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MANUFACTURE OF BIO-LABNEH USING ABT CULTURE AND BUFFALO AND SOY MILK MIXTURES

Magdy M. Ismail[1], Gehan A. Ghoneem[2], Naeem A. EL- Boraey[2], Mohamed M. Tabekha[2] and Hoda F. Elashrey[1]*

Address(es):
[1]Dairy Technology Department, Animal Production Research Institute, Agricultural Research Center, Dokki, Giza, Egypt.
[2]Food Industries Department, Faculty of Agriculture, Mansoura University, El-Mansoura, Egypt.

*Corresponding author: magdy250@yahoo.com

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ABSTRACT

The effect of using soy milk and ABT culture on various Labneh properties was investigated. Six treatments of Labneh were made from soy and buffalo milk and from their mixtures using classic and ABT-5 starters. Labneh made from soy milk had the lowest yield, acidity, redox potential (E_h), total solids (TS), fat, ash, TN, TVFA, saturated fatty acids (SFA), monounsaturated fatty acids (MUSFA), and total amino acids contents while buffalo's milk Labneh had the highest. Soy milk Labneh had the highest level of unsaturated fatty acids (USFA), polyunsaturated fatty acids (PUSFA) and linoleic acid and α-linolenic acid. Also, soy milk Labneh had the greatest count of *Str. thermophillus*, *L. acidophilus* and bifidobacteria. Utilization of soy milk only or mixed with buffalo milk in Labneh manufacture decreased the count of *L. bulgaricus*. Mixing of buffalo milk with soy milk increased the sensory evaluation scores of Labneh. Classic starter Labneh had higher values of yield, acidity, WSN, TVFA, SFA and lower values of pH than those of ABT Labneh. Using of ABT culture in Labneh manufacturing increased USFA and total free amino acids contents. ABT Labneh had higher numbers of *Str. thermophillus* than those of classic starter.

Keywords: Soy milk, ABT, Buffalo milk, Labneh

INTRODUCTION

In the Middle East region, concentrated yoghurt (Labneh) is highly appreciated and consumed with bread all year a round. Labneh is an important supplement to the local diet and provides vital elements for growth and good health (**Shamsia and El-Ghannam, 2012)**. According to Lebanese standards, Labneh is defined as a semisolid food derived from yogurt by draining away part of its water and water-soluble compounds **(Mohameed *et al.*, 2004)**. Usually, Labneh is prepared with two solids concentration. The first is 22% where Labneh is prepared to be consumed within two weeks and usually stored in refrigerators. The second is 40% and Labneh is stored in vegetable oil at room temperature and can be consumed within two years **(Keceli *et al.*, 1999)**.
On the other hand, soybean-based products are very popular and have attracted worldwide interest. China, the largest importing country to domesticate soybeans and a major global soybean grower and consumer, has extensive distributions of soybean accessions **(Li *et al.*, 2014)**. Soya milk is the biggest soya-based product consumed in the world, not only because of its potential health benefits but also as an alternative to cow milk targeting for lactose-intolerant individuals, those allergic to milk proteins, or those avoiding consuming milk for other reasons **(Reilly *et al.*, 2006)**. It is made from soaked soybeans by grinding, heating, and filtering **(Ikya *et al.*, 2013)**.
ABT culture (*Lactobacillus acidophilus*, *Bifidobacterium* spp., and *Streptococcus thermophilus*) was chosen for soymilk fermentation because of the positive interaction between probiotic strains *Bifidobacterium* spp., and *Lactobacillus acidophilus* **(Tamime *et al.*, 2005)**. Besides that, *Streptococcus thermophilus* is always the dominant strain in mixed cultures and shows the greatest growth regardless of the fermentable substrates, e.g. in cow milk, goat milk **(Božanić *et al.*, 2002)**, or in soymilk **(Božanić *et al.*, 2008)**. The healthy importance of probiotic bacteria was established from many decades ago. To overcome the loss of viability of probiotic bacteria, the present trend is to use starter cultures for yoghurt production with no *Lactobacillus delbrueckii* subsp. *bulgaricus*, such as ABT. *S. thermophiles*, which is less proteolytic than *L. bulgaricus*, is the main organism responsible for fermentation in ABT cultures **(Parmar, 2003)**. The objective of this study was to test the possibility of manufacturing of Labneh from soy and buffalo milk using classic and ABT cultures. Chemical composition, bacteriological parameters and sensory evaluation of Labneh were monitored.

MATERIALS AND METHODS

Milk, Soybeans and Starter Culture

Fresh buffalo's milk was obtained from private farm in Damiette Governorate, Egypt. Yellow soybeans (*Glycine max* (L.) were purchased from a local grocery in Damiette Governorate.
A commercial classic yoghurt starter containing *Streptococcus thermophillus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (1:1) and ABT-5 culture which consists of *S. thermophiles*, *Lactobacillus acidophilus* + *B. bifidum* (Chr. Hansen's Lab A/S Copenhagen, Denmark) were used. Starter cultures were in freeze-dried direct-to-vat set form and stored at –18°C until used.

Preparation of Soymilk

Beans of good quality were carefully selected and soaked overnight 12-18 h, at room temperature in ultrapure water contained 0.5% $NaHCO_3$. Once soaked, water was discarded and the grains were re-soaked in boiling water for 15 min then, hulls were removed under running water by manual rubbing. The peeled soybeans were next rinsed and drained with cold water several times. Of the water to be added to the soaked beans (1:6 beans: water ratios), about half was added at room temperature (23ºC) and blended with the beans at high speed for 10 min. The remaining water was heated to 80ºC and added to the slurry to enhance protein extraction. This mixture was blended for an additional 3 min. at high speed. The resultant slurry was filtered through 3 layers of cheese-cloth to remove coarse material (okara, which is mainly composed of insoluble fiber material). Thereafter, the isolated soymilk was boiled on a low heat for 5 min. to destroy trypsin inhibitor for improving flavor and cooled down to 25°C **(Ikya *et al.*, 2013)**.

Labneh Preparation

Labneh was made using the procedure normally used in homes. Fresh milk was heated to 85°C for 15 min., cooled to 40°C, inoculated with cultures (0.1 g/L of yoghurt mix) and incubated at 40°C for fully coagulation. The produced yoghurt was left at 4°C overnight, mixed and put into cloth bags which were hung for 24 h in a refrigerator to allow for whey drainage. The resulting Labneh was mixed

with 1.5% salt and transferred to 250g plastic cups, covered with polyethylene film and stored at 5°C for 28 days. Chemical, bacteriological and sensorial tests were occurred in Labneh samples in fresh and after 7, 14, and 21 days of refrigerated storage. Table 1 shows various Labneh treatments.

Table 1 Labneh treatments

Abbreviations	Treatments
A	Labneh made from soy milk and classic starter
B	Labneh made from buffalo's milk and classic starter
C	Labneh made from 75% buffalo's milk + 25% soymilk and classic starter
D	Labneh made from soy milk and ABT culture
E	Labneh made from buffalo's milk and ABT culture
F	Labneh made from 75% buffalo's milk + 25% soymilk and ABT culture

Chemical analysis

Total solids, fat, total nitrogen and ash contents of samples were determined according to **AOAC (2000)**. Titratable acidity in terms of % lactic acid was measured by titrating 10g of sample mixed with 10ml of boiling distilled water against 0.1 N NaOH using a 0.5% phenolphthalein indicator to an end point of faint pink color. pH of the sample was measured at 17 to 20°C using a pH meter (Corning pH/ion analyzer 350, Corning, NY) after calibration with standard buffers (pH 4.0 and 7.0). Redox potential was measured with a platinum electrode [model P14805-SC-DPAS-K8S/325; Ingold (now Mettler Toledo), Urdorf, Switzerland] connected to a pH meter (model H 18418; Hanna Instruments, Padova, Italy). Water soluble nitrogen (WSN) of Labneh was estimated according to **Ling (1963)**. Total volatile fatty acids (TVFA) were determined according to **Kosikowiski (1978)**.

Determination of fatty acids composition

The extraction of milk fat was done using the method of Rose-Gottlieb using diethyl ether and petroleum ether (Methodenbuch, Bd. VI VDLUFA-Verlag, Darmstadt, 1985). After that the solvents were evaporated on a vacuum rotary evaporator. For obtaining methyl esters of the fatty acids, sodium methylate (CH_3ONa) was used **(Jahreis *et al.*, 1997)**.

GC condition: Rtx-5 column (oven temperature 180°C, hold for 2 min; increase to 270°C at 10°C/min, hold for 4 min and total of running time 15 min. Carrier gas He 2.43 mL/min, flow rate of air 190 mL/min and flow rate H_2 80 mL/min. Injector temperature is 290ºC and temperature of flame ionization detector (FID) is 290ºC. Methyl laurat (10% in heptana) as a standard with injection volume 0.10 µL. The peak of sample chromatogram having the same retention time with the retention time of standard is the peak of fatty acid.

Determination of amino acids composition

Amino acid profile of fresh yogurt was performed following the protocol of **Walsh and Brown (2000)**. Hydrochloric acid (6 M) was added to the sample vial for a final concentration of 5 mg of protein/mL of HCl. Hydrolysis vial was placed in an ultrasonic cleaner and flushed with nitrogen gas before sealing under vacuum. Sample was placed in a heating block for 4 hr at 145°C. Afterwards, sample was removed from the heating block and allowed to cool before filtration through 0.2 µm filter. Sample was dried with nitrogen gas and dissolved in a dilution buffer. The prepared sample was analyzed for amino acid profile by running through Automated Amino Acid Analyzer (Model: L-8500 A, Hitachi, Japan). Areas of amino acid standards were used to quantify each amino acid in representative sample.

Microbial analysis

Labneh samples were analyzed for *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophiles* and *Lactobacillus acidophilus* counts according to the methods described by **Tharmaraj and Shah (2003)**. The count of bifidobacteria was determined according to **Dinakar and Mistry (1994)**. A mixture of antibiotics, including 2 g of neomycin sulfate, 4 g of paromomycin sulfate, 0.3 g of nalidixic acid, and 60 g of lithium chloride (NPNL, Sigma Chemical Co.), was prepared in 1 L of distilled water, filter-sterilized, and stored at 4°C until use. The mixture of antibiotics (5 ml) was added to 100 ml of MRS agar medium. Cysteine-HCl was added at the rate of 0.05% to decrease the redox potential of the medium. Plates were incubated at 37°C for 48 to 72 h under anaerobic condition.

Evaluation of sensory properties

The sensory properties of the Labneh samples were determined by a panel of judges who were familiar with the product using the hedonic scale where 1-9 represents dislike extremely to like extremely **(Tunde-Akintunde and Souley, 2009)**. The panelists analyzed the samples for colour, smell, texture, taste, appearance and mouth feel.

Statistical analysis

The obtained results were statistically analyzed using a software package **(SAS, 1991)** based on analysis of variance. When F-test was significant, least significant difference (LSD) was calculated according to **Duncan (1955)** for the comparison between means. The data presented, in the tables, are the mean (± standard deviation) of 3 experiments.

RESULTS AND DISCUSSION

Yield and chemical composition of Labneh as affected by milk and culture types

Data of the obtained yield (Table 2) show that significant differences could be detected between the various treatments. The yield values of fresh Labneh were higher in treatments made from buffalo milk as compared with that made from soy milk. These outcomes are in agreement with the findings of **Ammar *et al.*, (1999)** who stated that the yield of Labneh manufactured from buffalo milk was higher than that of cow or recombined milk. Raising the amount of salt added to Labneh's milk led to increase the yield and total solids of the product to some extent.

Utilization of ABT culture slightly lowered the yield values of Labneh apart from the type of milk used in manufacturing. It is seen from Table 2 that Labneh made from soy milk was relatively low acidic compared to that made from buffalo milk. Therefore incorporation of 25% soy milk with buffalo milk significantly (P-value; 0.0498) decreased the titratable acidity content of Labneh produced. Also, blending of soy milk with buffalo milk decreased E_h values of Labneh. The pH values had the opposite trend of acidity and E_h results. Acidity and E_h values of classic starter Labneh treatments were relatively higher while pH data were lower than those of ABT Labneh. Moreover, the rise in titratable acidity and E_h or drop in pH in classic starter Labneh was more than that observed in the ABT Labneh. This finding was in agreement with those of **Hussein (2010)** for yoghurt. Opposite outcomes were found by **El-Sayed *et al.*, (2013)** who reported that the pH decreased at similar rates within yoghurt treatments made using different combinations of normal yoghurt starter and probiotic *B. bifidum* and *L. plantarum.*

It can easily be observed from Tables 2 and 3 that there is a substantial effect of the milk type on TS, fat and ash contents of Labneh. Significant increases ($p<0.05$) in TS, fat and ash contents were obtained with using buffalo milk in Labneh production. Soy milk Labneh possessed the lowest TS, fat and ash contents. On the other side, classic starter Labneh gave TS, fat and ash results similar to Labneh prepared by ABT starter.

Significant ($p<0.05$) increasing in TN contents was noticed in Labneh made from buffalo milk as compared with that made from soy milk (Table 3). Accordingly, blinding of soy milk with buffalo milk lowered the TN contents of Labneh. TN contents of Labneh samples were not clearly affected by type of starter.

Table 2 Yield and physicochemical composition of Labneh during storage period

Properties	Treatments	Storage period (days)				Means
		Fresh	7	14	21	
Yield %	A	25.01	-	-	-	25.10^{d}±1.02
	B	34.57	-	-	-	34.57^{a}±1.13
	C	30.08	-	-	-	30.08^{bc}±1.42
	D	24.33	-	-	-	24.33^{d}±1.22
	E	33.41	-	-	-	33.41^{ab}±1.11
	F	29.15	-	-	-	29.15^{c}±1.09
Acidity %	A	0.91	0.98	1.09	1.25	1.06^{c}±0.02
	B	1.48	1.58	1.71	1.99	1.69^{a}±0.03
	C	1.34	1.43	1.54	1.76	1.52^{b}±0.05
	D	0.80	0.85	0.95	1.08	0.92^{c}±0.05
	E	1.37	1.45	1.55	1.78	1.54^{b}±0.03
	F	1.25	1.31	1.40	1.61	1.39^{b}±0.04
pH values	A	4.98	4.87	4.75	4.58	4.79^{a}±0.42
	B	4.42	4.26	4.05	3.68	4.10^{a}±0.28
	C	4.50	4.37	4.29	3.98	4.28^{a}±0.33
	D	5.11	5.05	4.94	4.80	4.97^{a}±0.53
	E	4.51	4.38	4.20	3.93	4.25^{a}±0.47
	F	4.60	4.49	4.37	4.17	4.41^{a}±0.60
E_h mV*	A	83.6	92.7	99.5	110.9	96.7^{e}±3.11
	B	131.5	149.2	176.1	198.7	163.9^{a}±2.89
	C	128.3	143.2	165.9	189.4	156.7^{b}±2.56
	D	74.1	81.9	91.6	99.4	86.7^{f}±4.01
	E	125.7	142.6	161.9	181.4	152.9^{c}±3.21
	F	110.0	119.2	139.6	161.8	132.6^{d}±3.42
TS %	A	27.80	27.93	28.23	28.40	28.09^{c}±1.23
	B	34.33	34.50	34.66	34.83	34.58^{a}±1.53
	C	32.17	32.33	32.46	32.63	32.40^{b}±1.85
	D	27.67	28.00	28.26	28.36	28.07^{c}±1.67
	E	34.73	34.89	35.13	35.20	34.99^{a}±1.57
	F	32.20	32.41	32.53	32.76	32.47^{b}±1.22

^{abcde} Letters indicate significant differences between Rayeb milk treatments

*mV: millivolts

A: Labneh made from soy milk and classic starter; B: Labneh made from buffalo's milk and classic starter; C: Labneh made from 75% buffalo's milk + 25% soymilk and classic starter; D: Labneh made from soy milk and ABT culture; E: Labneh made from buffalo's milk and ABT culture; F: Labneh made from 75% buffalo's milk + 25% soymilk and ABT culture

Properties	Treatments	Storage period (days)			
		Fresh	7	14	21
Yield %	A	25.10^{d}±1.02	-	-	-
	B	34.57^{a}±1.13	-	-	-
	C	30.08^{bc}±1.42	-	-	-
	D	24.33^{d}±1.22	-	-	-
	E	33.41^{ab}±1.11	-	-	-
	F	29.15^{c}±1.09	-	-	-
Acidity %	A	0.91^{dB}±0.01	0.98^{cB}±0.01	1.09^{cB} ±0.01	1.25^{cA} ±0.01
	B	1.48^{aB}±0.01	1.58^{aB}±0.01	1.71^{aA}±0.01	1.99^{aA}±0.01
	C	1.34^{bB}±0.01	1.43^{aB}±0.01	1.54^{bB}±0.01	1.76^{bA}±0.01
	D	0.80^{dB}±0.01	0.85^{cB}±0.01	0.95^{cA}±0.01	1.08^{cA}±0.01
	E	1.37^{bB}±0.01	1.45^{aB}±0.01	1.55^{bB}±0.01	1.78^{bA}±0.01
	F	1.25^{cB}±0.01	1.31^{bB}±0.01	1.40^{bB}±0.01	1.61^{bA}±0.01

Note: Mean (n = 3) ± SD; Means of properties, with different lower-case superscripts (a-d) in each column (according to treatment), are significantly different ($p< 0.05$); and with capital superscripts (A-B), within rows for each treatment, are significantly different ($p < 0.05$); (one-way ANOVA and Duncan's test. a or A—the highest content).

Just as TN values increased in buffalo milk Labneh, also WSN contents raised. Addition of 25% soy milk to buffalo milk decreased WSN concentration. On the other hand, WSN contents were higher in Labneh made using classic culture as compared with that made by ABT. This may be due to the high proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* **(Ammar *et al.*, 2014)**.

Buffalo milk Labneh had the highest levels of TVFA whereas Labneh made from soy milk possessed the lowest (Table 3). This may be attributed to the high fat content of buffalo milk. Addition of soy milk to buffalo milk slightly decreased TVFA values of Labneh. Also using of ABT culture in manufacturing of Labneh lowered TVFA content.

Table 3 Chemical composition of Labneh during storage period

Properties	Treatments	Storage period (days)				Means
		Fresh	7	14	21	
Fat %	A	7.4	7.4	7.4	7.5	7.4^{c}±0.12
	B	16.5	16.6	16.6	16.7	16.6^{a}±0.32
	C	14.1	14.1	14.2	14.2	14.1^{b}±0.52
	D	7.5	7.6	7.6	7.7	7.6^{c}±0.42
	E	16.4	16.5	16.5	16.6	16.5^{a}±0.63
	F	14.2	14.2	14.3	14.3	14.2^{b}±0.41
Ash %	A	1.48	1.53	1.60	1.63	1.56^{c}±0.05
	B	1.76	1.83	1.90	1.98	1.87^{a}±0.04
	C	1.66	1.76	1.79	1.86	1.77^{a}±0.03
	D	1.50	1.59	1.65	1.69	1.63^{c}±0.04
	E	1.73	1.79	1.86	1.94	1.83^{a}±0.02
	F	1.62	1.73	1.81	1.88	1.76^{ab}±0.01
TN %	A	1.36	1.38	1.39	1.41	1.38^{d}±0.02
	B	1.90	1.91	1.94	1.95	1.92^{ab}±0.03
	C	1.75	1.77	1.78	1.80	1.77^{c}±0.02
	D	1.35	1.37	1.39	1.40	1.38^{d}±0.04
	E	1.91	1.93	1.95	1.97	1.94^{a}±0.05
	F	1.77	1.78	1.80	1.81	1.79^{bc}±0.02
WSN %	A	0.335	0.357	0.385	0.430	0.377^{a}±0.007
	B	0.364	0.390	0.439	0.487	0.420^{a}±0.008
	C	0.352	0.376	0.415	0.460	0.401^{a}±0.005
	D	0.315	0.333	0.356	0.395	0.350^{a}±0.006
	E	0.358	0.380	0.421	0.464	0.406^{a}±0.005
	F	0.339	0.360	0.392	0.440	0.383^{a}±0.007
TVFA*	A	9.6	10.2	12.6	14.8	11.8^{c}±0.89
	B	14.4	16.0	18.8	21.6	17.7^{a}±0.75
	C	13.4	15.0	17.2	19.8	13.3^{ab}±0.87
	D	9.0	9.4	11.4	13.6	10.8^{c}±0.68
	E	14.0	15.4	17.8	20.4	16.9^{a}±0.55
	F	13.0	14.2	15.6	17.0	14.9^{b}±0.75

^{abcde} Letters indicate significant differences between Rayeb milk treatments

***** expressed as ml 0.1 NaOH 100 g^{-1} Labneh

Free fatty acids content (FFA) of Labneh

Data given in Tables 4 and 5 revealed that the saturated fatty acids (SFA) values of Labneh in all treatments were inversely proportional with the amount of unsaturated fatty acids (USFA). The concentration of SFA was higher than USFA in various Labneh treatments except samples A and D (soy milk Labneh). The results indicated that addition of 25% soy milk to buffalo milk markedly decreased the amount of SFA and inversely increased the amounts of USFA of Labneh. Value of SFA lowered by 9.17% while USFA content increased by 16.04% for sample C.

It could be viewed form Table 5 that utilization of ABT starter caused a markedly decrease in SFA and increase in USFA contents of fresh Labneh. Based on these results, combination of soy milk and ABT culture in one fermented dairy product like Labneh greatly reduced SFA content whereas highly raised USFA concentration. These findings are in agreement with the findings of **Caglayan.,** ***et al*****., (2014)** who found that the levels of USFA were slightly higher than SFA in probiotic Turkish yoghurt as compared with whole one. Lactic acid bacteria possess enzymes that are able to hydrolyze mono-, di- and triacylglycerols. The activity of the lipases depends strongly on the genera and bacteria species, as well as on the temperature and presence of calcium and magnesium ions **(Nurliyani** ***et al*****., 2014)**.

Decreasing of SFA and increasing USFA values in soymilk bio-Labneh obtained in our study raise the healthy benefit of this product because it is well known that unsaturated fatty acids are more important in human nutrition. **Siriwardhana** ***et al*****., (2012)** showed that n-3 PUFAs are known to have variety of health benefits against cardiovascular diseases (CVDs) including well-established hypotriglyceridemic and anti-inflammatory effects.

Generally, the most predominant SFA found in various Labneh treatments was palmitic acid (C_{16}) followed by stearic and myristic acids (C_{14}). The highest acid ratio of USFA differed between treatments. For samples A and D (soy milk Labneh), the major acid of USFA was linoleic (18:2 ω6) followed by oleic acid (18:1 ω9) whereas oleic acid was the corresponding one for samples B and E. For treatments C and F, the first acid of USFA was oleic while the next was lionleic acid.

Marked differences in monounsaturated (MUSFA) and polyunsaturated fatty acids (PUSFA) fatty acids values were noticed when soymilk was used in manufacturing of Labneh (Tables 4 and 5). Labneh made from buffalo milk had higher contents of MUSFA than that made from soy milk. Incorporation of 25% soy milk with buffalo milk also decreased the amounts of **MUSFA** of Labneh. On the complete contrary, soy milk Labneh had the greatest amounts of PUSFA as compared with buffalo milk one. Addition of soy milk to buffalo milk highly increased levels of **PUSFA.**

It should be pointed out that using of ABT culture in Labneh manufacturing didn't cause pronounced changes in the contents of MUSFA and PUSFA. In all Labneh treatments except A and D, MUSFA values were higher than those of PUSFA. Samples A and D had the opposite trend. Oleic acid was found to have the greatest concentration of MUSFA in various Labneh treatments. The dominant fatty acid of PUSSFA was linoleic acid followed by α-linolenic acid.

As it is well known, omega fatty acids are a group of essential fatty acids very important for human health. Soy milk Labneh (treatments A and D) characterized by very high contents of linoleic acid (omega-6) and α-linolenic acid (omega-3) as compared with Labneh made from buffalo milk (samples B and E). Because of the ultra-high contents of linoleic and α-linolenic acids in soy milk, replacement of 25% buffalo milk with soy milk highly increased the Labneh content from the two mentioned acids. Increasing rates were 333.67% for linoleic acid and 152.00% for α-linolenic acid when 25% soymilk was added to buffalo milk (sample C). Concerning oleic acid (omega-9), incorporation of soy milk with buffalo milk slightly decreased the concentration of this acid in Labneh produced. The slight decline in oleic acid content does not reduce the importance of the vast increase in linoleic and α-linolenic acids contents of Labneh when soymilk was used in manufacturing. The follow-up studies for many authors reported the healthy importance of linoleic and α-linolenic acids. **Simopoulos, (2002)** cleared that the beneficial health effects of omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were described first in the Greenland Eskimos who consumed a high seafood diet and had low rates of coronary heart disease, asthma, type 1 diabetes mellitus, and multiple sclerosis. Since that observation, the beneficial health effects of omega-3 fatty acids have been extended to include benefits related to cancer, inflammatory bowel disease, rheumatoid arthritis, and psoriasis. **Singh, (2005)** showed that essential fatty acids (EFAs) are required for maintenance of optimal health but they cannot be synthesized in the body and must be obtained from dietary sources.

Table 4 Effect of using soymilk on free fatty acids content of fresh Labneh

Fatty acids	C	Treatments					
		A	B	C	D	E	F
		Saturated fatty acids (SFA) %					
Caprylic	8:0	0.50	0.67	0.51	0.40	0.50	0.40
Capric	10:0	1.10	1.93	1.79	1.09	1.29	1.13
Undecanoic	11:0	-	0.42	0.28	-	0.20	0.20
Lauric	12:0	1.59	2.93	2.24	1.39	2.10	1.94
Tridecanoic	13:0	0.34	0.74	0.51	0.38	0.20	0.31
Myristic	14:0	2.93	10.39	9.87	3.10	10.57	9.84
Pentadecanoic	15:0	0.84	3.84	3.58	1.26	3.37	2.90
Palmitic	16:0	15.51	27.00	24.52	14.90	27.21	24.58
Heptadecanoic	17:0	0.47	2.00	1.86	0.24	2.48	2.17
Stearic	18:0	5.99	12.34	11.31	5.40	12.67	11.23
Arachidic	20:0	0.32	0.21	0.24	0.31	0.25	0.24
Behenic acid	22:0	0.31	0.19	0.20	0.34	0.18	0.17
Total		29.90	62.66	56.91	28.81	61.02	55.11
		Unsaturated fatty acids (USFA) %					
	12:1 ω5	0.50	0.58	0.54	0.53	0.56	0.35
5-Tetradecenoic (phytosteri)	14:1 ω5	-	0.34	0.46	-	0.56	0.50
	14:1 ω7	0.25	0.38	0.52	0.30	0.39	0.31
Myristioleic acid	14:1 ω9	0.40	0.34	0.63	0.44	0.36	0.34
	16:1 ω5	-	0.14	0.11	-	-	0.13
Palmitioleic	16:1 ω7	0.27	2.25	1.97	0.32	2.07	1.91
	16:2 ω4	-	0.41	0.30	-	0.39	0.34
Hexagonic	16:3 ω4	-	0.34	0.25	-	0.38	0.35
	18:1 ω4	-	0.21	0.20	-	-	0.20
Octadecosaenoic	18:1 ω5	-	0.48	0.47	-	0.30	0.31
Vaccienic	18:1 ω7	1.42	1.90	1.58	1.49	1.93	1.70
Oleic	18:1 ω9	21.00	24.39	22.17	20.74	23.90	23.10
	18:2 ω4	0.97	0.66	0.50	-	0.85	0.77
	18:2 ω5	-	0.24	0.20	-	0.38	0.35
Linoleic	18:2 ω6	38.20	1.99	8.63	39.79	2.84	10.04
	18:2 ω7	-	-	0.22	-	0.38	0.36
α-Linolenic	18:3 ω3	4.77	0.75	1.89	5.58	1.04	2.11
	18:3 ω4	-	0.13	0.15	-	0.15	0.15
Gamma linolenic	18:3 ω6	0.15	0.12	0.17	-	0.15	0.15
Octadecatetraenoic	18:4 ω3	0.21	-	0.18	-	0.44	0.21
Gadoleic acid	20:1 ω9	0.15	-	-	0.16	-	-
Eicosaenoic	20:1 ω11	-	-	0.23	-	0.43	0.21
Total		68.29	35.65	41.37	69.35	37.50	43.89
Non identified fatty acid		1.81	1.69	1.72	1.84	1.48	1.00

Utilization of soy milk in Labneh making lowered the concentrations of short-chain fatty acids **(C8 – C12)** (SCFA) (Tables 4 and 5). Also, ABT-Labneh had lower SCFA contents than that made by classic culture. In different Labneh treatments, the fatty acid lauric (C:12) was the predominant SCFA followed by capric acid (C10:0) and caprylic acid (C8:0). **Beshkova *et al.*, (1998)** found that the formation of volatile free fatty acids (C2-C10) was more active in the mixed yoghurt cultures than in the pure ones owing to the stimulating effect of protocol-operation between the two thermophillic species on the metabolic activities, which are responsible for the formation of free fatty acids. In fact, volatile acids is not only produced from lipolysis by lipases but also from several biochemical pathways including the fermentation of lactose or citrate and the degradation (oxidative deamination or decarboxylation) of amino acids (alanine and serine) which are the most important precursor of most volatile fatty acids **(Kneifel *et al.*, 1992; Beshkova *et al.*,1998)**.

Medium chain fatty acids **(C_{13} – C_{16})** (MCFA) of Labneh took the same trend of SCFA where using soy milk in manufacturing led to decreasing of the content from these fatty acids (Tables 4 and 5). It has also been cleared from Table 5 that MCFA content of Labneh didn't affect by culture type. In different Labneh treatments, the concentration of palmitic acid (C_{16}) was the highest of MCFA and was followed by the concentrations of myristic (C_{14}).

The levels of long chain fatty acids **(> C16)** (LCFA) were very high in soy milk Labneh as compared with buffalo milk one. Mixing of 25% soy milk with buffalo milk also increased the content of Labneh from these acids. Furthermore, LCFA concentrations increased by using ABT culture in Labneh making. Among all the long chain fatty acids determined, the concentration of oleic and stearic acids were the highest in various Labneh samples except A and D where the predominant acid of long chain fatty acids was linoleic acid followed by oleic acid. On a general note, the values of LCFA were higher than SCFA and MCFA in all Labneh samples. Similar results were found by **El-Kadi *et al.*, (2017)** who reported that utilization of ABT culture in yoghurt production slightly lowered MCFA but raised LCFA levels as compared with classic starter.

Table 5 Effect of using soymilk on free fatty acid indices ratios of fresh Labneh

Treatments	SFA	USFA	MUSFA	PUSFA	SCFA	MCFA	LCFA
A	29.90^{c}±1.11	68.29^{a}±2.10	23.99^{c}±1.12	44.30^{a}±2.34	3.69^{d}±0.75	20.54^{c}±1.42	75.77^{a}±0.89
B	62.66^{a}±1.20	35.65^{e}±1.99	31.10^{a}±1.52	4.55^{e}±0.44	6.53^{a}±0.89	46.17^{a}±1.53	46.80^{c}±1.32
C	56.91^{b}±1.31	41.37^{c}±1.75	28.88^{b}±1.41	12.49^{c}±1.20	5.46^{b}±0.56	42.72^{b}±1.33	51.42^{b}±0.78
D	28.81^{d}±1.14	69.35^{a}±1.86	23.98^{c}±1.32	45.37^{a}±3.53	3.41^{d}±0.23	20.70^{c}±1.62	75.89^{a}±0.98
E	61.02^{a}±1.15	37.50^{d}±1.05	30.50^{a}±1.60	7.10^{d}±1.24	4.25^{c}±0.52	45.40^{a}±0.99	50.35^{c}±1.34
F	55.11^{bc}±1.20	43.89^{b}±1.41	29.06^{b}±1.23	14.83^{b}±1.53	4.02^{c}±0.33	41.51^{b}±1.12	54.47^{b}±1.62

SFA: saturated fatty acids; USFA: unsaturated fatty acids; MUSFA: monounsaturated fatty acids (C:1); PUSFA: polyunsaturated fatty acids (C:2+ C:3); SCFA: short chain fatty acids (C8 to C12); MCFA: medium chain fatty acids (C13 to C16); LCFA: long chain fatty acids (> C16).

Free amino acids content (FAA) of Labneh

The change pattern of proteolytic activity during fermentation is of basic important for the degree of lactic acid bacteria. Proteinase activity was detected in several strains of lactobacilli and streptococci **(Zourari *et al.*, 1992)**. Results of Tables 6 and 7 illustrate the influence of using soy milk and ABT culture on the composition of FAA of fresh Labneh.

As shown in Tables 6 and 7, utilization of various kinds of milk or starter slightly impacted on the total free amino acids content of Labneh. Buffalo milk Labneh had the highest levels of the total free amino acids as compared to that prepared from soy milk. Mixing of 25% soy milk with buffalo milk has led to production of Labneh possessed the lowest total free amino acids content.

On the other side, utilization of ABT culture in Labneh making slightly raised the total free amino acids content. These results might be interpreted to mean that bacteria species has an effect on the content of amino acids in fermented dairy

products. In all Labneh samples, the highest level of total free amino acids was that of glutamic acid, which is responsible for protection from cardiovascular diseases **(Brosnan and Brosnan, 2013)**, followed by aspartic acid. On the contrary, methionine and cystine acids had the lowest content of total amino acids.

The concentrations of essential amino acids (EAA) were slightly higher in Labneh made from buffalo milk than those detected in Labneh made from soy milk. This is probably due to the high fat content of buffalo milk. Labneh made from mixtures 75% buffalo milk +25% soy milk possessed slightly lower EAA levels than those of other treatments. Also, Labneh made from the above mentioned mixture had low values of essential amino acids to total amino acids (E/T) among the different treatments.

Because of low content of soy milk from sulfur amino acids (methionine and cysteine), Labneh made from it contained lower levels of these acids than that made from buffalo milk. **Tabe and Higgins, (1998)** reported that legumes (e.g., soybean, pea, bean, chickpea, alfalfa, lentil, clover) are mainly limited in the contents of sulfur amino acids, methionine and cysteine. **Allman-Farinelli, (1998)** showed that compared with protein found in egg and milk, soy bean contains less of the sulphur amino acids methionine and cycteine but there is a sufficient amount to regard soya protein as a high quality protein source.

Table 6 Effect of using soymilk on free amino acids content (g/100mL) of fresh Labneh

Amino acids	Treatments					
	A	B	C	D	E	I
Aspartic (ASP)	1.54	1.10	1.09	1.55	1.13	1.11
Threonine (THR)	0.47	0.62	0.60	0.56	0.63	0.61
Serine (SER)	0.66	0.65	0.68	0.70	0.68	0.64
Glutamic acid (GLU)	2.55	2.37	2.40	2.57	2.38	2.41
Proline (PRO)	0.82	1.53	1.28	0.82	1.55	1.29
Glycine (GLY)	0.61	0.33	0.38	0.58	0.30	0.40
Alanine (ALA)	0.72	0.53	0.58	0.74	0.56	0.61
Valine (VAL)	0.83	0.96	0.90	0.83	0.93	0.94
Methionine	0.22	0.45	0.36	0.25	0.47	0.32
Isoleucine (ILE)	0.69	0.78	0.70	0.68	0.75	0.65
Leucine (LEU)	1.13	1.36	1.21	1.17	1.35	1.25
Tyrosine (TYR)	0.59	0.65	0.60	0.59	0.71	0.61
Phenylalanine (PHE)	0.69	0.79	0.72	0.73	0.80	0.73
Histidine (HIS)	0.41	0.49	0.45	0.42	0.55	0.47
Lysine (LYS)	0.95	1.15	1.07	0.93	1.18	1.04
Arginine (ARG)	1.04	0.65	0.74	1.07	0.60	0.70
Cystine (CYS)	0.22	0.27	0.26	0.21	0.29	0.25

It is apparent from the findings reported in Table 7 that bio-Labneh made using ABT had a little increasing of EAA than that made using classic starter. This may be due to the deference of bacteria species found in the starter. In all Labneh samples, the major essential amino acid was leucine followed by lysine. Methionine content was the lowest.

In contrast to trend of EAA, soy milk Labneh had the highest concentration of nonessential amino acid (Non-EAA) among different treatments. Utilization of ABT culture in Labneh preparation also increased the content of non-EAA. Generally, glutamic and aspartic acids were predominant in different Labneh treatments.

Leucine, isoleucine, and valine possess a similar structure with a branched-chain residue and therefore are referred to as branched-chain amino acids (BCAA) **(Layman and Baum, 2004)**. Free BCAAs, especially leucine, play a very important role in protein metabolism; leucine promotes protein synthesis and inhibits protein degradation via mechanisms involving the mammalian target of rapamycin **(Shimomura *et al.*, 2004)**. These findings suggest that leucine is not only a building block of proteins but also a modulator of protein metabolism.

Labneh made from buffalo milk had slightly higher amounts of total BCAA than that made from soy milk (Tables 6 and 7). Thus, incorporation of buffalo milk with soy milk increased the Labneh content of total BCAA as compared with that made from only soy milk. On the other side, no pronounced differences were noticed in concentrations of total BCAA between Labneh prepared by classic or ABT culture. Leucine was the abundant acid of total BCAA for various samples.

Table 7 Effect of using soymilk on free amino acid indices ratios of fresh Labneh

Treatments	Total amino acids (g/100mL)	Total EAA (g/100mL)	Total Non-EAA (g/100mL)	Total BCAA (g/100mL)	E/T (%)	Total BCAA/Total (%)
A	14.14^{a}±0.98	5.39^{ab}±0.23	8.75^{a}±0.52	2.65^{ab}±0.12	38.12^{c}±1.20	18.74^{b}±1.11
B	14.68^{a}±1.23	6.60^{a}±0.45	8.08^{a}±0.74	3.10^{a}±0.30	44.96^{a}±1.30	21.12^{a}±0.99
C	14.02^{a}±1.10	6.01^{a}±0.62	8.01^{a}±0.62	2.81±0.14	42.87^{b}±1.51	20.04^{ab}±0.85
D	14.40^{a}±0.78	5.57^{ab}±0.51	8.83^{a}±0.46	2.68^{ab}±0.21	38.68^{c}±1.62	18.61^{b}±1.30
E	14.86^{a}±0.89	6.66^{a}±0.87	8.20^{a}±0.52	3.03^{a}±0.51	44.82^{a}±1.63	20.39^{a}±1.24
F	14.03^{a}±1.12	6.01^{a}±0.23	8.02^{a}±0.43	2.84^{a}±0.21	42.84^{b}±1.44	20.24^{ab}±1.62

Total EAA: total essential amino acids; Total Non-EAA: total nonessential amino acids; Total BCAA: total branched-chain amino acids; E/T: Ratio of essential amino acids to total amino acids.

Microbial analysis of Labneh

Labneh samples made from soy and buffalo milk using classic or ABT cultures were analyzed microbiologically for *Streptococcus thermophillus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Data were recorded in **Table 8**. In various Labneh treatments, the counts of mentioned bacteria increased up to the seventh day then decreased till to the end of storage period. This reduction may be attributed to the high acidity produced by microbial fermentation **(Dave and Shah, 1997)**. Similar results were obtained by other authors but with some variations. **Ayyad *et al.*, (2015)** showed that the numbers of total viable count, *B. bifidum, L. acidophilus* and lactic acid bacteria of low fat Labneh increased up to the fourteenth day then decreased. Contrary, **Hamad *et al.*, (2014)** cleared that numbers of *L. acidophilus*, *S. thermophilus* and bifidobacteria gradually lowered during storage of Labneh.

Results in **Table 8** show that soy milk Labneh treatment had the greatest count of *Str. thermophillus* dislike Labneh made from buffalo milk which recorded the lowest count. This was the opposite of what was expected, because buffalo's milk is rich of elements and compounds more than soy milk, but may be soy milk as vegetarian milk contains some elements and compounds don't found or found with low levels in animal milk (buffalo) at the same time bacteria need that elements and compounds to grow well. As a sequence, blinding of 25% soy milk with buffalo or cow milk increased the count of *Str. thermophillus* in Labneh samples. In addition, loss of viability of these bacteria during storage period was lower in Labneh made from soy milk or mixtures of soy milk with buffalo milk comparing with that made from buffalo and cow milk. This may be due to the high acidity content and also the high acidity development rates in buffalo milk Labneh (Table 8). Loss of viability values of *Str. thermophillus* in samples A, B and C were 18.33, 37.50 and 22.03% respectively.

ABT Labneh treatments had the highest *Str. thermophillus* counts, meaning that the presence of *L. acidophilus* and *B. bifidum* clearly encouraged *Str. thermophillus* growth. This effect may be attributed to the low activity of acidity production of *L. acidophilus* and *B. bifidum* as compared with *L. bulgaricus* found in classic starter. Therefore, loss of survival values of *Str. thermophillus* were lower in ABT Labneh than those of classic starter one. Samples D, E and F had 5.55, 22.22 and 14.99% loss of survival of *Str. thermophillus* respectively.

With regard to *L. bulgaricus*, it is clear from **Table 8** that type of milk has had great effect on counts of *L. bulgaricus*. These bacteria didn't have the ability to grow well in soy milk Labneh. Thus the loss of viability during storage increased with using soy milk in Labneh manufacturing. The highest counts of *L. bulgaricus* were in buffalo milk Labneh. The obtained results agreed with **Sumarna, (2008)**.

Slight increases were observed in the viable number of *L. acidophilus* in Labneh made from soy milk only or mixed with buffalo milk. These results refer to using of soy milk in manufacturing of Labneh enhances the activity of *L. acidophilus*.

The loss of survival levels during Labneh storage were 19.35, 48.15 and 32.14% for samples D, E and F respectively. Similar results were found by **Hassanzadeh-Rostami *et al*., (2015)**.

It is clear from the results of Table 8 that bifidobacteria counts were higher in samples contained soy milk than those of buffalo milk which may be due to the activation effect of soy milk components on bifidobacteria. This means that our treatments had no worthless effect on these healthy bacteria. Furtherance of these results, the loss of viability rates of bifidobacteria throughout cold storage of Labneh also were lower in soy milk samples than other treatments. Loss of viability levels of samples D, E and F were 17.5, 32.43 and 18.42% respectively. **Kamaly, (1997)** stated that *B. longum* and *B. bifidum* exhibited proteolytic activity and were more pronounced in soy milk than in reconstituted skimmed milk.

It is necessary to maintain the probiotic bacteria numbers above 10^6 cfu.g^{-1} to give the beneficial probiotic effect. However the drop in the probiotic bacteria counts during storage time but they remained above 10^6 cfu.g^{-1} in all Labneh samples. This main that the viability of strains after the storage period was sufficient to yield numbers of beneficial organisms that were higher than the accepted threshold (10^6 cfu.g^{-1}) for a probiotic effect **(Gomes and Malcata 1998)**. One of the possibilities of high stability of bifidobacteria at refrigerated storage in these samples could be the absence of *Lb. delbrueckii* ssp. *bulgaricus* which is known to produce post acidification. Post acidification could have further inhibitory effect on the *S. thermophilus* counts.

Changes in sensory evaluation of Labneh

The results given in Table 9 described the influence of addition soy milk and using ABT culture on the sensory evaluation of Labneh. Buffalo milk Labneh gained the highest scoring points of color and appearance followed by soy milk one. These findings clarify the effect of milk type on properties of Labneh. The bright white color of buffalo milk desirable to the Egyptians makes any dairy product the leader among other milk types. Mixing 25% soy milk with buffalo milk slightly decreased color and appearance values of Labneh. These results correspond with **Osman and Abdel Razig (2010)**.

It is clear that the color and appearance scores of Labneh made using classic or ABT cultures were close to each other. Scores of color for fresh samples A and D were 8.50 and 8.50 respectively. These results are in agreement with those obtained by **Ammar *et al*., (2015)**.

Table 8 Effect of using soymilk and ABT-5 culture on starter bacteria counts of Labneh

Properties	Treatments	Storage period (day)				Means
		Fresh	7	14	21	
***Streptococcus thermophillus* (cfu×x10^5/g)**	A	60	84	70	49	65.7^{d}±3.12
	B	56	77	60	35	57.0^{f}±2.89
	C	59	81	65	46	62.7^{e}±3.11
	D	72	98	86	68	81.0^{a}±4.01
	E	63	86	72	49	67.5^{c}±2.98
	F	69	93	77	59	74.5^{b}±3.10
***Lactobacillus bulgaricus* (cfu×x10^5/g)**	A	51	60	41	17	42.2^{c}±2.55
	B	67	95	80	58	75.0^{a}±2.68
	C	61	83	69	49	65.5^{b}±3.52
	D	-	-	-	-	-
	E	-	-	-	-	-
	F	-	-	-	-	-
***Lactobacillus acidophilus* (cfu×x10^5/g)**	A	-	-	-	-	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	31	36	33	25	31.2^{a}±2.11
	E	27	30	25	14	24.0^{c}±2.62
	F	28	30	27	19	26.0^{b}±1.98
***Bifidobacterium bifidum* (cfu×x10^5/g)**	A	-	-	-	-	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	40	47	42	33	39.4^{a}±3.41
	E	37	42	35	40	34.7^{b}±3.25
	F	38	44	40	31	38.2^{a}±2.97

^{abcde} Letters indicate significant differences between Rayeb milk treatments

Not only the white color that makes buffalo's milk favorite to the Egyptians, but also riches of fat. Therefore, it was not surprising that buffalo milk Labneh also obtained the highest scores of smell, taste and mouth feel evaluation tests. On contrary, soy milk Labneh recorded the lowest levels because of a beany flavor. Incorporation of buffalo milk with soymilk markedly improved the smell, taste and mouth feel evaluation grades of Labneh.

Because ABT culture produces mild acidity as compared with classic culture **(Kurmann *et al*., 1992)**, using it in Labneh manufacture slightly improved the smell, taste and mouth feel properties. These findings agreed with that reported by **Abd El-Salam *et al*., (2011)**.

In the same trend of scores of color, appearance, smell, taste and mouth feel, scores of texture and body were higher in buffalo milk Labneh than those of soy milk one. Unlike other characterizes of sensory evaluation, texture and body properties of soy milk Labneh were almost similar to those of Labneh made from buffalo milk. On the other hand, the texture and body scores of ABT Labneh slightly lowered than classic starter Labneh.

The last organoleptic property cleared in Table 9 was overall acceptability. Based on the results of sensory evaluation referred to previously, buffalo milk Labneh gained the highest scores of overall acceptability. Mixing of buffalo milk with soy milk improved the scores of overall acceptability of resulted Labneh. Regardless of milk type, utilization of ABT culture slightly increased the overall acceptability scores of fresh Labneh and during storage period.

During storage period, a slight drop in color, appearance, smell, taste, mouth feel texture, body and overall acceptability scores of all Labneh samples was evident. Similar results were reported by **Salem *et al*., (2007)**.

CONCLUSIONS

It was found that synbiotic Labneh was successfully made from 75% buffalo milk and 25% soymilk mixture using of ABT culture. Utilization of soy milk reduced levels of saturated fatty acids and increased values of unsaturated fatty acids. Soy milk Labneh characterized by high amounts of omega 3 and 6. The recommended level of 10^7 cfu.g^{-1} of bifidobacteria as a probiotic was exceeded for synbiotic Labneh. The results of sensory attributes evaluation showed that Labneh made from buffalo and soy milk mixture was acceptable especially at the beginning of storage period.

Table 9 Effect of using soymilk and ABT-5 culture on sensory evaluation of Labneh

Properties	Treatments	Storage period (day)				Means
		Fresh	7	14	21	
Color	A	8.50	8.50	8.25	8.00	8.31^{c}±0.85
	B	10.00	10.00	9.50	9.00	9.62^{a}±0.67
	C	9.50	9.50	9.25	9.00	9.31^{b}±0.93
	D	8.50	8.50	8.25	8.00	8.31^{c}±0.75
	E	100	10.00	9.50	9.00	9.62^{a}±0.84
	F	9.50	9.50	9.25	9.00	9.31^{b}±0.75
Appearance	A	8.50	8.50	8.25	8.05	8.32^{d}±0.65
	B	10.00	10.00	9.75	9.10	9.71^{a}±0.88
	C	9.40	9.40	9.00	8.80	9.15^{c}±0.75
	D	8.50	8.50	8.25	8.00	8.31^{d}±0.74
	E	10.00	10.00	9.75	9.25	9.75^{a}±0.65
	F	9.50	9.50	9.20	9.00	9.30^{b}±0.55
Smell	A	8.15	8.15	7.90	7.50	7.92^{d}±0.43
	B	10.00	10.00	9.60	9.00	9.65^{a}±0.52
	C	9.50	9.50	9.25	9.00	9.31^{b}±0.63
	D	8.60	8.60	8.40	8.10	8.42^{c}±0.45
	E	10.00	10.00	9.75	9.25	9.75^{a}±0.58
	F	9.60	9.60	9.40	9.15	9.44^{b}±0.71
Taste	A	8.00	8.00	7.75	7.25	7.75^{e}±0.36
	B	10.00	10.00	9.50	9.00	9.62^{a}±0.65
	C	9.50	9.50	9.50	8.65	9.21^{c}±0.48
	D	8.50	8.50	8.30	7.75	8.26^{d}±0.42
	E	10.00	10.00	9.65	9.20	9.71^{a}±0.53
	F	9.60	9.60	9.45	9.00	9.41^{b}±0.44
Mouth feel	A	8.50	8.50	8.30	8.00	8.32^{d}±0.25
	B	10.00	10.00	9.40	8.70	9.52^{b}±0.23
	C	9.20	9.20	8.75	8.20	8.84^{c}±0.42
	D	8.60	8.60	8.45	8.20	8.46^{d}±0.33
	E	10.00	10.00	9.60	9.10	9.67^{a}±0.53
	F	9.30	9.30	8.90	8.40	8.97^{c}±0.61
Texture & Body	A	9.75	9.75	9.65	9.50	9.66^{b}±0.24
	B	10.00	10.00	9.80	9.55	9.84^{a}±0.71
	C	9.75	9.75	9.65	9.50	9.66^{b}±0.35
	D	9.50	9.50	9.40	9.30	9.42^{c}±0.62
	E	9.50	9.50	9.40	9.30	9.42^{c}±0.41
	F	9.50	9.50	9.40	9.25	9.41^{c}±0.43
Overall Acceptability	A	8.00	8.00	7.80	7.50	7.82^{d}±0.25
	B	10.00	10.00	9.55	9.00	9.64^{a}±0.36
	C	9.35	9.35	9.00	8.60	9.07^{b}±0.41
	D	8.50	8.50	8.35	8.10	8.36^{c}±0.62
	E	10.00	10.00	9.65	9.20	9.71^{a}±0.41
	F	9.45	9.45	9.15	8.80	9.21^{b}±0.52

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THE CONTENT OF POLYPHENOLS AND CHOSEN HEAVY METALS IN CHICKPEA SEEDS (*CICER ARIETINUM* L.) AFTER MICRONUTRIENTS APPLICATION INTO SOIL

Mária Timoracká and Alena Vollmannová*

Address(es): Ing. Mária Timoracká, PhD.,
Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Chemistry, Tr. A. Hlinku 2, 94901 Nitra, Slovak Republic, phone number: +421 37 641 4862.

*Corresponding author: maria.timoracka@uniag.sk

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ABSTRACT

The aim of work was the investigation of the input of chosen heavy metals into chickpea and polyphenols accumulation in seeds cultivated after Zn and Cu application into soil in model conditions. The soil used in the pot trial was uncontaminated. Chickpea (cv. Slovák) was harvested in full ripeness. With increased Zn (Cu) doses applied into the soil (0-500 and 0-100 mg.kg^{-1} for Zn and Cu, respectively), the strong statistical positive relationship between soil Zn (Cu) content and Zn (Cu) amount in seeds of chickpea was confirmed. Despite of high Zn (Cu) doses applied into the soil in model conditions, in all variants the determined Zn and Cu amount in chickpea was below the maximal allowed content in foodstuffs. Chickpea cv. Slovák accumulated high amounts of Pb and Cd (in all variants higher than hygienic limits). The contents of other tested heavy metals (Zn, Cu, Ni, Cr) were lower than hygienic limits. With increased Zn doses applied into the soil the polyphenols contents determined in seeds of chickpea were increased. With increased Cu doses applied into the soil the polyphenols contents determined in seeds of chickpea were decreased. The strong statistical relationship between soil Zn content and polyphenols amount (R=0.911) as well as the antioxidant capacity values (R=0.992) was confirmed. The moderate statistical linear negative relationship between soil Cu content and polyphenols content (R= - 0.671) and increased antioxidant capacity values in seeds of chickpea was confirmed.

Keywords: chickpea, metal contamination, zinc, copper

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an annual grain legume that is extensively cultivated for human consumption. Chickpea is a good source of carbohydrates (low glycaemic index) and protein, together constituting about 80% of the total dry seed mass **(Chibbar *et al.*, 2010)** in comparison to other pulses. Chickpea also contains important minerals (i.e. Ca, Mg, Zn, K, Fe and P) and vitamins (i.e. thiamine and niacin) **(Zia-Ul-Haq *et al.*, 2007)**, and is relatively free from antinutritional factors **(Muzquiz, Wood, 2007)**. Chickpea, similar to other legume seeds, must be soaked and/or cooked before consumption, a procedure that improves the flavor and palatability of the food product and increases its nutritional bioavailability by inactivaving antinutritional factors **(Chau *et al.*, 1997)**. Seeds of chickpea are helpful source of zinc and folate. Zinc is the main micronutrient in the soil that limits chickpea productivity **(Ahlawat *et al.*, 2007).** In general, each tonne of chickpea grain removes 38 g of Zn from the soil **(Ahlawat *et al.*, 2007)**. Among micronutrients, Zn deficiency is perhaps the most widespread **(Roy *et al.*, 2006)** and is common among chickpea-growing regions of the world.
The antioxidant capacity and antiproliferative effects of legumes are associated with the presence of phenolic compounds **(Dong *et al.*, 2007)**. Phenolic cpmpounds are known to exhibit a range of biological activities, including antibacterial, antioxidant and antiinflammatory properties **(Kamatou *et al.*, 2010)**. In studies with chickpea it was found that isolated hulls from a colored chickpea line contain large amounts of polyphenols and flavonoid compounds that exhibit high levels of antioxidant activity **(Segev *et al.*, 2010)**. On the other hand, common chickpea varieties with beige-colored seeds have low levels of total phenolic content, total flavonoid content and antioxidant activity determined by ferric reducing ability of the plasma **(Segev *et al.*, 2010)**. This variation in both seed coat color and antioxidant activity maked colored chickpea a strong potential model for studies of functional foods. Chickpea also contains isoflavones such as daidzein and genistein. In addition, high levels of genistein hexoside were detected recently in raw chickpea flours **(Aguilera *et al.*, 2011)**.
In our work, chosen micronutrients zinc and copper were added to the soil to reduce the intake of other heavy metals, especially of Cd or Pb - these toxic metals are present in the soil above the hygienic limit on most territory of the Slovak Republic - as well as to its consecutive accumulation in chickpea seeds and influence on total polyphenols creation and antioxidant activity of chickpea.

MATERIALS AND METHODS

Plant material

The cultivar of chickpea (*Cicer arietinum* L.) (cv. Slovak) was harvested in full ripeness and obtained from the Research Centre of Plant Production in Piešťany (Slovakia).

Soils samples

In the pot experiments the soil from locality Cakajovce (Slovak village located near to Nitra) was used. The soil was characterized by low supply of humus and the neutral soil reaction suitable for the legume cultivation. The used soil was characterized also by high content of potassium and phosphorus as well as by a very high content of magnesium. The soil used in the pot trial was uncontaminated. Only determined Cd content was on the level of limit value given by **Law N^{o} 220/2004** (valid in the Slovak Republic) for the soil extract by aqua regia as well as Pb content on the level of critical value given by **Law N^{o} 220/2004** (valid in the Slovak Republic) for the relationship between soil and plant. The values were far below threshold values proposed.

The experiment

The experiment was based on four replications in each variant. Five kilograms of soil was thoroughly mixed with a sand (1kg) and togheter were submitted into plastic bowl-shaped pots with foraminated bottom (average: 20 cm and height: 25 cm). The solution of $ZnSO_4.7H_2O$ was added to each pot to correspond to 0 (control, A variant), 40 (B variant), 250 (C variant), and 500 (D variant) mg Zn.kg^{-1} of soil.

The solution of $CuSO_4.5H_2O$ was added to each pot to correspond to 0 (control, A variant), 20 (B variant), 50 (C variant), and 100 (D variant) mg $Cu.kg^{-1}$ of soil. Individual doses were chosen by Decision of the Ministry of Agriculture of Slovak Republic N°531/1994-540 about the maximum exposure levels of risky compounds in the soil – hygienic limit (B variant), half dose of analytically significant contamination (C variant), analytically significant contamination (D variant).

Analytical methods

The chickpea seeds were decomposited with using of HNO_3 by microwave digestion in instrument MARS X-PRESS. The solutions were analyzed by flame AAS (AAS Varian AA Spectr DUO 240 FS/240Z/UltrAA). Gained results in $mg.kg^{-1}$ of dry mater were evaluated according to the **Food Codex of the Slovakia** valid in the Slovak Republic as well as according to **Commission Regulation 1881/2006**.

Total polyphenols

Total polyphenol content (TP) was determined using Folin-Ciocalteau reagent (FCR) according to **(Lachman *et al*., 2003)**. The solutions were measured at 765 nm using Shimadzu spectrophotometer (710, Shimadzu, Kyoto, Japan). The total polyphenols content was calculated as gallic acid equivalents (GAE) in milligrams per kilogram of dry matter.

The antioxidant activity (AA) was determined using free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to **(Brand-Williams *et al*., 1995)**. The solutions were measured at 515 nm in a Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan). Results were calculated as % inhibition of DPPH.

Statistics

All statistical analyses were carried out using the statistical software Statistica 12.0 (Statsoft, USA). Each analysis was done in six repetitions. Descriptive data analysis included mean, standard deviation. Mean comparisons between heavy metals content were done by the LSD-test, $P<0.05$.

RESULTS AND DISCUSSION

Two experiments were realised as the pot trials in the vegetation cage with the aims to investigate the effect of addition of increasing rates of selected micronutrients (zinc, copper) into the soil and its consecutive accumulation in chickpea seeds on total polyphenols and antioxidant activity.

The evaluation of heavy metals content

The graded Zn doses applied into the soil in the model conditions resulted in increased Zn content in seeds of chickpea harvested in the stage of full ripeness are presented in Table 1. The strong statistical relationship between soil Zn content and Zn amount in seeds of chickpea was confirmed (R= 0.889). In all variants the Zn content in seeds of chickpea was lower than hygienic limit. The determined Pb and Cd contents in control seeds were higher than maximal allowed amounts given by the legislative (by 1200% and 90%, respectively). The Zn, Cu, Pb and Ni contents were increased, the Cd content was decreased and the Cr content was only slightly changed. The strong positive statistical relationship between soil Zn content and Cu, Pb and Ni amount in seeds of chickpea was confirmed (R= 0.799, R= 0.880, R= 0.951, respectively). The negative linear statistical relationship between soil Zn content and Cd amount in seeds of chickpea was confirmed (R= - 0.774). Despite of decreased Cd content even in D variant the determined Cd amount in chickpea seeds was by 20% higher than hygienic limit.

Table 1 Heavy metals contents ($mg.kg^{-1}$) in seeds of chickpea cv. Slovak after Zn application into soil

Var.	Zn	Cu	Ni	Cr	Pb	Cd
A	31.63 (1.61)	6.43 (1.38)	0.70 (0.14)	0.93 (0.11)	2.58 (0.20)	0.19 (0.05)
B	35.60 (0.16)	5.05 (1.76)	0.95 (0.36)	0.80 (0.07)	2.50 (0.08)	0.14 (0.04)
C	47.85 (3.55)	9.65 (1.45)	1.45 (0.17)	1.10 (0.14)	3.00 (0.15)	0.14 (0.06)
D	49.08 (0.84)	9.15 (0.57)	1.65 (0.07)	0.90 (0.22)	3.00 (0.22)	0.12 (0.02)
Limit	50.0	15.0	3.0	4.0	0.2	0.1
Max.	-	-	-	-	0.2	0.1

Legend: The results show the mean value and the standard deviation (in parenthesis). Limit value for legumes according to the Food Codex of the Slovakia was shown. Max. value is maximal residue level according to Commission Regulation 1881/2006.

Wang *et al.* (2010) determined lower amounts of Cu and a similar Zn content in comparison to our results. By **Khan *et al.* (1998)** observed also zinc concentration in the shoot tissue of chickpea increased with an increase in Zn fertilisation and they confirm that low and high moisture regimes had no effect on critical Zn concentration. **Rutkowska *et al.* (2014)** reported the concentration of active zinc ions (Zn^{2+}) in the soil solution for the most part relies upon soil reaction when compared with other investigated physical and chemical soil properties. Higher Zn concentration can be projected in the soil solution of acidic soils when compared to soils with neutral soil reaction.

The graded Cu doses which have been applied to the soil in the model conditions resulted in increased Cu content in seeds of chickpea harvested in the stage of full ripeness are presented in Table 2. The strong statistical relationship between Cu content in the soil and Cu content in seeds of chickpea was confirmed (R= 0.963). In all variants the Cu content in seeds of chickpea was lower than the legislative determined limit value. In control variant only the established Pb content was by 125% higher than maximal allowed amounts given by the legislative regulation. In variants with Cu application the Cu, Ni, Cr and Cd contents were increased, whereas the Zn and Pb contents were decreased and the Zn content was only slightly changed. In D variant (with the highest Cu dose into the soil) the Cd content in chickpea seeds was by 50% higher than the hygienic limit. The positive statistical relationship between soil Cu content and Ni, Cr and Cd amount in seeds of chickpea was confirmed (R= 0.600, R= 0.822, R= 0.980, respectively). The negative statistical relationship between soil Cu content and Pb amount in seeds of chickpea was confirmed (R= - 0.706).

Table 2 Heavy metals contents ($mg.kg^{-1}$) in seeds of chickpea cv. Slovak after Cu application into soil

Var.	Zn	Cu	Ni	Cr	Pb	Cd
A	37.50 (0.12)	7.35 (0.11)	1.08 (0.08)	1.23 (0.13)	0.45 (0.05)	0.05 (0.01)
B	37.05 (0.11)	8.60 (0.10)	1.63 (0.04)	1.10 (0.07)	0.35 (0.11)	0.04 (0.01)
C	37.88 (0.15)	9.05 (0.17)	2.13 (0.23)	1.23 (0.08)	0.25 (0.05)	0.10 (0.01)
D	35.10 (0.12)	8.85 (0.11)	1.75 (0.05)	1.45 (0.11)	0.30 (0.07)	0.15 (0,03)
Limit	50.0	15.0	3.0	4.0	0.2	0.1
Max.	-	-	-	-	0.2	0.1

Legend: The results show the mean value and the standard deviation (in parenthesis). Limit value for legumes according to the Food Codex of the Slovakia was shown. Max. value is maximal residue level according to Commission Regulation 1881/2006.

The determined contents of Cu, Pb and Zn by **Salama, Radwan (2005)** in lentil and chickpea seeds were many times lower than those determined in our cultivar, and only Cd content determined by these authors was similar to our findings. **Głowacka (2014)** observed an interspecific facilitation between neighbouring plant species. Placement adjacent to the oat strip contributed to higher Cu content in the maize, while placement next to blue lupin increased the content of Fe and Zn. This makes it possible to eliminate or mitigate mineral deficiencies in the plants.

The total polyphenols content and antioxidant activity evaluation

Total polyphenols (TP) content and antioxidant activity (AA) determined in chickpea cv. Slovak harvested in the stage of full ripeness are presented in Table 3. The determined values of total polyphenol content ranged from 393 mg GAE.kg^{-1} (Cu application) to 1215 mg GAE.kg^{-1} (Zn application). The determined values of antioxidant activity (as DPPH inhibition) were in interval 4.33% (Cu application) - 11.15 % (Zn application).

Table 3 Total polyphenols content (mg.kg^{-1}) and antioxidant activity (% of DPPH inhibition)

Variant	Total polyphenol content		Antioxidant activity	
	Zn application	Cu application	Zn application	Cu application
A	742±10^{a}	672±10^{a}	10.32±1.60^{a}	4.80±0.90^{a}
B	1107±58^{b}	566±31^{b}	11.15±1.07^{b}	5.83±0.39^{b}
C	1215±46^{c}	574±66^{b}	9.33±1.04^{c}	4.93±0.80^{a}
D	661±24^{d}	393±12^{c}	5.77±1.50^{d}	4.33±0.53^{c}

Legend: The results show the mean ± the standard deviation. Values in the same column with different letters present significant differences $p < 0.05$.

With increased Zn doses applied into the soil the TP contents established in seeds of chickpea cv. Slovak were increased. The maximal TP content in seeds of chickpea seeds harvested in the stage of full ripeness was found at 250-300 mg Zn applied into 1 kg of the soil while after application of higher Zn doses into the soil a lower TP content in seeds of this legume was observed. The strong statistical relationship was confirmed between Zn content in the soil and TP content (R= 0.911) as well as AA values (R = 0.992) in chickpea seeds.

With increased Cu doses applied into the soil the TP contents determined in seeds of chickpea cv. Slovak were decreased. The moderate statistical linear negative relationship between soil Cu content and TP content in seeds of chickpea cv. Slovak was confirmed (R = -0.671).

With increased Cu doses applied into the soil the AA values determined in seeds of chickpea cv. Slovak were only slightly changed. The moderate statistical linear positive relationship between soil Cu content and AA value in seeds of chickpea cv. Slovak was confirmed (R = 0.621).

Our values of polyphenols and antioxidant activity are although much lower than reported by **Xu, Chang (2007**). However, they are in partial agreement with other authors **(Xu, Chang, 2008; Zia-Ul-Haq *et al*., 2008)**. The differences between results may be attributed to the differences in the sources of the samples.

CONCLUSION

The graded Zn doses applied into the soil in the model conditions resulted in increased Zn content in seeds of chickpea harvested in the stage of full ripeness. The strong statistical relationship between soil Zn content and Zn level in seeds of investigated chickpea cv. Slovak was confirmed.

With increased Cu doses applied into the soil the Cu contents determined in the fully rippened seeds of chickpea cv. Slovak were increased. The strong statistical relationship between soil Cu content and Cu amount in seeds was confirmed.

Graded Zn doses applied into the soil resulted in statistically significant increased Pb amounts and significantly decreased Cd amount in seeds of chickpea cv. Slovak, whereas inverse relationship for graded Cu doses applied into the soil and those elements was observed (decreased Pb amounts, increased Cd amount). The determined Pb and Cd contents in seeds were higher than maximal allowed amounts given by the legislative. The contents of other tested heavy metals (Zn, Cu, Ni, Cr) were lower than hygienic limits.

In experiment conditions, the total polyphenols contents determined in seeds chickpea cv. Slovak harvested in the stage of full ripeness were increased with soil enrichment with Zn. Moreover, the strong statistical correlation between the content of Zn added into the soil and the antioxidant activity values was confirmed.

Graded Cu doses applied into the soil resulted in increased AA values in seeds of chickpea cv. Slovak harvested in the stage of full ripeness, whereas inverse relationship for TP content was observed.

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THE EFFECT OF ADDED PUMPKIN FLOUR ON SENSORY AND TEXTURAL QUALITY OF RICE BREAD

Vikendra Dabash[1], *Iva Burešová**[1], *Marián Tokár*[2], *Michaela Zacharová*[1], *Robert Gál*[1]

Address(es): doc. RNDr. Iva Burešová, Ph.D.,
[1]Tomas Bata University in Zlín, Faculty of Technology, Department of Food Technology, nám. T. G. Masaryka 5555, 760 01 Zlín, Czech Republic, phone number: + 420 57 603 3333.
[2]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Storing and Processing of Plant Products, Tr. A. Hlinku 2, Nitra, Slovak Republic.

*Corresponding author: buresova@ft.utb.cz

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ABSTRACT

The impact of added pumpkin flour (1–10 g/100 g) on sensory and textural quality of rice bread was studied on rice biologically leavened rice bread. Specific volume, baking loss, crumb springiness, cohesiveness and resilience decreased with increasing amount of pumpkin flour. The opposite trend was found in crumb hardness and chewiness. Color measurements revealed that the lightness decreased and the greenness increased with increasing amount of pumpkin flour. Bread crumb and crust characteristics were not significantly impacted by the amount of added pumpkin flour. Additionally, the bread taste and flavor were decreased in breads with 8–10 g/100 g of added pumpkin flour, resulting in their lower overall acceptability. Weak impact of added pumpkin flour on sensory evaluation of rice bread may be related to a reduced amount of the panelists involved in this study. The addition of 1–7 g/100 g of pumpkin flour seems to be applicable to rice bread production. More extensive study will, however, be performed to describe the impact of added pumpkin flour on sensory quality of rice bread in more details.

Keywords: Gluten-free, fortification, crumb quality

INTRODUCTION

The nutritional quality of gluten-free (GF) breads is often decreased by low protein and fiber contents **(Rosell *et al.*, 2001)**. It may be increased by the addition of nutritionally valuable ingredients. Pumpkin (*Cucurbita moschata*) flour is known to be rich in carotene, pectin, minerals, vitamins and other substances beneficial to health such as phenolic phytochemicals (**Ptitchkina *et al.*, 1998; Kwon *et al.*, 2007; Dhiman *et al.*, 2009)**. Pumpkin has, moreover, potential to improve bland taste of rice bread due to its highly pleasure flavor and sweet taste. Additionally, pumpkin seeds proteins are known to increase in vitro protein digestibility of gluten free bread **(El-Soukkary, 2001)**, which may also positively impact bread quality. Pumpkin flour is one of the processed products of pumpkin fruit, which can be easily stored for long time and conveniently used in manufacturing of formulated foods. Pumpkin flour could be used to supplement cereal flours in bakery products to improve nutritional, physical and sensory qualities of these products **(Chi-Ho *et al.*, 2002**; **See *et al.*, 2007)**. The impact of pumpkin flour addition on nutritional quality of wheat bread was previously studied by **See *et al.* (2007)**. They concluded that rising amount of pumpkin flour resulted in decrease of loaf specific volume but increase of crumb hardness. Moreover, increasing level of pumpkin flour resulted in decreasing sensory acceptability. The positive effect on loaf volume and organoleptic acceptability of bread were also reported by **Ptitchkina *et al.* (1998)**.
The effect on added pumpkin flour on quality of gluten free rice bread has, however, not been described yet. Thus, the aim of this study was to investigate the impact of added pumpkin flour on sensory quality of rice bread, crumb color, as well as crumb characteristics measured by texture profile analysis. Disregarding the reduced amount of panelists involved in this study, the obtained preliminary results may indicate an applicability of pumpkin flour in gluten-free breadmaking.

MATERIAL AND METHODS

Flours

Fine rice flour was kindly provided by Adveni Medical, spol. s r.o. Brno, Czech Republic.

Bread preparation

A formula for dough preparation consisted of rice flour (100 g), water (110 g), sucrose (1.86 g), salt (1.50 g) and yeast (1.80 g). The amounts of 1 g, 2 g, 3 g, 4 g, 5 g, 6 g, 7 g, 8 g 9 g and 10.0 g of pumpkin flour were added on top of other ingredients. The amounts of the ingredients were related to 100 g of flour dry matter. Dry yeast was reactivated for 10 ± 1 min. in a sugar solution (35 ± 1 °C). The dough ingredients were placed into an Eta Exclusive Gratus mixer bowl (Eta, a.s. CZ) and mixed for 6 min. A 150 g of dough was scaled into bread pans and placed into a proofer for 20 min. at 30 ± 1 °C and 85% relative air humidity. The loaves were baked for 20 min. at 180 ± 5 °C in an oven MIWE cube (Pekass s.r.o. Plzeň, CZ). After baking, the breads were stored at room temperature for 2 h, and then analyzed. Each test was performed on dough samples prepared at least in three replicates. The given results are represented as mean values.

Textural properties of bread

Textural properties of bread crumb were measured using texture profile analysis (TPA) on a texture analyzer TA.XT plus (Stable Micro Systems Ltd., UK). TPA was performed on samples 35 mm in diameter and 10 mm in height obtained from the center of each loaf. The sample was placed onto the analyzer base and squeezed twice to 4 mm with the 75.0 mm diameter cylinder probe P/75. Test speed of probe was 1.00 mm/s. The crumb parameters (hardness, stickiness, elasticity, cohesiveness and chewiness) were determined using Exponent Lite software.

Sensory evaluation

The breads were subjected to sensory evaluation by a panel of 10 department staff and students, both male and female between the ages of 19 - 50 years. A nine point hedonic scale was used to evaluate the characteristics of bread crumb and crust. Sensory score range from 1: dislike extremely to 9: like very much was used. An extensive sensory evaluation performed by higher number of panelists will be performed to support the preliminary results obtained in this study.

Statistical analysis

The results were statistically analyzed using analysis of variance (ANOVA). The differences were tested on $\alpha = 0.05$ significance level using Fisher LSD test. Statistical analysis was accomplished using Statistica CZ9.1 software (Stat Soft Ltd., CZ).

RESULTS AND DISCUSSION

Baking loss of rice bread (15 %) was decreased to 12 % by the presence of pumpkin flour (Table 1). The observation may be related to high pumpkin flour ability to absorb water during dough preparation as well as during baking (**Rodge *et al.*, 2012)** decreasing the amount evaporated water. The bread baking loss forms the biggest losses in the technological process. The value of baking loss of rice bread with pumpkin flour decreases compared with the control bread sample, therefore the technological bread weight losses during rice bread production using pumpkin flour could be not significant. According to the experimental results, the values of bread dry off are similar for the control and experimental bread samples.

Specific loaf volume of rice bread (1.17 g/mL) was decreased by the presence of pumpkin flour to 1.00–1.16 g/mL, however significant ($P < 0.05$) decrease was observed only in bread containing 4 g/100 g (1.00 g/mL), 5 g/100 g (1.01 g/mL), and 10 g/100 g (1.04 g/mL) of pumpkin flour (Table 1). The reduction can be explained by high content of fiber in pumpkin flour **(Wang *et al.*, 2004)** since the fiber weakens dough structure, resulting in decrease of CO_2 retention in dough.

Table 1 The impact of added pumpkin flour on bread specific volume, baking loss and crumb characteristics evaluated by texture profile analysis

Pumpkin flour addition (g/100g)	Specific loaf volume (mL/g)	Baking loss (g/100g)	Hardness (N)	Springiness (%)	Cohesiveness (%)	Resilience (%)	Chewiness (N)
0	1.17±0.07^{d}	15±1^{d}	36±3^{a}	82±2^{e}	72±2^{b}	41±2^{c}	21±1.17^{a}
1	1.10±0.03^{bcd}	14±1^{cd}	43±7^{ab}	79±1de	71±2^{ab}	40±2^{bc}	24±4^{abc}
2	1.10±0.03^{bcd}	12±1^{abc}	43±12^{ab}	75±3^{bcd}	71±4^{ab}	40±5^{abc}	23±5^{ab}
3	1.10±0.09^{bc}	13±1^{abc}	44±4^{ab}	74±3^{bcd}	69±1^{ab}	40±1^{bc}	23±2^{ab}
4	1.00±0.10^{a}	12±1^{abc}	62±5^{cd}	75±3^{bcd}	68±1^{ab}	38±1^{abc}	32±4de
5	1.01±0.03^{a}	12±1^{abc}	53±9^{bc}	73±1^{abc}	71±7^{ab}	39±5^{abc}	27±3^{bcd}
6	1.16±0.08^{cd}	14±1^{cd}	39±6^{a}	77±2^{cd}	66±2^{a}	36±2^{a}	20±3^{a}
7	1.13±0.04^{cd}	13.0±0.5^{abc}	68±10^{d}	70±3^{a}	66±3^{a}	36±3^{ab}	31±7de
8	1.11±0.04^{bcd}	14.0±0.2^{bc}	61±10^{cd}	71±5^{ab}	70±3^{ab}	39±2^{abc}	30±6^{cde}
9	1.11±0.04^{bcd}	13±2^{abc}	71±10^{d}	73±7^{abc}	66±6^{a}	37±5^{ab}	34±6^{e}
10	1.05±0.05^{ab}	13±2^{abc}	60±4^{cd}	75±5^{cd}	68±2^{ab}	37±2^{ab}	31±3de

Crumb hardness is commonly used as an indicator of bread staling, and it is negatively co-related with bread quality **(Wang *et al.*, 2007)**. The hardness of rice bread rose with increasing amount of added pumpkin flour. Hardness of rice bread (35 N) was increased by the presence of pumpkin flour, however significant ($P < 0.05$) increase was observed only in the presence of 6 g/100 g, (38 N), 2 g/100 g (42 N) and 1 g/100 g (43 N) of pumpkin flour. Additionally, the rising amount of added pumpkin flour amount caused the creation of darker and greener crumb (Table 2).

Table 2 The impact of added pumpkin flour on crumb lightness *L**, red/green coordinate *a**, yellow/blue coordinate *b**

Pumpkin flour addition (g/100g)	L*	a*	b*
0	79.4±0.6^{f}	-0.07±0.08^{g}	14±0.3^{a}
1	76.8±0.9^{e}	-0.87±0.05^{f}	15±0.1^{b}
2	76±2^{e}	-1.00±0.2^{f}	15±0.3^{b}
3	74.6±0.5^{d}	-2.00±0.1^{e}	16±0.3^{c}
4	73.1±0.6^{c}	-2.3±0.1^{de}	17±1^{cd}
5	70±1^{b}	-2.6±0.2^{bc}	19±1^{e}
6	73.1±0.6^{c}	-2.5±0.1^{cd}	17±1^{d}
7	72.6±0.6^{c}	-2.3±0.3^{d}	18±1^{e}
8	70.0±0.6^{b}	-3.0±0.3^{a}	19±1^{e}
9	69.5±0.7^{b}	-3.1±0.2^{a}	19±1^{e}
10	67.7±0.8^{a}	-2.8±0.4^{ab}	20±1^{f}

Springiness, cohesiveness, and resilience were decreased by the presence of pumpkin flour (Table 1). However significant ($P < 0.05$) effect was observed only in the presence of 1–6 g/100 g. The observation may be related to dough weakening initiated by fiber, decreasing dough ability to trap leavening gas (**Plyer, 1988**).

Chewiness of rice bread (20%) was increased by the presence of pumpkin flour to 21–33 %, however significant ($P < 0.05$) increase were observed only in bread 1 g/100 g, 2 g/100 g, and 6 g/100 g. **Gomez *et al.* (2003)** also showed similar trend for breads with addition of fibers since they caused an increase in chewiness of tested breads.

Crumb color was found to be darker and less acceptable in breads containing pumpkin flour (Table 2). Darkness of the crumb was, moreover, directly related to the amount of pumpkin flour. The results of texture profile analysis indicated the significant impact of added pumpkin flour on rice bread crumb. The panelists, however, did not recognize any significant differences between samples (Table 3). Only taste and flavor was decreased by the presence of 8–10 g/100 g of pumpkin flour, resulting in lower overall acceptability of these breads. The impact of the presence of pumpkin flour on sensory evaluation of rice bread was weak. However, if backed by an extensive sensory study, this could be an important positive result, since pumpkin flour may improve nutritional quality of rice bread without a declining impact on bread sensory evaluation.

Table 3 Sensory evaluation of rice bread with added pumpkin flour

Pumpkin flour addition (g/100g)	Crust appearance and color	Crumb appearance and color	Crumb elasticity	Crumb porosity	Taste and flavor	Overall acceptability
0	7^{a}	5^{a}	4^{a}	4^{a}	6^{a}	6^{a}
1	7^{a}	6^{a}	5^{a}	5^{a}	6^{a}	6^{a}
2	7^{a}	6^{a}	5^{a}	4^{a}	6^{a}	6^{a}
3	6^{a}	6^{a}	5^{a}	4^{a}	6^{a}	6^{a}
4	7^{a}	6^{a}	5^{a}	4^{a}	6^{a}	6^{a}
5	7^{a}	6^{a}	4^{a}	4^{a}	5^{ab}	6^{a}
6	7^{a}	6^{a}	5^{a}	5^{a}	5^{ab}	6^{a}
7	6^{a}	6^{a}	5^{a}	5^{a}	6^{a}	6^{a}
8	7^{a}	5^{a}	5^{a}	5^{a}	4^{b}	5^{ab}
9	6^{a}	6^{a}	5^{a}	5^{a}	4^{b}	5^{ab}
10	7^{a}	5^{a}	5^{a}	4^{a}	4^{b}	4^{b}

CONCLUSION

The addition of pumpkin flour to rice flour at different levels modified the textural properties of the enriched breads. The impact on sensory evaluation was, however, weak. Pumpkin flour seems to be able to improve rice bread quality. An extensive sensory study is, however, necessary to back these preliminary results.

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OPTIMIZATION OF ALPHA AMYLASE FOR BETTER DOUGH PREPARATION

Flory Shobana Maria[1], *Ranjithkumar Rajamani*[2] *and Dinesh Bheeman*[*#1]

Address(es):
[1] Department of Biotechnology, Sree Narayanaguru College, Coimbatore, Tamil Nadu, India.
[2] Department of Biotechnology, Dr.N.G.P. Arts and Science College, Coimbatore, Tamil Nadu, India.

*Corresponding author: dinpree@mail.com

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ABSTRACT

Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. The present study mainly focused on screening of amylase producing *Bacillus subtilis*, production by solid-state fermentation using rice straw and banana pseudo stem and its optimization for amylase activity in dough preparation, effect on bread making and analysis of bread quality. Maximum production of amylase was obtained after 24hrs of incubation. The optimum pH for enzyme activity was found to be at pH 7 and the optimum temperature for the activity was found to be at the range of 30 – 70°C. The combination of 0.8g yeast and 300 U of amylase gave better results than enzyme alone for the better dough preparation.

Keywords: Amylase, *Bacillus subtilis*, solid state fermentation, optimization

INTRODUCTION

The industrial enzyme producers sell enzymes for a wide variety of applications and approximately more than 25% amylases represent as a class of industrial enzymes of the market **(Sidhu *et al*., 1997; Rao *et al*., 1998)**. Starch digesting amylase has found important application in bioconversion of starches and starch-based substrates **(Fogarty *et al*., 1983)**. Amylolytic enzymes are of great significance in biotechnological applications in food industry, amylases can be synthesized from numerous sources, like plants, animals and microorganisms. The enzymes from microbial sources commonly meet industrial demands and had made important contribution to the production of foods and beverages since, microbial amylases have almost completely replaced chemical hydrolysis of starch in starch processing industry **(Pandey *et al*.,2000a; Gupta *et al*., 2003; Kandra, 2003; Rajagopalan and Krishnan, 2008)**. Two major classes of amylases have been identified in microorganisms, namely, α-amylase and glucoamylase. α-Amylases (endo-1,4-a-D-glucan glucohydrolase) are extracellular enzymes that randomly cleave the 1,4-a-D-glucosidic linkages between adjacent glucose units in the linear amylase chain. Glucoamylase (exo-1,4-a-D-glucanm glucanohydrolase) hydrolyzes single glucose units from the nonreducing ends of amylose and amylopectin in a stepwise manner **(Rameshkumar and Sivasudha, 2011; Nigam and Singh, 1995)**. Alpha-amylase can produce by a wide spectrum of organisms, even though every source produces depend upon biochemical phenotypes that considerably differ in parameters such as pH and temperature optima as well as metal ion requirements. Submerged fermentation (Smf) has been a traditional way for production of industrially significant enzymes since long past due to multiple facilities like better control over environmental factors namely, pH, temperature, aeration, and moisture level. Cultures reported to be utilized for amylase production using SmF belong to a variety of *Bacillus* species like *Bacillus* sp.PN5, *Bacillus subtilis* JS-2004, *Bacillus* sp. IMD 435, *Bacillus* sp. I-3, *Bacillus caldolyticus* DSM405, *Bacillus licheniformis* GCBU-8 **(Saxena *et al*., 2007; Asgher *et al*., 2007; Hamilton *et al*., 1999; Goyal *et al*., 2005; Schwab *et al*., 2009; Haq *et al*., 2003)** since, Solid State Fermentation (SSF) replaces SmF as it mimics the natural habitat of microorganisms. SSF is alternative over SmF due to its low cost, lower energy requirement, simplicity, less water output, and lack of foam built up **(Couto and Sanroman, 2006; Pandey, 2003; Tanyildizi *et al*., 2007)**. Biotechnological innovations particularly in the area of fermentation technology and enzyme technology, SSF with agro wastes such as WB, RB, COC, and GOC has replaced the high cost media generally used in submerged fermentation for alpha amylase preparation and *Bacillus* species are frequently used for α-amylase production **(Mulimani and Ramalingam, 1999; Shukla and Kar, 2006; Vijayabaskar *et al*., 2012; Baysal *et al*., 2003; Mukherjee *et al*., 2009; Sodhi *et al*., 2005; Soni *et al*., 2003)**. The present study mainly focused on screening of amylase producing *Bacillus subtilis*, production by solid-state fermentation using rice straw and banana pseudo stem and its optimization for amylase activity in dough preparation, effect on bread making and analysis of bread quality.

MATERIAL AND METHODS

Isolation Of *Bacillus subtilis* from the environment

A cut piece of cut potato was buried about four inches deep, and covered with soil. After about 6-8 days, the potato was digged out; the soil was scarped off and was taken it to the lab in the ziploc bag or a plastic bag. The amylase producer was isolated from that soil sample. 10g of soil was suspended in 90ml of sterile distilled water, properly mixed. From the above, 10ml was transferred to 90ml of sterile distilled water. Two further dilutions were done in 90ml of sterile distilled water. 0.1ml of diluted sample was delivered in nutrient agar containing 1% (w/v) starch (corn starch) by means of pour plate techniques. The plate was incubated for 24 h at room temperature. Amylase producing colonies will have a clear area around them. To confirm, the plate was flood with Gram's iodine.

Optimization of pH for amylase activity

Nutrient agar containing1% (w/v) starch medium was prepared at different pH (3 - 10). The *Bacillus sp*. was streaked in the middle of the agar plates and incubated at room temperature for 24h. Amylase production was detected by the disappearance of blue colour around the colony when treated with iodine solution. Evaluation of the clear zones of each colony was estimated as radius (mm) of the clear zone minus the radius of the colony.

Bacterial amylase inoculation

Bacillus subtilis was inoculated into the bacteriological amylase production media (Bacteriological peptone –6gm, MgSO4.7H2O,-0.5gm, KCl-0.5gm, Starch-1gm Water -1000ml) and incubated at room temperature for 24 h.

Amylase production by solid state fermentation

Two sets of fermentation were carried out in 250ml Erlenmeyer flasks containing production media (Rice bran/ Banana pseudo stem -10g, Urea-0.05%, MgCl2 - 0.25%, KCl-0.25%,Sucrose-1%,Distilled water-30ml), which were autoclaved for 20min at 121℃. A cell suspension of 1% (v/w Rice bran/ Banana pseudo stem) containing viable cells/ml was used as inoculum. Incubation was carried out for 60 h at room temperature. Extraction was conducted using 10g fermented mass in 250ml conical flask. Soaking the fermented solid with a suitable solvent like glycerol, acetone, isopropyl alcohol and water for desired period. The crude extract was then squeezed out through cheese cloth. The clear extract obtained after centrifugation to remove insoluble, assayed for amylolytic activity.

Preparation of crude enzyme

Dialysis will remove residual sugars from the enzyme mixture. One end of a dialysis tube was tied and enzyme mixture was poured into dialysis tube. The other end was tied securely. Dialysis tube was put in distilled water in a beaker. The water was changed several times for 24h. Crude enzyme was poured into clean Universal bottles or screw cap tubes and stored in a freezer at about 0°C until needed. The dialysis sample that was collected in a sterile bottom flask was rotated in ice at 45°angles for the formation uniform thin layer of sample inside the flask and lyophilized. The enzyme powder collected from the flask was transferred into sterile vial and stored at 4°C.

Characterization of the crude enzyme

The effect of temperature on the crude amylase activity was assayed at temperature values ranging from 20°C to 90°C. The reaction mixture contained 0.1 ml of the crude enzyme in 1 ml of corn-starch (1% w/v). This was incubated for 30 mins at each chosen temperature. At room temperature, to 5ml of crude enzyme 0.5 ml of the extract was added and the enzyme activity was found by DNSA method and Bernfeld method **(Bernfeld, 1955)**.

Optimization of enzyme substrate concentration

One unit of enzyme activity was defined as the amount of enzyme that release 1µmole of reducing sugar as glucose per minute under the assay condition specified **(Soumen and Rintu, 2001)**. 1 to 10ml of 1% starch solutions were taken in different test tube and made up to 1ml with distilled water. 1ml of crude enzyme was added to the above starch solution. Incubated in water bath 60°C for 3min and the reaction was stopped by adding 1ml of DNSA reagent. The mixture was boiled for 5min and brought to room temperature then 10ml of distilled water was added and absorbance was measured at 540nm.

Optimization of enzyme concentration on dough preparation

The flour used was commercial standard white wheat flour, containing 30 ppm ascorbic acid, carbohydrate 73%, Protein 9.5%, fat 0.8%, crude fibers traces, minerals 0.3% and moisture contents 11.7%. Five different kinds of dough formulation were prepared for optimize the enzyme concentration. The above formulations prepared with 100g of wheat flour, 25ml of water containing 150 U, 300 U, 450 U, 0.8g yeast and 0.8g yeast with 300 U of amylase enzyme respectively. Then were kept for incubation at different period of time such as 0, 15, 30 minutes respectively. Six different dough formulations were given in the below table I.

Table 1 Six different dough formulations

Ingredients (in grams)	Types of Formulation					
	F1	F2	F3	F4	F5	F6
Flour	150	150	150	150	150	150
Dry yeast	-	1.2	-	-	1.2	1.2
Gluten	1	1	1	1	1	1
Amylase (Units)	-	-	200	400	200	-
Salt	3	3	3	3	3	3
Sugar	-	-	-	-	-	3
Water (ml)	75	75	75	75	75	75
Shortening	3	3	3	3	3	3

Analysis of bread

After baking the loaves volume were measured. Crumb stickiness, softness, and taste ware subjectively evaluated by well-experienced barking staff. A piece of crumb was squeezed between the thumb and two fingers for determine the stickiness of bread. Reducing sugar contents were analyzed by DNSA method. Softness was examined up to 7days. Reducing sugars were extracted from one-day bread crumb by mechanically stirring 2g crumb with 20ml distilled water at 25°C for 25 min and centrifuged at 12000 rpm for 15 min. The extracts were stored frozen if not used immediately. The resulted dates were calculated and tabulated.

RESULTS AND DISCUSSION

Screening of amylase producers

Clear zone around the colony were seen (figure not shown) when the plates were treated with iodine. This indicated the colonies are amylase producers. **Suman and Ramesh, (2010)** reported that the screening of purified cultures was done on MAM (Minimal agar media) supplemented with 1% starch the cultures growing in MAM were flooded with iodine solution and the zone of hydrolysis were obtained in the plates showing starch hydrolysis similar method in order to screen the microorganisms for amylase production.

Optimization of pH

pH and temperature have very important roles to play in amylase activity and stability. In this study the optimum pH range was found to be 7 but it works also in various pH ranges between 5 to 8. The colonies, which were grown in different pH media was treated with iodine solution. The zone of clearance was absorbed maximum in the pH 7 this indicate that the optimum pH for amylase production was 7 (Fig-1). Amylases are generally stable over a wide range of pH from 4 to 9 **(Vihinen and Mantsala, 1989)**; however, according to **Robyt *et al*. (**1971) amylases with stability showed in a narrow range of pH.

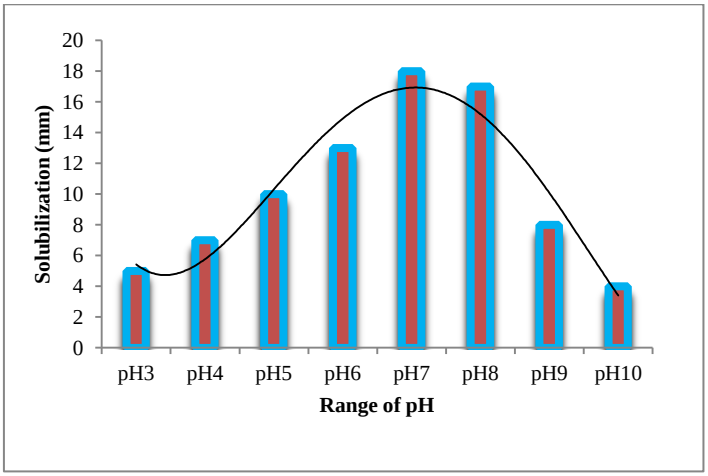

Figure 1 pH optimization by agar plate method

Solvent extraction

The extraction efficiency is critical to the recovery of the enzyme from the fermented biomass hence, selection of a suitable solvent is necessary. Different solvents selected were water, glycerol, and acetone. Among the solvent used glycerol gave the best result. Glycerol was found to be a suitable solvent to extract the extracellular amylase from the solid substrate fermentation medium. It showed the maximum release of 0.232 mg and 0.216 mg free glucose on banana pseudo stem and rice brawn substrate respectively followed by found to be isopropyl alcohol extraction (Table 2).

Table 2 Extraction of extra cellular enzyme from solid substrate by differed solvent systems

Type of solvent	Glycerol	Acetone	Isopropyl alcohol	Water
Free glucose (mg)/ 5 ml of Rice brawn as substrate (1%)	0.216	0.091	0.201	0.121
Free glucose (mg)/ 5 ml of Banana pseudo stem as substrate (1%)	0.232	0.086	0.226	0.176

Optimization of amylase enzyme activity

Effect of pH on amylase activity

The optimum pH was observed between the pH ranges of 5.0 - 8.0. After pH 8, a continuous decrease in enzyme activity was observed (Fig-2). Previous report

indicated the maximum production of amylase activity was obtained at pH of 7.0 **(Mukesh *et al*.,2012)**.

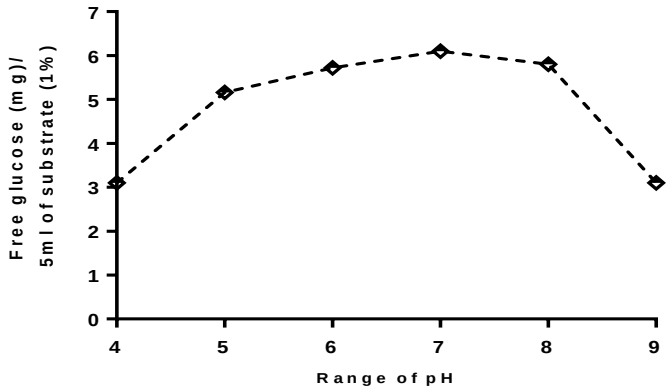

Figure 2 Effect of ph on amylase activity

Effect of temperature on amylase activity

The influence of temperature on amylase activity of the crude enzyme showed that enzyme activity increased progressively with increase in temperature from 20°C reaching a maximum at the range of 30 – 70°C. Above 70°C, there was a reduction in the amylase activity (Figure 3). Earlier studies indicated maximum amylase production occurred at the optimum growth temperature. The optimum temperature for enzyme activity was between 45°C and 55°C **(Mukesh *et al*., 2012)**. A reduction in enzyme activity was observed at values above 60°C. **Mukesh kumar *et al*. (2012)** reported the amylase activity attained its maximum at 50°C beyond which the enzyme activity was reduced gradually.

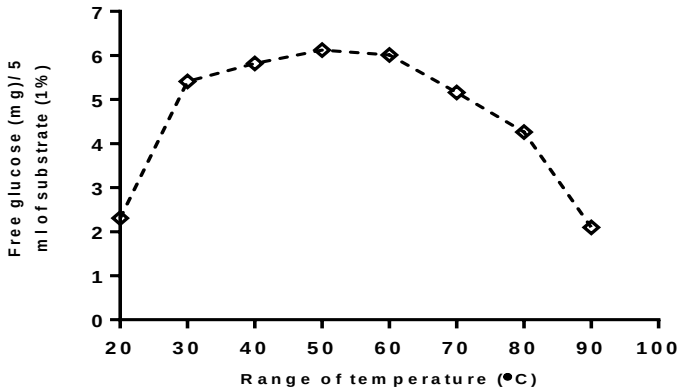

Figure 3 Effective on temperature on amylase activity

Effect of starch concentration on amylase activity

In the present study, amylase activity increased with increase in the starch concentration from 1ml to 10 ml of 1% starch concentration (Fig-4). The production of enzyme is greatly dependent on the condition of growth of the culture and composition of nutrient medium. The medium constituents have been predicted to play an important role in enhancing the production of enzyme from microbial sources **(Gupta *et al*., 2002).**

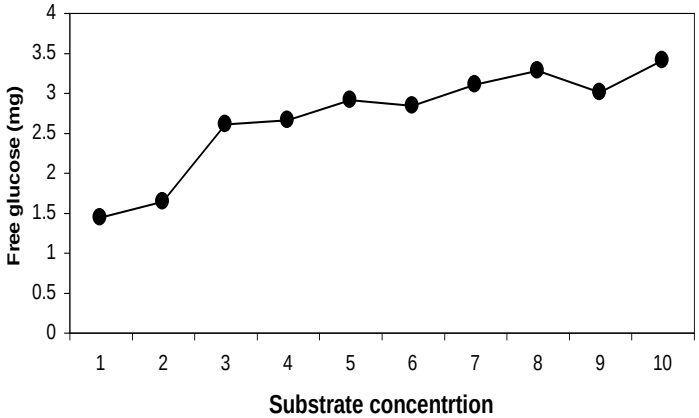

Figure 4 **Subtract concentration on amylase activity**

Optimization of enzyme concentration on dough preparation

The enzyme concentration was optimized based on the reducing sugar and incubation period. It was found to be 300 U for 30 min at 27°C in 100g of wheat flour. The combination of 0.8g yeast and 300 U of amylase gave better results than enzyme alone by means of dough volume. The detailed results were tabulated (Table- 3). Extra enzymes added to the dough improve control of the baking process, allowing the use of different baking processes, reducing process time, slowing-down staling, compensating for flour variability and substituting chemical additives **(Tramper *et al*., 2005).**

Table 3 Optimization of enzyme concentration on dough preparation

Enzyme/yeast	Flour gm	Incubation period	Reducing sugar (mg/gm of dough)
Amylase 150U	100	0 min	1.1
		15min	4.2
		30 min	6.7
Amylase 300U	100	0 min	1.82
		15 min	6.12
		30 min	9.62
Amylase 450U	100	0 min	2.27
		15 min	9.16
		30 min	13.72
Yeast 0.8g	100	0 min	0.42
		15 min	1.82
		30 min	3.62
Yeast 0.8g+300U amylase	100	0 min	2.16
		15 min	7.15
		30 min	10.82

Addition of α-amylase gave moderate crumb firmness after 7 days of storage than without enzyme product. The moisture content in breadcrumb has been shown to decrease during storage **(Herz, 1965)**. Addition of enzyme preparations caused significantly darker crusts (400U/150g of flour) compared to breads with 200 U and addition of yeast products. This can be explained by increased formation of reducing sugars when α-amylase is added and the estimated reducing sugar results were tabulated (Table-4) No differences were found between the other formulations like without enzyme, and without yeast products. The higher values for the intermediate resting time may be explained by assuming that, during the initial fermentation, the production of low-molecular weight sugars exceeded that metabolized by the yeast.

Table 4 estimation of reducing sugars of bread

Types of formulation	Reducing sugars (mg/gm of bread)
F1	3.1
F2	7.7
F3	14.23
F4	25.5
F5	20.6
F6	8.1

CONCLUSION

Amylase producing *Bacillus subtilis* was isolated from soil (potato tuber). The amylase enzyme activity was optimized at various parameters like pH, temperature and enzyme substrate concentration. It was found that the enzyme works wide ranges of pH (pH 5-8) and the temperature ranges from 30°C to 60°C. On SSF Banana pseudo stem reaches higher enzyme activity and glycerol was found to be a suitable solvent system for enzyme extraction. Crust darkness was significantly affected by addition of α-amylses. Independent of added α-amylase, resting time influences crust darkness, with maximum crust darkness obtained after 30 min. Addition of α- amylase reduced the dependence of loaf volume on mixing time, but gave slightly significant increase in bread volume. The present study concluded that the combination of α-amylase and yeast formulation showed better results compared with other formulations. So this enzyme system may be used under optimized concentration. Higher dosage of amylase gave high stickiness due to the formation of high levels of reducing sugars.

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EFFECT OF POMEGRANATE PEEL POWDER ON THE HYGIENIC QUALITY OF BEEF SAUSAGE

Ebied, A. Saleh[1], Alaa Eldin M. Morshdy[2], Abd-El-Salam E. Hafez[2], Mohamed A. Hussein[2], Eman S. Elewa[2], and Abdallah Fikry A. Mahmoud[2]*

Address(es):
[1]Food Control Department, Faculty of Veterinary Medicine, Damanhour University, Egypt.
[2]Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt.

*Corresponding author: abdallah.fikry90@gmail.com

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ABSTRACT

Pomegranate (*Punica granatum*) peel is a nutrient-rich byproduct whose production are extensively growing due to the exponential rise in the pomegranate juice production. Pomegranate juice and related products are directly added to foods due to their pleasant taste, palatability, and preservative effects. In this study, the effect of pomegranate peel powder was investigated at a concentration of 2.5 % and 5% on beef sausage stored at -18 ± 2°C. A significant effect on pH was detected at zero time where control, 2.5% and 5% had pH values of 6.18 ± 0.14, 5.87± 0.13 and 5.54 ± 0.17, respectively. Meanwhile, the significant effect of pomegranate peel powder on total volatile nitrogen (TVN) and thiobarbituric acid (TBA) appeared on 4th week. Total bacterial counts (TBC) and *Enterobacteriaceae* counts were reduced significantly on 1st and 3rd weeks in examined samples of different groups. Thus, in general, it can be concluded that addition of pomegranate peel powder is an effective tool to decrement pH, TVB-N, TBA and bacterial counts in oriental sausage.

Keywords: Beef sausage, Aerobic plate count, *Enterobacteriaceae*, TBA, TVB-N

INTRODUCTION

Sausages are comminuted processed meat products prepared from red meat, poultry or a combination of these with water, binders and seasoning. They are commonly stuffed into a casing, and may be cured, smoked or cooked. Sausages as one of the oldest varieties of meat processing in which meats go through various modification processes to acquire desirable organoleptic and keeping properties. The Sausage manufacture is a simple procedure of allowing meat to undergo series of controlled structural and chemical changes. These are basic to all cultures but the changes rely on varied methods of preparation and seasoning to achieve desired distinctive characteristics. Even though the size and scope of operation have experienced a remarkable level of change the principles and idea behind modern day sausage manufacture in achieving products of high organoleptic value and improved shelf life remain the same **(Savić, 1985).** Pomegranate (*Punica granatum*) from the Punicaceae family is an important commercial fruit crop that is extensively cultivated in parts of Asia, North Africa, the Mediterranean and the Middle East. Some studies have reported that different parts of the pomegranate fruit such as juice, peel, and seeds may act as potential antimicrobial agents **(Duman *et al*., 2009; Abdollahzadeh *et al*., 2011; Choi *et al*., 2011; Singh *et al*., 2014)**. Recently, higher antimicrobial and antioxidant activities of pomegranate peel extracts have been reported and therefore might be suggested as a safe natural option to synthetic antimicrobial agents **(Rosas-Burgos *et al*., 2017)**. Additionally, the pomegranate peel extract showed the highest punicalagin and ellagic acid concentrations that have been identified as the principal factor behind the pomegranate antimicrobial activity. On the other hand, the antioxidant activity of pomegranate juice is higher than other fruit juices **(Seeram *et al*., 2008)**. This antioxidant activity has been correlated to the highest content of phenolic compounds, including anthocyanins (3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin), ellagic acid, punicalin, punicalagin, pedunculagin and different flavonoids. Pomegranate rind is an inedible part obtained during the processing of pomegranate juice. Lately, the use of pomegranate juice and rind powder as a source of natural antioxidant in chicken patties had been investigated **(Naveena *et al*., 2008)**. Furthermore, **Devatkal *et al*. (2010)** have shown a significant antioxidant effect of extracts of pomegranate rind and seed powders. Therefore, the aim of our work was to improve the hygienic quality of the sausage by the increase of different concentrations of pomegranate peel powder.

MATERIALS AND METHODS

Preparation of beef sausage

Beef meat samples including boneless neck, chuck and rounds along with associated fats were obtained from local markets at Zagazig city, Egypt, and used for preparing beef sausage samples. All sub cut fat and inter-muscular fat were included as fat sources. The beef meat and fat tissue were transported to the laboratory using an icebox. Different ingredients used in preparing beef sausage samples e.g. table salt, starch and spices mixture such as black pepper, red pepper, nutmeg and ginger were obtained from local markets at Zagazig, Egypt. Beef sausage samples were made according to the method described by **Zaika *et al*. (1978)**. Meat and fat tissues were cut into small portions (approximately 6 cm long, 5 cm wide, and 4 cm thick). These cuts were ground to particles of about a rice size (2 mm x 4 mm), then the ingredients were blended to prepare sausage mixture emulsion, which was then stuffed by sausage filling machine previously washed by hot water and cased in mutton casings. Three groups of sausage were prepared, including control group, Group 1 and 2. The Control group consists of lean meat 70%, fat 12%, sodium chloride 2.3%, water 9.3%, garlic 1%, onion 1.2% and spices mixture 1.2%). The group 1 (G1) was similar to the control but after mixing 2.5% removed and replaced with 2.5% dried pomegranate peel powder then mixed again), meanwhile group 2 (G2), after mixing 5% removed and replaced with 5% dried pomegranate peel powder then mixed again). Finally, the prepared sausage samples were stored at -18 ± 2℃ in order to simulate the normal storage temperature used in the retail sausage outlets present in Egypt.

Chemical analyses

The determination of the pH values of different beef sausage samples were done according to the method described by **Defreitas *et al*. (1997)** as follows; a known weight of beef sausage sample (30 g) was blended with 100 ml distilled water and the pH of the slurry was then measured using a pH meter (HANNA Instrument, USA). On the other hand, determination of total volatile basic nitrogen (TVB-N) was carried out according to Conway's micro diffusion technique recommended by **(FAO, 1992)**. However, determination of thiobarbituric acid (TBA) was performed according to **(Kirk and Sawyer, 1991)**.

Bacteriological analyses

Preparation of samples for bacteriological examination

Sausage samples were prepared for microbiological analysis in accordance with **ISO 6887-1(2003).** For the Aerobic plate count **(Baumgart & Firnhaber, 1986);** One ml of each previously prepared serial dilution was carefully transferred into separate, duplicate, appropriately marked Petri dishes, and thoroughly mixed with about 15 ml of previously melted and adjusted (45 ± 1°C) plate count agar (Oxiod, CM325). After solidification, the inoculated plates as well as control one were inverted and incubated promptly for 48 ± 2 h at 37°C. The countable plates with 30-300 colonies were recorded and the total colony count per cm^2 was calculated. However, for the enumeration of *Enterobacteriaceae* **(ICMSF, 1978);** 0.1 ml from the original and the subsequent prepared dilutions were spread on surface of Petri dish in duplicate plates containing Violet red bile glucose agar (VRBGA), and incubated at 37°C for 24 hours. All large purple colonies were counted and the average number of *Enterobacteriaceae* per gram of sample was calculated and recorded.

RESULTS AND DISCUSSION

Effect of pomegranate peel powder on pH during freezing at -18 ± 2°C

The pH value is the important physicochemical characteristic to decide the quality and shelf life of sausage. The pH value of control, 2.5 and 5% pomegranate treated sausage at zero time was 6.18 ± 0.14, 5.87 ± 0.13 and 5.54 ± 0.17, respectively. There were significant effects ($p< 0.05$) of both treatments as compared to control samples. This direct effect related to the acidic pH of pomegranate peel powder (pH=3.75) as recorded by **(Ullah *et al.*, 2012).** Our results were in disagreement with **El-Nashi *et al.* (2015)** who found that no significant differences in pH values of different prepared beef sausage samples containing 0%, 1%, 2% and 3% of pomegranate peels powder. After elapsing of 8 weeks of storage at - 18 ± 2°C the mean value of pH was 6.27 ± 0.16, 5.9 ± 0.15 and 5.73± 0.19 for control, 2.5 and 5% pomegranate treated sausage, respectively. Results shown in the figure (1) indicated that freezing had no effect on pH value of the same group during storage weeks. The obtained results were corresponded with **Muela *et al.* (2010)** who suggested that the pH of fresh meat and frozen meat did not differ significantly. On contrary, **Kim and Lee (2011)** reported that frozen meat had a higher pH than fresh meat because of partial denaturation of the muscle proteins.

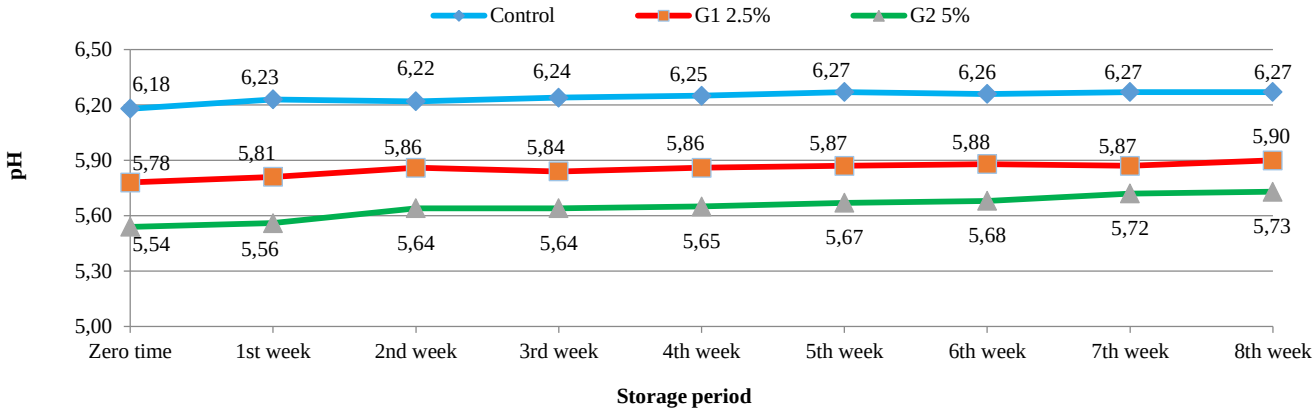

Figure 1 pH values in control and pomegranate treated groups (G1, 2.5%) and (G2, 5%) during freezing at -18 ± 2°C

Effect of pomegranate peel powder on total volatile nitrogen during freezing at -18 ± 2°C

From the results achieved in table (1), it could be noticed that total volatile nitrogen (TVN) of control, 2.5% and 5% pomegranate peel powder (PGPP) treated groups was 6.50 ± 1.23, 6.41 ± 1.23 and 6.39 ± 1.23 mg/100 g, respectively at zero time. There were no significant effects of pomegranate peel powder (PGPP) at zero time. The TVN values increased gradually with increasing frozen storage period. After elapsing of four weeks the recorded values were 16.00 ± 1.70, 11.00 ± 1.41 and 9.23 ± 1.12 mg/100 g for control, 2.5% and 5% pomegranate peel powder treated groups, respectively. By the 8th week, TVN values were 20.04 ± 4.10, 16.00 ± 3.45 and 14.33 ± 4.21 mg/100 g for control, 2.5% and 5% pomegranate peel powder treated groups, respectively. There were significant effects ($p< 0.05$) of pomegranate peel powder added at concentration of 2.5 and 5% after four and eight weeks of freezing at -18 ± 2°C. The increasing of TVN during freezing weeks was attributed to the continuous enzyme activity (**Berry *et al.*, 2008**). The obtained results were concurred with that obtained by **Ibrahim (2004)**. Additionally, the total volatile nitrogen values of all treatments were in the range of permissible level (< 20 mg /100 g) established by Egyptian standard specifications **(ESS, 2005)**.

Table 1 Total volatile basic nitrogen (TVB-N) mg/100g in control and treated sausage during freezing at -18 ± 2°C

Period	Control group				Treated group(G1) with 2.5%				Treated group(G2) with 5%			
	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
Zero time	5.40	7.80	6.50^{Ac}	1.23	5.40	7.80	6.41^{Ac}	1.23	5.40	7.80	6.39^{Ac}	1.23
4th week	12.00	19.20	16.00^{Ab}	1.70	8.00	13.10	11.00^{Bb}	1.41	7.40	11.20	9.23^{Bb}	1.12
8th week	13.20	23.20	20.04^{Aa}	4.10	11.23	19.30	16.00^{Ba}	3.45	9.47	17.36	14.33^{Ca}	4.21

Means carrying different superscript capital letters on the same column are significantly different ($P< 0.05$) on different group.
Means carrying different superscript small letters on the same row are significantly different ($P< 0.05$) on same group.

Effect of pomegranate peel powder on thiobarbituric acid (TBA) during freezing at -18 ± 2°C

As shown in table (2), the level of thiobarbituric acid of all treatments at zero time of control, 2.5% and 5% pomegranate powder treated groups was 0.27 ± 0.11 mg malonaldhyde/kg. There were no significant effects ($p> 0.05$) at zero time. As the frozen storage time progressed, the thiobarbituric acid values of all treatments increased gradually. However, the lowest TBA value was recorded for sausage contained 5% pomegranate peel powder, meanwhile the highest increment of TBA value was noted for control sausage which reached 0.84 mg malonaldhyde/kg after 8 weeks from the start of freezing storage (-18°C). Generally, the increases of TBA values that observed in all sausages treatments contained pomegranate peel powder were less than that found in the control sausage that might be explained by the ability of pomegranate peel powder to scavenge free radicals, and its antioxidant power (**Gil *et al.*, 2000).** TBA values of all samples after the eight weeks of storage were within the range of permissible level (< 0.9 mg malonaldhyde/kg for frozen sausage) set by Egyptian standard specifications **(ESS, 2005)**.

Table 2 Thiobarbituric acid (TBA) mg malondialdehyde/kg in control and treated sausage during freezing at -18 ± 2°C

Period	Control group				Treated group(G1) with 2.5%				Treated group(G2) with 5%			
	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
Zero time	0.21	0.39	0.27^{Ac}	0.11	0.21	0.39	0.27^{Ac}	0.11	0.21	0.39	0.27^{Ac}	0.11
4th week	0.56	0.79	0.62^{Ab}	0.15	0.35	0.54	0.46^{Bb}	0.08	0.28	0.49	0.39^{Bb}	0.10
8th week	0.82	0.87	0.84^{Aa}	0.09	0.48	0.67	0.59^{Ba}	0.10	0.38	0.49	0.42^{Ca}	0.13

Means carrying different superscript capital letters on the same column are significantly different (P< 0.05) on different group.
Means carrying different superscript small letters on the same row are significantly different (P< 0.05) on same group.

Effect of pomegranate peel powder on aerobic plate count (APC) during freezing at -18 ± 2°C

The data presented in figure (2) showed that the mean value of APC at zero time of control, 2.5% and 5% pomegranate treated groups was 5.65± 1.38, 5.59 ± 1.92 and 5.59 ± 1.92 $\log_{10}$ CFU/g, respectively. There were no significant effects related to the addition of pomegranate peel powder of different prepared beef sausage at zero time. The obtained data revealed that beef sausage samples treated with 2.5% and 5% concentrations of pomegranate peels powder, had a significant reduction on APC at 2nd and 3rd week, respectively. APC of prepared beef sausage (control, 2.5% and 5% pomegranate treated groups) was progressively reduced to 4.85 ± 0.64, 4.39 ± 0.51 and 4.10 ± 0.42 $\log_{10}$ CFU/g, respectively over the time of storage period. Moreover, the results showed a significant reduction at the 8th week ($p<0.05$) in both 2.5 and 5% pomegranate powder treated sausage. These results could be due to the antimicrobial effect of pomegranate peels powder especially when the concentration of pomegranate peels powder was increased. According to **Rosas-Burgos *et al.* (2017)**, the peel of the pomegranate fruits is a rich source of antifungal and antibacterial compounds such as ellagic acid and punicalagins (α and β), and this might be employed as a natural alternative to synthetic antimicrobial agents. The observed results seemed to be similar to the results of **El-Nashi *et al.* (2015)**, **Agourram *et al.* (2013)**, **Kanatt *et al.* (2010)** and **Al-Zoreky (2009)** who evaluated the antimicrobial characteristics of pomegranate peels. They found that pomegranate peels have an inhibition effect against gram positive and gram-negative bacteria. A gradual decrease was observed in APC of control beef sausage samples during the storage period. The count became 4.85 ± 0.64 $\log_{10}$ CFU/g at the end of storage period. On the other hand, the pomegranate 2.5 % and 5% treated groups had the same pattern of the gradual decrease and became 4.39 ± 0.51 and 4.10 ± 0.42 $\log_{10}$ CFU/g, respectively. The significant effect of freezing on reduction of APC appeared on control and pomegranate treated sausage in the 3rd and 5th week, respectively. Our results accorded with **(Ray and Bhunia, 2007)** who found that maximum lethality is seen with slow freezing where exposure to high concentrations is prolonged. Survival is greater with rapid freezing where exposure to these conditions is minimized. However, food-freezing processes are not designed to maximize microbial lethality but to minimize loss of product quality.

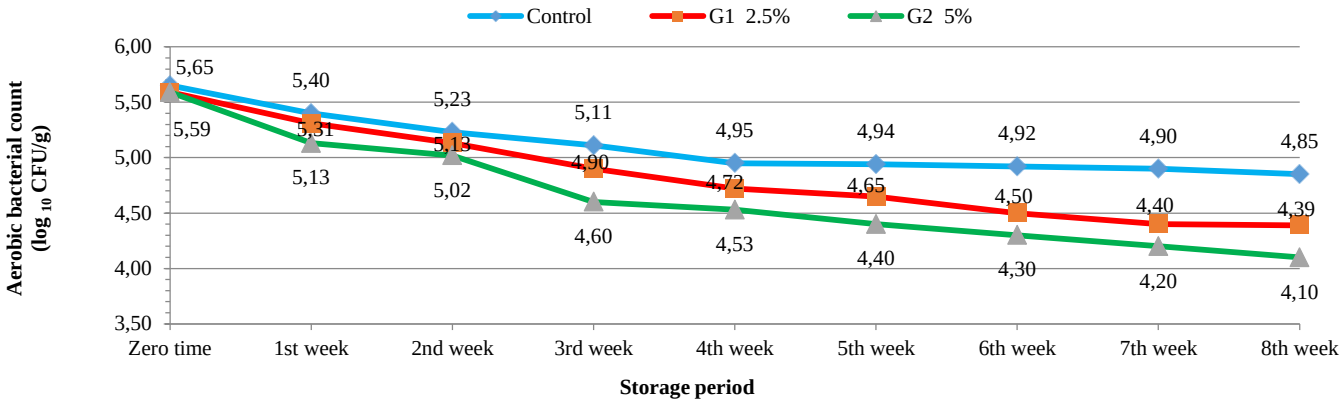

Figure 2 Aerobic bacterial counts ($\log_{10}$ CFU/g) in control and pomegranate treated groups (G1, 2.5%) and (G2, 5%) frozen at -18 ± 2°C

Effect of pomegranate peel powder on *Enterobacteriaceae* count during freezing at -18 ± 2℃

The data presented in figure (3) showed that the mean values of *Enterobacteriaceae* count at zero time of control, 2.5% and 5% treated groups were 3.18 ± 1.13, 3.12 ± 1.19 and 3.09 ± 0.98 $\log_{10}$ CFU/g, respectively. No significant effects related to the addition of pomegranate peel powder to the prepared beef sausage were detected at zero time. The obtained data revealed that, the beef sausage samples treated with different concentrations of pomegranate peels powder (2.5% and 5%) led to a significant reduction of *Enterobacteriaceae* count at 3rd week. Progressive reduction of the *Enterobacteriaceae* count over the time of storage period; whereas, in 8th week, the *Enterobacteriaceae* count of the control group, 2.5% and 5% of treated groups reached to 2.95 ± 0.65, 2.28 ± 0.87 and 2.20 ± 0.98 $\log_{10}$ CFU/g, respectively. Moreover, there were a significant effect at the 8th week ($p<0.05$) in both 2.5 and 5% pomegranate powder treated groups. The obtained results may be attributed to the antimicrobial effect of pomegranate peels. According to **Li *et al.* (2006)**, pomegranate peel powder is an important source of bioactive compounds such as phenolic compounds, which are secondary plant metabolites and possess antibacterial or antiviral activities (**Cai *et al.*, 2004**). The significant effect of freezing on reduction of *Enterobacteriaceae* appeared in control and pomegranate treated sausage in the 4th and 5th weeks, respectively. Furthermore, the freezing effect pronounced on *Enterobacteriacea*e count, where cold shock affect gram-negative bacteria than gram positive (**Dodd *et al.*, 2007**).

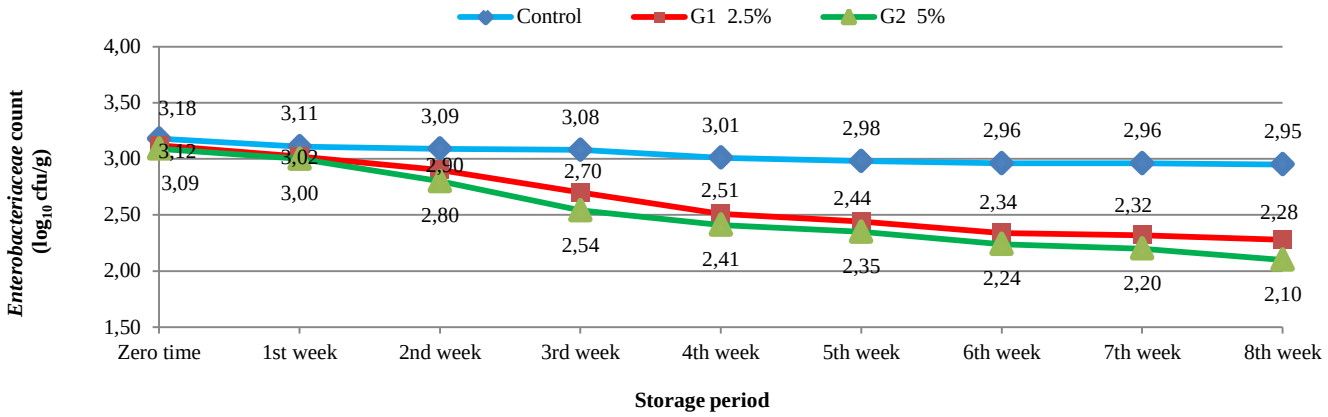

Figure 3 Enterobacteriaceae count (log $_{10}$ CFU/g) in control and treated groups (G1, 2.5%) and (G2, 5%) frozen at -18 ± 2℃

CONCLUSION

The aim of the presented study was to evaluate the impact of pomegranate peel powder (2.5 % and 5%) on beef sausage stored at -18 ± 2℃ . It was found that pomegranate peel powder had a substantial effect on pH, total volatile nitrogen (TVN) and thiobarbituric acid (TBA) over the storage period compared to the control group. Moreover, the total bacterial counts (TBC) and *Enterobacteriaceae* counts were reduced significantly in the pomegranate treated group. Therefore, the use of pomegranate peel powder is considered as effective food additive to decrement pH, TVB-N, TBA and bacterial counts in sausage. Further future studies are necessary to measure the color and organoleptic attributes of the sausages, in addition, the application of pomegranate peel powder alone or in combination with other antibacterial agents such as essential oils, organic acid salts to control foodborne pathogens in different food products.

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FERMENTED FRUIT JUICE PRODUCTION USING UNCONVENTIONAL SEASONAL FRUITS THROUGH BATCH FERMENTATION

*Pranita A D'Souza [1], Priyanka A.Naik [1], Shubashree C. Rao[1], Saujanya Vyas[1], Anusha M. Palan[1],Belrin Cornelio[1], Vinayaka B.Shet[*1], C.Vaman Rao[1]*

Address(es): Mr.Vinayaka B. Shet,
[1]NMAM Institute of technology (Affiliated to V.T.U, Belagavi), Department of Biotechnology Engineering, Nitte-574110,Udupi District,Karnataka, India, phone number:+919448452511.

*Corresponding author: vinayakabshet@gmail.com

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ABSTRACT

The attempt was made in the present work to ferment the juice of unconventional fruits. Jamun (*Syzgium cumini* L.), pomegranate, Cocoa (*Theobroma cacao L.*) were chosen for the study. Fruits were collected from local market and farmers. Juice was extracted from the fruits and initial sugar was maintained between 13 to 26 °Brix. Fermentation was carried out using *Saccharomyces cerevisiae* at room temperature. Fixed acidity estimated in terms of tartaric acid equivalent was determined in the range of 4.2 to 6.9g/L. Radical scavenging activity of the fermented juice was between the range of 1.42 to 1.96 mmol TE/L. Metals such as Cd, Cu, Mn, Ni, Pb and Zn were estimated. Residual sugar was within 3mg/ml. Ethanol concentration was estimated in the range of 5.25 to 10.67% (v/v).

Keywords: Jamun, Pomegranate, *Saccharomyces cerevisiae*, Ethanol

INTRODUCTION

Jamun (*Syzgium cumini* L.) is an evergreen tropical tree in the flowering plant family *Myrtaceae*, native to India and Indonesia. The other common names of Jamun are java plum, blackplum, jambul and Indian blackberry (**Chowdhury *et al.*, 2007**). India ranks second in production of Jamun in the world. The Jamun tree starts flowering in March-April. This is followed by fruiting (a berry) which appears in May- June. The berry is oblong, ovoid and shinning crimson black (rich in anthocyanin pigment, an anti-oxidant) when fully ripe. The fruits are a good source of iron and are used as an effective medicine against diabetes, problems related to heart, bleeding piles and liver trouble (**Satkar *et al.*, 2016**). The fruit and its juice and the seed contain a biochemical called 'jamboline' which is believed to check the pathological conversion of starch into sugar in case of increased production of glucose (**Chowdhury *et al.*, 2007**). Ripe Jamun contain approximately 83 % water with almost solids 14 % containing a mixture of fermentable sugar. The pulp of Jamun contains appreciable amounts of fermentable sugar, which can be used for alcoholic fermentation. In India alone wastage of Jamun is 0.5 MT due to rainfall, high temperature, humidity fluctuations, improper handling, inadequate storage facilities, inconvenient transport and microbial infections (**Jagtap *et al.*, 2015**). The food industry uses a variety of preservation and processing methods to extend the shelf life of fruits such that they can be consumed year round, and transported safely to consumers all over the world, not only those living near the growing region (**Barret and Lloyd,2011**).

The cultivation of pomegranate (**Reddy *et al.*, 2007**) is mainly extended to the Mediterranean basin, and in regions of Southern Asia, India, North and South America, where the high temperature allows the optimal fruit ripening. The pomegranate fruit has been used in folk medicine from ancient times as antimicrobial and as natural astringent for the treatment of diarrhea and harmful internal parasites. Nowadays, the research interest on pomegranate fruit is increased as a consequence of reports establishing its benefits on human health (**Faria and Calhau, 2011**). Ripe fruits are traditionally consumed fresh, also as topping in yogurt and salads, dried, as spice or acidic agent for the manufacture of chutney and curry, or processed. Pomegranate juice is consumed throughout the world because of its pleasant and unique aroma, flavour and color (**Ferrara *et al.*, 2011**).Besides sensory properties, pomegranate shows interesting nutritional and health-promoting features (**Viuda-Martos *et al.*, 2010**). In particular, the antioxidant properties of the fruit (**Seeram *et al.*, 2005**), which contains anti-carcinogenic (**Bell and Hawthorne, 2008**), antimicrobial (**Reddy *et al.*, 2007**), antiviral (**Kotwal, 2008**), anti-inflammatory (**Giménez-Bastida *et al.*, 2012**).In general, fruit wines are processed in the same way as wine made from grapes and significant compositional changes take part during winemaking (**Ginjom, D'Arcy, Caffin & Gidley, 2011; Heinonen, Lehtonen, & Hopia, 1998**). Both fermentations and ageing entail the transformation of native substances into secondary metabolites able to have an impact on the quality of the final product (**Duarte *et al.*, 2010; Ginjom, D'Arcy, Caffin, & Gidley, 2011; Yae *et al.*, 2007**). Furthermore, cultivars can also affect wine composition and not all may be useful for optimal fermentation purposes (**Towantakavanit, Park, & Gorinstein, 2011**). Thus, taking into account the great diversity existing among the sensorial and phytochemical properties of pomegranate cultivars (**Mena, García-Viguera *et al.*, 2011**), differences in the final quality of pomegranate varietal wines should be expected.Despite these considerations and the fact that some work on pomegranate fermented juices has already been performed (**Yae *et al.*, 2007; Zhuang *et al.*, 2011**), there is no substantial information on pomegranate wine composition, covering all stages of the pomegranate winemaking process.

Cocoa (*Theobroma cacao L.*) is world-wide known for its beans used in the manufacture of chocolate. For a long time the production and commercialisation of cocoa has been the basis for the economy of some Brazilian states, mainly Bahia. The cocoa pulp is a substrate rich in nutrients, which can be used in industrial processes for by-product manufacture (**Schwan & Wheals, 2004**). Cocoa can be readily fermented by yeasts such as *Saccharomyces cerevisiae*, producing an alcoholic beverage. *Saccharomyces cerevisiae* has been used in fermentative processes for thousands of years, according to the first historical stories of the production of beer and wine (**Rose, 1977; Demain, 2000; Ostergaard et al., 2000**). Because of commercial importance of this microorganism, strains with good fermentative characteristics have been selected and commercialised in dehydrated form and/or lyophilised to be employed in breweries, wineries and other industries (**Fleet & Heard, 1993; Colagrande *et al.*, 1994; Cappello *et al.*, 2004**). A range of environmental factors influences the production of metabolites and survival of yeasts during industrial fermentations. The main factors are temperature, pH, sugars concentration and acidity of fruit juice (substrate). In case of yeasts, temperature and tolerance of ethanol have an important influence on their performance (**Heard & Fleet, 1988**). Winemaking is a good example of the biotechnological evolution in the beverage production, passing from art to science-based technology.

Traditionally, cacao seed fermentation is a noncontrolled process initiated by microorganisms naturally occurring at fermentation sites,including yeasts,lactic and acetic bacteria,Bacilli and filamentous fungi. The diversity of yeasts associated with cacao seed fermentation is heterogenous, varying in terms of

location, producing country, climate conditions and fermentation method and duration.

In view of these many medicinal and therapeutic properties of Jamun, Pomegranate, cocoa and because of its short availability period, an attempt has been made in this study to preserve the unconventional seasonal fruit by fermenting its juice through batch fermentation using *Saccharomyces cerevisiae*.

MATERIAL AND METHODS

Mash preparation

Jamun

Jamun was collected from local city market of Mangaluru situated in Dakshina Kannada district, Karnataka State, India. Fresh jammun fruits collected were washed and peeled, extracting seeds. Extracted seeds were kept for drying in hot air oven at 71°C for 1-2hours. The pulp of the fruit along with its skin was crushed by mortar and pestle and kept in freezer to avoid contamination. The dried seeds were also crushed by mortar and pestle. The pulp, seeds and the skin were pasteurized at 70-80°C for 15 minutes along with 300mL of water in a flask. The boiled mash was plugged with cotton and allowed to cool.

Pomegranate

Bhagwa variety of pomegranate was collected from Hosadurga taluk situated in Karnataka state, India. The peels of pomegranate were removed and the seeds were excised from the pulp. The pulp was macerated using mortar and pestle. The initial sugar concentration in the mash was measured using the hydrometer and found to be 26 °Brix and the pH was adjusted suitable for the growth of yeast.

Cocoa

The cocoa was collected from plantation situated in Peruvai village of Dakshina Kannada district, Karnataka State, India. The cocoa seeds were cut into small pieces and ground to uniform slurry by pestle mortar. The slurry was further diluted with distilled water to reduce the turbidity (**Kocher *et al.*, 2011**). The pH and the sugar content are adjusted before fermentation. The crushed mixture was pasteurized in a conical flask at 70-80° C for 15 minutes along with 400mL of water.

Innoculum development and fermentation

The standardization of inoculum size is important as sugar consumption is balanced between biomass development and ethanol production (**Kocher, 2011**). Inoculation of the yeast culture into the mash depends on the time at which the yeast enters log phase which is determined in the growth kinetics study. Initial sugar concentration was estimated by hydrometer and pH by pH meter. Yeast from master culture was inoculated into MGYP liquid media from slant culture.10 ml of MGYP media having culture yeast *Saccharomyces cerevisiae* was pitched into the flask containing mash after 7 hours. The cotton plugged flask was kept in incubator shaker at a temperature of 31°C, 100 rpm for a span of 3 to 4 days for batch fermentation. Once the concentration of sugar decreased to less than 1g/litre, the fermentation was stopped. The fermented sample was clarified by centrifugation at 7000 rpm for 15 minutes (**Kocher, 2011**). The clarified sample was taken for analysis of parameters like pH, acidity, residual sugar, ethanol and metal concentration.

Analysis

pH

The initial pH of the sample is checked and adjusted to 3.5. The pH is also determined after fermentation by pH meter.

Soluble solid (sugar)

Hydrometer was used for the determination of initial sugar. The concentration was expressed in terms of °Brix. To bring the sugar concentration to required value chaptalization was carried out (**Reddy *et al.*, 2009; Kocher *et al.*, 2011**).

Titratable Acids

Titratable acidity (TA) was determined by titration of a strong base i.e 0.5N NaOH against 25 ml of sample to an end point of pH 8.2 using potentiometric titration (**Jacobson, 2006**).

Volatile acid

Fermented fruit juice was distilled at 118 °C in order to determine the amount of Volatile acid (VA).5 ml of distillate was titrated against 0.5N NaOH using phenolphthalein as indicator to determine volatile acid content (**Moura *et al.*, 2010**).

Determination of antioxidant activity

Fermented fruit juice was investigated for antioxidant activity by the 2, 2-diphenylpicrylhydrazyl (DPPH) method (**Seruga *et al.*, 2011**). 120µL of methanolic DPPH solution (1 mmol L) was mixed with 50 µL of fermented fruit juice sample and 1880µL of methanol. Incubation of the mixture was carried out in dark at room temperature for 15 min and at 517 nm the absorbance of this mixture (A_{Sample}) was measured against the blank sample (50µL of fermented fruit juice, 2000µL of methanol). The blank DPPH solution was freshly prepared (120µL of 1 mmol L DPPH, 1930 µL of methanol) and absorbance at 517 nm (A_{DPPH}) was measured. The calibration curve for Trolox, constructed by linear regression of absorbance value (A_{Trolox}) vs. Trolox concentration, was used to calculate the antioxidant activity of wine sample and to express their anti oxidant value in mmol of Trolox equivalents (mmol TE/L). Trolox standards with final concentration 0 – 2550 µmol/L in methanol were assayed under the same conditions as those used for the samples; i.e. 50µL of Trolox was mixed with 120µL of methanolic 1 mmol/L DPPH solution and 1880 µL of methanol. After 15 min, the absorbance at 517 nm (A_{Trolox}) against the prepared blank sample was measured. (**Seruga *et al.*, 2011**)

Metal analysis by AAS

Metal analysis was carried out by Atomic absorption Spectroscopy (GBC Avanta) after carrying out acid digestion of the sample. 3 ml of the sample was taken in 250 ml digestion flask, mixed with freshly prepared 3 ml of nitric acid and 3 ml of Hydrochloric acid, incubated at 60°C. Heating was continued until the solution becomes clear and colourless (**Woldemariam *et al.*, 2011**). The cooled digest was made up to 100 ml, by adding deionised ultra-pure water, stored in refrigerator for further analysis (**Nikolakaki *et al.*, 2002**). Standard metal solution of five different concentrations was prepared for calibration.

Residual sugar estimation

The concentration of residual sugars was estimated by the using the UV Vis spectrophotometer at 540 nm with 3, 5- DNSA reagent (**Miller, 1959**).

Quantitative estimation of ethanol

Ethanol was determined using potassium dichromate method (**Fletcher *et al.*, 2003**). 25 ml of centrifuged sample was distilled at 80°C. The distillate was used for ethanol estimation.

RESULTS AND DISCUSSION

Acidity

Volatile acidity (VA)

Amount of acetic acid present in fermented fruit juice is expressed as VA. The result reveals the presence of acetic acid in the fermented fruit juice. In the current investigation the volatile acidity estimated was 0.6 g/L,0.1g/L and 0.3g/L of acetic acid, respectively for jamun, pomegranate and cocoa fermented fruit juice. VA of pomegranate wine was reported between 0.26 to 0.36 g/L acetic acid **(Mena *et al.*, 2012)**

Titratable acidity (TA)

TA (g/L) =75 x N x (T/S) (1)

Where N is the normality of NaOH, T is the titer volume (in ml), S is the sample volume (in ml), and 75 is a constant. **Moura *et al.*, (2010)** reported TA of 7.05 to 9.83 g/L tartaric acid. **Vahl *et al.*, (2013)** reported TA in the range of 4.80 to 6.24 g/L tartaric acid.

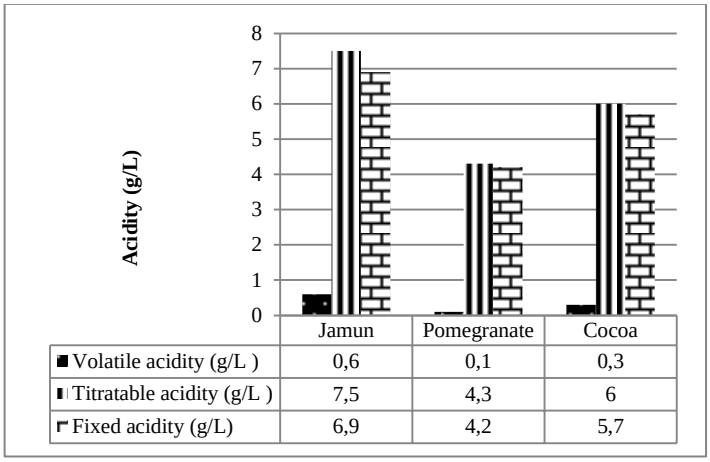
Current investigated revealed the presence of higher TA of 7.5 g/L in jamun fermented juice compared to 6.70 to 6.80g/L reported by **Arekar and Lee, (2015).**

Fixed acidity (FA)

Fixed acidity = Titratable acidity - Volatile acidity (2)

As per the Organisation of Vine and Wine (OIV) norms FA should not be less than 5g/L.

From the current investigation Jamun, cocoa fermented fruit juice meets the criteria.



	Jamun	Pomegranate	Cocoa
Volatile acidity (g/L)	0,6	0,1	0,3
Titratable acidity (g/L)	7,5	4,3	6
Fixed acidity (g/L)	6,9	4,2	5,7

Figure 1 Acidity of fermented fruit juices

Antioxidant assay

Cocoa fermented fruit juice exhibited strong antioxidant activity of 1.96 mmol TE/L. Least antioxidant activity revealed by Jamun. Radical scavenging activity of 80.56% to 81.90% was reported by **Arekar C. and S.S. Lee, (2015)** in jamun microvinification**.**

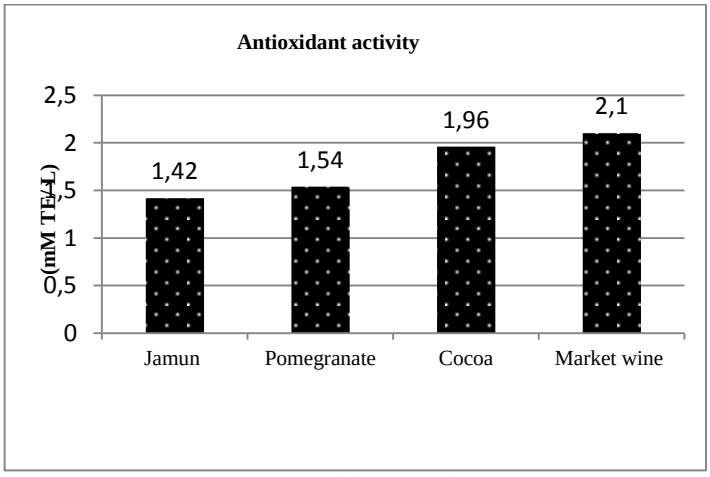
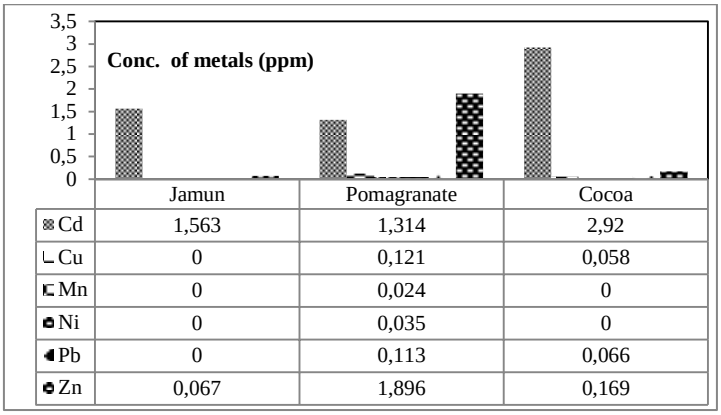


Figure 2 Radical scavenging activity of fermented fruit juice

Metals

Most metals are important for efficient alcoholic fermentation **(Pohl 2007)**. Cu, Fe and Mn are responsible for changes in stability and modification of the sensory quality of concoction after bottling. Fermented fruit juice of jamun contains metals like Zn and Cd with concentration of 0.067ppm and 1.563 ppm respectively. Several other metals like Cu, Mn, Pb and Ni showed negligible concentration. Even though pomegranate fermented juice has the metals such as Cd, Cu, Mn, Ni, Pb, Zn. Only Cd is beyond the accepted range of OIV standards. Cocoa fermented fruit juice consist of Cd, Cu, Pb, Zn. Cd exceeds the acceptable range of OIV.



	Jamun	Pomagranate	Cocoa
Cd	1,563	1,314	2,92
Cu	0	0,121	0,058
Mn	0	0,024	0
Ni	0	0,035	0
Pb	0	0,113	0,066
Zn	0,067	1,896	0,169

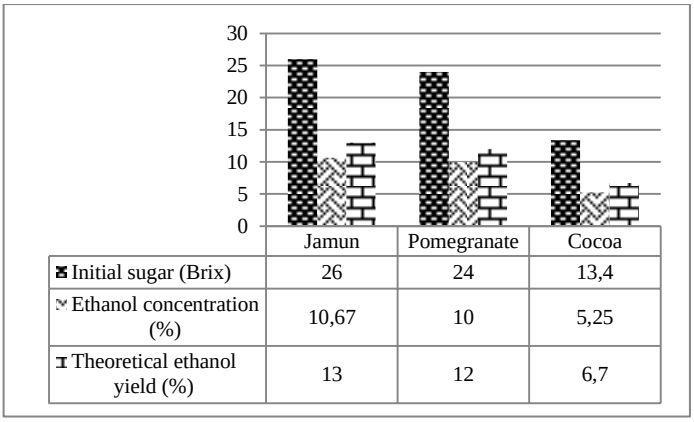
Figure 3 Concentration of metals in fermented fruit juice of Jamun, Pomegranate and Cocoa

Residual sugar

Amount of residual sugar present in all the fruit juice sample is within 3 mg/mL.

Ethanol

Ethanol concentration of various fermented fruit juices is given in Fig.4.Ethanol concentration in the range of 9.81 to 10.30 % was reported by **Arekar and Lee, (2015)** in jamun fermented juice**.** Current investigation reported 10.67% ethanol compare to rest of the fermented juice and also reported by literature. Higher ethanol concentration in pomegranate wine of 10.91 % (v/v) reported by **Berenguer et al., (2015).**



	Jamun	Pomegranate	Cocoa
Initial sugar (Brix)	26	24	13,4
Ethanol concentration (%)	10,67	10	5,25
Theoretical ethanol yield (%)	13	12	6,7

Figure 4 Theoretical and experimental ethanol concentration determined in fermented fruit juice

CONCLUSION

According to the study carried out it was found that the jamun pulp and seeds mash could be readily fermented by *Saccharomyces cerevisiae* thereby producing a promising alcoholic beverage. Residual sugar after fermentation was 2.1593 mg/mL. Fixed acidity was reported as 6.90 g/L which was greater than 5.5g/L therefore we could conclude that there was no contamination .The sample had an ethanol content of 10.67%.

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