Vol.65: e22210091, 2022
https://doi.org/10.1590/1678-4324-2022210091
ISSN 1678-4324 Online Edition

Article - Food/Feed Science and Technology

Probiotic Characterization of *Enterococcus* spp. Isolated from Raw Goat Milk

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Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Jéssica Caroline Bigaski Ribeiro

Received: 18-Fev-2021; Accepted: 03-Nov-2021.

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HIGHLIGHTS

- Lactic acid bacteria (LAB) from goat milk exhibited antimicrobial potential
- LAB with probiotic attributes were identified as *Enterococcus faecium*
- *Enterococcus* spp. showed no hemolysis and mild antibiotic resistance

Abstract: The aim of this research was to determine the probiotic potential and safety of lactic acid bacteria (LAB) isolated from raw goat milk. Gram positive and catalase negative bacteria were isolated from raw goat milk (n = 61) and identified as LAB. LAB isolates were screened for antimicrobial, probiotic and technological characteristics. LAB isolates showed antimicrobial activity against foodborne pathogens (Staphylococcus aureus, Escherichia coli and Salmonella Typhimurium) and high survival rate at pH 2 (93.54-100.38% after 4h), in the presence of 0.3% bile salts (100.85-108.96% after 4h) and simulated gastric fluid (74.16-80.13% after 3h). Three LAB isolates (1, 3 and 13) with high antimicrobial activity against all foodborne pathogens and probiotics characteristics were subjected to 16S rRNA sequencing and identified as Enterococcus faecium strains. Enterococcus spp. exhibited milk coagulation potential, amylolytic activity, susceptibility to antibiotics and no evidence of hemolysis. Enterococcus spp. isolated from goat milk showed probiotic and technological characteristics and can be used as a starter culture after further safety evaluation.

Keywords: Lactic acid bacteria; Probiotics; Antimicrobial activity; Raw goat milk; *Enterococcus* spp.

INTRODUCTION

Currently, the non-bovine milk accounts for more than 17% of total milk production worldwide with 133 million tons production per year. The goat milk contributes 13.5% to non-bovine milk production [1]. Goat milk is similar to human milk in composition and contains many bioactive compounds such as nucleotides, free amino acids and polyamines as in human milk, making it an alternative to baby food formulae [2]. Goat milk is preferred as it has lower allergenicity than cow milk, high digestibility and smaller size fat globules [3]. Goat milk is whiter in color than cow milk and contains higher level of vitamin A, due to the capability of goats to convert most of dietary carotene to vitamin A [4]. Lactic acid bacteria (LAB) isolated from dairy products exhibit probiotic characteristics and majority of the probiotic LAB belongs to Lactobacillus spp., Bifidobacterium spp. Enterococcus spp. and Lactococcus spp. [5]. Probiotics are live microorganisms that confer health benefits to the host, when administered in adequate amount. A probiotic should be able to tolerate low pH, gastric juice conditions, bile salts, colonize in the intestine, prevent colonization of pathogens in intestine and safe to use in diet [6]. Probiotics are nonpathogenic living organisms which are being used in foods to improve the normal microbiota of host intestine, regulate the immune system, prevent diarrhea, reduce hypertension and lower cholesterol level [7]. Enterococcus spp. are the group of LAB, which are facultative anaerobe, Gram positive, catalase negative and cocci-shaped. Enterococci are found in the intestinal tract of humans, various animals and in different food sources such as meat and milk as well [8]. Although the genus enterococcus has been used in food but some members of this group such as E. faecalis and E. faecium isolated from clinical sources are associated with various infections. Enterococci isolated from food sources are not associated with clinical infections, however, consumption of food carrying antibiotic resistant bacteria is a possible cause of transfer of this trait to the members of host microbiota [9]. Enterococcus spp. have been used as a starter culture for fermentation or as probiotics in various food products [10]. Among enterococci, Enterococcus faecium is reported to show beneficial effect in the treatment of irritable bowel syndrome, antibiotic associated diarrhea, immune regulation, and controlling serum cholesterol level in humans, by excreting deconjugated bile salts [11]. Various strains of E. faecium have been authorized by European Union to use as additives in feeding products [12]. The British 'Advisory Committee on Novel Foods and Processes' (ACNFP) have also authorized the use of E. faecium strain K77D in various fermented dairy products as a starter culture [13]. This study aims at the isolation and characterization of LAB with antimicrobial potential from goat milk obtained from local farms in Lahore, which can be utilized in food industry as a starter culture or potential probiotics in functional foods.

MATERIAL AND METHODS

Sample collection

The raw goat milk samples (n = 61) were collected in sterilized labelled bottles from different goat farms in Lahore city and transported to food microbiology laboratory in ice boxes (5±2 °C) for further isolation and characterization.

Isolation of LAB

The samples were subjected to serial dilution and spread plated on de Man, Rogosa and Sharpe agar (MRS, Oxoid, UK), incubated at 37 ºC both aerobically and anaerobically for 48-72 h. The representative colonies were selected from each MRS agar plate. Bacterial isolates were purified by streaking repeatedly on MRS agar medium. The isolated colonies were subjected to presumptive identification by catalase test and Gram staining [14].

Antimicrobial activity of LAB

The antimicrobial potential of twenty-five isolated LAB was evaluated by spot on lawn and well diffusion methods [15]. *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923 and *Salmonella* Typhimurium ATCC 14028 were used as indicator strains for the evaluation of antimicrobial potential of isolated LAB.

Spot on lawn assay

From overnight grown culture of LAB (10^7-10^8 CFU/mL), 5 µL was spot inoculated on freshly poured MRS agar plates and incubated at 37 ºC. After 24 h, plates were overlaid with 7 mL of 0.75 % (w/v) soft

nutrient agar (Sigma, Germany), previously inoculated with 100 µL of overnight grown indicator strains (10^7-10^8 CFU/mL). The plates were further incubated for 24-48 h at 37 ºC and diameter of inhibition zone around the spot was recorded.

Well diffusion assay

Putative probiotic cultures were grown overnight in MRS broth, were centrifuged (DLAB D3024R, China) at 13000 ×g for 10 min (4 ºC). After discarding the cell pellet, cell free supernatant (CFS) was collected and filter sterilized using 0.20 µm pore size filter. The neutralized (pH 6.5) and non-neutralized CFS were then freeze dried (Christ Alpha 1-2 LD plus, Germany), followed by reconstitution in sterile double distilled water to achieve 10-fold concentration. CFS was also incubated with pepsin (1mg/mL, Merck, Germany) for 2 h at 37 ºC and residual activity was measured by well diffusion assay to estimate the involvement of protein component in antimicrobial effect. The overnight grown indicator strains (100 µL of 10^7-10^8 CFU/mL) were spread on nutrient agar plates, separately. CFS (200 µL) was added into the wells (8 mm) made by sterile cork borer in nutrient agar plates. The plates were incubated for 24- 48 h at 37 ºC. The diameter of zone of inhibition was recorded.

Characterization of LAB

Tolerance to low pH and bile

Overnight grown cultures (10 mL) in MRS broth were centrifuged (Sigma 2-16PK, Germany) at 5000 ×g, 4 ºC for 15 min. The cell pellets were then washed twice with phosphate buffered saline (PBS, pH 7) and suspended in 10 mL respective MRS broth with pH 2 and 4, separately. The cultures were then placed in an incubator at 37 ºC for 4 h. Samples were taken at different time intervals (0, 2 and 4 h) and after serial dilutions spreaded on MRS agar plates, followed by incubation at 37 ºC. After 48 h, viable cells were counted [16]. For bile tolerance, cell pellets were washed with PBS and resuspended in 10 mL MRS broth containing 0.3% bile salt (Sigma-Aldrich, Germany). The broth without bile salt was taken as a control. After 0 and 4h of incubation at 37 ºC, samples were subjected to serial dilution and spread plated on MRS agar. The plates were incubated at 37 ºC. After 48 h, total viable cells were enumerated [16]. LAB survivability (%) was calculated by using Equation 1.

$$\text{Survival}\ (\%) = \frac{\text{Log CFU1}}{\text{Log CFU0}} \times 100 \quad (1)$$

CFU1: Viable count survived after incubation, CFU0: Initial viable count

Tolerance to simulated gastric juice

After centrifugation of overnight grown culture (10 mL) at 5000 ×g, 4 ºC for 15 mins, cells were harvested. After washing twice with PBS (pH 7.2), cells were resuspended in 10 mL PBS (pH 2) solution containing pepsin (3 mg/mL) and were incubated at 37 ºC. Samples were taken after 0, 1, and 3 h of incubation and spread plated on MRS agar [17]. After incubation at 37 ºC for 48 h, viable cells were enumerated.

Heat tolerance assay

Freshly grown cultures were centrifuged at 6000 ×g, 5 ºC for 15 min and washed twice with PBS, (pH 7.1) and resuspended in 10% (w/v) skim milk. Cell suspensions were subjected to heat shock treatment by placing in a water bath (Daihan scientific Co., Ltd, Korea) at 60 ºC for 5 min and subjected immediately to cooling in an ice bath. After incubation at 37 ºC for 48 h, heat resistance was analyzed by counting viable cells before and after exposure to heat [18].

Autoaggregation

Overnight grown cultures were centrifuged at 5000 ×g, 4 ºC for 15 min and cell pellets were suspended in PBS (pH 7.4). The bacterial suspension (4 mL) was vortexed for 10 s and incubated at 37 ºC. Samples were taken at 0, 3 and 24 h and their absorbance was recorded at 600 nm by UV-Vis spectrophotometer (Aurius 2000 series, Cecil instruments, England). Autoaggregation was calculated by using Equation 2 [19].

$$\text{A}\% = \frac{\text{At}}{\text{A0}} \times 100 \quad (2)$$

Where At is the absorbance at time t and A0 the absorbance at t= 0.

Coaggregation

Overnight grown cells (10 mL) were harvested by centrifugation at 5000 ×g for 10 min. After washing twice, the cell pellets were resuspended in PBS (pH 7.4). Equal volume (5 mL) of both pathogen strain *Salmonella* Typhimurium and cell suspension of LAB isolate were mixed in a test tube. After measuring primary absorbance at 600 nm by spectrophotometer, the test tubes were incubated at 37 ºC. After 24 h, the optical density was measured at 600 nm.

Percent coaggregation was determined by Equation 3 [20].

$$\text{Coaggregation}\% = \frac{(\text{A0} - \text{At})}{\text{A0}} \times 100 \quad (3)$$

Where At is the absorbance at time t and A0 the absorbance at t= 0.

Cell surface hydrophobicity

Overnight grown culture (10 mL) was centrifuged at 5000 ×g for 15 min and resuspended in PBS (pH 7.4). The primary absorbance of the cell suspension was observed at 600 nm. The cell suspension (3 mL) was transferred in a test tube and 1 mL xylene (Merck, Germany) was added. The test tube was vortexed for 2 min and left for 30 min at 25 °C to allow phase separation. The upper organic phase was carefully removed and the absorbance of the lower aqueous phase was measured at 600 nm. Cell surface hydrophobicity (CSH, %**)** was calculated by Equation 4 [21]:

$$\text{CSH}\% = \frac{(\text{A0} - \text{A})}{\text{A0}} \times 100 \quad (4)$$

Where A0 and A indicate the primary absorbance and absorbance after extraction with hydrocarbon, respectively.

Milk coagulation test

To check the milk coagulation activity, 1 mL of (1%; v/v) overnight grown LAB were inoculated into 99 mL of skimmed milk (10% w/v) and incubated at 37 ºC. After 24 h, milk coagulation was observed with naked eye [22].

Amylolytic activity

The overnight grown culture of LAB was streaked on modified MRS agar (containing 0.2%, w/v soluble starch, instead of glucose). The plates were incubated for 48-72h at 37 ºC. Few drops of Gram's iodine solution were then added on plates and plates were observed for clear halo zones (alpha–amylase production) [16].

Identification of selected strains by 16S rDNA sequencing

LAB isolates selected on the basis of antimicrobial potential and probiotic characteristics were further subjected to molecular identification. Chromosomal DNA was isolated and 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal forward P1 (F) (5'-CGAGAGTTTGATCCTGGTCAGAACGCT-3') and reverse primer P6 (R) (5'-CGTACGGCTACCTTGTTACGACTTCACCCC-3'). PCR amplification (Bio-Rad, USA) of selected strains was carried out as follows: 5 min at 94 ºC for initial denaturation followed by 35 cycles of 1 min at 94 ºC, 1 min at 55 ºC, 1 min at 72 ºC and a final extension for 10 min at 72 ºC. Amplified DNA was sequenced and the complete sequence of 16S rDNA genes were compared with those of BLAST's GenBank database. A neighbor joining method was used to construct the phylogenetic tree using MEGA 7 software.

Hemolytic activity

Fresh cultures of selected LAB isolates were grown overnight. A spot of 5 µL was inoculated on Columbia agar plates containing 5% of sheep blood (Oxoid, UK). The plates were placed in an incubator for 24- 48h at 37 ºC. The plates were examined for β- hemolysis (clear zones around colonies), α- hemolysis (green zones around colonies) or γ- hemolysis (no zones) [15].

Antibiotic susceptibility

Antibiotic susceptibility of LAB isolates was analyzed by disc diffusion assay, following the guidelines of Clinical and Laboratory Standard Institute [23]. The following commonly used antibiotics were tested; ampicillin (10 µg), vancomycin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), streptomycin (10 µg), septran (25 µg), gentamicin (10 µg), amikacin (30 µg) and chloramphenicol (30µg). Briefly, 100 µL of overnight grown LAB culture (10^8 CFU/mL) was spread plated on MRS agar. Antibiotics discs were placed on inoculated plates and the plates were incubated for 24 h at 30 ºC. The diameter of zone of inhibition was measured.

Statistical analysis

All experiments were conducted in triplicates. One-way analysis of variance (ANOVA) and Tukey's HSD tests were used to find the significant differences among treatments ($p < 0.05$) using SPSS statistical software package (SPSS, version 23.0, USA).

RESULTS

Isolation and presumptive identification of LAB

From 61 raw goat milk samples, 122 bacterial colonies were selected and subjected to Gram staining and catalase test. Out of 122, 25 isolates were Gram positive and catalase negative cocci (Table 1). These 25 isolates were then evaluated for antimicrobial potential against pathogenic strains of *E. coli*, *S. Typhimurium* and *S. aureus*.

Table 1. Lactic acid bacteria (LAB) isolated from raw goat milk

Isolate No.	Isolates Code	Growth Condition
1	S4	Anaerobic
2	S9	Aerobic
3	S11	Aerobic
4	S17A	Aerobic
5	S17B	Anaerobic
6	S19A	Aerobic
7	S19B	Anaerobic
8	S21A	Aerobic
9	S21B	Anaerobic
10	S22	Anaerobic
11	S25	Anaerobic
12	S43	Anaerobic
13	S49	Anaerobic
14	S51	Anaerobic
15	S52	Anaerobic
16	S56A	Aerobic
17	S56B	Anaerobic
18	S57	Aerobic
19	S58A	Aerobic
20	S58B	Anaerobic
21	S59	Aerobic
22	S60A	Aerobic
23	S60B	Anaerobic
24	S61A	Aerobic
25	S61B	Anaerobic

S indicates the sample number, A and B represent the aerobic and anaerobic cocci isolates of the same milk sample.

Antimicrobial activity of LAB

Spot on lawn assay

Out of 25 isolates, 16 showed clear zones of inhibition against test pathogens (*E. coli, S. aureus,* and *S.* Typhimurium). Isolate 1, 3, 4, 5, 8, 12, 13, 14, 16, 18, 19, 21, 22 and 23 showed remarkable inhibition against all pathogens (Table 2). Isolate 3 and 4 showed comparatively large zones of inhibition (33-38 mm) against all three pathogenic strains, whereas, isolates 6 and 15 showed antimicrobial activity against *E. coli* and *S. aureus*.

Table 2. Antimicrobial activity (inhibition zone in mm) of LAB isolates against foodborne pathogens

Isolates	Spot on lawn assay (mm)			Well diffusion assay (mm)		
	E. coli	***S. aureus***	***S.* Typhimurium**	***E. coli***	***S. aureus***	***S.* Typhimurium**
1	27.33 ± 0.58^{bA}	24 ± 2.00^{fghA}	25.33 ± 3.51^{cdeA}	18 ± 3.00^{aA}	20.33 ± 1.15^{aA}	25 ± 4.58^{aA}
3	33.33 ± 1.53^{aB}	38.33 ± 1.53^{abA}	35.33 ± 0.58^{abAB}	19.67 ± 3.06^{aA}	20.33 ± 4.17^{aA}	19.33 ± 2.08^{abA}
4	35.67 ± 0.58^{aA}	37.67 ± 2.5^{abcA}	35.00 ± 1.00^{abA}	-	17.33 ± 2.08^{aA}	17 ± 2.00^{bA}
5	22.67 ± 0.58^{bcdeB}	40.00 ± 0.00^{aA}	25.33 ± 3.51^{cdeB}	-	-	-
6	24.67 ± 0.58^{bcdB}	26.67 ± 0.15^{efgA}	-	-	-	-
8	23.00 ± 3.61^{bcdeA}	23.33 ± 3.31^{fghA}	20.00 ± 2.00^{eA}	-	-	-
12	25.33 ± 0.58^{bcdC}	30.33 ± 0.58^{cdefB}	35.53 ± 0.58^{abA}	-	-	-
13	23.67 ± 0.58^{bcdeB}	30.67 ± 0.50^{bcdefA}	29.33 ± 2.52^{bcA}	21.00 ± 3.61^{aA}	18.33 ± 0.58^{aA}	16.67 ± 1.53^{bA}
14	21.33 ± 5.86^{cdeA}	20.33 ± 5.58^{ghA}	29 ± 2.65^{bcdA}	-	-	-
15	26.33 ± 0.58^{bcA}	28.67 ± 0.53^{defA}	-	-	-	-
16	23.67 ± 1.53^{bcdeB}	25.00 ± 1.36^{fghB}	39.67 ± 0.58^{aA}	-	-	-
18	24.67 ± 1.53^{bcdA}	25.67 ± 1.5^{efgA}	28.33 ± 1.53^{bcdA}	-	18.00 ± 1.00^{aA}	15.67 ± 1.53^{bA}
19	18.00 ± 1.00^{eB}	17.67 ± 1.53^{hB}	21.67 ± 1.15^{deA}	-	-	-
21	33.67 ± 2.08^{aA}	33.00 ± 2.0^{abcdeA}	29.67 ± 1.53^{bcA}	-	-	-
22	20.00 ± 1.00^{deA}	26.00 ± 1.93^{efgA}	28.33 ± 2.89^{bcdA}	-	-	-
23	22.33 ± 1.53^{bcdeB}	35.33 ± 1.5^{abcdA}	28.33 ± 5.51^{bcdAB}	17 ± 1.00^{aA}	-	15 ± 2.65^{bA}

Different small superscript letters (a-h) within a column indicate that means are significantly ($p < 0.05$) different from each other, whereas, different capital superscript letters within a row indicate that means are significantly different from each other. The negative sign (–) indicates that antimicrobial activity was not detected.

Agar well diffusion assay

LAB isolates were also analyzed for their ability to produce antimicrobial substances by agar well diffusion assay (Table 2). Isolates 1, 3, and 13 showed inhibition zone against all three pathogens while, isolate 4, 18, and 23 showed inhibition zone against two pathogens. CFS of isolates 1, 3, 4, 13, 18 and 23 showed inhibition zones after heat and enzymatic treatment. Furthermore, no antimicrobial activity was observed by the neutralized CFS of LAB strains (1, 3, 4, 13, 18 and 23) indicating that antimicrobial activity was due to low pH of CFS and might be attributed to the organic acids.

Characterization of LAB isolates

LAB isolates (1, 3, 4, 13, 18 and 23) that showed antimicrobial activity by both, well diffusion and spot on lawn assay, were selected for further probiotic characterization.

Tolerance to low pH and bile

All the tested LAB isolates were resistant to low pH and survived at pH 2 and 4, however, isolate 23 was unable to survive at pH 2 (Table 3). Selected LAB isolates were able to grow in MRS supplemented with 0.3% bile salts with more than 95% survival rate. The survival rate of LAB isolates ranged from 100.85 ± 3.82 to 108.96 ± 4.55. Isolate 18 showed the maximum survival rate of 108.96 ± 4.55.

Tolerance to simulated gastric fluid (SGF)

After 1 h incubation in SGF, the highest survival rate was observed for isolate 18 (98.33 ± 2.93), while isolate 1 showed lowest survival rate (82.40 ± 2.18). Whereas, after 3 h of incubation in SGF, LAB isolate 23 showed highest survival rate (80.13 ± 3.45) and least survival rate was observed by isolate 4 (74.16 ± 3.06).

Heat tolerance

After heat treatment of LAB (60 ºC for 5 min), survival rates ranged from 95.26 ± 0.28 to 97.72 ± 0.43. The highest survival rate was observed by isolate 13 while isolate 1 was observed with lowest survival rate. The high survival rates of LAB after heat treatment indicated their potential to survive heat processing conditions. In this study, the cell death range of LAB (0.15 to 0.34 log) was lower than previously reported by Paéz and coauthors [18] that was 0.95 to 4.91 log for LAB isolates (commercial strains of, *L. paracasei, L. casei, L. acidophilus* and *L. plantarum*).

Autoaggregation

All isolated LAB showed high autoaggregation after 24 h as compared to 3 h of incubation at 37 ºC (Table 4). After 24 h incubation highest autoaggregation was shown by isolate 3 (55.36 %) and isolate 4 (51.45%). Isolate 13, 18 and 23 exhibited autoaggregation in the range of 30 to 48% while the least autoaggregation ability was shown by isolate 1 (28.45%) after 24 h.

Coaggregation

All tested LAB exhibited coaggregation after 2 h and 24 h incubation at 37 ºC (Table 4). After 2 h, isolates 3, 18, and 23 showed relatively high coaggregation ability; 9.44, 10.40, and 9.84% respectively. After 24 h of incubation, isolates 4 and 23 showed higher coaggregation ability with 59.27 and 58.15 %, respectively. Isolates 1, 3, 13, and 18 exhibited 47.16, 35.16, 44.33, and 50.87% coaggregation respectively.

Cell surface hydrophobicity

All of the LAB isolates exhibited cell surface hydrophobicity of less than 10% as shown in table 4. Isolate 13 showed the highest hydrophobicity (9.83%) followed by isolate 1 (9.21%), isolate 18 (9.16%) and isolate 3 (8.8%). Isolates 4 and 23 showed lower hydrophobicity (6.41 and 6.18% respectively).

Milk coagulation test

All the isolates (1, 3, 4, 13, 18, and 23) showed coagulation in skim milk after 24 h observation. No coagulation was observed in control. Previously all isolates of *Lactobacillus* spp. and *Bifidobacterium* spp. from buffalo milk were reported to exhibit milk coagulation activity [14].

Amylolytic activity

Amylolytic activities of selected LAB strains (1, 3, 4, 13, 18 and 23) are summarized in table 4. Isolate 1, 3, 13 and 23 showed amylolytic activity. Amylolytic activity is the ability to convert starch into sugar by action of acids or enzymes such as amylase.

Identification by 16S rDNA sequencing

All three potential probiotic isolates (1, 3 and 13) (based on antimicrobial and probiotic characteristics) were identified by 16S rDNA gene sequence and PCR amplification. The selected strains were identified as *Enterococcus faecium* strains. The phylogenetic tree of the identified strains was constructed by the neighbor-joining method using MEGA 7 software (Figure 1).

Hemolytic activity

None of the isolates when grown in blood agar exhibited hemolytic activity (β- or α- hemolytic activity). All selected strains (1, 3, and 13) were γ- hemolytic i.e. no hemolysis.

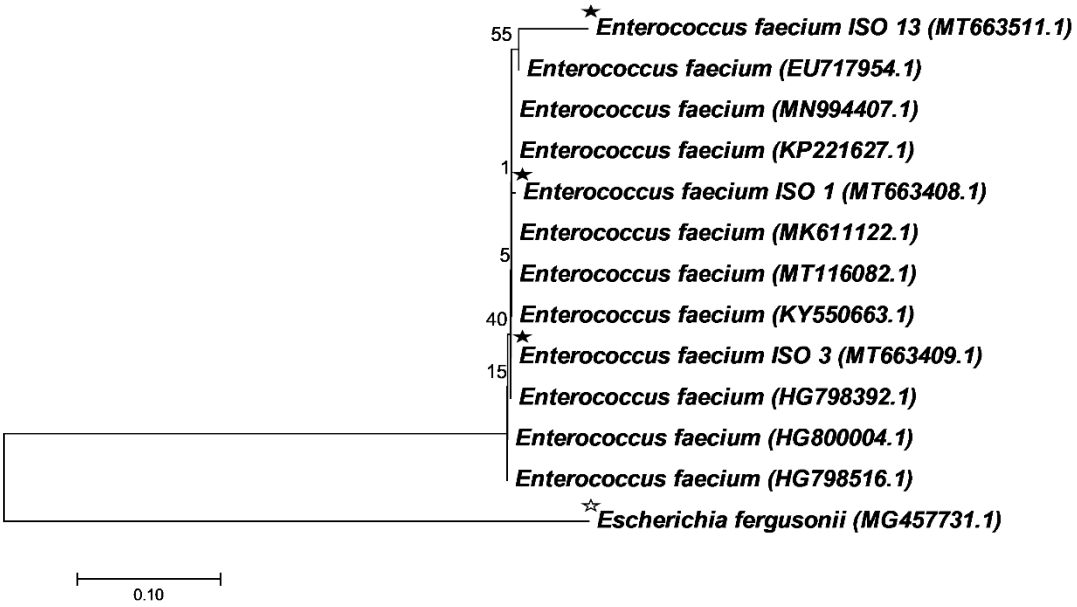

Figure 1. Neighbor joining phylogenetic tree based on 16S rDNA sequences. Accession numbers are presented in parenthesis. Filled stars indicate the lactic acid bacteria isolated from goat milk whereas, empty star indicates outgroup used for tree construction.

Antibiotic susceptibility test

Enterococcus spp. (goat milk isolates 1, 3 and 13) showed susceptibility to ampicillin, vancomycin, tetracycline, and chloramphenicol (Table 5). None of the isolates showed susceptibility to septran, whereas, isolate 3 and 13 showed no inhibition in growth after exposure to streptomycin and amikacin.

Table 3. Survival rate (%) of LAB isolates at low pH, in 0.30% bile concentration, simulated gastric fluid (SGF; pH 2 in the presence of pepsin) and after exposure to 60ºC for 5 min in 10% (w/v) skim milk.

Isolates	Acid tolerance				Bile tolerance (0.3% bile)	SGF with pepsin at pH 2		Heat resistance
	pH 2.0		pH 4.0					
	2h	4h	2h	4h	4h	1h	3h	
1	103.31 ± 2.24^{a}	96.85 ± 3.11^{a}	95.95 ± 1.26^{b}	80.79 ± 2.47^{c}	102.62 ± 3.23^{a}	82.40 ± 2.18^{c}	76.66 ± 3.55^{a}	95.26 ± 0.28^{a}
3	95.22 ± 4.92^{a}	93.54 ± 4.85^{a}	96.47 ± 2.15^{ab}	91.48 ± 4.97^{b}	100.85 ± 3.82^{a}	93.56 ± 1.98^{ab}	78.97 ± 4.23^{a}	95.56 ± 3.65^{a}
4	79.97 ± 2.96^{b}	97.25 ± 2.72^{a}	104.55 ± 4.55^{a}	100.26 ± 3.34^{ab}	103.20 ± 3.55^{a}	87.33 ± 2.79^{bc}	74.16 ± 3.06^{a}	96.30 ± 3.15^{a}
13	82.27 ± 3.48^{b}	100.38 ± 1.23^{a}	88.86 ± 2.62^{b}	108.40 ± 4.53^{a}	104.19 ± 0.69^{a}	91.99 ± 4.04^{ab}	78.94 ± 3.71^{a}	97.72 ± 0.43^{a}
18	96.34 ± 2.68^{a}	97.43 ± 4.76^{a}	90.30 ± 4.59^{b}	94.33 ± 4.61^{b}	108.96 ± 4.55^{a}	98.33 ± 2.93^{a}	74.23 ± 3.36^{a}	96.54 ± 2.79^{a}
23	-	-	97.21 ± 2.36^{ab}	97.67 ± 2.37^{ab}	103.80 ± 4.21^{a}	97.83 ± 3.66^{a}	80.13 ± 3.45^{a}	96.05 ± 1.00^{a}

Different small superscript letters (a-c) within a column indicate that means are significantly ($p < 0.05$) different from each other.

Table 4. Autoaggregation, coaggregation ability, cell surface hydrophobicity and amylolytic activity of LAB isolates

Isolates	Autoaggregation %		Coaggregation %		hydrophobicity %	Amylase activity
	3 h	24 h	2 h	24 h		Clear zones around colonies
1	8.5 ± 0.17^{c}	28.45 ± 0.30^{f}	7.49 ± 0.35^{d}	47.16 ± 0.85^{c}	9.21 ± 1.73^{a}	**+**
3	10.29 ± 0.12^{a}	55.36 ± 0.34^{a}	9.44 ± 0.29^{b}	35.16 ± 0.89^{e}	8.88 ± 2.03^{a}	**+**
4	9.31 ± 0.15^{b}	51.45 ± 0.28^{b}	8.39 ± 0.27^{c}	59.27 ± 0.35^{a}	6.41 ± 0.66^{a}	**-**
13	10.50 ± 0.39^{a}	30.49 ± 0.31^{e}	8.19 ± 0.15^{cd}	44.33 ± 1.07^{d}	9.83 ± 0.64^{a}	**+**
18	6.50 ± 0.32^{d}	47.22 ± 0.17^{c}	10.40 ± 0.33^{a}	50.87 ± 0.96^{b}	9.16 ± 2.07^{a}	**-**
23	9.64 ± 0.14^{b}	41.36 ± 0.46^{d}	9.84 ± 0.16^{ab}	58.15 ± 0.97^{a}	6.18 ± 2.29^{a}	**+**

Positive sign (+) indicates amylolytic activity was present, whereas negative sign (–) indicates that amylolytic activity was absent. Different small superscript letters (a-c) within a column indicate that means are significantly ($p < 0.05$) different from each other.

Table 5. Antibiotic susceptibility of LAB isolates

Isolates	Zone of inhibition (mm)								
	Ampicillin	Vancomycin	Tetracycline	Ciprofloxacin	Streptomycin	Septran	Gentamicin	Chloramphenicol	Amikacin
1	21.67 ± 2.08^{bC}	23.67 ± 1.53^{aC}	35.67 ± 3.06^{aA}	12 ± 2.00^{bD}	9.67 ± 1.15^{aD}	-	13 ± 2.00^{aD}	29.33 ± 0.58^{aB}	18.67 ± 0.58^{aC}
3	26.67 ± 2.08^{abA}	21.67 ± 0.58^{aB}	28.67 ± 1.15^{bA}	9.33 ± 1.15^{bC}	-	-	10.67 ± 1.53^{aC}	19.33 ± 1.15^{bB}	-
13	28.33 ± 2.08^{aB}	21.67 ± 0.58^{aC}	33.33 ± 2.08^{abA}	17.67 ± 1.53^{aD}	-	-	-	27 ± 1.73^{aB}	-

Different small superscript letters (a-c) within a column indicate that means are significantly ($p < 0.05$) different from each other, whereas, different capital superscript letters within a row indicate that means are significantly different from each other.

DISCUSSION

In this study, LAB isolated from raw goat milk exhibited antimicrobial potential against Gram positive and Gram-negative bacteria. Probiotics isolated from food products were reported to show antimicrobial potential against foodborne pathogens [16]. LAB strains isolated from food products were reported to inhibit the growth of both Gram negative and Gram-positive pathogenic bacteria [24]. The antimicrobial potential of the LAB strains is attributed to the production of antimicrobial metabolites including bacteriocins, organic acids and hydrogen peroxide, which inhibit pathogenic bacterial growth [19].

LAB strains isolated from camel milk were reported to exhibit resistance to pH 2 [25]. In this study LAB isolated from raw goat milk showed resistance to low pH and bile salts (0.3%), which corroborated with the findings of Hoque and coauthors [26], who reported that LAB isolated from yogurt survived in 0.05-0.3% bile salts. De Almeida Júnior and coauthors [27] reported that LAB isolated from goat milk showed resistance to pH 2 with a high survival rate ranging from 70 to 100%. LAB isolated from goat milk were able to survive in gastric juice, the results were similar to previous report, indicating that LAB from Ngari (a fermented food), survived gastric juice up to 3 h of incubation with a logarithm unit of 5.23 ± 0.02 and 6.2 ± 0.18 log CFU/mL [28]. LAB isolates from camel milk showed high autoaggregation potential (2.7±0.40 to 38.8 ± 0.05%) after 24 h of incubation than 3h incubation (1.6 ± 0.05 to 10.2 ± 0.25%) [25]. In this study LAB from raw goat milk showed high coaggregation potential, which was in accordance with Manhar and coauthors [29], who found that the probiotic bacteria isolated from goat milk showed high coaggregation (%) with *S. enterica* Typhimurium (55.84 ± 1.2) and *Listeria monocytogenes* (43.94 ± 0.6) after 24 h. Hydrophobicity of LAB isolates varies among different strains. Ding and coauthors [21], reported that hydrophobicity of LAB isolated from Tibetan yak milk ranged from 2.17 to 60.97%, whereas most of LAB strains exhibited less than 10% hydrophobicity. In the current study, hydrophobicity of goat milk LAB was in the range of 6.18-9.83%, the reported difference in the cell surface hydrophobicity might be due to the variation in expression of surface proteins either due to difference in strain or due to environmental conditions.

In this study, none of the *Enterococcus* strains was found to be hemolytic. Similar findings were reported by Mrkonjic and coauthors [30], that *Enterococcus* strains isolated from raw milk cheese did not exhibit hemolytic activity. Antibiotic resistance is one of the various factors to evaluate the safety of enterococci. Several studies have reported the resistance of *Enterococcus* spp. to various antibiotics. Hejazi and coauthors [31], reported that all *Enterococcus* strains isolated from cheese showed sensitivity to ciprofloxacin (except for two isolates), vancomycin, chloramphenicol and tylosin and were resistant to colistin, trimethoprim, sulfamethoxazole, ampicillin (except for one isolate), and nalidixic acid. Carasi and coauthors [9], reported that enterococci isolated from kefir showed no resistance against clinically important antibiotics such as vancomycin, ciprofloxacin, erythromycin. However further safety evaluation such as identification of virulence factor and detailed characterization should be carried out for commercialization of regional probiotic strains.

CONCLUSION

LAB isolates from goat milk exhibited remarkable antimicrobial and probiotic characteristics. LAB isolates were identified as *Enterococcus* spp. *Enterococcus* spp. isolated from goat milk can serve as suitable candidate for potential probiotic application in dairy and food industry. However further safety evaluation and detailed characterization should be carried out for commercialization of regional probiotic strains.

Funding: This research received no external funding.
Conflicts of Interest: The authors declare no conflict of interest

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