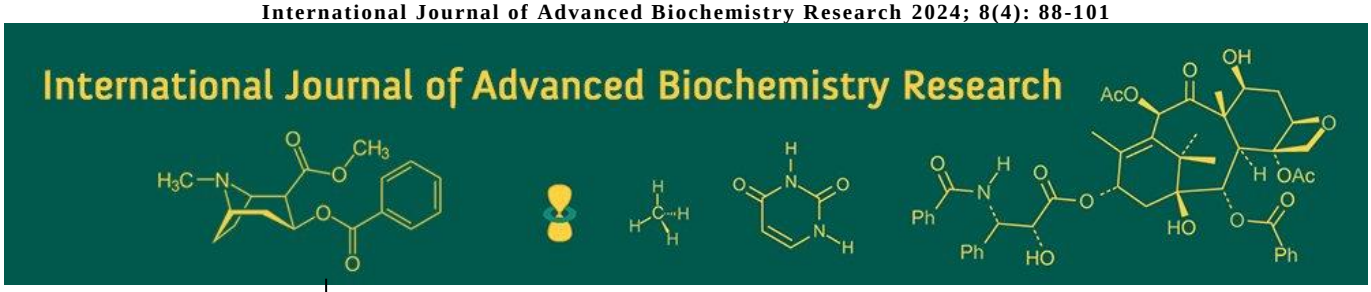

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Isolation, characterization and screening of lactic acid bacteria (Lab) from fruits for the production of probiotic beverage

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Abstract
The study was aimed to isolate, characterize and screen the efficient lactic acid bacteria (LAB) from different fruits which had probiotic properties. Naturally associated fifteen lactic acid bacterial isolates were isolated from different fruits. Isolation and identification of isolates were conducted according to the Bergey's manual (conventional method) and based on morphological, physiological and biochemical characterization. Morphologically distinct colonies were studied for their cell morphology. All LAB isolates were rod shaped, gram positive, non-motile, and non-endospore producers. Biochemical characterization of isolates showed negative results for catalase test whereas, they were positive for acid production, dextran production, casein hydrolysis, gelatin liquefaction and starch hydrolysis test. Utilization of different carbon sources by lactic acid bacterial isolates showed positive results. Isolates were screened for probiotic characters like tolerance to low pH (2.0, 2.5 and 3.0), bile salts (0.1, 0.2 and 0.3%) and phenol (0.1, 0.2 and 0.3%). Among all fifteen isolates, LAB-12 and LAB-14 showed maximum potential probiotic characters. Further these two isolates were used for the fermentation of watermelon juice.

Keywords: characterization, lactic, acid, bacteria, fruits, beverage

Introduction
In the last few decades, consumer demands for food products have changed considerably due to an increasing awareness on the beneficial effects of food on health. In this sense, the food industry has been directed to the production of functional foods. Foods that produce beneficial effects on health, in addition to their basic nutritional functions and probiotics are an important segment of this line (Zoumpopoulou *et al.*, 2017) [34].
Research on probiotic microorganisms has been moving towards the development of food supplementation. Thus, there is great interest from the food and pharmaceutical industries for isolation, identification and evaluation of lactic acid bacteria (LAB), native to foods with probiotic properties (Zielinska *et al.*, 2018) [33].
Consumption of probiotics by human or animal is an important aspect that needs to be carefully monitored. Probiotic populations should outnumber gut microbial populations by a wide margin in order to dominate the beneficial, native microbiota and reduce the harmful pathogenic microbiota. Since probiotics in probioticated food products must endure food processing and storage, this is a crucial aspect of their effectiveness. Since probiotics should retain a suitable level of viable cells during the product's shelf life without increasing manufacturing costs, their viability and stability in present marketing and technological problems for manufacturers (Doleyres and Lacroix, 2005) [5].
All the above facts were considered in the selection of potential probiotic strain to produce novel and good quality fermented watermelon beverages to combat the existing snag and confer health benefits to mankind. Hence, the present study titled "Isolation, characterization and screening of lactic acid bacteria (LAB) from fruits for the production of probiotic beverage" was attempted to obtain efficient probiotic strain to determine its suitability in various combination of treatments containing watermelon juice as basic raw substrate.

2. Materials and Methods

The present investigation was conducted in the Department of Agricultural Microbiology, College of Agriculture, University of Agricultural Sciences, Raichur during the year 2022-23 for isolation of the probiotic lactic acid bacterial isolates. This chapter deals with the experimental techniques and procedures adopted to carry out the experiments towards fulfilling the various objectives of this investigation. The materials used and the methods employed during the investigation are outlined in this chapter are given below under suitable headings.

2.1 Collection of fruit samples

Watermelon, banana, custard apple, tomato and papaya were procured from local market at Raichur for the isolation of lactic acid bacteria. Freshly harvested watermelon and fig fruits were procured from local market of Raichur for preparation of juice.

2.2 Isolation of lactic acid bacteria from fruits

Lactic acid bacteria were isolated from watermelon, banana, custard apple, tomato and papaya by standard plate count method using de Man, Rogosa and Sharpe agar (MRS) medium. From each sample, 1:10 dilution was subsequently made using sterile normal saline water (0.85%) followed by making a 10 fold serial dilution. The 0.1 ml from each dilution was then sub-cultured aseptically into MRS agar (Guessas and Kihal, 2004) [11] using pour plate technique. All plates were then incubated at 37 °C for 24 to 48 h in anaerobic condition to provide an optimal environmental for growing lactic acid bacteria.

Colonies differ in morphology, pigmentation, shape and size were subcultured in MRS broth. Initially all the isolates were examined for Gram staining. Only the Gram positive isolates were then purified by streak plate method on medium. The culture was kept in MRS agar slant and stored at 4 °C for further use (Hawaz, 2014) [13].

2.3 Reference lactic acid bacterial culture

Lactobacillus acidophilus (NCIM 2903) strain is used as reference strain.

2.4 Identification of lactic acid bacterial isolates

Identification of lactic acid bacterial isolates was done by studying their morphological characteristics and biochemical tests.

2.4.1 Colony Morphology of lactic acid bacterial isolates

Lactic acid bacteria formed different characteristic colonies on media, which could be a tool for preliminary identification. Each isolates were streaked on petriplates containing MRS medium and incubated for 48 h at 37 ºC for identification.

2.4.2 Microscopic observation of lactic acid bacterial isolates

Lactic acid bacterial isolates from watermelon, banana, custard apple, tomato and papaya were studied for their cell morphology and phenotypic characteristics under microscope.

2.4.3 Gram staining (Smith and Hussey, 2005) [26]

Gram staining was done by using the standard procedure. Briefly, a loop full of bacteria (24 h old culture) was transferred aseptically onto a clean glass slide and was heat fixed using a Bunsen burner. The smeared area was flooded with crystal violet for 1 min and washed with water. Gram's iodine solution was added on to smear for 60 seconds and washed with water. Slides were dipped in 95 percent ethyl alcohol for 45 seconds and then slides were allowed to air dry for alcohol to evaporate. Then safranin was used to stain the smears for 30 seconds. It was then blotted dry with a paper towel using firm pressure. The smear was examined with a compound microscope starting from the low power objective and finally the oil immersion lens.

2.4.4 Endospore staining (Murray *et al.*, 1994) [19]

The LAB cultures of 30 days old were used for endospore staining. A thin smear was made on a clean glass slide and air dried. The smear was flooded with malachite green (0.5% aqueous solution) and kept on hot water bath for seven minutes. Malachite green was not allowed to dry. Slides were washed with running water and counter stained with safranin for 30 seconds. Then washed, air dried and observed under 100X magnification.

2.4.5 Catalase test (Hitchins and Jinneman, 2011) [14]

A loop full of culture was transferred to a clean glass slide. A few drops of hydrogen peroxide (H_2O_2) reagent were put onto the sample using a pipette. The reaction was observed for bubbles/effervescence formation (positive reaction) while no bubbles formation indicated a negative reaction.

2.4.6 Starch hydrolysis (Yazdanparast, 1993) [31]

Starch agar medium was melted and poured to sterile petriplates and was allowed to solidify. Isolates were streaked and plates were incubated for 48 h at room temperature. Plates were flooded with iodine solution for 30 seconds, excess iodine solution was drained and observed for yellow clear zone around the streaked line.

2.4.7 Gelatin hydrolysis (Ewing, 1966) [6]

Nutrient gelatin stabs were inoculated with cultures and incubated for 48 h at 37 ºC. Test tubes were observed for gelatin hydrolysis by placing test tubes in refrigerator for 30 min until control test tube solidifies. At the end of refrigeration, the test tubes were observed if gelatin has been hydrolyzed. Solidification of stab is negative for gelatin hydrolysis by microorganism and non-solidification of gelatin is an indication of positive result for hydrolysis.

2.4.8 Dextran production (Garvie, 1960) [8]

Solidified plates of sucrose agar medium were streaked with cultures and plates were incubated at room temperature for 48 h and observations were recorded.

2.4.9 Utilization of different carbon sources

The experiment was conducted to study the utilization pattern of carbon sources *viz*., glucose, fructose, lactose, sucrose, maltose, galactose, mannitol, esculin, cellobiose and sorbitol by lactic acid bacterial isolates. Sterilized filtered solutions of different sugars were added to 5 ml of sterilized solution of phenol red broth and inoculated with lactic acid bacterial isolates with an initial cell density having 10^6 CFU/ml was incubated at 37 ºC. Sugar assimilation was observed for colour change from red to yellow.

2.4.10 Acid and gas production (Seeley and Vandemark, 1970)

The 48 h old lactic acid bacterial cultures were inoculated in to lactose broth with bromocresol purple. The tubes were incubated at room temperature for 48 h and observed for colour change from purple to yellow and bubble formation was observed in Durham's tube for gas production.

2.4.11 Casein hydrolysis (Aneja, 2012) [2].

Skim milk agar medium was prepared, poured to petriplates and allowed to solidify. Isolates were streaked and plates were incubated at room temperature for 48 h and observations were recorded by looking at the clear zone around the streaked line.

2.5 Screening of lactic acid bacterial isolates for probiotic properties

2.5.1 Screening of lactic acid bacterial isolates for acid and bile salt tolerance (Tambekar and Bhutada, 2010) [29]

Acid and bile salt resistance of isolated LAB were assayed using the method of Tambekar and Bhutada (2010) [29] with slight modification. For acid tolerance, strains were grown overnight on MRS broth at 37 °C. One hundred micro liters (100 µl) of each overnight cultures was inoculated separately into MRS broth adjusted to pH 2, 2.5, 3 with 5 M HCl and pH 6.0 (which served as control) and incubated anaerobically at 37 °C for 24 h. The growth of LAB in the adjusted low pH broth was used to designate isolates as a acid tolerant, and growth was measured after 24 h of incubation using spectrophotometer, read the optical density at 600 nm against uninoculated broth.

For bile salt tolerance, the acid-resistant strains were selected and tested for their resistance to bile salt. In this case, bile salt (0.1%, 0.2%, 0.3% w/v) was added to the MRS broth. Growth was measured after 24 h of incubation using spectrophotometer, read the optical density at 600 nm against uninoculated broth.

2.5.2 Screening of lactic acid bacterial isolates for NaCl and phenol tolerance (Reuben *et al.*, 2019) [22]

NaCl tolerance of isolates were tested. In this case, NaCl (0%, 2%, 4%, 6% w/v) was added to the MRS broth. Growth was measured after 24 h of incubation using spectrophotometer, read the optical density at 600 nm against uninoculated broth.

Phenol tolerance of isolates were tested. In this case, phenol (0%, 0.1%, 0.2%, 0.3% v/v) was added to the MRS broth. Growth was measured after 24 h of incubation using spectrophotometer, read the optical density at 600 nm against uninoculated broth.

2.6 Antagonistic activity (Tagg *et al.*, 1976) [28].

The antagonistic activity of the lactic acid bacterial isolate is studied against *E. coli.* To detect antimicrobial activity of the LAB isolates, *E. coli* grew in nutrient broth at 37 °C for 24 h are used. Actively grown culture of the test organisms were mixed 2.5% ($2.5x10^7$ CFU/ml) with melted nutrient agar and poured in sterile petri dishes and allowed to solidify. A 1 cm wide ditch was cut in the agar across the centre of the dish. The cell-free supernatant of LAB isolate was pipetted out into the ditch. When the mixture solidified, the plates were first incubated at 4 °C for 60 min to allow the test material to diffuse in the agar and then incubated at 37 °C for 18 h. After incubation, the diameter of the clear zone is measured in milli meters from the centre of the well.

2.7 Antibiotic susceptibility (Charteris and Kelly, 1994) [4].

Disc diffusion method was used to determine the antibiotic susceptibility. Antibiotic discs (Himedia) used were consists of ampicillin (10 µg), amoxyclav (30 µg), cephataxime (30 µg), co-trimoxazole (25 µg), gentamicin (10 µg), tobramycin (10 µg). Antibiotic discs were used to determine antibiotic sensitivity of lactic acid bacteria. The discs were placed on the solidified agar surface and plates were incubated at 37 °C for 24 h.

2.8 Molecular identification of probiotic cultures of lactic acid bacteria

The strains of promising probiotic LAB were identified by 16S rRNA.

3. Results and Discussion

Source and details of microbial cultures used in the experiment

3.1 Isolation and identification of lactic acid bacterial cultures

Lactic acid bacteria were isolated from different fruit samples by standard plate count method, using de Mann, Rogosa and Sharpe (MRS) agar media. The dominant and recurring colonies were purified by repeated streaking on agar medium. Thus fifteen LAB isolates obtained were subjected to various morphological and biochemical characterization for identification. The data pertaining to the source and isolates are presented in Table 1.

3.1.1 Morphological characterization of lactic acid bacterial isolates

Phenotypic identifications are the first step in the selection of probiotic bacteria. Hence, fifteen isolates of lactic acid bacterial isolates obtained were primarily subjected to various morphological characterization presented in Table 1. In the present investigation, all the colonies of lactic acid bacterial isolates were small in size, smooth appearance, white to creamy white, round and translucent with convex elevation (Plate 1). Similar observations were also reported by Yelnetty *et al.* (2014) [32]. Sixteen LAB isolates were isolated and observed rod shape, small to medium size and light creamy white to white colour colonies. Pampangouda (2021) [20] also observed similar colony morphology. He isolated ten LAB isolates from papaya and carrot, all the colonies were small in size, creamy white to white colour and oval to round in shape.

Table 1: Colony characteristics of lactic acid bacterial isolates from different fruits

SL. No	Source	Isolates	Colony morphological characteristics			
			Colour	Size	Shape	Elevation
1	Watermelon	LAB-1	Milky white	Small	Round	Convex
2		LAB-2	Milky white	Small	Round	Convex
3		LAB-3	Milky white	Small	Round	Convex
4	Fig	LAB-4	Creamy white	Small	Round	Convex
5		LAB-5	Creamy white	Small	Round	Convex
6	Papaya	LAB-6	Creamy white	Small	Round	Convex
7		LAB-7	Creamy white	Small	Round	Convex
8	Custard apple	LAB-8	Creamy white	Small	Round	Convex
9		LAB-9	Creamy white	Small	Round	Convex
10	Banana	LAB-10	Milky white	Small	Round	Convex
11		LAB-11	Milky white	Small	Round	Convex
12		LAB-12	Milky white	Small	Round	Convex
13	Tomato	LAB-13	Creamy white	Small	Round	Convex
14		LAB-14	Creamy white	Small	Round	Convex
15		LAB-15	Creamy white	Small	Round	Convex

3.1.2 Microscopic identification of lactic acid bacterial isolates

In the present study, the lactic acid bacterial isolates were identified based on Bergey's Manual of Determinative Bacteriology.

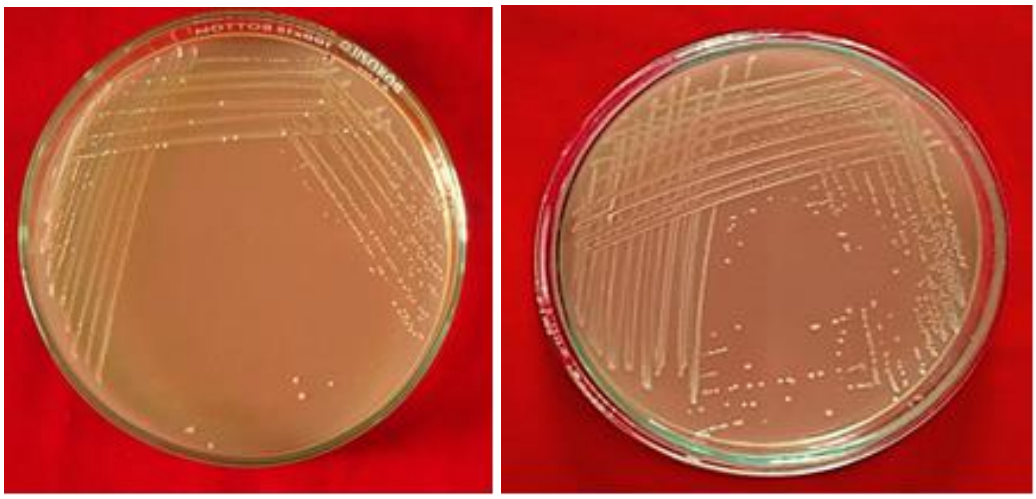
Lactic acid bacterial colonies showing different morphological characteristics on the MRS agar plates were selected for further characterization. The detailed microscopic observations of the isolates are presented in Table 2.

The results showed that the fifteen LAB isolated cells were rod shape, Gram-positive, non-motile and non-endospore producers (Plate 2). Similar results of lactic acid bacterial isolates were reported by Ahmed and Kanwal (2004) [1]. Pampangouda (2021) [20] also observed Gram positive and non-motile LAB isolates in his experiment.

Table 2: Microscopic characterization of lactic acid bacterial isolates from different fruits

Sl. No	Isolates	Gram's reaction	Motility	Cell shape	Cell size	Endospore
1	LAB-1	+	Non motile	Rod shape	0.4 x 2.0	-
2	LAB-2	+	Non motile	Rod shape	0.6 x 1.7	-
3	LAB-3	+	Non motile	Rod shape	0.5 x 1.7	-
4	LAB-4	+	Non motile	Rod shape	0.5 x 1.8	-
5	LAB-5	+	Non motile	Rod shape	0.9 x 1.1	-
6	LAB-6	+	Non motile	Rod shape	0.6 x 1.3	-
7	LAB-7	+	Non motile	Rod shape	0.7 x 1.6	-
8	LAB-8	+	Non motile	Rod shape	0.5 x 1.3	-
9	LAB-9	+	Non motile	Rod shape	0.7 x 1.6	-
10	LAB-10	+	Non motile	Rod shape	0.9 x 1.6	-
11	LAB-11	+	Non motile	Rod shape	0.4 x 1.1	-
12	LAB-12	+	Non motile	Rod shape	0.5 x 1.6	-
13	LAB-13	+	Non motile	Rod shape	0.5 x 1.1	-
14	LAB-14	+	Non motile	Rod shape	0.3 x 1.2	-
15	LAB-15	+	Non motile	Rod shape	0.9 x 1.1	-

Note: +: Positive, -: Negative



LAB-12 LAB-14

Plate 1: Pure cultures of lactic acid bacterial isolates and reference strain on MRS agar

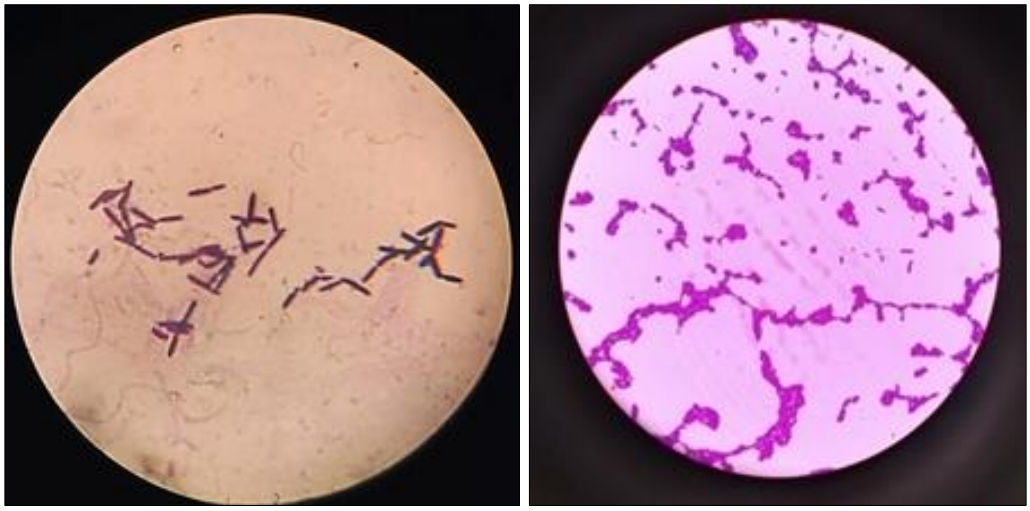


Plate 2: Microscopic characterization of lactic acid bacterial isolates

Biochemical characterization of lactic acid bacterial isolates

Biochemical characterization of microorganisms is important for many reasons as it helps to classify and identify bacteria based on their nutritional and metabolic capabilities.

Biochemical characterization of lactic acid bacterial isolates includes catalase test, gas production, acid production, starch hydrolysis, gelatin hydrolysis, casein hydrolysis and dextran production. These primary tests were carried out following standard protocols and the results are presented in Table 3.

Table 3: Biochemical characterization of lactic acid bacterial isolates from different fruits

SL. No.	Isolates	Biochemical characteristics						
		Catalase test	Gas production	Acid production	Starch hydrolysis	Gelatin hydrolysis	Casein hydrolysis	Dextran production
1	LAB-1	–	–	+	+	+	+	+
2	LAB-2	–	–	+	+	+	+	+
3	LAB-3	–	–	+	+	+	+	+
4	LAB-4	–	–	+	+	+	+	+
5	LAB-5	–	–	+	+	+	+	+
6	LAB-6	–	–	+	+	+	+	+
7	LAB-7	–	–	+	+	+	+	+
8	LAB-8	–	–	+	+	+	+	+
9	LAB-9	–	–	+	+	+	+	+
10	LAB-10	–	–	+	+	+	+	+
11	LAB-11	–	–	+	+	+	+	+
12	LAB-12	–	–	+	+	+	+	+
13	LAB-13	–	–	+	+	+	+	+
14	LAB-14	–	–	+	+	+	+	+
15	LAB-15	–	–	+	+	+	+	+

Note: +: Positive, -: Negative.

3.2.1 Catalase test

Effervescence was not observed with addition of three percent hydrogen peroxide solution to the inocula of lactic acid bacteria placed on the slide because of the presence of peroxidases. The 15 isolates were catalase negative and results are presented in Table 3 (Plate 3). The results are similar to that of research work carried out by Sirisha *et al.* (2021) [25]. She isolated 30 lactic acid bacteria from the fermented foods, out of 30 isolates 20 isolates were catalase negative.

3.2.2 Gas and acid production:

Gas and acid production is an important characteristic used in the differentiation of lactic acid bacterial cultures based on the mode of sugar fermentation under standard conditions. Colour of lactose broth with bromocresol purple as pH indicator changed from violet to yellow indicated the acid production and bubble formation in Durham's indicates the positive results for gas production. All the fifteen isolates were positive for acid production and negative for gas production (Plate 9). So, all the isolates belong to the homo fermentative lactic acid bacteria group which coverts sugars almost quantitatively to lactic acid. The experimental results are in conformation with observations recorded by Khalil and Anwar (2016) [16]. They characterised and screened LAB isolates for the acid production. Isolates like *Streptococcus uberis, L. cellobiosus* and *L. delbrueckii* produced highest quantity of acid after 6 h of incubation. Whereas some of the LAB isolates from fermented products were positive and some were negative for gas formation (Monika *et al.*, 2017) [18].

3.2.3 Starch hydrolysis

Determination of amylase production potentials of LAB isolates was carried out using starch hydrolysis test. Occurrence of clear zone around the streaked line was

considered as positive for starch hydrolysis. All the fifteen isolates were positive for starch hydrolysis indicating that they are able to utilize starch as carbon source (Plate 5). The results of experiment are similar to that of research work carried out by Anusha (2018) [3] where, all LAB isolates were able to produce amylase to hydrolyse the starch.

3.2.4 Gelatin hydrolysis

All the fifteen LAB isolates were found positive for gelatin liquefaction indicating the gelatinase enzyme activity (Plate 9). The results were in accordance with Khalil and Anwar (2016) [16]. They found that, isolates like *Streptococcus thermophiles* and *Streptococcus faecium* were able to liquefy the gelatin.

3.2.5 Casein hydrolysis

Occurrence of clear zone around streaked line was considered as positive for casein hydrolysis. All the isolates were positive for casein hydrolysis, that indicating isolates were able to use casein as nitrogen source (Plate 7). The results of this experiment are similar to that of research work carried out by Anusha (2018) [3]. Isolated lactic acid bacterial isolates were able to hydrolyse casein.

3.2.6 Dextran production

All fifteen isolates produced slime like substance after 48 h of inoculation on sucrose agar medium. Hence, all isolates were positive for dextran production (Plate 6). The results of this experiment are similar to that of research work carried out by Anusha (2018) [3]. She found that the lactic acid bacterial isolates were positive for exopolysaccharide (dextran) production.

Plate 3: Catalase test

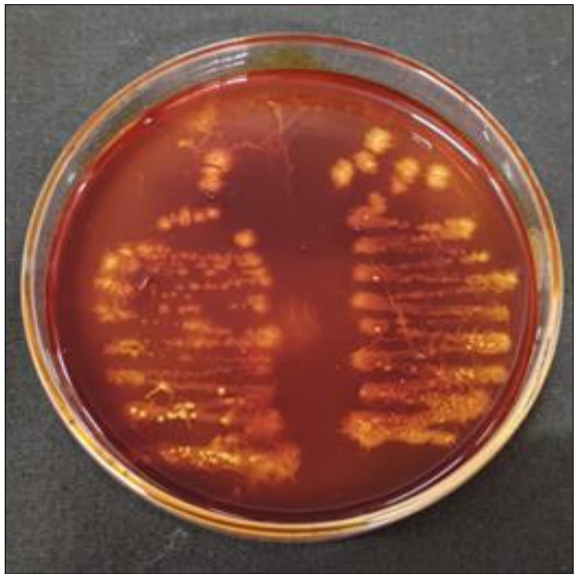


Plate 4: Starch hydrolysis test

Plate 5: Dextran production test

Plate 6: Casein hydrolysis test

Plate 7: Gas and acid production test by lactic acid bacterial isolates

Plate 8: Gelatin hydrolysis by lactic acid bacterial isolates

3.2.7 Utilization of different sugars by lactic acid bacterial isolates

The details of carbohydrates fermentation by lactic acid bacterial isolates are furnished in Table 4. The change in the colour of broth indicates sugar fermentation by isolates (Plate 9).

Plate 9: Utilization of carbon source by lactic acid bacterial isolates

All lactic acid bacterial isolates grew well in carbon sources *viz*. glucose, dextrose, sucrose, galactose, lactose, maltose and mannitol. Isolates like LAB-4, LAB-5, LAB-9, LAB-12 and LAB-14 grew well in fructose whereas, other isolates were unable to use fructose as their carbon source. However, all the fifteen isolates were failed to grow in inositol, esculin, cellobiose, xylose and arabinose.

The experimental results are in conformation with observations recorded by Patil *et al.* (2010) [21]. They isolated lactic acid bacteria from cucumber and curd, all the isolates were able to utilize carbon sources like lactose, dextrose, sucrose, maltose, galactose and fructose. Khalil and Anwar (2016) [16] isolated lactic acid bacteria from milk and yoghurt. In their study they found that Strain Y-GW and Y11a reflected similar carbohydrate fermentation pattern with exception in arabinose and inulin fermentation.

Table 4: Utilization of different carbon source by lactic acid bacterial isolates from different fruits

Sl. No	LAB isolates	Carbon source												
		Glucose	Dextrose	Lactose	Sucrose	Galactose	Fructose	Esculin	Inositol	Maltose	Mannitol	Cellobiose	Xylose	Arabinose
1	LAB-1	+	+	+	+	+	–	–	–	+	+	–	–	–
2	LAB-2	+	+	+	+	+	–	–	–	+	+	–	–	–
3	LAB-3	+	+	+	+	+	–	–	–	+	+	–	–	–
4	LAB-4	+	+	+	+	+	+	–	–	+	+	–	–	–
5	LAB-5	+	+	+	+	+	+	–	–	+	+	–	–	–
6	LAB-6	+	+	+	+	+	–	–	–	+	+	–	–	–
7	LAB-7	+	+	+	+	+	–	–	–	+	+	–	–	–
8	LAB-8	+	+	+	+	+	–	–	–	+	+	–	–	–
9	LAB-9	+	+	+	+	+	+	–	–	+	+	–	–	–
10	LAB-10	+	+	+	+	+	–	–	–	+	+	–	–	–
11	LAB-11	+	+	+	+	+	–	–	–	+	+	–	–	–
12	LAB-12	+	+	+	+	+	+	–	–	+	+	–	–	–
13	LAB-13	+	+	+	+	+	–	–	–	+	+	–	–	–
14	LAB-14	+	+	+	+	+	+	–	–	+	+	–	–	–
15	LAB-15	+	+	+	+	+	–	–	–	+	+	–	–	–

Note: +: Positive, -: Negative.

3.2.8 Growth of lactic acid bacterial isolates in different temperature

Lactic acid bacterial isolates inoculated on MRS agar were further characterized by conducting the following test. Growth response at different temperatures was done by keeping the inoculated plates in the incubator adjusted to 4 °C, 20 °C, 37 °C, 45 °C and 55 °C for 48 hours. The data pertaining to the growth of LAB isolates at different temperatures are depicted in Table 5.

The fifteen isolates were chosen for their growth response when exposed to different temperatures. All the isolates showed dense growth at 37 °C and medium growth was observed at 45 °C. However, all the isolates were unable to show their growth on agar medium when incubated at 4 °C

and 55 °C. Isolates LAB-7, LAB-10 and LAB-11 were able to grow at 20 °C whereas, other isolates were unable grow at this temperature. These observations are on par with research conducted by Khalil and Anwar (2016) [16]. They found that LAB isolates Y8 and Y19B produced luxuriant growth at 27 °C to 45 °C, Strain Y1A showed optimum growth at 37 °C and 45 °C with little growth at 10 °C.

Table 5: Growth of lactic acid bacterial isolates at different temperature

Sl. No	Isolates	Temperature				
		4 ℃	20 ℃	37 ℃	45 ℃	55 ℃
1	LAB-1	-	-	+++	+	-
2	LAB-2	-	-	+++	+	-
3	LAB-3	-	-	+++	+	-
4	LAB-4	-	-	+++	+	-
5	LAB-5	-	-	+++	+	-
6	LAB-6	-	-	+++	+	-
7	LAB-7	-	+	+++	+	-
8	LAB-8	-	-	+++	+	-
9	LAB-9	-	-	+++	+	-
10	LAB-10	-	+	+++	+	-
11	LAB-11	-	+	+++	+	-
12	LAB-12	-	-	+++	+	-
13	LAB-13	-	-	+++	+	-
14	LAB-14	-	-	+++	+	-
15	LAB-15	-	-	+++	+	-

Note: +: Medium growth, +++: Dense growth, -: No growth.

3.3 Screening of lactic acid bacterial isolates for probiotic properties

Lactic acid bacterial isolates were screened for probiotic properties like pH tolerance, bile salt tolerance, NaCl tolerance and phenol tolerance.

3.3.1 Evaluation of lactic acid bacterial isolates for pH tolerance

The survival of probiotics in gastrointestinal tract is always considered as prime determinant apart from the health benefits. In stomach, under harsh acidic condition (pH 1.5-2.5) most of the ingested microbes get inactivated or loss their viability. Hence, survivability at low pH conditions is an important probiotic property (Veerapagu and Jeya 2017) [30]. The pH tolerance of lactic acid bacterial isolates were carried out at four different pH (2, 2.5, 3 and 6.5) levels. The data pertaining to pH tolerance of lactic acid bacterial isolates is furnished in Table 6. The experiment was conducted to evaluate the effect of pH at 2.0, 2.5 and 3.0. The results showed that all the 15 bacterial isolates grew even at a low pH of 2. The probiotic growth in terms of optical density decreased with the decrease in pH. All the LAB isolates grew better at 3.0 pH, the highest OD value was recorded by isolate LAB-12 (0.35) followed by LAB-14 (0.33) after 24 h. The least growth was recorded by LAB-11 (0.15) at pH 3.0.

Similar observations were also found by Grosu-Tudor and Zamfir (2012) [10]. They isolated six lactic acid bacterial isolates from Romanian fermented vegetables and isolates were investigated for acid tolerance at pH 2.0, 3.0 and 4.0. All the six isolates were resistant to pH 3.0 and 4.0 after 24 h of incubation. Handa and Sharma (2016) [12] studied the effects of low pH (1, 2 and 3) on the growth and viability of *L. plantarum* F222, which showed remarkable survival of 90.4%, after 180 min at pH 1.0, whereas, at pH 2.0 and 3.0 it showed survival of 97.2% and 99.4%. Acidity is the most detrimental factor affecting growth and viability of lactic acid bacteria because their growth was down significantly below pH 4.5.

Table 6: Screening of lactic acid bacterial isolates for pH tolerance

Sl. No.	Isolates	OD (600 nm) value at 24 h			
		2 pH	2.5 pH	3 pH	6.5 pH
1	LAB-1	0.15	0.17	0.19	1.04
2	LAB-2	0.14	0.15	0.17	0.89
3	LAB-3	0.12	0.14	0.16	0.97
4	LAB-4	0.10	0.13	0.19	1.23
5	LAB-5	0.11	0.14	0.17	1.11
6	LAB-6	0.13	0.17	0.25	0.98
7	LAB-7	0.11	0.15	0.28	1.04
8	LAB-8	0.12	0.19	0.20	1.13
9	LAB-9	0.15	0.18	0.21	0.87
10	LAB-10	0.12	0.15	0.19	1.10
11	LAB-11	0.10	0.12	0.15	1.08
12	LAB-12	0.23	0.32	0.35	1.96
13	LAB-13	0.16	0.17	0.20	1.20
14	LAB-14	0.21	0.29	0.33	1.60
15	LAB-15	0.11	0.14	0.31	1.18
16	*Lactobacillus acidophilus*	0.20	0.27	0.30	1.56

3.3.2 Evaluation of lactic acid bacterial isolates for bile salt tolerance: Tolerance to bile salt is important for the probiotic strains to grow and survive in the digestive tract. Hence, bile salt tolerance test for LAB isolates was conducted. The results of bile salt tolerance of lactic acid bacterial isolates are furnished in Table 7 illustrated. The experiments were conducted to evaluate the effect of bile salt concentrations at 0, 0.1, 0.2 and 0.3 percent. Concentration of 0.3 percent of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Goldin *et al.*, 1992) [9].

The growth of isolates was measured in terms of OD at 600 nm. The results recorded that all LAB isolates grew better at 0.3 percent bile salt concentration, the highest OD value was recorded by isolate LAB-12 (0.49) followed by LAB-14 (0.41) and the least recorded by LAB-15 (0.29) after 24 h of incubation.

The results are on par with the observations recorded by Kim *et al.* (1999) [17]. They examined the effect of bile concentration in the range of 0 to 0.4% on *Lactobacillus lactis* and reported good growth upto 0.3% but growth was inhibited at 0.4%. Grosu-Tudor and Zamfir (2012) [10] isolated six lactic acid bacteria from Romanian fermented vegetables and were investigated for bile salt tolerance (0.3% and 0.5%). All isolates were showed good growth at 0.3 percent concentration after 24 h of incubation. At 0.5 percent concentration, only two isolates proved to be resistant to bile salts. Srinu *et al.* (2013) [27] isolated six lactic acid bacteria and isolates showed good survivability at high bile salt concentration (0.3-2.0%).

Table 7: Screening of lactic acid bacterial isolates for bile salt tolerance

SL. No.	Isolates	OD (600 nm) value at 24 h			
		0%	0.1%	0.2%	0.3%
1	LAB-1	1.11	0.53	0.41	0.31
2	LAB-2	1.06	0.51	0.47	0.39
3	LAB-3	1.17	0.55	0.40	0.30
4	LAB-4	1.43	0.50	0.43	0.38
5	LAB-5	1.12	0.51	0.47	0.31
6	LAB-6	0.98	0.53	0.46	0.39
7	LAB-7	1.44	0.50	0.45	0.38
8	LAB-8	1.02	0.49	0.41	0.33
9	LAB-9	0.93	0.51	0.43	0.36
10	LAB-10	1.80	0.52	0.46	0.39
11	LAB-11	1.89	0.50	0.47	0.36
12	LAB-12	3.12	0.68	0.57	0.49
13	LAB-13	1.26	0.51	0.51	0.37
14	LAB-14	2.97	0.62	0.56	0.41
15	LAB-15	1.28	0.53	0.79	0.29
16	*Lactobacillus acidophilus*	2.88	0.60	0.55	0.40

3.3.3 Evaluation of lactic acid bacterial isolates for NaCl tolerance

The NaCl test for lactic acid bacterial isolates was conducted and results are furnished in Table 8 illustrated. The experiment was conducted to evaluate the effect of salt concentrations like 0, 2, 4 and 6 percent. The growth of isolates was measured in terms of OD at 600 nm. The results recorded that all LAB isolates grew better even at 6 percent NaCl concentration, the highest OD value was recorded by isolate LAB-12 (0.81) followed by LAB-14 (0.62) and the least was recorded by LAB-3 (0.10) after 24 h of inoculation.

The results are in conformation with the observations recorded by Reuben *et al.* (2019) [22]. They investigated the tolerance of LAB isolates to NaCl concentration. LAB isolates grew well at 6.5% NaCl concentration with OD value more than 0.500 and there was very weak growth at 10.0% NaCl.

Table 8: Screening of lactic acid bacterial isolates for NaCl tolerance

SL. No.	Isolates	OD (600 nm) value at 24 h			
		0%	2%	4%	6%
1	LAB-1	1.02	1.12	0.19	0.14
2	LAB-2	0.90	1.16	0.78	0.16
3	LAB-3	1.17	1.26	0.51	0.10
4	LAB-4	1.23	1.12	0.67	0.28
5	LAB-5	2.42	1.29	0.85	0.36
6	LAB-6	1.18	0.44	0.06	0.12
7	LAB-7	1.54	0.70	0.79	0.21
8	LAB-8	1.06	0.99	0.50	0.18
9	LAB-9	0.87	0.98	0.30	0.14
10	LAB-10	2.35	1.25	0.55	0.18
11	LAB-11	1.92	0.94	0.63	0.29
12	LAB-12	3.19	1.64	1.48	0.81
13	LAB-13	1.20	1.05	1.07	0.36
14	LAB-14	2.99	1.44	1.20	0.62
15	LAB-15	1.06	0.97	0.17	0.28
16	*Lactobacillus acidophilus*	2.83	1.40	1.12	0.60

3.3.4 Evaluation of lactic acid bacterial isolates for phenol tolerance: Phenol tolerance test for lactic acid bacterial isolates was conducted and results are furnished in Table 9. The experiment was conducted to evaluate the phenol tolerance of lactic acid bacterial isolates at 0, 0.1, 0.2 and 0.3 percent. The results for phenol tolerance of LAB isolates grew better at 0.3 percent, the highest OD value was recorded by LAB-12 (0.79) followed by LAB-14 (0.69) and the least growth was recorded by LAB-1 (0.30) after 24 h of incubation.

The results are in conformation with the observations recorded by Reuben *et al.* (2019) [22]. They investigated the tolerance of LAB isolates to phenol concentration from 0.1-0.4%. LAB isolates were able to tolerate 0.4% phenol with OD values of more than 1.0. The viability of all the isolates showed significant difference with respect to phenol concentration.

3.4 Antagonistic activity

Antagonistic activity against indicator strain (pathogen) *Escherichia coli ATCC 10536*, was tested with the efficient lactic acid bacterial isolates designated as LAB-12, LAB-14 and *Lactobacillus acidophilus* (reference strain). Cell free supernatant adjusted to pH 6.5 was used to examine the inhibition activity of cell-free supernatants of the isolates against the pathogenic bacteria. Lactic acid bacterial isolates as well as reference strain exhibited the zone of clearance in each plate indicating the antagonistic activity against *Escherichia coli*. The zone of inhibition showed by LAB-12, LAB-14 and *Lactobacillus acidophilus* was 9.5 mm, 5.2 mm and 12 mm respectively (Plate 10). The zone of inhibition was measured in mm and tabulated in Table10.

The experimental results are in confirmation with the results reported by Reuben *et al.* (2019) [22], they conducted an experiment to assess the antagonistic activity of LAB isolates using the agar well diffusion assay and 18 isolates displayed inhibition activities. Wider zones of inhibition were exhibited by LAB isolates against *E. coli* ranging from 17±0 to 20.0±0 mm while the least zones of inhibition ranging from 12.5±0.71 to 17±0 mm. Fahathabad and Eslamifer (2011) [7]. They isolated lactic acid bacterial strains from the tea leaves (*Camellia sinensis*) and they examined the antagonistic activity of the identified *L. paraplantarum* and it inhibited the growth of *Salmonella typhi, E. coli, Staphylococcus aureus, Enterococcus fecalis* and *Citrobacter* sp. successfully. Furthermore, this strain could produce extracellular tannase which is beneficial property for probiotic activity.

Table 9: Antagonistic activity of efficient lactic acid bacterial isolates against *E. coli*

Sl. No	Isolates	Zone of inhibition (mm) against *E. coli* (test pathogen)
1	LAB-12	9.50
2	LAB-14	5.20
3	*Lactobacillus acidophilus*	12.00

3.5 Antibiotic susceptibility

The antibiotic susceptibility of lactic acid bacterial species has received high attention, which reflects their status as non-pathogenic commensal bacteria that enjoy a time honoured reputation as health promoters in the human gastrointestinal tracts (Salminen *et al.*, 1993) [23].

Lactic acid bacterial isolates designated as LAB-12, LAB-14 and *Lactobacillus acidophilus* (reference strain) were assayed for their susceptibility to 6 antibiotics. Disc diffusion method was used to determine the antibiotic susceptibility. Antibiotic discs (Himedia) were used consists of ampicillin (10 µg), amoxyclav (30 µg), cephataxime (30 µg), co-trimoxazole (25 µg), gentamicin (10 µg) and tobramycin (10 µg). The plates were incubated at 37 ℃ for 24 hours.

They exhibited high susceptibility to 5 antibiotics including inhibitors of cell wall synthesis (ampicillin, amoxyclav, Cephataxime), protein synthesis (gentamicin), nucleic acid synthesis (co-trimoxazole, and metronidazole) and an aminoglycoside (tobramycin). In addition, all the isolates were resistant to amoxyclav. LAB-12 and LAB-14 including reference strain were highly susceptible to ampicillin (10 µg) and co-trimoxazole (25 µg) with wider zone of inhibition 41, 36, 39 and 34, 24 28 respectively (Plate 11). Furthermore, results confirm that, the susceptibility of lactic acid bacterial isolates as non-pathogenic. Hence, can be used for the fermentation of watermelon juice. The data related to the susceptibility of lactic acid bacterial isolates to antibiotics are mentioned in the Table 10.

The present finding agrees with Justo *et al.* (2013) [15] they concluded that *Lactobacillus* species tend to be more susceptible to antibiotics that inhibit the synthesis of proteins, indicating their status as non-pathogenic probiotic bacteria.

Table 10: Evaluation of antibiotic susceptibility for efficient lactic acid bacterial isolates

		Zone of inhibition (mm)					
Sl. No	Isolates	A (10)µg	Ac (30)µg	Ce (30)µg	Co (25)µg	G (10)µg	Tb (10)µg
1	LAB-12	41	2.2	12	34	28	16
2	LAB-14	36	0	10	24	22	11
3	*Lactobacillus acidophilus*	39	0	18	28	25	14

Note: A - Ampicillin, Ac - Amoxyclav, Ce - Cephotaxime, Co - Co-Trimoxazole, G - Gentamicin, Tb - Tobramycin

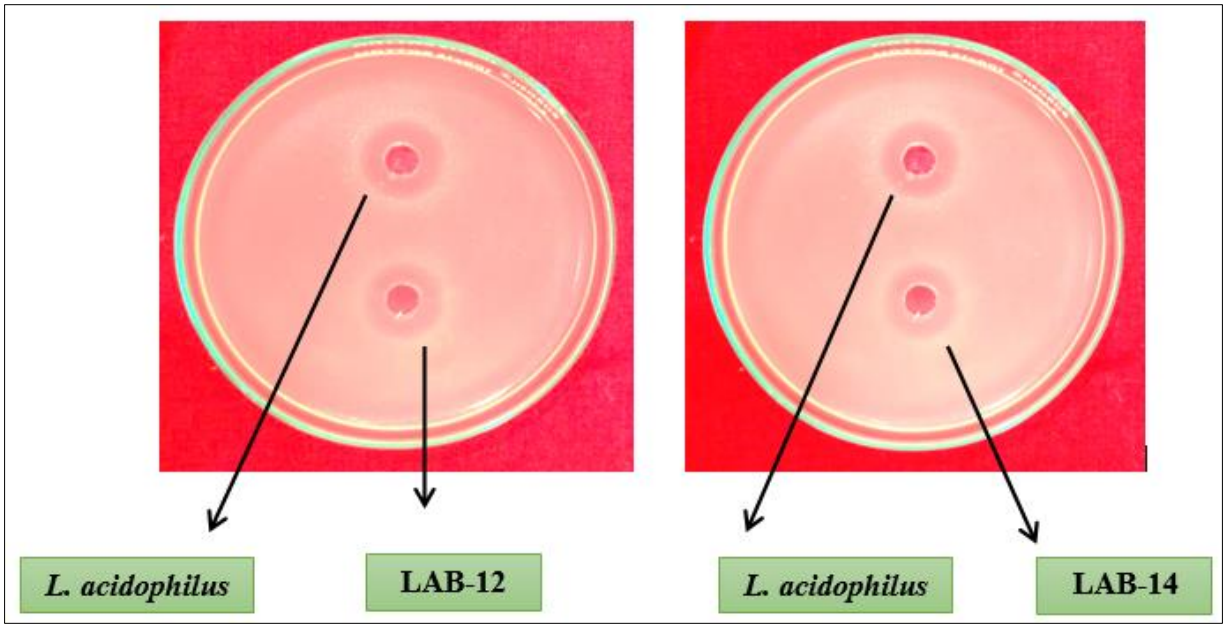


Plate 10: Antagonist activity of LAB-12, LAB-14 and *L. Acidophilus* against *E. coli*

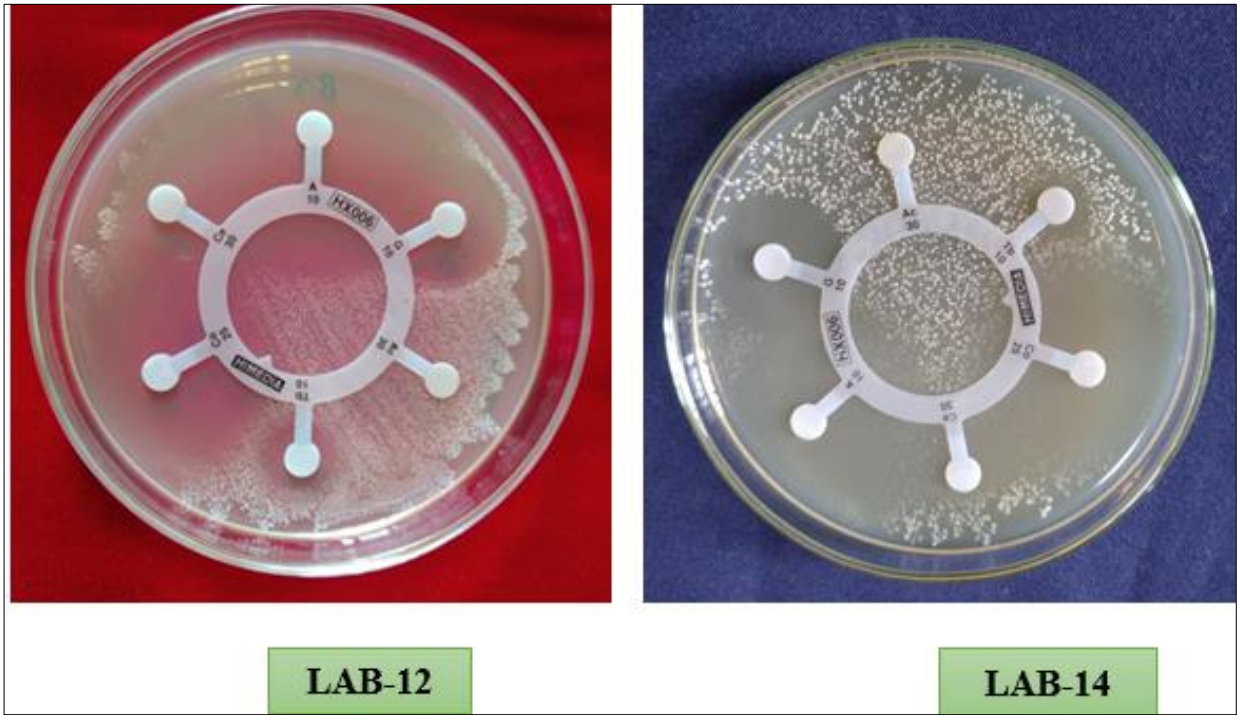


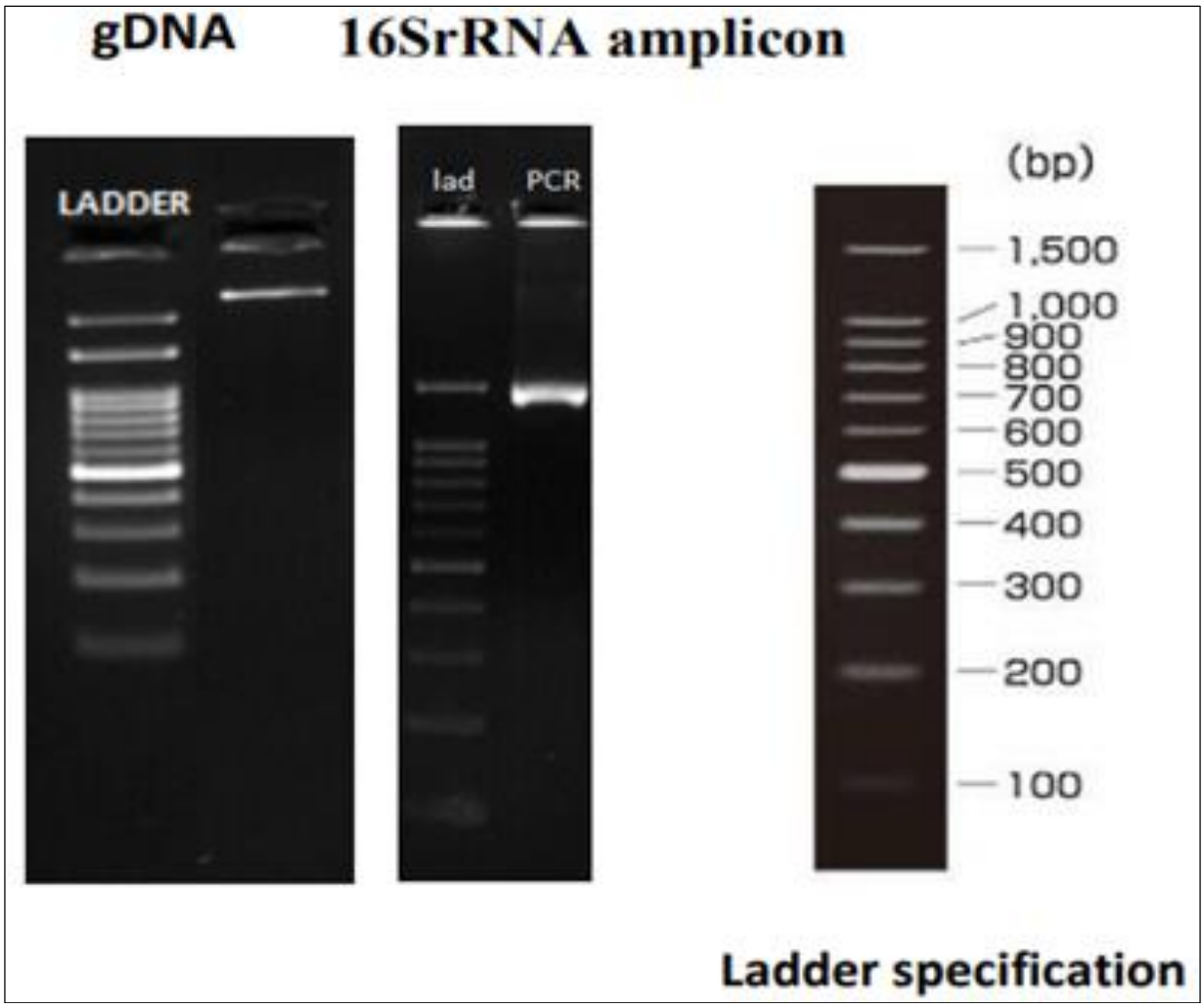
Plate 11: Antibiotic susceptibility of LAB-12 and LAB-14

3.6 Molecular identification of LAB-12 isolate

The molecular identification of LAB strains was conducted by 16S rRNA, because similar physiological profiles are often shown by phylogenetically related LAB species, which makes it inadequate to only rely on biochemical methods for identification. In the present investigation, fifteen isolates of lactic acid bacteria were isolated from different fruits. Out of fifteen isolates LAB-12 was found to show the promising probiotic characteristics such as low pH tolerance, bile salt tolerance, NaCl tolerance and phenol tolerance. Hence, LAB-12 isolate was further subjected to molecular characterization to identify at species level

Amplification of gDNA and 16S rRNA

Fragment of 16S rRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants.



Forward Sequence data

TGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACG
CATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
GTTGAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGC
GAAGAACCTTACCAGGTCTTGACATCTTCTGCCAAT
CTTAGAGATAAGACGTTCCCTTCGGGGACAGAATG
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATTATCAGTTGCCAGCATTCAGTTGGGCACTCT
GGTGAGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGACCT
GGGCTACACACGTGCTACAATGGACGGTACAACGA
GTTGCGAAGTCGTGAGGCTAAGCTAATCTCTTAAA
GCCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCC
TACATGAAGTTGGAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACACCATGAGAGTTTGTAACACC
CAAAGCCGGTGAGATAACCTTCGGGAGTCAGCCGT
CTAAGGTGGGACAGATGATTAGGGTGAAGTCGTAC
AAGGGGGAAACCCGGGACGA

Reverse Sequence Data

AGGGCGGAAACCCTCCAACACTTAGCACTCATCGT
TTACGGCATGGACTACCAGGGTATCTAATCCTGTTC
GCTACCCATGCTTTCGAGCCTCAGCGTCAGTTACAG
ACTAGACAGCCGCCTTCGCCACTGGTGTTCTTCCAT
ATATCTACGCATTCCACCGCTACACATGGAGTTCCA
CTGTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCGA
TGCACTTCTCCGGTTAAGCCGAAGGCTTTCACATCA
GACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCA
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CGCCGCGGGATCATCCAGAAGTGATAGCCGAAGCC
ACCTTTCAAACAAAATCCATGCGGATTTTGTTGTTA
TACGGTATTAGCACCTGTTTCCAAGTGTTATCCCCT
GCTTCTGGGCAGATTTCCCACGTGTTACTCACCAGT
TCGCCACTCGCTTCATTGTTGAAATCAGTGCAAGCA
CGTCATTCAACGGAAGCTCGTTCGACTTGCATGTAT
TAGGCATGCCGCCAGCGTTCGTCCTGAGCCAGAAT
CAAACTCTAAAA

Phylogenetic tree

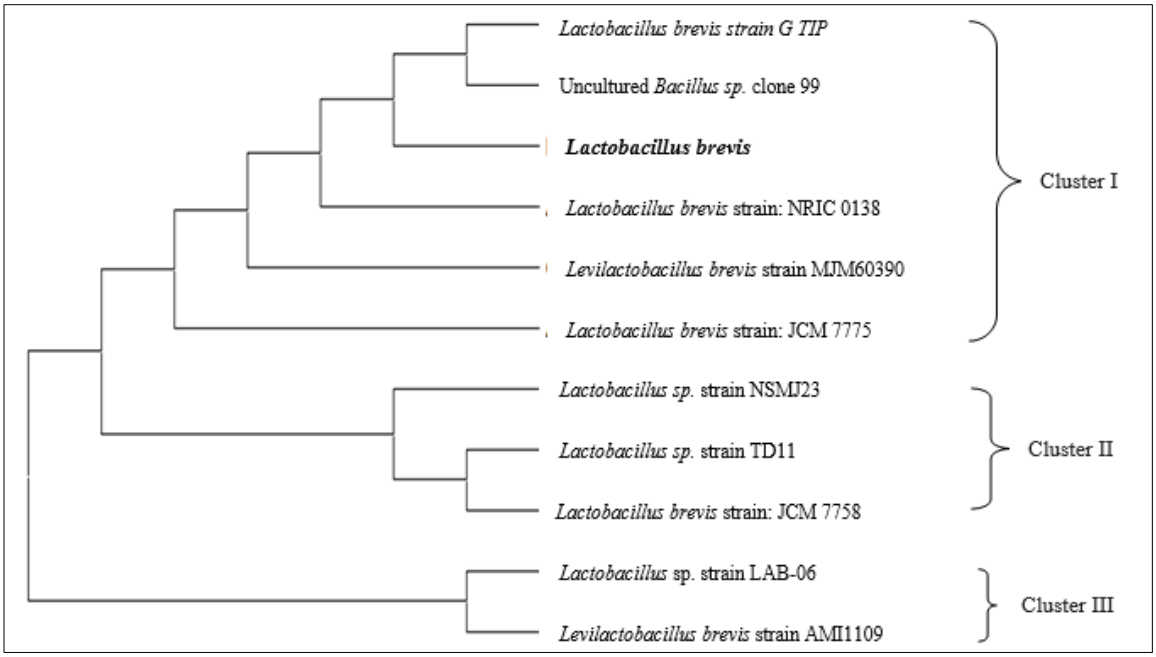

Fig 1: Phylogenetic tree analysis

Results and Discussion

The phylogenetic tree was constructed by using MEGA 11 and the tree was mid rooted. The tree was clustered into three groups, cluster I contains *Lactobacillus brevis strain G TIP*, Uncultured *Bacillus* sp. clone 99, *Lactobacillus brevis*, *Lactobacillus brevis* strain: NRIC 0138, *Levilactobacillus brevis* strain MJM60390, *Lactobacillus brevis* strain: JCM 7775, the second cluster contains *Lactobacillus* sp. strain NSMJ23, *Lactobacillus* sp. strain TD11, *Lactobacillus brevis* strain: JCM 7758 and third cluster contains *Lactobacillus* sp. strain LAB-06 and *Levilactobacillus brevis* strain AMI1109. Highest similarity was observed in the cluster I.

Sample which was labelled as LAB-12 was found as *Lactobacillus brevis*, which showed high similarity based on nucleotide homology and phylogenetic analysis.

Conclusion

The shift in consumer preferences towards functional foods, particularly those enriched with probiotics, reflects a growing awareness of the link between diet and health. This trend has spurred significant interest from industries in isolating and evaluating probiotic strains, especially lactic acid bacteria (LAB). However, ensuring the viability and stability of probiotics in food products throughout processing and storage remains a critical challenge for manufacturers. The study detailed here, "Isolation, characterization and screening of lactic acid bacteria (LAB) from fruits for the production of probiotic beverage," addresses these complexities by meticulously isolating and identifying LAB strains, such as *Lactobacillus brevis*, with high potential for use in novel probiotic beverages. This research is a crucial step towards developing effective, quality-controlled functional foods that offer substantial health benefits to consumers.

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