In vitro antioxidant and angiotensin-converting enzyme inhibitory activity of fermented milk with different culture combinations

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ABSTRACT

This study investigated the effects of *Lactobacillus plantarum* (Lp) and *Bifidobacterium animalis* ssp. *lactis* (Ba) in co-cultures with *Streptococcus thermophilus* (St) on changes in the acidification profile, proteolytic activity, peptide production, in vitro antioxidant activity, and angiotensin-converting enzyme (ACE) inhibitory properties of fermented milks during 21 d of storage at 4°C. The pH values and proteolysis in all batches showed a gradual decrease and increase during storage, respectively. The ACE-inhibitory activity and total antioxidant capacity of all co-fermented milk samples followed a similar pattern, with maximum values on d 6 of storage. The St starter, in conjunction with Ba or Lp or both, enhanced proteolysis, peptide generation, and ACE-inhibitory and antioxidant activity, but decreased pH values compared with St alone. The St-Ba-Lp samples showed higher DPPH• (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity, hydroxyl radical scavenging activity, and total antioxidant capacity, but similar superoxide anion scavenging activity compared to St-Ba or St-Lp samples. The St-Ba samples showed higher DPPH• radical scavenging activity but lower hydroxyl radical scavenging activity than St-Lp samples. In the ACE-inhibitory assays, the St-Lp samples exhibited relatively low activity among the co-fermented milks, digested or not. The presence of Ba and Lp in fermentation together did not affect ACE-inhibitory activity in undigested fermented milks compared with the presence of Ba alone, and St-Ba-Lp fermented milks demonstrated an increase in ACE-inhibitory activity after simulated gastrointestinal digestion in storage. Pepsin digestion largely improved ACE-inhibitory activity, except in St-Lp samples, in which the activity was reduced. Further hydrolysis by trypsin reduced final activity in digestion. This study suggests that co-cultured fermentation with probiotics

improves in vitro antioxidant and ACE inhibition activity in fermented milks, and this effect is partly due to the higher proteolytic activity of probiotics.

Key words: co-fermentation, proteolysis, antioxidant ability, angiotensin-converting enzyme inhibitory activity

INTRODUCTION

Oxidation in aerobic organisms produces large amounts of free radicals and active oxygen species, which can damage all types of cellular macromolecules and lead to cell death and tissue damage (Unal and Akalin, 2006). Oxidation also increases the risk of atherosclerosis, arthritis, diabetes, and cancer (Sarmadi and Ismail, 2010) in humans. Angiotensin-converting enzymes (ACE) can stimulate the production of angiotensin-II (a vasoconstrictor), which induces the release of aldosterone and elevates blood pressure (Saito et al., 2000; Turner and Hooper, 2002). It has been reported that ACE plays a key role in the regulation of blood pressure (Hernández-Ledesma et al., 2003; Chen et al., 2014). Therefore, it is of scientific and practical interest for researchers to investigate how to inhibit ACE and improve the body's antioxidant capacity for health benefits.

Over the past 2 decades, extensive studies about the ACE-inhibitory activity of fermented milks produced by lactic acid bacteria (LAB) have been carried out (Quirós et al., 2005; Chen et al., 2014; Solieri et al., 2015). Various ACE-inhibitory peptides have been identified and characterized in dairy products (Pihlanto-Leppälä, 2001; Seppo et al., 2003). As well, casein-derived peptides and whey hydrolysates have a strong ability to chelate with transition metals and scavenge free radicals (Nishino et al., 2000; Fardet and Rock, 2018; Khan et al., 2019). Compositions such as polysaccharides from LAB possess antioxidant activity (Zhang et al., 2013), and organic acids released during fermentation may be a small source of antioxidants (Correia et al., 2005). Farvin et al. (2010) stated that the antioxidant stability of fermented dairy products could be ascribed mainly to antioxidant peptides, and

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that these peptides, as electron donors, could react with free radicals to form more stable products.

Lactobacillus plantarum and *Bifidobacterium* spp. are used most frequently as probiotics, and are good candidates for producing fermented milks (Gomes and Malcata, 1999; Li et al., 2017). Milk fermented by *L. plantarum* displayed 14.7 to 48.9% 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) inhibition rate (Abubakr et al., 2012) and approximately 89% ACE-inhibitory activity (Chen et al., 2018). Milk fermented with *B. longum* exhibited antioxidant activity (Gagnon et al., 2015) and stronger ACE-inhibitory activity than other LAB (Gonzalez-Gonzalez et al., 2011). The findings of these studies suggest that metabolites of these 2 strains have strong antioxidative and ACE-inhibitory functions.

We assumed that the combined use of starters could enhance antioxidant and ACE-inhibitory activity. Only a few studies have investigated the effects of antioxidant activity and ACE inhibition with starter combinations. Nakamura et al. (1995) demonstrated that the tripeptides Val-Pro-Pro and Ile-Pro-Pro in sour milk by co-cultures with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* had hypotensive effectiveness. Madhu et al. (2012) found that yogurt prepared by fermentation with *Lactobacillus plantarum*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Streptococcus thermophilus* had more than 80% DPPH[•] inhibitory activity. In a previous study, we found that fermented milk from *S. thermophilus* in co-cultures with a 2:1 ratio of *L. plantarum* to *B. animalis* ssp. *lactis* produced abundant amounts of peptides as a result of strong proteolysis (our unpublished data). Therefore, we hypothesized that combinations of cultures may have the potential to release bioactive peptides with antioxidant and ACE-inhibitory activity. Elucidating changes in antioxidant and ACE-inhibitory activity as a result of the interaction of these microorganisms is important for the development of health foods. However, available information about the interactive effects of *S. thermophilus* with *L. plantarum* and *B. animalis* ssp. *lactis* on ACE-inhibitory and antioxidant activity for fermented milk has not been reported. The objectives of this study were to investigate the effects of various culture combinations of *S. thermophilus*, *L. plantarum*, and *B. animalis* ssp. *lactis* on proteolysis and on antioxidant and ACE-inhibitory activity in fermented milk during refrigerated storage.

MATERIALS AND METHODS

Bacterial Strains

Freeze-dried probiotic strains of *Lactobacillus plantarum* CICC-20263 (Lp) and *Bifidobacterium animalis*

ssp. *lactis* CICC-21717 (Ba) were provided by the China Center of Industrial Culture Collection (Beijing, China). The Lp and Ba were activated in de Man, Rogosa, and Sharpe (MRS) broth (Hopebio, Qingdao, China) at 37°C for 24 h under anaerobic conditions. After 2 successive transfers, the activated cultures were further inoculated into a milk medium for 6 to 7 h at 37°C, which allowed for an initial population of 10⁷ cfu/mL after milk inoculation. Commercial starter cultures of *Streptococcus thermophilus* (St) in a direct vat set form, with an activity of 250 Danisco units, were obtained from Danisco (Kunshan, China).

Fermented Milk Preparation

Fermented milks were produced using 4 culture combinations: St, St-Ba, St-Lp, and St-Ba-Lp (the ratio of Ba to Lp was 1:2). In all treatments, St was added at 0.1% (wt/vol) of milk (approximately 10⁸ cfu/mL), and Ba and Lp were added alone or simultaneously at an initial concentration of 10⁷ cfu/mL of milk to commence fermentation. During preparation, fresh full-cream milk (New Hope Dairy, Chengdu, China) was pasteurized at 90°C for 10 min, followed by cooling to 45°C. Then, the milk was mixed well after the addition of starter cultures according to the designated treatments. Sterilized glass cups (100 mL) were filled with the mixture and incubated at 42°C until the pH value decreased to 4.6. All of the fermented milk samples were transferred to a refrigerator and stored at 4 ± 1°C. Samples from different treatments were analyzed on d 1, 3, 6, 9, 15, and 21 of storage.

pH Measurement

The pH value of the fermented milk was measured using a pH meter (Sanxin, Shanghai, China) at room temperature (20 ± 2°C).

Proteolytic Activity

The degree of protein hydrolysis was evaluated using the α -phthaldialdehyde (OPA) method of Church et al. (1985). Each portion of fermented milk (2 g) was thoroughly mixed with 1 mL of deionized water, and then 5 mL of 0.75 M trichloroacetic acid was added and mixed vigorously. After 10 min standing, the mixture was centrifuged at 4,000 × *g* for 10 min at 4°C to obtain the supernatant. To measure proteolytic activity, 200 μ L of the supernatant was added to 4 mL of OPA reagent [50 mL of 100 mM sodium tetraborate, 5 mL of 20% (wt/wt) SDS, 80 mg of OPA dissolved in 2 mL of methanol, and 200 μ L of β -mercaptoethanol were

mixed and diluted to a final volume of 100 mL with deionized water], mixed well, and reacted at room temperature ($20 \pm 2^\circ\text{C}$) for 10 min. Absorbance was read using a spectrophotometer (Aoyi, Shanghai, China) at 340 nm. The same volume of deionized water (200 μL) was added to the OPA reagent, and the absorbance of this sample served as a control.

Peptide Performance

Peptide properties were evaluated by following the method reported by Nielsen et al. (2009), with a slight modification. Fermented milks were centrifuged at $5,000 \times g$ for 10 min (4°C). The supernatants were collected, and the pH value was adjusted to 7.5 using 1 M NaOH. The suspension (whey) was filtered through a $0.45\text{-}\mu\text{m}$ filter and used to determine peptide performance.

A volume of 20 μL of whey was loaded into and detected by reversed-phase HPLC (LC1260; Agilent, Santa Clara, CA). Peptides were separated on a C_{18} column ($4.6\text{ mm} \times 250\text{ mm}$, $5\text{ }\mu\text{m}$; Waters Symmetry, Milford, MA) at 30°C by a linear elution gradient of HPLC-grade acetonitrile (Mreda, Beijing, China) in 0.1% (vol/vol) HPLC-grade trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO) from 0 to 48% between 10.0 and 90.0 min. The flow rate was 1 mL/min, and peptide peaks were analyzed under a wavelength of 210 nm with a UV detector. The peptide contents of the fermented milk were evaluated by the amount of peptides present by summing the 210 nm peak areas in the peptide profiles. Peptide areas within 10.0 to 90.0 min of retention time were determined.

Determination of Antioxidant Activity

Preparation of Whey. The whey samples were separated following the method according to Virtanen et al. (2007). The pH value of fermented milk was adjusted to 4.6 with 1 M HCl, and followed by centrifugation at $10,000 \times g$ for 10 min at 4°C . The harvested supernatant was filtered through a $0.45\text{-}\mu\text{m}$ filter.

DPPH Radical Scavenging Ability. The DPPH $^\bullet$ scavenging activity of fermented milk was determined as described by Li et al. (2009). Briefly, 3 mL of fresh DPPH (Sigma-Aldrich) solution (0.2 mM in 95% methanol) with 1 mL of whey sample or 95% methanol (control) were mixed well and allowed to settle for 20 min at room temperature ($20 \pm 2^\circ\text{C}$). After centrifugation at $10,000 \times g$ for 5 min at 4°C , absorbance was measured at 517 nm (Aoyi). Ascorbic acid at a concentration of 0.1 mg/mL was used as positive control. The following equation was used to calculate DPPH $^\bullet$ scavenging activity:

Scavenging ratio (%) =

$$(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100,$$

where A represents the absorbance.

Hydroxyl Radical Scavenging Activity. The method described by de Avellar et al. (2004) was employed to evaluate the ability to scavenge hydroxyl radicals. In brief, a reaction system containing 1.5 mL of 5 mM phenanthroline (Aladdin, Shanghai, China) solution, 2.0 mL of test samples or 0.05 M phosphate buffer (pH 7.4), 1.0 mL of FeSO_4 (7.5 mM), 1 mL of 0.1% H_2O_2 (vol/vol), and 4.5 mL of deionized water was incubated at 37°C for 60 min. The absorbance at 536 nm was monitored by spectrophotometer (Aoyi). Ascorbic acid at a concentration of 0.1 mg/mL was used as positive control. The following equation was used to calculate hydroxyl radical scavenging activity:

$$\text{Scavenging ratio (\%)} = (A_S - A_1)/(A_0 - A_1) \times 100,$$

where A_S is the absorbance in the presence of the sample and H_2O_2 , A_1 is the absorbance without the sample, and A_0 is the absorbance without the sample and without H_2O_2 .

Superoxide Anion Scavenging Ability. Superoxide anion scavenging activity was measured using the method of Liu et al. (2014). A portion of 4.5 mL of Tris-HCl buffer (50 mM, pH 8.2) and 4.2 mL deionized water was incubated at 25°C for 20 min, and then 0.5 mL of preheated whey sample and 0.3 mL of pyrogallol (3 mM) dissolved in 10 mM HCl (as a control) were added and shaken vigorously. The optical densities of the reaction mixtures were determined at 320 nm (Aoyi). Data were collected at 30-s intervals for 270 s and graphed. The slope of the curve was the autoxidation rate. Ascorbic acid (0.1 mg/mL) was used as a positive control. The following equation was used to calculate superoxide anion radical scavenging activity:

$$\text{Scavenging activity (\%)} = (\Delta_{A_0} - \Delta_A)/\Delta_{A_0} \times 100,$$

where Δ_{A_0} is the autoxidation rate of control and Δ_A is the autoxidation rate of the sample.

Total Antioxidant Capacity. The total antioxidant capacity (T-AOC) of whey samples was determined at 520 nm (Aoyi) using kits provided by Jiancheng Bioengineering Institute (Nanjing, China). Ascorbic acid (0.1 mg/mL) was used as a positive control.

In Vitro ACE-Inhibitory Activity

Simulated Gastrointestinal Digestion. Gastrointestinal digestion of fermented milk was simulated

using pepsin and trypsin according to the method reported by Laparra et al. (2003), with minor modifications. Fermented milk was adjusted to pH 2.0 with 6.0 M HCl, and pepsin (Sigma-Aldrich) was added to the fermented milk at an enzyme:substrate ratio of 1:100 (wt/wt). After 2 h of digestion at 37°C and continuous stirring, the pepsin was inactivated by adjusting the pH to 6.8 with 0.4 M NaOH. Trypsin (Sigma-Aldrich) was added at a ratio 1:100 (wt/wt) of enzyme to substrate. After 2 h of digestion at 37°C, enzyme activity was terminated by heating for 10 min at 95°C. Samples were digested with pepsin or trypsin alone, or in a sequential digestion of pepsin followed by trypsin, and subjected to analysis for ACE-inhibitory activity.

Determination of ACE-Inhibitory Activity. A total of 5 mL of fermented milk samples or digested samples were centrifuged at $5,000 \times g$ for 15 min (4°C). Then, the supernatant was harvested and the pH was adjusted to 8.3 with 1 M NaOH. Afterward, the suspension was centrifuged at $14,000 \times g$ for 5 min at 4°C. The collected supernatant was filtered through a 0.45- μ m hydrophilic filter and subjected to analysis.

The ACE-inhibitory activity was evaluated following the methods of Cushman and Cheung (1971). In each assay, 60 μ L of sodium borate buffer (0.1 M) and 30 μ L of peptide extraction were mixed with an aliquot (200 μ L) of hippuryl-histidyl-leucine (Sigma-Aldrich) solution (5 mM hippuryl-histidyl-leucine in 0.1 M sodium borate buffer containing 0.3 M NaCl, pH 8.3) and incubated at 37°C for 10 min. The reaction was activated by adding 20 μ L of ACE (Sigma-Aldrich) solution (0.1 U/mL, pH 8.3) and incubated in a water bath (37°C) for 30 min. The reaction was terminated by adding 250 μ L of 1 M HCl. Then, 1.7 mL of ethyl acetate was added to the mixture to extract the released hippuric acid. After the samples were vigorously stirred for 10 s, they were centrifuged at $5,000 \times g$ for 15 min (4°C), and 1.4 mL of the organic phase was transferred to a fresh test tube. The ethyl acetate was evaporated to dryness on a boiling water bath. The residue was dissolved in 3 mL of deionized water, and absorbance was measured using a spectrophotometer (Aoyi) at 228 nm. The following equation was used to calculate ACE-inhibitory activity:

$$\text{ACE inhibitory activity (\%)} = \left(1 - \frac{C - D}{A - B}\right) \times 100,$$

where A is the absorbance with ACE but without the sample, B is the absorbance without ACE and the sample, C is the absorbance in the presence of ACE and the sample, and D is the absorbance with the sample but without ACE.

Statistical Analysis

All of the assays in this study were performed in triplicate. Data were presented as mean $\pm$ standard deviation. A P -value of less than 0.05 was selected as significant. All data were processed using 1-way ANOVA, the differences between pairs of treatments were calculated using Duncan's multiple comparison method, and the correlation analysis was conducted using LSD in SPSS 21.0 (IBM, Chicago, IL).

RESULTS AND DISCUSSION

pH Changes

The pH values of fermented milk decreased with storage time, and were significantly affected by the various strain combinations ($P < 0.05$), except on the first day of refrigerated storage (Table 1). All treatments exhibited a significant drop in pH values, and the maximum rate of pH decline occurred at d 6 to 9. It has also been reported that the pH values of fermented milk co-fermented by probiotics decreased continuously during storage (Casarotti et al., 2014; Tian et al., 2017). Fermented milk with St showed the highest pH value, and the St-Lp-Ba samples had the lowest values during storage. Interestingly, pH values in the St-Lp samples were lower than those in the St-Ba samples from d 9 to d 21; it may be that Lp has higher post-acidifying activity than Ba. Donkor et al. (2006) and Casarotti et al. (2014) noted that pH variations in fermented milk were related to proteolysis and appeared to depend on storage time and the post-acidifying activity of the starters.

Proteolysis in Fermented Milk

Milk proteins are hydrolyzed by extracellular proteinases released by LAB, resulting in rising levels of free amino groups as quantified by the OPA method (Church et al., 1985). Proteolysis data for milk protein during the 21 d of storage are plotted in Figure 1. Proteolytic activity in all fermented milk samples increased over the storage period. Similar findings have been reported by Ramchandran and Shah (2009) and Madhu et al. (2012). Fermented milk with co-cultures had stronger proteolytic activity than St treatment alone ($P < 0.05$), a further indication of the proteolytic activity of the probiotic strains. It was apparent that samples fermented by Lp demonstrated stronger proteolytic activity than those without Lp during storage, and this may further prove the capacity for protein hydrolysis of Lp. Moslehishad et al. (2013) found that *L.*

Table 1. pH value of fermented milk during 21 d of refrigerated storage at 4°C¹

Culture ²	Storage day					
	1	3	6	9	15	21
St	4.49 ± 0.02 ^{a,A}	4.48 ± 0.01 ^{ab,A}	4.46 ± 0.02 ^{b,A}	4.38 ± 0.01 ^{c,A}	4.35 ± 0.01 ^{d,A}	4.27 ± 0.01 ^{e,A}
St-Ba	4.49 ± 0.01 ^{a,A}	4.46 ± 0.02 ^{b,AB}	4.43 ± 0.01 ^{c,B}	4.36 ± 0.01 ^{d,B}	4.33 ± 0.01 ^{e,B}	4.26 ± 0.01 ^{f,A}
St-Lp	4.48 ± 0.00 ^{a,A}	4.46 ± 0.01 ^{b,AB}	4.42 ± 0.01 ^{c,BC}	4.35 ± 0.01 ^{d,C}	4.31 ± 0.01 ^{e,C}	4.21 ± 0.01 ^{f,B}
St-Ba-Lp	4.48 ± 0.01 ^{a,A}	4.44 ± 0.01 ^{b,B}	4.41 ± 0.01 ^{c,C}	4.34 ± 0.00 ^{d,D}	4.28 ± 0.00 ^{e,D}	4.21 ± 0.01 ^{f,B}

^{a-f}Means within the same row with different superscripts indicate significant differences among days of storage ($P < 0.05$).

^{A-D}Means within the same column with different superscripts indicate significant differences among starters ($P < 0.05$).

¹Data are expressed as mean ± SD.

²St = *Streptococcus thermophilus*; St-Ba = *S. thermophilus* and *Bifidobacterium animalis* ssp. *lactis*; St-Lp = *S. thermophilus* and *Lactobacillus plantarum*; St-Ba-Lp = *S. thermophilus* with 2:1 of *L. plantarum* and *B. animalis* ssp. *lactis*.

plantarum exhibited a high level of proteolytic activity among the tested strains. Shihata and Shah (2000) reported that *Bifidobacterium* spp. had lower proteolysis than other LAB. These distinctions in the proteolytic activity of strains may be the result of variations in their proteolytic systems.

Peptide Performance

Figure 2 shows the total areas of peptides in fermented milk throughout the storage period. The peptide areas for St-Ba, St-Lp, and St-Ba-Lp treatments rapidly elevated to a peak at d 6 ($P < 0.05$), followed by a reduction during the further storage; St samples exhibited their highest peak value at d 9. As expected, all fermented milk samples from co-cultures presented

more peptide contents than pure St cultures from d 1 to 21 of storage ($P < 0.05$). However, we found no observable difference in peptide area among co-cultures ($P > 0.05$) during storage. The proteolytic system involves a series of processes, including caseins being hydrolyzed into peptides and transported into cells, then further decomposed to a series of small peptides and amino acids by intracellular peptidases (Nielsen et al., 2009). Changes during proteolysis may result in the generation of more bioactive peptides (Shahidi and Zhong, 2008).

Antioxidant Ability

The antioxidant properties of fermented milk were estimated using 4 assay systems in vitro: DPPH• scav-

Figure 1. Changes in proteolysis of fermented milk during 21 d of refrigerated storage at 4°C. Error bars represent SD. Means with different lowercase letters (a-f) were significantly different for each type of fermented milk among days of storage ($P < 0.05$); means with different uppercase letters (A-C) were significantly different for a particular day of storage for each parameter ($P < 0.05$). St = *Streptococcus thermophilus*; St-Ba = *S. thermophilus* and *Bifidobacterium animalis* ssp. *lactis*; St-Lp = *S. thermophilus* and *Lactobacillus plantarum*; St-Ba-Lp = *S. thermophilus* with 2:1 of *L. plantarum* and *B. animalis* ssp. *lactis*.

Figure 2. Peptide area ($\times 10^5$ mAu-s) of fermented milk determined by HPLC during 21 d of refrigerated storage at 4°C. Error bars represent SD. Means with different lowercase letters (a-d) were significantly different for each type of fermented milk among days of storage ($P < 0.05$); means with different uppercase letters (A-C) were significantly different for a particular day of storage for each parameter ($P < 0.05$). St = *Streptococcus thermophilus*; St-Ba = *S. thermophilus* and *Bifidobacterium animalis* ssp. *lactis*; St-Lp = *S. thermophilus* and *Lactobacillus plantarum*; St-Ba-Lp = *S. thermophilus* with 2:1 of *L. plantarum* and *B. animalis* ssp. *lactis*.

enging activity, hydroxyl radical scavenging activity, superoxide anion radical scavenging activity, and total antioxidant capacity.

DPPH[•] Scavenging Activity. The DPPH[•] radical is a stable, long-lived, organic nitrogen radical, and the scavenging of DPPH[•] assay is commonly used to evaluate the antioxidant activity of products (Sharma and Bhat, 2009). Our findings (Figure 3) showed that probiotics increased DPPH[•] scavenging activity during storage. The antioxidant activity of the treatments demonstrated a down-upward tendency during storage. The highest DPPH[•] scavenging activity for all treatments occurred at d 1, and the lowest DPPH[•] scavenging activity was observed at d 9. Similar trends of DPPH[•] scavenging activity during storage for plain cow milk yogurt have been reported by Shori (2013) and Nikjooy and Hashemi (2015). The decrease in DPPH[•] scavenging activity is probably because of higher hydrolysis and cleavage in regions of bioactive peptides with antioxidant activity (Najaf et al., 2019). At 0.1 mg/mL, the scavenging activity of ascorbic acid in the present study was 79.12%. The co-cultured samples showed higher antioxidant potential than the St samples ($P < 0.05$). The St-Ba-Lp samples showed the highest DPPH[•] scavenging activity, followed by the St-Ba and St-Lp samples. The DPPH[•] scavenging activity was negatively correlated with peptide amounts ($P < 0.05$).

Figure 3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH[•]) radical scavenging activity of fermented milk during 21 d of refrigerated storage at 4°C. Error bars represent SD. Means with different lowercase letters (a-e) were significantly different for each type of fermented milk among days of storage ($P < 0.05$); means with different uppercase letters (A-D) were significantly different for a particular day of storage for each parameter ($P < 0.05$). St = *Streptococcus thermophilus*; St-Ba = *S. thermophilus* and *Bifidobacterium animalis* ssp. *lactis*; St-Lp = *S. thermophilus* and *Lactobacillus plantarum*; St-Ba-Lp = *S. thermophilus* with 2:1 of *L. plantarum* and *B. animalis* ssp. *lactis*.

Hydroxyl Radical Scavenging Activity. Hydroxyl radical is the most highly reactive oxygen radical known (Zhang et al., 2013). The scavenging activity of hydroxyl radical by the fermented milks increased to its highest values (90.35, 91.31, 94.21, and 95.62% for St, St-Ba, St-Lp, and St-Ba-Lp, respectively) on d 6 of storage, followed by a gradual decrease to between 80 and 84% on d 21 (Figure 4). Ascorbic acid demonstrated a scavenging activity of 70.93%. The order of hydroxyl radical scavenging activity in the fermented milks, from high to low, was St-Ba-Lp, St-Lp, St-Ba, and St ($P < 0.05$). Li et al. (2015) reported that co-culture increased the hydroxyl radical scavenging activity of fermented milk compared with a single LAB culture, similar to our findings. However, the changes in all fermented milks during storage with respect to hydroxyl radical scavenging activity were nonsignificant ($P > 0.05$) over 9 d of storage. It may be that the composition of the starter cultures did not have a lasting effect on hydroxyl radical scavenging activity over the storage period. However, the scavenging activity did have a strong positive correlation with peptide amounts ($P < 0.05$).

Superoxide Anion Radical Scavenging Activity. Superoxide anions are free radicals with unpaired electrons on oxygen, and they indirectly initiate lipid oxidation as a result of superoxide and hydrogen peroxide, serving as precursors of singlet oxygen and

Figure 4. Hydroxyl radical scavenging capacity of fermented milk during 21 d of refrigerated storage at 4°C. Error bars represent SD. Means with different lowercase letters (a-e) were significantly different for each type of fermented milk among days of storage ($P < 0.05$); means with different uppercase letters (A-D) were significantly different for a particular day of storage for each parameter ($P < 0.05$). St = *Streptococcus thermophilus*; St-Ba = *S. thermophilus* and *Bifidobacterium animalis* ssp. *lactis*; St-Lp = *S. thermophilus* and *Lactobacillus plantarum*; St-Ba-Lp = *S. thermophilus* with 2:1 of *L. plantarum* and *B. animalis* ssp. *lactis*.

Figure 5. Superoxide anion radical scavenging activity of fermented milk during 21 d of refrigerated storage at 4°C. Error bars represent SD. Means with different lowercase letters (a–d) were significantly different for each type of fermented milk among days of storage ($P < 0.05$); means with different uppercase letters (A–C) were significantly different for a particular day of storage for each parameter ($P < 0.05$). St = *Streptococcus thermophilus*; St-Ba = *S. thermophilus* and *Bifidobacterium animalis* ssp. *lactis*; St-Lp = *S. thermophilus* and *Lactobacillus plantarum*; St-Ba-Lp = *S. thermophilus* with 2:1 of *L. plantarum* and *B. animalis* ssp. *lactis*.

hydroxyl radicals (Karadag et al., 2009). As shown in Figure 5, the superoxide anion radical scavenging activity of all samples was time-dependent ($P < 0.05$), and values varied from 48.24 ± 2.44 to $65.50 \pm 0.72\%$, depending on the starter culture used. The superoxide anion radical scavenging activity of the fermented milk samples was higher than that of 0.1 mg/mL ascorbic acid (31.20%). The co-cultures demonstrated a favorable ability to eliminate superoxide anion radicals, and noticeably higher scavenging activity than that of the St samples ($P < 0.05$). In particular, the St-Ba-Lp samples showed the highest scavenging activity of superoxide anions over the 21 d of storage. Wang et al. (2006) reported that the superoxide anion radical

scavenging activity of soy milk prepared from both *S. thermophilus* and *B. infantis* was 65.04% after 24 h fermentation, whereas *S. thermophilus* single cultures exhibited only 58.34% activity. It is reasonable to postulate that *S. thermophilus* co-cultured with probiotics can resist oxidation of superoxide anions. We observed no significant relationship between scavenging activity and peptide content for the co-culture samples ($P > 0.05$), but the St samples showed positive correlations ($P < 0.05$). This finding suggests that co-fermentation of probiotics with *S. thermophilus* changed the metabolites with superoxide anion scavenging activity.

Total Antioxidant Capacity. The T-AOC of food depends on a variety of antioxidation mechanisms; any single antioxidant index cannot explain T-AOC. The T-AOC of all batches of fermented milks increased gradually up to d 6, and then decreased until d 21 (Table 2). Reduced antioxidant capacity is usually because of degradation of antioxidants (Yuksel et al., 2010). The T-AOC of the fermented milk samples was lower than that of ascorbic acid (6.82 U/mg protein). The co-cultured samples presented more effective T-AOC than the samples with St only ($P < 0.05$), but after 6 d, we found no significant differences among the treatments. The co-cultured samples improved the T-AOC of fermented milk, possibly because of microbial metabolic interactions, resulting in increased peptides with antioxidant activity. Statistical analysis demonstrated positive correlations between T-AOC and peptide content for the fermented samples throughout storage ($P < 0.05$) except for the St samples, which exhibited no significant positive correlation ($P > 0.05$). Archibald and Fridovich (1981) reported that the ability to produce superoxide dismutase could be one of the main factors accounting for the high superoxide anion scavenging effect of the *S. thermophilus* fermented milk. The T-AOC of fermented milk is related not only to peptide content, but also to some metalloproteins and amino acids.

Table 2. Total antioxidant capacity (U/mg of protein) of fermented milk during 21 d of refrigerated storage at 4°C¹

Culture ²	Storage day					
	1	3	6	9	15	21
St	$4.26 \pm 0.06^{b,C}$	$4.13 \pm 0.06^{b,C}$	$4.59 \pm 0.10^{a,C}$	$3.62 \pm 0.26^{c,A}$	$3.78 \pm 0.07^{c,A}$	$3.21 \pm 0.12^{d,A}$
St-Ba	$4.56 \pm 0.12^{b,B}$	$4.66 \pm 0.32^{b,B}$	$5.06 \pm 0.12^{a,B}$	$3.86 \pm 0.28^{c,A}$	$3.66 \pm 0.14^{c,A}$	$3.25 \pm 0.07^{d,A}$
St-Lp	$4.72 \pm 0.15^{b,B}$	$4.79 \pm 0.03^{ab,B}$	$5.15 \pm 0.14^{a,B}$	$3.70 \pm 0.25^{c,A}$	$3.52 \pm 0.44^{cd,A}$	$3.25 \pm 0.07^{d,A}$
St-Ba-Lp	$5.03 \pm 0.22^{b,A}$	$5.16 \pm 0.14^{b,A}$	$5.49 \pm 0.06^{a,A}$	$3.86 \pm 0.16^{c,A}$	$3.58 \pm 0.25^{cd,A}$	$3.45 \pm 0.12^{d,A}$
Ascorbic acid	6.82 ± 0.14					

^{a–d}Means within the same row with different superscripts indicate significant differences among days of storage ($P < 0.05$).

^{A–C}Means within the same column with different superscripts indicate significant differences among starters ($P < 0.05$).

¹Data are expressed as mean $\pm$ SD.

²St = *Streptococcus thermophilus*; St-Ba = *S. thermophilus* and *Bifidobacterium animalis* ssp. *lactis*; St-Lp = *S. thermophilus* and *Lactobacillus plantarum*; St-Ba-Lp = *S. thermophilus* with 2:1 of *L. plantarum* and *B. animalis* ssp. *lactis*.

Summary. The St-Ba-Lp samples had the highest total antioxidant activity, but the T-AOC of the fermented milks during storage did not change significantly between the St-Ba and St-Lp samples ($P > 0.05$). The St-Lp samples showed lower DPPH[•] scavenging activity but higher hydroxyl radical inhibition than the St-Ba samples, implying that the St-Ba and St-Lp samples used different mechanisms to resist oxidant attack. Sarmadi and Ismail (2010) noted that the antioxidative performance of the peptides from food proteins is closely related to the composition, structure, and hydrophobicity of the peptides. Based on these findings, we speculate that the development of free radical inhibition was connected to the specific proteolytic enzymes obtained from the microbial strains.

ACE-Inhibitory Activity

During the fermentation of dairy products, intense proteolysis takes place, resulting in the production of ACE-inhibitory peptides (Anne et al., 2010; Rai et al., 2017). These peptides can exert an antihypertensive effect in vivo if they reach the cardiovascular system in an activated state (Vermeirssen et al., 2004). Thus, these peptides need to resist complete degradation by

gastrointestinal proteases after oral administration. Peptides can be hydrolyzed, leading to the activation or inactivation of their ACE-inhibitory activity during gastrointestinal digestion (Vaštag et al., 2013; Jin et al., 2016).

All samples showed inhibitory activity against ACE (Table 3). A rising trend in ACE-inhibitory activity first emerged, followed by a decline, and this decreased ability suggested the possibility of cleavage of the structure of peptides with ACE-inhibitory ability during long-term hydrolysis (Hata et al., 1996). In contrast to the St samples, the co-cultures showed a higher ACE inhibition effect over the 21 d of storage. Up to 9 d, we detected no visible difference in ACE inhibition among the co-cultures. However, after 15 d of storage, the co-cultures containing Ba showed higher ACE-inhibitory activity than those without Ba, demonstrating the ACE-inhibitory activity of peptides from bifidobacteria (Ramchandran and Shah, 2008). We found highly positive correlations between peptide content and ACE-inhibitory effect for all fermented milks during storage ($P < 0.05$).

Pepsin treatment for fermented milks improved the ACE-inhibitory activity of gastric digests, with the exception of St-Lp. The increase in activity was closely

Table 3. Angiotensin-converting enzyme inhibition activity (%) of fermented milk during 21 d of refrigerated storage at 4°C¹

Culture ²	Storage day					
	1	3	6	9	15	21
Before digestion						
St	30.51 ± 0.97 ^{d,B}	41.25 ± 2.17 ^{b,B}	49.16 ± 2.87 ^{a,B}	40.96 ± 2.68 ^{b,B}	34.40 ± 2.30 ^{c,C}	29.75 ± 0.44 ^{d,C}
St-Ba	35.61 ± 1.25 ^{d,A}	51.19 ± 1.19 ^{b,c,A}	62.00 ± 4.71 ^{a,A}	52.02 ± 0.96 ^{b,A}	51.21 ± 1.06 ^{b,c,A}	47.72 ± 1.64 ^{c,A}
St-Lp	34.92 ± 1.87 ^{d,A}	50.00 ± 0.00 ^{b,A}	60.73 ± 2.00 ^{a,A}	50.62 ± 1.07 ^{b,A}	41.14 ± 0.47 ^{c,B}	35.37 ± 0.99 ^{d,B}
St-Ba-Lp	37.37 ± 2.63 ^{d,A}	51.61 ± 2.07 ^A	65.35 ± 0.30 ^{a,A}	52.00 ± 0.86 ^{b,A}	52.85 ± 2.51 ^{b,A}	47.17 ± 0.11 ^{c,A}
Gastrointestinal digestion						
St	21.40 ± 0.49 ^{c,D}	35.56 ± 0.57 ^{a,D}	35.73 ± 1.07 ^{a,D}	31.08 ± 0.22 ^{b,D}	21.61 ± 1.22 ^{c,D}	20.44 ± 0.76 ^{c,D}
St-Ba	33.47 ± 0.68 ^{d,B}	45.09 ± 0.62 ^{a,B}	44.23 ± 0.74 ^{a,B}	41.51 ± 1.41 ^{b,B}	37.56 ± 0.96 ^{c,B}	31.45 ± 0.20 ^{c,B}
St-Lp	28.43 ± 0.31 ^{c,C}	42.08 ± 1.01 ^{a,C}	42.67 ± 0.60 ^{a,C}	35.57 ± 3.08 ^{b,C}	30.97 ± 1.16 ^{c,C}	29.68 ± 0.17 ^{c,C}
St-Ba-Lp	34.79 ± 2.16 ^{d,A}	48.88 ± 2.40 ^{a,b,A}	51.44 ± 0.35 ^{a,A}	48.01 ± 1.42 ^{b,A}	40.14 ± 0.40 ^{c,A}	38.16 ± 1.04 ^{c,A}
Gastric digestion						
St	56.67 ± 2.79 ^{c,B}	67.28 ± 1.81 ^{a,B}	69.80 ± 0.47 ^{a,B}	63.06 ± 1.47 ^{b,B}	60.78 ± 0.81 ^{b,B}	55.13 ± 1.19 ^{c,C}
St-Ba	69.50 ± 1.02 ^{b,A}	72.19 ± 3.09 ^{b,AB}	75.54 ± 1.54 ^{a,A}	71.96 ± 0.83 ^{b,A}	66.31 ± 1.44 ^{c,A}	63.44 ± 0.06 ^{c,B}
St-Lp	39.11 ± 0.52 ^{c,C}	50.68 ± 4.58 ^{a,C}	54.25 ± 1.16 ^{a,C}	45.97 ± 0.44 ^{b,C}	41.80 ± 2.49 ^{c,C}	33.55 ± 0.22 ^{d,D}
St-Ba-Lp	68.04 ± 1.95 ^{c,A}	73.20 ± 1.16 ^{a,b,A}	75.87 ± 1.51 ^{a,A}	73.50 ± 0.80 ^{b,A}	68.50 ± 0.84 ^{c,A}	66.99 ± 1.43 ^{c,A}
Intestinal digestion						
St	28.68 ± 1.88 ^{d,D}	38.04 ± 1.94 ^{b,D}	44.58 ± 0.75 ^{a,D}	33.83 ± 1.40 ^{c,D}	27.65 ± 1.15 ^{d,D}	24.17 ± 1.50 ^{e,D}
St-Ba	35.64 ± 0.23 ^{e,B}	47.63 ± 0.89 ^{b,B}	53.02 ± 1.74 ^{a,B}	44.16 ± 1.30 ^{c,B}	39.05 ± 0.81 ^{d,B}	36.54 ± 0.17 ^{c,B}
St-Lp	33.46 ± 0.88 ^{d,C}	44.90 ± 1.26 ^{b,C}	50.27 ± 0.92 ^{a,C}	39.73 ± 1.02 ^{c,C}	33.43 ± 1.30 ^{d,C}	31.00 ± 0.48 ^{d,C}
St-Ba-Lp	36.19 ± 0.79 ^{e,A}	51.00 ± 0.96 ^{b,A}	62.63 ± 0.80 ^{a,A}	50.05 ± 2.51 ^{b,A}	44.52 ± 1.44 ^{c,A}	41.72 ± 1.14 ^{d,A}

^{a-e}Means within the same row with different superscripts indicate significant differences among days of storage ($P < 0.05$).

^{A-D}Means within the same column with different superscripts indicate significant differences among starters ($P < 0.05$).

¹Data are expressed as mean ± SD.

²St = *Streptococcus thermophilus*; St-Ba = *S. thermophilus* and *Bifidobacterium animalis* ssp. *lactis*; St-Lp = *S. thermophilus* and *Lactobacillus plantarum*; St-Ba-Lp = *S. thermophilus* with 2:1 of *L. plantarum* and *B. animalis* ssp. *lactis*.

related to the release of new ACE-inhibitory peptides during gastric digestion (Quirós et al., 2005; Jin et al., 2016), and also because some ACE-inhibitory peptides in fermented milk are resistant pepsin (Contreras et al., 2013). In contrast, the ACE-inhibitory peptides showed instability with trypsin treatment, and trypsin hydrolysis reduced the effectiveness of ACE-inhibitory peptides. We observed a similar phenomenon in samples digested by pepsin and then trypsin. This finding suggested that further digestion by pancreatic enzymes might weaken the ACE inhibition of gastric digests, resulting in no significant change in ACE-inhibitory activity in the final digest. Quirós et al. (2005) reported that ACE inhibition after simulated digestion was similar to or slightly lower than that of undigested peptides, except for peptide β -CN f (47–52) (DKIHPPF). Hernández-Ledesma et al. (2007) observed a moderate decrease in ACE-inhibitory activity for peptide fragments LQKW and LLF after simulated digestion. However, Jin et al. (2016) demonstrated that the ACE inhibition rate of the pancreatic digests was greater than that of yogurt and gastric digests. The St-Ba-Lp and St-Ba samples showed higher ACE-inhibitory activity than the St-Lp samples after applying different combinations of simulated digestive juices. Furthermore, we observed a relatively high ACE-inhibitory rate for all St-Ba-Lp samples compared with St-Ba samples after trypsin or pepsin–trypsin digestion, but found no differences in the untreated or pepsin-digested fermented milks. This finding was probably because microbial metabolic interactions resulting in the C-terminal residue of peptides produced by co-cultures of 3 strains involved more proline. Proline as the C-terminal residue could be primarily responsible for the high activity (Cheung et al., 1980; Quirós et al., 2005). Interestingly, the ACE inhibition of the St-Lp fermented milk was lower than that of the St fermented milk after pepsin digestion, implying that some of the active sequences of peptides from St-Lp samples were totally hydrolyzed after digestion (Vercruyssen et al., 2005). Similar results were reported by Quirós et al. (2005), who revealed that the loss of the C-terminal sequence RYL could be responsible for a decline in ACE-inhibitory properties.

Although we observed higher proteolytic capacity in St-Lp fermented milk than in St-Ba and no difference between St-Lp and St-Ba in terms of peptide production, the ACE inhibition of the St-Lp samples was lower than that of the St-Ba samples. This finding indicated that biological activity is not directly correlated with the proteolytic activity of starters. Instead, amino acid composition, as well as the size and sequence of these amino acids in peptides, may play a vital role in biological activity (Vermeirssen et al., 2003; Gómez-Ruiz et al., 2004; Salami et al., 2011).

CONCLUSIONS

This study showed that *S. thermophilus* co-cultured with *L. plantarum* or *B. animalis* ssp. *lactis* formed suitable combinations for exerting appreciable antioxidant and ACE inhibition in vitro. The peptide generation and ACE-inhibitory activity of co-fermented milk showed similar trends to T-AOC, and reached statistical peak values on d 6. The St-Ba-Lp starters demonstrated the highest activity for proteolysis, antioxidant activity, and ACE inhibition after digestion. The co-cultures of St-Ba and St-Lp exhibited similar trends and very similar T-AOC. The St-Ba co-culture showed higher DPPH radical scavenging activity but lower hydroxyl radical scavenging activity than the St-Lp co-culture. Pepsin digestion largely improved the ACE inhibition activity of the fermented milks, except for the St-Lp samples; however, hydrolysis by trypsin reduced final activity in digestion. This study suggests that combinations of probiotics in fermented milk could improve its functional components and activities, and the optimal shelf life is within 6 d after production.

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