RESEARCH ARTICLE

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

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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 37°C, for acidity produced by *Lactobacillus* cultures was ranged from 0.28 in 0 h to 3.21%LA after 48 h at 37°C 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 37°C. 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 found in the range of 35.32 to 53.43%, superoxide 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 α s1-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 α s1-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

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Streptococcus thermophilus, *Weissella paramensisenteroides*, and *Lactococcus lactis*. 84% of isolates were able to produce folate, 8% riboflavin and 8% both vitamins. Goat milk fermented with different LAB increase the biologically active peptides from corresponding sequences of the precursor protein. The health benefits of the bioactive peptides may be attributed to their demonstrated antimicrobial, antioxidant, antihypertensive, antithrombotic, immune-modulatory and opioid activities (Sharma et al. 2017; Parmar et al. 2018).

Generally, chronic diseases and ageing phenomena are relevant to the imbalance in free radical levels in the body. An excess of free radicals can cause lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA, and enzymes, thus shutting down cellular respiration (Rahal et al. 2014; Urso and Clarkson, 2003). To prevent foods from undergoing deterioration and to provide protection against serious oxidative related diseases, it is important to prevent the peroxidation of lipids and the formation of free radicals generating in the living body and foodstuffs. Lipid oxidation is inhibited by antioxidant agents (Chen et al., 2018; Kondyli et al. 2007). Artificial antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, and n-propyl gallate, exhibit strong antioxidant activity against several oxidation systems. However, the use of artificial antioxidants in foodstuffs is restricted or prohibited in some countries because of the potential risks for the living body. Antioxidants from natural sources are receiving increased attention. Food-derived peptides have been demonstrated to be the natural antioxidants without marked adverse effects. An increasing number of food protein hydrolysates and antioxidant peptides have been found to exhibit antioxidant activity (Samaranayaka and Li-Chan, 2011). Goat milk casein (GMC) differs greatly from bovine casein in content, peptide chain length, and Amino acid sequences (Kondyli et al. 2007; Ceballos et al. 2009). The study aims to evaluate the antioxidant activities, proteolytic activity and growth behaviour of *Lactobacillus* cultures during fermentation of goat milk.

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 $5 \pm 2^\circ\text{C}$.

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:

$$\text{Acidity (\% Lactic acid)} = 9 \times V \times NX$$

Where,

V = Volume (ml) of 0.1 [N] NaOH required for the titration,

N = Normality of NaOH solution and

X = Volume of milk (ml) taken for the titration

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

cooled (45°C) MRS agar was poured to each Petri plates. The content was mixed thoroughly by tilting and rotating the plates and allowed it to solidify and then add second layer (5-7 ml) of the same agar was poured completely over the solidified medium. Again allowed it to solidify, then incubated at 37°C for 24 h in the inverted position. Typical colonies were calculated and the counts were expressed as log CFU/ml.

Assessment of antioxidant activities (ABTS assay, Hydroxyl free radical scavenging assay and Superoxide free radical scavenging assay) of *Lactobacillus* cultures

Sample preparation

Fresh goat milk was collected. Then filtered through a muslin cloth and sterilized at 121°C (250°F) at 15 psi (pounds per square inch) for 15 minutes and stored at 5±1°C. The pure culture was activated by inoculating in sterilized goat milk at the rate of 2% and incubated at 37°C for 24 h. Further, sampling was carried out by inoculating each culture in 10 ml sterilized goat milk at the rate of 2% and incubated for 0 and 48 h at 37°C. After incubation of 0, 12, 24, 36 and 48 h, the fermented goat milk samples were centrifuged at 14,000 rpm for 30 min at 4°C (Eppendorf centrifuge, US). The supernatants were collected and filtered using 0.22µm syringe filter (Millex®-HV, MERK, Ireland) and stored further for evaluating the antioxidant activities.

ABTS assay (2, 2-Azino-bis (3-ethylbenzothiazoline 6-sulfonic acid) assay) of fermented goat milk

The radical scavenging capacity of *Lactobacillus* cultures was based on the capability of a compound to scavenge the stable ABTS radical. This assay was evaluated as per the method described by Hati et al. (2013) with some modification. The ABTS working solution was prepared in dark bottle by mixing 88 µL of 140 mM potassium persulphate with 5 mL of 7 mM ABTS stock solution and incubating overnight for the generation of radicals and was diluted with phosphate buffer saline (PBS) to adjust the absorbance at 734 nm to 0.7 ± 0.02. An aliquot of 200 µL of product supernatant was mixed with 2.3 ml ABTS in PBS solution and both were mixed for 10 sec. The decrease in absorbance at 734 nm was recorded over for 10 min at 10 sec (A_{Sample}) using Spectrophotometer (Systronics PC based double beam Spectrophotometer 2202, India). Unfermented goat milk supernatant was used as a control sample (A_{Control}). Free radical scavenging capacity of the peptides was measured using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \left(\frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100$$

Hydroxyl free radical scavenging assay of fermented goat milk

Antioxidant activity was carried out by using Hydroxyl free radical scavenging ability of the peptides following Li et al. (2008) 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:

$$\text{Hydroxyl radical scavenging activity (\%)} = \left[\frac{(A_s - A_1)}{(A_0 - A_1)} \right] \times 100$$

Where A_s is the absorbance of the sample; A_1 is the absorbance of the control containing 1, 10-phenanthroline, FeSO₄ and H₂O₂; and A_0 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 O₂⁻ 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-HCl buffer (0.05M, pH 8.3) was placed in a clean test tube; thereupon, 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):

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

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) $\pm$ 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 48h 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).

Titrateable acidity

Titrateable 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 titrateable 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 titrateable 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 titrateable 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. titrateable acidity) by *L. bulgaricus* NCDC 09 and *L. helveticus* V3 was higher than other strains. Higher viable counts were observed in *S. thermophilus* 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*

Fig. 1 Changes in pH of goat milk by *Lactobacillus* cultures at different incubation hours

Fig. 2 Changes in lactic acid production (%LA) by *Lactobacillus* cultures at different incubation hours in goat milk medium

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

Fig.3 *Lactobacillus* counts of different cultures at different incubation hours in goat milk medium

#Values with different superscripts differ significantly ($p < 0.05$), Hydroxyl free radical scavenging activity (%) Mean $\pm$ SD.

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 4°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 (IC_{50}) value of ABTS

activity of goat milk casein was 71.251 ± 2.747 $\mu\text{g/ml}$ and for goat milk casein hydrolysates was 0.449 ± 0.027 $\mu\text{g/ml}$. 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 (53.43%), followed by M9 (50.98%), NK9 (48.88%), M3 (46.93%) and M11 (35.32%) after 48 h at 37°C. 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

by *Lactobacillus casei* L61 and added the optimal additive amounts were 0.99% (w/v) calcium lactate, 0.21% (w/v) glucose, and 0.29% (w/v) casein peptone. They found that the hydroxyl free radical scavenging rate increased significantly ($p < 0.001$) from $56.50 \pm 0.57\%$ to $88.01 \pm 0.69\%$. In another study, Chen et al. (2019) investigated the hydroxyl free radicals scavenging activity of cheddar cheese during its ripening. They found that the activity of hydroxyl free radicals scavenging in cheese increased significantly in the first 4 weeks ($P < 0.05$), and reached the maximum of 43.86%, 46.19%, 46.66% and 47.43%. In the 20th and 24th weeks, all of them show the significant decrease ($P < 0.05$). But in the last 3 weeks, no significant differences were observed ($P > 0.05$). In the 16th week, there were no significant differences ($P > 0.05$) between mixed and individual probiotic cheese, however, the value of mixed one was significantly higher than that of control cheese ($P < 0.05$). In our study, we also found the same observation with the significant increase of hydroxyl free radicals-scavenging activity than the control (0 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

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

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

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

<i>Lactobacillus</i> Cultures	0h	12h	24h	36h	48h
M3	0.31 ± 0.22^a	07.70 ± 2.50^b	32.91 ± 0.85^c	40.15 ± 1.50^{hi}	46.93 ± 0.81^{lm}
NK9	0.31 ± 0.21^a	11.32 ± 1.07^c	33.08 ± 0.85^{ef}	43.20 ± 1.45^{jk}	48.88 ± 4.72^{mn}
M8	0.33 ± 0.22^a	05.96 ± 1.61^b	37.88 ± 2.00^{gh}	40.82 ± 0.71^i	53.43 ± 0.86^o
M9	0.31 ± 0.21^a	11.04 ± 1.12^c	38.80 ± 1.77^{hi}	44.83 ± 0.70^{kl}	50.98 ± 1.54^n
M11	0.32 ± 0.21^a	01.77 ± 1.05^a	19.95 ± 0.71^d	35.57 ± 2.80^{fg}	35.82 ± 1.96^e

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 37°C. 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 50°C (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.

Proteolytic (OPA) activity of fermented goat milk

The proteolytic (OPA) activity of all the five *Lactobacillus* cultures was presented in Table 4. From Table 4, it had been observed that proteolytic (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 proteolytic (OPA) activity of all the five *Lactobacillus* cultures was increased non-significantly with the time of incubation.

The proteolytic (OPA) activity of *Lactobacillus* cultures was found in the range of 7.30 to 9.96 mg/ml. M9 had exhibited highest proteolytic (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 37°C. It was also observed that the proteolysis was increased with an increase in the incubation time from 0 to 48 h.

Basically, Increase in proteolytic 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_3 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 proteolytic 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 proteolytic 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 proteolytic activity on skim milk agar. After its genotypic and phenotypic evaluation, found that *Lb.*

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

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

[#]Values with different superscripts differ significantly ($p < 0.05$), Superoxide free radical scavenging activity (%) Mean ± SD.

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

<i>Lactobacillus</i> 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*

*Values with different superscripts differ non-significantly ($p < 0.05$), Proteolytic activity (mg/ml) Mean ± SD.

delbrueckii had the best proteolytic activity (maximum 3 mm zone was hydrolysed). In order to isolate LAB from spontaneous fermented local goat milk, which had proteolytic activity and used for further fermentation of goat milk. Based on proteolytic activity, the isolates composed of two bacteria species namely *Lactobacillus plantarum* (YN 1.1, YN 1.3, YN 1.8 and isolates YN 2.25) and another bacteria was *Lactobacillus pentosus* (YN 1.6). Five isolates had proteolytic activity, where three of them had the best proteolytic activity namely *Lactobacillus plantarum* YN 1.1, *Lactobacillus plantarum* YN 1.3 and *Lactobacillus pentosus* YN 1.6 (Yelnetty et al., 2014). In a study, nineteen *Lactobacillus* isolated from Algerian goat's milk, 13 belonging to *Lb. plantarum*, three to *Lb. pentosus*, two to *Lb. rhamnosus* and one to *Lb. fermentum*, were examined *in vitro* used as adjunct culture in dairy products. The strains were tested for their proteolytic activity, sensory and safety properties. Strains LbMS16 and LbMS21 *Lb. plantarum* and LbMF25 *Lb. rhamnosus* presented the highest proteolytic activity (Ahmed and Bousmaha-Marroki, 2014).

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.

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