Effects of Inclusion of Allium sativum and Cinnamomum verum in Milk on the Growth and Activity of Lactic Acid Bacteria During Yogurt Fermentation

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Abstract: The present study investigated the effects of Allium sativum and Cinnamomum verum water extract on milk fermentation and subsequent changes in yogurt bacterial growth during fermentation. Three types of milk (cow, camel and goat) were incubated at 41°C with starter culture in the presence of either A. sativum or C. verum water extract until pH of yogurt was reduced to 4.5. The presence of A. sativum or C. verum water extract in all the three types of milk did not affect pH reduction as compared to respective plain milk (control) during fermentation. However, the presence of A. sativum in camel milk shorted the incubation period to 4 hours as compared to plain milk (5 hours). The proteolytic activity increased significantly in the presence of A. sativum or C. verum in all the three types of milk compared to respective control during fermentation. Cow milk in the presence of herbal extracts showed the highest (p<0.05) proteolytic activities of lactic acid bacteria among other treated milk. Viable cell counts (VCC) of Streptococcus thermophilus, Lactobacillus spp and Bifidobacterium bifidum increased in the presence of A. sativum or C. verum in all the three types of milk compared to respective control during fermentation. In conclusion, A. sativum and C. verum water extracts improved proteolytic activity and enhanced the growth of LAB in cow, goat and camel milk during fermentation.

Key words: Yogurt • Fermentation • Proteolytic activity • Lactic acid bacteria • Allium sativum • Cinnamomum verum

INTRODUCTION

Yogurt has long been known as a product with many desirable effects for health [1, 2]. The excellent sensory properties and the health benefits of yogurt [3] can be credited to the action of yogurt bacteria and their metabolites [1, 4]. Lactic acid bacteria (LAB) particularly Lactobacilli, Streptococci and Bifidobacteria are the most important microorganisms associated with the health status of human gastrointestinal tract which justifies the reason for calling them friendly bacteria. They are dependent on carbohydrates such as lactose and glucose for their energy sources and yield lactic acid as major end products. In yogurt production, changes in the milk substrate by LAB during fermentation are attributed to fermentation temperature, ingredients added during manufacturing, fermentative action of the inoculated starter cultures and the secretion of nutritional and chemical substances by the microorganisms [1]. Since conventional yogurt starter bacteria, Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus are very sensitive to survive passage through the low pH in gastric tract [5], the combination of live strains of L. acidophilus and species of Bifidobacterium to yogurt bacteria [1] have been widely used in yogurt manufacturing. A product is called yogurt if live bacteria are present in the final product. The viable number of probiotics in the final product was suggested to be at least 10⁶–10⁷ cfu/g to be accepted as the therapeutic minimum [6].

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Most yogurt are considered "ripe" somewhere in the pH range of 4.0-4.5, depending on how strong or mild a product is preferred [7]. The fermentation is terminated at pH 4.5 because this is the preferred pH in commercial dairy products. The pH values lower than 4.0 are undesirable because L. bulgaricus tends to produce excessive lactic acid, acetaldehyde and proteolytic by-products at such pH [8]. On the other hand, pH of about 4.5 can help maintain the yogurt throughout shelf life, maintain a mild flavour and a pleasant product appearance and eliminate the graininess that commonly develops during breaking and cooling of yogurt [7].

The traditionally prepared yogurt may be improved by the inclusion of materials such as soya protein, vegetables, sweet potato, pumpkin, plum and fish collagen [9-13] to enhance the flavour. In addition, some ingredients such as peppermint (Mentha piperita), dill (Anethum graveolens), basil (Ocimum basilicum) and neem (Azadirachta indica) showed remarkable improvement in the therapeutical properties of yogurt [14, 15]. Plants such as Allium sativum and Cinnamomum verum (also known as garlic and cinnamon) have unique propositional values to be considered as addition in yogurt making. Their bioactive compounds have great therapeutical values towards treatment of several diseases such as hypertension and diabetes [16, 17]. Previous studies showed that the addition of A. sativum or C. verum enhanced yogurt functional properties with anti-diabetic activity [18, 19]. Besides, the addition of A. sativum and C. verum did not affect the organoleptic properties of yogurt [11] although A. sativum may reduce the aroma score in cow milk yogurt but not camel milk yogurt [11]. The inclusion of these two herbs by virtue of the rich phytochemicals is expected to affect microbial growth during fermentation of milk. Thus, the present study investigated the effect of A. sativum and C. verum on LAB during fermentation of three types of milk (cow, goat and camel milk) by determining the acidification process, microbial growth and their proteolytic activity during milk fermentation.

MATERIALS AND METHODS

Plant Water Extraction Procedure: A. sativum or C. verum powder was mixed with sterile dH2O at the ratio of 1: 10 in a 250 ml bottle. The final volume of both herbal extracts was 0.1g/ml. The mixture was left for 12 hours [18, 19] in a water bath (70°C; Julabo, Model Sw-21c or Haake Model SWD 20) followed by centrifugation (1000 rpm, 15minutes at 4°C; Eppendoft 5804 R). The supernatant was harvested and used as herbal water extract in the making of herbal-yogurt.

Starter Culture Preparation: The preparation of starter culture was carried out using the method described by Shori and Baba [11]. Commercially available direct vat set (DVS) yogurt starter culture powder used in yogurt preparation consist of a mixture of Lactobacillus acidophilus LA-5, Bifidobacterium bifidum Bb-12, Lactobacillus casei LC-01, Streptococcus thermophilus Th-4 and L. delbrueckii ssp. Bulgaricus in the ratio of 4:4:1:1:1. Chilled pasteurized full cream milk (1 L) was pre-heated to 41°C. A small volume of milk (100ml) was placed into a sterilized beaker. The probiotic yogurt bacteria powder mix was added into milk. The mixture was stirred thoroughly and was then mixed evenly with the remainder of the milk followed by incubation for 12 hours at 41°C (Julabo, Model Sw-21c or Haake Model SWD 20). The yogurt formed was refrigerated (4°C) and used as starter culture within 3 days in the making of yogurt.

Yogurt Preparation: Pasteurized full cream milk (1 L) was heated to 41°C in a 3 L beaker. The milk was subsequently divided into 3 portions of 255 ml placed into 500 ml beaker. The first portion was used as plain-yogurt (control) after the addition of 30 ml dH2O and inoculation with 15g starter culture. The second and third portions were used to prepare A. sativum- and C. verum- yogurts by adding 30 ml of A. sativum or C. verum water extract (0.1g/ml) respectively and 15g of starter culture into each portion [18, 19]. Aliquots of 100 ml from each portion were placed into a disposable 150 ml plastic containers and these were held at 41°C (Julabo, Model Sw-21c or Haake Model SWD 20) until the required pH of 4.5 was reached. The acidification, proteolysis and viability of LAB and probiotics of cow, goat and camel milk both in the presence and absence of herbal water extracts were monitored by taking 10 ml samples for every 30 min for acidification measurement and every one hour for the other analysis until the pH reached 4.5.

Determination of pH: The changes of milk pH during fermentation were monitored as described by Shori and Baba [15].

Determination of Proteolytic Activity: Yogurt proteolytic activity was assessed during fermentation by measuring liberated free amino groups using o- phthalaldehyde (OPA) method [20].
Viable Cell Counts (VCC) of Lactic Acid Bacteria: The enumeration of lactic acid bacteria was carried out during fermentation at 41°C, using de Man, Rogosa and Sharp (MRS) agar for Lactobacillus spp enumeration [21]. Diluted yogurt (1ml) was mixed with 15ml of autoclaved melted MRS media (62 g/L dH2O, 45°C) and incubated in anaerobic atmosphere (37°C, 48 hours). M17 agar was used for Streptococcus thermophilus enumeration [22]. Diluted yogurt (0.1ml) was plated on solidified M17 media (48.25 g/ 950 ml dH2O with 50 ml of 10% w/v lactose solution) and incubated in aerobic atmosphere (37°C, 48 hours). MRS-LP agar was used for Bifidobacterium bifidum enumeration [23]. Diluted yogurt (1ml) was mixed with 15ml of autoclaved melted MRS-LP media and incubated in anaerobic atmosphere (37°C, 72 hours).

Statistical Analysis: All results presented as means of three independent replicates. Statistical analysis was performed using one way analysis of variance (ANOVA, SPSS 17.0), followed by Duncan’s post hoc test for mean comparison. The criterion for statistical significance was p<0.05.

RESULTS

Acidification Trend During Milk Fermentation: Cow milk did not show lag phase compared to other treated milk (goat and camel milk) both in the presence and absence of A. sativum or C. verum water extract (Figure 1). Both goat and camel milk samples started to show observable pH reduction after one hour of incubation. The presence of A. sativum or C. verum in all the three types of milk had no significant effect on pH reduction as compared to respective controls during fermentation. However, the addition of A. sativum in the camel milk resulted in shorter (240 min) incubation time as compared to plain milk (300 min, p<0.05; Figure 1 c).

Proteolytic Activity: The initial free amino groups of cow milk mixture with C. verum water extract showed no difference compared to cow milk alone. The free amino groups of cow milk + C. verum increased to 172.90±0.03 µg/g as compared to plain milk that increased to about 80.10±0.02 µg/g after 4 hours (Figure 2 a). The free amino groups in milk + A. sativum began with 166.51±0.1 µg/g and ended with 262.57±0.1 µg/g 4 hours after incubation (Figure 2 a). The initial free amino groups in goat milk before fermentation were 213.26±0.05 µg/g (Figure 2 b).

The presences of A. sativum or C. verum increased free amino groups in goat milk to 285.87±0.06 µg/g (p<0.05) and 235.83±0.05 µg/g (p>0.05) respectively. Proteolytic activity significantly increased after the 2nd hour of fermentation for goat milk and goat milk + C. verum. The proteolytic activity in goat milk + A. sativum decreased after the 1st hour followed by increased towards initial value during the next 3 hour (Figure 2 b). The initial free amino groups in camel milk were 268.97±0.03 µg/g as compared to plain milk that increased to about 368.23 µg/g to 470.66 µg/g by the end of fermentation (Figure 2 c).

The Growth of LAB During Milk Fermentation

Viable Cell Counts (VCC) of S. thermophilus: VCC of S. thermophilus in the mixture of cow milk with A. sativum or C. verum water extract was not different as compared to cow milk alone (Figure 3 a). The presence of herbal extracts did not affect the growth of S. thermophilus during the first hour of fermentation. However, the VCC of S. thermophilus increased (p<0.05) after the next 3 hours of fermentation. The VCC of S. thermophilus was the highest in cow milk + C. verum (2.70 x 10^6cfu/ml; p<0.05) followed by cow milk + A. sativum (2.60 x 10^6cfu/ml; p>0.05) and plain cow milk (2.40 x 10^6cfu/ml) at the end of fermentation. The initial VCC of S. thermophilus in goat milk (0.94 x 10^6cfu/ml) were not affected by the presence of A. sativum or C. verum water extract in milk (Figure 3 b). Fermentation of goat milk for one hour had no significant effect on S. thermophilus growth for all three treatments. However, the effects of addition of A. sativum or C. verum extract in goat milk on S. thermophilus VCC were significant after the 2nd hour to the end of fermentation as compared to goat milk alone (Figure 3 b). The initial S. thermophilus VCC of camel milk in the presence of A. sativum or C. verum water extract (1.31 x 10^6cfu/ml and 1.34 x 10^6cfu/ml respectively) were not different (p>0.05) from camel milk alone (1.14 x 10^6cfu/ml; Figure 3 c). The VCC of S. thermophilus did not change much (p>0.05) after 1 hour of fermentation but increased (p>0.05) after the 2nd hour of fermentation for all treated samples. The highest S. thermophilus VCC was seen at the end of fermentation with 3.1 x 10^6cfu/ml for both plain- and A. sativum- yogurts and 3.6 x 10^6cfu/ml for C. verum-yogurt.
Fig. 1: Changes of pH in a) cow milk, b) goat milk and c) camel milk in the absence (■) and presence of *A. sativum* (▲) or *C. verum* (●) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

Fig. 2: Changes in proteolytic activity (µg/g) in a) cow milk, b) goat milk and c) camel milk in the absence (■) and presence of *A. sativum* (▲) or *C. verum* (●) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.
Fig. 3: Changes in viable cell count (VCC) of *S. thermophilus* (10^6 cfu/ml) in a) cow milk, b) goat milk and c) camel milk in the absence (■) and presence of *A. sativum* (■) or *C. verum* (■) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

Fig. 4: Changes in viable cell count (VCC) of *Lactobacillus* spp. (10^6 cfu/ml) in a) cow milk, b) goat milk and c) camel milk in the absence (■) and presence of *A. sativum* (■) or *C. verum* (■) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.
Fig. 5: Changes in viable cell count (VCC) of *B. bifidum* (10^9 cfu/ml) in a) cow milk, b) goat milk and c) camel milk in the absence (■) and presence of *A. sativum* (□) or *C. verum* (□) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

**Viable Cell Count of Lactobacillus spp.:** The initial VCC of *Lactobacillus* spp was higher (p<0.05) in the mixture of cow milk with *A. sativum* or *C. verum* water extract (1.78 x 10^8 cfu/ml and 1.65 x 10^8 cfu/ml respectively) than in plain cow milk (1.19 x 10^8 cfu/ml; Figure 4 a). *Lactobacillus* spp grow at similar extent in cow milk and cow milk + *A. sativum* during the first two hours of fermentation. VCC of *Lactobacillus* spp for the next two hours of fermentation reduced for plain- and *A. sativum*-yogurts. *Lactobacillus* spp VCC in cow milk + *C. verum* increased (p<0.05) after two hours of fermentation but remained the same for the next two hours of fermentation. The initial VCC of *Lactobacillus* spp in goat milk was 1.44 x 10^8 cfu/ml (Figure 4 b). This value was increased to 2.12 x 10^8 cfu/ml and 1.72 x 10^8 cfu/ml (p<0.05) in the presence of *A. sativum* or *C. verum* water extract respectively. The VCC of *Lactobacillus* spp increased significantly (p<0.05) in all treatments from the 2nd hours of incubation onwards and were about 2 folds higher in all yogurts by the end of fermentation. The initial VCC of *Lactobacillus* spp in camel milk was 3.05 x 10^8 cfu/ml. The addition of *C. verum* extract into milk did not affect the initial VCC (Figure 4 c). However, camel milk + *A. sativum* showed higher (p<0.05) initial VCC of *Lactobacillus* spp (5.68 x 10^8 cfu/ml) than plain camel milk. Linear growth of *Lactobacillus* spp occurred after the first hours of incubation with the fastest growth shown by *A. sativum*- yogurt followed by *C. verum*- and plain- yogurts (Figure 4 c).

**Viable Cell Count of *B. bifidum*:** The *B. bifidum* VCC in cow milk at the start of fermentation were 1.7 x 10^8 cfu/ml. No difference in *B. bifidum* VCC was observed in cow milk + *A. sativum* or cow milk + *C. verum* (Figure 5 a). The VCC of *B. bifidum* increased (p<0.05) by the 1st hour of fermentation and reached maximum VCC by the 3rd hour of fermentation for all treatments. However, the VCC of *B. bifidum* reduced in *A. sativum*-yogurt (p<0.05) and in *C. verum*-yogurt (p>0.05) by the fourth hour of fermentation (Figure 5 a). The initial *B. bifidum* VCC in goat milk + *A. sativum* or *C. verum* (12.0 x 10^8 cfu/ml and 13.5 x 10^8 cfu/ml respectively) was higher than in goat milk alone (8.8 x 10^8 cfu/ml; Figure 5 b). *B. bifidum* VCC showed small increase (p>0.05) after the 1st hour of incubation in all treated milk. Incubation of goat milk to three hours enhanced (p<0.05) the growth of *B. bifidum* in goat milk + *A. sativum* (98.4 x 10^8 cfu/ml) and goat milk + *C. verum* (113.0 x 10^8 cfu/ml) compared to goat milk alone (46.8 x 10^8 cfu/ml). No further increase in *B. bifidum* VCC after the
4th hour of fermentation for plain- and A. sativum- yogurts (Figure 5 b). However, the VCC in C. verum-yogurt increased to 134.3 x 10^8 cfu/ml at the forth hour of fermentation. The B. bifidum VCC in the mixture of camel milk with A. sativum and C. verum was not significantly different from camel milk alone before fermentation and even after 1st hour of fermentation (Figure 5 c). The highest B. bifidum VCC in plain-yogurt was seen on the 2nd hour of fermentation (51.1 x 10^6 cfu/ml) followed by a gradual reduction to 20.0 x 10^6 cfu/ml (p<0.05) by the 5th hour of fermentation. The VCC of B. bifidum in yogurt increased (p<0.05) in the presence of A. sativum or C. verum water extract (196.1 x 10^6 cfu/ml and 255.5 x 10^4 cfu/ml respectively) by the end of fermentation (Figure 5 c).

DISCUSSION

Changes in pH During Fermentation: The pH reduction indicates different growth rates of LAB and probiotics [3]. LAB susceptibility to environmental stresses in logarithmic phase as compared to those in stationary phase previously reported in cow- [24-26], camel- [27, 28] and goat- [29-31] milk fermentation systems. The actual shape of fermentation curve depends strictly on several factors: the milk base, the present microorganisms and their associated enzymes in milk, starter culture, type and concentration of supplemented ingredients, milk heat treatment and incubation temperature [32]. In the present study, pH curve in camel and goat milk fermentation showed longer lag phase than cow milk and this could be attributed to the ability of camel and goat milk to resist changes in pH during fermentation [33, 34] due to inherent differential buffering capacity of milk [33, 34]. In addition, differential adaptation of LAB to a given growth media (cow, camel and goat milk) could be another reason of this delay [35]. The inclusion of A. sativum or C. verum water extract in three types of milk did not significantly influence pH reduction during fermentation. However, camel milk + A. sativum reached pH 4.5 after four hours, 1 hour less in fermentation time as compared to milk alone. This could be a consequence of higher (p<0.05) Lactobacillus spp. VCC (Figure 4 c) which produced relatively more lactic acids than plain milk during fermentation. On the other hand, the increase of fermentation time (5 hours) for camel milk despite higher (p<0.05) Lactobacillus spp. VCC than those in cow and goat milk (4 hours) could be explained by high buffering capacity in camel milk [36] compared to cow and goat milk.

Proteolytic Activity: Lactic acid bacteria are unable to synthesize essential amino acids and thus it is necessary for them to be capable of breaking down and efficiently utilize protein available from their surroundings [37]. The proteolytic system of dairy LAB consists of exocellular proteinases, membrane-bound aminopeptidases, intracellular exopeptidases and proteinases [38, 39]. The proteolytic activity of these enzymes yield polypeptides of various sizes, each with free amino groups that can be determined quantitatively using OPA method. Thus the free amino groups in yogurt indirectly reflect the proteolytic activity of LAB in different types of milk under the influence of additives such as A. sativum or C. verum. In the present study, the effectiveness of LAB to degrade milk proteins among the three types of milk in presence of herbal extracts occurred at the following sequence: cow milk + herbal extract > camel milk + herbal extract > goat milk + herbal extract. The initial free amino groups are significantly different between the three types of milk. The proteolytic activity was increased only in the presence of A. sativum and C. verum water extracts in cow milk but not in goat and camel milk during fermentation. Such differences have to be considered since the bacteria can find free substrate without the need of degrade proteins and peptides [40, 41]. The proteolytic activity in cow milk + A. sativum (0 hour) was 10 times more than in milk alone. This could be attributed to the higher amount of free amino groups in A. sativum water extract (22.4±0.1 µg/ml; data not shown). On the other hand, C. verum water extract showed only 10.2±0.1 µg/ml of free amino groups (data not shown) as compared to A. sativum water extract.

Viability of Lactic Acid Bacteria: The addition of probiotics to yogurt is a natural way of enhancing the functionality of yogurt [37]. There are two patterns of growth which can be observed in yogurt, one for Lactobacillus spp. and the other for S. thermophilus. S. thermophilus tend to grow faster during earlier fermentation (phase 1) due to liberation of amino acid and casein in milk. This is attributed to the accumulation of fermentation products such as lactic acid and acetic acid which stimulated the growth of Lactobacillus spp. [42]. The present study showed that non-significant differences in S. thermophilus VCC between milk and milk + A. sativum or C. verum water extract during the 1st hour of fermentation. This could be related to lag phase of the bacterial growth cycle which adapt themselves to growth conditions. However, the inclusion of A. sativum
or C. verum water extract in cow, camel and goat milk enhanced (p<0.05) the growth of LAB. Since the present study showed that proteolytic activity increased only during cow milk fermentation (Figure 2 a), it can be suggested that the herbal extracts could have provided essential growth factors possibly in form of peptides and amino acids to improve the growth of starter culture in the milk [37]. In addition, the potential of certain phenolic compounds act as prebiotics that improve LAB growth [43]. The growth of Lactobacillus spp. was the highest in camel milk+ herbal extracts followed by goat + herbal extracts and cow milk + herbal extracts. This could be related to high peptides and amino acids naturally available in milk as a result of proteolytic activity of indigenous bacteria, in addition to camel and goat milk proteins being much easier to be broken down by the proteolytic activity of bacteria than cow's milk [40, 44, 45]. Higher counts of B. bifidum (p<0.05) were found in the mixture of camel milk with A. sativum or C. verum water extract than in cow or goat milk mixture with these two herbal extracts. This is possibly related to high level of peptides and amino acids in camel milk [41] that improved the growth of bifidobacteria [38]. Besides, high level of lactoferrin in camel milk could enhance Bifidobacterium bifidum growth [46].

CONCLUSIONS

The addition of A. sativum or C. verum water extract in cow, goat and camel milk had no significant effect on the acidification during fermentation. However, the presence of these two herbs in milk enhanced the proteolytic activity during fermentation with the highest proteolytic action was seen in cow milk-yogurt. A. sativum and C. verum stimulated LAB growth in all the three types of yogurt during fermentation. Thus, A. sativum and C. verum may be used to support the growth of LAB in yogurt during fermentation.

Conflict of Interests: Shori, Amal Bakr as the corresponding author of the manuscript, do not have a direct financial relation that might lead to a conflict of interest for any of the authors.

REFERENCES


