

## Bioprospecting of probiotic lactic acid bacteria from jalebi batter and evaluation of their antidiabetic and antioxidant potential

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Diabetes has become the most commonly prevalent metabolic disorder worldwide. Though the medications are available for effective management of hyperglycemia due to diabetes but most of them have adverse side effects. Therefore, there is immense emphasis on the exploration of safer alternatives for managing diabetes. One such safe alternative may be the application of probiotics for the effectual control/management of diabetes. In the current study, sixteen lactic acid bacteria (LAB) were isolated from jalebi batter, a rarely explored ecological niche, for potential probiotics. These isolates were evaluated for their fundamental probiotic characteristics, with seven isolates demonstrating robust survival rates ranging from 87.6% to 96.8% in simulated gastrointestinal conditions. Additionally, these selected LAB isolates displayed noteworthy functional attributes viz. hydrophobicity, autoaggregation, and coaggregation capabilities. Furthermore, the LAB isolates were investigated for antibiotic susceptibility, antibacterial activity and antioxidant potential. Moreover, LAB isolates from the jalebi batter were examined for their inhibition potential against  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes via both intact cells (IC) and cell-free supernatants (CFS). Notably, LAB isolate J-7 exhibited the highest inhibition of  $\alpha$ -glucosidase (CFS: 72.27%; IC: 11.90%) and  $\alpha$ -amylase (CFS: 15.21%; IC: 13.04%), and also possessed other functionally desired probiotic attributes. The LAB isolate J-7 was identified based on 16S rDNA sequencing as *Limosilactobacillus fermentum*, and designated as *L. fermentum* PP556213 strain J-7.

**Keywords:** *Limosilactobacillus fermentum*,  $\alpha$ -amylase,  $\alpha$ -glucosidase, Autoaggregation, Hydrophobicity

Probiotics have been delineated as living microbes that impart health benefits to the host when consumed sufficiently. Probiotic bacteria majorly include various genera of lactic acid bacteria (LAB) such as *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Lactococcus*, *Pediococcus*, and others<sup>1</sup>, and species of *Bifidobacterium* such as *Bifidobacterium adolescentis*, *B. infantis*, *B. animalis*, *B. bifidum*, *B. lactis* and *B. longum*<sup>2</sup>. Several *Lactobacillus* spp. have been studied as potential probiotics<sup>3</sup>. The LAB probiotics are generally regarded as safe (GRAS) and carry the historical evidence of harmless consumption as they have been used widely for the production of various fermented foods<sup>4</sup>. Numerous studies have showed health enhancing and disease-managing properties of probiotics such as gastrointestinal diseases (irritable bowel syndrome, inflammatory bowel disease, diarrhea, Crohn's disease)<sup>1</sup>, anti-obesity, anti-pathogenicity, anti-inflammatory, anticancer, angiogenic, anti-allergic, hypocholesterolemic<sup>5</sup>, antidiabetic potential, maintaining mental health, and others<sup>6</sup>.

However, the health benefits of probiotics cannot be generalized, and strain specificity plays a pivotal role in imparting such beneficial effects on the host. Therefore, the isolation and characterization of novel probiotic LAB has always been an unremitting research thrust<sup>7</sup>. Numerous exotic sources are being delved into for the isolation of efficient LAB with probiotic potential, such as ethnic fermented foods, sausages, domestic fermented cereals, and many others<sup>8</sup>. LAB are a diverse group of Gram-positive, non-sporulating bacteria which are commonly used to produce fermented food products. A single or consortium of lactic acid bacteria is used to carry out the fermentation process<sup>7</sup>.

A variety of indigenous foods in India are prepared using traditional fermentation processes, such as idli, vada, dosa, dhokla and others<sup>9</sup>. Jalebi represents one such indigenously fermented food that is very popular and sold as sweetened confectionary product<sup>10</sup>. The traditional jalebi is prepared from a batter which is a blend of wheat flour and black gram, enriched with a precise quantity of yogurt and clarified butter. This mixture is combined with water until it forms a thick consistency and then left to naturally ferment in a

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covered container for 6-8 h in a warm environment<sup>10</sup>. Once adequately fermented, the batter is carefully deep-fried in edible oil, and excess oil is drained. Subsequently, the fried jalebi is immersed in sweet sugar syrup, rendering it a delectable dessert served hot<sup>10</sup>.

The microbial diversity of jalebi batter may exhibit significant variations due to various factors such as location, preparation methods, climatic conditions, and due to use of additional/other ingredients. The microbial diversity of jalebi batter may be explored for discovery of potentially novel probiotic strains with promising and unique health benefits<sup>11</sup>. Isolation and characterization of probiotic lactic acid bacteria has been attempted from diverse fermented products, but relatively few reports are available on isolating probiotic strains from jalebi batter<sup>10</sup>. To qualify as potential probiotic, the LAB must possess certain characteristics such as ability to withstand gut conditions (low pH, high bile salt concentration), to colonize efficiently<sup>7</sup>, autoaggregation, coaggregation with pathogenic bacteria, hydrophobicity, antimicrobial potential, antibiotic susceptibility and others<sup>6</sup>. Additionally, the potential probiotic strains must possess wide spectrum of health enhancing/disease managing bioactivities<sup>1,6</sup>.

Diabetes, a chronic disease is rising very swiftly throughout the world. Diabetes associated hyperglycemia is usually managed therapeutically by the available antidiabetic drugs such as biguanides, sulfonylureas, glinides,  $\alpha$ -glucosidase inhibitors, thiazolidinediones, and others either with or without insulin<sup>12</sup>. However, most of the available antidiabetic medications have serious side effects like lactic acidosis, ketoaciduria, myocardial infarction, hepatotoxicity, hypoglycemia, the possibility of medullary cancer, thyroid cancer, fluid retention and others<sup>13</sup>. Therefore, safer alternatives with no or minimal side effects are being investigated, and application of probiotics may represent one such choice for management of diabetes.

Probiotics may be considered as an adjuvant to the existing medications for managing diabetes as the probiotics can ameliorate symptoms of diabetes, such as polydipsia and polyphagia, and improve the glucose and insulin tolerance level<sup>14</sup>. Probiotics have been reported to decrease the insulin resistance, reduce plasma glucose level, and improve the intestinal permeability<sup>15</sup>. Furthermore, probiotics moderately inhibit  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes, and may help regulating blood glucose level. Also probiotics decrease the glycated

hemoglobin (HbA1c) level and improve insulin sensitivity and glycemic control<sup>16</sup>. Multi-species probiotics have also been shown to protect pancreatic islets and optimize gut microbiota structure, leading to tangible antidiabetic effects<sup>17</sup>. Thus, probiotics may offer a promising approach for the management/control of diabetes. Therefore, the present study was aimed to explore jalebi batter for isolation and evaluation of lactic acid bacteria for desired probiotic attributes and assessment of bio-functional properties of the selected ones for antidiabetic and antioxidant characteristics.

### Materials and Methods

All the media, chemicals, and reagents were purchased from Merck, Sigma-Aldrich, HiMedia, Qualigens, and others.

#### Isolation of lactic acid bacteria

The lactic acid bacteria (LAB) were isolated from jalebi batter, procured from the local market of Jammu. A uniform suspension of jalebi batter was inoculated in sterile de Man Rogosa Sharpe, (MRS) broth. After 24 h of incubation at 37°C (180 rpm), 1 mL of enriched broth was appropriately diluted in sterile saline and spread plated on MRS agar. The plates were incubated at 37°C under anaerobic conditions for 24 h. The pinhead colonies obtained on the plates were examined microscopically, Gram staining and catalase test for preliminary identification<sup>7</sup>. The selected LAB were sub-cultured further to obtain the purified LAB cultures.

#### Survival analysis of lactic acid bacteria in simulated gastrointestinal juice

The LAB isolates were evaluated for their survival ability in simulated gastrointestinal juice (SGJ). The LAB were inoculated in MRS broth and incubated overnight at 37°C with shaking at 180 rpm. The cultural broth was centrifuged (5000×g for 10 min), and cells were washed twice with sterile phosphate buffered saline (PBS) and then suspended in the same. The homogeneous cell suspension ( $10^8$ – $10^9$  cells/mL, Absorbance  $A_{600}$  0.9-1.0) was centrifuged at 12000×g, at 4°C for 5 min. The resultant bacterial pellet was reconstituted in 1.0 mL of filtered SGJ, and incubated at 37°C for 3 h. The SGJ-treated cells were spread-plated on MRS agar, and plates were incubated at 37°C for 48 h, and examined for growth. The survival of the LAB in artificial gastric conditions was estimated by colony forming units and expressed as log cfu/mL<sup>4</sup>.

### Hydrophobicity determination of lactic acid bacterial isolates

The adherence ability of LAB isolates or hydrophobicity was examined based on the bacterial adhesion to hydrocarbons (BATH). Two polar (chloroform, ethyl acetate) and one nonpolar (xylene) solvents were used to study the hydrophobicity of selected isolates. Freshly grown LAB culture in MRS broth was centrifuged (7500×g) for 10 min at 4°C. The bacterial cell pellet obtained was washed with PBS, and resuspended in the same. Then to the cell suspension (3.0 mL) 1.0 mL of either of the solvent was added, and allowed to stand at room temperature (25°C) for 10 min, then absorbance (580 nm) was examined ( $A_0$ ). The reaction mixture was left undisturbed for another 30 min for phase separation to take place, and then the upper aqueous layer was pipetted out to examine the absorbance<sup>8</sup>. Cell surface hydrophobicity was calculated using the equation 1:

$$\text{Hydrophobicity (\%)} = \frac{A_0 - A_i}{A_i} \times 100 \text{ Eq.} \quad \dots(1)$$

[ $A_i$  = final absorbance after 30 min, whereas,  $A_0$  = initial absorbance of reaction mixture]

### Autoaggregation and coaggregation potential of LAB isolates

For autoaggregation analysis, MRS broth grown LAB cell pellet was washed twice with PBS and resuspended in the same to attain the uniform cell density of  $10^8$ - $10^9$  cells/mL ( $A_{600}$  0.9-1.00). A 4.0 mL of bacterial cell suspension was vortexed and allowed to stand for 5 h. At an interval of 1 h, a 0.1 mL of sample was pipetted out from the top of the cell suspension, mixed with 3.9 mL of PBS, and examined for absorbance ( $A_{600}$ )<sup>18</sup>. The autoaggregation capability of LAB was calculated using the below mentioned formula.

$$\text{Autoaggregation (\%)} = 1 - \frac{A_t}{A_0} \times 100 \text{ Eq.} \quad \dots(2)$$

[where  $A_t$  = absorbance at 1 to 5 h, and  $A_0$  is absorbance at 0 h]

The coaggregation potential of LAB was determined for selected Gram-positive (*Streptococcus pneumoniae*, *Staphylococcus aureus*, *Micrococcus* sp.) and Gram-negative bacteria (*Alcaligenes denitrificans*, and *Klebsiella* sp.)<sup>7</sup>. The cell suspension of specific LAB and pathogenic bacteria were mixed and coaggregation potential was ascertained spectrophotometrically ( $A_{600}$ ) at the interval of 1 h up to 5 h. Individual LAB/pathogens cultures were used as the appropriate controls. The coaggregation was calculated by equation (3)

$$\text{Coaggregation (\%)} = \frac{\frac{A_{\text{path}+A_{\text{probio}}}}{2} - A_{(\text{path}+\text{probio})}}{\frac{A_{\text{path}+A_{\text{probio}}}}{2}} \times 100 \text{ Eq.} \quad \dots(3)$$

[where  $A_{\text{path}}$  and  $A_{\text{probio}}$  refer to absorbance (at 600 nm) of specific pathogenic or probiotic bacteria, and  $A_{(\text{path}+\text{probio})}$  refers to the absorbance of mixed pathogen and probiotic suspension]

### Inhibition potential of LAB for pathogenic bacteria

For assaying the antibacterial activity, the LAB isolates were grown in MRS broth for 24 h at 37°C. The cultures were centrifuged at 5000×g for 15 min, and the cell free supernatant (CFS) obtained was filtered using a 0.22 µm syringe filter, and examined for antibacterial activity against the bacterial pathogens (*Staphylococcus aureus*, *Micrococcus* sp., *Alcaligenes denitrificans*, *Klebsiella* sp., and *Streptococcus pneumoniae*). Each of the bacterial pathogens was grown in nutrient broth (NB) at the optimum growth conditions (37°C, 18 h, 180 rpm) and spread plated on nutrient agar. Then 6.0 mm wells were punctured in the agar plates and poured with 50 µL of CFS, followed by incubation of plates at 37°C for 24-48 h. The appearance of the zone of inhibition signifies the antimicrobial potential<sup>7</sup>.

### Antibiotic susceptibility assay of lactic acid bacteria

Antibiotic susceptibility of isolated LAB was evaluated by disk diffusion assay<sup>6</sup>. Briefly, a 24 h old LAB culture was spread plated on MRS agar and disks of various antibiotics viz. kanamycin, streptomycin, chloramphenicol, tetracycline, and erythromycin were gently placed on the spread plated bacterial lawn. The plates were given 24 h incubation at 37°C and examined for growth inhibition of bacteria around the disks<sup>6</sup>.

### Antioxidant potential of LAB isolates

The LAB were grown in MRS broth for 18 h at 37°C, and the cell free supernatant (CFS) obtained after centrifugation was examined for antioxidant potential based on free radical scavenging ability by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical. Also, superoxide anion scavenging ability of CFS was examined. For analyzing DPPH scavenging ability, 0.2 mmol ethanolic DPPH solution was mixed with bacterial CFS (1:2 v/v), and kept in a dark place for 30 min, and subsequently analyzed for absorbance ( $A_{517}$ ). The uninoculated broth was set as a blank solution, and a mixture of DPPH and deionized water equivalent to

the sample volume was used as a control<sup>7</sup>. The free radical scavenging ability was calculated as per the following formula

$$\text{DPPH free radical scavenging \%} = 1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100$$

[where  $A_{\text{sample}}$ =absorbance of DPPH and CFS,  $A_{\text{control}}$ =absorbance of DPPH and deionized water]

To analyse the ABTS scavenging ability, a 1:1 v/v, mixture of ABTS (7.0 mM) and potassium persulphate (2.45 mM) was prepared in distilled water and diluted to the absorbance of  $0.7 \pm 0.01$  at 734 nm<sup>7</sup>. Subsequently, the reaction was setup by mixing 300  $\mu$ L of CFS and 600  $\mu$ L of ABTS radical solution and placed in the dark for 10 min at room temperature, and then analyzed for absorbance ( $A_{734}$ ). Free radical scavenging ability was calculated using the formula given below

$$\text{ABTS free radical scavenging \%} = 1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100$$

[Where  $A_{\text{sample}}$ =absorbance of ABTS and CFS,  $A_{\text{control}}$ =absorbance of ABTS and deionized water]

For superoxide anion radical scavenging ability of the CFS, the reaction consisting of 0.8 mL of CFS and 0.2 mL Tris-HCL buffer (0.1 M, pH 8.0) was analyzed<sup>19</sup>. Absorbance was measured at 320 nm and subsequently 0.1 mL of 3 mM pyrogallol solution was added and again analyzed for absorbance at 320 nm. Scavenging of superoxide anions was determined as per the formula:

$$\text{Superoxide free radical scavenging \%} = 1 - \frac{A_{\text{test1}} - A_{\text{test2}}}{A_{\text{blank}}} \times 100$$

[ $A_{\text{test1}}$ = CFS with pyrogallol;  $A_{\text{test2}}$  = CFS without pyrogallol;  $A_{\text{blank}}$ = uninoculated MRS broth with pyrogallol]

#### Antidiabetic potential of probiotic lactic acid bacteria

The antidiabetic potential of probiotic LAB was assessed by evaluating the  $\alpha$ -amylase and  $\alpha$ -glucosidase potential.

##### *$\alpha$ -amylase inhibiting potential of LAB isolates*

The LAB cells and the cell free supernatant were examined for their  $\alpha$ -amylase inhibiting potential<sup>20</sup>. The freshly grown LAB were centrifuged followed by segregation of cell pellet and the cell free supernatant. The cell pellet was washed twice with PBS, and suspended in the same. A reaction was set up wherein 250  $\mu$ L of  $\alpha$ -amylase (0.5mg/mL) (1U/mL) was added to either 250  $\mu$ L of intact LAB cell suspension

or CFS of each LAB isolate. Acarbose (1.0 mg/mL) was used as the positive control. The reaction mixture was incubated at 27°C for 10 min followed by the addition of 0.25 mL of starch solution (1%, w/v), and again kept for 10 min at 27°C. The reaction was terminated by adding 3, 5-dinitrosalicylic acid (DNS) reagent, followed by heating for 5 min at 90°C. The reaction content was diluted five-fold with deionized water. The inhibition potential of LAB cells or the CFS was analyzed spectrophotometrically ( $A_{549}$ ). The  $\alpha$ -amylase inhibition was calculated as per the formula given

$$\alpha\text{-amylase inhibition \%} = \frac{A-B}{A} \times 100$$

[A is the absorbance of control; B is the absorbance of sample]

##### *$\alpha$ -glucosidase inhibition assay of LAB isolates*

For the assessment of  $\alpha$ -glucosidase inhibition, an appropriate amount of  $\alpha$ -glucosidase enzyme (1.0 IU/mL was dissolved in 100  $\mu$ L of 0.1 M PBS) was added to 50  $\mu$ L of either LAB cell suspension or the CFS, and the reaction mixture was incubated at 37°C for 10 min, followed by addition of 50  $\mu$ L of 5 mM p-nitrophenyl-d-glucopyranoside (pNPG), the substrate for  $\alpha$ -glucosidase. Acarbose was set as the positive control. The reaction between pNPG and  $\alpha$ -glucosidase enzyme was allowed to proceed at 37°C for 30 min, and then the reaction was terminated by adding 1.0 mL of 0.1 M  $\text{Na}_2\text{CO}_3$ . The  $\alpha$ -glucosidase activity was assayed spectrophotometrically ( $A_{400}$ ) based on conversion of colourless substrate (pNPG) into a yellow coloured product (p-nitrophenol). The enzyme inhibition was determined as per the given equation<sup>20</sup>.

$$\alpha\text{-glucosidase inhibition \%} = 1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \times 100$$

[ $A_{\text{control}}$ : reaction volume excluding acarbose, CFS, and intact cells;  $A_{\text{blank}}$ : solution devoid of substrate]

##### **Identification of LAB isolate with $\alpha$ -amylase and $\alpha$ -glucosidase inhibition potential**

Probiotic LAB isolate with proficient  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition potential was identified using 16S rDNA Sanger sequencing<sup>4</sup>. Bacterial genomic DNA was isolated (HiPurA, Himedia kit), and subjected to PCR-amplification using universal 16S rDNA primers (forward primer 5'-AGRGTGGATCCTGGCTCAG-3', reverse primer 5'-CGGCTACCTTGTTACGACTTT-3'). The amplified product was sequenced (Biologia Pvt. ltd. India). The sequence was analyzed for homology using the Basic Local Alignment Search Tool

(BLAST). Molecular Evolutionary Genetics Analysis 11(MEGA11), software was used for inferring the evolutionary relationship and construction of a phylogenetic tree.

## Results

Since, the health benefits of probiotics are strain specific, therefore, there is continuous research focus on isolation, evaluation, and identification of new effective probiotic strains from diverse sources. Among various sources for isolating probiotic lactic acid bacteria, fermented foods, due to their immense microbial diversity, have emerged as a promising pool of probiotic bacteria. Therefore, the current study explored the probiotic LAB from jalebi batter, a relatively less explored source. LAB isolates from jalebi batter were examined for probiotic attributes, and the selected ones were investigated for antioxidant and antidiabetic potential.

### Preliminary identification by gram staining and catalase test

The sixteen presumptive LAB which appeared as off-white pin head colonies on MRS agar, were isolated, purified, and found to be Gram-positive and catalase negative rods (Fig. 1).

### Survival analysis of lactic acid bacteria isolates in simulated gastrointestinal juice

The presumptive LAB isolates were initially screened for their endurance in simulated gastrointestinal juice (SGJ). Of sixteen isolates only seven viz. J-4, J-6, J-7, J-10, J-11, J-16, and J-17 illustrated the efficient survival in GIJ (87.6-96.8%). The LAB isolate J-17 (96.8%) exhibited highest survival in GIJ and lowest survival was exhibited by J-4 (87.6%) in comparison to untreated control which had 100% viability (Fig. 2). Survival ability of lactic acid bacteria defines the capability of LAB to hold up their viable count in the highly acidic gut lumen<sup>21</sup>.

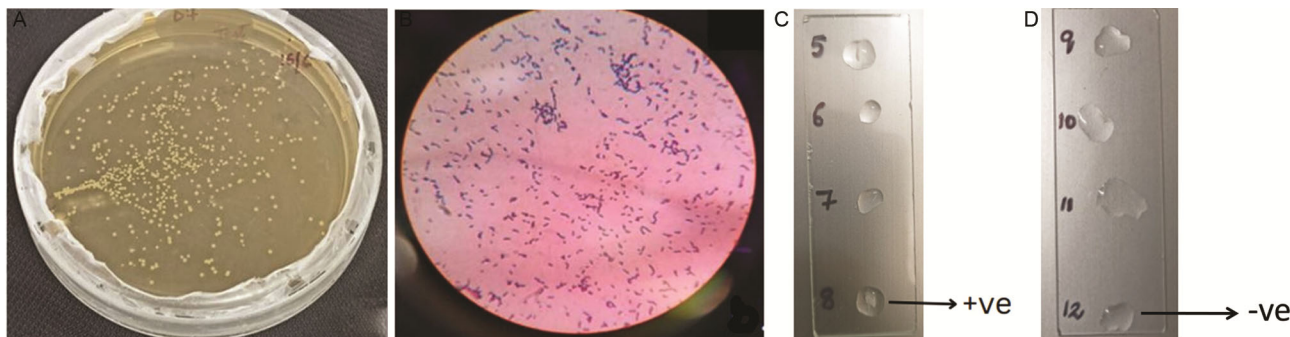


Fig 1 — (A) Colony morphology of lactic acid bacteria (LAB) isolates (B) LAB isolates under microscope after Gram staining (C) catalase positive LAB isolates (D) catalase negative LAB isolates

Most of the LAB isolates survived in the artificial gastrointestinal juice up to a significant level, and the selected isolates were further taken up for studying their other desirable probiotic attributes.

### Analysis of hydrophobicity of isolated lactic acid bacteria

One of the desired characteristic features of probiotic bacteria is their ability to adhere to the host gut lumen to ensure the persistent stay, thereby facilitating their benefitting attributes. The bacterial adhesion to hydrocarbon test (BATH) aids in assessing the physicochemical forces that are involved in probiotic adherence to the human GI tract<sup>22</sup>. All the LAB isolates under study demonstrated moderate to high adhesion towards xylene, LAB isolate J-16 exhibited highest degree of adhesion (85.75%) and was followed by J-7 (82.31%), J-17 (72.79%), and J-11 (63.50%), other isolates however, had low hydrophobicity (10.02-14.55%) with xylene. The adhesive ability of LAB towards xylene signifies hydrophobic nature of their cell surface. Hydrophobicity analysis with chloroform showed that some of the isolates had very high adhesion capacity, for instance isolates J-11, exhibited highest hydrophobicity of 94.2%. However, rest of the

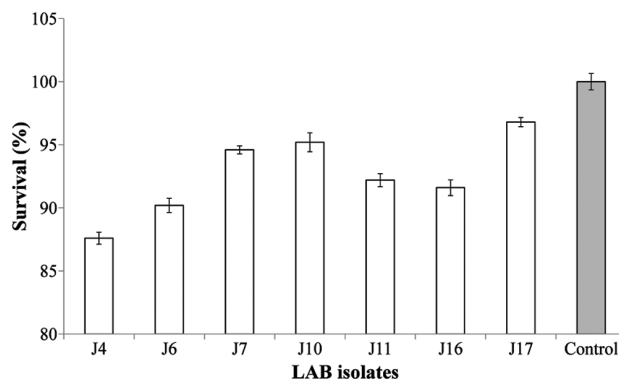


Fig 2 — Survival (%) of lactic acid bacteria (LAB) under simulated gastrointestinal conditions

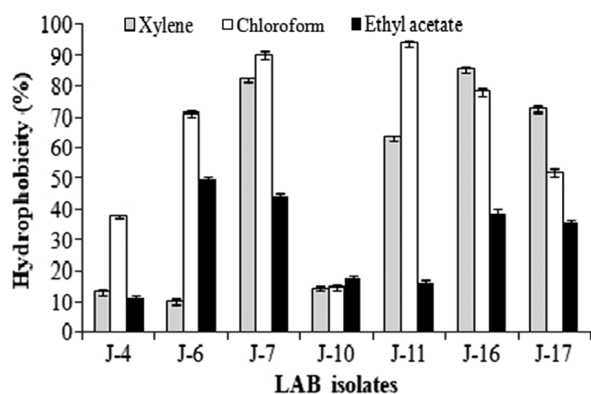


Fig 3 — Hydrophobicity (%) of lactic acid bacteria (LAB) with ethyl acetate, xylene and chloroform

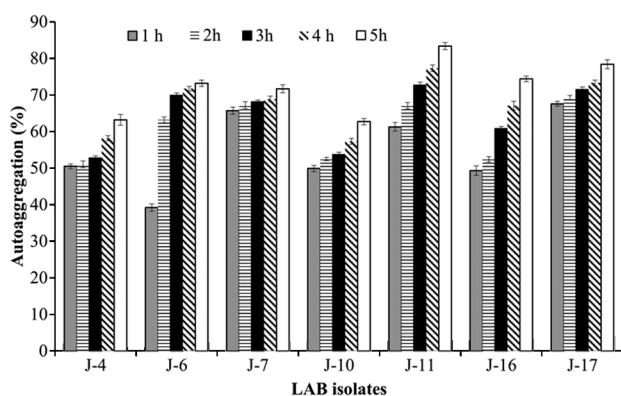


Fig 4 — Autoaggregation ability (%) of lactic acid bacterial isolates at different time intervals

isolates had moderate (J-17 52.5%, J-4 37.81%) or low hydrophobicity (J-10 14.8%). Hydrophobicity analysis of the LAB isolates with ethyl acetate showed that LAB isolates J-6, J-7, J-16, and J-17 had relatively higher values (37-49%) than the other isolates (Fig. 3).

#### Autoaggregation and coaggregation potential of LAB isolates

Autoaggregation of LAB isolates were observed for 5 h. The autoaggregation capability of LAB isolates varied in the array of 62.7% to 83.4% (Fig. 4). The LAB isolate J-11 had the highest autoaggregation of 83.4%, and the lowest autoaggregation capability was exhibited by J-10 i.e. 62.7% after 5 h of incubation at room temperature. Furthermore, autoaggregation increased with increased time of incubation signifying a direct relation between autoaggregation and time of contact.

The coaggregation property i.e. the ability of LAB to aggregate with dissimilar bacteria plays a prominent role in pathogen elimination from gut lumen by standing in as an obstruction for colonization<sup>7</sup>. Coaggregation

potential of LAB with different pathogens was analyzed over incubation period of 5 h (Table 1). LAB isolates J-4 and J-10 showed moderate coaggregation (39-50%) potential with all the five pathogenic bacteria examined. J-6 showed good coaggregation with *Micrococcus* sp. (77.56%) and *Klebsiella* sp. (74.52%) but moderate coaggregation with other pathogens (46.36-59.5%). Similar trend was shown by isolate J-7 but it showed good coaggregation with *S. pneumoniae* as well (78.46%). LAB isolates J-11, J-16, and J-17 exhibited relatively high coaggregation potential (65-83%) with most of the pathogens except with *S. aureus* where these isolates showed moderate coaggregation (37.68-41.67%). Thus, coaggregation capability of the LAB isolates with different bacterial pathogens was quite variable.

#### Antibiotic susceptibility and antimicrobial potential of probiotic isolates

The antibiotic susceptibility of isolated LAB was assessed by using different antibiotic discs. The LAB isolate J-6 showed resistance towards erythromycin and kanamycin, whereas, J7 exhibited resistant to kanamycin. The isolate J-11 had resistance for kanamycin and streptomycin. Moreover, the LAB isolates J-16 and J-17 were resistant towards kanamycin and streptomycin, respectively. The LAB isolates J-4 and J-10 were resistant to all the antibiotics examined (Table 2). Antimicrobial activity of probiotics refers to their potential capability to inhibit bacterial/fungal pathogens. Most probiotic isolates under study illustrated low to moderate antimicrobial activity towards the pathogenic bacteria examined in the study. The LAB isolates J-7 and J-16 showed maximum growth inhibition of the bacterial pathogens examined. The LAB isolates J-4 and J-10 exhibited the resistance towards all the tested antibiotics and showed negligible antimicrobial activity, therefore, five isolates J-6, J-7, J-11, J-16 and J-17 were chosen for further studies.

#### Antioxidant activity of isolated lactic acid bacteria

Oxidative stress refers to a condition where antioxidant and prooxidant equilibrium in the cell is distorted and results in several deleterious reactions such as lipid peroxidation, DNA hydroxylation, protein denaturation and apoptosis, consequently compromising the viability of cells<sup>23</sup>. The LAB isolates J-6, J-7, J-11, and J-16 exhibited high ABTS radical scavenging potential (76.30 to 77.60%). Isolate J-17 though exhibited antioxidant potential but slightly lesser than other isolates (65.60%) (Fig 5).

Table 1 — Coaggregation ability of lactic acid bacteria (LAB) isolates with different pathogenic bacteria

LAB isolates	Bacterial pathogens, Coaggregation ability (%)				
	<i>Micrococcus</i> sp.	<i>Staphylococcus aureus</i>	<i>Streptococcus pneumoniae</i>	<i>Alcaligenes denitrificans</i>	<i>Klebsiella</i> sp.
J-4	39.81±01	40.88±29	41.26±29	42.43±32	41.87±65
J-6	77.56±02	46.36±32	58.83±85	59.50±85	74.52±12
J-7	80.21±32	50.62±36	78.46±56	44.56±74	83.45±32
J-10	45.21±06	39.23±29	41.03±45	50.91±69	48.99±57
J-11	74.99±09	41.67±41	67.41±78	62.98±32	70.34±52
J-16	76.43±06	41.56±10	75.09±65	69.27±52	82.99±42
J-17	74.82±04	37.68±15	74.26±95	64.39±45	67.26±25

Table 2 — Antibiotic susceptibility of lactic acid bacteria (LAB) isolates against different antibiotics\*

LAB isolates	Chloramphenicol	Erythromycin	Tetracycline	Streptomycin	Kanamycin
J-4	R	R	R	R	R
J-6	S	R	S	S	R
J-7	S	S	S	S	R
J-10	R	R	R	R	R
J-11	S	S	S	R	R
J-16	S	-	S	S	R
J-17	S	S	S	R	S

[\*S-Susceptible; R-Resistant]

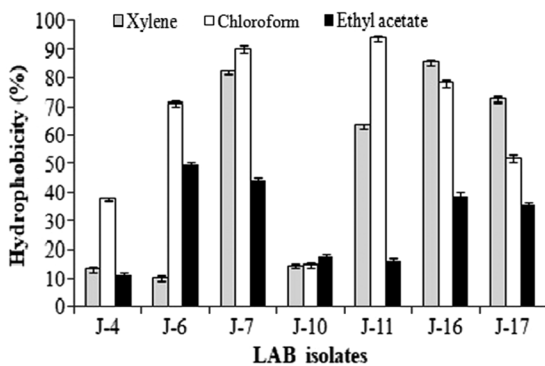


Fig 5 — Antioxidant activity (%) of selected lactic acid bacteria (LAB) isolates based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, superoxide anion radical scavenging assay and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) scavenging assay

According to the DPPH radical scavenging assay the high antioxidant activity was shown by isolate J-7 (79.14%), other isolates exhibited moderate antioxidant potential (48.68% to 60.14%) (Fig 5). Relatively similar antioxidant potential was observed for all the LAB isolates (30.10 to 31.97%) based on superoxide anion radical scavenging assay (Fig 5).

#### Antidiabetic potential of probiotic lactic acid bacteria

The LAB isolates that exhibited desired probiotic attributes were further examined for their antidiabetic potential by evaluating their capability to inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase, the major carbohydrate hydrolyzing enzymes. The inhibition of these enzymes restrains the rate of carbohydrate digestion,

consequently maintaining the blood glucose levels<sup>24</sup>. The cell free supernatant (CFS) and intact cells (IC) of LAB isolates exhibited varying level (5 to 15%) of  $\alpha$ -amylase inhibition. The LAB isolate J-7 showed highest inhibition potential (CFS: 15.21%; IC: 13.04%), followed by J-16 (CFS: 13.04%; IC: 10.14%), J-11 (CFS: 11.59%; IC: 11.59%), J-17 (CFS: 12.31%; IC: 5.07%) and J-6 (CFS: 11.59%; IC: 5.07%). The cell free supernatant was found to have higher inhibition potential than intact cells.

According to the  $\alpha$ -glucosidase inhibition test, the highest antidiabetic activity was shown by J-7 (CFS: 72.27%; IC: 11.90 %), followed by J-17 (CFS: 70.45%; IC: 6.40%), J-16 (CFS: 68.77%; IC: 10.40%), J-6 (CFS: 68.34%; IC: 7.20%) and J-11 (CFS 62.67%; IC 11.00%). Acarbose was used as a positive control, and it exhibited inhibition of 96.9%. For  $\alpha$ -glucosidase inhibition test, the CFS showed more antidiabetic activity than the intact cells (Fig 6).

#### Identification of most efficient antidiabetic probiotic isolate J-7

The LAB isolate J-7, which exhibited high antidiabetic potential and other desired probiotic attributes was identified based on 16S rDNA sequencing. Sequence analysis showed that the 16S rDNA sequence of isolate J-7 exhibited a high degree of similarity to that of *Limosilactobacillus fermentum* in the GenBank database (National Centre for Biotechnology Information, NCBI). The dendrogram was created using MEGA 11 software (Fig 7). The

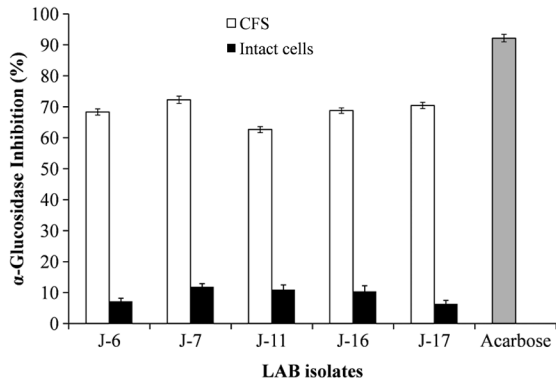


Fig 6 — Antidiabetic activity of lactic acid bacterial isolates based on  $\alpha$ -glucosidase inhibition by cell free supernatant; CFS, and intact cells

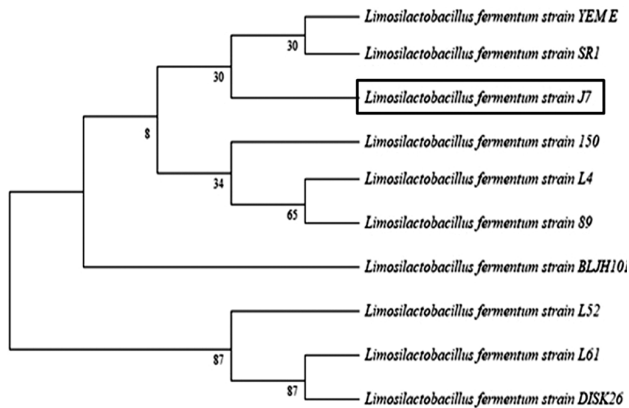


Fig 7 — Identification of probiotic isolate J-7 based on 16S rDNA sequence analysis

16S rDNA sequence has been submitted to GenBank database (accession number PP556213). The LAB isolate was identified, and designated as *Limosilactobacillus fermentum* J-7.

**Discussion**

Exploration of probiotic strains from exotic ecosystems is of principal importance, as the health-enhancing attributes of probiotics are extremely precise and strain-specific. Therefore, isolating the probiotics from unexplored niches such as fermented foods of different geographical regions holds great potential for developing new probiotics with diversified health benefits<sup>7</sup>. The diverse Gram-positive and catalase-negative LAB probiotics species have been isolated from jalebi, medhu vada, and kallappam batters<sup>24</sup>. Similarly, household cheese and fermented rice water were used to isolate different *Lactobacillus* species<sup>25</sup>. The awareness regarding health-promoting attributes of LAB probiotics has

grown, and this bacterial class has become the subject of ongoing research. As a result, greater focus is being placed on investigating unexplored niches for the isolation of the new/novel potential probiotic strains.

Probiotic bacteria play a crucial role in managing the gut health and improving the health status of the host. However, they face significant survival challenges during the gastrointestinal transition due to the harsh stomach conditions, such as low pH, gastric enzymes, and bile salts. Therefore, it is essential for probiotic to withstand the harsh GIT conditions<sup>21</sup>. Liang *et al.*<sup>26</sup> isolated *Lactocaseibacillus paracasei* (WFC 414) and *Lactiplantibacillus plantarum* (WFC 502) strains from fermented apple juice which exhibited high survival under artificial gastric juice i.e. 92% and 95%, respectively. Similarly, several LAB isolated from a Himalayan fermented milk product kalarie, showed a substantial survival of 94% to 99.0%<sup>7</sup>. However, in contrast, 22 selected LAB isolates of different genera (*Lactobacillus*, *Enterococcus*, and *Pediococcus*) from an Iranian snack food *Duimaj* showed low, moderate or high survival rates in simulated gastric juice (35.86–74.19%) and in artificial intestinal fluid (39.28–78.39%) up to 3 h<sup>27</sup>. Thus, survival of LAB in GIT may be strain-dependent.

The hydrophobicity of probiotic lactic acid bacteria plays a crucial role in their ability to adhere to hydrophobic surfaces of the intestinal epithelium, facilitating colonization in the host's gastrointestinal tract. Certain organic solvents like xylene, chloroform, or ethyl acetate may be used to evaluate bacterial hydrophobicity, and hence the affinity of LAB strains for hydrophobic substances. This assessment provides valuable insights into the potential attachment of probiotic lactic acid bacteria to the host's gastrointestinal mucosa<sup>4</sup>. *L. rhamnosus* NCDC953 and *L. reuteri* NCDC958, infant fecal isolates showed high hydrophobicity with chloroform (50.23% and 62.8%, respectively), than that with xylene (26.0% and 47.5% respectively) or ethyl acetate (48.5% and 30.8%) respectively<sup>28</sup>. *Lactobacillus casei* and *L. divergens* isolated from kpètè-kpètè (Benin opaque sorghum beer) exhibited high hydrophobicity with xylene (52.81%, and 50.70%, respectively) and with chloroform (65.75% and 37.16%, respectively)<sup>22</sup>. Six LAB isolates from kalarie showed varied level of hydrophobicity with xylene (11.10% to 87.80 %), chloroform (10.20% to 85.50%) or with ethyl acetate (12.70% to 59.10%)<sup>7</sup>. The hydrophobic nature

of probiotic cell surface enhances their biofilm formation ability. The cell aggregates tend to spend sufficient time in intestinal lumen because of their increased density, thus, improving the health status of the host<sup>22</sup>.

The aggregation of genetically similar bacteria is referred to as autoaggregation. The microbial ability to autoaggregate is necessary for colonization and is correlated with adhesion to the host gastrointestinal tract, achieving an effectively high cell number, thereby imparting health benefits<sup>6</sup>. The autoaggregation ability of probiotic bacteria isolated from various dairy products was observed to be in the range of 5.3% to 96% after 4 h of incubation<sup>29</sup>. The kalarie LAB isolates illustrated variable but relatively high degree of autoaggregation ability (74.5%-95.4%)<sup>8</sup>. The autoaggregation ability of three LAB isolates from kpètè-kpètè was found to be in range of 25.18% to 34.35%, where, *Lactobacillus casei* exhibited the highest aggregation ability while *E. faecalis* showed the lowest autoaggregation potential<sup>22</sup>.

The capacity of probiotic bacteria to coaggregate with other microbes, including harmful bacteria, is a crucial phenomenon that facilitates stable interactions and prevents pathogenic microorganisms from colonizing and surviving in the gastrointestinal tract (GIT). Coaggregation enables probiotic bacteria to attach and coaggregate with pathogens, contributing to maintain a balanced gut microbiota and preventing infections through competitive exclusion<sup>8</sup>. The coaggregation potential of *Enterococcus durans*, *Lactobacillus plantarum*, *Enterococcus faecium* and two strains of *Lactobacillus fermentum* with pathogenic bacteria *E. coli*, *L. monocytogenes*, *M. luteus* and *S. aureus* was studied. The *Lactobacillus fermentum* showed high coaggregation ability with *E. coli* (62.03%) and with *Enterococcus durans* (51.88%), while *Enterococcus faecium* exhibited high coaggregation potential with *S. aureus* (50.49%)<sup>30</sup>.

In another study, among 35 LAB isolates some didn't show coaggregation potential at all while some others (*Lb. plantarum* S70, *Lb. plantarum* S57, *Lb. casei* S81) displayed considerable coaggregation ability (57.4% to 63.4%) with different pathogens such as *B. cereus*, *S. aureus* and *Li. monocytogenes*<sup>29</sup>. The coaggregation of *L. brevis* gp104 with *S. aureus* ATCC 25923 was reported to be 48.6%<sup>31</sup>. Thus, coaggregation capability of LAB may be variable with different pathogens.

The probiotic bacteria intended for human/animal applications should not be resistant to antibiotics as

this trait may potentially be passed on to the pathogenic bacteria *via* horizontal gene transfer mechanisms<sup>8</sup>. However, intrinsic antibiotic resistance can be a desirable trait, as probiotics help to restore host gut microflora during a course of antibiotics<sup>4</sup>. The probiotic LAB strains of *Lactococcus lactis* subsp. *lactis* strains isolated from natural whey starter exhibited substantial susceptibility towards penicillin, vancomycin, ampicillin, and tetracycline, however, three strains had resistance towards gentamicin<sup>6</sup>. The Moroccan camel milk *Pediococcus* and *Enterococcus* LAB strains exhibited complete susceptibility towards tetracycline and penicillin, while some of the strains showed intermediate resistance towards kanamycin, gentamicin and erythromycin, but some others displayed resistance to vancomycin<sup>1</sup>. Similarly, LAB strains evaluated by Gupta & Bajaj<sup>8</sup> were entirely susceptible towards penicillin, ampicillin, tetracycline and erythromycin, few of them demonstrated resistance towards vancomycin. The probiotic isolate *L. plantarum* M-13 was susceptible to all the antibiotics examined. Therefore, probiotics must be first undertaken for comprehensive antibiotic resistance patterns before being considered for commercial applications.

The antimicrobial potential of probiotic bacteria is a vital aspect of their health benefitting attributes, as it aids in thwarting the proliferation of potential pathogens and helps to maintain gut microbiota homeostasis. The antagonistic activity of probiotic bacteria is attributed to synthesis and production of antimicrobial substances, such as hydrogen peroxide, natural protective organic acids and bacteriocins<sup>6</sup>. Chabi *et al.*<sup>22</sup> studied antibacterial effect of four LAB isolates against several pathogens. *Lactobacillus divergens* and *Limosilactobacillus fermentum* showed maximum inhibition towards all the tested pathogenic microorganisms, whereas, *Enterococcus faecalis* and *Lacticaseibacillus casei* exhibited moderate inhibition. In another study probiotic bacteria *Limosilactobacillus reuteri* NCDC953 and *Lacticaseibacillus rhamnosus* NCDC958 exhibited varied degree of antagonistic ability against different Gram positive and Gram negative pathogens<sup>17</sup>.

The reactive oxygen species (ROS) function inside the human body as mediators and regulators to maintain healthy cell functioning and appropriate signaling, but cause severe oxidative damage when their formation exceeds a certain threshold<sup>32</sup>. Antioxidants protect against oxidative damage by scavenging free radicals, chelating metal ions, enhancing antioxidant enzymes, and influencing gut microbiota. Probiotic LAB produces

various antioxidant compounds like exopolysaccharide, manganese ions, short peptides, and others<sup>7</sup>. As reported by Kang *et al.*<sup>23</sup> the ABTS radical scavenging potential of different LAB isolated from, Korean individuals and fermented foods was found in between 27.1% to 99.5%. The LAB isolates studied by Bangotra *et al.*<sup>7</sup>, exhibited highest ABTS radical scavenging potential of 78.46% to 90.60%. A probiotic strain *Lactobacillus reuteri* LR12 was reported to exhibit high antioxidant potential (89%) based on DPPH scavenging activity<sup>33</sup>. Similarly, *Lactiplantibacillus plantarum* 1K illustrated considerable DPPH scavenging potential (62.34%)<sup>32</sup>. In contrast, however, DPPH radical scavenging ability of LAB isolates was quite low and varied in the range of 2.55-6.88%<sup>34</sup>. Furthermore, analysis of antioxidant potential based on superoxide anion radical scavenging capability, several of the LAB strains from different sources were shown to possess a substantially high antioxidant activity (62% to 96%)<sup>35</sup>.

The probiotic bacteria *Lacticaseibacillus paracasei* ED25 isolated from different Turkish cheese (Beyaz Peynir) exhibited varying degree of free radical scavenging potential i.e. ABTS 37%, DPPH 49% and superoxide oxide free radical 38%<sup>36</sup>. The oxidative stress may cause various disorders, and probiotics may help neutralizing oxidative stress. Therefore, it is imperative that potential probiotic strains should be thoroughly investigated for their free radical scavenging potential.

The ability of probiotic bacteria to inhibit the  $\alpha$ -amylase and  $\alpha$ -glucosidase, enzymes involved in carbohydrate hydrolysis, and glucose metabolism play significant role in regulation of blood glucose level<sup>17</sup>.  $\alpha$ -amylase is an endogluconase which catalyzes the hydrolysis of  $\alpha$ -1, 4-glycosidic linkage of starch and glycogen molecules, thereby, producing disaccharides, whereas,  $\alpha$ -glucosidase facilitates the conversion of complex polysaccharides into monosaccharides like glucose<sup>24</sup>. Several species of *Lacticaseibacillus* and *Lactiplantibacillus* isolated from fermented foods namely, jalebi, medhu vada, and kallappam batters have been reported to exhibit  $\alpha$ -amylase inhibition in range of 18.79% to 63.42%<sup>24</sup>. Similarly, probiotic LAB isolated from indigenous fermented food of Darjeeling exhibited inhibitory activities against  $\alpha$ -glucosidase (34 to 39%) and  $\alpha$ -amylase (53 to 63%)<sup>37</sup>. The cell free supernatant of *P. pentosaceus* OBK05 isolated from buttermilk showed 61% inhibition of  $\alpha$ -glucosidase activity<sup>20</sup>. Similarly, the cell free supernatant, cell free extract and intact cells of *Lacticaseibacillus* and *Lactiplantibacillus*

species isolated from fermented foods exhibited  $\alpha$ -glucosidase inhibition in the range of 15.08 to 59.55%<sup>24</sup>. Shirkhan *et al.*<sup>38</sup> isolated *Lactobacillus delbrueckii*, from traditional fermented dairy products, and demonstrated its ability to inhibit the activity of  $\alpha$ -glucosidase by 25 to 50%. Inclusion of probiotics in daily diet routine may be helpful for managing diabetes because it can enhance insulin sensitivity, glycemic control, and general metabolic functions. However, more research is necessary on probiotics to find out the optimal probiotic dosage, course of treatment, and mode of action for the control/management of diabetes.

There are a number of conventional methods for identifying diverse potential probiotic strains, including microscopic analysis, culture morphology, and biochemical profiling. But, 16S rDNA sequencing is the most promising technique for identifying bacteria because it can distinguish between different bacterial strains and determine their evolutionary relationships.<sup>7</sup> The LAB probiotic *L. plantarum* M-13 was identified and reported from a fermented milk product kalarei<sup>8</sup>. Similarly, LAB strains *Lactobacillus plantarum* FT 12 and *Lactobacillus brevis* FT 6 were isolated from Malaysian fermented foods and identified using 16S rDNA sequencing<sup>39</sup>. The variety of Indian dishes, including jaggery and dhokla batter, were found as the potential source of *Lactiplantibacillus plantarum*<sup>21</sup>.

## Conclusion

The selected jalebi batter LAB isolates exhibited adequate GIJ tolerance (87.6 to 96.8%), and several desired functional probiotic attributes. Some of the isolates exhibited high hydrophobicity (xylene 72.79-85.75%, chloroform 71.39-94.2%), substantial autoaggregation (71.7 to 83.4%), coaggregation/antibacterial potential against several pathogens, antibiotic susceptibility, and free radical scavenging activity (ABTS 65.60-76.30%, DPPH 48.68-79.14%, superoxide anion 30.10-31.97%), and ability to inhibit carbohydrate hydrolyzing enzymes  $\alpha$ -amylase and  $\alpha$ -glucosidase *via*, cell free supernatant and intact cells. Isolate J-7 exhibited the maximum capability to inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase (antidiabetic potential) and possessed most of the functionally desired probiotic characteristics and was identified as *Limosilactobacillus fermentum* J-7. Thus, jalebi batter may be a potentially rich ecological source that may be explored for new proficient probiotics for promising applications in the food and pharmaceutical industries.

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## Conflict of interest

The authors declare that they have no competing interests.

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