Antioxidant activities, proteolytic activity and growth behavior of *Lactobacillus* cultures during fermentation of goat milk

Gauravkumar Panchal¹, Subrota Hati¹, VB Darji² and JB Prajapati¹

Received: 05 July 2019 / Accepted: 20 October 2019 / Published online: 27 February 2020
© Indian Dairy Association (India) 2020

**Abstract:** In the study, five *Lactobacillus* cultures i.e. *Lb. fermentum* (M3), *Lb. casei* (NK9), *Lb. rhamnosus* (M8), *Lb. rhamnosus* (M9) and *Lb. paracasei* (M11) were used for the fermentation of goat milk. Lactobacillus cultures were evaluated for their growth behavior on pH, acidity and *Lactobacillus* counts for different time periods. We found that pH reduction by *Lactobacillus* cultures in goat milk medium was ranged from pH 6.25 at 0 h to pH 3.06 after 48h at 37ºC, for acidity produced by *Lactobacillus* cultures was ranged from 0.28 in 0 h to 3.21%LA after 48 h at 37ºC and for lactic counts (log CFU/ml) were ranged from 3.87 log CFU/ml at 0 h to 9.03 log CFU/ml after 36 h incubation at 37ºC. Then, antioxidant activities (ABTS assay, Hydroxyl free radical scavenging assay and Superoxide free radical scavenging assay) as well as proteolytic activity (OPA method) were analyzed for different time periods and found that antioxidant activity (ABTS assay) of *Lactobacillus* cultures was in the range of 42.14 to 53.27%, hydroxyl free radical scavenging activity of *Lactobacillus* cultures was in the range of 42.14 to 53.27%, hydroxyl free radical scavenging activity of *Lactobacillus* cultures was in the range of 24.34% to 50.99% and proteolytic (OPA) activity of *Lactobacillus* cultures was found in the range of 7.30 to 9.96 mg/ml.

**Keywords:** Antioxidant activity, *Capra aegagrus hircus*, Fermented Goat milk, Proteolytic

**Introduction**

In Mesopotamia, part of today’s the Middle East, Goats (*Capra aegagrus hircus*) were the first species to be domesticated as livestock about 8000 BC. For centuries, humans have been used goats for many purposes (milk and meat) in all continents (Zervas and Tsiplakou, 2011). Goat milk production is an important part of the economy in many countries such as Spain, Switzerland, Italy, France, Turkey and New Zealand. On a global basis, different varieties of cheese, yoghurt, ice cream, fluid milk and milk powder are produced from goat milk. Goat milk production in the country has also increased from 3.6 to 4.7 million tons during the same period with an annual growth rate of 2.6 Per cent. The country stands first in goat milk production and is the second largest in goat meat production in the world by sharing 29 Per cent and 12 Per cent production, respectively (CIRG, 2015-2016).

Goat milk is nutritional and therapeutic food. Goat milk differs from cow or human milk in the context of higher digestibility, distinct alkalinity, higher buffering capacity and certain therapeutic in medicine and human nutrition (Park, 2009). Goat milk proteins may be digested more freely and their amino acids absorbed more efficiently than those of cow milk. Goat milk is considered to form a softer, more friable curd when acidified, which may be related to lower contents of αs1-casein and in the milk (Zenebe et al. 2014). Goat milk is good for ulcer treatment because it has better buffering capacity due to higher non-protein nitrogen (NPN) than cow milk (Park, 2009). The protein content of goat milk is quite similar to that of cow milk, although the caseins content in goat milk is slightly higher, and there is great homology between major proteins. However, β-casein is the major protein in goat milk (50 Per cent of total caseins), which is in contrast to cow milk where β-casein and αs1-casein are almost equally abundant, 37 Per cent and 30 Per cent, respectively (da Costa et al. 2014).

Many of the Lactic Acid Bacteria (LAB) were isolated from goat milk. Badis et al. (2004) isolated 725 lactic acid bacteria from raw goat milk of four Algerian races. They were phenotypically classified as *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus* and *Pediococcus*. Da Silva et al. (2016) too isolated riboflavin and folate producing 179 lactic acid bacteria from goat milk and cheeses from these predominance species are...
Materials and Methods

Materials and culture collection

In this study, Most of the bacteriological grade media, molecular biology grade chemicals and reagents were purchased either from Hi-media (Mumbai), Merck (Germany), Sigma (USA), Bio-Rad, Promega, Ameresco, MP Biomedicals. *Lb. fermentum* (M3), *Lb. casei* (NK9), *Lb. rhamnosus* (M8), *Lb. rhamnosus* (M9) and *Lb. paracasei* (M11) were procured from the Culture Collection of Dairy Microbiology Department, Sheth M. C. College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India. Sterilized reconstituted skim milk with 12% Total solids was used for the propagation of lactic culture and further stored at 5 ± 2 °C.

**Procurement of Goat milk** (*Capra aegagrus hircus*)

Goat milk of Surti breed (*Capra aegagrus hircus*) was procured from Instructional Livestock Farm Complex (ILFC), Veterinary College, AAU, Anand during the study.

**Determination of pH, acidity and lactic counts of fermented goat milk**

All the cultures were activated by growing in sterilized goat milk. The activated cultures were added to 100 ml of sterilized goat milk flasks at a rate of 2%. After mixing them thoroughly, culture flasks were incubated at 37°C for different time intervals i.e. 0, 6, 12, 24, 36 and 48 h. Then flasks were taken out for the determination of pH, titratable acidity and lactic counts.

**Determination of pH**

pH of fermented goat milk was determined as per the procedure described in Indian Standard (1961) with a calibrated pH meter (OAKTON pH700, India). Well mixed 10 ml fermented goat milk sample was put into a beaker and then pH was measured by immersing the pH meter probe into the fermented milk sample. Standard buffer solution of pH 4, 7 and 9 was used to calibrate the pH meter before measuring the sample.

**Determination of titratable acidity**

The titratable acidity was estimated by the procedure described in Indian Standard (1960). 10 ml sample was taken after each interval of 0, 6, 12, 24, 36 and 48 h into porcelain dish and an equal volume of lukewarm distilled water was added to it, then 1.0 ml phenolphthalein indicator was added and the contents of dish were titrated against 0.1 [N] NaOH till the appearance of light pink colour, which persisted for 30 seconds in the solution. Titratable acidity was calculated by the following formula:

\[
\text{Acidity} \% = \frac{9 \times V \times N}{X}
\]

Where,

\begin{align*}
V &= \text{Volume (ml) of 0.1 [N] NaOH required for the titration,} \\
N &= \text{Normality of NaOH solution and} \\
X &= \text{Volume of milk (ml) solution}
\end{align*}

**Determination of lactic counts**

*Lactobacillus* counts of fermented goat milk samples were determined as per the method described by IDF standards (146:2003). 1.0 ml sample was taken out from the tubes and added to 9 ml phosphate buffer tubes. Similarly, as per the required number of serial dilutions were prepared. 1.0 ml diluted sample from appropriate tubes was transferred to labelled sterile Petri plates (performed in duplicates), then 15-20 ml of melted and
Antioxidant activity was carried out by using Hydroxyl free radical scavenging assay of fermented goat milk (Hati et al., 2013) with some modification. A mixture of 3 ml phenanthroline (0.75mM) and 1.5 ml FeSO₄ (0.75mM) in phosphate buffer (pH 7.4) was prepared, and after adding 2 ml H₂O₂ (0.01%) and 1 ml supernatant, then the mixture was incubated at 37°C for 1 hour. Ultimately, the absorbance was evaluated at 536 nm. Hydroxyl free radical scavenging capacity of the peptides was evaluated by the following equation:

\[ \text{Hydroxyl radical scavenging activity} \% = \left( \frac{A_0 - A_S}{A_0 - A_C} \right) \times 100 \]

Where \( A_0 \) is the absorbance of the sample; \( A_S \) is the absorbance of the control containing 1, 10-phenanthroline, FeSO₄ and H₂O₂; and \( A_C \) is the absorbance of the blank containing 1, 10-phenanthroline and FeSO₄.

Superoxide free radical scavenging assay of fermented goat milk

This method is based on the ability of peptides to scavenge \( \text{O}_2^- \) through the production of a chromophoric compound during the reaction. This assay was followed by Liu et al. (2010) with some modification. In this method, 800 μl of supernatant along with 800 μl of Tris-HCI buffer (0.05M, pH 8.3) was placed in a clean test tube; thereafter, 400 μl of pyrogallol solution (1.5mM) was added. Finally, the absorption of the mixture was measured at 320 nm for 5 minutes at 25°C. Butylated hydroxytoluene (at 0-1mg/ml, final concentration) was used as a positive control (AC) to evaluate the superoxide free radical scavenging capacity of the sample (AS):

\[ \text{Superoxide radical scavenging} \% = \left( \frac{(\Delta A_C/\text{min}) - (\Delta A_S/\text{min})}{(\Delta A_C/\text{min})} \right) \times 100 \]

Assessment of proteolytic activity

The proteolytic activity of Lactobacillus cultures was optimized by measuring the peptide content through O-phthalaldehyde (OPA) method (Hati et al., 2015; Solanki et al., 2017). Lactobacillus cultures were activated by growing in sterilized goat milk. The activated cultures were added to 10 ml tubes of sterilized goat milk at the 2% rate of inoculation and incubated at different times intervals (0, 6, 12, 24, 36 and 48 h). Then the samples were taken out for the evaluation of peptide content (Proteolytic activity) after each interval. The degree of proteolysis during fermentation of milk was determined by measuring the release of free NH₃ groups following the O-phthalaldehyde (OPA) method. An aliquot of 3 ml from each fermented goat milk sample was mixed with 5 ml of 0.75% trichloroacetic acid (TCA) and vortexed for 1 min and then the mixture was filtered using Whatman no. 42 filter papers (UK). The filtrate (200 μl) was added to 3 ml of OPA reagent and after incubation at room temperature (20°C) for 2 min, the absorbance of the solution was measured by a Spectrophotometer (Systronics PC based double beam Spectrophotometer 2202, India) at 340 nm.
Statistical analysis

According to the statistical methods, all the study parameters were analyzed. Every experiment of the study was performed at least in triplicates with the results expressed as means (Average) ± standard deviations (SD). Statistical designs and software were used to analyze the experimental data. Using 5.0% level of significance and analysis of variance (ANOVA), the significant difference between the treatments was evaluated (Steel and Torrie, 1980).

Result and Discussion

Growth behaviour of Lactobacillus cultures in goat milk medium

The each Lactobacillus culture was inoculated at the rate of 2% in sterilized goat milk and then pH, titratable acidity (% Lactic acid) and Lactobacillus counts were evaluated at different time intervals (0, 6, 12, 24, 36 and 48 h) at 37°C. The pH reduction, titratable acidity (%LA) and Lactobacillus counts of individual Lactobacillus culture were determined.

pH

pH reduction by Lactobacillus cultures in goat milk was ranged from pH 6.25 at 0 h to pH 3.06 after 48 h at 37°C. M8 showed maximum pH reduction (pH 3.06) followed by M3 (pH 3.11), NK9 (pH 3.18), M9 (pH 3.32) and M11 (pH 3.42) after 48 h at 37°C (Fig. 1).

Titratable acidity

Titratable acidity was determined by calculating the amount of acidity developed up to 48 h of incubation by the formula given in (2.3.2). The acidity produced by Lactobacillus cultures was ranged from 0.28 in 0 h to 3.21%LA after 48 h at 37°C. During the growth in sterilized goat milk, M8 showed highest titratable acidity (3.18%LA), followed by M3 (3.17%LA), NK9 (3.05%LA), M9 (2.73%LA) and M11 (2.08%LA) after 48 h at 37°C (Fig. 2).

Lactobacillus counts

Lactobacillus counts (log CFU/ml) of all the Lactobacillus cultures were evaluated for 0, 6, 12, 24, 36 and 48 h at 37°C. Overall lactic counts (log CFU/ml) were ranged from 3.87 log CFU/ml at 0 h to 9.03 log CFU/ml after 36 h incubation at 37°C (Fig. 3). Different cultures treatment and time periods showed significant behaviour but the interaction of both varied non-significantly. Non-significant increases in viable counts were observed among the five Lactobacillus up to 24 h and growth was significantly (P<0.05) decrease after 48 h at 37°C in the study. M8 exhibited maximum growth (9.03 log CFU/ml), followed by M11 (9.00 log CFU/ml) up to 36 h than decrease after 48 h at 37°C. NK9 (8.98 log CFU/ml), M9 (8.62 log CFU/ml) and M3 (8.61 log CFU/ml) up to 24 h than decrease after 48 h at 37°C. In one study, Parmar et al. (2018) studied the growth and acidification of five selected lactic acid bacteria in heat-treated goat milk. Among five Lactobacillus cultures (L. rhamnosus (NK2), L. casei (NK9), L. fermentum (M5), L. paracasei (M16) and L. fermentum TDS030603 (MTCC 25067) (LF)) studied, during the growth in heat-treated goat milk, M5 showed highest titratable acidity (3.25%LA), followed by LF (3.17%LA), NK2 (3.13%LA), NK9 (2.88%LA) and M16 (1.72%LA) after 48 h at 37°C. Maximum pH reduction showed M5 (pH 3.10) followed by NK9 (pH 3.14), NK2 (pH 3.25), LF (pH 3.28) and M16 exhibited lowest pH reduction (pH 4.20) after 48 h at 37°C. Significant increases in viable counts were observed among the five lactic acid bacteria up to 12 h than growth was significantly (P<0.05) decrease after 48 h at 37°C in the study. M16 exhibited maximum growth (9.35 log CFU/ml), followed by LF (8.82 log CFU/ml), NK9 (8.80 log CFU/ml), NK2 (8.65 log CFU/ml) and M5 (8.18 log CFU/ml) up to 12 h than decrease after 48 h at 37°C except M5 as we found.

Similar kind of result was also found by Solanki et al. (2017) that studied the growth and acidification of nine selected lactic acid bacteria in heat treated camel milk. Among nine lactic acid cultures (Lb. rhamnosus (NS4 and NS6), Lb. acidophilus (298), Lb. helveticus (V3), Lb. acidophilus (015), Str. thermophilus (MD2), Lb. bulgaricus (09), Lactococcus lactis ssp. lactis (NK6) and Lb. fermentum (LBF)) studied, during the growth in heat treated camel milk, V3 showed highest titratable acidity (2.548%LA), followed by 09 (2.487%LA), LBF (2.450%LA), 015 (2.422%LA), NS6 (1.732%LA), 298 (1.333%LA), NS4 (1.221%LA), MD2 (0.836%LA) and NK6 (0.785%LA) after 48 h at 37°C. Maximum pH reduction showed V3 (pH 3.16) followed by 09 (pH 3.17), LBF (pH 3.27), 015 (pH 3.30), NS6 (pH 3.44), 298 (pH 3.55), NS4 (pH 4.00), MD2 (pH 4.49) and NK6 exhibited lowest pH reduction (pH 4.79) after 48 h at 37°C. Maximum lactic count (log CFU/ml) exhibited by NS6 (11.62 log CFU/ml) followed by 09 (11.33 log CFU/ml), NK6 (10.51 log CFU/ml), MD2 (10.40 log CFU/ml), V3 (10.20 log CFU/ml), NS4 (10.15 log CFU/ml), 015 (10.00 log CFU/ml), 298 (10.00 log CFU/ml) and LBF (9.40 log CFU/ml).

In another study, Hati et al. (2013) studied the growth and acidification of eight selected lactic acid bacteria in skim and soy milk. Among eight lactic cultures (S. thermophilus MD2, L. helveticus V3, L. rhamnosus NS6, L. rhamnosus NS4, L. bulgaricus NCDC 09, L. acidophilus NCDC 15, L. acidophilus NCDC 298 and L. helveticus NCDC 292) studied, L. bulgaricus NCDC 09 and S. thermophilus MD2 decreased the pH of skim and soy milk in greater extent. Acid production (i.e. titratable acidity) by L. bulgaricus NCDC 09 and L. helveticus V3 was higher than other strains. Higher viable counts were observed in S. thermophiles MD2 and L. helveticus V3. All the tested lactic acid bacteria performed better in skim milk as compared to soy milk. Hati et al. (2015) studied the growth performance of Lactobacillus
rhamnosus (NS4 and NS6), Lactobacillus helveticus MTCC 5463 (V3), Lactobacillus delbruckii (09), Enterococcus faecalis (ND3), Enterococcus faecalis (ND11) and Lactobacillus rhamnosus (SH8) by determining viable counts (log CFU/ml) and production of Lactobacillus acid measured by decline in pH in skim milk inoculated at the rate of 1% and incubated at 37°C for 12 h. It was observed that NS4 lowered down the pH at a maximum level compared to V3, ND3 and SH8. However, it was also observed
that NS4 produced maximum acidity compared to V3, ND3, SH8 and I4. Viable counts of all the cultures were measured after 12 h of incubation at 37°C. From the study, it was also found that NS4 gives the highest viable cell counts 10.68 log CFU/ml than the other bacterial isolates at this specified growth conditions. V3 also showed higher viable cell counts and NS6 was relatively exhibited lesser bacterial counts compared to other isolates in MRS agar medium. It was concluded that viable cell counts, pH and acidity varies due to the use of different strains (Hati et al., 2015) as similar to our study.

Antioxidant activity (ABTS assay) of fermented goat milk

The antioxidant activity generally indicates the relative ability of antioxidants to scavenge the free radicals generated in the aqueous phase. The ABTS is generated by reacting a strong oxidizing agent (e.g., potassium permanganate or potassium persulfate) with the ABTS salt (Hati et al. 2013). From Table 1, it had been observed that antioxidant activity was differing significantly (P<0.05) with incubation periods. Also, there was a significant difference (P<0.05) observed within the cultures. Also, it was found that the antioxidant activity of all the five Lactobacillus cultures was increased significantly with the time of incubation.

The antioxidant activity (ABTS assay) of Lactobacillus cultures was found in the range of 42.14 to 53.27%. NK9 had exhibited highest antioxidant activity (53.27%), followed by M8 (51.99%), M9 (50.55%), M3 (45.85%) and M11 (42.14%) after 48 h at 37°C.

Studies revealed the agreement with our work that Rahmawati and Suntornsuk (2016) found the antioxidant activity of goat milk yoghurt increase or constant during fermentation proceed or storage at 4R°C up to 21 days. The value of ABTS activity in per cent inhibition was almost 19% and then decrease after 21 days of storage. Similarly, Moreno-Montoro et al. (2017) evaluated the antioxidant activity of fermented goat milk. Different fractions of whey i.e. whey, cation exchange membrane retentate R, permeate P of two fermented skimmed goat milk (ultra-filtered (UF) goat milk fermented with the classical starter bacteria or with the classical starter plus the Lactobacillus plantarum C4 probiotic strain) were assessed. The maximum value reaches was up to 0.4 μmol Trolox equivalents per mL for ABTS radicals. In one study, Freire et al. (2017) evaluated ABTS activity of fermented goat milk beverage by Lactobacillus rhamnosus and Streptococcus thermophilus with or without addition of grape pomace on gut microbiota and showed that Antioxidant activity of formulation-1 (Goat milk, Sugar and Grape juice) was 418.02 ± 16.14 mmol TE g⁻¹ and formulation-2 (Goat milk, Sugar, Grape pomace extract and Grape juice) was 743.78 ± 23.88 mmol TE g⁻¹.

Li et al. (2013) evaluated the antioxidant activities of goat milk casein and goat milk casein hydrolysates (hydrolysed by using a combination of neutral and alkaline proteases). They found that half-maximal inhibitory concentration (IC₅₀) value of ABTS
activity of goat milk casein was 71.251 ± 2.747 µg/ml and for goat milk casein hydrolysates was 0.449 ± 0.027 µg/ml. It indicating that goat milk casein hydrolysates is a good antioxidant compound with strong free radical scavenging activity compared with goat milk casein. One possible reason is that some peptides of goat milk casein hydrolysates are electron donors, which could react with free radicals, convert them to more stable products, and terminate the radical chain reaction.

Hydroxyl free radical scavenging activity of fermented goat milk

Hydroxyl radicals are reactive oxygen species that begin peroxidation of lipid membranes. Hydroxyl radicals are one of the most damaging free radicals in the body and can be an important mediator of damage to cell structures, nucleic acids, lipids and proteins. Hydroxyl free radical scavenging assay measures the relative ability of an antioxidant to scavenge the free radical generated in the aqueous phase (Li et al. 2008).

Hydroxyl free radical scavenging activity of all the five Lactobacillus cultures was presented in Table 2. From Table 2, it had been observed that hydroxyl free radical scavenging activity was differing significantly (P<0.05) with incubation periods. Also, there was a significant difference (P<0.05) observed within the cultures. Also, it was found that the hydroxyl free radical scavenging activity of all the five Lactobacillus cultures was increased significantly with the time of incubation. The hydroxyl free radical scavenging activity of Lactobacillus cultures was found in the range of 35.32 to 53.43%. M8 had exhibited highest hydroxyl free radical scavenging activity of 53.43%, followed by M9 (50.98%), NK9 (48.88%), M3 (46.93%) and M11 (35.32%) after 48 h at 37ºC. It was observed that the percentage of hydroxyl free radical was increased with an increase in the incubation time from 0 to 48 h.

Similar kind of observation was shown by Shu et al. (2018) reported hydroxyl free radical scavenging activity in goat milk fermented cultures was presented in Table 2. From Table 2, it had been observed that hydroxyl free radical scavenging activity was differing significantly (P<0.05) with incubation periods. Also, there was a significant difference (P<0.05) observed within the cultures. Also, it was found that the hydroxyl free radical scavenging activity of all the five Lactobacillus cultures was increased significantly with the time of incubation. The hydroxyl free radical scavenging activity of Lactobacillus cultures was found in the range of 35.32 to 53.43%. M8 had exhibited highest hydroxyl free radical scavenging activity of 53.43%, followed by M9 (50.98%), NK9 (48.88%), M3 (46.93%) and M11 (35.32%) after 48 h at 37ºC. It was observed that the percentage of hydroxyl free radical was increased with an increase in the incubation time from 0 to 48 h.

Superoxide free radical scavenging activity of fermented goat milk

Superoxide free radical scavenging assay measures the relative ability of an antioxidant to scavenge the free radical generated in the aqueous phase. The original pyrogallol (1,2,3-trihydroxy benzene) method, which was developed specifically for superoxide dismutase, is widely used for measuring superoxide-scavenging of other antioxidants spectrophotometrically at 320 nm (Liu et al. 2010).

Superoxide free radical scavenging activity of all the five Lactobacillus cultures was depicted in Table 3. From Table 3, it had been observed that superoxide free radical scavenging activity was differing significantly (P<0.05) with incubation periods. Also, there was a significant difference (P<0.05) observed within the cultures. Also, it was found that the superoxide free radical scavenging activity of all the five Lactobacillus cultures was increased significantly with the time of incubation. The superoxide free radical scavenging activity of Lactobacillus cultures was found in the range of 35.32 to 53.43%. M8 had exhibited highest superoxide free radical scavenging activity of 53.43%, followed by M9 (50.98%), NK9 (48.88%), M3 (46.93%) and M11 (35.32%) after 48 h at 37ºC. It was observed that the percentage of superoxide free radical was increased with an increase in the incubation time from 0 to 48 h.

Table 1 Antioxidant activity (%) of Lactobacillus cultures (ABTS assay)

| Lactobacillus Cultures | 0h   | 12h  | 24h  | 36h  | 48h  |
|------------------------|------|------|------|------|------|
| M3                     | 2.51±0.76<sup>a</sup> | 10.19±1.34<sup>b</sup> | 20.87±2.00<sup>d</sup> | 31.62±1.76<sup>f</sup> | 45.85±4.07<sup>i</sup> |
| NK9                    | 2.49±0.77<sup>a</sup> | 15.45±0.50<sup>c</sup> | 29.79±1.77<sup>ef</sup> | 44.41±1.70<sup>i</sup> | 53.27±1.54<sup>h</sup> |
| M8                     | 2.48±0.76<sup>a</sup> | 15.06±1.91<sup>c</sup> | 20.87±2.00<sup>de</sup> | 40.48±1.58<sup>h</sup> | 51.99±1.01<sup>h</sup> |
| M9                     | 2.49±0.76<sup>a</sup> | 14.67±1.84<sup>c</sup> | 26.74±2.77<sup>e</sup> | 33.33±1.93<sup>ef</sup> | 50.55±1.34<sup>h</sup> |
| M11                    | 2.49±0.77<sup>a</sup> | 04.98±1.04<sup>c</sup> | 15.06±2.00<sup>de</sup> | 23.03±1.02<sup>de</sup> | 42.14±7.16<sup>de</sup> |

Table 2 Hydroxyl free radical scavenging activity (%) of Lactobacillus cultures

| Lactobacillus Cultures | 0h   | 12h  | 24h  | 36h  | 48h  |
|------------------------|------|------|------|------|------|
| M3                     | 0.31±0.22<sup>a</sup> | 07.70±2.50<sup>b</sup> | 32.91±0.85<sup>c</sup> | 40.15±1.50<sup>de</sup> | 46.93±0.81<sup>lm</sup> |
| NK9                    | 0.31±0.21<sup>a</sup> | 11.32±1.07<sup>b</sup> | 33.08±0.85<sup>de</sup> | 43.20±1.45<sup>de</sup> | 48.88±4.72<sup>dm</sup> |
| M8                     | 0.33±0.22<sup>a</sup> | 05.96±1.61<sup>b</sup> | 37.88±2.00<sup>de</sup> | 40.82±0.71<sup>de</sup> | 53.43±0.86<sup>de</sup> |
| M9                     | 0.31±0.21<sup>a</sup> | 11.04±1.12<sup>de</sup> | 38.80±1.77<sup>de</sup> | 44.83±0.70<sup>de</sup> | 50.98±1.54<sup>de</sup> |
| M11                    | 0.32±0.21<sup>a</sup> | 01.77±1.05<sup>de</sup> | 19.95±0.71<sup>de</sup> | 35.57±2.80<sup>de</sup> | 35.82±1.96<sup>de</sup> |
radical scavenging activity of all the five *Lactobacillus* cultures was increased significantly with the time of incubation.

The superoxide free radical scavenging activity of *Lactobacillus* cultures was found in the range of 24.34% to 50.99%. NK9 had exhibited highest superoxide free radical scavenging activity (50.99%), followed by M3 (48.68%), M8 (41.14%), M9 (36.13%) and M11 (24.34%) after 48 h at 37ºC. It was observed that the percentage of superoxide free radical was increased with an increase in the incubation time from 0 to 48 h. At 0 h, there was no superoxide free radical scavenging activity found in fermented goat milk produced by *Lactobacillus* cultures.

Similarly kind of observation reported by Shu et al. (2017). They optimized rate of addition of prebiotic was inulin (0.6%), xylo-oligosaccharide (0.6%), galacto-oligosaccharide (0.6%) and fructo-oligosaccharide (0.4%) and value of superoxide free radical were 21.09%, 18.20%, 27.61% and 29.92%, respectively. In another study, superoxide radical scavenging activity of fermented goat milk product was evaluated by Liu et al. (2016). They compared the antioxidant properties of cow milk, goat milk, cow milk kefir and goat milk kefir and showed that maximum (4.0 mg/ml) dose level of kefir gives better superoxide radical scavenging activity i.e. almost 70% for both kefir compare to milk. This same kind of observation found in our case because fermentation was increase superoxide radical scavenging activity compares to control. In one study, Shu et al. (2016) optimized the different condition for best superoxide free radical scavenging activity for goat milk casein hydrolysates by alcalase. The value of superoxide free radical scavenging activity at different condition was like, at temperature 50ºC (36.08%), at pH 8 (85.36%), at enzyme to substrate ratio 1.5% (43.01%), at 150 min hydrolysis time (46.13%) and also found increase in superoxide free radical scavenging activity with time of hydrolysis as we seen in our study.

### Protopolytic (OPA) activity of fermented goat milk

The protopolytic (OPA) activity of all the five *Lactobacillus* cultures was presented in Table 4. From Table 4, it had been observed that protopolytic (OPA) activity was differing significantly (P<0.05) with incubation periods. Also, there was a significant difference (P<0.05) observed within the cultures. It was found that the protopolytic (OPA) activity of all the five *Lactobacillus* cultures was increased non-significantly with the time of incubation.

The protopolytic (OPA) activity of *Lactobacillus* cultures was found in the range of 7.30 to 9.96 mg/ml. M9 had exhibited highest protopolytic (OPA) activity (9.96 mg/ml), followed by M3 (9.08 mg/ml), NK9 (8.62 mg/ml), M8 (8.37 mg/ml) and M11 (7.30 mg/ml) after 48 h at 37ºC. It was also observed that the proteolysis was increased with an increase in the incubation time from 0 to 48 h.

Basically, Increase in protopolytic activity with the different incubation periods was directly proportional to the number of amino acids required by the *Lactobacillus* cultures during their growth phases based upon which the release of free NH-groups. It was also reported that the extent of proteolysis showed to be the time and strain-dependent (Donkar et al. 2007). In agreement of our work, Parmar et al. (2018) evaluated the protopolytic activity of goat milk fermented by NK9 (*Lb. casei*) and LF (*Lb. fermentum TDS030603 (MTCC25067)). They optimized the rate of inoculation (1.0, 1.5 and 2.0 %) and incubation period (0, 6, 12, 24 and 48 h) by OPA method. They found that 2% inoculation rate and 48 h of incubation gives the best protopolytic activity for both cultures i.e. for NK9 (7.598 mg/ml) and LF (9.709 mg/ml). Karthikeyan et al. (2018) isolated eight LAB from goat milk. They screened that isolates on the basis of protopolytic activity on skim milk agar. After its genotypic and phenotypic evaluation, found that *Lb.*

### Table 3 Superoxide free radical scavenging activity (%) of *Lactobacillus* cultures

| *Lactobacillus* Cultures | 0h   | 12h  | 24h  | 36h  | 48h  |
|--------------------------|------|------|------|------|------|
| M3                       | 0.00±0.00<sup>a</sup> | 0.00±0.00<sup>a</sup> | 16.43±2.84<sup>c</sup> | 32.07±1.84<sup>c</sup> | 48.68±1.05<sup>c</sup> |
| NK9                      | 0.00±0.00<sup>a</sup> | 0.43±0.75<sup>c</sup> | 21.29±2.67<sup>a</sup> | 29.73±0.69<sup>c</sup> | 50.99±2.30<sup>c</sup> |
| M8                       | 0.00±0.00<sup>a</sup> | 0.88±0.88<sup>c</sup> | 10.80±2.62<sup>b</sup> | 25.82±1.86<sup>c</sup> | 41.14±2.64<sup>b</sup> |
| M9                       | 0.00±0.00<sup>a</sup> | 0.17±0.29<sup>c</sup> | 08.74±2.93<sup>b</sup> | 17.55±1.10<sup>c</sup> | 36.13±2.81<sup>b</sup> |
| M11                      | 0.00±0.00<sup>a</sup> | 0.00±0.00<sup>a</sup> | 02.73±2.38<sup>a</sup> | 15.84±2.22<sup>c</sup> | 24.34±1.83<sup>c</sup> |

<sup>a</sup>Values with different superscripts differ significantly (p < 0.05), Superoxide free radical scavenging activity (%) Mean ± SD.

### Table 4 Protopolytic activity (mg/ml) of *Lactobacillus* cultures (OPA activity)

| *Lactobacillus* Cultures | 0h   | 12h  | 24h  | 36h  | 48h  |
|--------------------------|------|------|------|------|------|
| M3                       | 2.49±0.03* | 4.69±0.37* | 6.40±0.55* | 7.05±0.37* | 9.08±0.93* |
| NK9                      | 2.30±0.15* | 5.49±0.82* | 6.76±0.43* | 7.81±0.78* | 8.62±0.93* |
| M8                       | 2.40±0.11* | 5.06±0.72* | 6.11±0.54* | 6.93±0.52* | 8.37±1.04* |
| M9                       | 2.33±0.11* | 5.72±1.12* | 7.35±0.33* | 9.11±1.28* | 9.96±0.67* |
| M11                      | 2.37±0.02* | 4.01±0.27* | 5.03±0.85* | 6.34±0.48* | 7.30±0.69* |

<sup>*</sup>Values with different superscripts differ non-significantly (p < 0.05), Protopolytic activity (mg/ml) Mean ± SD.
**Conclusions**

*Lb. rhamnosus* (M8) showed highest pH reduction, maximum acidity and *Lactobacillus* counts i.e. 3.06, 3.18% LA and 9.03 log CFU/ml, respectively during the fermentation of goat milk after 48h at 37°C than other cultures. Antioxidant activity (ABTS assay) of *Lactobacillus* cultures were found in the range of 42.14 to 53.27% and maximum antioxidant activity was shown by *Lb. casei* (NK9) culture. The hydroxyl free radical scavenging activity of *Lactobacillus* cultures was found in the range of 35.32 to 53.43% and highest was observed in *Lb. rhamnosus* (M8) culture. The superoxide free radical scavenging activity of *Lactobacillus* cultures was found in the range of 24.34% to 50.99% and the maximum was presented by *Lb. casei* (NK9) culture. The proteolytic (OPA) activity of *Lactobacillus* cultures was found in the range of 7.30 to 9.96 mg/ml and the maximum was exhibited by *Lb. rhamnosus* (M9). However, M8, NK9 and M9 could be used for the development of functional fermented goat milk.

**Acknowledgments**

We are thankful to Instructional Livestock Farm Complex (ILFC), Veterinary College, AAU, Anand for providing goat milk during the study.

**References**

Ahmed M, Bousmaha-Marroki L (2014) Lactobacilli isolated from Algerian goat’s milk as adjunct culture in dairy products. Brazilian Arch Biol Technol 57: 1678-4324

Badis A, Guetarni D, Moussa Boudjema B, Henni DE, Kihal M (2004) Identification and technological properties of lactic acid bacteria isolated from raw goat milk of four Algerian races. Food Microbiol 21: 579-588

Ceballos LS, Morales ER, Adarve GDG, Castro JD, Martinez LP, Sanz MR (2009) Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. J Food Compos Anal 22: 322-329

Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Zhao L (2018) Inflammatory responses and inflammation-associated diseases in organs. Oncotarget 9: 7204-7209

Chen P, Liu L, Zhang X, Massounga Bora AF, Li X, Zhao M, Wang Y (2019) Antioxidant activity of Cheddar cheese during its ripening time and after simulated gastrointestinal digestion as affected by probiotic bacteria. Int J Food Prop 22: 217-228

CIRG (2015-2016) Annual Report. Executive Summary. Published by Director, ICAR-CIRG, Makhdoom, Farah, Mathura, 281122, UP, 1-175.

da Costa WKA, de Souza EL, Beltrao-Filho EM, Vasconcelos GKV, Santi-Gadelha T, de Almeida Gadelha CA, Magnani M (2014) Comparative protein composition analysis of goat milk produced by the Alpine and Saanen breeds in northeastern Brazil and related antibacterial activities. PLoS One 9: e93361

Da Silva FFP, Biscola V, Jean Guy LeBlanc JG, Melo Franco BDG (2016) Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk. Food Sci Technol 71: 155-161

Donkar ON, Henriksson A, Vasiljevic T, Shah NP (2007) Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin converting enzyme inhibitory activity in fermented milk. Le Lait 86: 21-38

Freire FC, Adorno MAT, Sakamoto IK, Antoniassi R, Chaves ACSD, dos Santos KMO, Sivieri K (2017) Impact of multi-functional fermented goat milk beverage on gut microbiota in a dynamic colon model. Food Res Int 99: 315-327

Hati S, Patel N, Mandal S (2013) Comparative growth behaviour and bio functional activity of lactic acid bacteria during fermentation of soy milk and bovine milk. Probiotics Antimicrob Proteins 5: 233-286

Hati S, Sreeja V, Solanki J, Prajapati JB (2015) Influence of proteolytic lactobacilli on ACE inhibitory activity and release of bioactive peptides. Indian J Dairy Sci 68: 1-8

IDF International Dairy Federation (146:2003) Yogurt-Identification of characteristic microorganisms (*Lactobacillus delbrueckii supsp. bulgaricus* and *Streptococcus thermophilus*). http://www.dairyinfo.gc.ca/index_e.php?s1=fil-idf&s2=pub&s3=iso. Accessed 23 May 2019

Indian Standards (1960) Methods of test for dairy industry part-I rapid examination of milk. Indian Standards Institution, New Delhi (1479).

Indian Standards (1961) Methods of test for dairy industry part-II chemical analysis of milk. Indian Standards Institution, New Delhi (1479).

Karthikeyan G, Palanisamy A, Madheshwar RV, Sudhakar N (2018) Milk clotting and proteolytic activity of protease enzyme from *Lactobacillus delbrueckii* isolated from raw goat milk. Aust J Pharm Biol 1: 15-26

Kondili E, Katsiari MC, Voutsinas LP (2007) Amino acid composition and nutritional value of goat milk from the indigenous Greek breed. Milchwissenschaft 62: 164-166

Li Y, Jiang B, Zhang T, Mu W, Liu J (2008) Antioxidant and free radical-scapenging activities of chickpea protein hydrolysate (CPH). Food Chem 106: 444-450

Li Z, Jiang A, Yue T, Wang J, Wang Y, Su J (2013) Purification and identification of five novel antioxidant peptides from goat milk casein hydrolysates. J Dairy Sci 96: 4242-4251
Liu M, Bayjanov JR, Renckens B, Nauta A, Siezen RJ (2010) The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics 11: 36-43

Liu R, Xing L, Fu Q, Zhou GH, Zhang WG (2016) A review of antioxidant peptides derived from meat muscle and by-products. Antioxidants 5: 32-45

Mahdi C, Untari H, Padaga MC (2018) Identification and Characterization of Bioactive Peptides of Fermented Goat Milk as a Sources of Antioxidant as a Therapeutic Natural Product. International Conference on Chemistry and Material Science. doi:10.1088/1757-899X/299/1/012 014/pdf

Moreno-Montoro M, Olalla-Herrera M, Rufián-Henaes JA, Martínez RG, Miralles B, Bergillos T, Jauregi P (2017) Antioxidant, ACE-inhibitory and antimicrobial activity of fermented goat milk: activity and physicochemical property relationship of the peptide components. Food Funct 8: 2783-2791

Park YW (2009) Bioactive components in goat milk. In: Park YW (ed) Bioactive components in milk and dairy products, 1st edn. Wiley-Blackwell, Hoboken, NJ, USA, pp. 43-81

Parmar H, Hati S, Sakure A (2018) In vitro and in silico analysis of novel ACE-inhibitory bioactive peptides derived from fermented goat milk. Int J Pept Res Ther 24: 441-453

Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, Dharma K (2014) Oxidative stress, prooxidants, and antioxidants: the interplay. Biomed Res Int 2014: 1-19

Rahmawati IS, Suntornsuk W (2016) Effects of fermentation and storage on bioactive activities in milks and yoghurts. Procedia Chem 18: 53-62

Samaranayaka AGP, Li-Chan ECY (2011) Food-derived peptidic antioxidants: A review of their production, assessment and potential applications. J Funct Foods 3: 229-254

Sharma G, Rout PK, Kaushik R, Sing G (2017) Identification of bioactive peptides in goat milk and their health application. Adv Dairy Res 5: 191-196

Shu G, Shi X, Chen L, Kou J, Meng J, Chen H (2018) Antioxidant peptides from goat milk fermented by Lactobacillus casei L61: preparation, optimization, and stability evaluation in simulated gastrointestinal fluid. Nutrients 10: 797-810

Shu G, Wang Z, Chen L, Zhang Q, Xin N (2017) Enzymolysis technology optimization for production of antioxidant peptides from goat milk casein. J Lucian Blaga 21: 51-60

Shu G, Zhang B, Zhang Q, Wan H, Li H (2016) Effect of temperature, pH, enzyme to substrate ratio, substrate concentration and time on the antioxidative activity of hydrolysates from goat milk casein by alcalase. Food Technol 20: 29-38

Solanki D, Hati S, Sakure A (2017) In Silico and in vitro analysis of novel angiotensin i-converting enzyme (ace) inhibitory bioactive peptides derived from fermented camel milk (Camelus dromedarius). Int J Pept Res Ther 19: 275-380

Steel RGD, Torrie JH (1980) Principles and procedure of statistics- a biometrical approach. Mcgraw Hill Kogakusha Ltd., Japan

Urso ML, Clarkson PM (2003) Oxidative stress, exercise, and antioxidant supplementation. Toxicology 189: 41-54

Yelnetty A, Purnomo H, Mirah A (2014) Biochemical characteristics of lactic acid bacteria with proteolytic activity and capability as starter culture isolated from spontaneous fermented local goat milk. J Nat Sci Res 4: 2224-3186

Zenebe T, Ahmed N, Kabela T, Kebede G (2014) Review on medicinal and nutritional values of goat milk. Acad J Nutr 3: 30-39

Zervas G, Tsiplakou E (2011) The effect of feeding systems on the characteristics of products from small ruminants. J Small Ruminant Res 101: 140-149