Angiotensin-converting enzyme inhibitory activity of *Lactobacillus helveticus* strains from traditional fermented dairy foods and antihypertensive effect of fermented milk of strain H9

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ABSTRACT

Hypertension is a major global health issue which elevates the risk of a large world population to chronic life-threatening diseases. The inhibition of angiotensin-converting enzyme (ACE) is an effective target to manage essential hypertension. In this study, the fermentation properties (titratable acidity, free amino nitrogen, and fermentation time) and ACE-inhibitory (ACEI) activity of fermented milks produced by 259 *Lactobacillus helveticus* strains previously isolated from traditional Chinese and Mongolian fermented foods were determined. Among them, 37 strains had an ACEI activity of over 50%. The concentrations of the antihypertensive peptides, Ile-Pro-Pro and Val-Pro-Pro, were further determined by ultra performance liquid chromatography with quadrupole-time-of-flight mass spectrometry. The change of ACEI activity of the fermented milks of 3 strains exhibiting the highest ACEI activity upon gastrointestinal protease treatment was assayed. Fermented milks produced by strain H9 (IMAU60208) had the highest in vitro ACEI activity ($86.4 \pm 1.5\%$), relatively short fermentation time (7.5 h), and detectable Val-Pro-Pro ($2.409 \pm 0.229 \mu\text{M}$) and Ile-Pro-Pro ($1.612 \pm 0.114 \mu\text{M}$) concentrations. Compared with the control, a single oral dose of H9-fermented milk significantly attenuated the systolic, diastolic, and mean blood pressure of spontaneously hypertensive rats (SHR) by 15 to 18 mmHg during the 6 to 12 h after treatment. The long-term daily H9-fermented milk intake over 7 wk exerted significant antihypertensive effect to SHR, but not normotensive rats, and the systolic and diastolic blood pressure were significantly lower, by 12 and 10 mmHg, respectively, compared with the control receiving saline. The feeding of H9-fermented milk to SHR resulted in a significantly

higher weight gain at wk 7 compared with groups receiving saline, commercial yogurt, and captopril. Our study identified a novel probiotic *L. helveticus* strain originated from kurut sampled from Tibet (China), which is a valuable resource for future development of functional foods for hypertension management.

Key words: fermented milk, *Lactobacillus helveticus*, angiotensin-converting enzyme inhibitory activity, antihypertensive, spontaneously hypertensive rats

INTRODUCTION

Hypertension is a major global health issue. It is a predisposing factor for stroke, as well as cardiovascular and kidney diseases. The health condition of high blood pressure affects around one-third of the western population and over 40% of Chinese adults aged 45 or older (Kearney et al., 2005; Feng et al., 2013). Angiotensin-converting enzyme (ACE; peptidyl dipeptide hydrolase, EC 3.4.15.1) is a key physiological regulation of blood pressure. Angiotensin-converting enzyme raises blood pressure by converting the inactive decapeptide angiotensin I to the potent vasoconstrictor octapeptide angiotensin II, as well as inactivating the vasodilating nonapeptide bradykinin (FitzGerald et al., 2004). Therefore, ACE has become one of the most effective targets for modern medical treatment for hypertension nowadays (Speth and Karamyan, 2008). Moreover, ACE inhibitors (ACEI) are frequently used in therapy to reduce morbidity and mortality of patients with hypertension.

Epidemiological studies have shown that frequent intake and regular consumption of milk and dairy products do not only offer numerous nutritional benefits, but also lower the risk of high blood pressure (Jauhiainen et al., 2010a; Luo et al., 2010). Blood pressure level is negatively correlated to the quantity of dairy product consumption, and the antihypertensive effect is likely conferred by the milk proteins and peptides. Fermented milks contain a particularly large number and wide diversity of peptides, and many possess ACEI

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and antihypertensive activities. Most lactic acid bacteria (**LAB**) are auxotrophic for several AA, which are to be acquired from the direct environment. Therefore, to meet the AA requirement for maintenance and growth, many dairy LAB members have evolved in the milk environment to form highly sophisticated proteolytic and peptide-uptake systems to ensure efficient breakdown of proteins and uptake of the subsequently released subpeptide fragments. These subpeptides or peptides are mainly produced by the proteolytic strains of the LAB.

In Takano (1998), 16 strains from 7 different LAB species commonly used in fermented dairy products were tested for the antihypertensive activity in spontaneously hypertensive rats (**SHR**). Only milks fermented by several *Lactobacillus helveticus* strains, but not *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* ssp. *lactis*, *Streptococci thermophilus*, *Lactobacillus lactis* ssp. *lactis*, and *Lactobacillus lactis* ssp. *cremoris*, showed positive antihypertensive activity accompanied by the identification of the ACEI peptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP). *Lactobacillus plantarum* is another potential species bearing such activity (López-Fandiño et al., 2006). Among the various LAB species, the species *L. helveticus* is often reported to have a high proteolytic activity; therefore, it is deemed as a good candidate for producing fermented milks with high ACEI activity (Yamamoto et al., 1994; Fuglsang et al., 2003). Some *L. helveticus* strains, such as CP790, LBK16H, R211, R389, LMG11474, CHCC641, and CCCH637, have been used in the production of antihypertensive-fermented milk foods (López-Fandiño et al., 2006). Up to the present, the most characterized ACEI peptides found in fermented milk products and hydrolysates are IPP and VPP, which have been shown to be hypertensive in both humans (Ishida et al., 2011; Nakamura et al., 2011; Cicero et al., 2013) and rats (Sipola et al., 2001, 2002; Jauhiainen et al., 2010b). The ability of individuals *L. helveticus* to produce antihypertensive peptides is most likely related to the completeness and efficiency of their proteolytic system (Griffiths and Tellez, 2013). For example, *L. helveticus* CNRZ32 was known to possess ACEI activity and it was found to have as many as 21 proteolytic system-related genes by microarray analysis (Smeianov et al., 2007). As the proteolytic capacity of *L. helveticus* is strain-specific, it is, therefore, of interest and is indeed necessary to screen for suitable strains based on some of these criteria (such as the ability of the strain to produce VPP and IPP peptides during fermentation, in vitro and in vivo ACEI activity of the fermented products) for future development of novel functional products for management of hypertension.

Certain traditional fermented milk products, including koumiss (fermented mare milk) and kurut (fermented yak milk), were reported to exhibit ACEI activity and beneficial hypertensive effect (Sun et al., 2009; Chen et al., 2010). However, very few studies have performed on screening and characterizing the strains responsible for such activity in these products, in particular in the local homemade fermented dairy-based foods from Mongolia and China. Mongolians generally have a high consumption of dairy products. In contrast, traditionally fermented dairy products are only common in certain provinces in China, where the ethnic minority populations usually reside. These provinces include Inner Mongolia, Xinjiang, Qinghai, Tibet, and Yunnan.

In previous works, our laboratory has collected a variety of homemade traditional fermented products from various locations of Mongolia and China. The types of fermented products included kurut, koumiss, fermented camel and cow milks, as well as dairy fan and cake (fermented dairy products from Yunnan province). From these works, 259 *L. helveticus* strains were isolated. The principal aim of the current study was to screen for and to characterize these novel *L. helveticus* strains for their ACEI activity and ability to produce IPP and VPP. Furthermore, the antihypertensive effect of the fermented milks produced with a selected strain was assessed in SHR and normotensive (Wistar-Kyoto; **WKY**) rats. Data generated from the current study will serve as further evidence on the in vivo hypertensive effect of fermented milks. Moreover, novel strains characterized in this study will be valuable resources for future development of functional products for hypertension management.

MATERIALS AND METHODS

Original Source of *L. helveticus* Strains

In our previous work (Liu et al., 2009; Airidengcaিকে et al., 2010; Sun et al., 2010a,b,c; Bao et al., 2011; Yu et al., 2011), 259 strains of *L. helveticus* were isolated from different traditional fermented dairy products in China and Mongolia, including koumiss (fermented mare milk), kurut (fermented yak milk), fermented camel and cow milks, and dairy fan and dairy cake (traditional fermented dairy products in Yunnan Province of China). The sampling sites, type of fermented products and number of isolated strains are listed in Table 1. These strains were identified as *L. helveticus* by a combination of traditional physiological and biochemical identification methods together with 16S rRNA gene sequence analysis, as published in previous reports (Table 1).

Table 1. Sample type, sampling site and number of isolated *Lactobacillus helveticus* strains

Sample type	Sampling site	Number of isolated <i>L. helveticus</i> strains	Reference
Koumiss	Inner Mongolia	11	Sun et al., 2010a
	Xinjiang	99	
	Qinghai	20	
	Mongolia	3	
Kurut	Tibet	17	Yu et al., 2011 Airidengcaিকে et al., 2010
	Qinghai	14	
Fermented camel milk	Mongolia	22	Sun et al., 2010b
Fermented cow milk	Tibet	15	Sun et al., 2010c Airidengcaিকে et al., 2010
Dairy fan	Yunnan	56	Liu et. al., 2009
Dairy cake	Yunnan	2	Bao et al., 2011
Total		259	

Bacterial Stocks and Preparation of Fermented Milks

The 259 strains of *L. helveticus* were preserved as a freeze-dried powder at the Lactic Acid Bacteria Collection Centre of Inner Mongolia Agricultural University. The *L. helveticus* strains were activated by 2 rounds of overnight passage at 37°C in 11% (wt/wt) sterile reconstituted skim milk containing 2% glucose and 1.2% yeast extract before being used as starter cultures. The reconstituted milk was prepared by sterilizing 11% (wt/wt) skim milk powder (NZMP Ltd., Wellington, New Zealand) at 95°C for 10 min.

Bacterial growth was determined by spectrophotometric measurement of optical density (OD) at 600 nm. The viable bacterial count was determined by setting up a standard curve correlating the OD at 600 nm and plate count on de Man, Rogosa, and Sharpe agar. Fermented milks were made in flask cultures inoculated with seed cultures of the tested strains of *L. helveticus* at a concentration of 5×10^6 cfu/mL. Inoculated milk cultures were incubated at 37°C for fermentation until pH 4.5 was reached. The finished fermented milks were centrifuged at $6,000 \times g$ for 10 min at 4°C. The supernatants were collected for subsequent determination of ACEI activity, free amino nitrogen (FAN), titratable acidity [Thorner degrees (°Th)], and VPP and IPP contents.

Chemical and Microbiological Analyses

The sample FAN content was measured by the method of Church et al. (1983). The titratable acidity (°Th) was determined according to method 947.05 of AOAC International (1997). The viability of *L. helveticus* in the fermented milks was enumerated using the method described by Ishii (2003). Briefly, serial dilutions of milk samples were prepared and fixed volumes of diluents were plated out on plate count agar with bromocresol purple and cycloheximide agar (Eiken Chemical

Co. Ltd., Tokyo, Japan). The bacterial viability was expressed in colony-forming units per milliliter.

Determination of In Vitro ACEI Activity

The ACEI activity was measured using the HPLC method described by Chen et al. (2010) with some modifications. The substrate hippuryl-L-histidyl-L-leucine (HHL) and rabbit lung powder containing ACE were obtained from Sigma Chemical Co. (St. Louis, MO). Both HHL and ACE were separately dissolved in 100 mM Na-borate buffer (pH 8.3) containing 300 mM NaCl. The assay was performed by incubating a mixture of 50 µL of milk supernatant sample and 50 µL of HHL (10 mM) solution at 37°C for 2 min. Then, 50 µL of ACE (0.010 U/mL) solution was then added and the mixture was further incubated at 37°C for 30 min. The reaction was stopped by heating the mixture in an 85°C water bath for 10 min to inactivate the enzyme. Afterward, 150 µL of deionized water was added before 20 µL of this solution was directly injected onto a Zorbax C₁₈ column (4.6 × 250 mm, particle size 5 µm; Agilent, Santa Clara, CA) to separate the product, hippuric acid, from HHL. The column was eluted with 75% acetonitrile in water (vol/vol) containing 0.1% trifluoroacetic acid at a flow rate of 1.5 mL/min using a pump; the eluent was monitored at 228 nm. The column temperature was controlled at 30°C.

The inhibition was calculated from the following equation:

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

where C_c , C_b , and C_s were the concentrations of hippuric acid without the tested sample (control), without ACE (blank), and with both ACE and the tested sample (sample), correspondingly.

Characterization of Milk ACEI by Protease Digestion

To characterize the nature and the resistance to gastrointestinal proteases of the ACEI materials present in the fermented milks, samples were digested alone either with pepsin or trypsinase, or a sequential digestion of pepsin followed by trypsinase (Figure 1), before assaying for ACEI activity as described. The procedures for enzyme digestion were modified from Chen et al. (2010).

Quantification of VPP and IPP by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry

Separations of VPP and IPP in the supernatants from the fermented milk samples were performed on an ultra-performance liquid chromatography system (Waters, Milford, MA) connected to a quadrupole time of flight instrument (Q-ToF, Waters, Manchester, UK). The ultra-performance liquid chromatography system was equipped with a binary gradient pumping system, a photo diode array detector set at 220 nm, and an automatic injector (Waters). The 100×2.1 mm BEH C_{18} column (Waters) was used in these experiments. The injection volume was 4 μ L. Solvent A was a mixture of water-formic acid (100:0.1, vol/vol) and solvent

B contained acetonitrile-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 0.5 mL/min.

Data were acquired from 100 to 800 (m/z), using a desolvation temperature of 300°C, source temperature of 100°C, cone voltage of 45 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 centroided during acquisition using an external reference comprising a 2-ng/mL solution of leucine enkephalin infused at 10 μ L/min, generating a molecular ion [M^+] at m/z 556.2771.

The peptides IPP and VPP were prepared by a conventional 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis method with a 431A peptide synthesizer (Applied Biosystems Inc., Darmstadt, Germany). The purity of the synthesized peptides was verified by analytical HPLC coupled to MS.

Assessment of Antihypertensive Effect in Rats

The study protocol was approved by the Ethical Committee of Inner Mongolia Agricultural University

Figure 1. Procedures of gastrointestinal protease digestion of sample before angiotensin-converting enzyme (ACE) inhibitory activity determination.

(Hohhot, China). Rats were obtained from River Laboratory Animal Co. Ltd. (Beijing, China). They were maintained at a temperature of $22 \pm 2^\circ\text{C}$, humidity of $55 \pm 5\%$, with 12-h light-dark cycles, and with tap water and standard chow diet for rats (Animal Center of Inner Mongolia University, Hohhot, China) provided ad libitum during the experiments. All animals were acclimatized for 1 wk before the experiment. The hypertensive effect of a single oral dose and long-term administration of fermented milks were tested in 2 separate sets of experiments. Both experiments were randomized controlled trials. Randomization of rat treatment was accomplished by random digit generation by Excel (Microsoft, Redmond, WA).

Twenty-four 15-wk-old male SHR, weighing 260 ± 16 g, were used for the single dose oral administration study. Rats were divided into 3 groups ($n = 8$ for each group), including the control (received 0.9% NaCl in distilled water), H9 (received fermented milk produced with *L. helveticus* H9), and yogurt (received commercially available yogurt) groups. Each rat was given a single oral gavage of 15 mL/kg of BW of the respective treatment. Although the dose of 5 mL/kg of BW of fermented milk was given to the experimental rats in a similar study (Nakamura et al., 1995), the concentrations of VPP and IPP in the fermented milks produced by the strains used in the current study seemed to be a lot lower. As the effect of milk antihypertensive peptides is dose-dependent, an increased dose of fermented milk was administered to the rats. The systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate of the rats were measured using a tail-cuff apparatus (BP-98A, Softron, Tokyo, Japan; Chen et al., 2007). These parameters were monitored at 0 (before treatment), 2, 4, 6, 8, 12, and 24 h postadministration. Before the measurement, the rats were kept at 37°C for 10 min to make the pulsations of the tail artery detectable.

For the long-term administration experiment, 60 male 7-wk-old SHR (weighing 130 ± 6 g) and 15 7-wk-old male WKY rats (weighing 151 ± 26 g) were used. The SHR were divided into 4 groups (each of 15 rats), including the control (received 0.9% NaCl in distilled water), H9 (received *L. helveticus* IMAU60208/H9-fermented milk), yogurt (received commercial yogurt), and captopril (received the ACEI, captopril, dissolved in distilled water) groups. All WKY rats were given *L. helveticus* H9-fermented milk. All animals were given a daily oral gavage of the respective treatment for 7 continuous weeks. The administered dosage for the control, H9, yogurt groups, and the WKY rats was 15 mL/kg of BW, whereas the captopril group received 20 mg/kg of BW. The BW of the rats were taken weekly. The SBP and DBP were also monitored weekly using

the tail-cuff method (model BP-98, Softron). After the last measurements were taken in wk 7, rats were euthanized. The whole BW and left ventricle mass of each rat were recorded. The left ventricular mass index (LVMI; mg/g) of the rats was further determined as the ratio of the weight of left ventricle (mg) to the whole BW (g).

Statistical Analysis

All the studied parameters were measured in triplicate. Data were expressed in mean $\pm$ SEM. Statistically significant differences between sample groups were evaluated with ANOVA. The *P*-values less than 0.05 were considered as statistically significant differences between sample groups. Analysis of variance and correlation analysis were performed with the SAS version 9.00 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Fermentation Properties and In Vitro ACEI Activity of *L. helveticus* Strains

In the current study, we characterized the fermentation properties (titratable acidity, FAN, and fermentation time) and in vitro ACEI activity of 259 *L. helveticus* strains previously isolated from traditional Chinese and Mongolian fermented foods. The mean $\pm$ SEM of titratable acidity, FAN, ACEI activity, and fermentation time of the 259 *L. helveticus* strains were $101.73 \pm 12.52^\circ\text{Th}$, 8.32 ± 1.13 mmol/L, $28.9 \pm 23.7\%$, and 20.1 ± 7.8 h, respectively. During milk fermentation, the major milk proteins are degraded into a diverse array of peptides resulting from a combinatory action of microbial and endogenous milk enzymes (mainly plasmin). Microbial-based enzymes are mostly sourcing from the LAB indigenously present in the raw materials or from the starter cultures. *Lactobacillus helveticus* was chosen to be the target LAB to be analyzed in our study because of their known excellent extracellular proteinase activity and ability to release specific antihypertensive peptides into fermented milk during the fermentation process (Wakai and Yamamoto, 2012).

Out of the 259 tested strains, 37 (14.3%) were found to have in vitro ACEI activity of over 50%. The level of ACEI activity was comparable to some other fermentation studies (Muguerza et al., 2006; Sun et al., 2009). As the principal objective of the current study was to identify strains with high ACEI activity, 37 strains were ranked based on this criterion (Table 2). The first fermented milk with documented antihypertensive activity was marketed by the Japanese Calpis company (Tokyo, Japan) under the trade name of Amiiru S, which was

produced by using *L. helveticus* CP790 and *Saccharomyces cerevisiae*. Two ACEI tripeptides, VPP and IPP, of casein origin, were shown to be responsible for the in vivo antihypertensive properties of the drink (Bünning et al., 1983; Riordan, 2003; Corradi et al., 2006; Zinovia and Athanassios, 2010). Therefore, the concentrations of VPP and IPP in the respective fermented milks produced by the 37 strains exhibiting over 50% of ACEI activity were further determined. Among these 37 strains, 16 contained both VPP and IPP, whereas 15 had neither of the peptides. The other 5 strains were found to have a detectable level of only either VPP or IPP. The majority (4 out of 5) of strains with high ACEI activity (over 80% ACEI inhibition) were able to produce both VPP and IPP peptides. These results are in agreement with other previous studies showing that

the high ACEI activity was likely attributed by these 2 peptides (Bünning et al., 1983; Riordan, 2003; Corradi et al., 2006; Zinovia and Athanassios, 2010). However, the fact that 16 out of the 37 strains showed positive ACEI activity, but were negative in VPP and IPP, indicated that the in vitro ACEI activity might also be contributed by factors other than these 2 peptides.

Data of ACEI activity, VPP and IPP concentrations, titratable acidity, FAN, and time required for fermentation for the 37 strains are listed in Table 2. Although sharing a relatively high ACEI activity, the different strains exhibited considerable variation in their fermentation properties in terms of the titratable acidity (ranged from 76.44 ± 2.60 to $116.05 \pm 1.95^{\circ}\text{Th}$), FAN concentration (ranged from 5.67 ± 0.27 to 12.30 ± 1.65 mmol/L), and time required to end fermentation

Table 2. Properties of fermented milks produced by *Lactobacillus helveticus* strains showing over 50% of in vitro angiotensin-converting enzyme inhibitory (ACEI) activity

Strain number	Titratable acidity ($^{\circ}\text{Th}$) ¹	Free amino N (mmol/L)	Val-Pro-Pro concentration (μM)	Ile-Pro-Pro concentration (μM)	ACEI activity (%)	Fermentation time (h)
IMAU60208	110.31 ± 0.13	7.80 ± 0.33	2.409 ± 0.229	1.612 ± 0.114	86.4 ± 1.5	7.5
IMAU30046	101.31 ± 0.00	7.80 ± 0.09	1.853 ± 0.236	1.531 ± 0.108	83.3 ± 5.3	16.5
IMAU60205	104.02 ± 11.14	9.36 ± 0.27	0.721 ± 0.061	ND ²	82.3 ± 3.4	34.0
IMAU10142	96.71 ± 7.16	8.54 ± 0.02	2.006 ± 0.162	1.186 ± 0.078	81.0 ± 1.4	32.3
IMAU30005	101.31 ± 3.26	7.05 ± 0.09	0.360 ± 0.034	0.370 ± 0.063	80.2 ± 2.8	15.5
IMAU60207	100.71 ± 12.30	8.49 ± 0.27	2.538 ± 0.217	1.312 ± 0.121	79.0 ± 1.1	12.3
IMAU50079	95.78 ± 0.00	7.76 ± 0.00	ND	ND	78.5 ± 0.3	29.7
IMAU60210	95.32 ± 4.69	8.34 ± 0.87	3.304 ± 0.180	1.390 ± 0.164	77.9 ± 1.8	11.3
IMAU70168	116.05 ± 1.95	8.88 ± 0.07	ND	ND	77.3 ± 4.2	33.5
IMAU60211	91.18 ± 1.17	11.52 ± 0.84	2.603 ± 0.847	1.604 ± 0.280	77.2 ± 0.4	11.3
IMAU60204	91.18 ± 5.86	10.65 ± 0.75	2.368 ± 0.028	0.869 ± 0.060	74.5 ± 1.0	10.7
IMAU30028	92.56 ± 0.00	6.75 ± 0.05	ND	ND	73.8 ± 0.3	21.0
IMAU50010	104.07 ± 3.26	7.27 ± 0.09	2.121 ± 0.114	ND	72.8 ± 8.3	25.0
IMAU60201	94.50 ± 1.17	12.30 ± 1.65	2.467 ± 0.258	1.767 ± 0.372	72.0 ± 2.8	12.0
IMAU20076	110.98 ± 1.89	8.99 ± 0.51	ND	ND	71.4 ± 4.5	7.0
IMAU50019	113.47 ± 6.77	9.19 ± 0.18	ND	ND	71.2 ± 0.2	42.4
IMAU60220	84.55 ± 0.00	7.92 ± 0.90	0.872 ± 0.294	ND	70.7 ± 6.8	24.7
IMAU40088	107.30 ± 1.30	8.84 ± 0.02	ND	ND	67.5 ± 5.7	17.0
IMAU60066	103.78 ± 8.32	5.67 ± 0.27	2.029 ± 0.261	1.732 ± 0.353	67.1 ± 2.6	10.7
IMAU60212	96.16 ± 5.86	5.97 ± 0.30	2.387 ± 0.025	1.124 ± 0.032	64.6 ± 2.0	11.2
IMAU50024	102.23 ± 1.30	8.28 ± 0.04	ND	ND	64.4 ± 0.6	21.0
IMAU30087	91.18 ± 1.30	7.42 ± 0.02	ND	ND	63.6 ± 2.0	22.0
IMAU50151	91.18 ± 2.60	7.39 ± 0.07	1.451 ± 0.059	1.038 ± 0.072	62.4 ± 4.9	25.0
IMAU60117	108.54 ± 2.99	7.26 ± 1.44	2.632 ± 0.283	1.206 ± 0.196	58.7 ± 4.8	11.7
IMAU10149	95.51 ± 0.65	7.80 ± 0.02	ND	ND	58.4 ± 0.9	17.0
IMAU30003	114.30 ± 4.56	7.86 ± 0.01	2.194 ± 0.108	1.671 ± 0.081	58.2 ± 5.4	11.0
IMAU60227	87.45 ± 2.93	6.24 ± 1.50	ND	ND	57.5 ± 5.3	12.3
IMAU30023	102.69 ± 1.43	10.10 ± 0.39	ND	0.428 ± 0.048	57.2 ± 5.2	23.0
IMAU30041	99.01 ± 5.86	10.20 ± 0.16	ND	ND	57.2 ± 1.6	21.0
IMAU60224	107.76 ± 2.34	10.32 ± 2.01	ND	ND	57.1 ± 3.7	30.0
IMAU50064	95.32 ± 0.65	7.72 ± 0.02	ND	0.311 ± 0.054	55.4 ± 4.5	42.4
IMAU30134	105.92 ± 2.60	7.48 ± 0.01	1.236 ± 0.093	0.436 ± 0.093	55.1 ± 2.7	16.0
IMAU30109	96.71 ± 0.00	7.53 ± 0.00	ND	ND	53.9 ± 1.6	32.3
IMAU60206	91.18 ± 2.35	8.28 ± 1.47	2.256 ± 0.018	0.954 ± 0.023	52.7 ± 3.3	14.5
IMAU50011	76.44 ± 2.60	6.19 ± 0.07	ND	0.692 ± 0.066	52.0 ± 0.8	25.0
IMAU30016	103.15 ± 1.30	6.65 ± 0.00	ND	ND	50.5 ± 1.8	15.7
IMAU50013	109.14 ± 6.51	7.68 ± 0.18	ND	ND	50.0 ± 6.6	42.4

¹ $^{\circ}\text{Th}$ = Thorner degrees.

²ND = not detectable; data are expressed as mean $\pm$ SEM.

Figure 2. Correlation between angiotensin-converting enzyme inhibitory (ACEI) activity and pH, optical density (OD), titratable acidity (TA), and free amino nitrogen (FAN) of fermented milk samples. A positive or negative correlation is represented by a correlation coefficient greater or smaller than 0, respectively. Two asterisks (**) represent a statistically significant correlation ($P < 0.01$).

(ranged from 7 to 42.4 h). Together, our results confirm the fact that the fermentation and ACEI properties are strain-specific.

Furthermore, correlation between the in vitro ACEI activity and the monitored properties of fermented milks produced by the 259 *L. helveticus* strains was determined (Figure 2). Significant positive correlations were found between the in vitro ACEI activity with titratable acidity and FAN ($P < 0.01$), whereas the ACEI activity was negatively correlated with the pH value ($P < 0.01$). However, no significant correlation was found between the in vitro ACEI activity and OD at 600 nm. The increase in titratable acidity and FAN were possibly related to the bacterial fermentative activities particularly associated with the release of acidic metabolites into the fermentation medium and milk protein degradation. The positive correlation of ACEI activity and FAN may hint to a peptide-based enzyme

inhibitory mechanism. Both VPP and IPP are 2 of the most studied milk peptides known to inhibit ACE, but another lactotripeptide, leucine-proline-proline, has also been found to exhibit such activity. Moreover, other casein-derived peptides, such as α_{s1} -CN f(90–94) (RYLGY), α_{s1} -CN f(143–149) (AYFYPEL), and α_{s2} -CN f(89–95) (YQKFPQY), displayed both in vitro ACEI and in vivo hypotensive activities (reviewed by Jäkälä and Vapaatalo, 2010).

Characterization of Fermented Milk ACEI by Protease Digestion

To test the gastrointestinal protease resistance and further characterize the nature of the ACEI in the fermented milks, milk samples from IMAU60208, IMAU30046, and IMAU30005 were digested with pepsin, trypsinase and pepsin-trypsinase. Milk samples from

Table 3. Effect of protease treatment on the angiotensin-converting enzyme inhibitory (ACEI) activity of fermented milk of three selected strains¹

Strain number	Untreated control	Pepsin	Trypsinase	Pepsin followed by trypsinase
IMAU60208	86.4 ± 1.5 ^{b,x}	93.4 ± 5.8 ^{a,x}	91.4 ± 1.1 ^{ab,x}	94.2 ± 1.7 ^{a,x}
IMAU30046	83.6 ± 5.3 ^{a,x}	79.0 ± 3.4 ^{a,y}	81.7 ± 3.8 ^{a,y}	81.6 ± 2.5 ^{a,y}
IMAU30005	80.2 ± 2.8 ^{a,x}	84.7 ± 2.4 ^{a,y}	82.9 ± 4.0 ^{a,y}	85.3 ± 3.1 ^{a,y}

^{a,b}Means in the same row with different superscripts are significantly different ($P < 0.05$).
^{x,y}Means in the same column with different superscripts are significantly different ($P < 0.05$).
¹ACEI activity is expressed as percentage inhibition ± SEM.

these 3 strains were selected because of their high ACEI activity, over 80%, and their relatively fast fermentation time, which is an important fermentation criterion to consider for practical food application. Interestingly, the ACEI activity of the fermented milk responded differentially upon protease treatment (Table 3). Milk samples from IMAU60208 showed a significant increase in ACEI activity regardless of the tested protease type (pepsin, trypsinase, or sequential treatment of both proteases), whereas IMAU30005 exhibited a general but insignificant increase in ACEI activity as compared with the undigested control. In contrast, upon protease treatment, the ACEI activity of protease-digested milk of IMAU30046 slightly reduced, though insignificantly. Our results indicated that the ACEI peptides of *L. helveticus* IMAU60208- and IMAU30005-fermented milks were enhanced after digestion with gastrointestinal proteases, possibly due to a further release of active peptides or materials during the digestion process. According to Fujita et al. (2000), based on the response to treatment with ACE or gastrointestinal proteases, ACEI peptides can be classified into 3 groups: the true inhibitor (activity unchanged upon ACE or gastrointestinal protease treatment), substrate (activity reduced upon ACE or gastrointestinal protease treatment), and pro-drug (activity increased upon ACE or gastrointestinal protease treatment) types. From our experimental observation, the ACE inhibitors in *L. helveticus* IMAU60208- and IMAU30005-fermented milk most likely belonged to the pro-drug type or a mixture of the pro-drug and true inhibitor.

More importantly, the resistance of the milk ACEI to these proteases and their ability to remain active even after the treatment suggest a high likelihood of in vivo bioavailability and functionality. The availability of in vitro assay ACEI activity greatly facilitates the screening for potential antihypertensive fermented milk products, strains, and peptides. Large-scale screening can be performed relatively easily and at a low cost as compared with in vivo assay. However, results from in vitro and in vivo functional analyses may sometimes be contradictory to each other due to the bioavailability of the peptides. Therefore, experiments were subsequently performed in rats to confirm the in vivo functional antihypertensive effect of the fermented milk.

In Vivo Feeding Experiments

Spontaneously hypertensive rat is an inbred strain developed by selective breeding of the WKY stock for higher blood pressure (Ely and Turner, 1990). The SHR are the most commonly used animal model for studying hypertension. Owing to the high cost of the in vivo experiments, only the fermented milk produced by

strain H9 (IMAU60208, isolated from kurut of Tibet of China) was selected for further testing for its antihypertensive effect subjected to short-term and long-term administration in rats.

Antihypertensive Effect of Single Dose Oral Administration of H9-Fermented Milk

To test the hypertensive effect of short-term administration, a single oral dose of 0.9% NaCl (control), fermented milk produced by *L. helveticus* (strain IMAU60208 or H9) or commercial yogurt was fed to the SHR rats (Figure 3). All 3 groups showed a general reduction in SBP, DBP, and mean blood pressure 12 h after the treatment, which might have been caused by the natural physiological response of the animals as previously reported (Muguerza et al., 2006; Miguel et al., 2007; Quirós, et al., 2007). However, only H9, but not the control or commercial yogurt groups, showed a significant antihypertensive effect (attenuation of 15 to 18 mmHg in SBP, DBP, and mean blood pressure; $P < 0.05$) 6 to 12 h after administration of H9-fermented milk. The maximum level of blood pressure reduction was at 8 h postadministration. The blood pressure resumed to the untreated level after 24 h. No significant change in the heart rate in all the groups ($P > 0.05$) was observed, suggesting that the treatments exerted no adverse effect to the circulatory system of the SHR.

The level of antihypertensive effect of H9-fermented milk seems to be less potent, but with a longer effective time as compared with other previous studies. For example, Nakamura et al. (1995) evaluated the antihypertensive effect of yogurt calpis in SHR. The SBP of the rats significantly decreased after feeding for 6 to 8 h with the maximum decrease of 21 mmHg. Similar to our results, the blood pressure returned to the original level after feeding for 24 h. Takano (1998), Miguel et al. (2007), and Yamamoto et al. (1999) reported an antihypertensive range of attenuation of 6.3 to 41.2 mmHg after a one-time feeding of fermented milk of *L. helveticus* strains CPN4, CP190, and CP191 or the active peptide purified from the yogurt; the effective time was 6 to 10 h. The relatively low efficacy level but prolonged effective duration of antihypertensive effect observed in our study may be explainable by the high resistance and stability of the ACEI peptides in the H9-fermented milk to the gastrointestinal protease digestion and its requirement to be processed by gastrointestinal protease before the release of the maximum activity. These interesting features merit further study, relating in particular to their stability and bioavailability in simulated gastric juices and when passing through the digestive tract in vivo.

Figure 3. The effect of single oral dose of yogurt on blood pressure and heart rate of spontaneously hypertensive rats. Data are shown in mean $\pm$ SEM; SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate. Spontaneously hypertensive rats (SHR) received 0.9% NaCl (control), *Lactobacillus helveticus* H9 (H9)-fermented milk, or commercial yogurt. An asterisk (*) indicates a significant difference between the treatment and saline control groups at the same time point ($P < 0.05$).

Antihypertensive Effect of Long-Term Oral Administration of H9-Fermented Milk

The antihypertensive effect of long-term administration of H9-fermented milk was assessed and compared with animals administered 0.9% NaCl, commercial

yogurt, or captopril (an ACEI) in SHR rats (Figure 4). Both the SBP and DBP of all the SHR increased gradually throughout the 7 wk of the experiment. Such an increase was a natural feature of SHR, with their blood pressure rising from the age of 6 wk until 22 to 36 wk (Mujumdar et al., 2001). As the SHR used

in this experiment were 8 wk old at the start of the experiment, they were still at the blood pressure elevation phase throughout the experiment. Therefore, the antihypertensive effect was only comparable between sample groups within the same time point.

Throughout the 7 monitored weeks, both the SBP and DBP of the positive control group, administered with captopril, were significantly lower than the saline control group ($P < 0.05$). Captopril is a pure form of proline-derived ACE inhibitor used for treating hypertension and other cardiovascular conditions, thus a strong antihypertensive effect was expected and was demonstrated. A significant antihypertensive effect for both SBP (detectable from wk 5) and DBP (detectable from wk 6) was observed after the continuous administration of H9-fermented milk, as compared with the control and commercial yogurt groups (Figure 4). Relative to the control group receiving saline, the SBP and DBP in SHR administered with H9-fermented milk were 12 and 10 mmHg lower, respectively, after feeding for 5 wk, and the effect lasted consistently until

the experiment ended. The long-term administration of H9-fermented milk did not reduce the blood pressure in WKY rats (Figure 4), suggesting that it had no adverse effect on normal blood pressure. The antihypertensive effect of H9-fermented milk was lower than that of the values reported in 2 other similar studies, ranging from 17 to 21 mmHg; however, this was most likely due to the administration of a much smaller amount of H9-fermented milk and, hence, IPP and VPP peptides in our study (around 2–5 mL per rat daily depending on the weight of the animals versus oral ad libitum administration in other studies). Several reports have shown that the antihypertensive effect of milk peptides is dose-dependent (de Leeuw et al., 2009; Hirota et al., 2011).

Effect of Long-Term Oral Administration of H9-Fermented Milk on LVMI

Long-term hypertension may cause pathological changes in target organs, such as the heart, brain,

Figure 4. The effect of long-term yogurt and captopril administration on the systolic (SBP) and diastolic (DBP) blood pressure of rats. Spontaneously hypertensive rats (SHR) received 0.9% NaCl (control), *Lactobacillus helveticus* H9 (H9)-fermented milk, commercial yogurt, or captopril treatment. All Wistar-Kyoto (WKY) rats received H9-fermented milk. Data are shown in mean $\pm$ SEM; an asterisk (*) indicates a significant difference between the treatment and saline control groups of the SHR at the same time point ($P < 0.05$).

Figure 5. The effect of long-term yogurt and captopril administration on the left ventricular mass index (LVMI) of rats. Spontaneously hypertensive rats received 0.9% NaCl (control), *Lactobacillus helveticus* H9 (H9)-fermented milk, commercial yogurt, or captopril treatments, whereas all Wistar-Kyoto rats (WKY) received H9-fermented milk. Data are shown in mean $\pm$ SEM; an asterisk (*) and two asterisks (**) represent significant differences between the treatment and saline control groups at $P < 0.05$ and 0.01 , respectively.

and kidney. Hypertensive left ventricular hypertrophy is an independent risk factor for all cardiovascular complications and hypertensive heart disease, eventually resulting in heart muscle ischemia, left ventricular insufficiency, congestive heart failure, arrhythmia, and sudden death. The SHR are known to develop left ventricular hypertrophy at the age of 10 wk (Mujumdar et al., 2001), which make it an interesting model to investigate whether consuming dairy products confers a protective effect on such a condition. Left ventricular mass index is an important indicator for assessing the

pharmacodynamic of the drug treatment for hypertension. Therefore, the LVMI of SHR and WKY rats were determined at wk 7 (Figure 5). The LVMI of the SHR fed H9-fermented milk, commercial yogurt, or captopril were significantly lower than that of the control group (receiving 0.9% NaCl; $P < 0.05$), whereas the LVMI of the normal blood pressure WKY rats were significantly lower than that of the essential hypertensive rats ($P < 0.01$), confirming that a long-term intake of a fermented dairy product was effective in reducing the extent of left ventricular hypertrophy development.

Figure 6. The effect of long-term yogurt and captopril administration on BW of rats. Spontaneously hypertensive rats received 0.9% NaCl (control), *Lactobacillus helveticus* H9 (H9)-fermented milk, commercial yogurt, or captopril treatments, whereas all Wistar-Kyoto (WKY) rats received H9-fermented milk. Data are shown in mean $\pm$ SEM; an asterisk (*) represents significant difference between the treatment and saline control groups at $P < 0.05$.

Effect of Long-Term Oral Administration of H9-Fermented Milk on BW

The BW of SHR and WKY rats began to diverge at the age of 4 wk, and the rate of weight gain was much lower in SHR as compared with WKY rats (Dickhout and Lee, 1998). Interestingly, by the seventh week of our feeding experiment, SHR receiving H9-fermented milk were significantly heavier than rats from other groups ($P < 0.05$; receiving the saline, commercial yogurt, and captopril), which did not differ from one another (Figure 6). Our results suggest that the antihypertensive effect of H9-fermented milk was not due to weight loss of the SHR. More importantly, the H9-fermented milk was able to improve the retardation of weight gain in the SHR, indicating a possible improvement of their general health condition. This weight gain effect was not observed in the very similar study by Sipola et al. (2001). Conversely, a previous study reported that the long-term feeding with *L. helveticus*-fermented milk increased bone mineral density and bone mineral content in relation to BW in growing rats (Narva et al., 2004). The significance and mechanism for the weight gain effect observed in our study remains to be determined.

CONCLUSIONS

Our results have shown that traditional fermented products are excellent sources for functional LAB with ACEI and antihypertensive activities. Based on sequential experiments assaying the in vitro ACEI activity, change in ACEI activity upon gastrointestinal protease treatment, and fermentation properties, the novel potential probiotic *L. helveticus* H9 (IMAU60208) strain was selected. By feeding *L. helveticus* H9-fermented milk to essential hypertensive rats for once or multiple times over 7 wk, in vivo beneficial antihypertensive effects were observed. The current study has provided further scientific evidence on the in vivo function of consuming probiotics-based fermented dairy products, and identified a valuable starter strain for future development of antihypertensive functional food products.

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