Int.J.Curr.Microbiol.App.Sci (2017) *6*(12): 1670-1676

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 12 (2017) pp. 1670-1676**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.612.188

Angiotensin Converting Enzyme Inhibitory Activity from Fermented Goat Milk Produced with Different Lactic Acid Bacteria

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Keywords

Goat milk, Lactic acid bacterial cultures, Hypertension, ACE inhibitory activity, Fermentation.

Article Info

Accepted:
12 October 2017
Available Online:
10 December 2017

A B S T R A C T

A study was conducted to investigate the potential of different lactic acid bacterial cultures to release angiotensin converting enzyme (ACE) inhibitory peptides from goat milk. Fermented milk samples were prepared by using seven different lactic acid bacterial cultures such as *Lactobacillus helveticus, L. rhamnosus, L. delbruckii* subsp. *bulgaricus, L. plantarum, L. acidophilus, L. casei and L. paracasei subsp. paracasei.* The fermented milk samples were analysed for pH, titratable acidity, lactic acid bacterial count, proteolytic activity and ACE inhibitory activity. The pH of fermented milk samples ranged from 4.535±0.074 to 5.921±0.153. Titratable acidity ranged from 0.276±0.011 to 0.833±0.043 per cent lactic acid. Lactic acid bacterial count ranged from 9.079±0.064 to 10.042±0.056 log cfu/mL. Proteolytic activity varied from 0.415±0.029 to 0.619±0.049 and ACE inhibitory activity ranged from 65.121±0.963 to 90.700±1.276 per cent. The maximum ACE inhibitory activity was observed in goat milk fermented with *L. plantarum* 417.

Introduction

Milk proteins are good source of peptides with diverse biological activities. These bioactive peptides are latent or encrypted within the primary structure of native protein and can be released by enzymatic hydrolysis during gastrointestinal digestion or milk fermentation with lactic acid bacteria. They have anti oxidative, anti-carcinogenic, antimicrobial, immuno modulatory, antithrombotic, mineral binding and angiotensin converting enzyme (ACE) inhibitory activities (Nandhini *et al.*, 2012). ACE is a dipeptidyl carboxy peptidase, key enzyme which plays a major role in regulation of blood pressure in the renin-angiotensin-aldosterone system and kinin nitric oxide system. ACE raises blood pressure by converting the inactive angiotensin I (decapeptide) to the potent vasoconstrictor angiotensin II (octapeptide). It also inactivates the vasodilator, bradykinin which leads to increase in blood pressure. ACE inhibitory peptides were isolated from different food sources, such as soy proteins, fish proteins, egg proteins and maize and milk proteins. However, milk proteins appear to be the most important source of these bioactive peptides identified so far.

The aim of this study was to determine the ACE inhibitory activity from goat milk fermented with different lactic acid bacterial cultures

Materials and Methods

Goat milk required for the study was collected from University Goat and Sheep farm, Mannuthy. The starter cultures such as *Lactobacillus helveticus* (NCDC 288), *Lactobacillus bulgaricus* (NCDC 009), *Lactobacillus rhamnosus* (NCDC 024), *Lactobacillus plantarum* (NCDC 417), *Lactobacillus casei* (NCDC 297), *Lactobacillus paracasei* subsp. *paracasei* (NCDC 022) and *Lactobacillus acidophilus* (NCDC 015) were procured from National Collection of Dairy Cultures, NDRI, Karnal, India.

Preparation of fermented milk

Goat milk was filtered to remove the extraneous matter and laboratory pasteurization was carried out at 68ºC for 30 minutes. Then the milk was cooled to the inoculation temperature and inoculated with 3 percent of the cultures. Incubation was done at 37ºC for 14 hrs for the cultures *L. plantarum* NCDC 417, *L. casei* NCDC 297, *L. paracasei* subsp. *paracasei* NCDC 022, *L. acidophilus* NCDC 015. Milk samples fermented with *Lactobacillus helveticus* NCDC 288, *Lactobacillus bulgaricus* NCDC 009 *and Lactobacillus rhamnosus* NCDC 024 were incubated at 42ºC for 14hrs. Then the fermented milk samples were stored at refrigerated temperature.

Determination of pH, titratable acidity and lactic acid bacterial count of fermented milk

pH of the fermented milk was determined by using digital pH meter (Systronics pH meter). Titratable acidity was determined by the method suggested by (Ranganna, 2005). The acidity was expressed in terms of per cent lactic acid. Lactic acid bacterial count was done by pour plate method by using de Mann Rogosa Sharpe agar (MRS agar). The plates were incubated at 37ºC for 24hrs for *L. plantarum, L. acidophilus, L. casei and L. paracasei* subsp *paracasei.* Samples inoculated with L. *helveticus, L. bulgaricus and L. rhamnosus* were incubated at 42ºC for 24 hrs. After the incubation period, Lactic acid bacterial count was done by using a colony counter.

Determination of proteolytic activity

Measurement of proteolytic activity was done by Quantitative method (Ophthaldialdehyde method) as per Hati *et al.*, (2015). An aliquot of 2.5 ml of activated cultures were added to 5 ml of 0.75% trichloro acetic acid (TCA) and allowed to stand for 10 min and the mixture was filtered using Whatman filter paper 42. Then 150 µl of filtrate obtained from filtration process was transferred to 3 ml of OPA reagent and mixed properly. After incubation for 2 min at room temperature, the absorbance of solution was measured spectro photometrically at 340 nm. The proteolytic activities of bacterial cultures were expressed as absorbance of free amino acid groups measured at 340 nm.

Determination of ACE inhibitory activity

The Angiotensin Converting Enzyme inhibitory (ACE-I) activity was determined according to the method of Ramchandran *et al.*, (2008). Briefly, 0.005 M Hippuryl-L Histidyl-L-leucine (HHL) was diluted in 0.1 M borate buffer containing 0.3 M NaCl at pH 8.3, as a substrate for the rabbit lung ACE (0.1 units/mL). Approximately, 60 µl of the borate buffer was added to 200 µl of the substrate solution followed by the addition of

30 µl of the filtered sample and pre incubated at 37 °C for 5 min. The next step was addition of exactly 20 µl of the enzyme solution (ACE) and incubation at 37 °C for 30 min. The reaction was stopped by adding 250µl of 1M HCl solution and then mixing with 1.7 mL ethyl acetate. After 10 minutes incubation at room temperature, 1.4 mL of the ethyl acetate layer was removed and dried on a boiling water bath and then in an oven at 80°C for 30 min. The remaining residue after the removal ethyl acetate was discarded. The dried hippuric acid in the test tube was dissolved in 1 mL of deionised water and the absorbance of the resulting solution was measured at 228 nm using a UV spectrophotometer. The percent inhibition was calculated by using the following formula:

ACE inhibition = 1- (C-D/A-B) ×100

Where,

A is the absorbance in the presence of ACE and without sample,

B is the absorbance without ACE and sample,

C is the absorbance with ACE and ACE-inhibitory component, and

D is the absorbance with sample but without ACE.

Results and Discussion

pH

Mean pH of goat milk samples fermented with different lactic acid bacterial cultures are presented in Table 1. pH values of fermented milk samples ranged from 4.535±0.074 to 5.921±0.153. Milk fermented with *L. acidophilus* 015 showed highest pH value whereas, milk fermented with *L. helveticus* 288 showed lowest pH value. Hati *et al.*, (2015), reported that pH of fermented milk samples ranged from 3.8 to 4.8 for different strains like *L. helveticus (MTCC 5463), L. rhamnosus (NS4 and NS6) and L. bulgaricus* (009). In their study the cultures were inoculated at 1 percent level. In the present study the cultures were inoculated at 3 percent level. However, the selected cultures in the present study had low acid production potential. Hence, the fermented milk samples showed high pH values.

Titratable acidity

The mean titratable acidity of milk samples fermented with different lactic acid bacterial cultures are presented in Table 2. Titratable acidity of fermented milk samples ranged from 0.276±0.011 to 0.833±0.043 percent lactic acid. The highest titratable acidity was noticed in *L. helveticus* NCDC 288 and lowest titratable acidity was noticed in *L. acidophilus* 015. Hati *et al.*, (2015) reported that milk fermented with *L. bulgaricus (NCDC 009), L. rhamnosus NS4 and NS6, L. helveticus had 0.6*, 0.78, 0.97 and 0.831 percent lactic acid respectively. Shu *et al.*, (2015) also reported similar findings. Titratable acidity of fermented milk samples varied due to the difference in bacterial species, strains, inoculum level, and incubation time.

Lactic acid bacterial counts

Mean Lactic acid bacterial count of milk samples fermented with different lactic acid bacterial cultures are presented in Table 3. Lactic acid bacterial count of fermented milk samples ranged from 9.079±0.064 to 10.042±0.056 log cfu/mL. Highest Lactic acid bacterial count was noticed in milk fermented with *L. bulgaricus* 009 and the count was 10.042±0.056 log cfu/mL. The lowest count was noticed in milk fermented with *L. paracasei* subsp *paracasei* 022 and it was

9.079±0.064 log cfu/mL. Nejati *et al.*, (2013) found that lactic acid bacterial count of fermented milk prepared with *L. plantarum* PU11 was log cfu/mL.

Table.1 Mean pH of goat milk fermented with different lactic acid bacterial cultures

S.NO	CULTURE NO	INNOCULAM SIZE (3 PER CENT)
1.	*Lactobacillus helveticus* 288	4.535±0.074[a]
2.	*Lactobacillus bulgaricus* 009	5.613±0.093[de]
3.	*Lactobacillus rhamnoasus* 024	4.971±0.172[b]
4.	*Lactobacillus plantarum* 417	5.240±0.077[bc]
5.	*Lactobacillus casei* 297	5.326±0.094[cd]
6.	*Lactobacillus paracaseissp.paracasei* 022	5.695±0.119[de]
7.	*Lactobacillus acidophilus* 15	5.921±0.153[e]
F value		16.186[**]
p-value		<0.001

Each value is a mean of six observations with SE
Means with different superscripts within same column differ significantly (p<0.001)
**Significant at 0.01 level

Table.2 Titratable acidity of goat milk fermented with different lactic acid bacteria

S.NO	CULTURE NO	INNOCULAM SIZE (3 PER CENT)
1.	*Lactobacillus helveticus* 288	0.833±0.043[d]
2.	*Lactobacillus bulgaricus* 009	0.41±0.027[b]
3.	*Lactobacillus rhamnoasus* 024	0.589±0.048[c]
4.	*Lactobacillus plantarum* 417	0.566±0.026[c]
5.	*Lactobacillus casei* 297	0.526±0.056[c]
6.	*Lactobacillus paracaseissp.paracasei* 022	0.366±0.042[ab]
7.	*Lactobacillus acidophilus* 15	0.276±0.011[a]
F-value		22.007[**]
p-value		<0.001

Each value is a mean of six observations with SE
Means with different superscripts within same column differ significantly (p<0.001)
**Significant at 0.01 level

Table.3 Lactic acid bacterial count of goat milk fermented with different lactic acid bacterial cultures

S.No	Culture No	Inoculum Size (3 per cent)
1.	*Lactobacillus helveticus* 288	9.355 ± 0.042^{b}
2.	*Lactobacillus bulgaricus* 009	10.042 ± 0.056^{e}
3.	*Lactobacillus rhamnoasus* 024	9.695 ± 0.025^{d}
4.	*Lactobacillus plantarum* 417	9.570 ± 0.062^{cd}
5.	*Lactobacillus casei* 297	9.258 ± 0.081^{b}
6.	*Lactobacillus paracasei* subsp. *paracasei* 022	9.079 ± 0.064^{a}
7.	*Lactobacillus acidophilus* 15	9.426 ± 0.046^{bc}
F-value		31.196^{**}
p-value		<0.001

Each value is a mean of six observations with SE
Means with different superscripts within same column differ significantly (p<0.001). **Significant at 0.01 level

Table.4 Proteolytic activity of goat milk fermented with different lactic acid bacterial cultures

S.No	Culture No	Inoculum Size (3 per cent)
1.	*Lactobacillus helveticus* 288	0.415 ± 0.029^{a}
2.	*Lactobacillus bulgaricus* 009	0.513 ± 0.019^{b}
3.	*Lactobacillus rhamnoasus* 024	0.418 ± 0.027^{a}
4.	*Lactobacillus plantarum* 417	0.519 ± 0.019^{b}
5.	*Lactobacillus casei* 297	0.500 ± 0.013^{ab}
6.	*Lactobacillus paracasei* subsp. *paracasei* 022	0.619 ± 0.049^{c}
7.	*Lactobacillus acidophilus* 15	0.506 ± 0.032^{b}
F-value		5.623^{**}
p-value		<0.001

Each value is a mean of six observations with SE
Means with different superscripts within same column differ significantly (p<0.001). **Significant at 0.01 level

Table.5 Angiotensin converting enzyme inhibitory activity of goat milk fermented with different lactic acid bacterial cultures

S.No.	Culture No.	Inoculum Level (3 per cent) (per cent)	Inoculum Level (3 per cent) **(IC_{50} value mg/mL)**
1.	*Lactobacillus helveticus* 288	65.121 ± 0.963^{a}	0.082
2.	*Lactobacillus bulgaricus* 009	87.075 ± 1.709^{d}	0.033
3.	*Lactobacillus rhamnoasus* 024	88.660 ± 1.774^{d}	0.031
4.	*Lactobacillus plantarum* 417	90.700 ± 1.276^{d}	0.028
5.	*Lactobacillus casei* 297	68.196 ± 1.974^{a}	0.073
6.	*Lactobacillus paracaseissp.paracasei* 022	81.876 ± 2.191^{c}	0.043
7.	*Lactobacillus acidophilus* 15	76.363 ± 1.786^{b}	0.054
F-value		35.053^{**}	
p-value		<0.001	

Each value is a mean of six observations with SE
Means with different superscripts within same column differ significantly (p<0.001). **Significant at 0.01 level

Proteolytic activity

Mean proteolytic activity of different lactic acid bacterial cultures are presented in Table 4. The Proteolytic activity varied from 0.415±0.029 to 0.619±0.049.The highest proteolytic activity (0.619±0.049) was observed in milk fermented with *Lactobacillus paracasei* subsp. *paracasei* 022. Milk fermented with *Lactobacillus helveticus* 288 showed the lowest proteolytic activity (0.415±0.029). The extent of proteolysis varied among species and found to be strain dependant. Donkor *et al.,* (2007a) reported that proteolytic activity of *L. helveticus was* 0.592. In the present study proteolytic activity of *L. helveticus* was 0.415. The variation in proteolytic activity can be attributed to the difference in strains.

ACE inhibitory activity

Mean ACE inhibitory activity of milk samples fermented with different lactic acid bacterial cultures are presented in Table 5. ACE inhibitory activity ranged from 65.121±0.963 to 90.700±1.276 per cent. The highest ACE inhibitory activity was observed in goat milk fermented with *Lactobacillus plantarum* 417. The value was 90.700±1.276 per cent. The lowest ACE inhibitory activity was observed in goat milk fermented with *Lactobacillus heleticus* 288 and the activity was 65.121±0.963 per cent. Nandhini *et al.,* (2012) have studied the ACE- inhibitory activity and antioxidant properties of goat milk fermented with *Lactobacillus plantarum.* They have reported that the ACE inhibitory activity of the fermented milk was 88.96%. In a study conducted by Hati *et al.,* (2015) milk fermented with *Lactobacillus rhamnosus and Lactobacillus delbruckii* exhibited highest proteolytic activity and ACE inhibitory activity. Shu *et al.,* (2015) reported that ACE inhibitory activity of milk fermented with *L. plantarum* varied from 77.95 to 81.25%. In this study *Lactobacillus plantarum* (417) had ACE inhibitory activity of 90.70 % at 3% inoculum level. There was significant positive correlation between proteolytic activity and ACE inhibitory activity.

Goat milk fermented with different lactic acid bacterial cultures had ACE inhibitory activity ranging from 65.12 to 90.70%. Among seven LAB cultures, milk fermented with *Lactobacillus plantarum* (NCDC 417) showed maximum ACE inhibitory activity followed by *Lactobacillus rhamnosus* (NCDC 024). There was a significant positive correlation between proteolytic activity and ACE inhibitory activity. There is a great potential to develop functional dairy products with antihypertensive activity by incorporating specific lactic acid bacterial cultures capable of releasing ACE inhibitory peptides in the preparation of fermented dairy products.

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How to cite this article:

Sathya, P., K. Radha, C.T. Sathian and Srinivasan, C. 2017. Angiotensin Converting Enzyme Inhibitory Activity from Fermented Goat Milk Produced with Different Lactic Acid Bacteria. *Int.J.Curr.Microbiol.App.Sci.* 6(12): 1670-1676. doi: https://doi.org/10.20546/ijcmas.2017.612.188