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Weissella spp. from traditionally fermented ponni rice: Exopolysaccharide production and probiotic attributes for starter culture development

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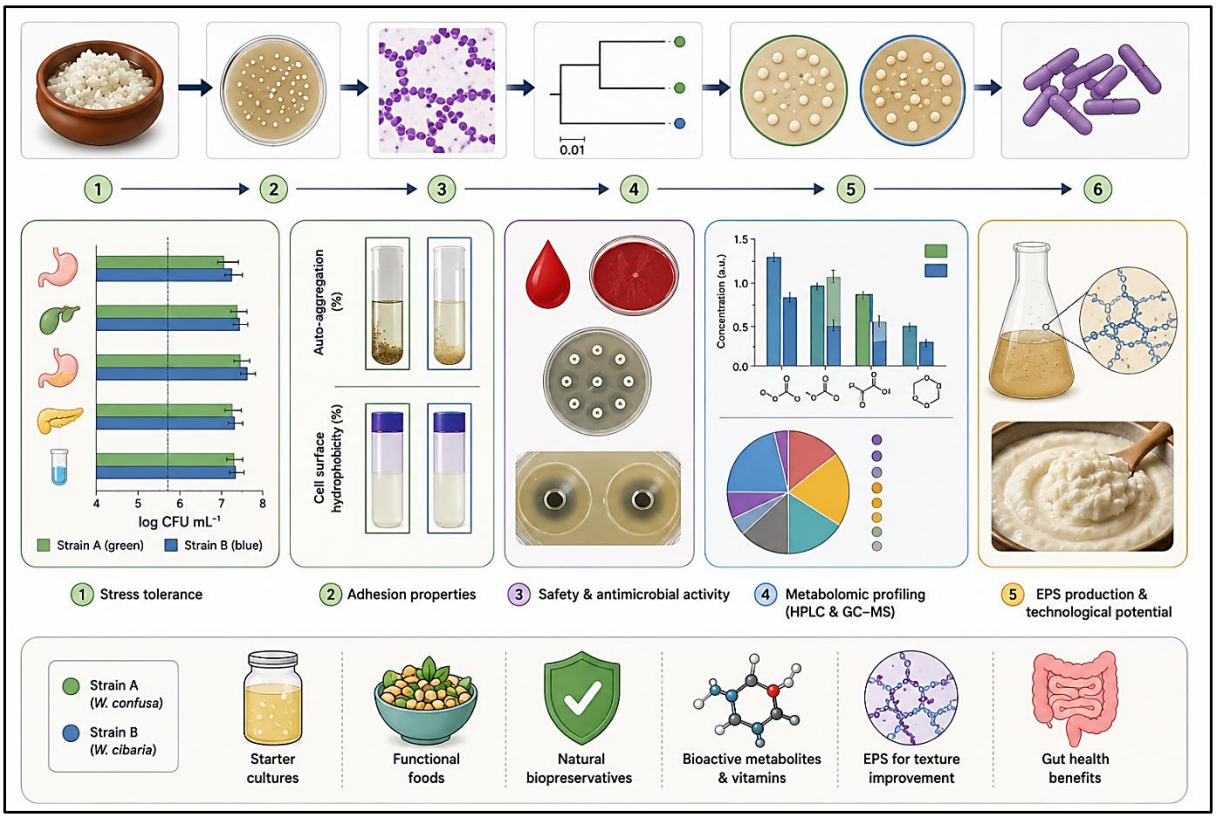
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Abstract

Probiotic autochthonous bacteria isolated from fermented foods represent potential candidates for functional starter cultures development. In the curent study, *Weissella confusa* and *Weissella cibaria* were identified as lactic acid bacterial isolates from traditionally fermented Ponni rice using 16S rRNA gene sequencing. Both isolates showed probiotic properties, efficient maintaining viable bacterial populations of > 5.8 log CFU mL^{-1}under acidic conditions (pH 3.0), bile salts, simulated gastric juice, and pancreatin. *W. cibari*a exhibited greater tolerance to gastrointestinal stress. *W. confusa* had significantly higher auto-aggregation (43.65 ± 0.06%), cell surface hydrophobicity (37.76 ± 0.32%) and exopolysaccharide production (165.95 ± 2.34 mg glucose equivalent L^{-1}). Both isolates were found to be γ-haemolytic, in terms of their susceptibility to clinically relevant antibiotics, and produced heat-stable antimicrobial metabolites with inhibition zones measuring ~18-25 mm against *Staphylococcus aureus* and *Salmonella typhi*. HPLC analysis indicated the production of lactic, acetic acid (~1.0 g L^{-1}) tartaric acid (~0.2gL^{-1}) and vitamin profiling suggested the presence of pantothenic acid (vitamin B5) in both *Weissella* isolates. GC-MS analysis of *W. confusa* indicated that the isolate is capable of producing several bioactive metabolites, such as azulene (19.85%), tetradecanoic acid (15.58%), cis-11-eicosenamide (11.45%), and pentadecanoic acid (4.10%). These results indicate that the two autochthonous *Weissella* isolates show complementary probiotic and metabolic characteristics, with *W. confusa* showing better exopolysaccharide production and metabolite diversity, which validates its use as a multifunctional probiotic starter culture and natural bio preservative for cereal-based fermented foods.

Keywords: *Weissella confusa*; *Weissella cibaria*; fermented Ponni rice; probiotics; metabolomics; functional foods.



Graphical abstract. Workflow illustrating the isolation, molecular identification, probiotic characterization, metabolomics profiling, exopolysaccharide production, and technological potential of *Weissella* isolates.

Introduction

Fermented foods are enriched with beneficial microorganisms that enhance food quality and produce bioactive metabolites with significant health benefits (Carvalho & Conte-Junior, 2023; Hasan et al., 2014). Regular consumption of fermented foods improves the composition of the gut microbiota, intestinal health, immune modulation, and reduces the risk of gastrointestinal disorders (D. Kim et al., 2026; Leeuwendaal et al., 2022; Padhi et al., 2024). Lactic acid bacteria (LAB) are the most abundant microbiota in fermented foods and are of interest as starter cultures and probiotics because of their ability to produce bioactive metabolites that enhance the quality and functionality of foods (Khan & Ahmad, 2020). *Lactobacillus*, *Lacticaseibacillus, Lactiplantibacillus,* and *Bifidobacterium* are commonly used commercial probiotics (Kaewarsar et al., 2023; J. Lee et al., 2024; Saarela et al., 2002). However, many strains have variable gastrointestinal survivability, limited colonization efficiency, and inconsistent functional performance, resulting in compromised probiotic efficacy and limited application in diverse food matrices (Lau & Quek, 2024; Palanivelu et al., 2022). Hence, the search for indigenous probiotic microorganisms with superior functional properties and greater adaptability is inevitable (Da Silva Vale et al., 2023).

Weissella, a genus within the family Leuconostocaceae in the order Lactobacillales, has received considerable attention as an emerging probiotic genus owing to its ecological diversity, metabolic versatility, and functional probiotic potential (Fusco et al., 2015; Teixeira et al., 2021). Recent studies have reported the functional diversity and biological characteristics of *Weissella strains,* including antioxidant, immunomodulatory, cholesterol-regulatory, antimicrobial, and exopolysaccharide-producing properties (Fhoula et al., 2018, 2022; Unnikrishnan, 2021; Unnikrishnan & Sreeja, 2025). However, these beneficial properties are specific to each strain and hence, a thorough evaluation of their functional characteristics and safety is essential prior to their exploitation in functional food and probiotic applications. Despite their promising probiotic potential, the widespread use of *Weissella* is limited by the lack of recognition from major regulatory agencies and hence requires strict strain-specific safety assessments, especially their haemolytic activity and antibiotic susceptibility (Fhoula et al., 2022; Liu et al., 2025).

The primary focus of research on *Weissella* has been on isolates from fermented vegetables, dairy products, sourdough, kimchi, and fermented meat products (Bian et al., 2020; J. K. Lee et al., 2023). Earlier studies have reported *Weissella confusa* from Ranjit rice (S. Nath et al., 2020; Tohno et al., 2012) and probiotic LAB, including *Pediococcus pentosaceus* and *Lactobacillus plantarum*, from Ponni rice (Kowsalya et al., 2022; Ouili et al., 2025). However, studies on the integrated probiotic, safety, antimicrobial, and metabolomic characterization of autochthonous *Weissella* species isolated from traditionally fermented Ponni rice are still limited. The identification of strain-specific metabolites may facilitate the unearthing of novel bioactive compounds associated with probiotic functionality and thus provide mechanistic support for probiotic selection (S. Nath et al., 2020; Verma & Rout, 2022). This knowledge deficit necessitates the systematic isolation and strain-specific characterization of autochthonous *Weissella* to identify safe and functionally superior probiotic candidates for food biotechnology and functional food applications (Apostolakos et al., 2022; Chaichana et al., 2026).

In this context, the present study focused on isolating and identifying autochthonous *Weissella* spp. from traditionally fermented Ponni rice to extensively evaluate their probiotic potential. To the extent of our knowledge, this is one of the first comprehensive studies to report the isolation and identification of *Weissella* species from traditionally fermented Ponni rice (S. Nath et al., 2020; Tohno et al., 2012; Zhang et al., 2026). The study will contribute to the current understanding of *Weissella* species from cereals and support their further use as multifunctional probiotic cultures, natural bio preservatives, and functional starter cultures for future fermented foods.

2. Materials and Methods

2.1 Materials and Chemicals

All microbiological media, analytical-grade chemicals, and reagents used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), unless specified otherwise. HPLC-grade solvents (ethanol and sulphuric acid) were purchased from SRL Chemicals (Mumbai, India). Organic acids (lactic, citric, acetic, and tartaric acids, and ethanol) were used for HPLC calibration. *Lactiplantibacillus plantarum* (MTCC 1746), procured from the Microbial Type Culture Collection (MTCC), CSIR–Institute of Microbial Technology, Chandigarh, India, was used as the reference probiotic strain.

2.2 Collection of Fermented Ponni Rice Samples and Isolation of Lactic Acid Bacteria

LAB were obtained by fermenting freshly cooked parboiled *Oryza sativa* subsp. *indica* (Ponni rice) overnight in potable water at room temperature. Fermented samples were collected aseptically, homogenized, serially diluted, and plated on MRS agar. After incubation at 37°C for 48 h, morphologically different colonies were cultured to obtain pure colony. Pure colonies were maintained on MRS agar plates at 4°C and routinely sub cultured in MRS broth for further analyses. Standard microbiological methods were used for the isolation and screening of probiotic LAB from fermented cereal-based foods (Makut et al., 2022; Tohno et al., 2013).

2.3 Biochemical and Molecular Characterization of the Isolates

2.3.1 Morphological and Biochemical Characterization

The purified isolates were examined for colony morphology, gram staining, and microscopic characteristics. Standard biochemical assays for preliminary identification were performed for indole production, methyl red (MR), Voges-Proskauer (VP), triple sugar iron (TSI) utilization, motility, and starch hydrolysis, according to the criteria described in Bergey's Manual of Systematic Bacteriology (Rahman et al., 2021; Roy et al., 2023). Further growth characteristics were studied on selective and differential media such as MacConkey agar, Eosin Methylene Blue (EMB) agar, and Milk Cetrimide agar. Comparative phenotypic analysis was performed using *L. plantarum* (MTCC 1746) as the reference strain.

2.3.2 Growth Curve Analysis

The growth kinetics of the selected isolates and reference strain were studied under standard culture conditions. Pure colonies cultivated on MRS agar were inoculated into 5 mL of MRS broth and incubated overnight at 37°C under shaking condition (150 rpm). The overnight cultures were then transferred to 100 mL of fresh MRS broth. The OD was measured at 600nm for every 2 h from the inoculation till the end in order to monitor the growth profile of the

bacteria. Growth curves were plotted to compare the growth characteristics of the isolates with those of the reference strain (Kumoro et al., 2020; Mahrous et al., 2013).

2.3.3 Molecular Identification by 16S rRNA Gene Sequencing

Molecular characterization of the selected isolates was performed by partial sequencing of 16S rRNA gene. Genomic DNA template was obtained by suspending fresh bacterial colonies in sterile distilled water, followed by thermal lysis at 100°C for 1 min. The 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′). PCR products were purified according to the manufacturer's protocol using the HiPurA PCR Product Purification Kit and subjected to Sanger sequencing. The sequences obtained were compared with the NCBI database using the BLAST algorithm for preliminary identification. Representative sequences were aligned using ClustalW in MEGA version 11, and phylogenetic relationships were inferred using the neighbor-joining (NJ) method with 1,000 bootstrap replicates (Matsumoto & Sugano, 2013; Nasika & Runthala, 2021; Polatoglou et al., 2025).

2.4 Evaluation of Probiotic Properties

2.4.1 Acid Tolerance

Acid tolerance was evaluated by suspending overnight bacterial cultures in 5 mL phosphate-buffered saline (PBS) adjusted to pH 3.0 and 7.0 using sterile 1 N HCl. Each tube was inoculated with 0.5 mL of the culture and incubated at 37°C for 3 h. At 1-h intervals, 100 µL aliquots were spread onto MRS agar plates and incubated at 37°C for 24–48 h. Viable cells were enumerated as colony-forming units (CFU mL^{-1}), and acid tolerance was determined by comparing bacterial survival at pH 3.0 and pH 7.0 (Amin et al., 2023).

2.4.2 Bile Salt Tolerance

Bile salt resistance of the isolates was assessed after exposure to physiological concentration of bile salts. Overnight cultures were inoculated into MRS broth supplemented with 0.3% (w/v) bile salts. MRS broth without bile was used as the control. The cultures were grown at 37°C with shaking. Samples (50-100 µL) were collected immediately after inoculation and after 3 h, serially diluted, and spread on MRS agar plates. Viable colonies were counted after incubation at 37°C for 24-48 h and expressed as CFU mL^{-1}. Bacterial survival in bile-containing medium was compared to that in the control medium (Xue et al., 2026).

2.4.3 Sodium Chloride (NaCl) Tolerance

The osmotic stress tolerance of the isolates was determined by culturing the isolates in MRS broth containing 0.5%, 1%, and 5% (w/v) NaCl. MRS broth without NaCl supplementation was used as the control. The inoculated cultures were incubated at 37 °C for 24 h, followed by quantification of bacterial growth by measuring the optical density at 600 nm using a UV–Visible spectrophotometer. The ability of the isolates to withstand osmotic stress was assessed by comparing their growth in NaCl-supplemented media with that of the (T. Nath et al., 2021).

2.4.4 Pancreatin Tolerance

The resistance of the isolates to digestive enzymes was assessed in pancreatin-supplemented medium. Overnight bacterial cultures were inoculated into MRS broth with 5% pancreatin. MRS broth without pancretin was used as the control. The cultures were shaken at 37°C for

24h. After incubation, 100 µL aliquots were spread on MRS agar plates and incubated for 24–48 h. Viable colonies were counted using a colony counter, and bacterial survival rate was expressed as CFU mL^{-1} (Pan et al., 2009).

2.4.5 Simulated Gastric Juice Tolerance

Tolerance to simulated gastric juice was determined using an artificial gastric solution composed of 3 g L^{-1} pepsin, 7 mM KCl, 45 mM $NaHCO_3$, and 125 mM NaCl. From the overnight cultures, cell pellets were washed and resuspended in 0.1 M PBS (pH 6.6) (centrifugation at 5000 rpm for 15 min). A 500 µL aliquot of the bacterial suspension was transferred into 5 mL of simulated gastric juice. The bacterial suspension inoculated into MRS broth without simulated gastric juice was used as control. Samples were collected immediately following inoculation and after 3 h of incubation at 37°C. Appropriate dilutions were plated on MRS agar and incubated for 24–48 h. The viable colonies were enumerated as follows. (Hassan et al., 2020).

The survival percentage was calculated using the following equation:

$$\text{Survival rate (\%)} = \frac{\text{CFU}_a}{\text{CFU}_c} \times 100$$

Where CFU_a = viable count obtained after exposure to simulated gastric juice and CFU_c = viable count in the control.

2.4.6 Cell Surface Hydrophobicity

The potential for bacterial adhesion to intestinal epithelial surfaces was indirectly assessed by determining the hydrophobicity of the cell surfaces. The overnight cultures were centrifuged at 6000 rpm for 10 min, and the cell pellets were washed twice with 0.1 M PBS (pH 6.6) and resuspended in 5 mL of the same buffer. The initial absorbance of the bacterial suspension was measured at 600 nm (A_0). Then, 3 mL of the suspension was mixed with 1 mL of hexane, vigorously vortexed for 2 min, and allowed to stand for 1 h for phase separation. The absorbance of the aqueous phase (A_1) was measured at 600 nm (Jacobsen et al., 1999).

The cell surface hydrophobicity was determined using the following equation:

$$\text{Cell Surface Hydrophobicity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

2.4.7 Cellular Auto-Aggregation Assay

Overnight cultures were centrifuged at 5000 rpm for 10 min and washed twice with PBS (pH 7.2), followed by resuspended in fresh PBS. The initial absorbance (A_0) of the suspension was measured at 600 nm. The suspensions were then incubated for 2 h at 37 °C to allow bacterial aggregation. The absorbance of the upper suspension (A_1) was measured at 600 nm after incubation (Sreenadh et al., 2022).

Auto-aggregation was determined using the following equation:

$$\text{Cellular Auto-aggregation (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

2.5 Evaluation of Safety Attributes

2.5.1 Haemolytic Activity

Fresh bacterial cultures were inoculated onto 5% sheep blood agar and incubated at 37°C for 48 h. The plates were examined for haemo lysis reactions around bacterial colonies. The isolates were β-haemolytic when complete lysis of erythrocytes produced a clear transparent zone, α-haemolytic when partial hemolysis produced a greenish discoloration around the colonies, and γ-haemolytic when no haemolytic zone was detected. Haemolytic activity was determined as recommended by the international probiotic evaluation guidelines (Krausova et al., 2019).

2.5.2 Antibiotic Susceptibility Assay

The selected *Weissella* isolates were subjected to antibiotic susceptibility test using the Kirby-Bauer disc diffusion method. Overnight cultures were spread evenly (approximately 10^8 CFU mL^{-1}) on Mueller–Hinton (MH) agar plates where the antibiotic discs were placed aseptically on the agar surface. The antibiotics tested were amoxicillin (10 µg), clindamycin (2 µg), erythromycin (10 µg), gentamicin (10 µg), penicillin (10 µg), and vancomycin (30 µg). The diameter of the inhibition zones (mm) was measured after incubation at 37°C for 24–48 h, and the susceptibility profiles were interpreted according to the established criteria for lactic acid bacteria (Han et al., 2024).

2.5.3 Antagonistic Activity of Cell-Free Culture Supernatant

The antagonistic activity of the selected isolates was determined using the agar well diffusion assay. The *Weissella* isolates and *L. plantarum* were cultured in 5 mL MRS broth at 37°C for 18–36 h. Cell-free supernatants (CFS) were obtained by centrifugation and filtered through 0.22 µm disc filter membrane. Portion of the CFS was boiled for 5 min for heat treatment. Environmental isolates of gram-negative *Salmonella typhi* (1×10^8 CFU mL^{-1}) and a food borne pathogen *Staphylococcus aureus* were used as indicator organisms. These indicator strains were spread on to Mueller–Hinton agar plates. Wells were made with a sterile cork borer, and 50–100 µL of untreated, filtered, and heat-treated CFS were added to each well, with sterile distilled water as the negative control. After incubation at 37°C for 24 h, the antagonistic or antimicrobial effect was determined by measuring the diameter of the zones of inhibition (mm) around each well (Sharma et al., 2025).

2.6 Analysis of Organic Acids

Organic acid and vitamin production by the selected *Weissella* isolates and reference strain was analyzed using high-performance liquid chromatography (HPLC). Cultures were grown in MRS broth at 37°C for 48 h, and the CFS of the strains obtained after centrifugation were filtered through 0.22 µm membrane disc filters. Organic acid profiling was performed using a Waters 2535 HPLC system equipped with a Waters 2414 refractive index detector and an Agilent Hi-Plex H column (300 × 7.8 mm, 8 µm) maintained at 60°C. The mobile phase consisted of 5 mM H_2SO_4 at a flow rate of 0.7 mL min^{-1} under isocratic conditions. Standard solutions of ethanol, lactic acid, acetic acid, citric acid, and tartaric acid (5 g L^{-1}) were used for calibration, and the organic acids were identified by comparing the retention times of the sample peaks with those of the corresponding standards (Y. Wang et al., 2022).

Vitamin production was performed using a Waters 2535 HPLC system equipped with a Waters 2998 photodiode array (PDA) detector and a C18 reversed-phase column. Standard solutions

of thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), and cyanocobalamin (B12) were used for the calibration. Vitamins were identified by comparing their retention times and UV absorption spectra with those of authenticated standards and quantified using the external standard method, with concentrations expressed as mg L^{-1} (Y. Wang et al., 2021).

2.7 GC–MS Analysis of Volatile Organic Compounds

The volatile metabolites produced by *W. confusa* 3 isolate were analyzed using gas chromatography–mass spectrometry (GC–MS). Cell-free culture supernatants obtained after 48 h of incubation were extracted with an organic solvent, concentrated, filtered through 0.22 µm membrane filters, and analyzed using GC–MS. Chromatographic separation was performed on an Agilent 8890 Gas Chromatograph coupled with an Agilent 5977B Mass Selective Detector fitted with a fused-silica capillary column. Helium was used as the carrier gas. The injector was used in the split mode, and mass spectra were obtained under electron impact (EI) conditions. Compounds were identified by comparing their mass spectra with those in the NIST Mass Spectral Library, and their relative abundances were calculated from the respective chromatographic peak areas (Cai et al., 2026; Tuccillo et al., 2022).

2.8 Production and Quantitative estimation of EPS

Overnight culture of *W. confusa* 3 (2%, v/v) was inoculated into 100 mL MRS broth supplemented with 2% (w/v) sucrose and incubated statically at 30°C for 48 h. Following incubation, the cultures were centrifuged at 8,000 × g for 15 min at 4°C to remove bacterial cells. Three volumes of chilled 95% ethanol were added to the cell-free supernatant, and the mixture was incubated overnight at 4°C to precipitate EPS. The precipitated EPS was collected by centrifugation at 10,000 × g for 20 min, dissolved in distilled water, and treated with an equal volume of 10% (w/v) trichloroacetic acid (TCA) for 2 h to remove residual proteins. After centrifugation, the supernatant was re-precipitated with three volumes of chilled ethanol, and the purified EPS pellet was dissolved in distilled water (Dubey & Jeevaratnam, 2015).

For quantitative estimation, 1 mL of purified EPS solution was mixed with 1 mL of 5% phenol, followed by the rapid addition of 5 mL of concentrated sulphuric acid. The reaction mixture was allowed to stand for 20 min at room temperature, and the absorbance was measured at 490 nm using a UV–visible spectrophotometer. Glucose (0–100 µg mL^{-1}) was used as the standard for calibration, and the EPS concentration was expressed as mg glucose equivalent (GE) L^{-1} of culture supernatant. All experiments were performed in triplicates (Moselhy et al., 2023; Yang et al., 2023).

2.9. Characterization of EPS

2.9.1. Qualitative detection of EPS by Congo red assay

Qualitative assessment of EPS production by *W. confusa* 3 was carried out using Congo red agar assay. Fresh overnight cultures were streaked on MRS agar supplemented with 5% (w/v) sucrose and 0.08 g L-1 Congo red and incubated at 37°C for 48 h. The colony morphology was observed visually after incubation. Colony positive for EPS production was identified by black mucoid appearance (Adesulu-Dahunsi et al., 2018).

2.9.2. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to study the morphology of purified EPS. Purified EPS after ethanol precipitation and freeze-drying were gently mounted onto aluminium specimen stubs using double-sided conductive carbon tape and subsequently sputter-coated with a thin layer of gold to enhance electrical conductivity. The samples were analysed with a TESCAN VEGA3 scanning electron microscope at an accelerating voltage of 10 kV. Micrographs at different magnifications were taken to study the surface morphology, porosity and structural organization of the EPS matrix (Yu et al., 2023).

2.9.3. Fourier-transform infrared (FTIR) spectroscopy

FTIR spectroscopy was used to determine the characteristic functional groups of the purified exopolysaccharide (EPS). The EPS sample was dried completely to remove any residual moisture and ground to a fine powder. The dried sample was mixed with spectroscopic grade potassium bromide (KBr) and pressed into a transparent pellet. The FTIR spectrum was obtained on a PerkinElmer Spectrum 100 FTIR spectrometer in the mid-infrared (MIR) configuration with the KBr accessory. The spectrum was recorded in the range 4000-450 cm-1 with a resolution of 4 cm-1. The wavenumbers of the absorption bands were used to assign the corresponding EPS functional groups (Bhowmik et al., 2025).

2.10 Statistical Analysis

All experiments were performed in triplicates, and the results are presented as the mean ± standard deviation (SD). Statistical analysis were performed using IBM SPSS Statistics (Version 27.0). Differences among experimental groups were evaluated using one-way analysis of variance (ANOVA), followed by an appropriate post hoc multiple comparison test to determine statistically significant differences between treatment means. A probability value of $p < 0.05$ was considered statistically significant throughout the study. Graphical representations were generated using OriginPro 2024 software.

3. Result and Discussion

3.1 Isolation and preliminary identification of bacterial isolates

Bacterial isolates present in overnight-fermented Ponni rice samples were recovered by serial dilution and plated on MRS agar for the isolation of lactic acid bacteria (LAB). Approximately 50 morphologically distinct colonies were obtained and evaluated for their antagonistic activity against food borne bacterial pathogens. Preliminary screening identified 10 isolates exhibiting potential antagonistic activity, which were tentatively assigned to the genera *L. plantarum* (n = 4), *Streptococcus* (n = 2), and *Weissella* (n = 4) based on colony morphology and preliminary phenotypic characteristics. From the four presumptive *Weissella* isolates, two isolates (*W. confusa*3 and *W. cibaria*4) with stable colony morphology, reproducible phenotypic features after successive subculturing, and consistent antagonistic activity were selected for detailed biochemical, molecular, and functional characterization. The selected *Weissella* isolates produced small, circular, mucoid, creamy white colonies on MRS agar, consistent with the colony morphology previously described for presumptive *Weissella* isolates (Fig.1) (Makut et al., 2022).

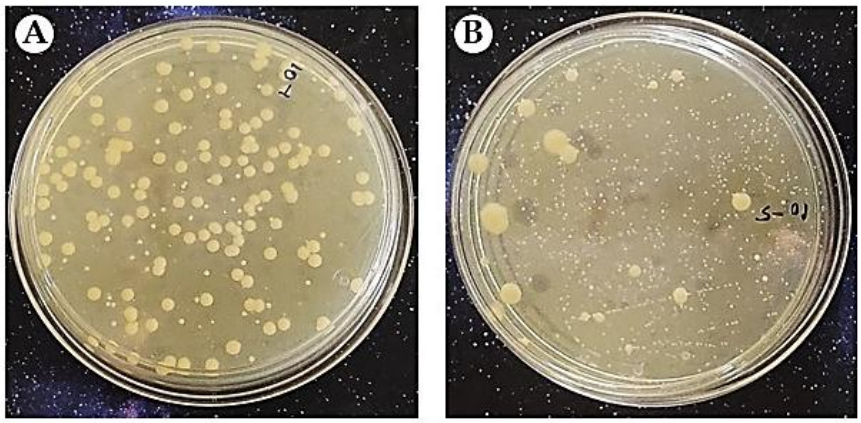


Figure 1. Pure colonies of *Weissella* isolates recovered from fermented Ponni rice on MRS agar. **(A)** *W. confusa* 3. **(B)** *W. cibaria* 4.

Microscopic examination following gram staining showed that both isolates were gram-positive, non-motile and exhibited coccal and coccobacillary morphologies, respectively (Fig. 2). Both selected isolates were aerotolerant facultative anaerobes and exhibited consistent growth at 30 and 37 °C under static and shaking culture conditions, demonstrating their physiological adaptability to cereal fermentation environments (Lin et al., 2020). These preliminary morphological and physiological characteristics facilitated their subsequent biochemical analyses.



Figure 2. Gram-stained morphology: **(A)** Control strain (*L. plantarum*), **(B)** FPRW 3 showing coccal morphology, and **(C)** FPRW 4 showing coccobacillary morphology.

Table 1 presents the biochemical characteristics of *W. confusa* 3, *W. cibaria* 4, and the reference probiotic strain (*L. plantarum*). Both isolates and the reference strain were positive for methyl red and triple sugar fermentation but negative for Voges-Proskauer, MacConkey agar, EMB agar, and milk cetrimide agar tests. *W. confusa* 3 differed from *W. cibaria* 4 and *L. plantarum* by exhibiting positive reactions in the indole and starch hydrolysis tests. *W. cibaria* 4 exhibited a biochemical profile similar to that of the reference strain, except for its coccobacillary morphology. The biochemical characteristics obtained in this study are consistent with those of *Weissella* isolates obtained from fermented foods (Liu et al., 2024). In particular, the positive starch hydrolysis by *W. confusa* 3 was in contrast to the negative starch hydrolysis reported by *Weissella* sp. isolated from fermented foods, indicating strain-specific metabolic differences among cereal-derived isolates (Unnikrishnan, 2021).

Table 1: Biochemical characteristics of the LAB isolated from fermented Ponni rice

Test	*L. plantarum* (Control)	FPRW 3	FPRW 4
Gram stain	G^{+ve}, cocci	G^{+ve}, coccal	G^{+ve}, coccobacilli
Indole test	–	+	–
Methyl red	+	+	+
Voges–Proskauer	–	–	–
Motility test	Non-motile	Non-motile	Non-motile
Triple sugar assay	+	+	+
MacConkey agar growth	–	–	–
EMB agar growth	–	–	–
Starch hydrolysis	–	+	–
Milk cetrimide agar growth	–	–	–

3.2 Molecular identification

The 16S rRNA gene sequences of *W. confusa* 3 and *W. cibaria* 4 were submitted to the NCBI GenBank database under the accession numbers OR826681.1 (VGSASTRA3) and OR826684.1 (VGSASTRA4) respectively. The phylogenetic relationship between the two autochthonous *Weissella* isolates were inferred using the 16S rRNA gene sequences together with representative *Weissella* reference strains retrieved from the NCBI database, using *L. plantarum* as the outgroup (Fig. 3A). The topology clearly resolved the sequences into distinct clades corresponding to the recognized *Weissella* species with moderate to high bootstrap support (22–100%). The isolate VGSASTRA3 (*W. confusa* 3) clustered closely with the *W. confusa* lineage, whereasVGSASTRA4 (*W. cibaria* 4) grouped with the *W. cibaria* lineage. Both isolates formed a well-supported subclade with a bootstrap value of 100%, indicating a close evolutionary relationship between the two isolates, despite their assignment to different species. This subclade was positioned adjacent to the reference strains *W. confusa* NR_113258.1, *W. confusa* NR_040816.1 and *W. cibaria* NR_036924.1, which were supported by bootstrap values of 97% and 93%, confirming the taxonomic placement of the isolates within the genus *Weissella*. The remaining *Weissella* species formed separate clusters, with *W. diestrammenae*, *W. kandleri* and *W. coleopterorum* constituting one lineage, whereas *W. soli*, *W. muntiaci* and *W. oryzae* formed another distinct lineage. *L. plantarum* was clearly separated from the *Weissella* clade, validating its suitability as an outgroup. The relative similarity of these isolates to their respective reference strains validates the molecular identification of these isolates by BLAST analysis and confirms the presence of genetically authentic *Weissella* members in fermented Ponni rice. This outcome aligns with prior studies that have reported these two species to have high 16S rRNA sequence similarity and often cluster together in phylogenetic analyses because of the conserved nature of the 16S rRNA gene (Paul, 2023).

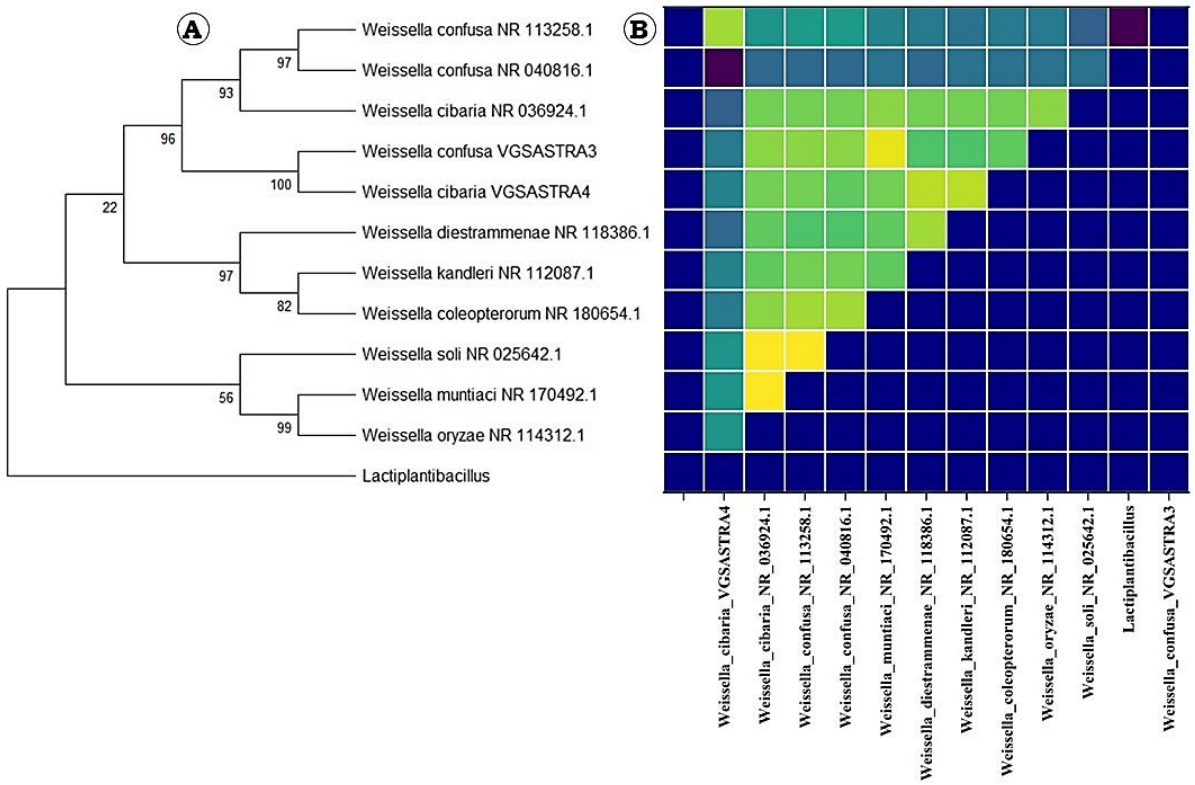


Figure 3. **(A)** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the taxonomic position of *W. confusa* 3 (VGSASTRA3) and *W. cibaria* 4 (VGSASTRA4) with respect to reference species of the genus *Weissella*. Bootstrap (%) values are shown at the nodes. *Lactiplantibacillus* sp. was used as an outgroup. **(B)** Heatmap shows pairwise sequence similarity of the *Weissella* isolates and reference strains based on 16S rRNA gene sequence analysis. Warmer colours represent higher similarity.

3.2.1 Heatmap analysis

The pairwise sequence similarity heatmap (Fig. 3B) supports the phylogenetic analysis by demonstrating the genetic relationships between the selected taxa. Darker blue colours represent lower sequence similarity, while progressively lighter green and yellow colours represent higher sequence similarity. The heatmap revealed a high sequence similarity between VGSASTRA3 and VGSASTRA4, presumably resulting from their close clustering in the phylogenetic tree. Similarly, high sequence similarity was found between both isolates and the reference strains *W. confusa* NR_113258.1, *W. confusa* NR_040816.1 and *W. cibaria* NR_036924.1, confirming their close evolutionary relationship. Relatively lower sequence similarity were found between the isolates and more distantly related *Weissella* species including *W. diestrammenae*, *W. kandleri*, *W. coleopterorum*, *W. muntiaci*, *W. oryzae and W. soli*. A significant variation was observed between the *Weissella* isolates and the outgroup *L. plantarum*, which was also represented by the lowest sequence similarity. The heatmap was an additional quantitative presentation of the pairwise genetic relationships of the selected taxa. The high sequence similarity obtained between the two isolates and the corresponding reference strains further support the phylogenetic results and demonstrate high sequence conservation within the genus. Conversely, the lower sequence similarity between the isolates and other *Weissella* species, the greater the evolutionary divergence, and the distinct separation from *L. plantarum* confirms the taxonomic distinction between these two genera.

3.2.2 Bacterial growth curve

The bacterial growth profiles of *W. confusa* 3, *W. cibaria* 4, and the reference strain *L. plantarum* showed characteristic bacterial growth behaviour with lag, exponential, and stationary phases (Fig. 4). All three strains showed a short period of adaptation (lag phase, 0-2 h) before active growth. *L. plantarum* showed rapid growth, with an evident exponential phase (2-12 h) and an earlier entry into the stationary phase (12-36 h) than *Weissella* isolates. In contrast, *W. confusa* 3 exhibited a distinct exponential phase (2-18 h) and a stable stationary phase (18-36 h). *W. cibaria* 4 showed relatively slow growth with a long exponential phase (2-30 h) and a gradual stationary phase (30-36 h). None of the strains showed an appreciable decline in growth during the incubation period, indicating the maintenance of cell viability under the experimental conditions. *W. confusa* 3 showed better growth performance than *W. cibaria* 4, although both isolates showed growth characteristics consistent with those reported for probiotic *Weissella* strains (Quattrini et al., 2020). Similar strain-dependent variations in growth kinetics have been reported for *Weissella* isolates from fermented foods, where differences in adaptation, growth rate, and biomass accumulation reflect intrinsic physiological and metabolic diversity (Chaichana et al., 2026). Nath et al., (S. Nath et al., 2020) observed that *W. confusa* isolated from fermented sour rice exhibited efficient growth and a prolonged stationary phase under optimal culture conditions, supporting its probiotic potential. Similarly, Rahman et al., (Rahman et al., 2021) and Lakra et al., (Lakra et al., 2020) observed strain-specific growth behaviour in *W. confusa* and *W. cibaria*, respectively, although they belong to the same genus. In the present study, the growth kinetics demonstrated that both isolates can achieve rapid biomass accumulation, supporting their suitability for industrial starter culture production.

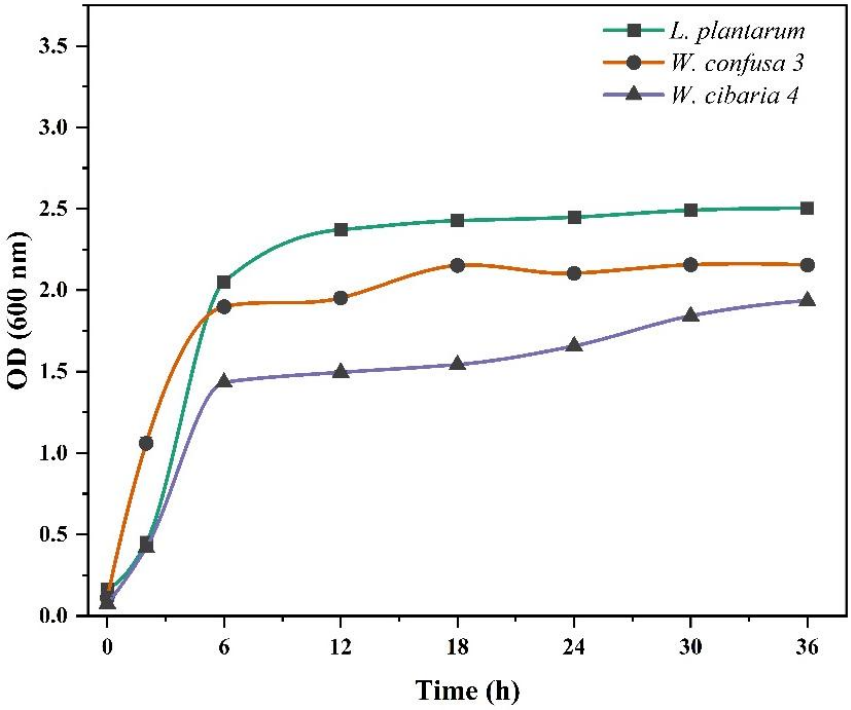


Figure 4. Growth curves of *W. confusa* 3, *W. cibaria* 4 and the reference probiotic strain *L. plantarum* in MRS broth at 37 ºC. Bacterial growth was monitored by measuring optical density at 600 nm over a 36-h incubation period.

3.3 Evaluation of probiotic properties

3.3.1 Tolerance to simulated gastrointestinal conditions

The probiotic characteristics of the selected autochthonous *Weissella* isolates was evaluated by assessing their tolerance to acidic pH, bile salts, sodium chloride, and simulated gastric juice (Fig. 5). All three strains maintained viable populations above 5.8 log CFU mL^{-1} under the evaluated gastro intestinal stress conditions, indicating their ability to withstand conditions simulating passage through the upper gastro intestinal tract. However, both *Weissella* isolates showed similar tolerance with only strain-dependent differences, whereas *L. plantarum* consistently showed the highest survival rate. A marginal reduction in viable counts was observed after exposure to acidic conditions (pH 3.0) for 2 h. *L. plantarum* showed the highest viability (6.17 ± 0.00 log CFU mL^{-1}), followed by *W. confusa* (5.98 ± 0.00 log CFU mL^{-1}) and *W. cibaria* (5.94 ± 0.01 log CFU mL^{-1}) (Fig. 5A), indicating that both autochthonous isolates can effectively tolerate gastric acidity. Similarly, exposure to 0.3% bile salts resulted in slight reductions in viability, with *W. cibaria* (5.96 ± 0.03 log CFU mL^{-1}) showing a higher tolerance to bile salts than *W. confusa* (5.88 ± 0.02 log CFU mL^{-1}) (Fig. 5B). Bacterial growth under osmotic stress gradually decreased with increasing NaCl concentrations. *W. cibaria* thrived better at low to moderate salinity (0–1% NaCl), and *L. plantarum* had the highest growth at 5% NaCl (Fig.5C). *W. cibaria* demonstrated marginally higher tolerance than *W. confusa*, as viable counts were negligibly reduced (0.03–0.07 log CFU mL^{-1}) when exposed to simulated gastric juice (Fig. 5D).

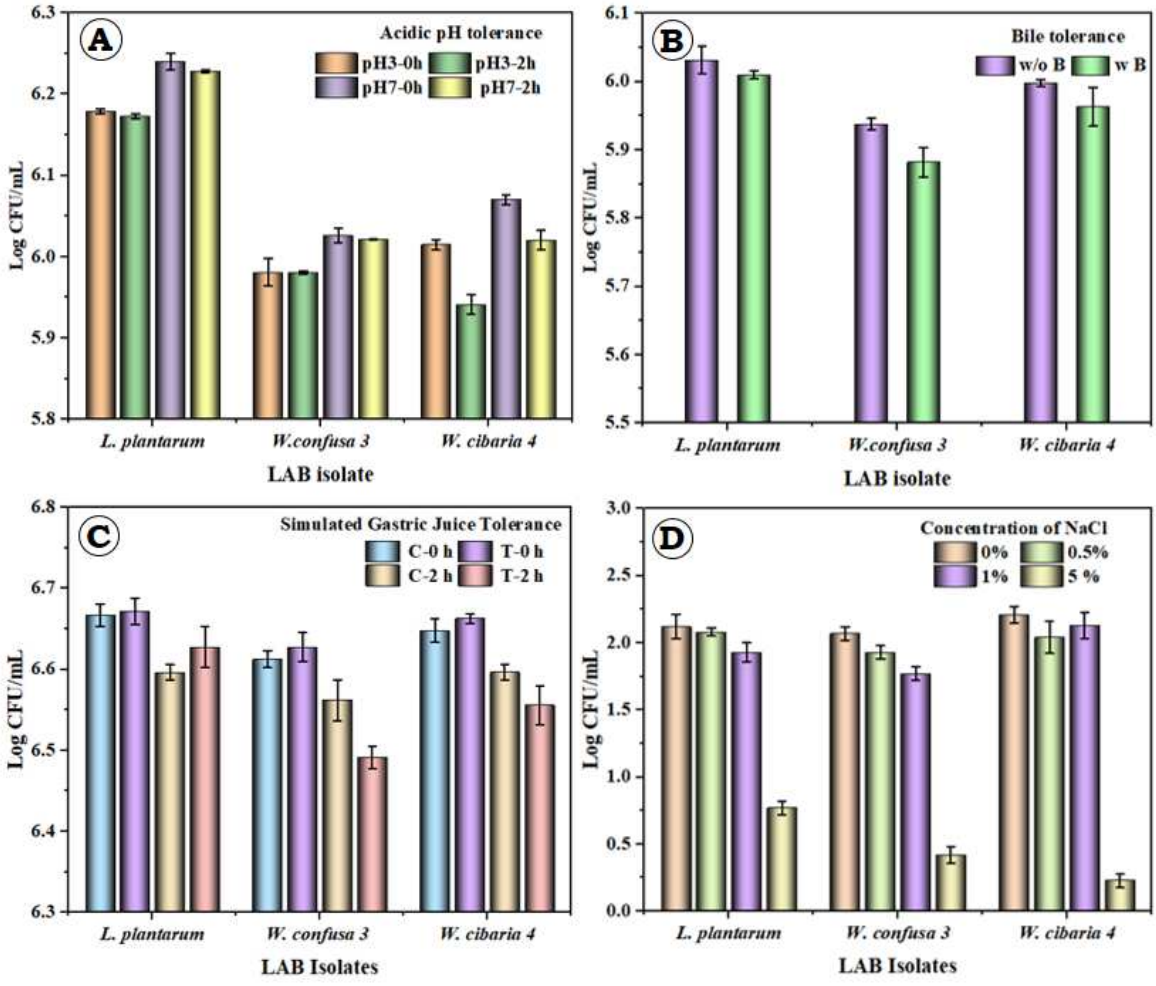


Figure 5. Evaluation of probiotic stress tolerance of *W. confusa* 3 and *W. cibaria* 4 compared with *L. plantarum* **(A)** Acid tolerance at pH 3 and pH 7 after 0 and 2 h of incubation. **(B)** Bile salt tolerance in the absence (w/o B)

and presence (w B) of bile salts. **(C)** Survival in simulated gastric juice after 0 and 2h of incubation. **(D)** Growth under different NaCl concentration (0, 0.5, 1 and 5%). Data is presented as mean ± standard deviation.

A prerequisite to successful gastrointestinal transit and transient colonization is the capacity of probiotic bacteria to endure acidic pH, bile salts, and gastric enzymes (Azhar et al., 2020). The high survival rates of both *Weissella* isolates are consistent with previous findings that reported cereal-derived *Weissella* strains as promising probiotic candidates that can maintain their viability under simulated gastrointestinal conditions (Fusco et al., 2023; Reyes-Portillo et al., 2025). Acid tolerance in LAB is associated with the maintenance of intracellular pH homeostasis, F_0F_1-ATPase system activation, amino acid decarboxylation pathways, and stress-response proteins that minimize acid-induced cellular damage (J. Wang et al., 2025). Similarly, bile salt resistance has been associated with membrane adaptation, bile salt hydrolase activity, and metabolic reprogramming that maintain membrane integrity under detergent stress (Qin et al., 2025). The higher bile and gastric tolerance of *W. cibaria* is another example of the strain specificity of probiotic functionality within the genus *Weissella*. Moderate tolerance to high NaCl concentrations also supports the technological suitability of these isolates for cereal-based fermentations, where osmotic stress commonly occurs during processing (Huang et al., 2022; M. Kim et al., 2018). The tolerance of the isolates to gastrointestinal stress is a general indication of their suitability for use as probiotic starter cultures (Oana et al., 2023).

Exposure to pancreatin had only a marginal effect on bacterial viability during the incubation period. The 48 h viable counts were 7.17 ± 0.02, 6.99 ± 0.01, and 6.97 ± 0.04 log CFU mL^{-1} for *L. plantarum*, *W. cibaria* 4 and *W. confusa* 3, respectively (Fig. 6A). All strains were metabolically active during the incubation period, suggesting that they were capable of tolerating intestinal digestive enzymes, an important characteristic of probiotic bacteria since contact with pancreatic enzymes is the last barrier to colonization in the intestine (J. Kim et al., 2025). The long-term survival of both autochthonous *Weissella* isolates is consistent with recent findings showing that probiotic *W. cibaria* strains retain metabolic activity and functional properties after treatment with pancreatin (Rahman et al., 2021; Unnikrishnan & Sreeja, 2025). *L. plantarum* is the most resistant reference strain; however, the similar performance of *W. cibaria* and *W. confusa* further confirmed their ability to with stand sequential gastrointestinal stress, thus strengthening their probiotic potential.

Figure 6B shows the cell surface hydrophobicities of the selected LAB strains. The highest hydrophobicity was observed for *W. confusa 3* (37.76 ± 0.32%), followed by *W. cibaria 4* (36.91 ± 0.15%) and *L. plantarum* (35.19 ± 0.21%). The difference between the two *Weissella* isolates was relatively low, with *W. confusa* presenting approximately 2.3% higher hydrophobicity than *W. cibaria*. Both isolates had hydrophobicity values slightly higher than those of the reference strain. The hydrophobicity of bacterial cell surfaces is defined as the affinity of bacterial cells for hydrophobic surfaces and is often used as an indirect indicator of their ability to interact with intestinal epithelial cells (Shalsh et al., 2023). The cell surface hydrophobicities is modulated by several cell surface components, such as lipoteichoic acids, surface proteins, and extracellular polysaccharides, which together contribute to bacterial adhesion and persistence (González-García et al., 2022; Pugazhendhi et al., 2022). However, hydrophobicity assays provide only indirect evidence of adhesion and should be complemented with epithelial cell adhesion studies. The hydrophobicity results, coupled with the good gastrointestinal stress tolerance observed in the present study, further substantiate the probiotic capability of both autochthonous *Weissella* isolates. Figure 6C shows the auto aggregation

abilities of the selected LAB strains. Among the strains tested, *W. confusa* 3 showed the highest auto aggregation (43.65 ± 0.06%), followed by *W. cibaria* 4 (39.71 ± 0.35%) and *L. plantarum* (38.32 ± 0.07%). *W. confusa* 3 showed an aggregation ability of about 9.9% higher than *W. cibaria* 4, and both autochthonous isolates presented aggregation capacities similar or higher than the reference probiotic strain. Auto aggregation is considered an essential functional property of probiotic bacteria because it allows self-association of bacteria, formation of biofilms, and may increase persistence in the gastrointestinal tract by decreasing the colonization of pathogens (Horrocks et al., 2023; Shu et al., 2023). The better aggregation of *W. confusa* 3 suggests stronger cell-to-cell interactions that may be associated with differences in extracellular polysaccharides and surface-associated proteins. The higher hydrophobicity exhibited by *W. confusa* 3 is in accordance with its higher ability to auto aggregate, which indicates improved surface-associated properties that could be advantageous for initial adhesion to the intestinal mucosa. Similar strain-dependent differences in auto aggregation have also been described for cereal-derived *Weissella* isolates, suggesting a wide variation in adhesion-related properties even within the same species (Krausova et al., 2019).

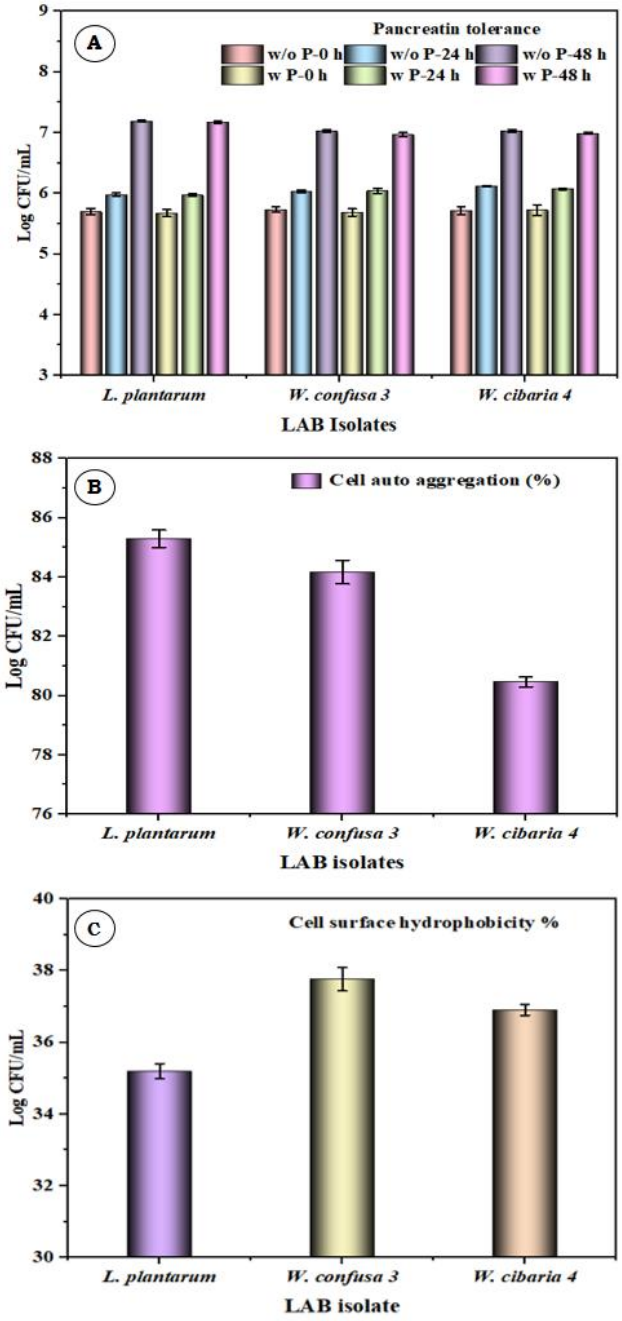


Figure 6. Assessment of probiotic of *W. confusa 3* and *W. cibaria 4* in comparison with *L. plantarum.* **(A)** Pancreatin tolerance determined after 0, 24 and 48h of incubation in the absence (w/o P) and presence (w P) of pancreatin. **(B)** Auto-aggregation ability of the LAB isolates. **(C)** Cell surface hydrophobicity of the LAB isolates. Data are presented as mean ± standard deviation.

3.4 Safety evaluation

3.4.1 Haemolytic activity and antibiotic susceptibility

The selected isolates were assessed for safety which is determined by their haemolytic activity (Fig. 7) and antibiotic susceptibility (Fig. 8). Both *W. confusa* 3 and *W. cibaria* 4 exhibited (Fig. 7B&C) **γ**-haemolytic (non-haemolytic) behaviour, with no zones of erythrocyte lysis surrounding the colonies, similar to the reference strain *L. plantarum* (Fig. 7A). Antibiotic susceptibility testing showed that both isolates were highly susceptible to clindamycin

gentamicin, and erythromycin, whereas comparatively smaller inhibition zones were observed for amoxicillin, penicillin, and vancomycin (Fig. 8B&C). The reference strain exhibited broader susceptibility to all the antibiotics tested. The absence of haemolytic activity is one of the principal safety criteria recommended for probiotic microorganisms because haemolysins are recognized as virulence factors associated with pathogenic bacteria (Allende et al., 2025; Binda et al., 2020).

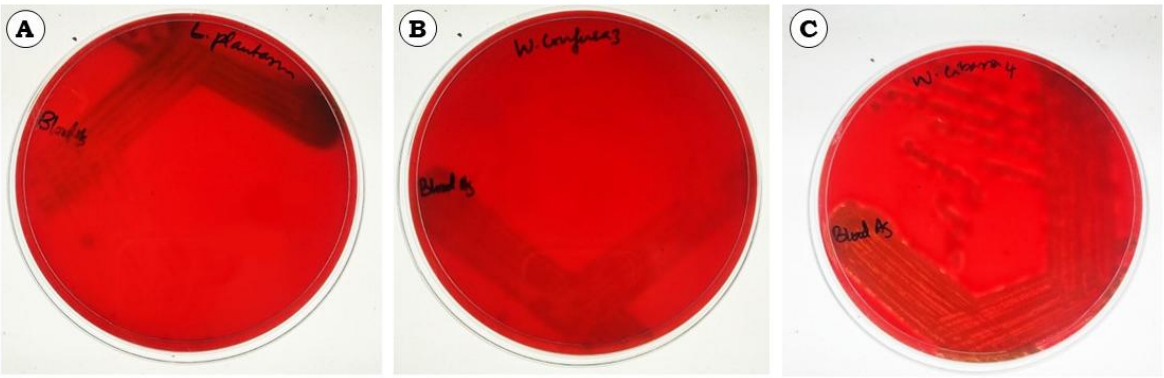


Figure 7. Haemolytic activity of probiotic bacterial isolates on blood agar. **(A)** *L. plantarum* (reference strain), **(B)** *W. confusa* 3, and **(C)** *W. cibaria 4 were* cultured on 5% blood agar plates. No strains exhibited clear (β-hemolysis) or greenish (α-hemolysis) zones around the colonies, indicating γ-haemolytic (non-haemolytic) activity and suggesting an absence of haemolytic potential.

Similarly, antibiotic susceptibility profiling is important for identifying strains that are unlikely to harbour transferable antimicrobial resistance determinants (Sanders et al., 2023). The susceptibility of both *Weissella* isolates to clinically important antibiotics, such as gentamicin, erythromycin, and clindamycin, supports their favourable safety profile. The comparatively lower susceptibility observed for vancomycin and penicillin may represent intrinsic characteristics in line with earlier reports on several lactic acid bacteria Chaichana et al., (Chaichana et al., 2026) rather than acquired resistance. However, confirmation through genomic analysis is required. Both, the haemolysis and antibiotic susceptibility results support the safe use of the selected *Weissella* isolates in future probiotic and food applications.

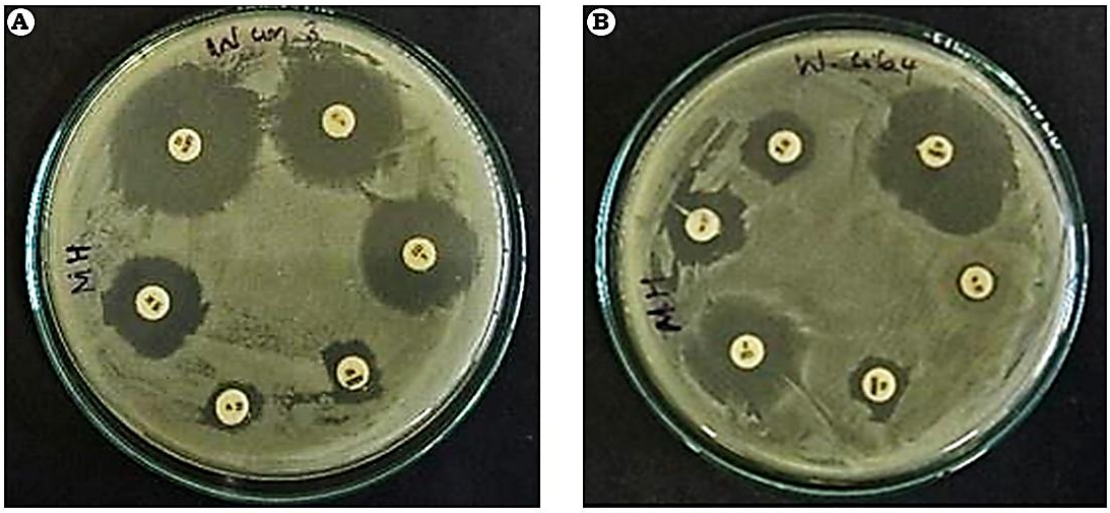


Figure 8. Antibiotic susceptibility profiles of the *Weissella* isolated determined by the Kirby-Bauer disc diffusion assay on Mueller-Hinton agar. **(A)** *L. plantarum* (reference strain), **(B)** *W. confusa* 3, and **(C)** *W. cibaria 4* zone of inhibition surrounding the antibiotic disc indicate susceptibility of the isolates to the tested antibiotic.

3.5 Antagonistic activity

The CFS of selected strains with antagonistic property is shown in Fig. 9. Unfiltered, filter-sterilized, and heat-treated CFS from both *Weissella* isolates produced inhibition zones ranging from 15-20 mm against *S. aureus* and *S. typhi*.

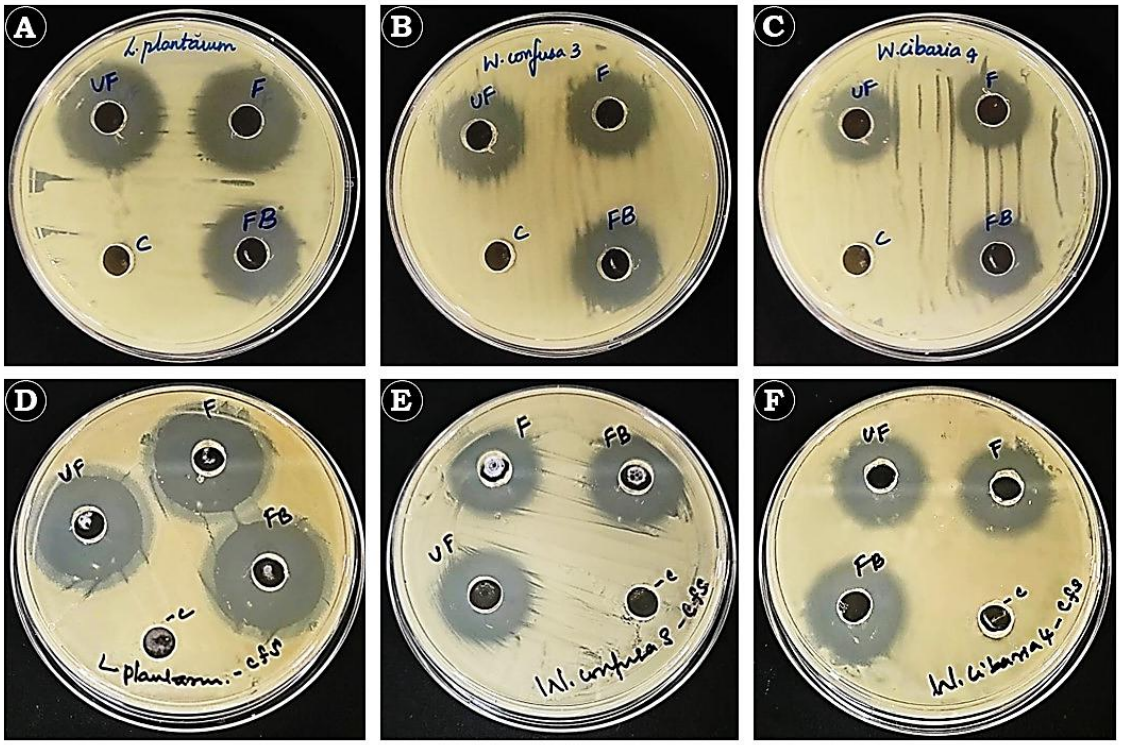
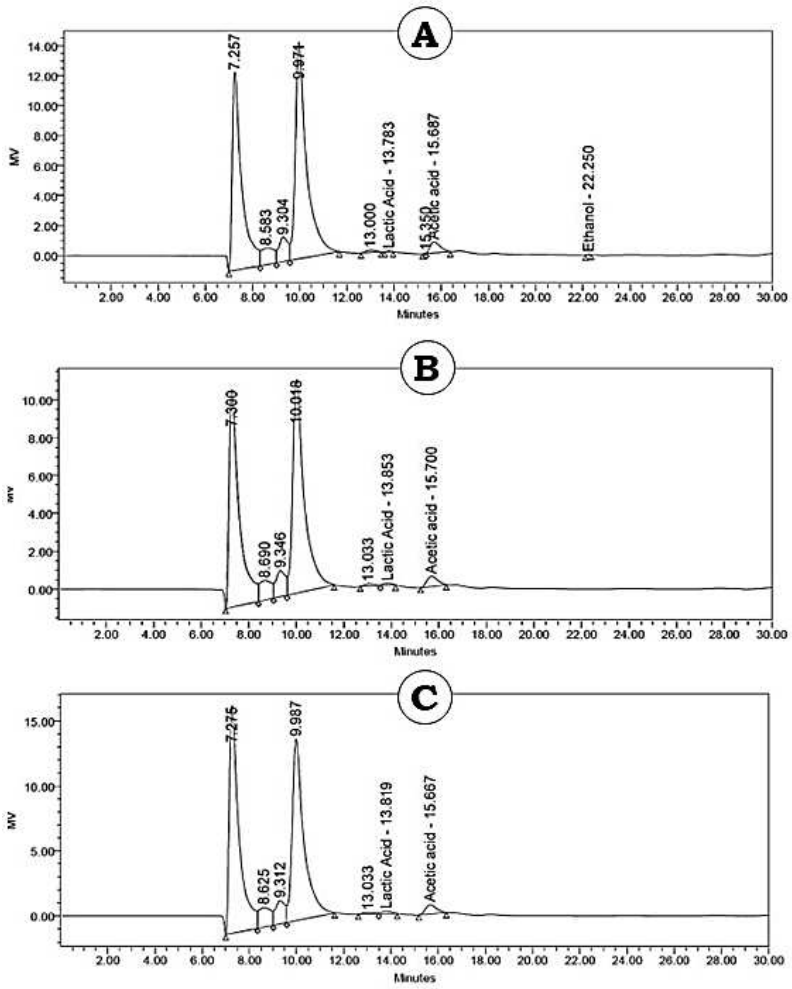


Figure 9. Antagonistic activity of lactic acid bacteria against bacterial pathogens. Antimicrobial activity of *L. plantarum* (A and D), *W. confusa* 3 (B and E), and *W. cibaria* 4 (C and F) against *S. aureus* (A–C) and *S. typhi* (D–F), as determined by the agar well diffusion assay.

The persistence of antimicrobial activity after boiling indicates that the inhibitory compounds retained their activity following heat treatment. The broad-spectrum antagonistic activity observed in this study demonstrates the ability of the selected *Weissella* isolates to produce extracellular metabolites capable of suppressing enteric pathogens (Salamatullah et al., 2021). Similar antimicrobial activity has been reported for probiotic *Weissella* strains isolated from fermented cereals and vegetables, where inhibition has been associated with the combined effects of organic acids, bacteriocin-like substances, hydrogen peroxide, and other extracellular metabolites (Badea et al., 2022; Fusco et al., 2023). Heat treatment retained their antimicrobial activity, indicating that the inhibitory compounds have high thermal stability (Andriyani et al., 2021). However, further purification and biochemical characterization are required to identify the specific molecules responsible. Both autochthonous *Weissella* isolates showed strong antagonistic activity, good gastrointestinal tolerance, and a safety profile, suggesting their use as multifunctional probiotic cultures and natural bio preservative candidates.

HPLC profiling of organic acids

Fig. 10 shows the HPLC chromatograms of the CFS of *W. confusa 3*, *W. cibaria 4* and the reference strain *L. plantarum*, and the quantified metabolites are presented in Table 2.



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607 **Figure .10** HPLC chromatograms of organic acids produced by the probiotic strains **(A)** *L. plantarum* (control),
608 **(B)** *W. confusa* 3 and **(C)** *W. cibaria* 4. Peaks corresponding to lactic acid, acetic acid, ethanol and other
609 metabolites are indicated by their respective retention times.

610 Both *Weissella* isolates produced lactic, acetic, and tartaric acids and ethanol. *L. plantarum*
611 produced lactic, acetic, and tartaric acids, but no detectable ethanol. Citric acid was produced
612 by *W. cibaria* in trace amounts (<0.1 g L-1). Among the strains tested *L. plantarum* (~2.0 g L^{-1})
613 maximum lactic acid concentration, which was approximately double that of the *Weissella*
614 isolates (~1.0 g L^{-1} each). In contrast, *W. confusa* 3 and *W. cibaria* 4 produced higher levels of
615 acetic acid (0.923 and 0.865 g L-1, respectively), tartaric acid (~0.2 g L-1), and ethanol,
616 resulting in metabolite profiles different from that of the reference strain. The metabolite
617 profiles confirmed the heterofermentative character of the two autochthonous *Weissella* isolates
618 compared to the predominantly homofermentative metabolism of *L. plantarum*.

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Table 2: Organic compounds obtained from the CFS of *L. plantarum, W. confusa* 3 and *W. cibaria* 4.

Organic compounds	***L. plantarum***	***W. confusa* 3**	***W. cibaria* 4**
Acetic acid (g/L)	0.606	0.923	0.865
Citric acid (g/L)	-	-	0.047
Ethanol (g/L)	-	0.526	0.55
Lactic acid (g/L)	2.317	1.232	0.99
Tartaric acid (g/L)	0.042	0.155	0.259

One of the characteristics of the phosphoketolase pathway is the co-production of lactic acid, acetic acid, and ethanol, which is indicative of a broader metabolic capability that may be involved in both flavour development and microbial inhibition during fermentation (Nabeta et al., 2019; Yang et al., 2023). The environmental pH is reduced by organic acids, notably lactic and acetic acid, helping with the inhibition of spoilage and pathogenic microorganisms, and the additional formation of ethanol can further improve this antimicrobial effect (Heng et al., 2025). These results align with the strong antagonistic activity of *Weissella* CFS against *S. aureus* and *S. typhi*, indicating that the combined role of several fermentation metabolites could be implicated in the observed inhibitory effects. Thus, the diverse metabolite profiles of *W. confusa* 3 and *W. cibaria* 4 suggest their potential as multifunctional probiotic cultures with promising applications in functional foods and natural bio preservation.

HPLC analysis of B-group vitamins

HPLC analysis of the culture extracts from *L. plantarum*, *W. confusa* 3 and *W. cibari*a 4 was performed at 250 nm, and the chromatograms were compared with authentic standards of vitamins B1, B2, B5, B6 and B9. All three isolates exhibited a predominant peak between 2.64 and 2.68 min, which closely matched the retention time of the vitamin B5 (pantothenic acid) standard (2.755 min at 250 nm and 2.761 min at 358 nm), suggesting the presence of pantothenic acid in the bacterial extract. The major peak represented 97.85% of the total chromatographic area, 80.27% in *W. confusa* 3, and 98.86% in *W. cibari*a, indicating that vitamin B5 was the dominant vitamin-like metabolite produced by these isolates (Fig. 11).

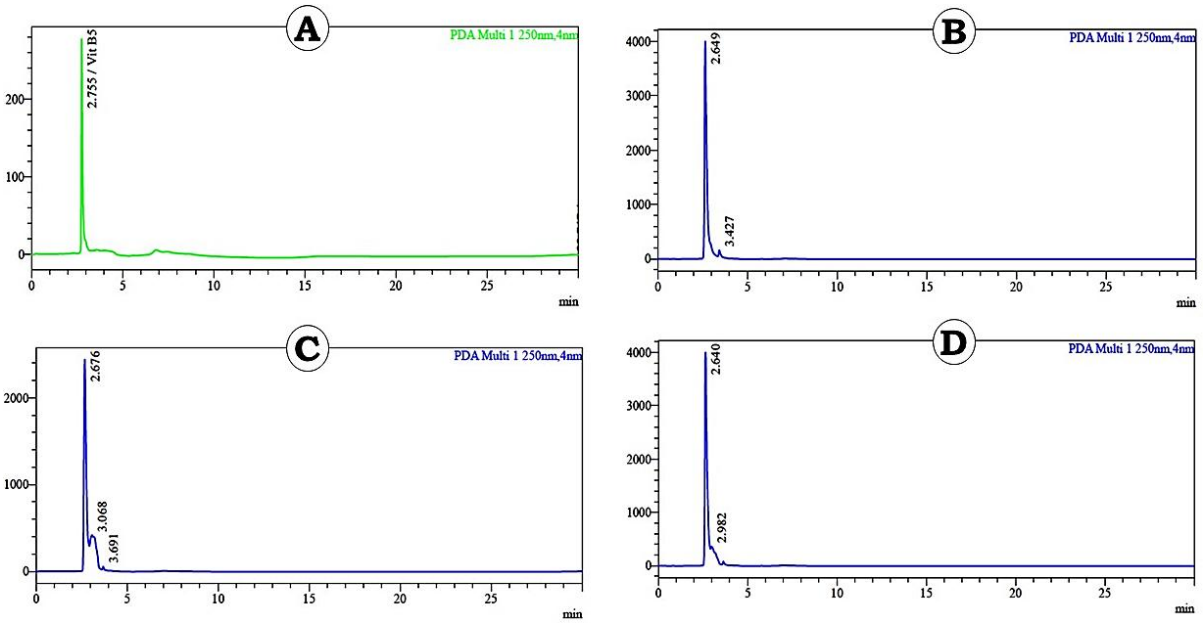


Figure 11. Representative HPLC chromatograms (250 nm) comparing probiotic isolates with the authentic vitamin B5 standard. **(A)** Vitamin B5 (pantothenic acid) standard, **(B)** *L. plantarum*, **(C)** *W. confusa* 3, and **(D)** *W. cibaria* 4. The major peaks detected in all three isolates exhibited retention times (2.640–2.676 min) closely corresponding to the vitamin B5 standard (2.755 min), suggesting the presence of pantothenic acid in the samples.

Additional minor peaks were detected in *L. plantarum*, *W. confusa* 3 and *W. cibari*a 4. However, their retention times did not correspond sufficiently with those of the vitamin B1, B2, B6, or B9 standards to allow reliable identification. Among the isolates, *W. cibari*a 4 exhibited the simplest chromatographic profile, followed by *L. plantarum*, whereas *W. confusa* 3 exhibited additional low-abundance metabolites. The consistent occurrence of a dominant B5-like peak across all three probiotic strains suggests a common ability to synthesize pantothenic acid, which may contribute to the nutritional enhancement of fermented food (Imami, 2021). Nevertheless, as the identification was based solely on retention time comparison, the presence of vitamin B5 should be regarded as tentative, and confirmation by co-injection with authentic standards or LC–MS/MS analysis is required for a definitive identification.

Table 3: Major HPLC peaks of *L. plantarum*, *W. confusa* 3 and *W. cibari*a 4 compared with authentic vitamin standards for the tentative identification of pantothenic acid (vitamin B5).

Sample	Major RT (250 nm)	Major RT (358 nm)	Relative peak area (%)	Tentative Identification
L. plantarum	2.649	2.638	97.85	Pantothenic acid
W. confusa 3	2.676	2.665	80.27	Pantothenic acid
W. cibaria 4	2.640	2.632	98.86	Pantothenic acid

GC–MS analysis of volatile metabolites

Gas chromatography–mass spectrometry (GC-MS) analysis was performed for *W. confusa* 3 based on its functional performance compared to that of *W. cibaria*. The chromatogram revealed 50 distinct peaks corresponding to the extracellular metabolites (Fig. 12).

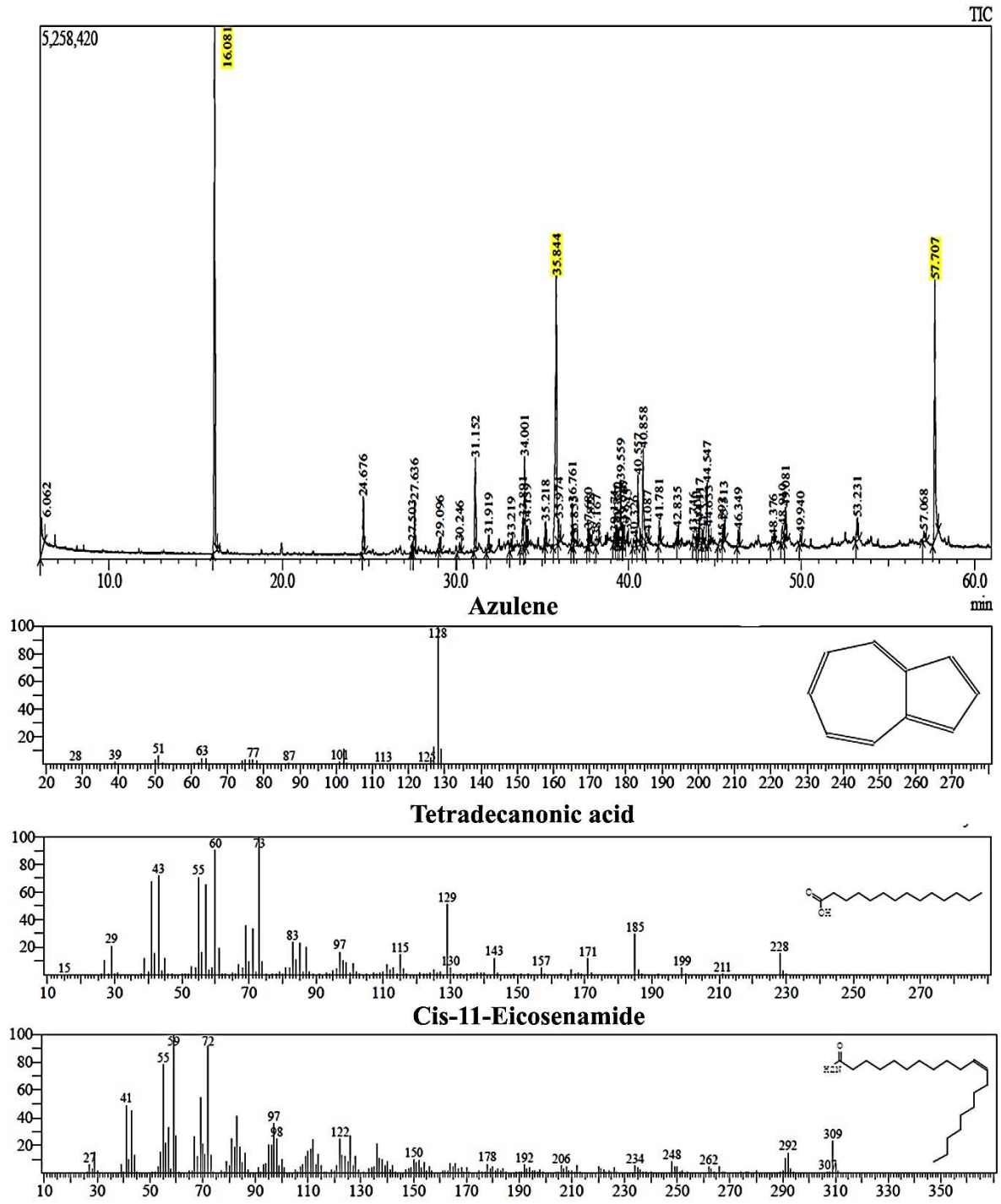


Figure 12. GC–MS total ion chromatogram of *W. confusa* 3, showing the major metabolites. The corresponding mass spectra and chemical structures of azulene, tetradecanoic acid, and cis-11-eicosenamide are shown below.

W. confusa 3 exhibited a metabolite profile enriched with fatty acids and lipid-derived bioactive compounds. The predominant biologically relevant metabolites were azulene (19.85%), tetradecanoic acid (15.58%), pentadecanoic acid (4.10%), and cis-11-eicosenamide (11.45%). Azulene is a naturally occurring bicyclic aromatic compound widely recognized for its anti-inflammatory, antioxidant and antimicrobial activities (Ayaz et al., 2020; Bakun et al., 2021; Rahmawati et al., 2020). The production of azulene-related metabolites has been associated with protection against oxidative stress and enhancement of the functional properties of natural

products (Knight et al., 2025). Tetradecanoic acid (myristic acid) is an important saturated fatty acid involved in membrane lipid biosynthesis and has been reported to inhibit several gram-positive microorganisms (Oepen et al., 2021). Pentadecanoic acid is an odd-chain fatty acid that is increasingly recognized as a biomarker of dairy fermentation and has demonstrated anti-inflammatory and metabolic health benefits (Fu et al., 2021; Yang et al., 2025). Another important metabolite, cis-11-eicosenamide, belongs to the fatty acid amide family and exhibits antimicrobial, anti-inflammatory, and surface-active properties (Malki & Touati, 2019; Takenouchi & Murata, 2022). The predominance of these lipid-derived metabolites suggests that *W. confusa* 3 possesses an active fatty acid biosynthetic pathway capable of producing compounds with potential nutraceutical and functional food applications. The broader metabolite diversity observed in autochthonous *W. confusa* 3 reflects its versatile fermentative metabolism and complements HPLC-based organic acid profiling. The presence of numerous volatile metabolites with reported antimicrobial, antioxidant, flavouring, and industrial relevance suggests that the isolate possesses a metabolically active phenotype capable of generating multiple functional biomolecules during fermentation.

Production and characterization of Exopolysaccharides by *W. confusa*

The ability of *W. confusa* 3 isolates to produce EPS was first assessed based on colony morphology on MRS agar. In the primary screening, many isolates showed the characteristic mucoid phenotype, which is indicative of extracellular polysaccharide production (Fig. 13A).

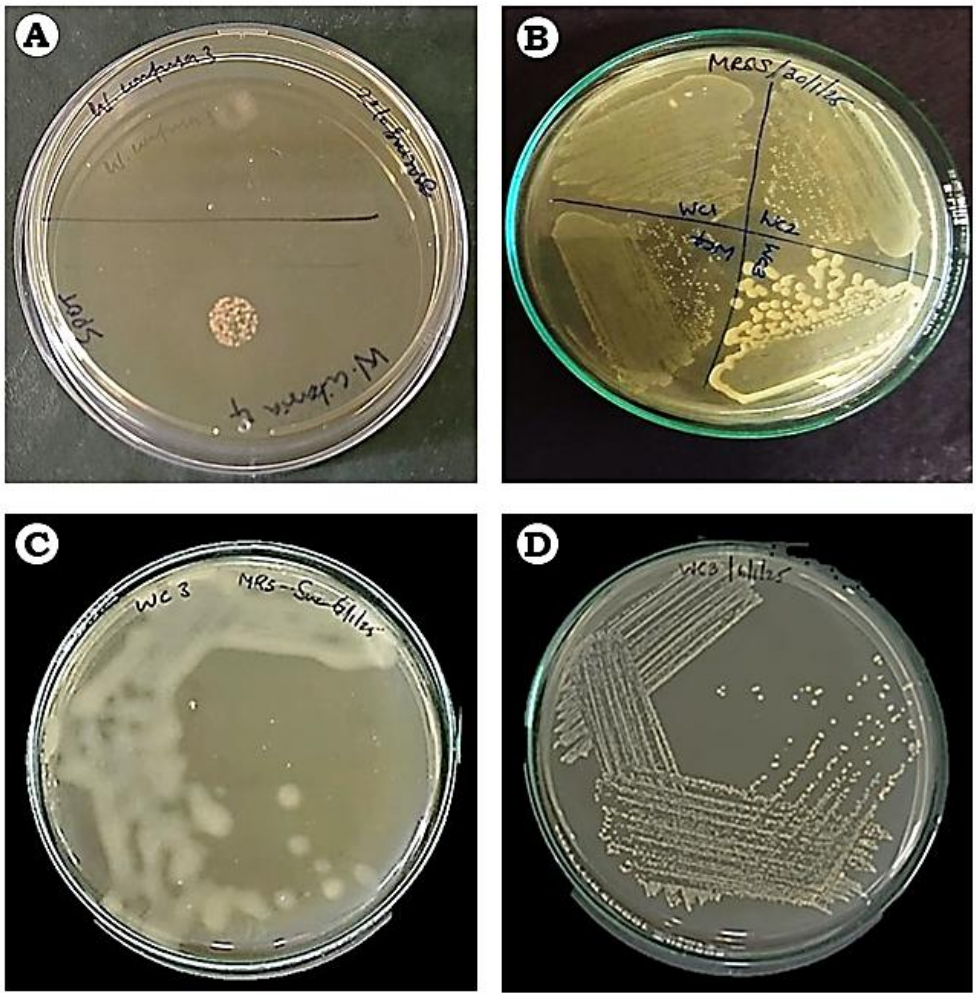


Figure 13. Representative colony morphology of *W. confusa* 3 on MRS agar supplemented with sucrose **(A, B)** and dextrose **(C, D)** for qualitative screening of exopolysaccharide (EPS) production. **(A)** Mucoid colony morphology on MRS– sucrose agar, **(B)** isolated colonies on MRS– dextrose agar, **(C)** confluent streak growth on MRS–sucrose agar, and **(D)** streak growth on MRS–dextrose agar.

The colony morphology of *W. confusa* 3 was stable after successive subcultures, with a creamy-white, smooth and highly mucoid colony phenotype (Fig. 13B) confirming the stability of the EPS-producing phenotype. The availability of carbohydrates is a vital factor in EPS biosynthesis; therefore, the isolate was further cultured on MRS agar medium with 5% (w/v) dextrose and 5% (w/v) sucrose. Growth on dextrose yielded moderately mucoid colonies (Figure 13C), but supplementation with sucrose produced thicker, glossier and more viscous colonies (Figure 13D), indicative of increased EPS synthesis. Sucrose is considered the preferred substrate for the glucansucrase-mediated production of exopolysaccharides in *Weissella* and other lactic acid bacteria, as it is a direct glucosyl donor in the synthesis of extracellular glucan (De Vuyst & Degeest, 1999; Lynch et al., 2018).

The qualitative observations were confirmed by quantitative estimation. *W. confusa* 3 produced significant amount of EPS (165.95 ± 2.34 mg glucose equivalent L-1). The increased EPS productivity of *W. confusa* 3 was consistent with the increased auto-aggregation and cell surface hydrophobicity during probiotic characterization, which indicated that EPS was involved in bacterial adhesion and persistence under the gastrointestinal conditions. Similar correlations between EPS production and improved probiotic functionality have been described for several *Weissella* and *Lactobacillus* species (Oleksy-Sobczak et al., 2020; Patel et al., 2012).

The initial confirmation of EPS production of *W. confusa* 3 was obtained through the Congo red assay (Fig. 13A). The isolate showed typical Congo red-positive phenotype on MRS-sucrose agar confirming EPS secretion (Freitas et al., 2011). Further study of the surface morphology of EPS produced by *W. confusa* 3 was carried out by SEM (Fig. 13B–C). At higher magnification (Fig. 13B), a highly porous 3D architecture of the EPS was observed, consisting of interconnected cavities and an irregular polymeric network. Pores of different diameters were uniformly distributed in the polymer matrix, indicating the presence of a broad extracellular polymer network within the structure. Figure. 13C shows the aggregated sheet-like structures with rough, uneven surfaces and compact polymer clusters, which demonstrate the natural tendency of polysaccharide chains to aggregate during dehydration. Similar porous and heterogeneous morphologies have been reported for EPS produced by *Weissella* spp. and other lactic acid bacteria, reflecting differences in molecular organization, purification procedures and drying conditions (Ruas-Madiedo et al., 2010; Yadav et al., 2024).
The porous microstructure of EPS is advantageous for numerous applications as it provides a high specific surface area, enhanced water-binding capacity and improved hydration characteristics. These structural features are responsible for the desirable rheological properties including higher viscosity, gel formation, emulsification and moisture retention. Therefore, microbial EPS are beneficial as natural hydrocolloids and texture modifiers in fermented foods. In addition, the polymeric network leads to the generation of stable matrices that can lead to the improvement of the product consistency and shelf-life, and to substitute synthetic stabilisers (Yadav et al., 2024). The positive Congo red reaction coupled with SEM observations suggests that *W. confusa* 3 is an effective producer of extracellular polysaccharides. Members of the

genus *Weissella*, are known to produce glucan-type homopolysaccharides, mainly dextran, by extracellular glucansucrase enzymes when grown on sucrose-containing media (Adesulu-Dahunsi et al., 2018; Yadav et al., 2024).

Previous studies have shown antioxidant, immunomodulatory, cholesterol lowering, prebiotic and antimicrobial properties of EPS. In addition, EPS is involved in bacterial adhesion, biofilm formation and protection against gastrointestinal stresses, thus enhancing probiotic persistence and functionality (Adesulu-Dahunsi et al., 2018; Yadav et al., 2024). From the industrial perspective, the efficient ability of W. confusa 3 to produce EPS greatly increases its value as a functional starter culture. Moreover, microbial EPS are gaining interest for use in edible coatings, encapsulation systems, pharmaceutical formulations, biomedical scaffolds and biodegradable packaging materials because of their biodegradability, non-toxicity and excellent biocompatibility (Yadav et al., 2024). Thus, the high EPS production by *W. confusa* 3 indicates its high potential for the development of clean-label functional foods and other high-value biotechnological applications.

FTIR analysis of exopolysaccharide

The FTIR spectrum of EPS produced by *W. confusa* 3 (Fig. 14D) showed typical absorption bands at 3417.88, 2925.10, 1645.78, 1456.46, 1366.44, 1276.92, 1155.10, 1120.00, 1018.56, 942.24, 919.24, 856.79, 762.25, and 704.44 cm^{-1}, suggesting the carbohydrate-rich nature of the polymer.

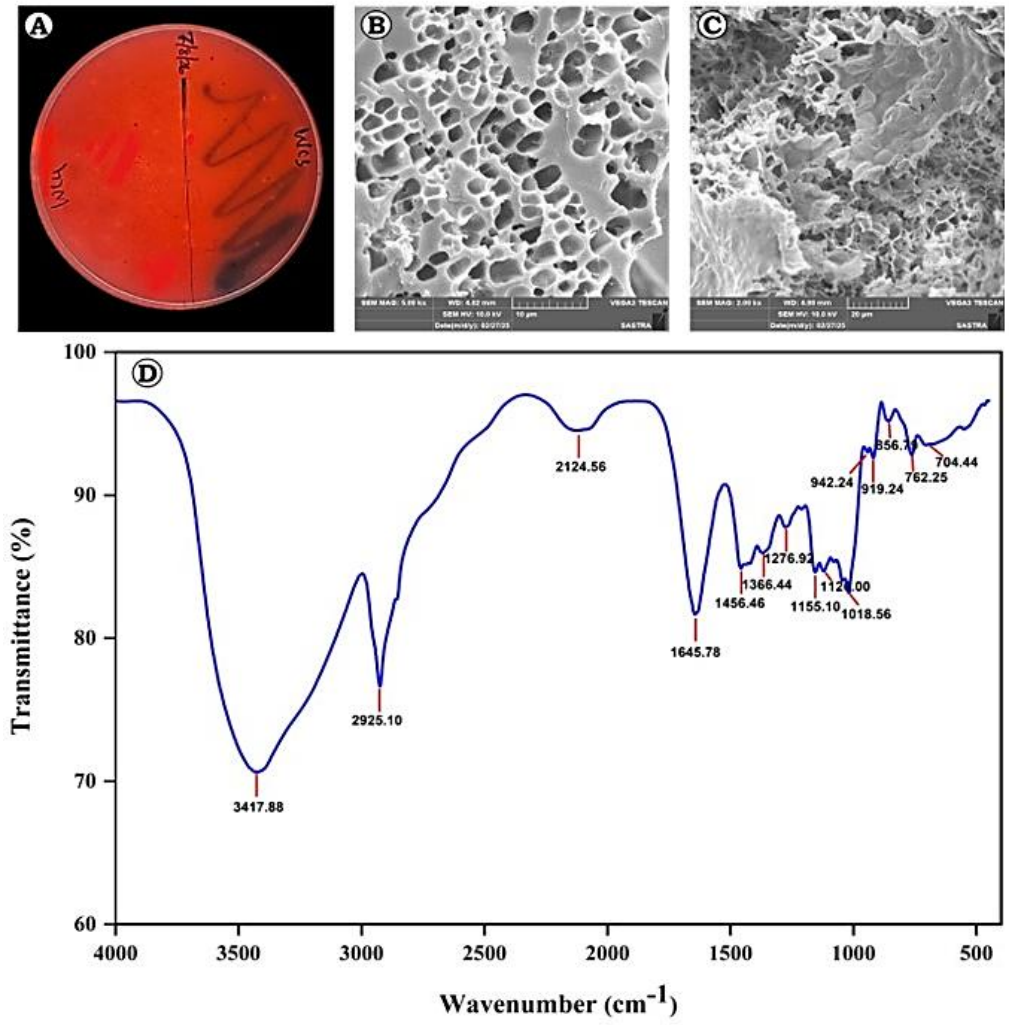


Figure 14. Characterization of exopolysaccharide (EPS) produced by *W. confusa* 3: **(A)** EPS-producing colony on agar medium; **(B–C)** scanning electron micrographs showing the surface morphology of the purified EPS; **(D)** FTIR spectrum of the purified EPS showing characteristic absorption bands associated with hydroxyl, C–H, and glycosidic/carbohydrate functional groups.

The broad band at 3417.88 cm^{-1} was attributed to O-H stretching, representing the hydroxyl group and hydrogen bonding typical of polysaccharides. The band at 2925.10 cm^{-1} was assigned to aliphatic C-H stretching vibrations (Adesulu-Dahunsi et al., 2018; Dubey & Jeevaratnam, 2015). The absorption at 1645.78 cm^{-1} is attributed to O–H bending of bound water molecules present in the polysaccharide matrix. The bands at 1456.46 and 1366.44 cm^{-1} were attributed to C–H deformation vibrations, while the band at 1276.92 cm^{-1} was assigned to C–O-related vibrations (Ma'unatin et al., 2020). The characteristic carbohydrate fingerprint region with prominent bands was observed at 1155.10, 1120.00, and 1018.56 cm^{-1}, which are primarily related to C-O, C-O-C, and C-C-O vibrations of sugar residues (Dubey & Jeevaratnam, 2015; Zhao et al., 2023). Additional evidence for the existence of glycosidic bonds linked to carbohydrate residues was observed at 942.24, 919.24, and 856.79 cm^{-1}. The EPSs produced by *W. confusa* have been reported to have similar fingerprint-region absorptions. The weak bands at 762.25 and 704.44 cm-1 could be due to skeletal or ring vibrations of carbohydrate residues. The weak signal at 2124.56 cm-1 is not considered diagnostic of the EPS structure, and was not assigned to a specific functional group. The

spectral pattern is consistent with the previously reported *Weissella* EPSs (Adesulu-Dahunsi et al., 2018; Dubey & Jeevaratnam, 2015; Zhao et al., 2023).

Conclusion

In this study, the indigenous isolates *W. confusa* 3 and *W. cibaria* 4 from traditional fermented Ponni rice have complementary probiotic properties appropriate for functional food applications. Both isolates tolerated simulated gastrointestinal conditions, showed a favourable safety profile, and produced antimicrobial metabolites active against enteric pathogens. *W. confusa* exhibited better auto-aggregation, cell surface hydrophobicity and EPS production, whereas *W. cibaria* showed relatively higher tolerance to gastrointestinal stresses. HPLC analysis revealed the production of organic acids and the tentative presence of pantothenic acid (vitamin B5) in the fermented product. GC–MS analysis of *W. confusa* revealed the presence of several bioactive metabolites with reported antimicrobial and nutraceutical properties. In summary, the findings support the potential use of these indigenous *Weissella* isolates as multifunctional probiotic starter cultures and natural bio preservatives for cereal-based fermented foods. Whole-genome sequencing, LC–MS/MS confirmation of vitamin metabolites, in vivo validation, and pilot-scale fermentation studies are required to establish their safety, functionality, and industrial applicability.

Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Data will be made available upon reasonable request.

CRediT authorship contribution statement:

Velvizhi Devi Rajendran: Conceptualization, Visualization, Methodology, Data curation, and Writing- Original draft. **Bharati Priya Ravichandran:** Data curation, Software, Writing-Original draft. **Poojasri S:** Methodology, Formal analysis. **Nikhileswari Senthilvel:** Methodology, Formal analysis. **Ananth. S**: Writing – review & editing. **Saisubramanian Nagarajan**: Result analysis & review. **Gowdhaman Dharmalingam:** Conceptualization, Writing-review and editing, Investigation, Resources, Validation and Supervision.

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