.00	4.00	4.50	5.00	3.50
max	4.50	4.50	5.00	5.00	4.00
CV%	6.93	6.66	6.19	0.00	7.53
The Korbáčik cheese made after the third week of cheese ripening					
mean	5.00	5.00	4.83	5.00	4.83
S. D.	0.00	0.00	0.29	0.00	0.29
S.E.	0.00	0.00	0.17	0.00	0.17
min	5.00	5.00	4.50	5.00	4.50
max	5.00	5.00	5.00	5.00	5.00
CV%	0.00	0.00	5.97	0.00	5.97
t - test	+	+	-	-	+

Note: $p > 0.05$; $+ p \leq 0.05$.

The statistical analysis of the acidity (°SH) showed significant differences ($p < 0.05$) among the lump cheese after first week of ripening and after third week of ripening. Average acidity was 98.27 °SH after first week of ripening and 108.33 °SH after third week of ripening in the lump cheese.

The statistical analysis of the MDA content showed significant differences ($p < 0.05$) among the lump cheese after first week of ripening and after third week of

ripening. Average concentration of malondyaldehyde (MDA) was 0.37 mg.kg⁻¹ after first week and 0.59 mg.kg⁻¹ after third week of ripening in the lump cheese. Variability of MDA concentration was higher in the lump cheese after third week after ripening (CV% 21.49) and it ranged from 0.51 to 0.73 mg.kg⁻¹.

Physical and chemical parameters of the Korbáčik cheese are shown in the Table 2. The statistical analysis of the moisture showed significant differences ($p < 0.001$) among

Korbáčik cheese made from raw material ripened one and three weeks. Average moisture of the Korbáčik made from cheese ripened one week was 44.73% and of the Korbáčik made from cheese ripened three weeks was 53.73%. Similar with our results, **Owini and Osman (2009)** reported values of moisture between 45.4 and 48.5% for steamed cheese. **Maldonado et al. (2013)** found out opposite our results higher moisture content (48.12 – 53.55%).

The statistical analysis of the dry matter value showed significant differences ($p < 0.001$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average value of dry matter of the Korbáčik made from cheese ripened one week was 55.27% and of the Korbáčik made from cheese ripened three weeks was 46.27%. Dry matter content of the Korbáčik made from cheese ripened one week ranged from 54.90 to 55.90% and of the Korbáčik made from cheese ripened three weeks ranged from 45.80 to 47.05%.

The statistical analysis of the fat content showed significant differences ($p < 0.01$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average fat content of the Korbáčik made from cheese ripened one week was 22.67% and of the Korbáčik made from cheese ripened three weeks was 20.20%.

The statistical analysis of the fat content in dry matter showed significant differences ($p < 0.001$) among Korbáčik cheese made from raw material ripened one and three weeks. Average fat content in dry matter of the Korbáčik made from cheese ripened one week was 40.35% and of the Korbáčik made from cheese ripened three weeks was 43.66%. Fat content in dry matter in the Korbáčik made from cheese ripened one week ranged from 40.07 to 40.72% and in the Korbáčik made from cheese ripened three weeks ranged from 43.31 to 44.32%.

Maldonado et al. (2013) found out opposite our results higher fat content in dry matter (49.52 – 54.94%) in the steamed cheese.

Average NaCl content in the Korbáčik made from cheese ripened one week was 3.78% and in the Korbáčik made from cheese ripened three weeks was 2.93%. Variability of NaCl content was higher in the Korbáčik cheese made from raw material ripened three weeks (CV% 26.43) and it ranged from 2.15 to 3.70%. Higher variability of salts content associated with more variable thickness raw thread. **Ma et al. (2013)** found out opposite our results lower content of NaCl from 1.07 to 1.29% in the steamed cheese.

The statistical analysis of the acidity ($^{\circ}\text{SH}$) showed significant differences ($p < 0.05$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average acidity of the Korbáčik made from cheese ripened one week was 99.00 $^{\circ}\text{SH}$ and of the Korbáčik made from cheese ripened three weeks was 109.73 $^{\circ}\text{SH}$.

The statistical analysis of MDA content showed significant differences ($p < 0.05$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average MDA content in the Korbáčik made from cheese ripened one week was 0.29 $\text{mg}\cdot\text{kg}^{-1}$ and in the Korbáčik made from cheese ripened three weeks was 0.36 mg/kg . MDA content was reduced by salting of the cheese in hot water. **Papastergiadis et al. (2014)** found out

content of MDA in steamed cheese in range from 0.20 to 0.66 $\text{mg}\cdot\text{kg}^{-1}$.

Results of sensory evaluation of Korbáčik cheese made from raw materials ripened 1 week and 3 weeks are shown in table 3. The sensory quality was analysed by Sensory descriptors and was defined from the appearance, aroma, flavor and texture evaluation by commission. Sensory evaluation was performed by 5 points system. The statistical analysis of the smell showed significant differences ($p < 0.05$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average point numbers for smell of Korbáčik cheese made from raw materials ripened 1 week was 4.17 and of Korbáčik cheese made from raw materials ripened 3 weeks was 5.00.

The statistical analysis of the taste showed significant differences ($p < 0.05$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average point numbers for taste of Korbáčik cheese made from raw materials ripened 1 week was 4.33 and of Korbáčik cheese made from raw materials ripened 3 weeks was 5.00.

Average point numbers for color of Korbáčik cheese made from raw materials ripened 1 week was 4.67 and of Korbáčik cheese made from raw materials ripened 3 weeks was 4.83.

Average point numbers for consistency of Korbáčik cheese made from raw materials ripened 1 and 3 weeks was the same 5.00.

The statistical analysis of the appearance showed significant differences ($p < 0.05$) among the Korbáčik cheese made from raw material ripened one and three weeks. Average point numbers for taste of Korbáčik cheese made from raw materials ripened 1 week was 3.83 and of Korbáčik cheese made from raw materials ripened 3 weeks was 4.83. **Romeih et al. (2002)** evaluated steamed cheese by 7 points method and they found out appearance 5.8 points, consistency 3.7, flavour 4.9 and odour 4.8 points.

Korbáčik cheese made from cheese aged 3 weeks was practically in all sensory parameters better evaluated than the Korbáčik cheese made from cheese aged one week.

CONCLUSION

The aim of this study was analysed the physical and chemical parameters in lump cheese and Korbáčik cheese. Sensory evaluation was performed only in Korbáčik cheese. There was compared quality of lump cheese and Korbáčik cheese made from lump cheese ripened one and three weeks. The statistical analysis of the moisture showed significant differences among Korbáčik cheese made from raw material ripened one and three weeks. The statistical analysis of the dry matter value showed significant differences among the Korbáčik cheese made from raw material ripened one and three weeks. The statistical analysis of the fat content showed significant differences among the Korbáčik cheese made from raw material ripened one and three weeks. Average fat content in dry matter of the Korbáčik made from cheese ripened one week was lower in comparison with the Korbáčik made from cheese ripened three weeks. Average NaCl content in the Korbáčik made from cheese ripened one week was higher in comparison with the Korbáčik made

from cheese ripened three weeks. The statistical analysis of the acidity showed significant differences among the Korbáčik cheese made from raw material ripened one and three weeks. Average MDA content in the Korbáčik made from cheese ripened one week was significantly lower in comparison with the Korbáčik made from cheese ripened three weeks. Korbáčik cheese made from cheese aged 3 weeks was practically in all sensory parameters better evaluated than the Korbáčik cheese made from cheese aged one week.

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APPLICATION OF LAVENDER AND ROSEMARY ESSENTIAL OILS IMPROVEMENT OF THE MICROBIOLOGICAL QUALITY OF CHICKEN QUARTERS

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ABSTRACT

The aim of the present work was monitoring of chicken quarters microbiological indicators after treatment by ethylenediaminetetraacetate (EDTA), lavender (*Lavandula angustifolia* L.) and rosemary (*Rosmarinus officinalis* L.) essential oil, stored under vacuum packaging, at $4 \pm 0.5^\circ\text{C}$ for a period of 16 days. The following treatments of chicken quarters were used: Air-packaging control samples, control vacuum-packaging samples, vacuum-packaging with EDTA solution 1.50% w/w, control samples, vacuum-packaging with *Lavandula angustifolia* essential oil at concentrations 0.2% v/w and vacuum-packaging with *Rosmarinus officinalis* essential oil at concentration 0.2% v/w. The quality assessment of all samples was established by microbiological analysis. Sampling was carried out after certain time intervals: 0, 4, 8, 12 and 16 days. Chicken quarters were stored under vacuum packaging, at $4 \pm 0.5^\circ\text{C}$ during experiment. Microbiological analyses were conducted by using standard microbiological methods. Anaerobic plate count were determined using Plate Count Agar, after incubation for 2 days at 35°C under anaerobic condition. *Pseudomonas* spp. were determined on Pseudomonas Isolation agar after incubation at 48 h at 25°C . For lactic acid bacteria were inoculated into Rogosa and Sharpe agar after incubation 48-78 h at 37°C in an aerobic atmosphere supplemented with carbon dioxide (5% CO_2). For members of the family *Enterobacteriaceae* violet red bile glucose agar were used and samples were incubated at 37°C for 24 h. The initial APC value of chicken quarter was $3.00 \log \text{CFU.g}^{-1}$ on 0 day. The number of anaerobic plate count ranged from $3.00 \log \text{CFU.g}^{-1}$ in all tested group on 0 day to $6.11 \log \text{CFU.g}^{-1}$ on 16 day in control group stored in air condition. The initial LAC value of chicken quarter was $3.00 \log \text{CFU.g}^{-1}$ on 0 day. The number of lactic acid bacteria ranged from $3.00 \log \text{CFU.g}^{-1}$ in all tested group on 0 day to $3.58 \log \text{CFU.g}^{-1}$ on 16 day in control group stored in air condition. The initial *Enterobacteriaceae* genera value of chicken quarter was $2.00 \log \text{CFU.g}^{-1}$ on 0 day. Presences of these bacteria were found on all groups at 16 days. The results of this present study suggest the possibility of application the *Lavandula angustifolia* and *Rosmarinus officinalis* essential oil as natural food preservatives and potential sources of antimicrobial ingredients for food industry.

Keywords: chicken quarters; microorganisms; lavender and rosemary essential oils; vacuum; EDTA

INTRODUCTION

Chicken meat has many desirable nutritional characteristics such as a low lipid content and relatively high concentration of polyunsaturated fatty acids (Bourre, 2005). Fresh meat products are usually marketed at refrigerated temperatures ($2 - 5^\circ\text{C}$). Spoilage of raw meat may occur in two ways during refrigeration: microbial growth and oxidative rancidity (Sebranek et al., 2005; Zelenáková et al., 2010). Spoilage of fresh poultry meat is an economic burden to the producer and it leads to the development of methods to prolong the shelf-life and overall safety/quality which is the major problem faced by poultry processing industry (Petrou et al., 2012).

Furthermore, meats might get contaminated with microorganisms while butchering or during the manufacturing process, though the tissues of healthy

animals would be sterile at the time of slaughter. These microorganisms bring about undesirable quality changes in meats, especially with respect to lactic acid bacteria, a major bacterial group associated with meat spoilage. When large numbers of microorganisms are present in raw meat, there will be changes such that it becomes unappealing and unsuitable for human consumption (Gram et al., 2002; Doulgeraki et al., 2012).

Plants and plants products have been claimed to have health-promoting effects, which may be related to the antioxidant activity *in vivo* (Ivanišová et al., 2013; Ivanišová et al., 2015 a,b).

At present, meat industry uses chemical additives in several meat processes to prevent the growth of food-borne pathogens and extend the shelf life of refrigerated storage. Since concern over the safety of chemical additives has

arisen in recent years, consumers increasingly demand the use of natural products as alternative preservatives in foods (Govaris et al., 2010). Herbs and spices, which are important part of the human diet, have been used for thousands of years in traditional medicine and to enhance the flavor, color and aroma of foods. In addition to boosting flavor, herbs and spices are also known for their preservative (Nielsen and Rios, 2000), antioxidative (Shobana and Naidu, 2000), and antimicrobial roles.

The plants of *Lamiaceae* family are a rich source of polyphenols and hence could possess strong antioxidant properties (Gulluce et al., 2007). *Lavandula latifolia*, known as Spike lavender or Portuguese lavender, is a flowering plant in the family *Lamiaceae*, native to the western Mediterranean region, from central Portugal to northern Italy (Liguria) through Spain and southern France. *L. latifolia* is a strongly aromatic shrub growing to 30 – 80 cm tall. The leaves are evergreen, 3 – 6 cm long and 5 – 8 mm broad. The flowers are pale lilac, produced on spikes 2 – 5 cm long at the top of slender, leafless stems 20 – 50 cm long. The flowers and leaves of this plant are used as an herbal medicine, in the form of an herbal tea. Lavender essential oil contains the most popular aromatic herbal compounds and is widely used in food, perfume and pharmaceutical industries (Kim and Lee, 2002). Lavender essential oil has a soothing and calming effect on the nerves, relieving tension, depression, panic, hysteria and nervous exhaustion in general and is effective for headaches, migraines and insomnia. It is also very beneficial for problems such as bronchitis, asthma, colds, laryngitis, halitosis, throat infections and whooping cough. Many pharmacological benefits such as anticonvulsant, anxiolytic effect, antioxidant and anticholinesterases properties, anti-bacterial, antioxidant, anti-inflammatory, antimicrobial activity, antifungal activity, have been associated with this essential oil. Therefore, many researchers focus on analyzing Lavender essential oil. It is shown that the main chemical composition of Lavender essential oil depends on genotype, environment, processing and extraction methods (Jalali-Heravi et al., 2015).

Rosmarinus officinalis L. (rosemary) is an aromatic plant belonging to *Lamiaceae* family (Begum et al., 2013). Rosemary has been used for thousands of years for both culinary and medicinal purposes, due to its aromatic properties and health benefits. The biological activities of this plant are mainly related to the phenolic and the volatile constituents (Babovic et al., 2010; Teixeira et al., 2013; Arranz et al., 2015) such as carnosol, carnosic acid and rosmarinic acid present in the extract of rosemary and α -pinene, bornyl acetate, camphor and eucalyptol present in the essential oil of this plant (Babovic et al., 2010; Teixeira et al., 2013; Arranz et al., 2015). Minor components may have a potential influence on the biological activity due to the possibility of synergistic effect among their components (Hussain et al., 2010). *R. officinalis* L. can be used fresh, dried or as tea infusion.

The essential oil and the extract of rosemary can be obtained for application in food packaging, aromatherapy and medicine treatment (Peter, 2004; Szumny et al., 2010; Amaral et al., 2013; Barreto et al., 2014). Rosemary is used for cooking as flavoring, in the

preservation of foods, cosmetics (Peter, 2004; Sasikumar, 2012) or in folk medicine for anti-inflammatory, diuretic and antimicrobial applications (Teixeira et al., 2013; Arranz et al., 2015). Currently, rosemary has been widely investigated as food additive. This aromatic plant can be added directly to food or incorporated in food packaging, performing as antimicrobial and antioxidant agent (Liptajová et al., 2010; Ribeiro-Santos et al., 2015).

The aim of this study was to investigate the effects of lavender and rosemary essential oils, ethylenediaminetetraacetate in combination with vacuum packaging, on the microbiological indicators of chicken quarters.

MATERIAL AND METHODOLOGY

Preparation of samples

To evaluate the antimicrobial activity of essential oils the chicken quarters with skin of each experimental group was taken. The chicken quarters fresh samples were prepared as follow: for air-packaging (AC, control samples) chicken quarters fresh meat was packaged to polyethylene bags and stored aerobically in refrigerator; for vacuum-packaged (VPC, control samples) chicken quarters fresh meat was packaged to polyethylene bags and stored anaerobically in vacuum and in refrigerator; for vacuum-packed samples with EDTA solution 1.5% w/w (VPEC, control samples) chicken quarters fresh meat was treated with EDTA for 1 min and packaged to polyethylene bags and stored anaerobically in vacuum and in refrigerator; for vacuum-packed samples with *Lavandula angustifolia* L. 0.20% v/w (VP+LAO) chicken quarters fresh meat was treated with lavender oil for 1 min and packaged to polyethylene bags and stored anaerobically in vacuum and in refrigerator; for vacuum-packed samples with *Rosmarinus officinalis* L. 0.20 % v/w, (VP+ROO) chicken quarter fresh meat was treated with rosemary oil for 1 min and packaged to polyethylene bags and stored anaerobically in vacuum and in refrigerator (4 ±0.5°C). For sample packaging a vacuum packaging machine type VB-6 (RM Gastro, Czech Republic) was used and each sample were packed immediately after treatment. A stock solution of 500 mM concentration of EDTA was prepared by diluting 186.15g in 1 L distilled water (EDTA, C₁₀H₁₄N₂O₈·Na₂·2H₂O), 99.5% purity, analytical grade, (Invitrogen, USA). A final concentration of 50 mM EDTA solution was prepared from the stock solution. The pH of the solution was adjusted to 8.0 with the addition of the appropriate quantity of NaOH solution. Lavender and rosemary essential oil (Hanus, Nitra, Slovakia) was added to coat chicken quarter surface (both sides) of each sample using a micropipette. Final concentration of 0.2% v/w of EO was used for treatment.

Microbiological analysis

Approximately 10 g (10 cm²) of the chicken quarter was sampled using sterile scalpels and forceps, immediately transferred into a sterile stomacher bag, containing 90 mL of 0.1% peptone water (pH 7.0), and homogenized for 60 s in a Stomacher at room temperature. Sampling was carried out after certain time intervals: 0, 4, 8, 12 and 16 days. Chicken quarters were stored under vacuum packaging, at

4 ±0.5°C during experiment. Microbiological analyses were conducted by using standard microbiological methods. Anaerobic plate count (APC) were determined using Plate Count Agar (PCA, Oxoid, UK), after incubation for 2 days at 35°C under anaerobic condition. For *Pseudomonas* spp. enumerations, 0.1 mL from 1:10 prepared serial dilutions (0.1% physiological solution) of chicken homogenates was spread onto the surface of solid media. *Pseudomonas* were determined on Pseudomonas Isolation agar (PIA, Oxoid, UK) after incubation at 48 h at 25°C. For lactic acid bacteria enumeration, a 1.0 mL sample were inoculated into Rogosa and Sharpe agar (MRS, Oxoid, UK) after incubation 48 – 78 h at 37°C in an aerobic atmosphere supplemented with carbon dioxide (5% CO₂). For members of the family *Enterobacteriaceae*, a 1.0 mL sample was inoculated into 10 mL of molten (45°C) violet red bile glucose agar (VRBL, Oxoid, UK). After setting, a 10 mL overlay of molten medium was added and samples incubated at 37°C for 24 h. The large colonies with purple haloes were counted. All plates were examined for typical colony types and morphology characteristics associated with each medium applied for incubation.

RESULTS AND DISCUSSION

The main driving force for the growth of worldwide food industry is the scope and range of food preservation and shelf life extension technology (Sadaka et al., 2013). Active packaging is gaining increasing attention from researchers and the industry due to its potential to provide quality and safety benefits. Active packaging is a type of packaging that changes its conditions as a way to extend life or enhance safety or sensory properties while maintaining food quality (Martucci et al., 2015). In view

of the health concerns expressed by consumers and current environmental problems, research is now focusing on the development of sustainable packaging materials based on annually renewable natural biopolymers such as polysaccharides and proteins (Gomez-Estaca et al., 2010).

The consumer's desire for natural ingredients and for chemical preservative-free foods has increased the popularity of natural antimicrobial agents (Sadaka et al., 2013). In this framework, the addition of essential oils to biopolymer films as natural bacteriostatics could be an interesting election. Essential oils have well-recognized properties, such as antimicrobial (Gende et al., 2010; Teixeira et al., 2013 a,b), antibacterial (Min and Oh, 2009; Teixeira et al., 2013 a,b) and antioxidant properties (Burt, 2004; Danh et al., 2012; Kacániová et al., 2012).

Anaerobic plate count (APC) values for the tested groups of chicken quarter are showed in Figure 1. The initial APC value of chicken quarter was 3.00 log CFU.g⁻¹ on 0 day. The number of anaerobic plate count ranged from 3.00 log CFU.g⁻¹ in all tested group on 0 day to 6.11 log CFU.g⁻¹ on 16 day in control group stored in air condition. In control group stored in air condition the number of APC ranged from 3.00 log CFU.g⁻¹ on 0 day to 6.11 log CFU.g⁻¹ on 16 day. In control group stored under vacuum packaging ranged from 3.00 log CFU.g⁻¹ on 0 day to 6.05 log CFU.g⁻¹ on 16 day. In control group stored under vacuum packaging and EDTA treatment APC ranged from 3.00 log CFU.g⁻¹ on 0 day to 5.75 log CFU.g⁻¹ on 16 day. In the group with lavender essential oil treatment number of APC ranged from 3.00 log CFU.g⁻¹ on 0 day to 5.21 log CFU.g⁻¹ on 16 day and in group with with rosemary essential oil treatment ranged from 3.00 log CFU.g⁻¹ on 0 day to 4.98 log CFU.g⁻¹ on 16 day.

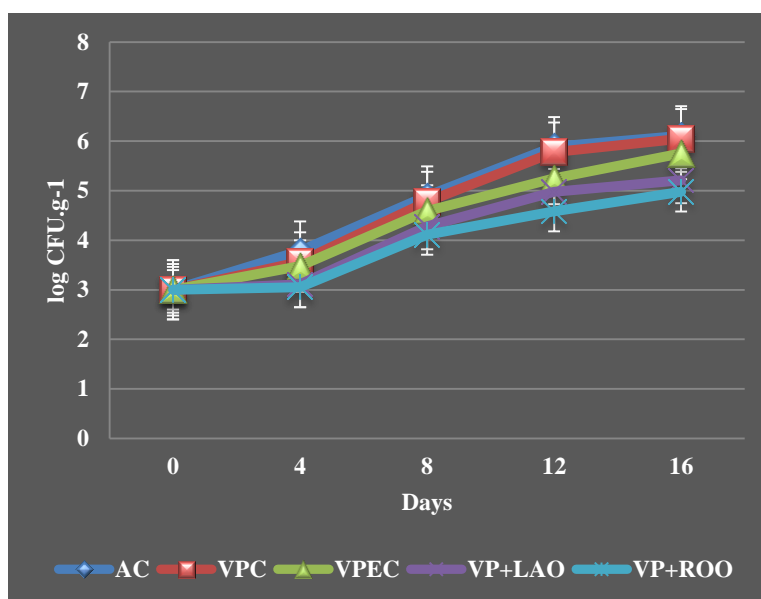


Figure 1 Changes (log CFU.g⁻¹) in population of anaerobic plate count in chicken quarter stored in air (AC); stored under vacuum (VPC); stored under vacuum packaging with EDTA (VPEC); stored under vacuum packaging with *Lavandula angustifolia* L. 0.20% v/w (VP+LAO); stored under vacuum packaging with *Rosmarinus officinalis* L. 0.20 % v/w, (VP+ROO).

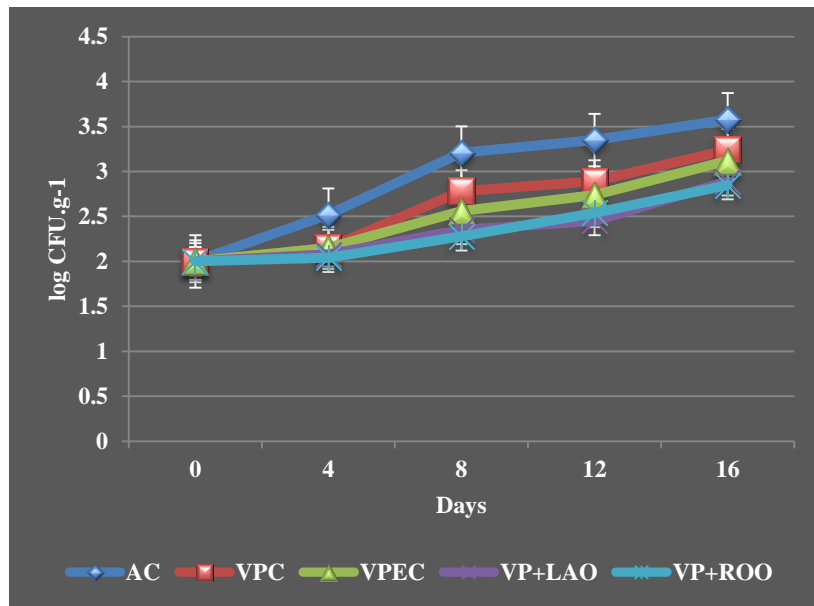


Figure 2 Changes ($\log \text{CFU.g}^{-1}$) of lactic acid bacteria in chicken quarter stored in air (AC); stored under vacuum (VPC); stored under vacuum packaging with EDTA (VPEC); stored under vacuum packaging with *Lavandula angustifolia* L. 0.20% v/w (VP+LAO); stored under vacuum packaging with *Rosmarinus officinalis* L. 0.20 % v/w, (VP+ROO).

Lactic acid bacteria (LAB) values for the tested groups of chicken quarter are showed in Figure 2. The initial LAC value of chicken quarter was $2.00 \log \text{CFU.g}^{-1}$ on 0 day. The number of lactic acid bacteria ranged from $2.00 \log \text{CFU.g}^{-1}$ in all tested group on 0 day to $3.58 \log \text{CFU.g}^{-1}$ on 16 day in control group stored in air condition. The number of lactic acid bacteria ranged from $2.00 \log \text{CFU.g}^{-1}$ in all tested group on 0 day to $3.58 \log \text{CFU.g}^{-1}$ on 16 day in control group stored in air condition. In control group stored in air condition the number of LAB ranged from $2.00 \log \text{CFU.g}^{-1}$ on 0 day to $3.58 \log \text{CFU.g}^{-1}$ on 16 day. In control group stored under vacuum packaging ranged from $2.00 \log \text{CFU.g}^{-1}$ on 0 day to $3.24 \log \text{CFU.g}^{-1}$ on 16 day. In control group stored under vacuum packaging and EDTA treatment LAB ranged from $2.00 \log \text{CFU.g}^{-1}$ on 0 day to $3.12 \log \text{CFU.g}^{-1}$ on 16 day. In the group with lavender essential oil treatment number of LAC ranged from $2.00 \log \text{CFU.g}^{-1}$ on 0 day to $2.89 \log \text{CFU.g}^{-1}$ on 16 day and in group with with rosemary essential oil treatment ranged from $2.00 \log \text{CFU.g}^{-1}$ on 0 day to $2.85 \log \text{CFU.g}^{-1}$ on 16 day.

The addition of rosemary or thyme EO to fine paste meat products has been effective against aerobic bacteria and LAB (Viuda-Martos et al., 2010).

The antimicrobial effects of different spice extracts in raw chicken meat during storage for 15 days at 4°C were studied in Radha Krishan et al. (2014) work. Raw chicken meat was treated with BHT (positive control), *Syzygium aromaticum*, *Cinnmomum cassia*, *Origanum vulgare*, and *Brassica nigra* extracts and the different combinations as well as the results were compared to raw chicken meat without any additive (negative control). The antimicrobial activities of spice extracts were determined.

The total viable counts, Lactic Acid Bacteria counts, *Enterobacteriaceae* counts, *Pseudomonas* spp. cunts were

determined at a gap of 3 days interval for a period of 15 days. The bacterial counts of groups with spice samples were lower than control samples during storage.

EOs have been shown to possess antibacterial and anti-fungal activities against several microorganisms associated with meat, including gram-negative and gram-positive bacteria (Karabagias et al., 2011). Many recent studies have been conducted to examine the effects of EOs obtained from sour-ces such as oregano, rosemary, thyme, sage, basil, turmeric, coriander, ginger, garlic, nutmeg, clove, mace, savory, and fennel, when used alone or in combination with other EOs and/or preservation methods, in order to improve the sensory qualities and extend the shelf life of meat and meat products (Goulas and Kontominas, 2007). Additionally, extracted EOs and oleoresins are preferred over crude spices in the meat in-dustry due to their better stability during storage, microbial safety, high concentration of flavor components, reduced storage space, ease of handling, no seasonal variation, and standardization (Jayasena and Jo, 2013).

Numerous experimental applications of EOs as antimicrobial agents in meat and meat products are summarized. Skandamis et al., (2002) reported that EOs from clove, oregano, rosemary, thyme, and sage have high inhibitory activity, particularly against gram-positive bacteria, rather than gram-negative bacteria (Marino et al., 2001).

Chicken treated with thyme EO has been reported to show remarkable inhibition of lactic acid bacteria (LAB) growth until the end of storage. This agrees well with the findings of Gutierrez et al., (2009) on the inhibitory action of these oils against *E. coli* and *Salmonella* spp. in food as well as in *in vitro* models.

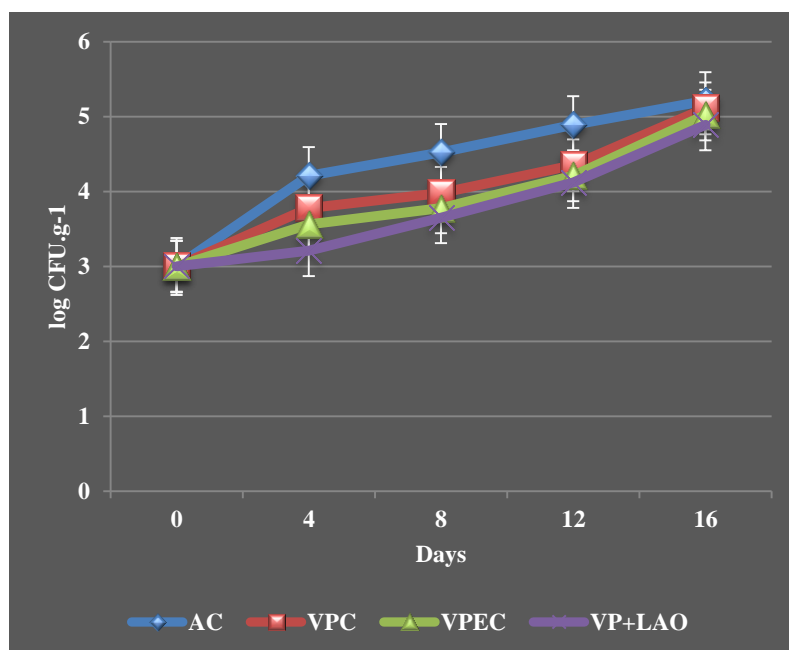


Figure 3 Changes ($\log \text{CFU.g}^{-1}$) in population of *Enterobacteriaceae* genera in chicken quarter stored in air (AC); stored under vacuum (VPC); stored under vacuum packaging with EDTA (VPEC); stored under vacuum packaging with *Lavandula angustifolia* L. 0.20% v/w (VP+LAO); stored under vacuum packaging with *Rosmarinus officinalis* L. 0.20% v/w, (VP+ROO).

Clove, cinnamon, pimento, and rosemary EOs effectively inhibited the growth of meat spoilage bacteria. Further, cumin, garlic, oregano, and black pepper EOs considerably inhibited the growth of meat spoilage organisms. Similar results had been reported previously by Aureli et al., (1992), who examined the inhibitory effect of EOs against various pathogens and spoilage bacteria.

Enterobacteriaceae genera values for the tested groups of chicken quarter are showed in Figure 3. The initial *Enterobacteriaceae* genera value of chicken quarter was $2.00 \log \text{CFU.g}^{-1}$ on 0 day. Presences of these bacteria were found on all groups at 16 day.

The presence of *Pseudomonas* spp. bacteria in this study were not found in all tested groups.

Main spoilage bacteria including *Pseudomonas*, *Acinetobacter*, *B. thermosphacta*, *Moraxella*, *Enterobacter*, *Lactobacillus* spp., *Leuconostoc* spp., *Proteus* spp. etc, yeast and mold decompose meat and meat products and develop unpleasant quality characteristics (Fратиanni et al., 2010; Lucera et al., 2012) when they grow in large number in these perishable products.

The mechanism of action for the antimicrobial activity of spice and plants extracts is not fully understood, however, membrane disruption by phenolics and metal chelation by flavonoids are considered to inhibit the growth of microorganisms. Some researchers have reported that phenolic compounds from different plant sources could inhibit various food-borne pathogens, and the total phenolic content have been highly correlated with antibacterial activity (Shan et al., 2007). The antimicrobial activities of phenolic compounds may involve multiple modes of action. For example, phenolic compounds can degrade the cell wall, disrupt the cytoplasmic membrane,

cause leakage of cellular components, change fatty acid and phospholipid constituents, influence the synthesis of DNA and RNA and destroy protein translocation (Shan et al., 2007).

CONCLUSION

Lavender and rosemary essential oils exhibited good antimicrobial properties against anaerobic bacteria, lactic acid bacteria and *Enterobacteriaceae* genera in 0.2% concentration. Meat is highly subject to microbial deterioration, which ultimately leads to safety and quality issues if the meat is not properly handled and preserved. Several plant-derived EOs can be effectively used in meat as natural alternatives to synthetic food additives, particularly as effective antimicrobial agents.

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COMPOSITION OF FATTY ACIDS IN SELECTED VEGETABLE OILS

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ABSTRACT

Plant oils and fats are important and necessary components of the human nutrition. They are energy source and also contain fatty acids – compounds essential for human health. The aim of this study was to evaluate nutritional quality of selected plant oil – olive, rapeseed, pumpkin, flax and sesame; based on fatty acid composition in these oils. Fatty acids (MUFA, PUFA, SFA) were analyzed chromatography using system Agilent 6890 GC, injector multimode, detector FID. The highest content of saturated fatty acids was observed in pumpkinseed oil (19.07%), the lowest content was found in rapeseed oil (7.03%), with low level of palmitic and stearic acids and high level of behenic acid (0.32%) among the evaluated oils. The highest content of linoleic acid was determined in pumpkinseed (46.40%) and sesame oil (40.49%); in these samples was also found lowest content of α -linolenic acid. These oils have important antioxidant properties and are not subject to oxidation. The richest source of linolenic acid was flaxseed oil which, which is therefore more difficult to preserve and process in food industry. In olive oil was confirmed that belongs to the group of oils with a predominantly monosaturated oleic acid (more than 70%) and a small amount of polysaturated fatty acid. The most commonly used rapeseed oil belongs to the group of oils with the medium content of linolenic acid (8.76%); this oil also showed a high content of linoleic acid (20.24%). The group of these essentially fatty acids showed a suitable ratio $\sum n3/n6$ in the rapessed oil (0.44).

Keywords: plant oil; fat; nutritional quality; fatty acid; SFA; PUFA; MUFA

INTRODUCTION

According to statistical data, the proportion of fat content in total energy intake of an adult man is in developed countries even 42%. Nutrition experts agree that fats in food should be no more than 30 – 35% of total energy daily intake, while one third should be in the form of animal fat and two thirds should constitute a high-quality vegetable fats and oils. The development of new kinds vegetable oils with functional properties use a components of oil seeds with health benefits to human body (Augustín, 2010).

Edible fats and oils are carriers of the important biological factors which relate to the presence of essential fatty acids. Vegetable oils are the primary source of essential fatty acids. Although the fats can be described as one of the basic nutritional components and they perform specific functions in the human body, currently they are perceived as a component associated with increased risk of disease. The problem is not in fats, but their over-consumption, especially in the unbalanced intake of fatty acids (Babinská and Bederová, 2012). The human body is capable of producing all the fatty acids which it needs except for two: linoleic acid (LA) (omega-6 fatty acid) and alpha-linolenic acid (ALA) (omega-3 fatty acid). These we should be adopt in the diet. Both of these fatty acids are necessary for growth and development, but they are used in the creation of other fatty acids (e.g. arachidonic acid (AA) is formed from LA). Given that the conversion to the omega-3 fatty acids - eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is limited, it is recommended

that a diet should contain their source. ALA and LA are found in oils from the various kinds of seeds (Lunn and Theobald, 2006). Essential fatty acids are able to absorb sun light. With absorption is improving their ability to react with oxygen, for this reason they are chemically very active. The biggest enemy of essential fatty acids is light. It causes bitterness of oil. The light causes the destruction of vital biological properties of these acids (Komprda, 2007). Fats accepted by food should contain saturated, monounsaturated and polyunsaturated fatty acids. Saturated acid should cover <10% of the available energy from food, polyunsaturated acids n-6, 4 – 8% on average, about 5% (Velíšek and Hajšlová, 2009). Omega-3 fats belong to the group called essential fatty acids. They are highly specific, unsaturated with three double bonds. These includes mainly the alpha-linolenic acid (ALA) and gamma-linolenic acid (GLA), but perhaps more important are eicosapentaenoic acid (EPA), which has five double bonds and is made of ALA and docosahexaenoic acid (DHA) which has six double bonds. Conjugated linoleic acid (CLA) belongs to the omega-3 fatty acids and it has exceptional effect, because it moderates the devastating effect of physical overload and stress. It is also involved in the development of obesity prevention. DHA has beneficial effect on vision and contribute to the brain development in children. The alpha-linolenic acid (ALA) is a part of the linseed oil. This oil also contains significant amounts of linoleic acid, which is one of the omega-6 acids group (Fořt, 2011). Komínková (2012) report, that the omega-3 acids are necessary for the production of

prostaglandins. The beneficial effect of omega-3 fatty acids for our bodies is to have a preventive effect on the many diseases caused by modern lifestyles. It reduce the risk of cardiovascular disease, modify of the blood pressure, improve the elasticity of blood vessels and reduce the level of cholesterol and blood coagulation. Different countries recommend varying amounts of omega-3 fatty acids from 0.5% to 2% of energy intake. The recommended intake of ALA is about 0.6 – 1.2% of daily energy intake or 1 – 2 g per day. It can be achieved by consuming fatty species of fish once or twice a week with occasional substitution sunflower oil by rapeseed oil (Frej, 2014; Nitrayová et al., 2014). Omega-3 and omega-6 fatty acids are important components of cell membranes and they are precursors of several substances in the body, which are involved in a blood pressure regulation and an inflammatory process. Omega-3 fatty acids are promoted to prevent the heart disease. Nowadays, interest about the role of omega-3 fatty acids is increase especially in relation to prevention of the diabetes and some types of cancer (Lunn and Theobald, 2006). Many studies about fatty acids have shown the exceptional importance of correct ratio of omega-6 and omega-3 fatty acids. Estimated long-term ratio of LA : ALA in human food is about 3 : 1 to 2 : 1. Most experts agree that this ratio is ideal for human body and its functions. In the human body, LA and ALA compete for metabolism by the enzyme $\Delta 6$ -desaturase. It has been suggested that this is important to health, as too high an intake of LA would reduce the amount of $\Delta 6$ -desaturase available for the metabolism of ALA, which may increase the risk of heart disease. This was supported by data showing that over the last 150 years, intakes of omega-6 have increased and intakes of omega-3 have decreased in parallel with the increase in heart disease. Thus, the concept of an “ideal” ratio of omega-6 to omega-3 fatty acids in the diet was developed (Simopoulos, 2008). However, the ratio that is associated with a reduced risk of heart disease has not yet been identified and some experts now suggest that the ratio is less important – what we should be more concerned with is the absolute levels of intake (Stanley et al., 2007).

The aim of our work was to analyze selected vegetable oils in terms of fatty acids representation and on the basis of obtained results evaluate their nutritional quality.

MATERIAL AND METHODOLOGY

Biological material

Vegetable oils were purchased from specialty store in the Slovak Republic (Olive oil, Olive oil Minerva, Rapeseed oil, Flaxseed oil, Pumpkinseed oil, Sesame oil).

Fatty acids analysis

Fatty acids of oils were analyzed chromatographically by Agilent 6890 GC system, multi Mode injector, FID detector, Agilent Technologies, USA). Lipid fraction was hydrolyzed (saponified) by hydroxide into glycerol and free fatty acids (FFAs). FFAs were esterified with methanol (methylation) to the corresponding fatty acid methyl esters (FAMES). FAMES were analyzed, i.e. separated and identified using gas chromatography-flame ionization detector (GC-FID).

Chemicals

All chemicals were analytical grade. We used 10 mg.mL⁻¹ of 37-component FAME standard mixture in methylene chloride containing C4 to C24 FAMES (2 to 4% of relative concentration). Producer Supelco, catalog number 47885-U.

Fatty acid methyl esters (FAMES) preparation method

Weigh 200 mg.mL⁻¹ fat sample into test tube (with ground joint neck). Using a pipette, add 5 mL of *n*-hexane to the test tube. The sample is subsequently dissolved by mixing. Add 1 mL of 2N potassium hydroxide in methanol. Test tube is shaken intensively and then put in water bath heated for 60°C for 30 s. After shaking the tubes, leave them rest for 1 min. Add 2 mL of hydrogen chloride and gently shake the content. After reaching the equilibrium state and layer separation, the upper layer containing FAMES is pipetted carefully, filtered through anhydrous Na₂SO₄ and used for GC FID analysis. Before the analysis, 50 μ L of FAME solution is diluted with 950 μ L of *n*-hexane. Elution time of separated analytes is the quality indicator. Chromatograms of samples are compared to the standard chromatogram. On the contrary, the area under the peak of monitored analyte is the indicator of quantity. For quantitative evaluation is usually being used the method of internal standardization which assumes that all of the sample components are recorded on chromatogram what covers the total surface of peaks (100%). Areas under the unitary peaks (of individual FAMES) represent the mass percentage of specific fatty acid from the total fatty acids content of sample.

RESULTS AND DISCUSSION

Lipids are known as important food elements which play an important role in human nutrition, e.g. for the overall health and organism development. According to Venclová, (2010) fatty acids are nutritionally the most significant lipid component. Vegetable fats and oils are common and essential constituents of human food where they act like a substantial part of energy source and they also contain essential fatty acids which are necessary for proper functioning of human body (Gustone, 2011). According to the literature reports, palmitic and stearic acid are the most important and widespread saturated fatty acids (SFA) present in animal fats. On the other hand, lauric and myristic acid are the main ingredients of palm fruits and seeds fat. We recorded the highest amount of SFA (19.07%) in pumpkin seed oil (Table 2). In comparison with other samples, the highest content of stearic acid (6.62%) was measured in this oil (Table 1). Equally high was also the palmitic acid content (11.61%) and this oil was the only one where we observed the presence of myristic acid (0.11%). On the contrary, the rapeseed oil showed the lowest content (7.33%) of saturated fatty acids, with low values of palmitic and stearic acid (Table 2). However, behenic and lignoceric acid are in this oil presented in the highest amount. Velišek and Hajšlová, (2009) also report the presence of behenic acid in rapeseed oil up to 2%.

The total saturated fatty acid content ranged in our evaluated oils from 7.33% to 19.07%. SFA are chemically very stable and change either by prolonged heating or at

high temperatures. The human body uses SFA to the length of 12 carbon atoms, mainly for the energy production.

They are recommended to people with digestive problems or liver disorders, because they are not stored in body as fat and also due to their easy digestibility. According to Keresteš et al., (2011), SFA with longer chain (above 14 carbons) have a tendency to agglomerate with negative effect to the cardiovascular disease occurrence. SFA are present especially in animal fats. Pursuant to the above mentioned author, SFA plug our tissues by sticking the red blood cells which causes their mobility deterioration and therefore disruption of their ability to supply oxygen to the cells and tissues (hypoxia). The length of SFA carbon chain sets their melting point and stiffness. The longer is fatty part of the molecule, the greater is their tendency to agglomerate. On the basis of the recommended daily amount, the SFA income should not exceed 10% (Kissová, 2009; Bowden, 2011). In animal fats and vegetable oils, most frequent are unsaturated fatty acids (UFA) with straight chain formed by 10 – 36 carbon atoms. Very common are monounsaturated and polyunsaturated fatty acids (MUFA and PUFA) with 16 – 18 carbons. Unsaturated fatty acid content varies in a very wide range, from more than 90% of all fatty acids in rapeseed oil to less than 10% in coconut oil. In comparison with animals, there is far greater variety in plants UFA composition. Like the other animals and plants, human being does not only receive fatty acids through food, but is also able to synthesize the SFA and certain UFA. However, contrary to the plants, he cannot synthesize the n-6 (linoleic) and n-3 (linolenic) PUFA therefore we have to receive them through food. In terms of use and composition, vegetable fats and oils are most often divided into groups with similar content of fatty acids. Olive oil belongs to the group of oils with predominant oleic acid and small amount of PUFA. As the table 1 shows, the oleic acid content was in both analyzed samples above 70% (73.61% and 74.79%) which is the highest% from all of the assessed oils. Olives contain around 50% of oil. The content of other fatty acids there is

significantly lower what guarantees longer shelf life and limpidity of olive oil at low temperatures. It is used in food industry as a traditional salad oil with excellent oxidative stability. The research of Blahová, (2011) suggests its great antioxidant effects as it prevents cholesterol oxidation to harmful form. Rapeseed oil belongs to the group of oils with medium linolenic acid content. Our sample reached the value around 8% (8.76) which is rather high number. Dejong and Lanari, (2009) state linolenic acid content may vary in the range of 6-14%. In the sample of rapeseed oil we also measured a relatively high percentage of another essential fatty acid - linoleic acid (20.24%). However, the most represented PUFA in our sample is oleic acid (58.8%) what coincides with the data of several authors (Šmidrkal et al., 2008; Velíšek and Hajšlová, 2009). From the content of ω-3 and ω-6 fatty acids is also apparent ideal $\sum n3/n6$ ratio in the rapeseed oil (0.44 - tab. 2). Thanks to high acid content, rapeseed oil is used as liquid component of fat blends for the production of emulsified fats and shortenings. Fat content in the oilseed rape ranges from 40 to 44%. Oilseed rape is alongside with sunflower our most widespread oil crop, while cultivated are only varieties with reduced erucic acid content (up to max. 2%). According to the mentioned classification, sesame oil belongs to the group of oils without linolenic acid and with high essential linoleic acid content. This also implies a significantly high $\sum n6/n3$ fatty acid ratio (136.2). Linoleic acid in our sesame oil sample reached value 40.94% from all of the fatty acids. Oleic acid achieved similarly high amount in the sesame oil (40.75%) and arachidonic acid reached the highest percentage from all of the evaluated oils. Blahová, (2011) points out to strong antioxidant effect of sesamol which may be found in Indian sesame seeds. Sesame oil has a soft taste and does not subject to oxidation. Pumpkin seed oil belongs to the same group as sesame oil, while we measured there the highest essential linoleic (46.4%) and lowest linolenic acid content (0.17%). $\sum n6/n3$ fatty acid ratio is much higher (275.68) than in sesame oil.

Table 1 Fatty acids composition in selected vegetable oils [%].

Fatty acids [%]	Evaluated oils					
	Sesame oil	Flaxseed oil	Olive oil	Olive oil Minerva	Rapeseed oil	Pumpkin seed oil
Myristic acid	0	0	0	0	0	0.11
palmitic acid	9.56	5.38	11.01	11.91	4.68	11.61
palmitoleic acid	0.14	0.07	0.59	0.79	0.14	0.11
heptadecenoic acid	0	0	0	0	0	0.07
cis-10-heptadecenoic acid	0	0	0	0	0.11	0
stearic acid	5.85	4.29	2.62	2.85	1.60	6.62
oleic acid	40.75	17.51	74.79	73.61	58.81	32.68
linoleic acid	40.94	15.11	7.01	6.51	20.24	46.40
α-linolenic acid	0.30	56.02	0.69	0.68	8.76	0.17
Arachidonic acid	0.62	0.14	0.45	0.48	0.55	0.46
cis-11-eicosenoic acid	0.16	0.11	0.33	0.28	1.11	0.08
cis-11,14-eicosenoic acid	0	0	0	0	0.08	0
behenic acid	0.11	0.11	0.13	0.15	0.32	0.12
lignocericacid	0.09	0	0	0	0.17	0.07
cis-4,7,10,13,16,19-docosahexaenoic acid	0	0	0	0	0.15	0

Table 2 Oil assessment from the viewpoint of the fatty acids summary composition.

Summary composition of FA [%]	Evaluated oils					
	Sesame oil	Flaxseed oil	Olive oil	Olive oil Minerva	Rapeseed oil	Pumpkin seed oil
PUFA	41.24	71.13	7.69	7.19	29.23	46.57
MUFA	41.05	17.69	75.72	74.69	60.16	32.87
SFA	16.24	9.92	14.22	15.40	7.33	19.07
∑ n3/n6 ratio	0.01	3.71	0.10	0.10	0.44	0.003
∑ n6/n3 ratio	136.20	0.27	10.19	9.58	2.28	275.68

Harperová, (2004) refers to extraordinary positive effects of pumpkin seed oil to digestive tract. Traditional flaxseed oil is one of the oils with high α -linolenic acid content. This acid is the main component of leaves, especially of algae and higher plants photosynthetic apparatus. In flaxseed oil is commonly in a quantity up to 65% and our sample showed amount of 56.02% what only confirms very high presence of α -linolenic acid in this oil. Brát, (2014) also points out to high α -linolenic acid content, which may in terms of flaxseed oil shelf life cause some problems during food processing. If the oil is not stored cool resp. without light and oxygen access, this will result in fatty acids change to undesirable trans-isomers. Flaxseed oil is primarily the richest omega-3 fatty acids source, which mainly serve as immunity and endocrine glands support and they also ease the water excretion via kidneys. High \sum n3/n6 ratio (56.02/15.25) was confirmed in our sample too. The presence of oleic acid in flaxseed oil sample represented the lowest number (17.51%) from all of the evaluated oils and so did palmitoleic acid (0.07%).

CONCLUSION

Plants oils are important energy source and their composition is necessary for the human body. They are a source of unsaturated fatty acids, phytosterols, vitamin E. Each type of oil is specific in composition. Their common features normally associated with a high percentage of unsaturated fatty acids, and the absence of cholesterol.

From the evaluation of plant oils, the highest content of saturated fatty acids was measured in pumpkinseed oil (19.7%). The relatively low proportion of saturated fatty acids was reflected in rapeseed oil (7.3%). This oil was represented the broadest range of saturated fatty acids.

From the unsaturated fatty acid had a high presence of oleic acid, olive oil (more than 70%) and rapeseed oil (more than 50%), reflected on the values of MUFA (75.7% in olive oil) and 60.2% rapeseed oil. These oils also had the lowest content of polyunsaturated fatty acid of 7.7% PUFA in olive oil and 29.2% rapeseed oil. From the results it can be confirmed that the pumpkin and sesame oil are

classified in the group of oils rich for linolenic acid. This indicated also a high ratio \sum n6 / n3 fatty acids (275.7 to 136.2 pumpkinseed oil and sesame oil). PUFA highest proportion (71.13%) was shown in flaxseed oil, in which the ALA content was found at 56%, but this can cause problems during processing and storage of flaxseed oil. From this reason is necessary storage this oil in the dark place, which decrease change for trans-isomers.

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HONEY CHARACTERISTICS AFTER EXTRACTION AND HALF-YEAR STORAGE

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Margita Čanigová, Peter Haščík

ABSTRACT

The aim of the study was to analyze the fresh honey after extracting and after half-year storage at room temperature. Overall, we analyzed 10 samples of rape (*Brassica napus*) honey coming from district Vranov nad Topľou located in the eastern Slovakia. The analysis consisted of the evaluation of the physico-chemical parameters (water content, free acidity and electrical conductivity) and microbiological evaluation (total plate count (TPC), counts of coliform bacteria, lactic acid bacteria, sporulating microorganisms and microscopic fungi). Water content, free acidity and electrical conductivity were measured according to IHC (2009), namely these parameters were detected by refractometer, titration and conductometer, respectively. We used dilution plating method for microbiological analysis. Fresh rape honey contained $18.3 \pm 1.0\%$ of water. Free acidity of fresh rape honey was 12.7 ± 2.0 meq.kg⁻¹ and electrical conductivity was 0.14 mS.cm⁻¹. After half a year of storage, water content and electrical conductivity decreased nonsignificantly and free acidity increased nonsignificantly. Stored honey samples meet the requirements of Decree 41/2012 and 106/2012. From microbiological point of view, fresh rape honey showed relatively high microbial counts. Mean values of TPC, sporulating microorganisms, lactic acid bacteria and yeasts exceeded 2.00 log cfu/g. All spotted microbial groups decreased in the stored honey comparing with the fresh honey. We found significant ($p < 0.01$) differences of TPC, lactic acid bacteria and yeasts comparing the fresh and stored honey samples. Evaluating microbiological parameters, one sample of stored honey did not meet the requirements of Codex Alimentarius SR (2014). TPC exceeded the limit value. Based on the results we can conclude that all samples meet the requirements for good quality honey. Microbial counts in the honey decreased gradually. Probably, various microorganisms have important role in creation of the honey from the nectar of plants, but non-sporulating microorganisms die in the ripe honey.

Keywords: honeybee, rape, microorganism, electrical conductivity

INTRODUCTION

Today, honey is one of the last untreated natural foods and the consumption of honey differs strongly from country to country (Bogdanov, 2015). Research and education in this field may be the good tools to rediscovery of these interesting products in our homes.

Honey is classified by its floral source from which the nectar is collected by the honey bee. Unifloral honey is made primarily from the nectar of one type of common flower in which the bees have access (Chaven, 2014).

Rape (*Brassica napus* L. and other species, hybrids and varieties) is largely cultivated in Europe for the seed, used for oil production. It is very attractive to bees both for nectar and pollen and in Central and Eastern European countries represents one of the most important spring sources, giving rise to large amounts of very pure unifloral honey (Oddo et al., 2004).

Rape honey is popular in Slovakia because of its interesting properties. Rape honey has pleasant taste and it is suitable for formation of creamed honey.

In general, ripe honey is commonly considered to be relatively sterile foodstuff. The properties of honey that

make it effective against bacterial growth are: high sugar content, low water activity, gluconic acid, which creates an acidic environment and hydrogen peroxide (Khan et al., 2007). Olofsson and Vásquez (2008) suggested that honey be considered as a fermented food product because of the lactic acid bacteria (LAB) involved in honey production.

Nectar is converted into honey through a maturation process, in which a considerable water loss (40 to 70% of nectar initial weight) is the most prominent feature (Ruiz-Argueso and Rodríguez-Navarro, 1975). The nectar sugars probably act as inducers for the resident honey stomach microbiota, enhancing their numbers, with the enhancement depending on the types of flowers that the honeybees visited; and the bacteria are added during the process by which nectar becomes honey (Olofsson and Vásquez, 2008). Moreover, honey can play a bifidogenic role in detoxifying mycotoxins and decrease their detrimental effects including tissue necrosis (Sidoo-Atwal and Atwal, 2012). Vidová et al. (2013) explain the bifidogenic substances as agents that can support the growth of *Bifidobacterium* sp. (*B. longum* ssp. *longum*,

B. breve, *B. pseudolongum*, *B. longum* ssp. *infantis* and *B. animalis* ssp. *lactis*) and *Lactobacillus* sp. (e. g. *L. acidophilus*, *L. casei*, *L. reuteri*, *L. plantarum*). **Ruiz-Argueso and Rodriguez-Navarro (1975)** isolated *Gluconobacter* sp., *Lactobacillus* sp., *Zymomonas* sp. and occasionally several types of yeasts from ripening honey. **Olofsson and Vásquez (2008)** discovered LAB in the fresh honey, namely *Lactobacillus kunkeei*, *L. buchneri* and *Bifidobacterium asteroides* and they confirmed origin of these bacteria in honeybee stomach. **Endo and Salminen (2013)** found specific group of LAB - FLAB (fructophilic lactic acid bacteria) in fresh honey, namely *Lactobacillus kunkeei*. **Bogdanov (2015)** concludes that in general, honey shows prebiotic activity and fresh honey (about to 2-3 months old) contains probiotic bacteria.

The aim of the study was to compare the basic physico-chemical parameters and microbial counts in the fresh and stored rape honey from one Slovak region.

MATERIAL AND METHODOLOGY

We analysed 10 apiary samples of blossom honey. Samples were obtained directly from beekeepers from 7 villages of Vranov nad Toplou district. Figure 1 show the region in eastern part of Slovakia, where the samples originated, together with detailed sampling places (names of villages). Length of sampling area was 30 km. Samples originated mainly from nectar of rape fields with additional nectar of fruit trees. Detailed characteristic of evaluated samples is in Table 1. Samples were collected aseptically from honey vessels immediately after honey extraction. Samples were stored in refrigerator before the first analysis (2 weeks after extraction). Then, samples were stored at room temperature in dark place. The second analysis was undertaken after half a year of storage.

The evaluation was divided into 2 parts: physico-chemical and microbiological analysis.

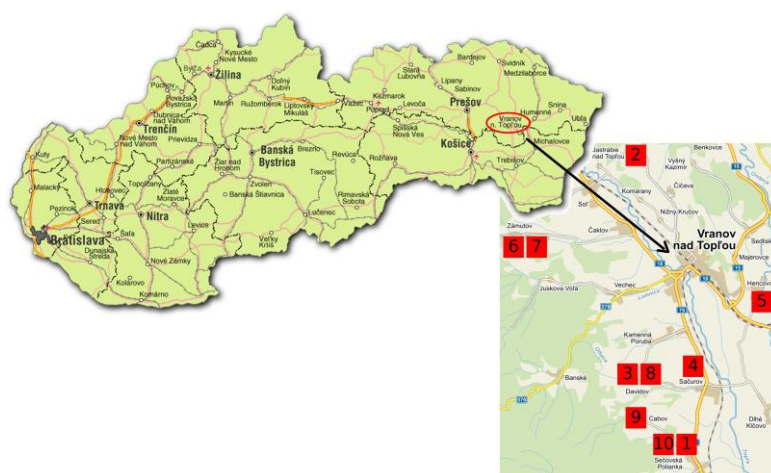


Figure 1 Sampling places on the map of Slovakia, 1 – 10 are number of samples.

Table 1 Characteristic of evaluated honey samples.

No.	Botanical or.	Geographical or.	Ext.
1	rape + fruit trees	Secovska Polianka	05/14
2	rape + fruit trees + pine	Jastrabie nad Toplou	05/14
3	rape + fruit trees	Davidov	05/14
4	rape + fruit trees	Sacurov	05/14
5	rape + fruit trees	Hencovce	05/14
6	rape + fruit trees	Zamutov	05/14
7	rape + fruit trees	Zamutov	05/14
8	rape + fruit trees	Davidov	05/14
9	rape + fruit trees	Cabov	05/14
10	rape + fruit trees	Secovska Polianka	05/14

Note: No. – number of sample, or. – origin, Ext. – date of extraction.

Table 2 Cultivation conditions used for microbiological analysis.

Microbial group	Medium	Inoculation	O ₂ requirement	Conditions of cultivation	
				Temperature	Time
TPC	GTY	pouring	aerobic	30 °C	48-72 h
CB	VRBL	pouring	aerobic	30 °C	24 h
LAB	MRS	pouring ¹	aerobic ¹	37 °C	48-72 h
SM	NA 2	pouring ²	aerobic	25 °C	48-72 h
MF	GYCH	pouring	aerobic	25 °C	5-7 days

Note: TPC – total plate count, cultivated on GTY – agar with glucose, tryptone and yeast extract (HiMedia); CB – coliform bacteria, cultivated on VRBL – violet red bile lactose agar (HiMedia); LAB – lactic acid bacteria, inoculated by ¹double-pouring (decrease of oxygen in medium), cultivated on MRS – de Man, Rogosa and Sharpe agar (HiMedia), SM – sporulating microorganisms, inoculated by ²pouring – after heat shock (at 80 °C for 10 min), cultivated on NA – nutrient agar no. 2 (HiMedia); MF – microscopic fungi, cultivated on GYCH – agar with glucose, yeast extract and chloramphenicol (HiMedia).

Physico-chemical analysis consisted of water content, free acidity and electrical conductivity determinations. The parameters were detected according to **IHC (2009)**. Water content was measured by Abbe refractometer (Krüss). Free acidity was measured by titration as follows: We mixed honey (10 g) with distilled water (75 mL). We added 2 – 3 drops of phenolphthalein. The solution was titrated with 0.1 mol.L⁻¹ sodium hydroxide to creation of pink colour. The consumption of 0.1 mol.L⁻¹ sodium hydroxide was multiplied by 10 to expression of free acidity in meq.kg⁻¹. Electrical conductivity was measured by conductometer Mini-Digi Conductivity Meter OK-113 (Rudelkis) as follows: We dissolved an amount of honey, equivalent to 20 g anhydrous honey, in distilled water. We transferred the solution to a 100 mL flask and make up to final volume with distilled water. We measured conductance (mS) of solution and multiplied by cell constant of the conductivity cell (cm⁻¹) to expression electrical conductivity in mS/cm. All physico-chemical measurements were performed in duplicate with mean value expression.

Microbiological analysis consisted of total plate count (TPC), counts of coliform bacteria, lactic acid bacteria

(LAB), sporulating microorganisms and microscopic fungi determinations. We used dilution plating method. Basic dilution (10⁻¹) was made by homogenizing 5 g of the sample with 45 mL of saline solution with peptone (0.85% sodium chloride, 0.1% bacteriological peptone, pH 7.0 ± 0.2) for 30 min. Then, we prepared a 10⁻² dilution according to the principle of the ten-fold dilution system. We inoculated the 10⁻¹ and 10⁻² dilutions for all spotted microbial groups onto sterile Petri dishes. Plates were cultivated under specific conditions. The characteristics of methods used are shown in Table 2. After cultivation, plate counts were calculated as cfu/g and converted into the common logarithms and results were presented as log cfu/g. Counts of microscopic fungi were expressed separately for yeasts and separately for microscopic filamentous fungi. The detection limit of this method was 10 CFU.g⁻¹ (1.00 log CFU.g⁻¹).

RESULTS AND DISCUSSION

The physico-chemical quality of evaluated honey after extraction and half-year storage is reported in Table 3.

Appearance of stored rape honey samples is showed in

Table 3 Physico-chemical quality of fresh and stored honey.

	Fresh honey (n = 10)	Honey stored for 6 months (n = 10)
Water content [%]	18.3 ±1.0	17.5 ±0.9
Free acidity [meq.kg ⁻¹]	12.7 ±2.0	13.0 ±2.6
Electrical conductivity [mS.cm ⁻¹]	0.14 ±0.02	0.13 ±0.02

Note: There were no significant differences between values in rows according to the t-test (p <0.05).

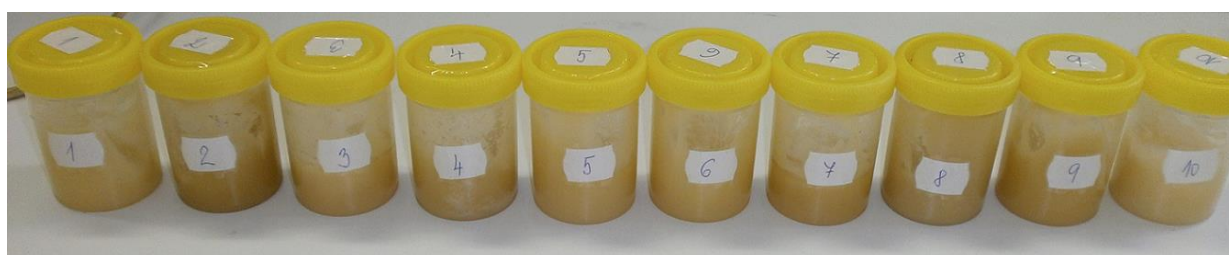


Figure 2 Samples of rape honey (photo: Bacikova, 2014).

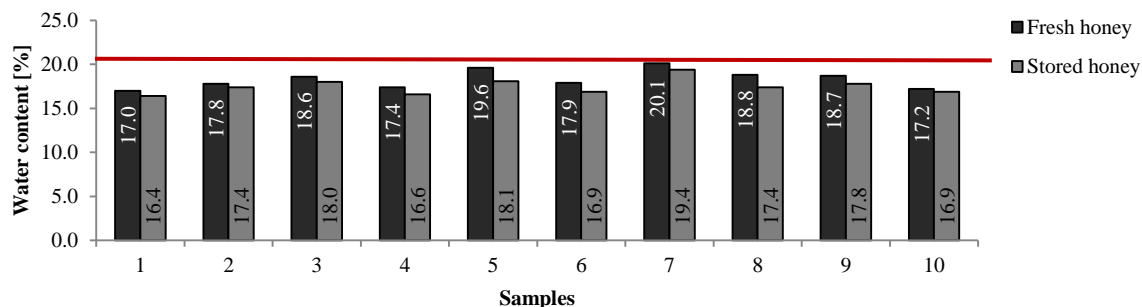


Figure 3 Water content in fresh and stored honey.

Table 4 Microbiological quality of fresh and stored honey [$\log \text{cfu.g}^{-1}$].

	Fresh honey		Stored honey (after 6 months)	
	n	mean \pm SD	n	mean \pm SD
Total plate count	10	2.70 ^a \pm 0.78	10	1.62 ^b \pm 0.57
Coliform bacteria	2	1.24 \pm 0.34	0	ND
Sporulating microorganisms	8	2.02 ^a \pm 0.77	9	1.66 ^a \pm 0.57
Lactic acid bacteria [MRS]*	10	2.91 ^a \pm 0.73	8	1.24 ^b \pm 0.25
Yeasts	9	2.27 ^a \pm 0.39	3	1.26 ^b \pm 0.24
Microscopic filamentous fungi	9	1.92 \pm 0.61	1	ND

Note: n – number of samples with detectable microbial counts, SD – standard deviation, ND – not detected.

Different letters in the same row indicate statistical differences at $p < 0.01$ according to the t-test.

* MRS is medium used for lactic acid bacteria isolation, but yeasts grown on medium, too. From fresh honey samples we cultivated colonies mainly consisted of long rods and rod groups, occasionally of yeast cells (evaluated by microscopic view). From stored honey we cultivated only colonies consisted of yeast cells (evaluated by microscopic view).

the Figure 2. Colour of fresh rape honey is light yellow, sometimes lemon colour, it crystallizes very fast and result of crystallization is white colour, gentle crystals and solid, very hard consistency (Kukurová et al., 2009). Castro-Vázquez et al. (2012) confirmed the importance of the use of low temperatures in order to keep the quality of physico-chemical parameters during the honey storage period. In general, recommended temperature for honey storage is max 20 °C.

During storage, water content, free acidity and electrical conductivity were without significant differences. Our results of physico-chemical quality are comparable with the data of 715 European rape honeys, reported by Oddo et al., (2004), who found mean values of water content $17.0 \pm 1.1\%$, free acidity $10.3 \pm 2.1 \text{ meq.kg}^{-1}$ and electrical conductivity $0.19 \pm 0.05 \text{ mS.cm}^{-1}$.

Water content is important parameter in term of honey shelf life. Figure 3 shows water content in evaluated samples of Slovak rape honey. Limit value is maximum 20% according to Decree 41/2012 and Codex Stan (2001). All samples (except sample no. 7 after extraction) met the requirements of the above mentioned standards.

Chaven (2014) states that honey with low water content (17.1 – 18.0%) and relatively high yeast spores content (max 10^3 cfu.g^{-1} , i. e. $3.00 \log \text{ cfu.g}^{-1}$) will remain stable. Association of Slovakian beekeepers (SZV) constitutes

Standard for quality and grade of the SZV no. 1/2006.

All Slovakian beekeepers, who want to use the trademark SLOVENSKÝ MED (SLOVAKIAN HONEY) have to meet the additional criteria (e. g. water content: max 18%). Water content over 18% was found in 5 out of 10 (50%) fresh samples and in 2 out of 10 (20%) stored samples.

All honeys are acidic (pH 3.5 – 5.5), due to the presence of organic acids that contribute to the honey flavour and stability against microbial spoilage (Bogdanov et al., 2004). Limit value for free acidity is 50 meq.kg^{-1} according to Decree 41/2012 and Codex Stan (2001). All samples met the requirement.

Electrical conductivity is parameter, which is suitable to differentiation between the blossom and honeydew honeys. Electrical conductivity of blossom honeys (except e. g. chestnut honey) must be maximum 0.8 mS.cm^{-1} (Decree 106/2012; Codex Stan, 2001). Electrical conductivity of all evaluated samples was typical for blossom honeys. At present electrical conductivity is the most useful quality parameter for the classification of unifloral honeys, which can be determined by relatively inexpensive instrumentation (Bogdanov et al., 2004).

The microbiological quality of fresh and stored rape honey is reported in Table 4. Significant ($p < 0.01$) differences were found between fresh and stored honey in TPC, counts of LAB and yeasts.

Limit value for TPC is 10^2 CFU.g⁻¹ (2.00 log CFU.g⁻¹), according to **Codex Alimentarius SR (2014)**. Obtained results of TPC in fresh and stored honeys are showed in Figure 4. Limit value for TPC was exceeded in 9 out of 10 (90%) fresh honey samples and in 1 out of 10 (10%) stored honey samples. **Sinacori et al. (2014)** found TPC values, which are comparable to the stored rape honey in this study. According to **Snowdon and Cliver (1996)**, counts of bacteria in finished honeys range normally from 1 to 5000 CFU.g⁻¹ (0 – 3.70 log CFU.g⁻¹) and lower numbers are achieved by additional industrial treatment. We

confirmed the importance of the honey age in microbial evaluating of the honey.

According to **Codex Alimentarius SR (2014)**, limit value for coliform bacteria in honey is 10^2 CFU.g⁻¹ (2.00 log CFU.g⁻¹). Coliform bacteria were detected in 2 out of 10 (20%) fresh honey samples, only at low level, below limit value. After 6 months storage at room temperature, coliform bacteria were not detected in evaluated honeys. **Sinacori et al., (2014)** found the members of *Enterobacteriaceae* family only in 2 from 38 honeys. Coliform bacteria are present in honey

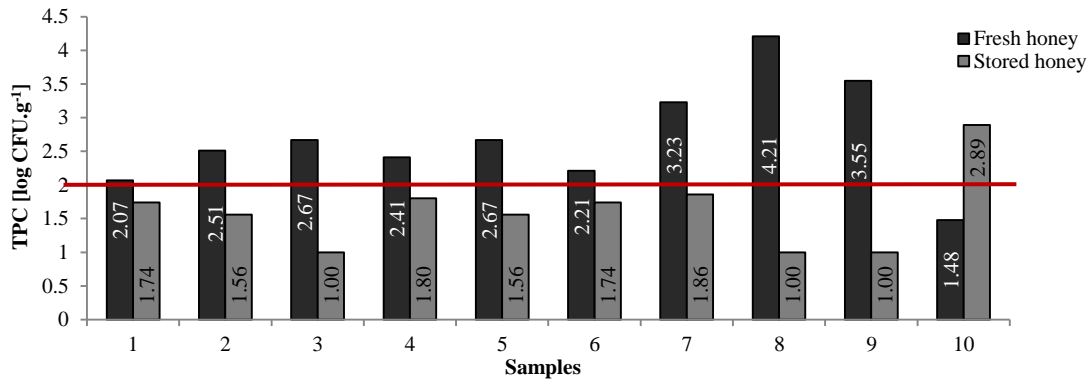


Figure 4 Total plate count in fresh and stored honey.

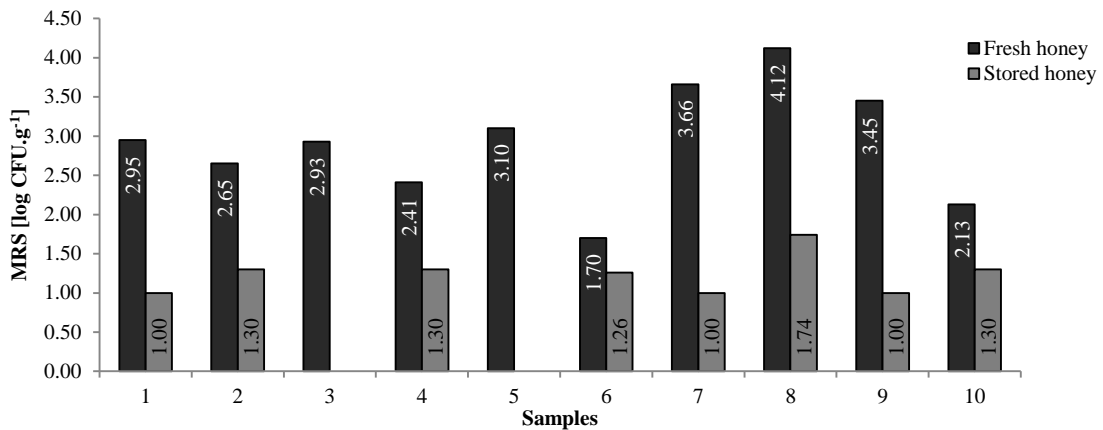


Figure 5 Microorganisms, cultivated on MRS medium, in fresh and stored honey.

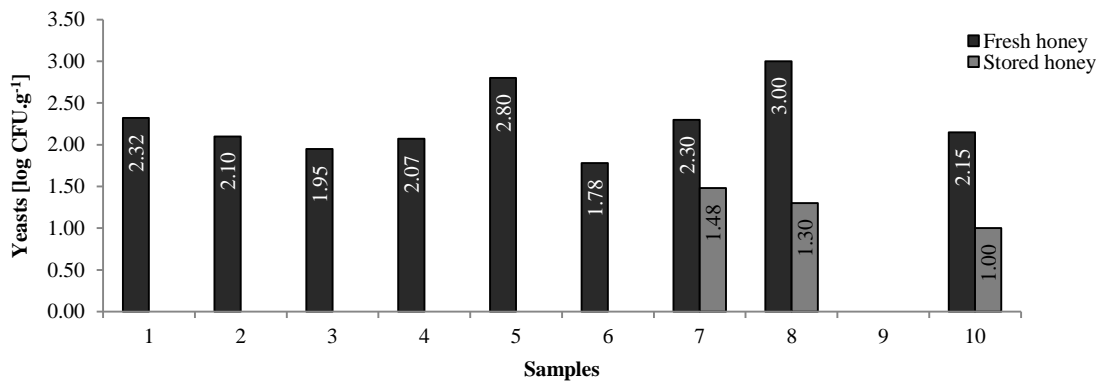


Figure 6 Yeasts in fresh and stored honey.

occasionally. In general, their high number in foodstuffs indicates faecal contamination.

Counts of sporulating microorganisms were without significant differences between the fresh and stored honey. **Sinacori et al. (2004)** analysed honey for sporulating microorganisms. They found no clostridia, but bacilli (*Bacillus amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. thuringiensis*, *B. licheniformis*, *B. megaterium*, *B. pumilus* and *B. simplex*) were frequent.

Obtained results of microorganisms, cultivated on MRS medium, in the fresh and stored honey are reported on Figure 5. Significant ($p < 0.01$) difference was found between the fresh and stored honey. MRS medium is used for lactic acid bacteria cultivation. Cultivated isolates were checked by microscopic views. Cells from colonies originating in fresh honey were mainly the rods and group of rods. Occasionally the yeast cells were occurred. MRS medium is rich of nutrients and yeasts can grow on it, when they are not inhibited by other microorganisms (e. g. by LAB). From stored honey, we cultivated only yeasts on MRS medium. Nevertheless, we observed the presumptive LAB cultivated only from fresh honey samples. The results are in accordance with **Olofsson and Vásquez (2008)**, **Endo and Salminen (2013)** and **Bogdanov (2015)**. *Gluconobacter* and *Lactobacillus* populations decrease as ripening proceeds; the number of bacteria decreases from stomach nectar to higher-moisture honey to low-moisture honey (**Ruiz-Argueso and Rodriguez-Navarro, 1975**).

Counts of yeasts in fresh and stored honey are showed on Figure 6. We observed significant ($p < 0.01$) decrease of yeast count after storage. According to **Chaven (2014)**, wide variety of yeast may be recovered from unprocessed honey; it is predominantly osmophilic yeast varieties such as *Zygosaccharomyces rouxii* or *Z. bailii*, which are of relevant concern for honey processing. Presence of microscopic filamentous fungi was found in 9 out of 10 (90%) fresh honey samples and only in 1 out of 10 (10%) stored honey samples.

Fermentation is the only microbiological alteration to which honey is susceptible. Only osmophilic yeasts can grow in the high sugar concentrations, but their presence is ubiquitous in honey, nectar, hive interiors, dust and soils. Below 18% moisture content there is a little probability of fermentation, but even at concentration below 17.1% the risk of fermentation cannot be completely excluded. This aspect of fermentation depends on factors such as the quantity of yeasts and other growing factors – honey temperature and the distribution and availability of water following crystallization (**Krell, 1996**).

The nutritional and health enhancing properties of unifloral honeys is quite a new field of research (**Bogdanov, 2015**).

CONCLUSION

Obtained data of physico-chemical parameters (water content, free acidity and electrical conductivity) were typical for rape honey. In the stored honey, we recorded small decrease of water content and electrical conductivity and small increase of free acidity. However, the changes were without significant differences.

Presence of lactic acid bacteria in fresh rape honey from eastern Slovakia was confirmed by cultivating method with microscopic views.

Counts of spotted microbial groups decreased significantly during the storage. Probably, it indicates continued ripening in the honey package after extracting.

Next research plans are centered on testing of various fresh honeys and identification of microbial isolates, mainly presumptive members of lactic acid bacteria, by molecular-biological methods.

Presence of coliform bacteria, yeasts and primarily lactic acid bacteria in fresh honey can play an important role in the future trends of nutrition, but detailed research is needed because of potential risks.

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Map 1 : Slovak republic map [on line] [cit. 2015-03-15]
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Map 2 : Google maps [on line] [cit. 2015-03-2015]
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THE EFFECT OF DIETARY ALFALFA MEAL ON THE CHICKEN MEAT QUALITY

Jana Tkáčová, Peter Haščik, Mária Angelovičová, Adriana Pavelková, Marek Bobko

ABSTRACT

The purpose of this study was to investigate the effect of feed mixtures with proportion of alfalfa meal 4% on body weight of broiler chickens, fat content their meat and oxidative stability meat fat under storage conditions. Final hybrid Cobb 500 chickens were used in the experiment. The broiler chickens were fed with feed mixtures starter from the 1st to the 18th day, grower from the 19th to the 31st day and finisher from the 32nd to the 38th ad libitum. An alfalfa meal of 4% was added in the feed mixtures of experimental group. In our experiment, we used alfalfa meal, which was made from *Medicago sativa* L. harvested in the bud's phase. The carcasses of broilers were stored at -18°C for a period of nine months. The average weight of chickens at the end of our experiment, the control group was 1685.6 g. In experimental group with addition of 4% alfalfa meal in feed mixtures, the chickens weighed 1709.6 g. The difference in body weight between the groups was not statistically significant ($p > 0.05$). The average fat content in chicken meat was lower in the group with a share of 4% alfalfa meal $2.33\text{ g}\cdot 100\text{g}^{-1}$ compared to the fat content in meat chickens control group $2.59\text{ g}\cdot 100\text{g}^{-1}$. Differences in fat content in meat between the groups were not statistically significant ($p > 0.05$). In our experiment, the average peroxide value of fat in meat under storage conditions 9 months at -18°C was lower in the group with a share of 4% alfalfa meal $2.42\ \mu\text{mol O}_2\cdot\text{g}^{-1}$ compared with an average value of peroxide number $5.79\ \mu\text{mol O}_2\cdot\text{g}^{-1}$ in the control group. *Medicago sativa* L. is an interesting object for research. It is characterized by high content of protein and biologically active substances that are effective for the promotion of health, and also an improvement the nutritional value and technological properties of the poultry food, when is used in feed mixtures.

Keywords: dietary alfalfa meal; chicken meat; storage; fat; peroxide number.

INTRODUCTION

Medicago sativa L. is one of the cheapest sources of protein from the aspect of high yields and low production costs (Radović et al., 2009). It is feedstuff with high fiber and with low metabolizable energy (Donalson et al., 2005). *Medicago sativa* L. is a readily available, high protein, high fiber feedstuff with one of the slowest rates of passage through the avian system (Garcia et al., 2000). *Medicago sativa* L. is valuable for chemical composition. It has a high crude protein content (Lupašku, 1988), with well-balanced contents in amino acids (Sen et al., 1998; Dinic et al., 2005; Markovic et al., 2007a; Ponte et al., 2004b; Jiang et al., 2012). Crude protein depends on the vegetative growth phase at harvest time and may vary within the range from 200 to $240\text{ g}\cdot\text{kg}^{-1}$ (Radović et al., 2009).

Medicago sativa L. is an important source of vitamins (Jiang et al., 2012), such as β -carotene and another 10 vitamins (Lupašku, 1988; Kindschy, 1991; Sen et al., 1998), various microelements too. The animals need these nutrients for normal growth and development (Marković et al., 2007b).

The β -carotene, xanthophylls and flavonoids in alfalfa are responsible for the high antioxidant properties (Aziz et al.,

2005). In particular, β -carotene is an important bioactive substance in alfalfa that is a precursor of vitamin A and retinoid, which has been defined as an important molecule in animal nutrition (Schweigert et al., 2002). Many authors investigated the effect of *Medicago sativa* L. on production properties, the color of subcutaneous fat and meat quality of broiler chickens (Han and Parsons, 1990; Ponte et al., 2004b; Donalson et al., 2005).

By Sen et al., 1998; Ponte et al., 2004a carotenoids and xanthophylls have cause that poultry carcasses have desirable yellow color.

Medicago sativa L. also contains high levels (2 to 3% of dry matter) of saponins, which have been shown to have hypocholesterolemic, anticarcinogenic, antiinflammatory, and antioxidant properties (Klita et al., 1996; Rao and Gurfinkel, 2000; Francis et al., 2002; Ponte et al., 2004a).

Alfalfa meal must be used in feed mixtures in limited quantities for maintain a high production of broiler chickens (Guenther et al., 1973).

Medicago sativa L. contains significant amounts of bioactive substances that exhibit antioxidant properties. Antioxidant is targeted against oxidation. The role of antioxidants is to protect lipids against radical peroxidation (Lauro, 1991).

Lipid oxidation is a major cause of quality deterioration of processed and unprocessed foods. Secondary oxidation products include aldehydes, ketones, hydrocarbons, and alcohols, among others. Secondary products of oxidation are generally odor-active, whereas primary oxidation products are colorless and flavorless (Akoh and Min, 2002).

Hydrogen peroxide can also arise directly from oxygen, for example by glucose oxidase (Rybár, 2002). Hydrogen peroxide, which is produced by superoxide dismutase or by direct enzymatic production (amino oxidase, glucose oxidase, and other) has a very important role in the initiation of lipid peroxidation. Its other function is creating the hydroxyl free radicals which oxidize all biological molecules. It is managed by a glutathione peroxidase in tissue. Hydrogen peroxide can be decomposed also by catalase (Kanner et al., 1987).

Guenther et al. (1973) and Dansk (1971) recommend the using the limited quantity of alfalfa meal in feed mixtures, which attribute mainly to the high fiber content. Many studies includes the effect of the addition of alfalfa meal in feed mixtures of laying hens (Güçlü et al., 2004; Mourao et al., 2006; Olgun and Yildiz, 2015; Varzaru et al., 2015), but few authors investigate the addition of alfalfa meal in broiler feed mixtures (Carasco and Bellof, 2013).

The purpose of this study was to investigate the effect of feed mixtures with proportion of alfalfa meal 4% on body weight of broiler chickens, fat content their meat and oxidative stability meat fat under storage conditions.

MATERIAL AND METHODOLOGY

Final hybrid Cobb 500 chickens were used in the experiment. The hybrid of the chickens is intended for meat production. The experiment was conducted on a commercial poultry farm. In poultry hall was created space for experimental broiler chickens in accordance with the requirements of Council Directive 43/2007/EC. Directive determines the maximum density of housing chickens on deep litter, which are intended for meat production. The litter consisted of wood chips, which were covered with adjusted straw. Space was divided into two equal parts. In each section were placed 100 birds of one-day-old. Own experimental feeding technology was used in the experiment. The broiler chickens were fed with feed mixtures starter from the 1st to the 18th day, grower from the 19th to the 31st day and finisher from the 32nd to the 38th ad libitum. An alfalfa meal of 4% was added in the feed mixtures of experimental group. The feed mixtures

were produced by Act. no. 440/2006 Coll. on feed mixtures in according to requirements nutrient content and energy for broiler chickens. The feed mixtures were without antibiotics and coccidiostats in control and experimental groups.

Carcass dissection was carried out at Department of animal products evaluation and processing, FBFS, SUA in Nitra. The carcasses of broilers were stored at -18 °C for a period of nine months. Chemical analyzes meat samples were carried out at Department of food hygiene and safety, FBFS, SUA in Nitra. Lipid extraction was carried out using a device Det Gras N Selecta P (JP Selecta S.A., Barcelona, Spain). Peroxide value was determined in fat so obtained. The peroxide value is determined by measuring the amount of iodine, which is formed by the reaction of the hydroperoxides formed in fat with iodide ion under acidic conditions. A thiosulphate concentration of 0.01 mol.L⁻¹ was used for analysis. The liberated iodine is titrated with sodium thiosulphate 0.01 mol.L⁻¹. Based on the usage of sodium thiosulfate in the analyzed sample and in blank test it determined the amount of O₂ µmol.g⁻¹.

Statistical evaluation of the results was carried out by the system program SAS Enterprise Guide version 1.5. We were used the Student's t-test to compare statistical difference between two groups of data.

RESULTS AND DISCUSSION

Medicago sativa L. is an important feed material that is used in nutrition for all kinds of animals intended for food production. It is characterized by a relatively high content of essential nutrients and certain biologically active substances. Currently, the extract from alfalfa meal begins to use in human medicine. Alfalfa meal is sources of many minerals and vitamins, especially vitamins of group B, vitamin C and E, contains flavonoids, phenolic acids, xanthophylls, carotenoids and other. It has different effects, such as the ability to eliminate uric acid in patients with gout; potassium can help to excrete sodium, and to participate in the reduction of cholesterol levels in blood and tissue. It participates in the acid-base balance, in inhibition the activity of inflammatory enzymes and acts as an antioxidant.

We investigated the effect of alfalfa meal on meat quality of broiler chickens in our experiment. Hunt and Bethke (2011) reported variation in riboflavin content of up to 25% depending on the phase of vegetation, which plays an important role in selecting the right collection green *Medicago sativa L.* – correct phase of vegetation for the purpose of producing alfalfa meal. In our experiment, we

Table 1 Statistical evaluation of body weight.

Index	Group	
	Control	Experimental
SD	139.82	166.85
c _v , %	8.3	9.76
t-test	p > 0.05	

Note: Control group – without alfalfa meal, Experimental group – 4% alfalfa meal in feed mixture, SD – standard deviation, c_v – coefficient of variation

Table 2 Average dry mater and fat content of chicken meat* and peroxide number.

Group		Index		
		Dry mater, g.100g ⁻¹	Fat**, g.100g ⁻¹	Peroxide number, μmol O ₂ .g ⁻¹
Control	Mean	27.89	2.59	5.79
	SD	1.85	0.81	1.24
	c _v , %	6.62	31.07	17.76
Experimental	Mean	28.56	2.33	2.42
	SD	1.54	0.52	0.75
	c _v , %	5.37	22.14	7.4

Note: *Chicken meat was stored under storage conditions at -18 °C 9 months, ** (Tkáčová, 2013) Control group – without alfalfa meal, Experimental group – 4% alfalfa meal in feed mixture, SD – standard deviation, c_v – coefficient of variation.

used alfalfa meal, which was made from *Medicago sativa* L. harvested in the buds stage.

Dong et al., (2007) investigated the effect of alfalfa extract obtained in the growing phase during flowering. Natural extract of *Medicago sativa* L. contains polysaccharides (18.63%), triterpenoids, saponins (5.58%) and flavonoids (5.89%). The experiment results showed that the extract of alfalfa decreased deposition of abdominal fat ($p < 0.05$) and increased immunity (humoral immunity and cellular immunity) without adversely affecting the production of chickens intended for meat production. These authors give a direct relation to fat storage and immunity.

The average weight of chickens at the end of our experiment, the control group was 1685.6 g. The Similar results found Haščík et al., (2010). A body weight of their experimental chickens Cobb 500 at their age 42 days was 1629.15 g, while the chickens were fed a standard commercial feed. The similar results of body weight of broiler chickens at age 42 days found also Liptaiová et al., (2011). Their experimental broiler chickens weighted 1591.0, 1603.0, 1651.0 and 1698.0 g, respectively. In our experimental group with addition of 4% alfalfa meal in

feed mixtures, the chickens weighed 1709.6 g. The difference in body weight between the groups was not statistically significant ($p > 0.05$). Higher body weight of broiler chickens of the same age found Angelovičová et al., (2012) in their experiments. Final body weight of their experimental broiler chicken was 2010.24 g and 2019.12 g, respectively.

Jiang et al., (2012) in a study they found that the addition of alfalfa meal did not have any effect on growth performance of Muscovy ducks from the 14th to the 49th day of age. Ducks given 3, 6, and 9% alfalfa meal had significantly higher dressing percentage and lower abdominal fat percentage compared with those given no alfalfa meal. Ducks given 9% alfalfa meal had higher breast meat percentage compared with those given no alfalfa meal. In other experiments, it would be appropriate to investigate also fiber, proteins and saponins of alfalfa meal from aspect of its use in feed mixtures. Their content can be a significant factor affecting the results of experiments. *Medicago sativa* L. is rich in fiber content, and is most often added to poultry diets as a source of xanthophylls for pigmentation, or as a source of so – called unidentified growth factors (Leeson and Summers, 2005).

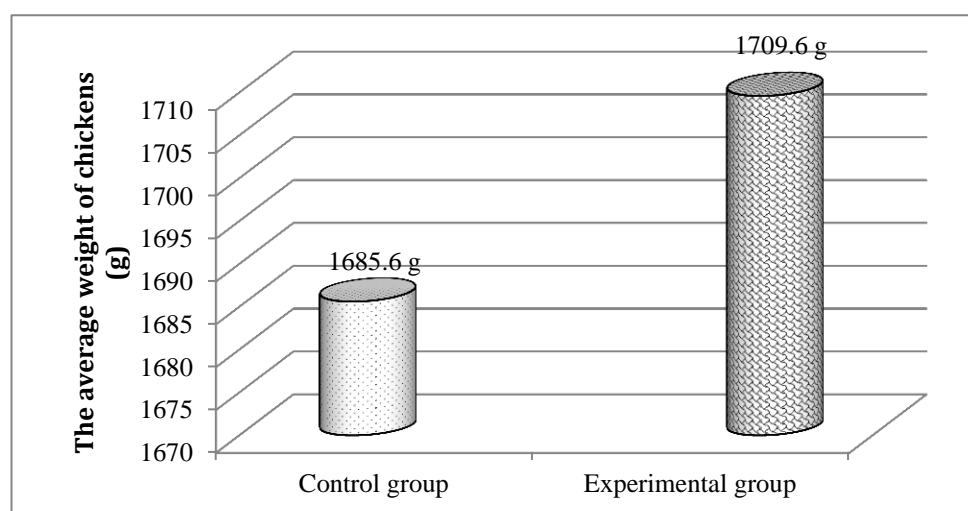


Figure 1 Average final body weight of broiler chickens.

Note: Control group – without alfalfa meal, Experimental group – 4% alfalfa meal in feed mixture.

Fat content and peroxide number of chicken meat

In our experiments, we used meat samples for determining the dry matter, which consisted of the same amount of breast and thigh muscles, and skin of 1 cm² with subcutaneous tissues.

The average dry matter content in the meat of chickens for meat production was in the group with a share of 4% alfalfa meal 28.56 g.100g⁻¹ and in control group 27.89 g.100g⁻¹. The differences in dry matter content of meat between the groups were not statistically significant ($p > 0.05$).

The average fat content in chicken meat was lower in the group with a share of 4% alfalfa meal 2.33 g.100g⁻¹ compared to the fat content in meat chickens control group 2.59 g.100g⁻¹. Differences in fat content in meat between the groups were not statistically significant ($p > 0.05$) (Tkáčová, 2013). In contrast to our experiment, in which we studied the fat content of the chicken meat, Angelovičová and Semivanová (2012) investigated the fat content in the breast muscle. A breast muscle contained lower fat 0.88 g.100g⁻¹, 1.05 g.100g⁻¹, respectively, than chicken meat. Dong et al., (2007) investigated the effect of alfalfa extract obtained in the growing phase during flowering. Natural extract of *Medicago sativa L.* contained polysaccharides (18.63%), triterpenoids saponins (5.58%) and flavonoids (5.89%). The results of experiment showed that the extract of *Medicago sativa L.* decreased deposition of abdominal fat ($p < 0.05$) and increased immunity (humoral immunity and cellular immunity) without adversely affecting production. These authors state that between fat storage and immunity is a direct relation. Interesting results related to investigation of the alfalfa meal effect 4% in feed mixtures of broiler chickens obtained Bobko et al., (2012) on the baking losses of meat. Baking losses were lower (30.72%) in group with application of alfalfa meal in comparison with control group (31.66%), without significant differences between groups ($p > 0.05$). Haščík et al., (2010) noted that the baking losses are often influenced by the chemical composition of muscle tissue, especially by the fat in muscle of animals.

In our experiment, the average peroxide value of fat in meat under storage conditions 9 months at -18 °C was lower in the group with a share of 4% alfalfa meal 2.42 μmol O₂.g⁻¹ compared with an average value of peroxide number 5.79 μmol O₂.g⁻¹ control group. Tichivangana and Morrissey (1985), Ruiz et al., (1999) reported that the rate of oxidation of fats in meat also depends on the presence of oxidants and antioxidants, which puts high emphasis on quality selection of raw materials – selection of *Medicago sativa L.* for the alfalfa meal production. Many studies were focused on the impact of *Medicago sativa L.* on the egg yolk quality of laying hens, but little attention is paid to the impact of *Medicago sativa L.* on meat quality of broiler chickens. This may be due to the fact that *Medicago sativa L.* is rich in fiber content, which may adversely affect the body weight gains. Laudadio et al., (2014) eliminated this property in experiment. The combination of sieving and air classification of alfalfa meal was effective in separating protein and fiber from starting material. Low-fiber alfalfa meal was found to contain appreciable content of nutrients;

in fact, the sieving and air classification processes improved crude protein and reduced crude fiber and neutral detergent fiber level compared with untreated meal. This mixture was used in feed mixture for laying hens. We hypothesize for the future adjustment path alfalfa meal by the way, and its use for broiler chickens. Some authors investigated the effect of alfalfa meal in combination with other additives. Ponte et al., (2004a) investigated the potential use of cellulase and xylanase to increase the nutritional value of *Medicago sativa L.* for broiler chickens. They found that a high proportion of alfalfa meal 20% for the purpose of application of cellulase and xylanase in chickens causes no apparent health problems and contributes significantly to the coloration of subcutaneous fat chicks, especially yellow pigments, while red and rosé unwanted pigments are significantly reduced. Ziegelhoffer et al., (1999) investigated transgene *Medicago sativa L.*, which had incorporated the genes in their bacterial cellulose gene. The authors recommended based on the results of their experiment, this type of *Medicago sativa L.* as suitable for the production of alfalfa meal.

Hunt and Bethke (2011) show the variations in the content of riboflavin up to 25% depending on the vegetative growth phase.

Further research is needed regarding alfalfa meal, as relates to the effect of the dietary fiber and a saponin and its effect on the growth ability of broiler chickens, as well as the possibility reducing of the fiber and increasing the protein.

Antioxidants already in low concentrations significantly delay or prevent oxygenation of oxidizable constituents (Halliwell and Gutteridge, 2001).

The fatty acids deposited in animal tissues are derived from various sources: from endogenous synthesis or directly from the feed, and from microbial synthesis or modification in the digestive tract. In non-ruminants such as pigs and poultry the dietary fatty acids more directly influence the body fat composition, making nutrition an effective tool to manipulate animal lipid composition (Scheeder, 2006).

In meat, triacylglycerols, phospholipids, and cholesterol are the main substrates for lipid oxidation (Márquez-Ruiz et al., 2014).

Some of these secondary products can be toxic to humans and are responsible for the undesirable rancid odor typical of oxidized oils (Decker et al., 2010; Kolakowska and Bartosz, 2014).

Oxidation may be initiated by the formation of lipid peroxides. Initial phases of lipid oxidation can be detected by measuring the peroxide value, which quantifies the levels of peroxides and hydroperoxides formed at that stage (Bennett et al., 2014).

CONCLUSION

Medicago sativa L. is an interesting object for research. It is characterized by high content of protein and biologically active substances that are effective for the promotion of health, and also an improvement the nutritional value and technological properties of the poultry food, when is used in feed mixtures. *Medicago sativa L.* uses in feed mixtures for poultry as alfalfa meal. The use this feed material in

feed mixtures for poultry is restricted by high fiber content. Further researches needed regarding alfalfa meal, as relates to the effect of the dietary fiber and saponins and its effect on the growth ability of broiler chickens, as well as the possibility reducing of the fiber and increasing the protein and using biologically active substances.

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IDENTIFICATION AND DIFFERENTIATION OF RICINUS COMMUNIS L. USING SSR MARKERS

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ABSTRACT

The castor-oil plant (*Ricinus communis* L.), a member of the spurge family (Euphorbiaceae), is a versatile industrial oil crop that is cultivated in many tropical and subtropical regions of the world. Castor oil is of continuing importance to the global specialty chemical industry because it is the only commercial source of a hydroxylated fatty acid. Castor also has tremendous future potential as an industrial oilseed crop because of its high seed oil content, unique fatty acid composition, potentially high oil yields and ability to be grown under drought and saline conditions. Knowledge of genetic variability is important for breeding programs to provide the basis for developing desirable genotypes. The aim of this study was to assess genetic diversity within the set of 60 ricin genotypes using 10 SSR primers. Ten SSR primers revealed a total of 67 alleles ranging from 4 to 9 alleles per locus with a mean value of 6.70 alleles per locus. The PIC values ranged from 0.719 to 0.860 with an average value of 0.813 and the DI value ranged from 0.745 to 0.862 with an average value of 0.821. Probability of identity (PI) was low ranged from 0.004 to 0.018 with an average of 0.008. A dendrogram was constructed from a genetic distance matrix based on profiles of the 10 SSR loci using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 60 diverse accessions of castor bean was clustered into six clusters. We could not distinguish 2 genotypes grouped in cluster 1, RM-96 and RM-98, which are genetically the closest. Knowledge on the genetic diversity of castor can be used to future breeding programs of castor.

Keywords: castor; genetic diversity; molecular markers; simple sequence repeat; SSR

INTRODUCTION

Castor (*Ricinus communis* L.) is a cross-pollinated diploid ($2n = 2x = 20$) species belonging to the family Euphorbiaceae and genus *Ricinus*. Castor is an important industrial oilseed crop. Its seed oil has multifarious applications in production of wide industrial products ranging from medicines to lower molecular weight aviation fuels, fuel additives, biopolymers and biodiesel (Ogunniyi, 2006). Castor seeds contain around 50 – 55% oil which is rich in an unusual hydroxy fatty acid, ricinoleic acid which constitutes about 80 – 90% of the total fatty acids (Jeong and Park, 2009).

Knowledge of genetic variability is important for breeding programs to provide the basis for developing desirable genotypes. Genetic variability in castor bean has been studied using molecular techniques, including amplified fragment length polymorphism (AFLP) (Pecina-Quintero et al., 2013), random amplified polymorphism DNA (RAPD) (Vivodík et al., 2014), single nucleotide polymorphism (SNP) markers (Foster et al., 2010), simple sequence repeat (SSR) (Tan et al., 2014), start codon targeted polymorphism (SCoT) and inter simple sequence repeat (ISSR) (Kallamadi et al., 2015). Pecina-Quintero et al., (2013) used four different AFLP primer pairs. In total, the four combinations of selective primers amplified 430 products, of which 228 were polymorphic. Vivodík et al., (2014) used 8 RAPD markers to detect genetic variability among the set of 40

castor genotypes. Amplification of genomic DNA of 40 genotypes, using RAPD analysis, yielded in 66 fragments, with an average of 8.25 polymorphic fragments per primer. Foster et al., (2010) analyzed the population genetics of *R. communis* in a worldwide collection of plants from germplasm and determined the population genetic structure of 676 samples using single nucleotide polymorphisms (SNPs) at 48 loci. The goal of Tan et al., (2014) was to develop a more complete panel of SSR markers that can be used to construct a genetic map of castor bean and to examine genetic variation in this plant. The present investigation of Kallamadi et al., (2015) has been undertaken to assess the extent of genetic diversity in 31 accessions of castor using ISSR and SCoT primers. Among the DNA markers, SSR markers have been used intensively to analyse genetic diversity. These markers are favourable as they exhibit high locus-specificity, high levels of variability, robustness towards genotyping, and a co-dominant mode of inheritance (Woodhead et al., 2005). So far, several investigations on the discrimination between crop genotypes using SSR markers have been carried out by Siripiyasing et al., (2013); Fayyaz et al., (2014); Kanwal et al., (2014); Polat et al., (2015); Yousaf et al., (2015).

This study investigates the genetic diversity among 60 castor genotypes using 10 SSRs markers.

MATERIAL AND METHODOLOGY

Plant material and DNA extraction:

A total 60 castor genotypes (called RM-45 – RM-105) obtained from the breeding station Zeainvent Trnava Ltd. (Slovakia), were used in this study. DNA of 60 genotypes of castor was extracted from leaves of 10 day old seedlings using the Gene JET Plant Genomic DNA Purification Mini Kit. Each sample was diluted to 20 ng with TE buffer (10 mM Tris–HCl, pH 8.0 and 0.1 mM EDTA, pH 8.0), stored at -20 °C and resolved on agarose gel with the standard lambda DNA for determining the DNA concentration.

SSR analysis: Amplification of SSR fragments was performed according to **Bajay et al., (2009, 2011)** (Table 1.). Polymerase chain reaction (PCR) were performed in 25 µl of a mixture containing 10.5 µl H₂O, 12.0 µl Master Mix (Genei, Bangalore, India), 0.75 µl of each primer (10 pmol) and 1 µl DNA (100 ng). Amplification was performed in a programmed thermocycler (Biometra, Germany) and amplification program consisted of an initial denaturing step at 94 °C for 1 min, followed by 35 cycles of amplification [94 °C (1 min), 1 min at the specific annealing temperature of each primer pair (Table 1), 72 °C (1 min)] and a final elongation step at 72 °C for 10 min. Amplification products were confirmed by electrophoresis in 7% denaturing polyacrylamide gels and silver stained and documented using gel documentation system Grab-It 1D for Windows.

Data analysis:

Data obtained from SSR analysis were scored as presence (1) or absence (0) of fragments for each castor genotype and entered into a matrix. Based on the similarity matrix, a

dendrogram showing the genetic relationships between genotypes was constructed using unweighted pair group method with arithmetic mean (UPGMA) by using the SPSS professional statistics version 17 software package. For the assessment of the polymorphism between castor genotypes and usability of SSR markers in their differentiation diversity index (DI) (**Weir, 1990**), the probability of identity (PI) (**Paetkau et al., 1995**) and polymorphic information content (PIC) (**Weber, 1990**) were used.

RESULTS AND DISCUSSION

Ten SSR primers were used for cultivar identification and estimation of the genetic relations among 60 ricin genotypes. We analyzed 60 genotypes of ricin, because the company Zeainvent Trnava gave us such a database of genotypes. All 10 SSR primers generated clear banding patterns with high polymorphism (Figure 1.). Ten SSR primers revealed a total of 67 alleles ranging from 4 (Rco15) to 9 (Rco05) alleles per locus with a mean value of 6.70 alleles per locus (Table 2). Results indicated the presence of wide genetic variability among different genotypes of castor. Variations in DNA sequences lead to polymorphism. Greater polymorphism is indicative of greater genetic diversity. The PIC values ranged from 0.719 (Rco15) to 0.860 (Rco05) with an average value of 0.813 and the DI value ranged from 0.745 (Rco15) to 0.862 (Rco05) with an average value of 0.821 (Table2). 100% of used SSR markers had PIC and DI values higher than 0.7 that means high polymorphism of chosen markers used for analysis. Probability of identity (PI) was low ranged from 0.004 (Rco05) to 0.018 (Rco15) with an average of 0.008 (Table 2).

Table 1 List ofSSR primers (**Bajay et al., 2009, 2011**).

Marker name	Ta (°C)	Repeat motif	Sequence of the primer (5' - 3')
Rco02	60	(AC) ₁₂	F: CTAGCTTTGGGGCACAGTC R: GGAAAATAGGTGCGTATGAAAC
Rco05	60	(TG) ₆ (GA) ₂₂ (GAA) ₄	F: AGCCAGAAATTGGAAAAGA R: CAAACCCAAGCAAACCTCA
Rco06	60	(TG) ₁₁	F: GGGTGAAAATGAAGAGATTGG R: ATAACCCGTGAAGCATGGAC
Rco08	60	(TG) ₁₀	F: CGTGTGTCTGTGTGCATGTC R: CCTCAACCCTTTGCTGTTTC
Rco09	60	(AC) ₁₁	F: CCAACTCCCTTGTCTGCAA R: GTGAATGGCAAGCAGCAAT
Rco13	62	(GA) ₂₃	F: GGTGCTTCCAGAAATTCAGTT R: GGAGGGGAAAGACAGGATTC
Rco15	60	(AG) ₁₈	F: CACGCACGTTAAAGCAAAC R: GCGAAGAAACCAAAATGGAG
Rco18	60	(CA) ₁₇	F: AGGGGATAAGCGTGATATG R: CCGTTATGAAAAGGAAAGCA
Rco20	60	(TC) ₂₃	F: CCAAAGGAATGTGGGACTC R: TGTGGAGAGGATGAAGAGGAA
Rco22	62	(AAAC) ₃ (AC) ₉ (TC) ₅	F: ATCCGCCGACAATAGCAG R: GCAACACTCTCTCCCTGAA

Note: Ta- annealing temperature.

Table 2 List of SSR primers, total number of bands and the statistical characteristics of the SSR markers used in castor.

Marker name	Number of alleles	DI	PIC	PI
Rco02	7	0.834	0.827	0.007
Rco05	9	0.862	0.860	0.004
Rco06	5	0.792	0.778	0.010
Rco08	7	0.834	0.830	0.006
Rco09	6	0.814	0.806	0.007
Rco13	8	0.839	0.835	0.006
Rco15	4	0.745	0.719	0.018
Rco18	8	0.841	0.838	0.006
Rco20	6	0.818	0.809	0.008
Rco22	7	0.832	0.826	0.006
Average	6,70	0.821	0.813	0.008

Note: DI- diversity index, PIC- polymorphic information content, PI- probability of identity.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

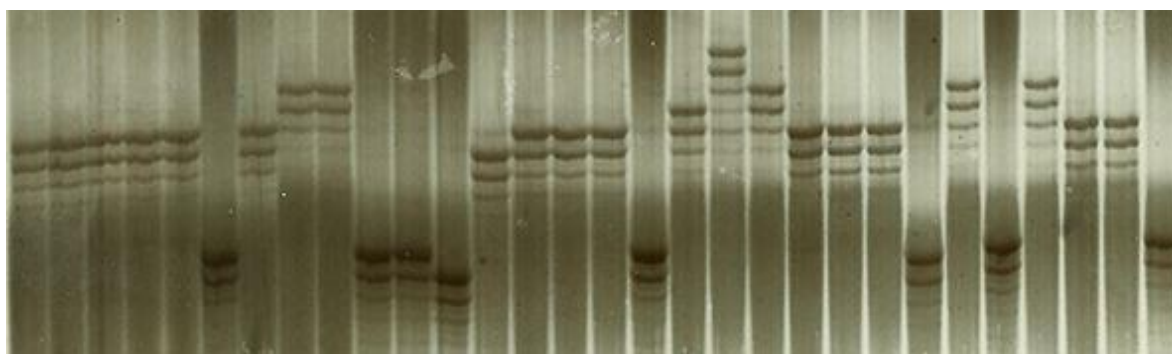


Figure 1. PCR amplification products of 30 genotypes of castor produced with SSR marker Rco-13. Lanes 1 - 30 are castor genotypes RM-45 – RM-74.

A dendrogram was constructed from a genetic distance matrix based on profiles of the 10 SSR loci using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 60 diverse accessions of castor bean was clustered into six clusters. Cluster 1 contained 13 genotypes, cluster 2 included 7 genotypes of ricin and cluster 3 contained 4 genotypes of ricin- RM-45, RM-46, RM-47 and RM-74. Cluster 4 included 15 genotypes and cluster 5 contained 8 genotypes. The last cluster 6 included 13 genotypes of ricin (Figure 2.). We could not distinguish 2 genotypes grouped in cluster 1, RM-96 and RM-98, which are genetically the closest.

Similar results detected **Pecina-Quintero et al., (2013)** who used seven SSR markers and the profiles generated were collectively able to discriminate among 82 *R. communis* accessions and the six controls. The number of alleles ranged from four to eight (Rco23) with an average of 5.5 per marker. **Kyoung-In et al., (2011)** used 28 SSR loci revealed polymorphisms in a castor bean collection consisting of 72 accessions. A total of 73 alleles were detected, with an average of 3.18 alleles per locus, and the

polymorphism information content (PIC) ranged from 0.03 to 0.47 (mean = 0.26). Values for observed (HO) and expected (HE) heterozygosity ranged from 0.00 to 0.19 (mean = 0.11) and from 0.04 to 0.54 (mean = 0.31), respectively. **Gedil et al., (2009)** used only six primers (SSRY26, SSRY40, SSRY47, SSRY 61, SSRY52, and SSRY 189) for analysis of castor. The 6 SSR primers produced amplification products with alleles ranging from 1 to 2 for the parents and the hybrids. The present investigation of **Tan et al., (2013)** was to assess the genetic diversity in 58 accessions of castor. Seventy alleles were detected among the somaclones and their donors, with an average of 2.1 alleles per locus. Each genotype had one or two alleles per locus. Shannon's information index ranged from 0.050 to 0.954 (mean 0.578) and Nei's expected heterozygosity values ranged from 0.017 to 0.587 (mean 0.439). Based on the profiles of the SSR loci, a dendrogram was constructed using the unweighted pair-group method with an arithmetic average (UPGMA). The 53 somaclones and five donors were divergent and clustered into six groups with a similarity coefficient of 0.878. **Dhingani et al., (2012)** used 9 SSR primers for

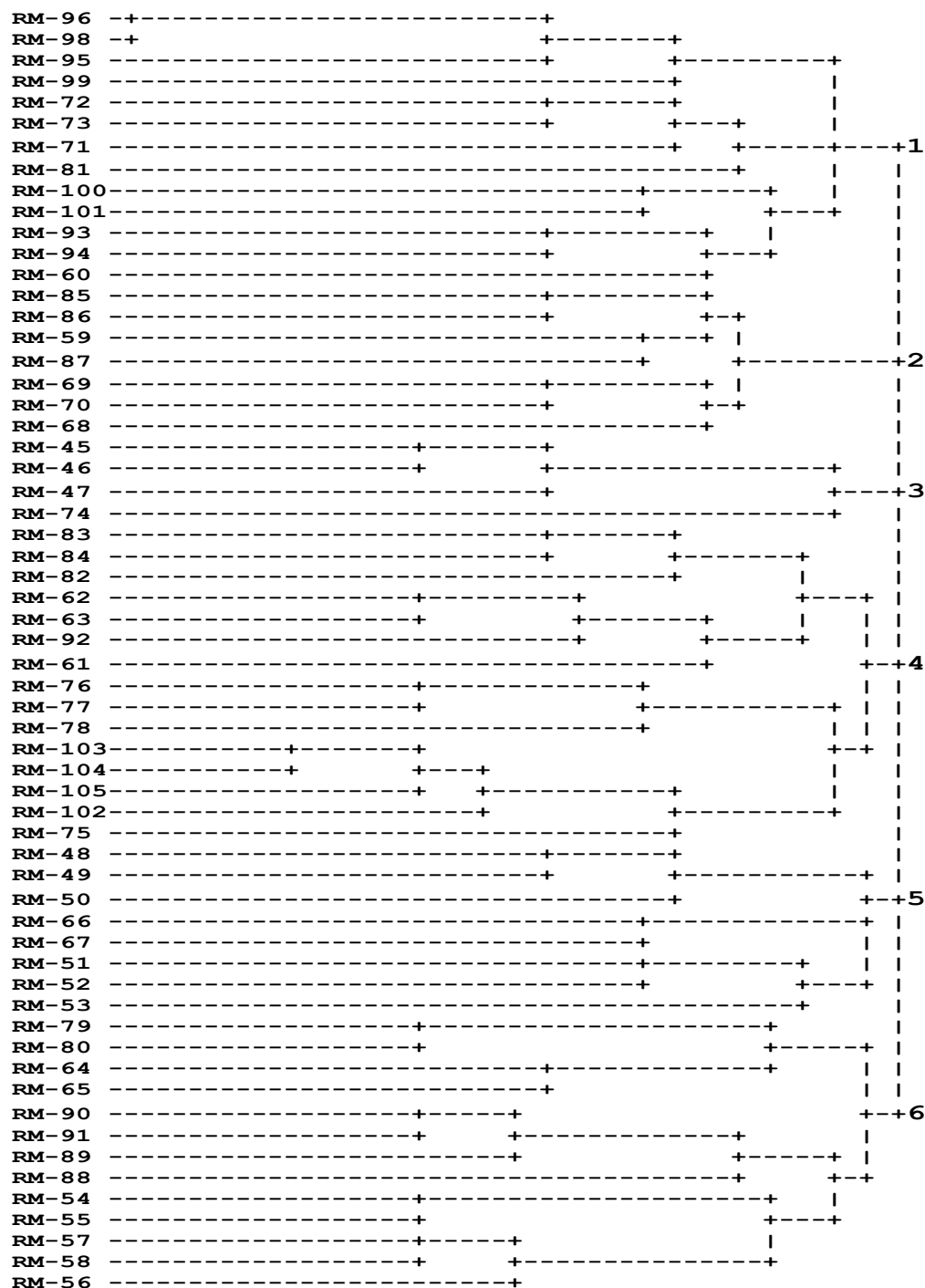


Figure 2. Dendrogram of 60 castor genotypes prepared based on 10 SSR markers.

analysis of genetic diversity of castor. SSR analysis yielded 16 fragments, of which 11 were polymorphic, with an average PIC value of 0.87. SSR molecular markers have been used in population genetic studies Yang et al., (2013); Žiarovská et al., (2013); Ahmad et al., (2014); Aslam et al., (2014); Maršáľková et al., (2014).

CONCLUSION

In conclusion, a high level of genetic diversity exists among the castor accessions analyzed. According to

analysis, the collection of 60 diverse accessions of castor bean was clustered into six clusters. We could not distinguish 2 genotypes grouped in cluster 1, RM-96 and RM-98 which are genetically the closest. A SSR marker system is a rapid and reliable method for cultivar identification that might also be used in quality control in certified seed production programs, to identify sources of seed contamination, and to maintain pure germplasm collections.

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EFFECT OF CHROMIUM NICOTINATE ON OXIDATIVE STABILITY, CHEMICAL COMPOSITION AND MEAT QUALITY OF GROWING-FINISHING PIGS

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ABSTRACT

The effect of different organic sources of Cr on growth, feed efficiency and carcass value is known but there is a lack of information between chromium nicotinate (CrNic) and pork quality. Therefore, purpose of this research was to investigate the effects of CrNic on chemical composition, quality and oxidative stability of pork meat. In the study, pigs of Large White breed (40 pcs) were used. The pigs were divided into two groups, namely the control and the experimental of 20 pcs with equal number of barrows and gilts. The pigs were fed the same diet which consisted of three feed mixtures applied at the different growth phases, from 30 – 45 kg OS-03, 45 – 70 kg OS-04 and 70 – 100 kg OS-05. The pigs were allowed *ad libitum* access to feed and water. The diet of experimental group was supplemented with 0.75 mg.kg⁻¹ CrNic in the form of chromium-inactivated yeast *Saccharomyces cerevisiae*. The fattening period in pigs lasted from 30 to 100 kg. The chromium supplementation led to a significantly higher content of chromium in *longissimus thoracis muscle* (LT) of experimental pigs. In addition, the results showed a statistically significant difference ($p \leq 0.05$) in retention of chromium in the LT, monounsaturated and omega-3 polyunsaturated fatty acids content in experimental group compared with control. Moreover, there was highly significant ($p \leq 0.05$) difference in essential fatty acids, as well as in oxidative stability in 7 days, among the groups. The highly significant differences were also observed among sexes, namely in total water, protein and intramuscular fat contents, colour CIE b* in both times, and oxidative stability. However, physical-technological parameters (pH, drip loss, shear force and meat colour) were not affected when pigs were fed the supplement. On the whole, the positive effect of chromium nicotinate in most of investigated parameters may be beneficial not only for pork industry but also for consumers.

Keywords: chromium nicotinate; meat quality; chemical composition; oxidative stability; pork

INTRODUCTION

Chromium (Cr) is known to be an essential trace mineral element (Lien et al., 2005; Kim et al., 2009). Trivalent chromium is a component of glucose tolerance factor and plays an important role in the metabolism of lipids, carbohydrates, proteins, nucleic acids (Amoikon et al., 1995; Lindemann et al., 1995; Real et al., 2008; Wang et al., 2009; Jiajun et al., 2011) and cholesterol in the body of animals (Jacela et al., 2009). However, most grains and feedstuffs are deficient in Cr and must be supplemented with a bioavailable source of Cr (Bunting, 1999). Animals cannot utilize glucose when chromium is deficient in feed (Tang et al., 2001).

It is generally accepted that organic sources of Cr like chromium picolinate and chromium nicotinate are utilized more efficiently than inorganic Cr sources (Page et al., 1993; Matthews et al., 2001), such as chromium chloride (Jacela et al., 2009). Chromium picolinate has been demonstrated to exhibit a significant number of health benefits in animals (Shrivastava et al., 2002). Recent studies have shown that chromium picolinate decreases backfat thickness and the rate of fat deposition, increase the carcass leanness (Boleman et al., 1995; Wenk et al., 1995; Arvizu et al., 2011), and stimulate muscle

development in pigs (Mooney and Cromwell, 1995; Jackson et al., 2009).

On the other hand, no effect on shear force (Waylan et al., 2003) and sensory traits (Dikeman, 2007; Sales and Jančík, 2011) was detected. Chromium picolinate also increases farrowing rate and total number of pigs born alive (Real et al., 2008). Further, the chromium propionate and nicotinate are the potential organic source of chromium (Matthews et al., 2003; Matthews et al., 2005). Chromium nicotinate improved feed efficiency (Dikeman, 2007) and had significant effect on carcass and meat quality of pigs (Štefanka, et al., 2013).

Supplemental chromium nanoparticle (CrNano) has shown beneficial effects on carcass characteristics and pork quality in finishing pigs (Wang and Xu, 2004). Research with four organic sources (Cr-tripicolinate, Cr-propionate, Cr-methionine, Cr yeast) in concentration 5000 µg.kg⁻¹ of Cr was reported (Lindemann et al., 2008). The effects of the forms of Cr fed on the meat quality and the carcass measurements were minimal. Selenium-yeast combined with chromium-yeast has positive effect on performance and carcass composition of finishing lambs (Domínguez-Vara et al., 2009).

Also, enhancement of the immune function and increasing carcass quality by dietary chromium supplementation have been reported in several studies (Lien et al., 2001; Xi et al., 2001; Shelton et al., 2003; Dikeman, 2007; Wang et al., 2007; Jacela et al., 2009; Zhang et al., 2011). The aim of the experiment was to verify the effect of chromium-nicotinate on the physical-chemical and technological parameters of the quality, nutritional value and oxidative stability of pork.

MATERIAL AND METHODOLOGY

Animals and diets

The experiment was carried out in an Experimental Centre near the Department of Animal Husbandry at the Slovak University of Agriculture in Nitra. In the study, 40 pigs of Large White breed were used. The genotype of all pigs on the marker RYR-1 (malignant hyperthermia syndrome) was analysed by the DNA test. All the experimental animals were detected as homozygous dominant (NN).

The pigs were divided into a control group and an experimental group (each of 20 animals) with equal number of barrows (S_1) and gilts (S_2). Both, control ($G_1 = \text{Cont}$) and experimental group ($G_2 = \text{CrNic}$) of pigs were fed the same diet which consisted of three feed mixtures applied at the different growth phases, from 30 – 45 kg OS-03, 45 – 70 kg OS-04 and 70 – 100 kg OS-05 (Table 1). The diet of experimental group was supplemented with 0.75 mg.kg^{-1} nicotinate (CrNic) in the form of chromium-inactivated yeast *Saccharomyces cerevisiae* fermented on the substrate which was from natural resources with a higher content of chromium during the whole fattening period. The pigs were housed in an environmentally controlled finishing barn with two pigs in each pen. They were allowed *ad libitum* access to feed and water. The fattening period in pigs lasted from 30 to 100 kg. The growth performance of pigs was controlled by

weighing with an accuracy of 0.5 kg. The weighing was realised in two-week intervals (30 – 90 kg) and one-week intervals (90 – 100 kg).

Slaughter and sample collections

The slaughtering and the carcass dissection of pigs were carried out in the slaughterhouse of Experimental Livestock Centre near the Department of Animal Husbandry. The pigs were slaughtered at an average live weight of 102.5 kg and the dissection of carcasses was done according to standard practices STN 466164. Carcasses were chilled at 3 – 4 °C overnight. The samples (100 g) for chemical analysis and determination of some meat quality traits were taken from LT on right half-carcass 24 hours *post mortem*. The place of sampling was above the last thoracic vertebra. After that, the samples were labelled and stored frozen at $-19^\circ\text{C} \pm 0.5^\circ\text{C}$ for 14 days until analysis.

Chemical analysis

The chemical composition of pork in LT was determined from samples of muscle homogenate (50 g) using the FT IR method (Nicolet 6700). The analysis of infrared spectra of muscle homogenate was done by the method of molecular spectroscopy.

The principle of the method was the absorption of infrared radiation by the transition the sample, in which were changes in rotation vibrational energy states of molecules in response to changes in dipole moment of the molecule. The analytical output was an infrared spectrum which was a graphical display of functional dependence of the energy, usually expressed in percentage of the transmittance (T) or in units of absorbance (A) on the wavelength of the incident radiation. The transmittance (throughput) was defined as the ratio of the intensity of the radiation that passed through the sample (I) and the intensity of the radiation emitted by the source (I_0). The

Table 1 Composition of basal diet and nutrient content.

Trait	$G_1 = \text{Cont}$			$G_2 = \text{CrNic}$		
	OS-3	OS-4	OS-5	OS-3	OS-4	OS-5
Barley (%)	26.5	26.0	26.0	26.5	26.0	26.0
Wheat (%)	26.0	24.4	26.0	26.0	24.4	26.0
Corn (%)	17.7	26.3	27.0	17.7	26.3	27.0
Soybean meal (%)	26.5	20.0	15.2	26.5	20.0	15.2
Wheat bran (%)	0.0	0.0	3.0	0.0	0.0	3.0
Mineral and vitamin supplement (%)	3.0	3.0	2.8	3.0	3.0	2.8
Fodder acid (%)	0.3	0.3	0.0	0.3	0.3	0.0
Nutrient composition						
Drymatter (%)	90.74	90.17	90.81	90.74	90.17	90.81
Crude protein (%)	15.28	11.65	11.46	15.28	11.65	11.46
Metabolisable energy, MJ	13.55	13.38	13.06	13.55	13.38	13.06
Lysine (g)	9.48	7.41	6.30	9.48	7.41	6.30
Chromium added ($\mu\text{g.kg}^{-1}$)	-	-	-	750	750	750
Chromium analysed ($\mu\text{g.kg}^{-1}$)	132	147	135	744	752	724

Legend: G_1 – control group; G_2 – experimental group; OS – growth phases of pigs.

absorbance was defined as the common logarithm of $1/T$. The energy dependence on the wavelength was logarithmic; the wave number was used, which was defined as the reciprocal of the wavelength, and thus the energy dependence of the wave number will be a linear function. Individual groups of fatty acids ($g \cdot 100g^{-1}$ FAME, Fatty Acid Methyl Ester) were determined from the muscle homogenate of LT in the Laboratory of gas chromatography at Faculty of Natural Sciences (Comenius University, Bratislava, Slovakia).

Preparation of fatty acid methyl esters

Small amount (4 – 5 g) of muscle tissue was sampled and homogenized by grinding. From the obtained homogeneous mixture, 1 g sample was collected. After that, 4 mL of mixture for the extraction was used, chloroform: methanol (2:1) and the sample was shaken for 1h. After extraction, 2 mL of saline solution (0.9% NaCl) was added and shaken again for 10 minutes. After few minutes, it was taken approximately 2 mL of lower layer which was subsequently centrifuged. From the adjusted sample, it was collected 1 mL for the transesterification.

Discovery Ag-Ion SPE pre-separation columns were developed for the separation of methyl esters according to the degree of saturation of fatty acids using a method of Kramer et al., (2008).

Meat quality measurements

The physical characteristics of meat quality were measured in the laboratory conditions of the Experimental Centre near the Department of Animal Husbandry, SUA in Nitra. The meat colour was determined on the cut of the LT above the last thoracic vertebra 24 h *post mortem* using a spectrophotometer CM-2600d. Commission Internationale de l'Éclairage (CIE) L^* , a^* , and b^* values were determined using the CIE Lab space with a D65 illuminate.

The actual acidity - $\log \text{molc./H}^+ / - \text{pH}$ muscle was assessed 45 minutes and 24 hours *post mortem* using combined micro-capillary electrodes (portable acidometer brand Sentron-Titan).

For determination of drip loss, the methodology described by Honikel (1998) was used. In time 24 h to 48 h *post mortem*, a sample (approximately 50 g) was taken from the LT, placed in vacuum plastic bags and hung in the refrigerator at 4 – 6 °C.

After 7 day-storage at temperature 4 ± 1 °C, the Warner-Bratzler shear force was analysed. The samples were heated to temperature of 71 ± 1 °C for 30 minutes and then cut for chips in 1x1 cm across fibers. Shear force was determined using the device Chatillon.

DNA analysis of RYR1 gene

DNA was determined by the salting-out method according to Miller et al., (1988) for the laboratory conditions of Department of Genetics and Animal Breeding Biology. The samples of DNA were stored frozen for the later analysis. The purified DNA was then used for PCR-RFLP analysis of the RYR1 gene. To amplify specific sectors of a gene RYR1, the following

oligonucleotide primers FOR and REV taken from the work of Kaminski et al., (2002) were used.

TBA method

Procedure for the sample preparation and determination of MDA was done according to the method of Marcinčák et al., (2009). A ground sample (1.5 g) was weighed in a 50 ml centrifuge tube and 1 mL EDTA (complex-forming agent) was added immediately. After gentle agitation, 5 mL 0.8% BHT was added, and the tube was gently shaken again. Just before homogenization, 8 mL 5% TCA was added to the tube and homogenization was carried out for 30 s at maximum speed. After homogenization, the sample stood for 10 minutes and then it was centrifuged for 5 min (3500 rpm, 4 °C). After centrifugation, the top hexane layer was discarded and the bottom layer was filtered through Whatman filter paper No. 4 into a 10 mL volumetric flask and diluted to volume with 5% TCA. After that, a 1 mL of TBA was added to the tube of 4 mL sample. The samples and MDA standards were incubated in a water bath for 90 min at 70 °C. After cooling in an ice bath, samples were incubated at room temperature for 30 min and extinction of samples was measured by UV-spectrophotometer at a wavelength of 532 nm.

Preparation of calibration curve

From the stored MDA solution, 1 mL was pipetted to 25 mL volumetric flask and added 0.1 mol^{-1} HCl. The resulting MDA working solution with a concentration of 0.1748 $\mu\text{g} \cdot \text{mL}^{-1}$ was used to preparation of the calibration curve.

Statistical analysis

The parameters of meat quality were statistically evaluated by statistical methods described by Grofik and Flak (1990) and by statistical package Statistix, Version 8 and 9 (Anonymous, 2001). At first, the basic statistical characteristics, means (\bar{y}) and standard deviations (SD) of analysed traits were computed. The differences of analysed traits between studied groups (G_i), sex (S_j), their interactions (GS) and pens (P_k) were evaluated by two-factor analysis of variance (AOV) with repeated measurements/animals on pens factor. The colour of meat was evaluated by three-factor AOV, with these same factors and factor time (24 hours and 7 days). The linear regression method was used for describing the dependence of oxidative stability of LT muscle after Cr-supplementation on time of storage.

RESULTS AND DISCUSSION

Chemical parameters

Supplementation of the diet with 0.75 $\text{mg} \cdot \text{kg}^{-1}$ chromium nicotinate resulted in a significantly higher content of chromium in the LT of experimental pigs than that of control pigs (0.199 vs. 0.153 mg) as shown in Table 2. The percentage of total water content in LT muscle was the same in both control pigs and pigs fed chromium. The percentage of total protein content was lower in the control group compared with the experimental, but the effect was not significant.

Table 2 Means \pm SD of analysed traits in *longissimus thoracis* muscle of pigs according to group of treatment.

Trait	G ₁ = Cont		G ₂ = CrNic		Total	
	\bar{y}	SD	\bar{y}	SD	\bar{y}	SD
Chromium (mg.kg ⁻¹)	0.152	0.017	0.199	0.023	0.176	0.031
Total water (%)	72.20	0.63	72.20	0.72	72.20	0.67
Protein (%)	24.29	0.60	24.58	0.65	24.44	0.63
Intramuscular fat (%)	1.88	0.94	1.59	0.54	1.734	0.77
Monounsaturated fatty acids (g.100g ⁻¹ FAME)	51.63	2.22	53.61	2.21	52.62	2.41
Polyunsaturated fatty acids	9.59	2.06	10.76	1.84	10.18	2.02
ω3 polyunsaturated fatty acids	0.420	0.072	0.469	0.059	0.445	0.070
ω6 polyunsaturated fatty acids	8.84	2.37	9.87	1.56	9.35	2.05
Essential fatty acids	6.65	1.43	7.80	1.16	7.23	1.41
Eicosapentaenoic acid	0.107	0.054	0.150	0.073	0.128	0.067
Docosahexaenoic acid	0.043	0.029	0.062	0.035	0.052	0.033
pH ₁ - log molc. (H ⁺)	6.22	0.10	6.19	0.12	6.21	0.11
pH ₂₄ - log molc. (H ⁺)	5.71	0.07	5.74	0.07	5.72	0.07
Drip loss (24 hours) %	5.78	2.70	5.98	2.58	5.88	2.61
Colour (24 hours) CIE L*	58.16	2.19	57.97	1.93	58.06	2.047
CIE a*	4.19	4.69	4.37	3.55	4.28	4.10
CIE b*	3.783	7.891	0.055	7.437	1.919	7.800
Colour (7. day) CIE L*	58.66	2.35	57.95	2.67	58.30	2.51
CIE a*	6.19	3.44	9.09	5.10	7.64	4.53
CIE b*	6.35	7.86	1.36	8.12	3.86	8.28
Shear force (W-B) (kg)	4.56	0.83	4.46	1.11	4.51	0.97
Oxidative stability (mg.kg ⁻¹) 1. day	0.032	0.027	0.030	0.033	0.031	0.030
3. day	0.097	0.071	0.056	0.049	0.077	0.063
5. day	0.204	0.180	0.122	0.082	0.163	0.144
7. day	0.314	0.208	0.161	0.095	0.238	0.177

Note: \bar{y} – mean; SD – standard deviation; G₁ – control group; G₂ – experimental group.

According to **Jacela et al., (2009)**, chromium increases the carcass leanness causing enhanced deposition of dietary protein in muscle cells, what also happened in our experiment. Dietary Cr supplementation in dos of 0.2 μg.g⁻¹ could promote protein deposition (**Zhang et al., 2011**).

Some studies (**Wang et al., 2007; Wang et al., 2009**) noted that addition of Cr from the chromium nanocomposite or chromium picolinate increased the concentrations of the total protein in the serum ($p \leq 0.05$). In our study, the biggest difference was shown in the percentage of the intramuscular fat. Control pigs reached a higher intramuscular fat content than experimental group but the difference was not significant, what is consistent with the results of **Wenk et al., (1995); Xi et al., (2001); Jacela et al., (2009)** and **Mrázová et al., (2013)**.

Similarly, **Page et al., (1993)** and **Jackson et al., (2009)** reported that the Cr supplementation decreased the 10th-rib backfat and increased the percentage of lean meat. Other research has shown similar results in lambs that organic chromium reduces the dorsal fat and meat fat content (**Arvizu et al., 2011**). **Domínguez-Vara et al., (2009)** indicated that fat content and retained fat in carcass lambs showed a linear reduction as Cr-yeast increased.

According to study of **Štefanka et al., (2013)**, addition of selenium with chromium nicotinate has reduced cholesterol content in the pork muscles. In our experiment, the differences in the fatty acids content in intramuscular fat of LT (g.100 g⁻¹ FAME) are presented in Table 2. There are highly significant differences caused by sex in total water, protein and intramuscular fat content.

Table 3 Means \pm SD of analysed traits in *longissimus thoracis* muscle of pigs in subgroups according to group of treatment and sex.

Trait	G ₁ = Cont				G ₂ = CrNic			
	G ₁ S ₁ = Barrows		G ₁ S ₂ = Gilts		G ₂ S ₁ = Barrows		G ₂ S ₂ = Gilts	
	\bar{y}	SD	\bar{y}	SD	\bar{y}	SD	\bar{y}	SD
Chromium (mg.kg ⁻¹)	0.154	0.010	0.151	0.022	0.202	0.023	0.196	0.024
Total water (%)	71.92	0.50	72.48	0.65	71.88	0.62	72.51	0.69
Protein (%)	23.94	0.65	24.63	0.29	24.43	0.59	24.7	0.69
Intramuscular fat (%)	2.51	0.78	1.25	0.61	1.69	0.49	1.49	0.60
Monounsaturated fatty acids	51.53	1.88	51.72	2.62	53.89	2.93	53.34	1.26
Polyunsaturated fatty acids	9.25	1.82	9.93	2.31	10.43	1.78	11.0	1.93
ω3 polyunsaturated fatty acids	0.424	0.080	0.416	0.067	0.468	0.058	0.47	0.064
ω6 polyunsaturated fatty acids	8.54	1.96	9.13	2.80	9.54	1.48	10.2	1.64
Essential fatty acids	6.35	1.49	6.95	1.393	7.61	1.27	7.99	1.07
Eicosapentaenoic acid	0.114	0.064	0.100	0.044	0.165	0.072	0.134	0.074
Docosahexaenoic acid	0.037	0.027	0.049	0.031	0.063	0.039	0.061	0.033
pH ₁ - log molc. (H ⁺)	6.19	0.10	6.25	0.10	6.22	0.16	6.16	0.08
pH ₂₄ - log molc. (H ⁺)	5.71	0.09	5.71	0.03	5.77	0.09	5.70	0.03
Drip loss (24 hours) (%)	5.65	3.27	5.92	2.17	5.55	2.32	6.41	2.88
Colour (24 hours) CIE L*	57.97	2.01	58.34	2.45	58.73	1.59	57.2	2.01
CIE a*	1.82	3.50	6.56	4.64	4.54	3.83	4.20	3.44
CIE b*	8.56	6.31	-1.00	6.39	0.47	7.59	-0.36	7.67
Colour (7. day) CIE L*	59.10	2.57	58.21	2.15	58.41	2.83	57.4	2.57
CIE a*	4.50	1.82	7.87	3.90	9.37	5.67	8.80	4.74
CIE b*	10.51	6.20	2.20	7.32	1.69	8.19	1.03	8.47
Shear force (W-B) (kg)	4.62	1.05	4.51	0.57	3.97	1.16	4.95	0.87
Oxidative stability (mg.kg ⁻¹)1.day	0.028	0.007	0.035	0.038	0.024	0.015	0.037	0.044
3.day	0.084	0.059	0.111	0.083	0.038	0.014	0.07	0.064
5.day	0.135	0.086	0.273	0.225	0.087	0.034	0.15	0.101
7.day	0.248	0.119	0.381	0.259	0.118	0.039	0.20	0.115

Note: \bar{y} – mean; SD – standard deviation; G₁ – control group; G₂ – experimental group; G₁S₁, G₁S₂, G₂S₁, G₂S₂ – subgroups.

The content of monounsaturated fatty acids was significantly lower in the control (51.63 g) compared with the experimental group (53.61 g). Also, the content of the essential fatty acids in the control group was significantly lower than that of experimental group (6.65 g vs. 7.80 g), see Tables 2 and 3. On the other hand, the chromium supplementation resulted in the significant increase ($p \leq 0.05$) of omega 3-polyunsaturated fatty acids in experimental pigs compared with the control ones (0.47 vs. 0.42 g). **Lien et al., (2001)** suggest that the carcass of the pigs that received the chromium picolinate supplemented diet (400 µg/kg) contained less oleic acid (C18:1) and total unsaturated fatty acids ($p \leq 0.05$). The total saturated fatty acid content in chromium fed group was higher than that in control.

Physical and technological quality of pork

The results for the physical and technological quality of pork are presented in Table 2 and Table 3. There were not

statistically significant effects of chromium supplementation on pH, drip loss, shear force and meat colour 24 h *post mortem* observed.

These results are in agreement with findings of the other studies (**Page et al., 1992; Matthews et al., 2003; Wang and Xu, 2004; Lindemann et al., 2008; Arvizu, 2011; Sales and Jančík, 2011; Bednářová et al., 2014**). Also, **Waylan et al., (2003)** identified no effect of Cr-nicotinate supplementation on sensory traits and pH values in *longissimus* muscle. **Boleman et al., (1995)** showed that sensory and shear force values were not affected by chromium picolinate.

On the other hand, addition of selenium with chromium nicotinate significantly increased shear force (**Štefanka et al., 2013**). Further, the reports of **Dikeman (2007)** were similar to our results and showed no differences for *longissimus* colour display or shear force in pigs supplemented by chromium nicotinate.

Table 4 Means squares (MS) of two factor analyses of variance with repeated observations on nested factor pen in the main group factor of analysed traits of pigs.

Trait	Error				
	Group, G	(Pen:Group) E (P:G)	Sex, S	Group*Sex GS	Error, e
	$f_{G=1}$	$f_{E(P:G)} = 18$	$f_S = 1$	$f_{GS} = 1$	$f_e = 18$
Chromium (mg.kg ⁻¹)	0.02120*	0.00022	0.00023	0.00002	0.00064
Total water (%)	0.00001	0.4916	3.5641*	0.0130	0.2745
Protein (%)	0.8702	0.4385	2.4701*	0.3764	0.2269
Intramuscular fat (%)	0.8526	0.4853	5.3729*	2.8409*	0.3082
Monounsaturated fatty acids (g.100 g ⁻¹ FAME)	39.4420*	5.3852	0.3349	1.3690	4.8917
Polyunsaturated fatty acids (g.100g ⁻¹ FAME)	13.6890	3.2606	4.5024	0.0022	4.5266
ω3 polyunsaturated fatty acids(g.100 g ⁻¹	0.0245*	0.0051	0.00006	0.00030	0.0040
ω6 polyunsaturated fatty acids (g.100g ⁻¹	10.5987	4.0605	3.8751	0.0133	4.2309
Essential fatty acids (g.100g ⁻¹ FAME)	13.2250*	1.2891	2.3426	0.1188	2.1528
Eicosapentaenoic acid (g.100g ⁻¹ FAME)	0.0181	0.0054	0.0048	0.00085	0.0029
Docosahexaenoic acid (g.100g ⁻¹ FAME)	0.0037	0.0009	0.00034	0.00047	0.0012
pH ₁ - log molc. (H ⁺)	0.0055	0.01386	2.500E-06	0.0378	0.01143
pH ₂₄ - log molc. (H ⁺)	0.0081	0.0038	0.0093	0.0141	0.0054
Drip loss (24 hours) (%)	0.3706	6.4221	3.1866	0.8791	8.1158
Colour (24 hours) CIE L*	0.3553	1.7234	3.3466	9.0155	6.5847
CIE a*	0.3098	17.1412	48.4880	64.6176*	13.0161
CIE b*	138.9430	69.4600	270.2440**	190.3140*	29.0670
Colour (7. day) CIE L*	4.9914	7.2778	8.0910	0.00272	5.6227
CIE a*	83.9551	21.9907	19.6420	38.5926	14.6029
CIE b*	249.4500	85.8650	201.1070*	146.0390*	29.5270
Shear force (W-B) (kg)	0.1026	1.1620	1.8387	2.9921*	0.60178
oxidative stability (mg.kg ⁻¹) 1. day	0.00001	0.00076	0.0010	0.00007	0.0011
3. day	0.0169	0.00502	0.0100	0.00025	0.0023
5. day	0.0687	0.0185	0.1082*	0.0116	0.0163
7. day	0.2349**	0.0221	0.1207*	0.0053	0.0260

Note: * $F_{0,05}(1, 18) = 4.4139$.

Some other reports have indicated that Cr-propionate may improve some aspects of pork quality (Shelton et al., 2003; Matthews et al., 2005; Jackson et al. 2009). The decrease of the drip loss by Cr-picolinate was reported by O'Quinn et al., (1998). Study of Xi et al., (2001) showed the decrease of drip loss in pig muscles after Cr-nanoparticle supplementation what is different comparing our result.

In our study, an effect of chromium supplementation on some meat colour parameters 7 days *post mortem* was determined. The values of meat colour were not significantly different between experimental and control group. The differences between sexes in CIE b* in 24 hours and 7 days were significant. The means of meat colour CIE L*, a* and b* in LT 24 hours and 7 days by groups, sex and time are presented in Table 6. It was found out that the differences between analysed groups, sexes and time were not significant for CIE L*. The results showed that there was a highly significant difference

between Time and interaction Group x Time in parameter CIE a*. Highly significant difference in CIE b* was caused by differences of Sex and Time. Also, there was a significant interaction in Group x Sex.

Oxidative stability

The effect of dietary chromium supplementation on the antioxidative stability of LT muscle is presented in Tables 2, 3 and 5. It was showed a highly significant difference between groups in 7th day (0.161 mg.kg⁻¹ in G₂ group vs. 0.314 mg.kg⁻¹ in G₁ group). The means and standard deviations of the oxidative stability of LT for total observations in the 1st, 3rd, 5th and 7th day, for groups and sex and also for subgroups G_iS_j are presented in Table 5.

The linear regression parameter estimates, the corresponded analyses of variance and significance of differences between elevations and slopes of oxidative stability of LT muscle are presented in Table 4.

Table 5 Means \pm SD of oxidative stability (mg.kg^{-1}) of *longissimus thoracis* muscle.

Source	n	\bar{y}	SD	Source	\bar{y}	SD
Total	160	0.1271	0.1429			
Day 1	40	0.0309	0.0298	Day 5	0.1629	0.1444
3	40	0.0766	0.0638	7	0.2378	0.1773
G ₁			G ₂			
Day 1	20	0.0315	0.0269	Day 1	0.0303	0.0331
3	20	0.0972	0.0712	3	0.0561	0.0489
5	20	0.2043	0.1804	5	0.1215	0.0816
7	20	0.3144	0.2079	7	0.1612	0.0945
G ₁	80	0.1619	0.1770	S ₁	0.0951	0.0898
G ₂	80	0.0923	0.0855	S ₂	0.1590	0.1759
G ₁ S ₁	40	0.1238	0.1118	G ₂ S ₁	0.0665	0.0464
G ₁ S ₂	40	0.2000	0.2190	G ₂ S ₂	0.1180	0.1062

Note: n – number; \bar{y} – mean; SD – standard deviation; G₁ – control group, G₂ – experimental group; S₁ –barrows, S₂ – gilts; G₁S₁, G₁S₂, G₂S₁, G₂S₂ – subgroups.

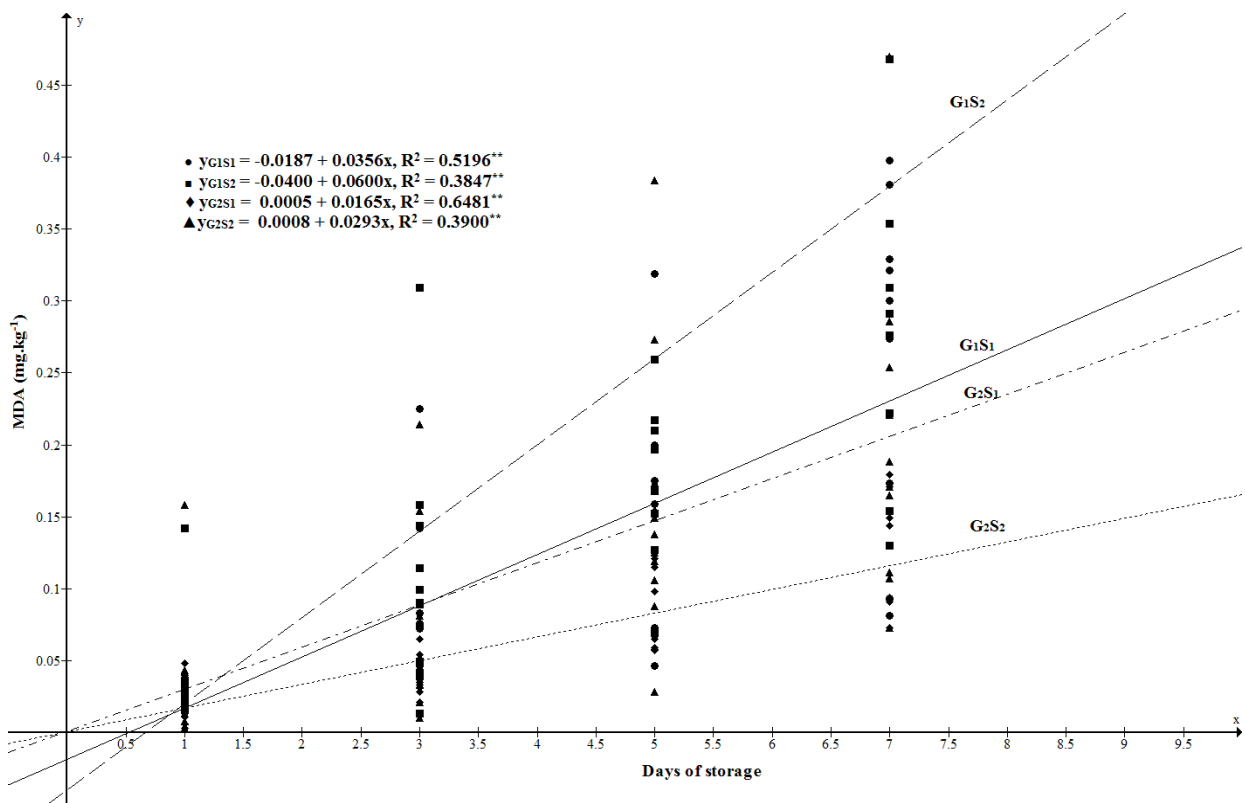


Figure 2 Linear regressions of oxidative stability of *longissimus thoracis* muscle after Cr-supplementation for subgroups G_iS_j, (G₁ = Contr, G₂ = CrNic, S₁ = barrows, S₂ = gilts).

The dependence of oxidative stability on time of storage ($x = t = 1, 3, 5,$ and 7 days) was highly significant. The deviations from linearity, i. e. nonlinearity (NonLin) were not significant. The dependence of oxidative stability had a pure linearity form. The comparison between groups and sexes showed significant or highly significant differences between elevations and slopes (Table 4).

There was a similar situation by comparison of sexes (S_j) in analysed groups (G_i). Figure 1 and 2 illustrate the linear functions. The coefficients of determination R² were

highly significant. The slope in the G₁ = Cont was two times higher than slope in the G₂ = CrNic group ($b_1 = 0.0478$ vs. 0.0229 MDA). The reverse situation was observed by comparison of sexes.

Recent research indicates that there are two mechanisms for chromium to affect the pork quality. It is an effect on carbohydrate metabolism or effect on stress. The investigation has shown that Cr-propionate and Cr-picolinate increases insulin sensitivity (Amoikon et al., 1995; Matthews et al., 2001). We can assume that

Table 6 Means of meat colour CIE L*, a* and b* in *longissimus thoracis* muscle.

Source	Hours/day	CIE L*	CIE a*	CIE b*
Group	1. – 24 h	58.406	5.186	5.068
	2. – 7. d	57.958	6.726	0.707
	SE Mean	0.378	0.949	1.944
Sex	1. – 24 h	58.552	5.057	5.308
	2. – 7. d	57.813	6.859	0.467
	SE Mean	0.512	0.726	1.160
Time	1. – 24 h	58.061	4.279	1.919
	2. – 7. d	58.303	7.637	3.856
	SE Mean	0.286	0.395	0.323

Note: h – hour, d –day.

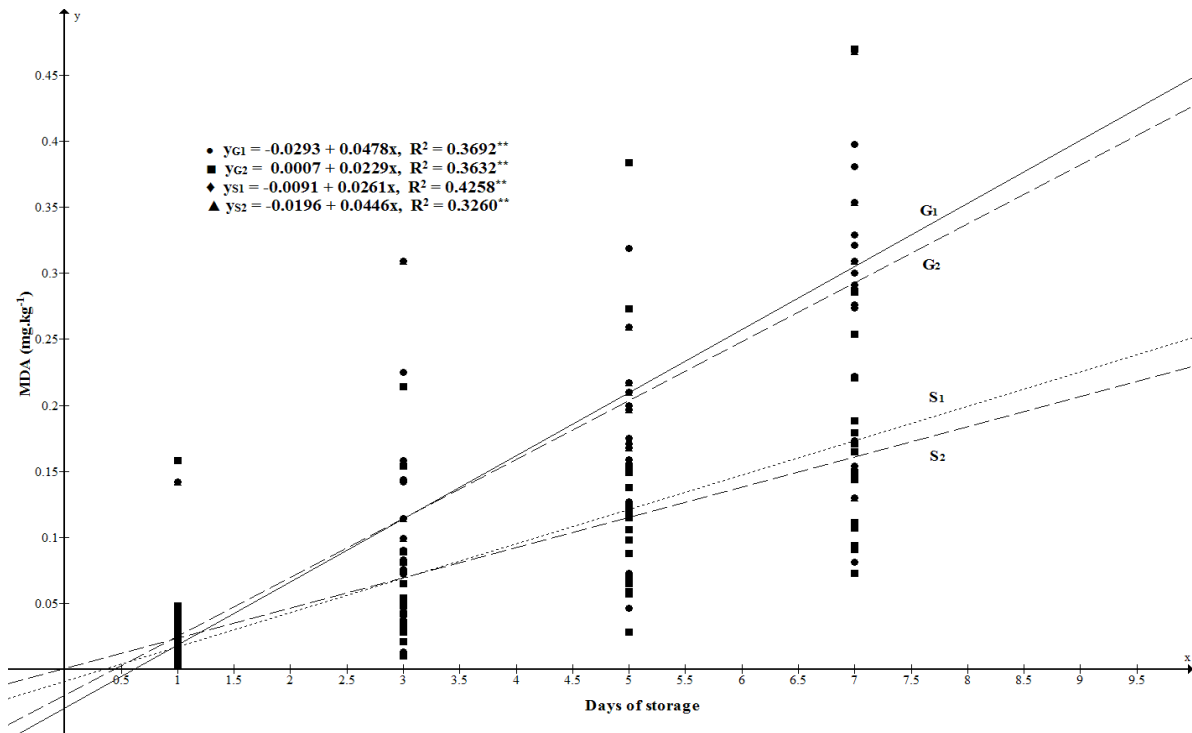


Figure 1 Linear regressions of oxidative stability of *longissimus thoracis* muscle after Cr-supplementation for groups G_i and S_j, (G₁ = Contr, G₂ = CrNic, S₁ = barrows, S₂ = gilts).

Cr-nicotinate may have a similar effect. Some studies (Ward et al., 1994; Berrio et al., 1995) have suggested decreasing insulin binding in certain tissues (porcine hepatic) and increasing in the other ones (adipocytes, red blood cells).

These findings would be indicated that the Cr may affect glycolytic potential in muscles and subsequently impact the pork quality. The glycolytic potential of muscle tissue also plays an important role in the preslaughter stress. Some research has mentioned that Cr may partially mitigate the effect of short-term stress (National Research Council, 1997).

CONCLUSION

According to the results obtained *in vivo* experiment, it can be concluded that the supplementation of organic chromium as chromium nicotinate (0.75 mg.kg⁻¹) in the

pig diet resulted in a higher retention of chromium in LT. The dietary addition of organic chromium to growing and finishing diets for pigs caused a higher content of monounsaturated fatty acids and essential fatty acids in intramuscular fat of LT. However, the feeding with the supplementation increased the content of polyunsaturated and omega 3 fatty acids in the experimental group of pigs. On the other hand, the chromium nicotinate have no effect on the chemical composition of meat and meat quality traits, except with some colour parameters after 7-days storage. The Cr-addition had significantly positive impact on the oxidative stability of pork during its storage. It could be demonstrated that Cr consistently affects pork quality, so that may be beneficial from pork industry and consumer point of view. However, more research is needed to investigate the consistency in which the chromium nicotinate may improve the pork quality.

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PHYTOESTROGENS DIETARY INTAKE AND HEALTH STATUS OF RETIREE FROM MIDDLE-NORTH SLOVAKIA REGION

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ABSTRACT

Phytoestrogens found in foods of plant origin presents chemical substances that possess a wide range of biochemical benefits. It has been found that they contribute in different health related problems. A wide range of commonly consumed foods contain appreciable amounts of phytoestrogens. Consumption of diet rich to phytoestrogen acts as a protective factor against many diseases such as cardiovascular diseases, post-menopausal symptoms in the context of osteoporosis, cancerous illnesses of colon, prostate and breast. Three main classes of phytoestrogens covers: isoflavones, lignans and coumestans. Selected nine major phytoestrogens had been analyzed simultaneously in the same foods. Questionnaire designed to determine intake frequency as well as amount of selected foods and the most common diseases presented in the population has been used to find relationships between dietary habits and health status. Evaluation of selected goals in the present study has been realized in cooperation with 140 respondents in retired age (divided into Males – covered by 34 individuals and Females – 106 individuals), coming from middle-north Slovakia region. On the base of collected data it can be concluded, that evaluated population is presented by high values of lignans intake and particularly secoisolariciresinol, mainly caused by relative high proportion of cereals and linseed in the diet. Furthermore, the relationship between phytoestrogens intake and eating habits as well as its contribution in protection against selected diseases was demonstrated.

Keywords: phytoestrogen, nutrition, intake, health

INTRODUCTION

Phytoestrogens presents photochemical substances that are found in foods of plant origin and possess a wide range of biochemical and health benefits. The interest in plant derived estrogens or phytoestrogens has recently been increased by the realization that hormone replacement therapy is not as safe or effective as previously thought (Hays et al., 2003). A wide range of commonly consumed foods contain appreciable amounts of phytoestrogens. Three main classes of phytoestrogens covers: isoflavones, lignans and coumestans. Phytoestrogens exhibits oestrogenic and antioestrogenic effect due to their similarity in structure to oestrogen (Tham et al., 1998; Zava and Duwe, 1997). The antiestrogenic activity of phytoestrogens may be partially explained by their competition with endogenous 17 β -estradiol for estrogen receptors (Martinez-Campos et al., 1986; Martin et al., 1978). One of the most important representatives are isoflavones, natural non-steroidal molecules of similar structure to 17- β oestradiol and selective oestrogen receptor modulators (Yildiz et al., 2005). Isoflavones contains phenolic ring which is essential for binding to oestrogen receptor. According to this, they have lower affinity for serum protein and better bioavailability at receptor site (Mishra, Mishra, and Devanshi 2011). Epidemiological studies have revealed that average intake of isoflavones in Japanese population is about 50 mg.day⁻¹ (Messina, 1995); rest of Asia has an average consumption

of 25 to 45 mg.day⁻¹; while the western consumption is less than 5 mg.day⁻¹ (Coward et al., 1961). Consumption of phytoen rich diet as seen in traditional Asiatic societies is protective against many diseases (Mishra et al., 2011). Phytoestrogens consist of more than 20 compounds, important sources of isoflavones are soy beans, they present the richest source of isoflavones and contains highest concentration of isoflavones, up to 300 mg per 100 mg. Common isoflavone fractions present in soya are genistein (4', 5, 7-trihydroxy-isoflavon) which constitutes 50% of soya isoflavone, diadzein (4', 7- dihydroxy isoflavone) 40% along with their betaglycosides – genistein, diadzein; and glycetein (7, 4'- dihydro-6-methoxyisoflavone) remaining 5 to 10%. Wheat, Bengal gram, moong beans, chick peas, cherries, parsley, apples, alfalfa and red clover are other sources of isoflavones. Lignans: are found in oil seeds such as flax seeds (linseed), rye, millet, sesame and sunflower seeds besides legumes, pulses, and whole grains. Sunflower seeds, alfalfa and clover are rich sources besides bean sprouts of coumestans. Soya sprout is a potent source of coumestans (Murkies et al., 1998; Tham et al., 1998). According to a number of epidemiological and clinical studies in this area, phytoestrogens have been generally accepted to have a beneficial, rather than a deleterious effect in humans. Potential health benefits of phytoestrogens may be attributable to metabolic properties that do not involve estrogen receptors, such as influence on enzymes, protein

synthesis, cell proliferation, angiogenesis, calcium transport, Na⁺/K⁺ adenosine triphosphatase, growth factor action, vascular smooth muscle cells, lipid oxidation, and cell differentiation (Adlercreutz and Mazur, 1997; Knight and Eden, 1996). Much of the research on the health effects of soyfoods has focused on postmenopausal women. In large part, this is because the soybean is such a rich source of isoflavones, a group of naturally-occurring plant chemicals that possess estrogen-like properties (Franke et al., 1998). As a result, some men are reluctant to eat soyfoods because of the mistaken belief that isoflavones exert feminizing effects. However, not only is this concern without scientific merit, but there is a large amount of evidence suggesting adding soyfoods to the diet can greatly benefit men by reducing the risk of prostate cancer and heart disease. There is also very preliminary evidence that consuming soyfoods might protect against male pattern baldness (Lai et al., 2013). However, it is difficult to recommend specific amounts of dietary phytoestrogen to prevent specific chronic diseases (Tham et al., 1998). Several recent animal studies indicate either teratogenic or protective effects of phytoestrogens with respect to fetal development (Zhao et al., 2010; Xing et al., 2010; Jefferson et al., 2009). Estimating dietary intake of phytoestrogens has been a challenge for various studies. Improvements in analytic techniques (Kuhnle et al., 2007) and published data provided an opportunity to improve estimation of dietary phytoestrogen intake.

The objective of our study was to estimate intake of the phytoestrogens, such as: lignans, isoflavones, coumestans and specific compounds within selected group of retiree from middle-north Slovakia region.

MATERIAL AND METHODOLOGY

Questionnaire

A package of 60 questions has been carefully prepared and developed on the basis of literature previously published in scientific journal (Thompson et al., 2006). The major questions covers: age; sex; dietary habits (focused to selected foods which are marked as the most common phytoestrogens sources) and health status represented by three major diseases such as cardiovascular disease, cancer and diabetes. A daily intake of selected foods was calculated on the basis of comparison of estimated dose provided by respondents and pattern of the food size published in the literature (FHCRC, 2015). A total number of respondents (140) is presented by 34 males and 104 females.

Analysis

Answers according to mentioned questions have revealed several important things. A part devoted to nutrition was processed to detailed list containing daily dosage of selected foods according to which overall daily intake (the values expressed in mg.day⁻¹) of phytoestrogens as well as individual substances (isoflavones: genistein, daidzein, glycitein, formononetin; lignans: secoisolariciresinol, matairesinol, pinoresinol, lariciresinol; coumestan:

coumestrol) was calculated individually on the base of age and sex. In this respect, as valuable source of information used to calculate values was published scientific literature. Other important section of the questionnaire was focused to health status of respondents, where the occurrence of selected diseases was expressed in percentage.

RESULTS AND DISCUSSION

Daily intake of selected nine substances (formononetin, daidzein, genistein, glycitein, matairesinol, lariciresinol, pinoresinol, secoisolariciresinol, coumestrol) belongs to phytoestrogens family had been calculated according to data collected from dietary questionnaire, realised on 140 (divided into Males – covered by 34 individuals and Females – 106 individuals) respondents retired, coming from middle-north Slovakia region. Questionnaire was designed to determine intake frequency as well as amount of selected food, fruits and vegetables and presence of the most common diseases found in population of higher age. According to findings rising from the measured data presented by Table 1 – 4, Secoisolariciresinol has been intended as phytoestrogen compound represented by the highest value irrespective to age range and sex (Table 1). On the other hand, coumestrol was presented by the lowest counts for all age ranges and sex. Integrated part of this population study was screening, focused to evaluation of food, vegetables and fruits intake, those which are reach to phytoestrogens and are typical for local market. Respondents of male sex in the age range 50 – 60 declared high consumption of cereals, this intake considerably falls down with increase of the age (Table 2). According this fact, cereals presents the main proportion of phytoestrogens source for men of the age between 50 and 60 years old. Dietary sources generally marked as others/snacks are considered as other significant part for men of the age between 61 – 70. Partially similar trend was recorded for female respondents of selected years groups, where the high intake of others/snacks brings a high values of phytoestrogen intake as described in the Table 2, except females of the age over 80. As the group marked as others/snacks presents an important dietary source for evaluated individuals from 50 to 80 years old, especially for women, detailed list of evaluated products is posted in the table 3, as well as calculated proportion of these sources on daily intake of phytoestrogens. Sesame seeds present the most important phytoestrogens sources for respondents of male sex except those, who are 61 – 70 years old. In that case, linseed was found as major phytoestrogen source. For female significant high intake of linseed brings high values of phytoestrogens daily intake except those, who are older than 80. A separate part of this population study presents questionnaire, which is focused to health status overview, where the most common diseases are listed in the table 4. Cardio-vascular diseases were found as dominant in high percentage (67%) occurrence in the case of 50 – 60 years old male population.

Table 1 Daily intake of phytoestrogens (mg) according to active compound.

Age	50 – 60	61 – 70	71 – 80	81 – 90
MALE				
Value (S.D.)				
FOR	0.013 (0.008)	0.009 (0.011)	0.005 (0.004)	NR
DAI	0.011 (0.007)	0.117 (0.249)	0.005 (0.003)	NR
GEN	0.023 (0.015)	0.227 (0.472)	0.011 (0.008)	NR
GLY	0.007 (0.005)	0.012 (0.021)	0.001 (0.001)	NR
MAT	0.057 (0.03)	0.026 (0.029)	0.012 (0.009)	NR
LAR	0.151 (0.084)	0.169 (0.358)	0.057 (0.036)	NR
PINO	0.387 (0.385)	0.458 (0.982)	0.187 (0.166)	NR
SECO	8.003 (7.279)	10.03 (30.255)	1.109 (1.276)	NR
COU	0.003 (0.002)	0.002 (0.004)	0.001 (0.001)	NR
Phytoestrogens Σ	8.655 (7.815)	11.05 (32.381)	1.388 (1.504)	NR
FEMALE				
Value (S.D.)				
FOR	0.012 (0.0142)	0.008 (0.012)	0.011 (0.013)	0.002 (0.001)
DAI	0.582 (1.347)	0.257 (0.658)	0.673 (2.415)	0.004 (0.002)
GEN	1.105 (2.468)	0.533 (1.234)	1.303 (4.518)	0.013 (0.008)
GLY	0.055 (0.103)	0.025 (0.055)	0.07 (0.235)	0.001 (0.001)
MAT	0.051 (0.073)	0.034 (0.082)	0.051 (0.102)	0.015 (0.003)
LAR	0.596 (1.094)	0.307 (1.144)	0.506 (1.295)	0.06 (0.018)
PINO	0.693 (0.847)	0.736 (2.344)	1.324 (3.68)	0.183 (0.12)
SECO	64.701 (136.644)	23.68 (111.525)	39.416 (102.643)	0.046 (0.019)
COU	0.009 (0.017)	0.003 (0.014)	0.006 (0.013)	0.001 (0.001)
Phytoestrogens Σ	67,804 (142,6072)	25.583 (117.068)	43.36 (114.914)	0.325 (0.173)

Note: * Abbreviations: FOR- formononetin; DAI- daidzein; GEN- genistein; GLY- glycitein; MAT- matairesinol; LAR- lariciresinol; PINO- pinoresinol; SECO- secoisolariciresinol; COU- coumestrol; NR- no respondents.

Table 2 Daily intake of phytoestrogens (mg) from main sources.

Age	50 – 60	61 – 70	71 – 80	81 – 90
MALE				
Value (S.D.)				
Soy	NA	0.327 (0.739)	NA	NR
Leguminous plants	0.006 (0.000)	0.011 (0.023)	0.003 (0.001)	NR
Vegetable	0.337 (0.237)	0.231 (0.132)	0.205(0.155)	NR
Fruit	0.045 (0.034)	0.041 (0.051)	0.015 (0.010)	NR
Cereals	7.972 (7.285)	1.626 (3.865)	1.079 (1.283)	NR
Others-snack	0.296 (0.319)	8.817 (28.381)	0.087 (0.140)	NR
FEMALE				
Value (S.D.)				
Soy	2.105 (4.390)	0.740 (1.892)	1.988 (7.220)	NA
strukoviny	0.028 (0.065)	0.005 (0.007)	0.003 (0.002)	0.001 (0.000)
Vegetable	0.431 (0.321)	0.320 (0.246)	0.404 (0.270)	0.267 (0.154)
Fruit	0.033 (0.030)	0.035 (0.047)	0.036 (0.059)	0.019 (0.013)
Cereals	3.126 (2.748)	2.005 (3.168)	2.203 (4.333)	0.031 (0.041)
Others-snack	56.876 (144.514)	22.479 (115.043)	38.728 (108.041)	0.007 (0.004)

Note. * NR- no respondents; NA- no answer.

Identical percentage of 29 values was found for the male population of 61 – 70 years old in the case of cardiovascular diseases and others (covers individual/combination of surgery and/or selected diagnosis such as allergy, osteoporosis, etc.). For female presence of cardio-vascular diseases was also found for all selected ages with the highest percentage (39%) occurrence between the age of 71 and 80. A group

described as others covers the major value of 58% in the interval 50 to 60 years old.

Present study utilized published data on the phytoestrogen content of foods to estimate phytoestrogen daily intake by the population from selected region. These estimations fill the data gap and concern the effects of phytoestrogen intake on consumers at the age of retirees. On the base of literature sources, the specific foods identified as important phytoestrogen sources and those,

Table 3 Daily intake of phytoestrogens (mg) from other sources.

Age	50 – 60	61 – 70	71 – 80	81 – 90
MALE				
Value (S.D.)				
Green Tea	0.052 (0.037)	0.009 (0.012)	0.004 (0.006)	NR
Beer	NA	0.001 (0.001)	0.001 (0.002)	NR
Red Wine	0.001 (0.001)	0.015 (0.032)	0.008 (0.014)	NR
White Wine	NA	0.002 (0.005)	0.002 (0.005)	NR
Peanuts	0.001 (0.001)	0.002 (0.003)	0.001 (0.005)	NR
Sesame seeds	0.217 (0.307)	0.321 (1.059)	0.056 (0.126)	NR
Linseed	NA	8.448 (27.341)	NA-	NR
FEMALE				
Value (S.D.)				
Green Tea	0.008 (0.012)	0.008 (0.013)	0.011(0,011)	0.002 (0.003)
Beer	NA	0.001 (0.001)	0.001 (0.001)	NA
Red Wine	0.001 (0.002)	0.005 (0.012)	0.005 (0.019)	NA
White Wine	0.001 (0.001)	0.001 (0.002)	NA	NA
Peanuts	0.001 (0.001)	0.001 (0.002)	NA	NA
Sesame seeds	0.280 (0.449)	0.554 (2.481)	1.132 (4.048)	NA
Linseed	56.569 (144.133)	21.894 (112.770)	37.564 (104.169)	NA

* NR- no respondents; NA- no answer.

which are available on local market and known as ingredients or a part of traditional meals, were analyzed according to published data and personalized questionnaire. Measurements of nine different phytoestrogens on different sets of foods have been made. The advantage of the study is that it could utilize recent data generated by single laboratories, on a wide range of foods common to Slovak diet. The mechanism of phytoestrogen action to human body is based on structural similarities between estradiol and the isoflavones, lignans, and coumestans. It suggests that these compounds may exert their effects through binding with estrogen receptors, acting estrogenically or antiestrogenically depending on endogenous estrogen levels and the type of receptor (α or β) in specific body tissues (Kinjo et al., 2004). Individual phytoestrogens may differ in activities such as inhibition of tyrosine kinase (Youngren et al., 2005), DNA topoisomerases II and growth factors (Gordaliza et al., 2000), alteration of enzymes involved in estrogen synthesis and metabolism, such as aromatase (Brooks and Thompson 2005) as well as antioxidant activities (Kitts et al., 1999) or antiangiogenesis (Fotsis et al., 1995). Thanks

to above mentioned facts, Webb and McCullough (2005) and couple of other authors point to potential health benefits of phytoestrogens obtained from food in reducing the risk of cancer, cardiovascular disease, osteoporosis, and menopausal symptoms. When the presence of selected diseases is compared to overall phytoestrogens intake measured individually for male and female respondents divided into four age intervals (Table 1 vs. Table 4), it is clear, that counting effect of phytoestrogens intake and eating habits partially protects the population against selected diseases, which are typically found at the retirees of slovak population. Moreover, this is in accordance to various studies such as the study presented by Adlercreutz (2007), where the author is describing how phytoestrogens, especially the ligans plays a defensive role against human cancer diseases. For a more precise determination of selected or total phytoestrogen daily intake value it is important to keep it in mind, that although some foods such as beverages, vegetables or fruits contain low concentrations for example of lignans, they are consumed in large amounts so they can contribute significantly to lignan intake. In respect of these,

Table 4 Presence of selected diseases according to age of respondents.

Age	50 – 60	61 – 70	71 – 80	81 – 90
MALE				
Cardio-vascular	67%	29%	20%	NR
Cancer	NA	5%	NA	NR
Diabetes	NA	10%	20%	NR
Others	NA	29%	30%	NR
FEMALE				
Cardio-vascular	25%	34%	39%	33%
Cancer	NA	4%	NA	NA
Diabetes	8%	4%	9%	NA
Others	58%	25%	22%	NA

* NR- no respondents; NA- no answer.

Horn-Ross et al., (2000) described that, coffee and orange juice contributed about 40% of total lignan intake in postmenopausal women of the United States. A fruit, vegetables and beverages presents 7%, 24% and 37% of total lignan intake in the Dutch population (Milder et al., 2005). Relatively low amounts of soy intake presented by our respondents is in accordance to the fact, that in western diets soy beans do not contribute substantially to the diet (Keinan-Boker et al., 2004). Low doses of coumestrol intake were recorded by the present study, what is caused by naturally lower concentrations. The same conclusion in relation to this substance was published by Thompson et al., (2006). Average intake (summary for male & female) of isoflavones at retirees of selected Slovakia region is represented by following values: 0.0226 (50 – 60 age intervals); 0.1485 (61 – 70 age intervals); 0.2599 (71 – 80 age intervals) and 0.005 mg.day⁻¹ (over 81). Presented values are in accordance to conclusion identified by Coward et al., (1961) about the intake of western consumers less than 5 mg.day⁻¹ and apparently lower than those found in Japanese population (50 mg.day⁻¹) presented by Messina (1995); or population of Asia (a range between 25 to 45 mg.day⁻¹) (Coward et al., 1961).

CONCLUSION

In conclusion, the present study summarized eating habits and health status of selected Slovak population in combination with estimation of potential effect of phytoestrogen sets intake to diseases frequency. Selected nine major phytoestrogens had been analyzed simultaneously in the same foods. Moreover, according to data reached from questionnaire it is clear, that cereals and linseed are the major contributors to phytoestrogen intake of evaluated consumers. This fact is in relation to high values of lignans and particularly secoisolariciresinol, which contributes significantly on phytoestrogen intake of evaluated population. This information packages we developed can be applied directly by future studies focused to personalizing the nutrition of retirees or designing high phytoestrogen diets in clinical trials that are related not only to cancer but also to other hormone-related diseases. If such knowledge is combined by more details like genetical background and metabolism of consumer, possitive effect of diet optimalsed in phytoestrogens content may brings a useful tool for reaching of desired health status.

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ASSESSING EXPRESSION OF TAS2R16 RECEPTOR ON THE TONGUE OF ELDERLY PERSONS

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ABSTRACT

In conducted study, we assessed expression of TAS2R16 receptor gene on the tongue of elderly persons. The TAS2R16 receptor belongs to family of G-protein coupled bitter taste receptors and is expressed in type 2 taste cells, which are a part of taste buds. The taste buds are distributed across the tongue's surface on the specialised structures called papillae. The TAS2R16 receptor mediates bitter taste in response to β -glucopyranosides such as salicin. The purpose of conducted study was to examine, whether the ageing process influence gene expression and hence the perception of taste at the molecular level. Ageing process is often related to either decreased or total lost perception of taste qualities. It is due to physiological changes in the oral cavity. The changes in taste cell membranes involve altered function of ion channels and receptors, which ultimately lead to decreased tasting ability of elderly people. In addition, various causes, such as oral and systemic diseases, drug administration, lifestyle (i.e. smoking) and some oral conditions (wearing dentures, dental caries and coated tongue), may exacerbate this issue. Loss of taste may become a large factor in reduction of appetite, which may lead to malnutrition. To accomplish the objective of this study, we recruited ten elderly persons. One 25-year old human was used as calibrator. We used non-invasive scraping method for collecting taste cells from fungiform papillae of each subject. A multiplex TaqMan real-time PCR was performed to amplify cDNA of TAS2R16 and PGK1 genes, whereas the last one served as housekeeping gene. The TAS2R16 gene expression for elderly persons relative to that of young one was calculated according to the $2^{-\Delta C_t}$ formula. Results pointed out to increased expression of TAS2R16 gene by 2-fold in 5th and 8th seniors. It is assumed that they perceive more intense bitterness from salicin at the molecular level than 25-year old person. The 2nd, 3rd, 7th and 10th elderly persons have had decreased expression level about 70%, whereas in case of 6th one that was even about 90%. It is supposed that these subjects, in particular last one, respond to salicin very weakly. This data may show evidence of almost total loss of taste. The causes and consequences are discussed in more detail.

Keywords: TAS2R16; elderly person; bitter taste

INTRODUCTION

Humans can distinguish five basic taste qualities, which are bitter, sweet, sour, umami and salty, as well as newly discovered and potentially accepted taste qualities, for instance, metallic, electrical, fatty and watery (Liman et al., 2014; Fábíán et al., 2015; Chaudhari and Roper, 2010). Detection of taste stimuli (compounds) in the oral cavity is provided by thousands of taste buds, which are arranged on the tongue papillae (Gravina et al., 2013). Additionally, the taste buds are on the soft palate, larynx, and pharynx (Behrens et al., 2007).

Each taste bud consists of approximately 50 – 100 taste receptor cells (TRCs) (Dotson et al., 2012). There are four types of TRCs: type 1, 2, 3 and 4 (basal cells) (Bachmanov et al., 2014). Type 4 cells are round, are located at the bottom of the taste buds, and are considered to be progenitor cells of other types of TRCs (Yamamoto and Ishimaru, 2013). Types 1, 2 and 3 (also referred to as dark, light and intermediate, respectively) are mature TRCs possessing microvilli at the apical ends (Chandrashekar et al., 2006; Cvijanovic et al., 2015).

These TRCs express proteins that participate in taste transduction. Some of these proteins are inserted into the

cell membrane to form taste receptors (Reed et al., 2006). The microvilli of TRCs project through „taste pore“ into the oral cavity, where interact with taste stimuli via taste receptors (Chandrashekar et al., 2006; Cvijanovic et al., 2015). TRCs convert chemical stimulus into an output signal that is sent to the brain via the afferent gustatory neurons to evoke taste perception (Medler, 2015).

Type 2 cells express type of G-protein coupled receptors for detecting sweet, bitter, and umami tastes (Yamamoto and Ishimaru, 2013; Niki et al., 2010), whereas type 3 cells are supposed to express channel type receptors, involved in mediating of sour taste (Chaudhari and Roper, 2010; Behrens and Meyerhof, 2011). However, it is not yet known which TRCs are specifically responsible for the salty taste sensation. According to Fábíán et al., (2015) it is likely that type 2 cells also do that in a specific way.

Up to now, the criteria for accepting any protein as a receptor, have been fulfilled by that one for sweet and umami (T1R), bitter (T2R) and salty (ENaC) taste. The sour taste receptors are still unknown, although some channel-type candidates have been proposed (ASICs,

PKDs, etc.) (Bachmanov et al., 2014; Fábíán et al., 2015).

In contrast to sweet and umami taste, which have evolved to recognize a limited subset of nutrients, in particular sources of energy such as saccharides and proteins, bitter taste has the onerous task of preventing the ingestion of a large number of structurally distinct toxic compounds (Chandrashekar et al., 2006). This is supported by fact that while just three genes exist in the TAS1R gene family (which is responsible for the receptors for both sweet and umami taste) (Feeney et al., 2011) over 25 genes from TAS2R family encode functional bitter taste receptors (Behrens and Meyerhof, 2013).

On the other hand, bitter sensations are also mediated by number of phytonutrients found in fruit, vegetable, coffee and green tea (i.e. phenols, flavonoids, isoflavones, terpenes, glucosinolates, isothiocyanates), which are reported to have antioxidant and anticancer properties and a wide spectrum of tumor-suppressing activities (Drewnowski and Gomez-Carneros, 2000; Trembecká et al., 2013).

As regard the ligands, the bitter taste receptors exhibit heterogeneous molecular receptive ranges. Some of them are narrowly tuned to 2 or 4 bitter-tasting compounds, whereas others are promiscuously activated by numerous ligands (Chaudhari and Roper, 2010). However, ligands for some receptors (TAS2R41, -42, -45, -48 and -60) remain still unknown (Niki et al., 2010).

In this study, we performed TaqMan real-time PCR in order to examine the expression level of TAS2R16 receptor gene on the tongue of elderly persons, in relation to the young human. The purpose was to find out, whether the ageing process influence gene expression level and hence the perception of taste at the molecular level. The TAS2R16 receptor mediates bitter taste in response to β -glucopyranosides such as salicin (Bufe et al., 2002).

The studies has shown that sensitivities to salty and bitter tastes show more substantial decreases in ageing process than do sensitivities to sour and sweet tastes (Feng et al., 2013). Taste disorders, in particular loss of taste, are often underestimated, but can have an unfavourable fallout on the health of older people, such as loss of appetite, changes in food preferences, anorexia, weight loss and malnutrition, consequently exacerbating their chronic diseases and related morbidity and mortality (Imoscopi et al., 2012).

MATERIAL AND METHODOLOGY

Participants

Ten elderly persons (mix of males and females) were recruited to participate in the study. One 25-year old healthy volunteer (male) was employed as control (i.e. calibrator – C). After a complete explanation of the study to the subjects, written informed consent was obtained from every participant.

Sample collection

We adopted a scraping method to collect tissue samples for this study. It is so minimally invasive technique that it is often used to collect tissue samples from the oral cavity. Using a tongue scraper, we obtained the epithelium specimen from tip, dorsum and foliate papilla in the

tongue. None of the participants had anything to eat or drink for at least 90 min before scraping the tongue surface. The mixture of scrapped taste cells from each subject (cca 50 μ L) was labelled with identifying code Sample from 25-year old human was labelled with number 0, whereas samples from elderly persons were labelled with numbers from 1 to 10. The samples were inserted into eppendorf tube with pre-pipetted volume (150 μ L) of stabilising solution RNALater (Sigma-Aldrich).

Preparation of cDNA

Total RNA was isolated using Nucleospin RNA II (Macherey-Nagel) with on-column DNA digestion according to the manufacturer's instructions. RNA concentration and quality was assessed spectrophotometrically using DS-11 FX+ device (DeNovix). Totally 100 ng of RNA was reverse transcribed using a mixture of random hexamer and oligo-dT oligonucleotide primers with ImProm-II Reverse Transcription System (Promega) following the recommendations of the manufacturer. Identical reactions omitting reverse transcriptase were performed to generate negative control templates.

Quantitative real-time PCR

Gene specific primers and TaqMan probe were used to amplify TAS2R16 gene (forward, 5'-CTGGCCTCCACCATCTTTC-3'; reverse, 5'-TGCAGTGACCAGTGC TATGAT-3'; TaqMan probe, 5'-TCATGGCATCACTGACCAAGCAGA-3') and housekeeping gene β -phosphoglycerate kinase 1 (PGK1) (forward, 5'-GGTGCTCAACAACATGGAGATTG-3'; reverse, 5'-GCTTTGGACATTAGGTCTTTGACA-3'; TaqMan probe, 5'-TCTCTGTTTGATGAAGAGGGAGCCA-3'), in a same tube (duplex reaction). Sequences for both genes were designed using Web programme RealTime PCR Design Tool (Biosearch Technologies) and synthesized by Generi Biotech (Czech Republic).

Amplification was performed on TOptical Gradient 96 device (Analytik Jena, Germany). The reaction mixture contained both primers for PGK1 at a concentration of 0.3 pmol. μ L⁻¹ and both primers for TAS2R16 at a concentration of 0.5 pmol. μ L⁻¹. In all cases, the probes for both genes were used at a final concentration of 0.1 pmol. μ L⁻¹.

For the real-time PCR reaction, 100 ng of cDNA template was incubated with qPCR ProbesMaster with UNG/lowROX clear mix containing dNTPs with dUTP and hot start DNA polymerase (Jena Bioscience, Germany) in a final volume of 25 μ L. Cycling parameters were as follows: 50 °C for 2 min for UNG treatment, followed by 95 °C for 2 min for initial denaturation, followed by 45 cycles of 15 s at 95 °C, 45 s at 60 °C. Each cDNA (+RT) and RNA (-RT) sample was tested in triplicate. As negative controls, reactions were performed using water or products of the cDNA reaction performed in the absence of the reverse transcriptase enzyme. Raw data were acquired and processed with the qPCR Software 3.0 (Analytik Jena, Germany) and further analysed with Microsoft Excel. Gene expression relative to that of PGK1

was calculated according to the $2^{-\Delta C_T}$ formula (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

The C_T (obtained in real-time PCR) and relative gene expression values are shown in Table 1. The last ones are also presented graphically on Figure 1. The TAS2R16 expression level of calibrator was set to unity and the relative expression levels of all the other samples were given in relation to the calibrator sample (i.e. x-fold either the increase or decrease in relation to the calibrator).

Increased expression of TAS2R16 gene around by 2-fold has been observed in 5th and 8th elders. It is assumed that they perceive more intense bitterness from salicin at the molecular level than 25-year old human. This may suggest that either density of taste buds was not decreased, or involvement some mechanism, contributing to up-regulated expression of TAS2R16 gene on the tongue.

Since the phytochemicals taste bitter, people with higher expression level of bitter taste receptors might not prefer food with their higher content (Drewnowski and Gomez-Carneros, 2000). Thus, such people must involuntary refuse the food with numerous beneficial effects. For instance, salicin is precursor of acetylsalicylic acid (aspirin) and has pharmacological effects on treatment of fever, pain, and inflammation (Kim et al., 2015).

On the other hand, in some cases (2nd, 3rd, 7th and 10th elders) the gene expression decreased about 70%. Moreover, we have noticed even more than 90% reduction of TAS2R16 gene expression (6th elder). He expressed only 0.08-fold amount of TASR16 gene, compared to calibrator. We suppose that these seniors, in particular last one, are responding on salicin very weakly. This data may show evidence of almost total loss of taste, the causes of which are discussed below.

Table 1 Recorded C_T values for TAS2R16 and PGK1 gene, calculated relative expression of TAS2R16 gene.

Sample	C_T	C_T	ΔC_T	ΔC_T	Mean ΔC_T	$SD^* \Delta C_T$	$\Delta \Delta C_T$	$SD^* \Delta \Delta C_T$	% KD**
	TAS2R16	PGK1							
C	23.22	26.14	-2.92	7.57	7.17	0.55	1.00	0.08	0.00
	23.29	26.18	-2.89	7.41					
	23.39	26.10	-2.71	6.54					
1	30.1	32.00	-1.90	3.73	4.04	0.51	0.56	0.07	-43.71
	30.01	32.22	-2.21	4.63					
	30.2	32.11	-1.91	3.76					
2	29.11	30.70	-1.59	3.01	2.03	1.25	0.28	0.17	-71.67
	29.19	28.51	0.68	0.62					
	29.2	30.50	-1.30	2.46					
3	30.5	32.69	-2.19	4.56	4.43	0.23	0.62	0.03	-38.23
	30.41	32.60	-2.19	4.56					
	30.4	32.46	-2.06	4.17					
4	28.95	28.41	0.54	0.69	0.84	0.25	0.12	0.03	-88.27
	29.1	28.61	0.49	0.71					
	28.74	28.91	-0.17	1.13					
5	27.39	31.12	-3.73	13.27	14.02	0.99	1.95	0.14	95.35
	27.26	31.03	-3.77	13.64					
	27.27	31.19	-3.92	15.14					
6	31.88	31.65	0.23	0.85	0.54	0.39	0.08	0.05	-92.48
	32.83	32.25	0.58	0.67					
	32.21	28.85	3.36	0.10					
7	34.02	34.39	-0.37	1.29	1.58	0.41	0.22	0.06	-77.96
	33.05	34.09	-1.04	2.06					
	33.55	34.03	-0.48	1.39					
8	24.36	28.46	-4.10	17.15	16.71	1.20	2.33	0.17	132.89
	24.55	28.49	-3.94	15.35					
	24.43	28.57	-4.14	17.63					
9	22.44	24.83	-2.39	5.24	4.69	0.49	0.65	0.07	-34.68
	22.64	24.81	-2.17	4.50					
	22.28	24.39	-2.11	4.32					
10	28.41	26.97	1.44	0.37	1.24	0.79	0.17	0.11	-82.65
	28.24	28.80	-0.56	1.47					
	27.08	28.00	-0.92	1.89					

* Standard deviation.

** Percent knockdown of TAS2R16 expression.

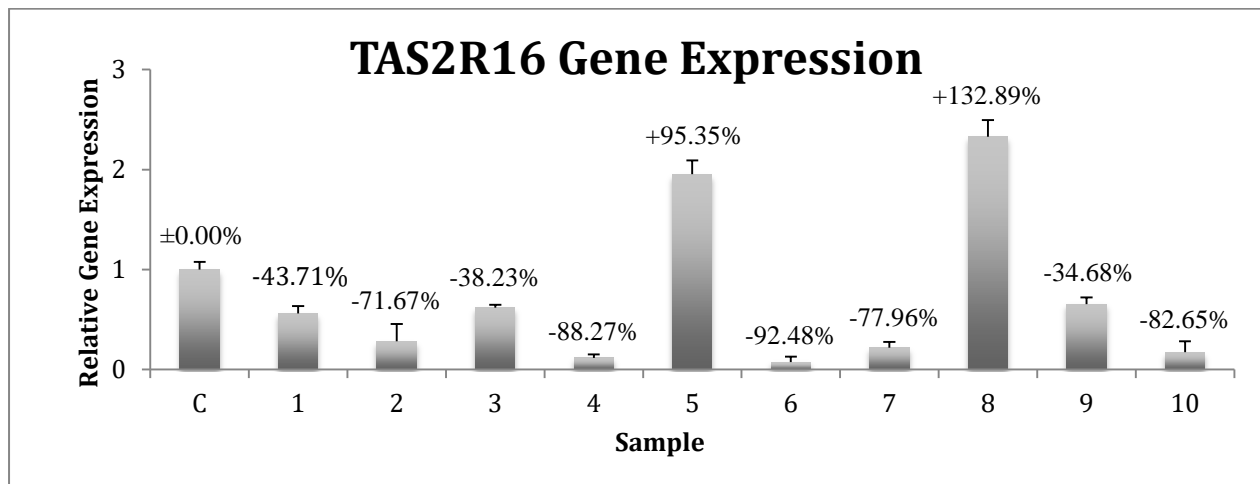


Figure 1 Expression level of TAS2R16 receptor on the tongue of elderly persons in relation to young human.

The issue of taste lost has been associated with normal ageing, drug administration, oral (dental caries, stomatitis) and systemic diseases. The last ones include neurological (Alzheimer, epilepsy), cardiovascular (hypertension), endocrinal (diabetes mellitus types 1 and 2, hypothyroidism), gastrointestinal, kidney, liver, respiratory and viral diseases, as well as some kind of cancer (lung, breast, stomach) (Boyce and Shone, 2006; Feng et al., 2013; Imoscopi et al., 2012; Ikeda et al., 2008).

As regards ageing process, numerous physiological changes in the oral cavity are observed that contribute to taste loss. These include: a) thinning and drying of oral mucosa due to declining keratinisation, b) thinning of the epithelial structure, c) atrophy of salivary glands or disappearing of acini (replaced by adipose and fibrous connective tissue) and e) diminishing of taste buds' density (Imoscopi et al., 2012). Therefore, the changes in taste cell membranes involve altered function of ion channels and receptors, which ultimately lead to decreased tasting ability of elderly people (Boyce and Shone, 2006).

Further, some oral conditions, such as wearing dentures, dry mouth and coated tongue, may cause taste impairment. Many elderly persons have poor oral health, characterized by heavy plaque accumulation, mucosal inflammation, and high dental caries activity (Solemdal et al., 2012; Imoscopi et al., 2012).

In addition to ageing process and diseases, the proper lifestyle is also important to maintain taste sensitivity. For instance, Aoki et al., (2014) compared expression of TAS2R genes (including TAS2R16) between group of elderly smokers and non-smokers. They demonstrated significantly lower expression levels of TAS2Rs in individuals who smoked cigarettes. Furthermore, a significant positive correlation ($p = 0.496$) between age and expression of TAS2R was observed in non-smokers. This study revealed that smoking in seniority lead to decreased sensitivity to bitter-tasting food.

Besides the smoking, alcohol also interferes into taste perception, because impairs intestinal absorption of zinc and vitamin A, both of which are essential to gustatory function (Imoscopi et al., 2012).

A loss of taste reduces the joy of eating nutritious, flavoursome foods. Taste disorders may become a large

factor in reduction of appetite, which may lead to malnutrition (Ikeda et al., 2008). Nutritional deficiencies of vitamin B12 also contribute to the taste dysfunction (Boyce and Shone, 2006; Feng et al., 2013). Consequently, protein malnutrition and deficits of zinc, selenium and vitamin B6 aggravate dysregulation of the immune system among older individuals. These seniors are more frequently predisposed to infectious diseases, with serious health implications, compared to healthy ones (Brownie, 2006).

CONCLUSION

To conclude, we demonstrated TAS2R16 gene expression on the tongue of elderly persons. In majority of tested subjects we found out decrease in expression level of this gene. This may point out to inability to detect salicin, i.e. to partial loss of taste function. This disorder might be caused by several factors such as normal ageing process and diseases. Taste loss is serious issue, which may have psychological implications, resulting in malnutrition and impaired health status.

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THE EFFECT OF INTENSIFICATION FACTORS TO TOTAL ANTIOXIDANT ACTIVITY OF HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM* L.) AND LINGONBERRY (*VACCINIUM VITIS-IDAEA* L.)

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ABSTRACT

Public attention is increasingly drawn to the protective effects of natural antioxidants against civilization diseases. An important source of antioxidants are berries, which until recently has received little attention, but the latest research towards the right to it. The phenolic profile and quantitative composition of blueberries as well as the corresponding antioxidant activity of blueberries is well documented. The aim of this paper was the determination of the relationship between different methods of fertilization and total antioxidant activity of six selected varieties of blueberries and five varieties of lingonberries. Each sample of blueberry varieties (Bluejay, Nelson, Bluecrop, Patriot, Berkeley and Brigitta) and lingonberry varieties (Koralle, Ida, Sanna, Linnea and Sussi) were collected from the research station Krivá in Orava. The values of total antioxidant activity of the extracts of studied varieties of blueberry after organic fertilization ranged from 27.15 to 52.25 $\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox. After mineral fertilization, the values of total antioxidant activity of the extracts of studied varieties of blueberry ranged from 21.27 to 51.00 $\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox. In the control treatment, the values of total antioxidant activity of the extracts of studied varieties of blueberry ranged from 26.99 to 54.15 $\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox. The values of values of total antioxidant activity of the extracts of studied varieties of lingonberry after organic fertilization ranged from 42.49 to 60.27 $\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox. After mineral fertilization, the values of total antioxidant activity of the extracts of studied varieties of lingonberry were in the interval from 38.85 to 55.15 $\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox. In the variant without fertilization, the values of total antioxidant activity of the extracts of studied varieties of lingonberry ranged from 37.16 to 65.11 $\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox. The application of organic fertilizer has a positive effect to increasing values of antioxidant activity in blueberries and lingonberries. It should be noted that the value of the total antioxidant activity is significantly influenced by the rainfall.

Keywords: cultivars; blueberries; lingonberries; antioxidant capacity; organic fertilizer; mineral fertilizer

INTRODUCTION

Rational and efficient use of acid, low fertile soils below the mountain areas in Slovakia is currently very difficult. Due to negative changes in the environment and lifestyle of today's population, is rapidly increasing diversity of lifestyle diseases. Therefore, in many countries is preferred "Food for the Future", supporting health food, and their place in the food system and health is very important. They are preserving foods as much as possible the original biologically valuable substances, produced in an environmentally friendly way.

Public attention is increasingly drawn to the protective effects of natural antioxidants against civilization diseases. An important source of antioxidants are berries, which until recently has received little attention, but the latest research towards the right to it. Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. There are two basic categories of antioxidants, namely, synthetic and natural. In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution, whereas

natural antioxidants can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid (Larson, 1988; Hudson, 1990; Hall and Cuppett, 1997). Thus, the interest in natural antioxidants has increased considerably (Löflier, 1991). The high content of flavonoids, which include anthocyanins, is one indicator of antioxidant activity. Antioxidative properties of anthocyanins protect cells of the body against harmful action of oxygen radicals (Poráčová et al., 2011).

From the biological point of view is an antioxidant a compound that at low concentrations in the reaction with reactive forms a relatively stable and non-toxic products, thereby protecting cells, tissues and entire organism against oxidative damage by free radicals (Šilhár et al., 2004). "Antioxidant power" is the term food as a gate capacity of the human body against the action of free radicals, preventing degenerative diseases are resulting from continuous operation of the oxidative stress (Di Majo et al., 2008). Result antioxidant activity is protection of biologically important molecules and, ultimately, cells,

tissues and the whole organism from oxidative damage by free radicals. The antioxidants can protect the fetus from damage and mutations prevent cancer development, protect certain enzymes vitamins (Kyselovič, 2002).

Lingonberry (*Vaccinium vitis-idaea* L.) and blueberry (*Vaccinium corymbosum* L.) are flowering plants belonging to the large genus of *Vaccinium*. Lingonberry is small, thickly branched, has not deciduous perennial shrub and is closely related to the blueberry. Blueberries and lingonberries is worldwide famous as the healthy and desirable fruit and are one of the richest sources of antioxidants in our diet (Mendelová et al., 2013; Kähkönen et al., 1999). Currently, the consciousness receiving fruit lingonberries and blueberries due to the high amounts of phenolic compounds, and hence a high antioxidant activity (Prior et al., 1998). Small forest fruits, for example: blueberries, lingonberries, strawberries and blackberries Beattie et al., (2005) called "food superstars" with a protective effect against heart disease, cancer, and aging. Bioactive wild blueberry extract are full of anthocyanins and proanthocyanidins and have a significant antioxidant activity (Smith et al., 2008). Blueberry and lingonberry extract prevent against the oxidation of lipids in liposomes, and to diminish the level of LDL cholesterol (Kale et al., 2006).

Antioxidant capacity of blueberries is influenced by various factors (temperature, pH and oxidation) and is compared with raspberries and wild strawberries about three times higher, but the vitamin C content of about 4-fold lower (Kalt et al., 1999). Prior et al., (1998) considered blueberries as one of the richest sources of antioxidant phytonutrients and have also confirmed the linear relationship between antioxidant capacity and total anthocyanins and polyphenols. Chemically, the antioxidants are considered to be all substances which prevent the oxidation of other compound with reactive (oxidant) that is itself oxidized. The result of antioxidant activity protect biological important molecules, and ultimately cell, tissue, and whole organism from oxidative damage species (Ďuračková, 1998).

The aim of this work is to provide information on the impact of intensification factors for antioxidant activity in fruit extracts of lingonberry and blueberry.

MATERIAL AND METHODOLOGY

The experiments were based on the research station Krivá in the Orava region, which is located in the north of Slovakia. The average of temperature in this area is 6 °C and rainfall is 900 mm. The experimental area with varieties of lingonberries and blueberries is located on a slope with 10° inclination and NW exposure at an altitude of 628 – 634 meters. In the experiment we watched five varieties of lingonberry (Koralle, Ida, Sanna, Linnea and Sussi) and 6 varieties of blueberry (Bluejay, Nelson, Bluecrop, Patriot, Berkeley and Brigitta). Fruit samples for analyses were collected in August, years 2013 – 2014 and subsequently analysed at the Slovak Agricultural University in Nitra.

Lingonberries and blueberries were fertilized:

Fertilization was carried out in two different variants (mineral and organic fertilizer). The third variant (control)

was without fertilizing. Fertilization by mineral fertilizers (ammonium sulfate, potassium sulfate, and superphosphate) was carried out in the spring of the five varieties of the first variant, in a ratio of 30 kg of N, 10 kg P and 30 kg of pure nutrients.ha⁻¹.year⁻¹. Nitrogen fertilization was divided into two parts. Half of the total dose in the spring (in the first half of April) and the other half at the end of June. P and K have been applied in one dose during each spring. Five varieties in the second variant was fertilized with Hoštický organic fertilizer with application at the beginning of vegetation (in the first half of April) at 1 kg per 10 m² and during the vegetation period (in the second decade of June) dose of 0.8 kg to 10 m². Hoštický organic fertilizer containing fermented cow and horse manure, horn and natural guano coming from the droppings of seabirds. It is a natural product containing 5% of N, 3.5% P₂O₅, K₂O 1% and 0.5% MgO.

Evaluation of the values of antioxidant activity in the fruits of lingonberry and blueberry:

Method PCL (photochemiluminescence) - The combination of fast photochemical generation of superoxide radicals with sensitive luminometric detection. It was measured luminescence of luminol remaining after the reaction of radicals with antioxidants of the samples. It allows the measuring of antioxidant activity of the hydrophilic (ACW) and lipophilic components (ACL).

ACW - aqueous extract; calibration for solutions from 0.5 to 4.0 nmol AK / 10 µL.

ACL - methanol extract; calibration for solutions from 0.5 to 4.0 nmol Trolox / 10 µL.

Repeatability RSD = 9.8% for n = 6

RESULTS AND DISCUSSION

Table No. 1 and Fig. 1 and 2 show the results of the average values of total antioxidant activity in six varieties of blueberry in various variants of fertilization.

During fertilization by Hoštický organic fertilizer (OF), the highest average value of antioxidant activity (AA) in year 2013 of blueberry had a variety Bluejay (32.6 µg.mg⁻¹ eq. Trolox) and the lowest variety Bluecrop (27.15 µg.mg⁻¹ eq. Trolox). In year 2014, during fertilization by OH, the highest average value AA had a variety Bluecrop (52.25 µg.mg⁻¹ eq. Trolox) and the lowest variety Berkeley (42.55 µg.mg⁻¹ eq. Trolox).

During fertilization by mineral fertilizer (MF), the highest AA in year 2013 of blueberry had a variety Bluejay (28.35 µg.mg⁻¹ eq. Trolox) and the lowest variety Bluecrop (21.27 µg.mg⁻¹ eq. Trolox). When fertilization variant with MF, the highest AA in year 2014 had a variety Bluejay (51 µg.mg⁻¹ eq. Trolox) and the lowest variety Nelson (36.5 µg.mg⁻¹ eq. Trolox).

The highest average value of AA was in the variant without fertilizing (WF) in year 2013 of blueberry had a variety Nelson (33.84 µg.mg⁻¹ eq. Trolox) and the lowest variety Berkeley (26.99 µg.mg⁻¹ eq. Trolox). In year 2014, the highest AA variant in WF had a variety Berkeley (54.15 µg.mg⁻¹ eq. Trolox) and the lowest variety Bluejay (38.25 µg.mg⁻¹ eq. Trolox). Our results correspond to the arguments authors Lima et al., (2009), who indicated similar levels of antioxidant activity in blueberries.

Table 1 Average values of antioxidant activity ($\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) in selected varieties of blueberries (*Vaccinium corymbosum* L.) determined by method PCL.

Variety	Fertilization	Antioxidant activity in $\mu\text{g}/\text{mg}$ eq. Trolox	
		Year 2013	Year 2014
Bluejay	WF	30.00 \pm 1.76	38.25 \pm 0.65
	MF	28.35 \pm 1.66	51.00 \pm 0.20
	OF	32.60 \pm 0.98	43.14 \pm 0.57
Nelson	WF	33.84 \pm 0.84	49.95 \pm 0.15
	MF	28.23 \pm 0.19	36.50 \pm 0.00
	OF	31.00 \pm 0.30	47.65 \pm 0.25
Bluecrop	WF	28.75 \pm 0,36	50.25 \pm 0.15
	MF	21.27 \pm 0.84	46.90 \pm 0.60
	OF	27.15 \pm 0.27	52.25 \pm 0.85
Patriot	WF	28.11 \pm 2.28	38.40 \pm 0.60
	MF	25.91 \pm 1.84	48.95 \pm 0.25
	OF	29.15 \pm 0.12	50.25 \pm 0.55
Berkeley	WF	26.99 \pm 0.02	54.15 \pm 0.55
	MF	26.83 \pm 2.34	48.70 \pm 1.10
	OF	30.92 \pm 0.50	42.55 \pm 0.05
Brigitta	WF	27.71 \pm 0.38	47.75 \pm 0.45
	MF	25.47 \pm 1.12	48.90 \pm 0.30
	OF	29.40 \pm 1.70	49.80 \pm 1.10

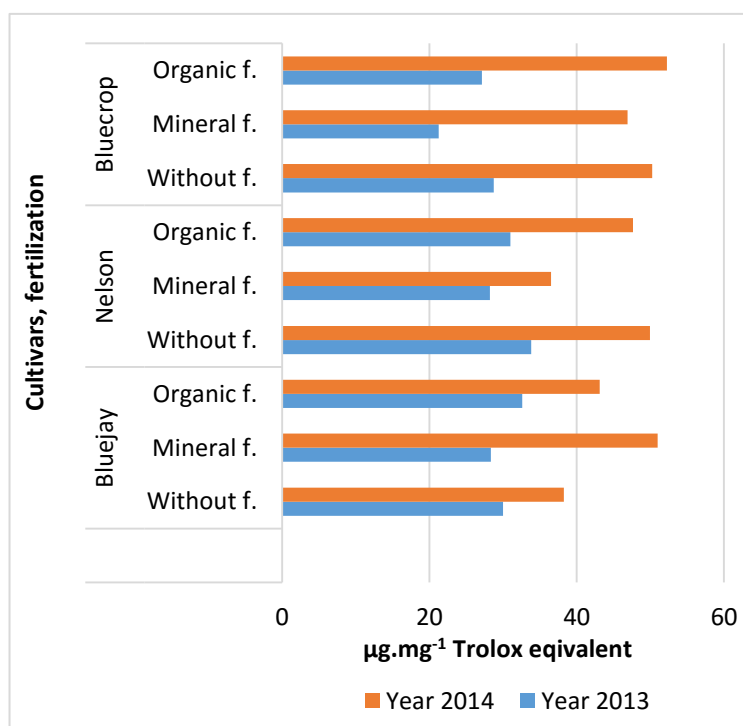


Figure 1 Average values of antioxidant activity ($\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) in selected varieties of blueberries (*Vaccinium corymbosum* L.).

From the average values of antioxidant activity in the variant with the application of organic fertilizer may be made the following order monitored varieties of

blueberries: Bluecrop > Patriot > Brigitta > Nelson > Bluejay > Berkeley.

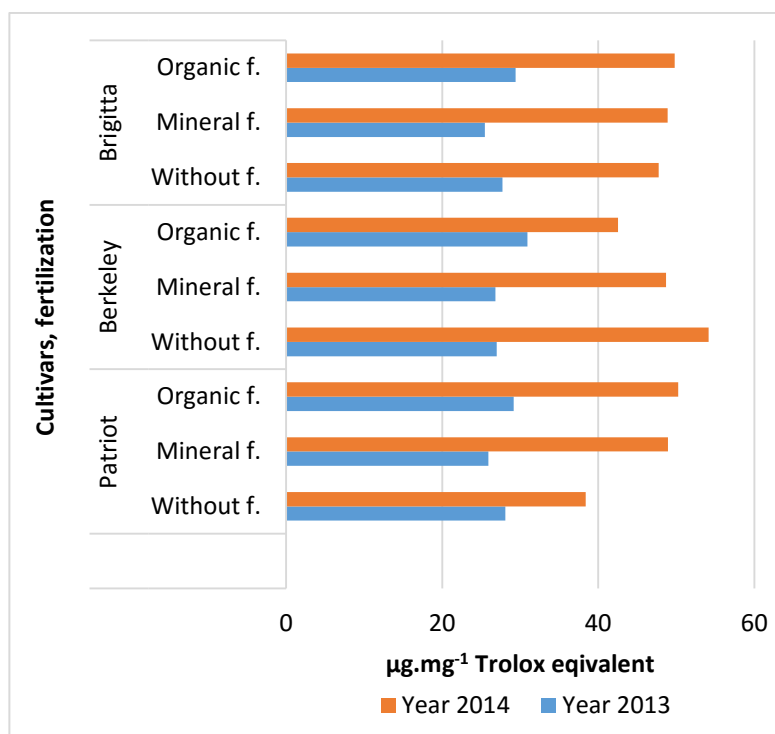


Figure 2 Average values of antioxidant activity ($\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) in selected varieties of blueberries (*Vaccinium corymbosum* L.).

Table 2 Average values of antioxidant activity ($\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) in selected varieties of lingonberries (*Vaccinium vitis-idaea* L.) determined by method PCL.

Variety	Fertilization	Antioxidant activity in $\mu\text{g}/\text{mg}$ eq. Trolox	
		Year 2013	Year 2014
Koralle	WF	37.32 \pm 1.38	38.13 \pm 0.52
	MF	44.36 \pm 1.02	43.37 \pm 1.86
	OF	44.33 \pm 1.80	47.59 \pm 1.78
Ida	WF	65.11 \pm 1.43	56.33 \pm 2.51
	MF	46.42 \pm 0.58	55.15 \pm 0.14
	OF	60.27 \pm 1.62	48.74 \pm 0.66
Sanna	WF	38.61 \pm 1.64	51.34 \pm 1.28
	MF	46.57 \pm 0.71	54.08 \pm 1.51
	OF	50.12 \pm 1.14	53.87 \pm 3.11
Linnea	WF	44.77 \pm 1.56	51.02 \pm 1.76
	MF	38.85 \pm 0.71	52.94 \pm 1.31
	OF	42.49 \pm 1.28	54.67 \pm 0.32
Sussi	WF	37.16 \pm 1.22	42.68 \pm 1.14
	MF	45.37 \pm 0.36	52.10 \pm 0.08
	OF	47.26 \pm 0.52	49.38 \pm 0.64

From the average values of antioxidant activity in the variant with the application of mineral fertilizer may be made in the following order monitored varieties of blueberries: Bluejay > Berkeley > Patriot > Brigitta > Bluecrop > Nelson.

From the average values of antioxidant activity in the variant without application of fertilizer may be made in the following order monitored varieties of blueberries: Nelson > Berkeley > Bluecrop > Brigitta > Bluejay > Patriot.

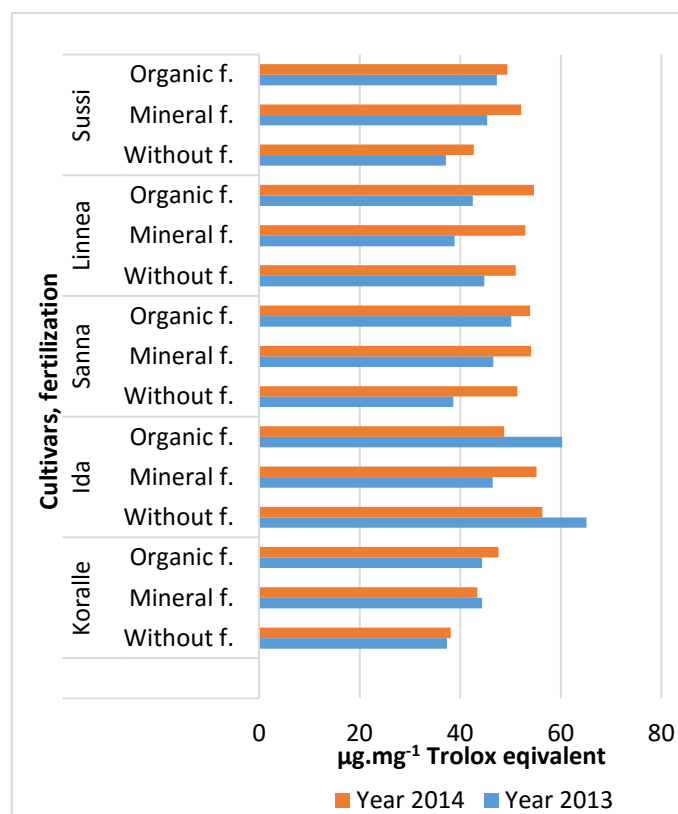


Figure 3 Average values of antioxidant activity ($\mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) in selected varieties of lingonberries (*Vaccinium vitis-idaea* L.).

From the average values of antioxidant activity all varieties may be made in the following order monitored varieties of blueberries Berkeley > Brigitta > Nelson > Bluecrop > Bluejay > Patriot.

From the average values of antioxidant activity of blueberries all varieties may be made in the following order the fertilization methods: organic fertilization > without fertilization > mineral fertilization.

Table 2 and Figure 3 show the results of the average values of antioxidant activity in five varieties of lingonberry in various variants of fertilization.

During fertilization by Hořtický organic fertilizer (OF), the highest average value of antioxidant activity (AA) in year 2013 of lingonberry had a variety Ida ($60.27 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) and lowest variety Linnea ($42.49 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox). In year 2014, during fertilization by OH, the highest average value AA had a variety Linnea ($54.67 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) and lowest variety Koralle ($47.59 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox).

During fertilization by mineral fertilizer (MF), the highest AA in year 2013 of lingonberry had a variety Sanna ($46.57 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) and lowest variety Linnea ($38.85 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox). In year 2014, during fertilization by MF, the highest AA had a variety Ida ($55.15 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) and lowest variety Koralle ($43.37 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox).

The highest average value of AA was in the variant without fertilizing (WF) in year 2013 of lingonberry had a variety Ida ($65.11 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox) and the lowest variety Sussi ($37.16 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox). In year 2014, the highest AA variant in WF had a variety Ida ($56.33 \mu\text{g}\cdot\text{mg}^{-1}$

eq. Trolox) and lowest Koralle variety ($38.13 \mu\text{g}\cdot\text{mg}^{-1}$ eq. Trolox).

From the average values of antioxidant activity in the variant with the application of organic fertilizer may be made the following order monitored varieties of lingonberries: Ida > Sanna > Linnea > Sussi > Koralle.

From the average values of antioxidant activity in the variant with the application of mineral fertilizer may be made in the following order monitored varieties of lingonberries: Ida > Sanna > Sussi > Linnea > Koralle.

From the average values of antioxidant activity in the variant without application of fertilizer may be made in the following order monitored varieties of lingonberries: Ida > Linnea > Sanna > Sussi > Koralle.

From the average values of antioxidant activity may be made in the following order monitored varieties of lingonberries Ida > Sanna > Linnea > Sussi > Koralle.

From the average values of antioxidant activity of lingonberries all varieties may be made in the following order the fertilization methods: organic fertilization with variant > variant with mineral fertilizers > variant without fertilization.

The average value of the total antioxidant activity in cultivated varieties of blueberries was 21.5% lower than in cultivated varieties of cranberries. We confirmed with author **Zheng and Wang (2003)**, who reported that blueberries have higher antioxidant capacity than lingonberries.

CONCLUSION

The presented research aimed at the effects of two fertilizer application treatments on the total antioxidant

activity in the fruit of some varieties of highbush blueberry (*Vaccinium corymbosum* L.) and lingonberry (*Vaccinium vitis-idaea* L.). The research results showed that the antioxidant activity was higher at the treatments with an organic fertilizer application. It was concluded that the values of total antioxidant activity can be influenced by the fruit variety, the soil properties (e.g. grain size, nutrient content, humus) and the climatic conditions.

1. The most positive effects on the overall mean antioxidant activity in the high-bush blueberry fruit were found at the treatment with the "Hoštické" organic fertilizer applied. The highest values of antioxidant activity were recorded at this treatment throughout the research years (2013 and 2014). The least positive impact on the overall mean antioxidant activity of high-bush blueberry fruit was recorded at the treatment with mineral fertilizer application.
2. From the point of view of average values of antioxidant activity of the extracts in year 2013 and year 2014 may be made in the following order monitored varieties of blueberries: Berkeley > Brigitta > Nelson > Bluecrop > Bluejay > Patriot.
3. The most positive impact on the total mean antioxidant activity in the fruit of lingonberry was found also at the treatment with the "Hoštické" organic fertilizer application, what may be considered a notable factor of influence on the human body. The least positive effects on the total antioxidant activity were recorded at the zero fertilizer application treatment (the control).
4. From the point of view of average values of antioxidant activity of the extracts in year 2013 and year 2014 may be made in the following order monitored varieties of lingonberries: Ida > Sanna > Linnea > Sussi > Koralle.
5. The high value antioxidant activity was influenced by rainfall, which was reflected in year 2014, when it had been raining a lot during the growing season compared to 2013. This rainfall was reflected up to twice as high values in most varieties of blueberries and lingonberries.

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BEE BREAD – PERSPECTIVE SOURCE OF BIOACTIVE COMPOUNDS FOR FUTURE

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ABSTRACT

Bee bread is product with long history used mainly in folk medicine. Nowadays, bee bread is growing in commercial interest due to its high nutritional properties. The objective of this study was to determine biological activity of ethanolic extract of bee bread obtained from selected region of Ukraine – Poltava oblast, Kirovohrad oblast, Vinnica oblast, Kyiv oblast, Dnepropetrovsk oblast. The antioxidant activity was measured with the radical scavenging assays using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as well as phosphomolybdenum assay. Total polyphenol content was determined with Folin-Ciocalteu reagent and total flavonoid content by aluminium-chloride method. Secondary was also evaluated antimicrobial activity in bee bread samples with disc diffusion method and minimum inhibitory concentrations. Antioxidant activity expressed as mg TEAC per g of dry weight (Trolox equivalent antioxidant capacity) was the highest in bee bread from Poltava oblast in DPPH and also phosphomolybdenum method. Samples of bee bread contained high levels of total polyphenols (12.36 – 18.24 mg GAE – gallic acid equivalent per g of dry weight) and flavonoids (13.56 – 18.24 µg QE – quercetin equivalent per g of dry weight) with the best values of bee bread from Poltava oblast. An elevated level of antioxidant potential in the bee bread determines its biological properties, which conditioned of the biological active substances. The best antibacterial activity of bee bred with disc diffusion method was found against *Bacillus thuringiensis* CCM 19. The antibacterial activity inhibited by the bee bread extract in the present study indicate that best minimal inhibition concentration was against bacteria *Escherichia coli* CCM 3988 and *Salmonella enterica* subs. *enterica* CCM 3807.

Keywords: antioxidant activity; pollen; flavonoids; polyphenols; antimicrobial activity

INTRODUCTION

Bee bread is a product of the hive obtained from pollen collected by bees, to which they added honey and digestive enzymes and subsequently stored in the combs, starting a lactic fermentation which gives it greater power conservation (Zuluaga et al., 2015). This type of lactic acid fermentation is similar to that in yoghurts (and other fermented milk products) and renders the end product more digestible and enriched with new nutrients (Krell, 1996). The process of bee bread formation starts with gathering of pollen, then a bee mixes it with flower nectar or honey and saliva, and carries to the beehive, where non flying bees fill the mixture into honeycomb cells for ¾ of the cell volume. Residual cell volume is filled with honey, thus protecting the pollen mass from oxygen. An anaerobic lactic fermentation process takes place and bee bread is forming. Bee bread differs from pollen by lower pH (3.8 – 4.3), it contains less proteins and fats, but more carbohydrates and lactic acid. Bee bread has a better bioavailability because the walls of pollen, which cannot be destructed by gastrointestinal liquids, have been partly destructed by fermentation and the functionally and energetically rich content of pollen can be assimilated and used easier (Mizrahi and Lensky, 1997; Fatrcová-Šramková et al., 2010). A proper hive management

promotes bee-bread collection, aimed at marketing it for human consumption since it can be considered as food supplement due to its content of a wide range of nutrients. One of the contributions to their high nutritional value is the presence of significant amounts of proteins, vitamins and phenolic compounds as natural antioxidants. The potential application of "bee bread" as a food and as a nutraceutical supplement depends in large part on its chemical composition which varies directly with the flora of the region and the time of collection by the bees (Čeksterytė et al., 2008). Bee bread differs from pollen by lower pH (3.8 – 4.3), it contains less proteins and fats, but more carbohydrates and lactic acid. Bee bread has a better bioavailability because the walls of pollen, which cannot be destructed by gastrointestinal liquids, have been partly destructed by fermentation and the functionally and energetically rich content of pollen can be assimilated and used easier (Mizrahi and Lensky, 1997). Bee bread has antimicrobial, antioxidant hepatoprotective, immunomodulating and antiradiation activity, adaptogenic properties. It stimulates protective forces of a human body, normalizes metabolism, has a positive influence on the liver, nervous and endocrine system functions, and enhances regeneration of tissues, physical and mental persistence of a human body (Bogdanov, 2015).

The aim of study was to determine biological activity of selected bee bread samples – antioxidant activity, total polyphenols and flavonoids content. Secondary was also to determine antimicrobial characteristic of these samples.

MATERIAL AND METHODOLOGY

Biological material

Bee bread was obtained from selected region of Ukraine (Poltava oblast, Kirovohrad oblast, Vinnica oblast, Kyiv oblast, Dnepropetrovsk oblast), by patent technology developed by research teams Department of beekeeping, National University of Life and Environmental Sciences of Ukraine, Kyiv. Before the measurement samples were crushed to the powder using mortar and store at 4°C in refrigerator.

Chemicals

All chemicals were analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

Sample preparation

0.1 g of bee bread was extracted with 20 mL of 80% ethanol for 2 hours. After centrifugation at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min, the supernatant was used for measurement (antioxidant activity, polyphenols, flavonoids).

Antioxidant activity

Radical scavenging activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sánchez-Moreno et al., 1998). The extracts (0.5 mL) were mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). Absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10-100 mg.L⁻¹; R² = 0.988) was used as the standard and the results were expressed in mg.g⁻¹ Trolox equivalents.

Reducing power

Reducing power of samples was determined by the phosphomolybdenum method of Prieto et al., (1999) with slight modifications. The mixture of sample extract (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90°C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10-1000 mg.L⁻¹; R²=0.998) was used as the standard and the results were expressed in mg.g⁻¹Trolox equivalents.

Total polyphenol content

Total polyphenol content of potato extracts was measured by the method of Singleton and Rossi, (1965) using Folin-Ciocalteu reagent. 0.1 mL of each sample extract was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 min. in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25-250 mg.L⁻¹; R²=0.996)

was used as the standard and the results were expressed in mg.g⁻¹ gallic acid equivalents.

Total flavonoid content

Total flavonoids were determined using the modified method of (Willett, 2002). 0.5 mL of sample extract was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 ml of 1 M sodium acetate and 4.3 mL of distilled water. After 30 min. in darkness the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (0.01 – 0.5 mg.L⁻¹; R² = 0.997) was used as the standard and the results were expressed in µg.g⁻¹ quercetin equivalents.

Antimicrobial activity

Microbial strains

Four strains of microorganisms were tested in this study, including two Gram-negative bacteria (*Escherichia coli* CCM 3988, *Salmonella enterica* subs. *enterica* CCM 3807), two Gram-positive bacteria (*Bacillus thuringiensis* CCM 19, *Staphylococcus aureus* subs. *aureus* CCM 4223). All tested strains were collected from the Czech Collection of microorganisms. The bacterial suspensions were cultured in the nutrient broth (Imuna, Slovakia) at 37 °C.

Disc diffusion method

Antimicrobial activity of each bee bred extract was determined by a disc diffusion method. Briefly, 100 µL of the test bacteria were grown in 10 mL of fresh media until they reached a count of approximately 10⁵ cells.mL⁻¹. Then 100 µL of the microbial suspension was spread onto Mueller Hinton agar plates. The extracts were tested using 6 mm sterilized filter paper discs. The diameters of the inhibition zones were measured in millimeters. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in at least triplicate. Filter discs impregnated with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentrations (MICs)

MICs were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation (CLSI, 2014) in Mueller Hinton broth (Bioline, Italy). Briefly, the DMSO plant extracts solutions were prepared as serial two-fold dilutions obtaining a final concentration ranging between 0.5-2048 µg.mL⁻¹. After that each well was inoculated with microbial suspension at the final density of 0.5 McFarland. After 24 h of incubation at 37 °C, the inhibition of microbial growth was evaluated by measuring the well absorbance at 450 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 microwell plates were measured before and after experiment. Differences between both measurements were evaluated as growth. Measurement error was established for 0.05 values of absorbance. Wells without plant extracts were used as negative controls of growth. Pure DMSO was used as negative control. This experiment was done in eight-replicates for a higher accuracy of the MICs of used medical plant extracts.

Statistical analysis

The basic statistical analyzes were realized in SAS programming packages (THE SAS SYSTEM V 9.2.). Correlation coefficients were calculated by CORR analysis (SAS, 2009).

RESULTS AND DISCUSSION

Antioxidant activity

In the DPPH radical-scavenging method, a compound with high antioxidant potential effectively traps the radical, thereby preventing its propagation and the resultant chain reaction (Brand-Williams et al., 1995). DPPH is a stable free radical that is dissolved in ethanol and its purple color shows a characteristic absorption at 515 nm. Antioxidant molecules scavenge the radical by hydrogen donation and the colour from the DPPH assay solution becomes light yellow resulting in a decrease in absorbance (Silva et al., 2012). As shown Fig. 1 all tested samples had effect to trap DPPH radical, with the best value in bee bred from Poltava oblast (15.78 mg TEAC.g⁻¹) and Vinnica oblast (14.62 mg TEAC.g⁻¹). High antioxidant activity also reported (Zuluaga et al., 2015), which evaluated polyfloral Colombian bee bread with ABTS method; values from their study range from 46.1 to 76.3 μmol Trolox/g. In spite of the relevance of bee bread as an antioxidant substance, there is not enough systematic information about the antioxidant activity and profile of bioactive compounds of bee bread.

Phosphomolybdenum method is used to measure the reductive ability of antioxidant, and it is evaluated by the transformation of Mo(VI) to Mo(V) where, the ability of samples to reduce Mo may be attributed from hydrogen donation from phenolic compounds which is also related to presence of reducing agent (Huda-Faujan et al., 2009). The reducing ability of the bee breads (Fig. 1) was in the order: bee bread from Poltava oblast > bee bread from Kyiv oblast > bee bread from Vinnica oblast > bee bread from Kirovohrad oblast > Dnepropetrovs oblast. Similar like DPPH method, the best values were determined in sample from Poltava region. Barros et al., (2007) demonstrated that the reducing properties are generally associated with the presence of reductones, which had been shown to exert antioxidant action by breaking the free radical chain by donating the bread hydrogen atom. Higher level of polyphenols in bee bread could act as reductone where these compounds could react with free radicals by converting them to more stable products and terminating the radical chain reaction (Oh et al., 2013). Siddiqui et al., (2012) claimed antioxidants chelate and disengage transition metals, thereby preventing such metals from participating in the initiation of lipid peroxidation and oxidative stress through metal catalyzed reaction.

On the basis of the above findings, bee bread seems to be attractive as an important source of antioxidants for the food and pharmaceutical industries. The differences observed between the antioxidant activities of the tested samples may be attributed to the presence of natural antioxidants, mainly phenolic compounds that differed depending on the region where they were collected (Sati et al., 2013; Tlili et al., 2014).

Total polyphenol and flavonoid content

Phenolic compounds are considered among the largest contributors to the antioxidant potential of natural food products. Total polyphenol content (Table 1) in bee-bread ranged from 12.36 to 25.4 mg GAE.g⁻¹. The highest value was observed in sample from Poltava region. Nagai et al., (2004) also determined high level of total polyphenols in bee bread and also reported that bee bread can be applied more as health food and medicine. Zuluaga et al., (2015) determined in Colombian bee bread values from 2.1 to 13.7 mg GAE.g⁻¹ of polyphenols. The information about spectrum of polyphenol compounds in bee bread is missing, but we can expect, that bee bread contains similar polyphenols like bee pollen. It is also potential, that and bee bread can contain new type of polyphenols. According to Fanali et al., (2013) in bee pollen, polyphenolic compounds are commonly glycosylated, esterified, present in free forms or combined with other pollen components. Bonvehi et al., (2001) reported that bee pollen is rich for gallic acid, vanillic, protocatechuic, *p*-coumaric acid, hesperidin, rutin, luteolin, apigenin, kaempferol, quercetin and isorhamnetin.

Total flavonoid content (Table 1) in observed samples of bee bread ranged from 13.56 to 18.24 μg QE.g⁻¹. The highest value, similarly like polyphenol content was observed in sample from Poltava region. Flavonoids are the secondary components of most importance in bee bread and influence the visual appearance of the grain (pigmentation) and flavour (astringency and bitterness) (DeGrandi-Hoffman et al., 2013). In pollen grains, most of flavonoids exist as glycosides, known as aglycones, being quercetin the major compound. Although there is not a recommended daily ingest for flavonoids, it is suggested an intake of about 200 – 100 mg per day. Zuluaga et al., (2015) determined total flavonoid content in Colombian bee bread from 1.9 to 4.5 mg QE.g⁻¹. It is very difficult determine average total flavonoid content in bee bread generally. Zuluaga et al., (2014) reported that bee pollen contains higher content of total flavonoids with compare to bee bread due to possible differences in botanical origin of pollen and also the fact that a degradation of the outer layer of the grain makes more available bioactive compounds to degrade by environmental conditions. These authors also published that in Colombian region was established average content of flavonoids in 5.16 mg.g⁻¹ (QE) of bee pollen. The separation of the individual polyphenols and flavonoids and detection of the other antioxidants will be necessary for evaluate of biological activity of bee bread in future.

Antimicrobial activity

Bee bread samples showed a potential activity against the growth of both gram positive and gram negative bacteria which was resistant to antibiotics. This would be a very interesting approach to control more dangerous species of micro-organism in medical sciences. Because of the development of resistance by the microorganisms to common antibiotics, it has become necessary to search for an alternative approach dealing with this situation. It had been suggested that natural products are preferable to synthetic ones (Abouda et al., 2011).

Results of antibacterial testing with disc diffusion method (Figure 2) showed that higher antibacterial activity was

found against *Bacillus thuringiensis* in sample from Vinnica oblast, Kyiv oblast and Dnepropetrovsk oblast. The higher inhibition zone was found in sample from Kirovohrad oblast against bacteria *Escherichia coli*. The higher antimicrobial activity against *Salmonella enterica* subs. *enterica* was found in sample from Kyiv oblast. Samples of natural bee-bread from different aromatic and medicinal plants were studied for their antimicrobial activities on antibio-resistant bacterial strains isolated from human pathology. Four samples of bee-bread were collected from different regions in Morocco. Dilutions of bee-bread from 1/2, 1/4, 1/8 and 1/16 were tested by the agar well diffusion method on various strains of bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa*. Results revealed that most of strains were inhibited by the dilution 1/2 and 1/4. The gram positive bacteria were more sensitive to bee-bread and bee-pollen than gram negative bacteria. All the samples showed strong antimicrobial activities on the bacterial strains, which were first tested for their resistance to antibiotics (Abouda et al., 2011). The best antimicrobial activity (Tab. 2) MIC50 was found in sample from Poltava region where minimal inhibition concentration ($6.40 \mu\text{g}\cdot\text{mL}^{-1}$) against gram negative bacteria; very good antibacterial activity were also found in same sample against bacteria in MIC90 ($6.40 \mu\text{g}\cdot\text{mL}^{-1}$). In generally all tested samples against all tested bacteria

had antibacterial influence.

Statistical analysis

Using Pearson correlation coefficients was verified correlation (Table 3) between antioxidant activity determined by DPPH and phosphomolybdenum method and total polyphenol and flavonoid content. The strong correlation dependence (0.95) was found between antioxidant activity (DPPH) and polyphenol content and also between flavonoid content and antioxidant activity (phosphomolybdenum method) (0.89). Between two different methods for determining the antioxidant activity, was determined the mean linear relationship (0.54). Based on these results, it can be concluded that polyphenols and flavonoids have a strong impact on the antioxidant activity of bee bread.

CONCLUSION

In conclusion, the results of this study demonstrate that bee bread is very good source of bioactive compounds not only with antioxidant but also antimicrobial effect. The best results were observed in most of parameters in sample from Poltava oblast. Bee bread can be use more in future not only in medicine, pharmacy but also in food industry. For confirmation of biologically effect is necessary more and intensive study, *in vivo* test for evaluating bioactive components and digestibility properties; very important is also determining some negative compounds which can

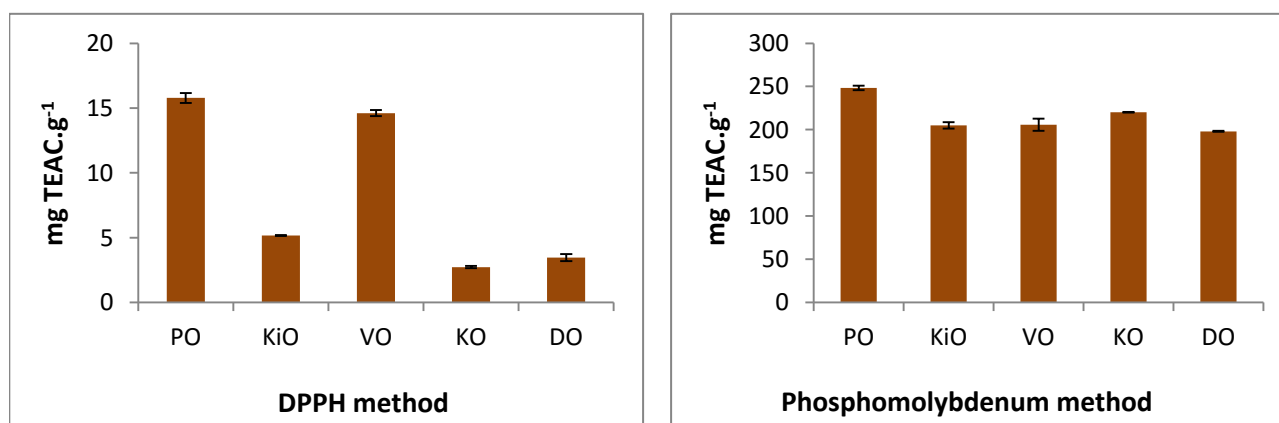


Figure 1 Radical scavenging activity and reducing power of bee bread (TEAC – Trolox equivalent antioxidant capacity PO – Poltava oblast, KiO – Kirovohrad oblast, VO – Vinnica oblast, KO – Kyiv oblast, DO – Dnepropetrovsk oblast.

Table 1 Total polyphenol and flavonoid content in bee bread.

Sample	Total polyphenol content (mg GAE.g ⁻¹)	Total flavonoid content (μg QE.g ⁻¹)
Poltava oblast	25.44 ±0.22	18.24 ±0.08
Kirovohrad oblast	19.96 ±0.59	15.25 ±0.04
Vinnica oblast	20.88 ±0.34	13.56 ±0.04
Kyiv oblast	12.36 ±0.34	15.35 ±0.09
Dnepropetrovsk oblast	13.47 ±0.56	14.04 ±0.03

Note: GAE – gallic acid equivalent; QE – quercetin equivalent; ± standard deviation.

Table 2 The antimicrobial activity of bee bread (MIC, µg.mL⁻¹)

	PO		KiO		VO		KO		DO	
	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
<i>Escherichia coli</i>	6.40	6.84	12.81	13.64	12.81	13.64	12.81	13.64	12.81	13.64
<i>Salmonella enterica</i>	6.40	6.84	12.81	13.64	12.81	13.64	12.81	13.64	8.53	9.54
<i>Staphylococcus aureus</i>	12.81	13.64	12.81	13.64	17.07	19.08	25.58	27.20	12.81	13.64
<i>Bacillus thuringiensis</i>	12.81	13.64	12.81	13.64	12.81	13.64	25.58	27.20	17.07	19.08

Note: PO – Poltava oblast, KiO – Kirovohrad oblast, VO – Vinnica oblast, KO – Kyiv oblast, DO – Dnepropetrovsk oblast; ± standard deviation.

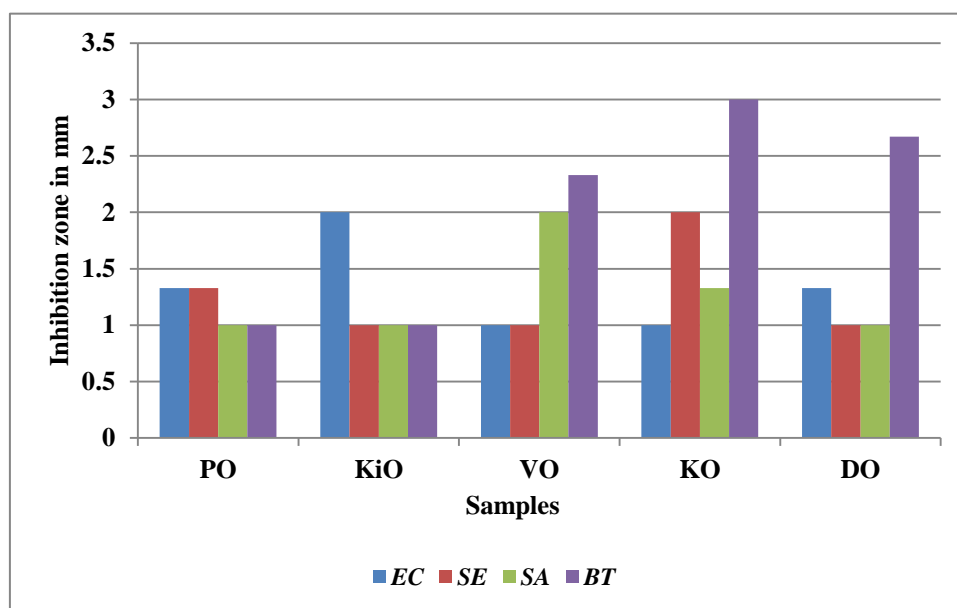


Figure 2 Antimicrobial activity of bee bread against bacteria.

Note: (EC-*Escherichia coli* CCM 3988, SE-*Salmonella enterica* subs. *enterica* CCM 3807, BT-*Bacillus thuringiensis* CCM, SA- *Staphylococcus aureus* subs. *aureus* CCM 4223); PO – Poltava oblast, KiO – Kirovohrad oblast, VO – Vinnica oblast, KO – Kyiv oblast, DO – Dnepropetrovsk oblast.

Table 3 Results of correlation analysis

Sign/Marker	Phosphomolybdenum method	Polyphenol content	Flavonoids content
DPPH method	0.54*	0.95***	0.35
Phosphomolybdenum method		0.63*	0.89***
Polyphenol content			0.54*

$p \leq 0,001$ ***; $\leq 0,05$ *; $> 0,05$ -

decrease the quality of bee bread (heavy metal, radionuclide, and microbes). Results in this work can be an important tool for recognizing bee bread as being a beneficial source of natural nutrients.

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