



Research Article

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**Effect of ascorbic acid, incubation temperature and inoculum size on ACE inhibitory activity in fermented goat milk by *Lactobacillus bulgaricus* LB6**

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**ABSTRACT**

The aim of this study was to investigate the effect of four factors including goat milk powder, ascorbic acid, incubation temperature and inoculum size on ACE inhibitory peptides in fermented goat milk by *Lactobacillus bulgaricus* LB6. The concentration of goat milk powder was 10%, 12%, 14%, 16% and 18%, ascorbic acid was 0.01%, 0.03%, 0.05%, 0.07% and 0.09%, incubation temperature was 27 °C, 32 °C, 37 °C, 42 °C and 47 °C and inoculum size was 3%, 4%, 5%, 6% and 7%, respectively. The results were as follows: The concentration of goat milk powder, ascorbic acid, incubation temperature and inoculum size had a significant effect on ACE inhibition and growth of *Lactobacillus bulgaricus* LB6 in fermented goat milk ( $p < 0.05$ ). The optimal concentrations of goat milk powder and ascorbic acid were 14% and 0.03% and the optimal temperature and inoculum size were 37 °C and 5% for ACE inhibition, corresponding ACE inhibitions were 74.02%, 89.64%, 74.98% and 74.32%, respectively, respectively.

**Keywords:** ACE inhibitory peptide; *Lactobacillus bulgaricus*; goat milk powder; ascorbic acid

**INTRODUCTION**

Hypertension is a risk factor for coronary heart disease [1-2]. Angiotensin-I converting enzyme (ACE; EC. 3.4.15.1) plays an important physiological role in regulating blood pressure [3-4], it is a dipeptidyl carboxypeptidase that raise blood pressure by generates the vasoconstrictor angiotensin-II and inactivates the vasodilator bradykinin [5-6]. Inhibition of ACE may exert an antihypertensive effect as a consequence of the decrease in angiotensin II as well as an increase of bradykinin[7]. Several synthetic ACE inhibitors have been developed such as captopril, enalapril since the discovery of ACE inhibitors in snake venom, which are currently used extensively in the treatment of hypertension [8-9]. Although synthetic ACE inhibitors are effective as antihypertensive drugs, they have certain side effects such as pruritic rash, loss of taste, and proteinuria [10]. In the respect, functional foods containing ACE inhibitory peptides have recently received considerable attention [11].

Lactic acid bacteria are among the most important groups of microorganisms used in food fermentations [12-13]. Lactic acid bacteria are known to produce inhibitors of ACE in foods containing protein during fermentation. The inhibitors are formed by the bacterial proteinase when lactic acid bacteria hydrolyze milk proteins into many peptides. Proteolysis plays an important role since the inhibitors formed are peptides [14]. The proteolytic enzymes of lactic acid bacteria are quite non-specific, and therefore protein is cleaved at several places. Recently, researchers have reported that ACE inhibitors can be released by proteolysis of milk during fermentation by different lactic acid bacteria, such as *Lactobacillus helveticus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. lactis ssp. lactis*, *L. lactis ssp. Cremoris* and *Enterococcus faecalis*[15-31]. In our previous study, 28 probiotic *Lactobacillus* strains were used to ferment goat milk to screen for production ACE-inhibitory peptides, the results showed that 4 strains including *Lactobacillus reuteri*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus* and *Lactobacillus helveticus* were especially significant as producers of ACE-inhibitory peptides [17]. The effect of carbon source (glucose and lactose) and salts (calcium lactate,  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  and  $\text{KH}_2\text{PO}_4$ ) on ACE inhibitory activity in fermented goat milk by

*Lactobacillus bulgaricus* LB6 was investigated [32]. In the present study, our objective was to investigate the effect of four factors including goat milk powder, ascorbic acid, incubation temperature and inoculum size on ACE inhibitory peptides in fermented goat milk by *Lactobacillus bulgaricus* LB6 in order to provide reference for further optimization.

## EXPERIMENTAL SECTION

### Materials and reagents

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

### Microorganism and their activation

Pure cultures of *Lactobacillus bulgaricus* LB6 was supplied by the College of Life Science & Engineering, Shaanxi University of Science & Technology. Stock cultures were stored at  $-20^{\circ}\text{C}$  in freeze-dried powder. *Lactobacillus bulgaricus* LB6 was activated successively three times in MRS broth (Haibio, Qindao, China) at  $37^{\circ}\text{C}$  for 24 h prior to use.

### Preparation of fermented goat milk

The goat milk was carried out with different systems for 12h: (1) reconstituted goat milk at different concentration (10%, 12%, 14%, 16% and 18%); (2) reconstituted goat milk with ascorbic acid at different concentration (0.01%, 0.03%, 0.05%, 0.07% and 0.09%); (3) reconstituted goat milk at different temperature ( $27^{\circ}\text{C}$ ,  $32^{\circ}\text{C}$ ,  $37^{\circ}\text{C}$ ,  $42^{\circ}\text{C}$ ,  $47^{\circ}\text{C}$ ) and (4) reconstituted goat milk at different inoculum size.

### Measurement of ACE inhibitory activity

The whey fraction from the fermented goat milk was used to measure the ACE inhibitory effect. Aliquots of the fermented goat milk were collected, vigorously stirred and centrifuged at  $5000\times g$  for 15 min to obtain the corresponding whey fractions. The supernatants collected were filtered through a Xinhua filter and used to determine their ACE inhibitory activity. ACE inhibitory activity was measured by a spectrophotometric assay according to the method of Cushman and Cheung (1971) with slight modifications [33-34]. Added  $80\mu\text{L}$  of each sample to  $200\mu\text{L}$  sodium borate buffer ( $0.1\text{mol/L}$ , pH 8.3) containing NaCl ( $0.30\text{mol/L}$ ) and HHL ( $5\text{mmol/L}$ ). Then, ACE ( $20\mu\text{L}$ ,  $0.1\text{U/mL}$ ) was added and the reaction mixture was incubated at  $37^{\circ}\text{C}$  for 30 min. The reaction was terminated by adding  $250\mu\text{L}$   $1\text{mol/L}$  HCl. Adding 1.7 mL ethyl acetate to extract the hippuric acid formed and evaporated at  $120^{\circ}\text{C}$  for 30 min, redissolved in 2mL deionized water after cooled at room temperature, then the absorbance was measured at an optical density of 228 nm using an UV-spectrophotometer (Shanghai Spectrum Instruments Co., Ltd, Shanghai, China). The activity of each sample was measured in triplicate and done averaging. The ACE inhibitory rate was calculated using the following formula: ACE inhibition (%) =  $(A - B) / (A - C) \times 100\%$ , 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.

### Measurement of viable cell counts, pH and titration acidity

Serial dilutions of the fermented goat milk made in saline water (0.9%, w/v, NaCl) containing 0.1g/L peptone were spread onto MRS agar plates and incubated for 48 h at  $37^{\circ}\text{C}$ . All dilutions were plated in triplicate. Enumeration was performed by manual counting, whenever possible the mean numbers from two different dilutions were used, and results were expressed as colony forming units per milliliter (CFU/ml) of fermented milk. 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 ( $^{\circ}\text{T}$ ) described, respectively.

## RESULTS AND DISCUSSION

### Effect of whole goat milk powder on ACE inhibitory activity in fermented goat milk

The whole goat milk powder was mixed with distilled water and the concentrations of reconstituted goat milk were 10%, 12%, 14%, 16% and 18%, respectively. After pasteurization and cooling to  $37^{\circ}\text{C}$ , The inoculum size of *Lactobacillus bulgaricus* LB6 was 5% and cultured at  $37^{\circ}\text{C}$  for 12h. The samples were taken out for determining ACE inhibition, viable count, pH value and titration acidity. The results were shown in Figure 1 and 2.

As shown in Figure 1, the viable cell counts of *Lactobacillus bulgaricus* LB6 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 *Lactobacillus bulgaricus* LB6 in fermented goat milk increased from

$1.95 \times 10^8$  CFU/ml at 10% goat milk powder to  $3.89 \times 10^8$  CFU/ml at 18% goat milk powder, but the ACE inhibition increased from 70.00% at 10% goat milk powder to 74.02% at 14% goat milk powder, then decreased to 33.95% at 18% 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 14-18% 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 lead to excessive protein inhibit production of proteolytic enzymes, thereby caused a reduction in ACE inhibitory peptide and lead to a decline in the rate of ACE inhibition. Zhang showed that the concentration of bovine milk powder will 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 [35], the optimal concentrations of bovine milk powder was 12% and the optimal concentrations of goat milk powder for ACE inhibition in our study was 14%.

As shown in Figure 2, the pH first increased and then decreased and titration acidity increased with the increase of the concentration of goat milk powder. The titration acidity gradually increased from 147 °T at 10 % goat milk powder to 218 °T at 18% goat milk powder, then decreased to 97.20 °T, but the pH increased from 147 °T at 10 % goat milk powder to 218 °T at 18% goat milk powder increased from 4.00 at 10 % goat milk powder to 4.08 at 14% goat milk powder, then decreased to 4.06.

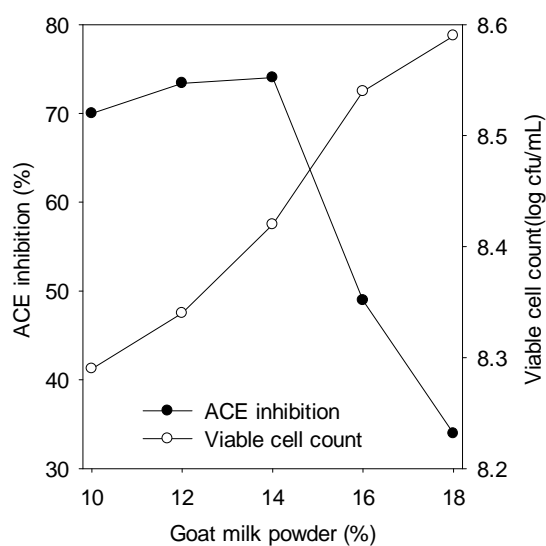


Fig.1 Effect of concentration of goat milk powder on ACE inhibition and viable counts of *Lactobacillus bulgaricus LB6* in fermented goat milk

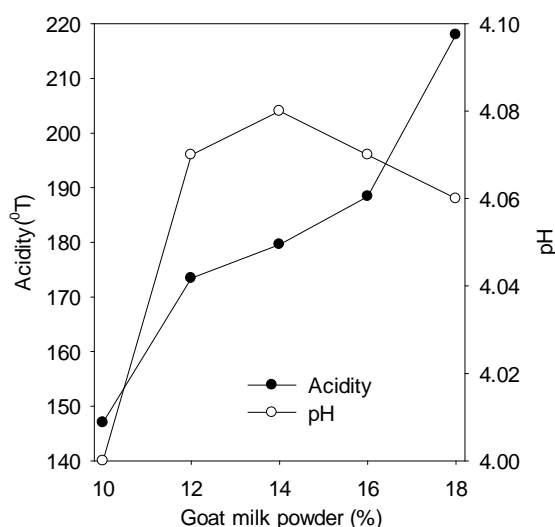


Fig.2 Effect of concentration of goat milk powder on acidity and pH in fermented goat milk

**Effect of ascorbic acid on ACE inhibitory activity in fermented goat milk**

The ascorbic acid was added to pasteurize reconstituted goat milk and the concentrations were 0.01%, 0.03%, 0.05%, 0.07% and 0.09%. The results were shown in Figure 3 and 4.

As shown in Figure 3, the ACE inhibition and viable counts of *Lactobacillus bulgaricus* LB6 in fermented goat milk first increased and then decreased with the concentration of ascorbic acid increasing. the ACE inhibition increased from 80.53% at 0.01% ascorbic acid to 89.64% at 0.03% ascorbic acid, then decreased to 59.71% at 0.09% ascorbic acid, the viable counts of *Lactobacillus bulgaricus* LB6 increased from  $1.0 \times 10^8$  CFU/ml at 0.01% ascorbic acid to  $1.48 \times 10^8$  CFU/ml at 0.05% ascorbic acid, then decreased to  $1.35 \times 10^8$  CFU/ml at 0.09% ascorbic acid. *Lactobacillus bulgaricus* is a facultative anaerobic and can grow under aerobic and anaerobic conditions, but has different metabolic pathway. Ascorbic acid can be used as an oxygen scavenger in the process of anaerobic culture. The results showed that ascorbic acid in the low concentration may promote growth of *Lactobacillus bulgaricus* LB6 and production of ACE inhibitory peptides, but ascorbic acid in high concentrations will inhibit the growth of *Lactobacillus bulgaricus* LB6 in goat milk and production of ACE inhibitory peptides, which may be related to the the different metabolic pathways of *Lactobacillus bulgaricus* LB6 under different oxygen conditions. The optimal concentrations of ascorbic acid for ACE inhibition and the viable cell counts of *Lactobacillus bulgaricus* LB6 were 0.03% and 0.05%, respectively.

The pH decreased and titration acidity increased with the increase of the concentration of ascorbic acid from Figure 4, but the pH and titration acidity variation had no significant difference ( $p > 0.05$ ), which showed that ascorbic acid had no significant influence on production of lactic acid by *Lactobacillus bulgaricus* LB6.

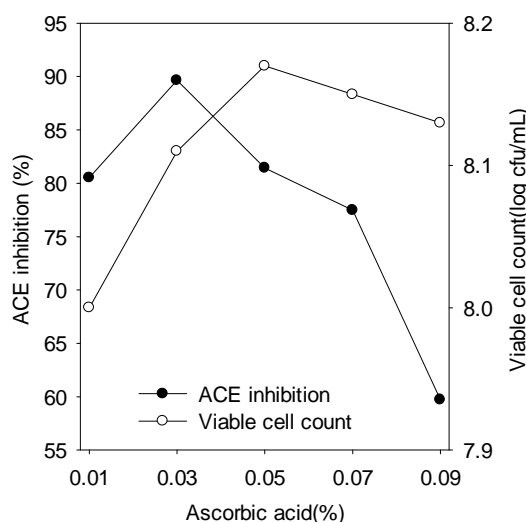


Fig.3 Effect of ascorbic acid on ACE inhibitory rate and viable cell count in fermented goat milk

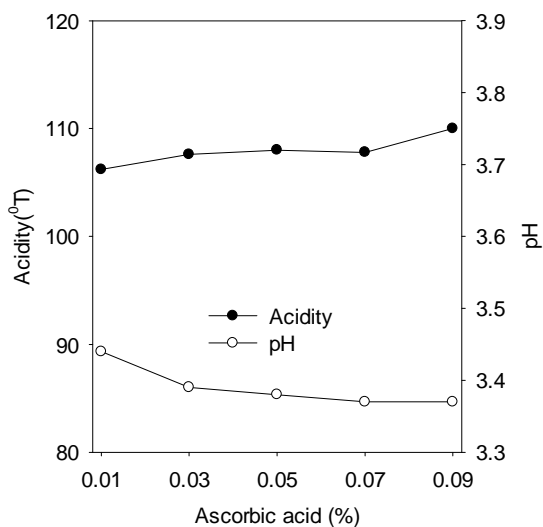
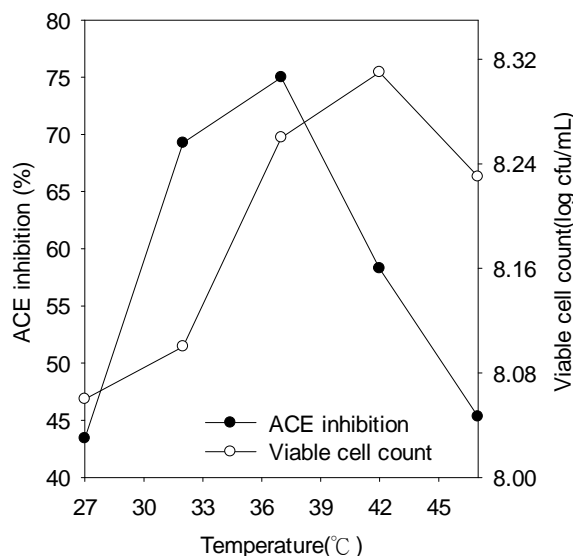


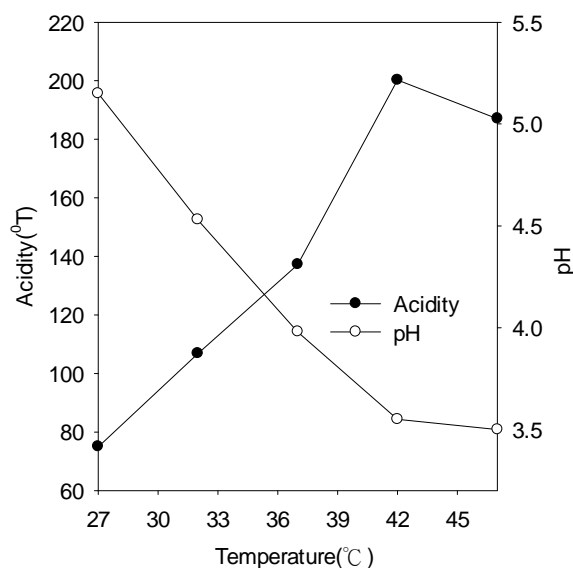
Fig.4 Effect of ascorbic acid on acidity and pH in fermented goat milk

**Effect of temperature on ACE inhibitory activity in fermented goat milk**

The *Lactobacillus bulgaricus* LB6 was inoculated into the 14% pasteurized reconstituted goat milk at 5% inoculum size and cultured at different temperature (27°C, 32°C, 37°C, 42°C, 47°C) for 12h, respectively. The results were shown in Figure 5 and 6.



**Fig.5 Effect of temperature on ACE inhibitory rate and viable cell count in fermented goat milk**



**Fig.6 Effect of temperature on acidity and pH in fermented goat milk**

As shown in Figure 5, the ACE inhibition and viable counts of *Lactobacillus bulgaricus* LB6 in fermented goat milk first increased and then decreased with the incubation temperature increasing. the ACE inhibition increased from 43.41% at 27°C to 74.98% at 37°C, then decreased to 45.32% at 47°C, the viable counts of *Lactobacillus bulgaricus* LB6 increased from  $1.15 \times 10^8$  CFU/ml at 27°C to  $2.04 \times 10^8$  CFU/ml at 42°C, then decreased to  $1.70 \times 10^8$  CFU/ml at 47°C. The temperature had a significant effect on the growth of *Lactobacillus bulgaricus* LB6 and activity of proteolytic enzyme. When the temperature was low, the metabolic activity of *Lactobacillus bulgaricus* LB6 was weak, grew slowly and enzyme activity was not strong, which led to low ACE inhibition and viable counts of *Lactobacillus bulgaricus* LB6; with temperature increasing, *Lactobacillus bulgaricus* LB6 quickly grew and the enzyme activity increased gradually, which led to increase ACE inhibition and viable counts of *Lactobacillus bulgaricus* LB6, the viable counts of *Lactobacillus bulgaricus* LB6 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 *Lactobacillus bulgaricus* LB6 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 *Lactobacillus bulgaricus* LB6 were 37°C and

42°C, respectively. Jiang compared that ACE inhibitory activity in co-fermented bovine milk by *Lactobacillus helveticus* and *Lactobacillus casei* under 37°C and 42°C and found that 37°C was more beneficial to the production of ACE inhibitory peptides [36], which was consistent with the results of this paper.

The pH decreased, titration acidity first increased and decreased with the increase of temperature from Figure 6, the incubation temperature on pH and titration acidity in fermented goat milk by *Lactobacillus bulgaricus* LB6 had significant difference ( $p < 0.05$ ). The pH decreased from 5.15 at 27°C to 3.50 at 47°C, The titration acidity gradually increased from 75.00 °T at 27°C to 200.20°T at 42°C then decreased to 187.00°T, which showed that incubation temperature could promote production of lactic acid by *Lactobacillus bulgaricus* LB6.

#### Effect of inoculum size on ACE inhibitory activity in fermented goat milk

The *Lactobacillus bulgaricus* LB6 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 7 and 8.

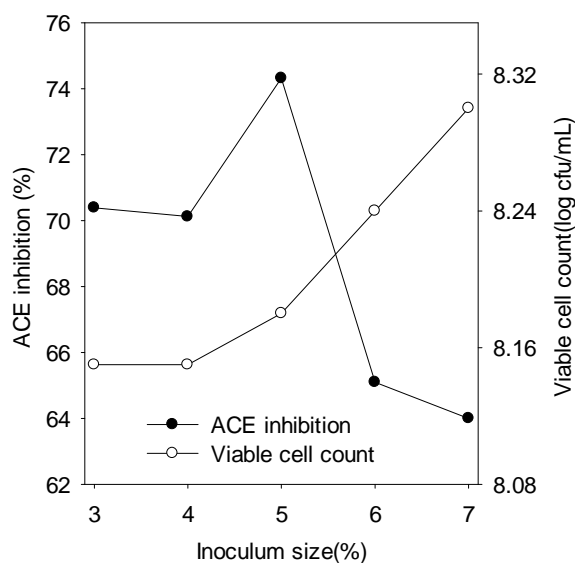


Fig.7 Effect of inoculum size on ACE inhibitory rate and viable count

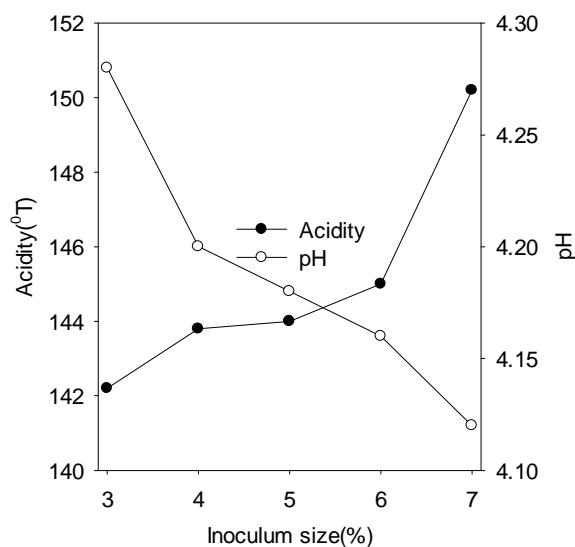


Fig.8 Effect of inoculum size on acidity and pH in fermented goat milk

As shown in Figure 7, the viable cell counts of *Lactobacillus bulgaricus* LB6 in fermented goat milk increased, but the ACE inhibition in fermented goat milk first increased and then decreased with the inoculum size increasing. The viable cell counts of *Lactobacillus bulgaricus* LB6 in fermented goat milk increased from  $1.41 \times 10^8$  CFU/ml at 3% inoculum size to  $2.00 \times 10^8$  CFU/ml at 7% inoculum size, but the ACE inhibition increased from 70.39% at 3% inoculum size to 74.32% at 5% inoculum size, then decreased to 64.00% at 7% inoculum size. This may be due to the gradual increase of viable cells of *Lactobacillus bulgaricus* LB6, the nutrients in reconstituted goat milk were not

meet the growth need of *Lactobacillus bulgaricus* LB6, which lead to goat milk protein and peptides were hydrolyzed by proteolytic enzyme in *Lactobacillus bulgaricus* LB6 to smaller peptide fragments or even a single amino acid to meet their growth, and some amino acid residues with ACE inhibitory peptides may also be decomposed and led to reduction of ACE inhibition in fermented goat milk.

the pH first increased and titration acidity increased in fermented goat milk with the increase of the inoculum size from Figure 8, the titration acidity and pH value showed the opposite trend, titration acidity and inoculum size was positive correlation, while the pH value was negatively correlated with the inoculum size, inoculum size had no significant influence on titration acidity and pH value in fermented goat milk ( $p > 0.05$ ).

### CONCLUSION

The concentration of goat milk powder, ascorbic acid, incubation temperature and inoculum size had a significant effect on ACE inhibition and growth of *Lactobacillus bulgaricus* LB6 in fermented goat milk ( $p < 0.05$ ). The optimal concentrations of goat milk powder and ascorbic acid were 14% and 0.03% and the optimal temperature and inoculum size were 37 °C and 5% for ACE inhibition, corresponding ACE inhibitions were 74.02%, 89.64%, 74.98% and 74.32%, respectively.

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