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Original Article

Isolation and characterisation of the biological traits of millet-derived lactic acid bacteria

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Abstract

Millets are sustainable, native foods with high nutritional content. Nowadays, beneficial bacteria derived from millets have gained increased interest among food industries and consumers due to the development of starter cultures for fermentation, which are alternatives to non-dairy substitutes. This study explored the potential beneficial activity of lactic acid bacteria (LAB) isolated from six millet cultivars. 16S rDNA sequencing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis were used to identify LAB strains. Further, biochemical characterisations were assessed for resistance to phenol, bile salts, acid, and NaCl, along with metabolite production using high-performance liquid chromatography (HPLC). Antibacterial, antioxidant, and anti-inflammatory properties were determined by biological characterisations. Among 12 isolated strains, *Lactococcus lactis* (SM02), *Weissella cibaria* (NM01 and SM01), and *Enterococcus casseliflavus* (BM01) from millets showed positive probiotic characteristics. These LAB isolates produced lactic acid in a range of 9,000–12,000 mg/L by utilising glucose. All LAB isolates were able to grow under acidic pH (4–5), presence of upto 0.5% bile salt, 0.3% phenol, and 5%–8% NaCl. Additionally, the cell-free supernatant of *L. lactis* (SM02) decreased the pro-inflammatory cytokine IL-6 levels in murine macrophage cell line compared to *W. cibaria* and *E. casseliflavus*. This study emphasises the potential of millet-derived *L. lactis* for developing non-dairy formulations and functional foods, paving the way for sustainable health while maintaining ecological, social, and economic resources for future generations.

Keywords: bioactives, fermentation, lactic acid bacteria (LAB), millets, Plant-based food

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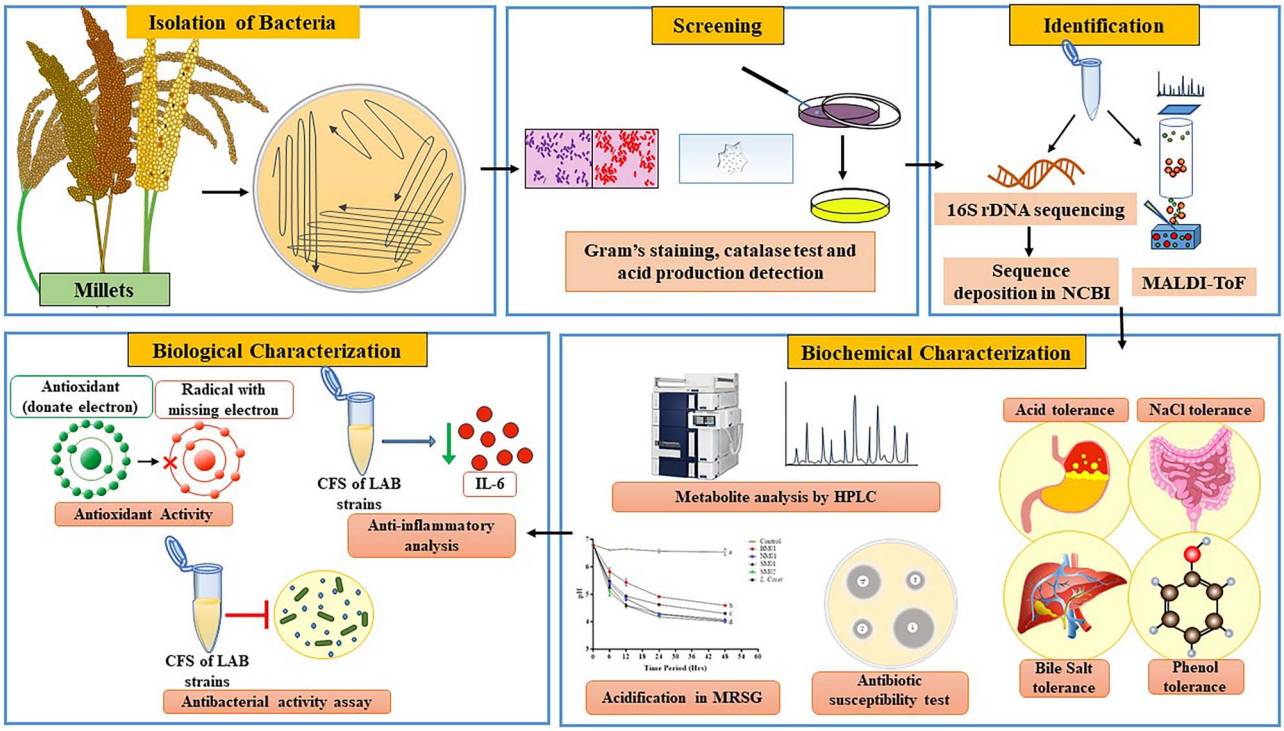
Graphical abstract



Introduction

Millets are ancient crops that have been cultivated worldwide, especially in Asia, Africa, and Eastern and Central Europe. Millets are consumed as a traditional staple food due to their nutritional benefits and gluten-free nature. They are classified as (1) major millets: finger, sorghum, and pearl; (2) minor millets: foxtail, proso, kodo, barnyard, little, browntop; and (3) pseudo-millets: buckwheat and amaranth. Millets are cereal-type food crops, characterised by containing high fibre, low glycaemic index, high concentration of micronutrients, bioactive flavonoids, and small peptides (Kumar et al., 2021). The advent of high-yielding varieties of cereals such as rice and wheat devalued millet usage in the late 1960s. However, millets are more nutritious and healthier traditional grains, which might substitute refined carbohydrates and can immensely contribute to therapeutic dietary adaptation and diversity (Kane-Potaka et al., 2021, Kane-Potaka & Kumar, 2019).

Due to their high nutritional value and capability to thrive in challenging climates, millets are currently gaining popularity as foods themselves and/or as ingredients for food product development throughout the globe (Kane-Potaka & Kumar, 2019). Millets are extensively used in India, China, and African countries, such as Niger, Nigeria, Ethiopia, Sudan, etc., in various food types such as fermented (dough and beverages) and unfermented breads, stiff and thin porridges (Ramashia et al., 2025). However, fermented millet-based products are less consumed due to lack of characterised microbial flora such as *Saccharomyces*, *Lactobacillus, Lactococcus, Weissella, Pediococcus, Enterococcus, Leuconostoc, Streptococcus, Propionibacterium*, *etc.*, which are beneficial both in food processing and better human health (Leeuwendaal et al., 2022, Tamang et al., 2016). Hence, there is a need for isolation, exploration, and characterisation of microbes from each millet source that might have potential beneficial attributes and provide a high-quality organoleptic property to fermented foods, i.e., lactic acid bacteria (LAB).

Lactic acid bacteria are heterogeneous, Gram-positive and ubiquitous group of bacteria, which are basically found in plants, animals, meat, and dairy sources. LAB produces various metabolites, such as lactic acid, organic acids, peptides, etc., by microaerophilic or anaerobic fermentation conditions, which have various health benefits for humans. Some of these metabolites can provide relevant nutritional, technological, and sensorial properties to the fermented foods (Ruiz Rodríguez et al., 2019, Wang et al., 2021). Around the world, the majority of probiotic products available in the market are dairy-based. The use of dairy-based probiotic products has limitations due to lactose intolerance, milk cholesterol content, and vegan diets (De Bellis et al., 2021, Küçükgöz & Trząskowska, 2022). In addition, the probiotics from dairy sources might be evolved and adapted to dairy-based raw materials and the fermentation process. Thus, nowadays, increasing vegan and plant-based diets and concerns about the use of dairy-based food bring about a shift in potential microbial sources from dairy to non-dairy alternatives such as plants. These food-grade microbes derived from plant-based sources will suit well for fermentation of respective plant-based raw material and potentially yield the right flavour, texture, aroma, and other functional qualities. The demand for plant-based microbes and non-dairy product alternatives is increasing, and the value of global plant-based product markets is expanding every year. The size of the global dairy substitute market was assessed as US$13,021 million in 2018 and is expected to rise to approximately US$35,805 million by 2026 (Rasika et al., 2021).

Further, studies have explored millet-derived LAB and their characteristics. In a recent study, Gouthami et al. (2024) isolated LAB from different millets and identified them as *Weissella cibaria* and *Weissella confusa*. These strains have shown probiotic potential

by growing in acidic pH (4.5), and in the presence of NaCl and bile salt (Gouthami et al., 2024). Similarly, a study isolated 15 LAB strains from fermented finger millet. Among them, five bacteria, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Enterococcus faecium*, and *Pediococcus acidilactici*, were able to survive under gastric conditions, indicating their probiotic efficacy (Divisekera et al., 2019). Among 18 LAB strains obtained from fermented broomcorn millet porridge, *Levilactobacillus brevis* showed higher probiotic potential by surviving under gastrointestinal conditions (Wang et al., 2022b). However, the isolation and characterisation of millet-based LAB have been studied by different research groups, revealing visible diversity among the millet-based bacterial isolates. This diversity indicates the influence of geographical and environmental factors, which play important roles in sample quality and their microbial composition (Wang et al., 2022a). In our study, we have isolated and identified different LAB strains from millet samples from Karnataka, India. Further, we have evaluated their functional efficiency, fermentation potential, and production of metabolites. In addition, biological activities such as antibacterial, antioxidant, and anti-inflammatory activities of the isolates were assessed by different methods.

Materials and methods

Raw material and isolation of millet-based microbes

Diverse types of millets such as foxtail (*Setaria italica*), finger (*Eleusine coracana*), proso (*Panicum miliaceum*), little (*Panicum sumatrense*), kodo (*Paspalum scrobiculatum*), and barnyard (*Echinochloa esculenta*), which are commonly cultivating in Karnataka, were procured from farmers of Gulur (12.4667° N, 76.9667° E), Karnataka, India (harvested in the year 2021). The microbes were isolated from whole millet seeds and sprouts (soaked in water overnight and placed in cotton cloth for 48 hr at room temperature) through the following method: 0.5 *g* of ground seeds and sprouts were placed in 0.85% NaCl (saline) solution for 8 hr at room temperature. Respective samples were streaked into Man, Rogosa, and Sharpe (MRS) (HiMedia, Mumbai) agar plates and incubated at 30 °C for 24–48 hr. Morphologically distinct colonies were selected and subsequently subcultured twice into MRS agar plates (with incubation at 30 °C for 24–48 hr) to obtain pure cultures. Isolated microbes were stored in 20% glycerol stock at −80 °C for further use.

Characterisation and acid production detection assay

To achieve and distinguish LAB from other microbial strains, basic Gram's staining, catalase test, and preliminary colour change acid assays (to test their ability to produce acid) were performed. In brief, 12 bacterial isolates obtained from different millets were inoculated into MRS agar plates containing 0.01% bromocresol purple and incubated at 30 °C for 24 hr. The microbes that change the colour of bromocresol purple from purple to yellow were considered as acid-producing bacteria (Kim et al., 2021b). The commercial strain, *Lacticaseibacillus casei* (Orla-Jensen) Zheng et al. (ATCC 393), obtained from the American Type Culture Collection, was used as a positive control (the strain is referred to as "*L. casei*" throughout the manuscript). Acid-producing bacteria were considered for further experiments.

Identification of selected bacterial strains using the MALDI-TOF technique

Four acid-producing bacteria were selected for MALDI-TOF Biotyper Sirius (Bruker Daltonics, Bremen, Germany) identification. MALDI-TOF identification was carried out as described by Sedó Molina et al. (Sedó Molina et al., 2022). Briefly, a single colony of selected bacteria were inoculated into 1 ml MRS broth and incubated for 16–24 hr at 30 °C. After incubation, the cultures were centrifuged at 4,000 *g* for 1 min, and the supernatant was discarded. The pellets were washed with MilliQ water (or 0.85% saline) twice, and the remaining proteins were removed from the media by spinning. Proteins from the bacterial pellets were extracted by treating them with MilliQ water and 96% (vol/vol) ethanol solution in a 1:3 ratio followed by vortex and centrifugation. The pellets were mixed with 99% (vol/vol) formic acid and 50 µl of pure acetonitrile and centrifuged at 15,330 *g* for 3 min. Supernatants were collected into fresh tubes. About 1 µl of the extracted protein solutions were spotted in the MALDI-TOF target plate, and 1 µl of matrix mixture (containing 475 µl of miliQ water, 500 µl of pure acetonitrile, 25 µl of pure trifluoroacetic acid, and α-cyano-4-hydroxycinnamic acid) was added on the dried sample spots. Then, the MALDI target plates were placed in a MALDI-TOF Biotyper Sirius and processed. The identification of genus/species of microbes, by using MALDI-TOF Biotyper, is through the analysis of the protein (*m/z*) spectra of each sample and then comparing them to a database of spectral information. The reliability of the results is confirmed based on the score values given by the instrument, which range from 1 to 3. The score values below 1.69 are considered not reliable, whereas scores between 1.70 and 1.99 indicate that identifications are reliable only on the genus level. Identifications on genus as well as species level are considered reliable if the scores are above 2 (Sedó Molina et al., 2022).

16S rRNA gene sequencing

The MALDI-TOF identified LAB were further confirmed by 16S rRNA gene sequencing. Genomic DNA were extracted from four identified bacterial strains using Qiagen QIAwave DNA Blood & Tissue Kit (Cat. No./ID: 69556) according to the manufacturer protocol. In brief, the isolates were grown overnight at 30 °C in 2 ml MRS broth. The cells were harvested by centrifugation at 20,000 *g* for 5 min. About 180 µl of enzymatic lysis buffer (20 mM Tris–HCl pH 8.0, 2 mM ethylenediaminetetraacetic acid (EDTA), 1.2% triton X-100, lysozyme- 20 mg/ml) was added to the cells and vortexed for 10–20 s. The samples were incubated at 37 °C for 30 min. About 25 µl proteinase K was added to the incubated samples and followed by the addition of 200 µl buffer AL (Lysis buffer) and vortexed. All the samples were incubated at 56 °C for 30 min, and then 200 µl of 100% ethanol was added. The samples were vortexed and loaded into the Mini spin columns. The columns were centrifuged at 10,000 *g* for 1 min. At this stage, the DNA is selectively bound to the membrane of spin columns, and then the columns are washed twice with 500 µl buffer AW1 (Washing buffer 1), followed by buffer AW2 (Washing buffer 2). The genomic DNA samples were eluted from Mini spin columns into fresh tubes by adding 200 µl of buffer AE (Elusion buffer) and centrifuged at 10,000 *g* for 1 min. The eluted DNAs were stored at 4 °C for further analysis. 16S rRNA gene-targeted PCR was performed by using the universal primers 27F (forward primer) and 1492R (reverse primer). The conditions followed for PCR consist of an initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Polymerase chain reaction (PCR) products were purified and sequenced using the Sanger sequencing method at QTLomics Technologies Pvt Ltd, Bangalore, India. The PCR conditions used for Sanger sequencing involve initial denaturation at 96 °C for 5 min, 25 cycles of denaturation at 96 °C for 30 s, hybridisation at 50 °C for 30 s, and elongation at 60 °C for 90 s. Sequencing was performed

using an ABI 3130 Genetic Analyser. The sequences obtained were compared with the NCBI (nt) database using basic local alignment search tool and submitted to the GenBank sequence database.

Growth study of LAB strains

The growth pattern of isolated LAB strains was studied based on biomass productivity according to the method explained by (Leska et al., 2022) with some modifications. Briefly, four LAB isolates, namely *L. lactis.*, *W. cibaria.*, and *Enterococcus casseliflavus*, were grown overnight in MRS broth at 30 °C. After incubation, the culture pellets were collected by centrifugation at 4,000 *g* for 10 min. Pellets were subjected to a subsequent wash with 0.8% saline, and optical density was measured. The growth study was carried out by inoculating the respective LAB cultures (equals to 0.1 OD_{600}) into fresh MRS broth and OD_{600} was measured at different time intervals, i.e., 2, 4, 6, 8, 10, 24, and 48 hr, by using UV Spectrophotometer (Thermo Scientific AQ8000, India).

Acidification by LAB strains

The acidification property of the four isolated LAB strains and positive control *L. casei* was monitored (0 to 48 hr) based on the method described by Petrut et al. (2019) with some modifications to analyse their utilisation of carbohydrate (glucose) and the ability to produce organic acids, which results in a pH drop. This assay was carried out in sterile MRS broth. All inoculation in triplicate was initiated at pH 6.8 with 0.1 OD600 culture as mentioned in growth study of LAB strains section. The inoculated cultures were kept in a shaking incubator at 30 °C with 150g for 48 hr. MRS broth without any inoculation was kept as a negative control. The pH of all samples was measured with a Digital pH metre (Analab, India) at 0, 6, 12, 24, and 48 hr after inoculation.

Quantification of carbohydrates and organic acids by HPLC

Quantification of substrate and metabolites produced by the selected four LAB strains, along with the positive control *L. casei* in MRS broth, was performed by HPLC. The utilisation of glucose by LAB isolates and the production of lactic acid, acetic acid, and ethanol were analysed. The LAB strains were cultured in MRS broth at 30 °C for 24–48 hr. Cultures were centrifuged, the supernatants collected at 0, 24, and 48 hr and filtered using 0.22 μm filters into HPLC vials. The Aminex HPX-87H HPLC column (Bio-Rad, Hercules, CA) with a Shodex RI-101 refractive index detector (Showa Denko K.K., Tokyo, Japan) was used for the detection and quantification. The column temperature was maintained at 60 °C, and 5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.5 ml/min. By comparing with the HPLC standard curves, which were made by measurement of analytically pure standards in 5 mM H_2SO_4, the glucose and metabolites in the samples were identified and quantified (Madsen et al., 2021; Sedó Molina et al., 2022).

Study of attributes of LAB

To determine the ability of isolated LAB strains to grow in gastrointestinal conditions such as acidic pH (acidic condition in the human gut), the presence of bile salt (small intestine environment), phenol (bacterial inhibition), and NaCl (Osmotic stress) were investigated based on the method described by (Kunchala et al., 2016).

Acid tolerance of isolated and control LAB strains was studied in MRS broth with pH adjusted (using 3 M HCl) to 2, 3, 4, 5, 6, and 6.8 (in triplicate). Growth was monitored by measuring OD at 600 nm for time intervals of 0, 6, 12, and 24 hr. Growth and inoculation conditions were the same as those mentioned in growth study of LAB strains section.

The bile salt tolerance of isolated and control LAB strains was determined in MRS broth containing 0.3%, 0.5%, and 0.8% of bile salt (Sigma Aldrich, USA). Growth was monitored by measuring OD at 600 nm for time intervals of 0, 6, 12, and 24 hr. Growth and inoculation conditions were the same as those mentioned in growth study of LAB strains section.

Phenol tolerance of isolated and control LAB strains was determined in MRS broth containing 0.2%, 0.3%, 0.4%, and 0.5% phenol (Sigma Aldrich, USA). Growth was monitored by measuring OD at 600 nm for time intervals of 0, 6, 12, and 24 hr. Growth and inoculation conditions were the same as those mentioned in growth study of LAB strains section.

NaCl tolerance of isolated and control LAB strains was determined in MRS broth containing 4%, 5%, 8%, and 12% NaCl (Sigma Aldrich, USA). Growth was monitored by measuring OD at 600 nm for time intervals of 0, 6, 12, and 24 hr. Growth and inoculation conditions were the same as those mentioned in growth study of LAB strains section.

Antibacterial activity test

The antibacterial activity of LAB isolates was tested against the food pathogens *Escherichia coli* EFR02 and *Salmonella enterica typhimurium* MTCC1251 (Procured from CSIR-Central Food Technological Research Institute, Mysore, India) by the microtitre plate assay (Divyashree et al., 2021). Cell-free supernatants (CFS) were obtained from 24 hr grown LAB cultures at 30 °C by centrifugation and filtration using 0.2 μ filters. Different concentrations of CFS (15%, 20%, and 25%) were tested against the pathogenic bacteria, which were grown overnight in nutrient broth at 37 °C. Pathogenic bacteria inoculated to 0.1 initial OD_{600} to microtitre plate and different concentrations of CFS were added. The total volume in each well was made up to 200 μl by using nutrient broth (NB). Pathogens in NB without CFS treatment were taken as positive control. Growth was monitored in a microtitre plate by measuring OD at 600 nm after 24 hr of incubation. The total inhibition percentage of pathogenic bacteria was calculated by the following equation:

$$\text{Inhibition Percentage} = \left[\frac{\text{OD of positive control} - \text{OD of test sample}}{\text{OD of positive control}}\right] \times 100 \quad (1)$$

Antibiotic susceptibility test

The Kirby-Bauer diffusion test was performed to detect the antibiotic susceptibility of LAB strains and followed the method from (Hamdaoui et al., 2024) with some modifications. In brief, overnight grown LAB strains in MRS broth with 0.3 OD_{600} were evenly spread onto MRS agar plates and placed the following antibiotic discs: ampicillin (10 μg), chloramphenicol (30 μg), amikacin (30 μg), and vancomycin (30 μg) (HiMedia, Mumbai). Plates were incubated at 30 °C for 24 hr. The zone of inhibition was measured, and the results were interpreted based on the Zone Size Interpretative Chart provided by HiMedia.

Radical scavenging assay by 2,2′-azobis(2-amidinopropane) dihydrochloride method

The antioxidant activity of CFS of LAB isolates was investigated by using 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). AAPH, a free radical generating compound, reacts with oxygen and produces peroxyl and alkoxy radicals. When

dichlorodihydrofluorescence diacetate (DCFH-DA) and AAPH interact, reactive oxygen species (ROS) generated from AAPH react with DCFH-DA and oxidise it into a fluorescent molecule 2,7′-dichlorofluorescein (DCF). The fluorescence emitted by DCF is directly proportional to the concentration of ROS. Briefly, 20 μl of each of the samples was mixed with 50 μg of DCFH-DA and 50 mM AAPH in a 96-well plate. The total volume in each well made up to 100 μl by using the vehicle (MRS broth). About 100 μM ascorbic acid (Vitamin C) and Vitamin E were taken as positive control. CFS of *E. coli* culture in nutrient broth was also used along with the samples for a comparative analysis (NB broth taken as vehicle). The OD_{530} was measured using an EnSpire 2300 Multimode Plate Reader (PerkinElmer) after 1 hr incubation at 37 °C.

Cytotoxicity and anti-inflammatory analysis

The cytotoxicity and anti-inflammatory effects of the CFS of LAB isolates were analysed in J774A.1 mouse macrophage cell line. J774A.1 cells were maintained and cultured in Dulbecco's modified eagle medium (DMEM) high glucose medium supplemented with 10% foetal bovine serum, L-glutamine, and 1% penicillin–streptomycin solution at 37 °C in 5% CO_2. The cells were seeded at the density of 0.08×10^6 cells/well in 96-well plates and allowed for 80% confluency. Later, the cells were pre-treated with CFS samples at 1:10 dilution for 1 hr, followed by lipopolysaccharide (LPS) stimulation for 6 hr. Plain MRS broth was used as a vehicle, and LPS treatment without any CFS samples was taken as a positive control. For a comparative analysis, the CFS of *E. coli* culture was also used in the study, along with the other samples. Cultured supernatants were collected after 6 hr stimulation and stored at −80 °C for enzyme linked immunosorbent assay (ELISA). Cells were added with 100 μl (1 mg) of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) reagent to measure cell viability. After 20–30 min of incubation, dimethyl sulfoxide (DMSO) was added, and OD_{570} was measured. The cell viability percentage was calculated by using the following formula:

$$\textit{Cell Viability}\ (\%) = \left[\frac{\text{OD of the sample}}{\text{OD of the control}}\right] \times 100 \qquad (2)$$

The cell-cultured supernatants were measured for secretory levels of pro-inflammatory cytokines, IL-6, and TNF-α, using ELISA Kits (R&D Systems) according to the manufacturer's protocol.

Statistical analysis

All experiments were performed in triplicate (i.e., three replicates in each experiment) on three occasions, and the results were expressed as $M \pm SD$. Statistical analyses were measured by one-way analysis of variance, unpaired *t*-test, and multiple comparison test (Tukey's test) using GraphPad Prism version 9.3.1 (GraphPad Software, Boston, USA), and a significance level of $p < .05$ was considered.

Results and discussion

Microbes isolated from millets

A total of twelve bacteria were isolated from millets and sprouted millets, which were subjected to the preliminary test by Gram's staining, catalase, and acid production. All isolated bacterial strains were Gram-positive and rod-shaped, whereas BM01 and SM02 were cocci. Further, the ability to produce acids was explored by culturing these strains on MRS agar plates supplemented with bromocresol purple (Supplementary Figure 1). Acid-producing strains turn the media's purple colour to yellow, whereas other strains did not change its colour. Among the 12 isolates, four were positive for acid production, and only these strains were considered for further studies (Supplementary Table 1).

These four LAB strains were identified as *Enterococcus sp.*, *Weissella cibaria*, and *Lactococcus lactis* by MALDI-TOF Bio-Typer analysis and also confirmed by 16S rRNA gene sequencing. The identified sequences were deposited in GenBank, and accession numbers were obtained (Table 1). LAB strains *W. cibaria* and *Weisella confusa* from different millet samples were isolated, and their probiotic potential was evaluated by Gouthami *et al.* (Gouthami et al., 2024). The diversity of LAB strains present in traditional Taiwanese millet beverage was studied by Chao et al. in 2019, and the following species were determined as predominant strains: *L. lactis*, *Levilactobacillus brevis*, *Lactococcus garvieae*, *W. cibaria*, *E. faecium*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Leuconostoc carnosum*, *Pediococcus stilesii*, and *Weissella soli* (Chao et al., 2013). However, potential probiotic bacteria obtained from pearl millet and sorghum belong to the genera *Bacillus*, *Sphingobacterium*, and *Brevibacterium* (Kunchala et al., 2016). This diversity observed among LAB strains from different millet samples might be due to the differences in the source of samples, climatic conditions, geographic area, and other environmental factors (Sedó Molina et al., 2022).

Growth of isolated LAB and acidification in MRS broth

By utilising glucose from MRS broth, all LAB isolates: *Enterococcus sp.* (BM01), *W. cibaria* (NM01), *L. lactis* (SM02), and control *L. casei* except *W. cibaria* (SM01), attained exponential growth phase within 6 hr in MRS broth and reached stationary phase soon after. *W. cibaria* strain SM01 was in exponential phase for up to 4 hr and then shifted to the stationary phase. Supplementary Figure 2A represents the growth pattern of LAB isolates in MRS broth.

All four LAB isolates cultured in MRS broth were able to reduce pH below 5 within 24 hr. The highest pH drop after 48 hr was observed with *L. lactis* (SM02), followed by *W. cibaria* (NM01 & SM01), which were 4, 4.03, and 4.08, respectively. *E. casseliflavus* (BM01) and positive control *L. casei* were shown to have a comparatively higher pH (Supplementary Figure 2B).

Utilisation of sugars and quantification of acids and ethanol

Glucose plays an important role in human metabolic activities, and its homeostasis is essential for the effective functioning of several organs and tissues. Numerous *in vitro* and *in vivo* studies have proven that probiotics can regulate blood glucose levels and prevent the development of metabolic disorders by affecting gut microorganisms and metabolite production (Ma et al., 2023). Glucose utilisation and metabolite production by all four millet-based LAB strains were analysed by HPLC (Figure 1). Complete utilisation of glucose was observed with BM01, whereas the strains SM01, SM02, and *L. casei* (positive control) consumed glucose to the level below 0.2 g/L from the initial concentration of ∼11 g/L. NM01 did not utilise the sugar completely. In contrast, different strains of *L. lactis* obtained from insect microbiomes showed an incomplete utilisation of glucose (∼50%) in a study conducted by Sedó Molina et al. (Sedó Molina et al., 2022). Conversion of glucose to lactic acid was efficient in all four strains, ranging from 0.95 to 1.2 g/L. More than 11 g/L lactic acid production was observed with BM01, SM01, and SM02, whereas with NM01, it was ∼9.6 g/L. Higher acetic acid production was observed with SM02 and control, followed by BM01, SM01, and NM01. All strains produced ethanol, with SM01 and SM02 being the highest and least ethanol-producing strains, respectively, among all four LAB isolates.

Table 1. Comparison of the bacterial identification results obtained by MALDI-TOF biotyper and 16 s rRNA gene sequencing.

Isolates name	MALDI-TOF biotyper		16 s rRNA gene sequencing	
	Identified strain	Log score	Identified strain	Accession no.
BM01	*Enterococcus faecalis*	2.39	*Enterococcus casseliflavus*	PP355677
NM01	*Weissella cibaria*	2.49	*Weissella cibaria*	PP355678
SM01	*Weissella cibaria*	2.47	*Weissella cibaria*	PP355679
SM02	*Lactococcus lactis*	2.35	*Lactococcus lactis*	PP355680

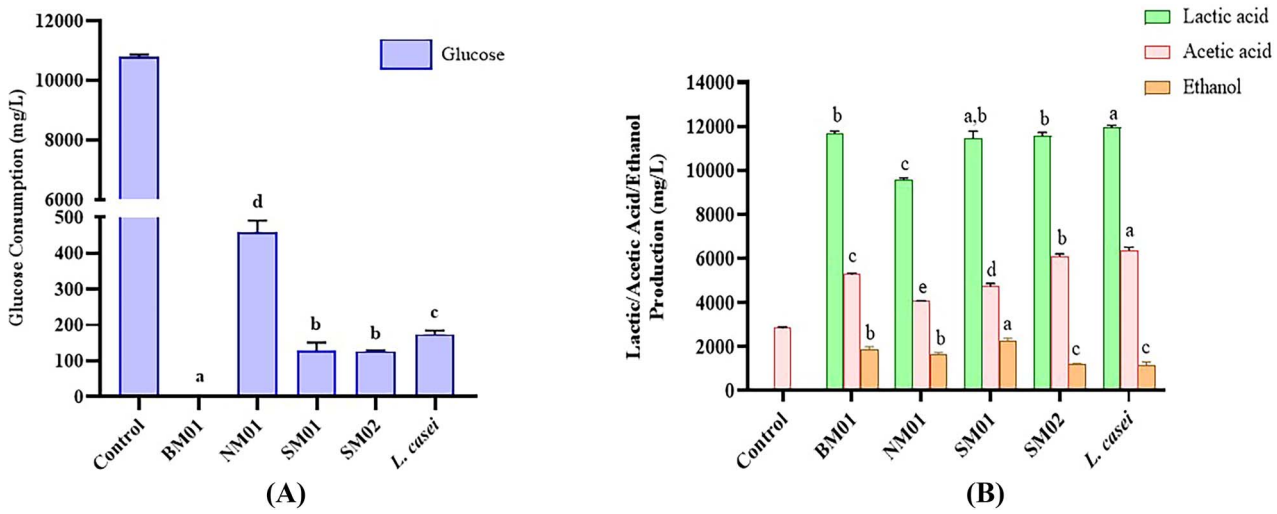


Figure 1. (A) Glucose utilisation and (B) metabolite production by lactic acid bacteria isolates in Man, Rogosa, and Sharpe broth after 48 hr incubation at 30 °C and determined by HPLC. The same letters above each mean indicate no significant difference based on an unpaired *t*-test with a 5% probability ($p < .05$).

Based on the carbohydrate metabolism and fermentation products, LAB can be divided into homofermentative and heterofermentative. Homofermentative bacteria produce lactic acid (>90%) as the major end product of carbohydrate metabolism, whereas heterofermentative bacteria produce other metabolites such as ethanol, acetic acid, and CO_2 along with lactic acid. Though *L. lactis* is well known as a homofermentative bacterium, under certain circumstances (oxygen-rich environment or slow metabolised carbon source), it can shift into a heterofermentative nature (Kowalczyk & Bardowski, 2007; Passerini et al., 2013; Xiao, 2021). This might explain the acetic acid and ethanol production observed with the *L. lactis* SM02 strain in this study. Further, studies on the effects of LAB on glucose metabolism in type 2 diabetes (T2D) patients show a significant reduction in fasting plasma glucose, suggesting that the glucose utilisation ability of LAB strains could be a therapeutic option to improve T2D mellitus in patients through better metabolic control (Kocsis et al., 2020; Zhang et al., 2016). Glycemic control through the intake of probiotic fermented milk was observed in T2D patients, along with increased levels of acetic acid (Tonucci et al., 2017). A study conducted by Wang et al. (2020) in mice models proven that higher glucose utilisation capability of intestinal probiotics can serve as a better treatment option for management of diabetes (Wang et al., 2020).

Millet-derived LAB show tolerance to acid, bile salt, phenol, and NaCl

As per protocol by the Food and Agriculture Organisation of the United Nations and the World Health Organisation (WHO) (2001), every potential probiotic strain should be effectively identified both phenotypically and genotypically, followed by a number of experiments that evaluate its survival capacity and functional characteristics, as mentioned earlier. Some of the key challenges that orally administered probiotics face in the GI tract are acidity, the presence of bile salts, and pancreatic enzymes. A potential probiotic strain must be able to withstand these stressful environments in order to survive in the GI tract (Shokryazdan et al., 2014). In this study, the ability of isolated LAB strains to combat the gastrointestinal conditions, was analysed by: (1) mimicking acidic condition of the human gut (subjected to various pH conditions), (2) mimicking small intestine environment with bile salt (subjected to range of bile slat condition), (3) testing the survivability and inhibition of LAB under phenol stress (subjected to range of phenol condition), and (4) testing the NaCl tolerance by osmotic stress (subjected to range of NaCl condition) based on the method described by Kunchala et al. (Kunchala et al., 2016).

The ability of selected LAB isolates to grow under different pH conditions showed higher growth potential at pH 5–7. BM01 and SM01 were unable to grow at pH 4, whereas NM01 and SM02 showed higher growth after 24 hrs compared to the positive control *L. casei* (Figure 2A). All isolates were unable to show higher growth potential below pH 3, but they might be able to survive in this stressful condition for a few hours. All isolates showed significant growth in the presence of up to 0.5% bile salt from 0 to 24 hr incubation. SM02 showed higher growth potential than *L. casei* under 0.5% bile salt conditions. None of the strains grew under 0.8% bile salt (Figure 2B). In contrast, LAB strains obtained from African fermented pearl millet slurries, which belong to the genera *Pediococcus* and *Lactobacillus*, were able to survive at acidic

pH and in the presence of 0.3% bile salt (Turpin et al., 2011). Optimum gastric pH ranges from 1.5 to 3.5, and bile salt content in the small intestine ranges from 0.2% to 0.3%, but it can vary based on the host and the quantity and type of food consumed (Divisekera et al., 2019; Menconi et al., 2013). In our study, all LAB isolates can grow in the intestine in terms of their pH and bile salt tolerance, whereas confirmation of their survival in gastric conditions for a few hours demands further analysis.

The ability of LAB strains to tolerate the presence of phenol was analysed, and all four strains showed a significant increase in growth up to 0.3% of phenol concentration. BM01 and SM02 were able to grow in the presence of 0.4% phenol. However, their growth potential is significantly reduced compared to the positive control *L casei* (Figure 3A). The NaCl tolerance test for all LAB isolates showed growth potential with 8% NaCl. A significant increase in growth from 0 to 24 hr in all isolates (except SM01 at 8%) was observed with 4%, 5%, and 8% NaCl. None of the strains showed growth at 12% NaCl (Figure 3B). Tolerance of LAB strains to 0.3%–0.4% phenol and 8% NaCl concentration ensures their probiotic potential to survive in intestinal conditions. In a similar study, five LAB strains, including *L. lactis*, obtained from fermented finger millet, showed promising probiotic efficacy by growing in upto pH 1.5, 1.5% bile salt, 0.4% phenol, and 12% NaCl (Divisekera et al., 2019).

CFS of LAB strains inhibits pathogens

The antibacterial activity of selected LAB isolates against *E. coli* EFR02 and *Salmonella typhimurium* MTCC 1251 showed higher inhibition in 25% CFS compared to 15% and 20% CFS. 25% CFS of SM01 (98.8 ± 0.5%), followed by NM01 (88.3 ± 3.1%), and SM02 (77.5 ± 3.0%) showed potential antibacterial activity against *E. coli*, whereas BM01 (41.16 ± 2.89%) had lower inhibition potential compared to all other isolates. All the LAB isolates showed higher inhibition against *S. typhimurium* MTCC 1251 with 15%, 20%, and 25% CFS. 100% inhibition was observed with 25% CFS of NM01, SM01, and SM02, whereas BM01 showed 76.5 ± 1.2% inhibition (Supplementary Figure 3). Potential antimicrobial activity of LAB isolates (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) against foodborne pathogens, *S. typhimurium, E. coli, Staphylococcus aureus*, and *Listeria monocytogenes*, was reported by (Yang et al., 2018). Natural antimicrobial substances produced by LAB (bacteriocins) and the pH drop caused by organic acids such as lactic, acetic, and formic acid play an important role in the inhibition of pathogenic bacteria (Ibrahim et al., 2021). Additionally, Nora Hamdaoui et al. (2024) reported the antibacterial effects of different *L. lactis* strains due to synthesis of antibacterial metabolites, including lactic acid, diacetyl, hydrogen peroxide, and protein (e.g., bacteriocin) (Hamdaoui et al., 2024). The antibacterial activity of LAB isolates observed in this study might be due to either bacteriocin production or pH drop or both, which demands more studies, such as whole genome sequencing, for genomic level confirmation.

LAB strains show resistance to antibiotics

Antibiotic resistance is an essential feature of probiotics for survival in the GI tract. However, harbouring intrinsic, non-transmissible genetic factors for antibiotic resistance is considered a major safety assessment for probiotics (Abdel Tawab et al., 2023; Zheng et al., 2017). In this study, the antibiotic resistance of the LAB strains against ampicillin, chloramphenicol, amikacin, and vancomycin was carried out using the agar disc diffusion method and the zone of inhibition was analysed after 24 hr of incubation. BM01, SM01, and the positive control *L. casei* showed susceptibility to ampicillin, vancomycin, and chloramphenicol, whereas they were resistant to amikacin. Meanwhile, NM01 and SM02 were susceptible to ampicillin and chloramphenicol and resistant to both amikacin and vancomycin (Supplementary Table 2). Several studies reported different antibiotic resistance patterns as probiotic characteristics. According to a previous report, LAB strains from Egyptian fermented food with probiotic potential showed resistance to vancomycin and susceptibility to chloramphenicol (Abdel Tawab et al., 2023). However, genetic-level analysis is necessary to confirm the absence of transmissible genetic factors related to antibiotic resistance, which will ensure the safety of LAB strains to use as probiotics.

CFS derived from LAB strains elevated antioxidant activity

The antioxidant activity of LAB strains was studied using CFS samples, and all the undiluted samples showed higher radical scavenging activity on AAPH. 1:10 dilution of samples was unable to scavenge AAPH, whereas 1:5 dilution samples of SM02 and NM01 showed a better scavenging activity than BM01 and *L. casei* (Figure 4). The radical scavenging activity of different concentrations of vitamin E and vitamin C provided as supplementary data (Supplementary Figure 4). Eight food-derived strains, belonging to the genera *Pediococcus, Leuconostoc, Weissella, Levilactobacillus,* and *Latilactobacillus,* showed significant antioxidant potential by the inhibition of nitric oxide (NO) (Kim et al., 2022). According to Amadou et al. (2013), the radical scavenging activity and antibacterial activity exhibited by fermented foxtail millets are due to the presence of foxtail millet-derived peptide fractions with sequences: Ser-Gly-Tyr-Tyr-Met-His, Leu-Gly-Thr-Phe-Gln-Asn, and Leu-His-AlaLeu-Leu-Leu (Amadou et al., 2013). Several studies from past decades have shown that different types of LAB may exhibit varying mechanisms of antioxidant potential, such as metal ion chelation (Lee et al., 2005), antioxidant enzymes system (e.g., superoxide dismutase) (Kullisaar et al., 2002), metabolites (Ahire et al., 2013), and various signalling pathways (Gao et al., 2013; Wang et al., 2017). Thus, the exhibited antioxidant activity of the LAB samples in the current study might be due to any of these mechanisms.

CFS derived from LAB strains reduced inflammatory response (*in vitro*)

The CFS of all five isolates were subjected to analysis of their cytotoxicity effect by MTT assay. All samples showed higher cell viability of J774A.1 mouse macrophage cell, in the presence and absence of LPS (Figure 5A). The highest cell viability was observed with *L. casei*, followed by BM01.NM01, SM01, and SM02 showed comparatively less growth in the presence of LPS. However, there was no significant difference ($p < .05$) observed between samples. The result obtained with CFS of *E coli* is given in the supplementary data (Supplementary Figure 5).

The anti-inflammatory roles of LAB have been reported by several studies, which help to regulate inflammatory conditions in the host. In this study, the anti-inflammatory effects were analysed by treating the J774A.1 cell line with CFS samples and detecting the IL-6 and TNF-α levels by using ELISA. SM02 showed a significant reduction of the pro-inflammatory cytokine IL-6 when compared to other samples and the positive control (LPS). There was no significant difference observed between the positive control and NM01, SM01 and *L. casei*, whereas BM01 showed a higher level of IL-6 production (Figure 5B). Significantly higher levels of TNF-α were obtained with the CFS of BM01, SM01 and *L casei* in

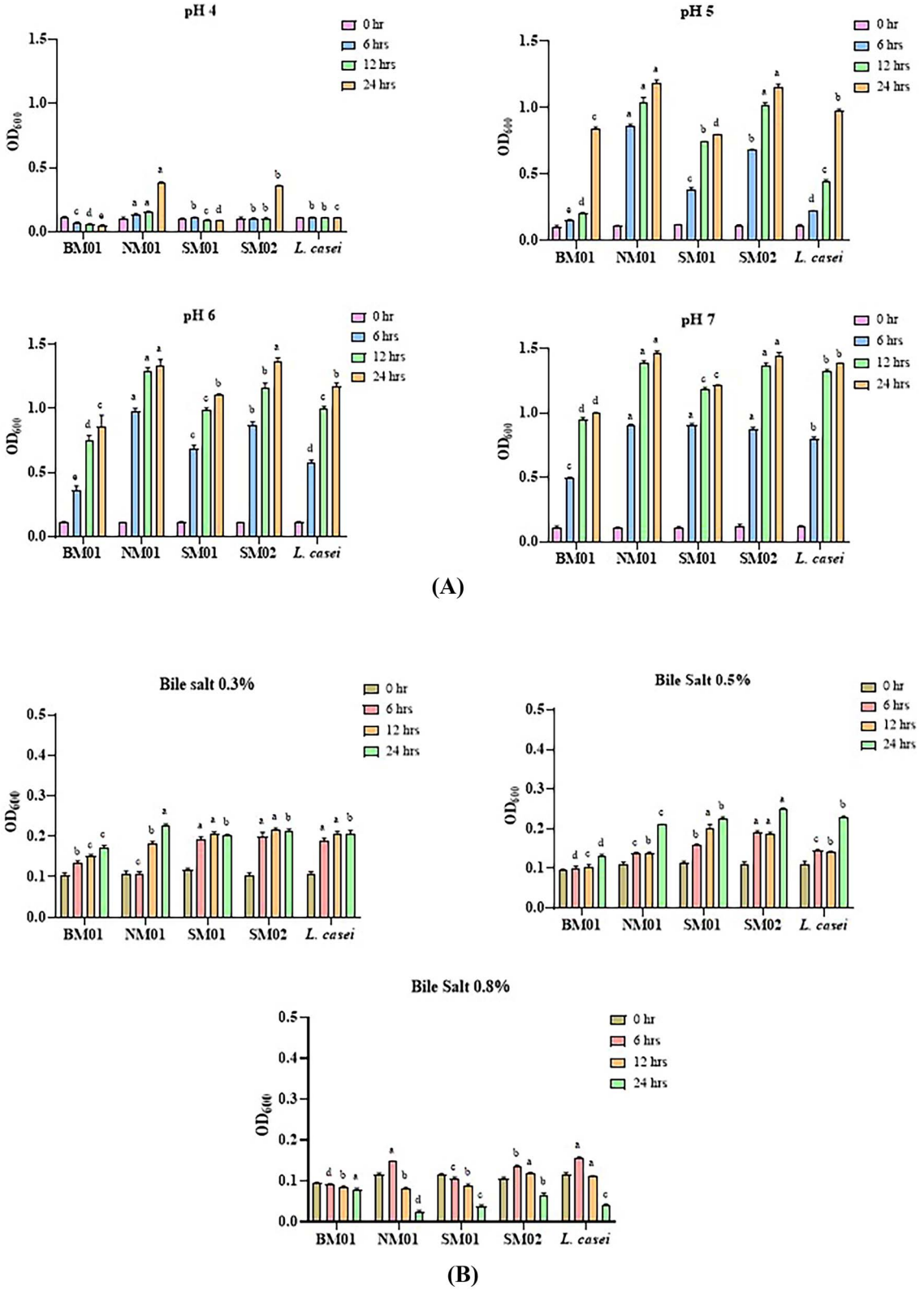


Figure 2. Growth pattern of lactic acid bacteria isolates in (A) different pH and (B) bile salt. The same letters above each mean indicate no significant difference based on multiple comparison test (Tukey's test) with a 5% probability ($p < .05$).

comparison with LPS treatment. There was no significant difference between the LPS control and NM01 and SM02 (Figure 5C). The results of *E coli* treatments are given in the supplementary data (Supplementary Figure 6). Treatment of Caco-2 cells with *L. lactis* NCDO 2118 strain showed a potential anti-inflammatory effect by downregulating IL-1β-induced IL-8 secretion (Luerce et al., 2014). Upregulation of anti-inflammatory cytokine IL-10 and reduction of Th2 cytokines IL-4 and IL-5 in a murine model of inflammation

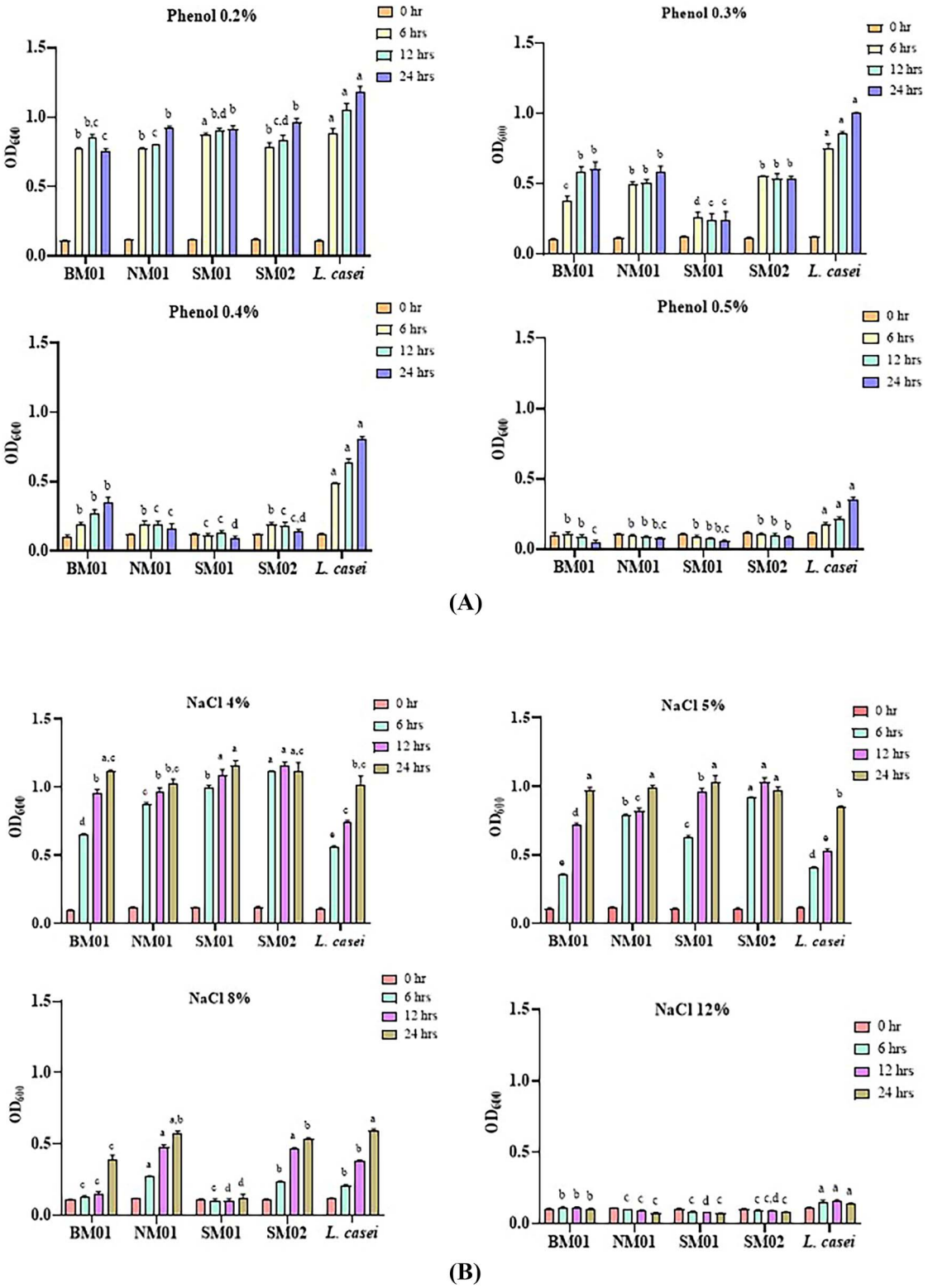


Figure 3. Growth pattern of lactic acid bacteria isolates in the presence of (A) phenol and (B) NaCl. The same letters above each mean indicate no significant difference based on multiple comparison test (Tukey's test) with a 5% probability ($p < .05$).

by the probiotic *Bacillus* strains (*Bacillus subtilis* and *Bacillus amyloliquefaciens*) were reported by Kim et al. (Kim et al., 2021a). The regulation of inflammatory conditions by probiotics occurs through different mechanisms such as T regulatory cells (Treg) maturation, induction of IL-10, and regulation of nuclear factor kappa-B (NF-κB), Toll-like receptor (TLR) and mitogen-activated protein kinase (MAPK) pathways (Cristofori et al., 2021; Plaza-Díaz et al., 2017).

To provide an overall pre-evaluation of the safety of biological agents such as LAB, the qualified presumption of safety (QPS)

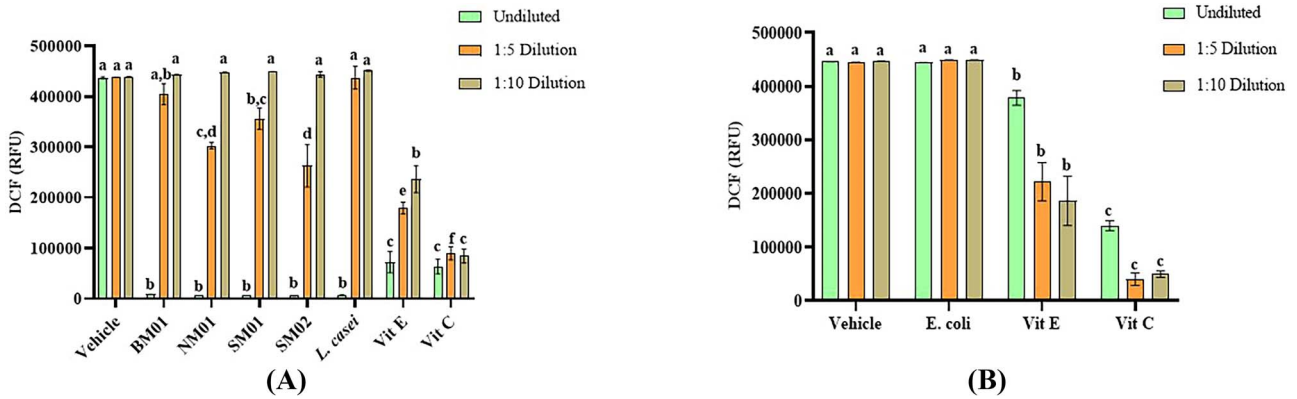


Figure 4. Radical scavenging activity of cell-free supernatants of lactic acid bacteria (LAB) isolates obtained by the 2,2′-azobis(2-amidinopropane) dihydrochloride method. (A) Antioxidant effect of LAB isolates along with Man, Rogosa, and Sharpe broth as vehicle and (B) antioxidant effect of *Escherichia. coli* with NB broth as vehicle. The same alphabet above each mean indicates no significant difference based on multiple comparison test (Tukey's test) with a 5% probability ($p < .05$).

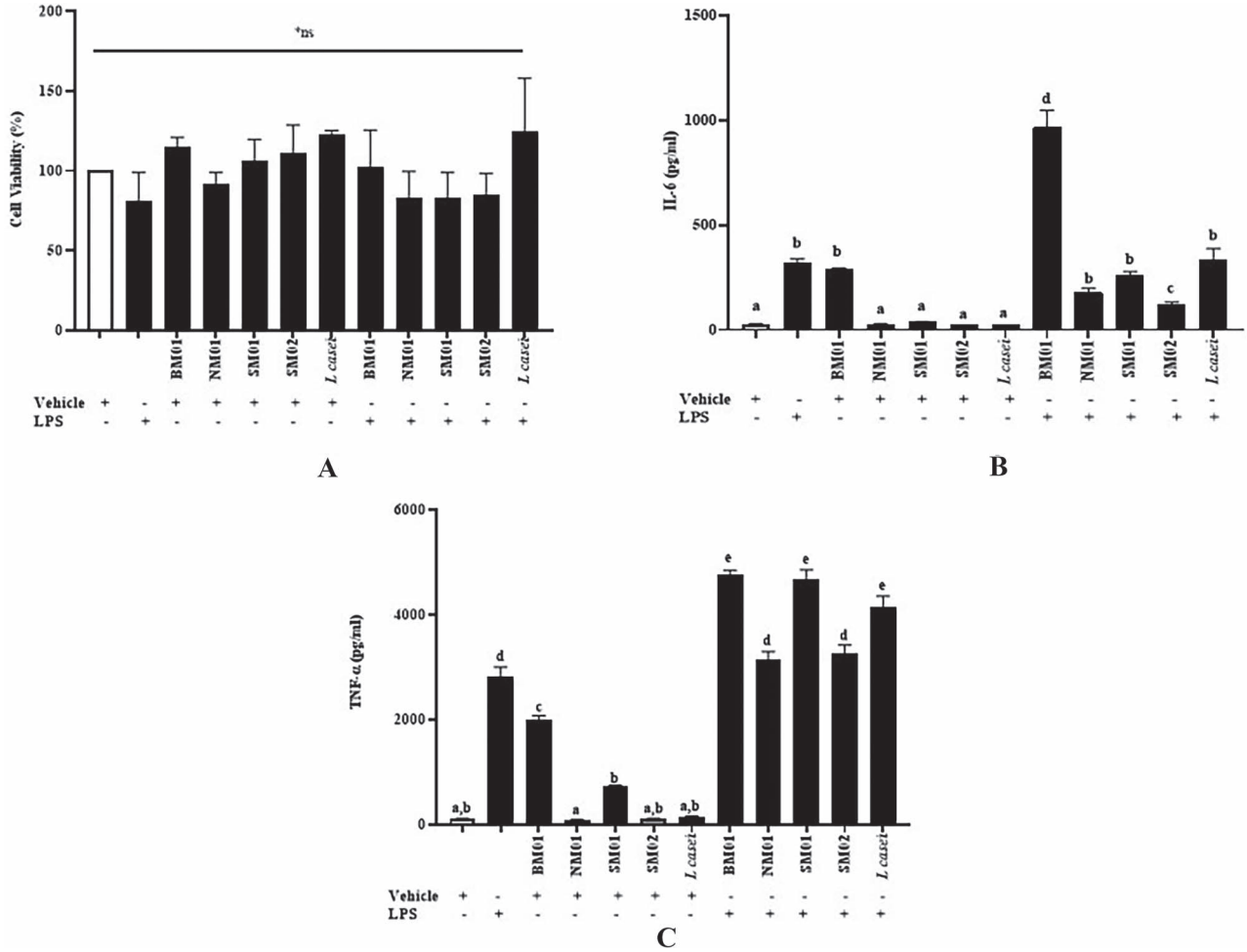


Figure 5. (A) Cell viability of J774A.1 cell after the treatments with cell-free supernatant (CFS) of lactic acid bacteria (LAB) isolates. Treatment with all the samples was able to maintain the cell viability. (B) Production of proinflammatory cytokine IL-6 after the treatments with CFS of LAB isolates. Treatment with the sample SM02 showed higher anti-inflammatory properties with significant differences obtained between samples based on multiple comparison test (Tukey's test) with a 5% probability ($p < .05$). (C) Production of TNF-α was not reduced by the treatment with LAB CFS samples. No significant difference obtained between samples based on multiple comparison test (Tukey's test) with a 5% probability ($p < .05$). The same alphabet above each mean indicates no significant difference.

approach was established by the European Food Safety Authority (EFSA), which addresses the safety concerns for the environment, humans, and animals. Among the four LAB strains, *L. lactis* SM02 (from little millet) falls under the QPS, which makes it an interesting candidate for utilisation in fermented food and beverages as a potential starter culture. However, the antibiotic resistance of SM02 against amikacin and vancomycin raises safety concerns about using the strain as a beneficial microbe due to

the chances of antibiotic resistance gene transfer to pathogenic bacteria (Li et al., 2020). This demands more safety assessments, including whole genome sequencing, before considering *L. lactis* SM02 as a potential probiotic strain. Further, the utilisation of *W. cibaria* and *E. casseliflavus* as probiotics was reported by several studies (Akbari et al., 2021; Kang et al., 2019; Kang et al., 2020; Safari et al., 2016; Yu et al., 2019) without a confirmation of their safety attributes by EFSA. Studies are required to determine whether millet-based LAB strains have beneficial attributes for fermented non-dairy functional food.

Conclusion

LAB isolated from millet are identified in this study as a possible non-dairy microbe with promising functional characteristics. Among the isolates, *L. lactis*, *W. cibaria*, and *E. casseliflavus* showed strong antibacterial and antioxidant properties in addition to notable probiotic potential, including resistance to bile salts, acidic pH, phenol, and NaCl. Additionally, these strains produced significant amounts of lactic (9,000–12,000 mg/L) and acetic (1,000–3,000 mg/L) acids through their efficient metabolism of glucose. Interestingly, only *L. lactis* (SM02), which is classified under the Qualified Presumption of Safety, substantially decreased pro-inflammatory cytokine IL-6, offering promising clinical and industrial applications. These results highlight the potential of millet-based LAB in developing plant-based sustainable solutions. Further, more studies such as whole genome sequencing are needed to confirm the safety attributes of the *L. lactis* for clinical usage and product development in later stages.

Supplementary material

Supplementary material is available at *International Journal of Food Science and Technology* online.

Data availability

The sequences of 16S rRNA sequencing were deposited at GeneBank-NCBI under the accession number: PP355677, PP355678, PP355679, and PP355680.

Author contributions

Mrudula M. Mohanan (Investigation, Formal analysis, Methodology, Writing—review & editing, Writing—original draft), Radhakrishna Shetty (Conceptualization, Investigation, Methodology, Supervision, Writing—review & editing), Prerana S. Bhat (Methodology, Investigation, Formal analysis), Vidyaranyapura S. Deepashree (Methodology, Investigation, Formal analysis), Rajesh Kumar Thimulappa (Writing—review & editing, Conceptualization, Funding acquisition), Claus Heiner Bang-Berthelsen (Writing—review & editing, Supervision), and Kiran Kumar Mudnakudu-Nagaraju (Conceptualization, Supervision, Writing—review & editing, Funding acquisition, Project administration).

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Conflicts of interest

The authors state that they have no conflict of interest.

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