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Research Article

ASSESSMENT OF SAFETY CRITERIA, PROBIOTIC POTENTIAL AND OTHER HEALTH ATTRIBUTES OF LACTIC ACID BACTERIA ISOLATED FROM KANJI

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ABSTRACT. Most health benefits of fermented foods and beverage can be traced to Lactic Acid Bacteria (LAB). In this study, the probiotic potential of LAB isolated from fermented carrot and beet root beverage, known as Kanji, was evaluated. The changes in physicochemical and microbial parameters were recorded during the course of fermentation (7 days). The prepared beverage showed pH 3.8, titrable acidity 2.78% and 4509-6752 ppm total dissolved solids. From the 130 isolates obtained on De Man Rogosa Sharpe agar, only 4 isolates were identified as potential probiotic strains. Three of these strains were identified as *Lactobacillus brevis* and one as *Leuconostoc mesenteroides*. All these strains showed absence of virulence factors and exhibited no antagonistic activity against *E. coli* (normal flora of intestine). Though multi-drug resistance was observed in all 4 strains, it may be intrinsic as detected commonly in LAB. They showed tolerance to 11% NaCl, 1% bile, pH up to 8 (for 3 h), temperature between 25°C to 40 °C and 0.6% phenol. Significant auto aggregation (64%-71%) and hydrophobicity (47%-76%) were also observed by these strains. Only one of the four LAB (MAP 29) produced exopolysaccharide and lipase enzyme. In addition, they also showed antimicrobial activity against enteropathogens and β-lactamase producing uropathogens with zones of inhibition ranging from 15 to 25 mm. They also possessed additional health attributes like cholesterol and oxalate degradation, antioxidation potential and the ability to utilize prebiotic inulin. Based on all these observations, the extraordinary potential of all LAB strains to be used as a probiotic is evident from this study.

Keywords: *Lactobacillus brevis, Leuconostoc mesenteroides, exopolysaccharide, auto aggregation, hydrophobicity, antagonistic activity, virulence.*

INTRODUCTION

The social and economic development of the world is accompanied by stress and exhaustion in humans. These conditions have gradually increased the reported incidences of heart attack, hypertension, cancer and many other chronic disorders. To a large extent, this scenario has become worse due to our dependence on comfort meals (like ready to cook recipes) and junk food. Several studies have also demonstrated how urbanization has challenged our health conditions leading to decreased immunity and increased intestinal disorders [1, 2]. Amidst the health issues arising from unhealthy food habits, probiotics are a hopeful panacea for the compromised immune system [3]. Probiotics are living microbial supplements that can be ingested to improve intestinal microbial balance

[4]. The intestinal flora is the key to optimum metabolism in the entire animal kingdom. Hence, intestinal health plays a major role in boosting immunity and managing metabolic disorders.

Although the intestinal flora can balance itself with the help of our routine diet and surrounding environment, it is often compromised by following an extreme urban lifestyle. To help maintain this balance, several dairy and non-dairy probiotics are available in the market in the form of tablet/ capsule and drinks, or simply as natural food products like curd, kefir (cultured milk) and sauerkraut. Although dairy probiotics are classically desirable, over 65% world population is lactose intolerant [5]. Many other individuals have reduced absorption of lactose, are intolerant to fat and cholesterol, or allergic to milk proteins. Hence, the products from plant origin serving as valuable alternative, is much suitable and desirable option. Among the non-dairy probiotics, the fruit and vegetable based products are gaining importance because of their high nutritive value and immune boosting ability [6]. In addition, probiotics are beneficial in treating intestinal infections, lactose intolerance, diarrhea, vaginal infection and constipation [7]. The lactic acid bacteria (LAB), in probiotics, improve the bioavailability of minerals, amino acids and trace elements, and enhance protein solubility up to 50%. They also reduce serum cholesterol levels and repress the activation of pro-carcinogenic compounds [8].

Kanji is one such fermented probiotic drink of North India with high nutritive value. Unfortunately, it is much less popular than other probiotic food and drinks in India. It is prepared by fermenting carrots and beetroots together along with crushed mustard seed, hot chilli powder and salt. Similarly, probiotic drinks are also prepared by fermenting carrot or beetroot individually. Many researchers have studied the effect of various strains on bioavailability of various nutrients including minerals (calcium, phosphorus, iron), vitamins (folate, cobalamin, betacarotene, betaine and vitamin C) and sugar levels during fermentation of carrot and beetroot juice. They reported that the extent of bioavailability of above nutrients was strain specific and the type of acid produced on metabolism [9, 10, 11]. The use of Kanji is also reported in Ayurveda, over 3000 year old Indian medicinal system [12].

At present, limited studies have been reported on the biochemistry of Kanji and LAB associated with its fermentation [13, 14]. Hence, the current study was undertaken to isolate potential probiotic strains from Kanji, based on their survival at low pH, cell surface characteristics (hydrophobicity and aggregation) and other probiotic criteria (cholesterol assimilation, oxalate degradation). In addition, their safety on application and antimicrobial activity against pathogens were investigated to provide an overview of the probiotic potential of the isolated strains.

MATERIALS AND METHODS

Fermentation of Kanji

To prepare the fermented probiotic drink, carrots (*Daucus carota* sub sp. *sativus*) and beetroots (*Chenopodiaceae)* were procured from local vegetable market (Virar, Mumbai, India). They were sorted manually to select the healthy vegetables, washed with water and disinfected with 0.01% potassium metabisulphite solution. The skin of vegetables was peeled and they were cut in equal sized (~ 3x 1 cm) pieces. The ingredients (and their proportion) used in the preparation of Kanji were carrots (23%) and beetroots (10%), common salt (2%), coarsely ground mustard seeds (1.5%) and red chilli powder (0.015%). These ingredients were mixed in potable water (500 mL), boiled for few minutes and used for the preparation of fermented beverage. During fermentation, the pot was covered with foil paper and kept at room temperature (20±5 °C) for 7 days. Three such batches were set up for the isolation of lactic acid bacterial strains.

Analysis of Physicochemical Characteristics of Kanji

The physicochemical parameters were analyzed at regular intervals (alternate days) during the fermentation. The organoleptic evaluation of naturally fermented kanji was done based on appearance, taste, color, aroma and flavor. The pH and Total Dissolved Solids (TDS) were estimated using a digital pH meter and TDS meter (HiMedia) respectively. The titrable acidity, expressed in terms of lactic acid, was estimated using a standard protocol [15].

Analysis of Microbiological Characteristics of Kanji

The enumeration of microbial flora was done on De Man Rogosa Sharpe (MRS) agar (HiMedia) plates containing 3% NaCl. To distinguish acid producing bacteria from other bacteria, 1% of $CaCO_3$ was added to the MRS agar plates and incubated under anaerobic conditions at 37 °C for 48h. The isolates were further tested for their homo/hetero fermentative nature and their biochemical characteristics were studied [16]. Other suspected bacteria were isolated on Nutrient Agar (NA), Yeast Extract Peptone Dextrose Agar (YEPDA), Acetobacter agar and MacConkey’s agar.

Evaluation of Probiotic Potential of Isolated Lactic Acid bacteria

The suspension of bacterial strains isolated on MRS plates was adjusted to 0.5 McFarland cell density (~$1.5x10^8$ CFU/mL). Rapid screening of acid and bile tolerance was done by observing growth of test strains in 10 mL of MRS broth adjusted to pH 3 and containing 0.3% (w/v) bile salt, after incubation at 37°C for 24h. The medium with good turbidity was selected and LAB were enumerated on MRS agar plates. The strains exhibiting over 90% of growth, under above conditions, were selected for further investigation of their probiotic potential [17]. To determine the sensitivity of selected strains, their growth efficiency were further evaluated at different pH (1 to 9), temperature (25 °C, 30 °C, 37 °C, 40 °C) and bile salt concentration (0.3, 0.5, 0.8, 1.0 and 2.0%). The growth was assessed by measuring optical density at 600 nm [18, 19]. Since phenols can be formed in the intestines by bacteria that deaminate few aromatic amino acids, its tolerance is a preferred characteristic among probiotic strains. In the present study, the tolerance of test strains to 0.4 to 0.6% phenol was tested. Similarly, they were evaluated for their tolerance to high salt concentration (3 to12%) to prevent contamination of fermented product. The ability of selected strains to metabolize lactose to lactic acid was determined using titrimetric method suggested by Jung et al. [15].

Identification of Potential Probiotic Strains

Based on above characteristics, the most promising probiotic strains were identified by 16s rRNA sequencing which was done at Sai Biosystem Private limited, Raghuji Nagar, Nagpur.

Evaluation of Standard Safety Criteria for LAB and Determination of their Antibacterial Activity

The potential probiotic strains were evaluated for their susceptibility to common antibiotics [20]. Octadiscs containing the following antibiotics were used to study the antibiotic resistance pattern of probiotic strains:

Ticarcillin/Clavunalic acid (85 μg), Oxytetracycline (30 μg), Ceftriaxone (30 μg), Cefuroxime (30 μg), Nalidixic acid (30 μg), Amoxicillin (30 μg), Cefadroxil (30 μg), Norfloxacin (10 μg), Cefoperazone (75 μg), Ceftazidime (30 μg), Polymyxin B (300μL), Ampicillin/Sulbactum (20 μg), Cefotaxime (30 μg), Piperacillin (100 μg), Chloramphenicol (30 μg), Ciprofloxacin (5 μg), Ceftizoxime (30 μg), Tetracycline (30 μg), Ofloxacin (5 μg), Gentamycin (10 μg), Amikacin (30 μg), Gatifloxacin (10 μg), Cefepime (30 μg) and Cotrimoxazole (25 μg). *E. coli* ATCC 25922 (obtained from Hinduja Hospital, Mumbai) was used as a standard quality control strain.

The virulence factors like gelatinase activity and siderophore production were also studied on NA plates containing 1% gelatin and 2mM Chrome Azurol S (CAS) dye solution respectively. The hemolytic activity was detected by spot inoculating the test strains on blood agar plates. A known hemolytic *Staphylococcus aureus* strain was used as control [21].

Determination of Antagonistic and Antibacterial Activity of Probiotic Strains

The antagonistic activity of LAB towards 31 microbial strains (including 9 enteropathogens, 20 β-lactamase producers, 1 *E. coli* and 1 fungal spp.) was detected using standard protocol described by Choi et al. [22]. The antibacterial activity against these isolates was also determined on NA plates supplemented with 0.001% Triphenyl Tetrazolium Chloride (TTC). The agar well diffusion method was used with cell free supernatant of probiotic strains to test the antibacterial activity [23]. To rule out the interference of organic acids, the cell suspensions were neutralized with 1 M NaOH before test.

Evaluation of Cell Surface Characteristics of Selected Probiotic Strains

The selected probiotic strains were studied for cell surface characteristics like exopolysaccharide (EPS) production, hydrophobicity and aggregation. The EPS production was checked by growing the isolates on MRS plates supplemented with 5% sucrose to detect mucoid colonies. The EPS production was confirmed by capsule staining and Molisch test. The hydrophobicity test was done by blending the probiotic strains with hydrocarbon solution (toluene and xylene) and observing for extent of separation of mixture in 2 phases [24]. It was calculated using the equation.

$$Hydrophobicity\ (\%) = \frac{A - B}{A}\ x\ 100$$

Where, A and B are the absorbance of the samples before blending and after phase separation respectively.

Auto aggregation was calculated based on the difference in optical density of cells pre grown in MRS, and same cells re-suspended in equal volume of MRS broth (centrifuged and removed in above step), after keeping the mixture at stationary conditions at 37 °C for 2 h. Aggregation was calculated as:

$$Aggregation\ (\%) = \frac{A - B}{A}\ x\ 100$$

Where, A is the absorbance of pre grown sample and B is the absorbance of re suspended sample.

Evaluation of Additional Health Attributes of Selected Probiotic Strains

In addition to general characteristics of probiotic strains, various other attributes were examined in this study. The oxalate utilization ability of selected strains was screened on calcium oxalate agar plates by observing clear zone around the colonies [25]. The cholesterol lowering capability was determined as described by Liong and Shah [26] using the ferric ammonium sulfate method with the help of MRS media prepared with 0.1% cholesterol. The cholesterol removal rate from MRS media was calculated using the formula

$$Cholesterol\ removal\ rate = \frac{Mu - Mi}{Mu}\ x\ 100$$

Where, Mu and Mi are the absorbance of un-inoculated and inoculated samples respectively.

The hydroxyl radical scavenging activity of cell free extract of strains grown in MRS broth for 24h was determined by sulfosalicylic acid method [27]. To carry out this method, 1 mL of bacterial cell or cell free extract (~10^8 CFU/mL) was mixed with the reaction mixture containing salicylic acid (6 mM/L), $FeSO_4$ (2 mM/L), and H_2O_2 (6 mM/L) and incubated at room temperature for 20 min. The absorbance was determined at 510 nm. The hydroxyl radical scavenging activity was expressed as:

$$\text{Hydroxyl radical scavenging rate} = 1 - \frac{As - A}{Ac}\ x\ 100$$

Where, As is the absorbance of the sample containing bacterial cells, Ac is the absorbance of cell free extract and A is the absorbance of the control sample without H_2O_2.

Besides the above characteristics, the utilization of prebiotic (inulin) and enzyme activity of selected strains for amylase, lipase, phytase and protease was also studied. The details of each assay are represented in Table 1.

***Table 1**. Enzyme Assays of Selected Probiotic Strains*

Enzyme activity	**Method**	**Observation/ Inference**
Amylase	Spot inoculation onto modified MRS agar (2% starch instead of glucose)	Clear zone of amylase activity was detected after flooding the plates with Lugol’s iodine solution
Lipase	Spot inoculation onto modified MRS agar containing 1% olive oil and 1% Arabic gum	Lipase positive colonies were detected by the presence of zone of precipitates around the colonies

Table 1. *(Continues).*

Enzyme activity	**Method**	**Observation/ Inference**
Phytase	Spot inoculation onto Phytate medium [composition in g/L: glucose (15), calcium phytate (5), NH_4NO_3 (5), KCl (0.5), $MgSO_4.7H_2O$ (0.5), $MnSO_4.7H_2O$ (0.2%), $FeSO_4.7H_2O$ (0.01) and agar (20)]	Phytase positive colonies were detected by the presence of zone of precipitates around the colonies
Protease	Spot inoculation onto skimmed milk agar	Clear zones surrounding colony indicated proteolytic activity

Statistical Analysis

All studies were carried out in triplicates and the results were reported as mean ± standard deviation.

RESULTS AND DISCUSSION

Analysis of Physicochemical Characteristics of Kanji

The changes in physicochemical characteristics of Kanji, observed over a period of 7 days, are represented in Fig. 1. It shows a decrease in pH from 6.8 to 3.5. This was further confirmed by the increase in observed titrable acidity from 0.15% to 2.7% lactic acid. The TDS content also showed a significant increase of 33.21% over 7 days indicating optimum metabolism of dissolved solids. All these observations indicated healthy parameters for a fermentation batch. The overall organoleptic properties were found to be ideal for naturally fermented kanji.

The acidity during fermentation imparts flavor and aroma to the beverage. The shelf life of kanji is enhanced due to the diverse LAB that increases during fermentation. Under normal conditions, it can be stored up to 3 months without the need for preservatives. Both carrots and beet roots are immensely nutritive and rich sources of β carotene and betacyanin respectively. On fermentation, the nutritive value of kanji is improved significantly and it also provides a soothing effect. The health benefits of kanji include increased immunity, improved metabolic and antioxidation functions and healthy skin [28].

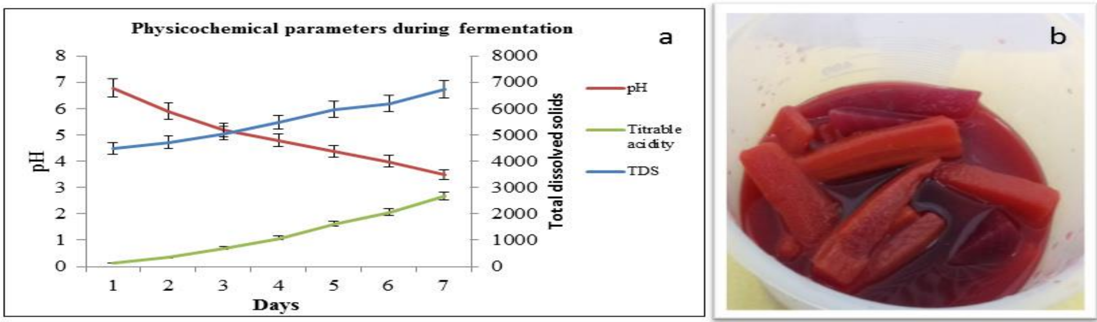

Fig. 1. *(a) Changes in Physicochemical Characteristics of Kanji Over a Period of 7 days (b) Fermented Beverage Kanji*

Analysis of Microbiological Characteristics of Kanji

The microbial load of kanji increased significantly (from 0.44 x 10^6 CFU/mL to 1.255x10^9 CFU/mL) during the first 5 days of fermentation. As the pH dropped below 4.4 on day 5, the microbial count also reduced significantly, basically enriching the lactic acid and other fermentative bacteria to promote fermentation of Kanji. The microbial load was found to be 9.09x10^9 CFU/mL on day 10. Also, *Bacillus* sp. dominated the initial stages of fermentation with considerable gram negative and few yeast colonies. However, the gram positive organisms persisted from day 3 onwards, throughout day 10. Similar trends are reported during the fermentation of sauerkraut, kimchi (Korean fermented cabbage) and Shalgam (Turkish beverage) in published literature [29, 30, 31].

On day 10, 130 distinct isolates were obtained on MRS plates. Among these, 59 isolates were catalase negative and gram positive. Zourari et al. [32] indicated that the LAB are facultative anaerobes, and show a preference to anaerobic conditions. They cannot synthesize porphyrins and consequently do not synthesize cytochromes or catalase. Hence, these isolates were characterized as LAB and screened for their probiotic potential. On further characterization of these cultures, we observed that 83.05% isolates solubilized $CaCO_3$ efficiently, 50.84% were heterofermentative and 49.16% were homofermentative. The observed biochemical characteristics and tolerance to pH and bile salt of all 59 isolates are represented in Table 2.

Evaluation of Probiotic Potential of Isolated Lactic Acid Bacteria

Based on the observed characteristics of fermentative bacteria and rapid screening for survival in gastrointestinal tract (at pH 3 and 0.3% bile) represented in Table 2, four isolates (Fig. 2) were identified as potential probiotic strains of LAB. The probiotic characteristics of these four strains (i.e., JN 3, JN 24, MAP 26 and MAP 29) are represented in Table 3. Three of the four isolates (JN 3, JN 24 and MAP 26) were identified as *Lactobacillus brevis* and one as *Leuconostoc mesenteroides* (MAP 29). They exhibited tolerance to 11% NaCl, 1% bile, pH up to 8 (for 3 h), temperature between 25 °C to 40°C and 0.6% phenol. The utilization of lactose by selected strains and lactic acid production was determined after 48 h incubation in our study. The probiotic strains produced 1.7 to 2.7 g lactic acid/ 20mL culture medium.

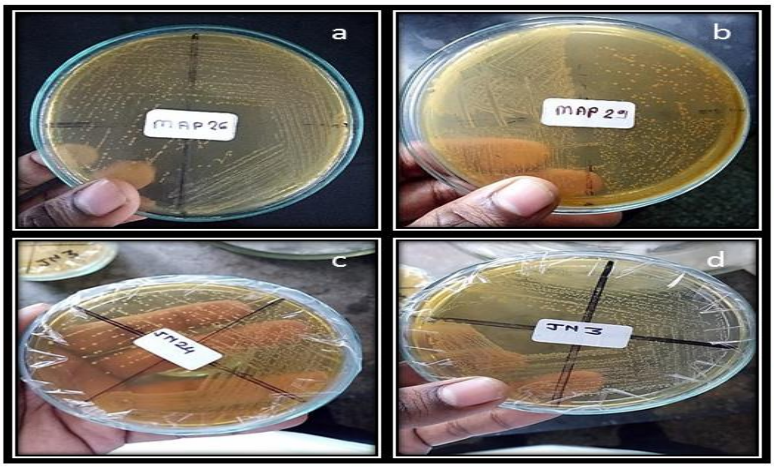

***Fig. 2**. Isolated colonies of [a] MAP 26 (L. brevis) [b] MAP 29 (L. mesenteroides) [c] JN 24 (L. brevis) and [d] JN 3 (L. brevis)*

***Table 2**. Characteristics of Catalase Negative and Gram Positive Bacteria Isolated from Kanji on Day 10 of Fermentation*

Sr. No	**Isolates**	**Morphology**	**Motility**	**Sugar fermentation**				**$CaCO_3$ solubility**	**Homo / Hetero fermentation**	**Tolerance (in % Survival)**	
				Glucose	**Lactose**	**Xylose**	**Sucrose**			**pH 3**	**0.3% Bile salt**
1.	MAP 3	Cocci in cluster	Non motile	+ **G**	-	+	+	+	Hetero	47.5816	23.56828
2.	MAP 13	Short rods	Non motile	+	+	+	+	+	Homo	38.08455	39.70944
3.	MAP 22	Cocci	Non motile	+	-	+	+	+	Homo	65.34023	49.87593
4.	MAP 24	Cocci	Non motile	+ **G**	-	+	+	+	Hetero	97.86982	28.55297
5.	**MAP 26**	**Cocco bacillus**	**Motile**	+ **G**	+	+	+	+/-	Hetero	99.49376	96.78511
6.	**MAP 29**	**Cocci in chain**	**Non motile**	+ **G**	+	+	+	+	Homo	99.07407	97.04348
7.	MAP 30	Cocci in chain & cluster	Non motile	+	+	+	+	+	Homo	67.23997	59.93976
8.	MAP 31	Cocci in cluster	Non motile	+	-	+	+	+	Homo	49.13753	60.69959
9.	MAP32	Cocci in solitary & chain	Non motile	+	+	+	+	+	Homo	83.27787	48.7234
10.	MAP 33	Cocci	Non motile	+	-	+	+	+	Homo	59.88526	63.0719
11.	MAP 35	Cocci in chain	Non motile	+	-	+	+	+	Homo	74.16471	68.36903
12.	MAP 36	Cocci	Non motile	+	+	+	+	+	Homo	86.49835	32.6
13.	MAP 38	Cocci	Non motile	+	-	-	+	+	Homo	71.27394	31.59722
14.	JN 1	Rods in solitary	Non motile	+ **G**	+	+	+	+/-	Hetero	92.36184	80.11364
15.	JN 2	Short rods	Motile	+	-	+	+	+	Homo	98.08678	38.68955
16.	**JN3**	**Rods in solitary**	**Motile**	+ **G**	+	+	+	+	Hetero	98.90421	91.89189
17.	JN 4	Short rods	Non motile	+ **G**	+	+	+	+	Hetero	98.52735	43.23144
18.	JN 5	Short rods	Non motile	+ **G**	+	+	+	+	Hetero	75.47771	61.50235
19.	JN 6	Short rods	Motile	+ **G**	+	+	+	+	Hetero	96.39929	31.07256

Key: + Growth; - No growth; + G growth with gas

***Table 2**. (Continues)*

Sr. No	Isolates	Morphology	Motility	Sugar fermentation				$CaCO_3$ solubility	Homo / Hetero fermentation	Tolerance (in % Survival)	
				Glucose	Lactose	Xylose	Sucrose			pH 3	0.3% Bile salt
20.	JN 7	Rods in chain	Non motile	+	-	+	+	+	Homo	67.15096	46.90265
21.	JN 8	Short rods	Motile	+	-	+	+	+	Homo	97.33096	73.2
22.	JN 9	Short rods	Motile	+ **G**	+	+	+**G**	+	Hetero	97.84099	54.22886
23.	JN 10	Short rods in chain	Motile	+ **G**	+	+	+	+	Hetero	95.68345	60.61856
24.	JN 11	Short rods	Non motile	+ **G**	+	+	+	+/-	Hetero	43.29974	70
25.	JN 12	Rods and cocci	Non motile	+ **G**	+	+	+	+	Hetero	97.84123	21.19816
26.	JN 13	Short rods	Motile	+ **G**	+	+	+	+	Hetero	12.0532	35.92493
27.	JN 14	Long rods	Non motile	+ **G**	+	+	+	+	Hetero	93.96483	48.64865
28.	JN 15	Short rods	Motile	+	-	+	+	+/-	Homo	99.06199	47.64468
29.	JN 16	Short rod in solitary & chain	Motile	+	+	+	+	+	Homo	71.42857	49.42149
30.	JN 17	Rods in solitary	Motile	+ **G**	+	+	+	+	Hetero	13.9834	27.41935
31.	JN 18	Cocci in cluster	Non motile	+	-	+	+	+	Homo	14.27146	32.54237
32.	JN 19	Cocco bacillus in solitary	Non motile	+ **G**	+	+	+**G**	+/-	Hetero	29.53325	52.60417
33.	JN 20	Short rod in chain	Non motile	+ **G**	+	+	+	+	Hetero	30.18057	38.81279
34.	JN 21	Short rod in chain & solitary	Non motile	+ **G**	-	+	+	+	Hetero	39.55943	32.64463
35.	JN 22	Short rod in chain	Motile	+	-	+	+	+	Homo	16.43101	65.66265
36.	JN 23	Short rod in solitary	Motile	+ **G**	-	+	+	+	Hetero	13.99586	53.55987
37.	**JN 24**	**Short rod in chain**	**Motile**	+ **G**	-	+	+	+/-	Hetero	97.0867	96.88474

Key: + Growth; - No growth; + G growth with gas

***Table 2**. (Continues)*

Sr. No	Isolates	Morphology	Motility	Sugar fermentation				$CaCO_3$ solubility	Homo / Hetero fermentation	Tolerance (in % Survival)	
				Glucose	Lactose	Xylose	Sucrose			pH 3	0.3% Bile salt
38.	JN 25	Cocci in solitary	Non motile	+	+	+	+	+	Homo	56.45514	72.58065
39.	JN 26	Short rods	Non motile	+ **G**	+	+	+	+	Hetero	92.09139	38.15261
40.	NAI 1	Cocci in cluster	Non motile	+	+	+	+	+/-	Homo	72.54747	19.39891
41.	NAI 2	Short rods	Motile	+ **G**	+	+	+	+	Hetero	94.84067	8.970976
42.	NAI 3	Short rod in chain & solitary	Non motile	+ **G**	+	+	+	+/-	Hetero	47.10547	33.54232
43.	NAI 4	Short rod in chain & solitary	Non motile	+	+	+	+	+	Homo	96.25293	38.25758
44.	NAI 5	Short rod in solitary	Motile	+ **G**	+	+	+	+	Hetero	34.76899	47.34607
45.	NAI 6	Cocco bacillus	Non motile	+ **G**	-	+	+	+/-	Hetero	93.46127	46.15385
46.	NAI 7	Short rod in chain & solitary	Motile	+	-	+	+	+	Homo	99.48301	40.25974
47.	NAI 8	Short rod	Motile	+	+	+	+	+	Homo	35.62909	53.49345
48.	NAI 9	Cocco bacillus	Non motile	+	+	+	+	+	Homo	28.33767	44.07989
49.	NAI 10	Diploid cocco bacillus	Non motile	+	+	+	+	+	Homo	13.36441	17.45636
50.	NAI 11	Short rods	Non motile	+ **G**	+	+	+	+	Hetero	22.41206	46.40625
51.	NAI 12	Rods in solitary	Motile	+ **G**	+	+	+	+	Hetero	81.69844	55.26676
52.	NAI 13	Rods in solitary	Motile	+ **G**	+	+	+	+/-	Hetero	83.34527	35.10204
53.	NAI 14	Rods in chain	Non motile	+ **G**	+	+	+	+	Hetero	75.61154	22.58065
54.	NAI 15	Short rods	Motile	+ **G**	+	+	+	+/-	Hetero	98.9291	43.00885
55.	NAI 16	Short rod in chain & solitary	Non motile	+	+	+	+	+	Homo	92.23712	58.60113

Key: + Growth; - No growth; + G growth with gas

Table 2*. *(Continues)

Sr. No	Isolates	Morphology	Motility	Sugar fermentation				$CaCO_3$ solubility	Homo / Hetero fermentation	Tolerance (in % Survival)	
				Glucose	Lactose	Xylose	Sucrose			pH 3	0.3% Bile salt
56.	NAI 17	Cocco bacillus	Non motile	+	+	+	+	+	Homo	87.96581	44.15157
57.	NAI 18	Rods in chain	Motile	+	+	+	+	+	Homo	90.39688	45.46649
58.	NAI 19	Short rods	Motile	+ **G**	+	+	+	+	Hetero	87.8185	75.35714
59.	NAI 20	Rods in chain	Non motile	+ **G**	+	+	+	+	Hetero	94.0526	51.2605

Key: + Growth; - No growth; + G growth with gas

***Table 3**. Observed characteristics of lactic acid bacteria with optimum probiotic potential*

Lactic acid bacteria	Identification	Tolerance		Lactose utilization and Lactic acid production (g/20mL)	Viability at different		
		pH 3	0.3% Bile		Temperature	pH and Bile salt concentration	NaCl and phenol concentration
MAP 26	*Lactobacillus brevis*	99.49	96.78	1.805	Growth was observed at 25°C, 30°C, 37°C and 40°C	All isolates tolerated up to pH 8 and 1% bile salt	All isolates tolerated up to 11% NaCl and 0.6% phenol
MAP 29	*Leconostoc mesenteroides*	99.07	97.04	1.751			
JN3	*Lactobacillus brevis*	98.90	91.89	2.022			
JN 24	*Lactobacillus brevis*	97.08	96.88	2.726			

The basic criteria for selection of probiotic strains require their survival in the gastrointestinal tract by tolerating low pH and high bile salt concentration. However, the conditions of the gastrointestinal tract are unpredictable and hence difficult to standardize. They vary with diet, intestinal health and secretion of pancreatic enzymes [33]. Hence, additional parameters like tolerance to, elevated levels of NaCl, bile, phenol and, wide pH and temperature range, was tested in this study. Although the in vitro assessments may not mimic the accurate conditions in the gut ecosystem, it remains a powerful tool for rapid screening of potential probiotic strains. Besides, the in vivo studies are extremely time consuming and require good financial inputs. Hence, it is always practical to investigate the preliminary stages of new studies by simple and effective techniques. Also, the selection criteria for probiotic strains require testing of several parameters, most of which can be done using in vitro methods [18, 34]. All these parameters were tested in our study, and the selected strains fulfilled every criterion indicating their extraordinary potential as probiotic culture.

Similar to the present study, only 4 out of 90 LAB strains isolated from Ethiopian food products and 6 out of 40 LAB strains isolated from fermented Xinjiang cheese showed significant survival rate at pH 3 and 0.3% bile salt concentration for 3 h [35, 36]. In another study, 9 *Lactobacillus* sp. isolated from various sources tolerated pH 3 for 3 h, 0.3% bile salts for 4 h, and 1.9 mg/mL pancreatic enzymes for 3 h [17]. Based on these characteristics, they were selected as potential probiotic strains.

Evaluation of Standard Safety Criteria for Lactic Acid Bacteria

The selected strains of LAB showed varied degree of resistance towards different antibiotics used in the present study. All isolates were resistant to ampicillin and chloramphenicol, and sensitive to cotrimoxazole, cefadroxil and ofloxacin (Table 4). Although LAB are naturally resistant to several antibiotics, their multidrug resistance potential may facilitate the dissemination of resistance genes to pathogens in the gut. In order to clearly define the safety of isolated strains, it is necessary to verify the intrinsically coded and acquired resistance of test strains. This is because the resistance to glycopeptide, aminoglycoside and sulfamethoxazole is commonly reported among LAB strains. The *Lactobacilli* and *Leuconostoc* strains are intrinsically resistant to common antibiotics like bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitro–furantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin and trimethoprim/sulphamethoxazole [37, 38]. Also, the resistance to vancomycin and erythromycin is increasingly reported among LAB [38, 39]. In case the resistance is acquired, all the beneficial factors contributing to the efficacy of probiotic strains is annulled by this single factor. At the same time, the intrinsic resistance to antibiotics can benefit the host in events of infectious diseases [40]. In the current study, the qualitative test with Congo red dye indicated the absence of virulence plasmids in all four strains, indicating that the observed antibiotic resistance may be intrinsic. The cellular binding of the sulfonated diazo dye Congo red is associated with virulence in pathogens. It also correlates with the expression of outer membrane haemin binding proteins [41]. Among other virulence factors, all four strains tested negative for gelatinase activity, and production of siderophores and hemolysin (Fig. 3). The virulence factors are useful for distinguishing potentially pathogenic and non pathogenic LAB strains. Hence, the observations in our study indicate that all 4 LAB strains are safe for further in vivo studies, and can be regarded as suitable for application as probiotic strains based on standard criteria of *in vitro* model studies [42].

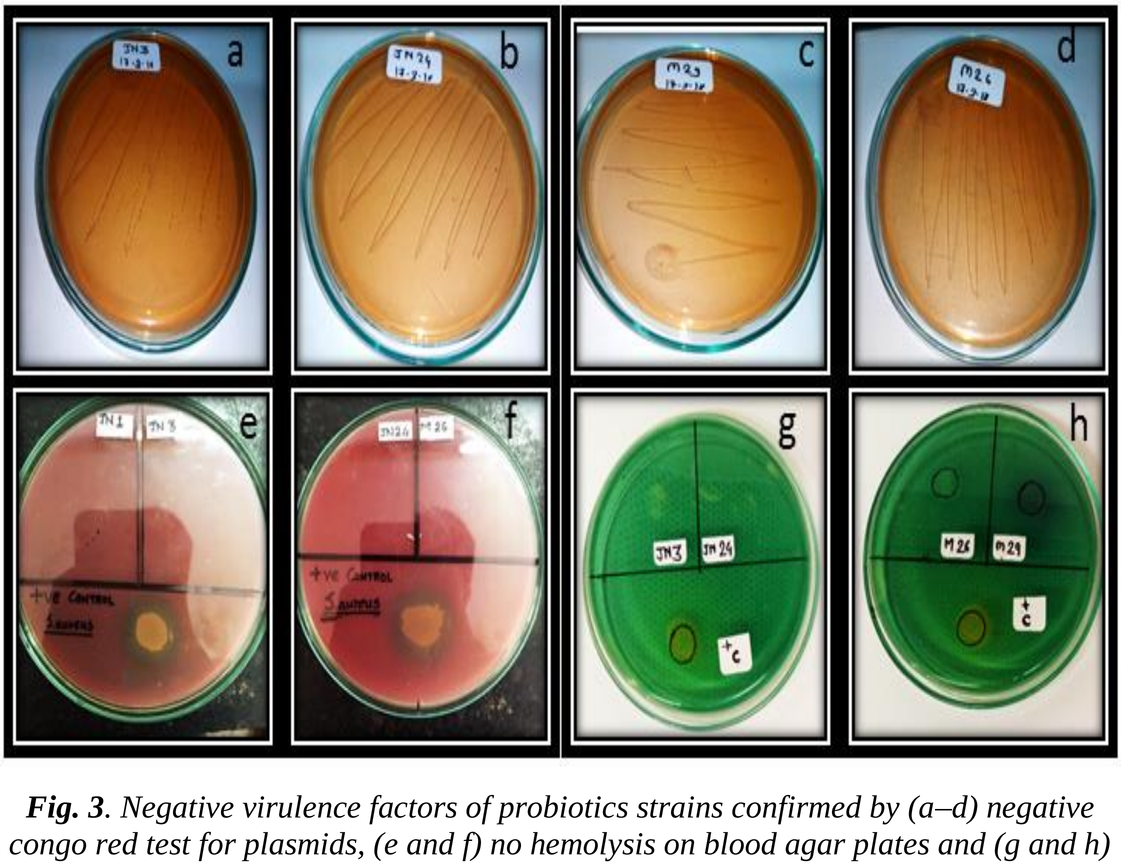

Fig. 3. *Negative virulence factors of probiotics strains confirmed by (a–d) negative congo red test for plasmids, (e and f) no hemolysis on blood agar plates and (g and h) No yellow zone of siderophore producers on CAS agar*

***Table 4**. Antibiotic resistance profile of probiotic strains*

Antibiotic	Symbol	Strength (µg)	JN 3	JN 24	MAP 26	MAP 29
Ticarcilin/clavunalic acid	TT	85	R	S	R	R
Oxytetracyclin	OX	30	S	S	S	S
Ceftriaxone	RP	30	R	S	R	R
Cefuroxime	CB	30	R	R	R	R
Nalidixic acid	NA	30	R	R	R	R
Norfloxacin	NX	10	R	R	R	R
Amoxycillin	AG	30	R	R	R	R
Cefadroxil	CU	30	S	S	S	S
Cefoperazone	CP	75	S	R	R	R
Ceftazidimine	FG	30	R	R	R	R
Polymyxin-B	PB	300 µL	R	R	R	R
Ampicillin / Sulbactam	AS	20	R	R	R	R
Cefotaxime	CF	30	S	S	S	S
Piperacillin	PC	100	S	S	S	S
Chloramphenicol	CH	30	R	S	R	R
Ciprofloxacin	RC	5	R	S	R	R
Ceftizoxime	CL	30	R	S	R	R
Tetracycline	TE	30	S	S	R	R
Ofloxacin	ZN	5	S	S	S	S
Gentamycin	GM	10	S	R	R	R
Amikacin	AK	30	S	S	R	R
Gatifloxacin	GF	10	S	R	S	S
Cefepime	ZX	30	R	R	R	R
Co-trimoxazole	BA	25	S	S	S	S

Determination of antagonistic and antibacterial activity of lactic acid bacteria

The antagonistic activity of probiotic strains with β lactamase producing pathogens, a fungal isolate and *E. coli* was evaluated in our study. All four probiotic strains showed antagonistic activity against the pathogenic strains and no effect against non-pathogenic *E. coli*. The antibacterial activity of LAB strains was further evaluated against these isolates. Interestingly, all pathogenic isolates were inhibited by probiotic strains with zones of inhibition in the range of 17mm and 23mm (Fig. 4). The widespread resistance of Enterobacteriaceae towards β lactam antibiotics is a major global issue at present (besides low immunity). More than 60% pathogens causing respiratory and urinary tract infections (1st and 2nd most common infections) are Extended spectrum β lactamase (ESBL) producers, and the count is rapidly rising for Metallo β lactamase producers [43, 44, 45, 46]. Hence, it is practical to study the interference of these pathogens with newly isolated strains for application in humans and animals. In our study, 30 representative β lactamase producers including *E. coli*, *K. pneumoniae*, *Proteus* spp. and *Citrobacter* spp.

were inhibited by all 4 LAB strains. This finding is of great relevance amidst the widespread scenario of antibiotic resistance globally. The antifungal and antibacterial activities of LAB is previously reported and attributed to the production of various compounds like organic acids (including lactic acid), hydrogen peroxide, proteinaceous compounds, hydroxyl fatty acids, phenolic compounds, or selective antibacterial/antifungal compounds [47, 48, 49]. These observations are encouraging for the application of the isolated LAB strains in pharmaceutical as well as food industries.

The antibacterial activity of LAB against ESBL producers was recently reported by Mokhtar et al. [50] using agar diffusion method. The observed zones of inhibition were reported to be ≥13mm. In addition, the *Lactobacillus* sp. used in their study could inhibit biofilm formation and clear the preformed biofilms of pathogens. Similarly, the antibacterial activity of LAB has also been reported against virulent *E. coli* [51]. The observations of these studies along with our results strongly suggest the possible application of LAB as a substitute for antibiotics and growth promoters in agriculture and animal husbandry. A detailed review of above application is also reported by Vieco– Saiz et al. [52].

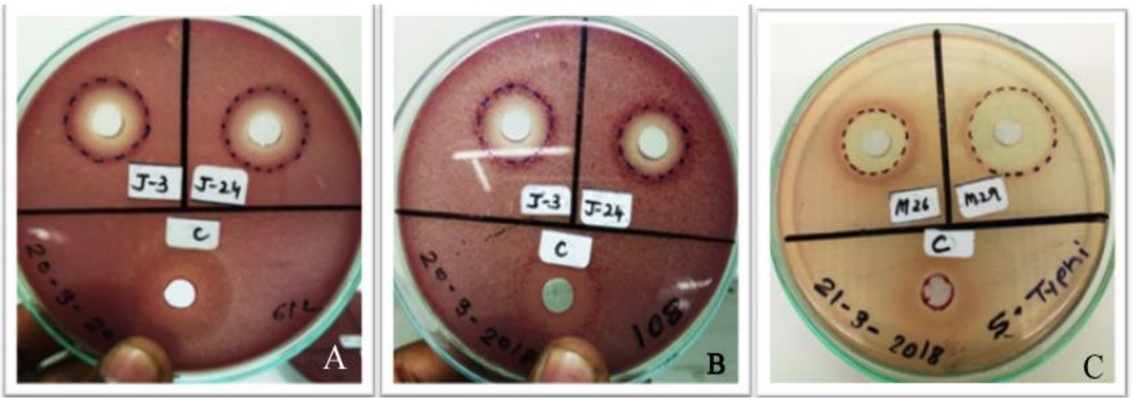

Fig. 4. *Antagonistic effect of probiotics strains against (A) ESBL producing uropathogen (E. coli); (B) MBL producing uropathogen (E. coli); and (C) Salmonella typhi (laboratory culture)*

Evaluation of Cell Surface Characteristics of Lactic Acid Bacteria

The cell surface characteristics like EPS production, auto aggregation and hydrophobicity are important factors responsible for colonization of the intestinal tract. These characteristics also help in competitive binding of probiotics strains that prevent the attachment of pathogens. In the current study, the aggregation and hydrophobicity of the isolates ranged between 64.74% to72.78% and 50% to76% respectively (Fig. 4). Based on literature review of published studies, auto aggregation and hydrophobicity over 40% may be considered as suitable for probiotic strains [53, 54]. In this study, the EPS production was observed only by MAP 29 LAB strain. The EPS production further aid in binding of probiotic strains to the intestinal cell surface. However, no correlation was observed between the three characteristics in our study. Although MAP 29 showed EPS production in addition to above characteristics, JN 24 showed better aggregation and hydrophobicity even in absence of EPS (Fig. 5). Similarly, no correlation between above factors was reported in Lactobacillus and Bifidobacterium sp. [55]. In contrast, Aslim et al. [56] reported that EPS producing *Lactobacillus delbrueckii* subsp. *bulgaricus* show better auto aggregation and hydrophobicity even after treatment with bile, which apparently affected the EPS non producers negatively. From a different perspective, a study by Dertli et al. [57] reported that EPS production may improve tolerance to stress factors in *Lactobacillus johnsonii* FI9785, but reduce cell adhesion in poultry hosts. In conclusion, the strain specific characteristics of LAB are easy to comprehend. Besides, the microbial polysaccharides are in great demand for various biotechnological processes at industrial level [58]. Hence, EPS producing LAB can be a gold mine for safe microbiological practices.

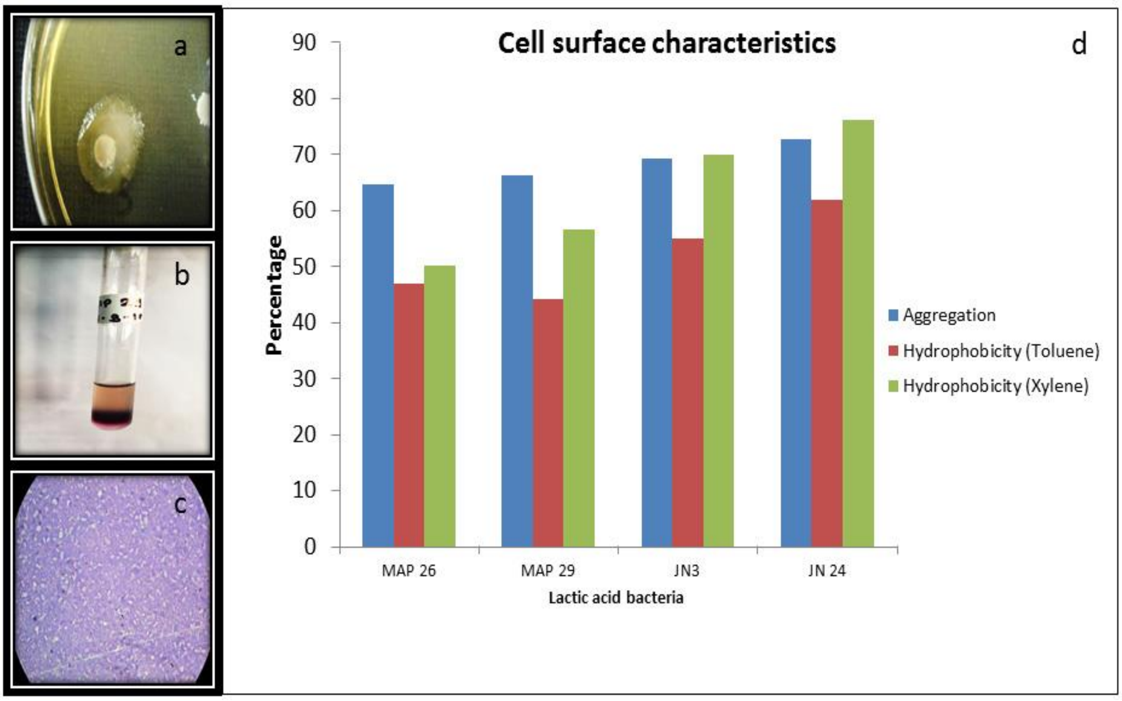

Fig. 5. *Cell surface characteristics of probiotics strains: MAP 29 strain showing (a) EPS production on MRS agar plates (b) Positive Molisch test and (c) colorless EPS on capsule staining (100x magnification), (d) auto aggregation and hydrophobicity of probiotics strains*

Evaluation of Additional Health Attributes of Lactic Acid Bacteria

Besides safety and probiotic potential of 4 LAB strains, additional health attributes were also investigated. All 4 strains showed antioxidant activity, and cholesterol and calcium oxalate degradation to different degree (Fig. 6). These characteristics are represented in Table 5. Cholesterol degradation was observed in the range of 55% and 67%. Calcium oxalate degradation was tested qualitatively by plate method, where clear zone of inhibition (13 mm to 30 mm) was observed by MAP 29, JN 3 and JN 24 whereas MAP 26 exhibited a very faint zone of clearance (26 mm). This may be due to partial degradation of calcium oxalate or interference of degraded compound with the growth of MAP 26 strain. Also, the hydroxyl radical scavenging activity was observed between 43% and 60%. In addition, MAP 29 strain showed lipase activity. Thus, our study reports great potential of four LAB strains for pharmaceutical applications in formulation of hypocholesterolemic agents, treatment of recurrent kidney stones, and potential antioxidants. Interestingly, all 4 isolates also utilized prebiotic source (inulin) and thus can be used to enhance the therapeutic value of probiotic food (especially dairy) products.

The hypocholesterolemic effects of LAB are most probably due to the assimilation of cholesterol, binding to cell surface or coprecipitation. Most *Lactobacillus* and *Bifidobacterium* sp. are capable of secreting Bile Salt Hydrolase (BSH) which deconjugates bile acid that can be excreted through faeces. The BSH thus helps in reduced binding of cholesterol to host cell surface. The free bile also acts as a biosurfactant and increases the binding of cholesterol to probiotic cell membrane [59, 60]. Many LAB strains also express catabolic enzymes like formyl CoA transferase and oxalyl CoA decarboxylase that aid in oxalate degradation. Among the novel therapies for recurrent kidney stones, oxalate degrading probiotic strains possess significant potential. Many researches have provided significant evidence for use of probiotics in treatment of kidney stones based on in vitro and in vivo studies [61, 62]. However, clinical trials have not yet confirmed any such claims.

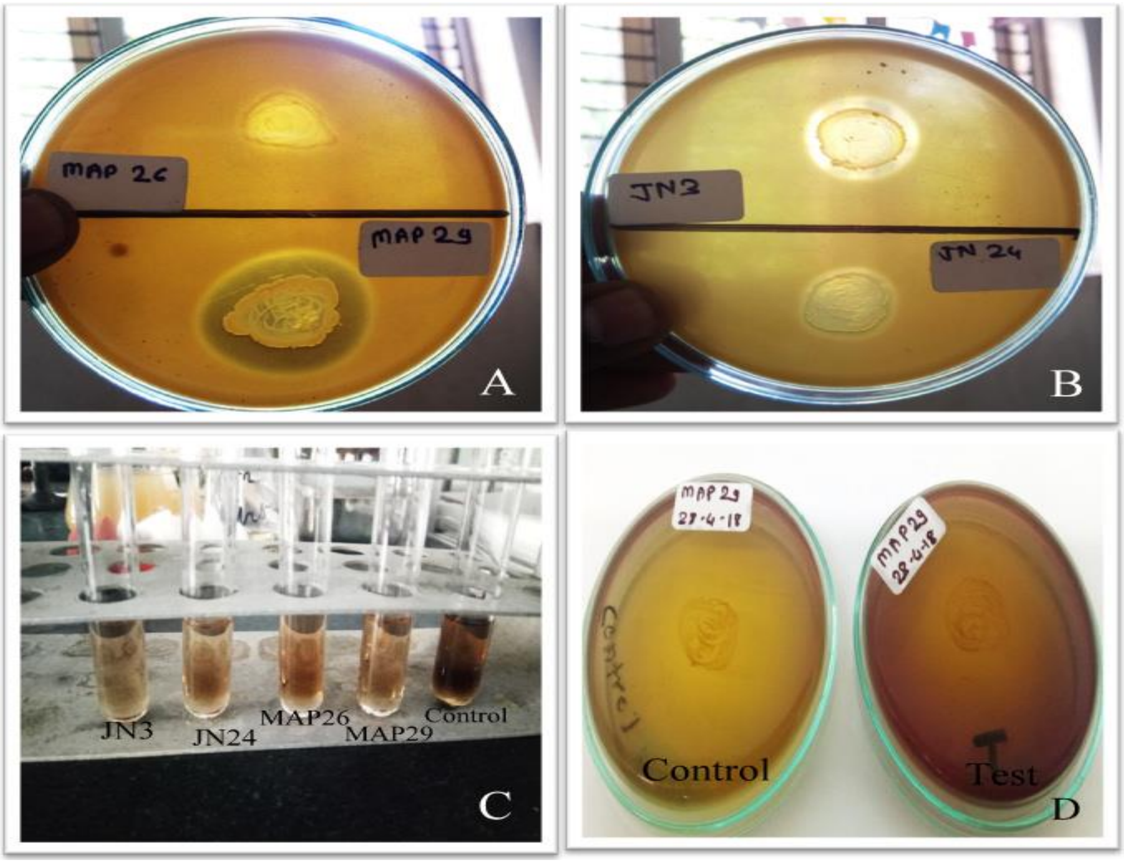

Fig. 6. *LAB strains showing (A and B) degradation of calcium oxalates (C) degradation of cholesterol and (d) utilization of inulin*

***Table 5**. Additional health attributes of lactic acid bacteria isolated in this study*

Lactic acid bacteria	Degradation of		Utilization of prebiotic (inulin)	Hydroxyl radical scavenging activity (%)
	Cholesterol (%)	Calcium Oxalate (Zone of inhibition)		
MAP 26 (*L. brevis*)	56.62393	26 mm (faint zone)	+	43.13725
MAP 29 (*L. mesenteroides*)	55.44872	30 mm	+	48.36601
JN3 (*L. brevis*)	66.34615	13 mm	+	58.82353
JN 24 (*L. brevis*)	69.01709	15 mm	+	60.78431

CONCLUSION

The immense health potential of probiotics is evident from many published studies including the present study. In addition to several health benefits of LAB strains isolated in this study, MAP 29 showed a unique characteristic of lipase production. Enzyme production from LAB is rarely reported in literature. The data in this study suggests the possible application of 4 LAB strains isolated from this beverage in probiotic formulations to help manage conditions like lactose intolerance or allergy to protein products after further studies. Other parameters like its efficacy in managing hypercholesterolemia and recurring kidney stones may also be explored based on the encouraging observations of the present study.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept: A.M.S., M.R.G., Design: A.M.S., M.R.G., Data Collection or Processing: A.M.S., M.R.G., Analysis or Interpretation: A.M.S., M.R.G., Literature Search: A.M.S., M.R.G., Writing: A.M.S., M.R.G.

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