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Effect of Carbon Source and Salts on Angiotensin I Converting Enzyme (ACE) Inhibitory Activity in Fermented Goat Milk by *Lactobacillus bulgaricus* LB6

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Lactic acid bacteria are known to produce Angiotensin I converting enzyme (ACE) inhibitory peptides during fermentation. The objective of this work was to investigate effect of carbon source (glucose and lactose) and salts (calcium lactate, $Ca(H_2PO_4)_2$ and KH_2PO_4) on ACE inhibitory activity in fermented goat milk by *Lactobacillus bulgaricus LB6*. The addition of carbon source was 0.10, 0.30, 0.50, 0.70 and 0.90%, calcium lactate was 0.05, 0.06, 0.07, 0.08 and 0.09%, $Ca(H_2PO_4)_2$ and KH_2PO_4) were both 0.01, 0.03, 0.05, 0.07 and 0.09%. The results were as follows: The carbon source and salts on ACE inhibition and viable cell counts of *Lactobacillus bulgaricus LB6* in fermented goat milk had a significant effect($p<0.05$), the salts all had a significant inhibition on growth of *Lactobacillus bulgaricus LB6* but had a significant promotion on ACE inhibition in low concentration ($p<0.05$). The lactose was prior to glucose and $Ca(H_2PO_4)_2$ was prior to calcium lactate and KH_2PO_4 when taking ACE inhibition, viable cell counts and titration acidity into account, the optimal concentrations of lactose and $Ca(H_2PO_4)_2$ was 0.50% and 0.03%, respectively.

Key words: ACE inhibitory peptide; Goat milk; *Lactobacillus bulgaricus*; Carbon source; Calcium lactate.

Hypertension is a very important risk factor for the development of cardiovascular diseases, which is one of main causes of mortality (Duprez *et al*, 2002). The angiotensin converting enzyme (ACE, EC. 3.4.15.1) plays an important physiological role in regulating blood pressure and raise blood pressure by converting angiotensin-I to the potent vasoconstrictor angiotensin-II (Leclerc *et al*, 2002; Ondetti *et al*, 1977; Skeggs *et al*., 1956). Therefore, inhibition of ACE activity is considered to be a useful therapeutic approach in the treatment of hypertension. Although synthetic ACE inhibitors are effective as antihypertensive drugs, they have certain side effects. In this respect, functional foods with blood pressure-lowering properties have recently received considerable attention (Mohamed *et al*, 2010; Roy *et al*, 2010).

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Recently, some 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 and antihypertensive peptides have been produced using different lactic acid bacteria (Maryam *et al*, 2013), such as *Lactobacillus helveticus, L. acidophilus, L. casei, L. plantarum, L. rhamnosus, Lactobacillus bulgaricus, L. lactis ssp. lactis, L. lactis ssp. Cremoris* and *Enterococcus faecalis* (Algaron *et al*,2004; Ashar *et al* 2003; Chen *et al*, 2012; Fuglsang *et al*., 2003; Gobbetti *et al*., 2000; Hernandez-Ledesma *et al*.,2004; Hou *et al*, 2013; Leclerc *et al*., 2002; Muguerza *et al*.,2006; Nakamura *et al*., 1995; Quiros *et al*., 2007; Robert *et al*., 2004; Rodríguez-Figueroa *et al*,2010; Rokka *et al*., 1997; Shuangquan *et al* 2008; Vermeirssen *et al*., 2003; Yamamoto *et al*., 1994a, b, 1999). Other natural milk compounds that are associated with reduction of blood pressure are calcium and potassium (Groziak & Miller, 2000), but the effect of the salts containing

calcium and potassium on the ACE inhibitory acrivity was not studied.

In our previous study, 28 probiotic Lactobacillus strains, including 6 *Lactobacillus acidophilus* strains, 3 *Lactobacillus reuteri* strains, 5 *Lactobacillus rhamnosus* strains, 8 *Lactobacillus bulgaricus* strains, 2 Lactobacillus casei strains, 1 *Lactobacillus paracasei* strain, 2 *Lactobacillus plantarum* strains and 1 *Lactobacillus helveticu* strain, were used to ferment goat milk to screen for production ACE-inhibitory peptides, the results showed that 20 strains had ACE inhibitory activity and among them of 4 strains including *Lactobacillus reuteri LT33*, *Lactobacillus bulgaricus L6*, *Lactobacillus rhamnosus LR22* and *La*ctobacillus *helveticus LH69* were especially significant as producers of ACE-inhibitory peptides (He *et al*, 2012). In this study, Effect of carbon source (glucose and lactose) and salts (calcium lactate, $Ca(H_2PO_4)_2$ and KH_2PO_4) on ACE inhibitory activity in fermented goat milk by *Lactobacillus bulgaricus LB6* was investigated to obtain the optimum adding of carbon source and salts and provided reference for further optimization.

MATERIALS AND METHODS

Materials and reagents

Whole goat milk powder was purchased from Shaanxi Redstar Dairy Co., Ltd. (Weinan, China). ACE (extracted from rabbit lung acetone powder) and Hippuryl-histidyl-leucine (Hip-His-Leu) were bought from Sigma Chemical Co. (St Louis, MO, USA), carbon source (glucose and lactose) and salts (calcium lactate, $Ca(H_2PO_4)_2$ and KH_2PO_4) were purchased from Xi'an Luosenbo Biotechnology Co., Ltd. (Xi'an, China), All chemicals used were of analytical grade unless otherwise specified.

Microorganism and its activation

The strain used in this study was *Lactobacillus bulgaricus LB6* obtained from the College of Life Science and Engineering, Shaanxi University of Science and Technology. The strain was stored at –20°C in freeze-dried powder. *Lactobacillus bulgaricus LB6* was activated successively three times in rehydrated MRS broth (Hopebio, Qindao, China) at 37°C for 24 h prior to use.

Preparation of fermented goat milk

All fermentations were carried out by triplicate using 11% (w/v) reconstituted skim goat milk (RSGM) according to Muguerza *et al*. (2006). The goat milk was previously pasteurized in a water bath pot at 90°C for 15 min. an inoculum of 5% from *L. bulgaricus LB6* in MRS broth was transferred into 10 mL of RSGM (pre-culture) and then incubated at 37æ%C for 12h. The viable cell counts of *L. bulgaricus LB6* in the fermented goat milk was counted using MRS agar (Hopebio, Qindao, China).

Measurement of ACE inhibitory activity

The whey fraction separated from the fermented milk was used for testing the ACE inhibitory activity. Aliquots of the fermented goat milk were collected, vigorously stirred and centrifuged at 1000×g for 20 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 some modifications as described below. This method is based on the reaction of hydrolysis of N-Hippuryl-His-Leu (HHL) into hippuric acid (HA) and His-Leu (HL) catalysed by the ACE. The activity of ACE was measured in terms of HA produced over time. Added 80µL of each sample to 200µL sodium borate buffer (0.1mol/L, pH 8.3) containing NaCl (0.30 mol/L) and HHL (5mmol/L). Then ACE (20µL, 0.1U/mL) was added and the reaction mixture was incubated at 37°Cfor 30 min. The reaction was terminated by adding 250µL 1mol/L HCl. 1.7 mL ethyl acetate was used to extract the hippuric acid formed and evaporated at 120°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. The activity of each sample was tested in triplicate and done averaging. 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.

Measurement of viable cell counts, pH and titration acidity

The number of viable cells was determined by plating on triplicate MRS nutrient agar plates serial dilutions of milk samples made in saline water (0.9%, w/v, NaCl) containing 0.1g/L peptone. The inoculated plates were incubated at 37°C for 48 h under anaerobic conditions. Viable cell counts were performed by manual counting in those plates containing between 20 and 200 colonies and when possible, two different dilutions were considered to calculate the average of colony forming units per millilitre expressed as log cfu/mL (He *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 after mixing the fermented goat milk sample with 10 ml of hot distilled water (~90°C) and titration with 0.1N NaOH using a 0.5% phenolphthalein indicator to an end point of faint pink color.

RESULTS

Effect of glucose on ACE inhibitory activity in fermentated goat milk by L. bulgaricus LB6

The glucose was added to pasteurize reconstituted goat milk and the concentrations were 0.10, 0.30, 0.50, 0.70 and 0.90%, respectively. The inoculum size was 5% and cultured at 37 °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 ACE inhibition in fermented goat milk increased, but the viable cell counts of *Lactobacillus bulgaricus LB6* in fermented goat milk first increased and then decreased with the concentration of glucose increasing. The ACE inhibition in fermented goat milk increased from 37.09% at 0.1% glucose to 80.50% at 0.9% glucose, but the viable cell counts of *Lactobacillus bulgaricus LB6* increased from 4.17×10^7CFU/ml at 0.1% glucose to 5.89×10^7CFU/ml at 0.5% glucose, then decreased to 4.18×10^7 CFU/ml at 0.9% glucose, which indicates glucose in the low range of concentration(0.1-0.50%) can promote the growth of *Lactobacillus bulgaricus LB6*, but glucose in high concentrations will inhibit the growth of *Lactobacillus bulgaricus LB6* in goat milk, the inhibition may be due to increase in cell osmotic pressure resulted from increase of the glucose concentration. The increase of ACE inhibition in fermented goat milk may be due to glucose concentration on the metabolism of the cells had a positive impact, thereby promoting the hydrolysis of protease. As shown in Figure 2, the pH decreased and titration acidity increased with the increase of the concentration of glucose. The titration acidity gradually increased from 80.60 °T at 0.10 % glucose to 99°T at 0.70% glucose, then decreased to 97.20°T, but the pH decreased increased from 3.77 at 0.10 % glucose to 3.42 at 0.90% glucose. The optimal concentrations of glucose for ACE inhibition and the viable cell counts of *Lactobacillus bulgaricus LB6* were 0.9% and 0.5%, respectively.

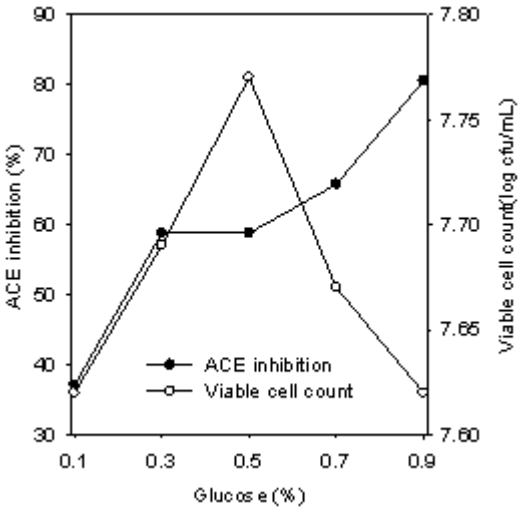

Fig.1. Effect of glucose on ACE inhibition and viable cell count in fermented goat milk

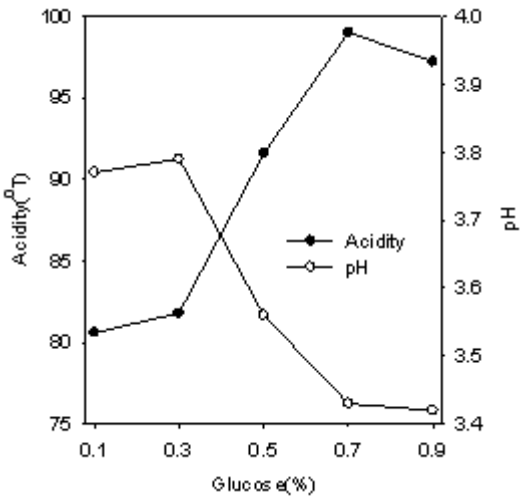

Fig. 2. Effect of glucose on acidity and pH in fermented goat milk

Effect of lactose on ACE inhibition and viable count in fermented goat milk by *L. bulgaricus* LB6

The lactose was added to pasteurize reconstituted goat milk and the concentrations were 0.10, 0.30, 0.50, 0.70 and 0.90%. The results were shown in Figure 3 and 4.

The ACE inhibition and viable cell counts of *L. bulgaricus LB6* in fermented goat milk were both first increased and then decreased with the increase of lactose concentration from Figure 3. The ACE inhibition in fermented goat milk first increased from 19.18% at 0.10% lactose to 72.73% at 0.70% lactose, then decreased to 65.71% at 0.90% lactose. The viable counts of *L. bulgaricus LB6* in fermented goat milk increased from 2.34×10^7 CFU/ml at 0.10% lactose to 3.72×10^7 CFU/ml at 0.70% lactose, then decreased to 2.75×10^7 CFU/ml at 0.90% lactose, which indicated that addition of lactose could promote the growth of *L. bulgaricus LB6* and production of ACE inhibitory peptide. With lactose increasing, the titration acidity in fermented goat milk first increased from 99.80°T at 0.10% lactose to 103.40 at 0.50% lactose, then decreased to 101.00°T, which indicated that addition of lactose on titration acidity and pH value in fermented goat milk had no significant change(p>0.05), the optimal concentration of lactose was 0.50% for ACE inhibition and the viable cell counts of *Lactobacillus bulgaricus LB6*.

Effect of calcium lactate on ACE inhibition and viable count in fermented goat milk by *L. bulgaricus* LB6

The calcium lactate was added to pasteurize reconstituted goat milk and the concentrations were 0.05, 0. 06, 0.07, 0.08 and 0.09%. The results were shown in Figure 5 and 6.

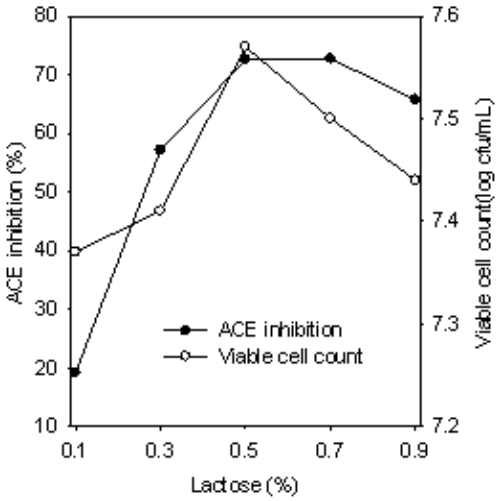

Fig. 3. Effect of lactose on ACE inhibition and viable cell count in fermented goat milk

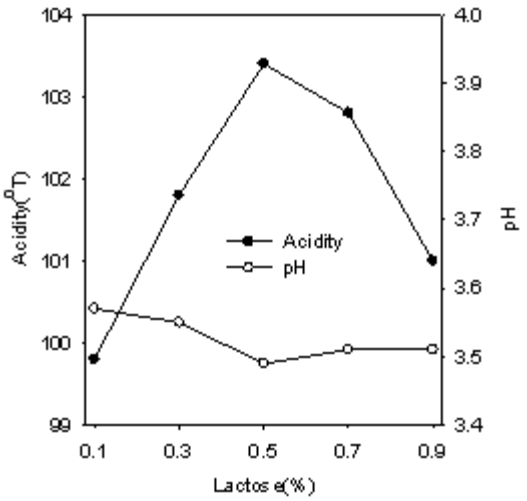

Fig. 4. Effect of lactose on acidity and pH in fermented goat milk

The ACE inhibition and viable cell counts of *L. bulgaricus LB6* in fermented goat milk showed opposite changes with the increase of calcium lactate concentration from Figure 5. The ACE inhibition decreased from 80.32% at 0.05% calcium lactate to 57.04% at 0.9% calcium lactate, while the viable counts of *L. bulgaricus LB6* in fermented goat milk increased from 3.31×10^7CFU/ml at calcium lactate 0.5% to 6.61×10^7CFU/ml at calcium lactate 0.8%, then decreased to 6.03×10^7CFU/ml at calcium lactate 0.9%, which indicated that addition of calcium lactate could promote growth of *L. bulgaricus LB6*, but inhibit the production of ACE inhibitory peptide in high concentration. With calcium lactate increasing, the titration acidity and the pH value had no significant change(p>0.05) from figure 8, the range of titration acidity was 192.0~202.4°T and the pH value increased from 3.78 at 0.5% calcium lactate to 3.88 at 0.9% calcium lactate. The optimal concentrations of calcium lactate for ACE inhibition and the viable cell counts of *Lactobacillus bulgaricus LB6* were 0.50% and 0.80%, respectively.

Effect of $Ca(H_2PO_4)_2$ on ACE inhibition and viable count in fermented goat milk by *L. bulgaricus* LB6

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%. The results were shown in Figure 7 and 8.

With the concentration of $Ca(H_2PO_4)_2$ increasing, The viable counts of *L. bulgaricus LB6* in fermented goat milk gradually decreased from

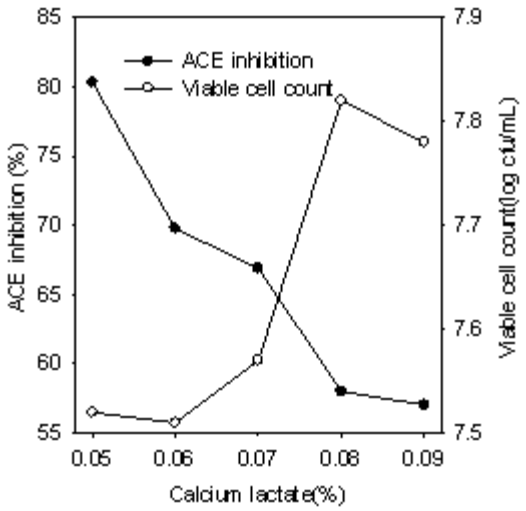

Fig. 5. Effect of calcium lactate on ACE inhibition and viable cell count in fermented goat milk

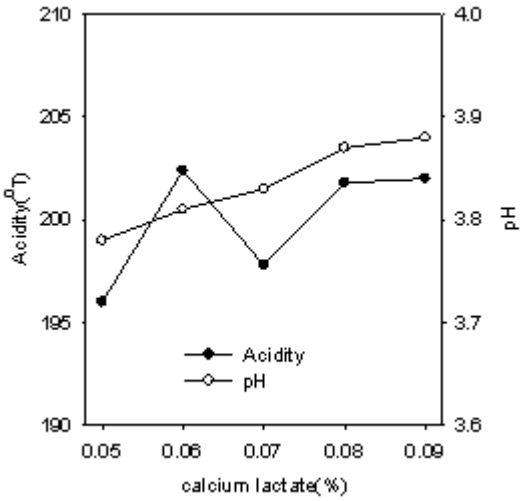

Fig. 6. Effect of calcium lactate on acidity and pH in fermented goat milk

4.27×10^7CFU/ml at 0.01% $Ca(H_2PO_4)_2$ to 2.14×10^7 CFU/ml at 0.09% $Ca(H_2PO_4)_2$, while ACE inhibition first increased from 70.18% at 0.01% $Ca(H_2PO_4)_2$ to 76.71% at 0.03% $Ca(H_2PO_4)_2$ and then decreased to 64.73% at 0.09% $Ca(H_2PO_4)_2$ from figure 7, which indicated that addition of $Ca(H_2PO_4)_2$ in goat milk inhibited the growth of *L. bulgaricus LB6*, but promoted production of ACE inhibitory peptides in low concentration. With $Ca(H_2PO_4)_2$ increasing, the titration acidity in fermented goat milk increased

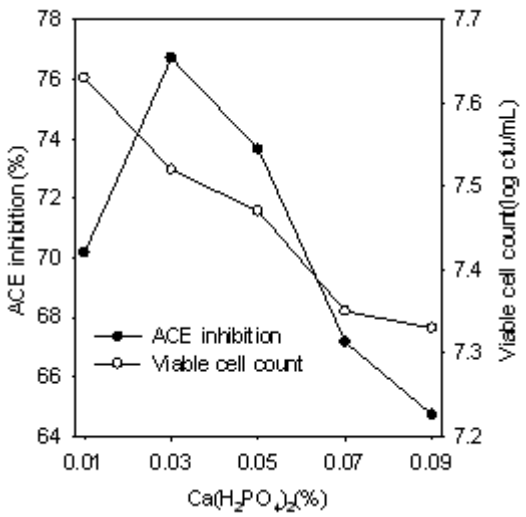

Fig. 7. Effect of $Ca(H_2PO_4)_2$ on ACE inhibition and viable count in fermented goat milk

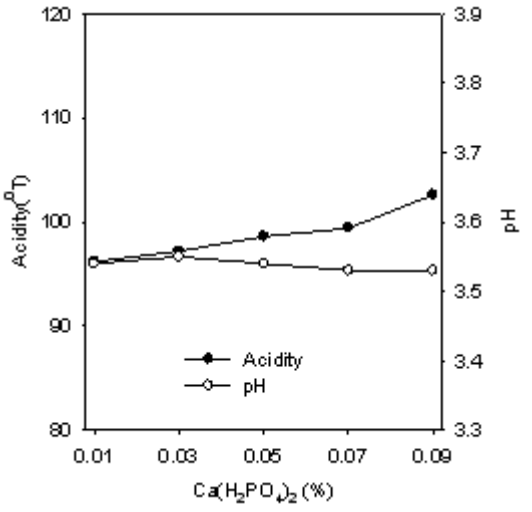

Fig. 8. Effect of $Ca(H_2PO_4)_2$ on acidity and pH in fermented goat milk

from 96.20°T at 0.01% $Ca(H_2PO_4)_2$ to 102.60°T at 0.09% $Ca(H_2PO_4)_2$ from figure 8, but the titration acidity and the pH value had no significant change(p>0.05). The optimal concentrations of $Ca(H_2PO_4)_2$ for ACE inhibition and the viable cell counts of *Lactobacillus bulgaricus LB6* were 0.03% and 0.01%, respectively.

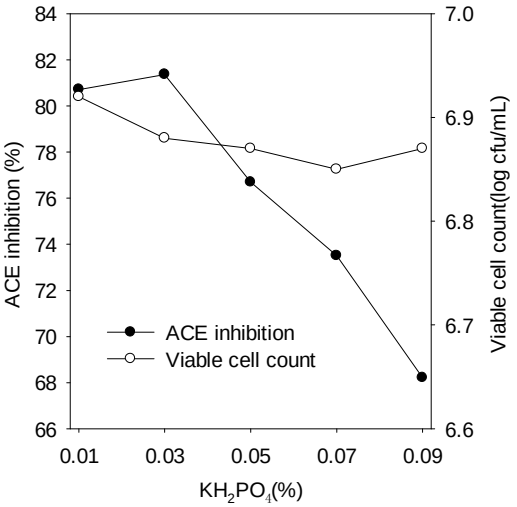

Fig. 9. Effect of KH_2PO_4 on ACE inhibition and viable count in fermented goat milk

The viable counts of *L. bulgaricus LB6* in fermented goat milk gradually decreased from 8.32×10^7CFU/ml at 0.01% KH_2PO_4 to 7.08×10^7 CFU/ml at 0.09% KH_2PO_4 with the concentration of KH_2PO_4 increasing, while ACE inhibition first increased from 80.71% at 0.01% KH_2PO_4 to 81.36% at 0.03% KH_2PO_4 and then decreased to 68.21% at 0.09% KH_2PO_4 from figure 9, which showed that addition of KH_2PO_4 in goat milk inhibited the growth of *L. bulgaricus LB6*, but promoted production of ACE inhibitory peptides in low concentration. With KH_2PO_4 increasing, the titration acidity in fermented goat milk increased significantly (p<0.05),which increased from 107.60°T at 0.01% KH_2PO_4 to 169.20°T at 0.07% KH_2PO_4 then decreased to 160.80°T at 0.09% KH_2PO_4 from figure 10, while the pH value had no significant change(p>0.05) , which showed that goat milk has a good buffer capacity. The optimal concentrations of KH_2PO_4 for ACE inhibition and the viable cell counts of *Lactobacillus bulgaricus LB6* were 0.03% and 0.01%, respectively.

Effect of KH_2PO_4 on ACE inhibition and viable count in fermented goat milk by *L. bulgaricus* LB6

The KH_2PO_4 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 Figure9 and 10.

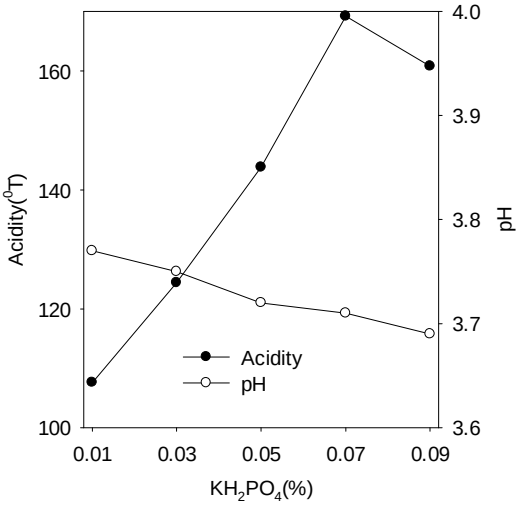

Fig. 10. Effect of KH_2PO_4 on acidity and pH in fermented goat milk

DISCUSSION

Two kinds of carbon including glucose and lactose on ACE inhibition and viable cell counts of *Lactobacillus bulgaricus LB6* in fermented goat milk had a significant effect(p<0.05) and on titration acidity and pH value had no significant change(p>0.05), the lactose was prior to glucose when using ACE inhibition and viable cell counts as responses and the optimal concentrate of lactose was 0.50%.

Ionic calcium has also been reported to possess antihypertensive activity in vivo, but the underlying mechanism is not yet clears (Griffith *et al*, 1999). Ionic calcium released upon milk acidification during fermentation is also known to exert hypotensive activity (Allen, 1982; Hebert *et al*, 2010). Calcium can enhance the hypotensive activity of certain fermented dairy products either alone or in combination with other hypertensive bioactive components in these foods (Gonzalez-Gonzalez *et al*, 2011), it was found that the ionic

calcium released during milk fermentation could contribute to the ACE-inhibitory activity. Three kinds of salts including calcium lactate, $Ca(H_2PO_4)_2$ and KH_2PO_4 in the study on ACE inhibition and viable cell counts in fermented goat milk had a significant effect($p<0.05$). The addition of calcium lactate and $Ca(H_2PO_4)_2$ on titration acidity and pH value had no significant change($p>0.05$), but addition of KH_2PO_4 on titration acidity had a significant effect($p<0.05$), so $Ca(H_2PO_4)_2$ was prior to calcium lactate and KH_2PO_4 when using ACE inhibition, viable cell counts and titration acidity as responses and the optimal concentrate of lactose was 0.03%.

CONCLUSION

The carbon source (glucose and lactose) and salts (calcium lactate, $Ca(H_2PO_4)_2$ and KH_2PO_4) on ACE inhibition and viable cell counts of *Lactobacillus bulgaricus LB6* in fermented goat milk had a significant effect($p<0.05$), the salts all had a significant inhibition on growth of *Lactobacillus bulgaricus LB6* but had a significant promotion on ACE inhibition in low concentration ($p<0.05$). The lactose was prior to glucose and $Ca(H_2PO_4)_2$ was prior to calcium lactate and KH_2PO_4 when taking ACE inhibition, viable cell counts and titration acidity into account, the optimal concentrations of lactose and $Ca(H_2PO_4)_2$ was 0.50% and 0.03%, respectively.

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