International Journal of Veterinary Sciences and Animal Husbandry 2023; SP-8(5): 140-146

ISSN: 2456-2912
VET 2023; SP-8(5): 140-146

www.veterinarypaper.com
Received: 20-04-2023
Accepted: 06-06-2023

K Kavitha
Department of Dairy Science, College of Veterinary and Animal Science, Pookode, Kerala, India

T Magna
Department of Dairy Science, College of Veterinary and Animal Science, Pookode, Kerala, India

CT Sathian
Department of Dairy Science, College of Veterinary and Animal Science, Mannuthy, Kerala, India

K Radha
Department of Dairy Science, College of Veterinary and Animal Science, Mannuthy, Kerala, India

S Renjith
Department of Veterinary Biochemistry, College of Veterinary and Animal Science, Pookode, Kerala, India

M Arshath
Department of Dairy Science, College of Dairy Science and Technology, Pookode, Kerala, India

V Namratha
Department of Livestock products technology, College of Veterinary and Animal Science, Pookode, Kerala, India

Corresponding Author:
K Kavitha
Department of Dairy Science, College of Veterinary and Animal Science, Pookode, Kerala, India

Analysis of lactic acid bacteria present in raw samples of milk in Wayanad district of Kerala

K Kavitha, T Magna, CT Sathian, K Radha, S Renjith, M Arshath and V Namratha

DOI: https://doi.org/10.22271/veterinary.2023.v8.i5Sc.727

Abstract
Lactic acid bacteria are the bacteria present in milk and milk samples and they are group of catalase-negative, nonspore forming microorganisms. Upon gram staining Lactic acid bacteria (LAB) are gram positive and rod shaped. The genera *Lactococcus* and *Lactobacillus* are frequently linked to milk and dairy products. A total number of 50 fresh raw milk samples of 200 ml each was collected from various sources in Wayanad district of Kerala like ILFC Pookode, local households and nearby dairy chilling units, aseptically in sterile autoclavable bottles. Our study mainly focusses on the species level identification of *Lactobacillus* spp. and *Lactococcus* spp. We used two type of agar in our research work, for the isolation of *Lactobacillus* spp. de Man Rogosa and Sharpe (MRS) agar was used and M17 agar was used for the isolation of *Lactococcus* spp. 50 samples each were cultured on de Man Rogosa and Sharpe (MRS) agar and M17 agar. Colonies having typical characteristics were taken for preliminary identification by Gram staining and Biochemical tests. Purity of culture were maintained by obtaining pure culture. 16S rRNA gene sequencing was used for further identification of lactic acid bacteria (of *Lactobacillus* and *Lactococcus*) upto species level. The study was successful in species level identification of LAB from raw milk. We can conclude that raw sample milk contains abundance of lactic acid bacteria.

Keywords: Lactic acid bacteria, milk, Man Rogosa and Sharpe

1. Introduction
Humans have been consuming animal milk for about 6, 000 years and it forms an important part of their daily eating habits. Humans consume various types of milk including cow, buffalo, goat, camel, yak, etc. [1].
Milk and milk products are a well-known source of lactobacilli. Lactic acid bacteria (LAB) belong to various genera of the Lactobacillaceae family. Lactic acid bacteria are generally represented as gram-positive, non-spore-forming, non-pigmented, catalase-negative, and oxidase-negative organisms [2].
Lactic acid bacteria includes generas *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, *Weisella*, *Lactoshaera*, *Melisococcus*, *Oenococcus*, *Pediococcus*, *Microbacterium*, *Bifidobacterium* [3].
LAB produce various substances such as Bacteriocin, Lactic Acid, Ethanol, Formic Acid, Acetone, Hydrogen Peroxide, Diacetyl, Exo-polysaccharides etc. [4].
Lactic acid bacteria and their metabolites have major role in improving microbial quality as well as in biological preservation. [5].
To initiate desired fermentation under controlled conditions, LAB starter cultures are purposefully added to the milk."Generally Recognized as Safe" group of lactic acid bacteria are of genera *Lactococcus*, *Bifidobacterium* and *Lactobacillus* [6].
These cultures affect the production, flavor, and texture of fermented foods so they are unavoidable in the dairy industry [7].
Native source of LAB is the raw milk and can be used for preparation of various milk products [8].
New useful strains of LAB can be discovered with knowledge of the composition and amount of LAB in raw milk that can be used as starting cultures for the development of a new, high-

quality product as well as for new applications.
In our research work, the 16S rRNA sequencing technique is used to discover the species of isolated LAB. By comparing the sequences of the molecular bases, especially the 16S rRNA gene, it is possible to determine the relationship between the bacteria.

2. Materials and Methods

2.1 Collection of sample

A total number of 50 fresh raw milk samples of 200 ml each was collected from various sources like ILFC Pookode, local households and nearby dairy chilling units, aseptically in sterile autoclavable bottles from Wayanad district of Kerala. Milk samples were serially numbered from 1-50 on the collection bottles.

2.2 Isolation of bacteria

Serial dilution method was done with Phosphate Buffered Saline for isolation of LAB [9, 10]. One ml of the sample was mixed with 9 ml of phosphate buffered saline, and a dilution was prepared until 10^{-7}. Then, 0.1 ml of the solution was transferred to a sterile petri dish containing MRS and M17 agar. The spread-plate method was followed.
Plates were then incubated at optimum conditions for LAB growth. MRS agar plates were incubated anaerobically at 28-30 °C for 24-48 hours and M17 agar plates were kept at incubation temperature of 30-35 °C for 48 hours. All the agar plates were placed in inverted manner in incubator until the bacterial growth become evident. After incubation isolated colonies were restreaked on agar plates until pure cultures were isolated.

2.3 Characterization of Isolates

All the pure cultures isolated from dairy samples were studied for their morphological and staining reaction:
Morphological examination was carried out by examining colony characteristics of all the isolates. Staining method followed was Gram staining (1984).

2.4 Biochemical characteristics

The biochemical tests performed are catalase test and oxidase test.

2.5 Storage of isolates

Thirty percentage (v/v) glycerol was used to store the selected LAB isolates at -20 °C.

2.6 16S rRNA sequencing of bacteri

PCR was used for amplification of DNA of selected LAB isolates and 16S rRNA gene sequencing was done for the identification of LAB (9).
The procedure for 16S rRNA sequencing is as follows:

2.6.1 DNA Isolation

DNA from samples were extracted using HiPurA Multi-Sample DNA Purification Kit.
The extracted DNA were stored at –20 °C for future research.

2.6.2 Amplification of 16S rRNA gene

PCR was employed for amplification of 16 srRNA gene. Two universal 16S rRNA primers were used for PCR.

Table 1: Details of PCR primers used for detection of LAB

Set	Primer name	Sequence 5'-3'	Target region	Product size	Reference
1	16SrRNAFor	5'- AGAGTTTGATCCTGGCTCAG-3'	16S rRNA	1500 bp	(10)
2	16SrRNA Rev	5'-ACGGCTACCTTGTTACGACTT-3'	16S rRNA	1500 bp	(10)

The reaction volume was 50 µl containing 25 µl of PCR Master Mix 2X (50 U/ml Taq polymerase, 400 µM dNTPs, and 3 mM MgCl2), 5 µl of each forward and reverse primer (10 µM each, IDT synthesised), 5µl of DNA, and 15 µl of nuclease-free water. The amplification conditions were: an initial denaturation time of 4 minutes at 94 °C, a subsequent denaturation time of 1 minute at 94 °C, a primer annealing time of 1 minute at 58 °C, an extension time of 2 minutes at 72 °C for 35 cycles, and a final extension time of 7 minutes at 72 °C. The reactions were carried out in a thermal cycler.

2.6.3 Electrophoresis of the amplified PCR product

A 10 µl of the PCR-amplified product was electrophoresed for 40 minutes at 100 volts in 1X TAE buffer on an agarose gel stained with 1.5 percent (w/v) ethidium bromide. The gel was then visualized and documented using a gel documentation system. As a molecular weight marker, a DNA ladder (100 bp+) was employed.

2.6.4 Sequencing of the PCR Products

PCR product of 6 random LAB isolates from both MRS and M17 agar was outsourced to Protein Design Private Limited in Bengaluru, Karnataka, for sequencing. Samples.

2.6.5 Sequence alignment of the 16S rRNA gene

The 16S rRNA gene sequences of the isolates were compared using the BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). After then, these sequences were matched to those of homologous sequences.

3. Results and Discussion

The present study was conducted to analyse the species of the Lactic acid bacteria (LAB) present in raw sample of milk in Wayanad district of Kerala using 16 srRNA sequencing as tool.
For this study 50 fresh raw milk samples were collected from various sources like ILFC Pookode, local households and nearby dairy chilling units. LAB isolates were obtained by culturing in MRS and M17 agar. Identification and biochemical tests of LAB were performed. DNA amplification of selected isolates was done by 16SrRNA sequencing. The results are presented in the sections below:

3.1 Isolation and identification of lab

The serial dilution (10-dilution procedure) was used to isolate specific lactic acid bacteria from raw milk samples. One milliliter of the material was combined with nine milliliters of phosphate buffered saline to create a dilution that was then diluted to a final concentration of 10-7. A sterile petri dish containing MRS and M17 agar was next loaded with 0.1 ml of the solution and then spread-plate technique was used.
After complete solidification, invert the prepared Petri dish and incubate in an incubator at the optimum conditions for each of the agar plates.

Until bacterial growth was visible, the MRS plates were then incubated anaerobically at 28–30 ºC for 24–48 hours. M17 agar plates were likewise maintained for 48 hours inverted at their incubation temperature of 30-35 ºC till bacterial growth occurred. Colonies were randomly selected, picked up, and purified by streaking on respective plates.

3.2 Colony characteristics of isolated LAB

Observations of colony morphology were done after getting a pure culture in a Petri dish.

Colony observations were put into groups based on size, pigmentation, edge formation and elevation.

Table 2: Characteristics of a LAB colony that was isolated using MRS agar

Observation	Type of colony	Type of colony	Type of colony
Size of colony	Small (Fig 1)	Medium (Fig 2)	Large (Fig 3)
Sample number of milk sample in which LAB colony is present	3, 6, 16, 19, 20, 26, 28, 30, 32, 33, 36, 37, 39, 42, 45, 46	1, 5, 11, 24, 35	2, 4, 7, 8, 9, 10, 12, 13, 14, 15, 17, 18, 21, 22, 23, 25, 27, 29, 31, 34, 38, 40, 41, 43, 44, 47, 48, 49, 50
Pigmentation	Nil	Nil	Nil
Edge formation	Circular	Circular	Circular
Elevation	Flat	Raised	Raised

Fig 1: Small sized colonies generated by LAB that were isolated using MRS agar

Fig 2: Medium sized colonies generated by LAB that were isolated using MRS agar

Fig 3: Large sized colonies generated by LAB that were isolated using MRS agar

Colony observations were in accordance with the findings of researchers [11].

Table 2: Characteristics of a LAB colony that was isolated using M17 agar

Observation	Type of colony	Type of colony	Type of colony
Size of colony	Small (Fig 4)	Medium (Fig 5)	Large (Fig 6)
Sample number	2, 3, 4, 6, 7, 9, 12, 18, 23, 25, 27, 29, 32, 36, 37, 41, 43, 45, 46, 47, 48	28	1, 5, 8, 10, 11, 13, 14, 15, 16, 17, 18, 19, 26, 29, 30, 31, 33, 35, 38, 40, 42, 44, 49, 50
Pigmentation	Nil	Nil	Nil
Edge formation	Circular	Circular	Circular
Elevation	Flat	Raised	Raised

Fig 4: Small sized colonies generated by LAB that were isolated using M17 agar

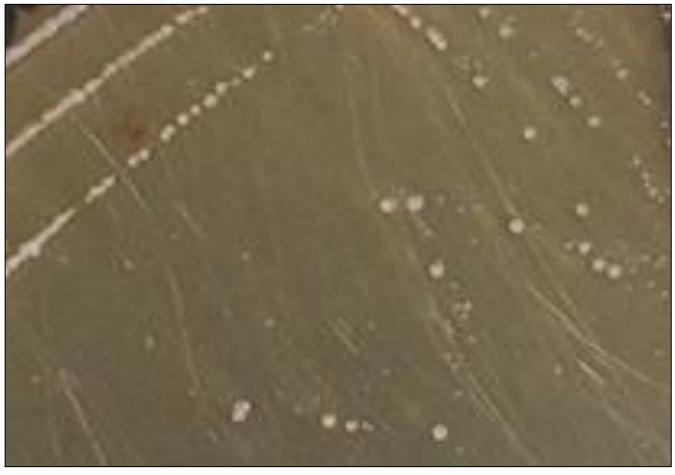


Fig 5: Medium sized colonies colonies generated by LAB that were isolated using M17 agar

Fig 6: Large sized colonies generated by LAB that were isolated using M17 agar

Colony observations were in accordance with the findings of researchers [11].

3.3 Morphology and staining reaction

In MRS agar, out of 50 LAB isolates, all the isolates were rod shaped and occurred as singles, pairs, or clusters. All the LAB isolated using MRS Agar showed a positive gram staining result; hence, all the LAB isolated are Gram positive (Fig. 7 and Fig. 8)**.** All the isolates had violet colour after gram staining. This was in accordance in observation by researchers [12].

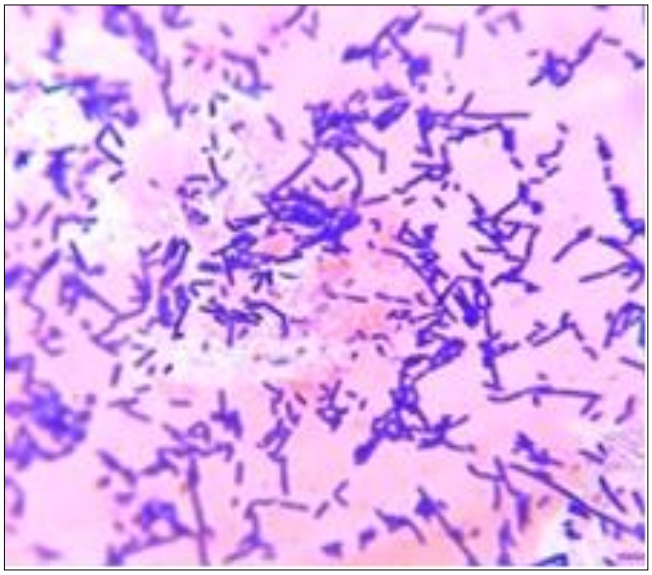


Fig 7: LAB isolates from MRS agar under oil immersion

All 50 isolates isolated from M17 agar were cocci shaped and occurred as singles, pairs, or clusters. All the isolates showed a positive gram staining result; hence, all LAB isolates are Gram positive these observations were similar to findings by researchers [13]. They isolated LAB from milk, curd and colostrum using MRS agar and observed rod shaped bacteria.

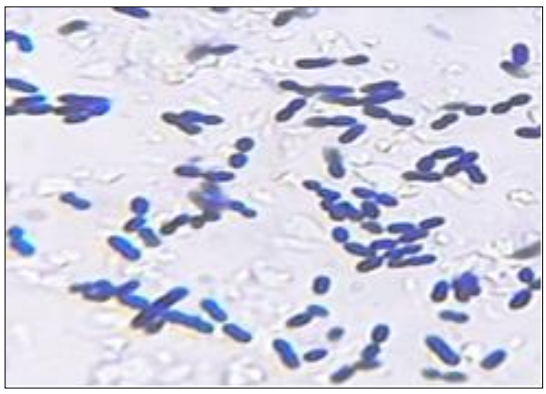


Fig 8: LAB isolates from M17 agar under oil immersion

Similar observations were made by researcher [14] that *Lactococcus* spp. are lactic acid bacteria with coccoid shapes.

3.4 Results on biochemical tests

3.4.1 Catalase test

All the LAB isolates were catalase negative. This matches with observations of researchers [15]. According to these researchers, lactic acid bacteria fail to produce the catalase enzyme, which converts hydrogen peroxide into water and oxygen and thus yields a negative catalase test result.

3.4.2 Oxidase test

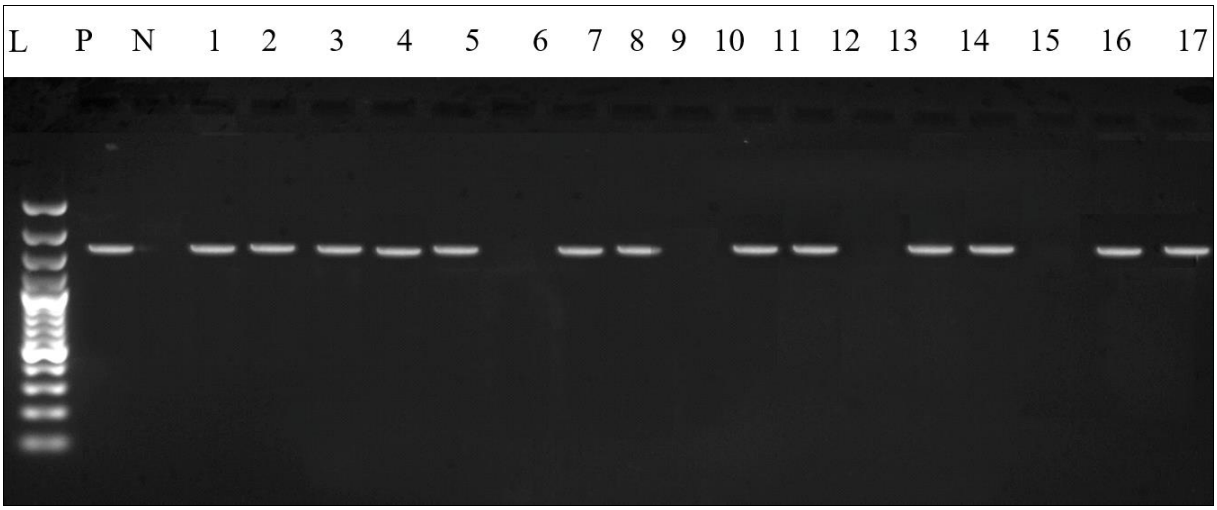
All the LAB isolates were oxidase negative. Researchers [16] explained this phenomenon that in the oxidase test, tetramethyl p-phenylene diamene dihydrochloride, could not be oxidised by the bacterial cultures to produce the purplecoloured compound. Hence LAB were oxidase negative.

3.5 16 Sr RNA sequencing

3.5.1 16S rRNA gene amplification of LAB isolates by Polymerase chain reaction (PCR)

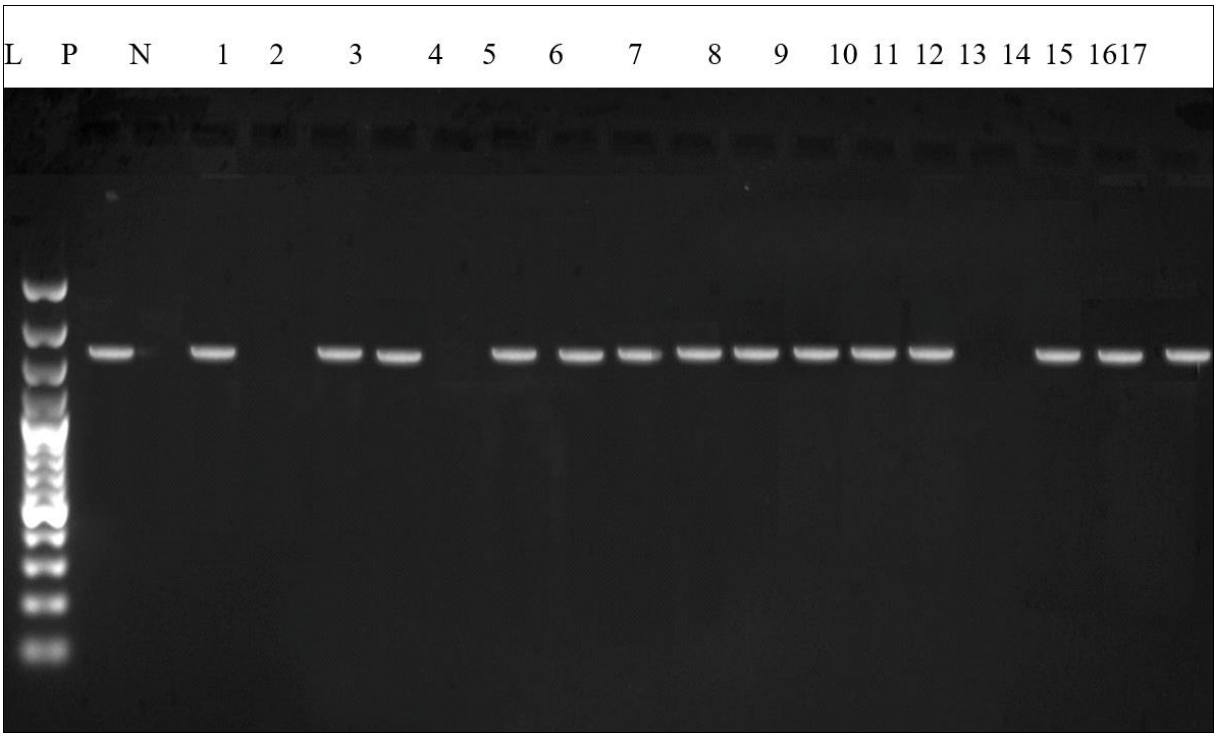
3.5.1.1 Gel documentation of PCR amplicons of LAB

Out of LAB isolated from 50 samples in MRS agar, 29 samples shown positive result for PCR**.**



L-Ladder
P-Positive control
N-Negative control
1, 2...17-Samples
Out of LAB isolated from 50 samples in M17 agar, 27 samples shown positive result for PCR.

Fig 9: Gel documentation of PCR amplicons of LAB from MRS agar.



L-Ladder
P-Positive control
N-Negative control
1, 2...17-Samples

Fig 10: GEL Documentation of PCR amplicons of LAB from M17 agar

3.5.1.2 Sequence of the 16S rRNA gene of LAB isolates:
3.5.1.2.1 16S rRNA sequencing of LAB from MRS:
Three random samples with sample numbers 32, 38 and 40 was sent for sequencing.
LAB isolated from sample number 32 in MRS agar exhibited 99.25percentage similarity with *Lactobacillus plantarum* strain 2.7.6 (MK611339.1) of Brazil by BLAST analysis.
LAB isolated from sample number 38 in MRS agar exhibited 98.97 percentage similarity with *Lactoplantibacillus plantarum* strain 155 (OP901765.1) and strain HBUAS51486 (OP024124.1) of Turkey and China respectively. This isolate has 98.97% similarity with *Lactoplantibacillus plantarum* strain HBUAS51486 (OP024124.1).
LAB isolated from sample number 40 in MRS agar exhibited 99.09 percentage similarity with *Lactobacillus plantarum* strain 1588(MT597491.1) of China.
3.5.1.2.2 16 S rRna sequencing of LAB isolated from M17 agar
Three random samples of sample number 20, 22, 27 were sent for sequencing. BLAST analysis was done to identify the 16S rRNA gene.
LAB isolated from sample number 20 in M17 agar exhibited 97.72 percentage similarity with *Lactococcus garvieae* strain RCB59 (KT260271.1) and GXLA20200825-5(OKO67770.1)

of India and China respectively.
LAB isolated from sample number 22 in M17 agar exhibited 99.56 percentage similarity with *Lactococcus garviea* strain IMAU98489 (MW135264.1) of China.
LAB isolated from sample number 27 in M17 agar exhibited 98.6 percentage similarity to *Lactococcus garvieae* strain GXLA20200825 (OK067770.1) of China.

4. Conclusion
The study was conducted to analyse the species of the Lactic acid bacteria (LAB) present in raw sample of milk in Wayanad district of Kerala using 16 srRNA sequencing as molecular tool.
In raw milk, a vast variety of lactic acid bacteria have been found. Therefore, raw milk can offer enormous amounts of lactic acid bacteria and thus enhance health.

5. References

1. Tapeshova SZ, Tokabasova AK, Atalikhova GB. Isolation and study of lactic acid bacteria cultures, yeast of natural cooking kumis starter for the goat's milk. Biological Science. 2013;12:68-71.
2. Sarangdhar M, Dipak V, Sheela S. Isolation and Identification of lactobacilli from raw milk samples obtained from Aarey Milk Colony. International Journal of Scientific and Research Publications. 2015;5(4):1-5.
3. Holzapfel WH, Haberer P, Geisen. Microorganisms in food and nutrition. American Journal of Clinical Microbiology. 2001;73:365s-373s.
4. Rodriguez E, Arques JL, Rodriguez R, Nunez M, Medina M. Reuterin production by lactobacilli isolated from pig feces and evaluation of probiotic traits. Letters of Applied Microbiology. 2003;37:259-263.
5. OSullivan L, Ross RP, Hill C. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie. 2002;84:593-604.
6. Salminen S, Deighton MA, Benno Y, Gorbach SL. Lactic acid bacteria in health and disease. Lactic Acid Bacteria: Microbiology and Functional Aspects; c1998. p. 211-54.
7. Li CT, Peng HJ. of Department of Bacteriology, GuangxiVeterinary Research Institute, No. 50 Youaibei Road, Nanning, Guangxi 530001, China; c2021.
8. Tulini FL, Hymery N, Haertlé T, Le Blay G, De Martinis EC. Screening for antimicrobial and proteolytic activities of lactic acid bacteria isolated from cow, buffalo and goat milk and cheeses marketed in the southeast region of Brazil. Journal of Dairy Research. 2016 Feb;83(1):115-124.
9. Bin M, Bahieldin A, Alharbi MG, Al-Masaudi S, Al-Jaouni SK, Harakeh SM, *et al*. Isolation, Characterization Molecular and Probiotic Potential of Lactic Acid Bacteria in Saudi raw and fermented Milk. Compl Alt. Med. 2018;20:1-12.
10. Malik V, Devi U, Yadav RNS, Mahanta J. Isolation, identification and antibacterial properties of *L. plantarum* from 'Khorisa', a traditional food product of Assam. International Journal of Pharmaceutical Res. and Health Care. 2014;4(4). ISSN No: 2249-5738.
11. Cappuccino JG, Sherman N. Microbiology: A Laboratory Manual, Eds J. G. Cappuccino & N. Sherman, State University of New York, Rockland Community College; c2001.
12. Rahmawati N, Syukri M, Darmawi D, Zachreini I, Yusuf M, Idroes R. February. Identification of lactic acid bacteria from etawa goat milk kopelma Darussalam Village, Banda Aceh. In IOP Conference Series: Earth and Environ. Sci. 2021;667(1):012022. IOP Publishing.
13. Padmavathi T, Bhargavi R, Priyanka PR, Niranjan NR, Pavitra PV. Screening of potential probiotic lactic acid bacteria and production of amylase and its partial purification. Journal of Genetic Engineering and Biotechnology. 2018 Dec 1;16(2):357-62.
14. Teuber M. The genus *Lactococcus*. In The genera of lactic acid bacteria. Boston, MA: Springer US; c1995. p. 173-234.
15. Tadesse G, Ashenafi M, Ephraim E. Survival of *E. coli* O157: H7 Staphylococcus aureus, Shigella flexneri and Salmonella spp. in fermenting Borde', a traditional Ethiopian beverage. Food control. 2005;16(2):189-196.
16. Thakur M, Deshpande HW, Bhate MA. Isolation and identification of lactic acid bacteria and their exploration in non-dairy probiotic drink. International Journal of Curr. Microbiol. Appl. Sci. 2017;6:1023-1030.