Effects of glycinin basic peptide on physicochemical characteristics and microbial inactivation of pasteurized milk

Guo-Ping Zhao,* Ying-Qiu Li,*¹ Gui-Jin Sun,* and Hai-Zhen Mot†

*School of Food Science & Engineering, Qilu University of Technology, No. 3501 University Road of Changqing District, 250353, Jinan, Shandong Province, China

†School of Food Science, Henan Institute of Science and Technology, Xinxiang, 453003, China

ABSTRACT

The effects of glycinin basic peptide (GBP) on physicochemical characteristics and microbial inactivation of pasteurized milk were investigated over 21 d of storage at 4°C. Sensory properties, total bacterial count, pH, alcohol levels, lactose content, and protein changes of pasteurized milk differentially treated with GBP were analyzed periodically during refrigerated storage. Compared with the control, reductions for total bacterial count and specific bacterium (*Staphylococcus aureus*) in pasteurized milk treated with GBP during storage were found. However, sensory scores, pH, lactose, and protein contents of pasteurized milk treated with GBP were much higher than those of the control. A concentration of 0.015% (wt/vol) GBP could effectively inhibit the growth and reproduction of bacteria in pasteurized milk, enhance its sensory and physicochemical properties, and extend its shelf life to 15 d. Thus, GBP has good potential to be a natural milk preservative.

Key words: glycinin basic peptide, pasteurized milk, sensory property, physicochemical characteristic, microbial inactivation

INTRODUCTION

It is generally recognized that long-term use of chemical synthetic preservatives in the food industry may have potential risk to human health (Meyer et al., 2002; Li et al., 2014). Thus, studies of natural food preservatives have become a priority to prevent food from deterioration and to improve the safety of products in the food industry for decades (Menon and Garg, 2001; Seo et al., 2002; Conte et al., 2009). Natural food preservatives will play a crucial role in the future of the food industry, although there are many hurdles to be overcome (Davidson and Taylor, 2007).

Among all food preservatives, natural antimicrobial peptides and proteins have an important prospective role as food preservatives (Sitohy and Osman, 2010). Their broad-spectrum antimicrobial activities against bacteria allow them to be used as food preservatives to prolong the shelf life of food (Stevens et al., 1991; Shih et al., 2006; Teixeira et al., 2012). Mitra et al. (2011) evaluated the effectiveness of nisin Z in controlling the growth of bacteria in pasteurized milk. The use of esterified legume proteins to improve the quality of milk was investigated by observing changes in total bacterial count and other indicators (Sitohy and Osman, 2011). In addition, Chang et al. (2010) reported that ϵ -polylysine could protect roast beef from microbial contamination, enhancing its storage quality.

Glycinin basic peptide (GBP), isolated from soybean, is a basic subunit of soybean glycinin and has a molecular weight of 17 to 20 kDa. Sitohy et al. (2012) found that GBP could inhibit the growth of several spoilage bacteria. Li et al. (2015) showed that low concentrations of GBP could effectively inactivate bacteria, such as *Escherichia coli*, in which the inactivation mechanism was via interactions with the bacterial membrane structure.

Milk and milk products are high-protein beverages; thus, they are susceptible to contamination of pathogens and spoilage bacteria, especially *Staphylococcus aureus*, a common pathogen in milk and milk products (Griffiths and Phillips, 1990; Hotchkiss et al., 1999). Spoilage and deterioration of milk and milk products commonly lead to an increase in total bacterial count and changes in physicochemical features, including sensory properties, pH, lactose content, and protein (Barbano and Lynch, 2006; Mitra et al., 2011). Therefore, these features are often regarded as indicators of whether deterioration has occurred in milk and milk products. Saha et al. (2003) measured sensory properties, pH, protein content, and acidity of raw milk to demonstrate that hydrogen peroxide was suitable for extending its shelf life. Based on analysis of bacterial count and acidity, Haddadin et al. (1996) reported that natural lactoperoxidase could retain good quality char-

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¹Corresponding author: lyq@qlu.edu.cn or sdililyq@126.com

acteristics of raw milk and extend its shelf life during cold storage. In addition, indicators such as sensory properties, bacterial count, pH, and protein of milk were measured to study the preservative effect of additives on shelf life extension of fermented milk beverages (Hussain et al., 2014).

However, few investigations have been done into whether GBP affects the physicochemical characteristics of milk when it is used to supplement milk as a preservative. The purpose of this paper was to study the changes in microbiological and physicochemical features (sensory properties, bacterial count, pH, alcohol test, lactose content, and protein) in pasteurized milk treated with GBP and to evaluate the preservative effects of GBP on pasteurized milk stored at 4°C.

MATERIALS AND METHODS

Reagents and Chemicals

Tryptone, peptone, yeast extract, beef extract, glycine, glucose, and agar for microbial analysis were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Tris-HCl, SDS, β -mercaptoethanol, bromophenol blue, and Coomassie Brilliant Blue R250 for protein analysis were all from Sigma (St. Louis, MO). All other reagents and chemicals were of analytical grade.

Preparation and Treatment of Pasteurized Milk with GBP

The GBP powder was prepared in the laboratory following the method described by Li et al. (2015). Milk was purchased from a local dairy farm in Jinan, China, and pasteurized at 85°C for 5 min, after which it was called pasteurized milk. Pasteurized milk was treated with GBP to obtain final GBP concentrations of 0, 0.005, 0.010, 0.015, and 0.020% (wt/vol). All samples were stored at 4°C for 21 d. Sensory scores, total bacterial count, pH, alcohol test, lactose content, and *S. aureus* inactivation were measured periodically on 0, 3, 6, 9, 12, 15, 18, and 21 d, respectively. Protein changes were measured every 6 d.

Sensory Evaluation

The sensory properties of pasteurized milk were evaluated using the method described by Küçükgülmez et al. (2013) with some modifications, using 7 panelists trained to be familiar with the characteristics of pasteurized milk. The panelists scored the sensory properties, including appearance, odor, texture, taste, and overall acceptability, using a 9-point descriptive scale

(1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely).

Measurement of Total Bacterial Count

The total bacterial count of pasteurized milk stored at 4°C was determined as described by Li et al. (2014) with some modifications. Plate count agar medium consisted of 0.5% tryptone, 0.25% yeast extract, 1% glucose, 1.5% agar, and 96.75% sterile distilled water. Every 3 d, serial dilutions of pasteurized milk (1.0 mL) in sterile distilled water were mixed with plate count agar medium in Petri dishes and incubated at 30°C for 48 h to get total bacterial colonies. The total bacterial colonies were counted and transformed into the logarithm of the number of colony-forming units ($\log_{10}$ cfu/mL).

Determination of pH in Pasteurized Milk

The pH of pasteurized milk under each treatment was determined with a precision pH meter (PB-10, Sartorius, Goettingen, Germany), as described by Li et al. (2014).

Alcohol Test of Pasteurized Milk

Three-milliliter aliquots of milk sample were mixed with 3 mL of 70% (vol/vol) alcohol in a dish and observed for 10 to 20 s. If flocculent precipitates appeared in the milk, the milk was regarded as alcohol positive, indicating spoilage of the pasteurized milk. Otherwise, the sample was determined to be alcohol-negative milk (Demirbas et al., 2008).

Determination of Lactose Content of Pasteurized Milk

The lactose content of pasteurized milk was measured using the method described by Ning et al. (2014) with some modifications. An aliquot of 0.05 mL of pasteurized milk was mixed with 1.25 mL of 31 g/L potassium ferrocyanide and 1.25 mL of 40 g/L zinc sulfate solution, causing proteins to precipitate in a 25-mL colorimetric tube. Next, 2.5 mL of color former (1% phenol, 5% sodium hydroxide, 1% picric acid, and 1% sodium bisulfite mixed in a 1:2:2:1 ratio) was added to the resulting mixture and boiled for 5 min to produce chromogen. Then, the chromogenic mixture was cooled to room temperature (25°C) and diluted with distilled water to 25 mL in volumetric flask. The precipitated protein from the chromogenic mixture was removed by centrifugation ($12,000 \times g$, 10 min, 4°C). The absor-

balance of the supernatant was then measured at 520 nm and transformed into lactose content (mg/mL).

SDS-PAGE Electrophoresis

The SDS-PAGE electrophoresis of protein in pasteurized milk was performed according to the method described in Li et al. (2015) with some modifications. An aliquot (1 mL) of pasteurized milk was diluted with 5 mL of distilled water and mixed with 6 mL of sample buffer (pH 6.8; 1 M Tris-HCl, 50% glycerol, 10% SDS, 10% β -mercaptoethanol, and 0.1% bromophenol blue). The mixture was boiled for 5 min, cooled to 25°C, and loaded onto a 3% stacking gel over a 15% resolving gel. The resulting protein was electrophoresed at 15 mA for 1 h on the stacking gel and at 30 mA for 5 h on the resolving gel. The gel was dyed with staining solution (1.25 g of Coomassie Brilliant Blue R250, 454 mL of 50% methanol, and 46 mL of glacial acetic acid) for 4 h and decolorized with decolorizing agent (75 mL of glacial acetic acid, 50 mL of methanol, and 875 mL of distilled water). After 4 d, the protein bands were visualized on the gels.

Analysis of *S. aureus* Inactivation

Staphylococcus aureus (ATCC 6538) was obtained from the Culture Collection, Qilu University of Technology. Freeze-dried cells were activated according to the ATCC guidelines. The strain was maintained on slants of beef extract peptone medium (0.3% beef extract, 1% peptone, 0.5% NaCl, 2% agar, and 96.2% sterile distilled water). One single colony of culture was inoculated from the beef extract peptone medium into a flask containing 100 mL of sterile beef extract peptone broth. The inoculated broth was then incubated at 37°C in a water bath with shaking (130 rpm) for 10 h to achieve the stationary phase. The final cell concentration was approximately $8 \log_{10}$ cfu/mL. The cells were centrifuged at $5,000 \times g$ at 4°C for 5 min and washed 3 times by resuspension in sterile distilled water. The washed cells were finally resuspended in pasteurized milk at an initial concentration of approximately $5 \log_{10}$ cfu/mL. The resulting pasteurized milk was treated with GBP to obtain final concentrations of 0, 0.005, 0.010, 0.015, and 0.020%, respectively, and then stored at 4°C. Every 3 d of storage, GBP-treated pasteurized milk was incubated onto Baird-Parker selective agar medium (1% tryptone, 0.5% beef extract, 0.1% yeast extract, 1% sodium pyruvate, 1.2% glycine, 0.14% lithium chloride, 2% agar, and 95.36% sterile distilled water) at 37°C for 48 h. The *S. aureus* colonies were counted and transformed into $\log_{10}$ cfu/mL.

Statistical Analysis

The experiments were performed in triplicate. Mean values, standard errors, and ANOVA were performed using SPSS17.0 software (SPSS Inc., Chicago, IL). Graphs were made in Excel 2007 (Microsoft Office Excel 2007 for Windows, Microsoft Corp., Redmond, WA). Analysis of variance was used to determine the significance of treatments. Duncan's method was used for multiple comparison corrections, and significance was reported at the 5% level ($P < 0.05$).

RESULTS AND DISCUSSION

Effect of GBP on Sensory Properties of Pasteurized Milk

The sensory scores of pasteurized milk treated with different concentrations GBP are given in Table 1 over the storage period of 21 d at 4°C. Milk with sensory scores lower than 4.0 was considered unacceptable for human consumption (Küçükgülmez et al., 2013). As can be seen in Table 1, a significant decline ($P < 0.05$) was observed in all sensory scores of the control samples with increasing storage time. On d 9 of cold storage, the control had sensory scores of approximately 4.0 and was considered to have deteriorated. The control was assessed to be in the state of putrefaction, achieving sensory scores of approximately 3.3 on d 12. At the same time, 0.005 and 0.010% GBP-treated pasteurized milk had slightly acceptable scores of approximately 5.5 and 5.9. However, all sensory scores of pasteurized milk treated with 0.015 and 0.020% GBP were approximately 6.0 and 6.4, well within the acceptable range when evaluated on d 15. On d 18 of storage, appearance, odor, texture, taste, and overall acceptability of all samples presented unacceptable scores except the 0.020% GBP-treated sample. These results showed that GBP could improve sensory properties of pasteurized milk during storage and that 0.015 and 0.020% GBP had significantly ($P < 0.05$) preservative effects on pasteurized milk over 15 d of storage. Duncan's method of ANOVA demonstrated no differences in the sensory properties of pasteurized milk treated with 0.015 or 0.020% GBP. Thus, 0.015% GBP was the optimal concentration for use in pasteurized milk, with a shelf life of 15 d.

Osman et al. (2013) reported that a solution of 11S glycinin was highly effective at maintaining normal sensory properties of milk. Sitohy et al. (2012) found that the basic subunits of glycinin (i.e., GBP) had better antimicrobial effect than 11S glycinin in spite of the former's attachment to the acidic subunits. In this study, GBP showed a strong preservative effect on

Table 1. Effect of different concentrations of glycinin basic peptide (GBP) on sensory properties of pasteurized milk after storage at 4°C (mean ± SE)

Item	Treatment	Storage time (d)									
		0	3	6	9	12	15	18	21		
Appearance	Control	9.0 ± 0.06 ^a	8.0 ± 0.12 ^b	6.6 ± 0.15 ^c	4.0 ± 0.05 ^d	3.1 ± 0.06 ^e	2.8 ± 0.14 ^f	2.0 ± 0.18 ^f	1.4 ± 0.08 ^g		
	0.005% GBP	9.0 ± 0.00 ^a	8.3 ± 0.01 ^{ab}	7.3 ± 0.15 ^b	6.6 ± 0.09 ^{bc}	5.2 ± 0.14 ^d	3.9 ± 0.17 ^e	2.7 ± 0.17 ^f	2.0 ± 0.09 ^f		
	0.010% GBP	9.0 ± 0.06 ^a	8.4 ± 0.08 ^a	7.7 ± 0.07 ^b	6.9 ± 0.12 ^c	5.8 ± 0.05 ^d	4.2 ± 0.08 ^e	3.0 ± 0.07 ^f	2.5 ± 0.10 ^f		
	0.015% GBP	9.0 ± 0.04 ^a	8.8 ± 0.13 ^a	8.5 ± 0.06 ^a	7.9 ± 0.10 ^{ab}	6.8 ± 0.08 ^b	5.9 ± 0.16 ^c	4.1 ± 0.05 ^d	3.2 ± 0.2 ^e		
Odor	0.020% GBP	9.0 ± 0.02 ^a	9.0 ± 0.05 ^a	8.8 ± 0.03 ^a	8.3 ± 0.09 ^{ab}	7.2 ± 0.12 ^b	6.0 ± 0.11 ^c	5.0 ± 0.10 ^d	4.2 ± 0.05 ^e		
	Control	9.0 ± 0.01 ^a	8.0 ± 0.06 ^b	6.4 ± 0.00 ^c	4.2 ± 0.1 ^d	3.3 ± 0.07 ^e	2.7 ± 0.10 ^f	2.1 ± 0.16 ^g	1.6 ± 0.19 ^g		
	0.005% GBP	9.0 ± 0.02 ^a	8.4 ± 0.07 ^{ab}	7.5 ± 0.10 ^b	6.7 ± 0.05 ^c	5.3 ± 0.12 ^d	3.8 ± 0.06 ^e	2.7 ± 0.05 ^f	2.1 ± 0.12 ^f		
	0.010% GBP	9.0 ± 0.04 ^a	8.5 ± 0.08 ^a	7.8 ± 0.06 ^{ab}	7.0 ± 0.01 ^b	5.7 ± 0.11 ^c	4.2 ± 0.15 ^d	3.1 ± 0.20 ^e	2.5 ± 0.07 ^f		
Texture	0.015% GBP	9.0 ± 0.01 ^a	8.8 ± 0.10 ^a	8.4 ± 0.07 ^a	7.9 ± 0.05 ^{ab}	6.9 ± 0.09 ^{bc}	6.0 ± 0.00 ^c	4.1 ± 0.10 ^d	3.4 ± 0.12 ^e		
	0.020% GBP	9.0 ± 0.00 ^a	8.8 ± 0.04 ^a	8.8 ± 0.06 ^a	8.4 ± 0.10 ^{ab}	7.4 ± 0.13 ^b	6.1 ± 0.09 ^c	5.3 ± 0.04 ^d	4.3 ± 0.13 ^e		
	Control	9.0 ± 0.02 ^a	7.9 ± 0.05 ^b	6.3 ± 0.10 ^c	4.1 ± 0.12 ^d	3.2 ± 0.01 ^e	2.7 ± 0.08 ^e	2.0 ± 0.05 ^f	1.3 ± 0.13 ^g		
	0.005% GBP	9.0 ± 0.04 ^a	8.5 ± 0.10 ^a	7.6 ± 0.07 ^b	6.5 ± 0.02 ^c	5.5 ± 0.12 ^d	3.9 ± 0.15 ^e	2.6 ± 0.09 ^f	2.0 ± 0.03 ^f		
Taste	0.010% GBP	9.0 ± 0.06 ^a	8.6 ± 0.12 ^a	7.8 ± 0.15 ^b	7.1 ± 0.07 ^{bc}	5.8 ± 0.08 ^c	4.2 ± 0.07 ^d	3.0 ± 0.16 ^e	2.3 ± 0.11 ^f		
	0.015% GBP	9.0 ± 0.01 ^a	8.7 ± 0.03 ^a	8.5 ± 0.06 ^a	7.9 ± 0.03 ^{ab}	6.8 ± 0.15 ^b	5.9 ± 0.16 ^c	4.0 ± 0.20 ^d	3.3 ± 0.08 ^e		
	0.020% GBP	9.0 ± 0.00 ^a	8.9 ± 0.07 ^a	8.8 ± 0.05 ^a	8.5 ± 0.12 ^{ab}	7.6 ± 0.10 ^b	6.3 ± 0.01 ^c	5.4 ± 0.04 ^d	4.2 ± 0.11 ^e		
	Control	9.0 ± 0.08 ^a	7.8 ± 0.15 ^b	6.4 ± 0.18 ^c	4.0 ± 0.06 ^d	3.1 ± 0.14 ^e	2.5 ± 0.15 ^e	1.8 ± 0.08 ^f	1.3 ± 0.22 ^g		
Overall acceptability	0.005% GBP	9.0 ± 0.14 ^a	8.4 ± 0.06 ^a	7.8 ± 0.12 ^b	6.6 ± 0.04 ^c	5.5 ± 0.08 ^d	3.9 ± 0.10 ^e	2.5 ± 0.15 ^f	1.9 ± 0.13 ^f		
	0.010% GBP	9.0 ± 0.10 ^a	8.5 ± 0.09 ^a	7.9 ± 0.11 ^{ab}	7.2 ± 0.16 ^{bc}	5.9 ± 0.08 ^c	4.1 ± 0.00 ^d	2.9 ± 0.01 ^e	2.2 ± 0.20 ^f		
	0.015% GBP	9.0 ± 0.17 ^a	8.7 ± 0.12 ^a	8.4 ± 0.10 ^a	7.9 ± 0.21 ^{ab}	7.0 ± 0.13 ^b	5.9 ± 0.22 ^c	4.1 ± 0.06 ^d	3.3 ± 0.18 ^e		
	0.020% GBP	9.0 ± 0.06 ^a	8.9 ± 0.12 ^a	8.8 ± 0.15 ^a	8.5 ± 0.05 ^{ab}	7.5 ± 0.26 ^b	6.4 ± 0.10 ^c	5.6 ± 0.08 ^d	4.2 ± 0.17 ^e		
Overall acceptability	Control	9.0 ± 0.09 ^a	7.9 ± 0.16 ^b	6.4 ± 0.13 ^c	4.0 ± 0.06 ^d	3.3 ± 0.04 ^e	2.7 ± 0.05 ^e	1.9 ± 0.08 ^f	1.2 ± 0.25 ^g		
	0.005% GBP	9.0 ± 0.04 ^a	8.4 ± 0.06 ^a	7.8 ± 0.12 ^b	6.6 ± 0.14 ^c	5.5 ± 0.08 ^d	3.9 ± 0.06 ^e	2.5 ± 0.10 ^f	1.9 ± 0.13 ^f		
	0.010% GBP	9.0 ± 0.10 ^a	8.5 ± 0.07 ^a	7.9 ± 0.11 ^a	7.2 ± 0.17 ^b	5.9 ± 0.18 ^c	4.1 ± 0.10 ^d	2.9 ± 0.08 ^e	2.2 ± 0.06 ^f		
	0.015% GBP	9.0 ± 0.07 ^a	8.7 ± 0.12 ^a	8.4 ± 0.16 ^a	8.0 ± 0.11 ^{ab}	7.0 ± 0.23 ^b	6.0 ± 0.22 ^c	4.1 ± 0.06 ^d	3.3 ± 0.10 ^e		
	0.020% GBP	9.0 ± 0.06 ^a	8.9 ± 0.13 ^a	8.8 ± 0.16 ^a	8.5 ± 0.15 ^{ab}	7.5 ± 0.16 ^b	6.4 ± 0.15 ^c	5.6 ± 0.08 ^d	4.2 ± 0.05 ^e		

^{a-g}Means within a row with different superscripts differ ($P < 0.05$).

pasteurized milk. These results suggested GBP of 11S glycinin played a crucial role in its preservative effect on pasteurized milk.

Effect of GBP on Total Bacterial Count in Pasteurized Milk

Total bacterial count in pasteurized milk was affected by the addition of GBP at different concentrations over 21 d of storage at 4°C, as presented in Figure 1. The $\log_{10}$ cfu/mL values of the control increased from 2.28 to 10.55 as the storage time increased from 0 to 21 d. On the other hand, the $\log_{10}$ cfu/mL values of GBP-treated pasteurized milk increased from 2.28 to 6.06 during storage over the same amount of time. On d 15, pasteurized milk treated with 0.005, 0.010, 0.015, and 0.020% GBP had 6.03, 5.87, 4.59, and 4.52 $\log_{10}$ cfu/mL, respectively, whereas the value of control already reached 8.44 $\log_{10}$ cfu/mL. These results demonstrated that GBP had a clear antibacterial effect on pasteurized milk. An ANOVA of the data in Figure 1 showed that the antibacterial activities of 0.015 and 0.020% GBP were significantly ($P < 0.05$) different from the

other concentrations (0.005 and 0.010%). Moreover, no significant difference ($P > 0.05$) was found between 0.015 and 0.020% GBP in suppressing the $\log_{10}$ cfu/mL of pasteurized milk samples. In addition, sensory analysis also showed that pasteurized milk treated with 0.015 and 0.020% GBP was well within the acceptable range on d 15. On d 21, the $\log_{10}$ cfu/mL values of all samples exceeded 6.06, which were considered unacceptable (milk with greater than 6 $\log_{10}$ cfu/mL was regarded as degenerated milk; Desmasures et al., 1997). These results indicated that addition of 0.015 and 0.020% GBP could significantly inhibit the growth and reproduction of bacteria, thus extending the shelf life of pasteurized milk to 15 d with refrigeration.

Effect of GBP on pH of Pasteurized Milk

A decrease in pH has a negative effect on the quality of milk during storage, especially when considering sensory characteristics, such as odor, taste, and texture. The decline in the pH of milk is attributed to the lactic acid produced by microbial decomposition of lactose (Olasupo et al., 1996). The pH of fresh milk is approxi-

Figure 1. Effect of different concentrations of glycinin basic peptide (GBP) on total bacterial count in pasteurized milk after storage at 4°C of storage. Values are means $\pm$ SE.

Figure 2. Effect of different concentrations of glycinin basic peptide (GBP) on pH of pasteurized milk after storage at 4°C. Values are means $\pm$ SE.

mately 6.7, and milk is regarded as corrupted when the pH is lower than 6.4 (Mahgoub et al., 2011).

Changes in the pH of pasteurized milk treated with different concentrations of GBP over 21 d of storage at 4°C are shown in Figure 2. The pH values of all pasteurized milk samples appeared to follow a downward trend with increasing storage time. The pH of the control decreased from 6.68 to 5.80 over 21 d of storage at 4°C. The phenomena of whey exudation and casein precipitation were observed when the pH of milk reached 5.80. However, the pH of GBP-treated pasteurized milk decreased from 6.68 to 6.41, which showed a slower decline than that of the control during the same storage period. By d 9, the pH of the control had reached 6.38, indicating deterioration of the pasteurized milk, whereas the pH of the pasteurized milk treated with the lowest concentration of GBP (0.005%) was still 6.51, which was still considered to be fresh milk. On d 15, the pH of pasteurized milk treated with GBP concentrations of 0, 0.005, 0.010, 0.015, and 0.020% was 6.12, 6.37, 6.40, 6.50, and 6.56, respectively. It was clear that the 0, 0.005, and 0.010% GBP-treated pasteurized milk had deteriorated, whereas 0.015 and 0.020% GBP-treated pasteurized milk were still fresh. An ANOVA (Figure 2) showed that 0.015 and 0.020% GBP-treated

milk maintained pH significantly better up to d 15 ($P < 0.05$), and no noticeable difference was found in the preservative effects of 0.015 and 0.020% GBP-treated milk ($P > 0.05$). These results demonstrated that 0.015 and 0.020% GBP could inhibit the decrease in pH and prolong the storage time of pasteurized milk to 15 d, which was consistent with the sensory evaluation.

The tendency of the pH of pasteurized milk to decrease in this research was in agreement with the finding of Cotter and Hill (2003), who reported that the decrease in pH was due to the growth of bacteria in the milk. Mahgoub et al. (2011) reported that esterified legume proteins decreased the declining pH of milk and thereby extended its shelf life.

Effect of GBP on Alcohol Test of Pasteurized Milk

An alcohol test can indicate high or low acidity of milk to aid in determining its freshness. High acidity of pasteurized milk indicates excessive lactic acid in milk. Adding 70% alcohol to high-acid milk results in milk protein precipitation. This phenomenon is called an alcohol positive test, which is an important sign of milk deterioration (Demirbas et al., 2008).

Table 2. Effect of different concentrations of glycinin basic peptide (GBP) on the alcohol test of milk after storage at 4°C

Treatment	Storage time (d)							
	0	3	6	9	12	15	18	21
Control	–	–	–	+	+	+	+	+
0.005% GBP	–	–	–	–	–	+	+	+
0.010% GBP	–	–	–	–	–	+	+	+
0.015% GBP	–	–	–	–	–	–	+	+
0.020% GBP	–	–	–	–	–	–	–	+

The effect of different GBP concentrations on the alcohol test of pasteurized milk within 21 d of storage is shown in Table 2. On d 9, the untreated pasteurized milk presented an alcohol positive. On d 15, the pasteurized milk samples treated with 0.005 and 0.010% GBP were also an alcohol positive; however, those treated with 0.015 and 0.020% GBP were still an alcohol negative. Alcohol-positive phenomena were not observed in pasteurized milk treated with 0.015 and 0.020% GBP until d 18 and 21, respectively. These results showed that GBP concentrations of 0.015 and 0.020% could preserve pasteurized milk during storage over 15 d, which was consistent with evaluations of sensory properties and pH.

Effect of GBP on the Lactose Content of Pasteurized Milk

The lactose content in milk is also an important indicator of milk quality: the lower the lactose content, the worse the quality of milk (Osman et al., 2013). The lactose content of pasteurized milk treated with different concentrations of GBP over 21 d of storage at 4°C is shown in Table 3. The lactose content of GBP-treated pasteurized milk declined at a much slower rate (from 54.18 to 40.98 mg/mL) compared with the control (from 54.18 to 17.78 mg/mL) over time in storage. Furthermore, the lactose content of pasteurized milk samples treated with high GBP decreased more slowly than those treated with low GBP concentrations during the same storage period. On d 15, all pasteurized milk samples treated with 0, 0.005, 0.010, 0.015, and 0.020% GBP had 25.20, 39.19, 40.56, 44.33, and 45.85 mg/mL of lactose, respectively. The lactose content of GBP-treated pasteurized milk was much higher than that of the control samples (25.20 mg/mL). Moreover, 0.015 and 0.020% GBP concentrations in pasteurized milk maintained significantly higher lactose content ($P < 0.05$). These results demonstrated that GBP had a potent preservative effect on pasteurized milk, and 0.015% GBP was an optimal concentration to prolong the shelf of pasteurized milk to 15 d.

Table 3. Effect of different concentrations of glycinin basic peptide (GBP) on the lactose content (mg/mL) of pasteurized milk after storage at 4°C (mean ± SE)

Treatment	Storage time (d)								
	0	3	6	9	12	15	18	21	
Control	54.18 ± 0.036 ^a	47.67 ± 0.010 ^b	41.49 ± 0.045 ^c	34.88 ± 0.015 ^d	29.45 ± 0.010 ^e	25.20 ± 0.035 ^f	21.55 ± 0.066 ^g	17.78 ± 0.060 ^h	
0.005% GBP	54.18 ± 0.032 ^a	50.69 ± 0.043 ^b	48.23 ± 0.031 ^b	46.75 ± 0.057 ^c	43.38 ± 0.010 ^d	39.19 ± 0.067 ^e	37.32 ± 0.061 ^f	34.00 ± 0.026 ^g	
0.010% GBP	54.18 ± 0.041 ^a	51.41 ± 0.026 ^b	49.63 ± 0.055 ^b	47.09 ± 0.010 ^c	44.55 ± 0.060 ^d	40.56 ± 0.060 ^e	39.03 ± 0.015 ^f	36.97 ± 0.033 ^g	
0.015% GBP	54.18 ± 0.010 ^a	53.79 ± 0.045 ^a	51.92 ± 0.056 ^{ab}	50.41 ± 0.058 ^b	47.96 ± 0.080 ^c	44.33 ± 0.044 ^c	41.11 ± 0.021 ^d	38.86 ± 0.095 ^e	
0.020% GBP	54.18 ± 0.043 ^a	54.12 ± 0.049 ^a	52.37 ± 0.080 ^a	50.45 ± 0.036 ^{ab}	48.37 ± 0.059 ^b	45.85 ± 0.030 ^c	43.44 ± 0.093 ^d	40.98 ± 0.027 ^d	

^{a-h}Means within a row with different superscripts differ ($P < 0.05$).

Figure 3. Electrophoretic patterns of pasteurized milk treated with different concentrations of glycinin basic peptide (GBP) and stored at 4°C. Samples stored for 0, 6, 12, and 18 d are labeled at the top. For each sample, the 6 lanes are as follows: 1 = marker, 2 = 0.020% GBP, 3 = 0.015% GBP, 4 = 0.010% GBP, 5 = 0.005% GBP, and 6 = 0% GBP. Molecular weights (kDa) of markers are indicated on the left, and corresponding proteins are indicated on the right. Color version available online.

Osman et al. (2013) reported that the soybean 11S protein subunit had a positive effect on maintaining the lactose content of milk. Glycinin basic subunit (i.e., GBP), which is the basic chain of the 11S subunit, played an important role in milk preservation (Sitohy et al., 2012).

Effect of GBP on Protein Content in Pasteurized Milk

The protein content in milk, especially casein, is a critical indicator in the evaluation of the quality of milk. In addition, whey in milk is a complete protein containing 8 essential AA. During storage, bacterial growth could make milk proteins decompose, therefore decreasing the quality of the milk (Saha et al., 2003).

Figure 3 shows gel electrophoresis of milk samples. Lane 1 was loaded with the marker, and lanes 2 through 6 were loaded with proteins from pasteurized milk treated with 0.020, 0.015, 0.010, 0.005, and 0% GBP. The molecular weights of IgG, α_{s1} -casein, and α_{s2} -casein were approximately 60, 32, and 27 kDa, respectively. We observed a decrease of protein content in pasteurized milk with increasing storage time. However, protein content in GBP-treated pasteurized milk, especially casein, decreased more slowly than that of the control after storage. On d 6, small protein fragments were found lower than the whey and casein bands in all pasteurized milk. On d 12, casein content in pasteurized milk treated with 0.020, 0.015, 0.010, 0.005, and 0% GBP decreased with decreasing GBP concentration. The same phenomenon was found with whey protein content. After 18 d of storage, the casein

and whey contents in pasteurized milk with 0, 0.005, and 0.010% GBP had decreased remarkably, and low-molecular-weight proteins also disappeared gradually. On the other hand, in 0.015 and 0.020% GBP-treated pasteurized milk, the content of casein, whey, and low-molecular-weight proteins hardly changed. The decline or disappearance of proteins in milk might be due to bacterial decomposition of milk proteins. Osman et al. (2013) reported similar findings that glycinin treatment of milk could maintain casein content, thus preventing the deterioration of pasteurized milk.

Effect of GBP on Inactivation of *S. aureus* in Pasteurized Milk

The effects of different concentrations GBP on *S. aureus* in pasteurized milk over the storage period are presented in Figure 4. *Staphylococcus aureus* count hardly changed or decreased a little (approximately 4.7 log₁₀ cfu/mL) compared with initial inoculation of 5 log₁₀ cfu/mL in GBP-untreated pasteurized milk during 21 d storage time. However, in pasteurized milk treated with 0.005, 0.010, 0.015, and 0.020% GBP, the count of *S. aureus* was 3.45, 3.03, 2.58, and 2.33 log₁₀ cfu/mL on the third day of storage, respectively. Moreover, these low levels of *S. aureus* population were kept during the following storage time in GBP-treated pasteurized milk. An ANOVA (Figure 4) showed these data (3.45, 3.03, 2.58, and 2.33 log₁₀ cfu/mL) were significantly different from 5 log₁₀ cfu/mL of initial inoculation ($P < 0.05$). These results showed that *S. aureus* did not grow and replicate at 4°C. But GBP could inactivate or kill *S. aureus* in pasteurized milk.

Figure 4. Effect of different concentrations of glycinin basic peptide (GBP) on *Staphylococcus aureus* in pasteurized milk after storage at 4°C. Values are means $\pm$ SE.

Sitohy et al. (2012) reported that a GBP concentration of 0.5% reduced the population of *Bacillus subtilis*, *S. enteritidis*, and *Listeria monocytogenes* by 1.78, 2.73, and 1.65 log₁₀ cfu/mL, respectively. Our study demonstrated that GBP at a concentration of 0.020% decreased the population of *S. aureus* by 2.75 log₁₀ cfu/mL. These results suggested that *S. aureus* might be more sensitive to GBP than *Bacillus subtilis*, *S. enteritidis*, and *Listeria monocytogenes*.

CONCLUSIONS

Treatment of pasteurized milk with GBP over 21 d of storage at 4°C resulted in significant effects on sensory scores, total bacterial count, alcohol test, pH, lactose content, protein content, and *S. aureus* inhibition, which correlated with increasing concentrations of GBP. These results revealed that 0.015% GBP could inhibit the growth and reproduction of microorganisms and improve sensory properties as well as physicochemical attributes of pasteurized milk, indicating that its deterioration was prevented. Therefore, GBP can be used as a natural preservative to extend the shelf life of milk.

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