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Traditionally fermented beverages fishtail palm toddy and banana pseudo-stem sap as reservoirs of *Leuconostoc mesenteroides* with probiotic potential

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Abstract

Traditional fermented foods are integral to India's culinary heritage and represent long-standing empirical knowledge of naturally occurring microorganisms. Fishtail palm toddy (*Caryota urens* L.) and banana pseudo-stem sap are plant-based fermented foods traditionally consumed in rural Karnataka, yet their native lactic acid bacteria remain poorly characterized. This study investigated these foods as potential reservoirs of probiotic microorganisms. Six bacterial isolates obtained from fishtail palm toddy and banana pseudo-stem sap were identified as *Leuconostoc mesenteroides* using 16S rRNA gene sequencing and phylogenetic analysis. All isolates were Gram-positive, catalase-negative, heterofermentative, and bile-salt tolerant. They exhibited desirable probiotic properties, including carbohydrate fermentation, acid tolerance, favourable antibiotic susceptibility, non-haemolytic activity, broad-spectrum antibacterial activity, survival under simulated gastrointestinal conditions, cell-surface hydrophobicity, auto-aggregation, and co-aggregation with enteric pathogens. Isolates from fishtail palm toddy showed comparatively greater tolerance to acidic pH, high salt concentrations, and elevated temperatures. Among the strains, FBN7 and BPS4 demonstrated superior functional characteristics. These findings establish traditional fermented foods as indigenous non-dairy sources of functionally competent *L. mesenteroides* and provide a microbiological basis for preserving traditional fermentation practices and exploring these strains for future applications in probiotic and functional food development.

Abbreviations:

NaCl: Sodium chloride; EPS: Exopolysaccharide; LAB: Lactic Acid Bacteria; NaOH: Sodium hydroxide; H_2O_2: Hydrogen peroxide; MRS Broth: De Man, Rogosa and Sharpe Broth; KOH: Potassium hydroxide; NCBI: National Centre for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; SGF: Simulated Gastric Fluid; SIF: Simulated Intestinal Fluid; CFU: Colony-Forming Unit; PBS: Phosphate-Buffered Saline; OD: Optical Density; EFSA: European Food Safety Authority.

Introduction

India possesses a rich and diverse heritage of traditional fermented foods and beverages, many of which remain

scientifically underexplored despite their promising potential as reservoirs of novel probiotic microorganisms. These traditional foods are deeply embedded in local culture and are commonly prepared using ethnic knowledge systems passed down through generations (Rawat *et al*., 2018). Plant-derived fermented beverages represent an important component of the indigenous food heritage of rural and agrarian communities. Such seasonal, locally prepared foods reflect long-standing empirical practices of food selection, natural fermentation and preservation. Beyond their nutritional relevance, indigenous fermented foods are closely associated with community traditions, ecological sustainability and local health beliefs, thereby offering valuable opportunities for scientific documentation and bioprospecting of beneficial microbial strains with probiotic potential (Jha *et al*., 2021; Tamang, 2022).

Fishtail palm toddy is a traditional plant-derived fermented beverage obtained from the tapped inflorescence sap of *Caryota urens* and is an important indigenous beverage consumed in Southern India and parts of Southeast Asia. This beverage is traditionally collected and naturally fermented under local environmental conditions and is valued not only as a seasonal refreshment but also as a culturally significant product of rural palm-based livelihoods. Owing to its nutrient-rich composition and spontaneous microbial succession, fishtail palm toddy provides a favourable ecological niche for beneficial lactic acid bacteria (LAB) with potential probiotic relevance (de Paula *et al*., 2014 ; Somashekaraiah *et al*., 2019).

Similarly, banana pseudo-stem sap, obtained from the water-rich inner tissues of *Musa* spp., has been associated with rural food practices and folk-health traditions in India, Malaysia, and Sri Lanka (Gupta *et al*., 2022). These plant-based substrates are exposed to fluctuating pH, osmotic conditions, and nutrient availability during natural fermentation, which may favour the selection of stress-tolerant microorganisms with desirable probiotic traits, such as acid and bile tolerance, adhesive ability, and environmental resilience (Shriniketan & Puranik, 2019).

Lactic acid bacteria constitute an important microbial group in traditional fermented foods, where they contribute to preservation, improved digestibility, nutrient bioavailability and functional quality (Ray *et al*., 2016). Among LAB, *Leuconostoc mesenteroides* is a heterofermentative species known for producing exopolysaccharides, organic acids and antimicrobial metabolites, which may enhance both the technological and

health-promoting properties of fermented foods. However, compared with extensively studied probiotic genera such as *Lactobacillus* and *Bifidobacterium*, *Leuconostoc mesenteroides* from non-dairy, plant-based traditional fermentations remains insufficiently explored (Cele *et al*., 2022). Therefore, the present study was undertaken to isolate, identify and characterize *Leuconostoc mesenteroides* strains from fishtail palm toddy and banana pseudo-stem sap collected from Kodagu and Mandya districts of Karnataka, India. Further, the probiotic attributes of the selected isolates, including gastrointestinal tolerance, antimicrobial activity, safety profile and adhesion-related properties, were evaluated to assess their suitability for functional food and therapeutic applications.

Materials and methods

Materials

Xylene, n-hexadecane and hydrochloric acid were procured from Merck, USA. De Man, Rogosa and Sharpe (MRS) broth, glycerol, hydrogen peroxide (H_2O_2), sheep blood agar, sample collection vials, Muller-Hinton agar, agar-agar, antibiotic discs, sodium hydroxide (NaOH), sodium chloride (NaCl), pepsin, pancreatin, ox gall and all other analytical-grade reagents were obtained from HiMedia Laboratories, Mumbai, India.

Test organisms

Reference pathogenic strains used for antibacterial assays included *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 443), *Listeria monocytogenes* (MTCC 1143) and *Salmonella enterica* serovar Typhi (MTCC 733). These cultures were procured from the CSIR–Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained as per standard microbiological procedures.

Sample collection

Fishtail palm toddy from Kodagu and banana pseudo-stem sap from Mandya, Karnataka, were collected from identified farmers. The sap was obtained by cutting the pseudo-stem, covering the incision with polythene and aseptically collecting the sap after 1 h. It was exposed to sunlight for 4-5 h to promote fermentation. Both samples were transported on ice and refrigerated at 4°C until analysis.

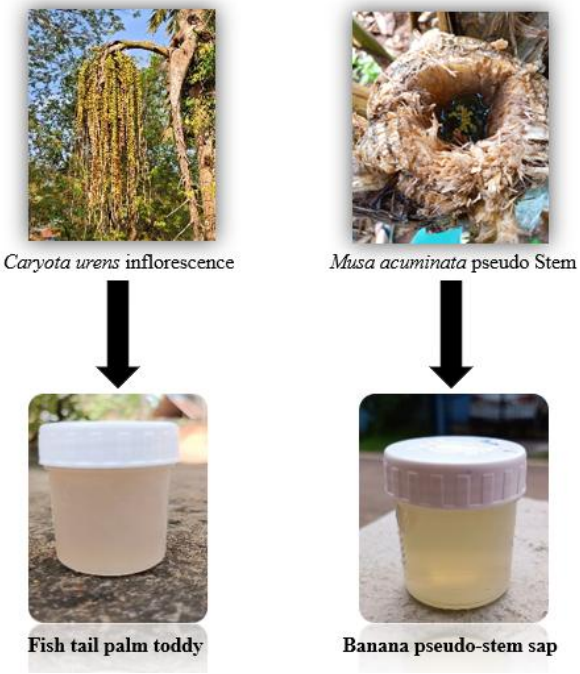

Fig. 1- Collection of fishtail palm toddy and banana pseudo-stem sap from their respective plant sources found in Karnataka, India.

Preparation of isolates

Each sample (1 mL) was mixed with 9 mL of 0.85% sterile saline and serially diluted. Dilutions were plated on MRS agar and incubated anaerobically at 37°C for 24 h. Six distinct LAB colonies were selected, sub cultured in MRS broth, purified by repeated streaking, and preserved on MRS slants (4°C) and as glycerol stocks (-20°C) for further analysis.

Molecular identification of isolated probiotics from 16s rRNA sequencing

Molecular identification was performed according to a previously described method with minor modifications as described by **Kharnaior & Tamang (2023)**. Isolates were cultured in MRS broth at 37 °C for 24 h, and genomic DNA was extracted using the phenol–chloroform method. The 16S rRNA gene (~1.46 kb) was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′). PCR conditions comprised initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1–5 min, with a final extension at 72 °C for 7 min. Amplicons were separated on 1% (w/v) agarose gels at 100 V for 20 min, visualized under UV light, purified using a Promega PCR purification kit, and commercially sequenced. Sequence similarity was determined using NCBI

BLAST, followed by multiple sequence alignment in BioEdit. Phylogenetic analysis was conducted in MEGA X using the neighbour-joining method with 1,000 bootstrap replicates.

Preliminary characterization of isolated probiotic strains

Preliminary identification of the *Leuconostoc mesenteroides* isolates was performed as described by (Mulaw *et al*., 2019) based on their morphological, physiological and biochemical characteristics. Gram staining, potassium hydroxide (KOH) test and catalase reaction were used for biochemical characterization. Tolerance to sodium chloride (3–7%) and bile salts (0.3% and 0.5%) was assessed by culturing isolates in MRS broth supplemented with respective concentrations of NaCl or ox gall, followed by incubation at 37 °C for 24 h. Growth response under different environmental conditions was evaluated by incubating cultures at 10 °C, 37 °C and 45 °C, and at pH values ranging from 2.0 to 4.0. Carbohydrate fermentation profiles were determined using MRS broth containing individual sugars as carbon sources. Cell viability and growth kinetics under these conditions were quantified by the plate count method.

Safety assessment of probiotics

Antibiotic resistance

Antibiotic susceptibility was evaluated by the disc diffusion method (Sengun *et al*., 2025). A 50 µL aliquot of the 24 h MRS culture was spread on MRS agar plates and antibiotic discs (ampicillin, vancomycin, streptomycin, kanamycin, chloramphenicol, erythromycin, tetracycline and ciprofloxacin) were placed aseptically. Plates were incubated at 37 °C for 24–48 h and the diameters of inhibition zones were measured. Sensitivity was interpreted as follows: ≤14 mm = Resistant (R); 15–19 mm = Intermediate (I); ≥20 mm = Sensitive (S).

Haemolytic resistance

Haemolytic behaviour was assessed on 5% (w/v) sheep blood agar (Yasmin *et al*., 2020). Plates were incubated at 37 °C for 48 h and haemolysis patterns were recorded. Colonies producing clear zones were categorized as β-haemolytic, green zones as α-haemolytic and those without visible zones as γ-haemolytic. Only γ-haemolytic strains were considered non-pathogenic and safe for probiotic use.

Antibacterial activity

The antibacterial effectiveness of the samples was evaluated using the agar well diffusion method (Huligere *et al*., 2023). The indicator bacteria were adjusted to a 0.5 McFarland standard (~10^8 CFU/mL)

and evenly distributed on Mueller-Hinton agar dishes. Holes with a diameter of 6 mm were created with clean cork borers, and 100 µL of sterile supernatant from each sample was placed into the holes. A suitable antibiotic was used as the reference standard. The plates were kept at 37 °C for 24 hours, and the diameter of the zones of inhibition was recorded in millimetres.

Gastrointestinal tolerance assay

Gastrointestinal tolerance was evaluated using a previously described method (Bindu & Lakshmidevi, 2021). Simulated gastric fluid (SGF) was prepared with 0.1% (w/v) pepsin, 0.5 M HCl, and sterile water, adjusted to pH 2.0. Simulated intestinal fluid (SIF) contained 0.1% (w/v) pancreatin and 0.3% (w/v) bile salts in sterile water adjusted to pH 7.4. Cultures grown in MRS broth at 37 °C for 24 h were adjusted to 10^8–10^9 CFU/mL. One millilitre of culture was mixed with 9 mL of SGF or SIF and incubated at 37 °C for 1, 2, and 4 h. SGF-exposed samples were neutralized with phosphate-buffered saline (pH 7.4). At each interval, samples were serially diluted in PBS, plated on MRS agar, and incubated at 37 °C for 24 h to determine viable counts. Cultures maintained in PBS served as untreated controls.

Adherence-related properties

Cell surface hydrophobicity

Hydrophobic properties were assessed using the microbial adhesion-to-hydrocarbon (MATH) test (Kaushik *et al.,* 2009), with adjustments. Overnight MRS cultures were spun down (6,000 rpm, 10 minutes, 4 °C), rinsed, and suspended in PBS (A_0 at 600 nm). A volume of one millilitre of the suspension was combined with an equal amount of xylene or n-hexadecane and left at 37 °C for 10 minutes without stirring. Following the separation of phases, the optical density of the aqueous layer (A_1) was gauged at 600 nm and hydrophobicity was determined using the formula: % Cell Surface Hydrophobicity = (1 - A1/A0) × 100

Auto-aggregation and Co-aggregation assays

Cell aggregation properties were evaluated according to previously described method (Zommiti *et al.,* 2017**).** Overnight cultures of *L. mesenteroides* were adjusted to 10^8–10^9 CFU/mL in sterile saline. For auto-aggregation, 1 mL of bacterial suspension was incubated at 37 °C for 4 h without shaking. For co-aggregation, equal volumes (1:1) of *L. mesenteroides* and each pathogen (*E. coli, S. Typhi, B. subtilis*) were mixed and incubated similarly. After incubation, samples were centrifuged (6000 rpm, 10 min), the upper phase was

measured at 600 nm and aggregation percentages were calculated using:

Auto aggregation (%) = [(OD initial – OD final) / OD initial] × 100

Co-aggregation (%) = $(1 - A_1/A_0) \times 100$

where A_0 is the average absorbance of individual strains and A_1 is the absorbance of the mixed suspension after incubation.

Statistical analysis

The experiments were carried out three times, and the findings were presented as average ± SD. Data analysis was done with GraphPad Prism software (version 8.0) using one-way ANOVA, and significance was determined at $p < 0.05$.

Results

Molecular identification and genetic diversity of LAB strains

Molecular identification based on 16S rRNA gene sequencing confirmed that all six selected isolates belonged to the species *Leuconostoc mesenteroides* (Table 1). BLAST analysis against sequences available in the NCBI GenBank database showed high homology with reference *L. mesenteroides* strains, with sequence identity ranging from 96.23% to 99.89%. Among the isolates, FBN7 exhibited the highest similarity (99.89%), followed by FBN3 (99.57%), BPS22 (99.53%), and both FBN15 and BPS4 (99.25%), whereas BPS5 showed comparatively lower identity (96.23%). Phylogenetic analysis based on 16S rRNA gene sequences using the neighbor-joining method further supported the genetic affiliation of these isolates with *L. mesenteroides* (Fig. 2). The dendrogram revealed that FBN7 and BPS4 clustered closely together with a bootstrap value of 86%, and this cluster further grouped with FBN15 with strong bootstrap support (100%). FBN3 was positioned close to this cluster, while BPS5 and BPS22 formed comparatively distant branches, indicating a certain level of intraspecies genetic variability among the isolates. Overall, the molecular and phylogenetic analyses confirmed that the isolates recovered from fishtail palm toddy and banana pseudo-stem sap were members of *L. mesenteroides* and demonstrated moderate genetic diversity within the species.

Table 1- Molecular identification of potential probiotic *Leuconostoc mesenteroides* isolates based on 16S rRNA gene sequencing

Sl.no	Isolates code	Identified Strain	NCBI accession no	% identity

1	FBN3	*Leuconostoc mesenteroides*	PV124360	99.57%
2	FBN7	*Leuconostoc mesenteroides*	PV124363	99.89%
3	FBN15	*Leuconostoc mesenteroides*	PV124369	99.25%
4	BPS4	*Leuconostoc mesenteroides*	PV130037	99.25%
5	BPS5	*Leuconostoc mesenteroides*	PV124350	96.23%
6	BPS22	*Leuconostoc mesenteroides*	PV124358	99.53%

All isolates were identified through 16S rRNA sequencing, and BLAST analysis confirmed homology with reference *L. mesenteroides* sequences in NCBI GenBank.

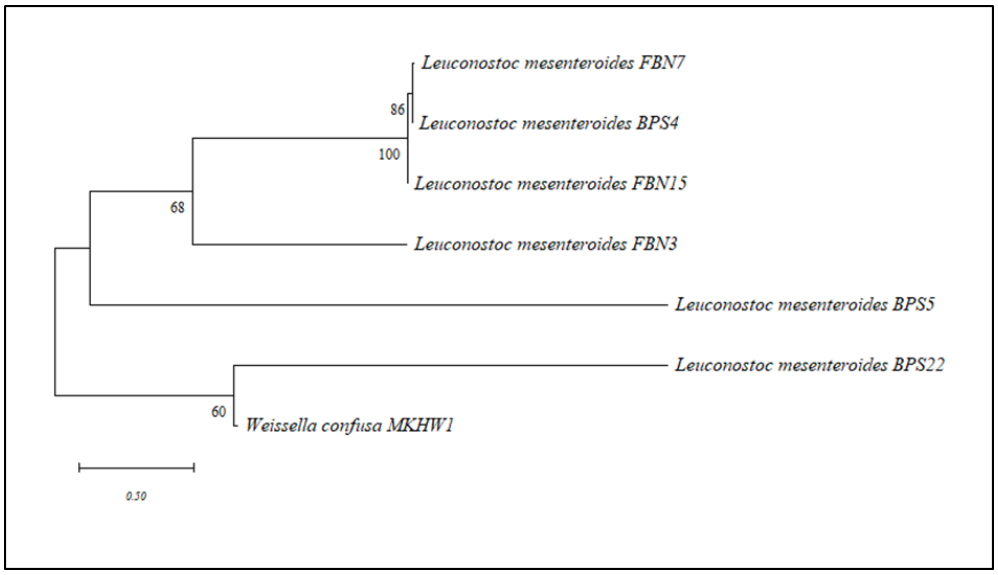

Fig.2- Phylogenetic relationships among *Leuconostoc mesenteroides* isolates obtained from fishtail palm toddy and banana pseudo-stem sap; constructed using the neighbor-joining method based on 16S rRNA gene sequences. Bootstrap values (based on 100 replications) indicate the confidence in clustering.

Phenotypic and biochemical characterization of LAB strains

The phenotypic, biochemical and fermentative characteristics of the six *Leuconostoc mesenteroides* isolates were systematically evaluated and the results are summarized in (Table 2). All isolates were Gram-positive, coccoid in morphology, catalase-negative and exhibited positive growth in the KOH test, confirming their classification as lactic acid bacteria. Uniform growth was observed in the presence of 3% and 5% NaCl across all strains, while tolerance to 7% NaCl was restricted to isolates FBN3, FBN7 and

FBN15, indicating strain-dependent resistance to osmotic stress. Temperature tolerance assays revealed distinct growth patterns among the isolates. Strains BPS4, BPS5 and BPS22 demonstrated growth at 10 °C, whereas FBN3, FBN7 and FBN15, did not. All isolates grew optimally at 37 °C. Growth at 40 °C was observed exclusively in FBN3, FBN7 and FBN15, highlighting adaptive variability to elevated temperature conditions. Acid tolerance assessment showed that isolates FBN3, FBN7 and FBN15, survived at pH 2, while BPS4, BPS5 and BPS22 failed to grow under this highly acidic condition. However, all strains demonstrated robust growth at pH 3 and pH 4. Consistently, all isolates tolerated 0.3% and 0.5% bile salts, indicating potential survivability under gastrointestinal conditions. Carbohydrate fermentation profiling indicated that all isolates were heterofermentative and capable of fermenting glucose, maltose, fructose, sucrose, xylose, and arabinose. In contrast, the fermentation of lactose and raffinose was strain-specific; FBN3, FBN7 and FBN15, were able to ferment both the sugars, whereas BPS4, BPS5 and BPS22 were unable to utilize these substrates.

Table 2- Phenotypic, biochemical and fermentative properties of *L. mesenteroides* isolates

Test	Condition	FBN3	FBN7	FBN15	BPS4	BPS5	BPS22
Gram's staining		+	+	+	+	+	+
Morphology		Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase		-	-	-	-	-	-
Growth with KOH		+	+	+	+	+	+
NaCl Tolerance							
	3%	+	+	+	+	+	+
	5%	+	+	+	+	+	+
	7%	+	+	+	-	-	-
Temperature Tolerance							
	10°C	-	-	-	+	+	+
	37°C	+	+	+	+	+	+
	40°C	+	+	+	-	-	-
pH Tolerance							
	pH2	+	+	+	-	-	-
	pH3	+	+	+	+	+	+
	pH4	+	+	+	+	+	+

Bile Salt Tolerance							
	0.3%	+	+	+	+	+	+
	0.5%	+	+	+	+	+	+
Fermentation							
Type		**Hetero**	**Hetero**	**Hetero**	**Hetero**	**Hetero**	**Hetero**
Glucose		+	+	+	+	+	+
Maltose		+	+	+	+	+	+
Fructose		+	+	+	+	+	+
Sucrose		+	+	+	+	+	+
Xylose		+	+	+	+	+	+
Arabinose		+	+	+	+	+	+
Lactose		+	+	+	-	-	-
Raffinose		+	+	+	-	-	-

Data shown are mean ± SD of triplicate values of three independent experiments. Note: +: Positive, −: Negative, Hetero: Hetero fermentative.

Antibiotic sensitivity profile of *Leuconostoc mesenteroides* isolates

The antibiotic susceptibility patterns of the *Leuconostoc mesenteroides* isolates (FBN3, FBN7, FBN15, BPS4, BPS5 and BPS22) were assessed using disc diffusion assays and the results are summarized in (Table 3). All isolates exhibited uniform sensitivity to ampicillin (10 µg), indicating the absence of resistance to β-lactam antibiotics. A similar sensitivity trend was observed for chloramphenicol (30 µg) across all strains. Erythromycin (15 µg) demonstrated high efficacy, with all isolates being sensitive, except for FBN15, which exhibited an intermediate response. The isolates demonstrated strong susceptibility to tetracycline (30 µg), with five strains showing sensitivity, while BPS22 exhibited intermediate sensitivity. In the case of ciprofloxacin (30 µg), strain-dependent variation was observed; FBN15 and BPS5 were sensitive, whereas FBN3, FBN7, BPS4 and BPS22 showed intermediate responses. Consistent intrinsic resistance to vancomycin (30 µg) and streptomycin (10 µg) was observed across all isolates. Kanamycin (30 µg) susceptibility varied among strains, with FBN7, FBN15, BPS4 and BPS5 being sensitive, FBN3 showing intermediate sensitivity and BPS22 exhibiting resistance.

Table -3 Antibiotic sensitivity profile of *L. mesenteroides* isolates

Antibiotics	Concentration	FBN3	FBN7	FBN15	BPS4	BPS5	BPS22
Ampicillin	10µg	S	S	S	S	S	S
Vancomycin	30µg	R	R	R	R	R	R
Erythromycin	15µg	S	S	I	S	S	S
Tetracycline	30µg	S	S	S	S	S	I
Ciprofloxacin	30µg	I	I	S	I	S	I
Streptomycin	10µg	R	R	R	R	R	R
Chloramphenicol	30µg	S	S	S	S	S	S
Kanamycin	30µg	I	S	S	S	S	R

Data shown are mean ± SD of triplicate values of three independent experiments. S: sensitive, R: resistant, I: Intermediate

Hemolytic activity of *Leuconostoc mesenteroides* isolates

Table-4 Haemolytic activity of *L. mesenteroides* isolates

	Strain	Positive		Negative
		Alpha	Beta	Gamma
Control	***E.coli***		O	
Test (Isolates)	**FBN3**			O
	FBN7			O
	FBN15			O
	BPS4			O
	BPS5			O
	BPS22			O

Data shown are mean ± SD of triplicate values of three independent experiments. O: Overserved, Alpha: Alpha haemolysis, Beta: Beta haemolysis and Gamma: Gamma haemolysis.

The hemolytic potential of the isolated *Leuconostoc mesenteroides* strains was evaluated based on their appearance as clear (β-hemolysis), greenish (α-hemolysis) and no hemolytic zones (γ-hemolysis) on blood agar plates. The positive control (*Escherichia coli*) exhibited a clear hemolytic response, confirming the assay's validity (Table 4). In contrast, none of the tested *Leuconostoc mesenteroides* isolates (FBN3, FBN7, FBN15, BPS4, BPS5 and BPS22) showed α- or β-hemolysis. All

isolates consistently demonstrated γ-hemolysis, characterized by the absence of a hemolytic zone around the colonies, supports their biological safety.

Antibacterial activity of LAB strains

The antibacterial activity of the *Leuconostoc mesenteroides* isolates was assessed using the agar well diffusion assay (Table 5). All strains inhibited *Staphylococcus aureus*, producing zones of 10.3–13.0 mm, with FBN7 showing the highest activity (13 ± 1.0 mm). Inhibition of *Bacillus subtilis* was minimal (7.9–8.1 mm) and absent in BPS4, BPS5 and BPS22, indicating strain-dependent differences in antimicrobial metabolite production. Activity against Gram-negative pathogens varied considerably. FBN7 (18.3 ± 1.1 mm) and FBN15 (17.6 ± 0.5 mm) displayed the strongest inhibition of *Escherichia coli*, although all isolates were less effective than the antibiotic control. Notably, all strains exhibited consistently high inhibitory activity against *Listeria monocytogenes* (22.0–23.6 mm), which was comparable to that of the positive control (22.6 ± 1.5 mm). Inhibition of *Salmonella typhi* ranged widely, with BPS4 showing the greatest activity (18.6 ± 1.5 mm), whereas BPS5 and FBN15 produced only weak inhibition (6.6–6.7 mm).

Table 5- Antibacterial activity of *L. mesenteroides* isolates against selected pathogens

Isolates	**Pathogens**				
	***S.aureus* (mm)**	***B.subtils* (mm)**	***E.coli* (mm)**	***L.monocytogenes* (mm)**	***S.Typhi* (mm)**
CTRL	23.33 ± 0.5	21.6 ± 1.1	29 ± 1.0	22.6 ± 1.5	21.3 ± 2.8
FBN3	11.33 ± 2.3	8.0 ± 0.0	17.3 ± 0.5	23.3 ± 1.5	13.3 ± 2.0
FBN7	13 ± 1.0	8.1 ± 0.1	18.3 ± 1.1	23.6 ± 1.1	16.1 ± 2.7
FBN15	12 ± 2.0	7.9 ± 0.1	17.6 ± 0.5	23.0 ± 1.0	06.6 ± 0.1
BPS4	12.6 ± 2.3	-	8.0 ± 0.1	22.6 ± 0.5	18.6 ± 1.5
BPS5	11.6 ± 2.0	-	7.8 ± 0.0	22.0 ± 1.0	06.7 ± 0.3
BPS22	10.3 ± 0.5	-	7.7 ± 0.6	22.1 ± 0.7	14.3 ± 0.5

Data shown are mean ± SD of triplicate values of three independent experiments. Note: CTRL-Ciprofloxacin (30µg) ZOI, zone of inhibition in mm, and indicates no ZOI detected.

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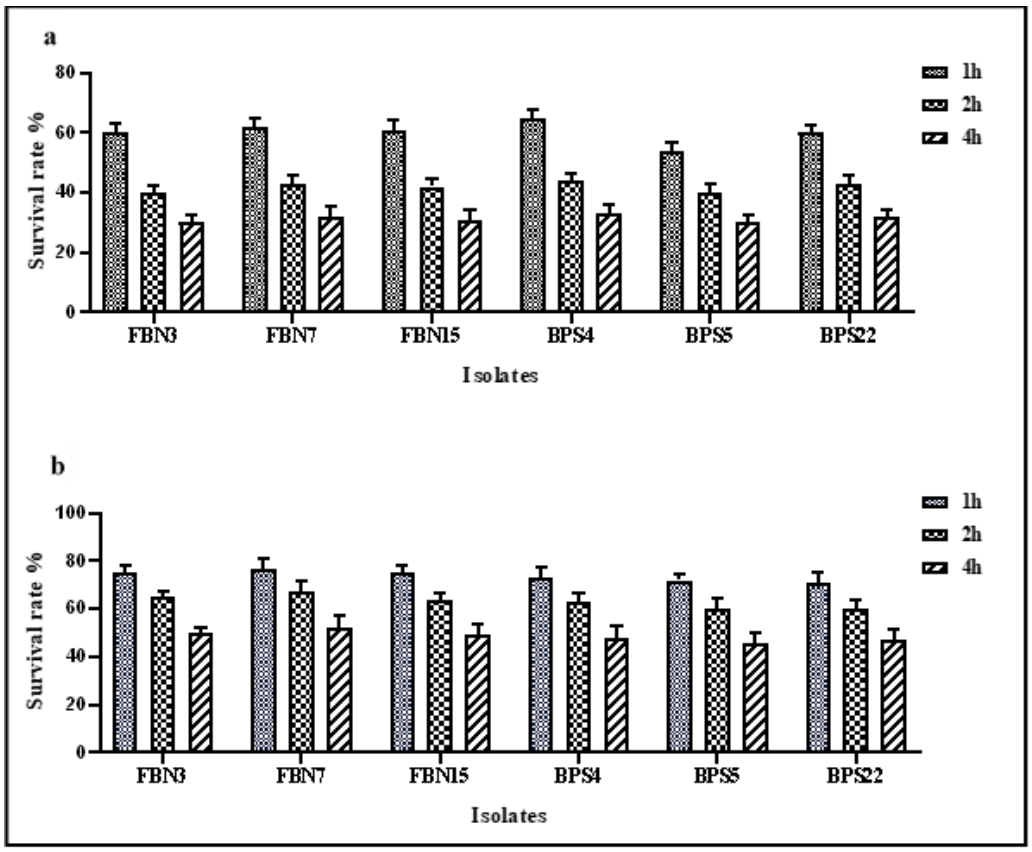

Fig.3- Gastrointestinal tolerance of *Leuconostoc mesenteroides* isolates under simulated conditions. (**a**) Gastric enzyme tolerance; (**b**) Intestinal enzyme tolerance. Data are mean ± SD of triplicate experiments representing survival rates at 1 h, 2 h, and 4 h.

The tolerance of *Leuconostoc mesenteroides* isolates to simulated gastrointestinal conditions was evaluated to assess their survival during gastric and intestinal transit (Fig. 3a–b). All strains demonstrated substantial acid tolerance at pH 2.0 with pepsin, maintaining 54–65% viability after 1h and gradually decreased with time. FBN7 and BPS4 showed the highest gastric survival (62% and 65% respectively), while BPS5 exhibited comparatively lower tolerance (54%) (Fig. 3a). Under simulated intestinal conditions (bile salts + pancreatin), all isolates displayed a time-dependent decline in viability; however, survival remained above 60% at 2 h for most strains, with FBN7 and BPS4 performing best within their respective groups. After 4 h, viability decreased to 46–52%, with FBN7 retaining the highest survival (~52%) (Fig. 3b). Overall, all isolates maintained around 50% viability after prolonged exposure, indicating strong physiological resilience and the potential to reach the colon in

sufficient numbers to exert probiotic functionality.

Adhesion properties of LAB strains

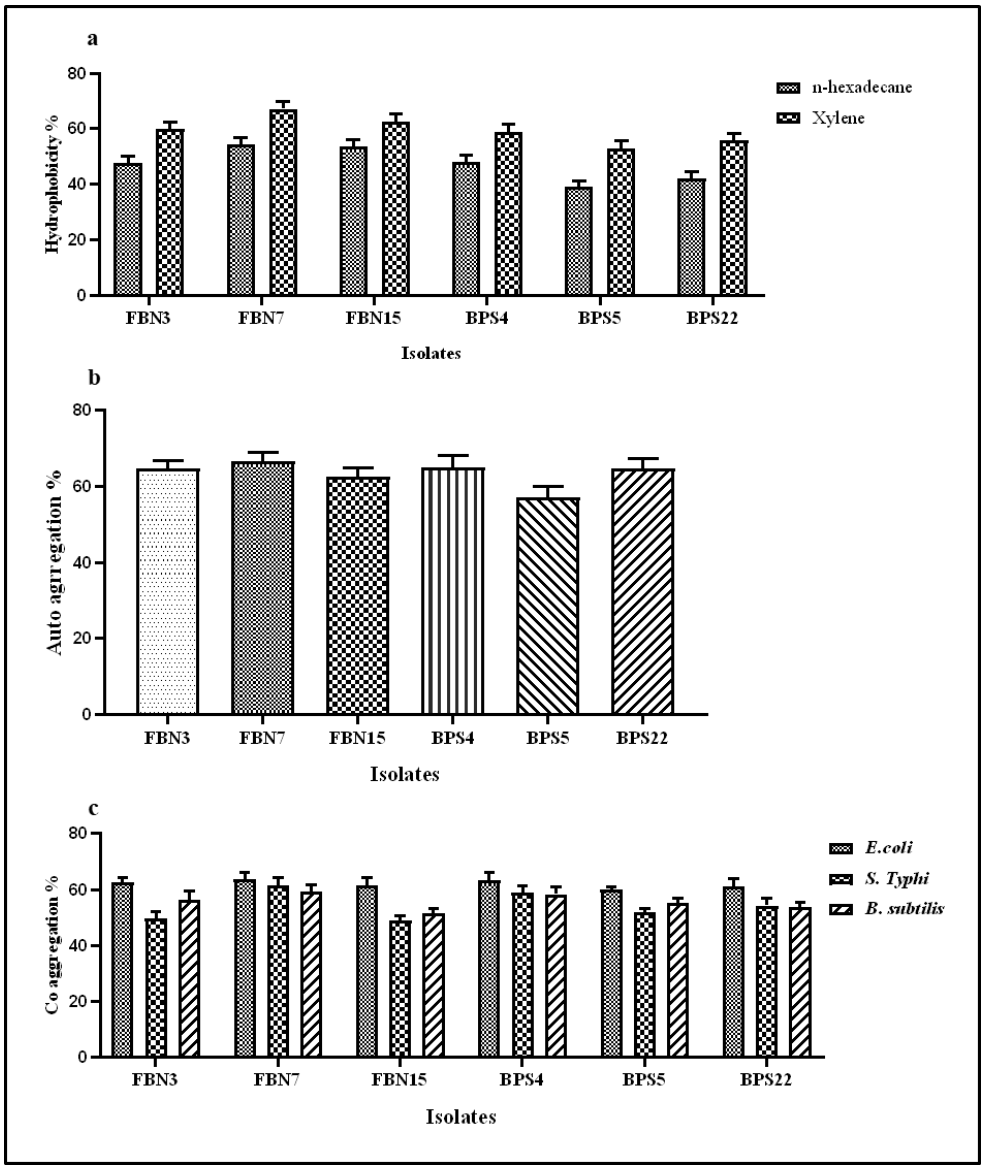

Fig.4- Adhesion properties of *Leuconostoc mesenteroides* isolates. (**a**) Surface hydrophobicity with n-hexadecane and xylene; (**b**) Auto-aggregation of isolates; (**c**) Co-aggregation with *Escherichia coli*, *Salmonella typhi* and *Bacillus subtilis*. Data are expressed as mean ± SD of triplicate experiments.

The adhesion-related traits of the *Leuconostoc mesenteroides* isolates were assessed through hydrophobicity, auto-aggregation and co-aggregation assays

(Fig. 4a–c). The adhesion-related traits of the *Leuconostoc mesenteroides* isolates were assessed through hydrophobicity, auto-aggregation and co-aggregation assays (Fig. 4a–c). Hydrophobicity measurements using n-hexadecane and xylene showed strain-dependent variability, with FBN7 exhibiting the highest hydrophobicity of 54.4% and 67.30 % respectively, while among banana pseudo-stem isolates, BPS4 showed the highest of 48.2% and 58.83% respectively (Fig. 4a). Auto-aggregation values ranged between 57–66%, with FBN3, FBN7, BPS4 and BPS22 demonstrating the strongest self-aggregation (~65%), indicative of efficient cell–cell interaction capacity (Fig. 4b). All isolates displayed measurable co-aggregation with *Escherichia coli*, *Salmonella typhi* and *Bacillus subtilis*, although the extent of interaction varied across strains (Fig. 4c). FBN7 and BPS4 showed the highest co-aggregation efficiencies (>63% with *E. coli* and >58% with *S. Typhi* and *B. subtilis*), while the remaining isolates exhibited moderate co-aggregation (48–60%). These adhesion-related properties, particularly the strong hydrophobicity and co-aggregation displayed by FBN7 and BPS4, highlight their potential to competitively exclude pathogens and contribute to effective colonization within the gastrointestinal environment.

Discussion

The predominance of *Leuconostoc mesenteroides* among isolates recovered from traditionally fermented fishtail palm toddy and banana pseudo-stem sap demonstrates that indigenous fermentation practices create ecological conditions favourable for the establishment of specific lactic acid bacteria. Unlike controlled industrial fermentations that rely on defined starter cultures, spontaneous fermentation is governed by naturally occurring microbial succession, in which physicochemical changes within the substrate progressively select microorganisms capable of adapting to acidic, nutrient-limited, and competitive environments (Wolfe & Dutton, 2015; Tamang *et al*., 2020). Molecular characterization based on 16S rRNA gene sequencing confirmed that all six isolates belonged to *Leuconostoc mesenteroides*, indicating that traditionally fermented fishtail palm toddy and banana pseudo-stem sap constitute important ecological reservoirs of this lactic acid bacterium. Most isolates showed high sequence similarity to reference strains, whereas the lower identity of BPS5 suggested possible strain-level divergence requiring confirmation through higher-resolution molecular methods (Chun *et al.,* 2017). Phylogenetic analysis further revealed

intraspecific diversity; isolates FBN7, BPS4, and FBN15 clustered closely, whereas FBN3, BPS5, and BPS22 formed distinct branches. This heterogeneity may reflect differences in substrate composition, fermentation conditions, and traditional processing practices, highlighting indigenous fermented beverages as valuable sources of genetically diverse and potentially beneficial microorganisms (Marco *et al*., 2021).

The genetic heterogeneity of the isolates was reflected in their phenotypic and metabolic variation, indicating strain-specific adaptation to the ecological conditions of the two fermented substrates. All isolates were Gram-positive, catalase-negative cocci, consistent with the characteristics of *Leuconostoc* spp (Hemme & Foucaud-Scheunemann, 2004). However, differences in acid, salt, and temperature tolerances indicated distinct physiological responses. Isolates from fishtail palm toddy generally exhibited greater stress tolerance than those from banana pseudo-stem sap, possibly due to adaptation to the progressive accumulation of organic acids and ethanol during toddy fermentation. Such exposure may promote cross-protective mechanisms against multiple environmental stresses (Benmechernene *et al.,* 2013 & Luo *et al.*, 2021). All isolates displayed heterofermentative metabolism and utilized glucose, maltose, fructose, sucrose, xylose, and arabinose (Yu *et al*., 2021), whereas lactose and raffinose fermentation was limited to FBN3, FBN7, and FBN15, confirming strain-dependent metabolic versatility. These strain-dependent properties suggest that selected isolates are promising candidates for fermented food applications and probiotic formulations.

The safety profiles of the isolates were evaluated alongside their stress tolerance. All strains were susceptible to β-lactam antibiotics and chloramphenicol but exhibited the expected intrinsic resistance to vancomycin and aminoglycosides, consistent with the characteristics of *Leuconostoc* species (Salvetti *et al.,* 2021). No atypical or multidrug-resistance patterns were detected, reducing concerns regarding transferable antibiotic resistance and supporting their potential use in food applications. Furthermore, all isolates displayed γ-haemolysis, indicating the absence of erythrocyte lysis. This non-haemolytic phenotype provides additional evidence of their preliminary biosafety and conforms to the safety criteria generally applied to lactic acid bacteria intended for human consumption (Icer *et al*., 2023). Further all the isolates exhibited antibacterial activity against both Gram-positive and Gram-negative pathogens,

with the strongest inhibition observed against *Listeria monocytogenes* and moderate activity against *Staphylococcus aureus* and *Escherichia coli*. The pronounced anti-listeria activity may be associated with bacteriocin production by *Leuconostoc mesenteroides* (Shao *et al*., 2020). The cell-free supernatants of all isolates exhibited inhibitory effects to varying degrees, confirming the production of antibacterial metabolites, like organic acids, hydrogen peroxide and bacteriocin-like substances, which are common features of lactic acid bacteria (LAB) (Prabhurajeshwar & Chandrakanth, 2019). These differences in inhibitory activity demonstrated strain-specific variation within *L. mesenteroides* and are in line with previous reports that the antimicrobial activity of strains isolated from fermented plant and dairy products depends on their bacteriocin production and acidification capacity (de Paula *et al.,* 2014)

Finally, all the isolates maintained substantial viability under simulated gastric and intestinal conditions, with several retaining more than 50% of their initial viable counts after prolonged exposure to acid and bile. This resilience suggests effective stress-response mechanisms and the potential to survive gastrointestinal transit (Schifano *et al.,* 2021). Adhesion-related properties varied among strains. Differences in cell-surface hydrophobicity may reflect variations in surface proteins, lipoteichoic acids, and exopolysaccharides, confirming the strain-dependent nature of epithelial adhesion (Grajek *et al.,* 2016). The isolates also exhibited auto-aggregation, which may promote intestinal persistence and competitive exclusion of pathogens, consistent with previous findings for *Leuconostoc mesenteroides* strains from fermented foods (de Paula *et al.,* 2014). Strong-to-moderate coaggregation with enteric pathogens further indicated their potential to limit pathogen adhesion and colonization. These observations align with earlier studies emphasizing the role of probiotic coaggregation in enhancing gut health and preventing enteropathogenesis (Schifano *et al*., 2021).

Conclusion

The present study demonstrates that fishtail palm toddy and banana pseudo-stem sap are non-dairy, plant-based, indigenous fermented beverages harbouring *Leuconostoc mesenteroides* strains with strong probiotic potential. The isolates showed tolerance to simulated gastrointestinal conditions, non-haemolytic behaviour, antibiotic susceptibility, pathogen inhibition and favourable adhesion-related properties. Among the isolates, FBN7 and BPS4 exhibited

superior performance and warrant further investigation as potential starter cultures, bio-preservatives, and probiotic adjuncts. These findings scientifically validate the microbial value of traditional fermentation practices and support their documentation and conservation. However, whole-genome safety assessment, in vivo validation, technological evaluation and food-matrix compatibility studies are essential before commercial application.

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Conflict of interest

The authors declare no conflict of interest

Data availability

All data are available on reasonable request to the corresponding author

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