The therapeutic effects of fermented milk with lactic acid bacteria from traditional Daqu on hypertensive mice

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ABSTRACT

Lactic acid bacteria (LAB), a type of microorganism widely used in functional foods, has gained notable research attention in recent years. Certain strains possess the proteolytic ability to release potentially antihypertensive peptides from dairy proteins, which prompted us to explore the LAB strains from an under-studied and unique ingredient, Daqu. We screened for 67 strains of LAB strains from traditional Daqu using the calcium dissolution ring method. Sixteen strains exhibiting angiotensin-converting enzyme inhibition (ACE-I) activity exceeding 50% were chosen for 16S rDNA sequencing and safety assessment. It is noteworthy that Enterococcus faecium CP640 and Lacticaseibacillus rhamnosus CP658 exhibited significant ACE-I activity, which was the result of strain fermentation in reconstituted skim milk. These 2 strains did not exhibit hemolytic activity or antibiotic resistance. They also did not produce biogenic amines and showed high survival rates in the gastrointestinal tract. Additionally, Enterococcus faecium CP640 and Lacticaseibacillus rhamnosus CP658 fermented milk exhibited a notable reduction in blood pressure levels in spontaneously hypertensive rats (SHR) compared with negative controls in SHR. Importantly, no adverse effect was observed in normal Wistar-Kyoto rats. Through the analysis of physiological, serum, and urine-related indicators, it was observed that Enterococcus faecium CP640 and Lacticaseibacillus rhamnosus CP658 have the potential to promote weight gain in SHR, alleviate excessive heart rate, improve renal function indicators, and effectively regulate blood sugar and uric acid levels in SHR. These 2 strains showed optimal properties in lowering blood pressure and have the potential to be used in functional dairy products in the future.

Key words: probiotic, lactic acid bacteria, angiotensin-converting enzyme, Daqu, hypertension

INTRODUCTION

Hypertension, a prevalent clinical condition worldwide, is characterized by elevated arterial blood pressure (systolic pressure ≥140 mm Hg or diastolic pressure ≥90 mm Hg). In the 21st century, hypertension continues to be an important risk factor contributing to the global burden of disease (Fursov et al., 2013). The medical field offers various preventive and treatment approaches for hypertension. Nevertheless, a complete radical cure for hypertension is currently unavailable, and most hypertensive patients rely on long-term use of antihypertensive medications. Unfortunately, millions of patients remain unresponsive to various antihypertensive drugs, leading to incomplete recovery (Carey et al., 2018). Consequently, early prevention and control of hypertension through a range of methods becomes crucial in effectively reducing its incidence.

When dealing with hypertension, a global disease, numerous studies have focused on targeting the elevated activity of angiotensin-converting enzyme (ACE) as the primary therapeutic approach. Chemical ACE inhibitors, including captopril, lisinopril, and enalapril, have been synthesized and employed as antihypertensive agents in clinical settings. Despite their ability to effectively reduce blood pressure, these inhibitors have been associated with various side effects (such as altered taste perception, skin rash, dry cough, and so on). Additionally, there are also shortcomings such as high price and poor compliance (Boschin et al., 2014). Given the escalating prevalence of hypertension, particularly in its severe form, there is a growing demand for more efficient and safer natural remedies for its treatment. The ACE inhibitors derived from food sources have demonstrated blood pressure-lowering activity (Fadda et al., 2017) and hold notable potential as alternatives to chemosynthetic drugs in clinical practice. These
natural ACE inhibitors can be employed for the prevention and initial treatment of high blood pressure, while also providing abundant nutritional resources for individuals seeking a healthy and low-risk approach (Li et al., 2017).

Lactic acid bacteria (LAB) are considered crucial bacterial strains in the food industry. In recent years, LAB strains have gained notable popularity and usage as probiotics in various domains, including food, medicine, and related fields. They have shown promising potential in areas such as cancer inhibition (Dehghani et al., 2021), serum cholesterol reduction (Aziz et al., 2017), diabetes treatment (Zhang et al., 2023), and obesity management (Fan et al., 2023). Baijiu, also known as Chinese white liquor, is a traditional distilled spirit deeply rooted in Chinese culture. It is renowned for its strong aroma, rich flavor, and historical significance. At the heart of Baijiu production lies a vital ingredient called “fermentation starter” or “Daqu” in Chinese (Sakandar et al., 2020; Shi et al., 2022). Daqu is a unique blend of microorganisms and grains that initiates and drives the fermentation process, shaping the distinctive characteristics of Baijiu (Sakandar et al., 2020; Xia et al., 2023). It comprises a diverse microbial population that secretes a range of vital enzymes, facilitating the decomposition of raw materials and the production of various flavor compounds (Fan et al., 2019). Sakandar et al. (2020) further noted that the microbial community is the main driving factor in the fermentation process, which may include mold, yeast, lactic acid bacteria and actinomycetes, among others. Lactic acid bacteria strains exhibit a high abundance and are the predominant species found in Daqu (Xiao et al., 2021). Therefore, Daqu serves as a promising source for screening potential probiotic strains. Researchers from different countries have successfully identified LAB probiotic strains from diverse sources such as dairy (Chen et al., 2014; Georgalaki et al., 2017; Li et al., 2017), infant feces (Ait Seddik et al., 2016), and coastal anchorage (Das et al., 2016). However, our objective is to identify potent probiotic LAB strains with marked blood pressure-lowering capabilities from traditional Daqu and investigate their potential for reducing blood pressure in spontaneously hypertensive rats (SHR). This research aims to provide fundamental scientific insights for the future development and application of these strains in functional dairy products.

**MATERIALS AND METHODS**

The animals used in this study complied with the guidelines for the care of laboratory animals and related guidelines. See the Animal Experiment to Analyze the Antihypertensive Effect of LAB Strain Fermented Milk section for more information.

**Isolation and Screening of LAB Strains**

Daqu (Chengdu Shuzhiyuan Liquor Co. Ltd.) was ground to a powder, and an appropriate amount of Daqu powder (10 g) was dissolved in 90 mL of sterilized saline and diluted in gradient. Using the calcium dissolution ring method (Dumitru et al., 2019), each 0.1 mL of diluent was coated on a solid MRS+CaCO₃ (0.3% CaCO₃) plate and incubated at 37°C for 24 h. Colonies with obvious transparent circles and gram-positive staining on the medium were selected, and purified until single bacteria appeared. The colonies were selected and stored in 30% glycerol tubes at −70°C.

**Preparation and Activity Determination of ACE Enzyme in Rabbit Lungs**

The ACE enzyme preparation solution was prepared following the method outlined by Cushman and Cheung (1971), with suitable modifications. Freshly slaughtered rabbit lung tissue weighing 20 g, obtained from a local farmers’ market, was sliced and thoroughly rinsed with pre-cooled potassium phosphate buffer to eliminate blood residues. The washed rabbit lung slices were then homogenized by blending them with the same buffer at a ratio of 1:10 (wt/vol) for 3 min. Lung tissue was centrifuged and solubilized with sodium deoxycholate. Dialysis was performed using a 20-fold volume of phosphate buffer, using a dialysis tube (Spectrum Medical Industries Inc., Houston, TX) with a molecular weight cutoff of 12–14 kDa, at 4°C for 24 h with buffer replacement every 6 h. Dialysis extracts were used for ACE enzyme activity assay.

The ACE activity in the rabbit lung extract was measured using the following procedure. A total of 250 μL of N-[(N-(benzoylglcyl)-L-histidyl]-L-leucine (HHL; Shanghai Hengyuan Biochemical Reagents Co. Ltd., LR) solution was combined with 70 μL of ACE preparation and incubated at 37°C for 30 min. The reaction was stopped by adding 250 μL of 1 mol/L HCl. Subsequently, 1 mL of ethyl acetate was added, mixed, and extracted for 20 s. The mixture was then centrifuged at 3,000 × g and 4°C for 20 min. The top layer was collected and ethyl acetate removed by 1 h evaporation in a 100°C water bath within the chemical hood. The resulting residue was dissolved in 1 mL of HPLC-grade water, and the absorbance was measured at 228 nm (A). A blank solution was prepared in the same manner, with the HCl mixed immediately before adding ACE. One unit of ACE activity is defined as the quantity necessary to catalyze the formation of 1 μmol of equine uric acid from the previously prepared HHL solution in 1 min at 37°C under standard assay conditions. The ACE enzyme activity was calculated according to the following formula:
ACE activity = (A − A₀) × 5.6 × 10⁻³,

where A represents the light absorption of the reaction mixture at 228 nm, and A₀ represents the light absorption of the blank solution at 228 nm.

After testing, the specific enzyme activity of ACE extracted from rabbit lung was determined to be 0.85 U/mg. Following freeze-drying, it was set at 0.1 U/mL.

Isolation and Purification of ACE-Inhibition Peptides

Following the activation of LAB strains, they were inoculated into liquid MRS medium at a 1% inoculum amount and cultured under the same conditions for 24 h. The fermentation supernatants were collected by centrifugation at 3,000 × g for 10 min at 4°C and suspended in sterile saline (0.9% NaCl) to achieve an optical density of 600 nm of 1. Then, 2% of the inoculum was added to sterile reconstituted skim milk powder (11%, wt/vol) and incubated at 37°C for 48 h. After incubation, the mixture was centrifuged at 10,000 × g for 10 min at 4°C. The pH of the solution was adjusted to 4.6 using 1 mol/L NaOH and subjected to centrifugation for another round of centrifugation at 7,000 × g for 10 min at 4°C. The resulting supernatant was further adjusted to pH 8.3 using 1 mol/L NaOH and subjected to centrifugation once more (7,000 × g, 10 min; Xia et al., 2020). They were then filtered through a 0.45 μm PVDF filter, followed by freeze-drying. The freeze-dried sample was stored at 4°C (Georgalaki et al., 2017).

Determination of ACE-Inhibition Activity

Three sets of tubes, labeled as a, b, and c, were prepared for the experiment. In group a, 120 μL of HHL solution with a concentration of 5 mmol/L was mixed with 20 μL of ACE inhibition (ACE-I) peptides; group b consisted of 120 μL of HHL solution and 20 μL of borate buffer; After adding 120 μL of HHL solution and 20 μL of ACE-I peptide in group c, 150 μL of HCl was immediately added to terminate the reaction. The 3 test tubes were then simultaneously incubated at 37°C for 5 min in a water bath. Twenty microliters of ACE enzymes (0.1 U/mL) were added to a and b groups and the water bath was again at 37°C for 1 h. Immediately after the water bath, 150 μL of HCl was added to group a and group b to terminate the reaction. Following the addition of 20 μL of ACE-I peptide to group b, and 20 μL of borate buffer to group a and group c, 1.0 mL of ethyl acetate (Sigma Aldrich Trading Co. Ltd., Shanghai, China) was added to all 3 groups. The mixture was then mixed for 20 s and centrifuged at 3,000 × g for 20 min at 4°C. The top layer was collected, and the solvent was evaporated in a 100°C water bath in the chemical hood for 1 h. The remaining residue was dissolved in 1 mL of HPLC-grade water, and the absorbance at 228 nm was measured using a spectrophotometer. The ACE-I activity was calculated according to the following formula:

ACE-I (%) = (A_b − A_a)/(A_b − A_c) × 100,

where A_a, A_b, and A_c are added lactate bacteria ACE-I peptide, blank control, and inactive LAB ACE-I peptide at 228 nm absorbance, respectively.

Inhibitory activity was expressed as the percent of ACE inhibition at 0.2 mg/mL peptide concentration and as the peptide concentration required to inhibit 50% of the original ACE activity (IC₅₀).

Identification of Selected LAB Strains by 16S rDNA Sequencing

The strain genome DNA was extracted by the test kit (chloroform, Chengdu Fuji Biotechnology Co. Ltd.), and then PCR amplification was performed (Ayyash et al., 2018). The PCR primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were employed during amplification. After sequencing and obtaining the results, a BLAST online (https://blast.ncbi.nlm.nih.gov/Blast.cgi) comparison was performed to confirm the strain classification.

Simulated Gastrointestinal Tract Survival of Strains

First, 0.1 g of peptone, 0.3 g of yeast extract, 0.2 g of NaCl, 0.04 g of glucose, and 0.3 g of pepsin (Shanghai Boao Biotechnology Co. Ltd.) were added into 100 mL of distilled water. The mixture was then thoroughly stirred and dissolved. Subsequently, the pH was adjusted to 3.0, and the solution was filtered through a 0.40-μm filter membrane to eliminate bacteria to obtain an artificial gastric juice.

Subsequent to this, a volume of 100 mL of artificial intestinal fluid was formulated. To this end, 0.1 g of trypsin (Shanghai Boao Biotechnology Co. Ltd.), 0.2 g of sodium taurocholate, and 0.24 g of sodium bicarbonate were dissolved within 100 mL of distilled water. The pH of this solution was calibrated to 6.8. Further refinement ensued with the adjustment of the final cholesterol content to a range of 70 to 100 μg/mL. The solution was ultimately subjected to filtration through a 0.40-μm filter membrane.

The test procedure employed the method described by Xiao et al. (2019), with suitable modifications. The activated seed solution was centrifuged at 3,000 × g at 4°C for 15 min, and the resulting supernatant was
discarded. After 3 washes, the bacterial suspension was resuspended in sterile normal saline (0.9% NaCl) and adjusted to an optical density of 600 nm of 1.9. The bacterial suspension was mixed with artificial gastric juice at a ratio of 1:9 and incubated on a shaker at 37°C and 150 rpm for 2 h. After incubation, the mixture was centrifuged under the same conditions for 15 min. The supernatant was collected, and an equal volume of artificial intestinal juice was added. The mixture was further incubated under the same conditions for 3 h. The viable bacteria were counted using the dilution coated plate method, and the number of viable bacteria was measured at 0, 2, and 5 h. The survival rate of LAB strains was calculated according to the following formulas:

\[
\text{LAB survival rate} (\%) = \left( \frac{A_{2h}}{A_{0h}} \right) \times 100;
\]

\[
\text{Survival rate in intestinal fluid of LAB} (\%) = \left( \frac{A_{5h}}{A_{2h}} \right) \times 100;
\]

\[
\text{LAB mimic gastrointestinal tract survival} (\%) = \left( \frac{A_{5}}{A_{0h}} \right) \times 100,
\]

where \( A_{0h} \) represents the number of live bacteria in the culture solution when just being inserted into artificial gastric juice, \( A_{2h} \) indicates the number of live bacteria after 2 h of culture, and \( A_{5h} \) indicates the number of live bacteria cultured in artificial intestinal solution for 3 h.

**Hemolytic Activity**

Modifications were made to the method described by Niu (2017). A sterilized 0.6 mm filter sheet was gently placed on the surface of 5% defibrated sheep blood agar plates (Beckman Biotechnology Co. Ltd.). Then, 0.02 mL of the activated 24-h seed solution was absorbed and dropped onto the filter paper. The plate was subsequently incubated at 37°C for 48 h. Observations were made during and after the incubation period.

**Ability of Biogenic Amine Production**

Biogenic amine evaluation was conducted following the method described by Das et al. (2016), with appropriate modifications. After activation, the strains were inoculated into MRS liquid medium at a 1% inoculation level, along with the addition of 0.005% pyridoxal-5-phosphate (Shanghai Hanxiang Biotechnology Co. Ltd.) and 0.1% precursor AA (Sichuan Jiayinglai Technology Co. Ltd.). After 6 activations, the strains were inoculated at a 10% (vol/vol) level into a modified medium containing histidine, lysine, arginine, and ornithine. The culture medium was sealed with paraffin oil and incubated at 37°C for 3 d. The appearance of a light blue or purple color in the culture medium indicates the production of corresponding biogenic amines by the experimental strains.

**Determination of Antibiotic Susceptibility**

According to the method described by Das et al. (2016), antibiotic susceptibility was tested using the disk diffusion method. The strains were activated for 24 h and then 0.1 mL was evenly spread on MRS solid medium. After the surface of the plates dried, antibiotic disks (Beckman Biotechnology Co. Ltd.) were gently placed on the surface of the medium. The plates were then incubated at 37°C for 24 h to observe the formation of clear zones.

**Determination of pH, Titratable Acidity, Viable Bacteria Number, Proteolytic Activity, and ACE-I Activity During Milk Fermentation**

Two strains were inoculated into skim milk powder purchased at a local market and fermented under their respective optimal conditions. The fermentation process of LAB strains is depicted in Supplemental Figure S1 (https://doi.org/10.6084/m9.figshare.24633171.v1; Jiang et al., 2023). For the CP640 strain, the optimal fermentation conditions included an inoculum size of 2.5%, fermentation temperature of 37°C, fermentation time of 36 h, and milk powder concentration of 16%. As for the CP658 strain, the optimized fermentation conditions were an inoculum size of 2.5%, fermentation temperature of 37°C, fermentation time of 48 h, and milk powder concentration of 16% (Supplemental Tables S1, S2, and S3; https://doi.org/10.6084/m9.figshare.24633171.v1; Jiang et al., 2023).

Samples were collected every 4 h from the fermentation process to determine the viable bacteria count, ACE-I activity, pH, titratable acidity, and proteolytic activity (Amani et al., 2017). The relationship between ACE-I activity and pH, titratable acidity, viable cell count, and protein hydrolysis activity was investigated using Pearson correlation analysis.

**Stability of ACE-I Peptide in Vitro**

Initially, an HCl solution with a pH of 3 was formulated, and 1% pepsin was incorporated based on mass fraction to create the simulated gastric juice (SGF).

Subsequently, a 50 mmol/L KH₂PO₄ solution was prepared, and the pH was adjusted to 6.8. Following
Effect of LAB Strain Fermented Milk Animal Experiment to Analyze the Antihypertensive

To characterize the properties of ACE-I peptides and their resistance to gastrointestinal proteases in fermented milk, SGF and SIF were prepared. The determination method followed the study by Chen et al. (2010) with some modifications. The experiment was divided into 4 treatments: Normal fermentation of the strain to produce fermented milk, and ACE inhibition activity was measured by sampling as a blank control; Testing the digestion of ACE-I peptides in the gastric tract. The pH of fermented milk was adjusted to pH = 3.0 using 1 mol/L HCl. Then, it was mixed with the configured SGF at a ratio of 1:10 (Hasan et al., 2006; Megías et al., 2009), and incubated at 37°C for 3 h. Digestion was terminated in boiling water bath for 10 min, and ACE-I activity was measured at last. To test the digestion of ACE-I peptides in the intestine, the strain was first adjusted to pH = 6.8 in fermented milk with the same conditions, and the subsequent operation was similar to gastric digestion. The effect of pepsin digestion first followed by trypsin digestion was tested. The operation method was the same as above. After simulated gastric juice was treated and digestion was terminated at high temperature, the pH was adjusted immediately for SIF digestion, and finally the changes in its ACE-I activity were measured.

Animal Experiment to Analyze the Antihypertensive Effect of LAB Strain Fermented Milk

Male SHR and Wistar-Kyoto (WKY) rats (7 wk of age, weighing 180–200 g) were purchased from Beijing Victoria Lihua Experimental Animal Technology Co. Ltd. The 24 SHR rats were divided into 4 groups with 6 rats each, whereas the 12 WKY rats were divided into 2 groups with 6 rats each. The animals would be properly labeled and housed in a clean animal room at the Laboratory Animal Center with free access to food and tap water. The room was maintained at a temperature of 22 ± 2°C, humidity of 55 ± 5%, and a 12-h day-night cycle. The rats underwent a 7-d adaptation period, during which their blood pressure was measured to ensure stability. After the blood pressure stabilized, gavage administration was performed at a fixed time every day, with one gavage session per day for a duration of 4 wk. The methods of gavage are shown in Table 1, and the captopril tablets used were obtained from Chengdu Laogouyaooa Pharmacy. The BW of the rats was measured weekly. The tail sleeve method (BP-98, Softron) was used to monitor systolic pressure (SBP), diastolic pressure (DBP), and heart rate (blood pressure monitor: Shanghai Yuyan Scientific Instrument Co. Ltd.).

One day before the end of the experiment in the fourth week, a 12-h urine collection was performed. Following a 12-h fasting period, the rats were intraperitoneally injected with 30 mg/kg of 1.5% pentobarbital sodium for active extraction of abdominal blood and serum separation. The serum samples were stored at −80°C for further analysis. The blood glucose (GLU) levels in the serum were measured using a blood glucose meter (Jiangsu Yuyue Medical Equipment & Supply Co. Ltd.); The levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and uric acid (UA) in the serum were measured using rat ELISA kits specifically designed for TC, TG, HDL-C, LDL-C, and UA. The concentration of microalbumin (ALB) in the urine was determined using a rat microalbuminuria (MAU) ELISA kit. The ELISA kits mentioned above were provided by Nanjing Jiancheng Bioengineering Institute. All of the experimental procedures involving animals were conducted in accordance with animal laws and relevant guidelines (Chengdu Senwei Experimental Animal Co. Ltd.; License number: SYXX [Chuan] 2020–0232).

Table 1. Groups and gavage doses of rats in animal experiment

| Animal                | Group                      | Treatment feeding
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Spontaneously</td>
<td>Experimental group CP640</td>
<td>CP640 fermented milk</td>
</tr>
<tr>
<td>hypertensive rats</td>
<td>Experimental group CP658</td>
<td>CP658 fermented milk</td>
</tr>
<tr>
<td></td>
<td>Positive control group</td>
<td>Captopril tablets</td>
</tr>
<tr>
<td></td>
<td>Negative control group</td>
<td>Normal saline</td>
</tr>
<tr>
<td>Wistar-Kyoto rats</td>
<td>Blank group CP640</td>
<td>CP640 fermented milk</td>
</tr>
<tr>
<td></td>
<td>Blank group CP658</td>
<td>CP658 fermented milk</td>
</tr>
</tbody>
</table>

The gavage dose was consistent among the groups, with a dosage of 20 mg/kg per day.

Statistical Analysis

All measurements were repeated independently in triplicate. Statistical comparisons were made when applicable using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). SPSS 21.0 (IBM, Armonk, NY) statistical software was used for correlation analysis. Differences were considered significant at $P < 0.05.$
RESULTS AND DISCUSSION

**Determination of ACE-I Activity and 16S rDNA Sequencing**

A total of 67 LAB strains exhibiting transparent rings and positive Gram stains in MRS+CaCO₃ medium were screened and numbered sequentially as CP601-CP667. The ACE-I activity of 67 strains was measured using a spectrophotometric method (Table 2). The results showed that the ACE inhibition rate of the 58 strains ranged from 4.67% to 76.48% and 9 strains did not exhibit significant ACE-I activity. Among them, 16 strains exhibited an inhibition rate greater than 50%, with 4 strains (CP647, CP653, CP640, and CP658) showing an inhibition rate greater than 70%. Additionally, the CP653 strain demonstrated the highest ACE inhibition activity at 76.48% ± 3.16%, IC₅₀ was 0.131 mg/mL. In the analysis of 16 LAB strains with ACE-I activity >50%, the results revealed that the strains can be classified into 5 species (Figure 1): Lactobacillus acidipiscis (CP644, CP653, CP636), Leuconostoc mesenteroides (CP634, CP635, CP642), Lactocaseibacillus rhamnosus (CP667, CP658), Enterococcus lactis (CP638, CP651, CP657, CP639), and Enterococcus faecalis (CP640, CP647, CP661, CP645). The similarity among the strains ranged from 99.72% to 100.00% (Supplemental Table S4; https://doi.org/10.6084/m9.figshare.24633171.v1; Jiang et al., 2023).

In this study, bacteria with high ACE-I activity were mainly found in Enterococcus and Lactobacillus species. In a study conducted by Georgalaki et al. (2017), among 20 strains of bacteria involved in the fermentation of skim milk, 8 strains of Enterococcus and 4 strains of Lactobacillus exhibited ACE-I activity exceeding 70%. These findings are consistent with our research results, indicating that Enterococcus and Lactobacillus possess favorable characteristics in terms of ACE inhibition. In a similar study, the IC₅₀ value displayed by milk fermented with Lactoccaseibacillus casei PRA205 was 3.9 times lower compared with milk fermented with Lb. rhamnosus PRA331. (Solieri et al., 2015). It is speculated that the variations in ACE-I activity among different LAB strains may be attributed to individual differences and their ability to hydrolyze proteins and generate specific peptides. In the study by Moslehhishad et al., the lowest IC₅₀ value was observed in the <5 kDa peptide fraction obtained after hydrolyzing milk proteins with Lb. rhamnosus protease (Moslehhishad et al., 2013). It has been established that most of the antihypertensive peptides are short peptides with Pro residues at the C-terminus (Mizuno et al., 2004). Short peptides containing Pro at the C-terminus exhibit resistance to digestive enzymes and

### Table 2. Determination of angiotensin-converting enzyme inhibition (ACE-I) activity of 67 strains of lactic acid bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>ACE-I (%)</th>
<th>IC₅₀ (mg/mL)</th>
</tr>
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<tbody>
<tr>
<td>CP601</td>
<td>22.34 ± 2.42</td>
<td>0.418 ± 0.037</td>
</tr>
<tr>
<td>CP602</td>
<td>42.34 ± 2.62</td>
<td>0.236 ± 0.018</td>
</tr>
<tr>
<td>CP603</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CP604</td>
<td>12.34 ± 2.56</td>
<td>0.810 ± 0.063</td>
</tr>
<tr>
<td>CP605</td>
<td>18.2 ± 1.86</td>
<td>0.549 ± 0.059</td>
</tr>
<tr>
<td>CP606</td>
<td>26.57 ± 3.12</td>
<td>0.376 ± 0.024</td>
</tr>
<tr>
<td>CP607</td>
<td>17.21 ± 3.12</td>
<td>0.581 ± 0.037</td>
</tr>
<tr>
<td>CP608</td>
<td>25.94 ± 1.48</td>
<td>0.386 ± 0.052</td>
</tr>
<tr>
<td>CP610</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CP611</td>
<td>26.24 ± 2.31</td>
<td>0.381 ± 0.033</td>
</tr>
<tr>
<td>CP613</td>
<td>32.34 ± 2.43</td>
<td>0.309 ± 0.025</td>
</tr>
<tr>
<td>CP614</td>
<td>6.14 ± 2.35</td>
<td>1.629 ± 0.035</td>
</tr>
<tr>
<td>CP615</td>
<td>41.14 ± 3.15</td>
<td>0.243 ± 0.015</td>
</tr>
<tr>
<td>CP617</td>
<td>36.64 ± 3.16</td>
<td>0.273 ± 0.017</td>
</tr>
<tr>
<td>CP618</td>
<td>38.54 ± 2.15</td>
<td>0.259 ± 0.024</td>
</tr>
<tr>
<td>CP620</td>
<td>10.74 ± 2.96</td>
<td>0.931 ± 0.063</td>
</tr>
<tr>
<td>CP621</td>
<td>43.14 ± 3.18</td>
<td>0.232 ± 0.015</td>
</tr>
<tr>
<td>CP622</td>
<td>41.34 ± 3.31</td>
<td>0.242 ± 0.015</td>
</tr>
<tr>
<td>CP623</td>
<td>19.44 ± 2.22</td>
<td>0.514 ± 0.032</td>
</tr>
<tr>
<td>CP625</td>
<td>31.26 ± 2.24</td>
<td>0.320 ± 0.029</td>
</tr>
<tr>
<td>CP626</td>
<td>33.21 ± 2.25</td>
<td>0.301 ± 0.019</td>
</tr>
<tr>
<td>CP627</td>
<td>7.44 ± 2.52</td>
<td>1.344 ± 0.107</td>
</tr>
<tr>
<td>CP628</td>
<td>49.34 ± 3.23</td>
<td>0.203 ± 0.017</td>
</tr>
<tr>
<td>CP629</td>
<td>34.44 ± 3.26</td>
<td>0.298 ± 0.018</td>
</tr>
<tr>
<td>CP630</td>
<td>40.54 ± 2.68</td>
<td>0.247 ± 0.018</td>
</tr>
<tr>
<td>CP632</td>
<td>17.56 ± 2.18</td>
<td>0.569 ± 0.052</td>
</tr>
<tr>
<td>CP633</td>
<td>64.74 ± 1.46</td>
<td>0.154 ± 0.021</td>
</tr>
<tr>
<td>CP634</td>
<td>51.14 ± 2.36</td>
<td>0.196 ± 0.017</td>
</tr>
<tr>
<td>CP635</td>
<td>55.18 ± 3.14</td>
<td>0.181 ± 0.012</td>
</tr>
<tr>
<td>CP636</td>
<td>23.42 ± 2.61</td>
<td>0.427 ± 0.033</td>
</tr>
<tr>
<td>CP637</td>
<td>57.34 ± 3.32</td>
<td>0.174 ± 0.011</td>
</tr>
<tr>
<td>CP638</td>
<td>62.54 ± 2.65</td>
<td>0.160 ± 0.012</td>
</tr>
<tr>
<td>CP639</td>
<td>73.24 ± 3.15</td>
<td>0.137 ± 0.009</td>
</tr>
<tr>
<td>CP640</td>
<td>28.35 ± 2.55</td>
<td>0.353 ± 0.041</td>
</tr>
<tr>
<td>CP641</td>
<td>64.61 ± 2.53</td>
<td>0.155 ± 0.012</td>
</tr>
<tr>
<td>CP642</td>
<td>9.17 ± 1.62</td>
<td>1.091 ± 0.135</td>
</tr>
<tr>
<td>CP643</td>
<td>61.13 ± 1.78</td>
<td>0.164 ± 0.018</td>
</tr>
<tr>
<td>CP644</td>
<td>55.64 ± 2.61</td>
<td>0.180 ± 0.014</td>
</tr>
<tr>
<td>CP645</td>
<td>38.25 ± 2.64</td>
<td>0.261 ± 0.020</td>
</tr>
<tr>
<td>CP646</td>
<td>74.24 ± 2.24</td>
<td>0.135 ± 0.012</td>
</tr>
<tr>
<td>CP647</td>
<td>8.74 ± 1.62</td>
<td>1.144 ± 0.141</td>
</tr>
<tr>
<td>CP648</td>
<td>38.23 ± 2.16</td>
<td>0.262 ± 0.024</td>
</tr>
<tr>
<td>CP649</td>
<td>42.43 ± 3.42</td>
<td>0.236 ± 0.014</td>
</tr>
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<td>CP650</td>
<td>57.34 ± 3.32</td>
<td>0.174 ± 0.015</td>
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<td>CP651</td>
<td>39.25 ± 2.27</td>
<td>0.255 ± 0.022</td>
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<td>CP652</td>
<td>76.48 ± 3.16</td>
<td>0.131 ± 0.008</td>
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<td>CP653</td>
<td>31.56 ± 2.53</td>
<td>0.317 ± 0.025</td>
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<td>CP654</td>
<td>6.67 ± 2.32</td>
<td>2.141 ± 0.133</td>
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<td>CP655</td>
<td>14.34 ± 2.72</td>
<td>0.697 ± 0.051</td>
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<td>CP656</td>
<td>58.74 ± 2.23</td>
<td>0.170 ± 0.015</td>
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<td>CP657</td>
<td>75.24 ± 2.56</td>
<td>0.133 ± 0.010</td>
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<tr>
<td>CP658</td>
<td>36.14 ± 2.58</td>
<td>0.277 ± 0.021</td>
</tr>
<tr>
<td>CP659</td>
<td>20.31 ± 2.57</td>
<td>0.492 ± 0.038</td>
</tr>
<tr>
<td>CP660</td>
<td>66.18 ± 1.94</td>
<td>0.151 ± 0.016</td>
</tr>
<tr>
<td>CP661</td>
<td>44.63 ± 2.37</td>
<td>0.224 ± 0.019</td>
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<tr>
<td>CP662</td>
<td>38.24 ± 2.37</td>
<td>0.262 ± 0.022</td>
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<tr>
<td>CP663</td>
<td>10.14 ± 2.87</td>
<td>0.986 ± 0.060</td>
</tr>
<tr>
<td>CP664</td>
<td>47.32 ± 2.57</td>
<td>0.211 ± 0.016</td>
</tr>
<tr>
<td>CP665</td>
<td>40.25 ± 2.38</td>
<td>0.248 ± 0.021</td>
</tr>
<tr>
<td>CP666</td>
<td>61.62 ± 2.18</td>
<td>0.162 ± 0.015</td>
</tr>
</tbody>
</table>

Data are mean ± SD from triplicate experiments; IC₅₀ = median inhibitory concentration.
are more easily transported into the bloodstream (Ko- 
rhonen and Pihlanto, 2006). Gobbetti et al. reported the 
formation of ACE-I peptides released from milk 
proteins by starter cultures containing proline-specific 
peptidases during fermentation (Gobbetti et al., 2000). 
*Lacticaseibacillus rhamnosus* possesses a protein hydro-
lysis system that includes proline-specific peptidases, 
such as proline-specific aminopeptidase (PepR) and 
X-prolyl-dipeptidyl aminopeptidase (PepX; Paster et 
al., 2003; Savijoki et al., 2006). This may lead to the 
accumulation of bioactive ACE-inhibitory peptides in 
fermented milk. This indicates that *Lb. rhamnosus* 
is capable of producing effective ACE-inhibitory peptides 
from milk proteins, particularly from casein with a high 
level of proline residues and explains the reason why 
*Lb. rhamnosus* exhibited high ACE inhibition activity 
in this study.

**Survival of LAB Strains in Conditions Mimicking the Gastrointestinal Tract**

In Figure 2, it can be observed that CP651, CP634, 
CP640, and CP658 exhibit relatively high viability 
compared with the other strains. After a 2 h treatment 
in artificial gastric juice, all 16 strains still maintain a 
survival rate of over 50%, with bacterial counts ranging 
from $10^8$ to $10^9$ cfu/mL. However, upon adding artificial 
intestinal juice for a 3 h digestion, the viability of the 
LAB strains significantly decreases. Only a few strains 
manage to survive, with bacterial counts ranging from 
$10^5$ to $10^6$ cfu/mL. Although the numbers are lower, 
they still meet the requirements for exhibiting prebi-
otic abilities as probiotics. These results highlight the 
resilience of CP651, CP634, CP640, and CP658 strains 
to gastric digestion and their potential to survive and 
exert beneficial effects in the intestinal environment.

Lactic acid production during fermentation by LAB 
strains causes acidification of carrier matrix. This un-
dissociated organic acid enters the cell through simple 
diffusion and dissociate inside the cell due to high pH. 
This leads to cause acidic cytoplasm and damages 
DNA, proteins, and many vital biomolecules which are 
necessary for the cell’s viability (Wu et al., 2012). Low 
pH in gastric conditions also prevents the microbial 
colonization in the gastrointestinal tract (Pérez Mon-
toro et al., 2018).
Pérez Montoro et al. (2018) proposed that neutralization of cytoplasm by metabolism of AA is one of the mechanisms activated during acid stress by the probiotic bacteria. Amino acid decarboxylation produces ATP as well as neutralize acid by producing alkaline metabolites. Probiotics can boost the functioning of the F$_0$F$_1$ ATPase that uses ATP to propel the evacuation of H from the cell and so maintain pH homeostasis. *Lacticaseibacillus rhamnosus* GG increased F$_0$F$_1$-ATP synthase production, when grown in whey broth (5% hydrolyzed whey, 0.6% casein hydrolysate and 0.0015% MnSO$_4$, and water, pH 5.8). Furthermore, another study has shown that when *Lb. rhamnosus* is exposed to pH 4.8, the ClpE chaperone concentration was 2 times greater in the late lag phase of growth, demonstrating its defensive function in acidic stress (Koponen et al., 2012). In this experiment, under low pH conditions, it is speculated that *Lb. rhamnosus* CP658 achieved the highest survival rate by utilizing the mechanisms mentioned above.

**Evaluation of Hemolytic Activity, Ability of Biogenic Amine Production, and Antibiotic Susceptibility of LAB Strains**

It is necessary to perform safety analysis on the selected LAB strains to provide relevant safety data for their potential use in pharmaceuticals or food additives. Safety assessment is primarily conducted based on the following 3 aspects: hemolytic activity screening, evaluation of biogenic amine production, and antibiotic susceptibility testing.

The results of hemolytic activity and biogenic amine production experiments for the 16 strains of LAB strains are shown in Table 3. None of the 16 strains exhibited AA decarboxylation capability or produced biogenic amines during the cultivation process. Except for CP653 and CP639, which showed β-hemolysis, the other strains did not display hemolytic activity, indicating that these strains do not possess hemolytic capacity and pose no potential risk for hemolytic-related diseases. Overall, the majority of LAB strains in the Daqu are considered safe, nonpathogenic, and can be considered as potential probiotics.

The resistance testing of 16 LAB strains to common antibiotics (Table 4) revealed that the susceptibility to antibiotics varied significantly among the different strains. Most of the isolates demonstrated sensitivity to all the antibiotics tested, while 4 strains (CP644, CP634, CP639, CP645) exhibited resistance to specific antibiotics such as ceftriaxone, chloramphenicol, oxacillin, and vancomycin. These differences in sensitivity could be attributed to species and strain variations. Previous studies have suggested that the presence of β-lactamase in strains is often associated with resistance to β-lactam antibiotics, including ceftriaxone, ampicillin, and oxacillin (Chung et al., 2008). Similarly, resistance to aminoglycosides (gentamicin and streptomycin) and glycopeptides (vancomycin) in LAB strains is primarily attributed to innate resistance mechanisms, potentially involving impermeability of their cell membranes (Liasi et al., 2009). Additionally, some LAB strains have been reported to exhibit resistance to chloramphenicol, as supported by the findings of Sharma et al. (2014). It is worth noting that the source and geographical location of LAB can also influence their antibiotic sensitivity patterns in potential probiotic strains (Al Kassaa et al., 2014). Further investigation is required to elucidate the
Table 4. Experimental results of resistance of 16 strains of lactic acid bacteria to common antibiotics

| Antibiotic          | CP 638 | CP 639 | CP 640 | CP 641 | CP 642 | CP 643 | CP 644 | CP 645 | CP 646 | CP 647 | CP 648 | CP 649 | CP 650 | CP 651 | CP 652 | CP 653 | CP 654 | CP 655 | CP 656 | CP 657 | CP 658 | CP 659 | CP 660 | CP 661 | CP 662 | CP 663 | CP 664 |
|---------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Ampicillin (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Ceftriaxone (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Streptomycin (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Kanamycin (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Gentamicin (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Oxacillin (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Penicillin G (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Erythromycin (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Chloramphenicol (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Ciprofloxacin (µg/piece) | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      | S      |

S = sensitive; I = insensitive; R = resistant. The diameter of antibacterial zone ≥15 mm is highly sensitive (S), 5 to 15 mm is moderately sensitive (I), and <5 mm is insensitive (R), that is, strong resistance.

2Refer to Charteris et al. (1998) and Community and Labor Studies Institute (CLSI) for determination of antibiotic disk concentration.

During milk fermentation CP640 and CP658 were selected for further experiments due to their favorable gastrointestinal viability and ACE-I activity, after eliminating strains with hemolytic activity and antibiotic resistance.

As shown in Figure 3, during fermentation, both CP640 and CP658 strains exhibited a decrease in pH and an increase in titrated acidity (a). The production of lactic acid contributed to the pH reduction and the characteristic sour taste of yogurt. According to consumer preferences, acceptable acidity levels for yogurt can vary. Some individuals may enjoy yogurt with a surprisingly acidic pH as low as 3.0, whereas others may prefer a relatively neutral pH of 5.5. Commercially available yogurts typically have a pH range of 4.0 to 4.5, which falls within the acceptable acidity range for most consumers (Chen et al., 2018). The viable bacterial count of CP640 strain showed rapid growth during the logarithmic phase (4–16 h; b), and the pH dropped to 4.6 to 5.0, resulting in the curdling of the fermented milk. From 16 h to the stable phase, the growth rate and mortality were relatively stable, while the pH continued to decrease and the titrated acidity continued to increase (a). The ACE-I activity of CP640 strain increased rapidly from 0 to 16 h, reached a plateau from 16 to 20 h, and gradually decreased thereafter. The highest ACE-I activity was 77.74% (d). Similar trends were observed for CP658 strain, with its logarithmic phase occurring between 8 and 20 h (b). The strain entered the stable phase after 20 h, and its pH was lower in the later stage of fermentation compared with CP640 strain (a). The ACE-I activity of CP658 strain followed a similar growth pattern as CP640 strain but exhibited higher activity. The highest ACE-I activity was 79.54% (d). In summary, both CP640 and CP658 strains demonstrated favorable fermentation characteristics, including pH reduction, increased titrated acidity, rapid growth during the logarithmic phase, and significant ACE-I activity.
We employed the Pearson correlation coefficient to examine their correlations (Figure 4). The ACE-I activity is closely associated with these 4 characteristics. The ACE-I activity showed positive correlations with viable cell count ($\rho = 0.6$), acidity ($\rho = 0.6$), and protein hydrolysis activity ($\rho = 0.7$), while it exhibited a negative correlation with pH ($\rho = −0.8$). We derive from that during the logarithmic phase of bacterial growth (Figure 3 a), the rapid increase in viable bacteria leads to increased production of proteases or peptidases. These enzymes hydrolyze bovine milk protein, resulting in the generation of ACE-I peptides. As the number of viable bacteria continues to increase, the ACE inhibition activity gradually enhances. However, during the stable phase, the content of ACE-I peptides reaches its maximum. As the titration acidity increases, the activity of proteases or peptidases may be inhibited, and ACE-I peptides can be decomposed. This results in a decrease in ACE inhibition activity, as observed in the changes in proteolytic activity (Figure 3 c). This can also explain the strong negative correlation between ACE-I activity and pH. The slightly greater increase in ACE inhibition activity of the CP658 strain compared with the CP640 strain could be attributed to the lower titrating acidity of the CP658 strain in the later stage of fermentation.

**Stability of ACE-I Peptides in Vitro**

During the fermentation process of food using LAB strains, food proteins are hydrolyzed by cell wall-associated proteases into short peptides. Subsequently, these peptides are transported into the cell and further broken down by intracellular peptidases into various bioactive peptides (Nielsen et al., 2009). These bioactive peptides have been demonstrated by numerous studies to possess potential antihypertensive activity. Indeed, for probiotic bacteria to successfully pass through the host’s gastrointestinal system, they must overcome several key obstacles, and one of these obstacles is the presence of proteases in the human gastrointestinal tract.

To test the gastrointestinal protease resistance and further characterize the properties of ACE-I peptides in fermented milk, milk samples fermented from CP640 and CP658 strains are subjected to digestion with...
pepsin, trypsin, and pepsin-trypsin. Interestingly, the ACE-I activity of the fermented milk from the 2 strains shows different responses to protease treatment (Figure 5). In the case of CP640 strain, regardless of the type of protease used, the fermented milk samples exhibit a significant increase in ACE-I activity compared with the undigested controls. This finding aligns with the results reported by Li et al. (2017) in their study. In contrast, the ACE-I activity of CP658 fermented milk is significantly reduced after protease treatment. This suggests that the ACE-inhibiting peptides in CP640 fermented milk are enhanced following digestion with gastrointestinal proteases, possibly due to the further release of active peptides or other active substances during digestion. However, the activity of ACE-inhibiting peptides in CP658 fermented milk decreases, which could be attributed to the hydrolysis of a small number of ACE-inhibiting peptides by gastrointestinal enzymes. The ACE-inhibiting substances produced by LAB in fermented milk are peptide-based, and digestion by host or microbial-derived proteases during gastrointestinal transit may lead to the inactivation of ACE-inhibiting substances or the release of bioactive peptides from dietary proteins, thereby increasing their bioavailability (Vermeirssen et al., 2003). In the digestive system, pepsin breaks down leucine residues and C-terminal aromatic AA, while trypsin preferentially attacks positively charged C-terminal AA, such as arginine and lysine (Neurath, 1957).

Maeno et al. identified an antihypertensive peptide (Lys-Val-Leu-Pro-Val-Pro-Gln) with high ACE-I activity after gastrointestinal enzyme digestion (Maeno et al., 1996). In similar studies, Saavedra and Quiros et al. discovered bioactive peptides in fermented milk, such as Leu-His-Leu-Pro-Leu-Pro-Leu, Val-Val-Pro-Phe, Leu-Thr-Gln-Thr-Pro-Val-Pro-Phe, and Val-Arg-Glu-Pro-Phe-Pro-Ile-Val. These peptides exhibited enhanced ACE-I effects when cultured with pepsin or pancreatic extract (Saavedra et al., 2013). The ACE-inhibiting peptides can be classified into 3 groups based on their response to ACE or gastrointestinal protease treatment: true inhibitors (no change in activity after protease treatment), substrates (decreased activity after protease treatment), and prodrugs (increased activity after protease treatment; Iroyukifuji et al., 2000). Based on our experimental observations, the ACE-I peptide in CP640 is likely a prodrug-type inhibitor, while the ACE-I peptide in fermented milk produced by CP658 is likely a substrate-type inhibitor.
Animal Experiment to Analyze the Antihypertensive Effect of LAB Strain Fermented Milk

Weight Change. In our study, it was observed that the weight of rats in each group increased over time (Figure 6). Specifically, WKY rats showed significantly higher weight gain compared with SHR starting from the second week of age. This difference in BW between SHR and WKY rats is consistent with previous research findings. It is worth noting that SHR are generally lighter than age-matched WKY rats, and this characteristic is associated with hypertension in gram progeny resulting from the cross between SHR and WKY rats (Xie et al., 2005). The exact reasons for this difference in BW between the 2 rat strains are not clear and require further investigation. The observed difference may indicate an underlying metabolic irregularity in SHR, which aligns with our study findings. Furthermore, it was noticed that the weight gain of SHR given CP640 and CP658 fermented milk was higher than that of the negative control group, although not statistically significant. This suggests that lactobacilli fermented milk may have a positive effect on alleviating delayed weight gain in SHR, potentially leading to improved overall health. A previous study has reported that long-term consumption of *Lactobacillus Swiss* fermented milk increased bone mineral density and bone mineral content in growing rats (Narva et al., 2004).

The exact significance and underlying mechanisms of the weight gain effects observed in our study require further investigation.

Heart Rate Change. After 14 d of gavage, significant differences in heart rate changes were observed between the CP640 and CP658 fermented milk group, the captopril positive control group, and the negative control group (Figure 7). The heart rate decreased slowly, indicating that ACE-I peptides can alleviate the symptoms of rapid heart rate in SHR. Additionally, no significant difference is observed in the blank group before and after gavage, suggesting that the ACE-I peptides produced by the experimental strain have no adverse effects on the heart rate of rats with normal blood pressure.

Blood Pressure Change. The SBP and DBP of SHR administered fermented milk produced by CP640 and CP658 strains show a consistent changing trend. To provide a more direct view of the blood pressure changes, the changes in SBP are presented on a weekly basis, indicating increases or decreases (Figure 8).

The SBP of all SHR gradually increases over the course of 4 wk. This continuous rise in blood pressure is a characteristic feature of SHR, as their blood pressure remains elevated from 6 to 36 wk of age (Mujumdar et al., 2001). As the experiment begins with SHR at 8 wk of age, they are already in a hypertensive state throughout the duration of the study. Therefore, the
antihypertensive effect can only be compared between sample groups at the same time point to assess their relative effectiveness.

The positive controls treated with captopril have significantly lower SBP than the normal saline controls \( (P < 0.05) \). Captopril, a pure proline-derived ACE inhibitor, is used in the treatment of hypertension and other cardiovascular diseases and therefore is expected and demonstrated to have potent antihypertensive effects. Compared with the negative control group, a significant antihypertensive effect in the SBP (discovered from wk 3) of the rats after continuous application of CP640 and CP658 fermented milk, and the effect lasted until the end of the experiment. At the end of the experiment, the 2 strains decreased blood pressure by 18.27 and 17.13 mm Hg, respectively, compared with the negative control group. Long-term administration of CP640 and CP658 fermented milk have no significant effect on blood pressure of WKY rats, indicating that there is no adverse effect on normal blood pressure. The antihypertensive effect of fermented milk of CP640 strain is higher than that of fermented milk of CP658 strain, although it is not significant.

**Analysis of Serum and Urine.** In addition to basic physiological changes, such as BW, blood pressure, and heart rate, this study also collected arterial blood and urine samples during the gavage of SHR. The collected samples were used to assess changes in various metabolic parameters, including serum glucose (GLU), total cholesterol (CHOL), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), UA, and urinary ALB. These parameters provide insights into the metabolic profile and potential comorbidities associated with hypertension. While there may be some variations between the experimental results and the pathogenesis of human hypertension, the similarities in metabolic pathways between SHR and hypertensive patients make these measurements relevant for understanding the potential effects of CP640 and CP658 fermented milk on metabolic health.

The results of the study show that there are some changes in serum and urine parameters of SHR fed

![Figure 6. Effect of long-term gavage of fermented milk on BW of spontaneously hypertensive rats. eg CP640 = experimental group CP640; eg CP658 = experimental group CP658; nc = negative control; pc = positive control; bg CP640 = black group CP640; bg CP658 = black group CP658. Data are the means of triplicate experiments, and error bars indicate SD. * indicates a significant difference compared with the negative control \( (P < 0.05) \).]
fermented milk and captopril tablets compared with those fed normal saline. However, most of these changes are not statistically significant (Table 5). The levels of CHOL, TG, HDL-C, and LDL-C did not show significant differences between the experimental group and the control group. However, the concentrations of GLU and UA in the experimental group fed CP640 and CP658 fermented milk were significantly lower than those in the negative control group. Additionally, the ALB content was significantly higher in the experimental group compared with the negative control group. These changes indicate that ACE-I peptides may have a beneficial effect on renal function and can help in controlling blood glucose and UA levels in SHR.

Table 5. Test results of serum and urine of rats (in mmol/L unless otherwise noted)

<table>
<thead>
<tr>
<th>Item</th>
<th>eg CP640</th>
<th>eg CP658</th>
<th>pc</th>
<th>nc</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU</td>
<td>13.42 ± 1.35*</td>
<td>14.72 ± 2.14*</td>
<td>12.70 ± 1.45*</td>
<td>16.36 ± 2.35</td>
</tr>
<tr>
<td>CHOL</td>
<td>1.15 ± 0.11</td>
<td>1.16 ± 0.08</td>
<td>1.11 ± 0.09</td>
<td>1.14 ± 0.12</td>
</tr>
<tr>
<td>TG</td>
<td>1.32 ± 0.06</td>
<td>1.33 ± 0.04</td>
<td>1.32 ± 0.03</td>
<td>1.31 ± 0.03</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.30 ± 0.08</td>
<td>0.31 ± 0.09</td>
<td>0.35 ± 0.12</td>
<td>0.36 ± 0.14</td>
</tr>
<tr>
<td>LDL-C</td>
<td>0.14 ± 0.02</td>
<td>0.23 ± 0.01</td>
<td>0.29 ± 0.02</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>UA (µmol/L)</td>
<td>131.67 ± 6.54*</td>
<td>134.36 ± 7.24*</td>
<td>137.68 ± 6.12*</td>
<td>147.24 ± 8.31</td>
</tr>
<tr>
<td>ALB (mg/L)</td>
<td>0.48 ± 0.06*</td>
<td>0.54 ± 0.04*</td>
<td>0.40 ± 0.03</td>
<td>0.35 ± 0.08</td>
</tr>
</tbody>
</table>

GLU = glucose; CHOL = total cholesterol; TG = triglycerides; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; UA = blood uric acid; ALB = urinary microalbumin; eg CP640 = experimental group CP640; eg CP658 = experimental group CP658; nc = negative control; pc = positive control. Data are mean ± SD from triplicate experiments.

* indicates a significant difference compared with the negative control ($P < 0.05$).
By MRS+CaCO₃ plate and Gram staining, 67 strains of gram-positive *Lactobacillus* were screened from Daqu, which could produce a clear calcium-soluble ring. Sixteen strains with ACE-I activity over 50% were selected. These strains belonged to 5 species as determined by 16S rRNA sequencing. Safety evaluations showed that the LAB strains were mostly nonpathogenic. *Enterococcus faecalis* CP640 and *Lb. rhamnosus* CP658 were chosen for fermentation in milk due to their strong ACE-I activity and gastrointestinal survival. Long-term gavage of CP640 and CP658 fermented milk in SHR and WKY rats resulted in significant blood pressure reduction in SHR without adverse effects in WKY rats. The fermented milk also improved weight gain, heart rate, renal function, blood sugar, and UA levels in SHR. These findings provide guidance and a scientific basis for understanding the blood pressure-lowering mechanism and the development of probiotic products for blood pressure control.

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