# Probiotic potential of β-galactosidase-producing lactic acid bacteria from fermented milk and their molecular characterization

MAHANTHESH VASUDHA[1], CHAKRA S PRASHANTKUMAR[1], MALLIKA BELLURKAR[2], VISHWAS KAVEESHWAR[2] and DEVARAJA GAYATHRI[1]

[1]Department of Studies in Microbiology, Davangere University, Davangere, Karnataka 577007; [2]Central Research Laboratory, SDM College of Medical Sciences and Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, Karnataka 580009, India

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**Abstract.** Probiotics have attained significant interest in recent years as a result of their gut microbiome modulation and gastrointestinal health benefits. Numerous fermented foods contain lactic acid bacteria (LAB) which are considered as GRAS and probiotic bacteria. The present study aimed to investigate indigenous LAB from homemade fermented milk samples collected in remote areas of Karnataka (India), in order to isolate the most potent and well-adapted to local environmental conditions bacteria, which were then evaluated using a step-by-step approach focused on the evaluation of probiotic traits and β-galactosidase-producing ability. LAB were screened using 5-bromo-4-chloro-3-indole-D-galactopyranoside (X-Gal) and *O*-nitrophenyl-β-D-galactopy ranoside (ONPG) as substrate, and exhibited β-galactosidase activity ranging from 728.25 to 1,203.32 Miller units. The most promising isolates were selected for 16S rRNA gene sequence analysis and identified as *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Lactiplantibacillus pentosus* and *Lactiplantibacillus* sp. Furthermore, these isolates were evaluated by *in vitro, viz*., survival in gastrointestinal tract, antibiotic susceptibility, antimicrobial activity, cell surface characteristics, and haemolytic activity. All eight isolates demonstrated strong adherence and prevented pathogen penetration into HT-29 cells, indicating potential of the bacteria to scale up industrial level production of milk products for lactose intolerants.

*Correspondence to:* Professor Devaraja Gayathri, Department of Studies in Microbiology, Davangere University, Shivagangothri, Davangere, Karnataka 577007, India
E-mail: gayathridevaraja@gmail.com

Dr Vishwas Kaveeshwar, Central Research Laboratory, SDM College of Medical Sciences and Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, Karnataka 580009, India
E-mail: vishwas.kaveeshwar@sdmmedicalcollege.org

*Abbreviations:* ATCC, American type culture collection; BLAST, Basic Local Alignment Search Tool; CFS, cell-free supernatants; DMEM, Dulbecco's modified Eagle's medium; DNA, deoxyribonucleic acid; LAB, lactic acid bacteria; LI, lactose intolerance; LP, lactase persistence; MIC, minimum inhibitory concentration; MRS, de Man, Rogosa and Sharpe agar; MTCC, microbial type culture collection; NCBI, National Center for Biotechnology Information; ONPG, *O*-nitrophenyl-β-D-galactopyranoside; PBS, phosphate-buffered saline; X-Gal, 5-bromo-4-chloro-3-indole-D-galactopyranoside

*Key words:* probiotic, β-galactosidase, LAB, X-Gal, ONPG, lactose intolerance, HT-29 cells, GenBank

## Introduction

Lactose intolerance (LI) is a typical condition of dairy food intolerance, that occurs generally when lactase activity is decreased in the brush border of human small intestinal mucosa. LI prevalence shows diversity among regions, human populations, continents and across the globe (1,2). It has been observed that 70% of the global human population exhibit transient lactase activity without LI symptoms, which is influenced by nutritional and genetic factors (1). Prevalence of lactase non-persistence condition in Asian and African countries ranges between 80-100%, however, among Northern European countries the prevalence of LI is observed to be very low (2,3). Furthermore, hypolactasia in the Asian continent has rarely been reported, while in the Western world it is relatively prevalent (1). In the Indian subcontinent, particularly the northern region, the frequency of maldigesters was reported to be 48% per 200 subjects during a breath test, while in the southern region it was observed to be higher (66%) (4). It has been documented that, Indo-Aryan migration brought the lactase persistence (LP) trait to northern India, which was later spread by intermixing of the native population (2). Thus, it is of interest to study whether the distribution of the genetic marker responsible for the LP trait varies between northern and southern Indian populations. In addition, in Northwest Russia lactase non-persistence ranges between 16-23% (1-4). Thus, LI management is a worldwide issue in terms of public health management.

The most essential enzyme in the dairy sector for developing low-lactose food stuffs to overcome LI is β-galactosidase (EC 3.2.1.23) and it is commercially manufactured from microorganisms such as bacteria, yeast, and fungus. Although chemically-synthesized enzymes are gaining significance, bacterial enzymes are preferred as they exhibit high activity and stability (5). β-galactosidase enzyme is produced in the small intestine and its deficiency can cause LI. Diarrhea, stomach discomfort, distention, flatus, and borborgygmi are typical symptoms of LI, affecting both infants and adults, and manifest after 30-120 min of lactose absorption (6). Primary deficiency, also known as hypolactasia, is characterized by partial or complete lack of β-galactosidase in the small intestine of children of various ages, while 70% of the population of the world exhibit evidence of usual symptoms in late adolescence and adulthood. Damage to the small intestine induced by an overgrowth of enterobacteria in secondary deficiency results in the destruction of small intestinal cells. Despite the fact that β-galactosidase is a non-inducible enzyme, it is found in the jejunum and produced in the microvillus membrane of the small intestine, where lactose digestion leads to the production of glucose and galactose monosaccharide, and is absorbed by enterocytes (7).

Probiotics are live microorganisms, which when administered in an adequate amount, confer health benefits to the host (FAO/WHO) (8). The mechanism of action of beneficial organisms include, competition with pathogens for adhesion and nutrients, and in addition to the production of antimicrobial metabolites, enhance host immunity against pathogens in the gut. The association between human health and probiotic gut microbiota has been thoroughly studied, with a particular emphasis on homeostatic and barrier function (9). A wide range of metabolites are produced from probiotic bacteria, including nonspecific fatty acids, and highly specific bacteriocins with antimicrobial properties. Previous studies have shown that probiotic bacteria are progressively renowned as a means for alleviating intestinal disorders and treatments have been successful in mouse models for certain clinical intestinal disorders (10,11). The most prominent probiotics are *Lactiplantibacillus* and *Bifidobacteria* genera (10,11) which can be used for prevention and management of numerous disorders including diarrhea, rotaviral diarrhea, *Helicobacter pylori* infection, hyperlipidemia, colitis, acute and chronic gastroenteritis, irritable bowel syndrome, inflammatory bowel disease, cirrhosis, pouchitis, vaginosis and maldigestion-related conditions such as LI, milk protein allergy and soy protein allergy (6).

In the present study, some of the potential lactic acid bacterial isolates were used to alleviate LI and analysed for probiotic potentiality. β-galactosidase-producing lactic acid bacteria (LAB) were isolated and assessed for acid and bile tolerance, antibiotic susceptibility, antimicrobial activity, auto-aggregation and co-aggregation abilities, cell-surface hydrophobicity, and HT-29 cell adhesion and invasion assays. The 16S rRNA gene consists of highly conserved nucleotide sequences that can be used to distinguish closely related bacterial species and to determine the taxonomy and phylogeny of unknown bacteria by comparing the obtained sequence to known sequences of other bacteria in the GenBank database (12,13). Thus, molecular characterization of selected LAB isolates using 16S rRNA sequence analysis was performed.

## Materials and methods

*Isolation of LAB from homemade curd samples.* A total of 30 homemade curd samples were collected from different rural regions of Karnataka state, India and the samples were stored at 4˚C until further use. With all aseptic precautions, the samples were homogenized, serially diluted (tenfold), 0.1 ml of the sample was plated on de Man Rogosa Sharpe (MRS) agar (Himedia Laboratories Pvt, Ltd.) and incubated for 24 to 48 h at 37˚C. Bacterial colonies developed on MRS media were serially subcultured by following microdilution technique and pure cultures were preserved at 4˚C/MRS agar slants.

*Screening of β-galactosidase-producing LAB.* A total of 450 LAB isolates were inoculated with MRS agar medium supplemented with 60 µl X-Gal (20 mg/ml in DMSO; Himedia Laboratories Pvt, Ltd.) as a chromogenic substrate and 10 µl of iso-propyl-thio-β-D-galactopyranoside (IPTG) (Himedia Laboratories Pvt, Ltd.) as an inducer for the β-galactosidase. Following incubation for 48 h at 37˚C, development of blue colonies indicated β-galactosidase enzyme activity (10,14).

*Quantitative assay for β-galactosidase.* β-galactosidase assay of eight isolates was performed (15,16). Briefly, selected isolates were centrifuged at 12,000 x g for 5 min at 4˚C (Eppendorf AG 22331; Eppendorf SE) and washed twice in phosphate-buffered saline (PBS) and cells were adjusted to 1.0 (560 nm). Furthermore, cells were permeabilized with 50 µl of toluene/acetone (1:9 v/v) (HiMedia Laboratories Pvt, Ltd.), vortexed for 7 min and then 100 µl of cell suspension was added to a tube consisting of 900 µl of phosphate buffer and 200 µl of *O*-nitrophenyl-β-D-galactopyrano side (ONPG; 4 mg/ml) solution (Himedia Laboratories Pvt, Ltd.). Additionally, after a 15-min incubation period at 37˚C, 0.5 ml of 1 M $Na_2CO_3$ was added to terminate the reaction, and absorbance values at 420 and 560 nm (NanoDrop 2000C UV-Spectrophotometer; Thermo Fisher Scientific, Inc.) were recorded, with β-galactosidase activity represented in Miller units:

$$\beta - \text{galactosidase activity} = 1{,}000\,\frac{(A1_{420} \times 1.75 \times A2_{560})}{(15\ \text{min} \times 1\ \text{ml} \times A1_{560})}$$

Where, $A1_{560}$ denotes the absorbance before the test and $A2_{560}$ denotes the absorbance of the reaction mixture.

*Phenotypic and molecular characterization.* β-galactosidase-producing isolates were identified by colony characteristics *viz.,* size, shape, color and texture. Furthermore, the isolates were subjected to biochemical tests and characterized based on Bergey's manual of systematic bacteriology (17). Furthermore, carbohydrate fermentation was performed for species level identification using glucose, fructose, sucrose, galactose, lactose, maltose, cellobiose, xylose, arabinose, rhamnose, mannitol, and sorbitol sugars as previously described (18,19).

*Identification by 16S rRNA gene sequence.* From the selected bacterial isolates, DNA was isolated using the CTAB protocol (20) and the extraction was confirmed by electrophoresis using 0.8% agarose gel. The PCR reaction

mixture contained 400 ng of forward primer and 400 ng reverse primer, 4 $\mu$l 10X dNTPs (2.5 mM each), 10 $\mu$l of DNA polymerase assay buffer and 3U of 1 $\mu$l Taq DNA polymerase enzyme (Sigma-Aldrich; Merck KGaA). For 16S rRNA gene amplification, prokaryotic universal primers [Pair 1: ~1,500 bp amplification, forward primer (395), 5'-GGATGAGCCCGCGGCCTA-3' and reverse primer (396), 5'-CGGTGTGTACAAGGCCCGG-3'; Pair 2: ~1,300 bp amplification, forward primer (63F), 5'-CAGGCCTAACAC ATGCAAGTC-3' and reverse primer (1387R), 5'-GGCGGA TGTGTACAAGGC-3'] were used and this experiment was performed at CellKraft Biotech Pvt, Ltd., and designed, using an ABI thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the program: Denaturation at 94˚C for 5 min, followed by 35 cycles at 94˚C for 1 min, annealing at 55˚C for 1 min, and extension at 72˚C for 2 min, and a final extension for 7 min at 72˚C. Amplification was confirmed by electrophoresis of PCR products using 1% agarose gel and then sequenced by Sanger sequencing method with an ABI 3130 genetic analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). 16S rRNA sequences were compared using the Basic Local Alignment Search Tool (BLASTn) program of the National Center for Biotechnology Information (NCBI) database. Furthermore, the multiple sequence alignment was aligned using the MUSCLE program (https://www.drive5.com/muscle/) and the phylogenetic tree was constructed by the neighbor-joining method using MEGA-X software (https://www.megasoftware.net/). Partial nucleotide sequences of 16S rRNA of the identified *Lactiplantibacillus* isolates were deposited in the NCBI/GenBank (12,13).

*In vitro evaluation for potential probiotic bacteria. In vitro* studies were undertaken to evaluate the probiotic potentiality of β-galactosidase-producing isolates as per FAO/WHO (8). All eight isolates were subjected to simulated gastric juice and bile tolerance tests. The simulated gastric juice contained 0.3% w/v pepsin and 0.5% w/v NaCl (Himedia Laboratories Pvt, Ltd.), at pH 2 or 4. A total of 1 ml of cell suspension was centrifuged (12,000 x g, 5 min at 5˚C), inoculated into 10 ml of gastric juice with pH 2 or 4, incubated at 37˚C for 3 h and cell viability was measured as colony-forming units (CFU) by plating technique, and the percentage of survival was calculated as follows:

$$\text{Survival rate } (\%) = \frac{\text{CFU per ml in pH 2 or 4}}{\text{CFU per ml in contol}} 100$$

Bile tolerance was determined by inoculating each strain (1% v/v) into MRS broth with 0.3% (w/v) of bile salt (Oxgall; Himedia Laboratories Pvt, Ltd.) and incubated for 3 h at 37˚C. Viability was measured as CFU by plating technique and compared with the control (without bile salt) (13,21).

$$\text{Survival rate } (\%) = \frac{\text{CFU per ml in bile salt}}{\text{CFU per ml in contol}} 100$$

Pancreatic enzyme tolerance was calculated according to a study by Rashmi and Gayathri (13), with slight modifications. Overnight cultures were centrifuged at 6,000 x g (20 min at 5˚C), inoculated into simulated pancreatic juice (SPJ; bile 3 g/l and pancreatin 0.1 g/l, sodium phosphate dibasic heptahydrate 50.81 g/l, and NaCl 8.5 g/l in a $KH_2PO_4$ buffer at pH 8.0; Himedia Laboratories Pvt, Ltd.), incubated at 37˚C for 3 h and the percentage of survival was calculated as follows:

$$\text{Survival rate } (\%) = \frac{\text{CFU per ml in SPJ}}{\text{CFU per ml in contol}} 100$$

*Hemolytic activity*. Sheep blood agar (Himedia Laboratories Pvt, Ltd.) was used for inoculation of selected LAB isolates and incubated for 48 h at 37˚C, and then plates were observed for α, β, or γ hemolysis (22,23).

*Hydrophobicity*. For the hydrophobicity assay, two different solvents *viz.,* non-polar solvent-xylene and polar solvent-chloroform was used. Centrifuged cells (12,000 x g for 5 min at 5˚C) were suspended in 50 mM $K_2HPO_4$ (pH 6.5) buffer and adjusted to OD 1.0 at $A_{600}$ nm and 3 ml of the bacterial suspensions were mixed with 1 ml of solvent and allowed to stand at room temperature for 20 min (13,15). The percentage of bacterial adhesion to the solvent was measured using the aqueous phase at 600 nm.

$$\text{Hydrophobicity } (\%) = \frac{(A_0 - A)}{A_0} 100$$

Where, $A_0$ and A are the absorbance before incubation and after incubation, respectively.

*Antimicrobial activity against human pathogenic bacteria*. Antimicrobial activity using crude secondary metabolites of LAB against selected pathogenic bacteria was performed using the agar well diffusion technique. Cell-free supernatants (CFS) of each bacterial isolate were prepared and adjusted to pH 6.5. Certain selected strains of pathogenic bacteria *viz., Escherichia coli* (MTCC no. 433), *Staphylococcus aureus* (ATCC no. 6538), *Pseudomonas aeruginosa* (ATCC no. 9027), *Salmonella abony* (ATCC no. BAA2162) and *Listeria monocytogenes* (*L. monocytogenes*; MTCC no. 1143), were purchased from the American Type Culture Collection (ATCC) and Microbial Type Culture Collection (MTCC; IMTech), and 0.1 ml of pathogens were inoculated onto Mueller Hinton agar media (Himedia Laboratories Pvt, Ltd.). Furthermore, 7-mm diameter wells were made in the agar plates and 100 $\mu$l of CFS was placed into these wells, and finally the inhibition zone was measured (mm) after 48 h of incubation at 37˚C.

*Antibiotic sensitivity test*. Antibiotic disc diffusion method was performed according to Kumara *et al* (23) with modifications. A total of 0.1 ml of each selected isolate was inoculated onto MRS agar media and antibiotic disc containing penicillin (P) 10 mcg, erythromycin (E) 15 mcg, ampicillin (AMP) 10 mcg, amikacin (AK) 30 mcg, ofloxacin (OF) 5 mcg, cefixime (CFM) 5 mcg, ciprofloxacin (CIP) 5 mcg, and azithromycin (AZM) 15 mcg (Himedia Laboratories Pvt, Ltd.) were placed and incubated for 24 h at 37˚C. Subsequently, the diameter of the inhibition zone was measured in mm.

*Auto-aggregation and co-aggregation assays*. The ability of bacteria to auto-aggregate and co-aggregate was assessed according to Armas *et al* (22) with slight modifications. Stationary phase cells were centrifuged (5,000 x g for 15 min at 5˚C), pellets were washed thrice and suspended in PBS and the OD was adjusted to 1 (equivalent to $10^9$ CFU m/l)

at 600 nm. The bacterial suspension (4 ml) was incubated at 37˚C and monitored at a different time intervals (0 to 5 h) and 0.1 ml of upper suspension was removed and mixed with 3.9 ml of PBS. The percentage of absorbance was then measured at 600 nm using the following formula:

$$\text{Autoaggregation (\%)} = 1 - \left(\frac{A_t}{A_0}\right) \times 100$$

Where, $A_t$: Absorbance of the upper layer mix at a particular time (1 to 5 h).

$A_0$: Absorbance at time zero.

To determine co-aggregation, equal volumes (5 ml; 1:1) of each selected isolate and pathogens [*E. coli* (MTCC no. 433) and *L. monocytogenes* (MTCC no. 1143] were incubated together at 37˚C for 5 h without disturbance. The absorbance was then calculated at 600 nm and the percentages of co-aggregation were determined as follows:

$$\text{Co} - \text{aggregation (\%)} = 1 - A_{mix}\frac{(A_{\text{probiotic bacteria}} + A_{\text{pathogen}})}{2}100$$

Where, $A_{\text{probiotic bacteria}}$: Absorbance of the *Lactiplantibacillus* isolates as control,

$A_{\text{pathogen}}$: Absorbance of the pathogen as a control

$A_{mix}$: Absorbance of both probiotic bacteria and the pathogen in a single tube.

*Adhesion assay.* Bacterial adhesion with human colon cancer cells was performed (18,24) with some modifications. The human HT-29 cell line (ATCC no. HTB-38; ATCC) was used, and the cell culture work was carried out at the Central Research Laboratory, SDM College of Medical Sciences and Hospital (Dharwad, India). Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (both from Himedia Laboratories Pvt, Ltd.) were used to grow HT-29 cells in 12-well flat-bottom cell culture plates until they reached 80% confluence. Prior to the experiment, HT-29 cells were washed gently with PBS twice. Subsequently, the selected eight isolates were centrifuged (5,000 x g for 15 min at 4˚C) and suspended in DMEM without antibiotics to provide approximately $10^9$ CFU ml of the bacterial suspension. Additionally, 200 $\mu$l of each strain was added to separate wells and incubated for 2 h at 37˚C in 5% $CO_2$ atmosphere. The HT-29 cells were then washed twice in sterile PBS to remove non-adherent bacteria before being lysed in 2 ml of 0.1% Triton X-100 in PBS. Cell lysates were tenfold serially diluted and plated with MRS agar and incubated for 24 h at 37˚C. The percentage of adherence was expressed using the formula:

$$\text{Adhesion (\%)} = \frac{\text{CFU of adhered bacteria per ml}}{\text{CFU of initial bacteria per ml}}100$$

*Gram's staining for cell adhesion assay.* The adhesion of all eight isolates to HT-29 cells in cell culture plates was assessed using the methanol fix technique for microscopic analysis. Each well received 3 ml of methanol, which was allowed to stand for 10 min. Furthermore, fixed cells were stained with Gram's solution (at 28˚C for 5 min) and examined under oil immersion objective (Olympus Corporation) (24,25).

*Invasion assay. In vitro* methods evaluated the ability of the eight selected isolates to inhibit the colonization of human pathogens with intestinal cells (13,26). HT-29 cells and the eight isolates were inoculated at a concentration of $10^9$ CFU per well in antibiotic-free DMEM and incubated for 2 h. *L. monocytogenes* (MTCC no. 1143 and *E. coli* (MTCC no. 433) ($10^9$ CFU/well) were inoculated into each well with the antibiotic-free medium and incubated for 1 h at 37˚C with 5% $CO_2$. Furthermore, extracellular bacteria in the well were eliminated by transferring DMEM to 10% FBS with streptomycin 100 U/ml and incubating for 1 h. Subsequently, 2 ml of 0.1% Triton X-100 in PBS was used to lyse the treated HT-29 cells. Additionally, to count invading bacteria, 0.1 ml of $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions of cell lysates were inoculated with brain heart infusion agar (Himedia Laboratories Pvt, Ltd.). Invasion assays were performed on cell lines that had only been exposed to pathogens. The invasion percentage was determined using the following formula:

$$\text{Invasion (\%)} = \frac{\text{Control pathogen (CFU per ml)} - \text{Probiotic bacteria with pathogen (CFU per ml)}}{\text{Control pathogen (CFU per ml)}}100$$

*Statistical analysis.* All experiments were conducted in triplicate and the results were reported as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc.). Differences between multiple groups were compared using one-way ANOVA with post hoc Tukey's multiple comparison tests, Brown-Forsythe test and Bartlett's test and two-way ANOVA of grouped multiple t-tests. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Isolation and screening of β-galactosidase-producing bacteria.* A total of 450 LAB isolates were isolated from homemade curd samples collected from various regions of Karnataka (12.97 N 77.50 E), India and all isolates were screened for the β-galactosidase enzyme by qualitative assay using X-gal plates (Fig. 1A). Development of blue-green colored bacterial colonies were selected and the presumptive tests confirmed that selected isolates were Gram-positive, non-motile, non-spore producing *Bacillus* and the carbohydrate fermentation pattern varied among the isolates (Fig. 2). Based on the quantitative assay of β-galactosidase activity level ranging from 728.25 to 1,203.32 (U/ml) Miller units ($P<0.05$), it was revealed that *Lactiplantibacillus sp.* GV66 had the highest value of 1,203.32 (U/ml) and *L. fermentum* GV254 had the lowest value of 728.25 (U/ml) Miller units.

*Identification of β-galactosidase probiotic isolates by 16S rRNA gene sequence.* Molecular characterization employing 16S rRNA gene sequence analysis was performed for eight most potential β-galactosidase-producing isolates. To estimate an approximate phylogenetic association, the acquired nucleotide sequences were compared with existing nucleotide gene sequences from GenBank using the BLAST tool. Furthermore, nucleotide sequences were aligned using MUSCLE, and a phylogenetic tree was constructed using the neighbor-joining method in the MEGA-X software. All eight isolates belonged to phylum Firmicutes, showing the highest similarity with the genera *Lactiplantibacillus*. Fig. 3 illustrates the phylogenetic tree, in which GV54, GV64, GV69, GV418 showed 99% and

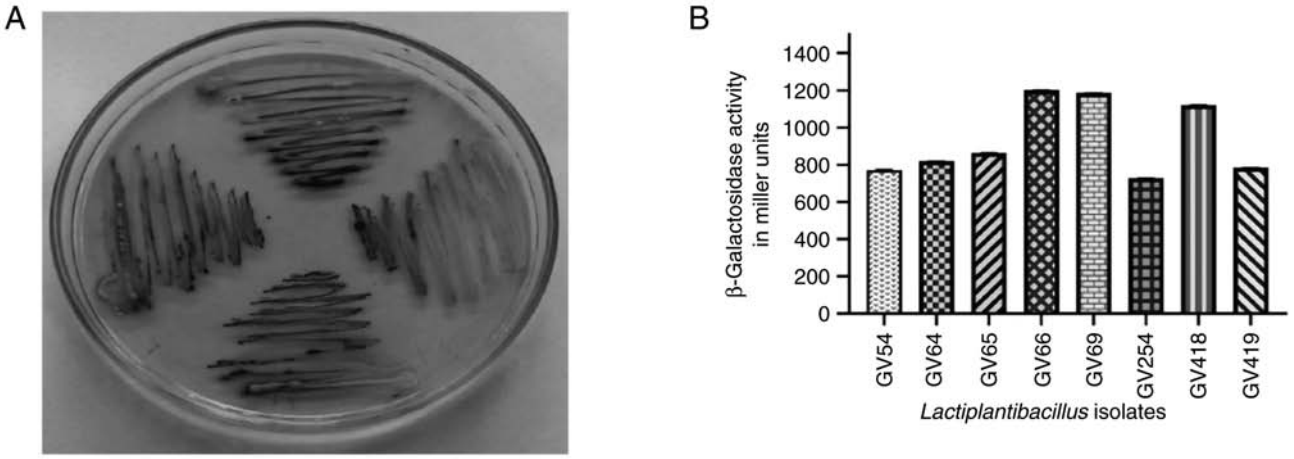


Figure 1. (A) Bacterial colonies (deep grey colour) were identified on a de Man, Rogosa and Sharpe agar plate containing 5-bromo-4-chloro-3-indole-D-galactopyranoside, for β-galactosidase production. (B) β-Galactosidase production and bar graph represents β-galactosidase activity of *Lactiplantibacillus* isolates in Miller units. Bar graph was plotted incorporating the sample mean (n=3) and error bar (standard deviation of individual isolate).

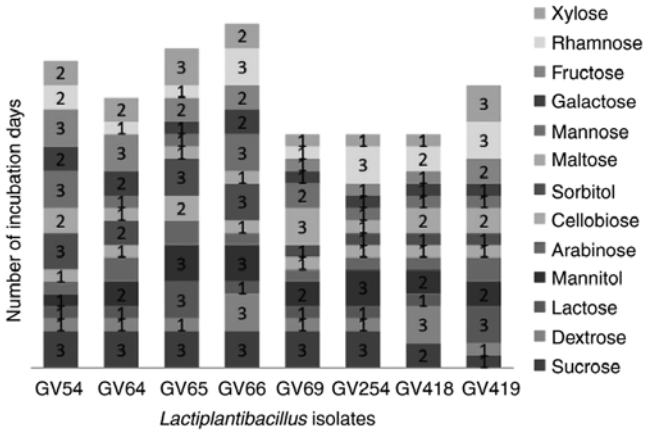


Figure 2. Carbohydrate fermentation profile of *Lactiplantibacillus* isolates.

GV419 showed 98% similarity with *L. plantarum*. However, GV66 showed 98% similarity with *Lactiplantibacillus* sp., and GV65 showed 99% similarity with *L. pentosus*, whereas GV254 showed 100% similarity with *L. fermentum*. Furthermore, nucleotide sequences were deposited in the GenBank database.

*In vitro evaluation of potential probiotic bacteria*. All eight identified β-galactosidase-producing isolates were subjected to probiotic characterization, in which the isolates exhibited considerably less tolerance to gastric juice at pH 2, while at pH 4 the survival rate was increased, and among them *L. plantarum* GV64 exhibited the highest tolerance (82.6%) and *L. plantarum* GV54 exhibited the lowest tolerance (17.77%). For bile tolerance *L. plantarum* GV418 showed the highest tolerance (99.93%) and *L. plantarum* GV54 showed the lowest tolerance (44.68%) to bile juice after 4 h of incubation. The percentage of tolerance of simulated gastric juice and bile juice is illustrated in Fig. 4. In the pancreatic enzyme tolerance test, the survival rate ranged from 54 to 77.33%, and among them *L. plantarum* GV54 exhibited the highest tolerance (77.33%) and *L. plantarum* GV418 exhibited the lowest tolerance (54%) after 4 h of incubation (Fig. 4). The survival percentage of the selected eight isolates in gastric, bile and pancreatic juices confirmed the resistance to upper gastrointestinal conditions. All eight isolates exhibited γ hemolytic activity and were demonstrated as non-pathogenic.

*Hydrophobicity*. The results of assessment of hydrophobicity indicated that the eight isolates were hydrophobic, as revealed in Fig. 5A. *L. plantarum* GV419 (63%) and *L. plantarum* GV69 (56%) with maximum affinity, whereas *Lactiplantibacillus* sp. GV66 (9%) and *L. plantarum* GV64 (15%) with minimal affinity to xylene and chloroform, respectively.

*Antimicrobial activity*. The findings of the agar well diffusion method indicated that the eight isolates have an antagonistic impact on human pathogens. All isolates exhibited a zone of inhibition ranging from 4.66±0.57 to 27.00±0.00 mm, whereas *L. plantarum* GV64 was resistant to *L. monocytogenes* (MTCC no. 1143) and *Lactiplantibacillus* sp. GV66 and *L. plantarum* GV69 were resistant to *Pseudomonas aeruginosa* (ATCC no. 9027) (Table I).

*Antibiotic sensitivity*. Antibiotic discs were used to assess antibiotic sensitivity/resistance of the eight isolates, and the assessed antibiotics suppressed the growth of *L. fermentum* GV254 and *L. plantarum* GV418. On the other hand, *L. plantarum* GV54 was cefixime-resistant; *L. plantarum* GV64 was resistant to ofloxacin, cefixime, and ciprofloxacin; *L. pentosus* GV65 was amikacin-resistant; *Lactiplantibacillus* sp. GV66 was ofloxacin-resistant; *L. plantarum* GV69 was resistant to ampicillin, ofloxacin, and cefixime; and *L. plantarum* GV419 was resistant to ofloxacin and cefixime [measured in terms of diameter (mm); Table II].

*Auto-aggregation and co-aggregation assays*. The percentage of auto-aggregation was measured after every hour of incubation, and *L. plantarum* GV54 exhibited the highest rate of auto-aggregation (81%), while *L. plantarum* GV69 and *L. plantarum* GV419 showed moderate auto-aggregation (61 and 71%, respectively). *L. pentosus* GV65 exhibited minimal

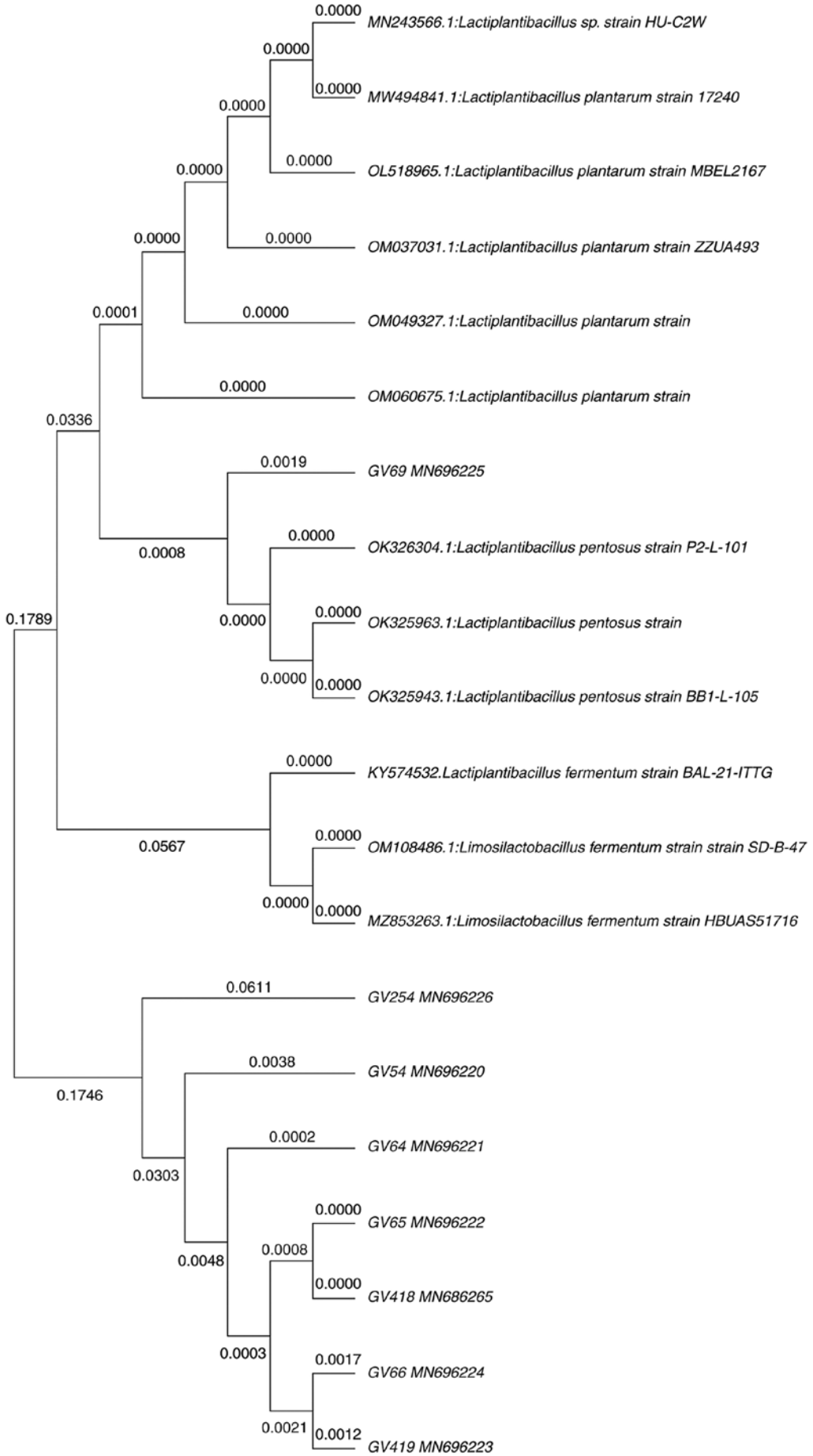


Figure 3. Phylogenetic neighbor-joining tree based on the 16S rDNA sequences (~1,300-1,500 bp) of the eight selected lactic acid bacteria strains of curd samples based on the results of alignment. The scale bar represents 0.10 nucleotide substitutes per position.

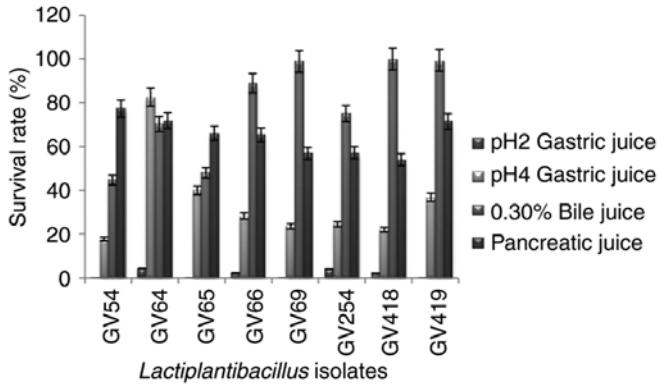


Figure 4. Gastric, bile, and pancreatic juice tolerance tests of *Lactiplantibacillus* isolates, expressed as percentages [the bar graph was plotted incorporating the sample mean (n=3) and error bar of individual isolates].

auto-aggregation 50% (Fig. 5B). Furthermore, the eight isolates exhibited co-aggregative properties with both the pathogens, *E. coli* (MTCC no. 433) and *L. monocytogenes* (MTCC no. 1143), after 5 h of incubation at 37˚C. The rate of co-aggregation of *E. coli* and *L. monocytogenes* ranged between 10 and 28%, and 14 to 42%, respectively. *L. monocytogenes* exhibited higher co-aggregation compared to *E. coli*. Notably, *L. plantarum* GV419 exhibited higher co-aggregation (28.67%) of *E. coli* compared to *L. monocytogenes*, while *L. plantarum* GV64 (42.71%) exhibited higher co-aggregation of *L. monocytogenes* compared to *E. coli* (Fig. 5C).

*Adhesion assay*. Adhesion assay of eight potential isolates to human colorectal adenocarcinoma intestinal epithelial HT-29 cells was determined and graphically represented in Fig. 5D. Bacterial adherence ability with *Lactiplantibacillus* sp. GV66 was 99.43% and *L. fermentum* sp. GV254 exhibited considerably less adhesion at 81.14%. The adhesion of all eight isolates was further verified by direct microscopic observation (Fig. 6A).

*HT-29 cell invasion assay*. All eight isolates were investigated for the suppression of *E. coli* and *L. monocytogenes* intracellular invasion, as revealed in Fig. 6B. The inhibition percentage ranged from 3.26 to 37.41% and 2.56 to 31.31%, respectively. *L. plantarum* GV54 had a low percentage of invasion for both pathogens (3.26 and 2.56%). *Lactiplantibacillus sp*. GV66 and *L. plantarum* GV64 exhibited the highest percentage of invasion of *E. coli* (37.41%) and *L. monocytogenes* (31.31%), respectively.

## Discussion

Fermented foods containing LAB are traditionally used in daily food intake. Curd, prepared by fermentation of milk with an inoculum of previously made curd, is used in most households in India, where it constitutes a significant part of the daily diet. The LAB that ferment the milk are likely to differ slightly in each household as there is no standardized starter culture used to prepare the curd. Although curd is considered to contain probiotics, there is little documentation in this line. The present study was undertaken to evaluate the LAB from homemade curd in southern India for probiotic properties. Probiotic diversity is very vast, therefore, in the present study, potential LAB isolates were selected, which are capable of eliminating or reducing LI.

The key findings in the present study successfully revealed the most promising isolates (GV54, GV64, GV65, GV66, GV69, GV254, GV418, and GV419) with probiotic characteristics and β-galactosidase production. LAB that are found in fermented foods aid lactose digestion by increasing the activity of the β-galactosidase enzyme with distinct health advantages (19). It has been reported that lactose from milk digested by β-galactosidase to hydrolyse glucose and galactose, is absorbed by enterocytes and used as an energy source (4). LI symptoms can be managed with dairy products supplemented with β-galactosidase-producing probiotics, which also inhibit human pathogen adhesion (2). Hence, selected isolates were preferred for β-galactosidase enzyme activity with X-Gal and ONPG as substrates (14). While, *Lactococcus casei* A13 exhibited negative or zero enzyme activity, *L. delbrueckii subsp. bulgaricus* Db1 exhibited 2,053±25 Miller units (15), which was the highest enzyme activity reported, and served as a positive control. In the present study, β-galactosidase production was highest (1,203.32 Miller units) in *Lactiplantibacillus* sp GV66 with 58% of enzyme activity compared to the positive control. Therefore, this isolate is a potential β-galactosidase product and hence the strain can be further improved for industrial production of β-galactosidase.

API50 CHL and 16S rRNA sequence analysis may also be used to identify LAB (27,28). Kumara *et al* (23) used 16S rRNA gene sequence analysis to identify four LAB isolates, all of which were demonstrated to be *L. fermentum*. The present study identified eight potential isolates, by molecular characterization using 16S rRNA gene sequencing, including *L. plantarum* GV54, GV64, GV69, GV418 and GV419, *Lactiplantibacillus* sp. GV66, *L. pentosus* GV65, *L. fermentum* GV254, and deposited them in the GenBank database.

*Lactiplantibacillus* sp. have been termed probiotic bacteria due to their ability to survive in gut conditions such as gastric juice and bile, and exhibit pancreatic enzyme tolerance, as well as intestinal epithelial cell adhesion (29). However, the aforementioned attributes are not completely the same in *in vitro* testing as found in the *in vivo* gut system, but it is still a valuable tool for rapid screening of possible probiotic strains. *In vivo* investigations are more expensive and time-consuming than *in vitro* studies for evaluating the probiotic characteristics of unknown bacteria; hence, *in vitro* testing is selected as an alternative (21). Although, an *in vivo* validation for the above results is a limitation in this study, further animal studies are in progress. Bacteria must be able to survive in intestinal conditions for considerably long periods to be classified as probiotics (30).

*L. fermentum* HM3 isolate exhibited better acid tolerance at pH 3 for 3 h than the reference strain *L. casai Shirota* (31). In addition, Vinderola and Reinheimer (15) reported that *S. thermophilus* exhibited tolerance while *L. lactis* and *L. delbrueckii subsp. Bulgaricus* exhibited tolerance at pH 2 and 3. Hsieh *et al* (32) reported that *Lactobacillus* strains TSP05, TSF331 and TSR332 were resistant to gastric

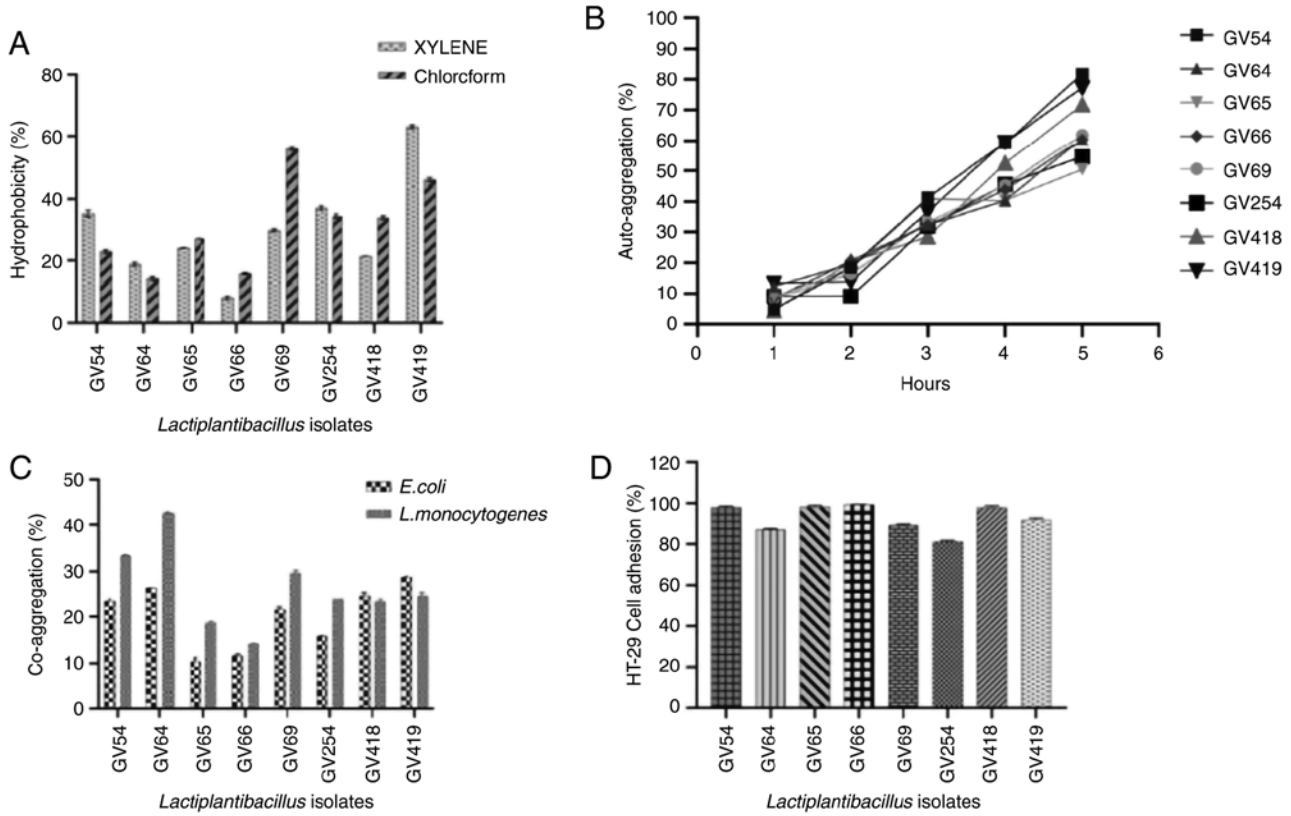


Figure 5. (A) Hydrophobicity of *Lactiplantibacillus* isolates with xylene and chloroform. (B) Auto-aggregation expressed as a percentage after each hour of incubation. (C) Co-aggregation expressed as a percentage after 5 h of incubation with *E. coli* and *L. monocytogenes*. (D) HT-29 cell adhesion of *Lactiplantibacillus* isolates expressed as a percentage after 3 h of incubation. *E. coli*, *Escherichia coli*; *L. monocytogenes*, *Listeria monocytogenes*.

acid at pH 3.5 in MRS medium for 3 h, and with regard to the cell viability measured as CFU/ml, *Lactobacillus* TSF331 exhibited the highest viability ($4.45x10^9$) in gastric conditions. In the present study, *Lactiplantibacillus* strains exhibited better acid tolerance at pH 4 than pH 2 for 4 h. Among them, *L. plantarum* GV64 exhibited the highest tolerance (82.6%) and *L. plantarum* GV54 showed the lowest tolerance (17.77%). Several studies have reported that 0.3% (w/v) bile salt concentration in the human gut also varies according to diet and the level of pancreatic enzyme secretion (33). Succi *et al* (34) also reported that most of the LAB showed resistance to a 0.5% bile concentration, and Byakika *et al* (35) examined whether the probiotic bacteria were tolerant to 1% bile salt. Hsieh *et al* (32) also reported that 0.3% bile was used for a bile tolerance test and *Lactobacillus* TSR332 exhibited the highest viability ($4.74x10^8$ CFU/ml) indicating high tolerance to bile salt. In the present study, a 0.3% bile concentration was used and eight isolates that exhibited favourable bile tolerance (99.93%) were selected. Among them, *L. plantarum* GV418 showed the highest tolerance (99.93%) and *L. plantarum* GV54 exhibited the lowest tolerance (44.68%). Pancreatic enzymes digest various carbohydrates, proteins, and lipids in the human diet (33). According to Shokryazdan *et al* (31), LAB strains were incubated for 3 h in growth media containing pancreatic enzymes, and the viability of the *L. brevis* strain differed. All eight isolates of the present study, exhibited high pancreatic tolerance in this investigation, and the degree of tolerance differed among strains. As aforementioned, the assessed LAB isolates were able to survive at gastric pH. Notably, to increase 100% viability of lactic acid bacterial cells, either encapsulation or coating with inert biomolecules can be performed to improve the likelihood of survival.

The eight isolates in the present study with the highest hydrophobicity demonstrated maximum adherence to xylene and chloroform solvents, with *L. plantarum* GV419 showing 63% adhesion to xylene and *L. plantarum* GV69 exhibiting 56% adhesion to chloroform. The adherence of fecal isolates to the affinity for n-hexadecane and toluene was examined in another study (25). The greatest hydrophobicity of *L. brevis CCMA 1284, L. plantarum CCMA 0743, L. plantarum CCMA 0359* was reported by Fonseca *et al* (36), and Behbahania *et al* (37) analyzed *L. plantarum* strain L15, which showed 54% adherence to solvents. This suggests that the hydrophobic and hydrophilic appendages, as well as other macromolecule components, contribute to the cell surface mosaic's complexity, resulting in hydrophobicity differences toward hydrocarbons.

The ability of auto-aggregation and co-aggregation increased with time, reaching a maximum level at 24 h of incubation rather than at 5 h (38). The auto-aggregation ability of the *L. plantarum* strain was shown to be the highest in a similar study reported by Fonseca *et al* (36), which revealed that *L. paracasei CCMA 0504* and *L. paracasei CCMA 0505* exhibited the highest percentage of auto-aggregation. All eight isolates in the present study exhibited variable values of auto-aggregation. Notably, *L. plantarum* GV54 showed the highest rate of auto-aggregation with 81%, and *L. plantarum* GV69 and *L. plantarum* GV419 exhibited moderate

Table I. Antimicrobial activity against human pathogenic bacteria.

| Isolates | Human pathogenic bacteria with zone of inhibition in diameter (mm) | | | | |
|---|---|---|---|---|---|
| | *Escherichia coli* (MTCC no. 433) | *Staphylococcus aureus* (ATCC no. 6538) | *Pseudomonas aeruginosa* (ATCC no. 9027) | *Salmonella abony* (ATCC no. BAA2162) | *Listeria monocytogenes* (MTCC no. 1143) |
| GV54 | 12.33±0.57 | 12.66±0.57 | 9.33±0.57 | 16.00±1.0 | 10.33±0.57 |
| GV64 | 4.66±0.57 | 11.00±1.00 | 7.33±0.57 | 10.33±0.57 | ND |
| GV65 | 12.66±0.57 | 11.33±0.57 | 12.33±0.57 | 12.33±0.57 | 13.33±0.57 |
| GV66 | 17.66±0.57 | 9.33±0.57 | ND | 14.66±0.57 | 9.33±0.57 |
| GV69 | 27.00±0.00 | 14.66±0.57 | ND | 12.33±0.57 | 18.66±0.57 |
| GV254 | 23.33±0.57 | 11.33±0.57 | 13.66±0.57 | 12.33±0.57 | 9.33±0.57 |
| GV418 | 23.66±0.57 | 7.66±0.57 | 15.00±0.00 | 13.66±0.57 | 10.66±0.57 |
| GV419 | 23.00±0.00 | 9.66±0.57 | 12.33±0.57 | 15.6667 | 9.00±1.00 |

Zone of inhibition in mm. ND, not detected.

Table II. Assessment of antibiotics for *Lactiplantibacillus* isolates with probiotic potentiality.

| Isolates | Zone of inhibition in diameter (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AMP | AK | OF | P | CFM | CIP | E | AZM |
| GV54 | 10.33±0.57 | 11.66±1.52 | 9.66±0.57 | 10.66±0.57 | ND | 12.66±0.57 | 29.33±0.15 | 24.66±0.57 |
| GV64 | 11.00±0.00 | 12.33±0.57 | ND | 13.66±0.57 | ND | ND | 30.00±00 | 26.33±1.15 |
| GV65 | 9.33±0.57 | ND | 10.33±0.57 | 12.66±0.57 | 9.66±0.57 | 12.33±0.57 | 30.00±00 | 24.33±0.57 |
| GV66 | 6.00±0.00 | 15.66±0.57 | ND | 12.00±0.00 | 10.33±0.57 | 10.33±0.57 | 25.66±0.57 | 20.00±00 |
| GV69 | ND | 14.66±0.57 | ND | 10.66±0.57 | ND | 10.33±0.57 | 29.66±0.57 | 24.66±0.57 |
| GV254 | 12.33±0.57 | 14.66±0.57 | 13.00±0.00 | 10.00±0.00 | 10.00±0.00 | 14.66±0.57 | 27.66±0.57 | 23.33±0.57 |
| GV418 | 12.33±0.57 | 14.00±0.00 | 8.66±0.57 | 7.66±0.57 | 13.33±0.57 | 9.00±0.00 | 30.00±00 | 23.00±00 |
| GV419 | 8.00±0.00 | 14.66±0.57 | ND | 7.33±0.57 | ND | 8.33±0.57 | 24.66±0.57 | 17.66±0.57 |

Zone of inhibition in mm. AMP, ampicillin; AK, amikacin; OF, ofloxacin; P, penicillin; CFM, cefixime; CIP, ciprofloxacin; E, erythromycin; AZM, azithromycin; ND, not detected.

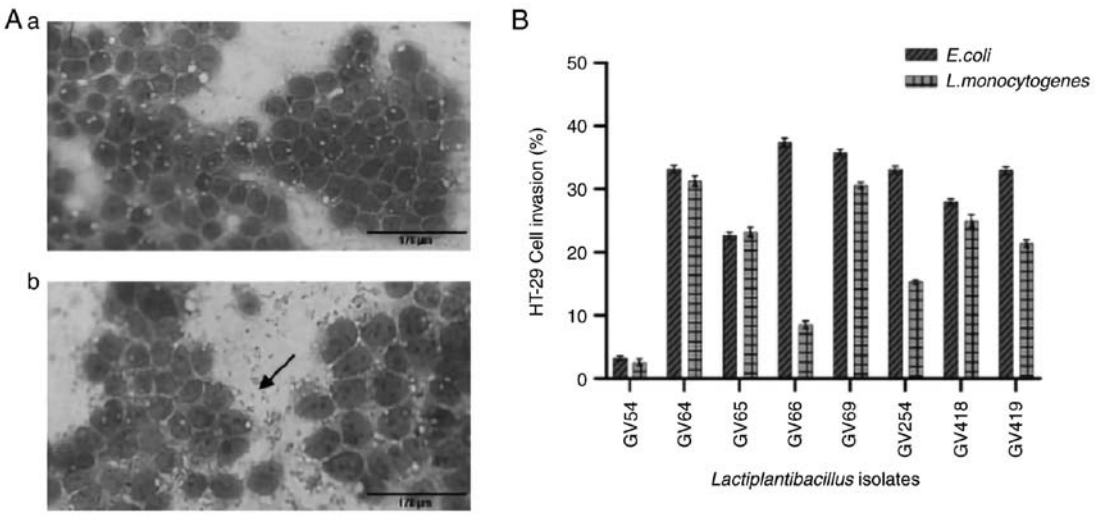


Figure 6. (Aa) Control HT-29 cell line monolayer and (Ab) arrow showing adhered Gram-positive *Lactiplantibacillus* isolate to HT-29 cells (magnification, x1,000). (B) HT-29 cell invasion of human pathogens *E. coli* and *L. monocytogenes* expressed as a percentage after 3 h of incubation with *Lactiplantibacillus* spp. *E. coli*, *Escherichia coli*; *L. monocytogenes*, *Listeria monocytogenes*.

auto-aggregation with 61 and 71%, respectively. Furthermore, all isolates exhibited co-aggregative properties, and among them, *L. plantarum* GV419 exhibited a high co-aggregation (28.67%) with *E. coli* while *L. planatrum* GV64 (42.71%) with *L. monocytogenes*.

The antimicrobial activity against human pathogens is considered as a main characteristic of probiotic strains that maintain gut health (21). All eight isolates were antagonistic against human pathogens in the present study, with the zone of inhibition ranging from 4.66±0.57 to 27.66±0.57 mm. Asha and Gayathri (18) assessed *Lactiplantibacillus* strains from curd samples for their antibacterial activity against *E. coli, V. cholerae, Klebsiella* strains, *Proteus* strains, and *S. dysenteriae*. An additional role of the *Lactiplantibacillus* strain is to inhibit the colonization of pathogenic bacteria to human and animal intestines by secreting several biochemical compounds and enzymes to prevent various infections. Kumara *et al* (23) reported that *L. fermentum* inhibited colonization of *S. ebony, S. aureus, E. coli, P. aeruginosa,* and these bacteria were susceptible to gentamycin, chloramphenicol, cefoperazone, ampicillin, and resistant to ciprofloxacin and vancomycin. The susceptibility of *Lactiplantibacillus* strains to antibiotics, using the disc diffusion method, was investigated in the present study and it was revealed that these strains were susceptible to penicillin, cefixime, ofloxacin, ciprofloxacin, amikacin, wherein *Lactiplantibacillus* sp. GV66 was ofloxacin-resistant, and *L. plantarum* GV69 was resistant to ampicillin, ofloxacin and cefixime.

The assessed strains were susceptible to at least one of the antibiotics that would prevent the formation of cell wall and proteins. Two strains of *L. paracasei* were mildly sensitive or susceptible to lincomycin, azithromycin, and penicillin, according to Fonseca *et al* (36), and *L. brevis* CCMA1284 strain was resistant to these three antibiotics. Of note, if a gene transfer process was involved, then antibiotic resistance would become a dangerous scenario. Alhough, this process may not be communicable and would not be a unique criteria of the microbial genus or species, it would however be a sort of alerting condition. Furthermore, minimum inhibitory concentration (MIC) values for antibiotic susceptibility of the *Lactobacillus* strains were tested against antibiotics, including ampicillin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol (31) The MIC values of the reference strain and the *Lactobacillus* strains were less than the MIC breakpoint values. Antibiotic resistance genes have accumulated in probiotics, due to the widespread use of probiotics in combination with antibiotics to restore gut flora. There are significant clinical risks if these resistance genes are transferred from probiotics to pathogens in the colon. The antibiotic sensitivity of a few antibiotics used in the present study were found to be within the European Food Safety Authority (EFSA) breakpoint values for all the examined gluten-hydrolysing bacteria and were thus deemed safe (13). In concurrence with the aforementioned evidence, gentamycin, chloramphenicol, cefoperazone, ampicillin, ciprofloxacin and vancomycin antibiotics were selected to ensure the safety and efficacy of the assessed probiotic bacterial isolates.

Probiotic bacteria exhibit cell line attachment and can colonize with intestinal epithelial cells in order to establish themselves in the gut (31,36). The HT-29 cell line and the selected eight isolates were used in the present study for cell adhesion assay, with *Lactiplantibacillus* sp. GV66 exhibiting strong adhesion at 99.43% and *L. fermentum* GV254 exhibiting comparatively less adhesion at 81.14%. In other studies, *Lactiplantibacillus* strains exhibited strong adherence abilities to the Caco-2 and HT-29 cell lines (39). Rashmi and Gayathri (13) investigated whether gluten-hydrolyzing bacteria adhered to the Caco-2 cell line and inhibited cell invasion by *E. coli* and *L. monocytogenes*. Byakika *et al* (35) used the goat ileum to assess cell adhesion, and Behbahani *et al* (37) used scanning electron microscopy to identify that the adhesion level of the *L. plantarum* strain L15 to Caco-2 cells was 12%. Aissi *et al* (24) employed HT-29, Caco-2 and INT-407 cells, as well as *Bifidobacterial* strains, and microscopically studied them. HT-29 cell invasion assay by *E. coli* and *L. monocytogenes* was performed in the present study using an *in vitro* approach. *L. plantarum* GV54 had a low percentage of invasion by the pathogens *E. coli* and *L. monocytogenes* (3.26 and 2.56%). By contrast, *Lactiplantibacillus sp*. GV66 and *L. plantarum* GV64 exhibited the highest percentage of invasion of *E. coli* (37.41%) and *L. monocytogenes* (31.31%), respectively, thus revealing their significant potential as probiotic bacteria.

Vinderola and Reinheimer (15) assessed the β-galactosidase activity in *L. delbrueckii substrains bulgaricus*, *L. acidophilus,* and other *Lactiplantibacillus* stains ranging from 1,301 to 2,053 Miller units. Gheytanchi *et al* (14) also reported the β-galactosidase enzyme activity in *L. delbrueckii substrains bulgaricus* and *L. casei* (ranging from 867 to 1,966 U/ml) isolated from cheese. *Lactiplantibacillus* strains with substantial β-galactosidase activity were identified in the present study; among them *Lactiplantibacillus* sp. GV66 had the highest value at 1,203.32 (U/ml) Miller units. All of these positive traits of *Lactiplantibacillus* sp. render this strain ideal for use in probiotic formulations, either alone or in combination with other advantageous probiotic-bacterial isolates. *Lactiplantibacillus* sp. that produces β-galactosidase could be used as a probiotic supplement to help individuals with LI. Thus, the use of probiotics may lead to a promising method in prevention or management of LI. In addition, it is possible to improve and optimize the enzyme activity and development of milk products with potential probiotics/enzymes for the management of LI.

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**Availability of data and materials**

Sequence data that support the findings of the present study have been deposited in GenBank with the primary accession no. MN686265: MN696220-MN696226 (https://www.ncbi.nlm.nih.gov/search/all/?term=MN68626 5:MN696220-MN696226 [accn]). All data used or analyzed during the present study are included within this article. All

other data are available from the corresponding authors upon reasonable request.

### Authors' contributions

MV executed the planned experimental work, wrote the manuscript and performed the data analysis. DG conceived and designed the study, as well as acquired and analysed the data. VK, CSP and MB drafted the work, and revised it critically for important intellectual content. DG and VK confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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