Effects of ultra-high pressure treatment on angiotensin-converting enzyme (ACE) inhibitory activity, antioxidant activity, and physicochemical properties of milk fermented with *Lactobacillus delbrueckii* QS306

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ABSTRACT

The present study investigated the influence of ultra-high pressure (UHP) treatment on angiotensin-converting enzyme inhibitory (ACEI) activity and quality of milk fermented with *Lactobacillus delbrueckii* QS306 after storage. By varying treatment pressure, duration of pressure treatment, and duration of fermentation, optimal process parameters for the UHP treatment of milk fermented with QS306 to enhance ACEI activity were found to be 400 MPa, 10 min, and 48 h, respectively. The degree of ACE inhibition of the fermented milk was $91.63 \pm 0.02\%$. After UHP treatment, ACEI activity, apparent viscosity, concentrations of polypeptides and volatile aromatic substances, umami, and richness had increased significantly, bitterness and astringency were significantly reduced, and antioxidant properties were maintained. In addition, UHP fermented milk maintained a high level of ACEI activity and good quality during storage. Thus, the data represent a valuable reference for improving the storage quality of fermented milk and research for the future development of dairy products with high ACEI activity.

Key words: ultra-high pressure, angiotensin-converting enzyme inhibitory activity, *Lactobacillus delbrueckii* QS306, fermented milk

INTRODUCTION

Hypertension is a serious medical condition that increases the risk of heart, brain, kidney, and other diseases (Kearney et al., 2005). Synthetic drugs are often prescribed because of their excellent effectiveness and low cost, but they can cause adverse effects such as extremely low blood pressure, edema, dry coughs, and erectile dysfunction (Wilson et al., 2004; Kumari et al., 2011; Udenigwe and Mohan, 2014). Conversely, natural inhibitors regulate hypertension, providing mild

alleviation of the condition without significant side effects. Hence, over the past 2 decades, researchers have focused on the extraction of antihypertensive peptides from food proteins, principally because of their low cost and lack of toxicity.

Fermented dairy products provide comprehensive nutrition and have a unique flavor. They are more easily absorbed and utilized by humans than milk, a dairy product well known for its beneficial properties toward human health. Fermented milk products are rich in protein, vitamins, minerals, and other nutrients, including functional polypeptides that are released during fermentation or hydrolysis, including those with cholesterol-lowering, antioxidant, and anti-hypertensive properties (Sakandar and Zhang, 2021). Angiotensin-converting enzyme (ACE) is a Zn-metalloproteinase that removes a dipeptide from the decapeptide angiotensin-I, producing the potent vasoconstricting octapeptide angiotensin-II (Memarpoor-Yazdi et al., 2012). Several polypeptides originating from plant, milk, and animal proteins exhibit substantial therapeutic performance. Six highly active peptides (Leu-Tyr, Pro-Tyr, Tyr-Gln, Ala-Pro-Ser-Tyr, Arg-Gly-Gly-Tyr, and Leu-Val-Ser) from wheat gluten oligopeptides have been identified, 4 of which have dual activities, and most which were novel (Liu et al., 2021). The amino acid sequence VISDEGVTTH was isolated and identified from *Ruditapes philippinarum* fermented with *Bacillus subtilis* var. natto and found to significantly reduce hypertension in animals when administered as an oral treatment (Chen et al., 2018). An economical and effective method for the production of bioactive peptides is fermentation with *Lactobacillus* (Raveschot et al., 2020). A small number of commercialized ACE inhibitory peptide antihypertensive dairy products are currently on the market, such as Ameal and Calpis in Japan and Evolus in France, which use 2 ACE inhibitory (ACEI) peptides, VPP and IPP (Nakamura et al., 1995), commonly found in milk fermented by various *Lactobacillus helveticus* strains.

Ultra-high pressure (UHP) treatment is a nonthermal machining technology. The first studies on milk

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processing using high pressure date back as far as the end of the nineteenth century (Hite, 1899). Ultra-high pressure treatment can dissociate casein micelles at a pH of less than 6.7 and concentration of less than 4% (wt/wt), whereas β -LG is the most pressure-sensitive whey protein due to the presence of free thiol groups (Munir et al., 2019). The processing of milk using high pressure can modify its high-abundance proteins to improve their functional properties (Balci and Wilbey, 1999; López-Fandiño, 2006).

Lactobacillus delbrueckii QS306 rapidly produces acid, has a strong capability to decompose proteins, and produces an agreeable flavor, and the resultant fermented milk has good ACEI activity. We have previously isolated and purified a pentapeptide sequence, LPYPY, from fermented milk samples using *L. delbrueckii* QS306 (Wu et al., 2019). At present, the use of UHP in food is mostly focused on the processing of fruit, vegetables, and meat and meat substitutes, rather than on dairy products. Ultra-high pressure treatment has been shown to maintain or improve the quality of food and can modify macromolecules such as proteins. For the first time in the present study, UHP was used to enhance the ACEI activity of milk fermented with *L. delbrueckii* QS306 and its quality following storage.

MATERIALS AND METHODS

Materials

Lactobacillus delbrueckii QS306 was initially isolated from Xilin Gol League traditionally fermented milk (yogurt fermented by herdsmen of Inner Mongolia). It was provided for the present study by the National Food Research and Development Team of the College of Food Science and Engineering, Inner Mongolia Agricultural University (Hohhot, PRC). Angiotensin-converting enzyme (EC 3.4.15.1, from rabbit lung) and hippuryl-L-histidyl-L-leucine were obtained from Sigma-Aldrich. All other chemical reagents were obtained commercially and were of analytical or chromatographic grade.

Cultivation of Strains and Preparation of Fermented Milk

Lactobacillus delbrueckii QS306 was cultured for 3 generations in de Man, Rogosa, and Sharpe broth (Guangdong Huankai Microbial Sci. & Tech. Co. Ltd.) at 37°C for 24 h. The cells were collected by centrifugation ($3,000 \times g$ for 15 min at 4°C) and resuspended in sterilized saline (approximately 10^8 cfu/mL). The fermentation medium was prepared with a slight modification of the method of Li et al. (2020). Skim milk

powder containing 33% protein (11%; wt/vol) was purchased from Fonterra Co. Ltd. Bacterial cultures were added to fermentation medium (11%; wt/vol) at a concentration of 3% (vol/vol) and then incubated at 37°C.

High-Pressure Processing

Influence of Single UHP Treatment on the ACEI Activity of Fermented Milk. Fermented milk (FM) was placed in 100-mL polyethylene terephthalate (PET) bottles, sealed on a super-clean table (SW-GJ-2D, Suzhou Zhijing Purification Equipment Co. Ltd.), and then treated at room temperature at ultra-high pressure. Five levels of pressure (0, 200, 300, 400, 500, or 600 MPa) were applied to the samples (HPP600 Mpa/5 L, Baotou Kefa High Pressure Tech. Co. Ltd.), with a treatment time of 5 min. Three parallel pressure gradients were selected for each pressure. Fermented milk before and after treatment was stored at 4°C and the activity of ACEI peptide measured over time.

Influence of the Duration of a Single UHP Treatment on Fermented Milk ACEI Activity. Fermented milk was placed in 100-mL PET bottles and sealed on a super-clean table. The single factor optimal pressure was used for UHP treatment for 0, 5, 10, 15, 20, or 25 min, using 3 parallel time gradients for the single-factor time test. Fermented milk before and after treatment was stored at 4°C, and ACEI peptide activity was measured as time progressed.

Influence of Duration of Fermentation on a Single UHP Treatment on Fermented Milk ACEI Activity. Fermented milk was placed in 100-mL PET bottles and sealed on a super-clean table. A 3% (vol/vol) bacterial culture was added to sterile reconstituted skim milk (11%; wt/vol) and incubated at 37°C for 24, 48, 72, and 96 h. Samples fermented for varying durations were treated at single-factor optimal pressure and for the optimal duration at a single ultra-high pressure. All experiments were performed in triplicate. Fermented milk before and after treatment was stored at 4°C, and ACEI peptide activity was measured over time.

Orthogonal Experiment

On the basis of single-factor tests, optimal pressure, treatment time, and sample fermentation duration of ACEI activity of UHP fermented milk (UHPFM) were selected for an orthogonal test. An $L_9 (3^4)$ orthogonal table was selected for experimental design, with optimal process parameters for UHP fermentation of milk determined through use of ACEI activity as the evaluation index. The factor levels are displayed

in Supplemental Table S1 (<https://data.mendeley.com/datasets/cv24vxzck9/1>).

ACE Inhibitory Activity and Peptide Concentration

The ACE inhibitory activity and peptide concentrations were calculated using the method described by Wu et al. (2019). The quantity of ACE inhibition at a given peptide concentration required to inhibit 50% of the original ACE activity was used to represent inhibitory activity.

Antioxidative Properties

Scavenging Rate of Hydroxyl Radicals ($\cdot\text{OH}$).

A 1-mL aliquot of milk sample was placed into a test tube, to which 1-mL aliquots of 9 mmol/L ferrous sulfate, 9 mmol/L salicylic acid in ethanol, and 8.8 mmol/L hydrogen peroxide were added, respectively. The tubes were mixed and centrifuged (10 min, $3,300 \times g$, 4°C), and protected from light at room temperature for 15 min. For the supernatant, absorption of light at 510 nm (A_1) was measured against a distilled water blank (A_0) using a microplate reader (Synergy H1, BioTek):

$$\cdot\text{OH scavenging rate (\%)} = [(A_0 - A_1)/A_0] \times 100\%.$$

Scavenging Rate of DPPH (1,1-diphenyl-2-picrylhydrazyl) Free Radical. The free radical scavenging activity of each sample was evaluated with 1,1-diphenyl-2-picrylhydrazyl (DPPH) using a method described by Sahgal et al. (2009). A 1-mL sample of treated milk was placed in a test tube, to which 1 mL of 0.2 mmol/L DPPH $\cdot$ ethanol solution was added. It was placed in the dark at room temperature for 15 min, after which the absorbance of light at 517 nm was measured using a microplate reader (Synergy H1, BioTek). The activity was compared against ethanol, in which 1 mL of 60% ethanol was mixed with 1 mL of 0.2 mmol/L DPPH $\cdot$ ethanol (A_C). Both were referenced against 60% ethanol as a blank:

$$\text{DPPH free radical scavenging rate (\%)} = [1 - (A_i - A_j)/A_C] \times 100\%,$$

where A_C = absorption without milk sample, A_i = absorption value of milk sample, and A_j = background absorption without DPPH free radical.

Oxygen Free Radical ($\text{O}_2^{\cdot-}$) Scavenging Rate. The oxygen free radicals in each sample were determined essentially according to the method of Ji et al. (2015). Diethylenetriamine pentaacetic acid (3

mmol/L, 1 mL), Tris (hydroxymethyl) aminomethane (Tris-HCl, pH 8.2, 150 mmol/L, 2 mL), and pyrogallol (1.2 mmol/L, 1 mL) were added to a test tube containing 0.5 mL of either milk sample (A_1) or distilled water (A_0). After incubation in a constant-temperature water bath at 25°C for 10 min, the absorbance of the mixture was measured at 325 nm using a microplate reader (Synergy H1, BioTek):

$$\text{Oxygen free radical clearance rate (\%)} = [(A_0 - A_1)/A_0] \times 100\%,$$

where A_0 = absorption of the blank group, and A_1 = absorption of the sample.

Reducing Power. The reducing activity of the samples was determined essentially according to the method of Oyaizu (1986), using potassium ferricyanide. Phosphate-buffered saline (0.2 mol/L, pH 6.6, 2 mL) and a potassium ferricyanide solution (1%, 2 mL) were pipetted into a test tube and a 1-mL milk sample added, which was then incubated at 50°C in a water bath for 20 min. Trichloroacetic acid (10%, 2 mL) was then added and the tube centrifuged at $3,300 \times g$ for 15 min at 4°C for 20 min. A 2.0-mL aliquot of the supernatant was removed, to which 2.0 mL of deionized water and 0.4 mL of 0.1% ferric chloride were added and incubated in a water bath at 50°C for 10 min. The absorbance of the mixture at 700 nm was measured using a microplate reader.

Amino Acid Analysis

The amino acid composition of FM was determined in accordance with the method of Wen et al. (2021), with modifications. One milliliter of each sample was mixed with 1 mL of 5% sulfosalicylic acid, placed at room temperature for 15 min, and then centrifuged at $5,500 \times g$ for 15 min at 4°C. The supernatant was discarded, and the precipitate was resuspended in 20 mL of 0.02 mol/L HCl, after which it was filtered through a 0.22- μm membrane (Millex Syringe Filter, PES, Sterile, 0.22- μm pore size, 13-mm diameter, Millex-GP PES Millipore Express membrane, hydrophilic). The amino acid composition was then determined using an automated amino acid analyzer (S433D, Sykam Co. Ltd.).

Acidity and Viable Counts

Changes in acidity and viable cell counts during storage were monitored in FM. Titratable acidity (Thorner degrees, °T), pH value, and viable count for each sample

were determined in accordance with the method of Zha et al. (2021). Lethality rate was calculated as follows:

$$L = [(L_{FM} - L_{UHPFM})/L_{FM}] \times 100\%,$$

where L represents the lethality rate (%), L_{FM} the viable cell count in FM (cfu/mL), and L_{UHPFM} the viable count in UHPFM (cfu/mL).

Apparent Viscosity

Viscosity was measured using a rotational rheometer (RS6000, Haake GmbH). The apparent viscosity of 7 mL of mixed FM was measured at room temperature, with a scan of shear measured from 1 to 100 s.

Sensory Evaluation

Color Measurement. The change in color of the samples was measured with a digital colorimeter (CR-20, Konica Minolta Optics Inc.) using the CIE L^* (luminance), a^* (green-red), and b^* (blue-yellow) scales (Cheng et al., 2021). Difference in color (ΔE) was calculated as $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$.

Taste Analysis Using Electronic Tongue. Taste was assessed using an electronic tongue (SA402B, INSENT) consisting of 5 taste sensors (for sourness, bitterness, astringency, saltiness, and umami, respectively). The reference solution consisted of an odorless sample of 0.3 mM tartaric acid and 30 mM KCl. Fermented milk and UHPFM were centrifuged at $3,000 \times g$ for 10 min at 4°C, after the supernatant was assayed, in accordance with the method of Yu et al. (2021), with modifications. Measurements were repeated 5 times for each sample.

Analysis Using Electronic Nose. An electronic nose (PEN3, Aisense GmbH) equipped with a sensor array of 10 semiconductor metal oxide chemical sensors was used. Pooled samples (5 ± 0.01 g) were placed in a closed vial to allow the internal headspace to equilibrate for 0.5 h, after which the vapor was analyzed in accordance with the method of Cai et al. (2021), with modifications.

Statistics and Data Analysis

Data are expressed as means $\pm$ standard deviation and analyzed by one-way ANOVA, followed by Duncan's test. All statistical comparisons were conducted using SPSS version 19.0 software (IBM Corp.), with a threshold of significance of $P < 0.05$. Statistical analyses were performed using GraphPad Prism 6.01 (GraphPad Software Inc.) and Excel software (Microsoft Corp.).

RESULTS AND DISCUSSION

Effect of UHP on ACE Inhibitory Activity in Fermented Milk

As shown in Figure 1B, ACEI activity in FM increased significantly ($P < 0.05$) after treatment with UHP at different pressures. At a treatment pressure of 400 MPa, ACEI activity of UHPFM was $90.23 \pm 0.11\%$. Proteolysis at pressures of 200 to 400 MPa accelerated the release of the 3 peptides that have demonstrated antihypertensive effects in vivo (Quiros et al., 2007). The effect of the duration of UHP processing is displayed in Figure 1A. When exposed to UHP for 10 min, the ACEI activity of UHPFM was $86.80 \pm 0.14\%$, significantly greater than other durations. The change in ACEI activity with varying duration of fermentation of milk treated with UHP is presented in Figure 1C, first increasing then slowly decreasing, for fermentation times of 24, 48, 72, and 96 h, for which ACE inhibition rates were $81.30 \pm 0.31\%$, $88.66 \pm 0.26\%$, $86.20 \pm 0.27\%$, and $86.86 \pm 0.14\%$, respectively.

Based on the single-factor testing for pressure, duration of pressurization, and duration of fermentation for UHP treatment, an optimal orthogonal level was determined from ACEI activity, namely treatment pressure of 400 MPa, pressurization for 10 min, and fermentation for 60 h. The test results and variance analysis are displayed in Supplemental Table S2 (<https://data.mendeley.com/datasets/cv24vxzck9/1>).

The conditions for UHP treatment of FM were optimized by ACE inhibition rate. Based on the highest K-value score and the orthogonal combination detailed in Supplemental Table S2, the optimal combination of each factor in the orthogonal test was determined. Through this verification test, it was concluded that ACEI activity was the highest at a treatment pressure of 400 MPa, pressurization for 10 min, and fermentation for 48 h. The ranking of the effect of each factor was treatment time range > treatment pressure range > fermentation time range.

The effects of high hydrostatic pressure on the structure and profiles of peptides from hydrolyzed flaxseed protein have been investigated previously. Perreault et al. (2017) demonstrated that high hydrostatic pressure can induce dissociation of flaxseed proteins and produce higher molecular weight aggregates as a function of processing time.

Free Amino Acid Content of FM and UHPFM

The concentrations of free amino acids (FAA) in FM and UHPFM (400 MPa, 10 min, 48 h) are displayed in Table 1. After treatment with UHP, the concentra-

Figure 1. Effect of ultra-high pressure (UHP) treatment on angiotensin-converting enzyme inhibitory (ACEI) activity of fermented milk. (A) Duration of UHP, (B) processing pressure, (C) duration of fermentation of fermented milk treated with UHP. Error bars indicate SD of 3 independent experiments. a–d: Within a row, means with different letters are significantly different ($P < 0.05$).

tion of FAA was significantly greater in UHPFM than in FM ($P < 0.05$). The total quantity of FAA in the UHPFM group was 8.59 ± 0.54 mg/g, 7.10 ± 0.35 mg/g greater than that in the FM group (1.49 ± 0.19 mg/g). The FAA content of hydrophobic amino acids was significantly higher ($P < 0.05$) in the UHPFM group (3.34 ± 0.19 mg/g) compared with FM (0.54 ± 0.09 mg/g). Furthermore, the contents of essential (threonine, valine, leucine, isoleucine, methionine, phenylalanine, and lysine) and aromatic (phenylalanine and tyrosine) amino acids in UHPFM were 0.76 ± 0.05 mg/g and 1.18 ± 0.14 mg/g, respectively. The ACEI activity of FM increased after UHP treatment, with valine, alanine, tyrosine, phenylalanine, leucine, and proline concentrations of 0.39 ± 0.04 , 0.22 ± 0.02 , 0.48 ± 0.10 , 0.70 ± 0.04 , 0.96 ± 0.04 , and 0.71 ± 0.03 mg/g, respectively.

The abundance of amino acids can significantly influence the regulation of human physiology. For example, lysine, the first limiting amino acid, can affect the absorption and utilization of other amino acids in addition to their metabolism and immune function (Gu et al., 2011). Leucine, isoleucine, and proline, branched-chain amino acids, are important sources of metabolic energy (Nichols et al., 1998). The functional activity of peptides can be significantly affected by the type and

number of amino acids within their composition. Previous studies have suggested that ACEI peptides generally have a high proportion of hydrophobic amino acids, aromatic amino acids, and branched-chain amino acids, in addition to high concentrations of valine, alanine, tyrosine, phenylalanine, leucine, and proline (Kong and Xiong, 2006).

Antioxidant Capacity Assays

Four parameters of antioxidant activity were measured, namely DPPH, $-\text{OH}$, O_2^- , and reducing power assays (Figure 2). Four assays were used because previous studies have found limitations when a single assay is used (Makori et al., 2021). As shown in Figure 2, vitamin C, FM, and UHPFM exhibited antioxidant activity in each of the DPPH, $-\text{OH}$, O_2^- , and reducing power assays, confirming that FM and UHPFM exhibited antioxidant capability, principally due to the presence of probiotics and peptides.

The DPPH radical scavenging activity in vitamin C, FM, and UHPFM was 89.26%, 90.93%, and 80.21%, respectively. Both FM and UHPFM contained a large quantity of FAA and short peptides. As hydrogen donors, peptides can react with free radicals to form stable products, thus preventing a DPPH free radical

Table 1. Amino acid composition and concentrations in fermented milk¹

Amino acid ²	FM group		UHPFM group	
	Content (mg/g)	Proportion (% wt/wt)	Content (mg/g)	Proportion (% wt/wt)
Asp	0.13 ± 0.01	8.73 ± 0.71	0.81 ± 0.03	9.55 ± 1.22
Thr	—	—	0.39 ± 0.05	4.52 ± 0.60
Ser	—	—	0.48 ± 0.09	5.61 ± 1.10
Glu	0.35 ± 0.03	23.48 ± 1.46	0.57 ± 0.02	6.65 ± 0.78
Gly	—	—	0.39 ± 0.03	4.57 ± 0.52
Ala	—	—	0.22 ± 0.02	2.59 ± 0.43
Val	—	—	0.39 ± 0.04	4.63 ± 0.79
Met	—	—	—	—
Ile	—	—	0.36 ± 0.02	4.18 ± 0.41
Leu	0.15 ± 0.03	10.07 ± 2.03	0.96 ± 0.04	11.22 ± 1.14
Tyr	0.13 ± 0.03	8.94 ± 1.63	0.48 ± 0.10	5.57 ± 1.13
Phe	0.23 ± 0.04	15.67 ± 2.38	0.70 ± 0.04	8.27 ± 1.15
His	—	—	0.18 ± 0.02	2.07 ± 0.22
Lys	0.15 ± 0.04	10.29 ± 2.29	0.67 ± 0.02	7.83 ± 0.91
Arg	0.18 ± 0.02	11.86 ± 1.11	0.76 ± 0.05	8.87 ± 0.93
Pro	0.16 ± 0.02	10.96 ± 0.96	0.71 ± 0.03	8.28 ± 0.84
EAA	0.53 ± 0.11	36.03 ± 6.70	3.47 ± 0.21	40.65 ± 5.00
HAA	0.54 ± 0.09	36.7 ± 5.37	3.34 ± 0.19	39.17 ± 4.76
AAA	0.36 ± 0.06	24.61 ± 4.01	1.18 ± 0.14	13.84 ± 2.28
NCAA	0.48 ± 0.04	32.21 ± 2.17	1.38 ± 0.05	16.20 ± 2.00
PCAA	0.33 ± 0.06	22.15 ± 3.40	1.61 ± 0.09	18.77 ± 2.06
Total	1.49 ± 0.19	100.00	8.59 ± 0.54	100.00

¹FM = fermented milk; UHPFM = ultra-high pressure treated FM. Results are expressed as means ± SD (n = 3).

²HAA = hydrophobic amino acids; AAA = aromatic amino acids; NCAA = nonpolar amino acids; PCAA = polar amino acids.

chain reaction. The OH radical scavenging activity in vitamin C, FM, and UHPFM was 96.27%, 95.74%, and 95.89%, respectively. After peptides donate electrons to hydroxyl radicals, they are scavenged by reduction to $-OH$. We found no significant change in scavenging capacity after UHP treatment ($P < 0.05$). The UHPFM also retained the scavenging capability of FM to superoxide anions. After UHP treatment, the conformation of proteins changes, and so the degree of hydrolysis by the majority of enzymes increases. The products released by interaction with enzymes can affect the scavenging capability of free radicals, and milk protein is also influenced by pH and pressure. Sarmadi and Ismail (2010) believe that the antioxidant properties of peptides in food are closely associated with their composition, structure, and hydrophobicity.

ACE Inhibitory Activity of FM and UHPFM

The ACE inhibition by FM after UHP treatment increased significantly ($P < 0.05$), reaching $91.63 \pm 0.02\%$ (Figure 3A). During storage, the ACE inhibition rate decreased in both FM and UHPFM groups, although the UHPFM group retained high ACEI activity ($74.69 \pm 0.03\%$). High-pressure treatment induced structural changes in the proteins. Compared with FM, the peptide concentration of the UHP treatment group

was significantly higher ($P < 0.05$), and the proteolytic capacity of UHPFM remained higher than the FM group after storage. Treatment with UHP promotes the hydrolysis of proteins into FAA and oligopeptides.

Apparent Viscosity of FM and UHPFM

Apparent viscosity is a rheological index often used to evaluate and compare the fluidity of materials. Under the same conditions, differences in the internal structural characteristics of a sample can be measured. Figure 3B displays the apparent viscosity of FM and UHPFM after storage (0, 7, 15, and 30 d). The apparent viscosity of UHPFM was significantly higher than FM, indicating that, after UHP treatment, the globular protein aggregation of FM changed, causing the degree of gelation to be higher, with a greater level of crosslinking, increasing the viscosity of FM. Serra et al. (2008) conducted UHP and heat treatment in skim milk before fermentation and concluded that UHP caused the gelation hardness to be greater than that caused by heat treatment. The formation of gels under acidic conditions improved the texture and hardness of yogurt. As storage time increased, the apparent viscosity of fermented milk decreased steadily at different storage times, with characteristics of consistent decline. The apparent viscosity of UHPFM was essentially sta-

Figure 2. Antioxidant activity of fermented milk (FM) and ultra-high pressure treated fermented milk (UHPFM). The concentration of the vitamin C (Vc) positive control was 0.05 mg/mL for each method [1,1-diphenyl-2-picrylhydrazyl (DPPH), $-OH$, O_2^- , and reducing power assays]. Error bars indicate SD of the mean ($n = 3$).

ble at 4°C for 30 d, indicating that the treatment would extend the shelf life of FM. It has been reported that the mechanical properties of the acid-set gels prepared from high-pressure treated milk are greatly improved. Such changes have been shown to be dominated by increased gel rigidity, strength, and resistance to syneresis, associated with protein hydration and increased network chain density (López-Fandiño, 2006).

Acidity and Viable Counts of FM and UHPFM

Changes in acidity and viable bacterial counts in FM and UHPFM following storage are presented in Table 2. The pH value of FM decreased during storage, declining to approximately 3.44 ± 0.03 after 30 d. The pH of FM increased significantly after UHP treatment to 3.86 ± 0.04 , and increased to approximately 4.03 ± 0.07 after storage for 30 d. The decrease in pH in FM was caused mostly by the lactic acid bacteria fermenting lactose to produce acid (Sherbon, 1988). Buffers in milk comprise citrate, protein, phosphate, and carbonate, which also influence pH during fermentation. The pH value of UHPFM after storage was significantly higher than that of FM, the pH increasing due to increased numbers of basic amino acids (Lys, Arg) after UHP treatment, suggesting that UHP reduces the change in pH during storage, and enabling superior preservation of FM.

Titrateable acidity is the principal index of astringency in fermented milk. It represents the total number of acidic groups in the sample, including peptides and FAA residues. Maintaining titrateable acidity of 70 to 110°T has been demonstrated to improve the taste of FM (Donkor et al., 2006). For pressures exceeding 300 MPa, the viable counts were found to decrease, inhibiting the corresponding post-acidification phenomenon. The data indicate that UHP treatment resulted in low levels of post-acidification during storage (Table 2).

The viable counts in FM and UHPFM increased during early storage ($8.11 \log_{10}$ cfu/mL and $5.95 \log_{10}$ cfu/mL, respectively, after 7 d of storage). Thereafter, viable counts declined significantly. The viable count in a probiotic is an important parameter associated with healthcare-related functionality. Lourens-Hattingh and Viljoen (2001) have recommended the consumption of more than 10^6 cfu/mL. The present study demonstrated that viable counts in FM remained stable, consistently $>7.00 \log_{10}$ cfu/mL, close to the end of the storage period, whereas the count in UHPFM was $5.00 \log_{10}$ cfu/mL after 30-d storage. Tanaka and Hatanaka (1992) treated packaged fermented milk at 200 to 300 MPa for 10 min, which did not affect the texture of the FM or the number of active lactic acid bacteria. Viable counts decreased when exposed to a pressure of 400 MPa, although the lethality of UHPFM remained at a high level, consistently 94% toward the end of storage.

Figure 3. Angiotensin-converting enzyme inhibitory (ACEI) activity, apparent viscosity, flavor, and odor of fermented milk (FM) and ultra-high pressure (UHP) treated fermented milk (UHPFM) during storage. (A) Peptide concentration (PC) and ACEI activity. (B) Steady shear and dynamic viscoelastic properties of FM and UHPFM. (C) Flavor radar map of FM and UHPFM across storage time. Designations 0, 7, 15, and 30 indicate different storage times (d) before UHPFM and FM. Aftertaste-A = aftertaste-astringency; aftertaste-B = aftertaste-bitterness. (D) Odor radar range map of FM and UHPFM across storage time, 0–30 d. W1C = aromatic compounds; W5S = reacts to nitrogen oxide; W3C = ammonia used as an aromatic compound sensor; W6S = hydrogen; W5C = alkanes, aromatic compounds, less polar compounds; W1S = methane; W1W = reacts to sulfur compounds, otherwise sensitive to many terpenes and sulfur organic compounds, which are important for smell, limonene, pyrazine; W2S = alcohol, partially aromatic compounds; W2W = aromatic compounds, sulfur organic compounds; W3S = reacts to high concentrations, selective methane. Error bars indicate SD of the mean ($n = 3$).

Sensory Properties of FM and UHPFM During Storage

The changes in color of FM and UHPFM during storage are displayed in Table 3. The a^* , L^* , and b^* values of UHPFM did not change significantly. Following storage, the L^* value of UHPFM displayed an upward trend, whereas the values of a^* and b^* exhibited a downward trend, indicating that the brightness of UHPFM improved, with no apparent red or yellow phenomena. Color and luster are not apparently different, where overall difference in color (ΔE) < 2, as was the

case with FM and UHPFM here. Treatment with UHP effectively protected the color quality of FM. Studies have shown that UHP sterilization can break protein colloids, reduce their diameter, improve the transparency of fermented milk, and reduce its turbidity and whiteness (Serra et al., 2008).

As shown in Figure 3D, during storage, the aromatic compounds (W1C) of FM decreased, W5S (reacts to nitrogen oxide), W1W (reacts to sulfur compounds), and W2W (aromatic compounds, sulfur organic compounds) sensors displayed a clearly increasing trend. The abundance of benzene aromatic substances decreased,

Table 2. Change in acidity and viable counts of milk fermented by *Lactobacillus delbrueckii* QS306 during storage, and before and after ultra-high pressure (UHP) treatment¹

Item	Storage period (d)	Fermented milk	UHP fermented milk
pH	0	3.80 ± 0.03	3.86 ± 0.04
	7	3.74 ± 0.03	3.88 ± 0.05
	15	3.65 ± 0.02	3.97 ± 0.03
	30	3.44 ± 0.03	4.03 ± 0.07
TA ² (°T)	0	100.62 ± 0.29	95.21 ± 0.35
	7	108.61 ± 0.66	96.89 ± 0.09
	15	116.37 ± 1.96	99.93 ± 1.59
	30	134.77 ± 0.42	110.66 ± 0.71
Viable cell counts (log cfu/mL)	0	7.04 ± 0.02	4.85 ± 0.01
	7	8.11 ± 0.02	5.95 ± 0.01
	15	7.18 ± 0.03	5.92 ± 0.02
	30	7.58 ± 0.03	5.00 ± 0.01
Lethality rate (%)	0	—	99.93 ± 0.50
	7	—	99.30 ± 0.50
	15	—	94.40 ± 0.67
	30	—	99.73 ± 0.33

¹Results are expressed as means ± SD (n = 3).²TA = titratable acidity.

whereas nitrogen oxides, sulfides, organic sulfides, and chlorides were in greater concentration. Following UHP treatment, the values from aromatic compound (W1C) sensors in FM increased significantly, but the concentrations of other substances were relatively stable. The results indicated that UHP treatment increased the concentration of aromatic substances in FM, whereas other substances did not change significantly during storage, indicating that UHP can improve the odor of FM, and was beneficial for its preservation.

As demonstrated in Figure 3C, during the storage of FM, its sourness, saltiness, and astringency increased significantly, and its sweetness and richness decreased significantly. After UHP treatment, the umami and richness of FM increased significantly, with good storage stability, whereas bitterness, saltiness, and astringency decreased significantly. Compounds that elicit an umami flavor include free L-amino acids, peptides, and their derivatives or reaction products.

Cheng (2010) and other researchers have demonstrated that the majority of the esters can reduce amines and fatty acid in fermented milk. The formation of ester compounds occurs through the combination of FAA and alcohols. After UHP treatment, the concentration of FAA in FM increased, thus promoting the formation of esters, causing astringency and bitterness to decrease. The UHP treatment modified the concentration of amino acids and peptides that create flavor in FM, changing its flavor. During storage, ACEI activity and the taste of the UHP treatment group were significantly enhanced compared with the FM group. The UHP treatment improved the taste of FM to some extent. The increased sourness indicated an increase in organic acid, resulting in an increase in titratable acidity of FM. Umami and saltiness increased during storage following UHP treatment, indicating that increased basic amino acids and inorganic salt levels resulted in increased pH.

Table 3. Change (Δ) in color of fermented milk (FM) and ultra-high pressure treated fermented milk (UHPFM) during storage¹

Item	Storage period (d)	L^*	a^*	b^*	ΔL^*	Δa^*	Δb^*	ΔE
FM	0	80.57 ± 1.14 ^a	-1.64 ± 0.21 ^c	6.87 ± 0.21 ^a	—	—	—	—
	7	79.70 ± 0.46 ^b	-1.14 ± 0.15 ^b	5.77 ± 0.50 ^b	—	—	—	—
	15	79.43 ± 1.80 ^{ab}	-2.30 ± 0.06 ^d	5.03 ± 0.42 ^{bc}	—	—	—	—
	30	78.57 ± 1.38 ^{ab}	-2.20 ± 0.10 ^d	4.47 ± 0.40 ^c	—	—	—	—
UHPFM	0	78.90 ± 0.50 ^{ab}	-1.47 ± 0.15 ^c	7.00 ± 0.26 ^a	-1.67	0.16	0.13	1.68
	7	78.20 ± 0.50 ^b	-0.87 ± 0.15 ^b	5.13 ± 0.51 ^{bc}	-1.5	0.26	-0.64	1.65
	15	79.77 ± 1.89 ^{ab}	-0.50 ± 0.30 ^a	4.43 ± 0.67 ^c	0.34	1.8	-0.6	1.93
	30	79.27 ± 0.51 ^{ab}	-2.20 ± 0.00 ^d	4.33 ± 0.35 ^c	0.7	0	-0.14	0.71

^{a-d}Within a row, means with different letters are significantly different ($P < 0.05$).¹Results are expressed as means ± SD (n = 3). CIE color scale: L^* = luminance; a^* = green-red; b^* = blue-yellow. ΔE = difference in color.

CONCLUSIONS

Treatment with UHP increased ACEI activity of *Lactobacillus delbrueckii* QS306 FM and slowed the post-acidification of FM during storage. Compared with FM, UHPFM displayed a higher FAA content and polypeptide concentration. The UHPFM maintained good antioxidant activity. Treatment improved the flavor and color of FM, beneficial to its perceived quality. Thus, UHP has considerable potential for improving ACEI activity and storage quality of milk fermented with *Lactobacillus delbrueckii* QS306.

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