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Research Article

Effect of Casein Hydrolyses, Ascorbic Acid, $CaCl_2$, Ca $(H_2PO_4)_2$, NaCl and Tween 80 on ACE Inhibitory Activity in Fermented Goat Milk by *Lactobacillus plantarum* LP69

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Abstract: Angiotensin I-Converting Enzyme (ACE) inhibitory peptides produced by Lactic acid bacteria during fermentation can lower hypertension. The objective of this study was to investigate the effect of casein hydrolyses (0.50, 0.60, 0.70, 0.80 and 0.90%, respectively), ascorbic acid (0.01, 0.03, 0.05, 0.07 and 0.09%, respectively), $CaCl_2$ (0.04, 0.05, 0.06, 0.07 and 0.08%, respectively), Ca $(H_2PO_4)_2$ (0.01, 0.03, 0.05, 0.07 and 0.09%, respectively), NaCl (0.30, 0.60, 0.90, 1.20 and 1.50%, respectively) and Tween 80 (0.02, 0.04, 0.06, 0.08 and 0.10%, respectively) on ACE inhibitory peptides fermented from goat milk by *Lactobacillus plantarum* LP69 using single factor experiment. The results were as follows: The optimal concentration of casein hydrolyses, ascorbic acid, $CaCl_2$, $(CaH_2PO_4)_2$, NaCl and Tween 80 for ACE inhibitory activity was 0.70, 0.03, 0.08, 0.05, 0.90 and 0.04% in fermented goat milk by *Lactobacillus plantarum* LP69, respectively. The casein hydrolyses, ascorbic acid, $CaCl_2$, Ca $(H_2PO_4)_2$, NaCl and Tween 80 had a significant influence on ACE inhibitory activity in fermented milk and growth of *Lactobacillus plantarum* LP69, the results are beneficial for further screening main factors by using fractional factorial designs.

Keywords: ACE inhibitory peptide, ascorbic acid, casein hydrolyses, goat milk, *Lactobacillus plantarum*

INTRODUCTION

Hypertension is a main risk factor for the development of cardiovascular diseases (Duprez *et al*., 2002), Angiotensin-I Converting Enzyme (ACE; EC 3.4.15.1)) is a dipeptidyl carboxypeptidase that regulates blood pressure by producing the vasoconstrictor angiotensin II and degrading the vasodilator bradykinin (Campbell, 1987). Therefore, inhibition of ACE activity is considered to be a useful therapeutic approach in the treatment of hypertension. Although synthetic ACE inhibitors such as captopril, enalapril, alacepril and lisinopril are effective as antihypertensive drugs, they have some side effects such as cough, taste disturbances and skin rashes (Ondetti, 1977; Patchett *et al*., 1980). Therefore, the researchers have great interest in searching for natural ACE inhibitors as alternatives to synthetic ones for safe and economical use.

Bioactive peptides can be released by enzymatic proteolysis of food proteins or fermentation of milk with lactic acid bacteria. ACE-inhibitory peptides were produced by the following lactic acid bacteria by fermenting cow milk or goat milk, such as *Lactobacillus helveticus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. lactis* ssp. lactis, *L. lactis* ssp. Cremoris and *Enterococcus faecalis* (Algaron *et al*., 2004; Ashar and Chand, 2003; Chen *et al*., 2012, 2013a; Flambard, 2004; Fuglsang *et al*., 2003b; Gobbetti *et al*., 2000; Hernández-Ledesma *et al*., 2004; 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; Shu *et al*., 2013a, b, 2014; Shuangquan and Taku, 2008; Vermeirssen *et al*., 2003; Yamamoto *et al*., 1994a, b, 1999). The Chr. Hansen collected some interesting lactic acid bacteria known to be proteolytic and to produce ACE-inhibitory peptides growing in milk (Flambard, 2003)

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 of 4 strains including *L. bulgaricus* LB6, *Lactobacillus reuteri* LT33, *Lactobacillus rhamnosus* LR22 and *Lactobacillus plantarum* LP69 (which was previously been mistaken as *Lac*tobacillus *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, b, 2014) and carbon and nitrogen source on ACE inhibitory activity in fermented goat milk by *L. plantarum* LP69 (Chen, *et al*., 2013b). In this study,

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Effect of casein hydrolyses, ascorbic acid, $CaCl_2$, Ca $(H_2PO_4)_2$, NaCl and Tween 80 on ACE inhibitory activity in fermented goat milk by *Lactobacillus plantarum* LP69 was investigated to provide reference for further optimization by using response surface methodology.

MATERIALS AND METHODS

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), casein hydrolyses, ascorbic acid, $CaCl_2$, Ca $(H_2PO_4)_2$, NaCl and Tween 80 were purchased from Xi'an Luosenbo Technology Co., Ltd. (Xi'an, China), All chemicals used were of analytical grade unless otherwise specified.

Microorganisms and its activation: *Lactobacillus plantarum* LP69 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. 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. After three successive transfers the cultures were finally transferred into sterile reconstituted skim goat milk to obtain approximately 10^8 Colony-Forming Units per milliliter (CFU/mL) for fermented milk manufacture.

Preparation of fermented goat milk: Reconstituted skim goat milk was pasteurized, inoculated with *Lactobacillus plantarum* LP69 and fermented at 37°C for 16 h. The whey was collected by centrifugation at 5000 g for 15 min. The viable counts of *L. plantarum* LP69 in the fermented milk was 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 in 2 mL deionized water after cooled to room temperature, then the absorbance was measured at an optical density of 228 nm. The activity of each sample was tested in triplicate and done averaging. The ACE inhibitory rate was calculated using the following formula:

$$\text{ACE inhibition (\%)} = (A-B) / (A-C) \times 100\%$$

where,

A = The optical density without the whey fraction
B = The optical density without ACE
C = The optical density in the presence of both ACE and the whey fraction

Measurement of viable cell counts, pH and titration acidity: The viable counts of *L. plantarum* LP69 were determined by using the pour plate technique. Briefly, 1 mL of each fermented 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 QL-901 vortex mixer (Qilinbeier, Haimen, China) for 30s, then spread onto MRS agar plates and incubated for 48-72 h at 37°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 (Chen *et al*., 2013b). The pH change in fermented goat milk was monitored by a pHS-3C pH metre (Shanghai Jinke, Shanghai, China) at the room temperature and titration acidity was determined according to the sodium hydroxide titration method and Jill Nieer degrees (°T) described, respectively.

RESULTS AND DISCUSSION

Effect of casein hydrolyses on ACE inhibitory activity in fermentated goat milk: The casein hydrolyses were added to pasteurized reconstituted goat milk and the concentration were 0.50, 0.60, 0.70, 0.80 and 0.90%, respectively. The results were shown in Fig. 1.

Figure 1, the viable counts of *L. plantarum* LP69 gradually decreased, ACE inhibition in fermented goat milk first increased and then decreased with the increase of casein hydrolyses concentration, which is different in fermented goat by *L. bulgaricus* LB6 (Shu *et al*., 2014). The viable counts of *L. plantarum* LP69 decreased from 3.20×10^9 CFU/mL at casein hydrolyses 0.5% to 1.68×10^9 CFU/mL at casein hydrolyses 0.9%, while ACE inhibition increased from 70.00% at casein hydrolyses 0.5 to 84.54% at casein hydrolyses 0.7%, them decreased to 60.90% at casein hydrolyses 0.9%, which indicated that addition of casein hydrolyses could promote the production of ACE inhibitory peptide at 0.5~0.7%, but inhibit growth of *L. plantarum* LP69 at 0.5~0.9% and production of ACE inhibitory peptide at 0.7~0.9%. The decline reason of ACE inhibition may be due to some components in casein hydrolyses was the product of hydrolysis of protease produced by *L. plantarum* LP69, which increased product concentration and generated feedback inhibition for enzyme activity, thereby led to inhibit the hydrolysis. With casein hydrolyses increasing, the titration acidity

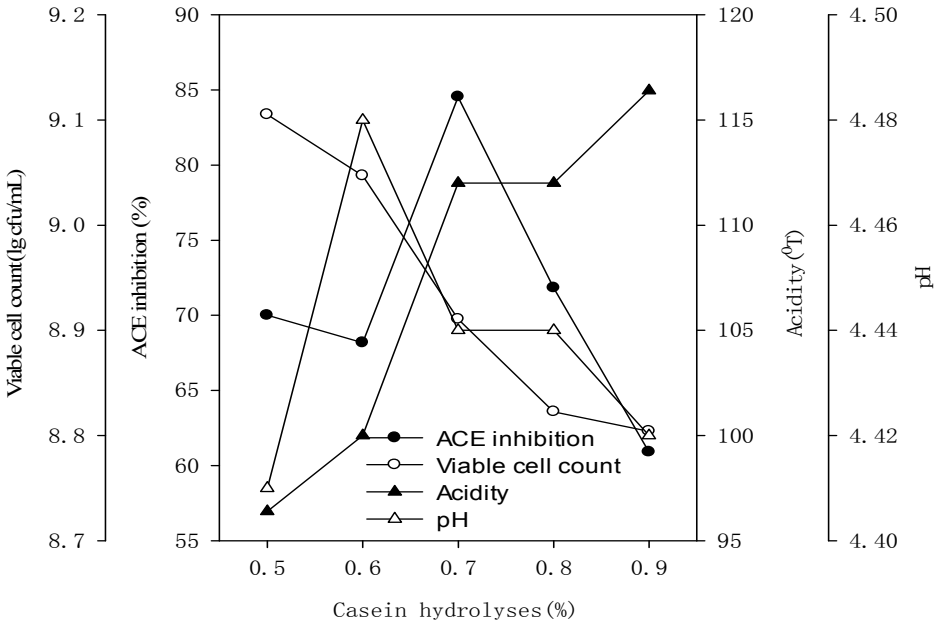

Fig. 1: Effect of casein hydrolyses on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

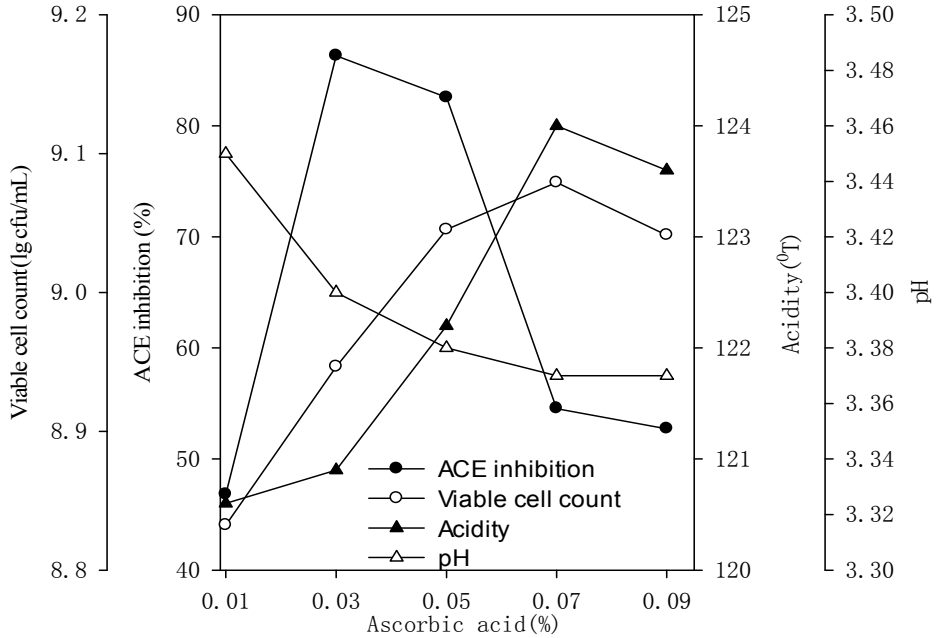

Fig. 2: Effect of ascorbic acid on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

in fermented goat milk increased significantly ($p<0.05$) from 96.4°T at casein hydrolyses 0.5% to 116.4°T at casein hydrolyses 0.9%, while the pH value had no significant change ($p>0.05$). The optimal concentration of casein hydrolyses were 0.5% for the viable counts of *L. plantarum* LP69 and 0.7% for ACE inhibition in fermented goat milk, respectively.

Effect of ascorbic acid on ACE inhibitory activity in fermentated 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%, respectively. The results were shown in Fig. 2.

Figure 2, the ACE inhibition and viable counts of *L. plantarum* LP69 in fermented goat milk were all first increased and then decreased with the concentration of ascorbic acid increasing, which had same trends in fermented goat by *L. bulgaricus* LB6 (Shu *et al*., 2013a). the ACE inhibition increased from 46.82% at 0.01% ascorbic acid to 86.31% at 0.03% ascorbic acid,

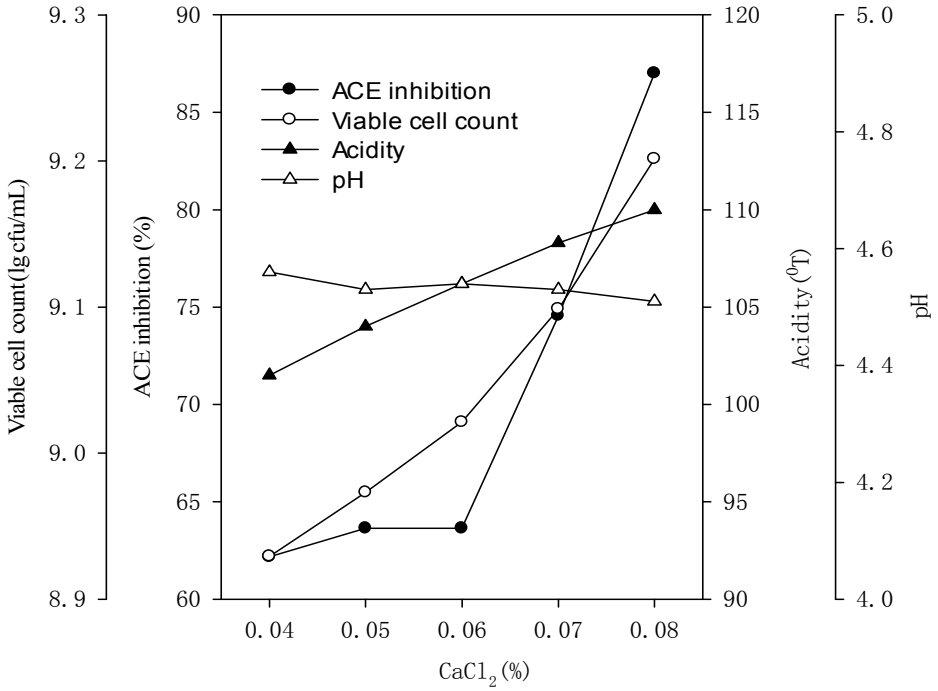

Fig. 3: Effect of $CaCl_2$ on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

then decreased to 52.73% at 0.09% ascorbic acid, the viable counts of *L. plantarum* LP69 increased from 4.68×10^8 CFU/mL at 0.01% ascorbic acid to 1.29×10^9 CFU/mL at 0.07% ascorbic acid, then decreased to 1.26×10^9 CFU/mL at 0.09% ascorbic acid. *L. plantarum* LP69 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 *L. plantarum* LP69 and production of ACE inhibitory peptides, but ascorbic acid in high concentrations will inhibit the growth of *L. plantarum* LP69 in goat milk and production of ACE inhibitory peptides, which may be related to the different metabolic pathways of *L. plantarum* LP69 under different oxygen conditions. The pH decreased and titration acidity increased with the increase of the concentration of ascorbic acid from Fig. 2, the variation of titration acidity had no significant difference ($p<0.05$), but the pH had significant difference ($p>0.05$). The optimal concentrations of ascorbic acid were 0.03% for ACE inhibition and 0.07% for the viable cell counts, respectively.

Effect of $CaCl_2$ on ACE inhibition and viable count in fermented goat milk: The $CaCl_2$ was added to pasteurize reconstituted goat milk and the concentrations were 0.04, 0.05, 0.06, 0.07 and 0. 08%, respectively. The results were shown in Fig. 3.

With the concentration of $CaCl_2$ increasing, the viable counts of *L. plantarum* LP69 and ACE inhibition in fermented goat milk were all gradually increased. The viable counts increased from 8.50×10^8 CFU/mL at 0.02% $CaCl_2$ to 1.59×10^9 CFU/mL at 0.08% $CaCl_2$ and ACE inhibition increased from 68.18% at 0.02% $CaCl_2$ to 87.01% at 0.08% $CaCl_2$ from Fig. 3, which indicated that addition of $CaCl_2$ in goat milk promoted the growth of *L. plantarum* LP69 and production of ACE inhibitory peptides. Gonzalez *et al*. (2011) found that the ionic calcium released during milk fermentation could contribute to the ACE-inhibitory activity by *Lactobacillus casei YIT* 9029 and *Bifidobacterium bifidum MF* 20/5, which was consistent with the results of this study. With $CaCl_2$ increasing, the titration acidity in fermented goat milk increased significantly ($p<0.05$) from 101.50°T at 0.02% $CaCl_2$ to 110.00°T at 0.08% $CaCl_2$, but the pH value had no significant change ($p>0.05$) from Fig. 3. The optimal concentrations of $CaCl_2$ for ACE inhibition and the viable cell count of *L. plantarum* LP69 were both 0.08%.

Effect of Ca $(H_2PO_4)_2$ on ACE inhibition and viable count in fermented goat milk: The Ca $(H_2PO_4)_2$ was added to pasteurize reconstituted goat milk and the concentrations were 0.01, 0.03, 0.05, 0.07 and 0. 09%, respectively. The results were shown in Fig. 4.

With the concentration of Ca $(H_2PO_4)_2$ increasing, The viable counts of *L. plantarum* LP69 in fermented goat milk gradually increased from 2.60×10^8 CFU/mL at 0.01% Ca $(H_2PO_4)_2$ to 8.94×10^8 CFU/mL at 0.09% Ca $(H_2PO_4)_2$, while ACE inhibition first increased from 56.36% at 0.01% Ca $(H_2PO_4)_2$ to 789.09% at 0.05% Ca $(H_2PO_4)_2$ and then decreased to 45.45% at 0.09%

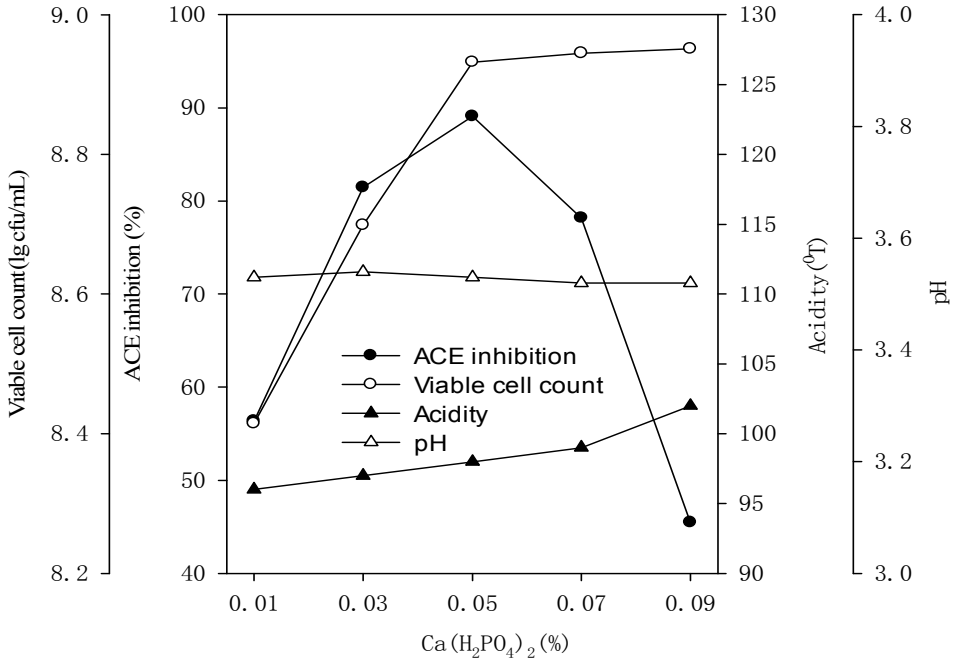

Fig. 4: Effect of Ca $(H_2PO_4)_2$ on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

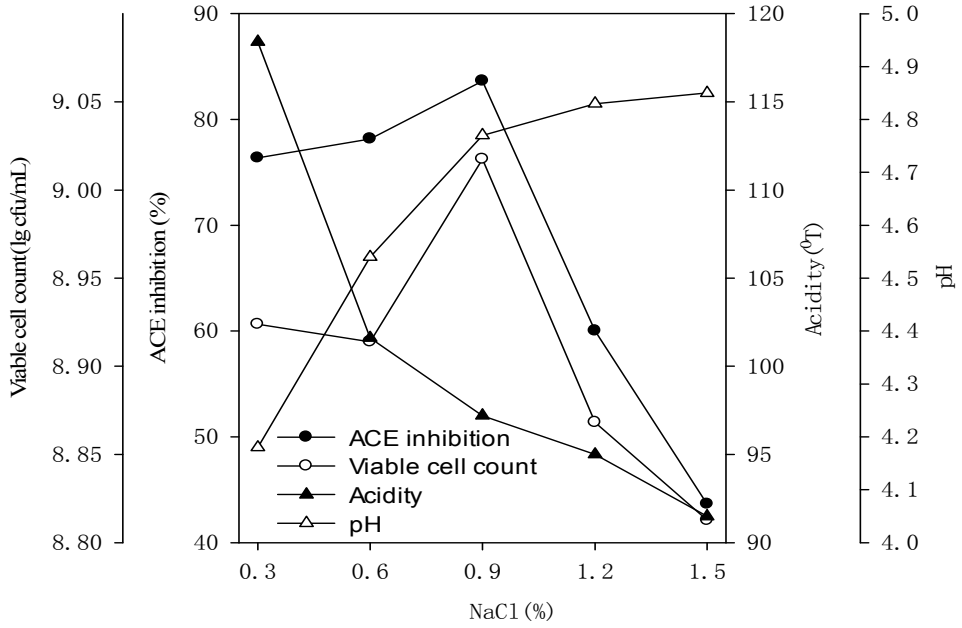

Fig. 5: Effect of NaCl on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

Ca $(H_2PO_4)_2$ from Fig. 4, which indicated that addition of Ca $(H_2PO_4)_2$ in goat milk promoted the growth of *L. plantarum* LP69, while promoted production of ACE inhibitory peptides in low concentration. With Ca $(H_2PO_4)_2$ increasing, the titration acidity and pH in fermented goat milk had no significant change ($p>0.05$) from Fig. 4. The optimal concentrations of Ca $(H_2PO_4)_2$ for ACE inhibition and the viable cell counts of *L. plantarum* LP69 were 0.05 and 0.09%, respectively.

Effect of NaCl on ACE inhibition and viable count in fermented goat milk: The NaCl was added to pasteurize reconstituted goat milk and the concentrations were 0.3, 0. 6, 0.9, 1.2 and 1.5%, respectively, the results were shown in Fig. 5.

The ACE inhibition and viable cell counts of *L. plantarum* LP69 in fermented goat milk both first increased and then decreased with the increase of NaCl concentration from Fig. 5. The ACE inhibition increased

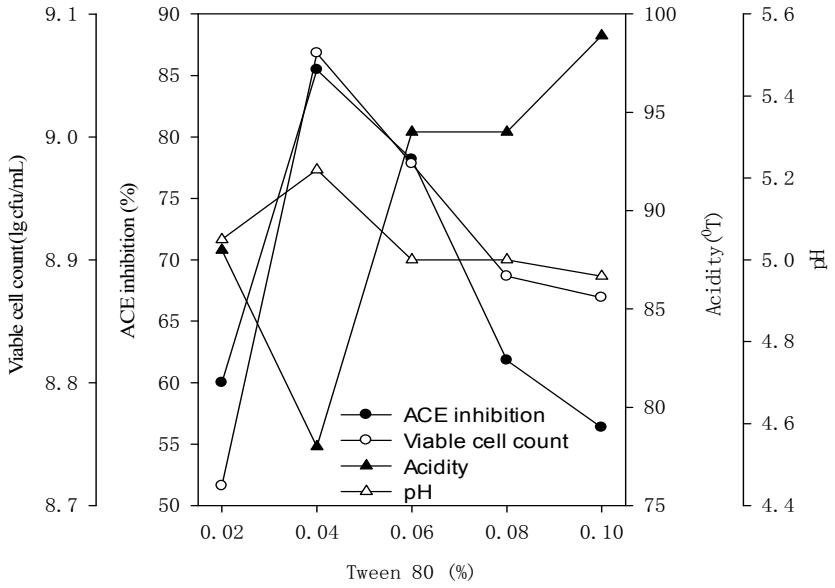

Fig. 6: Effect of tween 80 on ACE inhibition, viable cell counts, pH and titration acidity in fermented goat milk

from 76.36% at 0.3% NaCl to 83.64% at 1.5% NaCl, then decreased to 43.64%, the viable counts of *L. plantarum* LP69 in fermented goat milk increased from 1.33×10^9 CFU/mL at NaCl 0.3% to 1.65×10^9 CFU/mL at NaCl 0.9%, then decreased to 1.03×10^9 CFU/mL at NaCl 1.5%, which indicated that addition of NaCl could promote the production of ACE inhibitory peptide and growth of *L. plantarum* LP69 in low concentration. The titration acidity and the pH value showed opposite changes with NaCl increasing from Fig. 5, the titration acidity decreased from 118.4°T to 91.5°T and the pH value increased from 4.18 at 0.3% NaCl to 4.86 at 1.5% NaCl. The optimal concentrations of NaCl for ACE inhibition and the viable cell counts of *L. plantarum* LP69 were both 0.90%, which was also the physiological concentration for maintenance of cell osmotic equilibrium.

Effect of tween 80 on ACE inhibition and viable count in fermented goat milk: The Tween 80 was added to pasteurize reconstituted goat milk and the concentrations were 0.02, 0. 04, 0.06, 0.08 and 0.10%, respectively the results were shown in Fig. 6.

Figure 6, the ACE inhibition and viable counts of *L. plantarum* LP69 in fermented goat milk were both first increased and then decreased with the concentration of Tween 80 increasing. the ACE inhibition increased from 60.00% at 0.02% Tween 80 to 85.45% at 0.04% Tween 80, then decreased to 56.36% at 0.10% Tween 80, the viable counts of *L. plantarum* LP69 increased from 5.20×10^8 CFU/mL at 0.02% Tween 80 to 1.17×10^9 CFU/mL at 0.04% ascorbic acid, then decreased to 7.40×10^8 CFU/mL at 0.10% Tween 80. Tween 80 is a surfactant allowed to use in food, it can change the permeability of cell membranes and is helpful to transport nutrients into the interior, release metabolites into cell exterior and reduce feedback inhibition. To ferment goat milk by *L. plantarum* LP69, addition of Tween 80 could promote the production of ACE inhibitory peptide and growth of *L. plantarum* LP69 within 0.04% concentration. The pH decreased and titration acidity increased with the increase of the concentration of Tween 80 from Fig. 6, which showed a opposite changes. The optimal concentrations of Tween 80 were both 0.04% for ACE inhibition and the viable cell counts.

CONCLUSION

The casein hydrolyses, ascorbic acid, $CaCl_2$, Ca $(H_2PO_4)_2$, NaCl and Tween 80 had a significant influence on ACE inhibitory activity in fermented milk and growth of *Lactobacillus plantarum* LP69. Addition of casein hydrolyses could promote the production of ACE inhibitory peptide, but inhibit growth of *L. plantarum* LP69, ascorbic acid, Ca $(H_2PO_4)_2$, NaCl and Tween 80 in low concentration could promote the production of ACE inhibitory peptide, $CaCl_2$ could promote the production of ACE inhibitory peptide and growth of *L. plantarum* LP69. The optimal concentration of casein hydrolyses, ascorbic acid, $CaCl_2$, $(CaH_2PO_4)_2$, NaCl and Tween 80 for ACE inhibitory activity was 0.7, 0.03 0.08 0.05, 0.90 and 0.04% in fermented goat milk by *Lactobacillus plantarum* LP69, respectively.

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