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EVALUATION OF INHIBITORY MEASURES FOR FOOD SPOILER YEAST *CANDIDA KRUSEI* DURING FERMENTATION PROCESS BY CHEMICAL, BIOCHEMICAL AND NANOPARTICLE APPROACHES

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ABSTRACT

Screening of chemical, biochemical and biomolecule-nanoparticle methods for the inhibition of *Candida krusei* were evaluated without hampering the growth of dairy yeast *Kluyveromyces marxianus*. The effective inhibition was observed with the help of H_2O_2, *Williopsis saturnus*, at specific combination of pH and temperature (pH 5.0 and 40 °C) and Ag-KT4561 nanoparticles among the various methods used. However, the most efficient inhibition was observed with Ag-KT4561 nanoparticles. In general H_2O_2 works best at pH range 4.0 to 10.0 and at temperature 30 °C or above. H_2O_2 concentration of 4000 ppm at 45 °C and pH 5.5 exhibited significant inhibition of *C. krusei*, while *K. marxianus* remains unaffected. But, when used with lyophilized supernatant of *W. saturnus*, 2400 ppm H_2O_2 was effective. Further, nanoparticle with silver was synthesized to reduce the quantity of killer protein and enhance the efficiency of protein. Complete inhibition of *C. krusei* was observed at 350 μM of synthesized silver nano-particle (AgNPs) of the killer protein from *W. saturnus*, with little effect on *K. marxianus* concentration. A stability test confirms the effect of protein silver nanoparticles on *C. krusei* for more than 20 weeks without any change in pH and temperature. Thus, the nanoparticles could be potentially used for inhibition of *C. krusei* without affecting the growth of *K. marxianus* and the process could be run non-aseptically.

Keywords: H_2O_2, Spoiler yeast, Inhibition, Killer protein, Ag-KT4561 bio-molecule nanoparticles, Green chemistry

INTRODUCTION

Cheese whey (a byproduct of cheese processing industries) has been efficiently exploited for the production of single cell protein (SCP) over the years as cheese whey has an immense nutritional value **(Ayoola *et al.*, 2008; Carvalho *et al.*, 2013)**. It contains 4.5-5% (w/v) of lactose, 0.6-0.8% (w/v) of soluble proteins, 0.4-0.5% (w/v) of lipids and 8-10% (w/v) of mineral salts of the dried extract. Efficient utilization of cheese whey for SCP conversion reduces the biochemical oxygen demand (BOD) by 75% and thus decreases the disposal problem **(Eyster, 1950; Prazeres *et al.*, 2012)**. Mostly lactose-consuming organisms, such as *Kluyveroymyces* spp. and *Lactobacillus* spp. grow in cheese whey **(Koleva *et al.*, 2008; Orru *et al.*, 2010)**. Production of SCP from cheese whey serves dual purpose by reducing environmental pollution and generating a valuable product (i.e. proteinaceous biomass which is used as animal feed and food ingredients) **(Koleva *et al.*, 2008)**. Likewise, *Kluyveroymyces marxianus* has been grown in cheese whey as a mono-culture for SCP production **(Yadav *et al.*, 2012)**. However, on a large scale industrial process, contamination is a major problem for SCP production despite the treatment of large volume of cheese whey. To eliminate this problem, certain extreme fermentation parameters have been applied, such as low pH (3.0-4.0) and high temperature (40-45 °C). At these conditions, most of the pathogenic microorganisms cannot survive **(Maneesri and Maneesri, 2009; Ahariz *et al.*, 2010)** and therefore a safe food or feed product is ensured during fermentation process. Additionally, the extreme fermentation conditions help to make the process economical due to reduced operating cost of maintaining sterility.

However, certain food spoilers (contaminant) e.g. *Candida krusei* still survive under extreme conditions (pH 3.0 and 45 °C) **(Guo and Bhattacharjee, 2006)**. The opportunistic *Candida* species exist as commensal in healthy individuals **(Heard and Fleet, 1988)**. During the production of SCP, *C. krusei* emerges as a contaminant while it grows along with *K. marxianus* and this is a concern for food safety. *C. krusei* is known as a food contaminant and an opportunistic pathogen **(Siso, 1996; Hornbæk *et al.*, 2006; Maneesri and Maneesri, 2007; Kim and Lee, 2012)**. However, *C. krusei* is reported to be present in many dairy and fermented food products, but yet does not come under generally recognized as safe (GRAS) microorganism **(Walker and Dijck, 2006; Walker *et al.*, 2008)**. Therefore, the eradication of *C. krusei* is essential from food products to meet safety regulations.

Certain chemical and biochemical approaches were reported to employ for selective inhibition of *C. krusei*. The chemical (NaCl, H_2O_2) and the biological inhibitors (medicinal plants, such as *Lupinus angustifolis, Syzigium aromaticum* (clove) oil; nisin and *Williopsis saturnus* and synergistic effect of *W. saturnus* and H_2O_2) to inhibit *C. krusei* have been reported **(Ayoola *et al.*, 2008; Dingman, 2008; Adeniyi *et al.*, 2010; Da Silva *et al.*, 2011)**. It was reported that H_2O_2 inhibited *C. krusei* **(Morgulis *et al.*, 1926)**. Apparently, *C. krusei* has also been tested against a wide range of essential oils where ethanol 70% v/v served as control **(Nel *et al.*, 2006; Souza *et al.*, 2008; Waema *et al.*, 2009)**. Another significant approach to inhibit *C. krusei* was using NaCl; however it depends on the sensitivity of the organism and the concentration of NaCl used. The sensitive strain of *C. krusei* undergoes cell death at 2 M concentration of NaCl **(Aguiar and Lucas, 2000)**.

Yeasts such as *Aspergillus furnigatus*, *W. saturnus* (major yeast from yogurt) have the capacity to produce killer proteins **(Fang *et al.*, 2002; Brock, 2008)**. The mycotoxins/killer proteins produced by *W. saturnus* have a broad spectrum of inhibitory activity at wide range of pH and temperature **(Buzzini *et al.*, 2004)**. These could be used as the versatile anti-spoilage agents for food and feed production **(Kao *et al.*, 1999; Liu *et al.*, 2006)**. Another killer protein is nisin, which is used for food preservation and is produced by *Lactobacillus* spp. or lactic acid bacteria (LAB) **(Guwy *et al.*, 1999)**. Nisin is used to stabilize food products and is often added to the cheese for inhibiting toxin production by *Clostridium botulinum*. It was also reported to inhibit *C. krusei* efficiently **(Lowes *et al.*, 2000; Russell and Jarvis, 2001)**.

In certain industrial fermentation processes, stress of pH shock was encountered to inhibit certain food spoiler yeasts **(Siso, 1996; Pinheiro *et al.*, 2002)**. At pH 2.0, *C. krusei* did not grow well **(Lowes *et al.*, 2000)**. In a mixed culture, where the presence of other yeast strains was also reported, effective utilization of any inhibitor (i.e. chemical or biochemical inhibitor against *C. krusei*) depends entirely upon whether the other type of yeast was also inhibited by the specific inhibitor being used. Another effective way of inhibition of pathogens is by the usage of metal nanoparticles (NPs) or biomolecule based nanoparticles **(Dingman, 2008)**. It has already been observed that silver NPs can kill pathogens

at very low concentrations and biomolecule based nanoparticles do not exert any toxic effects on human cells. Apart from that, silver NPs do not cause any microbial resistance and also there is no specific site of action for inhibition of the microbial cells **(Panacek *et al*., 2009)**. Hence, the aim of the present study was to evaluate different inhibition methods to inhibit *C. krusei* alone as well as in a mixed culture system without affecting the growth of *K. marxianus*.

MATERIALS AND METHODS

Chemicals

Analytical grade chemicals were used in the experiments. NaCl (Quelab Lab Inc., Montréal, Canada), H_2O_2 (Laboratoire Mat, Québec), yeast Extract (Fisher Scientific, USA), malt extract (Oxoid Ltd., Basingstoke, England), meat peptone (Organotechnie SA., La Courneuve, France), glucose, ethyl alcohol 95% (Fisher Scientific, USA), agar (Quebact Lab Inc., Montréal, Canada), cheese whey (Agropur, Canada), and $AgNO_3$ (Fisher Scientific, Ottawa).

Microorganisms

K. marxianus strain used in the study was isolated and characterized from the SCP production plant using cheese whey as substrate. *C. krusei* strain was also isolated and identified as a contaminant during SCP production employing cheese whey. *W. saturnus* strain DBVPG 4561 was obtained from the Industrial Yeasts Collection DBVPG of Perugia (Italy). Strains were sub-cultured on YEPD (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) agar slants and stored at 4 ^oC for further use.

Inhibition studies for *C. krusei*

Chemical Methods

Inhibition by NaCl

Pre-culture broth of *K. marxianus* of $2.0x10^8$ CFU/mL and *C. krusei* of $4.0x10^7$ CFU/mL were prepared in 100 mL YEPD media in 500 mL Erlenmeyer flasks. The medium pH was adjusted to 3.5 and sterilized at 121 ^oC for 15 min. After sterilization, 1.5 M and 2 M NaCl were added in different sets of flasks. *C. krusei* is a non-lactose assimilating organism, while *K. marxianus* is a lactose assimilating organism. Therefore, sterilized YEPD media were inoculated with 30 µL (from stock culture) of *K. marxianus* and 50 µL of *C. krusei*. Inoculated flasks were incubated at 40 °C for 24 h. Samples were taken at regular intervals for the analysis of total cell count. Total cell concentration was measured using standard agar plate technique **(Logothetis *et al*., 2007; Goretti *et al*., 2009;Kosseva *et al*., 2009)**.

Inhibition by H_2O_2

A pre-culture was prepared for *C. krusei* and *K. marxianus* as above. After that, culture media of cheese whey powder 4.5% (w/v) and urea 0.22% (w/v) were prepared and pasteurized at 80 °C for 20 min. The pasteurized cheese whey culture media at different pH (3.5, 4.5, 5.5, and 6.0) was added to different 500 mL flasks and inoculated with 1% (v/v) inoculum of *C. krusei* and *K. marxianus*. Different concentrations of H_2O_2 (100, 200, 300 and 400 ppm) were added to these flasks. After inoculation, flasks were incubated at 28 °C and 40 °C in an orbital incubator shaker for 24 h. Samples were drawn at regular intervals to analyze the total cell count.

Simultaneously, two different set of experiments were conducted, where in the primary set of experiments the H_2O_2 concentration were varied (0, 300, 400, 500, 600 and 800 ppm) and applied directly on the fermenter broth containing *K. marxianus* and *C. krusei*, which was collected from commercial continuous SCP production plant. 100 mL of fermented broth of *K. marxinaus* ($3.0x10^6$ CFU/mL) severely contaminated with *C. krusei* ($1.8x10^6$ CFU/mL) was taken in 500 mL 2 sterilized flasks. Flasks were incubated at pH 3.5, 150 rpm and 40 °C in an incubator shaker.

In the secondary set of experiments, variation in H_2O_2 concentration (2400, 3200 and 4000 ppm) were considered and applied directly to the fermenter broth and flasks were incubated at pH 5.0, 150 rpm and 45 °C in an incubator shaker.

Biochemical Methods

Inhibition by S. aromaticum oil

A set of experiments were conducted in which 0.4% (v/v) of clove oil was added in fermenter broth which contains *C. krusei* and *K. marxianus*. The initial cell count of *C. krusei* and *K. marxianus* was $5.0x10^6$ CFU/mL and $6.0x10^6$ CFU/mL, respectively. The flasks were placed in an orbital incubator shaker at 28 °C at 150 rpm for 6 h. Sampling was performed at an interval of 2 h. Samples were analyzed for total cell count using standard agar plate technique.

Inhibition with nisin

The culture of *C. krusei* and *K. marxianus* were grown separately in MRS broth at 35 ^oC for 24 h. Bioassay MRS media with 0.75% of Bacto agar and 1% Tween-20 were prepared. Media were sterilized at 121 ^oC for 15 min. A solution of nisin (1,000 IU/mL) was prepared by adding 0.025 g of commercial nisin (Sigma-Aldrich, Milwaukee, USA) into 25 ml of sterile solution of 0.02 N HCl. Sterilized media were cooled down to 40 ^oC and inoculated with 1% (v/v) of the 24 h culture of *C. krusei and K. marxianus* in two sets (duplicate). Then the bioassay agar (25 mL) was aseptically poured into sterile petri dishes (100x15 mm) and allowed to solidify for 1 h. On each plate, four or five holes were bored, using a 7 mm outer diameter stainless steel borer with a slight suction. An aliquot (50 μL and 100 μL) of standard nisin solution was placed into a well and the bioassay agar plate was incubated right away at 35 ^oC for 24 h. The control for each plate was prepared using sterile distilled water in wells. Zone of inhibition was observed in control and test samples.

Inhibition study with *W. saturnus*

Preparation of W. saturnus culture broth

YEPD (100 mL) was prepared in 500 mL flasks and sterilized at 121 ^oC for 15 min. The sterilized flask was inoculated with loopful of *W. saturnus* and incubated in an orbital incubator shaker at 150 rpm and 28 ^oC for 48 h. Samples were taken at regular time intervals for total cell count.

Well assay method

Pre-culture of *C. krusei* was prepared in YEPD as described above using 1% (v/v) inoculum. After 24 h, *C. krusei* sample was diluted 10^2, 10^3 and 10^4 times in saline solution and different diluted samples were spread plated in YEPD agar plates. After spread plating, wells were made in agar plates using borer and 60 μL of *W. saturnus* (48 h) culture was added in each well. The plates were incubated in an orbital incubator at 28 ^oC for 24 h. The plates were visually observed after 24 h.

To differentiate the morphology of *C. krusei* from *W. saturnus,* Methylene Blue Citric-Phosphate agar (MBA) plates were prepared and spread plated using *C. krusei* and *W. saturnus*. Plates were incubated for 24 h at 28 ^oC and were visually examined to check the morphology.

Inhibition by W. saturnus

W. saturnus was grown in YEPD and cheese whey medium for 24 h. YEPD and cheese whey powder 4.5% (w/v) with 0.22% (w/v) urea were prepared in two flasks of 2 L capacity each containing 500 mL medium. After sterilization, each flask was inoculated with 2% (v/v) *W. saturnus* and incubated in an orbital incubator shaker at 150 rpm and 28 °C. The culture was harvested at 48 h. The culture broth was centrifuged at 10 000 x g and the supernatant was lyophilized to obtain the powder which contained extracellular proteins. The extracellular proteins specifically contain a particular protein KT4561 (~ 62 kDa protein), which has anti-mycotic activity **(Buzzini *et al*., 2004)**. Simultaneously, another set of flasks containing *W. saturnus* were grown, where no centrifugation was performed. Henceforth, the cultures were directly taken for lyophilization.

Pre-cultures were prepared by growing *C. krusei*, *K. marxianus* and *W. saturnus* in YEPD medium for 24 h. One hundred milliliters of fresh cheese whey powder 4.5% (w/v) with urea 0.22% (w/v) solution was added to each five hundred milliliters Erlenmeyer flask (two flasks) and pasteurized at 80 °C for 20 min. After pasteurization, media were aseptically adjusted to different pH (3.5 and 4.5) followed by inoculation with 1% (v/v) mixed culture (*C. krusei* and *K. marxianus*).

Inhibition by lyophilized supernatant from W. saturnus

Various concentrations of lyophilized supernatant of *W. saturnus* were considered (well plate assay method) and the zone was created by the inhibitory effect of the killer protein. The inhibition zones were measured after 24 h of incubation at 30 ºC. A linear equation (y = 0.30x-0.36) was sketched out between the diameter of the clear zone (measured in millimeters, x axis) and the logarithm of the quantity of the killer protein (measured in nanograms, y axis). This method was used to determine the killer protein concentration required for the inhibition of *C. krusei* which is similar to the technique mentioned in **(Chen *et al*., 2000)**. Lyophilized supernatant prepared in YEPD media was served as the control and lyophilized cheese whey was the experimental product.

Inhibition of C. krusei by synergistic effect of H_2O_2 and W. saturnus

To study inhibition of *C. krusei*, different H_2O_2 concentrations were used along with *W. saturnus* (entire organism lyophilized supernatant powder, as described

above). 300 ppm of H_2O_2 was used along with 1% (v/v) of *W. saturnus* (inoculum from pre-culture) for the inhibition of *C. krusei* in a mixed culture of *C. krusei* and *K. marxianus* at pH 6.0 and 28 °C. Similar sets of experiments were conducted with a variation in pH (3.5-4.5) at 28 °C.

Two different set of experiments were conducted, where in the primary set of experiments was conducted where lyophilized *W. saturnus* was used by varying the H_2O_2 concentration directly on the fermenter broth containing *K. marxianus* and *C. krusei*, collected from commercial continuous SCP production plant. 100 mL of fermented broth of *K. marxinaus* ($3.1x10^6$ CFU/mL) grossly contaminated with *C. krusei* ($1.5x10^6$ CFU/mL) was taken in 500 mL 2 sterilized flasks. The lyophilized powder of *W. saturnus* (200 mg/mL) along with different concentrations of H_2O_2 (2400 and 4000 ppm) was then added to each flask. Flasks were incubated at pH 5.0, 150 rpm and 40 °C in an incubator shaker.

Whereas in secondary set of experiments, about 400 mg/mL of lyophilized supernatant of *W. saturnus* was used along with 2400 ppm of H_2O_2 in a mixed culture by adjusting the pH of fermenter broth to 5.0. As the killer protein produced by lyophilized *W. saturnus* is highly effective at pH range of 4.5-10.0 and temperature from 25 to 45 °C **(Goretti *et al*., 2009)**. Flasks were kept at 150 rpm and 40 °C in an orbital incubator shaker.

Inhibition of *C. krusei* by Ag-KT4561 NPs

Synthesis of nanoparticles (Ag-KT4561) was carried out in the previous study **(Bhattacharya *et al*., 2015)**. However a bulk preparation of the same has been conducted in this study. During the scale-up process, 20 mL of 0.1 M $AgNO_3$ solution is continuously stirred along with 18 mL of *W. saturnus* supernatant at 25 °C for 48 h. Ag^+ ions were completely reduced at 48 h of stirring. After which the bulk nanoparticle solution was taken for lyophilization and the lyophilized product was tried against *C. krusei* in 4.5% (w/v) cheese whey and 0.22% (w/v) of urea. From the lyophilized product different concentration of Ag-KT4561 ranging from 10 µM - 1 mM were tried at pH 5.5 and 30 °C in shake flasks. In these experiments, mixed culture of *C. krusei* (2% (v/v)) and *K. marxianus* (2% (v/v)) were tested for 12 h and total cell concentration (CFU/mL) was measured at 3 h time intervals.

Analytical methods

Cell count

Total cell count as CFU (colony forming units) was estimated by standard agar plate technique in YEPD agar plates **(Nathan *et al*., 1978)**. The appropriately diluted samples were plated on agar plates and incubated at 30 °C overnight to form fully developed colonies. The colonies of *K. marxianus*, *C. krusei* and *W. saturnus* were identified based on its morphology by visible examination.

Protein estimation

The soluble protein concentration was determined by Lowry's method **(Lowry *et al*., 1951)** using bovine serum albumin as standard.

UV-Vis Spectroscopy

The bulk sample of $AgNO_3$ and *W. saturnus* supernatant were prepared at 48 h and samples were collected at every 6 h to analyze for nanoparticle formation at 300-700 nm in Spectrophotometer (Carry 100 Bio®, Varian USA).

Statistical method

For each set, samples were analyzed in triplicates and mean values are taken in account. Further standard deviation of the colonies in log units (Log_{10} CFU/mL) were calculated for each experimental point in Microsoft excel 2013 external package similar to the method of **De Oliveira *et al*., 2014**.

RESULTS

Inhibition by NaCl

Different concentrations (1.5- 2 M) of NaCl were tested to inhibit the growth of *C. krusei* in mono-culture and the results were presented in Figure 1. It was clear that NaCl concentration of 2 M showed significant inhibition of *C. krusei* compared to 1.5 M without having any effect on *K. maxianus*. This is due to the fact that *C. krusei* exhibited salt-stress **(Aguiar and Lucas, 2000)** and got killed at pH 3.5 and 40 °C. No inhibition of *C. krusei* was observed when NaCl concentration was less than 2 M at pH 3.5 and 40 °C.

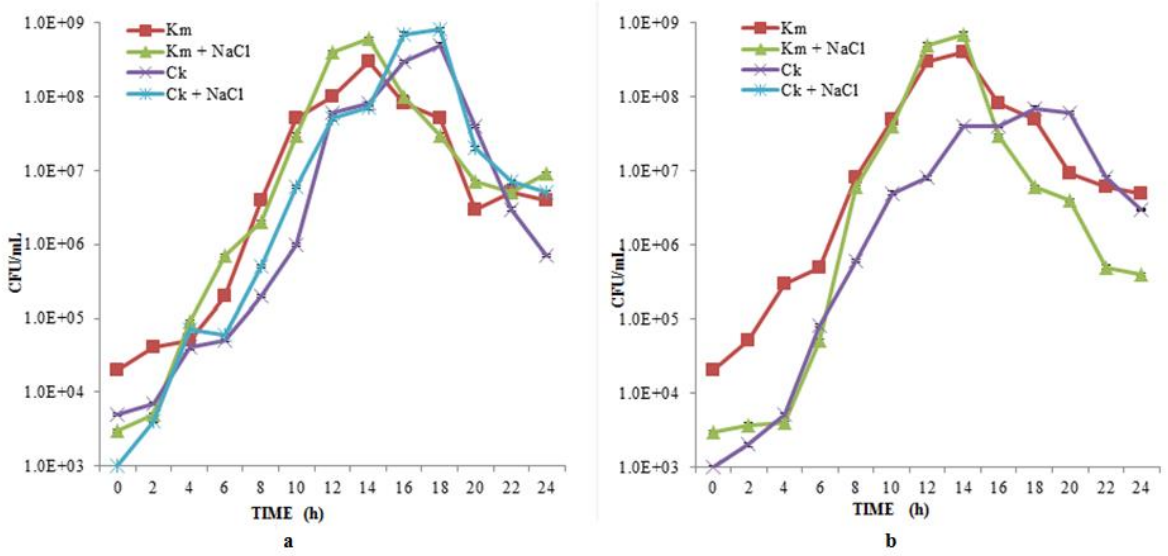

Figure 1 Impact of **a)**1.5 M NaCl and **b)** 2.0 M on *C. krusei* (Ck) and *K. marxinaus* (Km) in YEPD medium at pH 3.5 and 40 °C (Shake flask experiments)

Inhibition by H_2O_2

Viability of individual cultures of *C. krusei,* and *K. marxianus* at different concentrations of H_2O_2 (100 - 400 ppm) in cheese whey at pH 6.0 and 28 °C was studied (Table 1). It showed that *C. krusei* was not inhibited at lower concentration of H_2O_2. However, inhibition occurred at 300 ppm H_2O_2. *K. marxianus* was not inhibited at these concentrations of H_2O_2.

Table 1 Impact of various concentrations of H_2O_2 on *C. krusei* and *K. marxianus* in cheese whey powder at pH 6.0 and temperature 28 °C (Shake flask)

	Individual Organisms **(Log $_{10}$ CFU/mL) ± Standard Deviation**						
H_2O_2 (ppm)	**0**		**100**	**200**	**300**	**400**	
Time (h)	**Ck**	**Km**	**Ck**	**Ck**	**Ck**	**Ck**	**Km**
0	5.73±.07	4.83±.01	5.81±.01	5.81±.01	5.92±.01	5.15±.03	5.81±.02
12	6.52±.02	9.33±.01	6.08±.03	5.23±.01	5.40±.04	NG	9.26±.02
24	8.51±.02	8.31±.01	6.12±.05	5.18±.05	NG	NG	8.28±.02

Legend: NG- No Growth Observed, Ck – *C. krusei*, Km – *K. marxianus*

The inhibition of *C. krusei* in mixed cultures (*C. krusei* and *K. marxianus*) in cheese whey was studied and the results were presented in Figure 2. The concentration of H_2O_2 used was 300 ppm and 400 ppm (from previous results in Table 1). *C. krusei* was not inhibited at 300 - 400 ppm of H_2O_2. On the contrary, *C. krusei* dominated over *K. marxianus* in a mixed culture at 24 h.

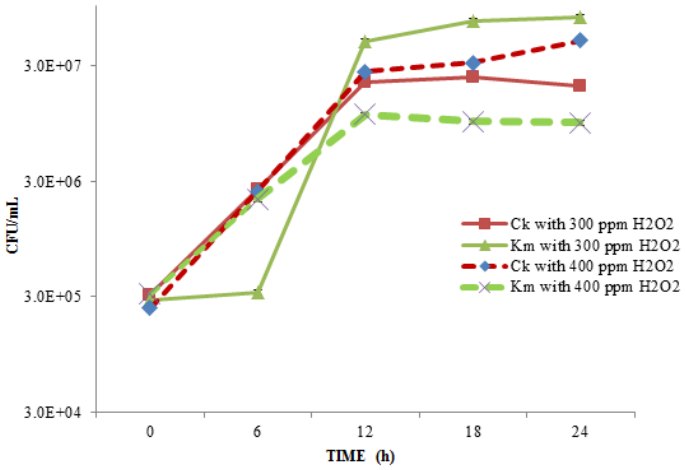

Figure 2 Impact of H_2O_2 on *C. krusei* (Ck), *K. marxianus* (Km) in cheese whey at pH 6.0 and 28 °C with 300 ppm and 400 ppm H_2O_2

Variations in pH along with 300 ppm H_2O_2 concentration

pH variations were carried out and it was lowered to 3.5 from 6.0 and studies of mixed culture (*C. krusei, K. marxianus* and *W. saturnus* 1% and 2% (v/v)) were also carried out maintaining similar parameters in cheese whey. *C. krusei* was not inhibited at these parameters, whereas growth of *K. marxianus* and *W. saturnus* remains unchanged.

A mixed culture study at pH 4.5 and 28 °C in cheese whey powder with 300 ppm H_2O_2 exhibited a partial inhibition of *C. krusei* (Figure 3a). However when bio-preservative *W. saturnus* was added at the similar condition, the growth of *C. krusei* decreased by one log-unit at 12 h (Figure 3b), but the growth accelerated after 12 h. As *W. saturnus* produces killer protein (KT4561) but the concentration of the killer protein remains low which is insufficient to inhibit *C. krusei* in a large-scale fermentation. Hence, this parameter could be considered for the inhibition of *C. krusei*.

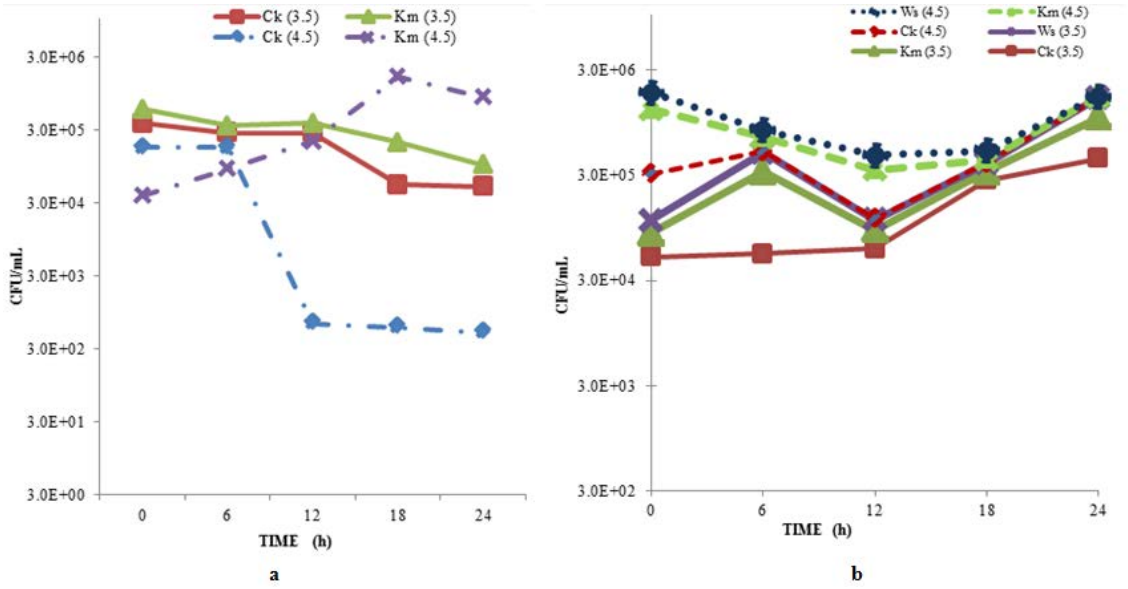

Figure 3 Impact of H_2O_2 and *W. saturnus* (Ws) on the mixed culture (*C. krusei* (Ck) and *K. marxianus* (Km)) in cheese whey at pH (3.5, 4.5) and 28 °C (Shake flask). **a)** With 300 ppm H_2O_2 only **b)** With 300 ppm H_2O_2 and 1 % (v/v) *W. saturnus*

C. krusei was efficiently inhibited in mixed cultures (*C. krusei* and *K. marxianus*) and (*C. krusei*, *K. marxianus* and *W. saturnus*) at pH 4.0, 28 °C and 400 ppm of H_2O_2. Lower CFU/mL of $1.7x10^3$ was observed for *K. marxianus* at pH 4.0 and 28 °C. On the contrary, when *C. krusei* was grown along with *K. marxianus* and *W. saturnus*, *K. marxianus* was observed at high CFU/mL of 2.4×10^3 (as compared to *C. krusei* when grown along with *K. marxianus*). *K. marxianus* showed remarkable growth at pH 5.5 and 40 °C rather than at other pH values, hence pH 4.5-5.5 was ideal for *K. marxianus*.

Higher ranges of H_2O_2 concentrations

After deducing the optimum amount of H_2O_2 used for the complete inhibition of *C. krusei*, similar concentration was applied for industrial scale fermenter broth to eliminate *C. krusei* without affecting the *K. marxianus*. So, 300 ppm of H_2O_2 was the optimum concentration for inhibiting *C. krusei* in the mixed culture in shake flask experiments. When concentration of H_2O_2 was increased from 300 to 800 ppm in the lab scale fermenter broth, no significant inhibition of *C. krusei* was observed at pH 3.5 and 40 °C (Table 2). *K. marxianus* degraded H_2O_2 at pH 3.5, making H_2O_2 ineffective for *C. krusei* inhibition **(Pinheiro *et al*., 2002)**. In fermented broth, higher concentration (2400 ppm, 3200 ppm, and 4000 ppm) of H_2O_2 was considered at pH 5.0 and 45 °C. Study was conducted for 6 h, as H_2O_2 got degraded into H_2O and O_2 after 6 h (Table 3). A very high concentration 4000 ppm of H_2O_2 finally could kill *C. krusei* completely in the fermented broth. Higher concentration of H_2O_2 was required due to simultaneous degradation of H_2O_2 by catalase action of *K. marxianus* **(Pinheiro *et al*., 2002)**.

Table 2 Impact of varying concentration of H_2O_2 on the mixed culture in the fermenter broth at pH 3.5 and 40 °C (Shake flask)

	H_2O_2 (ppm)											
	0		300		400		500		600		800	
	Individual Organisms (Log $_{10}$ CFU/mL) ± Standard Deviation											
Time (h)	Ck	Km	Ck	Km	Ck	Km	Ck	Km	Ck	Km	Ck	Km
0	7.88±.01	9.04±.02	8.26±.02	8.97±.02	8.42±.02	8.71±.01	7.17±.03	7.67±.01	6.02±.02	6.14±.05	6.17±.11	5.87±.02
3	7.91±.01	8.89±.01	8.34±.02	8.72±.01	8.12±.02	8.76±.01	6.71±.01	6.15±.05	6.47±.10	6.87±.01	5.34±.03	6.49±.02
6	8.18±.04	7.05±.01	8.18±.03	8.69±.01	7.96±.01	7.96±.01	6.85±.01	6.11±.03	6.04±.04	6.95±.04	5.36±.04	6.87±.02
9	7.78±.01	7.79±.01	8.28±.02	8.80±.01	8.18±.03	8.18±.03	6.77±.01	7.28±.03	6.85±.04	7.32±.07	6.04±.02	6.70±.08
12	7.18±.04	8.12±.06	8.80±.02	8.18±.02	8.32±.02	8.32±.02	6.72±.01	6.90±.01	8.25±.13	8.00±.02	6.47±.03	7.47±.02
24	8.40±.02	8.08±.09	8.45±.02	8.45±.03	8.42±.02	8.42±.02	8.04±.13	8.04±.02	8.45±.06	7.41±.07	8.41±.05	7.98±.02

Legend: Ck- *C. krusei*, Km – *K. marxianus*

Table 3 Impact of higher concentrations of H_2O_2 on the mixed culture in the fermenter broth at pH 5.0 and 45 °C (Shake flask)

Time (h)	H_2O_2 (ppm)					
	2400		3200		4000	
	Individual Organisms (Log $_{10}$ CFU/mL) ± Standard Deviation					
	Ck	Km	Ck	Km	Ck	Km
0	6.18±.04	6.50±.02	5.31±.02	6.31±.02	NG	5.18±.05
2	6.31±.01	6.58±.01	6.18±.03	6.42±.01	NG	5.31±.01
4	6.47±.02	6.57±.01	6.31±.01	6.48±.01	NG	5.31±.03
6	6.52±.01	6.81±.01	6.37±.02	6.54±.01	NG	5.39±.01

Legend: NG- No Growth, Ck – *C. krusei*, Km – *K. marxianus*

Inhibition by *S. aromaticum* oil

A study of the mixed culture (*C. krusei* and *K. marxianus*) at pH 3.5 and 28 °C along with various concentrations of clove oil was performed. It was observed that using clove oil concentration 0.5% (v/v) at pH 3.5 and 28 °C is ideal for *C. krusei* inhibition without affecting much the growth of *K. marxianus* ($1.6x10^7$ CFU/mL) in a mixed culture. However when concentration of clove oil was brought down to 0.4% (v/v) and was used in the fermented broth. *C. krusei* was inhibited at 0 h and *K. marxianus* ($1.7x\ 10^7$ CFU/mL) growth was unhampered at 6 h (Table 4). Clove oil 0.4% (v/v) at similar set of pH and temperature used above was ideal for *C. krusei* inhibition in a mixed culture. *Candida* are associated with infections as they form biofilms, *S. aromaticum* extracts worked against biofilm formation and thus, inhibit the growth of *C. krusei* **(Kim and Lee, 2012)**.

Table 4 The inhibition performed by using 0.4% (v/v) of clove oil at pH 3.5, 28°C in fermenter broth (100 mL)

Time (h)	Individual Organisms (Log $_{10}$ CFU/mL) ± Standard Deviation	
	Ck	Km
0	7.21±.03	6.78±.01
2	NG	6.91±.01
4	NG	7.08±.04
6	NG	7.26±.03

Legend: NG- No Growth Observed, Ck – *C. krusei*, Km – *K. marxinaus*

Inhibition by nisin

After 24 h of incubation the plates were observed and no yeast species were inhibited by nisin.

Inhibition by *W. saturnus*

A primary test was conducted to investigate the interaction between *W. saturnus* and *C. krusei,* along with *K. marxianus.* From the plate technique, it was concluded that *W. saturnus* could inhibit *C. krusei* but not *K. marxianus.* It is necessary to check whether *C. krusei* is an inducer for the production of the killer protein in *W. saturnus* or the latter naturally produces extracellular protein KT4561.

Usage of *W. saturnus* lyophilized powder

A minimum of 156 µg/mL of lyophilized protein in YEPD media is equivalent to 321.9 µg/mL of lyophilized protein in cheese whey needed for the inhibition of *C. krusei* (Table 5). *W. saturnus* did not show any effect below pH 4.5 and it grows well at 25-45 °C. Also *W. saturnus* grow well at pH 3.5 but failed to produce killer protein at the same pH.

Table 5 The inhibition zone created by the minimum concentration of the killer protein along with varying concentration from the lyophilized supernatant from *W. saturnus*

Media	Lyophilized supernatant concentration (mg/mL) of *W. saturnus*	Protein concentration (µg/mL) of killer protein	Inhibition zone formed by killer protein (cms) (Average + Standard Deviation)
Cheese whey	500	321	1.81±.03
	700	475	1.50±.08
Synthetic media (YEPD)	200	156	1.00±.01
	250	158	1.50±.02
	500	168	2.11±.03
	750	176	2.30±.02

Inhibition by synergistic effect of H_2O_2 and lyophilized *W. saturnus*/ supernatant from *W. saturnus*

4000 ppm of H_2O_2 could inhibit the growth of *C. krusei* in the fermented broth (mono-culture) obtained from continuous aerated fermentation (Table 3); and 200 mg/mL (killer protein concentration is 156 mg/L) was the concentration of lyophilized powder needed for the inhibition of *C. krusei* (obtained from Table 5). A synergistic effect of H_2O_2 and lyophilized powder of *W. saturnus* was studied. The set of experiments conducted at pH 5.0 and 40 °C, where 4000 ppm of H_2O_2 and 200 mg/mL of lyophilized *W. saturnus* powder was added. *W. saturnus* was highly effective in killing *C. krusei*, but in these set of experiments; such an inhibition did not occur because *W. saturnus* possesses peroxidase activity, which along with *K. marxianus* degraded H_2O_2 at a much faster rate than *K. marxianus* alone **(Buzzini *et al.*, 2004)**.

2400 ppm of H_2O_2 was considered along with 200 mg/mL of lyophilized supernatant of *W. saturnus* grown in cheese whey at pH 5.0 and 40 °C. At 24 h, cell concentration of *C. krusei* was reduced (Figure 4). By increasing the concentration of lyophilized supernatant of *W. saturnus* to 300 mg/mL, complete inhibition did not take place in a mixed culture. Simultaneously when 2400 ppm H_2O_2 and 400 mg/mL of lyophilized supernatant of *W. saturnus* was applied, H_2O_2 was degraded between 0-6 h because of the catalase-peroxidase enzymatic activity from *K. marxianus* and *W. saturnus*, but lyophilized supernatant of *W. saturnus* showed activity till 24 h. *C. krusei* (CFU/mL) lowered and showed drastic reduction in cell concentration at 24 h, whereas *K. marxianus* ($1.8x10^7$ CFU/mL) remained unaffected.

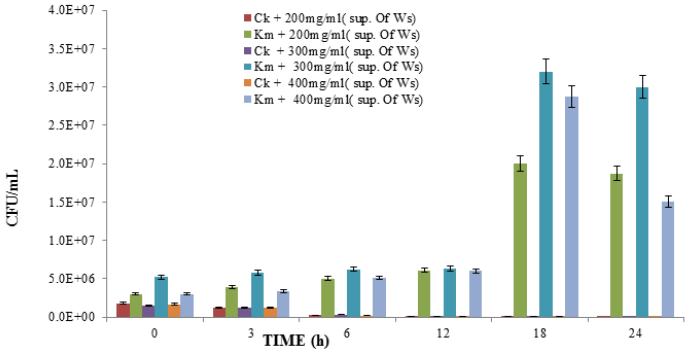

Figure 4 Impact of 2400 ppm H_2O_2 with 200 - 400 mg/mL (156 – 200 µg/mL killer protein) of lyophilized supernatant *W. saturnus* (Ws) powder on the mixed culture (*C. krusei* (Ck) and *K. marxianus* (Km)) in the fermenter broth at pH 5.0 and 40 °C (Shake flask)

Inhibition of *C. krusei* by Ag-KT4561 NPs

Higher concentration of the Ag-KT4561 was observed at 48 h than at 12 h (Figure 5a). Therefore NPs formed at 48 h were considered for this study. A concluding study of the mixed culture (*C. krusei* and *K. marxianus*) along with silver-KT4561 nanoparticles (Ag-KT4561NP) at pH 5.5 and 30 °C showed that 350 µM of Ag-KT461 could efficiently inhibit *C. krusei*. At concentration of 350 µM (Ag-KT4561), the conjugate consists of 1 ppm of reduced Ag.

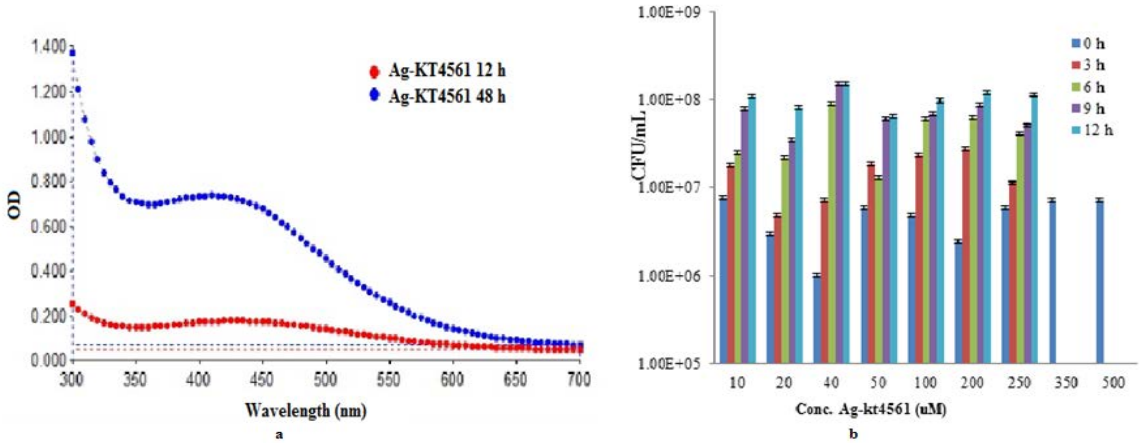

Figure 5 a) UV-VIS spectroscopy showed peak at 410 nm at 12 h and maximum at 48 h during bulk preparation of Ag-KT4561; **b)** A 12 h study of *C. krusei* when various concentrations of Ag-KT4561 was mixed with cheese whey

While growth curves of *K. marxianus* slightly decreases from $8.9x10^8$ to $2.6x10^8$ (Figure 5b). The decrease was less than a log-unit and this might be due to the presence of silver in the Ag-NPs. The other concentrations of the Ag-KT4561 used are as less as 10 µM and as maximum as 1 mM. In any food and feed grade products, a very high concentration of biomolecule based nanoparticle may be toxic for consumption but at a lower concentration of 350 µM (with 1 ppm of reduced silver ions), Ag-KT4561 is an efficient bio-preservative. Another effective approach to use biomolecule based nanoparticle is, no pH adjustment and no temperature adjustment is required. Ag^0 has anti-microbial effects against a wide range of pathogenic microorganisms and since Ag-KT4561 is a combination of killer protein from *W. saturnus* which specifically targets *C. krusei*. A synergistic effect of both (reduced Ag ion and killer protein) can kill *C. krusei* and *K. marxianus* remains partially affected (Figure 5b and 6a). A stability test of Ag-KT4561(350 uM) was performed on cheese whey till 12 h for 20 weeks and every time *C. krusei* was killed after being inoculated at 0 h and *K. marxianus* showed growth at a maximum of $2.3x10^8$ CFU/mL. Though *K. marxianus* growth was affected it did not perish away with the concentration of Ag in Ag-KT4561 (Figure 6b). A tabular representation (Table 6) shows the economics of bio-inhibitor (Ag-KT4561) production in a bulk amount of 20, 000L with a minimum of 1 ppm reduced silver ions.

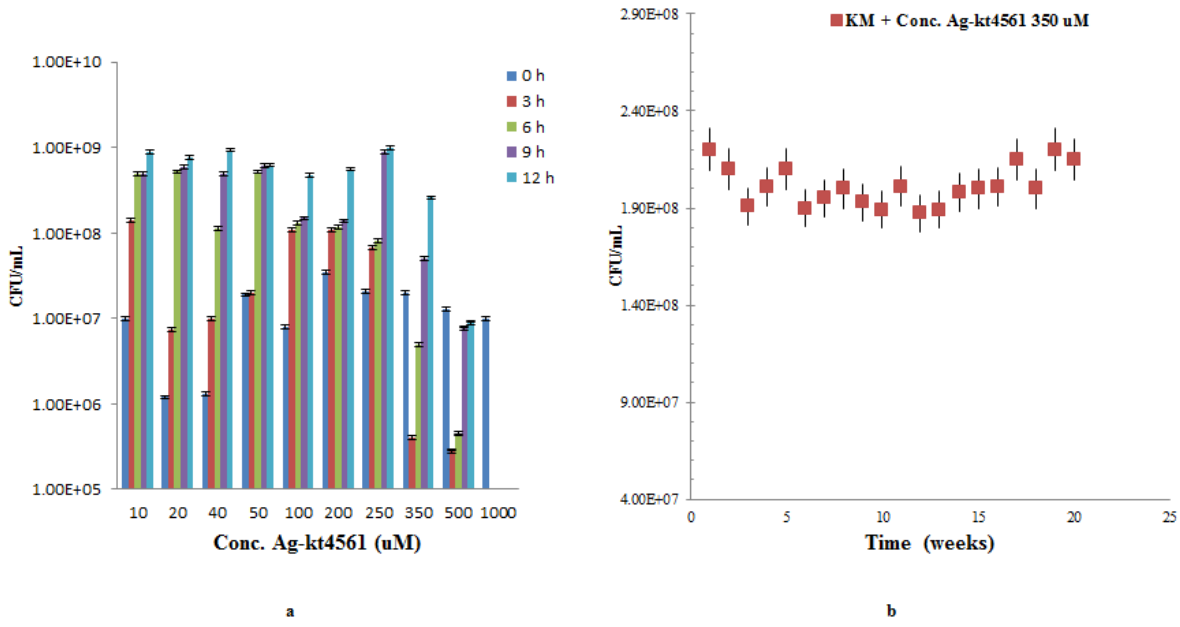

Figure 6 a) A 12 h study of *K. marxianus* when various concentrations of Ag-KT4561 was mixed with cheese whey; **b)** A stability test done for 20 weeks representing growth of *K. marxianus* (KM) at a minimum of 2.1 x 10^8 and no traces of *C. krusei*

Table 6 Bio preservative (Ag-KT4561 conjugate) production of 20,000 L

S. No	Items Required (amount)	Cost of Production (CAD $)
	Reagents and culture medium for stock	
1	Silver nitrate (271.6 g) for 40 L d.H_20	242
2	Culture medium of *W. saturnus* 360 L Preparation cost	306
3	Mechanical stirring of $AgNO_3$ reagent (36W Input power) for 48 h (~ 1.728 kwh)	12.19
4	Centrifugation (700W) for 15 mins (~ 0.175 kwh)	1.23
5	Freeze drying (1200W) for 24 h (~ 28 kwh)	197
	Total	760
6	10% cost of man-power	76

DISCUSSION

The results indicated that 2 M NaCl could effectively inhibit *C. krusei* in a monoculture of *K. marxianus* at temperature 40 °C, pH 3.5 without affecting the growth of *K. marxianus*. However, *C. krusei* showed lower NaCl tolerance than any other yeast species e.g. *Saccharomyces* had different sensitivity towards osmotic stress, but *C. krusei* was inhibited efficiently at 2 M without affecting the growth of *K. marxianus* **(Lynum and Nauth, 2000; Uchida *et al*., 2005)**. The reported concentration is used to discriminate *K. marxianus* as it is sensitive up to a concentration of 3 M NaCl. Stress-induced by salt induction results into two different phenomena, primarily, ion toxicity and secondly, osmotic stress. Apparently, other physiological changes can also take place such as: a) efflux of intracellular H_2O, i.e. total cell volume deduction; b) transient increase in glycolytic intermediates and finally triggering the hyper osmotic glycerol signaling pathway. Specific species, such as *Saccharomyces* and *Klyuveromyces* can develop systems to counteract to osmotic stress by NaCl. Special features of *Saccharomyces* and *Klyuveromyces* species are that they produce intracellular trehalose under stress conditions to maintain the membrane integrity and stabilizing the proteins **(Kuhn *et al*., 2004; Wang and Wu, 2008; Davey, 2011)**. However, in large-scale fermenters, it was not possible as it would lead to high utilization of NaCl for the inhibition of *C. krusei*. At industrial scale, such inhibitions performed by utilization of NaCl is difficult not because of the market price which is 16-20 USD per Kg; but the volume of NaCl required was more than 500 Kg for 40 000 L industrial reactor **(Goretti *et al*., 2009; Kosseva *et al*., 2009)**. Therefore, NaCl was not a suitable approach for *C. krusei* inhibition.

Apparently, H_2O_2 was found to be effective at 300 ppm when *C. krusei* inhibition was performed at shake flask level. In a shake flask study, it has been shown that 200 ppm could efficiently inhibit *C. krusei* **(Nel *et al*., 2006)**. Other yeasts, such as *W. saturnus* and *K. marxianus* have shown no inhibition in the presence of H_2O_2 as both the yeasts showed catalase activity. Maximum oxidative stress was observed in case of *Saccharomyces* spp. which was nearly 2-folds more than *K. marxianus* **(Kang *et al*., 2011)**. Every organism possesses specific antioxidant-defense systems. When *W. saturnus* was added along with *K. marxianus* and *C. krusei*, no inhibition of *C. krusei* was bound to happen as *W. saturnus* even possessed peroxidase activity **(Buzzini *et al*., 2004)**. Apart from catalase, when *K. marxianus* was introduced to H_2O_2 in the exponential phase, other enzymes, such as superoxide dismutase and glutathione reductase content were even increased to 2-fold **(Nilsson, 2011)**. These were specific antioxidant defensive agents present in *Klyuveromyces* spp. **(Meurman *et al*., 2007)**.

When pH was brought down from 6.0 to 3.5, it did not affect the inhibition of *C. krusei* either. However, catalase was widely active in a vast range of pH (3.5-10). Apparently, decomposition of peroxidase lowered the pH in the medium **(Pinto *et al*., 2009; Warnke *et al*., 2009; Guevara-Flores *et al*., 2010)**. While varying the pH, temperature was increased to 40 °C and *C. krusei* was efficiently inhibited. When it came to the effect of catalase activity on thermal capacity, 55 °C was the critical temperature and beyond which catalase enzyme was completely destroyed **(Erdei *et al*., 2011)**. The factors that shifted the physiological process of the glutathione reductases in *K. marxianus* showed higher pH of 6.8 along with temperature (37-40 °C) where it possesses more antioxidant activity **(Ghaly *et al*., 2005; Pinto *et al*., 2009)**.

In an aerated continuous fermenter, 4000 ppm of H_2O_2 was required without affecting the growth of *K. marxianus* and *W. saturnus* as both the microorganisms possessed catalase and peroxidase activity which efficiently degraded H_2O_2 during 6 h of inoculation. With such an extreme concentration of 4000 ppm of H_2O_2, *K. marxianus* still had the capacity to resist it. However when fermenter conditions were considered, it was well-stated that 100 ppm of H_2O_2 could cause corrosion of fermenter frame. The other catalysts for corrosion were O_2 and higher temperature **(Sathishkumar *et al*., 2010)**. Again, •OH, H_2O_2, O_2, H_2, •O_2 could interact with the surroundings; and therefore led to corrosive behavior of many materials including stainless steel **(McMahon *et al*., 2007; Siddique and Wahid, 2012)**. Though, H_2O_2 might be an inexpensive ingredient for *C. krusei* inhibition, fermenter inner body could have corrosive effects. Therefore, lower concentration of H_2O_2 might be an ideal approach. The use of chemicals depends entirely on the form of free radicals being produced and the damage they may or may not have on the fermenter.

Simultaneously, it was observed that 0.45% (v/v) clove oil could inhibit *C. krusei* without affecting other yeasts. The factor responsible for inhibiting the growth of *C. krusei* was eugenol. Eugenol is the component present in clove oil which can kill *C. krusei* at optimum concentrations **(Noori, 2012)**. *S. aromaticum* (clove oil) 0.4% (v/v) can efficiently bring down the concentration of *C. krusei* in a mono-culture of *K. marxianus*. However, on a large-scale fermentation, it would not be an approachable or economical aspect for inhibition. When the prices were compared, it is observed that 200 Kg of wholesale clove oil will cost USD 12 834.00, and definitely in large scale fermentations, the volume of clove oil required was 160 L, which will not only make the final product oily, but also very expensive. Therefore, it was not an ideal approach for *C. krusei* using clove oil. Similarly when nisin was used for the inhibition as it is one of the biochemical approaches, nisin could not inhibit *C. krusei* at all. Henceforth, the focus was shifted to *W. saturnus* killer protein.

W. saturnus was found to be an effective species which could kill *C. krusei* in a mono-culture of *K. marxianus* without having any implications on *K. marxianus*. Killer protein produced by KT4561 at a concentration of 200 mg/mL (where killer protein concentration is 156 ug/mL) when grown in glucose rich medium;

and 400 mg/mL when grown in a lactose-efficient medium (cheese whey) can inhibit *C. krusei*. The purpose of production of lyophilized supernatant from *W. saturnus* is to justify that *W. saturnus* produces naturally occurring extracellular killer protein KT4561, which strongly inhibited *C. krusei*. The killer protein produced by *W. saturnus* caused cell membrane damage and an independent energy link in between the cell wall receptor and KT4561 at the region of (1→6)-β-D-glucan complex **(Fang *et al*., 2002)**.

More efficient inhibition of *C. krusei* was possible if *W. saturnus* would have been grown in a glucose-rich medium where efficient production of the killer protein could have inhibited *C. krusei*. This study revealed a real understanding of the different microbial species dealt with and different behavioral patterns with respective to the varied inhibitors used for the inhibition of *C. krusei*. The factor to be considered when biochemical approaches such as H_2O_2 were used is whether it again had any effect on the organic matter present in cheese whey. As over the years, approaches have been made to protect the food and humans from consuming it against any oxidative damage. Free radicals such as hydroxyl, peroxyl, and superoxide have been bound to release when biochemical methods are used for inhibiting the food pathogens **(Erdemoglu *et al*., 2007)**.

Killer protein-based nanoparticle showed an effective inhibition for *C. krusei*. It was observed that 350 µM of Ag-KT4561 (with 1 ppm of Ag) could bring in effective inhibition of *C. krusei* within 3 h. But beyond 350 µM concentration could affect the growth *K. marxianus*. *K. marxianus* growth was affected due to the presence of Ag^o but killer protein has no effect on it. Other significant consideration was that no pH or temperature was adjusted, because Ag ion was effective against almost all pathogens. Other benefit of using biomolecule based nanoparticle was that metal nanoparticles were toxic for human or animal consumption but biomolecule based nanoparticles had shown no toxicity so far **(Nel *et al*., 2006; Da Silva *et al*., 2011)**.

CONCLUSION

Biomolecule based nanoparticle approach (Ag-KT4561) for inhibition of *C. krusei* served to be better method than other chemical and biochemical methods used in this study. Other suitable alternative approach might have been ultra-filtration, however, on an industry scale it was an expensive approach. Therefore, Ag-KT4561 was the effective and economic inhibitory approach towards *C. krusei* (non-*Candida albicans* spp.) and it even supported green chemistry. Although the composition of cheese whey was known, further verification and prolonged usage of killer protein-based silver nanoparticle to sustain the antimicrobial effect need to be investigated further.

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EFFECT OF COMMERCIALLY MANUFACTURED *KAPPA* SEMI-REFINED CARRAGEENAN (SRC) WITH DIFFERENT PHOSPHATE SALTS ON YIELD, TEXTURAL AND SENSORY PROPERTIES OF BEEF MEAT

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ABSTRACT

The phosphate salts such as SDP, TSP, SHMP, DSP, TPPP, TKPP and STPP were blended with semi-refined *kappa* carrageenan (SRC) individually and their aqueous solution was injected into the fresh beef meat. The highest weight gain (24%) in fresh meat was obtained from injection of blend made with SRC and STPP as compared to other blends and control sample after forzen storage. Similarly, it also showed low cooking loss (24%) with good testural and sensory proerties as compared to other blends. It can be concluded from the presnt study that blend of SRC and STPP can be used in beef meat for yield and quality improvement.

Keywords: Beef, Semi Refined Carrageenan, Phosphate salts, Sensory analysis, Texture

INTRODUCTION

Nowadays, trendy sophisticated lifestyle makes the people moving towards modern technology especially in food processing side. They are looking for the solutions which fulfil their day by day needs. As we know, perishable products like meat and dairy usage among people requires specific concentration. For this sake it has technology like infusion of hydrocolloids and salts to make the product such as beef, poultry, pork and fish with better flavour texture, tenderness and juiciness for the customer satisfaction. Generally salts having good functional properties using in meat and meat products, which acts as preservative, binding to water and fat, textural properties and increasing shelf life by controlling water activity **(Bess, 2011).** Cooking loss, texture deterioration, decrease shelf life are the important factors affected by the reduction of salt in meat products, and at the same time there were controversy in hypertension, so there was mandatory to control the salt limits in meat and meat products. Sodium phosphate is generally used to increase moisture retention and reduce oxidative rancidity in meat **(Baumert and Mandigo, 2005).** Today, the meat industry offers a variety of different formulations to meet different nutrition needs.

Wierbicki and Howker (1976) observed that phosphates and NaCl at different levels showed various effects on colour, quality and sensory characteristics of beef and found that at 3% salt, either 0.3% STPP or 0.217% TSPP with other curing ingredients was an acceptable limit for cut-and-formed smoked as well as cured ham **(Wierbicki and Howker, 1976). Baublits *et al.* (2005)** studied the effect of the addition of STPP, SHMP and TSPP at the concentration 0.2 and 0.4% along with 2% NaCl on color, quality, and sensory characteristics of beef and observed that STPP was the most effective phosphate type for improving the color at the concentration 0.4% at the injection rate 18% **(Baublits *et al.*, 2005). Torley *et al.* (2000)** had observed that TSPP (0.35%) and STPP (0.37%) on pale soft exudative (PSE) pork had small effects on the functional properties such as pH, cooking temperature and ionic strength than in normal pork meat and concluded that addition of polyphosphates only gave a lower cooking loss through texture.

Tetra sodium diphsphates are the most functional phosphates in meat products. They act on the actomyosin complex of meat protein and also have high pH value. It results in high protein solubility which induces good water binding capacity **(Molins, 1991; Zayas, 1997).** Short chain phosphates are used as improving emulsion water holding capacity and stability **(Feiner, 2006; Zayas, 1997)** whereas the long chain phosphates such as SHMP and STTP used for optimize solubility and functionality of meat products **(Alvarado and McKee, 2007; Anjaneyulu *et al.*, 1989; Offer and Trinick, 1983).** The addition of NaCl or STPP and a lower pH led to an increase in the metmyoglobin level **(Fernández-López *et al.*, 2004; Moiseev and Cornforth, 1997)** in meat. **Anjaneyulu *et al.* (1990)** had reported that application of phosphate blends on buffalo meat patties improved the level of emulsifying capacity, increased emulsion stability, yield of patties, water holding capacity and reduced cooking loss, then shrinkage of patties. The phosphates such as TSPP, STPP and SAPP with NaCl in various concentrations had played a main role on buffalo meat and that the effects of phosphate were always comparatively better than sodium blends and control **(Anjaneyulu *et al.*, 1990).** Phosphates with kappa-carrageenan salt on the low fat emulsified meatballs significantly affect the product cooking yield, adhesion, gumminess, chewiness, lipid content, hardness, viscosity, cohesiveness and brittleness. The combination of salt and polyphosphates had effects on the product's texture and overall acceptance **(Hsu and Chung, 2001).**

Carrageenans are highly flexible molecules that form helical structure which has ability to form a variety of different gels at room temperature. Red sea weeds are the sources of carrageenan i.e., linear anionic sulphated polymer of galactose and anhydrogalactose. They are used in the food industries such as canned meat, reduced fat products for its gelling characteristic, water binding properties and thickening **(Giese, 1992; Therkelsen *et al.*, 1993; Candogan and Kolsarici, 2003a,b; Bixler and Porse, 2011).** The function of carrageenan in meat and its adding to the low-fat meat products improves water retention, consistency, slicebility and texture. **DeFreitas *et al.* (1997)** evaluated the effects of κ-, ι-, and λ-carrageenan (CGNs) on the rheological properties, water loss, and ultra structure of salt-soluble meat protein (SSMP) gels and found that κ-CGN increased gel strength and water retention of SSMP. At 0.2 to 1.5% of carrageenan in turkey meat sausage caused reduction of emulsion stability and increased water holding capacity **(Ayadi *et al.*, 2009)** also addition of carrageenan to increase emulsion stability in low fat frankfurter was reported by Candogan and Kolsarici (2003a). Influence of carrageenan on sensory properties of sausages showed that it could improve sensory scores in beef sausages **(Xiong *et al.*, 1999).**

The present study was to evaluate the effect of blends prepared with commercially manufactured semi refined carrageenan (MK-250 is brand name of AquAgri for food application) and different phosphates on yield, textural and sensory properties of beef meat with different phosphate salts.

MATERIAL AND METHODS

Sample collection

Beef *biceps femoris* (breeds: Jersey; sex: male, age: 2 years) was purchased from local meat shop and it was kept in refrigerator (4°C) for about 2 hours till it was used for the experiment, then ligaments, tendons and extraneous tissues were removed as much as possible and cut into 100g pieces with almost similar shape using a meat cutter.

SRC & Phosphate Salts

Semi-refined carrageenan (MK-250 is a brand name of AquAgri for food application) with particle mesh size of 200 mesh (0.074mm) used was from stock of Aquagri Processing Private Limited Batch No-108/2015, Manamadurai, India. The salts of Sodium Phosphate dibasic (SDP), trisodium orthophosphate (TSP), Sodium hexa meta phosphate (SHMP), Sodium dihydrogen orthophosphate dehydrate (DSP), Tetra potassium pyrophosphate (TPPP), Di sodium hydrogen orthophosphate anhydrous (TKPP), Sodium tripolyphosphate (STPP) were purchased from LOBA Chemicals Private Limited, Mumbai, India. Water bath-250 W, Sigma Scientific Instrument (P) Ltd, Chennai, India, Blue Star Chest freezer, Model CHF 200 B, India, Sony Cyber shot, GPS- DSC- HX 200 were used in the present investigation.

Preparation of Brine solution

Brine solutions were freshly prepared and used. Seven phosphate salts viz. SDP, TSP, SHMP, DSP, TPPP, TKPP and STPP were mixed separately with semi-refined carrageenan (MK-250) at 1:3 ratio in chilled water (5^{o}C) and used.

Meat treatment

All Part of *biceps femoris* was usedpieces was injected with 30% of its initial weight with freshly prepared brine solution by using syringe and the meat piece without brine injection was treated as control sample. Both treated and control samples were stored in freezer at -18°C for two days. Then samples were taken out, allowed to thaw and weight gain was calculated after drip loss. The samples were subjected to cooking at optimum temperature level of 80º ±2°C. Each sample was tagged with the wooden card board with nylon thread for identity. The experiment was replicated and average of data obtained from two experiments was considered.

Analysis of Physicochemical parameters

pH of sample was measured by using an electrical automatic pH meter (Eutech Instruments, Malaysia). A few drops of distilled water was sprayed on meat and measured the pH by direct contact between the sensitive diaphragm of the electrode and meat tissue.
Moisture of meat samples was determined according to AOAC method **(AOAC, 1990).** Meat sample was weighed in pre-weighed crucibles and charred on a hot plate and then placed in a muffle furnace at 550^{o}C for 4 hours and total ash was measured as below:
Ash Content (%) = weight of residue after ashing (g)/ Weight of sample (g) *100

Yield calculation

The cooked yield was calculated in relation to the raw meat weight (before injection) **(Drummond and Da-Wen Sun, 2006)** using the following equation:
Cooked yield (%) = Cooked weight/ Raw weight *100

Textural analysis

Extract release volume (ERV) of meat was determined using the method described by **Jay (1964).** 20g of meat was homogenised with 100 ml of distilled water for 2 minutes. Then poured the homogenate directly into the funnel lined with Whatman filter paper No.1, folded thrice as to make eight sections and allowed the homogenate to seep between the folds. The volume collected in 15 min was considered for calculating ERV.
Meat swelling capacity (MSC) of meat was determined by method of **Leora *et al.* (2006).** 25g of meat was homogenised with 100 ml distilled water for 2 minutes. Then 35 ml of homogenate was centrifuged at 2000 rpm for 15 minute and collected the supernatant (S) and calculated the MSC as below.

% Meat Swelling Capacity = (35-S-7)/7 *100

Water holding capacity (WHC) of meat was measured using the method described by **Kauffman *et al.* (1986) and Trout (1998).** 0.5g of meat sample was weighed and placed in between filter papers and this in turn was placed between glass sheets weighing 1.58kg. Over it, a weight of 4.0kg weight was place, thus total weight including glass sheet was 5.58kg for 5min. The water from the meat was then absorbed on the filter paper and filter paper was dried. Then area of the filter paper marked with and meat was later determined using a compensatory planimeter. Taking the differences from the resulting areas of the sample from marked borderline on the filter paper (moisture) and meat and a ratio area marked borderline was expressed as water holding capacity (WHC) of the meat:

WHC % = (Area marked borderline – Area meat) * 100 / Area marked borderline

Sensory analysis

Ten panellists were chosen for the assessment of the sensory attributes of the cooked beef meat samples. The samples were coded with alphabets and served to the panellists in individually partitioned booths. Sensory property was evaluated using standard evaluation score card (9 hedonic scales). Statistical analysis was done according to method described by **Steel *et al.* (1996).**

RESULTS AND DISCUSSION

The weight extension in brine injected meat samples ranged between 16 to 24%. The highest weight gain was observed in brine prepared with STPP and injected in meat i.e. 24% and it was 21%, 20%, 19%, 17%, and 16% in TPPP, SHMP, TSP, SDP & TKPP and DSP blended with MK-250 and injected in meat samples respectively, thus the response of different phosphates in weight extension of beef meat follows as: TPPP >TPPP>SHMP>TSP>SDP & TKPP>DSP. In the control sample there was 4% weight loss was recorded. Similarly lowest cooking loss i.e. 34% observed with beef sample treated with STPP followed by TPPP (26%), TSP (28%), SHMP (30%), SDP & TKPP (34%) and DSP (35%) (Table 1). **Garcia *et al.* (2013)** and **Lee *et al.* (2014)** had reported that addition of kappa carrageenan improved the yield and textural characteristics of beef and similar effect was observed in pork by **Patrascu *et al.* (2013).** Blend of STPP also showed lowest cooking loss of 38.70% followed by TSP (39.49%) and in other phosphates, the cooking loss ranged between in 40.49% to 43.58% .and in control it was 47.91%. The net weight loss from raw meat to after cooking ranged from 24% to 35% in treated samples with STPP being lowest weight loss (24%) and in control meat sample the weight loss was 50% (Table 1). It has been reported in literature that decreasing cooking loss was observed when treating muscle with a brine solution **(Sheard *et al.*, 1999; Walsh *et al.*, 2010).**

Table 1 Yield improvement and cooking loss of beef meat injected with blend of SRC (MK- 250) and different phosphates *

Blend	Initial weight(g)	Weight after injection (g)	Weight after storage (after drip loss) (g)	Weight post cooking (g)	Weight after cooking loss (%)	Net loss for initial weight of 100g (%)
MK-250 + SDP	100	130	117	66	43.58	34
MK-250 + TSP	100	130	119	72	39.49	28
MK-250 +SHMP	100	130	120	70	41.66	30
MK-250 + DSP	100	130	116	65	43.96	35
MK-250 + TPPP	100	130	121	72	40.49	28
MK-250 + TKPP	100	130	117	66	43.58	34
MK-250 + STPP	100	130	124	76	38.70	24
Control	100	100	96	50	47.91	50

*Each value is a mean of duplicates

The pH of brine made with different phosphates ranged from 6.53 to 11.67 with the order of TSP (11.67) > TKPP (10.75) > SDP (10.20), STPP (10.17), TPPP (10.09), SHMP (7.75 and slight acidic pH in DSP (6.53). The pH of raw meat was 5.54 and phosphate salts ranged from slight acidic (DSP pH 6.53) to high alkali condition of TSP (pH 11.67). The pH of brine injected meat and cooked increased from pH of its raw meat samples but all within acidic condition (Table 2) including TSP injected meat. The pH of meat improves little after injection of phosphate salts, thereby structure of muscle protein is opened as to increase the water holding capacity in order to yield extended weight gain and decreasing cooking losses **(Knipe, 2003 and Molins, 1991).** It has also been reported by **Xiong *et al.* (1999)** that an increase in pH sharply enhanced the water binding

strength in beef sausage when injected with some gums including carrageenans along with salts.

Table 2 Physiochemical properties beef meat injected with blend of SRC (MK- 250) and different phosphates*

Meat Blends	Moisture			Ash			pH				
	Raw Meat	Injected Meat	Cooked Meat	Raw Meat	Injected Meat	Cooked Meat	Raw meat	Salts	Salts with MK-250	Injected Meat	Cooked Meat
MK-250 + SDP	56.80	64.07	63.92	0.58	0.63	0.98	5.54	8.98	10.20	6.17	6.55
MK-250 + TSP	56.78	69.34	67.09	0.58	0.68	0.97	5.54	11.86	11.67	6.48	6.78
MK-250 +SHMP	56.12	66.21	67.12	0.58	0.65	0.96	5.54	5.43	7.75	6.01	6.44
MK-250 + DSP	56.74	60.69	64.72	0.57	0.74	0.98	5.54	4.41	6.53	5.75	6.25
MK-250 + TPPP	56.86	60.46	63.09	0.58	0.62	0.97	5.54	10.01	10.09	6.04	6.42
MK-250 + TKPP	56.22	60.76	64.78	0.57	0.63	0.97	5.54	8.93	10.75	6.24	6.74
MK-250 + STPP	56.54	60.81	65.18	0.58	0.61	0.98	5.54	8.94	10.17	6.06	6.45
Control	56.80	56.80	60.02	0.58	0.58	0.97	5.54	-	-	5.56	6.04

*Each value is a mean of duplicates

Moisture and Ash content

The ash and moisture content of meat injected with brine and its respective control is given in Table 2. Moisture of raw meat ranged between 56.12 to 56.86% and ash content was 0.57 to 0.58%. Because of specificity of the blend created with SRC (MK-250), the injected meat and cooked meat had shown increasing the moisture and ash level when compare to the raw meat due to its higher water holding capacity. The moisture level in control sample was 56.80% and 60.02% and ash content was 0.58% and 0.97% in raw and cooked meat respectively. In the treated meat, TSP had highest moisture content of 69.34% and lowest moisture level was observed in meat injected with TPPP (60.46%). The range of ash content in treated meat was very short i.e. 0.61% to 0.74% with lowest level in STPP and highest in DSP treated meat. Similarly, in the case of respective cooked meat, SHMP treated meat showed highest moisture level of 67.12% and lowest moisture was observed with meat treated by TPPP i.e. 63.09%. The ash content of all the samples treated with different phosphate salts and control was within the range from 0.97 to 0.98%.

Textural properties

The textural properties of raw, injected and cooked beef meat are presented in Table 3 and Figure1-2. The average ERV values of raw beef were 54 ml, while water holding capacity was 28.34 % and meat swelling capacity was 40%. For injected beef meat, highest ERV value was observed with STPP treated meat i.e. 53% followed by TKPP (52%), control (52%), TPPP (51%), TSP (50%), DSP (49%), SDP (48%) and SHMP (48%) treated meat samples (Table 3 and Figure 3). Water holding capacity of the beef meat refers to its ability of retain water and it is affected by space in muscle and pH of the tissue. The WHC of injected meat ranged from 26.12 % to 44.89 % with the order of STPP (44.89%) > TPPP (43.52%) > SHMP (43.22%) and the lowest range as TSP (42.16%) > SDP (41.94%) >TKPP (40.71%) >DSP (40.50%) and control (26.12%) (Figure 4). **Verbeken (2003)** reported that increasing the concentration of carrageenan from 0 to 2% led to an increase in WHC of about 5% and most studies reported a better water retention in the presence of carrageenan **(Pietrasik and Duda, 2000; Pietrasik and Li-Chan, 2002; Pietrasik, 2003).** it was 52%, 51%, 50%, 49%, and 48% in

Table 3 Textural properties beef meat injected with blend of semi-refined carrageenan (MK- 250) and different phosphates*

Meat Blends	Raw meat			Injected meat			Cooked Meat		
	ERV (ml)	WHC (%)	MSC (%)	ERV (ml)	WHC (%)	MSC (%)	ERV (ml)	WHC (%)	MSC (%)
MK-250 + SDP	54	28.34	40	48	41.94	45.71	20	38.94	20.00
MK-250 + TSP	54	28.34	40	50	42.16	42.85	18	39.16	17.14
MK-250 +SHMP	54	28.34	40	48	43.22	45.71	17.5	40.12	22.85
MK-250 + DSP	54	28.34	40	49	40.50	48.57	18	37.50	14.28
MK-250 + TPPP	54	28.34	40	51	43.52	51.42	21	39.25	17.14
MK-250 + TKPP	54	28.34	40	52	40.71	48.57	18.5	37.71	18.57
MK-250 + STPP	54	28.34	40	53	44.89	42.85	20	40.82	28.57
Control	54	28.34	40	52	26.12	34.28	36.5	24.46	11.42

*Each value is a mean of duplicates

The MSC of injected beef meat as follows from highest to lowest TPPP (51.42%), DSP and TKPP (48.57%), SDP and SHMP (45.71), STPP and TSP (42.85%) and control (34.28%) (Figure 5). The cooked beef meat had lowest ERV value (18%) was found using DSP and TSP added with MK-250 while the highest ERV value is found in control 36.5 %. The highest WHC obtained for cooked meat using STPP with MK-250 (40.82%) and while the lowest WHC is for control (24.46). STPP had highest MSC value is 28.57% for STPP with MK-250 and lowest MSC is 11.42% for control. The TSP and TPPP has similar MSC value (17.14%).

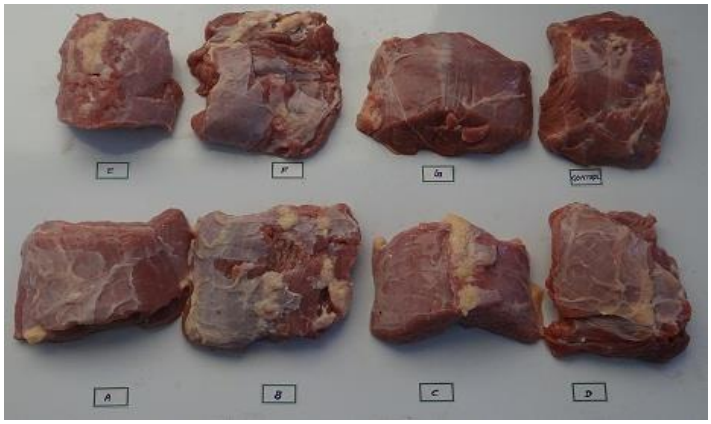

Figure 1 Texture of beef meat injected with blend of semi-refined carrageenan (MK- 250) and different phosphates

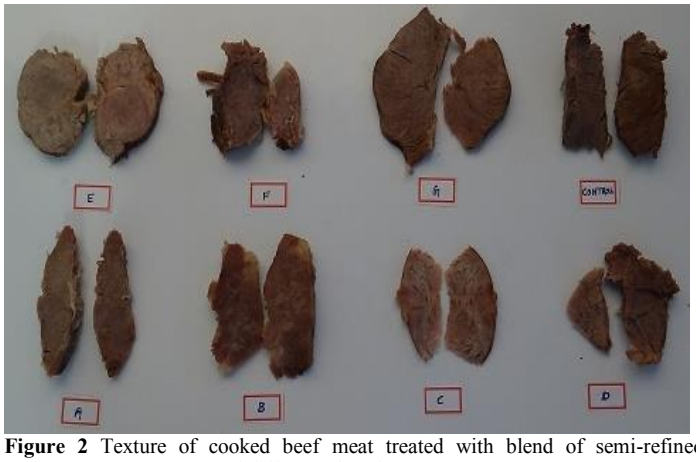

Figure 2 Texture of cooked beef meat treated with blend of semi-refined carrageenan (MK- 250) and different phosphates

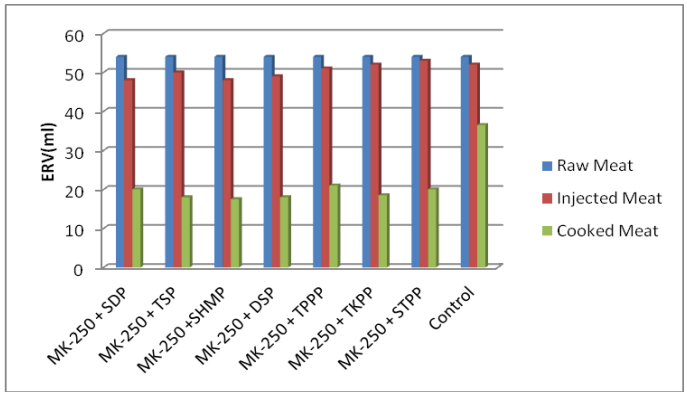

Figure 3 Effect of semi-refined carrageenan (MK- 250) and different phosphates on Extract Release Volume of beef meat

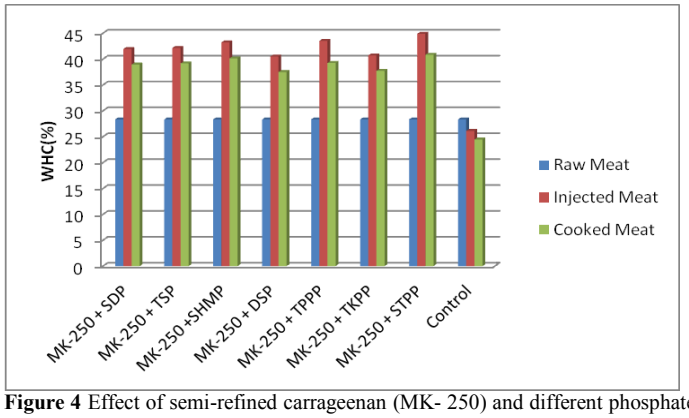

Figure 4 Effect of semi-refined carrageenan (MK- 250) and different phosphates on Water Holding Capacity of beef meat

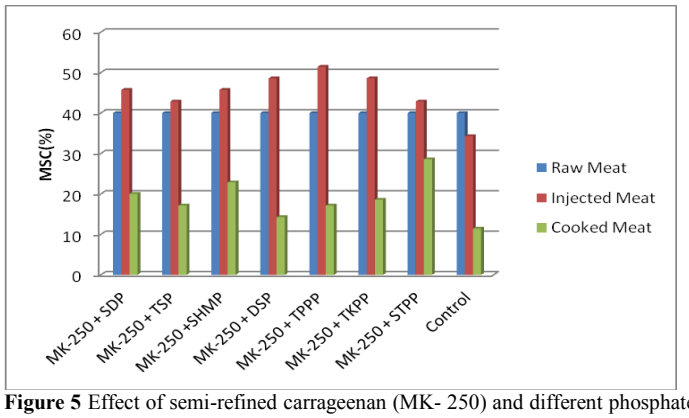

Figure 5 Effect of semi-refined carrageenan (MK- 250) and different phosphates on Meat Swelling Capacity of beef meat

Sensory properties

Palatability of treated along with control meat samples were evaluated by 10 panellists. Panellists gave feedback that acceptability all treated meat samples were very good as compared to control sample. On the basis of 9 hedonic scales the sensory analysis score card of SRC (MK-250) added with different phosphate salts on beef meat are showed in the Table 4. Brine injected samples had higher values in all sensory attributes like appearance, colour, odour, juiciness, texture, tenderness and flavour than control sample.

Table 4 Appearance and sensory analysis of beef meat injected with blend of SRC (MK- 250) and different phosphates

Blend/Traits	Appearance	Color	Odour	Juiciness	Texture	Tenderness	Flavor	Overall Palatability
MK-250 + SDP	6	6	6	5	8	7	7	6.4
MK-250 + TSP	6	7	6	6	7	7	7	6.5
MK-250 +SHMP	7	8	8	6	8	5	7	7
MK-250 + DSP	6	6	7	6	7	5	7	6.2
MK-250 + TPPP	6	7	7	6	7	6	8	6.7
MK-250 + TKPP	6	6	5	6	5	7	7	6
MK-250 + STPP	7	8	8	7	8	7	8	7.5
Control	6	6	5	6	5	6	6	5.7

In the aspects of appearance of meat injected with brine made up of STPP and TPPP got maximum scores among other treated samples and control was the least cored product. The brine made with salts such as TSP, SHMP and TPPP accounted very good status in colour, while brine of TKPP treated sample and control were in the last level. Odour and flavour was commonly shared the same level in the score card i.e. there was no such hyper variations among brine made with all the phosphate salts tested. In juiciness, mostly panellist preferred SHMP, TPPP and STPP over the other salts and the control product. These results are in agreement with literature reports that juiciness of meat increased when it was phosphated **(Baublits *et al.*, 2005; Miller and Harrison, 1965; McGee *et al.*, 2003)** an enhanced flavour in beef and pork **(Smith *et al.*, 1984; McGee *et al.*, 2003; Scanga *et al.*, 2000).** Texture and tenderness were mostly in the friendly zone as STPP scored recorded maximum level of point for both texture and tenderness followed by TSP, SHMP, TPPP, other phosphates and control. In the overall acceptability, panelists given maximum score to meat treated with brine made up of STPP and SRC (KM-250) followed by other brines SHMP, TPPP, TSP, SDP, DSP, TKPP and finally control. Therefore, STPP among all seven salts used for making brine with SRC (MK-250) performed well in terms of weight gain and improvements in texture and sensory when injected into beef meat.

CONCLUSIONS

Beef injected with blend of semi-refined carrageenan (MK-250) and phosphates like SDP, TSP, SHMP, DSP, TPPP, TKPP and STPP yielded higher weight gain with improved sensory properties like tenderness, juiciness, color and flavour as compared to control sample. Among seven phosphates tested, STPP performed well in terms of weight gain i.e. 24%, improved quality parameters and less cooking loss as compared to other phosphate salts, therefore, it can be concluded from the present investigation that there is potential use of blend made with STPP and semi-refined carrageenan (MK-250) in the beef processing industry.

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INFLUENCE OF MAGNETIC FIELD STIMULATION ON THE GROWTH AND BIOCHEMICAL PARAMETERS IN *PHASEOLUS VULGARIS L.*

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ABSTRACT

Plant response to magnetic field stimulation (MFS) is varied and dependent on many factors such as the intensity, time of exposition, or application form. It is known that high intensity MFS inhibits the growth and development of plants. However, weak MF stimulates many processes in plant cells. This study reports the effects of 130-mT magnetic field stimulation (MFS) on the growth and selected biochemical parameters in the common bean *(Phaseolus vulgaris L.)* in laboratory conditions. Our results indicated that the stimulation of plants by a weak permanent magnetic field (130 mT) increased mitotic activity in meristematic cells of the common bean (*P. vulgaris* L.). There was no influence of the 130-mT MFS on the development of aboveground plant parts; however, there was a marked increase in GPOX activity in the leaves after 130-mT magnetic field stimulation.

Keywords: Bean, magnetic field, growth, assimilation pigment, antioxidant enzyme

INTRODUCTION

Both magnetic and electromagnetic field influence the functioning of biological organisms. The literature describes the effects of electromagnetic field on seed germination **(Aksyonov *et al.*, 2001)** or antimicrobial activity **(Akinyele *et al.*, 2012).** Despite the extensive literature on the effects of magnetic field stimulation (MFS) on plants **(Pietruszewski *et al.*, 2001; Belyavskaya, 2004; Çelik *et al.*, 2009; Muszyński *et al.*, 2009; Jouni *et al.*, 2012)**, recognition and understanding of the mechanisms of action of the magnetic field on the plant organism is still a challenge for researchers. Identification of the mechanism of magnetic field stimulation may be a key in regulating the biological activity of plants **(Cakmak *et al.*, 2012)**. It has been well documented that magnetically treated water **(Alkassab and Albach, 2014)** or magnetic field stimulation exerts an effect on plants **(Belyavskaya, 2004; Çelik *et al.*, 2009; Muszyński *et al.*, 2009; Jouni *et al.*, 2012)**. However, the response of plants to magnetic field stimulation depends on the species, dose, or exposure time **(Pietruszewski *et al.*, 2001)**. The optimal magnetic field stimulates plant growth and development or seed germination **(Pietruszewski *et al.*, 2001)**. It was observed that weak magnetic field stimulation had a beneficial effect on seed germination in radish **(Novitskaya *et al.*, 2001)**, root formation in wheat **(Aksyonov *et al.*, 2001)**, or formation of somatic embryos in cultures of alfalfa (*Medicago sativa* L.) **(Dijak *et al.*, 1986)**. Moreover, studies on the meristematic cells of pea have shown that the magnetic field has an influence on metabolism and cellular division **(Belyavskaya *et al.*, 1992)**. However, **Lebedev *et al.* (1977)** showed a decrease in the fresh and dry weight of shoots and roots in barley seedlings. Moreover, the function of antioxidant enzymes (SOD and CAT) in *Glycine max* (L.) seedlings was intensified due to treatment with the magnetic field **(Çelik *et al.*, 2009)**.
Nowadays, the results obtained by researchers on the magnetic field stimulation are particularly promising and important. This is due to the huge interest in the environment-friendly technology and the possibility of obtaining increased yields without the use of fertilizers.
The bean *(Phaseolus vulgaris L.)* is often used for laboratory testing due to its rapid growth, a short period of vegetation, and relatively low cultivation requirements. The bean is an important legume constituting a major source of proteins, fibre, prebiotics, vitamin B, and micronutrients for humans worldwide. In addition, some species of legumes are used as animal fodder **(Câmara *et al.*, 2013)**. These properties have contributed to the selection of these plants for this study.
The objective of our study was to investigate the influence of weak permanent magnetic field stimulation (130 mT) on the growth and selected biochemical parameters of the common bean *(Phaseolus vulgaris L.)*.

MATERIAL AND METHODS

Seeds (20 seeds per 1 replication) of the common bean (*Phaseolus vulgaris* L.) were sown in seed trays on filter paper moistened with distilled water. The plant material was divided into two groups: the control (C) and experimental group (MF). Seeds from the control group (C) were growing in a natural magnetic field while the seeds of the test group (MF) were grown during the experimental period (14 days) in the presence of 130-mT permanent magnetic field (MF) perpendicular to the plants. Based on previous studies, we selected the induction of 130 mT, which gives the best results, for the magnetic field **(Kornarzyński *et al.*, 2004).** The seeds germinated at 20±2°C, at a 12/12 h photoperiod and an illumination of 300 lux in the vegetation chamber for 14 days. After this time, the plant material was used to the analyses. Plant breeding was conducted in the Department of Physics, University of Life Sciences in Lublin.

The biometric parameters

After 14 days of the experiment, the basic growth parameters: fresh weight, average length of roots and shoots, and the average length and width of leaves were analysed. Plant individual average length of roots and the aboveground parts of plants was measured with 0.1 cm precision. The fresh weight (FW) of seedling samples was measured with 10^{-5} g accuracy. The biometric measurements were performed in triplicate for selected 10 plants at the same physiological age.

Cell division

To study the mitotic activity, 14-day-old root meristems were fixed for 24 h in AA (92% EtOH and CH_3COOH, 3:1) and stained in 0.5% acetocarmine. After staining, the plant material was rinsed with dH_2O and macerated for 10 minutes in a solution: HCl (conc.) : 96% EtOH (1:1). The macerated tissues were placed in dH_2O for 10 minutes and then in 45% acetic acid for 10 minutes **(Clark, 1981).** The mitotic index (MI) was calculated as the percentage of proliferating cells among 1000 cells. For the analysis, a Leica DM 4000 B microscope was used.

The biochemical assays

The biochemical analyses were carried out on day 14 of the experiment and all measurements were performed in triplicate.

Measurement of assimilation pigments

The level of chlorophyll a (chl a), chlorophyll b (chl b), chlorophyll a+b (chl a+b), and carotenoids (car) was determined by the **Lichtenthaler and Wellburn** method **(1983)**. Leaf samples (0.5 g) were homogenized in 5 ml of 80% acetone chilled to 4°C. The homogenate was centrifuged and the precipitate was washed with cold acetone until complete chlorophyll extraction. The extract was supplemented with cold 80% acetone to 25 ml. The absorbance of the supernatant was read at wavelengths of 663 nm, 645 nm, 652 nm, and 470 nm in an Agilent Cary 60 UV-Vis spectrophotometer. The contents of pigments were calculated according to **Bruinsma (1963).** The extract was diluted with acetone and the absorption spectra were recorded in the range of 800 to 200 nm using the Agilent Cary 60 UV-Vis spectrophotometer.

Determination of the protein content

The protein content in the plant material (roots, shoots, and leaves) was determined according to **Bradford (1976)** using BSA as a standard. The measurement was performed after 15 min. of adding the dye concentrate (Bio-Rad Protein Assay) at a wavelength λ = 595±10 nm on the Agilent Cary 60 UV-Vis spectrophotometer.

Determination of guaiacol peroxidase activity (GPOX)

The measurement of guaiacol peroxidase activity GPOX (EC 1.11.1.70) was performed according to **Velikova *et al.* (2000)**. Frozen plant tissues (roots, shoots, and leaves) (0.5 g) were transferred to a cooled mortar and homogenized with 5 ml of 50 mM phosphate buffer pH 7.0 with 1 mM EDTA and 1% PVPP. The material was centrifuged at 15.000 rpm at 4°C for 20 min. in an MPW 350-R centrifuge. The reaction mixture contained 2750 ml of 1% guaiacol in 50 mM phosphate buffer at pH 7 and 100 ml supernatant. The reaction was initiated by addition of 150 µl 100-mM H_2O_2 to the reaction mixture. The absorbance was read at 470 nm on the Agilent Cary 60 UV-Vis spectrophotometer. The specific activity of the enzyme was expressed in $mM.min^{-1}.mg^{-1}$ protein.

Statistical analysis

The statistical analysis was performed using one-way ANOVA and Tukey's post hoc analysis for determination interaction significance at $p < 0.05$. The results were expressed as mean values ± standard deviation (SD).

RESULTS

The analysis of root growth of common bean (*Phaseolus vulgaris* L.) showed that the average length of the control roots was 16.72 cm and that of the MF-stimulated plants was 16.80 cm after 14 days of the experiment. There were no significant differences between these groups. Moreover, no significant influence of the 130-mT MF on the shoot and leaf growth was observed. The average shoot length and the average length and width of leaves of the MF-stimulated plants persisted at the control level. Additionally, the fresh weight (FW) of the MF-stimulated plants remained at the control level and there were no statistically significant differences between the analysed plant groups (Table 1).

Table 1 Average length [cm] of the primary organs, average length and width [cm] of the leaves, and average weight [g] of bean seedlings (*Phaseolus vulgaris* L.).

Description	Roots [cm]	Shoots [cm]	Leaf length [cm]	Leaf width [cm]	Seedling weight [g]
C	16.72 ± 1.40 a	25.63 ± 6.48 a	2.02 ± 0.17 a	1.29 ± 0.30 a	1.54 ± 0.43 a
MF	16.80 ± 2.38 a	25.44 ± 1.83 a	1.87 ± 0.22 a	1.30 ± 0.19 a	1.58 ± 0.26 a

Legend: C - control conditions, MF - 130-mT magnetic field stimulation. Mean ± SD, n = 30, p> 0.05. Numbers in columns marked with the same letters do not differ significantly.

On the contrary, the study showed that in bean plants (*P. vulgaris* L.) the stimulation with the magnetic field had a beneficial effect on the mitotic activity in the root meristem cells. The mitotic index (MI) value was 36.33% in the control roots, while the mitotic activity in the MF-stimulated roots was 57.8%, and this was a statistically significant increase by 59% in comparison to the control level. The average number of cells in the different phases of mitosis in the control plants was 64 in the prophase, 29 in the metaphase, 23 in the anaphase, and 247 in the telophase. In the MF-stimulated root meristems of bean plants, the following values were noted: 83 in the prophase, 35 in the metaphase, 17 in the anaphase, and 443 in the telophase. Importantly, a beneficial effect of MF on the number of cells was observed in the metaphase and telophase stages, and the numbers were increased by 23% and 79 %, respectively, in relation to the control (Table 2).

Table 2 Mitotic index (MI) [%] and the number of cells in the different phases of mitosis in root meristems of bean (*Phaseolus vulgaris* L.).

Description	MI [%]	Prophase	Metaphase	Anaphase	Telophase
C	36.33 ± 3.72 a	64.00 ± 6.39 a	28.67 ± 3.87a	23.33 ± 4.47 a	247.33 ± 26.03 a
MF	57.80 ± 1.31 b	82.67 ± 6.43 a	35.33 ± 3.15b	16.67 ± 5.03 a	443.33 ± 9. 45 b

Legend: C - control conditions, MF - 130-mT magnetic field stimulation. Mean ± SD, n = 1000, p> 0.05. Numbers in columns marked with the same letters do not differ significantly.

Continuous measurements of the chlorophyll absorption spectrum were also performed. The highest absorbance was in the ranges of 500 to 400 nm and 700 to 600 nm (Fig. 1).

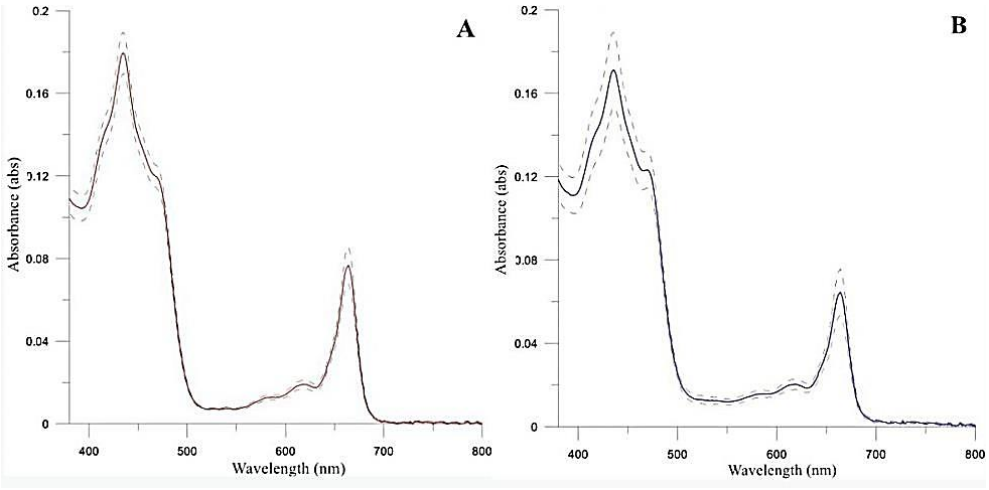

Figure 1 The assimilation pigment absorption spectra in bean (*Phaseolus vulgaris* L.) leaves. A - control conditions and B - in the presence of the 130-mT magnetic field (MF).

The measurement of the assimilation pigment content in the leaves (chl a, chl b, chl a+b, carotenoids) was performed on day 14 of the experiment. In the leaves of the common bean (*P. vulgaris* L.), the pigment content of the control was 0.21 $mg.g^{-1}$ FW (chl a), 0.09 $mg.g^{-1}$ FW (chl b), and 0.33 $mg.g^{-1}$ FW (chl a+b). The pigment content in the MF-stimulated leaves was 0.16 $mg.g^{-1}$ FW (chl a), 0.08 $mg.g^{-1}$ FW (chl b), and 0.26 $mg.g^{-1}$ FW (chl a+b). We noted that MFS did not influence significantly the chlorophyll content (Table 3). Similarly, the carotenoid content in the bean leaves was 0.13 $mg.g^{-1}$ FW in the control and 0.12 $mg.g^{-1}$ FW in the MF-stimulated leaves and this indicated that the carotenoid content in bean leaves exposed to MF remained at the control level (Table 3).

Table 3 The chl a, chl b, chl a+b and carotenoid content [$mg.g^{-1}$ FW] in leaves of bean (*Phaseolus vulgaris* L.).

Description	Chl a	Chl b	Chl a+b	Carotenoids
C	0.21 ± 0.05 a	0.09 ± 0.01 a	0.33 ± 0.10 a	0.13 ± 0.01 a
MF	0.16 ± 0.03 a	0.08 ± 0.01 a	0.26 ± 0.04 a	0.12 ± 0.01 a

Legend: C - control conditions, MF - 130-mT magnetic field stimulation. Mean ± SD, n = 3, p> 0.05. Numbers in columns marked with the same letters do not differ significantly.

Additionally, the effect of MFS on guaiacol peroxidase activity (GPOX) in the roots, shoots, and leaves of the common bean (*P. vulgaris* L.) plants was analysed. The experiment showed that GPOX activity in the bean roots was 17.1 U.mg^{-1} protein in the control and 20.67 U.mg^{-1} protein in the MF-stimulated plants and there were no statistically significant differences between these groups (Fig. 2).

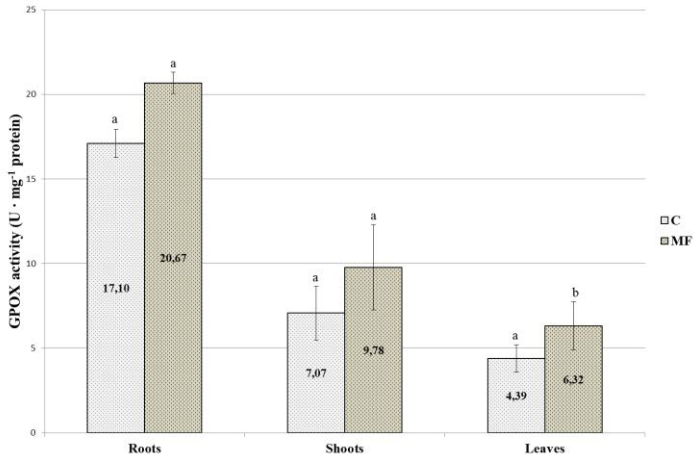

Figure 2 Guaiacol peroxidase activity (GPOX) in bean (*Phaseolus vulgaris* L.) organs. C - control conditions and MF - 130-mT magnetic field stimulation. Mean ± SD, n = 3, p> 0.05. Numbers in columns marked with the same letters do not differ significantly.

Similarly, there was no significant influence of MFS on the GPOX activity in the shoots. The study showed that the GPOX activity in the shoots of the control was 7.07 U.mg^{-1} protein and in the MF-stimulated plant 9.78 U.mg^{-1} protein. However, the leaves of the bean plants appeared to be highly sensitive to the 130-mT MF stimulation. The activity of GPOX in the control leaves was 4.39 U.mg^{-1} protein, and 6.32 U.mg^{-1} protein in the leaves exposed to 130 mT MF. A statistically significant increase (by 44%) in the activity of GPOX was observed in the bean leaves after stimulation with 130 mT MF (Fig. 2).

DISCUSSION

Magnetic field stimulation (MFS) of plants is one of environmental-friendly techniques used to improve crop yields and seed germination and the results obtained are promising **(Pietruszewski and Kania, 2010; Cakmak *et al*., 2012; Jouni *et al*., 2012)**. In this paper, the influence of permanent magnetic field stimulation (130 mT MF) on the growth and development of the common bean (*Phaseolus vulgaris* L.) was studied. In our experiment, there was no effect of the MF stimulation on the root growth of the common bean. However, **Vashisth and Nagarajan (2009)** found that 50-mT magnetic field stimulation led to a 42.4% increase in root length of sunflower. **Nagy *et al*. (2005)** also observed a 70% increase in the sunflower root length after MF stimulation in 0.01 Hz - 20 kHz and induction of 400 μT. Moreover, in *Lens culinaris*, a permanent magnetic field (120 mT, 10 min.) increased the root length by 58.96% **(Shabrangi and Majd, 2009)**. On the other hand, the results of this study showed that the 130-mT MF stimulation had a beneficial effect on cell mitotic activity in common bean (*P. vulgaris* L.) root meristems, and the mitotic index (MI) increased by 59%. Similar results were obtained by **Răcuciu (2011)**, who observed a 3-fold increase in the number of dividing root cells after 12 h stimulation of maize (*Zea mays*) with a 10-mT and 50 Hz magnetic field. Similarly, **Shabrangi *et al*. (2011)** observed a 3% increase in the mitotic activity in meristematic cells after 4-h exposure of maize (*Zea mays*) to MF stimulation (60 Hz, 3 mT). In our study, an increase in the number of cells in the metaphase or telophase stages by 23% and 79%, respectively, was also observed in bean plants exposed to 130-mT MFS. **Răcuciu (2011)** and **Fomicheva *et al*. (1992)** suggest that the increase in the mitotic activity of root meristems exposed to the magnetic field is caused by prolongation of the duration time of the different phases in the cell cycle and by the number of cells in the different stages of mitosis. This is in agreement with **Fomicheva *et al*. (1992)**, who also noted a prolonged time of the G1 and G2 phases in *Linum usitatissimum* L. by 51% and 33 %, respectively.

We also studied the influence of 130-mT MF stimulation on the growth and development of the aboveground plant parts. We noted that 130 mT MFS did not significantly influence the growth of shoots and leaves or fresh weight in bean plants. However, **Bilalis *et al*. (2013)** reported a 42% and 31% increase in leaf area in cotton (*Gossypium hirsutum* L. cv. *Campo*) after stimulation with a magnetic field of 3 Hz and 12.5-mT induction after 35 and 45 days, respectively. Moreover, **Yaycili and Alikamanoglu (2005)** observed an increase in the number of leaves by 36% in *Paulownia tomentosa* and by 40% in *Paulownia fortunei* after MF stimulation (2.9 mT to 4.9 mT). In turn, **Dardeniz *et al*. (2006)** obtained a 17% increase in shoot length of grapes (*Vitis vinifera*) after stimulation with 50 Hz and 0.15 mT MF.

To know better the molecular mechanisms involved in the plant growth processes after MF stimulation, spectrophotometric measurements of selected physiological and biochemical processes (photosynthesis pigment content or GPOX activity) were carried out. Photosynthesis is the main metabolic process in plant growth and development and thus very sensitive to environmental changes. The differences in chlorophyll content are often observed after treatment with various environmental factors (**Velikova *et al*., 2000)**. In the present study, we indicated that there was no significant effect of the 130-mT MFS on the assimilation pigment content in bean (*Phaseolus vulgaris* L.). The effect of MFS on the assimilation pigment content is unclear. **Atak *et al*. (2007)** showed that stimulation of soybean (*Glycine max* L.) with the magnetic field (2.9 mT to 4.6 mT) decreased the content of chl a, chl b, and chl a+b by 16%, 14%, and 14%, respectively. In contrast, the change in the exposure time resulted in an increase in the assimilation pigment content by 21% (chl a), 13% (chl b), and 18% (chl a+b). Similarly, **Răcuciu *et al.* (2008)** noted that permanent magnetic field stimulation (50 mT) for 14 days contributed to a 3% increase in the content of chl a + b in maize (*Zea mays*), but 100 mT MFS decreased the chl a + b content by 4%. Furthermore, **Muszyński *et al.* (2009)** noted that chlorophyll levels in *Triticum durum* seedlings were significantly modified by extremely low frequency magnetic field (f = 50 Hz, B = 15 mT), however, the chlorophyll a and b ratios remained unchanged.

The ROS scavenging enzymes: superoxide dismutase (SOD), catalase (CAT), and peroxidases (POX) are the main protective agents against ROS formation. SOD is the major enzyme involved in ROS detoxification, CAT is a key enzyme that eliminates H_2O_2, and POX catalyses the reaction of H_2O_2 degradation **(Jouni *et al*., 2012)**. Furthermore, guaiacol peroxidase (GPOX) [EC 1.11.1.7] is an enzyme of the peroxidase class that plays an important role in lignification, ethylene biosynthesis, or defence against pathogens and ROS **(Mika and Lüthje, 2003)**. In the present paper, we have demonstrated that 130-mT MFS has a beneficial influence on GPOX activity, which was manifested by a significant increase in GPOX activity by 44% in the bean leaves. Similarly, **Atak *et al.* (2007)** observed a significant increase in GPOX activity in soybean (*Glycine max* L.) leaves exposed to 2.9-4.6 mT MFS for 2.2 s and 19.8 s, but a better effect was obtained for the longer exposure time. Moreover, an electromagnetic field (275 kV) contributed to a significant increase in GPOX activity in mustard (*Brassica chinensis*) leaves, although a weaker electromagnetic field (33 kV) did not affect peroxidase significantly **(Maziah *et al*., 2012).** The increase in the GPOX activity that we noted in the bean (*Phaseolus vulgaris* L.) leaves may suggest increased potential of the plant defence system **(Jouni *et al*., 2012)**. Furthermore, the studies carried out by **Shabrangi *et al.* (2011)** showed that magnetic field stimulation (60 Hz, 3mT and 10 mT) increased the activity of other antioxidant enzymes, i.e. ascorbate peroxidase (APX) and superoxide dismutase (SOD) in maize (*Zea mays*) roots and shoots. SOD and CAT activity were also intensified in *Glycine max* (L.) seedlings due to the treatment with magnetic field **(Çelik *et al*., 2009).**

CONCLUSION

In conclusion, our results presented in this paper indicate that the 130-mT magnetic field stimulation promoted mitotic activity in meristematic cells in bean (*Phaseolus vulgaris* L.) roots. The development of the aboveground plant parts was slower, but at the control level. Similarly, the assimilation pigment content and GPOX activity in roots and leaves remained at the control level. However, there was a marked enhancement of GPOX activity in leaves after 130-mT MFS. Our results might be useful to expand the general knowledge about the mechanism of the magnetic field action on plants. Currently, these phenomena are still unclear and require further research.

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IMPROVEMENT OF *BORASSUS AKEASSII* WINES QUALITY BY CONTROLLED FERMENTATION USING *SACCHAROMYCES CEREVISIAE* STRAINS

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ABSTRACT

Palm wine produced traditionally and consumed by many people around the world and specifically in Burkina Faso posed health risks because of questionable quality of wine produced by mix culture fermentation and the use of antiseptics for the stabilization. In order to improve its quality, *Saccharomyces cerevisiae* strains isolated from *Borassus akeassii* wines and identified by amplification and RFLP analysis of the 5-8S-ITS region were used for in vitro fermentation of unfermented palm sap. The physicochemical characteristics of the sap were measured before and after fermentation process by High-Performance Liquid Chromatography (HPLC) and the microbiological quality were also performed. HPLC analysis showed that glucose and fructose concentration in palm sap were 37.0 and 27.6 g/L respectively, ethanol content was ranged between 2.76 and 5.31 % (g/mL) for controlled fermentation and 2.20 % (g/mL) for spontaneous fermentation. Lactic and acetic acids were ranged between 0.1 and 0.3 g/L and 1.5 and 1.6 g/L for controlled fermentation versus 2.5 and 3.1 g/L and the spontaneous fermentation respectively. Coliforms and *Staphylococcus aureus* were detected only in the unfermented palm sap and the wine fermented spontaneously.
Principal component analysis showed a good separation between spontaneous and controlled fermentation. Sterilization and controlled fermentation of the unfermented sap with palm wine *Saccharomyces cerevisiae* strains led to the improvement of palm wine quality.

Keywords: *Borassus akeassii* wine, Fermentation, improvement, quality, *Saccharomyces cerevisiae,* RFLP, HPLC

INTRODUCTION

Palm wine is an alcoholic beverage from the sap of various species of palm tree such as *Palmyra* and coconut palm (**Adeleke and Abiodun, 2010**). It is a sweet alcoholic beverage widespread in African, American and Asian tropical regions and which is obtained by spontaneous fermentation of sap tapped from palm trees such as *Elaeis guineensis* , *Raphia hookeri, Raphia vinifera* (**Ezeronye and Legras, 2005**) and *Borassus akeassii Bayton* (**Bayton et *al.*, 2006 ; Bayton and Ouédraogo, 2009**). Palm wine contains 300 calories/L, 0.5-2.0 g of proteins, considerable of vitamins, a major component of which is vitamin A, C and K helps consumers eye sight, protects and improves the eye sight (**Santiago-Urbina and Ruíz-Terán, 2014**). Many components of palm wine have been found previously in conventional wines. This wine is colorless and very sugary (**Obahiagbon and Osagie, 2007**) until sugars are fermented into alcohol then organic acids spontaneously. According to producers and consumers, palm wine obtained by mix culture (spontaneous) fermentation, gets inconsumable after 3 days (**Ouoba *et al.*, 2012**). Natural uncontrolled fermenting process led to unstableness and easy spoilage of this product quality (**Ngọc *et al.*, 2014**). This beverage produced traditionally is unstable, therefore exposed to alteration if fermentation is not controlled. The acidification and instability of palm wine during its fermentation need to be controlled in order to ensure its quality. Different antiseptics such as sorbic acid, diethylpyrocarbonate (DEPC) and sodium metabisulfite have been already used for stabilisation of palm wine (**Okafor, 1975 a**). Even though use of these antiseptics would be the efficient means for the stabilization of palm wine, they can pose health risks. In a former work, coliforms and *Staphylococcus aureus* were detected in natural palm wine (**Tapsoba et *al.*, 2011; 2014**). According **Olawale *et al.* (2010)**, sterilization and use of purified *Saccharomyces cerevisiae* in fermentation of palm sap could confer a more quality and hygienic palm wine. Because of questionable quality of palm wine produced by mix culture fermentation and the use of antiseptics for the stabilization towards the world and specifically in Burkina Faso, we proposed an improvement of this beverage quality by controlled fermentation of unfermented sap with selected strains of *Saccharomyces cerevisiae* isolated from palm wine. It has been to use active *S. cerevisiae* strains isolated from *Borassus akeassii* palm wine for fermentation of the unfermented sap, evaluate the microbiological and biochemical quality of palm wine produced secondly and to compare controlled fermentation and spontaneous.

MATERIAL AND METHODS

Sampling of palm crude sap

Palm crude sap of the same *B. akeassii* species was purchased in South-West of Burkina Faso where palm wine is largely tapped and very consumed. Two samples of 1 L of fresh palm sap were transferred in sterile plastic containers which were immediately immersed in an isothermal box, and brought to the laboratory and maintained at 4° C before the analysis.
Before fermentation, 10 mL of the unfermented palm sap were used for microbiological analysis and 15 mL were filtered and stored at -20° C for further analysis

Palm wine fermentation process

Preparation of the inoculums for fermentation

We used *Saccharomyces cerevisiae* strains YBPW7, YBPW13 and YBPW25 isolated from *Borassus akeassii* wines and identified by amplification and RFLP analysis of the 5-8S-ITS region (**Esteve-Zarzoso *et al.*, 1999**). Each strain was overnight grown aerobically in shake 10 mL flasks at 30 °C in YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose).

Fermentation process assays

The unfermented palm sap was subjected to flowing vapor sterilization as method described by **Clément (2012)**. The fermentation assays were carried out using *Saccharomyces cerevisiae* strains YBPW7, YBPW13 and YBPW25 isolated from palm wines to inoculate 250 mL palm sap collected. The Overnight culture each of strain was used to inoculate 250 mL of sterilized *Borassus akeassii* crude

sap at a density of 10^6cells / mL. Spontaneous (Mix culture) fermentation was at the same time carried out with the endogenous microorganisms (natural microflora). The experiments were performed at 30° C for 72 hours.
Palm wines produced were designed CF7, CF13, CF25 and SP respectively. Sterile samples were collected at 24 hours time intervals for further analysis.
Wine was filtered using cheese cloth as method described by **Kumar *et al.* (2012)** and stored at 4°C. Then 10 mL were used for microbiological analysis and 15 mL were stored at -20 ° C for analysis of physico-chemical parameters. Glucides, organic acids, glycerol and ethanol were measured by HPLC in the supernatant. The pH was also measured using a pH-meter (WTW 82362) at 25° C.

Biochemical and microbiological analyses of palm wines

Sugars, ethanol and organic acids analyses by High-Performance Liquid Chromatography (HPLC)

Glucose, ethanol, glycerol and organic acids in palm wines were determined by High-Performance Liquid Chromatography (HPLC-1 Agilent 1260) equipped of a degasser G132A, a quaternary pump G1311 A, a passor of samples G131 A, a furnace G131 A, a detector UV (G131A) to the variable wavelength, a refractometer G1382 A and a column Phenomenex-Rezex ROA-Organics Acid H^+ (Size 300 x 7.8 mm). For analysis, column effluents were monitored by an UV detector (G131A) set at 210 nm and a refractometer (G1382A). The mobile phase (0.0005 N H_2SO_4) filtered through a 0.2 µm Millipore membrane filter was used at a low rate of 0.6 ml/min and 25 µl of the prepared sample were automatically injected.
The detection of targeted compounds was performed by refractometry for glucose, fructose, ethanol, glycerol and succinate and by UV spectrophotometry at a wavelength set at 210 nm for pyruvate and acetate.

Analysis of microbiological quality of produced palm wines

In order to control the quality of palm wine produced, coliforms bacteria and *Staphylococcus aureus* counts were performed (**Norme ISO 7218, 2007**). Ten (10) mL of each sample were mixed with 90 mL of sterile peptone solution. Serial dilution was performed with the sterile peptone solution and 100 µL of decimal dilutions were plated in duplicate on Petri dishes. Chapman's Agar medium (Sigma Aldrich, USA) were used for *Staphylococcus aureus* counts and Violet Red Bile Agar (VRBL) (Biokar, France) for coliforms bacteria. Plates were incubated for 48 hours at 37±2°C *Staphylococcus aureus* counts and VRBL plates that were incubated at 30±2°C for total coliforms and 44±2°C for thermo-tolerant coliforms for 48 hours. Counts were expressed as colony forming units per mL (cfu/mL).

Principal Component Analysis (PCA) of different palm wines

The comparison of different process was performed by Principal Component Analysis using the package FactoMineR of Rcommander of R 3.2.3.

Data analysis

Data analyses were performed with R 3.2.3. Data obtained were subjected to an analysis of variance (ANOVA) using the Fisher's least significant difference (LSD) test to determine significant differences between each sample (wine or sample) ($P \leq 0.05$). Principal Component Analysis (PCA) was performed to compare the different types of fermentation by using the package FactoMineR of Rcommander.

RESULTS AND DISCUSSION

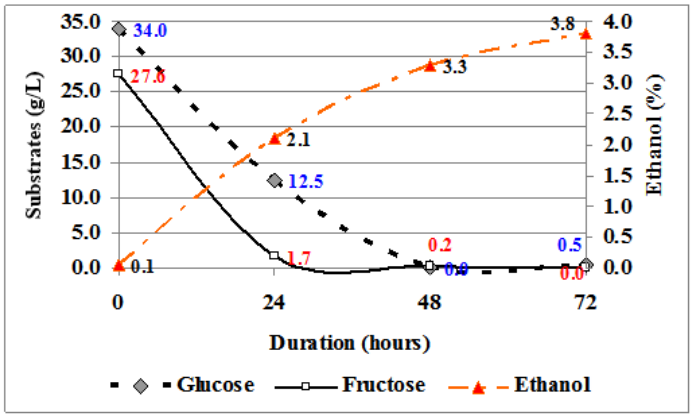
Strains used in this study were YBPW7, YBPW13 and YBPW25, isolated from *Borassus akeassii* wine. As presented in table 1, strains were identified as *S. cerevisiae* species by amplification and RFLP analysis of the 5-8S-ITS region (**Esteve-Zarzoso *et al.*, 1999**).

Table 1 Size (bp) of the PCR products and the restriction fragments

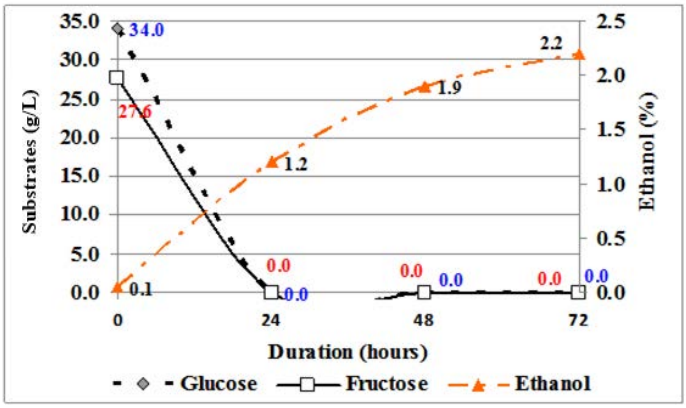
Strains	Fragments size (pb)	Restricted fragments size (pb)		Identification
		Hae III	Hinf I	
YBPW7	880	140-360	120-180-220-300	*S. cerevisiae*
YBPW13	850	140-380	120-180-220-300	*S. cerevisiae*
YBPW25	850	140-380	120-180-220-300	*S. cerevisiae*

Many authors reported that *S. cerevisiae* was the species as responsible for the fermentation and aroma of the wine (**Amoa-Awua *et al*,. 2007; Stringini *et al*., 2009; Ouoba *et al*., 2012**).

The kinetics of fermentation of palm sap by *S. cerevisiae* was presented in figure 1.



a. Controlled fermentation with *Saccharomyces cerevisiae* strains



b. Mix culture fermenation of *Borassus akeassii* sap by endegenous microorganisms

Figure 1 Kinetics of mix culture and controlled fermentation of *Borassus akeassii* palm wine

Substrates (glucose and fructose) detected in palm sap were consumed completely at 24 hours in the spontaneous (mix culture) fermentation as presented in figure 1a. According to **Opara et *al.* (2013)**, four micro-organisms were found to be frequently present during the mixed culture fermentation of palm sap. These micro-organisms in the order of succession are: Yeasts, *Micrococcus*, Lactic Acid bacteria and *Leuconostoc spp*.
We have analyzed substrates and metabolites present in palm sap and palm wines and the results are presented in table 2.

Table 2 Physico-chemical characteristics of crude sap and wines of *Borassus akeassii*

Samples	pH	Glucose (g/L)	Fructose (g/L)	Ethanol (%)	Glycerol (g/L)	Succinate (g/L)	Acetate (g/L)	Malate (g/L)	Lactate (g/L)	Pyruvate (g/L)
PWS	6.5	34.0	27.6	0.05	0.10	0.8	1.0	1.2	0.1	ND
SP	3.5	0.50	0.0	2.20	1.50	1.1	3.1	2.0	2.5	0.1
CF7	4.2	0.0	0.0	5.31	3.0	2.4	1.6	0.6	0.2	0.2
CF13	3.9	0.0	0.0	3.34	3.0	2.5	1.5	0.6	0.3	0.5
CF25	4.5	0.0	0.0	2.76	2.6	2.5	1.6	0.4	0.1	0.2

Legend: PWS: Palm wine sap; SP: Spontaneous fermentation; CFX: Controlled fermentation with strain YVPWX; ND: Not detected

Ethanol was also found in all samples that indicating the alcoholic fermentation. Ethanol content was ranged between 2.76 and 5.31 % for controlled fermentation versus 2.20 % for spontaneous fermentation. Glucose and fructose were about 34.0 g/L and 27.6 g/L respectively but sucrose was not detected in unfermented palm sap. There was an important production of glycerol in the controlled fermentation. Glycerol concentration was ranged between 2.6 and 3.0 g/L versus 1.5 g/L in spontaneous fermentation process. Glycerol was the major fermentation by-product of *Saccharomyces cerevisiae*, which indirectly contributes to the sensory character of wine (**Yalçin and Özba, 2006**).

The comparison of physicochemical characteristics of palm wines shows that the wine produced by mix culture fermentation is more acidic that those obtained by controlled fermentation. It has also been shown that the mix culture fermentation process is acidic as it progresses and there is proliferation of micro-organisms depending on the condition of the medium (**Opara *et al.*, 2013**). **Naknean *et al.* (2010)** reported that when fructose is available in wine and lactic acid bacteria are able to grow, they can produce equimolar amounts of lactic and acetic acids from fructose and this could constitute a serious source of acetic acid in wine.

The results of microbiological quality of palm sap and wine were presented in table 3.

Table 3 Microbiological characteristics of palm sap and wines of *Borassus akeassii*

Samples	TC (10^4)	FC (10^2)	*S. aureus* (10^5)
PWS	3.75	1.5	3.4
SP	1.75	0.75	1.5
CF7	ND	ND	ND
CF13	ND	ND	ND
CF25	ND	ND	ND

Legend: PWS: Palm wine sap; SP: Spontaneous fermentation; CFX: Controlled fermentation with strain YBPWX; ND: Not detected; TC: Total coliforms; FC: Thermotolerant coliforms

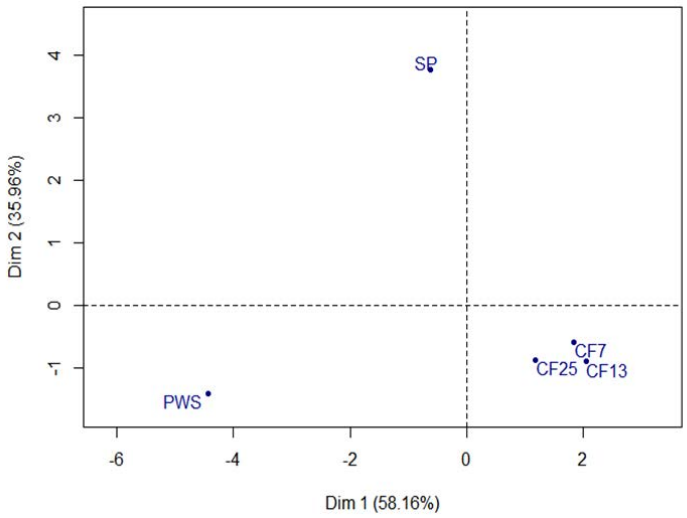
In the palm wine obtained by mix culture fermentation and the crude sap, we have detected coliforms and *Staphylococcus aureus* while in the controlled fermentation, they were not detected. The presence of these microorganisms revealed that unfermented palm sap used is collected under unhygienic condition (**Olawale *et al*,. 2010; Tapsoba et *al*., 2014**).

Bacteria and yeasts usually contaminate the juice as it is tapped and there are changes in biochemical composition of the palm wine (**Olawale *et al.*, 2010**). Normally, palm sap is a raw material to produce palm sugar syrup. Some factors affected the quality of palm sugar syrup such as processing method and quality of palm sap (**Phaichamnan *et al.*, 2010**).

Naknean *et al.* (2009) studied the effect of processing method on quality of palm sugar syrup. Flowing vapor sterilization could be important before fermentation of palm sap because the presence of potential endogenous microorganisms is avoided. The microbiological quality of palm sap becomes important to obtain a more quality and hygienic palm wine.

According to **Olawale *et al.* (2010)**, the sterilization and the use of purified *Saccharomyces* in fermentation of palm sap led to a more quality and hygienic palm wine. Of the yeasts responsible for palm wine fermentation, the predominant and best alcoholic fermenter was *Saccharomyces cerevisiae* (**Stringini *et al.*, 2009**).

Fermentation processes were compared using principal component analysis (PCA) as presented in figure 2. This analysis showed a good separation between fermentation processes. The PCA gives also an overview of the differences between the mix culture fermentation and controlled fermentation.



Legend: PWS**:** Palm wine sap; SP: Mixed culture fermentation; CFX: Controlled fermentation with *S. cerevisiae* strain X

Figure 2 Principal component analysis of palm wine fermentation

The wines produced by controlled fermentation (CF7; CF13 and CF25) were grouped together in the bottom right of the figure 2. The wine produced by mix culture fermentation (SP) was at the top of the figure while the unfermented sap (PWS) was shown in the bottom left. This analysis shows that there is a difference between the unfermented sap, the wine produced by mix culture fermentation and the wine obtained by controlled fermentation.

Figure 3 provides the flow chart for controlled fermentation for improved palm wine. Indeed, this diagram provides a wine, different of the wine obtained by mix culture fermentation. It enables the improvement of microbiological and physicochemical quality of palm wine.

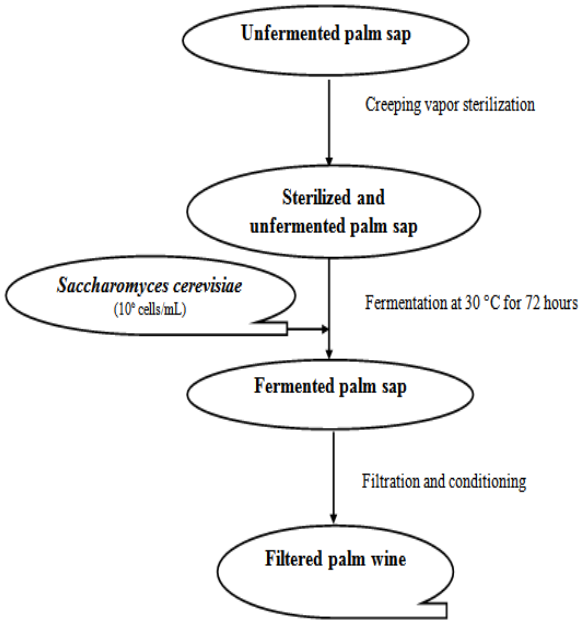


Figure 3 Flow chart (diagram) for controlled fermentation of palm wine

Flowing (Creeping) vapor sterilization eliminates undesirable microorganisms that could contaminate raw sap during its extraction process. According to the care applied in the collection of the crude sap, a first hypothesis on the presence of potential endogenous microorganisms in the sap was emitted (**Ben Thabet *et al.* 2010**).

CONCLUSION

Three *Saccharomyces cerevisiae* strains isolated from *Borassus akeassii* wines and identified by amplification and RFLP analysis of the 5-8S-ITS region were used for in vitro fermentation of unfermented palm sap.
This work confirmed that the use of active *Saccharomyces cerevisiae* strains for fermentation of palm sap gave a more quality and hygienic palm wine. Flowing vapor sterilization used during the production of conventional wines, can be used for the improvement of palm wine quality.

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PRESERVING THE QUALITY AND PROLONGATION THE SHELF-LIFE OF BEEF PACKED UNDER VACUUM OR MODIFIED ATMOSPHERE USING TERNARY ANTIOXIDANT BLEND

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ABSTRACT

Keeping the quality and prolongation the shelf-life of stored at 0 ± 0.5ºC packed under vacuum or modified (80%O_2/20%CO_2) atmosphere beef *m. semimembranosus* sprayed with 0.02% solution, containing 10 g.l^{-1} dihydroquercetin from Siberian larch (*Larix sibirica Ledeb*), 5 g.l^{-1} rosemary (*Rosmarinus officinalis*) extract and 1 g.l^{-1} L-ascorbic acid was studied. The experiments were carried out with five samples: control - air packaged; vacuum packaged; vacuum packaged and treated with 0.02% ternary antioxidant blend; packaged under modified atmosphere (80%O_2/20%CO_2); and packaged under rich in oxygen modified atmosphere, after spaying with 0.02% ternary antioxidant blend. Samples were stored 28 days (to 32 d *post mortem*) at 0 ± 0.5ºC. The pre-treatment of beef with ternary antioxidant blend preserve the sensory scores and colour properties of beef, and inhibited total microbial growth, and development of *Brochothrix termosphacta* and pathogens to the end of storage (28 d at 0 ± 0.5ºC), was found. The pre-treatment of beef with ternary antioxidant blend was not main factors which can affect the pH and free amino nitrogen changes in fresh beef. The pre-treatment of beef with 0.02% ternary antioxidant blend may be successfully used for preserving the quality and prolonging the shelf-life of beef *m. semimembranosus* packed under modified (80%O_2/20%CO_2) atmosphere. The shelf-life can extend with 75% compared to air packed meat, and with 7 days against only vacuum- or modified atmosphere packed beef.

Keywords: Natural antioxidants, vacuum, modified atmosphere packaging, meat, sensory properties, colour, microbiological status

INTRODUCTION

The main criterions in purchasing fresh beef on the market are its colour, flavour, odour and microbiological status (**Ercolini *et al.*, 2006**). The sensory properties are the most important factor determining the meat quality. That is way, the extension of the quality and shelf-life of packed chilled beef was one of necessity to meet the demands of consumers **(Lagerstedt *et al.*, 2011)**. In this respect, increasing attention was put on the packaging conditions. Vacuum packed meat changes its colour from bright-red to purple-brown during refrigeration **(Blixt and Borch, 2002)**. The reason is conversion of muscle oxymyoglobin in metmyoglobin **(Mancini and Hunt, 2005)**. By controlling the levels of residual oxygen in the package, prevention of this negative phenomenon is achieved. The rich in oxygen modified atmosphere packaging (MAP) preserves muscle oxymyoglobin two times longer, compared to air-packed meat **(Lagerstedt *et al.*, 2011)**. Unfortunately, the high-oxygen MAP system induces lipid and myoglobin oxidation and protein polymerization **(Kim *et al.*, 2010)**. Better oxidative, microbial and colour stability can be achieve when combined the effect of antioxidants and MAP **(Lund *et al.*, 2007; Rojas and Brewer, 2007)**.

For extending the shelf-life of the MAP (70%O_2/20%CO_2/10%N_2) beef was used rosemary or vitamin C solutions **(Djenane *et al.*, 2003)**. **Lund *et al.* (2007)** offered the protein and lipid oxidation in MAP (100% N_2, or 80%O_2/20%N_2) minced beef patties stored for 6 days in the dark at 4°C, to be inhibit by addition of rosemary extract or ascorbate/citrate (1:1) mixture. Natural antioxidants such as rosemary extracts **(Rohlik *et al.*, 2010, 2012)** and dihydroquercetin **(Semenova *et al.*, 2008; Bakalivanova and Kaloyanov, 2012)** have been proposed for prolongation of the shelf-life of meat products and especially the MAP beef **(Fernández-López *et al.*, 2005; Balev *et al.*, 2010)**.

Rosemary extracts (RE) were able to scavenge the free hydroxyl radicals converting them into stable products **(Djenane *et al.*, 2003; Brewer, 2011)**. The aqueous extract of rosemary (*Rosmarinus officinalis Linn.*) contains phenolic diterpenes (carnosic, carnosol, rosmanol, rosmadial, 12-methoxycarnosic acid, epi-, and iso-rosmanol) and phenolic acids (rosmarinic and caffeic) **(Brewer, 2011)**. While carnosic acid also has a single aromatic ring, it has two -OH groups that can serve as H donors. The vicinal -OH groups can chelate pro-oxidative metals thereby preventing oxidation. The polyphenols, rosmarinic acid has two aromatic rings, each with two -OH groups that are capable of donating H^+ and chelating metals. In lipid-based systems, carnosic acid and carnosol effectively chelate iron and scavenge peroxyl radical **(Djenane *et al.*, 2003; Lund *et al.*, 2007)**.

The dihydroquercetin (DHQ) is a dihydroflavonol. It is having properties of a powerful free radical-chain terminator **(Bakalivanova and Kaloyanov, 2012)**. The common characteristic of the flavonoids is the basic 15-carbon flavan structure ($C_6C_3C_6$). These carbon atoms are arranged in three rings (A, B, and C) **(Brewer, 2011)**. The free radical-scavenging potential of the dihydroquercetin includes: (1) Phenolic hydroxyls in flavonoids were the main active groups capable of scavenging $^{\bullet}$OH, (2) Hydroxyl groups in ring B and A were important $^{\bullet}$OH-scavenging active groups, (3) The ortho-dihydroxyl groups in ring A and/or B could greatly enhance the $^{\bullet}$OH-scavenging activity of the rings, (4) The hydroxyl groups on 3',4' position of ring B possessed the highest $^{\bullet}$OH-scavenging activity compared with hydroxyl groups in ring B, and was higher than that of hydroxyl groups in ring A **(Vladimirov *et al.*, 2009)**. The synergism and antagonism between quercetin and other chain-breaking antioxidants is possible, too **(Becker *et al.*, 2007)**.

L-ascorbic acid (AA) is a source of four -OH groups. AA can donate hydrogen to an oxidizing system, to chelate metal ions (i.e. Fe^{2+}), to scavenge free radicals, to quencher the $O_2^{-\bullet}$ radicals, and to acts as a reducing agent **(Brewer, 2011)**. At high levels (>1000 mg.kg^{-1}) AA shifts the balance between ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron, and acts as an oxygen scavenger. However, at low levels (<100 mg.kg^{-1}) it can catalyse oxidation in muscle tissue **(Yetella and Min, 2008)**. L-ascorbic acid can exert a synergistic effect when added along with polyphenolic antioxidants as play role of metals chelators **(Brewer, 2011)**.

The stored meat loses potential to oxygenate deoxymyoglobin. When beef is packaged under vacuum the oxygen content must be less than 0.05%. Addition of reducing agents is recommended to improve oxygen absorption **(Motoyama *et al.*, 2010)**. The inhibition of oxidative processes in beef using combination of

rosemary extracts, as free radical scavengers, which improves and enhances antioxidant effect of dihydroquercetin **(Silva *et al.*, 2002)** was still not discussed.
All the above-mentioned combinations of the treatment of MAP or VP meat with separate antioxidants have not shown a satisfactory effect when it comes to a more prolonged of 21 days storage at 0 - 4°C **(Fernández-López *et al.*, 2005; Kim *et al.*, 2010)**. In the available literature, we did not encounter information on the use of a combination of natural antioxidants and vitamin C, which exhibit a synergistic effect.
That is why, the objective of this study was to determine the effect of surface pre-treatment with of ternary antioxidant blend (TAB) containing dihydroquercetin extracted from Siberian larch *(Larix sibirica Ledeb)*, extract from rosemary (*Rosmarinus officinalis Linn.*), and L-ascorbic acid on the quality and shelf-life of (80%O_2/20%CO_2) modified atmosphere packed (MAP) or vacuum packed (VP) beef.

MATERIALS AND METHODS

The beef was supplied by the company "Unitemp" Ltd (Voyvodinovo village, district Plovdiv, Bulgaria). The carcass quarters were imported from slaughterhouse SC Nicbac ProdSRL (Loc. Nicolae Balcescu village, district Bacau, Romania). The carcasses were deboned, and the *m. semimembranosus* were packaged on the 4th day *post mortem*. The pH of meat was 5.36, and the temperature at the moment of packaging was around 1°C.
Three-component antioxidants blend solution was prepared as ten g DHQ and one g AA were dissolved in 25 ml 96% ethanol. Five g RE were emulsified in 20 cm^3 96% ethanol. Two liquids were mixed. The mixture was filled up to 1 dm^3 with 950 cm^3 bidistilled water.
The powdered dihydroquercetin extract (DHQ) from Siberian larch (*Larix sibirica Ledeb*) was purchased by Flavit Ltd. (Pushtino, Russia). It contains: 96% dihydroquercetin, 3% dihydrokempferol and 1% naringenin. The powdered rosemary extract (RE) was supplied by Aromena Ltd. (Sofia, Bulgaria). The content of flavonoids was approx. 42 $g.kg^{-1}$, and peroxide value (POV) = 0.658 ± 0.018 meqv $O_2.kg^{-1}$lipids. The L-ascorbic acid (AA) was purchased from Sigma Chemical Group Pty Ltd. (Balcatta, Perth WA, USA). All rest chemicals and reagents were purchased from E. Merck KGaA (Darmstadt, Germany).
The surfaces of the 50 kg beef *m. semimembranosus,* with temperature 6.3ºC, were sprayed with 1 dm^3 0.02% TAB. Samples were strained off for 60 min at 1.2°C, and were packaged in transparent polymer bags. The packaged samples were put into plastic boxes, labelled and stored at 0 ± 0.5°C before analysis. One part of examined samples was MAP (80%O_2/20%CO_2). The other part of experimental samples was VP. A packaging machine Yang SR1, model Polaris VAC, Ductto (Como via al Bassone, Italy) was used.
The experiments were carried out with 5 groups: control samples C - air packaged only, samples VP - vacuum packaged only, samples AVP - vacuum packaged and treated with 0.02% TAB, samples MAP - packaged under modified atmosphere (80%O_2/20%CO_2) only, and samples AMAP - packaged under rich in oxygen (80%O_2/20%CO_2) modified atmosphere, after spaying with 0.02% TAB. Samples were stored 28 days. The analyses were carried out on: 4 day *post mortem* (1 day of experiment), 11 day *post mortem* (7 day of storage), 18 day *post mortem* (14 day of storage), 25 day *post mortem* (21 day of storage), and 32 day *post mortem* (28 day of storage).The samples were obtained according ISO 3100-1:1991. Before analysis samples were stored at 0 ± 0.5°C no more than 6 h.
The panellists making sensory analysis participated in six training sessions and underwent performance testing as specified in guidelines developed by **Meilgaard *et al.* (2006)**. The panellists were passed the triangular test for differentiation of fresh and rancid meat taste, odor and colour. The beef roasts were evaluated by the panellists for aromatics (cooked beef/broth, cooked beef fat, chemical taste, serum/bloody and plum/prune), feeling factors (astringent, metallic and chemical burn) and basic tastes (salt, sour, bitter and sweet). The roast beef samples were also scored using 1-5 scale **(Larick and Turner, 1990)**. Examinations of beef samples were done after the packs opening. The chilled to 0°C samples were put in aluminium foil packs and grilled for 20 min at 200-250°C.
The colour measurement was made by colorimeter Konica Minolta model CR-410 (Konica Minolta Holding, Inc., Ewing, New Jersey, USA), purchased by Sending Inc. (Tokyo, Japan). By it was evaluated the brightness of the colour (L*), red (a*) and yellow (b*) color component **(Hunt *et al.*, 2012)**.
The modified titration method of Sørensen **(Lorenzo *et al.*, 2008)** was used for free amino nitrogen determination in beef samples.
pH value of the beef was determined using pH-meter MS 2004 (Microsyst Ltd., Plovdiv, Bulgaria), equipped by combined pH electrode Sensorex Combination Recorder S 450 CD (Sensorex pH Electrode Station, Garden Grove, CA, USA **(Young *et al.*, 2004)**.
The total aerobic plate count was determined by EN ISO/DIS 4833-2003 **(Cohen *et al.*, 2007)**. The *Escherichia coli* were estimated according ISO 16649-1:2001 **(Nastasijevic *et al.*, 2009)**. The *Salmonella* bacteria determinations were carried out following ISO 6579-2002 **(Piknová *et al.*, 2002)**, and the *Listeria monocytogenes* - using ISO 11290-2002 **(Scotter *et al.*, 2004)**. The *Brochothrix termosphacta* was determined by BSS ISO 13722:2002 **(Russo *et al.*, 2004)**, and of *Enterobacteriaceae spp.* - using BSS ISO 21528-1:2011 **(Ercolini *et al.*, 2006)**.
The data were analysed using factorial analysis of variance procedure (mixed procedure) of SAS Version 8.2 software package **(SAS Institute Inc., 2002)**. The model included the main effects and interactions of nine treatments and four storage times. Multiple tests were used to separate means at 95% significance level for each test.

RESULTS

Sensory evaluated properties

At four (C, VP, AVP, and MAP) from five examined samples steadily decreasing average scores for sensory evaluated beef *m. semimembranosus* surface colour during storage at 0 ± 0.5ºC were assessed. The only exception were samples AMAP, (Fig 1).
The colour of the samples VP was unacceptable after 28 days of refrigeration storage at 0 ± 0.5ºC (32 d *post mortem*). At the end of the experiment (28 d), the colour of the samples AVP and MAP remains acceptable and their marks undergoes reduction by 27 % and 36 % respectively. The reduction in samples VP was 57 %.
During the storage period (28 d at 0 ± 0.5ºC) the odour's scores of samples C, VP, AVP and MAP decreases significantly ($P < 0.05$). The only exception was samples AMAP, where the odour scores were very high throughout the studied period and the average values was not significantly ($P > 0.05$) different to the end of the experiment (Fig 1). Reduction of the smell average scores in samples MAP was 28.0%, while the established decrease in samples AVP was 16.3%.
Similarly to the results obtained for smell, in four (C, VP, AVP and MAP) from the five examined samples the scores for taste decreases during the storage period (28 d at 0 ± 0.5ºC). The only exception were the samples AMAP. In these samples (AMAP) the taste scores were found very high throughout the studied period and the average values did not significantly ($P > 0.05$) different by the end of the experiment (Figure 1). After 28 days of refrigeration storage at 0 ± 0.5ºC (32 d *post mortem*), the flavour of samples VP was completely unacceptable and unpleasant as scores were decreased by 57.1%. The flavour of samples AVP and MAP retained acceptable to the end of the experiment (28 d at 0 ± 0.5ºC). The reduction of the flavour scores in samples MAP was 32.0%, and in samples AVP - 18.4 %. Only the combination of the surface treatment of beef *m. semimembranosus* with 0.02% TAB, and MAP (80%O_2/20%CO_2), was able to preserve the good sensory properties and to extend the shelf-life of meat to 28 day (or up to 32 day *post mortem*). The MAP samples demonstrated good sensory properties up to 21st day after packaging (up to 25 day *post mortem*). During the storage period (28 d at 0 ± 0.5ºC) the highest decrease in sensory evaluated colour was established in vacuum packaged beef (Fig 1).

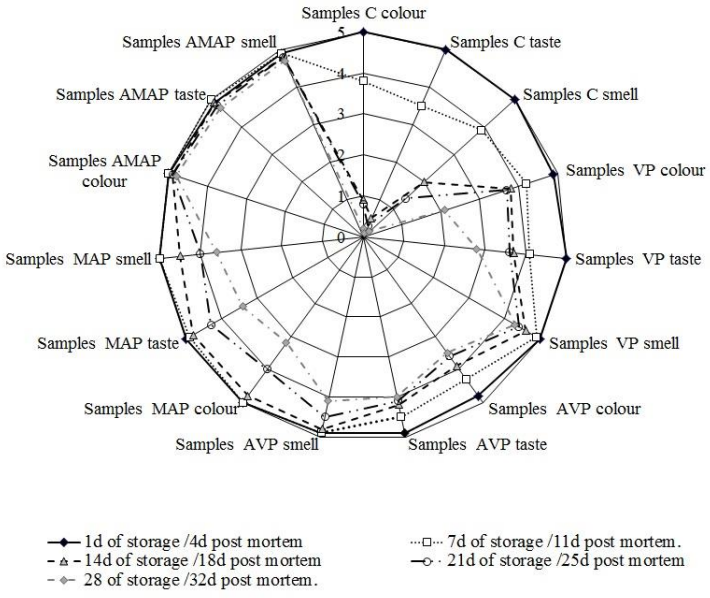

Figure 1 Effect of the ternary antioxidant blend superficial treatment on the sensory characteristics of beef *m. semimembranosus* packaged under vacuum and rich of oxygen modified atmosphere during storage at 0 ± 0.5ºC.
Legend: **samples C** - controls only air packaged; **samples VP** - vacuum packaged only; **samples AVP** - vacuum packaged and treated superficially with 0.02% tertiary antioxidant blend; **samples MAP** - packaged under modified atmosphere

($80\%O_2/20\%CO_2$) only, **samples AMAP** - packaged under rich in oxygen ($80\%O_2/20\%CO_2$) modified atmosphere, after superficial spaying 0.02% tertiary antioxidant blend

Instrumentally measured colour characteristics

The results obtained for instrumentally measured colour characteristics (Table 1) of beef *m.–semimembranosus* during storage at 0 ± 0.5°C correspond with the data from sensory analysis (Fig 1). During the refrigeration storage (28 d at 0 ± 0.5ºC) the colour lightness (L*) of control samples C significantly ($P \leq 0.05$) decrease with 7.33% comparing with initial values.

Table 1 Effect of the three-component antioxidant blend superficial treatment on the colour characteristics of the external surface of beef *m. semimembranosus* packaged under vacuum and rich of oxygen modified atmosphere during storage at 0 ± 0.5ºC

Samples	Storage time, days	The colour lightness, (L*)	Redness (a*)	Yellowness (b*)
Control samples C	1d	38.36 ^{a} ± 0.47	23.55 ^{a} ± 0.49	9.65 ^{a} ± 0.33
	7d	38.37 ^{a} ± 0.46	23.28 ^{a} ± 0.52	9.50 ^{a} ± 0.34
	14d	37.02 ^{b} ± 0.39	14.37 ^{b} ± 0.48	10.44 ^{b} ± 0.46
	21d	36.40 ^{b} ± 0.38	12.83 ^{c} ± 0.47	11.83 ^{c} ± 0.37
	28d	35.55 ^{c} ± 0.43	11.36 ^{d} ± 0.39	12.67 ^{d} ± 0.36
Samples VP	1d	38.36 ^{a} ± 0.47	23.55 ^{a} ± 0.66	9.65 ^{a} ± 0.33
	7d	38.14 ^{a} ± 0.42	23.26 ^{a} ± 0.50	9.63 ^{a} ± 0.59
	14d	37.27 ^{b} ± 0.38	20.40 ^{b} ± 0.63	6.77 ^{b} ± 0.49
	21d	37.09 ^{b} ± 0.34	20.19 ^{b} ± 0.53	6.65 ^{b} ± 0.37
	28d	36.36 ^{c} ± 0.33	19.14 ^{c} ± 0.45	5.90 ^{c} ± 0.35
Samples AVP	1d	38.36 ^{a} ± 0.47	23.55 ^{a} ± 0.49	9.65 ^{a} ± 0.33
	7d	38.47 ^{a} ± 0.44	23.63 ^{a} ± 0.51	10.58 ^{b} ± 0.50
	14d	38.36 ^{a} ± 0.51	22.40 ^{b} ± 0.50	10.51 ^{b} ± 0.44
	21d	37.86 ^{b} ± 0.45	21.30 ^{c} ± 0.43	9.39 ^{c} ± 0.39
	28d	36.89 ^{c} ± 0.42	20.85 ^{d} ± 0.48	8.67 ^{d} ± 0.32
Samples MAP	1d	38.36 ^{a} ± 0.47	23.55 ^{a} ± 0.39	9.65 ^{a} ± 0.33
	7d	39.96 ^{b} ± 0.46	24.09 ^{b} ± 0.36	10.26 ^{b} ± 0.56
	14d	39.33 ^{b} ± 0.42	23.86 ^{b} ± 0.45	11.49 ^{b} ± 0.58
	21d	38.42 ^{c} ± 0.32	21.01 ^{c} ± 0.46	12.64 ^{c} ± 0.47
	28d	34.77 ^{d} ± 0.39	16.78 ^{d} ± 0.48	14.53 ^{d} ± 0.68
Samples AMAP	1d	38.36 ^{a} ± 0.47	23.55 ^{a} ± 0.39	9.65 ^{a} ± 0.33
	7d	38.87 ^{a} ± 0.49	23.56 ^{a} ± 0.36	10.21 ^{a,b} ± 0.38
	14d	38.49 ^{a} ± 0.47	23.51 ^{a} ± 0.41	10.77 ^{b} ± 0.47
	21d	38.26 ^{a} ± 0.43	23.42 ^{a} ± 0.39	11.01 ^{b} ± 0.43
	28d	37.98 ^{a} ± 0.42	23.18 ^{a} ± 0.33	11.34 ^{b} ± 0.39

Means ± standard divinations
$^{a, b, c, d}$ – indexes show data with statistical different value in columns ($p < 0.05$)

Similarly to the control samples C, the colour lightness (L*) in samples VP decreases with 5.21%. However, on 28th day of storage the colour lightness (L*) of samples MAP was reduced with 9.36% ($P \leq 0.05$). A decrease by 3.83% in the L* value of samples AVP was estimated. Only the lightness (L*) of the samples AMAP does not change significantly ($P > 0.05$) throughout the 28 day period of the refrigeration storage at 0 ± 0.5°C. Our results was similar to the data obtained by **Ahn *et al.* (2002)** which determined beef patties containing antioxidants had higher L* values after 9th day of storage.

The colour redness (a*) in four of examined samples (C, VP, AVP, and MAP), decreases significantly ($P \leq 0.05$) during 28 days of storage at 0 ± 0.5ºC. The established decreases were with 51.76%, 18.73%, 11.46%, and 28.75% respectively, compared with initial values. For the studied period (28 days at 0 ± 0.5ºC) only the a*-values was of samples AMAP was unchanged ($P > 0.05$).

The results obtained for colour yellowness (b*) indicate different trends between beef samples during 28 days storage at 0 ± 0.5°C (Table 1). The b* values of control samples C and samples MAP increased significantly ($P \leq 0.05$) with 31.3%, and with 50.6%. Superficial pre-treatment of beef with 0.02% natural antioxidant mixture increases the b* values of rich in oxygen modified packaged samples AMAP only to 17.5%. Contrary, a decreasing of the b* values was observed in VP samples, and was less pronounced in samples AVP (Table 1). After 28th day of storage at 0 ± 0.5°C the colour yellowness (b*) in samples VP decrease with 38.9%, and in samples AVP – with 10.2% **(**Table 1).

pH value

The pH of experimental samples VP, AVP, MAP and AMAP increases very slightly, but significantly ($P \leq 0.05$) during 28 days of storage at 0 ± 0.5ºC (to 32nd day *post mortem*) (Table 2). The only exception were control samples C, with 18.21% increase of pH. At the end of the experiment (28 d at 0 ± 0.5ºC) maximal increase of the pH was established in samples MAP and AMAP ($P > 0.05$). For the studied period (28 d at 0 ± 0.5ºC) the pH of samples VP and AVP increase with 6.38% and 6.92% respectively.

Free amino nitrogen

A significant ($P \leq 0.05$) increasing of FAN was determined in all samples (Table 2). After 28 days of storage (32 d *post mortem*) FAN content in control samples C raised 27.8 times. The increase in experimental samples was from 15.2 (samples VP) to 15.8 times (sample AVP). During 28 days of refrigeration storage (0 ± 0.5ºC) the FAN content in test samples does not exceed 10 $mg.100g^{-1}$ meat. These results confirm the data from sensory estimated beef odour and flavour and were evidence for a proteolysis.

Table 2 Effect of the three-component antioxidant blend superficial treatment on the pH and free amino nitrogen content of beef *m. semimembranosus* packaged under vacuum and rich of oxygen modified atmosphere during storage at 0 ± 0.5ºC

Samples	Storage time, d	pH	Free amino nitrogen, $mg.kg^{-1}$ meat
Control samples C	1d	5.49 ^{a} ± 0.03	0.22 ^{a} ± 0.09
	7d	5.83 ^{b} ± 0.02	4.15 ^{b} ± 0.52
	14d	5.83 ^{b} ± 0.04	5.07 ^{c} ± 0.48
	21d	6.09 ^{c} ± 0.05	8.75 ^{d} ± 0.47
	28d	6.49 ^{d} ± 0.06	10.04 ^{e} ± 0.39
Samples VP	1d	5.49 ^{a} ± 0.03	0.22 ^{a} ± 0.09
	7d	5.54 ^{a} ± 0.04	3.86 ^{b} ± 0.50
	14d	5.56 ^{a} ± 0.05	4.69 ^{c} ± 0.63
	21d	5.61 ^{b} ± 0.04	5.29 ^{d} ± 0.53
	28d	5.62 ^{b} ± 0.02	6.04 ^{e} ± 0.45
Samples AVP	1d	5.49 ^{a} ± 0.03	0.22 ^{a} ± 0.09
	7d	5.63 ^{b} ± 0.03	3.94 ^{b} ± 0.51
	14d	5.64 ^{b} ± 0.04	4.87 ^{c} ± 0.50
	21d	5.66 ^{b} ± 0.04	5.83 ^{d} ± 0.43
	28d	5.75 ^{c} ± 0.04	6.42 ^{e} ± 0.48
Samples MAP	1d	5.49 ^{a} ± 0.03	0.22 ^{a} ± 0.09
	7d	5.47 ^{a} ± 0.04	3.68 ^{b} ± 0.36
	14d	5.78 ^{b} ± 0.03	4.63 ^{c} ± 0.45
	21d	5.81 ^{b,c} ± 0.04	5.32 ^{d} ± 0.46
	28d	5.87 ^{c} ± 0.04	6.01 ^{e} ± 0.48
Samples AMAP	1d	5.49 ^{a} ± 0.03	0.22 ^{a} ± 0.09
	7d	5.64 ^{b} ± 0.03	3.89 ^{b} ± 0.36
	14d	5.75 ^{c} ± 0.05	4.75 ^{c} ± 0.41
	21d	5.78 ^{c} ± 0.04	5.52 ^{d} ± 0.39
	28d	5.84 ^{d} ± 0.04	6.31 ^{e} ± 0.33

Means ± standard divinations
$^{a, b, c, d, e}$ – indexes show data with statistical different value in columns ($p < 0.05$)

Microbiological examinations

The standard examined microbiological indices for freshness and hygiene of samples were in the norms during whole 28th day period of storage at 0 ± 0.5°C (Table 3). These results was evidence that all examined samples meet the requirements of Regulation (EC) № 1441/2007. The only exception was found in control samples C on the 28th day of storage. In these samples a triple increase of the *E.coli* growth was determined.

The similar results were observed for the *Enterobacteriaceae spp.* In samples VP, AVP, MAP and AMAP the absence or very limited growth of *Enterobacteriaceae spp.*, determined between 14th and 28th day of storage at 0 ± 0.5ºC were found. In contrast, in control samples C was established systematic irregular increase in the *Enterobacteriaceae spp*, more pronounced after 21st day of storage when viewed 128.6 times more colonies, and further – on the 28th day – 653.6 times. However, the *Brochothrix thermosphacta* colonies increased significantly ($p \leq 0.05$), but on the end of storage (28th day) not exceed 50000 - 1890000 cfu.g in different samples. The most pronounced increase was rapidly detected in control samples C - 9450 times from the initial number. Ten-fold smaller number of *Brochothrix thermosphacta* colonies was determined in samples AMAP. The slowest increase of the *Brochothrix thermosphacta* colonies – only 250 times was found in samples AVP.

Table 3 Effect of the three-component antioxidant blend superficial treatment on the microbiological status of beef *m. semimembranosus* packaged under vacuum and rich of oxygen modified atmosphere during storage at 0 ± 0.5ºC

Samples	Storage time at 0 ± 0.5ºC, d	Total mesophilic aerobic and facultative anaerobic microorganisms, cfu/g Norm 5.10^5- 5.10^6 cfu/g	*Brochothrix termosphacta*, cfu/g Norm is not listed	*Enterobacteriaceae*, cfu/g Norm is not listed	*Escherichia coli*, cfu/g Norm *500 - 5000* cfu.g^{-1}	*Salmonella* spp., Presence in 25 g Norm: Absence in *25 g sample*	*L. monocytegenes*, Presence in 1 g Norm: Absence in *1 g sample*
Control samples C	**1d**	534 ^{a} ± 38	200 ^{a} ± 24	28 ^{a} ± 15	20 ^{a} ± 8	-	-
	7d	1938 ^{b} ± 28	970 ^{b} ± 37	50 ^{a,b} ± 8	40 ^{b} ± 7	-	-
	14d	321000 ^{c} ± 592	130000 ^{c} ± 146	60 ^{b} ± 10	50 ^{b} ± 10	-	-
	21d	962400 ^{d} ± 2608	784000 ^{d} ± 569	3600 ^{c} ± 187	3300 ^{c} ± 155	-	-
	28d	2314000 ^{e} ± 4183	1890000 ^{e} ± 1258	18300 ^{d} ± 433	15200 ^{d} ± 373	-	-
Samples VP	**1d**	534 ^{a} ± 38	200 ^{a} ± 24	28 ^{a} ± 15	20 ^{a} ± 8	-	-
	7d	1606 ^{b} ± 36	760 ^{b} ± 30	-	-	-	-
	14d	20300 ^{c} ± 384	25000 ^{c} ± 133	-	-	-	-
	21d	71400 ^{d} ± 548	50000 ^{d} ± 487	-	-	-	-
	28d	195200 ^{e} ± 837	130000 ^{e} ± 792	26 ^{a} ± 13	19 ^{a} ± 8	-	-
Samples AVP	**1d**	534 ^{a} ± 38	200 ^{a} ± 24	28 ^{a} ± 15	20 ^{a} ± 8	-	-
	7d	960 ^{b} ± 24	850 ^{b} ± 32	-	-	-	-
	14d	12300 ^{c} ± 348	6200 ^{c} ± 90	-	-	-	-
	21d	44600 ^{d} ± 894	7000 ^{d} ± 355	-	-	-	-
	28d	179000 ^{e} ± 1000	50000 ^{e} ± 669	14 ^{a} ± 9	10 ^{a} ± 7	-	-
Samples MAP	**1d**	534 ^{a} ± 38	200 ^{a} ± 24	28 ^{a} ± 15	20 ^{a} ± 8	-	-
	7d	1380 ^{b} ± 27	1240 ^{b} ± 95	-	-	-	-
	14d	4118 ^{c} ± 93	3200 ^{c} ± 55	-	-	-	-
	21d	321000 ^{d} ± 837	68000 ^{d} ± 852	-	-	-	-
	28d	981800 ^{e} ± 2049	121000 ^{e} ± 1653	-	-	-	-
Samples AMAP	**1d**	534 ^{a} ± 38	200 ^{a} ± 24	28 ^{a} ± 15	20 ^{a} ± 8	-	-
	7d	820 ^{b} ± 26	400 ^{b} ± 23	-	-	-	-
	14d	980 ^{c} ± 286	640 ^{c} ± 28	-	-	-	-
	21d	104200 ^{d} ± 707	12500 ^{d} ± 410	-	-	-	-
	28d	878800 ^{e} ± 3564	189000 ^{e} ± 806	27 ^{a} ± 11	16 ^{a} ± 6	-	-

Means ± standard divinations.
$^{a, b, c, d}$ – indexes show data with statistical different value in columns ($p < 0.05$) ***Legend:*** With the sign "**-**" is result marked "***is not detectable***".

DISCUSSION

Similarly to our results **Djenane *et al.* (2003)** found a significant reduction of the **sensory evaluated colour scores** after addition of rosemary and ascorbic acid mixture. Those authors **(Djenane *et al.*, 2003)** explain their findings by the rates of metmyoglobin formation and the extended shelf-life from about 10 to 20 days. Our results about sensory evaluated odor were confirmed and by findings of **Rojas and Brewer (2007)** which were determined the effect of 0.02% rosemary oleoresin on colour stability of cooked beef patties stored at 4ºC for 8 days. At the end of storage (28d at 0 ± 0.5°C) the determined light off-odor in samples VP, AVP and MAP, were associated with lipid oxidation rancidity, and described as a smell of wet cardboard. The MAP of beef gives brightly red colour on the meat surface, but high oxygen content in MAP leads to rancid taste, while meat is still attractive red. The high oxygen atmosphere promotes the myoglobin oxidation and prolongs the time for metmyoglobin formation on meat surface **(Kim *et al.*, 2010).** However, up to 21st day of storage samples AVP received comparatively higher sensory evaluated flavor scores. The main reason was the use of DHQ as inhibitor of lipid oxidation **(Semenova *et al.*, 2008)**, which in combination with rosemary **(Ahn *et al.*, 2002)** and ascorbic acid effectively preserve the bright red beef colour and reduce the worm over-flavor scores. The results obtained for colour brightness (L*) can be explained with properties of antioxidants used in TAB. Rich in phenolic components rosemary extract show strong activity and separating H^+ ions removed hydrogen peroxide and reactive oxygen radical species **(Brewer, 2011)**. L-ascorbic acid donate hydrogen, chelate metal ions (i.e. Fe^{2+}), scavenge free radicals and quencher the peroxyl ($O_2^{-\bullet}$) radicals **(Brewer, 2011)**. On the other hand, DHQ release H^+ protons and reduce prooxidant activity of metal ions with mobile valence such as iron and copper ions, "free" and heme iron **(Silva *et al.*, 2002)**.

The results obtained about colour redness (a*) confirm the hypothesis of **Lund *et al.* (2007)** who claim that in high oxygen atmospheres rosemary extract protected the fresh red meat colour. Our results are similar to **Akarpat *et al.* (2008)** research about use of hot-water rosemary extracts for prevention of colour changes in beef patties, and confirm **Rohlik *et al.* (2012)** findings about positive effect of RE addition on colour in the dried/cooked sausages. A TAB which is a powerful polyphenolic antioxidant and plays role of an inhibitor of free radical formation **(Silva *et al.*, 2002)** probably reduced velocity of the oxymyoglobin oxidation in AMAP samples. Similarly to our findings about colour stability **Berruga *et al.* (2005)** confirmed optimal levels in VP and MAP lamb *m. longissimus dorsi*. **Berruga *et al.* (2005)** confirm that after 7th day of refrigeration storage at 2°C, the b* value increased and a* value decreased. The results of the **Berruga *et al.* (2005)** regarding colour lightness (L*) were opposite of our findings. The analysis of those results showed that the combination between superficial treatment with 0.02% TAB, and MAP can stabilize L*, and a* values, and was able to minimize the increase of b* value of fresh beef comparing with vacuum and air packaging.

When commenting the pH of the control samples C it should be noted that they were in the process of deep deterioration with a strong sour smell, probably caused by the development of lactic acid bacteria **(Blixt and Borch, 2002)**. Under similar conditions, a higher pH can accelerate the respiratory activity of the muscle tissue and to form a purple deoxymyoglobin **(Rhee *et al.*, 2004)**. This explains the purple colour of VP samples. On the other hand, most myoglobin-reducing activity was recorded at pH 7.4 **(Hutchison *et al.*, 2010)**. The myoglobin reduction decreased with pH decline to 5.7 (the pH established at the end of the storage). The lower pH values in samples VP can be caused by more rapid development of anaerobes **(Blixt and Borch, 2002)**, or comparatively high activity of lactic acid bacteria **(Borch *et al.*, 1996)** under those conditions. Our results showed that the beef treatment with 0.02% TAB had no effect on the pH

changes of meat and the main factor for MAP beef spoilage was the growth of lactic acid bacteria. At comparatively low pH and higher osmotic pressure proteolytic enzymes, such as calcium-neutral proteases **(Hool and Corry, 2007)**, calpains **(Sazili *et al.*, 2004)**, or cathepsins **(Polidori *et al.*, 2001)** have been activated, in a result of exempted calcium ions in sarcoplasmic reticulum **(Geay *et al.*, 2001)**. Under those conditions bacteria associated with the spoilage of chilled meat, had negative effects such as sour off-flavour, discoloration, gas production, slime production and decrease in pH, and consist except of *Brochothrix thermosphacta*, *Carnobacterium spp., Lactobacillus spp., Leuconostoc spp.* and *Weissella spp.* **(Borch *et al.*, 1996)**.While the *Brochothrix thermosphacta* dominates in MAP beef **(Baranyi *et al.*, 1996)**, the main microflora in VP beef was probably lactic acid bacteria **(Blixt and Borch, 2002)**. More favourable microbiological results in VP beef can be explained by the suppression of *Brochothrix thermosphacta*, anaerobic, and lactic acid bacteria, *Pseudomonas* and *Enterobacteriaceae spp*. to levels that would not cause meat spoilage **(Blixt and Borch, 2002)**. The growth of aerobic microflora decreases in VP meat **(Motoyama *et al.*, 2010).** Compared to the control samples C, MAP ($80\%O_2/20\%CO_2$) with or without TAB treatment extend the shelf-life of beef with 14 days. Similarly to our findings **Djenane *et al.* (2003)** significantly reduced the rates of microbial growth using the mixture of rosemary and ascorbic acid. The rosemary extract influenced only on the growth of lactic acid bacteria **(Fernández-López *et al.*, 2005).**

CONCLUSIONS

The results obtained allowed us to conclude that the packaging of beef in modified atmosphere ($80\%O_2/20\%CO_2$), combined with preliminary superficial treatment with 0.02% TAB containing DHQ, RE, and AA may extend the shelf-life of beef stored at 0 ± 0.5°C till 32nd day *post mortem* (to 28th day of storage). This shelf-life is with 7 days (25%) longer in comparison with beef packed under rich in oxygen modified atmosphere or under vacuum. The combination of pre-treatment of beef with TAB and rich in oxygen MAP preserve the sensory properties, stabilize the L* and a* values, and provide slower increase of the b* value. The pre-treatment of beef with TAB and packaging in rich oxygen MAP inhibit the microbial growth and prevent meat spoilage.

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