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Valorization of buffalo milk proteins in yoghurt: Antioxidant activity and quality attributes

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Abstract

Antioxidants are used medically to reduce oxidative stress in humans. Foods that naturally contain these antioxidants may be better at alleviating oxidative stress. This study intended to explore the antioxidant activity of buffalo milk casein and whey subjected to enzymatic hydrolysis by using pepsin and trypsin enzymes. Also, different yoghurt treatments were produced from buffalo milk to explore their potential antioxidant characteristics. The casein and whey samples were separated from buffalo milk after rennet coagulation or after the biological acidification using *Lactobacillus sp*. The different casein types subjected to pepsin hydrolysis showed higher antioxidant activity than those subjected to trypsin enzyme, while the whey samples treated with trypsin enzyme exhibited higher antioxidant activity than those treated with pepsin. The different yoghurt treatments manufactured using buffalo milk with the addition of pepsin, trypsin, or *Lactobacillus sp.* showed significantly higher antioxidant activity in contrast with the treatment of the control at all time intervals.

Key words: casein, whey, *lactobacillus*, pepsin, trypsin

1. Introduction

Oxidative stress is defined as, "a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses" (**Betteridge, 2000)**. The ROS are naturally formed through oxygen metabolism as by-products, and they have many physiological functions (like, cell signaling). The ROS significantly increase due to several environmental stress causes (such as pollution, ultraviolet rays, heavy metals, and ionizing rays) and inflammatory drugs, causing damage to cells and tissues (**Pizzino *et al.*, 2017**).

Oxidative stress can be responsible for several harmful effects on cells and tissues, causing DNA damage (**Young and Woodside 2001**), cancer (**Valko *et al.*, 2006**), neurological disease (**Butterfield 2002**), cardiovascular disease (**Pacher *et al.*, 2007**), respiratory disease **(Caramori and Papi 2004**), rheumatoid arthritis **(Mahajan 2004**) and kidney diseases (**Galle 2001**).

Antioxidants are substances capable of safeguarding the body's cells from deterioration caused by oxidative stress, and if present in foods or the human body in very small concentrations, can block, manage, or slow down oxidative processes. This capacity helps to prevent degenerative diseases from developing and spreading throughout the body (**Shahidi and Zhong 2011**).

Many medical reports have shown the opposite effects of the excessive use of antioxidant supplements, and these supplements are not helpful

and should be replaced by natural foods **(Tyuryaeva and Lyublinskaya 2023).**

Buffalo is an important contributor to the global milk supply. Buffalo milk contains high concentrations of nutrients, including lipids, proteins, and minerals. Also, contains important bioactive components such as acetyl- l-carnitine and δ-valerobetaine, which have roles as anti-inflammatories, antioxidants, and promote health properties (**Liao *et al.,* 2025**).

Bioactive peptides with antioxidant roles can be liberated from buffalo milk proteins through enzymatic hydrolysis (**Kumar *et al.,* 2020** and **Shazly *et al.,* 2019).**

Yoghurt is considered a popular fermented milk globally **(Behare *et al.,* 2016).** Yoghurt manufactured using buffalo milk supplies significant nutritional and sensory advantages. The fat percentage of buffalo milk is noticeably higher than cow milk, but has a lower content of cholesterol. This is due to the profusion of polyunsaturated fatty acids content and the smaller diameter of fat globules **(Vargas-Ramella *et al.* 2021 and Akbal *et al.,* 2025).**

Even though buffalo milk supplies significant nutritional and functional properties, most of the research focuses on the bioactivities of cow's milk. There are few methodical studies on the potential bioactivities of buffalo milk and its products. Therefore, this research aimed to highlight the potential antioxidant properties of casein and whey separated from buffalo milk by rennet coagulation or after the biological acidification using *Lactobacillus sp*. The promising results of the antioxidant properties of buffalo milk casein and whey led to the making of yoghurt from buffalo milk with promising antioxidant properties.

2. Materials and Methods

Materials:

Raw fresh buffalo milk (7.5% fat, 3.94% protein, and 18.7% TS) was purchased from local farm in Qaha city, Kalyopiya Governorate, Egypt.

Cream (62% fat): The cream was obtained by the separation of buffalo raw milk to skimmed milk and cream (62 % fat) using a milk separator at the dairy experiment facility at Benha University's Faculty of Agriculture, Egypt.

Microorganisms:

The culture collection of the Dairy Science Department at Benha University's Faculty of Agriculture provided *Lactobacillus rhamnosus* Bu-Eg6 and *Lactobacillus paracasei* Bu-Eg3. These cultures underwent three activations on De Man, Rogosa, and Sharpe (MRS) broth (HIMEDIA, pvt. Ltd, India) and incubation for 24 h at 37°C before using in milk fermentation.

Yoghurt starter culture consist of ***Streptococcus thermophilus*** and ***Lactobacillus delbrueckii subsp. bulagaricus*** (DVS, YF-L811, Chr. Hansen) was purchased from Misr Food Additives (Mifad), Egypt, and was subjoined at a rate of 0.02% (w/v) of milk for making yoghurt.

Microbial Rennet: RENIPLUS 2250 IMCU/g, a coagulant enzyme of *Rhizomucor miehei* for food industrial use, was purchased from PROQOIGA S.A. POL INDS.BERGONDO

Pepsin enzyme (1:3000) was obtained from Qualikems Fine Chem Pvt. Ltd., Industrial Estate Nandesari, Vadodara, India.

Trypsin enzyme (1:250) was obtained from Techno Pharmchem, Bahadurgarh, Haryana, India.

2,2 Diphenyl-1-picryihydrazyl was obtained from Sigma-Aldrich, St. Louis, MO, USA.

Methods:

Separation of casein and whey after rennet coagulation:

The method of **Smith, (2001)** was used to separate casein and whey with slight modification as follows: fresh buffalo skimmed milk was subjected to 72 °C for 15 seconds heat-treatment, then immediate cooling to 35 °C, 0.02% calcium chloride was subjoined to the skimmed milk, then 2 ml of the diluted microbial rennet (1:1000) was subjoined and agitated for 2 min. Then, incubation at 37°C for 15 min. Subsequently, the coagulum was heat-treated to 60- 65°C for 30 min to stop the activity of the rennet enzyme. Centrifugation was applied to the coagulum (6000 rpm for 10 min at 4°C). The separated whey was stored in sterilized glass bottles, and the casein was washed 4 times with sterilized distilled water, then gathered in sterilized glass bottles. Both casein and whey were stored at -20°C until used.

Separation of casein and whey after the biological acidification:

The method of **Smith, (2001)** was used with modifications to separate casein and whey as described. Activated *Lactobacillus rhamnosus* Bu-Eg6 and *Lactobacillus paracasei* Bu-Eg3 were used to sour and coagulate the fresh buffalo skimmed milk as follows: portions (10 ml) of *Lactobacillus paracasei* Bu-Eg3 or *Lactobacillus rhamnosus* Bu-Eg6 were transferred into 1 L of heat-treated buffalo skimmed milk (72°C/ 15 sec. then, cooled to 37°C). The injected milk was incubated at 37°C for 20 h (until coagulation). Heat treatment was applied to the coagulum in order to stop the *Lactobacillus sp*. Activity (60- 65°C for 30 min). Then, the coagulum was subjected to centrifugation at 6000 rpm for 10 min at 4°C. The separated whey was stored in sterilized glass bottles, and the casein was washed 4 times with sterilized distilled water, then gathered in

sterilized glass bottles. Both casein and whey were stored at -20°C until further examination.

Enzymatic hydrolysis of casein and whey:

Both rennet & acid caseins and whey were hydrolyzed using pepsin and trypsin enzymes as characterized by **(Abdel-Hamid *et al.* 2017),** with alternations as follows: the casein was dissolved in 2% tri-sodium citrate buffer at a ratio of 3% (similar to the casein % in buffalo's milk). The trypsin enzyme was inserted at a rate of 1:250 to the casein or whey (after adjusting the pH to 8), and the pepsin enzyme was inserted at a rate of 1:3000 (after adjusting the pH to 2). Samples of enzymatic hydrolyzed caseins and whey were taken at 0, 2, 4, and 6 h for the antioxidant activity determination. To deactivate the enzymes' actions on casein and whey, samples were heat-treated for 10 min at 85°C, then left to cooldown to room temperature before examination. The control was casein or whey without being subjected to the enzymatic hydrolysis.

Determination of the antioxidant activity of the hydrolyzed caseins and whey:

The antioxidant activities of both rennet & acid caseins and whey were tested utilizing the method of (**Zhang *et al.*, 2013**) with many alterations. As follows, 4 ml of hydrolysed casein or whey were mixed with 1 ml of DPPH radical stock solution (1 mM in methanol), and the measurement of absorbance was done at 517 nm using a spectrophotometer (UV/VIS SPECTROMETER model T80+) immediately after vigorous mixing for 15 sec., then, left to react at 37°C for 30 min. in the dark, the absorbance was measured again at the same wavelength. The antioxidant activity% was calculated using the following equation:

$$antioxidant\ activity\ \% = [B - R/\ B] \times 100$$

Where *B* is the absorbance recorded immediately after mixing and *R* is the absorbance after 30 min of scavenging reaction at 37°C.

Production of yoghurt:

Yoghurt treatments were made as outlined by **Elalfy *et al.* (2011)** with alterations as follows: the raw fresh buffalo milk was skimmed into skim milk (<0.5% fat) and cream (62% fat) by using a milk separator (Alfa-Laval 29AI, Stockholm, Sweden). The cream (62% fat) was utilized to adjust the milk fat to 5.5%. The milk underwent two homogenization stages at 2500 and 500 psi, by using a homogenizer (Hoyers S.p.A via monferrato 52-20098 San Giuliano Milanese (MI) Italia), then heated up to 90°C for 10 min, subsequently was cooled to (45 ± 1°C), and splited into seven proportions (2 kgs each), The 1st proportion (C) used as a control (the starter culture was subjoined at a ratio of 0.02%). The trypsin enzyme was subjoined to the 2nd proportion (TR1) at a ratio of 1:250000, and the milk was incubated at 37°C for 3 h, then heated for 10 min to 85°C and cooled to 45°C to stop the trypsin action before adding the starter culture (0.02%). The trypsin enzyme was subjoined to the 3rd proportion (TR2) at a rate of 1:250000 + 0.02% of the starter culture. The pepsin enzyme was subjoined to the 4th proportion (PS1) at a rate of 1:3000000, and the milk was incubated at 37°C for 3 h, then heated to for 10 min 85°C and cooled to 45°C to stop the pepsin action before adding the starter culture (0.02%). The pepsin enzyme was subjoined to the 5th proportion (PS2) at a rate of 1: 3000000 + 0.02% of the starter culture. Activated *Lb. paracasei* Bu-Eg3 was subjoined to the 6th proportion (LP) at a ratio of 1% and incubated for 6 h at 37°C before adding the starter culture (0.02%). Activated *Lb. rhamnosus* Bu-Eg6 was subjoined to the 7th proportion (LR) at a ratio of 1% and incubated for 6 h at 37°C before adding the starter culture.

The resulting treatments were loaded in 120 g sterilized plastic cups and subsequently incubated until coagulation at 42 ± 1°C (pH attained ~ 4.6). The resulting yoghurt treatments were subjected to refrigerated storing at 5±1°C and analyzed for their quality attributes (*i.e*. chemical, microbiological, organoleptic), and antioxidant characteristics when fresh, 7 days, and after 14 days of refrigerated storage at 5±1°C.

Analysis of yogurt's chemical attributes:

Samples of the produced yoghurt treatments were examined for chemical quality attributes (*i.e.* fat%, total solids% (TS), and protein %) as outlined by the International Dairy Federation **(IDF) Standards 1991 a, b, and 1993**, respectively. The pH values were recorded using a pH meter (Adwa AD111, Romania) as outlined by **BSI (1985).**

Microbiological examinations of yoghurt:

The microbiological examinations of yoghurt treatments were determined when fresh, 7 and after refrigerated storage at 5±1°C for 14 days. Total lactic acid bacterial count was determined using Elliker medium (Singapore Bioscience PTE Ltd.) and incubation for 48h at 37°C according to **Elliker *et al.* (1956).** *Lactobacillus sp.* count was determined using MRS medium (Singapore Bioscience PTE Ltd.) according to **Deman et al.,(1960)** *Streptococcus thermophilus* count was done using M17 medium (HiMedia laboratories Pvt.Ltd.) according to **Coban *et al.*, (2024)** Yeast & Mould examination were done according to **APHA (2004)**. using potato Dextrose agar Medium. Coliform examination was carried out as outlined by **BSI (1993).**

The capability of yoghurt samples to hold water:

The procedure described by **Sodini *et al.* (2005)** was utilized to determine the capability to hold water (CHW, g/ Kg) for the produced yoghurt treatments as follows: 20 g of the sample (W) were centrifuged for

10 min at 10000 rpm and 4 °C. The whey separated (S) was weighed. The capability to hold water (CHW, g/ Kg) was calculated as follows:

CHW = [(W – S) / W] x 1000.

Organoleptic properties of the produced yoghurt:

Sensory evaluation of the produced yoghurt treatments were carried out by 10 Dairy scientists at Benha University's Faculty of Agriculture's Dairy Science Department, according to the method provided by **IDF (1995)**.

Determination of the antioxidant activities of the produced yoghurt extracts:

The yoghurt samples extracts were prepared when fresh, 7 and after 14 days of referigerated storage at 5±1°C as oulined by **Hernández-Ledesma *et al.* (2004)** with the following adjustments: 20 g of yoghurt were subjected to heating (90°C for 10 min) to halt the fermentation process, subsequently cooled to room temperature. The pH of the sample was regulated to 3.8 using 50% lactic acid, then centrifuged at 10000 rpm for 10 min. The supernatants' pH was regulated to 8.3, then centrifuged as described previously. The antioxidant activities of the yielding extracts were monitored as outlined by (**Zhang *et al.*, 2013)**.

Statistical analysis:

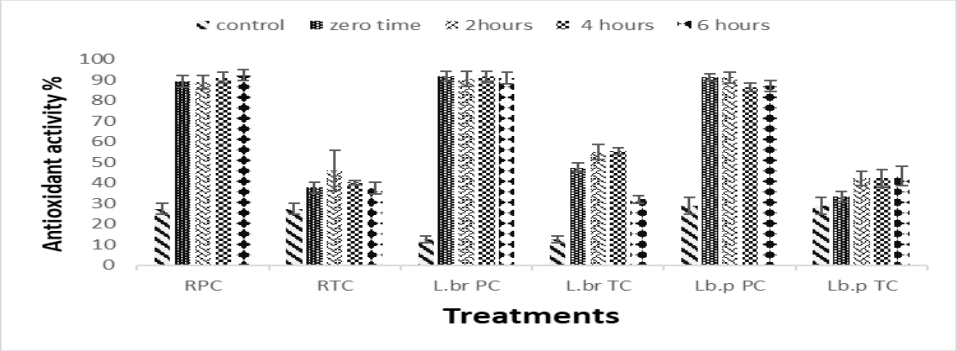
Three replicates were done for each experiment. The Statistical Package for the Social Sciences (SPSS, 31.0.2.0) was used to statistically analyse the collected data. Mixed ANOVA was done to explore the differences between treatments and between treatments & different time intervals.

3. Results and Discussion

Antioxidant activity of buffalo casein and whey:

The casein and whey of buffalo milk prepared either by rennet coagulation or the biological acidification using *Lactobacillus* strains, were subjected to enzymatic hydrolysis by pepsin and trypsin enzymes to explore their potential antioxidant activities (Fig 1).

The control casein precipitated by *Lb. paracasei* showed the highest antioxidant activity (28.96%) followed by the casein prepared by rennet coagulation (27.32%), and the casein precipitated by *Lb. rhamnosus* showed the lowest antioxidant activity (12.51%). All the casein treatments subjected to enzymatic hydrolysis showed significantly higher antioxidant activities in comparison to the control. The casein precipitated by *Lb. rhamnosus* at zero time of pepsin hydrolysis (Lbr PC) resulted the greatest antioxidant activity when compared to all the treatments at all times of hydrolysis (91.59%) followed by Lbp PC (91.07%) and RPC (89.31%). The results of antioxidant activities of different casein types showed a little increase or decrease after 2, 4, and 6 hrs of hydrolysis. It is noticed that the different casein types subjected to pepsin hydrolysis showed higher antioxidant activity than the casein subjected to trypsin enzyme. Figure (1). **Kumar *et al.*, (2020)** found that enzymatic hydrolysis of buffalo milk protein can produce bioactive peptides with potent antioxidant activity. **Shazly *et al.*, (2019)** found that hydrolyzing buffalo casein with alkaline protease and trypsin enzyme showed the highest antioxidant activities (*in vitro*). The antioxidant activities of casein could be due to the liberation of bioactive peptides by trypsin and pepsin. **Abdel-Hamid *et al.* (2017)** found that treating buffalo milk proteins with pepsin and trypsin liberates bioactive peptides with antioxidant activities. Many factors affect the antioxidant potential of hydrolyzed protein, such as the kind of protein, the amino acid sequences, and the type of proteolytic enzyme **(Wu *et al.*, 2012).** The highest antioxidant potential of buffalo milk casein hydrolyzed by pepsin compared to that hydrolyzed by trypsin can be attributed to the specificity of each enzyme (**Shazly *et al.*, 2019**). Pepsin breaks peptide bonds adjacent to hydrophobic and aromatic residues such as tryptophan, tyrosine, and phenylalanine. This cleavage releases peptides rich in hydrophobic scope (**Florkin 1957 and Li *et al.*, 2018**). On the other hand, trypsin cleaves peptide bonds of basic amino acids such as arginine and lysine at the carboxyl side, generating peptide fragments more polar and have less antioxidant potential (**Olsen *et al.*, 2004**). The buffalo casein is known to be rich in hydrophobic regions, which pepsin cleaves and liberates bioactive fragments with potent antioxidant activity. Moreover, pepsin liberates low molecular weight peptides from casein, which have superior antioxidant potential due to their diffusivity, solubility, and conductivity to reactive oxygen species. (**Santos *et al.*, 2024**).There were statistical differences between all treatments and control, either at zero time of enzymatic hydrolysis or after 2,4 and 6 hrs.

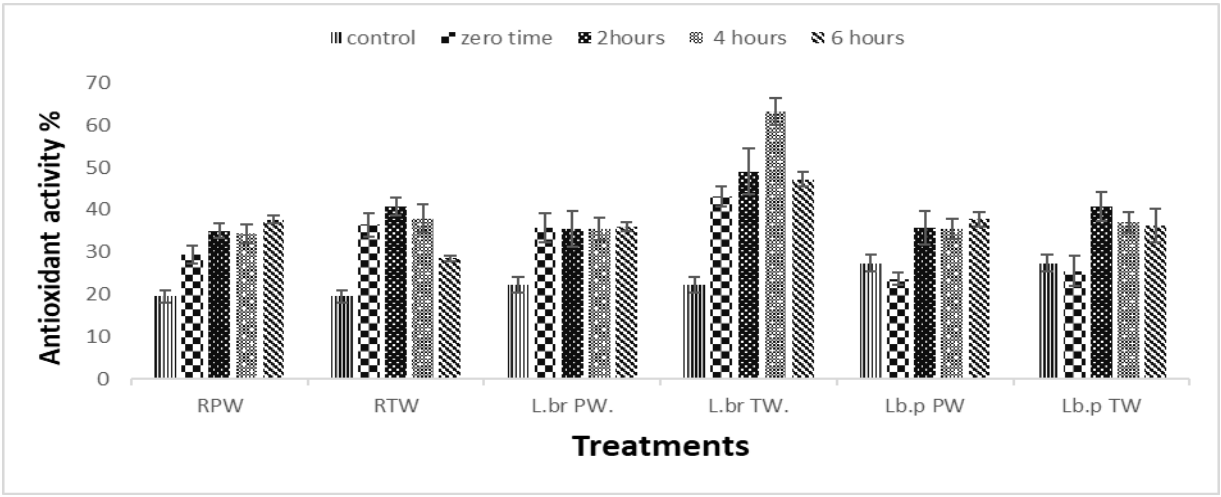


RPC, rennet casein hydrolyzed by pepsin; **RTC**, rennet casein hydrolyzed by trypsin; **Lbr PC**, *Lb. rhamnosus Bu-Eg6* casein hydrolyzed with pepsin; **Lbr TC**, *Lb. rhamnosus Bu-Eg6* casein hydrolyzed with trypsin**; LbpPC**, *Lb. paracasei Bu-Eg3* casein hydrolyzed with pepsin; **Lbp TC,** *Lb. paracasei Bu-Eg3* casein hydrolyzed with Trypsin.

Figure (1) Antioxidant activity % of buffalo's milk casein subjected to hydrolysis by pepsin and trypsin enzymes.

Whey obtained after the precipitation of casein by the biological acidification using *Lb. paracasei* showed the highest antioxidant activity when compared with that obtained by rennet coagulation or *Lb. rhamnosus* precipitation. All whey treatments subjected to enzymatic hydrolysis exhibited significantly higher antioxidant activities than the control and increased during all the durations of hydrolysis up to 6 h. It is noticed that the whey samples treated with trypsin enzyme exhibited higher antioxidant activity than those treated with pepsin enzyme at zero time, 2,4 and 6 h of hydrolysis. The antioxidant activities of whey could be due to the liberation of bioactive peptides by trypsin and pepsin enzymes.

There were statistical differences between all treatments and control, either at zero time of enzymatic hydrolysis or after 2,4 and 6 hr. The results of the antioxidant activity of buffalo milk whey treated with pepsin and trypsin are presented in Figure (2)



RPW, rennet whey hydrolyzed by pepsin; **RTW**, rennet whey hydrolyzed by trypsin**; Lbr PW**, *Lb. rhamnosus Bu-Eg6* whey hydrolyzed with pepsin; **Lbr TW**, *Lb. rhamnosus Bu-Eg6* whey hydrolyzed with trypsin; **Lbp PW**, *Lb. paracasei Bu-Eg3* whey hydrolyzed with pepsin; **Lbp TW**, *Lb. paracasei Bu-Eg3* whey hydrolyzed with Trypsin.

Figure (2) Antioxidant activity % of buffalo's milk whey subjected to hydrolysis by pepsin and trypsin enzymes.

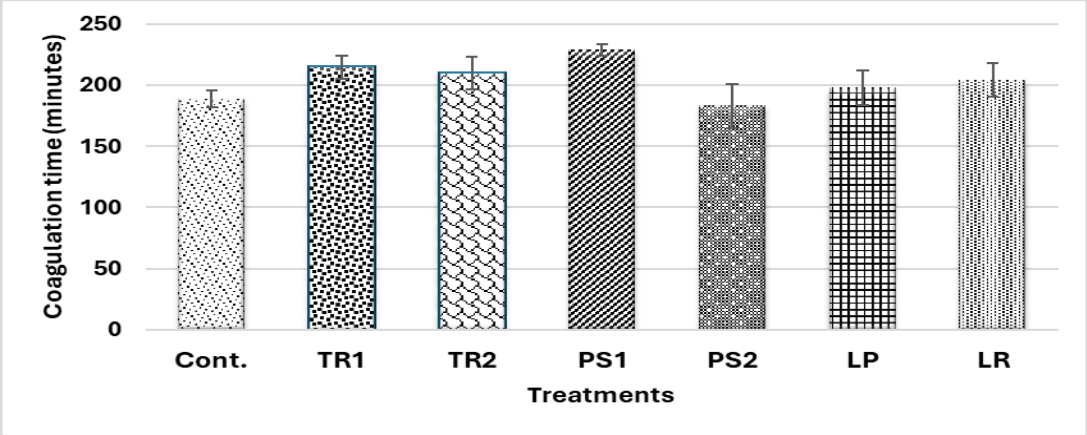
Qualities of yoghurt:

Coagulation time:

The coagulation time refers to the time from starting the incubation of yoghurt milk and ending when the required firm curd is attained. Figure (3) show the times of coagulation of the different treatments of the manufactured yoghurts. It is found that the PS2 had recorded the lowest time of coagulation (3:03 h: min.), followed by the control (3:09 h: min.) On the other side, PS1 and TR1 recorded the highest coagulation time (3:49, 3:35 h: min., respectively). **Lorenzen and Meisel (2005)**

found that treating yoghurt milk with trypsin enzyme can delay the fermentation progress and lower the production of lactic acid in comparison with non-treated milk. **Abdou *et al.* (2015)** and **El-Alfy *et al*., (2016)**, mentioned that the subjoining of some probiotic bacteria (***i.e Lb. casei***) may increase the coagulation time of yoghurt because of the potential suppression of the starter culture. Also, **Vinderola *et al.* (2002),** found that the presence of probiotic bacteria delays the multiplication of the starter culture. ***Lb. paracasei*** and ***Lb. rhamnosus*** cultures used in this study were found to have the probiotic behavior, **Elkhtab and Ismail (2021)**

Based on the one-way ANOVA point of view, there were no statistical analysis differences between PS2 and control, but there were statistical analysis differences between PS2 & control and the other treatments, but there were no statistical analysis differences between TR1, TR2, LP, and LR ($p < 0.05$)



Cont., control; **TR1**, yoghurt milk + trypsin enzyme and incubation at 37°C for 3 h before adding the starter culture; **TR2**, yoghurt milk + trypsin enzyme + the starter culture; **PS1**, yoghurt milk + pepsin enzyme and incubated at 37°C for 3 h before adding the starter culture; **PS2**, yoghurt milk + pepsin enzyme +starter culture; **LP**, yoghurt milk + *Lb. paracasei* Bu-Eg3 and incubated at 37°C for 6 h before adding the starter culture and **LR**, yoghurt milk+ *Lb. rhamnosus* Bu-Eg6 and incubated at 37°C for 6 h before adding the starter culture.

Figure (3): The impact of different treatments on the coagulation time of the produced types of yoghurt

pH values:

The effect of treating yoghurt milk with trypsin, pepsin and *Lactobacillus sp*, on the pH values of yoghurt treatments is presented in Table (1). Due to the continuous activity of the subjoined microorganisms, either starter culture or the subjoined *Lactobacillus sp*., the pH values were reduced through storage periods. Similar conclusions were reported by **El-Alfy et al. (2011), El-Alfy et al., (2016) and Saad & Elkhtab (2019).**

The effect of treatments on pH values exhibited no statistical difference between treatments when fresh but the effect of storage periods exhibited a statistically significant interaction between treatments and storage periods (7 and 14 days) on pH values ($p<0.05$) except for control ($p> 0.05$).

Table (1): The impact of treatments on pH values of the produced types of yoghurt

Storage periods (days)	Treatments						
	Cont.	**TR1**	**TR2**	**PS1**	**PS2**	**LP**	**LR**
Fresh	4.68^{a}	4.69^{a}	4.66^{a}	4.70^{a}	4.70^{a}	4.65^{a}	4.69^{a}
7	4.66^{a}	4.66^{a}	4.65^{a}	4.63^{a}	4.60^{b}	4.60^{a}	4.58^{b}
14	4.62^{a}	4.61^{a}	4.54^{b}	4.56^{b}	4.50^{c}	4.54^{b}	4.53^{b}

Cont., control; **TR1**, yoghurt milk + trypsin enzyme and incubation at 37°C for 3 h before adding the starter culture; **TR2**, yoghurt milk + trypsin enzyme + the starter culture; **PS1**, yoghurt milk + pepsin enzyme and incubated at 37°C for 3 h before adding the starter culture; **PS2**, yoghurt milk + pepsin enzyme +starter culture; **LP**, yoghurt milk + *Lb. paracasei* Bu-Eg3 and incubated at 37°C for 6 h before adding the starter culture and **LR**, yoghurt milk+ *Lb. rhamnosus* Bu-Eg6 and incubated at 37°C for 6 h before adding the starter culture.

* $^{a-b}$Mean values of different rows with different superscripts are significantly different ($P < 0.05$).

Chemical attributes of the produced yoghurt:

The chemical attributes of the treatments of yoghurt are presented in Table (2). Results show that

the T.S% of the produced yoghurt was slightly increased through the refrigerated at 5±1°C up to 14 days; this slight augmentation could be due to the possible evaporation and loss of moisture through the storage period as described by **El-Nagar & Shenana (1998),Basiony *et al.* (2015), El-Alfy *et al.* (2016), and Saad & Elkhtab (2019).**

Based on mixed ANOVA, there was no statistically significant difference either between treatments (p>0.05) or between treatments and storage time on the T.S% of the produced yoghurt (p>0.05).

Table (2): Chemical attributes of the produced yoghurt stored at 5±1°C up to14 days.

Storage period (days)	Treatments						
	Total solids %						
	Cont.	TR1	TR2	PS1	PS2	LP	LR
Fresh	17.9 [a]	17.9 [a]	18.0 [a]	18.1 [a]	18.02 [a]	17.91[a]	17.91 [a]
7	18.0 [a]	18.1 [a]	18.1 [a]	18.1 [a]	18.16 [a]	18.07 [a]	18.01 [a]
14	18.2 [a]	18.3 [a]	18.3 [a]	18.3 [a]	18.34 [a]	18.12 [a]	18.12 [a]
	Fat %						
Fresh	5.60 [a]	5.65 [a]	5.59 [a]	5.59 [a]	5.60 [a]	5.57 [a]	5.59 [a]
7	5.61 [a]	5.62 [a]	5.63 [a]	5.65 [a]	5.61 [a]	5.62 [a]	5.62 [a]
14	5.67 [a]	5.67 [a]	5.64 [a]	5.65 [a]	5.66 [a]	5.68 [a]	5.67 [a]
	Protein %						
Fresh	4.05 [a]	4.12 [a]	4.22 [a]	4.18 [a]	4.14 [a]	4.06 [a]	4.20 [a]
7	4.20 [a]	4.38 [a]	4.38 [a]	4.34 [a]	4.31 [a]	4.29 [a]	4.32 [a]
14	4.30 [a]	4.47 [a]	4.45 [a]	4.48 [a]	4.41 [a]	4.44 [a]	4.41 [a]
	Soluble nitrogen /total nitrogen %						
Fresh	15.44[a]	20.48[a,b]	20.88[a,b]	24.53[b]	23.79[a,b]	22.11[a,b]	21.73[a,b]
7	22.17[a]	22.05[a]	23.21[a]	27.19[a]	25.95[a]	25.06[a]	23.85[a]
14	23.35[a]	23.52[a]	24.26[a]	26.98[a]	27.14[a]	28.02[a]	27.19[a]
	Ash %						
Fresh	0.84 [a]	0.85 [a]	0.83 [a]	0.84 [a]	0.83 [a]	0.85 [a]	0.85 [a]
7	0.89 [a]	0.90 [a]	0.88 [a]	0.93 [a]	0.89 [a]	0.89 [a]	0.89 [a]
14	0.92 [a]	0.97 [a]	0.93 [a]	0.93 [a]	0.94 [a]	0.94 [a]	0.93 [a]

Cont., control; **TR1**, yoghurt milk + trypsin enzyme and incubation at 37°C for 3 h before adding the starter culture; **TR2**, yoghurt milk + trypsin enzyme + the starter culture; **PS1**, yoghurt milk + pepsin enzyme and incubated at 37°C for 3 h before adding the starter culture; **PS2**, yoghurt milk + pepsin enzyme +starter culture; **LP**, yoghurt milk + *Lb. paracasei* Bu-Eg3 and incubated at 37°C for 6 h before adding the starter culture and **LR**, yoghurt milk+ *Lb. rhamnosus* Bu-Eg6 and incubated at 37°C for 6 h before adding the starter culture.

∗ [a–b]Mean values of different row with different superscripts are significantly different (P < 0.05).

The fat % of yoghurt treatments recorded 5.6, 5.65, 5.59, 5.59, 5.57, and 5.59 when fresh for control, TR1, TR2, PS1, PS2, LP, and LR subsequently. There were little augmentations in the fat% for all yoghurt treatments through the refrigerated storage up to 14 days. This augmentation is related to the augmentation of T.S.

El-Alfy *et al.* (2016), Basiony *et al.* (2015) and Saad & Elkhtab (2019) mentioned that the augmentation of fat in yoghurt during refrigerated storage could be due some moisture evaporation which increases the total solids of yoghurt during storage.

From the statistical point of view, there were no statistical significant differences between the different yoghurt treatments samples when fresh or through the refrigerated storage.

The protein % of yoghurt treatments was increased slightly through storage up to 14 days at 5±1°C. The increase in protein% is caused by the

increase of T.S through storage. These results agree with that reported by by **Abdou *et al*., (2015), El-Alfy *et al*., (2016) ,, and Saad & Elkhtab (2019).**

No statistically significant differences were found either between treatments or between treatments and storage periods ($p>0.05$).

The soluble nitrogen/total nitrogen % (S.N/T.N %) of yoghurt treatments when fresh recorded 15.44, 20.48, 20.88, 24.53, 23.79, 22.11, and 21.73% for control, TR1, TR2, PS1, PS2, LP, and LR, respectively. It can be noted that the control exhibited the lowest S.N/T.N%. PS1 recorded the highest S.N/T.N% when compared to all the yoghurt treatments. The S.N/T.N% increased for all treatments through the refrigerated storage at 5±° C, but less for the control. The increase of S.N/T.N% in the control treatment could be regarded to the proteolytic system of the starter culture used. On the other hand, the obvious increase in S.N/T.N% for TR1, TR2, PS1, and PS2 could be due to the activity of pepsin and trypsin enzymes. Besides the proteolytic system of the starter culture. The increase of S.N/T. N% in LP and LR could be due to the proteolytic system of all the subjoined cultures. There was a statistically significant difference between samples when fresh, but there were no statistically significant differences between samples either after 7 or 14 days of refrigerated storage at 5±° C. These results are in line with those found by **Hassan *et al*., (2015) , Elalfy *et al*., (2016) and Ismail *et al*., (2016).**

From the obtained results, it can be observed that there were no obvious differences between all treatments of the produced yoghurt, but there was little augmentation in ash % after 7 and 14 days of refrigerated storage at 5±1°C. the little augmentation of ash% could be due to the augmentation of T.S% during the refrigerated storage period. These results are agreed with those reported by **El-Nagar and Brennan (2001) and El-Nagar *et al.* (2007).**

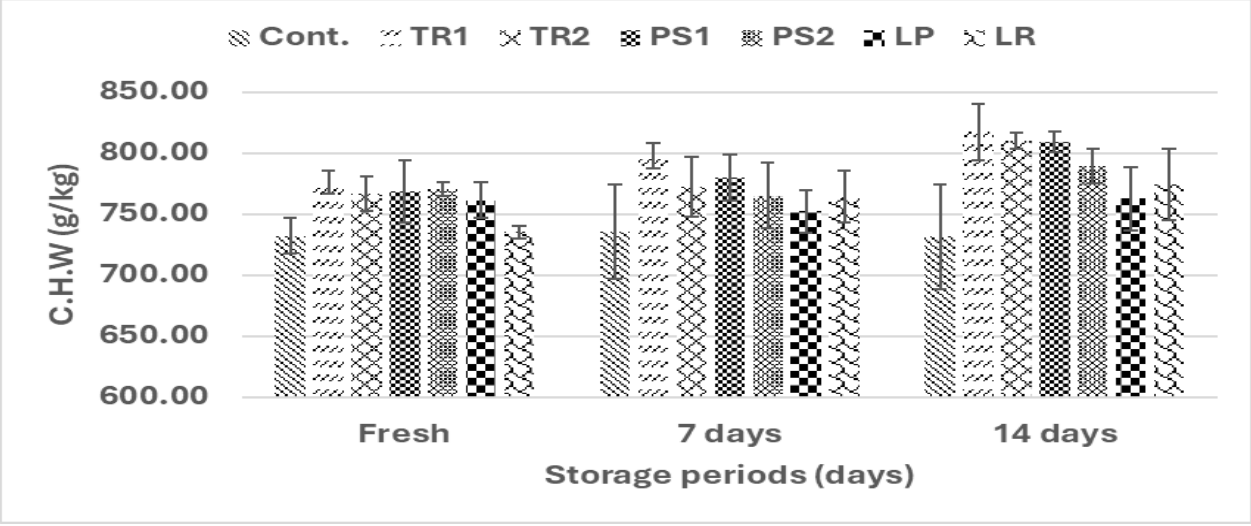
There was no statistically significant difference either between treatments ($p>0.05$) or between treatments and storage time on the ash% of the produced yoghurt ($p>0.05$).

The capability of holding water

The capability of holding water (CHW) represents how much wate can the curd hold inside its matrix and, it is a very important indicator of quality because it reflects the gel properties. **Chandan *et al*., 2006** reported that T.S (particularly fat and proteins %) and starter culture along with manufacturing and storage conditions are the main factors affecting CHW.

The capability of holding water increased in all treatments during the refrigerated storage **Figure (4)** but less in control. This increase could be regarded to the increase of The T.S during storage periods, which reflects the quality of treatments gel. These results agreed with that explained by (**Lucey, 2004).**

There was an effect of treatment on CHW of the produced yoghurt when it was fresh and after14 days but not after 7 days of refrigerated storage, on the other hand, there was no effect of storage periods on the CHW of the produced yoghurt.



Cont., control; **TR1**, yoghurt milk + trypsin enzyme and incubation at 37°C for 3 h before adding the starter culture; **TR2**, yoghurt milk + trypsin enzyme + the starter culture; **PS1**, yoghurt milk + pepsin enzyme and incubated at 37°C for 3 h before adding the starter culture; **PS2**, yoghurt milk + pepsin enzyme +starter culture; **LP**, yoghurt milk + *Lb. paracasei* Bu-Eg3 and incubated at 37°C for 6 h before adding the starter culture and **LR**, yoghurt milk+ *Lb. rhamnosus* Bu-Eg6 and incubated at 37°C for 6 h before adding the starter culture.

Figure (4) CHW differences of the produced yoghurts through refrigerated storage at 5±1°C up to 14 days.

Antioxidant activity of yoghurt treatments extracts:

Figure (5) show The antioxidant activity results of the produced yoghurt treatments extracts the different treatments of yoghurt extracts have greater antioxidant activities than the control extract, either when fresh or during 7 and 14 days of refrigerated storage at 5±1°C. The antioxidant activities when fresh were 16.95, 77.43, 63.88, 68.96, 74.63, 75.93 and 68.62% for control, TR1, TR2, PS1, PS2, LP and LR, respectively. The antioxidant δ-Valerobetaine (δVB) is a natural compound in ruminants' milk produced in the rumen by the microbial metabolization of free Nε-trimethyllysine (TML) present in vegetables **(D'Onofrio *et al.*, 2019** and **Liao *et al.*, 2025). Servillo *et al.*, 2018** found that buffalo milk contains the highest levels of δVB if compared with other ruminant or non-ruminant milk. **Kumar *et al.*, 2020** found that enzymatic hydrolysis of buffalo milk proteins can produce bioactive peptides with potent antioxidant activity. **Shazly *et al.*, 2019** found that hydrolyzing buffalo's casein with Alkaline protease and trypsin enzyme showed the highest antioxidant activities (*in vitro*). The antioxidant activities of the produced yoghurt could be due to the presence of δVB and liberation of bioactive peptides either by trypsin and pepsin enzymes or/and the natural protease system of the used starter culture. It is important to mention that the S.N/T.N% increased during the storage periods, which gave means more protein hydrolysis either by the used starter culture or the subjoined enzymes.There were statistical differences between all treatments and control, either when fresh or during 7 and 14 days of refrigerated storage at 5±1°C.

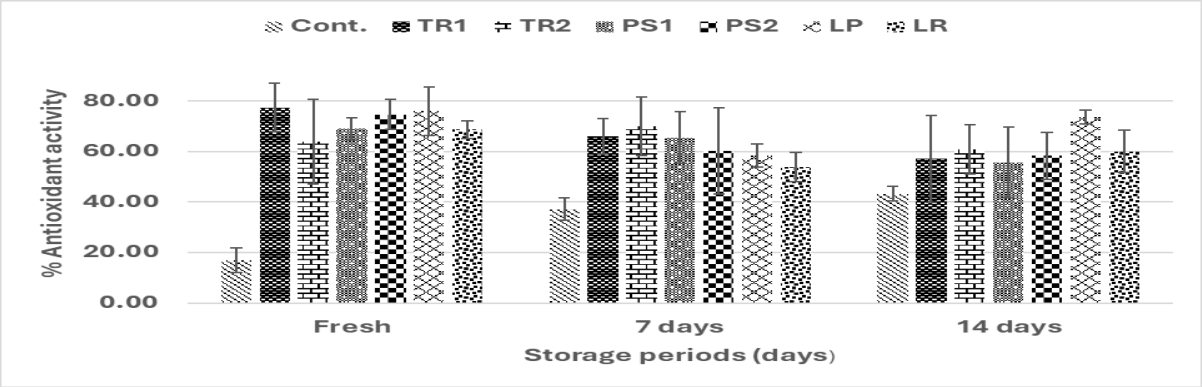


Figure (5) Antioxidant activity % of the produced yoghurt during refrigerated storage at 5±1°C up to 14 days

Microbiological examinations:

Microbiological examinations are important aspects in dairy microbiology to ensure the microbiological safety and quality of dairy products. The produced yoghurt treatments were examined for the total viable lactic acid bacteria (LAB), *Streptococcus thermophilus* and *Lactobacillus sp.* counts. Also, samples were examined for the presence of coliform bacteria and yeast & molds.

The changes of total viable LAB, *streptococcus thermophilus* and *lactobacillus sp.* counts through refrigerated storage at 5±1°C are shown in Figures (6, 7, 8) it could be concluded that the LAB of the produced yoghurt recorded 6.39, 6.30, 6.06, 6.29, 6.19, 8.59 and 8.39 log (cfu g^{-1}) for C, TR1, TR2, PS1, PS2, LP and LR respectively when fresh. By increasing the storage time of the yoghurt treatments up to 7 days, there was an increase of LAB counts to be 6.61, 6.49, 6.76, 6.41, 6.64, 8.67, and 8.53 log (cfu g^{-1}) for C, TR1, TR2, PS1, PS2, LP, and LR, in the same sequence. Then decrease was noticed after 14 days of storage and recorded 6.19, 6.13, 6.24, 5.92, 6.21, 8.17, and 8.47 log (cfu g^{-1}), respectively. These results are similar to those obtained **by Kebary and Kamely (1991)** and **Ibrahim *et al* (2004).**

Also, it was observed that the total viable LAB counts of LP and LR were higher than all other treatments, as a result of adding 1% *Lactobacillus paracasei* with yoghurt starter (LP) and 1% *Lactobacillus rhamnosus* with yoghurt starter (LR).

The results of ***streptococcus thermophilus*** counts of the produced yoghurt treatments recorded 8.56, 7.98, 8.17, 7.90, 7.86, 8.54 and 8.52 log (cfu g^{-1}) for C, TR1, TR2, PS1, PS2, LP and LR correspondingly when fresh. There was a decrease of ***streptococcus thermophilus*** log count after 7 days of refrigerated storage at 5±1°C and recorded 8.36, 7.81, 8.06, 7.49, 7.75, 8.43 and 8.34 log (cfu g^{-1}) in the same order. The decline continued after 14 days of refrigerated storage at 5±1°C to be 8.08, 7.67, 7.64, 7.30, 7.16, 7.99 and 8.18 log (cfu g^{-1}) in the same treatments sequence. The decrease of *streptococcus thermophilus* counts during storage of all treatments

may be attributed to their sensibility to the developed acidity. Similar results were found by **Abd-Elsalam *et al.* (2011)** and **El-Nager and Abd El-Tawab (2012).**

The counts of *lactobacillus sp.* when fresh recorded 5.76, 6.22, 6.52, 5.89, 5.95, 8.39, and 8.47 log (cfu g^{-1}) for C, TR1, TR2, PS1, PS2, LP, and LR, respectively. There was an increase of these counts after 7 days of storage at 5±1°C for all treatments to be 5.97, 6.41, 6.71, 6.12, 6.30,8.51 and 8.61 log (cfu g^{-1}) for C, TR1, TR2, PS1, PS2, LP, and LR, respectively. Decline has been noticed for these counts by the end of storage period to be 5.32, 5.78, 5.69, 5.49, 5.63, 8.10 and 7.92 log (cfu g^{-1}) in the same previous order. These results agree with **Abd-Elsalam *et al.* (2011)** and **Servili *et al.* (2011)**.

The different trend of increasing *lactobacillus sp.* count and decreasing *Streptococcus thermophilus* count after 7 days of refrigerated storage at 5±1°C may be regarded to the rise of acidity, which affects *Streptococcus thermophilus* growth, while *lactobacillus sp* tolerate, these results are similar to what was found by **El-Nager and Bernnan (2001)**.

Yeasts & moulds and coliforms were not detected in all yoghurt treatments, either fresh or during storage up to 14 days, which is due to the good hygiene conditions through the making of yoghurt treatments as well as the ability of lactic acid bacteria to produce antimicrobial compounds **(Gould 1991 and El-Nager 2009)**.

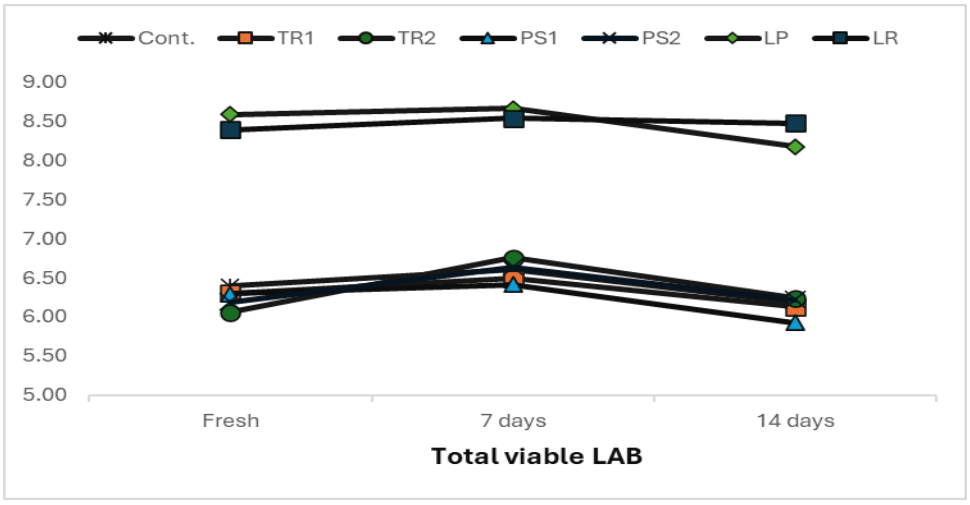


Figure (6): Microbiological analysis of the produced yoghurts during storage periods at 5±1°C up to 14 days (Total viable LAB)

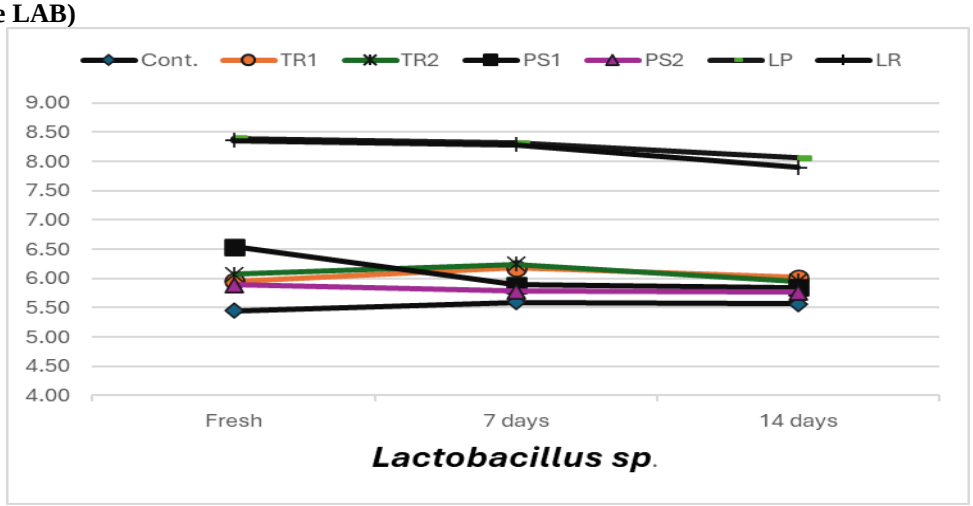


Figure (7): Microbiological analysis of the produced yoghurts during storage periods at 5±1°C up to 14 days (*Lactobacillus sp.*)

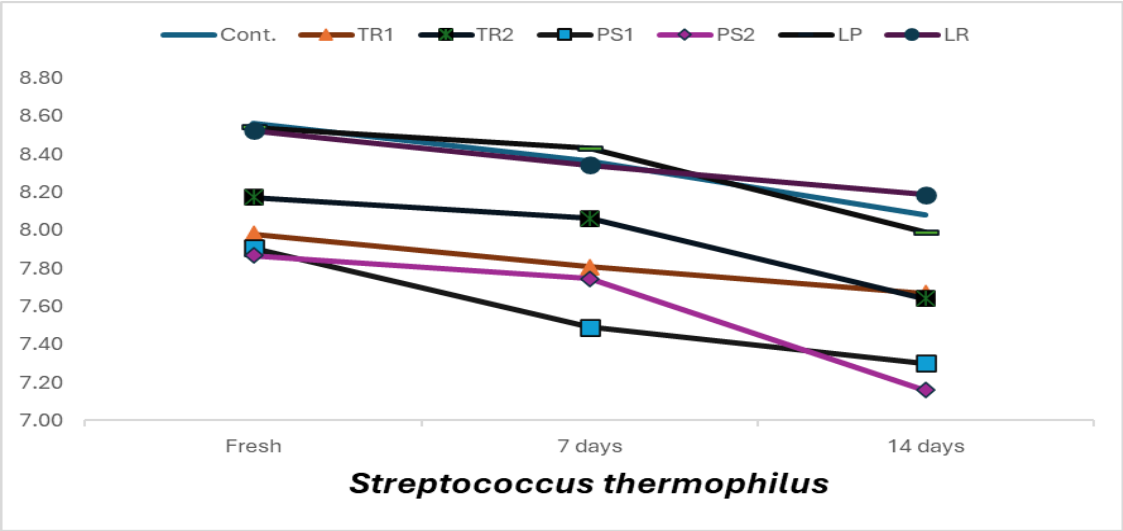


Figure (8): Microbiological analysis of the produced yoghurts during storage periods at 5±1°C up to 14 days (*Streptococcus thermophilus*).

Sensory evaluation:

Organoleptic description is considered one of the most important analyses of novel products to ensure acceptability and consumer contentment and is also considered a key factor in assessing the quality of such products.

The sensory analysis of the produced yoghurts was done when fresh, subsequently after 7& 14 days of refrigerated storage at 5±1°C. Results of the sensory attributes are presented in Table (3). The obtained results of flavor attribute when fresh showed that TR1 and LR recorded the highest scores (26/30) followed by TR2 (25.33/30) while PS1 and PS2 recorded 24.67/30, the control treatment recorded 24/30 followed by LP, which recorded the lowest score (23.67/30). The Flavor attribute was enhanced during the refrigerated storage after 7 and 14 days for all treatments. TR1, TR2, LP, and LR recorded 28.33/30 after 14 days; the rest of the treatments recorded 28.00/30. **Hemantha Kumar *et al.* (2001)** found that treating yoghurt milk with trypsin enzyme has a non-significant effect on the Flavor of yoghurt when compared to the control. **Lorenzen and Meisel (2005)** found that yoghurt made from milk treated with trypsin has better sensory characteristics, *i.e.*, taste and smell, when compared to the control. **Hekmat and Reid (2006)** concluded that the yoghurt made with *L. rhamnosus* GR-1 is as good as the standard yoghurt regarding the sensory attributes.

According to what concluded from the statistical analysis of Flavor data, results showed that there was a statistically significant interaction between the interventions when fresh (p = 0.01) and after 7 days of refrigerated storage (p = 0.02), but there were no statistically significant differences between treatments after 14 days of refrigerated storage (p =0.856).

Regarding the odor attribute, LR recorded the highest score when fresh (17/20) followed by TR1, TR2, PS1, and PS2 (16.67/20) while LP and the control treatment recorded the lowest scores, 16.33/20, 15.33/20, respectively. It was noticed that the odor attribute was enhanced through the storage periods up to 14 days for all treatments. Based on statistical analysis of odor data, results showed that there were no significant differences between treatments when fresh or after 7 and 14 days of refrigerated storage.

The aftertaste attribute for all treatments was very acceptable when fresh and was enhanced after 7 and 14 days of refrigerated storage, where all the treatments recorded 9/10 or higher. Based on statistical analysis of aftertaste data, results showed that there were no significant differences between treatments when fresh or after 7 and 14 days of refrigerated storage.

Concerning the body & texture characteristics, when fresh, the highest scores went to LP and LR (28.33/30), followed by TR1 (28/30), TR2 (27.67/30), PS2 (26.33/30), PS1 (26.00/30), and control (25.33/30). The body & texture scores were increased after 7 and 14 days of refrigerated storage for all treatments. **Hemantha Kumar *et al.* (2001)** found that the yoghurt produced from milk treated with trypsin had a soft body and a smooth texture.

According to the statistical analysis of body & texture data, results showed that there were a statistically significant interaction between the interventions when fresh (p = .004) but there were no statistically significant differences between treatments after 7 and 14 days of refrigerated storage.

Regarding the appearance of the produced yoghurt, all treatments were accepted when fresh,

with scores of 9/10, but the control treatment was slightly lower (8/10). Also, the appearance of all treatments was enhanced during refrigerated storage up to 14 days. Statistical analysis of appearance data showed that there were no significant differences between treatments either when fresh or during the refrigerated storage periods.

Regarding the total sensory evaluation scores, all samples were accepted when fresh or during refrigerated storage. TR1, LP, and LR scored 89/100 when fresh, followed by the rest of the treatments. The total score was increased for all the treatments during the storage periods due to the increase in the different sensory attributes. Statistical analysis of the total score of different yoghurt treatments showed that there were a statistically significant differences between treatments at fresh ($p < 0.001$). On the other hand, there were no statistically significant differences after 7 or 14 days of refrigerated storage.

Table (3) Sensory evaluation of the produced yoghurt during refrigerated storage at 5±1°C up to 14 day

properties	Storge period (days)	Cont.	TR1	TR2	PS1	PS2	LP	LR
Flavor (30)	Fresh	24.00^{a,b}	26.00^{b}	25.33^{a,b}	24.67^{a,b}	24.67^{a}	23.67^{a,b}	26.00^{b}
	7	26.67^{a,b}	27.67 ^{a,b}	26.33 ^{a,b}	25.33^{a}	25.33^{b}	28.00 ^{a,b}	27.67 ^{a,b}
	14	28.00^{a}	28.33^{a}	28.33^{a}	28.00^{a}	28.00^{a}	28.33^{a}	28.33^{a}
Odor (20)	Fresh	15.33^{a}	16.67^{a}	16.67^{a}	16.67^{a}	16.67^{a}	16.33^{a}	17.00^{a}
	7	16.67^{a}	18.33^{a}	18.33^{a}	18.00^{a}	18.00^{a}	17.00^{a}	18.67^{a}
	14	19.00^{a}	19.00^{a}	19.00^{a}	19.00^{a}	19.00^{a}	19.00^{a}	19.00^{a}
After taste (10)	Fresh	9.33^{a}	9.33^{a}	9.33^{a}	9.33^{a}	9.33^{a}	9.00^{a}	9.33^{a}
	7	9.67^{a}	9.00^{a}	9.33^{a}	9.33^{a}	9.00^{a}	9.33^{a}	9.33^{a}
	14	9.33^{a}	9.67^{a}	9.67^{a}	9.67^{a}	10.00^{a}	9.33^{a}	9.67^{a}
Body & texture (30)	Fresh	25.33^{a}	28.00^{b}	27.67^{a,b}	26.00^{a,b}	26.33^{a,b}	28.33^{b}	28.33^{b}
	7	27.00^{a}	28.33^{a}	28.00^{a}	26.67^{a}	27.00^{a}	28.33^{a}	28.33^{a}
	14	28.67^{a}	29.00^{a}	28.67^{a}	28.67^{a}	28.67^{a}	28.67^{a}	28.66^{a}
Appearance (10)	Fresh	8.00^{a}	9.00^{a}	9.00^{a}	9.00^{a}	9.00^{a}	9.00^{a}	9.00^{a}
	7	8.67^{a}	9.00^{a}	9.00^{a}	9.00^{a}	9.00^{a}	9.00^{a}	9.00^{a}
	14	9.67^{a}	9.67^{a}	9.67^{a}	9.67^{a}	9.67^{a}	9.67^{a}	9.66^{a}
Total (100)	Fresh	82.00^{a}	89.00^{b}	88.00^{b}	85.67^{a,b}	84.67^{a,b}	89.00^{b}	89.00^{b}
	7	88.67^{a}	92.33^{a}	91.00^{a}	88.33^{a}	90.00^{a}	92.00^{a}	93.00^{a}
	14	92.67^{a}	95.67^{a}	95.33^{a}	95.00^{a}	96.00^{a}	95.67^{a}	95.66^{a}

Cont., control; **TR1**, yoghurt milk + trypsin enzyme and incubation at 37°C for 3 h before adding the starter culture; **TR2**, yoghurt milk + trypsin enzyme + the starter culture; **PS1**, yoghurt milk + pepsin enzyme and incubated at 37°C for 3 h before adding the starter culture; **PS2**, yoghurt milk + pepsin enzyme +starter culture; **LP**, yoghurt milk + *Lb. paracasei* Bu-Eg3 and incubated at 37°C for 6 h before adding the starter culture and **LR**, yoghurt milk+ *Lb. rhamnosus* Bu-Eg6 and incubated at 37°C for 6 h before adding the starter culture.
Significant differences ($p < 0.05$) exist between means in the same row with different superscripts.

Conclusion:

From this research results, it can be concluded that enzymatic hydrolysis of buffalo milk proteins (casein and whey) using pepsin and trypsin enzymes can liberate antioxidants. Moreover, the production of yoghurt as described in this research may be a good source of naturally liberated antioxidants. The chemical, microbiological, the capability of holding water, and the organoleptic qualities of the produced yoghurt were found to be similar or better than the control samples up to 14 days of refrigerated storage at 5±°C. Further studies are required (*in vivo*) to ensure the potential antioxidant properties of buffalo casein, whey, and yoghurt made from buffalo milk.

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الاستفادة من قيمة بروتينات اللبن الجاموسي في الزبادي: النشاط المضاد للأكسدة وخصائص الجودة

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الملخص

تُستخدم مضادات الأكسدة طبيًا للحد من الإجهاد التأكسدي لدى الإنسان. وقد تكون الأغذية التي تحتوي على هذه المضادات بشكل طبيعي أكثر فعالية في تخفيف هذا الإجهاد. ركزت هذه الدراسة على استكشاف النشاط المضاد للأكسدة لكازين والشرش اللبن الجاموس بعد خضوعهما للتحلل الإنزيمي باستخدام إنزيمي الببسين والتربسين. كما تم تحضير أنواع مختلفة من الزبادي من اللبن الجاموسي لاستكشاف خصائصها المضادة للأكسدة. تم فصل عينات الكازين والشرش من اللبن الجاموسي بعد التجبن بالمنفحة أو بعد التحميض البيولوجي باستخدام بكتيريا *Lactobacillus sp.* أظهرت أنواع الكازين المختلفة التي خضعت للتحلل بالببسين نشاطًا مضادًا للأكسدة أعلى من تلك التي خضعت للتحلل بالتربسين، بينما أظهرت عينات الشرش المعالجة بالتربسين نشاطًا مضادًا للأكسدة أعلى من تلك المعالجة بالببسين. تم تحضير أنواع مختلفة من الزبادي من اللبن الجاموسي بإضافة الببسين أو التربسين أو بكتيريا *Lactobacillus sp.* أظهرت المعاملات المختلفة للزبادي نشاطًا مضادًا للأكسدة أعلى بشكل ملحوظ مقارنةً بالعينة الكنترول في جميع الفترات الزمنية.