Bioprospecting Medicinal Plants of Gujarat for Novel, Safe, and Functional Probiotic Lactic Acid Bacteria

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Abstract:

Plant-derived probiotics constitute a largely unutilized resource of micro-organisms with great functional and biotechnological diversity. The objective of this study was to obtain, screen and characterise lactic acid bacteria (LAB) from four species of medicinal plants (Carica papaya, Ficus benghalensis, Woodfordia fruticosa, and Phyllanthus emblica) from diverse agro-climatic zones in Gujarat, India. Thirty-two bacterial isolates were recovered by culturing on MRS medium and then subject to primary screening (gelatinase negativity and catalase negativity) leading to 10 isolates passing the initial screening. Biochemical tests confirmed that the isolates had characteristics consistent with probiotics (indole negativity, positive mixed-acid fermentation, urease negativity). Enzyme assays indicated that all shortlisted isolates produced universal proteases, while PL2 was found to produce lipase. Safety evaluations indicated that all isolates were γ-hemolytic and exhibited susceptibility to a wide range of clinically important antibiotics. Probiotic functional assays showed that isolates had differential tolerance to pH (2–4), NaCl (2–6%) and bile salts (0.5–1.5%). The range of antioxidant activity (DPPH) for all isolates was between 8% and 57%, with BF1 and BF5 exhibiting the most activity. Identification of the most frequently isolated species using MALDI-TOF mass spectrometry was conducted and included Enterococcus faecalis, Enterococcus faecium, Enterococcus casseliflavus, Enterococcus mundtii, and Lactococcus lactis. Therefore, plant-associated LAB are potentially safe probiotic candidates and also possess antioxidant activities and digestive enzymes, making them worthy of further research, including in vivo studies, and potential commercialisation.

1. Introduction:

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) define probiotics as 'live microorganisms (that), when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO 2002). Probiotic organisms and products have become increasingly more commercialized and researched since the last few decades (FAO/WHO, 2002). The gastrointestinal tract is inhabited by a diverse community of microbial organisms, which significantly influence immune responses, nutrient metabolism and the prevention of disease by protecting against enteric pathogens (Thursby & Juge, 2017). The disruption of the microbial community has been shown to be linked to metabolic disorders, inflammation, and infectious diseases; therefore, identifying effective probiotic strains is a significant area of study (Sánchez et al., 2017).

Research has primarily focused on fermented foods (especially, dairy) as the primary sources of probiotics and Lactobacillus and Bifidobacterial are two of the well-studied genera

from fermented dairy products. However, the plant kingdom, particularly the ethnobotanically important plant species, represents a largely unexamined ecological area from which to identify potential probiotic organisms (Sharma et al. 2020). Microorganisms that inhabit the surface of the human gastrointestinal tract may also originate from other sources. Many plants have co-evolved with the microorganisms that inhabit them which then may have developed functional characteristics to help them survive in the gastrointestinal environment of the human host, such as stress resistance, antimicrobial production, and enzyme production (Coman et al. 2022). India's rich Phyto diversity, particularly in the state of Gujarat, encompasses several medicinally significant plant species used in traditional Ayurvedic medicine. Among these, Carica papaya (papaya) is renowned for its digestive enzymes and antimicrobial phytochemicals; Ficus benghalensis (banyan tree) is valued for its antidiabetic and antioxidant properties; Woodfordia fruticosa (Dhawani) is prized for its antimicrobial and anti-inflammatory activities; and Phyllanthus emblica (Indian gooseberry/Amla) is celebrated globally for its exceptionally high ascorbic acid content and free radical scavenging capacity (Kumar et al., 2017; Srivastava et al., 2021). The microbial communities associated with these plants may therefore possess unique metabolic capabilities shaped by their bioactive phytochemical environment.

Probiotics must undergo rigorous safety evaluations before qualifying as a probiotic strain. As indicated by regulatory and scientific guidelines, probiotic candidate strains must meet the following criteria: be non-hemolytic, be susceptible to clinically-relevant antibiotics, and not have virulence factor-associated enzyme production (gelatinase) (European Food Safety Authority; EFSA, 2018). Additionally, a number of functional characteristics (i.e., acid and bile tolerance; antimicrobial; antioxidant; digestive enzyme) also indicate the commercial and therapeutic viability of the strains (Hill et al., 2014).

Accurate species identification of probiotic isolates is of equal importance. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry is the accepted standard for quick, high-throughput identification of microorganisms, and has allowed researchers to confidently differentiate microbial species and, in some cases, the same species at the strain level, using the ribosomal protein fingerprints (Seng et al., 2009; Smith et al., 2010).

The current study was conducted to meet the following objectives: (i) isolate LAB from four ethnobotanically important plants from Gujarat; (ii) present stepwise primary and secondary screening for safety, and functional assays; and (iii) identify the best candidates for probiotic application using MALDI-TOF mass spectrometry. The findings of this study will

provide information supporting the use of LAB from plants, as a novel, safe and functional source of probiotics.

2. Materials and Methods

2.1 Sample Collection

The purpose of this study was to systematically collect fresh plant materials (i.e., whole leaves, foliage, stems, and fruits) from four different ethnobotanical species and to gather examples of them in relation to the various geographic areas and agro-climatic zones in Gujarat, India. Carica papaya and Ficus benghalensis were collected during this research from an agro-ecosystem located in Ghani, District Tapi, Gujarat (20.932807° N,73.3066° E). In addition, samples of Woodfordia fruticosa were sourced from the mountainous forest region around Parnera, District Valsad (20.5543° N,72.9489° E), and Phyllanthus emblica was harvested along the areas prone to heavy rainfall in the forests of Vansda, District Navsari (20.7573° N,73.3552° E). The plant materials were collected under strict aseptic conditions to avoid cross-contamination and to keep the native epiphytic microbial populations intact using sterile surgical instrumentation. Each sample was transferred to and placed in airtight plastic containers, pre-sterilized, and stored in cooled (4 ℃) insulated transport containers before being transported to the laboratory for microbiological analysis and probiotic isolation.

2.2 Isolation of Microorganisms

The collected plant samples underwent surface sterilization according to the procedure outlined in Ghanbari et al. (2009). The surface sterilization procedure involves washing plant samples with 70% ethanol for 30 seconds and rinsing the samples twice with deionized water, eliminating epiphytic contaminants. After surface sterilization, plant samples were manually chopped into small fragments using a sterile scalpel in an aseptic environment and placed into sterile Petri dishes.

The chopped plant materials were added to MRS broth (HiMedia, India) and incubated at 37°C for 24-48 hours. After incubation, the samples were serially diluted (10-3 to 10-5, 1 mL of each dilution) and spread on MRS agar. After incubation at 37°C for 24-48 hours, distinct colonies were selected and continually sub-cultured on MRS agar until pure cultures were obtained. The purified cultures were characterized using Gram staining (Holt et al., 1994).

2.3 Primary Screening

2.3.1 Gelatinase Hydrolysis Test

The activity of gelatinase in all isolates was investigated in a Gelatin Nutrient Broth containing gelatin as the only source of carbon. Isolates were inoculated into a liquid medium (gelatin nutrient broth) and incubated at 37°C. After incubation, each tube was transferred to

an ice bath, and the medium in each tube was checked for solidification. If the medium did not solidify, it was assessed as gelatinase-positive (Mami et al., 2014). Isolates that had retained solidification of medium (gelatinase-negative) were selected for additional screening, due to the fact that gelatinase activity is recognized as a factor of virulence in potential probiotic strains.

2.3.2 Catalase Activity Test

Catalase activity was evaluated following the method described by Angmo et al. (2016). Briefly, two drops of a 3% (v/v) hydrogen peroxide solution were placed onto a clean glass slide. A single, isolated bacterial colony was subsequently transferred into the solution using a sterile nichrome wire loop. The immediate evolution of gas bubbles (effervescence), signifying oxygen production, was recorded as a positive result. Conversely, the absence of bubble formation indicated catalase-negativity. Because a lack of catalase activity is a biochemical hallmark of true lactic acid bacteria (LAB), only catalase-negative isolates were selected for subsequent phenotypic and genotypic characterization.

2.4 Biochemical Characterization of Bacterial Isolates

The shortlisted isolates were assessed for their biochemical characteristics using standard tests previously described (Angmo et al., 2016; Kavitha et al., 2013). Indole production was evaluated with Kovac's reagent and a positive result was indicated by the formation of a red ring at the liquid-air interface. The mixed acid fermentation (Methyl-Red) and acetoin (Voges-Proskauer) tests were performed to determine the fermentation characteristics of the isolates. The ability of the isolates to utilize citrate was determined on Simmons Citrate Agar; a positive result was indicated by colour change to blue after 24-48 hours. Urease activity was assessed using urea broth; a positive result was indicated by a colour change to pink. Triple Sugar Iron (TSI) Agar was used to assess the fermentation of carbohydrates and gas production. All sugar fermentation tests were performed using phenol-red sugar broths and Durham tubes; a positive result for sugar fermentation was indicated by a colour change to pink/yellow and the presence of gas in the Durham tube.

2.5 Analysis of Isolates for Enzyme Production

Potential production of enzymes was assessed in all isolates for amylase, protease, and lipase using established agar-diffusion methods (Thakkar et al., 2015; Dutta et al., 2015). Amylase activity was demonstrated on Starch Agar plates that had been flooded with Gram's iodine solution; a clear halo zone around colonies indicated the presence of starch hydrolysis. Protease activity was determined on Skim Milk Agar; a clear and transparent zone of hydrolysis around colonies was indicative of proteolytic activity. Lipase activity was observed on

Tributyrin Agar; clear haloes resulting from hydrolysis of triglycerides indicated the presence of lipolytic activity. All plates were incubated at 37°C for 24–48 hours.

2.6 Safety Assessment

2.6.1 Hemolytic Activity

Isolation of blood agar base supplemented with 5% (w/v) defibrinated sheep blood was performed to assess the hemolytic activity of isolates and was incubated at 37 °C for 24-48 hours. Hemolysis was classified as the following: α-hemolysis (partial hemolytic, greenish discoloration); β-hemolysis (complete hemolytic, clear zone) and γ-hemolysis (no hemolytic). The only strains that were considered safe were the γ-hemolytic strains (Asadi et al., 2022; Wei et al., 2022).

2.6.2 Antibiotic Susceptibility Test

Antibiotic susceptibility was assessed by disc diffusion method on MRS agar plates using the Kirby-Bauer method (Hudzicki, 2009). Isolates were plated uniformly on MRS agar plates and antibiotic discs (Penicillin-G (10 units), Streptomycin (10 mcg), Tetracycline (30 mcg), Chloramphenicol (30 mcg), Gentamicin (10 mcg), and Kanamycin (30 mcg)) were applied. After incubation at 37 °C for 24-48 hours, zones of inhibition were measured in millimetres.

2.7 Probiotic Potency Tests

2.7.1 Acid Tolerance Test

MRS Continuance was used to assess the tolerance of bacteria to acid. As follows: First, MRS Broth was modified at pH 1, 2, 3 and 4 with the addition of 1N HCl and 1N NaOH. Then, a culture of the relevant bacteria was inoculated into the pH modified MRS Broth and incubated for 48 hours at 37°C. After 24 hours of incubation, turbidity (OD 620nm) was determined for all treatments. Uninoculated MRS Broth was used as a negative control (Chetan et al., 2022)

2.7.2 NaCl Tolerance Test

MRS Broth was also modified by adding NaCl to assess the tolerance of the bacteria to NaCl at concentrations of 2%, 4%, 6%, and 8%. A culture of the relevant bacteria was prepared and inoculated into the MRS Broth and incubated as previously described at 37°C for 24 hours and turbidity (OD 620nm) was determined for all treatments and compared to the controls (Wang et al., 2024).

2.7.3 Bile Salt Tolerance Test

To determine bile salt tolerance of the lactic acid bacteria, the modified method of Ng et al. (2015) was employed. MRS Broth was supplemented with 0.5%, 1.0%, 1.5%, and 2.0% (w/v) oxgall bile salt. The lactic acid bacteria cultures were grown at 37°C for 24 hours and turbidity (OD 600nm) was determined for all cultures.

2.7.4 Antimicrobial Activity

To test the antimicrobial ability of the cell-free (CFS) supernatants of the lactic acid bacteria cell, an agar well diffusion test was done against four pathogenic indicator strains (Bacillus cereus, Staphylococcus aureus, Salmonella Typhimurium, and Escherichia coli) using Mueller Hinton Agar (MHA) and the pH of all CFS was adjusted to 6.5 with NaOH to eliminate any inhibitory effect from the lactic acid (Botthoulath et al., 2018). Following the agar plates being incubated for 24 hours at 37°C, the inhibition zone surrounding each well was measured in mm.

2.7.5 Antioxidant Activity (DPPH Radical Scavenging)

The antioxidant activity of the samples under examination was quantified by using the method of scavenging the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. A working solution of DPPH was formed by dissolving (0.003 g) DPPH in 100 mL methanol. Equal parts (200 µL) of the supernatant were mixed with 800 µL of DPPH, and incubated for 30 min at 37°C. The absorbance of the incubated sample, prepared by mixing supernatant with DPPH, was measured at 517 nm, using a UV-Visible spectrophotometer. The antioxidant activity in each sample was calculated using ascorbic acid as the standard. The formula for percent inhibition of DPPH was provided by Farzana et al. (2011) and Khan et al. (2021).

Inhibition (%) = [(Absorbance of control − Absorbance of test) / Absorbance of control] × 100

2.8 Identification by MALDI-TOF Mass Spectrometry

MALDI-TOF Mass Spectrometry was employed to identify potential probiotic candidates (Smith et al. 2010). Bacterial colonies grown on solid media were spotted directly onto a MALDI-TOF target plate, covered with a matrix solution (α-cyano-4-hydroxycinnamic acid), and then analysed by using a calibrated MALDI-TOF instrument. The mass spectra obtained in this manner were compared to reference databases for identification and species differentiation based on score values of 1.70 or greater for good genus identification; and greater than 2.00 for good species identification.

3. Results

3.1 Sample Collection and Isolation of Microorganisms

Our ethnomedicinally-important plants (Carica papaya, Ficus benghalensis, Woodfordia fruticosa, and Phyllanthus emblica) were collected from different regions within the state of Gujarat (Ghani, Parnera, and Vansda). The 32 bacterial isolates recovered from the MRS Agar plates were from mixed cultures. The morphology of bacterial colonies differed in colony size,

shape, texture, elevation, and pigmentation, reflecting the diversity of microorganisms present in and on the surface of the different plants studied.

3.2 Primary Screening: Gelatinase Hydrolysis and Catalase Activity

Ten isolates were chosen out of 32 due to their lack of gelatinase and catalase producing abilities, which would result in a lower level of virulence to the human host. The absence of enzymes that degrade gelatine would also diminish the chance of survival in an oxidative environment (the gastrointestinal tract) because true lactic acid bacteria cannot neutralize hydrogen peroxide through the mechanism of catalase.

Table 1. Results of Gelatinase Hydrolysis and Catalase Activity Tests

Isolate	Gelatinase Test	Catalase Test
PL1	Negative (−)	Negative (−)
PL2	Negative (−)	Negative (−)
PR7	Negative (−)	Negative (−)
PR11	Negative (−)	Negative (−)
BF1	Negative (−)	Negative (−)
BF2	Negative (−)	Negative (−)
BF4	Negative (−)	Negative (−)
BF5	Negative (−)	Negative (−)
FL1	Negative (−)	Negative (−)
FL2	Negative (−)	Negative (−)

3.3 Biochemical Characterization

All ten of these selection isolates (PL1, PL2, PR7, PR11, BF1, BF2, BF4, BF5, FL1, FL2) showed negative results for indole production, demonstrating that tryptophanase is not produced by these strains—this related to a safer organism. All ten isolates met the positive standard for MR, proving that all 10 are capable of mixed-acid fermentation of glucose, a characteristic of lactic acid bacteria. All ten strains were also negative in the VP test, thus demonstrating that no acetoin is produced by glucose fermentation. All isolates were negative in Simmons' Citrate test, as no citrated were used as a carbon source—this characteristic is typically found in LAB. All ten isolates were positive for glucose fermentation on TSI agar, demonstrating that they produced acid and gas when fermenting carbohydrates. All strains gave a positive reaction to fermenting maltose, mannitol, and xylose in sugar fermentation profiles. All isolates were negative for urease activity—indicating that the urease enzyme was produced, a critical safety concern. Table 2 provides a summary of the above information.

Table 2. Biochemical Characterization of Selected Isolates

Isolate	Indole	MR	VP	Citrate	TSI	Maltose	Mannitol	Xylose	Urease
PL1	–	+	–	–	+	+	+	+	–
PL2	–	+	–	–	+	+	+	+	–
PR7	–	+	–	–	+	+	+	+	–
PR11	–	+	–	–	+	+	+	+	–
BF1	–	+	–	–	+	+	+	+	–
BF2	–	+	–	–	+	+	+	+	–
BF4	–	+	–	–	+	+	+	+	–
BF5	–	+	–	–	+	+	+	+	–
FL1	–	+	–	–	+	+	+	+	–
FL2	–	+	–	–	+	+	+	+	–

3.4 Enzyme Production Profile

The proteolytic ability of all 10 isolates was confirmed by observing clear halos on Skim Milk Agar plates. The production of protease by probiotic organisms has been found to assist in protein digestion and to provide immunomodulatory properties. There were no isolates that produced any amylases on Starch Agar plates. Lipase production was limited to isolate PL2 based upon the observation of a clear halo on Tributyrin Agar plates. These findings are shown in Table 3.

Table 3. Enzyme Production Profile of Selected Isolates

Isolate	Amylase	Protease	Lipase
PL1	–	+	–
PL2	–	+	+
PR7	–	+	–
PR11	–	+	–
BF1	–	+	–
BF2	–	+	–
BF4	–	+	–
BF5	–	+	–
FL1	–	+	–
FL2	–	+	–

3.5 Safety Assessment

3.5.1 Hemolytic Activity

The determination of hemolytic reaction was conducted using blood agar plates. All ten isolates exhibited a γ-hemolytic reaction, which is characterized by the absence of hemolysis and a lytic zone surrounding an isolate. This is a critical safety factor, as isolates that do not lyse red blood cells may potentially be evaluated as viable probiotics, in accordance with international regulations.

3.5.2 Antibiotic Susceptibility

Antibiotic susceptibility assays demonstrated that all isolates were susceptible to Penicillin-G, tetracycline, Chloramphenicol and Gentamicin. All five isolates that exhibited resistance to Streptomycin included isolates PL1, PL2, BF2, BF4 and BF5. Isolates PL2, BF1, BF2, BF4 and BF5 were also resistant to Kanamycin. Results of the inhibition zones are presented in Table 4.

Table 4. Antibiotic Susceptibility Test – Zone of Inhibition (mm)

Antibiotic	PL1	PL2	PR7	PR11	BF1	BF2	BF4	BF5	FL1	FL2
Penicillin-G	S	S	S	S	S	S	S	S	S	S
Streptomycin	R	R	S	S	S	R	R	R	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	S	S	S	S	S	S	S	S	S
Kanamycin	S	R	S	S	R	R	R	R	S	S

Note: R = Resistant; values in mm represent zones of inhibition.

3.6 Probiotic Potency Tests

3.6.1 Acid Tolerance

Acid tolerance testing revealed that isolates PL1, BF1, and BF4 showed no growth at pH 1, indicating their inability to survive extreme acidity. However, all 10 isolates demonstrated progressive improvement in growth at pH 2, 3, and 4. The ability to survive at pH 2–3 is physiologically significant, as the human stomach typically maintains a pH of 1.5–3.5 in the fasted state. Isolates demonstrating growth at pH 2 and above are likely to survive gastric transit in sufficient numbers to exert probiotic effects.

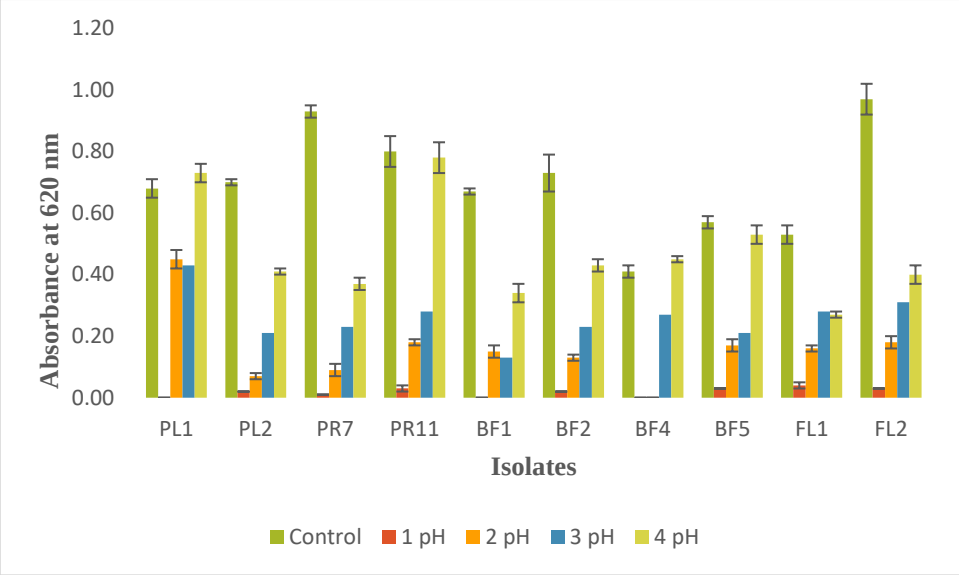

Figure 1: Acid tolerance profile of isolated lactic acid bacteria (LAB) strains.

3.6.2 NaCl Tolerance

Most isolates displayed optimal growth at 2% NaCl concentration. At 4% NaCl, growth responses were heterogeneous, with some isolates showing increased growth relative to 2% NaCl and others showing slight reductions. A general decline in growth was observed at 6% NaCl, and minimal to no growth was recorded at 8% NaCl across most isolates. This differential NaCl tolerance profile reflects the variability in osmoregulatory capacity among the isolates and has implications for their use as functional additives in fermented food products.

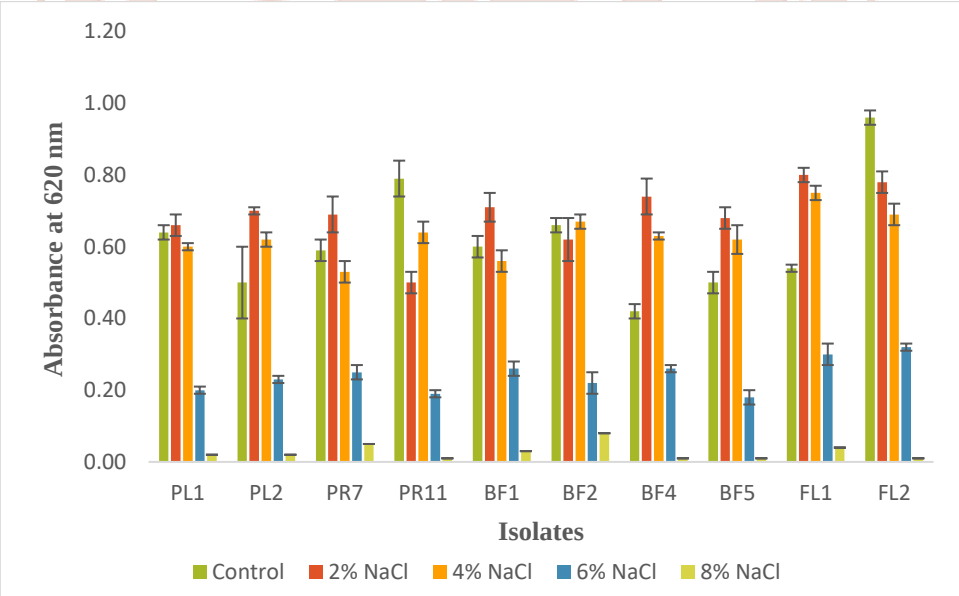

Figure 2: NaCl tolerance profiles of isolated lactic acid bacteria (LAB) strains.

3.6.3 Bile Salt Tolerance

Bile salt tolerance is a critical determinant of probiotic survival in the small intestine, where bile salt concentrations typically range from 0.3% to 2.0%. Most isolates-maintained growth at 0.5% bile salt concentration. Growth rates were variable at 1.0% bile salt, with significant inhibition observed at 1.5% for the majority of isolates. Minimal to no growth was observed at 2.0% bile salt across all isolates, suggesting that the strains have moderate bile tolerance sufficient for gastrointestinal transit at physiological bile concentrations.

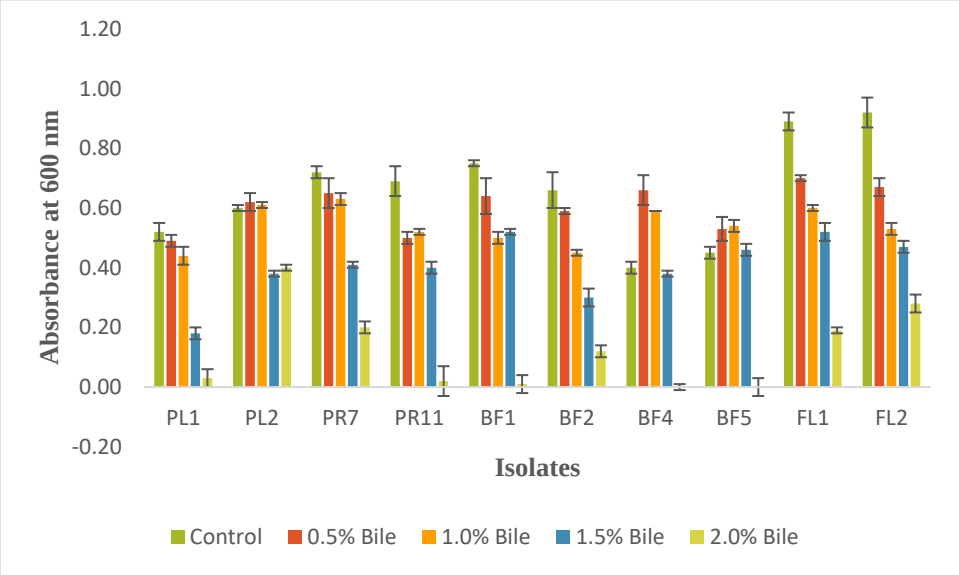

Figure 3: Evaluation of in vitro bile salt tolerance among potential probiotic LAB isolates.

3.6.4 Antimicrobial Activity

Cell-free supernatants of all 10 isolates did not produce detectable zones of inhibition against any of the four tested pathogenic indicator bacteria (Bacillus cereus, Staphylococcus aureus, Salmonella typhimurium, and Escherichia coli) on MHA plates at pH 6.5. The absence of antimicrobial activity at the tested conditions may be attributed to the neutralization of organic acids and insufficient concentration of bacteriocin-like inhibitory substances under the experimental conditions employed.

3.6.5 Antioxidant Activity

DPPH radical scavenging activity varied markedly among the 10 isolates. The highest antioxidant activity was recorded in isolates BF1 and BF5 (60% inhibition), while BF2 showed the lowest activity (5% inhibition). Other isolates showed intermediate scavenging activities. Higher DPPH scavenging activity reflects greater free radical neutralizing capacity, which has direct implications for host protection against oxidative stress-mediated diseases. The antioxidant potential observed in BF1 and BF5 is particularly noteworthy, as these isolates

originate from Ficus benghalensis—a species already recognized for its potent antioxidant phytochemical profile.

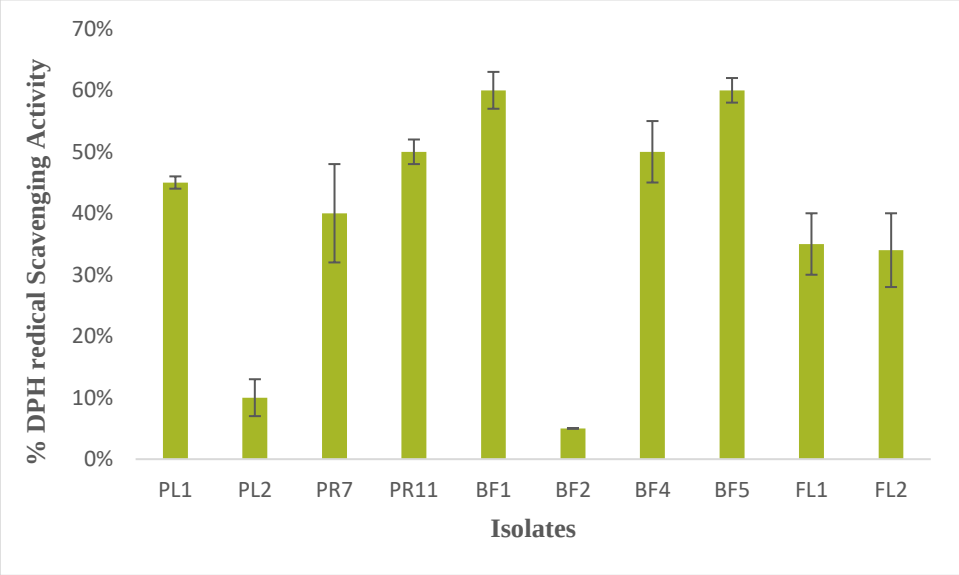

Figure 4: DPPH radical scavenging activity (%) of the isolated lactic acid bacteria (LAB) strains.

3.7 MALDI-TOF Mass Spectrometry Identification

MALDI-TOF mass spectrometry provided high-confidence species-level identification for all 10 candidate isolates (Table 5). The majority of isolates were identified as belonging to the genus Enterococcus, with species diversity including Enterococcus faecalis (PL2, PR7, BF2), Enterococcus faecium (FL1, FL2), Enterococcus casseliflavus (BF1, BF4, BF5), and Enterococcus mundtii (PL1). Isolate PR11 was identified as Lactococcus lactis. Score values ranged from 1.75 to 2.40, with the majority exceeding 2.00, indicating confident species-level identifications. The diversity of Enterococcus species identified—particularly E. faecium and E. casseliflavus—is consistent with existing literature documenting Enterococcus as widely distributed in plant-associated environments and as emerging probiotic candidates.

Table 5. MALDI-TOF Mass Spectrometry Identification of Probiotic Isolates

Sample ID	Best Match Organism	Score
PL1	*Enterococcus mundtii*	2.06
PL2	*Enterococcus faecalis*	2.40
PR7	*Enterococcus faecalis*	2.08
PR11	*Lactococcus lactis*	2.27
BF1	*Enterococcus casseliflavus*	1.78
BF2	*Enterococcus faecalis*	2.29

BF4	*Enterococcus casseliflavus*	2.12
BF5	*Enterococcus casseliflavus*	2.19
FL1	*Enterococcus faecium*	2.18
FL2	*Enterococcus faecium*	1.84

4. Discussion

The present study systematically explored four traditional medicinal plants of Gujarat as ecological niches for probiotic microorganisms, yielding 10 candidate LAB isolates that satisfied stringent safety and functional criteria. The use of MRS medium for selective enrichment and isolation is well-established for LAB recovery, given its formulation with nutrients and inhibitors that favour lactobacilli and enterococci over competing Gram-negative flora (De Man et al., 1960).

The selection of gelatinase-negative and catalase-negative isolates as primary criteria is scientifically grounded. Gelatinase production is classified as a virulence factor in Enterococcus faecalis due to its role in biofilm formation, tissue invasion, and host protein degradation (Dubin et al., 2011). Catalase negativity, conversely, is a defining physiological feature of LAB and reflects their fermentative metabolism and adaptation to oxygen-limited niches such as the intestinal mucosa (Kandler & Weiss, 1986). The biochemical profiles observed—particularly indole negativity, citrate non-utilization, urease negativity, and positive carbohydrate fermentation—are consistent with LAB characteristics and confirm the safety profile of selected isolates (Carr et al., 2002).

The universal protease production observed across all 10 isolates is a commercially and therapeutically relevant trait. Probiotic protease production has been associated with enhanced dietary protein digestion, modulation of mucosal immune responses, and reduction of allergenic food proteins (Donkor et al., 2007). The exclusive lipase activity in isolate PL2 may confer additional benefit in fat absorption and lipid metabolism. Conversely, amylase negativity across all isolates is not unexpected for Enterococcus and Lactococcus species, which are generally non-amylolytic (Schleifer & Kilpper-Bälz, 1984).

The γ-hemolysis pattern observed uniformly across all isolates is a critical safety milestone. β-hemolytic strains, particularly among Enterococcus spp., are considered potentially pathogenic and ineligible for probiotic use (Giraffa, 2002). The observed susceptibility of all isolates to Penicillin-G, Tetracycline, Chloramphenicol, and Gentamicin is consistent with intrinsic susceptibility profiles reported for Enterococcus and Lactococcus species and reduces the risk of antibiotic resistance gene dissemination (EFSA BIOHAZ Panel, 2012). The

resistance of certain isolates to Streptomycin and Kanamycin is noted; however, intrinsic aminoglycoside resistance in enterococci is well-documented and is typically chromosomally encoded rather than transmissible (Murray, 1990), which mitigates the safety concern.

The tolerance of isolates to pH 2–4 and bile salt concentrations of 0.5–1.5% indicates their potential to survive gastric and duodenal transit. While none of the isolates grew at pH 1—representative of the highly acidic fasting stomach environment—survival at pH 2 and above is considered sufficient for probiotic functionality, as food intake raises gastric pH above 2.0 during digestion (Charteris et al., 1998). NaCl tolerance up to 6% further supports the osmoregulatory fitness of these strains for survival in food matrices and the gastrointestinal environment.

The absence of detectable antimicrobial activity in cell-free supernatants against tested pathogens warrants consideration. Probiotic antimicrobial activity is mediated by multiple mechanisms including organic acid production, hydrogen peroxide, bacteriocins, and biosurfactants (Cotter et al., 2005). The pH adjustment to 6.5 used in this study eliminates organic acid contributions, thereby restricting detection to bacteriocin-like substances. The negative results observed may reflect the absence of bacteriocin production under the tested conditions or the requirement for co-culture conditions for their expression. Future studies employing bacterial co-culture assays without pH neutralization are recommended.

The DPPH antioxidant activities observed—particularly the 60% inhibition in BF1 (Enterococcus casseliflavus) and BF5 (Enterococcus casseliflavus) isolates—are consistent with emerging literature reporting antioxidant activity in Enterococcus species (Tuo et al., 2018). The plant of origin (Ficus benghalensis) is rich in flavonoids and polyphenols, which may have influenced the metabolic capabilities of these plant-associated microorganisms. Probiotic antioxidant activity is increasingly recognized as a mechanism for reducing oxidative stress-related pathologies including cardiovascular disease, type 2 diabetes, and neurodegeneration (Wang et al., 2017).

The dominance of Enterococcus faecalis, E. faecium, E. casseliflavus, and E. mundtii in the MALDI-TOF identification results is scientifically interesting. While E. faecalis and E. faecium harbor clinical strains with virulence factors, the non-pathogenic, gelatinase-negative, non-hemolytic isolates identified in this study represent the commensal/probiotic ecotype documented in traditional fermented food products and plant-associated environments globally (Franz et al., 2011). Lactococcus lactis (PR11) is an established probiotic and food-grade organism widely employed in cheese and fermented food production and recognized for its bacteriocin (nisin) production (Wiedemann et al., 2001).

5. Conclusion

This study demonstrates that the medicinal plants of Gujarat—specifically Carica papaya, Ficus benghalensis, Woodfordia fruticosa, and Phyllanthus emblica—harbor diverse LAB communities with significant probiotic potential. Through a comprehensive multi-step screening pipeline encompassing primary selection, biochemical characterization, enzyme profiling, safety assessment, and functional assays, 10 candidate strains were identified, predominantly belonging to Enterococcus faecalis, Enterococcus faecium, Enterococcus casseliflavus, Enterococcus mundtii, and Lactococcus lactis. The universal protease production, γ-hemolysis, antibiotic susceptibility to clinically important antibiotics, moderate acid and bile tolerance, and notable antioxidant activity—particularly in BF1 and BF5—collectively affirm their probiotic candidacy. Future research should focus on in vivo validation of the probiotic efficacy of these isolates in animal models, whole-genome sequencing for comprehensive virulence and resistance gene profiling, and assessment of their technological properties for functional food development.

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Conflict of interest:

The authors declare no conflict of interest regarding the research, authorship, or publication of this article. Both authors have contributed collaboratively to the work and the study's findings are independent of any commercial or financial interest.

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