JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ISOLATION AND CHARACTERIZATION OF THE PROBIOTIC POTENTIAL OF LACTIC ACID BACTERIA AND THEIR APPLICATION IN BIOFILM ERADICATION OF *PSEUDOMONAS AERUGINOSA* PA01

*Jahanvee Chanpura, Mansi Shukla, Maheshwari Patadia and Shilpa Gupte **

Address(es):
Department of Microbiology, Institute of Science & Technology for Advanced Studies & Research (ISTAR), The CVM University, Vallabh Vidyanagar- 388 120, Anand, Gujarat, India.

*Corresponding author: shilpagupte@aribas.edu.in, shilpa.gupte@cvmu.edu.in

https://doi.org/10.55251/jmbfs.9752

ARTICLE INFO

Received 31. 12. 2022
Revised 12. 11. 2025
Accepted 7. 8. 2026
Published xx.xx.201x

Regular article

OPEN ACCESS

ABSTRACT

Probiotic microorganisms were isolated from fermented curd, idli batter, lentils, human milk, and healthy infants' faecal matter. A total of 27 isolates were screened for acid and bile tolerance, with 12 isolates showing resistance to low pH and bile and nine isolates indicating significant viability during gastro-intestinal simulation. They were further investigated for probiotic properties where isolate B33 (from idli batter) with 66.57 ± 0.05% autoaggregation potential, isolate D25 (from curd) with 93.25 ± 0.60% hydrophobicity, and 75.25 ± 0.21% biofilm-forming potential were observed as significant isolates. Some isolates have shown positive lipolytic, amylolytic, and proteolytic activity and all the isolates have shown negative hemolytic and gelatinase activity. Antibiotic resistance was also analysed using the disc diffusion method, wherein isolates B11, B32, and B34 were most resistant to eight antibiotics. The antibacterial properties of cell-free supernatant (CFS) of isolates were determined against *Pseudomonas aeruginosa* PA01, *Bacillus cereus, Staphylococcus aureus,* and *Salmonella* Typhimurium by agar well diffusion method, wherein isolates B11, B32, D25, and B34 showed high antibacterial activity. Potential probiotic isolates D25 and B11 were identified using the 16S rRNA sequencing method, and they were identified as *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus,* respectively. Application of their CFS (20 µL) for biofilm eradication was also studied on the biofilm of *Pseudomonas aeruginosa* PA01 by 96-well microtiter plate assay, where isolate D25 showed 65 ± 0.20% biofilm eradication. The study suggested that both isolates were potential probiotics and could be applied in food preparation, animal feed, or the pharmaceutical industry.

Keywords: Probiotics, Characterization, Autoaggregation, Hydrophobicity, Biofilm eradication

INTRODUCTION

Probiotics have long been associated with health and well-being. With the current upward trend in health awareness, consumers' demand for highly nutritive products is increasing. The health benefits of microorganisms as probiotics have encouraged worldwide consumption. Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) defined probiotics as follows: "Live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host" (Vidhate et al., 2024). The strains most frequently used as probiotics are generally isolated from traditional fermented products, the gut, feces, and human breast milk (Wang et al., 2021). Lactic acid bacteria (LAB) are among the most important groups of bacteria used in the food industry. They have been consumed in dairy products by people all over the world, and most are classified as "generally recognized as safe" (GRAS) microorganisms (Reuben et al., 2020). Several species belonging to the genera of *Lactobacillus*, *Bifidobacterium, Streptococcus, Lactococcus*, *Carnobacterium, Leuconostoc,Pediococcus, Tetragenococcus, Weissella*, and some species of *Enterococcus* and *Escherichia coli* are reported as probiotics (Satish Kumar et al., 2013). Moreover, *Saccharomyces boulardii* is the only non-pathogenic yeast used as a probiotic (Pais et al., 2020; Pakbin et al., 2023).

Probiotic microorganisms provide many physiological, immunological, metabolic, genetic, and technological benefits to the host. There are various mechanisms by which probiotics act, such as bacteriocin production, lowering of the gut pH, competitive exclusion, competition for nutrients and attachment surfaces, stimulation of mucosal barrier function, and immunomodulation. For immunomodulation, evidences show the influence of probiotics in several aspects of acquired and innate immunity (Mora-Villalobos et al., 2020). The probiotic actions for immunomodulation include phagocytosis, IgA secretion, and modifying T-cell responses by enhancing and attenuating Th1 and Th2 responses (Yousefi et al., 2019). Moreover, potential probiotic strains should be non-toxic and non-pathogenic. They should be able to survive and proliferate at the target site, resist gastric acid and bile, adhere to the gastrointestinal tract, and antagonize pathogenic bacteria (Choi et al., 2018; Khan et al., 2021).They should also produce various antimicrobial compounds(Dowarah et al., 2018), exert anti-tumoral activity(Garbacz, 2022), protection against colon cancer (Drago, 2019),stimulation of the immune system (Pimentel et al., 2015), stabilization of gut microbiota (Sánchez et al., 2017), and improvement in lactose metabolism (Salvetti & O'Toole, 2017). Additionally, certain enzymes such as NADH oxidase, glutathione reductase, glutathione S-transferase, catalase, glutathione peroxidase, superoxide dismutase, and feruloyl esterase released by probiotic strains may contribute to the antioxidant property which play a key role in human health (Zaghari et al., 2020). Probiotics also improve digestion and absorption by secreting extracellular digestive enzymes such as amylases, proteases, and lipases (Ahmadifar et al., 2020).

Recently, with the emergence of antibiotic resistance, emphasis has been put on investigating probiotics and their products as alternatives to antibiotics. The antagonistic activity of probiotics against pathogens is brought about by producing effective antimicrobial compounds such as peptides that include competitive exclusion of pathogens, boosting the function of the intestinal barrier (Bazireh et al., 2020). Various probiotic strains have been identified to produce such peptides (bacteriocins) which act as antimicrobial agents against pathogenic microorganisms. Probiotics can also produce organic acids upon fermentation, which decreases the environmental pH and inhibits the survival of some pathogens that cannot tolerate acidic conditions (Hwanhlem et al., 2014). These organic acids can inhibit bacteria by penetrating the cell membrane and affecting its functions. They acidify the cytoplasm and inhibit acid-sensitive enzymes of the cells. Other than these bacteriocins and organic acids (lactic, acetic, pro- pionic and succinic acid), some other metabolites also contribute to the antimicrobial and antibiofilm activity of probiotic cells such as hydrogen sulfide and peroxide, ethanol, carbon dioxide, EPS and, biosurfactants. Probiotics may act on pathogenic biofilm by displacement, exclusion and competition with pathogens. The displacement includes disruption of the pre-formed pathogenic biofilms, exclusion includes pre-coating of a surface to inhibit pathogen adhesion and competition involves the co-culture of pathogenic cells with probiotics and/or their metabolites (Carvalho et al., 2021).

Hence, probiotics can be a useful tool for eradication of the pathogenic biofilms; in the present study, we aimed to isolate and screen potential probiotic organisms from different sources followed by their characterization and investigation of their biofilm eradication potential.

MATERIALS AND METHODS

Media and Chemicals

All chemical reagents and microbial media used in this study were purchased from Himedia Laboratories (Mumbai, India) and Loba Chemie (Mumbai, India) companies with analytical grades. Pathogenic culture *Pseudomonas aeruginosa* PA01 (MTCC 3541) was procured from Microbial Type Culture Collection (Chandigarh, India). *Bacillus cereus* (ATCC 14579), *Salmonella* Typhimurium (ATCC 23564), and *Staphylococcus aureus* (ATCC 25923) were procured from American Type Culture Collection (ATCC, USA). All pathogens were then activated in their specific media.

Isolation of Probiotic Microorganisms

Probiotic microorganisms were isolated from various samples such as fermented idli batter, curd, human milk, lentils, and an infant's fecal material. For the isolation three different idli batter and two different "Dahi" (curd) samples were collected from local street food sellers from Anand, Gujarat; lentils such as "Chola" (*Vigna unguiculate*), "Rajma" (*Phaseolus vulgaris*), "Toor" (*Cajanus cajan*), and "Mung" (*Vigna radiata*) were collected from the local market of Anand, Gujarat. The samples were fermented in anaerobiosis. Moreover, a mother-infant (one-month-old) pair was also selected. Then, an informed consent form was taken from the mother, and the mother's milk was collected by suction in a sterile container and the infant's fecal matter was also collected in a sterile container.
The samples were fermented in TGYE (Tryptone Glucose Yeast Extract) and MRS (De Man Rogosa Sharpe) broth at room temperature under shaking condition for 24-48 h while the mother's milk and infant's fecal samples were directly proceeded for isolation purposes. For the isolation samples were serially diluted and plated on TGYE and MRS media by spread-plate method and incubated at 37 °C for 24 h and next day plates were observed (Rameshgar et al., 2020). TGYE medium was used to isolate various probiotic strains other than those which can be easily isolated by the MRS medium. Colonies with different cultural characteristics were selected for further screening. Probiotic organisms were isolated from various samples like fermented curd, batter, lentils, mother milk and fecal samples.

Primary Screening of Isolated Organisms

Isolates were first evaluated for primary acid and bile tolerance because they should survive during gastrointestinal transit (Devi et al., 2020). For the same, cultures were inoculated in their respective broths (MRS or TGYE) and incubated at 37ºC for 24 h. Further, these actively grown cultures were inoculated (10%) in their respective broth, adjusted to pH 2.0 for acid tolerance, and supplemented with 2% bile (ox bile) with pH 8.0 for determination of bile tolerance. They were incubated for 0, 3, and 6 h at 37 ºC, and then the broth was centrifuged at 10,000 rpm for 5 min. The pellets were re-suspended in normal saline, and their optical density was measured at 600 nm. The isolates with higher growth were selected for the further work.

Secondary Screening of Isolates

Viability of isolate after gastric, intestinal, and gastrointestinal simulation

After primary evaluation, the isolates who showed good acid and bile tolerance were subjected to gastric, intestinal, and gastrointestinal simulation to determine their potential for surviving in *in vivo* gastrointestinal conditions. Selected isolates were subjected to gastric simulation suggested by (Diana et al., 2015) and intestinal simulation suggested by (Topçu et al., 2020) with certain modifications in the simulated juices' pH. Actively grown culture broths were centrifuged and the pellets were treated with simulated gastric juice (0.3% pepsin, 0.5% NaCl, pH 2.0) for 2 h and with intestinal juice (Solution A: 1.01% $NaHCO_3$, 0.2% NaCl, 0.1% Trypsin, pH 8.0; Solution B: 1.8% Ox-bile, pH 8.0; Solution A & B was mixed in the proportion of 2:1 and the final pH was adjusted to 8.0) for 4 h, at 37 ºC. The gastrointestinal simulation treatment of 6 h was also given to these isolates and for that after the incubation time of gastric simulation, the simulated gastric juice was replaced with simulated intestinal juice and further incubated for 4 h at 37 ºC. Thus, after the incubation period of 2 h (gastric simulation), 4 h (intestinal simulation), and 6 h (gastrointestinal simulation), the cultures were spread on TGYE or MRS plates by spread-plate method and incubated at 37 ºC for 24 h. The next day, the viability of probiotic isolates after the simulation treatment was determined in terms of $\log_{10}$ CFU/mL.

Determination of Morphological, Cultural, and Biochemical Characteristics

The morphological, cultural, and biochemical characteristics of various isolates were studied as per Bergey's Manual of Systematic Bacteriology. Biochemical tests, such as the indole production test, Methyl Red (MR) test, Vogas Proskauer (VP) test, citrate utilization test, nitrate reduction test, triple sugar iron agar test, urea test, hydrogen sulphide test and sugar fermentation were also performed for nine selected probiotic isolates.

Determination of Probiotic Potentials of Selected Isolates

Auto-aggregation

The auto-aggregation property was determined by a method described by (Aina et al., 2020), with certain modifications. Activated cultures were subjected to gastric, intestinal, and gastrointestinal simulation and then proceed further for centrifugation. Pellets were washed with sterile saline and re-suspended in 2 mL saline, which was equally distributed into two tubes (1 mL in each). From the first tube, 1 mL aliquot was directly used for measurement of initial OD at 620 nm (Ai). Another tube was spun at 2000 rpm for 2 min, and the supernatant was used to measure its final OD at 620 nm (Af). The percent auto-aggregation (AAG%) was calculated as:

$$\%\text{Autoaggregation} = \frac{A_I - A_f}{A_i} \times 100$$

Cell surface hydrophobicity

Cell-surface hydrophobicity was determined using the method by (Topçu et al., 2020), with certain modifications. Activated cultures were subjected to gastric, intestinal, and gastrointestinal simulation and then proceed further for centrifugation. Pellets were washed with sterile saline and re-suspended in 2 mL PUM (Phosphate Urea Magnesium) buffer, which was equally distributed into two tubes (1 mL in each). From the first tube, 1 mL aliquot was used for measurement of initial OD at 620 nm (Ai). 660 µL of xylene was added to another tube and vortexed for 90 sec. It was allowed to stand for 30 min at 37 °C for phase separation. After phase separation, the aqueous phase was carefully aspirated and transferred to another tube. It was allowed to stand for 30 min to remove residual xylene and then its final OD was measured at 620 nm (Af). The percent Cell Surface Hydrophobicity (% CSH) was calculated as:

$$\%\text{CSH} = \frac{A_i - A_f}{A_i} \times 100$$

Biofilm-forming potential

BFP was determined with the method suggested by (Bazireh et al., 2020), with certain modifications. Actively grown cultures were subjected to gastric, intestinal, and gastrointestinal simulation. Then, 10% inoculum of the simulated cultures were inoculated in a 96-well microtiter plate containing 200 µL of TGYE or MRS broth, supplemented with 0.25% glucose in triplicate, and incubated at 37 ºC for 24 h. Un-inoculated wells were maintained as control. After incubation, media were decanted and 250 µL methanol was added for fixation followed by rinsing with 250 µL of sterile distilled water. After rinsing, cells were stained with 1% crystal violet for 5 min. The excess stain was decanted and rinsed with sterile saline. The cell-bound stain was re-solubilized in 250 µL of 33% glacial acetic acid and the absorbance was measured using an ELISA reader at 595 nm. The percent Biofilm Formation Potential (%BFP) was calculated as:

$$\%\text{BFP} = \frac{\text{Final OD} - \text{Initial OD}}{\text{Final OD}} \times 100$$

Extracellular Enzyme Production by Isolates

Improvement in probiotic status includes many approaches and is not limited to the selection of technological beneficial properties but also comprises the production of extracellular digestive enzymes like lipase, amylase, and protease (Mostefa et al., 2018). The lipolytic, amylolytic, and proteolytic activity was determined by transferring the activated cultures of isolates on 3% tributyrin agar, 2% starch agar, and 3% casein agar plates, respectively. The plates were incubated at 37 °C for 24 h, and the next day, plates were observed for the clear zone around the colonies, which indicated extracellular enzyme production by probiotics.

In Vitro Safety Assessment of Isolates

Isolates were assessed for hemolytic and gelatinase activity to confirm their safety for consumption (Kondrotiene et al., 2020). Activated cultures of probiotics were streaked on 5% blood agar and 5% gelatin agar plates to determine the hemolytic and gelatinase activity. These plates were incubated at 37 °C for 24 h. The next day, blood agar plates were observed for hemolytic reaction, i.e., α-hemolysis (partial hemolysis with greenish discoloration surrounding bacterial colony), β-hemolysis (complete hemolysis with clear zone surrounding bacterial colony), and γ-hemolysis (no hemolysis) and gelatin plates were observed for the colonies surrounded by a clear zone, indicating a positive gelatinase activity by the probiotic organism.

Assessment of Antibiotic Sensitivity

Antibiotic sensitivity of isolates was determined according to the European Food Safety Authority's (EFSA) recommendations, using antibiotic susceptibility

testing octa-discs (Combi XIII, Hi-media, Mumbai) on MRS or TGYE agar plates seeded with the test probiotic organism (10^7–10^8 CFU/mL), as described by (Mohammad et al., 2020). The antibiotic discs were placed on the surface of the agar and kept undisturbed for 1 h at 4 °C to allow diffusion, followed by incubation at 37 °C for 24 h. The pattern was assessed against different antibiotic, namely penicillin (P- 2 units), tetracycline (TE- 10 mcg), co-trimoxazole (COT- 25 mcg), cloxacillin (COX- 5 mcg), cefradine (CH- 30 mcg), erythromycin (E- 10 mcg), lincomycin (L- 10 mcg), and cefuroxime (CXM- 30 mcg). The zone of inhibition was measured (mm) and probiotic isolates were categorized into resistant, intermediate, and sensitive as per CLSI standards (Clinical and Laboratory Standard Institute, 2003).

Determination of the Antibacterial Activity of CFS (Cell-Free Supernatant)

Antibacterial activity of CFS from activated probiotics was determined by agar well diffusion assay (Aina et al., 2020). Potential probiotic isolates were activated in their respective broths and grown cultures were centrifuged the next day at 8000 rpm, after which the supernatant was collected and filtered by a 0.2-µm syringe filter membrane. Pathogens such as *Pseudomonas aeruginosa* PA01*, Bacillus cereus, Staphylococcus aureus,* and *Salmonella Typhimurium* were inoculated in nutrient broth and incubated at 37 °C for 24 h for activation. 100 µL of these were inoculated in melted nutrient agar and poured on nutrient agar plates. 9-mm-diameter wells were made on each plate, wherein 100 µL of antibiotic streptomycin was poured as a positive control, and probiotic isolates' cell-free supernatants (CFS) were poured as test samples. Plates were placed undisturbed in the refrigerator for 30 min to allow diffusion and then incubated at 37 °C for 24 h. The next day, antagonism was determined by measuring the zone of inhibition around the wells.

Identification of Potential Probiotic Isolate

After the characterization of probiotic isolates, two potential probiotic isolates were selected and processed for genomic identification. The genomic identification was carried out by 16S rRNA nucleotide sequence analysis using universal 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers.

Application of CFS of Potential Probiotics for Biofilm Eradication

Probiotic isolates' biofilm eradication ability was determined by the method used by (Hossain et al., 2020). The activated culture of *P. aeruginosa* PA01 was inoculated (10% inoculum) in a 96-well microtiter plate containing 200 µL nutrient broth supplemented with 0.25% glucose in triplicate. To this plate, 20 µL of cell-free supernatant (CFS) of selected isolates was inoculated and the plate was incubated at 37 °C for 24 h. Wells without CFS inoculation, were maintained as positive control. After incubation, media was decanted and the biofilms were fixed with methanol. After that, the plate was rinsed with sterile distilled water, followed by staining with 1% crystal violet for 20 min. The excess stain was removed by washing the plate with saline. The cell-bound stain was re-solubilized in 33%-glacial acetic acid, the absorbance of which was measured using an ELISA reader at 595 nm. The percent biofilm eradication was calculated with reference to the control.

Statistical Analysis

All experimental measurements were repeated independently in triplicate, and the results were expressed as the mean ± standard deviation and evaluated by performing the analysis of variance (ANOVA) and Tukey's test, both considering the significance level of $P < 0.05$. The analysis was performed using MS Excel.

RESULTS AND DISCUSSION

Sample Collection and Isolation

A total of twenty seven lactic acid bacterial isolates were obtained from five different fermented foods (fermented curd, batter, lentils, mother milk and fecal) samples. The isolates were initially differentiated on the basis of their cultural and cellular morphological studies after which they were subjected to various physiological and biochemical tests. These isolates were Gram positive rods, cocci and short rods as well as the colonies were white, circular with entire margine. The results of morphological and cultural characterization are shown in online resource 1.

Primary Screening of Isolates for Acid and Bile Tolerance

Probiotic isolates should survive in gastric and intestinal transit. They should remain metabolically active and viable for providing health-promoting effects when they arrive in the colon. Therefore, probiotic isolates were screened for their acid and bile tolerance (Sharma et al., 2021). Out of 27 isolates, 12 isolates were found to be resistant to acid (pH 2.0) and bile (2%), as shown in Figure 1a & 1b respectively. Similarly, Huang et al., (2020) treated 24 different isolates at pH 2.5 for 3 h and with 1% bile salts for 4 h. Out of them, 12 isolates have shown acid-bile resistance. On the contrary, Kondrotiene et al., (2020) determined the bile tolerance with 24 h treatment at three different concentrations (0.3%, 0.5%, and 1% (w/v)), and acid tolerance with 3 h exposure of phosphate buffer saline (pH 2.5 with 5 M HCL). They observed two *L. lactis* strains with more than 50% resistance to low pH and more than 80% resistance to varis. Also, Mishra & Ghosh, (2018) found the highest survival of two strains of *E. faecalis* AG11 (99±0.26%) after 4 h incubation in intestinal transit and AG9 strain (93±0.87%) after 2 h incubation in gastric transit condition**.** Hence, the overall result shows that the acid and bile tolerance are intrinsic property of probiotics that may differ across the species.

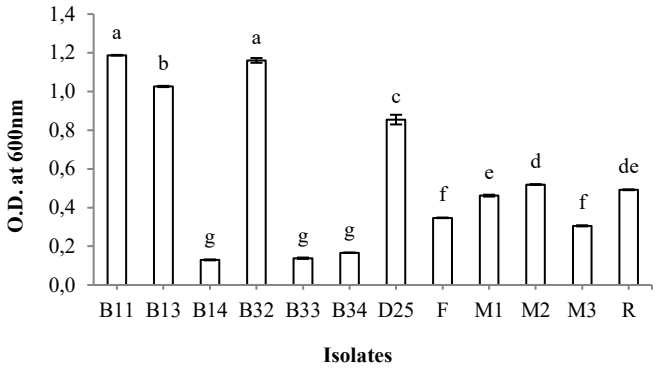

Figure 1a Acid tolerance of isolates after 6 h of incubation (pH 2.0). Different letters above the bars denote statistically significant differences at $p<0.05$.

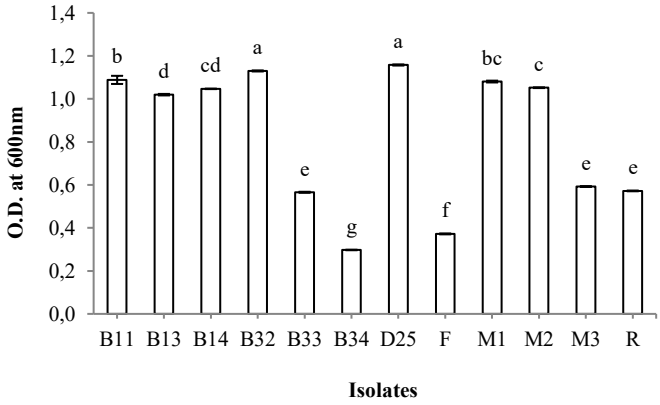

Figure 1b Bile tolerance of isolates after 6 h of incubation (2% Bile, pH 8.0). Different letters above the bars denote statistically significant differences at $p<0.05$.

Secondary Screening of Isolates for Survival after Simulation Treatment

For the *in vitro* selection of probiotic bacteria that may reflect certain *in vivo* effects, the isolates were further evaluated for gastric, intestinal, and gastrointestinal simulation. Here, from our 12 isolates, nine isolates have shown significant viability after incubation at 37 °C for 24 h, indicating good tolerance of isolates against acid and bile conditions prevalent in the gastrointestinal passage. Their survival was determined in $\log_{10}$ CFU/mL with simulation effect (Figure 2a, 2b & 2c). Similarly, (Gebru & Sbhatu, 2020) have studied 200 different isolates for their survival in GIT simulation, reporting that 44 isolates were resistant to GIT simulation with a high survival rate. (Kostelac et al., 2021) studied the survival of *L. plantarum* KO9 and *L. plantarum* M2 when treated with simulated gastric and intestinal fluids. At pH 3.0, there were no statistical differences in CFU mL^{-1} count compared with the control sample, but the lowest survival (76%) was observed at pH 1.5. On the other hand, both strains showed high tolerance to simulated intestinal fluids where *Lact. plantarum* KO9 showed no significant difference in survival at all three concentrations of bile salts, while *L. plantarum* M2 showed a decrease in viability by 2.8% and 5.7% at 1.5 mg mL^{-1} and 3.0 mg mL^{-1} bile salts concentration, respectively. Moreover, (de Souza et al., 2018) found all *L. fermentum* strains and six *L. casei* strains (SJRP38, SJRP135, SJRP144, SJRP148, SJRP150, and SJRP166) with good survival (> 4 $\log_{10}$ CFU/mL) when exposed to the simulated GIT condition for 360 min, which was equivalent to the passage of bacteria through the gastrointestinal tract of humans.

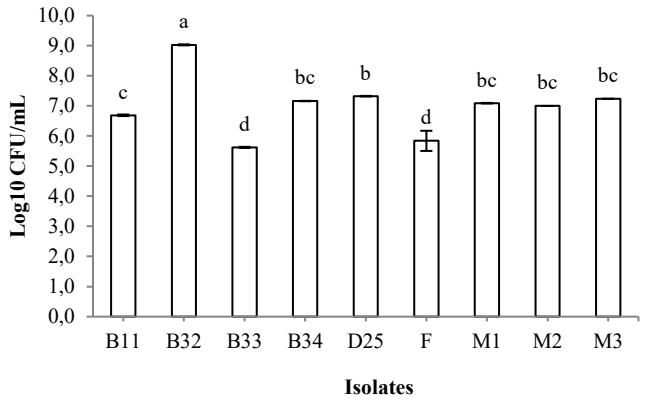

Figure 2a Viability of isolates after Gastric Simulation. Different letters above the bars denote statistically significant differences at $p < 0.05$.

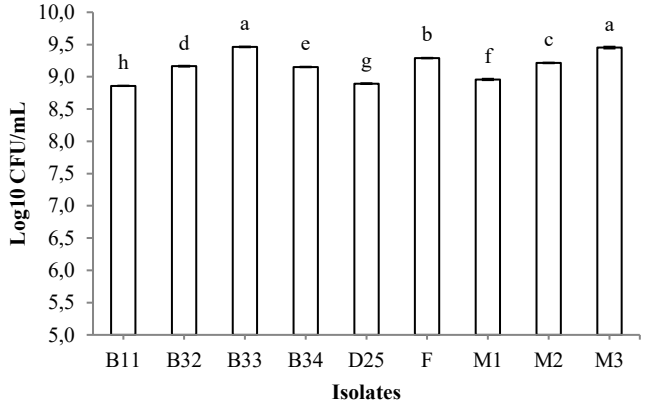

Figure 2b Viability of isolates after Intestinal Simulation. Different letters above the bars denote statistically significant differences at $p < 0.05$.

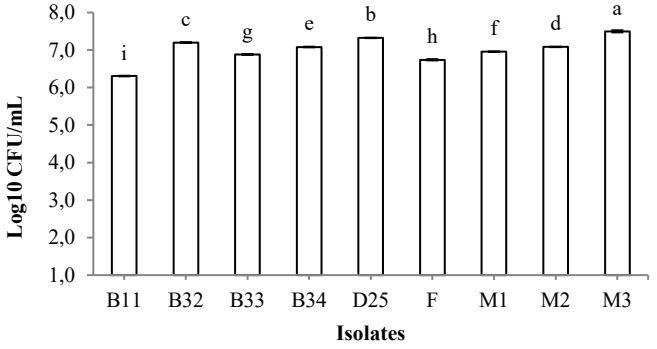

Figure 2c Viability of isolates after Gastrointestinal Simulation. Different letters above the bars denote statistically significant differences at $p < 0.05$.

Determination of Probiotic Potentials of Selected Isolates

Autoaggregation

Interactions among components present on cell surface such as proteins, carbohydrates, and lipids (teichoic acid) are generally believed to be associated with cell aggregation (Hossain et al., 2020). Autoaggregation of the nine selected isolates was determined with the effect of gastric, intestinal, and gastrointestinal simulation. The highest autoaggregation potential was shown by isolate B33 (66.57 ± 0.05%) followed by isolate M3 (61.91 ± 0.13%), D25 (60.76 ± 0.31%), B11 (57.34 ± 0.28%), B34 (53.78 ± 0.19%) and isolate B32 (49.05 ± 0.21%) as shown in Tab 1. It was observed that autoaggregation potential was less after 2 h of gastric simulation but it was increased with time in the case of intestinal (4 h) and gastrointestinal simulation (6 h). A similar kind of observation was noted by (Mohammad et al., 2020). They observed, among different 27 probiotic isolates, isolate *L. mindensis* SGMT22 has shown the highest autoaggregation ability (41.16%) while *E. faecalis* MPS15 showed the lowest after 3h incubation at 37 °C. Moreover, (Bhagat et al., 2020) determined the autoaggregation ability of *P. acidilactici* was maximum (77 ± 0.68%) and a gradual increase was reported after 24 h incubation at 37 °C. Similarly, (de Souza et al., 2018) observed *L.* casei SJRP166 and *L. fermentum* SJRP32 showed high autoaggregation, 60.97% and 96.18%, respectively without any simulation. Likewise, (Sui et al., 2021) have reported high (95.5%) autoaggregation for *L. plantarum* NF4 while low (15%) for *L. plantarum* NF1.

Table 1 Autoaggregation potential of isolates

Isolates	Gastric Simulation	Intestinal Simulation	Gastro-intestinal Simulation
B11	42.11 ± 0.44^{aA}	61.34 ± 0.39^{eB}	57.34 ± 0.28^{eC}
B32	6.11 ± 0.14^{fA}	57.24 ± 0.21^{fB}	49.05 ± 0.21^{eC}
B33	7.14 ± 0.13^{fA}	84.27 ± 0.16^{aB}	66.57 ± 0.05^{aC}
B34	41.58 ± 0.51^{aA}	77.90 ± 0.16^{cB}	53.78 ± 0.19^{dC}
D25	5.82 ± 0.98^{fA}	74.65 ± 0.24^{dB}	60.76 ± 0.31^{bC}
F	34.85 ± 0.32^{bA}	74.09 ± 0.15^{dB}	37.40 ± 0.13^{hC}
M1	11.51 ± 0.56^{eA}	56.16 ± 0.35^{fB}	39.31 ± 0.65^{gC}
M2	32.14 ± 0.34^{cA}	80.17 ± 0.67^{bB}	44.69 ± 0.34^{fC}
M3	27.77 ± 0.55^{dA}	76.48 ± 0.18^{cB}	61.91 ± 0.13^{bC}

Values of means followed by the same letter in lower and upper case do not differ statistically by the Tukey's test at 5% probability.

Cell surface hydrophobicity

Bacteria with a high percentage of hydrophobicity typically have a high capacity of adherence to the intestinal mucosal cells (Romero-Luna et al., 2020). The cell surface hydrophobicity is one of the significant physicochemical characteristics which can facilitate the first contact between the microorganisms and the host's intestinal wall cells (Oliveira et al., 2017). Among the nine different isolates, isolate D25 have shown highest hydrophobicity of about 93.25 ± 0.60% followed by M1 (89.12 ± 0.13%), B11 (88.41 ± 0.16%), B32 (85.41 ± 0.12%), B33 (78.39 ± 0.30%) and M2 (70.35 ± 0.28%) after 6 h of gastrointestinal simulation as shown in Table 2. (dos Santos Leandro et al., 2021) studied the hydrophobicity of 17 probiotic isolates and observed all strains with more than 55% of hydrophobicity with the exception of strains *L. fermentum* C4848.7 (10.03%) and *L. pseudomesenteroides* C4820.3 (24.83%) which showed lower hydrophobicity. (de Souza et al., 2018) have reported the hydrophobicity of *L. casei* strains which ranged between 9.66 and 69.36%, while hydrophobicity of other *L. fermentum* strains varied from 0.3% to 68.81%. Additionally, (El-Deeb et al., 2020) studied hydrophobicity with many *Lactobacillus* isolates with different hydrocarbons where isolate MF1 showed maximum affinity towards hexadecane. Among all the three different hydrocarbons taken under study; maximum adhesion was observed with hexadecane (49.6 ± 0.6%), followed by xylene (44.3 ± 0.5%) and toluene (41.6 ± 0.6%). Isolates MF1, MF2, MF3, IG1, IG2, and IG3 showed significant hydrophobicity than the other isolates such MF6, WD2, IG6, and WD6 which exhibited no hydrophobicity towards any of the three hydrocarbons. Besides, (Hashem & Hashem, 2021) studied the cell surface hydrophobicity of cells of the two *Lactobacillus* strains using the salt aggregation test (SAT). In this method, they mixed the bacterial cell suspensions individually on a glass slide with equal volumes of ammonium sulphate solution having different concentrations (0.5, 1.5, 2, and 4 mol/L). The SAT hydrophobicity value is defined as the lowest concentration of Ammonium sulphate that causes visible aggregation of the *Lactobacillus* cells. Based on these values, the probiotics were classified as high hydrophobic (< 0.9 mol/L), intermediate hydrophobic (0.9–1.5 mol/L), or hydrophilic (>1.5 mol/L). In their study, both the potential probiotic strains, i.e., *L. plantarum* ATCC 14917 and *L. acidophilus* ATCC 20552, have shown hydrophilic properties, as cell aggregates were formed with both tested *Lactobacillus* strains just after 1 min. at a salt concentration of 2 mol/L. Much larger aggregates are observed with *L. acidophilus* than *L. plantarum*.

Table 2 Cell surface hydrophobicity of isolates

Isolates	Gastric Simulation	Intestinal Simulation	Gastro-intestinal Simulation
B11	57.46 ± 0.17^{eA}	58.69 ± 0.38^{dB}	88.41 ± 0.16^{bC}
B32	75.30 ± 0.19^{cA}	58.55 ± 0.32^{dB}	85.41 ± 0.12^{cC}
B33	87.83 ± 0.18^{aA}	46.16 ± 0.11^{eB}	78.39 ± 0.30^{dC}
B34	70.50 ± 0.52^{dA}	60.84 ± 0.29^{cB}	64.70 ± 0.25^{fC}
D25	86.33 ± 0.32^{abA}	58.10 ± 0.18^{dB}	93.25 ± 0.60^{aC}
F	56.49 ± 0.21^{eA}	79.17 ± 0.44^{aB}	57.81 ± 0.29^{gC}
M1	26.70 ± 079^{gA}	35.84 ± 0.26^{fB}	89.12 ± 0.13^{bC}
M2	40.67 ± 0.43^{fA}	69.45 ± 0.07^{bB}	70.35 ± 0.28^{eC}
M3	84.62 ± 0.31^{bA}	25.85 ± 0.15^{gB}	13.55 ± 1.25^{hC}

Values of means followed by the same letter in lower and upper case do not differ statistically by the Tukey's test at 5% probability.

Biofilm-forming potential

Biofilm formation is an important property of being probiotic organisms as it helps the cells adhere to the gastrointestinal tract. Among the nine isolates, six isolates showed more than 45% BFP after 6 h of gastrointestinal simulation. Highest potential was observed in isolate D25 (75.25 ± 0.21%) followed by isolate B11 (61.84 ± 0.29%), B34 (60.89 ± 0.54%), M1 (55.35 ± 0.10%), B33 (47.66 ± 0.35%)

and F (46.15 ± 0.16%) as shown in Tab 3. Likewise, (Bazireh et al., 2020) studied 17 different probiotic isolates for their biofilm formation ability where all the isolates showed strong biofilm formation except four isolates (SA 135, ST 13, ST 67, and ST 126) which have shown modest biofilm formation. On the other hand, (Fredua-Agyeman et al., 2020) assessed the biofilm formatting ability of 25 different isolates using the Alvatex 3D scaffold insert. The confocal imaging of the 3D scaffold showed randomly aligned fibres and fibrous mesh at 60x magnification. This 3D scaffold was explored to simulate the structural matrix that is generally found in the gut and it will mimic the *in vivo* biofilm growth. All tested species formed biofilm comprised of both live and dead cells on the 3D scaffold. This biofilm-forming ability would increase the potential use of strains as probiotics, since this biofilm formation may prevent the adhesion of pathogens to the GIT membrane surfaces (Penaloza-Vazquez et al., 2019).

Table 3 Biofilm forming potential of isolates

Isolates	Gastric Simulation	Intestinal Simulation	Gastro-intestinal Simulation
B11	40.19 ± 0.12^{dA}	76.46 ± 0.04^{bB}	61.84 ± 0.29^{bC}
B32	40.79 ± 0.24^{dA}	28.80 ± 0.30^{eB}	45.52 ± 0.14^{eC}
B33	53.12 ± 6.65^{bcA}	49.44 ± 0.36^{eB}	47.66 ± 0.35^{dC}
B34	44.31 ± 0.24^{cdA}	64.64 ± 0.24^{dB}	60.89 ± 0.54^{bC}
D25	78.70 ± 0.24^{aA}	79.26 ± 0.21^{aB}	75.25 ± 0.21^{aC}
F	47.75 ± 0.05^{cdA}	68.33 ± 0.22^{cB}	46.15 ± 0.16^{eC}
M1	59.78 ± 0.21^{bA}	14.26 ± 0.51^{iB}	55.35 ± 0.10^{cC}
M2	52.45 ± 0.21^{bcdA}	20.41 ± 0.34^{hB}	39.94 ± 0.29^{hC}
M3	44.76 ± 0.10^{cdA}	30.96 ± 0.19^{fB}	42.26 ± 0.18^{gC}

Values of means followed by the same letter in lower and upper case do not differ statistically by the Tukey's test at 5% probability.

Extracellular Enzyme Production by Probiotic Isolates

Extracellular enzymes produced by probiotics may help in the digestion of food by the host, contribute towards energy generation by catalytic digestion of macromolecules present in food, and enhance fermented foods' organoleptic properties (Bhat & Bajaj, 2020). With reference to that, we have studied the extracellular enzyme production for our isolates. Lipolytic, amylolytic, and proteolytic activity of probiotic isolates was determined on tributyrin, starch, and casein agar plates, respectively. The zone around the colony was considered positive for enzyme production. Among the nine different isolates, isolate B34, F, M2, and M3 have shown positive results for lipase production. Similarly, isolates B32, D25, F, and M2 have shown positive results for amylase production and isolates B34, D25, F, M2, and M3 have shown positive caseinase activity (Tab 4). Similarly, (Mostefa et al., 2018) have determined the extracellular enzyme production ability of lactic acid bacteria. All lactic acid bacteria have shown significant protein-digesting ability. With the use of MRS media supplemented with 2% skimmed milk, *Aerococcus* spp. (isolates Lbm3, 10, 17, 18), *Enterococcus* sp. (isolate Lbm50), and *Weisella* sp. (isolate Lbm22) showed 17-22 mm of the zone of clearance, while MRS media supplemented with 10% skimmed milk; *Aerococcus* sp. (isolate Lbm19) *Enterococcus* spp. (Lbm46, 47, 49) showed a 10–15 mm zone of clearance on the plate. Additionally, (Ghanei-Motlagh et al., 2021) have reported that proteolytic, lipolytic, and amylolytic activities of probiotic organisms significantly ($p < 0.05$) increased in quorum quenching treatments when compared to the control group. In contrast, (Bhagat et al., 2020) earmarked the phytase production as an essential criterion of probiotics and carried out qualitative analysis of phytase in *P. acidilactici* SMVDUDB2, which showed an 8 mm zone that was higher than *L. rhamnosus* MTCC 1408 (5 mm) on phytase-screening medium (PSM) after 48 h at 37 °C. The quantitative analysis using spectrophotometer showed that the enzyme activity was 5.18 ± 0.09 U/mL for *P. acidilactici* SMVDUDB2 and 3.69 ± 0.12 U/mL for *L. rhamnosus* (MTCC 1408). Also, (Khan et al., 2021) have qualitatively determined the extracellular enzyme-producing ability of *B. licheniformis* and *B. subtilis* and observed that both strains were positive for the production of proteinase, amylase, lipase, and cellulase. Similarly, (Papamanoli et al., 2003) have determined the percentage of lipolytic activity from *L. sakei* (31%) and *L. curvatus* (42%) by hydrolysing tributyrin and confirmed that *Lactobacillus* species were weakly lipolytic. Additionally, (H. Lee et al., 2001) isolated three strains (A-4, L9, and L23) of *Lactobacillus* sp. and studied their amylolytic activity in a broth containing soluble starch as a sole carbon source. Among these three cultures, *L. acidophilus* L23 has shown high starch hydrolase activity and was selected for improving digestion of starch in pigs based on its hydrolysis activity of rawstarch.

***In Vitro* Safety Assessment of Isolates**

Hemolytic and gelatinase activity is an important aspect of probiotic safety (Vasiee et al., 2020). When all our nine isolates were streaked on blood agar and gelatin agar plates, they were found to be negative for both properties, as shown in Tab 4. A similar study was carried out by (Premasiri et al., 2021) and they have screened out isolates on the basis of their hemolytic activity, and those with positive hemolytic activity were not considered suitable for food supplementation. Out of 20 isolates, they observed 60% of isolates with positive hemolysis (35% were β-Hemolytic and 25% were α-Hemolytic) and 40% of isolates with negative hemolysis (ϒ-Hemolytic), which can be considered as safe organisms for live consumption. They also suggested these probiotics should be further assessed for *in vivo* toxicity and animal toxicity study for safety confirmation. (Pinto et al., 2020) have also determined the hemolytic activity of seven lactic acid bacteria on Columbia Agar plates with 5% defibrinated sheep blood, considering *Staphylococcus aureus* ATCC 25213 as a positive control. The results showed that none of their isolates showed positive hemolysis. Likewise, (Sabo et al., 2020) studied five probiotic isolates and none of the selected isolates have demonstrated β or α-hemolytic activities. Moreover, (Vasiee et al., 2020) confirmed the safety aspects of *P. acidilactici* IAH-5 by showing its negative DNase production or hemolytic activity. Likewise, (Kondrotiene et al., 2020) studied 13 different *L. lactis* strains, and none of them have presented positive hemolytic or gelatinase activity. The absence of these features supports the safety of selected *Lact. lactis* strains. Moreover, (Foulquié Moreno et al., 2003) studied the major factors involved in a potential health risk associated with the use of E*nterococci*, namely hemolysis. The six *E. faecium* strains and one *E. faecalis* showed γ-hemolytic activity in horses, sheep, and human blood agar plates, indicating the absence of hemolysis. Further, (Monteagudo-Mera et al., 2011) studied 12 probiotic strains of *E. faecalis* that showed that at least one of the virulence factors was positive, either haemolysin or gelatinase. In another study, 15 potential probiotic *lactobacilli* isolates were tested for their gelatinase activity, and all the isolates showed negative gelatinase activity compared to the positive control strain of *S. aureus* ATCC 25923 (Francois et al., 2011).

Table 4 Enzyme production by isolates

Probiotic Isolates	Lipolytic Activity	Starch Hydrolysis	Proteolytic Activity	Hemolytic Activity	Gelatinase Activity
B11	-	-	-	-	-
B32	-	+	-	-	-
B33	-	-	-	-	-
B34	+	-	+	-	-
D25	-	+	+	-	-
F	+	+	+	-	-
M1	-	-	-	-	-
M2	+	+	+	-	-
M3	+	+	+	-	-

Antibiotic Sensitivity Test of Isolates

All the isolates were subjected to antibiotic susceptibility test using different antibiotics. Among the nine isolates, isolates B11, B32, and B34 were resistant and B33, D25, and M1 were sensitive when tested against antibiotics such as penicillin, tetracycline, co-trimoxazole, cloxacillin, cefradine, erythromycin, lincomycin, cefuroxime, etc. (Tab 5). Antibiotic-resistant properties of these isolates may be useful for remaining stable against antibiotic exposure. Similarly, (Kang et al., 2020) investigated three *lactobacillus* strains for antibiotic susceptibility and found all of them resistant to chloramphenicol, streptomycin, and kanamycin, while strain LHL7 was different from LHL6 and CECT5716, being susceptible to ampicillin and resistant to tetracycline. *L. plantarum* KU200656 was found to be sensitive to tetracycline, ampicillin, chloramphenicol, and doxycycline, while it was resistant to streptomycin, gentamicin, kanamycin, and ciprofloxacin (J. E. Lee et al., 2021). Further, (Chen et al., 2020) have assessed antibiotic susceptibility of four probiotic strains against 30 antibiotics of nine different classes, and each strain showed different sensitivity to antibiotics. Isolate HSM- 1 showed resistance to amikacin, tetracycline, norfloxacin, and sulfamethoxazole, isolate HSM-10 was resistant to nine antibiotics; isolate HSM-14 was resistant to 10 antibiotics, and isolate HSM-18 exhibited resistance to kanamycin, neomycin, tetracycline, doxycycline, and sulfamethoxazole. Similarly, (Betancur et al., 2020) analyzed three probiotics isolated from Colombian native pigs for their susceptibility to five antibiotics commonly used in the swine industry. All three of them have shown resistance to antibiotics such as ciprofloxacin, tetracycline, doxycycline, and trimethoprim/ sulfamethoxazole, with the only exception being isolate CAM6, which showed intermediate resistance to trimethoprim/sulfamethoxazole. However, all LAB strains were susceptible to amoxicillin.

Table 5 Antibiotic sensitivity test of isolates

Antibiotics	B11	B32	B33	B34	D25	F	M1	M2	M3
Penicillin (P)	R	R	S	R	S	S	S	S	M
Tetracycline (TE)	R	R	S	S	S	S	S	S	S
Co-Trimoxazole (COT)	R	R	S	S	S	R	R	S	S
Cloxacilin (COX)	R	R	S	S	M	R	S	S	S
Cefradine (CH)	R	R	S	S	S	M	S	S	S
Erythromycin (E)	R	R	S	R	S	S	M	M	R
Lincomycin (L)	R	R	S	R	M	R	M	R	R
Cefuroxime (CXM)	R	R	R	R	S	S	S	R	M

Legend: (R: Resistance, M: Moderate, S: Sensitive)

Antibacterial Activity of CFS (Cell-Free Supernatant)

Several metabolic compounds, such as bacteriocin, bacteriocin-like inhibitory substances (BLIS), organic acids, exopolysaccharides (EPS), short-chain fatty acids (SCFA) and hydrogen peroxide are produced by lactic acid bacteria, which have antimicrobial effects (Bajaj et al., 2015). To determine the antimicrobial property of our isolates, we have taken cell-free supernatant of the nine isolates and checked their antibacterial activity against four pathogenic strains; two Gram-positive (*Bacillus cereus, Staphylococcus aureus*) and two Gram-negative (*Salmonella* Typhimurium, *Pseudomonas aeruginosa* PA01*).* Among the nine potential probiotics, isolates B11, B32, B33, B34, D25, and F showed significant antibacterial activity, from which B11, B32, B34, and D25 showed zone of inhibition against all four indicator pathogenic strains. The zone diameter of inhibition is shown in Tab 6, which shows the potential of probiotic isolates to inhibit pathogenic organisms. Similarly, (Karimi et al., 2020) have determined the antibacterial activity of *L. plantarum* against five different foodborne pathogens, such as *L. monocytogenes*, *S. enterica*, *P. aeruginosa*, *S. aureus*, and *E. coli*. No significant inhibition zone was observed against *L. monocytogenes* ($p<0.05$). However, inhibition zones against *Salm. enterica*, *P. aeruginosa*, *S. aureus,* and *E. coli* were 12.3 ± 0.5, 14.6 ± 0.5, 14.0 ± 1.0 and 12.6 ± 0.5 mm, respectively. (Jinendiran et al., 2017) screened out seven probiotic strains showing antagonistic activity, but only three isolates (S01, V03, and V04) exhibited promising antagonism against *Aeromonas hydrophila, Vibrio parahaemolyticus,* and *V. alginolyticus* in the agar well diffusion assay.

Table 6 Antimicrobial activity of isolates

Isolates	*Bacillus cereus*	*Staphylococcus aureus*	*Salmonella* Typhimurium	*Pseudomonas aeruginosa*
B11	11.67 ± 0.33^{cA}	12.00 ± 0.00^{dB}	16.33 ± 0.67^{bC}	12.67 ± 0.33^{bD}
B32	15.33 ± 0.67^{bA}	16.33 ± 0.33^{bcB}	0.00 ± 0.00^{cC}	12.33 ± 0.67^{bD}
B33	15.67 ± 0.33^{bA}	14.33 ± 0.33^{cdB}	0.00 ± 0.00^{cC}	15.67 ± 0.67^{aD}
B34	15.67 ± 0.33^{bA}	17.33 ± 0.67^{bB}	16.33 ± 0.33^{bC}	13.33 ± 0.33^{bD}
D25	18.67 ± 0.67^{aA}	14.67 ± 0.67^{bcB}	27.00 ± 0.58^{aC}	12.67 ± 0.33^{bD}
F	0.0 ± 0.00^{dA}	28.33 ± 0.67^{aB}	0.00 ± 0.00^{cC}	0.00 ± 0.00^{cD}

Values of means followed by the same letter in lower and upper case do not differ statistically by the Tukey's test at 5% probability.

Identification of Potential Probiotic Isolates

From the above observations, two potential probiotics, isolates D25 and B11, were identified by 16S rRNA sequence analysis, using universal primers. The phylogenetic tree (Figure 3a & 3b) was constructed by the neighbor-joining method. Isolate D25 was identified as *Lactiplantibacillus plantarum*. with accession number MW362778, and isolate B11 was identified as *Pediococcus pentosaceus,* with accession number MW362744.

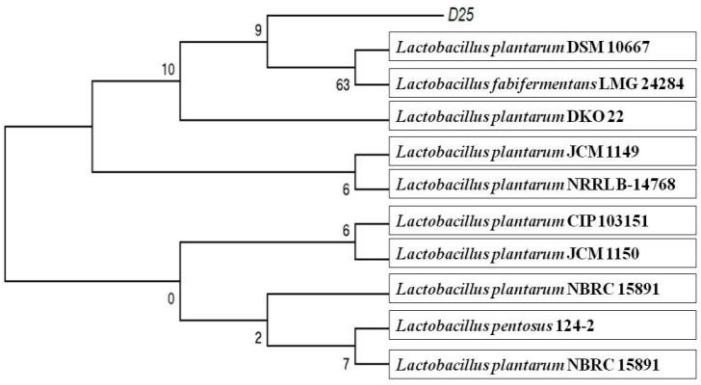

Figure 3a Phylogenetic tree of potential probiotic isolate D25

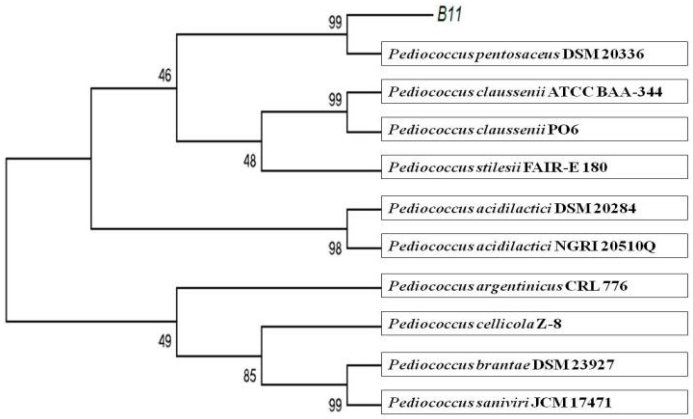

Figure 3b Phylogenetic tree of potential probiotic isolate B11

Application of CFS of Potential Probiotics for Biofilm Eradication

Both identified probiotic isolates B11 and D25 were further analysed for the biofilm eradication ability. Cell-free supernatant (CFS) of both isolates was used to determine their effect on the biofilm of *P. aeruginosa* PA01. Isolate D25 showed 65 ± 0.20% eradication, while isolate B11 did not show any significant effect on the biofilm of *P. aeruginosa* PA01. Similarly, (Singh et al., 2020) have observed that the cell-free extracts of *Lact. brevis* SII showed biofilm inhibition. Biofilm inhibition of *P. aeruginosa* and *Klebsiella pneumoniae* was found to be 52.63% and 22.2%, respectively. Likewise, (Riyaz Ali et al., 2021) have determined the antibiofilm activity (% inhibition activity) of the *Enterococcus* sp. against *E.coli* (33.0±4.0%,), *Vibrio harveyi* (41.0±3.2%,), *P. aeruginosa* (37.8±4.0%), and *S.aureus* (41.7±2.0%). The results suggested that the isolated culture was a potential biofilm inhibitor. Additionally, (Hashem & Hashem, 2021) have determined the antibiofilm activities of the two *Lactobacillus* supernatants using the tissue-culture plate assays. 50-72% and 74-85% biofilm reduction was observed against *Candida* spp. by *L. plantarum* and *L. acidophilus,* respectively. Further, (J. E. Lee et al., 2021) studied the CFS of probiotics, which exhibited an

anti-biofilm property against all pathogens except for *S.* Typhimurium. *L. plantarum* GG at 1/2 MIC exhibited 33.62%, 47.69%, and 38.50% anti-biofilm activities against *S.aureus*, *L.monocytogenes*, and *E.coli*, respectively, whereas *L.plantarum* KU200656 at 1/2 MIC exhibited 44.45%, 48.07%, and 52.61% anti-biofilm activities, respectively. Thus, they concluded that, at the 1/2 MIC, isolate KU200656 had higher anti-biofilm activities than isolate GG against *S.aureus* and *E. coli*. (Ji & Yang, 2021) have determined the cell viability of *Helicobacter pylori* mature biofilms after the treatment of CFS of *L. plantarum* LN66, clarithromycin (CLR), and levofloxacin (LVX) alone or in combination with the XTT (2, 3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt) method. Their results showed that LN66 CFS had a biofilm eradication effect on mature *H. pylori* biofilm; when used in combination with CLR; LN66 CFS significantly attenuated the eradication effect of CLR on biofilms. In contrast, when used with LV; LN66 CFS enhanced the disrupting effect of LVX. They speculated that different effects of CFS and antibiotic combinations in biofilm eradication might be related to the changes in EPS (proteins and polysaccharides), concluding that the combination of CFS and CLR might promote the secretion of EPS, while the combination of CFS and LVX might have the opposite effect.

CONCLUSION

In the present study, different probiotic organisms were isolated from various sources. They were screened and characterized for their primary and secondary probiotic characteristics. They have given significant gut adherence properties and produced extracellular enzymes which helped with digestion. Besides this, they have shown good antibiotic resistance towards many antibiotics and antibacterial activity against four potent pathogens. Therefore, their cell-free supernatant was applied for eradication of biofilm of *P. aeruginosa* PA01, which has shown efficient removal of biofilm. Thus, the overall study indicates that both isolates are potential probiotics and can be further applied in the preparation of functional food or the preparation of animal feed, aquaculture, and pharmaceutical industries.

Funding information: The authors declare that no funds, grants, or other support were received during the preparation of this work and manuscript.
Conflict of Interest: The authors have no competing interests to declare that are relevant to the content of this article.
Data Availability Statement: All data generated or analysed during this study are included in this article and in the supplementary data.

REFERENCES

Ahmadifar, E., Sadegh, T. H., Dawood, M. A. O., Dadar, M., & Sheikhzadeh, N. (2020). The effects of dietary Pediococcus pentosaceus on growth performance, hemato-immunological parameters and digestive enzyme activities of common carp (Cyprinus carpio). *Aquaculture*, *516*. https://doi.org/10.1016/j.aquaculture.2019.734656

Aina, F., Amin, Z., Sabri, S., Ismail, M., Chan, K. W., Ismail, N., Esa, N. M., Azmi, M., & Lila, M. (2020). Probiotic Properties of Bacillus Strains Isolated from Stingless Bee (Heterotrigona itama) Honey Collected across Malaysia. *International Journal of Environmental Research and Public Health*, *17*(278), 1–15.

Bajaj, B. K., Claes, I. J. J., & Lebeer, S. (2015). Functional mechanisms of probiotics. *Journal of Microbiology, Biotechnology and Food Sciences*, *04*(04), 321–327. https://doi.org/10.15414/jmbfs.2015.4.4.321-327

Bazireh, H., Shariati, P., Azimzadeh Jamalkandi, S., Ahmadi, A., & Boroumand, M. A. (2020). Isolation of Novel Probiotic Lactobacillus and Enterococcus Strains From Human Salivary and Fecal Sources. *Frontiers in Microbiology*, *11*(December), 1–12. https://doi.org/10.3389/fmicb.2020.597946

Betancur, C., Martínez, Y., Tellez-isaias, G., Avellaneda, M. C., & Velázquez-martí, B. (2020). In vitro characterization of indigenous probiotic strains isolated from colombian creole pigs. *Animals*, *10*(7), 1–11. https://doi.org/10.3390/ani10071204

Bhagat, D., Raina, N., Kumar, A., Katoch, M., Khajuria, Y., Slathia, P. S., & Sharma, P. (2020). Probiotic properties of a phytase producing Pediococcus acidilactici strain SMVDUDB2 isolated from traditional fermented cheese product, Kalarei. *Scientific Reports*, *10*(1), 1–12. https://doi.org/10.1038/s41598-020-58676-2

Bhat, B., & Bajaj, B. K. (2020). Multifarious cholesterol lowering potential of lactic acid bacteria equipped with desired probiotic functional attributes. *3 Biotech*, *10*(5), 1–16. https://doi.org/10.1007/s13205-020-02183-8

Carvalho, F. M., Teixeira-Santos, R., Mergulhão, F. J. M., & Gomes, L. C. (2021). The use of probiotics to fight biofilms in medical devices: A systematic review and meta-analysis. *Microorganisms*, *9*(1), 1–26. https://doi.org/10.3390/microorganisms9010027

Chen, T., Wang, L., Li, Q., Long, Y., Lin, Y., Yin, J., Zeng, Y., Huang, L., Yao, T., Abbasi, M. N., Yang, H., Wang, Q., Tang, C., Khan, T. A., Liu, Q., Yin, J., Tu, Q., & Yin, Y. (2020). Functional probiotics of lactic acid bacteria from Hu sheep milk. *BMC Microbiology*, *20*(1), 1–12. https://doi.org/10.1186/s12866-020-01920-6

Choi, A.-R., Patra, J. K., Kim, W. J., & Kang, S.-S. (2018). Antagonistic activities and probiotic potential of lactic acid bacteria derived from a plant-based fermented food. *Frontiers in Microbiology*, *9*, 1963.

de Souza, E. L., de Albuquerque, T. M. R., dos Santos, A. S., Massa, N. M. L., & de Brito Alves, J. L. (2018). Potential interactions among phenolic compounds and probiotics for mutual boosting of their health-promoting properties and food functionalities – A review. *Critical Reviews in Food Science and Nutrition*, *8398*, 1–15. https://doi.org/10.1080/10408398.2018.1425285

Devi, T. S., Prabina, B. J., Gomathy, M., & Kumutha, K. (2020). Isolation and Characterization of Lactic acid bacteria from homemade fermented foods for probiotic applications. *International Journal of Current Microbiology and Applied Sciences*, *9*(2), 1355–1362. https://doi.org/10.20546/ijcmas.2020.902.158

Diana, C. R., Humberto, H. S., & Jorge, Y. F. (2015). Probiotic Properties of Leuconostoc mesenteroides Isolated from Aguamiel of Agave salmiana. *Probiotics and Antimicrobial Proteins*, *7*(2), 107–117. https://doi.org/10.1007/s12602-015-9187-5

dos Santos Leandro, E., Ginani, V. C., de Alencar, E. R., Pereira, O. G., Rose, E. C. P., do Vale, H. M. M., Pratesi, R., Hecht, M. M., Cavalcanti, M. H., & Tavares, C. S. O. (2021). Isolation, Identification, and Screening of Lactic Acid Bacteria with Probiotic Potential in Silage of Different Species of Forage Plants, Cocoa Beans, and Artisanal Salami. *Probiotics and Antimicrobial Proteins*, *13*(1), 173–186. https://doi.org/10.1007/s12602-020-09679-y

Dowarah, R., Verma, A. K., Agarwal, N., Singh, P., & Singh, B. R. (2018). Selection and characterization of probiotic lactic acid bacteria and its impact on growth, nutrient digestibility, health and antioxidant status in weaned piglets. *PloS One*, *13*(3), e0192978.

Drago, L. (2019). Probiotics and colon cancer. *Microorganisms*, *7*(3). https://doi.org/10.3390/microorganisms7030066

El-Deeb, W. M., Fayez, M., Elsohaby, I., Ghoneim, I., Al-Marri, T., Kandeel, M., & ElGioushy, M. (2020). Isolation and characterization of vaginal Lactobacillus spp. In dromedary camels (Camelus dromedarius). In vitro evaluation of probiotic potential of selected isolates. *PeerJ*, *2020*(2), 1–19. https://doi.org/10.7717/peerj.8500

Foulquié Moreno, M. R., Callewaert, R., Devreese, B., Van Beeumen, J., & De Vuyst, L. (2003). Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. *Journal of Applied Microbiology*, *94*(2), 214–229. https://doi.org/10.1046/j.1365-2672.2003.01823.x

Francois, Z., Pierre Marie, K., Cresci, A., & Anyangwe Florence, F. (2011). PROBIOTIC PROPERTIES OF LACTOBACILLI STRAINS ISOLATED FROM RAW COW MILK IN THE WESTERN HIGHLANDS OF CAMEROON Water quality and protection of resources View project Development of non-dairy Probiotic beverages View project. *Innovative Romanian Food Biotechnology*, *9*(August 2015), 12–28.

Fredua-Agyeman, M., Ofosu-Boateng, M., Offei, A. O., Mehta, M., Gaisford, S., & Limayem, A. (2020). Study on the functional properties of potential indigenous probiotics isolated from human samples in West Africa. *LWT- Food Science and Technology*, *133*, 1–7. https://doi.org/10.1016/j.lwt.2020.109895

Garbacz, K. (2022). Anticancer activity of lactic acid bacteria. *Seminars in Cancer Biology*, *86*, 356–366.

Gebru, Y. A., & Sbhatu, D. B. (2020). Isolation and Characterization of Probiotic LAB from Kimchi and Spontaneously Fermented Teff (Eragrostis tef (Zucc.) Trotter) Batter: Their Effects on Phenolic Content of Teff during Fermentation. *BioMed Research International*, *2020*, 1–9. https://doi.org/10.1155/2020/4014969

Ghanei-Motlagh, R., Mohammadian, T., Gharibi, D., Khosravi, M., Mahmoudi, E., Zarea, M., El-Matbouli, M., & Menanteau-Ledouble, S. (2021). Quorum quenching probiotics modulated digestive enzymes activity, growth performance, gut microflora, haemato-biochemical parameters and resistance against Vibrio harveyi in Asian seabass (Lates calcarifer). *Aquaculture*, *531*, 1–13. https://doi.org/10.1016/j.aquaculture.2020.735874

Hashem, Z. S., & Hashem, A. S. (2021). An in vitro study on the antifungal and antibiofilm activities of probiotic bacteria against Candida species isolated from orthodontic appliances and dental caries. *Novel Research in Microbiology Journal*, *5*(2), 1176–1193.

Hossain, M. I., Mizan, M. F. R., Ashrafudoulla, M., Nahar, S., Joo, H. J., Jahid, I. K., Park, S. H., Kim, K. S., & Ha, S. Do. (2020). Inhibitory effects of probiotic potential lactic acid bacteria isolated from kimchi against Listeria monocytogenes biofilm on lettuce, stainless-steel surfaces, and MBEC™ biofilm device. *LWT-Food Science and Technology*, *118*, 1–9. https://doi.org/10.1016/j.lwt.2019.108864

Huang, J., Zhang, W., Hu, Z., Liu, Z., Du, T., Dai, Y., & Xiong, T. (2020). Isolation, characterization and selection of potential probiotic lactic acid bacteria from feces of wild boar, native pig and commercial pig. *Livestock Science*, *237*(235), 1–10. https://doi.org/10.1016/j.livsci.2020.104036

Hwanhlem, N., Chobert, J. M., & H-Kittikun, A. (2014). Bacteriocin-producing lactic acid bacteria isolated from mangrove forests in southern Thailand as potential bio-control agents in food: Isolation, screening and optimization. *Food Control*, *41*(1), 202–211. https://doi.org/10.1016/j.foodcont.2014.01.021

Ji, J., & Yang, H. (2021). In vitro effects of lactobacillus plantarum ln66 and antibiotics used alone or in combination on helicobacter pylori mature biofilm. *Microorganisms*, *9*(2), 1–19. https://doi.org/10.3390/microorganisms9020424

Jinendiran, S., Boopathi, S., Sivakumar, N., & Selvakumar, G. (2017). Functional Characterization of Probiotic Potential of Novel Pigmented Bacterial Strains for Aquaculture Applications. *Probiotics and Antimicrobial Proteins*, 1–12. https://doi.org/10.1007/s12602-017-9353-z

Kang, W., Pan, L., Peng, C., Dong, L., Cao, S., Cheng, H., Wang, Y., Zhang, C., Gu, R., Wang, J., & Zhou, H. (2020). Isolation and characterization of lactic acid bacteria from human milk. *Journal of Dairy Science*, *103*(11), 9980–9991. https://doi.org/10.3168/jds.2020-18704

Karimi, N., Alizadeh, A., Almasi, H., & Hanifian, S. (2020). Preparation and characterization of whey protein isolate/polydextrose-based nanocomposite film incorporated with cellulose nanofiber and L. plantarum: A new probiotic active packaging system. *LWT- Food Science and Technology*, *121*. https://doi.org/10.1016/j.lwt.2019.108978

Khan, M. I. R., Choudhury, T. G., Kamilya, D., Monsang, S. J., & Parhi, J. (2021). Characterization of Bacillus spp. isolated from intestine of Labeo rohita—Towards identifying novel probiotics for aquaculture. *Aquaculture Research*, *52*(2), 822–830. https://doi.org/10.1111/are.14937

Kondrotiene, K., Lauciene, L., Andruleviciute, V., Kasetiene, N., Serniene, L., Sekmokiene, D., & Malakauskas, M. (2020). Safety Assessment and Preliminary In Vitro Evaluation of Probiotic Potential of Lactococcus lactis Strains Naturally Present in Raw and Fermented Milk. *Current Microbiology*, *77*(10), 3013–3023. https://doi.org/10.1007/s00284-020-02119-8

Kostelac, D., Gerić, M., Gajski, G., Markov, K., Domijan, A. M., Čanak, I., Jakopović, Ž., Svetec, I. K., Žunar, B., & Frece, J. (2021). Lactic acid bacteria isolated from equid milk and their extracellular metabolites show great probiotic properties and anti-inflammatory potential. *International Dairy Journal*, *112*, 1–8. https://doi.org/10.1016/j.idairyj.2020.104828

Lee, H., Gilliland, S., & Carter, S. (2001). Amylolytic Cultures of Lactobacillus acidophilus : Potential Probiotics to Improve Dietary Starch Utilization. *Food Microbiology and Safety*, *66*(2), 338–344.

Lee, J. E., Lee, N. K., & Paik, H. D. (2021). Antimicrobial and anti-biofilm effects of probiotic Lactobacillus plantarum KU200656 isolated from kimchi. *Food Science and Biotechnology*, *30*(1), 97–106. https://doi.org/10.1007/s10068-020-00837-0

Mishra, A. K., & Ghosh, A. R. (2018). Characterization of Functional, Safety, and Probiotic Properties of Enterococcus faecalis AG5 Isolated From Wistar Rat, Demonstrating Adherence to HCT 116 Cells and Gastrointestinal Survivability. *Probiotics and Antimicrobial Proteins*, *10*(3), 435–445. https://doi.org/10.1007/s12602-018-9387-x

Mohammad, S. M., Mahmud-Ab-Rashid, N. K., & Zawawi, N. (2020). Probiotic properties of bacteria isolated from bee bread of stingless bee Heterotrigona itama. *Journal of Apicultural Research*, *0*(0), 1–16. https://doi.org/10.1080/00218839.2020.1801152

Monteagudo-Mera, A., Caro, I., Rodriguez-Aparicio, L. B., Rua, J., Ferrero, M. a., & Garcia-Armesto, M. R. (2011). Characterization of certain bacterial strains for potential use as starter or probiotic cultures in dairy products. *Journal of Food Protection*, *74*(8), 1379–1386. https://doi.org/10.4315/0362-028X.JFP-10-392

Mora-Villalobos, J. A., Montero-Zamora, J., Barboza, N., Rojas-Garbanzo, C., Usaga, J., Redondo-Solano, M., Schroedter, L., Olszewska-Widdrat, A., & López-Gómez, J. P. (2020). Multi-product lactic acid bacteria fermentations: A review. In *Fermentation* (Vol. 6, Issue 1). MDPI AG. https://doi.org/10.3390/fermentation6010023

Mostefa, N., Amar, A., & Boumédiène, K. M. (2018). PRELIMINARY PROBIOTIC POTENTIAL OF SELECTED AEROCOCCUS SPP., ENTEROCOCCUS SPP., AND WEISELLA SP. FROM ALGERIAN GUEDID. *Journal of Microbiology, Biotechnology and Food Sciences*, 1–8.

Oliveira, L. C., Saraiva, T. D. L., Silva, W. M., Pereira, U. P., Campos, B. C., Benevides, L. J., Rocha, F. S., Figueiredo, H. C. P., Azevedo, V., & Soares, S. C. (2017). Analyses of the probiotic property and stress resistance-related genes of Lactococcus lactis subsp. Lactis NCDO 2118 through comparative genomics and in vitro assays. *PLoS ONE*, *12*(4), 1–28. https://doi.org/10.1371/journal.pone.0175116

Pais, P., Almeida, V., Yılmaz, M., & Teixeira, M. C. (2020). Saccharomyces boulardii: What Makes It Tick as Successful Probiotic? *Journal of Fungi*, *6*(2). https://doi.org/10.3390/jof6020078

Pakbin, B., Allahyari, S., Dibazar, S. P., Zolghadr, L., Chermahini, N. K., Brück, W. M., Brück, T. B., & Mahmoudi, R. (2023). Effects of Probiotic Saccharomyces boulardii Supernatant on Viability, Nano-Mechanical Properties of Cytoplasmic Membrane and Pro-Inflammatory Gene Expression in Human Gastric Cancer AGS Cells. *International Journal of Molecular Sciences*, *24*(9). https://doi.org/10.3390/ijms24097945

Papamanoli, E., Tzanetakis, N., Litopoulou-Tzanetaki, E., & Kotzekidou, P. (2003). Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Science*, *65*(2), 859–867. https://doi.org/10.1016/S0309-1740(02)00292-9

Penaloza-Vazquez, A., Ma, L. M., & Rayas-Duarte, P. (2019). Isolation and characterization of Bacillus spp. strains as potential probiotics for poultry . *Canadian Journal of Microbiology*, *65*(10), 762–774. https://doi.org/10.1139/cjm-2019-0019

Pimentel, T. C., Madrona, G. S., Garcia, S., & Prudencio, S. H. (2015). Probiotic viability, physicochemical characteristics and acceptability during refrigerated storage of clarified apple juice supplemented with Lactobacillus paracasei ssp. paracasei and oligofructose in different package type. *LWT-Food Science and Technology*, *63*(1), 415–422.

Pinto, A., Barbosa, J., Albano, H., Isidro, J., & Teixeira, P. (2020). Screening of bacteriocinogenic lactic acid bacteria and their characterization as potential probiotics. *Microorganisms*, *8*(3), 1–12. https://doi.org/10.3390/microorganisms8030393

Premasiri, D. L. D. A. S., Rajawardana, D. U., Mudannayake, D. C., & Hewajulige, I. G. N. (2021). Isolation , Characterization and Identification of Industrially Beneficial Probiotic Lactic Acid Bacteria from Goat Milk. *The Journal of Agricultural Scinces- Sri Lanka*, *16*(2), 369–382.

Rameshgar, M., Marzdashti, M. R. G., Karoui, S. M. H., Fard, S. M. H., & Pour, S. R. T. (2020). Isolation, characterization and molecular identification of potential probiotic lactobacilli from gastrointestinal tracts of Capoeta razii and Aristichthys nobilis. *International Journal of Aquatic Biology*, *8*(6), 396–403. https://doi.org/10.22034/ijab.v8i6.1003

Reuben, R. C., Roy, P. C., Sarkar, S. L., Alam, A. S. M. R. U., & Jahid, I. K. (2020). Characterization and evaluation of lactic acid bacteria from indigenous raw milk for potential probiotic properties. *Journal of Dairy Science*, *103*(2), 1223–1237.

Riyaz Ali, L., Natarajan, B., Monisha, M., & Paari, K. A. (2021). Probiotic properties of enterococcus faecium isolated from gallus gallus domesticus and its anti–microbial, anti–biofilm and growth enhancing potential in danio rerio. *Journal of Fisheries and Environment*, *45*(1), 23–34.

Romero-Luna, H. E., Peredo-Lovillo, A., Hernández-Mendoza, A., Hernández-Sánchez, H., Cauich-Sánchez, P. I., Ribas-Aparicio, R. M., & Dávila-Ortiz, G. (2020). Probiotic Potential of Lactobacillus paracasei CT12 Isolated from Water Kefir Grains (Tibicos). *Current Microbiology*, *77*(10), 2584–2592. https://doi.org/10.1007/s00284-020-02016-0

Sabo, S. D. S., Mendes, M. A., Araújo, E. D. S., Muradian, L. B. D. A., Makiyama, E. N., LeBlanc, J. G., Borelli, P., Fock, R. A., Knöbl, T., & Oliveira, R. P. D. S. (2020). Bioprospecting of probiotics with antimicrobial activities against Salmonella Heidelberg and that produce B-complex vitamins as potential supplements in poultry nutrition. *Scientific Reports*, *10*(1), 1–14. https://doi.org/10.1038/s41598-020-64038-9

Salvetti, E., & O'Toole, P. W. (2017). The Genomic Basis of Lactobacilli as Health-Promoting Organisms. *Microbiology Spectrum*, *5*(3), 1–17. https://doi.org/10.1128/microbiolspec.bad-0011-2016

Sánchez, B., Delgado, S., Blanco-Míguez, A., Lourenço, A., Gueimonde, M., & Margolles, A. (2017). Probiotics, gut microbiota, and their influence on host health and disease. *Molecular Nutrition & Food Research*, *61*(1), 1600240.

Satish Kumar, R., Kanmani, P., Yuvaraj, N., Paari, K. A., Pattukumar, V., & Arul, V. (2013). Traditional Indian fermented foods: A rich source of lactic acid bacteria. In *International Journal of Food Sciences and Nutrition* (Vol. 64, Issue 4, pp. 415–428). https://doi.org/10.3109/09637486.2012.746288

Sharma, A., Lavania, M., Singh, R., & Lal, B. (2021). Identification and probiotic potential of lactic acid bacteria from camel milk. *Saudi Journal of Biological Sciences*, *28*(3), 1622–1632. https://doi.org/10.1016/j.sjbs.2020.11.062

Singh, V., Ganger, S., & Patil, S. (2020). Characterization of Lactobacillus brevis with Potential Probiotic Properties and Biofilm Inhibition against Pseudomonas aeruginosa. *Proceedings*, *66*(14), 1–7. https://doi.org/10.3390/proceedings2020066014

Sui, Y., Liu, J., Liu, Y., Wang, Y., Xiao, Y., Gao, B., & Zhu, D. (2021). In vitro probiotic characterization of Lactobacillus strains from fermented tangerine vinegar and their cholesterol degradation activity. *Food Bioscience*, *39*, 100843. https://doi.org/10.1016/j.fbio.2020.100843

Topçu, K. C., Kaya, M., & Kaban, G. (2020). Probiotic properties of lactic acid bacteria strains isolated from pastırma. *LWT- Food Science and Technology*, *134*, 1–9. https://doi.org/10.1016/j.lwt.2020.110216

Vasiee, A., Falah, F., Behbahani, B. A., & Tabatabaee-yazdi, F. (2020). Probiotic characterization of Pediococcus strains isolated from Iranian cereal-dairy fermented product: Interaction with pathogenic bacteria and the enteric cell line Caco-2. *Journal of Bioscience and Bioengineering*, *130*(5), 471–479. https://doi.org/10.1016/j.jbiosc.2020.07.002

Vidhate, P., Wakchoure, P., Borole, S., & Khan, A. A. (2024). Lactobacillus as probiotics: opportunities and challenges for potential benefits in female reproductive health. *American Journal of Translational Research*, *16*(3), 720.

Wang, X., Wang, W., Lv, H., Zhang, H., Liu, Y., Zhang, M., Wang, Y., & Tan, Z. (2021). Probiotic potential and wide-spectrum antimicrobial activity of lactic acid bacteria isolated from infant feces. *Probiotics and Antimicrobial Proteins*, *13*, 90–101.

Yousefi, B., Eslami, M., Ghasemian, A., Kokhaei, P., Salek Farrokhi, A., & Darabi, N. (2019). Probiotics importance and their immunomodulatory properties. *Journal of Cellular Physiology*, *234*(6), 8008–8018.

Zaghari, M., Sarani, P., & Hajati, H. (2020). Comparison of two probiotic preparations on growth performance, intestinal microbiota, nutrient digestibility and cytokine gene expression in broiler chickens. *Journal of Applied Animal Research*, *48*(1), 166–175. https://doi.org/10.1080/09712119.2020.1754218