Journal of Advanced Scientific Research

Available online through https://sciensage.info

ISSN
0976-9595
Research Article

ISOLATION AND IDENTIFICATION OF INDIGENOUS PROBIOTICS FROM DAIRY PRODUCTS AND HUMAN BREAST MILK

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Received: 16-08-2023; Accepted: 24-08-2023; Published: 30-09-2023

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ABSTRACT

Probiotics are live microbes that beneficially affect the human and many studies have proved their promising role as safe and natural therapeutics. The isolation of probiotics from indigenous sources may develop a new way to improved probiotic strains with precious medical relevance for human benefits. Hence the aim of the present study is to isolate probiotics from locally available various dairy products (Viz. raw milk of cow and buffalo and traditional homemade curds) and human breast milk and to identify them upto species level. A total of about 80 samples from human breast milk (n=30) and dairy products (n=50) were collected aseptically and screened for the probiotic microflora using standard microbiological methods. The colonies suspected for probiotics were picked up for morphological and biochemical identification which were further confirmed by molecular methods and application of bioinformatics. The preliminary results showed several different microflora viz. *Streptococcus, Staphylococcus, Lactobacillus, Diplococcus,* etc. About twelve (12) isolates were further confirmed to be of different species belonging to the Genus *Lactobacillus*. Therefore dairy products and human breast milk are considered as suitable natural sources for probiotic microorganisms especially *Lactobacillus spp.,* which are reported to show beneficial effects in humans.

Keywords: Probiotics, Indigenous sources, Dairy products, Human breast milk, *Lactobacillus spp*.

1. INTRODUCTION

Probiotics are "live microorganisms which when taken in adequate amount confer the health benefit on the host" [1]. Probiotics are found to be from decades and will be in use as long as they are consumed in the form of food supplements. Probiotics have been shown to be effective in various clinical conditions such as infantile diarrhoea, necrotizing enterocolitis, antibiotic associated diarrhoea, relapsing clostridium difficile colitis, Helicobacter pylori infections, inflammatory bowel disease etc. They have the ability to modulate host immune system, to strengthen the intestinal barriers, prevention of colon cancer and female urogenital and surgical infections [2], to manage lactose intolerance, reduction of cholesterol and blood pressure, reducing the inflammatory actions of body, beneficial effects on mineral metabolism especially bone stability and to prevent osteoporosis, suppression and control of pathogenic microorganisms growth are some of the beneficial health related effects of probiotics [3]. The most commonly used probiotics are Lacticacid bacteria (LAB) such as *Lactobacilli* and *Bifidobacteria* which have a major role in the longevityof human health.

LAB are generally associated with habitats rich in nutrients such as milk, cheese, meat, beverages and vegetables. However Lactic acid bacteria (LAB) are found to be naturally present in milk and milk products. Even though the useful strains for probiotic applications can be isolated from many different sources, the ideal sources are found to be human breast milk, raw milk of cow and buffalo and homemade curds, as these are considered as suitable and natural sources for probiotics.

Breast milk is considered as a vital source of nutrients for neonates with lot of health benefits [4]. Some of the potent probiotic bacteria isolated from human milk are *Lactobacillus fermentum, Lactobacillus rhamnosus, Lactobacillus gasseri* and *Enterococcus feacium*, etc. Breast milk containing LAB protects the infant and the mother

against pathogenic microbes that causes diseases and thus can be upgraded as a natural food of probiotic microflora for neonates and infants [5, 6].

The dairy relevant microorganisms originating from raw milk of animals such as cow and buffalo along with fermented foods such as curds are considered to be good sources of LAB with potential desirable properties for use in the production of novel probiotic products [7-11]. The most frequently isolated LAB from dairy products are *Lactobacillus, Enteroccoccus, Lactococcus, Leuconostoc, and Streptococcus* [12, 13].

Therefore the isolation and identification of Lactic acid bacteria (LAB) especially *Lactobacilli* from a complex microflora of these sources shows a promising role. The characterization of LAB is based on simple microbiological and biochemical methods which includes, morphology; cellular (shape, Gram staining) and colonial (color, form) characteristics, and carbohydrate fermentation profiles among different LAB isolates [14]. The DNA-based molecular methods are found to show significant increase in accuracy, quality and efficiency in the identification of Lactic acid bacteria [15]. Hence all these methods helps in the identification of different probiotics upto species level with high resolution.

Further literature studies suggests that probiotics from these sources are most potential candidates because they are found to adapt well to the environmental conditions prevailing in human gut and therefore are more competitive than probiotics from other sources. Hence aim of this study was to isolate probiotic bacteria from locally available sources and to identify them up to the species level.

2. MATERIAL AND METHODS

2.1. Sample Collection

A total of about 80 (eighty) samples from various sources viz. raw milk of Cow and Buffalo and human breast milk were collected aseptically along with traditional homemade curds and were screened for the presence of probiotics.

2.1.1. Collection of Dairy samples

About 50 samples [15 each from cow and buffalo milk (from milk vendors) and homemade curds (20)] were randomly collected in sterile bottles from different locations of Hyderabad city. Before collecting the samples, the milk vendors were asked to maintain aseptic conditions to avoid cross contamination of skin microflora from udders of animals.

Though, there are other dairy products like cheese, yoghurts or any fermented milks, the aim of the study is to isolate probiotics from locally available natural sources. Hence commercial products available in the market were not included for isolation of probiotics.

2.1.2. Collection of Human milk samples

2.1.2.1. Selection of subjects

About 30 lactating mothers were recruited from the post-natal ward of maternity hospital (Niloufer Mother and Child Hospital), located at Hyderabad and all of them voluntarily participated in the study. The Ethical Committee approval was obtained from the host institute, ICMR-National Institute of Nutrition (No. 06/11/2016) and also from maternity Hospital, Hyderabad (No: 21/01/2017). A signed informed consent was also obtained from all the participant subjects, before commencement of the study.

The demographic details and clinical data of each participant subject was recorded onto a specially designed study proforma. General characteristics of the lactating mothers recruited for the study with mean age (n=30) is 24.6 ± 3.88, height (cm), weight (Kgs) and BMI (weight in kg/Ht in m^2) are 154.26 ± 5.71, 56.46 ± 8.12, and 23.73 ± 3.66 respectively. *Inclusion criteria:* Healthy lactating women with full term pregnancy and absence of maternal or any pre or postnatal problems were randomly selected for sample collection. *Exclusion criteria:* Women who had received any antibiotics treatment during pregnancy or having any infectious diseases like HIV, Hepatitis etc. were excluded for this study.

2.1.2.2. Human milk collection

About 30 samples of human milk were aseptically collected from the recruited lactating mothers within first week of delivery. Before the collection of breast milk samples, the mammary areola was thoroughly cleansed with chlorohexidine to avoid skin microflora cross contamination [16]. During collection, first few drops of milk were discarded and further about 2-3 ml of samples were collected in sterile tubes by manual expression. All the collected samples (dairy and human milk) were transported in an ice box to the laboratory and stored at -20°C until further processing.

2.2. Isolation and Identification of Probiotic microorganisms

The collected samples were subjected for screening of microflora particularly probiotics using standard microbiological methods.

2.2.1. Isolation of Probiotic bacteria

About 1 ml/ 1 gm of sample (Human breast milk/cow or buffalo milk/curd) was serially diluted in 9 ml of sterile phosphate buffered saline (PBS) and made up to 10^{-1} to 10^{-5} dilutions. About 100µl of the diluted sample was plated on MRS agar plates (HiMedia- India) from dilutions 10^{-2} to 10^{-5}. The inoculated plates were further incubated anaerobically at 37°C for 24-48 hrs. After incubation, the culture plates were observed for various microbial colonies and number of colonies produced on each plate of different dilutions was recovered [17].

2.2.2. Identification of Probiotic bacteria

The isolated colonies suspected for probiotics were picked up and identified using standard morphological and biochemical methods [18] and confirmed by molecular methods and through application of bioinformatics.

2.2.2.1. Morphological identification

Colony morphology

The pure isolates were subjected to identification using macroscopic appearance for their morphological characteristics such as size, shape, margin, color and texture of the colonies were examined.

Gram staining

The various bacterial colonies were subjected to gram staining (kit supplied by Hi-Media) and observed under light microscope with 100X magnification.

2.2.2.2. Biochemical identification

The bacterial colonies positive in morphological identification were further subjected to various biochemical tests like motility, KOH, catalase and oxidase tests along with IMVIC tests (Indole, Methyl red, Voges Prausker and Citrate utilization), arginine hydrolysis, nitrate reduction tests and carbohydrate fermentation tests [19].

KOH test

Bacterial colony was treated with KOH to determine whether it is Gram positive or Gram negative. Cells were placed on microscopic slides, 3% KOH was added and the suspension mixed for 30 seconds with a platinum loop. For Gram negative bacteria the solution becomes viscous and mucoid whereas Gram positive bacteria show no reaction.

Catalase test

To determine catalase activity, an isolated colony was picked up and placed on a glass slide. After they had been in contact with atmospheric oxygen for at least 30 minutes, a few drops of 3% H_2O_2 (hydrogen peroxide) was added. For catalase-positive, immediate gas bubbles (O_2) production will be observed and catalase-negative strains show no gas production.

Oxidase test

A well-isolated colony with the help of an inoculation loop was picked up from a fresh bacterial culture plate. Then the colony was spread over the oxidase disc (Himedia laboratories) in a sterile environment and observed for color change. The dark purple colour appearance within 5-7 seconds, indicates positive reaction.

Indole test

The tryptone broth was inoculated with 100 µl of overnight grown bacterial culture and incubated at 37°C for 24-48 hrs. Then about 5 drops of Kovac's reagent was directly added to the tube. The formation of pink colour ring on top of the broth medium is considered to be positive.

MR-VP (Methyl Red- VogesProskauer) test

To 10 ml of MR-VP broth medium, 100µl of overnight grown bacterial culture was inoculated and incubated at 37°C for 24 hrs. After incubation the broth medium was separated into two test tubes. To one of the tube methyl red indicator (5 drops) was added and to another tube Barrit's reagent A (2 drops) and Barrit's reagent B (3 drops) were added. The change of broth medium to red after adding methyl red and to pink colour when added with Barrit's reagents, for Methyl Red and Voges Proskauer tests respectively are considered to be positive.

Citrate Utilization Test

Simmons citrate agar slants were prepared and allowed to come to the room temperature before inoculation. From a fresh overnight grown culture a single well-isolated colony was picked up with the help of an inoculation needle and streaked on the surface of agar slant and kept for incubation at 37°C for 24 hrs. After incubation, if growth was seen on the agar slants with change in color of medium to intense blue, indicated positive result for the test performed.

Arginine Hydrolysis test

Sterile arginine hydrolysis broth tubes were inoculated with pure fresh culture (1%) and incubated for 48 h at 37°C. Further, after incubation to each of the test

tubes 4-5 drops of Nessler's reagent was added and observed for color change. The change in color from yellow to orange indicated a positive result for arginine hydrolysis.

Nitrate Reduction Test

The trypticase nitrate broth was inoculated with bacterial culture (1%) and incubated for 24 h at 37°C. After incubation, 500ul of α-naphthylamine (0.5% in 5N Acetic acid) and sulphanilic acid (0.8% in 5N Acetic acid) were added to the broth tubes and observed for development of color within a minute. The appearance of pink or red color indicates positive reaction and the results for all the isolates were recorded accordingly.

Gas production from Glucose

To 10 ml of sterile glucose broth containing inverted Durham's tube, bacterial culture (1%) was inoculated and incubated at 37°C for 24-48 h. The appearance of gas in Durham's tube in the form of a hollow space indicated positive result.

Carbohydrate (Sugar) fermentation test

This test was done to identify/classify the bacteria to which the probiotics belong to, based on the utilization of carbohydrates by using Phenol red broth medium [20]. The sterile test tubes containing 5 ml of phenol red broth was added with individual carbohydrate discs (Himedia Laboratories). Further, 100µl of overnight grown bacterial culture was inoculated into the broth medium and incubated for 24 hrs at 37°C. Acid production by the bacteria by utilizing carbohydrates was evaluated at 24 hrs and 48 hrs of incubation.

2.2.2.3. Molecular characterization

Extraction of Genomic DNA

The extraction of genomic DNA from the bacterial culture was performed based on the modified protocol of Wright et al., 2017 [21]. Overnight grown bacterial culture (about 2ml) was harvested by centrifugation and dissolved the pellet with freshly prepared lysozyme (500µl) which was further incubated at 37°C for 45 mins. In the next step about 100 µl of 10 % SDS and after 30 sec 200 µl of 5M NaCl were added and the total mixture was incubated for about 1 hr. After incubation, from the bacterial cell lysate DNA was extracted by adding 800µl of Phenol-chloroform. Then the DNA extracted was precipitated with 70 % ethanol and further dissolved and stored in 50 µl of Tris- EDTA(TE) buffer and preserved at -20°C until further analysis. The concentration and purity of the extracted DNA was confirmed by agarose gel electrophoresis and also spectrophotometrically by measuring the absorbance at 260/280 nm using the nanodrop.

Genus identification

The genus level identification was carried out using PCR with genus specific primers. A 20µl reaction mixture consisted of 10 µl of 2 X Master mix (Norgen), 1 µl of each Forward and Reverse primers with concentration of 10 µM, 1 µl of DNA template (25ng) and 7µl of nuclease free water. The PCR amplification program consisted of a cycle of initial denaturation at 94°C for 5 min, followed by denaturation for 30 sec at 94°C, annealing for 1 min at 52°C, extension for 1min (each for 30 cycles) at 72°C and final extension of one cycle at 72°C for 8 min and at the end samples were cooled down at 4°C. Further, the amplified PCR products of DNA were electrophoresed at 100 V on agarose (1.5%) gel stained with ethidium bromide (10mg/ml) for 45 min and visualized under UV light in gel doc.

Primers used for PCR amplification

The primer sequences required for PCR amplification was adopted from Rekha R et al., 2006 [22].

Primers	Sequences	Product length
Lactobacillus-F	TGCCTAATACATGC AAGTCGA	318 bp
Lactobacillus-R	GTTTGGGCCGTGTC TCAGT	

Species identification

Sequencing: After identification of genus the amplified DNA products were further proceeded to Sanger sequencing for 16s rRNA gene sequence [Eurofins genomics (I) Pvt. Ltd.].

Preparation of consensus sequence: The sequences (Forward and Reverse) generated using Sanger sequencing were further assembled to create a single consensus sequence with the help of a bioinformatics software (DNA star Laser-gene).

Bioinformatics analysis: The consensus sequence obtained was used for species identification by BLAST (Basic local alignment search tool) in NCBI- GENBANK (genomic database). Further the alignment of multiple sequences by Clustal W was performed. Subsequently, the

neighbour joining phylogenetic trees by using MEGA (Molecular Evolutionary Genetic Analysis)-X software has been drawn for all the isolates [23-25].

2.3. Long-term preservation of the isolates

The isolated and confirmed bacterial species were preserved as glycerol stocks (prepared by mixing 500µl of sterile 80% glycerol with 500µl of pure culture) and stored at -80°C for future use.

3. RESULTS

The isolation of bacteria from the dairy and human breast milk samples showed diversified microflora viz. *Streptococcus, Lactobacillus, Staphylococcus, Diplococcus*, etc.

3.1. Identification by morphological methods

Morphologically the single colonies which were circular, transparent, white-creamish, pin point with entire margin and that showed Gram positive, rod shaped bacilli were picked up for further identification [Fig.1(a&b) and Table- 1].

3.2. Identification by biochemical methods

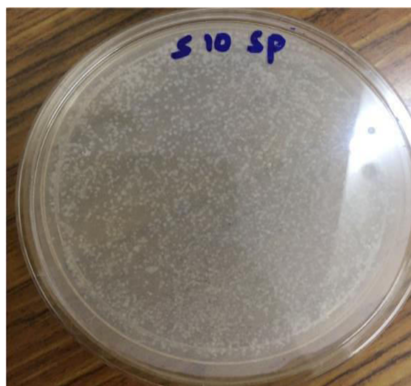
All the isolated strains were found to be catalase-negative (no bubbles formation), KOH- positive (no formation of viscous solution), oxidase-negative (no color change to dark purple), Nitrate reduction- negative (no appearance of pink or red color ring), Arginine hydrolysis-negative (no color change from yellow to orange), gas production from glucose- negative (no gas detected in inserted Durham tubes) (Table-2).

3.2.1. IMVIC tests

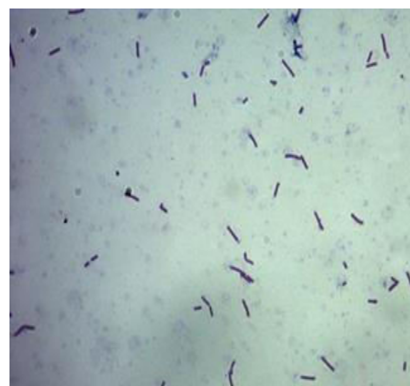
There was no indole ring formation after the addition of Kovac's reagent, when added with methyl red indicator MR-VP medium did not turn into red colour, no change in colour after addition of Barrit's reagents A and B for Voges-Proskauer test, and for citrate utilization test no colour change was observed. Hence almost all the isolates showed negative reactions to the IMVIC tests performed (Table-2).

3.2.2. Carbohydrate (Sugar) fermentation test

The change in color from red (alkaline) to orange/ yellow (acidic) was observed upon 24 hrs and 48 hrs of incubation which indicated positive reaction towards some of the sugars, Viz. Glucose, Sucrose, Fructose, etc.(Fig. 2) and hence identified to be of *Lactobacillus spp*. The utilization of different sugars by the isolated strains are mentioned in Table (3).



a) Culture plate showing bacterial colonies



b) Gram's staining showing bacilli

Fig. 1: Morphological Identification

Fig. 2: Showing carbohydrate utilization by the isolated strain no.6; 1-19 tubes: different sugars depicted in Table (3) and 20th tube: control.

Table 1: Morphological identification of isolates

Isolate No.	Motility	Gram's reaction	Cell shape	Pigment	Colony shape	Surface	Elevation	Spores	Colony Size
1	Non-motile	+	Bacilli	Creamish White	Round	Mucoid	Flat	-	0.1-0.5mm
2	Non-motile	+	Bacilli	white	Round	Glistening	Opaque	-	0.1-0.5mm
3	Non-motile	+	Diplobacilli	Cream	Round	Mucoid	Flat	-	0.1-0.5mm
4	Non-motile	+	Bacilli	white	Round	Glistening	Flat	-	0.1-0.5mm
5	Non-motile	+	Bacilli	white	Round	Glistening	Flat	-	0.1-0.5mm
6	Non-motile	+	Bacilli	white	Round	Mucoid	Opaque	-	0.1-0.5mm
7	Non-motile	+	Bacilli	white	Round	Mucoid	Flat	-	0.5-1mm
8	Non-motile	+	Bacilli	white	Round	Mucoid	Opaque	-	0.1-0.5mm
9	Non-motile	+	Diplobacilli	cream	Round	Mucoid	Flat	-	0.1-0.5mm
10	Non-motile	+	Bacilli	white	Round	Mucoid	Flat	-	0.5-1mm
11	Non-motile	+	Bacilli	white	Round	Glistening	Flat	-	0.1-0.5mm
12	Non-motile	+	Strepto-bacilli	white	Round	Glistening	Flat	-	0.1-0.5mm

Table 2: Biochemical identification of isolates

Isolate No.	Catalase	KOH	Oxidase	Gas from glucose	Nitrate reduction	Arginine hydrolysis	Indole	Methyl Red	Voges Prausker	Citrate utilization
1	-	+	-	-	-	-	-	-	-	-
2	-	+	-	-	-	-	-	+	-	-
3	-	+	-	-	-	-	-	-	-	-
4	-	+	-	-	-	-	-	-	-	-
5	-	+	-	-	-	-	-	-	-	-
6	-	+	-	-	-	-	-	-	-	-
7	-	+	-	-	-	-	-	+	-	-
8	-	+	-	-	-	-	-	-	-	-
9	-	+	-	-	-	-	-	-	-	-
10	-	+	-	-	-	-	-	+	-	-
11	-	+	-	-	-	-	-	-	-	-
12	-	+	-	+	-	+	-	+	-	-

Table 3: Carbohydrate utilization test for the isolates

S. No.	Name of the carbohydrate	1	2	3	4	5	6	7	8	9	10	11	12
1.	Glucose	++	++	++	++	++	++	++	++	++	++	++	++
2.	Lactose	++	++	++	++	++	++	++	++	++	++	++	++
3.	Fructose	++	++	++	++	++	++	++	++	++	++	++	++
4.	Galactose	+	++	++	++	++	-	+	-	++	+	++	++
5.	Dextrose	++	++	++	++	++	++	++	++	++	++	++	++
6.	Arabinose	-	+	+	+	+	-	-	-	+	-	+	+
7.	Maltose	-	++	++	++	++	-	-	-	++	-	++	++
8.	Mannose	++	++	++	++	++	-	++	-	++	++	++	++
9.	Mellibiose	-	++	++	++	++	-	-	-	++	-	++	++
10.	Sorbitol	-	++	++	++	++	++	-	++	++	-	++	-
11.	Sucrose	+	++	++	++	++	++	+	++	++	+	++	++
12.	Xylose	-	-	-	-	-	-	-	-	-	-	-	-
13.	Inositol	-	-	-	-	-	-	-	-	-	-	-	-
14.	Rhamnose	-	++	++	++	++	++	-	++	++	-	++	-
15.	Mannitol	-	++	++	++	++	++	-	++	++	-	++	+
16.	Salicin	-	++	++	++	++	++	-	++	++	-	++	-
17.	Cellobiose	-	++	++	++	++	-	-	-	++	-	++	-
18.	Trehalose	-	++	++	++	++	++	-	++	++	-	++	-
19.	Raffinose	-	++	++	++	++	+	-	+	++	-	++	++

(- No colour change, + Orange colour, ++ yellow colour)

Note: The change from red color to orange colour of phenol red broth was considered as weak fermentation (+), and from red colour to yellow colour as strong fermentation (++) and no change in colour of broth, indicated no fermentation (-) of that particular sugar.

3.3. Identification by molecular methods

3.3.1. Genomic DNA

The average concentration of DNA from the isolates was 649.12ng/μl. The mean value for absorbance (A260/280) was found between 1.8 and 2.0, which indicates that the purified genomic DNA of isolates was of good quality (Fig. 3).

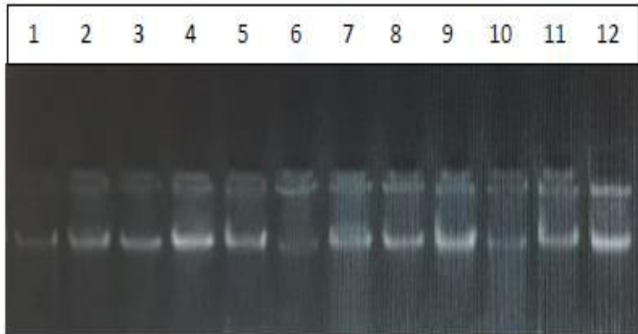


Fig. 3: Showing DNA of the isolates

3.3.2. Genus identification

About twelve (12) strains, isolated from various samples of dairy products and human milk, were identified to be of *Lactobacillus* genus by observing amplified PCR products of DNA at 318 bp (Fig. 4).

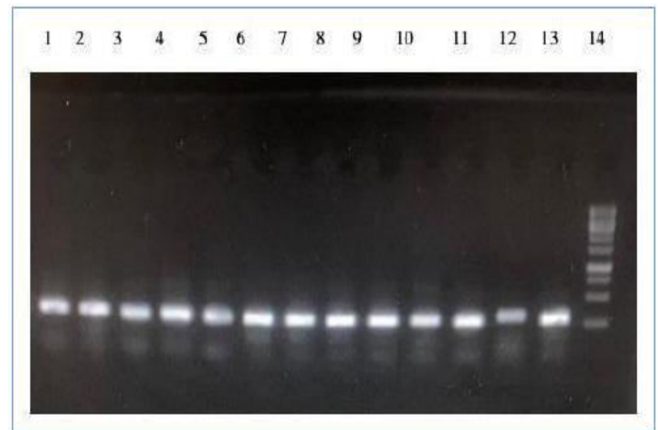


Fig. 4: Genus Specific PCR. Lane 1-12: PCR products of the isolates at 318 bp, Lane 13: Positive control, Lane 14: 1kb DNA Ladder.

3.3.3. Species identification

After performing BLAST of the consensus sequence in NCBI-GENEBANK, the top hit similarity strains across the species were considered as the best match. All the isolated strains were identified to be of different species belonging to the genus *Lactobacillus* (Table 4).

Further the cladogram or the neighbour joining phylogenetic trees were drawn using MEGA-X software for all the isolates and one of them is shown in Fig. (5).

The neighbour joining Phylogenetic tree of *Lactobacillus* isolate no.1

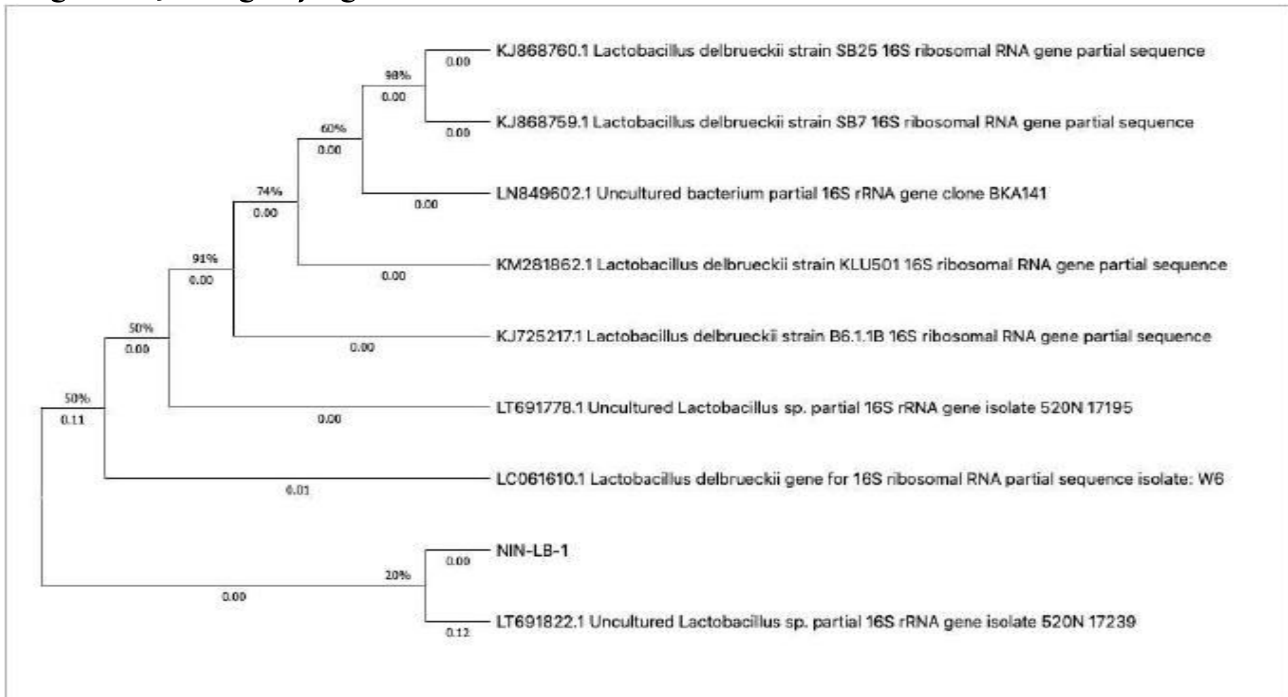


Fig. 5: The evolutionary history using the Neighbour-Joining method was inferred and the optimal tree with the sum of branch length is 0.24430533.

Table 4: Species level identification of isolates after blast in NCBI-GENBANK

S. No.	Sample Name	Source	Bacteria	Similarity
1	NIN-LB-1	Homemade curd	*Lactobacillus delbrueckii subsp.bulgaricus* CP049052.1	94.36%
2	NIN-LB-2	Homemade curd	*Lactobacillus plantarum* MH665778.1	96.87%
3	NIN-LB-3	Homemade curd	*Lactobacillus helveticus* KM506757.1	94.89%
4	NIN-LB-4	Homemade curd	*Lactobacillus acidophilus* MG654777.1	96.55%
5	NIN-LB-5	Homemade curd	*Lactobacillus acidophilus* HE793099.1	98.97%
6	NIN-LB-6	Cow milk	*Lactobacillus spp.* JF811579.1	95.11%
7	NIN-LB-7	Buffalo milk	*Lactobacillus delbrueckii* MW479188.1	86.11%
8	NIN-LB-8	Human milk	*Lactobacillus spp.* MG966424.1	79.20%
9	NIN-LB-9	Human milk	*Lactobacillus helveticus* MT781347.2	98.97%
10	NIN-LB-10	Human milk	*Lactobacillus delbrueckii* MN104797.1	82.56%
11	NIN-LB-11	Human milk	*Lactobacillus acidophilus* MG654777.1	95.61%
12	NIN-LB-12	Human milk	*Lactobacillus fermentum* HQ720139.1	99.32%

4. DISCUSSION

Probiotics are the beneficial microbes, when taken in adequate quantities provide beneficial effects on the health of the host [1]. Most of the probiotics include Lactic acid bacteria which are considered generally as safe microorganisms. Therefore the isolation of probiotics from indigenous natural sources viz. dairy products and humanbreast milk may develop a new way to improved probiotic strains with precious medical relevance for benefits in humans.

Breast milk is a natural source for nutrition, immune protection and developmental programming in infants. It is considered to be the continuous and excellent source of potential probiotic microorganisms like *Lactobacilli, Bifidobacterium, Lactococci, Streptococci, Enterococci, Pediococcus, Leuconostoc* species to the infant's gut [5]. Several reports suggested that human milk plays a major role in the development of gut microbiome in the new borns [26, 27]. Breast milk majorly contains probiotics especially Lactic Acid Bacteria viz. *Lactobacilli spp.* and *Bifidobacteria* which have tremendous bio therapeutic applications in humans.

Probiotics from human breast milk are known to protect against various infections in breast fed infants [28, 29]. Many studies have reported the beneficial role of breast milk bacteria against several pathogens, through the mechanismby competition for adhesion or production of various antimicrobial compounds such as bacteriocins, etc. [30, 31]. Therefore considering the efficiency of probiotics to colonize in human intestine, probiotics from human milk is of great importance in the establishment of gut microflora in infants and further these can also be used in the development of many probiotic based food products [31].

Milk of cow/buffalo and their products are most usually associated with probiotics, which provides supplements for the beneficial maintenance of the gut [32, 33]. Fermented foods especially curds (homemade) contain several species of *Lactobacillus* like *L. fermentum, L. acidophilus, L. casei,* etc. which converts lactose into lactic acid and these strains are found to show a wide variety of beneficial effects, for safe use in humans.

In various dairy products such as milk and curds, *Lactobacillus spp*. are found to be naturally grown and has status of GRAS microorganisms. Scientists [34, 35] have isolated some Lactic acid bacteria from milk and curds, and identified them as *Lactobacillus spp.* based on morphological and biochemical characterization. Tambekar and Bhutada, 2010[32] isolated some species of *Lactobacilli* viz., *L. plantarum, L.acidophilus, L. bulgaricus,L. fermentum and L. lactis* from cow and buffalo milk samples. Similarly Rasha et al., 2012; and Srinu et al., 2013 [36, 37] have isolated some strains of *Lactobacillus spp.* such as *L. acidophilus, L. lactis,* etc. from various dairy products and reported them as potential probiotics.

In the present study the isolation and screening of probiotics from locally available sources viz. human breast milk, raw milk of cow/buffalo and homemade curds was carried out. This was found similar to that of Samuel et al., 2016 [38] study where probiotics from different sources such as raw milk, curd and dosa batter etc. were isolated and identified, based on their morphological and biochemical characteristics. The results of morphological and biochemical tests in the

study were similar when compared to other studies, reported on *Lactobacillus* species [5, 17, 20, 39].

The probiotic bacteria are generally found capable to ferment different sugars which depends on their enzyme complement. The bacterial species and the type of carbohydrate are considered important during fermentation of food products, as they contributes in formation of flavor, aroma and to preserve the final products. *Lactobacillus* species are well known food fermenters,and thus ferments different monosaccharides and disaccharides [40]. In this study all the strains, fermented sugars such as sucrose, lactose and glucose which suggest that the isolates were capable to grow in different habitats by consuming a range of carbohydrates. Therefore based on the sugar utilization patterns, the isolates in the study were identified as belonging to different species of *Lactobacillus.*

The isolates confirmed for genus *Lactobacillus* in this study, were further proceeded for identification of species by 16s rRNA sequencing through BLAST search in NCBI- GENBANK. In a study Dickson (2005) [41] used novel species specific PCR assay, to identify different *Lactobacillus* species. According to FAO/WHO guidelines, identifying and analyzing probiotic strains through 16S rRNA sequencing can be considered as the most suitable technique when compared to other molecular methods [42]. Mancini et al., 2012 [43] reported in a study that, this technique was found to be an effective method in analyzing the *Lactobacillus* species, which were isolated from various fermented dairy products and milk. Therefore in the present study after performing bioinformatics analysis, all the identified species were found similar to many studies reported on dairy products and human milk microflora [35, 36, 44-46].

5. CONCLUSION

Current study revealed that among different micro flora screened from various sources and samples, about 12 isolates [Human Breast Milk (5), cow and buffalo milk (2) and Homemade curds(5)] were identified and confirmed as *Lactobacillus spp.* based on their morphological, biochemical and molecular methods. Thus the dairy products and human breast milk are considered as potential sources for probiotic microflora especially *Lactobacillus spp*. Since these isolates are original and indigenous they can easily adapt and colonize in the human intestine. However the isolated *Lactobacillus* strains from these sources are further need to be studied for their probiotic potential, which can be further used in the development of many probiotic based food supplements for their health benefits in humans.

6. ACKNOWLEDGEMENTS

The authors of the study acknowledge Indian Council of Medical Research (ICMR), New Delhi for providing Fellowship (JRF/SRF) to Ms. G. Sumalata for pursuing her Ph.D. Further we wish to thank The Director, National Institute of Nutrition (ICMR-NIN) for her continuous support. The authors also wish to thank the significant contribution of all the human participants, milk vendors, households for providing samples and co-operation for smooth completion of the research work.

Conflicts of Interest

The authors declare no competing interests.

Source of Funding

No funding or grants received for this research

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