American Journal of BioScience
2022; 10(3): 118-130
http://www.sciencepublishinggroup.com/j/ajbio
doi: 10.11648/j.ajbio.20221003.14
ISSN: 2330-0159 (Print); ISSN: 2330-0167 (Online)

Exploration of Probiotic Potential of Lactic Acid Bacteria Isolated from Different Food Sources

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To cite this article:
Vikas Jha, Chinmayi Sarang, Dattatray Sawant, Kavita Nalawade, Vrushali Dhamapurkar, Navdeep Kaur, Kabir Thakur, Shruti Amin, Pratyusha Mane, Aishwarya Marath. Exploration of Probiotic Potential of Lactic Acid Bacteria Isolated from Different Food Sources. *American Journal of BioScience*. Vol. 10, No. 3, 2022, pp. 118-130. doi: 10.11648/j.ajbio.20221003.14

Received: June 1, 2022; **Accepted**: June 20, 2022; **Published**: June 30, 2022

Abstract: The growing food adulteration and increasing antibiotic intake has led to deterioration of gut health in humans. Probiotics are feed supplements in the form of live microorganisms that have a positive impact on the host by improving intestinal microbial balance. They offer a variety of important and documented beneficial effects, including the prevention of various disorders and modulating the immune response. Lactic acid bacteria (LAB) are class of organisms possessing probiotic potential and hence, can be used for producing novel therapeutics for tackling the above-mentioned health concerns. This work focuses on isolation and exploration of probiotic potential of LAB which can confer health benefits when consumed in an adequate manner. A total of 48 bacteria were isolated on the Lactobacillus selection medium (LSB) from fourteen different food sources such as milk, milk-based products, and non-dairy fermented foods. The isolates were assessed for tolerance against various physiochemical conditions, antibiofilm activity, antimicrobial activity, antibiotic resistance, auto and co-aggregation, and hemolytic activity. Six potential LAB isolates exhibited tolerance against a higher concentration of bile acid, NaCl, phenol and stimulated gastric juice. The isolates were negative for gelatinase and hemolytic activity. All the six isolates showed antimicrobial activity against the test organisms, and antimicrobial resistance against the selected antibiotics. In addition to this, all LAB isolates indicated co-aggregation with test pathogens and adhesion to silicone oil and paraffin oil, respectively. Further, these isolates were found to be biofilm producers and exhibited anti-biofilm activity. The Lactic acid bacteria were found to fulfill the basic requirements of a probiotic bacteria and hence, can be used for human consumption resulting in various health benefits.

Keywords: Acid Tolerance, Antibiofilm, Antimicrobial, Fermented Foods, Lactic Acid Bacteria, Probiotics, Tolerance

1. Introduction

Probiotics are living organisms providing health benefits when consumed in an adequate amount. Lactic acid bacteria are non-sporulating Gram-positive, strictly fermentative bacteria with lactic acid as the main metabolic end product of carbohydrate fermentation [1]. *Lactobacillus, Bifidobacterium, Streptococcus and Enterococcus* genera are some of the commercially available probiotic bacteria [2]. LAB are found in a wide range of habitats, including oral cavities, fermented foods, vaginal tracts and Gastrointestinal tract (GI) of humans and animals. They offer various health benefits such as improved digestion, good immune system, curing inflammatory bowel diseases, easing of constipation, firming the mucosal barrier, treatment of antibiotic-associated and acute diarrhea, allergy-related conditions, hypertension and diabetes [3]. They are characterized by the production of lactic acid, antioxidants, organic acids, and antimicrobial compounds, controlling and improving intestinal microbial balance [3]. LAB are Generally Recognized as Safe (GRAS) as their consumption contributes to the digestive and metabolic processes, as well as

modulation of the local and systemic immune response [1]. It is necessary for probiotic strains to possess tolerance to gastrointestinal conditions such as higher concentration of bile, NaCl, phenol and varying pH. In addition to these prerequisites, they should also exhibit antimicrobial activity, intestinal mucosa adhesion and antibiotic resistance, lactic acid production, antagonism against pathogens, bile salt hydrolysis and absence of virulence genes and hemolytic activity [1, 2].

Extensive research is being conducted on LAB due to their involvement in most fermented foods, as well as their antimicrobial potential and probiotic characteristics including antitumor activity, reduction of lactose intolerance, decrease in serum cholesterol and stabilization of gut microflora. LAB strains can be used to inhibit mycotoxins such as aflatoxins, trichothecenes, and fumonisins from various food products during pre-harvest, production, and storage stages [4]. Due to their therapeutic benefits, lactic acid bacteria are becoming very popular in the dairy industry. Since supplementation of probiotic products lead to improved feed efficiency, growth rate and reduces intestinal infections, they have been widely used to manufacture feed additives to avoid antibiotic resistance [3]. They are widely used in the production of fermented foods and beverages as they provide both sensory qualities to the food and prevent spoilage of food [5].

Incorporating probiotics into a food matrix involves a number of technological hurdles that must be overcome. Temperature, acid, and bile, higher concentrations of specific ions or nutrient depletion, exposure to osmotic and oxidative stress in product matrices, and passage through the gastrointestinal transit are all stressors that can negatively influence the survival and functionality of these bacteria. In order to survive and become available in sufficient quantities to give their health advantages, probiotics must either adapt to such a dynamic environment or be safeguarded. Substantial increase has been observed in infections related to human Gastrointestinal tract due to food adulteration and development of antibiotic resistance by pathogenic strains. These health concerns can be overcome by application of probiotic organisms as they possess the ability to enhance gut health and also contribute in modulating immune response. The objective of the study was to isolate and characterize LAB from food sources like dairy products, non-dairy fermented food sources. After isolation, the isolates were subjected to various tests such as acid tolerance, antibiotic resistance, antimicrobial, antibiofilm activities, aggregation, co-aggregation, hemolytic activity, gelatinase liquefaction and cell surface hydrophobicity to investigate the probiotic potential of lactic acid bacteria.

2. Materials and Methods

2.1. Sample Collection

A total of six isolates were selected from samples such as fermented finger millet batter, fermented maize batter, colostrum milk, yogurt, fermented green chickpeas batter and cheese which were procured from a local market in Mumbai, India. Non-dairy food products were washed and soaked in water for up to six hours, grinded and incubated at room temperature for fermentation, while the dairy products were used directly. A three-day concoction of the samples was then collected for further screening of the lactic acid bacteria. The LAB isolates were detected using method described by Poornachandra Rao *et al.* [6]. Briefly, 30 g of the non-dairy and 20 mL of the dairy samples, were placed in sterile stomacher bags and vigorously homogenized. Initially, 1 mL of the sample stomachate was added to 9 mL of sterile phosphate buffered saline (PBS) and was further serially diluted. Then, 1 mL aliquots of the samples' suitable dilutions were plated onto Lactobacillus selection medium (HI Media, Mumbai, India) plate. The agar plates were incubated at 30°C and 37°C under aerobic and anaerobic conditions for 72 hours. To maintain anaerobic condition, anaerobic jars were used. After incubation, selective colonies were picked and further streaked onto fresh Lactobacillus selection media plates for further purification. Purified bacterial isolates were then maintained in Lactobacillus Selection base (LSB) broth (HI Media, Mumbai, India) at 4°C. The bacterial isolates from the maintained stocks were further analyzed based on gram nature, morphological and biochemical characterization.

2.2. Characterization of Isolates

The colony morphology of the bacterial isolates was determined visually on Lactobacillus Selection Agar base media (HI Media, Mumbai) and their motility was recorded using the hanging drop technique. Gram nature and the colony characteristics were determined via gram staining of the isolates. Initially, the isolates were characterized using certain biochemical tests such as catalase, oxidase, sugar fermentation, indole and H_2S production.

2.3. Identification Using 16S rRNA Sequencing

The molecular identification of the potential candidate was carried out by amplification and sequencing of 16S ribosomal RNA gene. 8F (5′-GGATCCAGACTTTGATYMTGGCTCAG) 907R (5′-CCGTCAATTCMTTTGAGTTT) universal primer were used for amplifying 16S rRNA gene, as per the protocol given by C et al. [7]. Briefly, the genomic DNA of the isolates were extracted using Cetyl trimethyl ammonium bromide (CTAB) buffer as per the method described by Chapela et al. [8] with little modification. The DNA that was extracted was utilized as a template for the PCR reaction. The reaction mixture for PCR was consisting of 2.5 mM MgCl2, 0.2 mM dNTP, 0.5 µM primers, 2.5 unit of Taq DNA polymerase, 50 ng of template DNA and 1X buffer. The reaction mixtures were incubated in thermocycler programmed with an initial denaturation at 96°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 5 minutes. The

resultant amplicons were analyzed on agarose gel for assessment of the amplification and purity. The amplicons were purified using a PCR purification kit and further taken for Sanger sequencing reaction. The FASTA file obtained from sequencing results was aligned with nucleotide database using BLAST Tool. The gene sequences were submitted to GenBank. The accession ID of the isolates are shown in Table 1.

Table 1. *Isolation Source and sequence analysis.*

SR. No.	Isolate	Scientific Name	Source	Gen Bank ID
1	LAB01	*Limosilactobacillus oris*	Fermented Finger millet Batter	MW578541
2	LAB02	*Lentilactobacillus buchneri*	Fermented Maize batter	MW578542
3	LAB03	*Pediococcus pentosaceus*	Colostrum milk	MW578543
4	LAB04	*Lactococcus lactis*	Yogurt	MW578544
5	LAB05	*Levilactobacillus brevis*	Fermented Green chick peas batter	MW578545
6	LAB06	*Lactiplantibacillus plantarum*	Cheese	MW578546

2.4. Characterization and Determination of the Probiotic Potential of LAB

2.4.1. Tolerance to Sodium Chloride, High Bile Salts, High Phenol Concentration and Simulated Gastric Fluid

The Minimum Inhibitory Concentration (MIC) of sodium chloride salt was determined by a 96-well microtiter plate assay for each selected isolate using the method described by Georgieva et al. [9]. Isolates were grown in LSB broth at 37°C for 48 hours. The concentrated NaCl salt solution was prepared separately and autoclaved. The 1% culture of each isolate was inoculated in 10 ml of fresh LSB broth adjusted to concentrations from 1.0% to 10%. The growth obtained at incubation temperature 37°C for 3 hours was hourly monitored spectrophotometrically at an optical density of 620 nm, control (1% inoculum in LSB broth).

Bile tolerance was carried out as described by Pacheco et al. [10]. Minimum Inhibitory Concentration (MIC) of the bile salt was determined using 96-well microtiter plate assay for each selected isolate (0.1%, 0.2%, 0.3%, 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0%). The 1% culture of each isolate grown for 48 hours was added into 10 ml of fresh LSB broth containing 0.1%, 0.2%, 0.3%, 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0% (w/v) bile salt concentration. The growth at incubation temperature 37°C was monitored at optical density 620 nm, at 0, 1, 2, 3, 4 and 5 hour interval. For control, 1% inoculum in LSB broth was used.

Phenol tolerance was carried out using the methodology provided by Mannan et al. [11]. Isolates were grown in LSB broth at 37°C for 48 hours. The 1% culture of each isolate grown for 48 hours was added into 10 mL of fresh LSB broth adjusted to concentrations 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1.0%, 3.0% and 5.0% by using sterile concentrated phenol solution. The growth at incubation temperature 37°C was monitored at optical density 620 nm, at 0, 1, 2 and 3-hours intervals against control (1% inoculum in LSB broth).

The simulated gastric fluid represents the environment for the human stomach acids. It is prepared by dissolving 2.0 g sodium chloride, 3.2 g pepsin, 7.0 mL HCl in 1 L of distilled water [12]. The selected six isolates were tested for gastric acid tolerance. Isolates were grown in LSB broth at 37°C for 48 hours. The 1% culture of each isolate grown for 48 hours was inoculated in 10 mL of simulated gastric fluid. The growth was observed under incubation temperature 37°C for 0, 1, 2, 3 and 6 hours monitored at an optical density of 620 nm, respectively.

2.4.2. Tolerance to Different Ph and Temperature

To evaluate survival at different pH, the 1% culture of isolate grown for 48 hours was inoculated in fresh LSB broth adjusted to pH 1.0, 2.0, 3.0, 4.0, 6.0 and 8.0 with 5N HCl and 1N NaOH. The culture growth was hourly monitored at an optical density of 620 nm for 3 hours against control (1% inoculum in LSB broth) [3].

The selected bacterial isolates were grown in LSB broth at 37°C for 48 hours. 1.0 mL inoculum from each isolate was then transferred onto LSB broth along with bromocresol purple indicator and incubated at varying temperatures, i.e., 25°C, 30°C, 37°C and 47°C for 48-72 hours. Growth was depicted with a color change from purple to yellow. The method followed was described by Samedi et al. [13].

2.4.3. Resistance to Antibiotics

The antibiotic susceptibility of selected isolates was assessed using the antibiotic disc diffusion method and agar well diffusion method as stated by Rose et al. [14]. Amoxycillin, Amikacin, Amphotericin B, Chloramphenicol, Ciprofloxacin, Ceftriaxone, Ceftazidime, Cefotaxime, Fusidic acid, Gentamicin, Imipnem, Kanamycin, Levofloxacin, Methicillin, Nalidixic acid, Nevobiocin, Netilmicin, Ofloxacin, Penicillin, Rifampicin, Trimethoprim, Vancomycin, Voriconazole and Lincomycin were used as test antibiotics. For the antibiotic disc diffusion method, the antibiotic discs were placed on the surface of the LSB media plate and were incubated at 37°C for 48 hours.

2.4.4. Antimicrobial Activity

In this study, the selected LAB isolates were evaluated for antimicrobial activity. The test organisms namely, *Acinetobacter Baumannii, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus mutants,* and *Serratia marcescens* were obtained from MTCC Chandigarh and some laboratory isolates were also used. To screen anti-microbial activity, selected isolates were inoculated in freshly prepared LSB broth and incubated at 37°C for 60-72 hours. At every 6 hours of incubation, the suspension of culture was centrifuged at 7,500rpm for 10 minutes at 4°C [15]. The supernatant was collected carefully in

a fresh tube, labeled, and stored at -80°C, which was then used for testing anti-microbial activity by the agar well diffusion method [16]. Test micro-organisms were grown in LB broth at 37°C for 24 hours. Plates were incubated at 37°C for 24 hours and were then observed for the clear zone and the diameter of the zone of inhibition was measured.

2.4.5. Gelatinase Liquefaction Activity

Gelatinase production was established by spot inoculating the 48 hours old cultures onto the surface of LSB media plates supplemented with 3% (w/v) gelatin. Plates were incubated at 37°C and 42°C for 48 hours and 25°C for 72 hours. After incubation, the plates were maintained at 4°C for 5 hours and the hydrolysis of gelatin was recorded by the formation of opaque halos around the colonies. The Gelatinase liquefaction technique used for this study was described by Silva et al. [17].

2.4.6. Hemolytic Activity

Isolated strains were screened for hemolysis activity on sheep blood agar (HI Media) plates containing 5% (v/v) blood. The isolates were grown at 37°C for 48 hours in LSB medium, streaked onto blood agar and incubated at 30°C for 24–48 hours. A clear zone around the colony indicated hemolytic activity [18].

2.4.7. In Vitro Cell Surface Hydrophobicity

Hydrophobicity was tested against three hydrocarbons (xylene, paraffin oil and silicon oil). The hydrophobicity assay and calculations (%) were carried out according to the protocol described by Rokana et al. [4]. The in vitro bacterial cell surface hydrophobicity of the selected LAB isolates was evaluated by measuring bacterial cell adhesion to hydrocarbons. The overnight cultures in LSB broth were harvested by centrifugation at 8,000 rpm at 4°C for 10 minutes and washed twice with PBS. Further, the cultures were resuspended in PBS buffer followed by absorbance (A_0) measurement at 600 nm. A cell suspension of about 3 mL was mixed with 1 mL of hydrocarbon (xylene, paraffin oil and silicon oil) and incubated at 37°C without shaking for 1 hour for separating the organic and aqueous phases. 1 mL aqueous phase was removed carefully, and the absorbance (A1) was measured at 600 nm. The percentage hydrophobicity was measured via a decrease in absorbance and calculated by the following formula:

$$\text{Percentage cell surface hydrophobicity} = \left(1 - \frac{A_1}{A_0}\right) \times 100$$

2.4.8. Test for Cell Auto-aggregation and Co-aggregation

Auto aggregation of isolates was evaluated using the method described by Maria Carmen Collado [19]. The LAB isolates were cultured in LSB medium at 37°C for 18 hours. The cultures were further harvested by centrifugation at 5000 rpm for 15 minutes, washed 3 times using PBS and resuspended in 2 mL of PBS at 600 nm OD. The bacterial suspension was incubated at room temperature and absorbance (At) was measured at different time intervals (0, 2, 4, 6, 10 and 24 h). During each time interval, 100 µl of upper layer of the bacterial suspension was transferred to a cuvette and absorbance (A) recorded at 600 nm.

In addition to this, the LAB isolates were subjected to co-aggregation assay using the method described. To evaluate co-aggregation capacity, the bacterial isolates were grown in LSB broth at 37°C for 48 hours. Bacterial cells from the culture suspension were harvested by centrifugation (7,500×g, 10 minutes, 4°C), washed twice with PBS and resuspended in the same buffer. The same volume of each LAB isolate and pathogenic strain were mixed and incubated at room temperature for 24 hours and the co aggregation percentages were monitored after incubation according to methods described by B. Del Re, et al. [20]. The percentage auto-aggregation and co-aggregation was determined using the following formula.

$$\text{Percentage auto} - \text{aggregation} = \left(\frac{A_t - A_1}{A_t} \times 100\right)$$

Where, A_t= absorbance at different time intervals;
A_1= absorbance of upper layer of bacterial suspension.

2.4.9. Biofilm Formation Assay

The LAB isolates were evaluated for their potential to produce biofilm using the crystal violet method [21]. A 100 µL of overnight LAB cultures were added into the microtiter polystyrene plate wells which were previously coated with 100 µL of LSB broth. The cells were allowed to adhere at 37°C for 24 hours. Followed by the incubation, the non-adherent cells were removed by washing the wells 3 times with 200 µL of PBS. The adhered cells were stained with crystal violet (100 µL/well, 0.1%, *w/v*, solution) for 30 minutes. Wells were then washed 5 times with PBS to remove the excess stain. After incubation at room temperature for 30 minutes, the absorbance was determined at 640 nm using a microtiter plate reader. The negative control included wells containing non-inoculated broth. Results were calculated by subtracting the absorbance of the negative control from the absorbance value documented for each inoculated well.

2.4.10. Antibiofilm Activity

According to the methods described by [22], the selected LAB isolates were subjected to anti-biofilm assay and the percentage anti-biofilm activity of each isolate was calculated. The test organisms namely, *Acinetobacter Baumannii, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus mutants, Serratia marcescens and Vibrio cholera* were obtained from MTCC Chandigarh and some laboratory isolates were also used. The test organisms were cultured overnight with fresh sterile tryptone soya broth (TSB) supplemented with 0.5% (w/v) glucose. A 100 µL of cultures of each bacterium was transferred to 96-well microtiter plates followed by the addition of 100 µL of LAB bacterial supernatants adjusted to pH 7.0 and were incubated for 24 hours at 37°C. After the incubation, the medium was discarded, and planktonic cells were removed from each well by gently washing twice with sterile phosphate-buffered saline. Further, the biofilms formed were fixed with 200 µL methanol for 10 minutes, stained with 200 µL 0.1% crystal violet for 10 minutes, and rinsed thrice with water gently. Crystal violet attached to the

biofilm samples was dissolved with 200 µL of 33% acetic acid. The absorbance was measured using a microplate reader at 590 nm as the value of biofilm formation. The positive control comprised of test organism grown on culture media whereas TSB medium with 0.5% (w/v) glucose was used as a negative control. The percentage of inhibition of biofilm production was calculated using the equation:

$$\left(\frac{A_{\text{growth control}} - A_{Sample}}{A_{\text{growth control}}}\right) \times 100$$

3. Results and Discussion

3.1. Isolation, Microscopic Evaluation, and Biochemical Evaluation of Lactic Acid-Producing Isolates

In this study, Lactic acid bacteria were isolated from Dairy beverages (milk, colostrum milk, buttermilk, yogurt, and cheese) and Non-dairy fermented products [Green gram, Finger millet (ragi), Dosa batter, Black gram, Maize, Wheat, Rice, Broom Corn (Sorghum) and Pearl millet (bajra)]. A total of six LAB isolates were obtained from samples such as fermented finger millet batter, fermented maize batter, colostrum milk, yogurt, fermented green chickpeas batter and cheese on LSB medium. Table 1 and Table 2 represent the sources of isolation of the Lactic acid bacteria and its morphological characterization, respectively. Further, biochemical characterization of the six isolated lactic acid bacteria was carried out. The biochemical traits of bacterial isolates were found to be positive for growth at different temperatures and in presence of bile salt. On the other hand, none of the lactic acid-producing isolates were positive for oxidase, catalase, indole, H_2S production, gelatin liquefaction (Table 3).

Table 2. *Morphological characterization of six lactic acid bacteria.*

Isolates	Size (mm)	Shape	Color	Margin	Elevation	Opacity	consistency	Gram nature
LAB 01	3mm	Circular	White	Entire	Convex	Opaque	Viscid	Positive
LAB 02	2mm	Circular	White	Entire	Convex	Opaque	Viscid	Positive
LAB 03	Pinpoint	Circular	White	Entire	Convex	Opaque	Moist	Positive
LAB 04	5mm	Filamentous	White	Undulate	Flat	Opaque	Dry	Positive
LAB 05	2mm	Circular	White	Entire	Convex	Opaque	Viscid	Positive
LAB 06	2mm	Circular	White	Entire	Convex	Opaque	Viscid	Positive

Table 3. *Biochemical characterization of lactic acid bacteria.*

Biochemical Test	LAB 01	LAB 02	LAB 03	LAB 04	LAB 05	LAB
Growth at 450C	+	+	+	+	+	+
Growth at 150C	+	+	+	+	+	+
Catalase	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Indole	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-
Bile salt	+	+	+	+	+	+

Key: (-) Negative, (+) Positive.

3.2. Molecular Identification of Selected Lactic Acid Bacteria

The genomic DNA was isolated and 16S rRNA sequencing was carried out for the selected LAB isolates. The 16S rRNA gene was amplified from all the isolated strains. Then, the amplicons were column purified and sequenced. The resultant nucleotide sequences were Basic Local Alignment Search Tool (BLAST) searched to check for sequence homology with known sequences in the National Center for Biotechnology Information (NCBI) database. According to which the isolates were identified as *Limosilactobacillus oris* (LAB01), *Lentilactobacillus buchneri* (LAB02), *Pediococcus pentosaceus* (LAB03), *Lactococcus lactis* (LAB04), *Levilactobacillus brevis* (LAB05), *and Lactiplantibacillus plantarum* (LAB06) respectively as shown in table 1.

3.3. Tolerance to High Bile Concentration and Gastric Acid

The acidic pH and bile salt of the stomach are known to hinder the survival of LAB in the GI tract of a host [3]. In this study, different levels of resistance to bile salt of six isolates were assessed. Also, tolerance of these isolates against the gastric acidic conditions was tested. According to which the selected LAB isolates were found to show maximum tolerance to 3% bile salt concentration. It was observed that tolerance to bile salt concentration decreases with increase in concentration. The results, for tolerance to different bile salt concentration, are shown in graphs Figure 1. Meanwhile, all the isolates showed an increase in absorbance at 600 nm after 30 minutes till 3 hours. After 3 hours and up to 6 hours of incubation, the absorbance started to decrease with time. The survival and tolerance of the LAB isolates to stimulated gastric fluid is shown in Table 4 and the

percentage survival of LAB isolates is shown in Figure 2. Thus, the selected Lab isolated were able to withstand high concentration of bile and gastric acid thus, qualifying as good probiotic candidates. The isolates were also found to differ in the survivability in acidic conditions. These results are majorly due to the species or strain-dependent acid-tolerance mechanism with certain bacterial proteins that might promote the resistance [3]. According to a previous study, the LAB population decreased by 0.5 to 5.2 log CFU/mL after 2 hours of incubation under gastric condition at pH 2.0 with pepsin. Meanwhile, minor reduction ranging from 0.0 to 1.5 log CFU/mL after 6 hours of incubation in the LAB population was observed under intestinal conditions with pH 8.0 with trypsin. The decrease in the tolerance leading to reduction in survival rate was due to oxgall, cholic, and taurocholic acids, the representative bile acids. It was also observed that Taurocholic acid showed greater inhibition as compared to oxgall and cholic acids [2].

Table 4. *Tolerance to Gastric Acid Stimulation.*

Time (minutes)	LAB01	LAB02	LAB03	LAB04	LAB05	LAB06
30	68.73 ± 0.05	50.53 ± 0.06	58.55 ± 0.06	55.54 ± 0.06	52.22 ± 0.05	48.67 ± 0.07
60	80.49 ± 0.08	72.30 ± 0.07	72.76 ± 0.07	76.81 ± 0.09	55.92 ± 0.03	52.68 ± 0.07
120	95.64 ± 0.06	92.39 ± 0.06	97.27 ± 0.08	90.76 ± 0.08	69.24 ± 0.09	95.67 ± 0.05
180	99.25 ± 0.08	98.54 ± 0.07	99.05 ± 0.05	99.45 ± 0.07	99.83 ± 0.05	99.64 ± 0.06
360	41.07 ± 0.07	61.84 ± 0.06	70.18 ± 0.09	54.46 ± 0.08	38.34 ± 0.08	55.78 ± 0.08

Values are mean (n = 3) ± standard error.

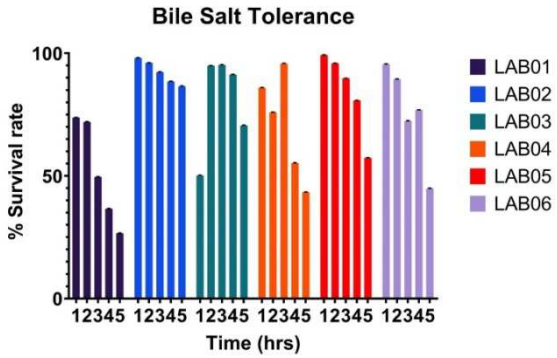

Figure 1. *Percentage Survival Rate of LAB Isolates in 10% Bile salt.*

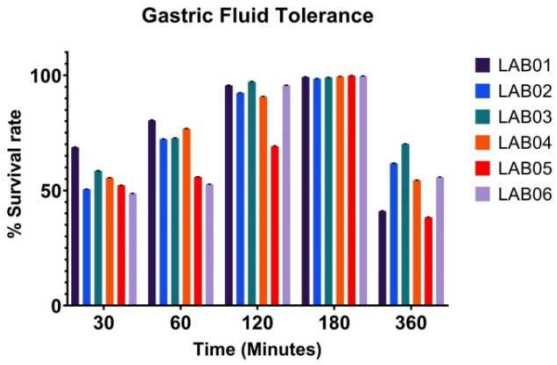

Figure 2. *Percentage survival rate of LAB isolates in Stimulated Gastric Fluid.*

3.4. Tolerance to Sodium Chloride, Phenol, and Different pH

Tolerance against sodium chloride, phenol concentrations and varying pH is a property for a probiotic organism to survive in extreme Gastrointestinal tract (GI) tract conditions. The high sodium chloride concentration provides an advantage to LAB as compared to the less tolerant bacteria. Since, it promotes the LAB to begin metabolism, which leads to acid production and further inhibits the growth of non-desirable bacteria [23]. The results, for tolerance to sodium chloride salt concentration, is shown in Figure 3. It was seen that all the isolates were able to survive 7% NaCl concentration. The absorbance at 600 nm decreased continuously with increase in salt concentration from 8% to 10%. Similar results were observed in earlier investigation where LAB strains isolated from poultry gastrointestinal tract showed excellent tolerance at 6.5% NaCl concentration but a reduction in survival rate was recorded at 10.0%. Therefore, the isolated LAB can be considered desirable as probiotics as well as for preservation in food and dairy industry [24].

Along with the tolerance to salts, the tolerance to phenol is an important factor for survival and growth of LAB in host intestinal tract. The gut bacteria have the capacity to deaminate aromatic amino acids derived from dietary proteins leading to phenol formation [4]. A tolerance to phenol was observed in the LAB isolates when evaluated for phenol tolerance. The results, for tolerance to phenol, are shown in graphs of Figure 4. According to which all the selected LAB isolates were found to show maximum tolerance up to 5% of phenol concentration. In a previous study, LAB isolates from poultry gastrointestinal tract exhibited tolerance to a phenol concentration of 0.4% and a decreased tolerance at higher concentrations [24]. Therefore, the six LAB isolates exhibiting tolerance at 5% concentration can prove to be better candidates for probiotics.

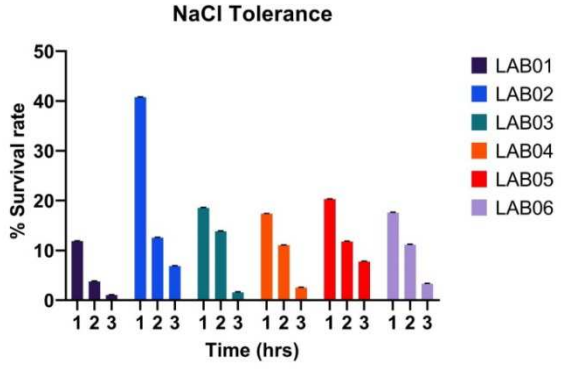

Figure 3. *Percentage Survival Rate of LAB Isolates in 10% NaCl.*

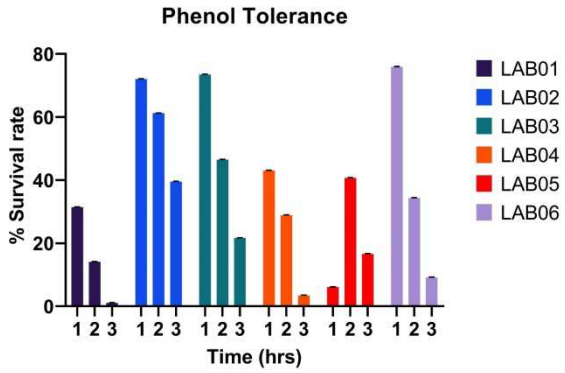

Figure 4. *Percentage Survival Rate of LAB Isolates in 5% phenol.*

Lactic acid bacteria are acidophilic in nature that is they are tolerant to low pH. However, their growth is affected due to presence of high concentration of free acids as they have growth inhibitory effects [25]. Since, resistance to low pH is one of the major selection criteria for probiotic organisms, tolerance of the selected LAB isolates was evaluated at different pH. According to the results, OD values at 600 nm, are shown as graphs for each LAB isolates in Figure 5. All the isolates showed increase tolerance at pH 3, 4, 6 and 8. Absorbance for LAB isolates was found to decrease with a reduction in pH indicating intolerance to pH 1 and 2. Prior studies have also shown growth at pH 3 with a significant reduction in survival at pH 2 except for *Lactobacillus lactis* and no growth at pH 1.5 [26].

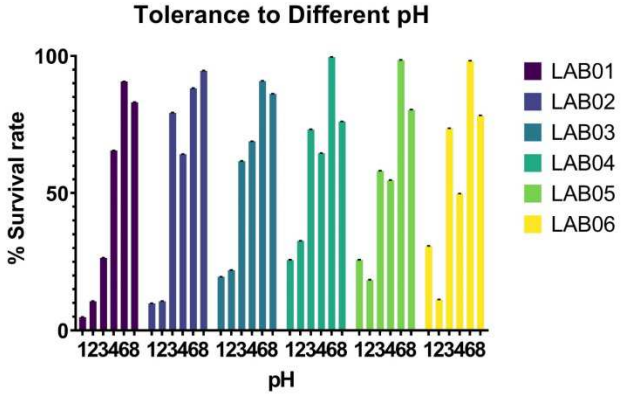

Figure 5. *Percentage Survival Rate of LAB Isolates at different pH after 4hr exposure.*

3.5. Growth at Different Temperature, Gelatinase Liquefaction and Hemolytic Activity

For Lactic acid bacteria to qualify as probiotic and to be regarded as safe it should not be harmful to the host [27]. Cytolysin, a bacterial toxin expressed by some LAB and *Enterococcus faecalis* exhibits hemolytic and bactericidal activities. The absence of cytolysin coding genes is considered as a benefit for application of LAB in dairy and food industry. Also, absence of hemolytic activity is an indicator of non-virulent strain of LAB making it an ideal candidate for a probiotic [18]. Therefore, the selected LAB isolates were subjected to hemolytic activity by growing them on sheep blood agar (HI Media) at different temperatures such as 25°C, 30°C, 37°C and 47°C. The isolates showed growth at all the above-mentioned temperatures. Meanwhile, earlier findings have revealed that majority LAB strains showed optimal growth at 37°C after 24 hours of incubation with a reduction in survival rate at 4°C and 55°C [24]. None of the LAB isolates showed hemolytic activity on sheep blood agar (HI Media). In addition to this, gelatinase liquefaction test was also conducted which is employed to check whether the selected isolate can break down gelatin. All the six isolates showed absence of gelatinase activity. The results obtained from growth at a different temperature, Gelatinase liquefaction activity, and hemolysis activities are shown in Table 5. Previously, similar results representing absence of hemolytic activity by majority of LAB strains have been reported [3]. Since all the selected isolates showed absence of hemolytic and gelatinous activity they can be considered as good probiotic candidates.

Table 5. *Growth at a different temperature, Gelatinase liquefaction activity, the Hemolytic activity.*

Isolates	Temperature in °C at 48hrs				Gelatinase Activity	Hemolytic Activity
	25	30	37	47		
LAB01	+	+	+	+	-	-
LAB02	+	+	+	+	-	-
LAB03	+	+	+	+	-	-
LAB04	+	+	+	+	-	-
LAB05	+	+	+	+	-	-
LAB06	+	+	+	+	-	-

Key: (-) No Growth, (+) Growth.

3.6. Resistance to Antibiotic

Studies have shown that probiotic organisms possess the ability to inhibit intestinal pathogenic bacteria and protect host from infections [3]. Lactic acid bacteria are highly beneficial organisms but however they may act as intrinsic or extrinsic reservoirs for antibiotic resistance genes thus having a high rate of developing resistance against variety of antibiotics [28]. The pattern of resistance and susceptibility to antibiotics for all the selected LAB isolates was studied by disc diffusion method and agar well diffusion method. The antibiotic resistance and susceptibility profile for selected LAB isolate are shown in Table 6. All the isolates showed resistance to Amphotericin B, Ceftazidime, Gentamicin, Kanamycin, Methicillin, Nalidixic acid, Netilmicin, Rifampicin, Vancomycin, Voriconazole whereas they were sensitive to Chloramphenicol, Ofloxacin and Penicillin. LAB01 exhibited resistance against all antibiotics except Chloramphenicol, Ceftriaxone, Cefotaxime, Imipnem, Levofloxacin, Ofloxacin and Penicillin. LAB02 isolate was found to be susceptible to Amikacin, Chloramphenicol, Ciprofloxacin, Fusidic acid, Imipnem, Nevobiocin, Ofloxacin, Penicillin and Lincomycin. Meanwhile, LAB03 exhibited resistant against Lincomycin, Amphotericin B, Ceftriaxone, Ceftazidime, Gentamicin, Imipnem, Kanamycin, Levofloxacin, Methicillin, Nalidixic acid, Netilmicin, Rifampicin, Trimethoprim, Vancomycin and Voriconazole. LAB04 showed

resistance against to all except Chloramphenicol, Fusidic acid, Imipnem, Nevobiocin, Ofloxacin, Penicillin and Lincomycin. LAB05 showed resistance against Amphotericin B, Ceftriaxone, Ceftazidime, Cefotaxime, Gentamicin, Kanamycin, Methicillin, Nalidixic acid, Netilmicin, Rifampicin, Vancomycin, Voriconazole and Lincomycin. LAB06 showed susceptibility to Chloramphenicol, Ciprofloxacin, Fusidic acid, Imipnem, Levofloxacin, Nevobiocin, Ofloxacin and Penicillin. LAB04 exhibited resistance against a large number of antibiotics whereas LAB05 exhibited sensitivity towards a large number of antibiotics used in the study. According to previous investigation, *Lactobacillus helveticus* and *Lactobacillus lactis* were found to be susceptible to ciprofloxacin, chloramphenicol, vancomycin, erythromycin, streptomycin, clindamycin, gentamicin, ampicillin, tetracycline and kanamycin whereas *Lactobacillus sakei* exhibited resistant against vancomycin, erythromycin, ampicillin, and tetracycline. Therefore, it can be concluded that majority LAB strains are resistant to Kanamycin, Gentamicin and Vancomycin [26].

Table 6. *Antibiotic susceptibility and resistance profile.*

Antibiotics			**LAB isolates Zone if inhibition in mm**					
Sr. No.	**Name**	**Conc.in µg**	**LAB001**	**LAB 02**	**LAB 03**	**LAB 04**	**LAB 05**	**LAB 06**
1	Amoxycillin	10	R	R	R	R	10	R
2	Amikacin	10	R	20	14	R	19	R
3	Amphotericin B	30	R	R	R	R	R	R
4	Chloramphenicol	30	15	15	15	16	14	16
5	Ciprofloxacin	30	R	20	23	R	17	22
6	Ceftriaxone	30	15	R	R	R	R	R
7	Ceftazidime	30	R	R	R	R	R	R
8	Cefotaxime	30	18	R	16	R	R	R
9	Fusidic acid	10	R	27	17	22	23	23
10	Gentamicin	10	R	R	R	R	R	R
11	Imipnem	10	34	31	R	30	31	32
12	Kanamycin	30	R	R	R	R	R	R
13	Levofloxacin	5	19	R	R	R	29	21
14	Methicillin	5	R	R	R	R	R	R
15	Nalidixic acid	30	R	R	R	R	R	R
16	Nevobiocin	30	R	12	12	15	12	13
17	Netilmicin	30	R	R	R	R	R	R
18	Ofloxacin	5	19	15	14	10	15	15
19	Penicillin	30	24	23	25	25	27	23
20	Rifampicin	5	R	R	R	R	R	R
21	Trimethoprim	5	R	R	R	R	19	R
22	Vancomycin	30	R	R	R	R	R	R
23	Voriconazole	30	R	R	R	R	R	R
24	Lincomycin	30	R	21	23	R	R	R

Key: R- Resistance.

Table 7. *Antimicrobial Activity.*

Test organisms	**Zone of clearance in mm for**					
	LAB01	**LAB02**	**LAB03**	**LAB04**	**LAB05**	**LAB06**
Acinetobacter Baumannii	14.03 ± 0.05	15.1 ± 0.08	11.93 ± 0.09	10.97 ± 0.05	14.03 ± 0.05	13.97 ± 0.05
Escherichia coli	21.07 ± 0.09	16.97 ± 0.05	14 ± 0.08	15.02 ± 0.06	19.98 ± 0.06	16.03 ± 0.05
Pseudomonas aeruginosa	16.04 ± 0.04	14.07 ± 0.09	16.05 ± 0.04	17.07 ± 0.09	17.1 ± 0.08	18.97 ± 0.05
Proteus mirabilis	16.04 ± 0.03	16.97 ± 0.05	14.07 ± 0.06	15.2 ± 0.08	20.05 ± 0.04	16.1 ± 0.08
Proteus vulgaris	13 ± 0.08	17.1 ± 0.08	12.95 ± 0.07	12.04 ± 0.04	14.09 ± 0.08	16.05 ± 0.05
Staphylococcus epidermidis	15.97 ± 0.05	15.05 ± 0.04	16.03 ± 0.05	16.97 ± 0.05	15.93 ± 0.09	15.05 ± 0.07
Staphylococcus aureus	15.99 ± 0.07	16.1 ± 0.08	12.07 ± 0.06	15.92 ± 0.06	17.93 ± 0.09	13.97 ± 0.05
Streptococcus mutants	17.99 ± 0.07	15.08 ± 0.06	11.07 ± 0.09	13.1 ± 0.08	16.97 ± 0.05	16.02 ± 0.02
Serratia marcescens	17.1 ± 0.08	14.93 ± 0.09	14.1 ± 0.08	16.03 ± 0.05	18.03 ± 0.05	19.02 ± 0.03

3.7. Antimicrobial Activity

Anti-microbial property of probiotics plays a major role in defense against pathogens. LAB are capable of producing bacteriocin which are bioactive peptides possessing antimicrobial activity towards Gram positive bacteria and pathogenic bacteria [29]. The antimicrobial activity of LAB is influenced by decrease in pH level, production of bacteriocin and competition for substrates. A considerable reduction in pH due to lactic acid production can lead to inhibition of certain pathogenic organisms [27]. All six selected LAB isolates were screened for their ability to have antibacterial activity against ten pathogenic test organisms namely *Acinetobacter Baumannii, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermidis, Staphylococcus*

aureus, Streptococcus mutants and *Serratia marcescens by* agar well diffusion assay, results of which is shown in Table 6. The supernatants of all six LAB isolates were found to be inhibitory against all these test organisms. LAB01 showed highest antimicrobial activity against *Escherichia coli* whereas LAB05 showed highest antimicrobial activity against *Escherichia coli* and *Proteus mirabilis*. Table 7 show the results recorded during antimicrobial activity of the LAB isolates. Figure 6 represents zone of inhibition of all the LAB isolates. According to a previous study performed on LAB isolates from pickles it was observed that LAB strains like *Lactobacillus plantarum* exhibit a broad spectrum antimicrobial activity against *Bacillus cereus, Escherichia coli, Shigella dysenteriae* and *Staphylococcus aureus* [27].

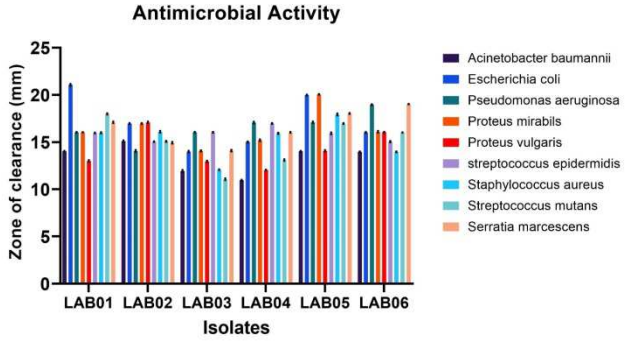

Figure 6. *Antimicrobial activity exhibited by selected LAB isolates on different test organisms.*

3.8. In-vitro Cell Surface Hydrophobicity

The bacterial cell surface hydrophobicity is one of the in-vivo properties, which can be studied in in-vitro experimental set up for understanding the probiotic nature. The level of adhesion of the bacteria is seen to increase with increased hydrophobicity. The capacity of the organisms to adhere to hydrocarbons determines their adherence to the epithelial cells in the colon [30]. Cell surface hydrophobicity of LAB isolates was carried out with hydrocarbons namely xylene, paraffin oil and silicone oil. The results of the in-vitro cell surface hydrophobicity are shown in Table 8. All the isolates showed adhesion to the given hydrocarbon out of which LAB01 and LAB03, relatively showed more adhesion to silicone oil and paraffin oil respectively. Figure 7 represents the percentage hydrophobicity exhibited by the selected LAB isolates. From a study performed by Gómez et al, it was observed that *Lactococcus lactis* (95.2 ± 0.09) and *Lactococcus lactis* (95.1 ± 0.13) exhibited excellent cell surface adhesion [26].

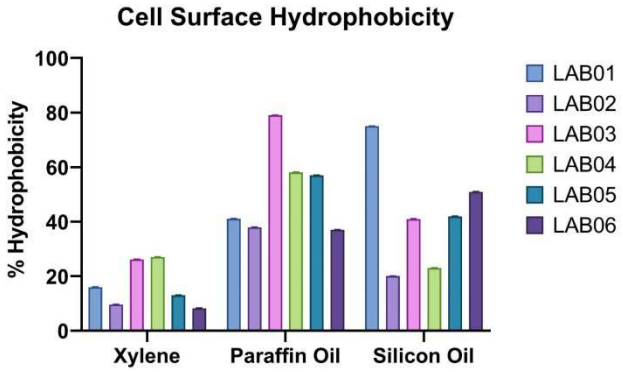

Figure 7. *Cell Surface Hydrophobicity of LAB isolates.*

Table 8. *In Vitro Cell Surface Hydrophobicity.*

	LAB01	LAB02	LAB03	LAB04	LAB05	LAB06
Xylene	15.93 ± 0.09	9.61 ± 0.09	26.10 ± 0.08	27 ± 0.08	12.99 ± 0.07	8.27 ± 0.05
Paraffin Oil	41.07 ± 0.09	37.93 ± 0.09	79.02 ± 0.06	58.07 ± 0.09	57.02 ± 0.02	37.05 ± 0.04
Silicon Oil	74.97 ± 0.05	20.03 ± 0.05	40.93 ± 0.09	23.02 ± 0.02	41.93 ± 0.09	50.94 ± 0.05

Values are mean (n = 3) ± standard error.

3.9. Auto-aggregation and Co-Aggregation Assay

Auto-aggregation and coaggregation are the properties of probiotics which inhibit surface colonization by pathogens. Auto-aggregation allows bacteria of the same strain to form clumps and adhere to a surface whereas coaggregation leads to the intercellular adhesion of different strains [31]. In this study, six LAB isolates were subjected to Auto-aggregation and Co-aggregation assay. The absorbance of bacterial suspension was seen to decrease with an increase in auto-aggregation of cells. Figure 8 represents the percentage auto-aggregation of selected LAB isolates that ranged from 60-95%. LAB02 isolate exhibited the highest auto-aggregation (95%) whereas LAB03 isolate showed a moderate auto-aggregation of 59%. Co-aggregation is the clumping of cells with different strains. All LAB isolates indicated co-aggregation with test pathogens (Figure 9). The LAB isolates showed highest co-aggregation with *Staphylococcus aureus* and *Proteus vulgaris*. Tables 9 and 10 represent the results obtained for auto-aggregation and co-aggregation of the selected LAB isolates, respectively. A previous study has confirmed that any potential aggregation phenotype is influenced by internal factors as well as the environment [32]. *Lactobacillus salivarius*, a LAB strain isolated from gastrointestinal tract of wild boar have shown highest autoaggregation percentage of 95.6 ± 4.61% at 24 hours [3]. Furthermore, LAB possessing the aggregation-promoting factors contribute to elimination of pathogens by maintaining the balance of gut microflora and the coaggregation mechanism [3]. Therefore, the isolated LAB in this study exhibiting the ability to auto aggregate and co-aggregate pathogens could promote good intestinal health and can be used as a probiotic source.

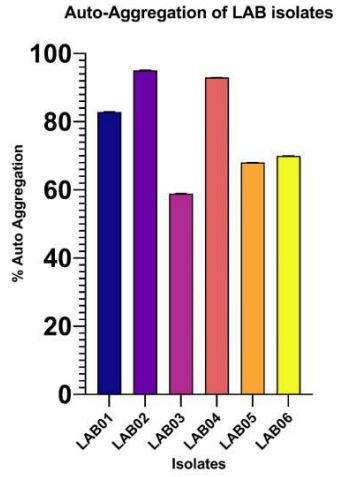

Figure 8. *Auto-aggregation of LAB isolates.*

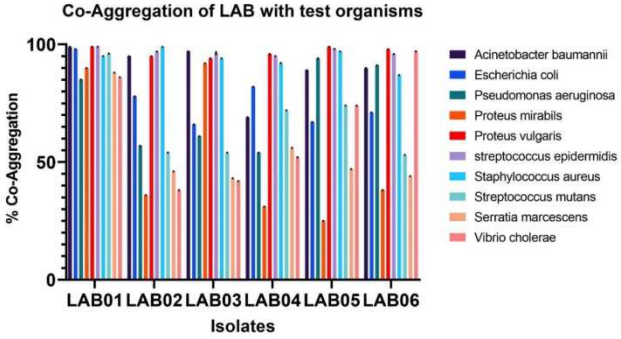

Figure 9. *Co-aggregation of LAB isolates.*

Table 9. *Auto-aggregation.*

	% Auto-aggregation
LAB01	82.90 ± 0.08
LAB02	95.07 ± 0.09
LAB03	58.9 ± 0.08
LAB04	92.97 ± 0.05
LAB05	68.3 ± 05
LAB06	69.97 ± 0.05

Values are mean (n = 3) ± standard error.

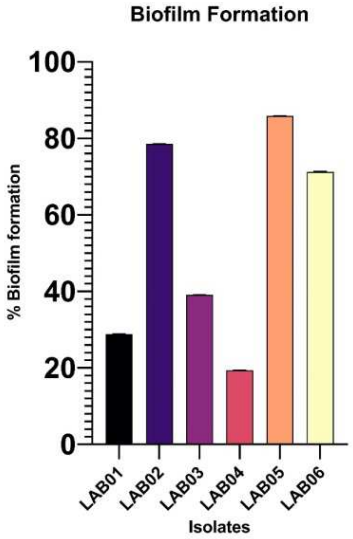

Figure 10. *Percentage biofilm formation by LAB isolates.*

Table 10. *Co-aggregation.*

	LAB01	LAB02	LAB03	LAB04	LAB05	LAB06
Acinetobacter baumannii	98.93 ± 0.09	94.97 ± 0.05	97.05 ± 0.07	69.06 ± 0.08	89.07 ± 0.05	89.93 ± 0.09
Escherichia coli	98.04 ± 0.04	77.93 ± 0.09	66.05 ± 0.04	82.05 ± 0.04	67.03 ± 0.05	71.07 ± 0.09
Pseudomonas aeruginosa	85.03 ± 0.05	56.93 ± 0.09	61.07 ± 0.05	54.05 ± 0.04	94.08 ± 0.06	91.08 ± 0.08
Proteus mirabils	89.98 ± 0.05	36.02 ± 0.02	92.03 ± 0.05	31.10 ± 0.08	25.07 ± 0.06	38.09 ± 0.08
Proteus vulgaris	99.05 ± 0.04	95.02 ± 0.03	94.07 ± 0.06	95.97 ± 0.05	99.05 ± 0.04	97.97 ± 0.05
streptococcus epidermidis	99.03 ± 0.05	96.97 ± 0.05	96.31 ± 0.04	95.06 ± 0.04	98.10 ± 0.08	95.97 ± 0.05
Staphylococcus aureus	95.05 ± 0.04	98.97 ± 0.05	94.07 ± 0.05	92.06 ± 0.06	97.04 ± 0.03	86.95 ± 0.07
Streptococcus mutans	96.10 ± 0.08	54.05 ± 0.06	54.03 ± 0.04	71.97 ± 0.05	74.08 ± 0.07	53.05 ± 0.04
Serratia marcescens	88.02 ± 0.02	46.08 ± 0.07	43.10 ± 0.08	56.08 ± 0.08	47.04 ± 0.06	44.03 ± 0.05
Vibrio cholerae	86.05 ± 0.04	38.10 ± 0.08	41.97 ± 0.05	52.08 ± 0.07	74.06 ± 0.06	97.05 ± 0.07

Values are mean (n = 3) ± standard error.

Table 11. *Biofilm Formation.*

	% Biofilm formation
LAB01	28.78 ± 0.08
LAB02	78.52 ± 0.06
LAB03	39.06 ± 0.05
LAB04	19.32 ± 0.07
LAB05	85.84 ± 0.07
LAB06	71.25 ± 0.08

Values are mean (n = 3) ± standard error.

3.10. Biofilm Formation and Anti-biofilm Activity

Biofilms formed by Lactic acid bacteria play a very important role as they act as barrier against other pathogenic microorganism by not allowing them to adhere to the mucosal surface [33]. Also, Biofilm producers secrete exopolysaccharide which provides health benefits to consumers in the form of non-digestible fiber or in enhancing the sensory properties of food [2]. All the selected LAB isolates were tested to check if they were able to form biofilms and the results of the same are provided in Table 11. The percentage of biofilm formation by each isolate is shown in Figure 10. Out of the six LAB isolates, LAB02 and LAB05 exhibited highest percentage of biofilm formation whereas LAB01 showed lowest percentage of biofilm formation. Earlier research performed by Gómez et al. have

shown promising results in case of biofilm formation by *Lactobacillus lactis* and *Lactobacillus helveticus* [26].

Studies have shown that Lactic acid bacteria produce bacteriocins and anti-adherence biosurfactant proteins against pathogenic bacteria. Probiotic LAB also suppresses virulence and propagation of infectious pathogens [21]. Biosurfactant production contributes in inhibiting the attachment of pathogens. Also, anti-biofilm forming properties of lactobacilli have been reported in previous studies, such as Lactobacillus delbrueckii against *Escherichia coli* and Lactobacillus brevis against Prevotella melaninogenica [26]. The LAB isolates were subjected to antibiofilm activity against the test organism including *Acinetobacter Baumannii, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus mutants, Serratia marcescens and Vibrio cholera.* All the isolates effectively inhibited the biofilm formed by *Acinetobacter baumannii.* Table 12 shows the results obtained for antibiofilm activity of the six LAB isolates. The anti-biofilm activity of LAB isolates was seen to be less effective against *Serratia marcescens*. According to the previous studies, LAB strains like *Lactobacillus plantarum* and *Lactobacillus helveticus* have exhibited antibiofilm activity against *Escherichia coli* and *Staphylococcus aureus*. Thus, it has been observed that certain LAB strains possess the ability to inhibit biofilms formed by gram-positive and gram-negative bacterium, but the abilities are strain dependent [34]. The Figure 11 represents percentage inhibition of biofilm formation by test organisms in presence of spent media of LAB isolates.

The secretion of antagonistic substances like surfactants, organic acids, bacteriocins, exopolysaccharides, lactic acid and hydrogen peroxide) as well as the creation of adverse environmental conditions for pathogens are some of the molecular mechanisms by which LAB can eradicate biofilms and inhibit pathogenic organism. They also reduce pathogenic biofilm formation by lowering ambient pH, indole synthesis and biofilm biomass. The competitive adherence of probiotics LAB to human tissues prevents harmful pathogens from invading them and causing infections [35].

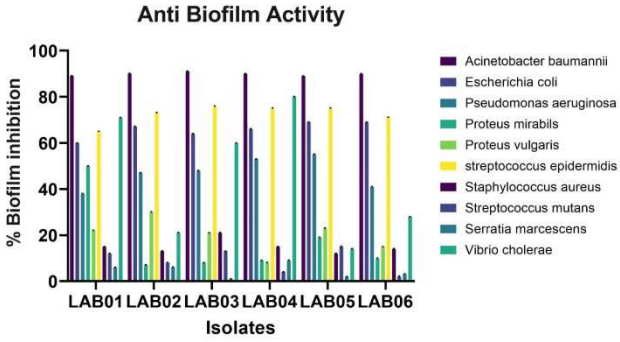

Figure 11. *Percentage biofilm inhibition by LAB isolates on selected test organisms.*

Table 12. *Anti-biofilm Activity.*

	LAB01	LAB02	LAB03	LAB04	LAB05	LAB06
Acinetobacter baumannii	89.09 ± 0.07	90.11 ± 0.08	91.10 ± 0.09	90.05 ± 0.05	89.03 ± 0.05	90.02 ± 0.07
Escherichia coli	59.98 ± 0.07	67.13 ± 0.09	63.99 ± 0.06	66.09 ± 0.08	69.07 ± 0.06	69.02 ± 0.06
Pseudomonas aeruginosa	38.01 ± 0.08	47.08 ± 0.06	48 ± 0.04	53.07 ± 0.05	55.10 ± 0.08	41 ± 0.08
Proteus mirabils	50 ± 0.08	7.10 ± 0.08	8.05 ± 0.05	9.03 ± 0.05	19 ± 0.08	9.97 ± 0.05
Proteus vulgaris	22.10 ± 0.08	30.07 ± 0.06	20.99 ± 0.07	8.10 ± 0.08	23.08 ± 0.06	14.97 ± 0.04
streptococcus epidermidis	65.01 ± 0.09	73.10 ± 0.08	75.97 ± 0.05	75.08 ± 0.06	75.10 ± 0.08	71.10 ± 0.08
Staphylococcus aureus	15 ± 0.08	13.1 ± 0.08	21.07 ± 0.05	15.05 ± 0.04	12.08 ± 0.05	14.08 ± 0.07
Streptococcus mutans	12 ± 0.08	8.10 ± 0.09	13.07 ± 0.05	4.07 ± 0.06	15.07 ± 0.05	2.09 ± 0.07
Serratia marcescens	5.97 ± 0.05	6.10 ± 0.08	1.06 ± 0.04	9.10 ± 0.08	1.97 ± 0.05	3.10 ± 0.08
Vibrio cholerae	70.97 ± 0.05	21.06 ± 0.05	59.97 ± 0.05	80.08 ± 0.06	14.09 ± 0.07	27.97 ± 0.05

Values are mean (n = 3) ± standard error.

4. Conclusion

Traditionally fermented dairy and non-dairy fermented products are found to be rich source of Lactic acid bacteria (LAB). LAB are catalase negative gram positive bacteria majorly used for probiotic purposes. The LAB were isolated from fermented dairy products and non-dairy fermented products. Probiotic characteristics like antimicrobial activity, auto-aggregation, and co-aggregation abilities, hemolytic activity, antibiofilm activity, antibiotic resistance and tolerance to high bile salts and gastric conditions were evaluated. All the isolates showed varied capability to survive in gastric conditions like high bile concentration, high acid concentration, low pH, and different temperatures. They also exhibited good anti biofilm and biofilm forming potential. Study isolates fulfilled several criteria to be used as probiotic microorganisms, including adherence to hydrocarbons, auto and co-aggregation activity as- well as susceptibility to some antibiotics. The isolates did not exhibit hemolysis and gelatinases activity proving to be an ideal candidate as a probiotic. Therefore, we conclude that LAB isolates are suitable to be used as probiotic microorganisms. However, identification of antimicrobial bioactive compounds needs to be performed. With an increasing concern of antibiotic resistance, appropriate screening strategies should be undertaken to improve the process of developing better probiotics and evaluation of the properties in vitro and in vivo. Therefore, attempts are made to isolate LAB bacteria possessing excellent probiotic characteristics

from fermented food sources.

Abbreviations

LAB, Lactic acid bacteria; GRAS, Generally Recognized as Safe; PBS, Phosphate buffer saline; LSB, Lactobacillus Selection base; MIC Minimum Inhibitory Concentration; LB, Luria Bertini, BLAST, Basic Local Alignment Search Tool; NCBI, National Center for Biotechnology Information; GI, Gastrointestinal tract, AGE, Agarose gel Electrophoresis; CTAB, Cetyl trimethyl ammonium bromide.

Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of Interest

The authors declare that they have no competing interests.

Data Availability Statement

1. All data generated or analysed during this study are included in this published article.
2. All the DNA sequences were published in NCBI Database.

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