ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Technol. Aliment. 14(2) 2015, 107–116
www.food.actapol.net pISSN 1644-0730 eISSN 1889-9594 DOI: 10.17306/J.AFS.2015.2.12

EFFECT OF INCUBATION TIME, INOCULUM SIZE, TEMPERATURE, PASTEURIZATION TIME, GOAT MILK POWDER AND WHEY POWDER ON ACE INHIBITORY ACTIVITY IN FERMENTED MILK BY *L. PLANTARUM LP69*

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ABSTRACT

Background. Angiotensin I converting enzyme (ACE) plays an important physiological role in regulating hypertension. Lactic acid bacteria are known to produce ACE inhibitory peptides which can lower hypertension during fermentation.
Methods. The effect of incubation time (0~36 h), inoculum size (3, 4, 5, 6 and 7%, v/v), temperature (25, 30, 35, 40 and 45°C), sterilization time (5, 10, 15, 20 and 25 min), concentration of goat milk powder (8, 10, 12, 14 and 16%, w/v) and whey powder (0.5, 0.6, 0.7, 0.8 and 0.9%, w/v) on ACE inhibitory peptides fermented from goat milk by *Lactobacillus plantarum LP69* was investigated using single factor experiment.
Results. The optimal incubation time, inoculum size, temperature, pasteurization time, goat milk powder and whey powder in fermented milk by *L. plantarum LP69* was 14 h, 3.0%, 35°C, 20 min, 14% and 0.70% for ACE inhibitory activity and 22 h, 3.0%, 40°C, 25 min, 16% and 0.60% for viable cell counts, respectively.
Conclusion. The incubation time, inoculum size, temperature, pasteurization time, goat milk powder and whey powder had a significant influence on ACE inhibitory activity in fermented milk by *Lactobacillus plantarum LP69*, the results are beneficial for further screening of main factors by using fractional factorial designs.

Key words: ACE inhibitory peptide, goat milk, *Lactobacillus plantarum*, inoculum size, pasteurization time, incubation time, whey powder

INTRODUCTION

Hypertension is a common disease, which leads to stroke, coronary heart disease, kidney dysfunction, disability and death (López-Fandiño et al., 2006), and it is often accompanied by obesity, hyperlipidemia, arteriosclerosis and coronary heart disease. The angiotensin converting enzyme (ACE, EC. 3.4.15.1) is a peptidyl dipeptide hydrolase and plays an important physiological role in regulating blood pressure (Leclerc et al., 2002). Therefore, inhibition of ACE activity is considered to be a useful approach in the treatment of hypertension.

Angiotensin converting enzyme inhibitors (ACE--inhibitors) plays an important role in the world of medicine. ACE-inhibitors prolong patients' life with ischemic heart disease, heart failure and impaired left ventricular contractility. Application of ACE inhibitors

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prevents adverse cardiac remodelling infarction after myocardialinfarction. Therefore, it is highly appreciated to research on the isolation and enhancement of its preparation. Studies on bioactive peptides are better planned and thereby, well documented.

Recently, certain functional foods containing ACE inhibitory peptides have been shown to act as an additional or alternative treatment in hypertension. Fermented milks containing many ACE-inhibitory peptides has been produced using different lactic acid bacteria or yeast, such as *Lactobacillus helveticus*, *L. casei*, *Lactobacillus casei* spp. *Pseudoplantarum*, *L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. lactis* ssp. *lactis*, *L. lactis* ssp. *Cremoris*, *Enterococcus faecalis* and *Clavispora lusitaniae KL4A* (Algaron et al., 2004; Ashar and Chand, 2003; Chen et al., 2012; Chaves-López et al., 2012; Fuglsang et al., 2003; Gobbetti et al., 2000; Hernández-Ledesma et al., 2004; Leclerc et al., 2002; Muguerza et al., 2006; Nakamura et al., 1995; Quirós et al., 2007; Robert et al., 2004; Rodríguez-Figueroa et al., 2010; Rokka et al., 1997; Shuang et al., 2008; Vermeirssen et al., 2003; Vishwanath and Purnima, 2014; Yamamoto et al., 1994a, 1994b, 1999), most studies were based on milk as a medium and seldom goat milk was used.

In our previous study, 28 probiotic Lactobacillus strains were used to ferment goat milk to obtain products with high Angiotensin I-converting enzyme inhibitory activity, the results showed that 20 strains had ACE inhibitory activity and among them 4 strains including *L. bulgaricus LB6*, *Lactobacillus reuteri LT33*, *Lactobacillus rhamnosus LR22* and *Lactobacillus plantarum LP69* (which was previously mistaken as *Lactobacillus helveticus*) were especially significant as producers of ACE-inhibitory peptides (Chen et al., 2012) and investigated carbon source, organic nitrogen source, salts and fermentation conditions on ACE inhibitory activity in fermented goat milk by *Lactobacillus bulgaricus LB6* (Shu et al., 2013a, 2013b, 2014). In this study, effect of incubation time, inoculum size, temperature, pasteurization time, concentration of goat milk powder and whey powder on ACE inhibitory activity in fermented milk by *Lactobacillus plantarum LP69* were investigated to provide reference for further optimization.

MATERIAL AND METHODS

Materials and reagents

Whole goat milk powder was purchased from Shaanxi Redstar Dairy Co., Ltd. (Weinan city, China). Hippuryl-histidyl-leucine (Hip-His-Leu) and ACE (extracted from rabbit lung, lyophilized powder) were bought from Sigma Chemical Co. (St Louis, MO, USA), whey powder (Hilmar 8010 WPC80, USA), was purchased from Xián Luosenbo Technology Co., Ltd. (Xián, China). All chemicals used were of analytical grade unless otherwise specified.

Microorganisms and activation

A pure culture of *Lactobacillus plantarum LP69*, isolated from inner yogurt and identified by 16S rDNA, was obtained from the College of Life Science and Engineering, Shaanxi University of Science and Technology. Stock cultures were stored at –20°C in freeze-dried powder prepared from 0.2 ml MRS broth containing *Lactobacillus plantarum LP69* and 0.2 ml 20% reconstituted skimmed milk by freeze-drying. The microorganism was activated successively three times in rehydrated de Mann Rogosa Sharpe (MRS) broth (Haibo media, Qindao, China) at 37°C for 24 h prior to use.

Preparation of fermented goat milk

125 g whole goat milk powder was added to water and attained 1000 ml reconstituted goat milk, then divided equally into 50 anaerobic tubes and pasteurized, inoculated with *Lactobacillus plantarum LP69*, and fermented at 37°C until coagulated, all fermentations were carried out by triplicate. The whey was collected by centrifugation at 5000 g for 15 min. The viable counts of *L. plantarum LP69* in the fermented milk were counted using de Man, Rogosa, Sharpe (MRS) agar (Haibo media, Qindao, China).

Measurement of ACE inhibitory activity

ACE inhibitory activity was measured by a spectrophotometric assay according to the method of Cushman and Cheung (1971) with some modifications. Added 80 µL of each sample to 200 µL sodium borate buffer (0.1 mol/L, pH 8.3) containing NaCl (0.30 mol/L) and HHL (5 mmol/L). Then, ACE (20 µL, 0.1 U/mL) was added and the reaction mixture was

incubated at 37°C for 30 min. The reaction was terminated by adding 250 μL 1 mol/L HCl. Adding 1.7 mL ethyl acetate to extract the hippuric acid formed and evaporated at 120°C for 30 min, redissolved it in 2 mL deionized water after cooled at room temperature, then the absorbance was measured at an optical density of 228 nm (OD_{280}). The activity of each sample was measured in triplicate. The ACE inhibitory rate was calculated using the following formula: ACE inhibition (%) = (A – B) / (A – C) × 100%, where A is the optical density without the whey fraction, B is the optical density without ACE and C is the optical density in the presence of both ACE and the whey fraction.

Determination of viable cell counts, pH and titration acidity in the fermented goat milk

The viable cell counts of *L. plantarum LP69* were determined by using the pour plate technique (Donkor et al., 2006), 1 ml of each fermented goat milk sample was added to 9 ml of saline water (0.9%, w/v, NaCl) containing 0.1 g/L peptone and water diluent followed by vortexing using autovortex mixer for 30 s. The resulting suspension was serially diluted in sterile 0.15% (v/v) peptone water (Oxoid) and 1 mL of the appropriate dilution was used for enumeration by the pour plate technique with MRS agar (Haibo media, Qindao, China) for 48–72 h at 37°C. All dilutions were plated in triplicate and results were expressed as colony forming units per milliliter (CFU/ml) of fermented milk (Chen et al., 2013). The pH in fermented goat milk was directly evaluated through a pH-meter (pHS-3C) at the room temperature and titration acidity was determined according to the sodium hydroxide titration method and Jill Nieer degrees (ºT) described, respectively.

RESULTS AND DISCUSSION

Effect of incubation time on ACE inhibitory activity in fermentated goat milk

The activated *L. plantarum LP69* at inoculum size 5% was transferred into 14% pasteurized reconstituted goat milk and cultured at 37°C for 36 h. The samples were taken out for determining ACE inhibition, viable cell counts, pH and titration acidity every 2 h for 0~24 h and every 3 h for 24~36 h. The results were shown in Figure 1.

The growth curve of *L. plantarum LP69* showed as "S" type, 0–2 h was a period of adjustment, the viable count of *L. plantarum LP69* was 8.49 × 10^6 CFU/mL

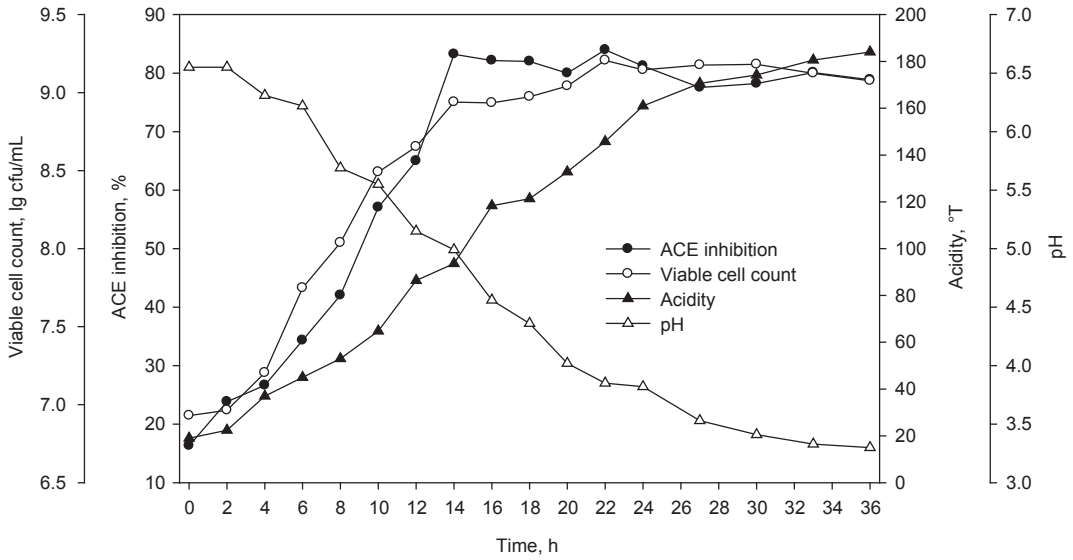

Fig. 1. Effect of incubation time on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

after introducing into milk and slowly increased, pH value decreased and the titration acidity increased slowly; 2–14 h was logarithmic phase for cell growth of *L. plantarum LP69*, the numbers of viable counts increased rapidly, changes of pH and acidity is large, when the pH value decreased to 4.6–5.0, the fermented milk began to appear curd; then *L. plantarum LP69* grew into the stable phase from 14 h. The viable count remained almost unchanged because the growth rate and death rate of *L. plantarum LP69* is basically the same, the value of pH continues to decline and titration acidity continued to increase, but the trend tended to be smooth. During the 36 h fermentation, the ACE inhibition in fermented goat milk increased rapidly in 0–14 h, tended to be smooth in 14–18 h, then decreased gradually from the beginning of 18 h, which was consistent with the growth regularity of *L. plantarum LP69*. The ACE inhibition increased with the increase in the number of live bacteria in the first 14 h when *L. plantarum LP69* was in logarithmic phase, the possible reasons was that the ACE inhibitory peptide content reached the maximum for the hydrolysis of goat milk protein by proteases or peptidase produced by *L. plantarum LP69*, but the proteases or peptidase was inhibited or the ACE inhibitory peptide was broken down when the titration acidity increased after 14 h, which led to ACE inhibition decrease. The ACE inhibition in fermented goat milk had no significant differences during 14~22 h. The ACE inhibitions, the viable count, pH and titration acidity in fermented goat milk by *L. plantarum LP69* was 83.21%, 8.7×10^8 CFU/ml, 4.99 and 93.6°T, but The ACE inhibition in fermented goat milk by *L. bulgaricus LB6* reached maximum (74.70%) at 12 h and the viable count, pH and titration acidity was 3.72×10^7 CFU/mL, 4.44 and 149.8°T (Shu et al., 2014), which showed *L. plantarum LP69* had higher ACE inhibition, grew faster but produced less lactic acid than *L. bulgaricus LB6*. Therefore, the incubation time was chosen as 14 h for further research on ACE inhibitory peptide produced by *L. plantarum LP69*, but the optimal incubation time for *L. bulgaricus LB6* was 12 h (Shu et al., 2014).

Effect of inoculum size on ACE inhibitory activity in fermentated goat milk

The *L. plantarum LP69* was inoculated into the 14% pasteurized reconstituted goat milk at different inoculum size (3, 4, 5, 6 and 7%), respectively. The results were shown in Figure 2.

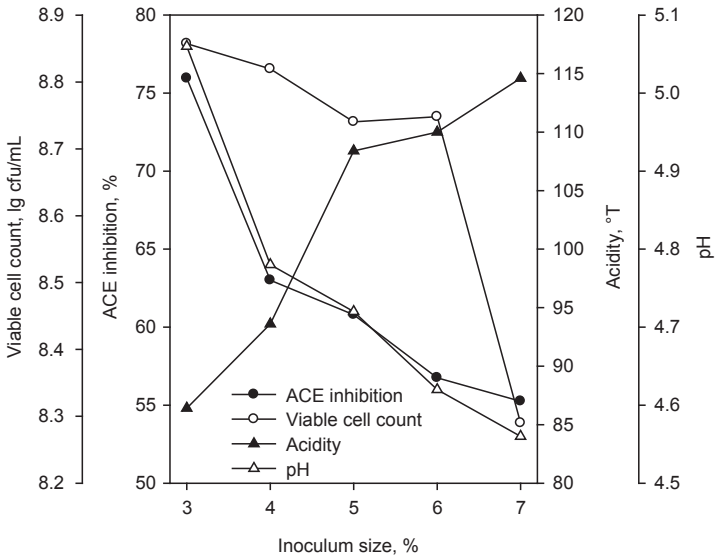

Fig. 2. Effect of inoculum size on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

As shown in Figure 2, both the viable cell counts of *L. plantarum LP69* and the ACE inhibition in fermented goat milk decreased with the inoculum size increasing. The viable cell counts of *L. plantarum LP69* in fermented goat milk decreased from 7.2×10^8 CFU/ml at 3% inoculum size to 1.95×10^8 CFU/ml at 7% inoculum size, and the ACE inhibition decreased from 75.95% at 3% inoculum size to 55.25% at 7% inoculum size. This may be due to the gradual increase of viable cells of *L. plantarum LP69*, the nutrients in reconstituted goat milk were not meet the growth need of *L. plantarum LP69*, which led to goat milk protein and peptides were hydrolysed by proteolytic enzyme in *L. plantarum LP69* to smaller peptide fragments or even a single amino acid to meet their growth, and some amino acid residues with ACE inhibitory peptides may have also been decomposed and led to reduction of ACE inhibition in fermented goat milk. The pH decreased and titration acidity increased in fermented goat milk with the increase of the inoculum size, the titration acidity and pH showed the opposite trend, titratable acidity and inoculum size was positive correlation, while the pH value was negatively correlated with the inoculum size. The optimal inoculum size was 3% for ACE inhibition and the viable cell count of *L. plantarum LP69*, the result was same as Liang Mei-yan and Chen Qing-sen (2009), who used *Lactobacillus helveticus* TS6024 to ferment cow milk, but the optimal inoculum size was 5% for ACE inhibition in fermented goat milk by *L. bulgaricus LB6* (Shu et al., 2013a, 2013b). The effect of inoculum size has different terms than incubation time with a large difference in pH from Figure 1 and 2, below 4.6 when tested inoculum size and incubation time at pH tested about 3.

Effect of temperature on ACE inhibitory activity in fermentated goat milk

The *L. plantarum LP69* was inoculated into the 14% pasteurized reconstituted goat milk at 5% inoculum size and cultured at different temperature (25, 30, 35, 40 and 45°C) for 12 h, respectively. The results were shown in Figure 3.

As shown in Figure 3, the ACE inhibition and viable counts of *L. plantarum LP69* in fermented goat milk first increased and then decreased with the incubation temperature increasing. The ACE inhibition increased from 31.75% at 25°C to 81.25% at 35°C, then decreased to 30.00% at 45°C, the viable counts of *L. plantarum LP69* increased from 2.15×10^8 CFU/ml

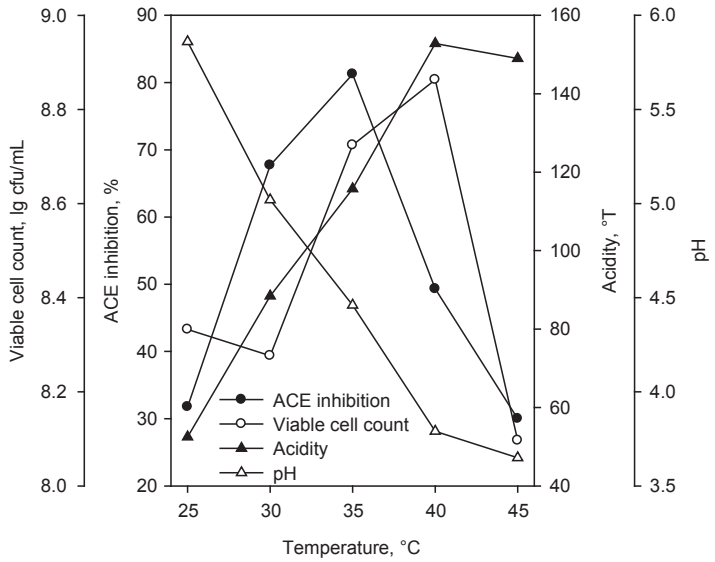

Fig. 3. Effect of temperature on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

at 25°C to 7.30×10^8 CFU/ml at 40°C, then decreased to 1.25×10^8 CFU/ml at 45°C. The temperature had a significant effect on the growth of *L. plantarum LP69* and activity of proteolytic enzyme. When the temperature was low, the metabolic activity of *L. plantarum LP69* was weak, grew slowly and enzyme activity was not strong, which led to low ACE inhibition and viable counts of *L. plantarum LP69*; with temperature increasing, *L. plantarum LP69* quickly grew and the enzyme activity increased gradually, which led to increase ACE inhibition and viable counts of *L. plantarum LP69*, the viable counts of *L. plantarum LP69* reached the maximum number at the optimum temperature, the ACE inhibition reached highest when protease enzyme activity was at optimum temperature; when temperature continued to rise beyond the optimal range, the enzymes in *L. plantarum LP69* were inhibited or destroyed, either cell growth or protease hydrolysis were subject to a certain degree of inhibition. So the optimal temperature for ACE inhibition and the viable cell counts of *L. plantarum LP69* were 35°C and 40°C, respectively.

The pH gradually decreased, titration acidity first increased and decreased with the increase of temperature from Figure 3, the incubation temperature on pH and titration acidity in fermented goat milk by *L. plantarum LP69* had significant difference ($p < 0.05$). The pH decreased from 5.86 at 25°C to 3.79 at 45°C. The titration acidity gradually increased from 52.50°T at 25°C to 152.80°T at 40°C then decreased to 149.00°T, but the viable counts of *L. plantarum LP69* was 1.25×10^8 CFU/ml at 45°C. The reason was maybe *L. plantarum LP69* grew faster at hign tempetature and produced more lactic acid, which led to lower pH value, but *L. plantarum LP69* gradually died with the extension of incubation time. The result showed that incubation temperature could promote production of lactic acid by *L. plantarum LP69*.

Effect of pasteurization time on ACE inhibitory activity in fermentated goat milk

The 14% reconstituted goat milk was pasteurized at 90°C for different time (5, 10, 15, 20 and 25 min, respectively). The results were shown in Figure 4.

As shown in Figure 4, the ACE inhibition in fermented goat milk first increased and then decreased, the viable counts of *L. plantarum LP69* increased, but the pH and acidity had no significant difference in fermented goat milk with the pasteurization time increasing. The ACE inhibition increased from 44.00%

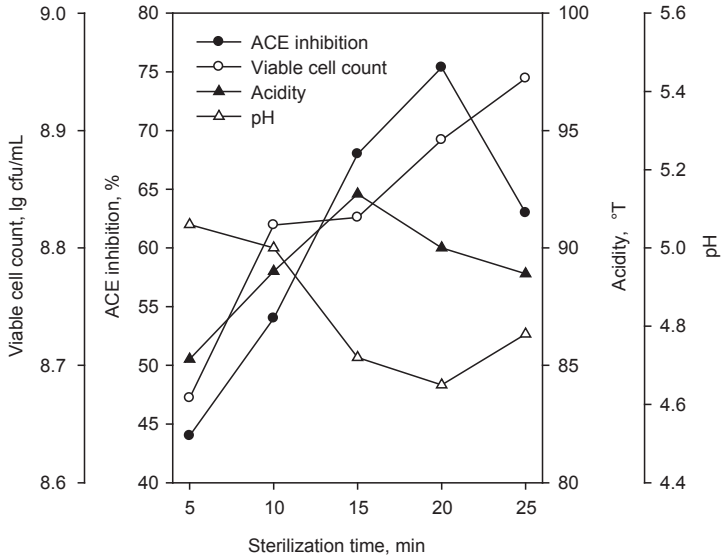

Fig. 4. Effect of pasteurization time on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

at 5 min sterilization time to 75.38% at 20 min pasteurization time, then decreased to 63.00% at 25 min, the viable counts of *L. plantarum LP69* increased from 4.70×10^8 CFU/ml at 5 min sterilization time to 8.80×10^8 CFU/ml at 25 min sterilization time, which indicated the pasteurization time had a significant effect on the growth of *L. plantarum LP69* and denaturing of protein in goat milk. The protein in goat milk denatured during pasteurization process, and the partly denatured protein was beneficial for the use of *L. plantarum LP69*, but it would effect the growth of *L. plantarum LP69* when the pasteurization time was too long, which led to excessive protein denaturation, so the optimal sterilizing time for ACE inhibition and the viable cell counts of *L. plantarum LP69* were 20 min and 25 min, respectively, but the optimal pasteurization time for ACE inhibition was 30 min for cow milk fermented by *Lactobacillus helveticus TS6024* (Liang and Chen, 2009).

Effect of whole goat milk powder on ACE inhibitory activity in fermentated goat milk

The whole goat milk powder was mixed with distilled water and the concentrations of reconstituted goat milk were 8%, 10%, 12%, 14% and 16%, respectively. After pasteurization and cooling to 37°C, *L. plantarum LP69* was inoculated and cultured at 37°C for 14 h, the results were shown in Figure 5.

As shown in Figure 5, the viable cell counts of *L. plantarum LP69* in fermented goat milk increased, but the ACE inhibition in fermented goat milk first increased and then decreased with the concentrations of goat milk powder increasing. The viable cell counts of *L. plantarum LP69* in fermented goat milk increased from 5.10×10^8 CFU/ml at 8% goat milk powder to 1.60×10^9 CFU/ml at 16% goat milk powder, but the ACE inhibition increased from 43.30% at 8% goat milk powder to 74.98% at 14% goat milk powder, then decreased to 58.76% at 16% goat milk powder, which indicated goat milk powder in the concentration of 10–14% can promote the increase of ACE inhibition, but goat milk powder in concentrations of 14–16% will inhibit production of ACE inhibitory peptide, the reason for that may be due to increase protein with the concentration goat milk powder from 10% to 14% and increase available substrate for enzymatic hydrolysis and promote production of ACE inhibitory peptide. With concentration of goat milk powder continued to increase and led to excessive protein inhibit production of proteolytic

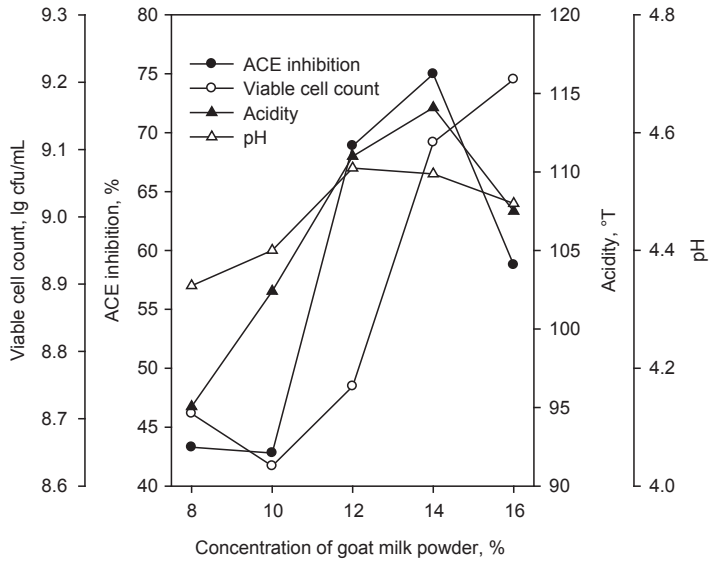

Fig. 5. Effect of concentration of goat milk powder on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

enzymes, thereby caused a reduction in ACE inhibitory peptide and led to a decline in the rate of ACE inhibition. Zhang showed that the concentration of bovine milk powder would affect the proteolytic activity and ACE inhibition of *Lactobacillus casei D400* and the ACE inhibition in fermented bovine milk first increased and then decreased with the concentrations of bovine milk powder increasing, the optimal concentrations of bovine milk powder was 12% and the optimal concentrations of goat milk powder for ACE inhibition in the study was 14%, which was the same as in our previous study (Shu et al., 2013a, 2013b). The pH change had no significant difference ($p < 0.05$) and titration acidity first increased with the increase of the concentration of goat milk powder from Figure 5. The titration acidity gradually increased from 95.06°T at 8% goat milk powder to 114.1°T at 16% goat milk powder, then decreased to 107.50°T.

Effect of whey powder on ACE inhibitory activity in fermentated goat milk

The whey powder was added to pasteurized reconstituted goat milk and the concentration was 0.50, 0.60, 0.70, 0.80 and 0.90%, respectively. The inoculum size was 5% and cultured at 37°C for 14 h, the results were shown in Figure 6.

As shown in Figure 6, the viable counts and ACE inhibition in fermented goat milk first increased and then decreased with the concentration of whey powder increasing, the viable counts reached maximum value (6.50×10^8 CFU/mL) at whey powder 0.60%, but ACE inhibition reached maximum value (84.27%) at whey powder 0.70%. The ACE inhibition gradually decreased in the cow milk fermented by *Lactobacillus casei* with the concentration of whey powder increasing (Jiang et al., 2011), which may be because the structure and content of whey protein in bovine and goat milk is different. The variation between pH and titration acidity had an opposite trend with the increase of the concentration of whey powder from Figure 6. The titration acidity first increased from 88.0°T at 0.50% whey powder to 93.5°T at 0.70% whey powder, then decreased to 83.0°T at 0.90% whey powder, but the pH decreased from 4.81 at 0.50% whey powder to 4.72 at 0.70% whey powder, then increased to 4.98 at 0.90% whey powder, which suggested that adding whey powder had no significant effect on acidity and pH in fermented goat milk. The optimal concentration of whey powder for ACE

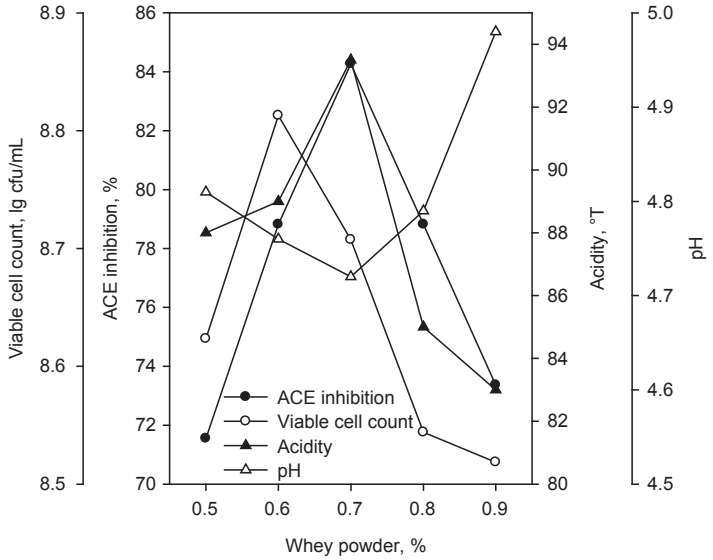

Fig. 6. Effect of concentration of whey powder on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

inhibition and the viable cell counts of *L. plantarum LP69* were 0.70% and 0.60%, respectively, which was in agreement with our previous study (Shu et al., 2014).

CONCLUSION

The optimal incubation time, inoculum size, temperature, pasteurization time, goat milk powder and whey powder in fermented milk by *L. plantarum LP69* was 14 h, 3.0%, 35°C, 20 min, 14% and 0.70% for ACE inhibitory activity and 22 h, 3.0%, 40°C, 25 min, 16% and 0.60% for viable cell counts, respectively. The ACE inhibitory activity and viable cell counts reached 74.70% and 1.61×10^9 CFU/ml, 75.95% and 7.2×10^8 CFU/ml, 81.25% and 7.30×10^8 CFU/ml, 75.38% and 8.8×10^8 CFU/ml, 74.98% and 1.60×10^9 CFU/ml, 84.27% and 6.50×10^8 CFU/ml under optimal condition mentioned above.

ACKNOWLEDGMENT

The project was partly supported by the Science and Technology Research Development plan project of Shaanxi Province (No. 2014K01-17-07), Shaanxi Provincial Education Department (No. 2013JK0747) and the science and technology plan project of Xián city (No. NC1317 (1)), Shaanxi province, China.

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Received – Przyjęto: 1.09.2014

Accepted for print – Zaakceptowano do druku: 12.02.2015

For citation – Do cytowania

Shu, G., Yang, H., Chen, H., Zhang, Q., Tian, Y. (2015). Effect of incubation time, inoculum size, temperature, pasteurization time, goat milk powder and whey powder on ACE inhibitory activity in fermented milk by *L. plantarum LP69*. Acta Sci. Pol. Technol. Aliment., 14(2), 107–116. DOI: 10.17306/J.AFS.2015.2.12