Fermentation characteristics and angiotensin I-converting enzyme–inhibitory activity of *Lactobacillus helveticus* isolate H9 in cow milk, soy milk, and mare milk

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ABSTRACT

Lactobacillus helveticus isolate H9 demonstrated high angiotensin I-converting enzyme (ACE)-inhibitory activity in previous research. Here, we evaluated the fermentation characteristics (pH, titratable acidity, free amino nitrogen, and viable bacterial counts), ACE-inhibitory activity, and contents of Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) peptides of stored yogurt (4°C for 28 d) fermented by *L. helveticus* isolate H9 (initially inoculated at 4 concentrations), from cow, mare, and soy milks. During storage, the pH and titratable acidity remained stable in yogurts produced from all milk types and all inoculation concentrations. The viable bacterial counts in all stored yogurts ranged between $10^{6.72}$ and $10^{8.59}$ cfu/g. The highest ACE-inhibitory activity (70.9–74.5%) was achieved at inoculation concentrations of 5×10^6 cfu/mL. The ACE-inhibitory tripeptides VPP and IPP as determined by ultra-performance liquid chromatography-tandem mass spectrometry were not produced in yogurt made from soy milk or mare milk. These evaluations indicate that *L. helveticus* H9 has good probiotic properties and would be a promising candidate for production of fermented food with probiotic properties.

Key words: *Lactobacillus helveticus* H9, fermentation characteristics, angiotensin-converting enzyme, cow, mare, and soy milks

INTRODUCTION

Lactobacillus helveticus belongs to a group of organisms collectively known as the lactic acid bacteria (LAB). This species has a generally recognized as safe (GRAS) status and is widely used in the dairy industry as a starter culture in the production of fermented milk products including yogurt (Giraffa, 2014). In a previous study, *L. helveticus* isolate H9 was selected from

259 *L. helveticus* isolates from traditional fermented dairy products, because it had high proteolytic activity when incubated in skim milk and was capable of producing the antihypertensive peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) during the fermentation process (Chen et al., 2014). The mechanism for the antihypertensive activity of these peptides is based on inhibition of the angiotensin I-converting enzyme (ACE; EC 3.4.15.1); ACE catalyzes the release of the His-Leu dipeptide from the angiotensin I C-terminus in vivo, resulting in production of octapeptide angiotensin II, a potent vasoconstrictor (Riordan, 2003). Furthermore, this enzyme hydrolyzes vasoactive bradykinin (Centeno et al., 2006) and stimulates release of aldosterone in the adrenal cortex (Cheung et al., 1980), which increases blood pressure. In fact, ACE inhibition is an excellent physiological target for treatment of clinical hypertension due to its involvement in the renin-angiotensin system (RAS) and kinin-nitric oxide system (KNOS).

Probiotics are defined as “living microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO 2001). Fermented dairy products, particularly yogurt-like products, have been marketed as probiotics for many decades. Although yogurt produced from cow milk is the most popular yogurt worldwide, the demand for alternatives is growing because of an increase in the prevalence of allergies to cow milk and because of increasing interest in the unique health benefits of other fermented products based on soy milk or mare milk (Park et al., 2006).

Soy milk is an aqueous extract from soybeans and is rich in protein (35–40%), minerals, and vitamins; it contains 8 EAA and is especially high in calcium, iron, thiamine, and riboflavin (Weingartner, 1987). Soy-based foods could provide unique health benefits to consumers because of their hypolipidemic, anticholesterolemic, and antiatherogenic properties; they could also reduce the risk of hormone-associated health disorders (Trindade et al., 2001). Mare milk has a different composition to cow milk; in particular, mare milk has a lower nitrogen

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content, lower casein-to-whey protein ratio, and higher content of lactose than cow milk (Bonomi et al., 1994). The richness and composition of whey proteins in mare milk make it more suitable for human nourishment and digestion than cow milk (Potočnik et al., 2011). In fact, mare milk is more similar to the milk of humans in its CP, salt, and lactose contents, and it is significantly lower in fat (Potočnik et al., 2011). Mare milk has been reported to have therapeutic effects in the treatment of hepatitis, chronic ulcers, and tuberculosis (Park et al., 2006).

Like fermented cow milk, soy milk and mare milk products are important sources of ACE-inhibitory and antihypertensive peptides (Pihlanto et al., 2010); these products include fermented soybean extract (Rho et al., 2009), douchi qu (a Chinese traditional fermented soybean food; Li et al., 2009), natto (a Japanese traditional food; Murakami et al., 2012), and koumiss (a traditional fermented mare milk product from Inner Mongolia; Chen et al., 2010).

During fermentation of milk by LAB, many reactions occur, including hydrolysis of milk proteins, a decrease in pH, an increase in viscosity, and production of bacterial metabolites; these reactions contribute to the flavor and, in some cases, the health-promoting properties of the resulting yogurt (Wang et al., 2009). The LAB are known to produce ACE inhibitors in various amounts during fermentation. The inhibitors are formed by bacterial proteinases when milk proteins, particularly casein, are hydrolyzed into peptides to provide the nitrogen sources necessary for bacterial growth (Pihlanto et al., 2010). Of all the LAB, *L. helveticus* produces the greatest abundance of intracellular enzymes, including cell-envelope proteinase, endopeptidases, aminopeptidases, and the X-prolyl dipeptidyl aminopeptidase, PepX (Exterkate, 1995), the latter of which releases ACE-inhibitory peptides into fermented milk drinks. Several ACE-inhibitory peptides have been isolated from milk fermented by *L. helveticus* (Fuglsang et al., 2003; Pihlanto et al., 2010).

The ACE-inhibitory activity of peptides produced by LAB has been demonstrated in vitro (Pihlanto et al., 2010), and a hypotensive effect has been demonstrated in both a hypertensive rat model (Jauhiainen and Korpela, 2007) and in human studies (Seppo et al. 2003; Tuomilehto et al., 2004). Maes et al. (2004) isolated the lactokinins ALPMHIR peptide, which can reduce blood pressure in hypertensive subjects and is an ACE-inhibitory peptide released by tryptic digestion of the milk protein β -LG. Chen et al. (2010) also isolated 4 ACE-inhibitory peptides (YQDPRLGPTGELD-PATQPIVAVHNPVIV, PKDLREN, LLLAHL, and NHRNRMMDHVH) from koumiss by enzyme hydrolysis. The best-characterized ACE-inhibitory peptides

are VPP and IPP, both of which are found in fermented milk. The antihypertensive effects of VPP and IPP have been demonstrated, including ACE-inhibitory activity in vitro (Stefanova et al., 2009) and decreased systolic blood pressure in spontaneously hypertensive rats (Jauhiainen and Korpela, 2007). However, evidence to date for ACE-inhibitory effects in humans is still not conclusive (Engberink et al., 2008; Usinger et al., 2009).

The objective of the present study was to investigate fermentation characteristics and ACE-inhibitory activity during storage of cow, soy, and mare milks fermented by *L. helveticus* isolate H9 at 4 inoculation levels. The aim was to provide useful baseline information on the potential of *L. helveticus* isolate H9 in the production of probiotic dairy products.

MATERIALS AND METHODS

Bacterial Isolates and Growth Conditions

Lactobacillus helveticus H9 (LABCC IMAU60208) was isolated from traditionally fermented yaks' milk from the Tibet Autonomous Region, China and preserved in the culture collection of the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China. The isolate was stored long-term in a reconstituted skim milk medium at -80°C . The reconstituted skim milk medium was prepared by sterilizing 11% (wt/wt) skim milk powder (NZMP Ltd., Wellington, New Zealand) containing 2% glucose and 1.2% yeast extract at 95°C for 10 min. Following retrieval from storage, the culture was activated by 2 rounds of overnight passage at 37°C in fresh skim milk medium before being used as a starter culture in experiments.

Fermented Milk Manufacture

Three milk powders were used: nonfat cow milk powder, mare milk powder, and nonfat soy milk powder (Table 1 for composition and source). Each was blended with water at 50°C to provide a protein content of 3.6 g/100 g, supplemented with 1.5 g/100 g of glucose, and pasteurized by heating to 85°C for 30 min (Wang et al., 2009; Zhou et al., 2009). The reconstituted milks were then cooled to the incubation temperature of 37°C and inoculated with *L. helveticus* H9 to achieve 4 different final concentrations: 2×10^6 , 5×10^6 , 1×10^7 , and 2×10^7 cfu/mL. The inoculated milk mixes were then poured into 250-g glass cups. Fermentation was allowed to proceed until a pH of 4.5 was achieved. Each resulting yogurt produced was then immediately cooled to 4°C in an ice bath and stored at 4°C for 28 d. The

Table 1. Composition (% wt/wt) of the 3 milk powders used and their source

Milk powder	Protein	Carbohydrate	Source
Cow	33	51 (lactose)	NZMP Ltd. (Wellington, New Zealand)
Soy	55	34 (predominant carbohydrate)	Monenzyme Research and Production Co. Ltd. (Ulaanbaatar, Mongolia)
Mare	26	56 (lactose)	Monenzyme Research and Production Co. Ltd.

pH value, titratable acidity (**TA**), free amino nitrogen content (**FAN**), viable counts of *L. helveticus* H9, ACE-inhibitory activity, and VPP and IPP contents were determined at 0, 7, 14, 21, and 28 d during refrigerated storage. The entire experiment was replicated 3 times.

Fermentation Characteristics

The pH was measured using a pHSJ-3F pH meter (Leici, Shanghai, China) with a combined glass electrode and temperature probe. Titratable acidity (°Th) was determined by titration with NaOH using phenolphthalein as the indicator (National Standards of the People's Republic of China, 1996). The FAN content of the sample was measured following the methods of Church et al. (1983). The viable counts of *L. helveticus* H9 were enumerated according to the methods of Tharmaraj and Shah (2003).

Measurement of ACE-Inhibitory Activity

The ACE-inhibitory activity was measured using HPLC following the methods of Chen et al. (2010) with some modifications. Each yogurt sample was centrifuged at $8,000 \times g$ for 10 min and the supernatant collected. The pH of the supernatant was adjusted to 8.3 followed by high-speed centrifugation ($12,000 \times g$) for 10 min, after which time the ACE-inhibitory activity was measured. The substrates used were hippuryl-L-histidyl-L-leucine (**HHL**) and rabbit lung powder containing ACE; both were obtained from Sigma Chemical Co. (St. Louis, MO). Each was separately dissolved in 300 mM NaCl and 100 mM Na-borate buffer (pH 8.3), respectively. The assay was performed by incubating a mixture of 50 μ L of yogurt supernatant with 50 μ L of HHL (10 mM) solution at 37°C for 2 min. To this, 50 μ L of ACE (0.010 U/mL) solution was added, and the mixture was incubated at 37°C for a further 30 min. The reaction was stopped by heating the mixture to 85°C in a water bath for 10 min to inactivate the enzyme. Finally, 200 μ L of EDTA (0.1 mol/L) solution and 150 μ L of deionized water were added. A 20- μ L sample of this solution was directly injected onto a Zorbax C₁₈ column (4.6 $\times$ 250 mm, particle size 5 μ m, Agilent, Palo Alto, CA) to separate the product and hippuric acid from HHL. The column was eluted with 22% (vol/

vol) acetonitrile in deionized water (containing 0.1% trifluoroacetic acid) at a flow rate of 1.0 mL/min using a pump, and the eluent was monitored at 228 nm. The column temperature was controlled at 30°C.

The level of inhibition was calculated from the following equation:

$$\text{ACE-inhibitory activity} = \frac{[(C_c - C_s)/(C_c - C_b)] \times 100\%,}{}$$

where C_c was the concentration of the buffer (control), C_s was the concentration of the reaction mixture (sample), and C_b was the concentration of the blank samples with adding solution before the reaction.

Quantification of VPP and IPP

Separation of VPP and IPP in the yogurt supernatants (following centrifugation at $8,000 \times g$ for 10 min) was achieved on an ultra-performance liquid chromatography (UPLC) system (Waters, Manchester, UK) connected to a quadrupole time-of-flight instrument (Q-Tof, Waters). The UPLC system was equipped with a binary gradient pumping system, a photo diode-array detector set at 220 nm, an automatic injector (Waters, Milford, MA), and a 2.1 mm $\times$ 100 mm BEH-C₁₈ column (Waters). The injection volume was 4 μ L. Solvent A was a mixture of water and formic acid (100:0.1, vol/vol) and solvent B was a mixture of acetonitrile and formic acid (100:0.1, vol/vol). Peptides were eluted with a linear gradient of solvent B in A going from 5 to 30% over 5 min at a flow rate of 0.4 mL/min.

Data were acquired in the 100 to 700 m/z range, using a desolvation temperature of 350°C, a source temperature of 100°C, a cone voltage of 30 V, and collision energy of 6 eV. The nitrogen desolvation and nebulizer gas flow rates were set to 600 and 50 L/h, respectively. The mass spectrometer was calibrated across the range 100 to 1,500 m/z using a solution of sodium formate. Data were adjusted during acquisition using an external reference comprising a 2 ng/mL solution of Leu-enkephalin infused at 10 μ L/min, generating a $[M^{+H}]$ at m/z 556.2771.

Standards for the peptides IPP and VPP were prepared by a conventional Fmoc solid-phase synthesis method with a 431A peptide synthesizer (Applied Bio-

systems Inc., Darmstadt, Germany). The purity of the synthesized peptides was verified by analytical HPLC coupled to MS.

Statistical Analyses

All experiments were performed in triplicate. All data are presented as means $\pm$ standard deviation. Significant differences between treatments were evaluated by ANOVA using the software package SPSS (version 16; SPSS/IBM, Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Fermentation Characteristics

Fermentation Times. We detected a significant effect of inoculation concentration on the time required for fermentation of all milk types by *L. helveticus* H9 to achieve the endpoint pH of 4.5 ($P < 0.05$); fermentation times were shorter at higher concentrations (Table 2). At the maximum *L. helveticus* H9 inoculation concentration of 2×10^7 cfu/mL, the time required to reach pH 4.5 was just 6, 6, and 9 h for cow, soy, and mare milk, respectively; mare milk had the longest fermentation time before pH 4.5 was achieved (Table 2). Samona et al. (1996) found that decreases in pH and increases in TA of soy milk fermented by *Bifidobacterium* species were faster at higher initial inoculation rates, which supports our findings for *L. helveticus* H9. Similarly, Wang et al. (2002) found that the initial inoculation rates of *Lactobacillus acidophilus* and *Bifidobacterium* species in fermented soy milk were positively correlated with the rate of decrease in pH.

pH and TA. Postacidification during storage resulted in gradual decreases in pH and increases in TA in yogurts produced from all 3 milk types, regardless of the inoculation concentration of *L. helveticus* H9 (Table 3). In fermented mare milk, only slight increases in TA were observed during the 28 d of storage; the final TA values of these samples were in the range of 100.99 to 130.38 °Th. However, dramatic increases in TA values occurred during storage of fermented cow and soy milks; at 0 d, TA values were in the range of 102.18 to 118.67 °Th and 39.02 to 43.80 °Th, respectively, but at the final assessment (d 28), the TA values ranged from 134.20 to 156.17 °Th and from 41.21 to 47.29 °Th for cow and soy milks.

Different compositions and buffering capacity of milk from different sources affect the nature of the final fermented yogurt product; the pH buffering capacity of milk is modulated primarily by proteins and free AA in the milk (Salaün et al., 2005). In our study, yogurt

Table 2. The time (h) taken for the pH of various milks to decrease to 4.5 during fermentation by *Lactobacillus helveticus* inoculated at 4 different concentrations¹

Milk type	Inoculation level (cfu/mL)				Attributes of milk before initiation of fermentation ²					
	2 × 10 ⁶	5 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	pH	TA (°Th)	FAN (mmol/L)	ACEI (%)	VPP	IPP
Cow	8.17 ^a	7.67 ^b	7.17 ^b	6.00 ^c	6.50 ± 0.00	17.24 ± 0.68	1.94 ± 0.22	4.20 ± 0.33	ND ³	ND
Soy	9.00 ^a	8.33 ^b	6.00 ^c	6.00 ^c	6.73 ± 0.01	12.98 ± 1.36	1.52 ± 0.40	11.98 ± 0.65	ND	ND
Mare	15.00 ^a	12.00 ^b	11.33 ^c	9.00 ^d	6.41 ± 0.01	34.37 ± 1.22	9.02 ± 0.84	25.60 ± 2.36	ND	ND

^{a-d}Means in a row with different superscript letters differ significantly ($P < 0.05$).

¹Data are expressed as means of triplicate determinations $\pm$ SD.

²TA = titratable acidity; FAN = free amino nitrogen content; ACEI = angiotensin I-converting enzyme inhibitory activity; VPP and IPP = contents of Val-Pro-Pro and Ile-Pro-Pro peptides.

³ND = not detected.

Table 3. Changes in pH and titratable acidity (TA) during storage for 28 d at 4°C of yogurt produced from various milks¹

Inoculum and milk type	0 d		7 d		14 d		21 d		28 d	
	pH	TA (°Th)	pH	TA (°Th)	pH	TA (°Th)	pH	TA (°Th)	pH	TA (°Th)
2 × 10⁶ cfu/mL										
Cow	4.44 ± 0.04 ^{a,v}	116.71 ± 0.03 ^{d,w}	3.92 ± 0.04 ^{d,v}	166.72 ± 5.00 ^{a,v}	4.11 ± 0.03 ^{b,v}	157.78 ± 2.85 ^{b,v}	4.03 ± 0.04 ^{c,v}	149.94 ± 1.41 ^{c,v}	4.02 ± 0.02 ^{c,w}	156.17 ± 6.24 ^{bc,v}
Soy	4.47 ± 0.04 ^{a,vw}	39.02 ± 0.00 ^{c,x}	4.21 ± 0.01 ^{a,v}	49.24 ± 1.32 ^{a,wx}	4.08 ± 0.04 ^{a,v}	42.65 ± 0.12 ^{b,x}	4.09 ± 0.00 ^{a,v}	43.94 ± 1.05 ^{b,w}	4.11 ± 0.01 ^{a,v}	42.18 ± 2.24 ^{b,w}
Mare	4.55 ± 0.02 ^{a,v}	139.07 ± 0.00 ^{a,v}	4.18 ± 0.04 ^{c,y}	121.58 ± 3.77 ^{d,v}	4.27 ± 0.01 ^{b,y}	131.31 ± 0.02 ^{b,v}	4.15 ± 0.01 ^{cd,y}	127.49 ± 1.22 ^{c,v}	4.13 ± 0.01 ^{d,y}	130.38 ± 2.02 ^{bc,v}
5 × 10⁶ cfu/mL										
Cow	4.35 ± 0.00 ^{a,v}	118.67 ± 0.00 ^{e,z}	3.95 ± 0.02 ^{c,v}	166.89 ± 2.62 ^{a,v}	4.03 ± 0.03 ^{b,w}	155.11 ± 0.83 ^{b,v}	4.01 ± 0.00 ^{b,v}	145.63 ± 0.47 ^{d,v}	4.04 ± 0.02 ^{b,vw}	149.83 ± 2.52 ^{c,v}
Soy	4.41 ± 0.01 ^{a,w}	40.79 ± 0.00 ^{c,w}	4.08 ± 0.01 ^{a,w}	51.98 ± 2.69 ^{a,w}	4.07 ± 0.01 ^{b,v}	46.45 ± 0.13 ^{b,w}	4.00 ± 0.01 ^{c,w}	45.34 ± 0.40 ^{b,v}	4.07 ± 0.01 ^{b,w}	41.21 ± 0.35 ^{c,w}
Mare	4.57 ± 0.01 ^{a,v}	116.95 ± 3.12 ^{a,w}	4.51 ± 0.02 ^{b,v}	100.20 ± 1.19 ^{c,x}	4.55 ± 0.01 ^{a,v}	104.50 ± 0.87 ^{b,y}	4.38 ± 0.00 ^{c,v}	103.41 ± 0.91 ^{bc,x}	4.39 ± 0.01 ^{c,v}	101.17 ± 3.01 ^{bc,x}
1 × 10⁷ cfu/mL										
Cow	4.44 ± 0.16 ^{a,v}	105.91 ± 0.07 ^{e,x}	3.97 ± 0.07 ^{b,v}	166.12 ± 3.71 ^{a,v}	4.12 ± 0.01 ^{b,v}	151.09 ± 1.36 ^{b,w}	3.99 ± 0.00 ^{b,v}	134.26 ± 6.68 ^{d,w}	4.02 ± 0.02 ^{b,w}	142.46 ± 0.98 ^{c,w}
Soy	4.42 ± 0.07 ^{bc,w}	43.80 ± 0.01 ^{a,v}	4.03 ± 0.00 ^{b,x}	57.43 ± 2.70 ^{a,v}	4.00 ± 0.01 ^{b,w}	46.96 ± 0.72 ^{b,w}	3.90 ± 0.02 ^{c,x}	45.25 ± 0.53 ^{bc,v}	3.98 ± 0.00 ^{b,x}	42.22 ± 2.86 ^{c,w}
Mare	4.48 ± 0.10 ^{a,v}	114.61 ± 0.01 ^{a,w}	4.29 ± 0.03 ^{b,x}	108.58 ± 1.16 ^{c,w}	4.40 ± 0.00 ^{a,w}	114.19 ± 0.16 ^{a,w}	4.23 ± 0.02 ^{b,x}	106.47 ± 0.78 ^{d,w}	4.22 ± 0.01 ^{b,x}	110.55 ± 1.00 ^{b,w}
2 × 10⁷ cfu/mL										
Cow	4.47 ± 0.00 ^{a,v}	102.18 ± 1.46 ^{d,y}	3.95 ± 0.01 ^{d,v}	174.71 ± 7.47 ^{a,v}	4.05 ± 0.03 ^{bc,w}	149.61 ± 0.32 ^{b,w}	4.02 ± 0.02 ^{c,v}	143.97 ± 4.85 ^{b,v}	4.07 ± 0.02 ^{b,v}	134.20 ± 1.65 ^{c,x}
Soy	4.54 ± 0.02 ^{a,v}	43.45 ± 1.25 ^{c,v}	3.90 ± 0.03 ^{bc,yz}	47.51 ± 1.32 ^{b,x}	3.92 ± 0.01 ^{b,x}	51.05 ± 1.38 ^{a,v}	3.87 ± 0.01 ^{c,y}	39.17 ± 0.43 ^{d,x}	3.91 ± 0.01 ^{b,y}	47.29 ± 3.02 ^{b,v}
Mare	4.56 ± 0.01 ^{a,v}	103.02 ± 1.17 ^{a,x}	4.38 ± 0.04 ^{c,w}	99.41 ± 1.59 ^{a,x}	4.44 ± 0.01 ^{b,x}	111.78 ± 0.11 ^{a,x}	4.31 ± 0.01 ^{d,w}	104.15 ± 0.38 ^{a,x}	4.34 ± 0.04 ^{cd,wz}	100.99 ± 1.17 ^{a,x}

^{a-e}Means in a row with different superscript letters differ significantly ($P < 0.05$).^{v-y}Means in a column and within a milk type with different superscript letters differ significantly ($P < 0.05$).¹Data are expressed as means of triplicate determinations ± SD.

from fermented mare milk had a stronger buffering capacity than yogurt from fermented cow milk because it had a higher content of FAN before fermentation began (Table 2). This explains why fermented mare milk showed a weaker capacity for postacidification during storage than either cow milk or soy milk (Table 3). The pH buffering capacity of proteins results from AA with basic and acidic side chains, and interactions among cations with functional groups in these side chains affects the pH buffering capacity (Salaün et al., 2005). This also explains why, in our study, the pH of fermenting mare milk decreased slowly, requiring 9 to 15 h to reach the end of fermentation (pH 4.5), which was longer than the time required for either fermenting cow milk or soy milk.

Microbiological Analysis and FAN. Viable bacterial counts and FAN content were monitored in yogurts produced from all 3 milk types during storage at 4°C for 28 d (Table 4). During storage, the viable bacterial counts of *L. helveticus* H9 in all milk types exceeded $10^{6.72}$ cfu/g regardless of the initial inoculation concentration; this indicates the probiotic potential of *L. helveticus* H9 in various fermented milk products.

To exert in vivo health benefits, probiotics must reach the intestinal tract alive. This requires them to remain viable in the food throughout the shelf life of the product and after consumption; that is, they must resist the acidic conditions of the stomach and the bile salts in the intestine (Kailasapathy and Rybka, 1997). Therefore, a viable bacterial count above 10^6 to 10^7 cfu/g is necessary to provide the reported “sufficient daily dose” of 10^6 to 10^9 viable bacteria (Vinderola et al., 2000). Our results showed that viable bacterial counts of *L. helveticus* H9 did decrease slightly ($10^{7.26}$ – $10^{8.49}$ cfu/mL to $10^{6.85}$ – $10^{8.26}$ cfu/mL) during yogurt storage, regardless of the inoculation level.

Interestingly, at all time points during storage, the viable bacterial counts of *L. helveticus* H9 in fermented mare milk were significantly ($P < 0.05$) lower when the initial inoculation concentration was 2×10^7 cfu/mL than when it was 2×10^6 cfu/mL, which is counterintuitive. A similar trend was observed for FAN content in fermented mare milk, which was also negatively correlated with the initial inoculation concentration. This may indicate that mare milk, generally, is not suitable for the growth of a wide range of bacteria and this could be due to the lack of free AA; ideally, therefore, yogurt cultures should have a proteolytic capacity. Both storage time (from 7 to 28 d) and *L. helveticus* H9 inoculation levels had significant effects on the FAN content of yogurt produced from cows' milk ($P < 0.05$). This could indicate that *L. helveticus* H9 has a high proteolytic capacity, producing more FAN at high inoculation concentrations than at low inoculation concentrations

and with increasing storage time. It has been shown previously that the main reason why LAB retain high activity and high survival rates during fermentation is their high protein hydrolysis capability (Donkor et al., 2007). Proteolysis is the most important biochemical process occurring in fermented milk products during fermentation and storage (Tamine and Deeth, 1980). The results of Dave and Shah (1998) indicated that addition of hydrolysed casein to cow milk promoted the growth of probiotic bacterial species. They suggested that this was because hydrolysis of nonprotein sources of nitrogen can stimulate the growth of isolates with an otherwise weak protein hydrolysis capability.

As seen for the pH and TA results, FAN content and viable bacterial counts were significantly higher ($P < 0.05$) in yogurt produced from mare milk than in that produced from the other 2 types of milk. The most plausible explanation for these findings is that mare milk has, intrinsically, more FAN than cow milk or soy milk (Table 1). In general, the viability of probiotic bacteria depends on isolate, storage conditions, and culture mixture. Both the relatively high viable bacterial counts and acidification rate of *L. helveticus* H9 in fermented soy milk samples indicate that *L. helveticus* H9 might have higher proteolytic activity in soy milk. In the LAB fermentation process, extracellular proteinases are involved in the initial degradation of caseins, yielding a large number of oligopeptides. Consequently, further breakdown by intracellular peptidases is critical to fulfill the needs for essential and growth-stimulating AA and peptides (Donkor et al., 2007). This proteolytic activity results in the release of bioactive peptides from specific AA sequences within the parent proteins and can provide physiological benefits (Yoshikawa et al., 2000).

ACE-Inhibitory Activity and Contents of VPP and IPP

Yogurt produced from cow milk had higher ACE-inhibitory activity than yogurt produced from either soy milk or mare milk (Table 5). Furthermore, VPP and IPP were only detected in cow milk and not in soy milk or mare milk (Table 5). This indicates that cow milk has constituents that can be utilized by *L. helveticus* H9 during fermentation to produce compounds with ACE-inhibitory activity, such as tripeptides (VPP and IPP) or gamma-aminobutyric acid, the latter of which have been reported in other studies (Sun et al., 2009a). Furthermore, ACE-inhibitory activity in yogurt produced from all 3 types of milk was stable and did not change significantly over the storage period ($P > 0.05$).

Previous studies on fermentation by probiotics of cow milk (Stefanova et al., 2009; Sun et al., 2009a;

Table 4. Changes in free amino nitrogen content (FAN; mmol/L) and viable bacterial counts (log cfu/g) during storage for 28 d at 4°C of yogurt produced from various milks¹

Inoculum and milk type	0 d		7 d		14 d		21 d		28 d	
	FAN	Viable count	FAN	Viable count	FAN	Viable count	FAN	Viable count	FAN	Viable count
2×10^6 cfu/mL										
Cow	$10.13 \pm 0.81^{b,v}$	$7.26 \pm 0.58^{b,w}$	$11.64 \pm 0.22^{a,w}$	$6.72 \pm 0.22^{c,w}$	$10.83 \pm 0.37^{b,v}$	$8.08 \pm 0.04^{a,v}$	$8.87 \pm 0.15^{c,w}$	$8.55 \pm 0.02^{a,v}$	$10.45 \pm 0.18^{b,v}$	$6.85 \pm 0.01^{bc,x}$
Soy	$4.17 \pm 0.18^{c,w}$	$8.49 \pm 0.02^{b,v}$	$4.75 \pm 0.31^{ab,w}$	$7.50 \pm 0.08^{c,x}$	$4.49 \pm 0.13^{bc,w}$	$8.63 \pm 0.10^{a,v}$	$4.40 \pm 0.40^{bc,v}$	$8.25 \pm 0.03^{c,v}$	$5.07 \pm 0.16^{a,v}$	$7.93 \pm 0.03^{d,v}$
Mare	$13.47 \pm 0.01^{d,v}$	$8.41 \pm 0.17^{b,v}$	$16.04 \pm 0.09^{c,w}$	$8.13 \pm 0.02^{c,vw}$	$17.38 \pm 0.75^{b,v}$	$8.59 \pm 0.05^{a,v}$	$18.91 \pm 0.22^{a,v}$	$8.34 \pm 0.01^{b,v}$	$17.37 \pm 0.04^{b,v}$	$8.26 \pm 0.04^{bc,v}$
5×10^6 cfu/mL										
Cow	$11.82 \pm 0.65^{bc,v}$	$8.05 \pm 0.00^{a,v}$	$13.99 \pm 0.48^{a,vw}$	$7.29 \pm 0.06^{b,w}$	$12.59 \pm 0.51^{b,v}$	$7.91 \pm 0.16^{a,v}$	$11.60 \pm 0.17^{cd,vw}$	$8.00 \pm 0.67^{a,vw}$	$10.89 \pm 0.26^{d,v}$	$7.29 \pm 0.05^{b,v}$
Soy	$3.82 \pm 0.01^{c,x}$	$7.72 \pm 0.01^{c,x}$	$4.66 \pm 0.20^{a,w}$	$7.63 \pm 0.03^{d,wx}$	$4.31 \pm 0.01^{b,wx}$	$8.54 \pm 0.03^{a,vw}$	$4.35 \pm 0.29^{b,v}$	$8.20 \pm 0.03^{b,v}$	$4.73 \pm 0.02^{a,v}$	$7.23 \pm 0.04^{c,x}$
Mare	$12.98 \pm 1.43^{b,vw}$	$8.36 \pm 0.01^{a,v}$	$18.11 \pm 1.44^{a,v}$	$8.36 \pm 0.02^{a,v}$	$18.72 \pm 1.33^{a,v}$	$8.40 \pm 0.13^{a,w}$	$17.42 \pm 1.22^{a,w}$	$7.90 \pm 0.70^{a,v}$	$16.48 \pm 3.37^{ab,v}$	$7.84 \pm 0.01^{a,x}$
1×10^7 cfu/mL										
Cow	$11.32 \pm 0.82^{b,v}$	$8.24 \pm 0.01^{a,v}$	$14.83 \pm 1.28^{a,v}$	$6.92 \pm 0.21^{b,w}$	$12.47 \pm 0.96^{b,v}$	$7.04 \pm 0.46^{b,w}$	$11.98 \pm 1.50^{b,vw}$	$6.72 \pm 0.55^{b,x}$	$11.59 \pm 1.44^{b,v}$	$7.18 \pm 0.06^{b,w}$
Soy	$3.63 \pm 0.13^{d,x}$	$7.74 \pm 0.02^{c,x}$	$6.80 \pm 0.07^{b,v}$	$7.72 \pm 0.12^{cd,w}$	$7.25 \pm 0.10^{a,v}$	$8.44 \pm 0.10^{a,wx}$	$4.10 \pm 0.07^{c,v}$	$7.90 \pm 0.03^{b,w}$	$4.06 \pm 0.19^{c,v}$	$7.59 \pm 0.03^{d,w}$
Mare	$13.40 \pm 0.17^{b,v}$	$8.36 \pm 0.03^{ab,v}$	$11.91 \pm 0.10^{cd,y}$	$7.85 \pm 0.04^{c,w}$	$14.67 \pm 1.02^{a,w}$	$8.38 \pm 0.02^{ab,w}$	$12.55 \pm 0.53^{bc,x}$	$8.43 \pm 0.19^{a,x}$	$11.12 \pm 0.57^{d,w}$	$8.24 \pm 0.02^{b,v}$
2×10^7 cfu/mL										
Cow	$10.89 \pm 1.20^{a,v}$	$7.73 \pm 0.55^{a,vw}$	$14.30 \pm 2.18^{a,v}$	$7.92 \pm 0.53^{a,v}$	$12.10 \pm 1.54^{a,v}$	$7.87 \pm 0.03^{a,v}$	$15.17 \pm 4.29^{a,v}$	$7.66 \pm 0.07^{a,w}$	$11.58 \pm 0.82^{a,v}$	$7.24 \pm 0.03^{a,vw}$
Soy	$5.11 \pm 0.19^{a,v}$	$7.78 \pm 0.01^{d,w}$	$4.11 \pm 0.44^{a,x}$	$8.12 \pm 0.01^{b,v}$	$4.17 \pm 0.23^{a,x}$	$8.29 \pm 0.11^{a,x}$	$4.06 \pm 0.45^{a,v}$	$7.80 \pm 0.08^{cd,x}$	$5.37 \pm 1.38^{a,v}$	$7.92 \pm 0.07^{c,v}$
Mare	$11.83 \pm 0.06^{c,w}$	$8.05 \pm 0.03^{a,w}$	$13.59 \pm 0.35^{a,x}$	$7.37 \pm 0.43^{b,x}$	$12.34 \pm 0.15^{b,x}$	$8.35 \pm 0.05^{a,w}$	$12.68 \pm 0.27^{b,x}$	$8.05 \pm 0.06^{a,v}$	$13.19 \pm 0.31^{a,w}$	$8.06 \pm 0.02^{a,w}$

^{a-c}Means in a row with different superscript letters differ significantly ($P < 0.05$).^{v-y}Means in a column and within a milk type with different superscript letters differ significantly ($P < 0.05$).¹Data are expressed as means of triplicate determinations $\pm$ SD.

Table 5. Changes in the ACE-inhibitory activity (ACEI; %) and VPP and IPP concentrations (μM) during storage at 4°C of yogurt produced from various milks¹

Inoculum and milk type	0 d			7 d			14 d			21 d			28 d		
	ACEI	VPP	IPP	ACEI	VPP	IPP	ACEI	VPP	IPP	ACEI	VPP	IPP	ACEI	VPP	IPP
2×10^6 cfu/mL															
Cow	75.1 $\pm$ 3.7 ^{a,v}	2.430 $\pm$ 0.103 ^{a,w}	1.299 $\pm$ 0.009 ^{c,w}	71.2 $\pm$ 4.0 ^{a,v}	2.583 $\pm$ 0.323 ^{a,w}	2.405 $\pm$ 0.173 ^{a,v}	70.4 $\pm$ 3.5 ^{a,vw}	2.473 $\pm$ 0.321 ^{a,w}	2.002 $\pm$ 0.123 ^{b,v}	71.3 $\pm$ 4.2 ^{a,v}	2.563 $\pm$ 0.145 ^{a,w}	2.084 $\pm$ 0.067 ^{b,vw}	70.7 $\pm$ 2.1 ^{a,v}	2.614 $\pm$ 0.086 ^{a,w}	2.111 $\pm$ 0.121 ^{b,x}
Soy	59.6 $\pm$ 6.2 ^{a,v}	ND ²	ND	64.9 $\pm$ 2.5 ^{a,v}	ND	ND	64.2 $\pm$ 4.1 ^{a,v}	ND	ND	61.2 $\pm$ 3.6 ^{a,v}	ND	ND	62.4 $\pm$ 2.6 ^{a,v}	ND	ND
Mare	56.6 $\pm$ 2.3 ^{a,v}	ND	ND	56.3 $\pm$ 3.3 ^{a,v}	ND	ND	59.3 $\pm$ 3.2 ^{a,v}	ND	ND	55.8 $\pm$ 2.2 ^{a,v}	ND	ND	57.1 $\pm$ 4.9 ^{a,v}	ND	ND
5×10^6 cfu/mL															
Cow	77.2 $\pm$ 1.5 ^{a,v}	2.811 $\pm$ 0.027 ^{a,v}	1.426 $\pm$ 0.178 ^{b,w}	73.1 $\pm$ 2.5 ^{a,v}	3.189 $\pm$ 0.323 ^{a,v}	2.244 $\pm$ 0.128 ^{a,v}	71.1 $\pm$ 4.6 ^{a,v}	3.045 $\pm$ 0.242 ^{a,v}	2.131 $\pm$ 0.438 ^{a,v}	70.9 $\pm$ 1.5 ^{a,v}	3.006 $\pm$ 0.184 ^{a,v}	2.275 $\pm$ 0.035 ^{a,v}	72.0 $\pm$ 6.7 ^{a,v}	2.907 $\pm$ 0.251 ^{a,v}	2.256 $\pm$ 0.127 ^{a,wx}
Soy	61.0 $\pm$ 6.9 ^{a,v}	ND	ND	62.0 $\pm$ 1.2 ^{a,vw}	ND	ND	61.7 $\pm$ 4.3 ^{a,vw}	ND	ND	61.2 $\pm$ 2.8 ^{a,v}	ND	ND	63.9 $\pm$ 5.3 ^{a,v}	ND	ND
Mare	60.1 $\pm$ 6.7 ^{a,v}	ND	ND	59.5 $\pm$ 2.6 ^{a,v}	ND	ND	58.0 $\pm$ 5.1 ^{a,v}	ND	ND	59.8 $\pm$ 3.0 ^{a,v}	ND	ND	57.9 $\pm$ 5.2 ^{a,v}	ND	ND
1×10^7 cfu/mL															
Cow	72.5 $\pm$ 4.7 ^{a,vw}	2.921 $\pm$ 0.168 ^{a,v}	2.565 $\pm$ 0.214 ^{a,v}	70.2 $\pm$ 2.2 ^{a,v}	3.021 $\pm$ 0.341 ^{a,vw}	1.785 $\pm$ 0.081 ^{c,w}	71.5 $\pm$ 3.6 ^{a,v}	3.106 $\pm$ 0.138 ^{a,v}	1.922 $\pm$ 0.122 ^{bc,v}	69.5 $\pm$ 3.2 ^{a,v}	3.245 $\pm$ 0.237 ^{a,v}	2.055 $\pm$ 0.005 ^{b,w}	71.2 $\pm$ 3.1 ^{a,v}	3.151 $\pm$ 0.060 ^{a,v}	2.655 $\pm$ 0.056 ^{ca,v}
Soy	57.0 $\pm$ 3.7 ^{a,v}	ND	ND	60.0 $\pm$ 9.6 ^{a,vw}	ND	ND	58.7 $\pm$ 1.8 ^{a,w}	ND	ND	55.6 $\pm$ 5.4 ^{a,v}	ND	ND	59.5 $\pm$ 4.8 ^{a,v}	ND	ND
Mare	58.7 $\pm$ 5.5 ^{a,v}	ND	ND	61.7 $\pm$ 3.1 ^{a,v}	ND	ND	59.5 $\pm$ 4.9 ^{ab,v}	ND	ND	58.9 $\pm$ 3.9 ^{ab,v}	ND	ND	55.4 $\pm$ 4.2 ^{b,v}	ND	ND
2×10^7 cfu/mL															
Cow	67.0 $\pm$ 3.0 ^{a,w}	2.769 $\pm$ 0.143 ^{c,v}	1.177 $\pm$ 0.143 ^{c,w}	65.1 $\pm$ 1.7 ^{a,vw}	3.119 $\pm$ 0.121 ^{a,vw}	2.111 $\pm$ 0.254 ^{ab,v}	65.2 $\pm$ 2.1 ^{a,w}	2.944 $\pm$ 0.069 ^{abc,v}	1.668 $\pm$ 0.197 ^{b,v}	67.6 $\pm$ 2.6 ^{a,v}	3.024 $\pm$ 0.068 ^{ab,v}	1.903 $\pm$ 0.210 ^{b,w}	66.0 $\pm$ 3.8 ^{a,v}	2.903 $\pm$ 0.061 ^{bc,v}	2.544 $\pm$ 0.349 ^{a,vw}
Soy	42.1 $\pm$ 0.7 ^{a,w}	ND	ND	56.2 $\pm$ 3.6 ^{a,w}	ND	ND	58.4 $\pm$ 2.2 ^{a,w}	ND	ND	55.9 $\pm$ 8.7 ^{a,v}	ND	ND	58.0 $\pm$ 2.5 ^{a,v}	ND	ND
Mare	58.1 $\pm$ 2.6 ^{a,v}	ND	ND	57.1 $\pm$ 4.0 ^{a,v}	ND	ND	58.6 $\pm$ 2.3 ^{a,v}	ND	ND	58.9 $\pm$ 1.8 ^{a,v}	ND	ND	54.3 $\pm$ 6.3 ^{a,v}	ND	ND

^{a-c}Means in a row with different superscript letters differ significantly ($P < 0.05$).^{v-y}Means in a column and within a milk type with different superscript letters differ significantly ($P < 0.05$).¹Data are expressed as means of triplicate determinations $\pm$ SD.²ND = not detected.

Chaves-López et al., 2012), soy milk (Rho et al., 2009), mare milk (Chen et al., 2010), goat milk (Minervini et al., 2009), and yak milk (Sun et al., 2009b) have demonstrated ACE inhibitory activity. In our study, ACE-inhibitory activity during storage, of yogurt produced from cow, soy, and mare milks ranged between 65.1 and 77.2%, 42.1 and 64.9%, and 54.3 and 61.7%, respectively. This result is in line with other studies on different isolates of *L. helveticus*; Pan and Guo (2012) found that milk fermented by *L. helveticus* LB10 at an inoculation concentration of 1×10^7 cfu/mL resulted in 75.46% inhibition of ACE activity, and Chen et al. (2010) demonstrated that 37 out of 259 *L. helveticus* isolates had an ACE-inhibitory activity of over 50%. The level and stability of ACE-inhibitory activity of *L. helveticus* H9 during storage indicates that it is a promising isolate for use in the production of different fermented milk products with antihypertension activity.

Several antihypertensive peptides that inhibit ACE activity have been isolated from different milk products (Donkor et al., 2007; Sun et al., 2009a; Chen et al., 2010). The inhibitors are formed by bacterial proteinase when LAB hydrolyse milk proteins, mainly casein, into peptides; because not all these peptides are used as nitrogen sources supporting bacterial growth, they can accumulate during fermentation (Leclerc et al., 2002). The proteolytic systems of LAB are quite nonspecific and, therefore, casein can be cleaved in several different places (Pihlanto et al., 2010). The AA sequence VPP is found in the primary structure of the bovine β -CN fragment 84–86 [β -CN(f84–86)] and the sequence IPP in β -CN(f74–76) and κ -CN(f108–110) (Pan et al., 2005). These AA sequences are cleaved by proteinase, with further modification by peptidases, from starter cultures during fermentation (Pan et al., 2005; Donkor et al., 2007). Furthermore, VPP and IPP are not digested by gastrointestinal enzymes and provide antihypertensive activity after they have been absorbed and enter the bloodstream (Ohsawa et al., 2008). Following absorption by the human body, VPP and IPP from fermented milk were effective in reducing the blood pressure of hypertensive patients (Donkor et al., 2007); some functional food products containing VPP and IPP have been commercialized, such as Calpis (Calpis Co. Ltd., Tokyo, Japan) and Evolus (Valio Ltd., Helsinki, Finland; Seppo et al., 2003). In our study, the VPP and IPP contents of yogurt during storage ranged from 2.430 ± 0.103 to 3.245 ± 0.237 $\mu\text{mol/L}$ and from 1.177 ± 0.143 to 2.655 ± 0.056 $\mu\text{mol/L}$, respectively. In our previous study, fermented skim milk produced by 37 *L. helveticus* isolates demonstrated in vitro ACE-inhibitory activity of over 50% and detectable levels of VPP (in 19 isolates) and IPP (in 19 isolates) that

ranged from 0.872 to 3.304 $\mu\text{mol/L}$ and from 0.311 to 1.767 $\mu\text{mol/L}$, respectively (Chen et al., 2014).

CONCLUSIONS

Our study assessed the fermentation characteristics and ACE-inhibitory activity of cow milk, soy milk, and mare milk fermentation and storage with *L. helveticus* H9. The viable bacterial counts of *L. helveticus* H9 remained above $10^{7.18}$ cfu/g in samples from all 3 milk types. The highest ACE-inhibitory activity (70.9–74.5%) was achieved with an inoculation concentration of 5×10^6 cfu/g, but VPP and IPP were only produced in cow milk, not in soy milk or mare milk. The ACE-inhibitory activity and VPP and IPP contents of cow milk were stable during storage. Based on the information reported here, *L. helveticus* H9 shows beneficial properties and is a promising candidate for use in commercial fermented food.

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