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Isolation and characterization of probiotic lactic acid bacteria from fermented traditional rice for potential applications in food and livestock production

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ABSTRACT

Probiotic lactic acid bacteria (LAB) are widely used in fermented food products and as feed additives for livestock, poultry, and fish. Fermented rice presents significant potential as an alternative carrier for probiotics. Therefore, this study aimed to isolate, identify, and characterize potential probiotic LAB strains from fermented traditional rice cultures. After primary isolation on De Man, Rogosa, and Sharpe (MRS) agar, presumptive isolates were first phenotypically characterized using Gram staining, catalase assay, endospore staining, and motility tests. Preliminary phenotypic tests identified 48 isolates as presumptive LAB. 16S rRNA sequencing analysis confirmed the presence of five species; *Lacticaseibacillus casei, Lacticaseibacillus paracasei*, and *Lacticaseibacillus rhamnosus*, notably, *Schleiferilactobacillus harbinensis*, and *Liquorilactobacillus vini* were identified for the first time in fermented rice. In the biochemical characterization, none of the isolates produced H_2S, and all exhibited a homofermentative glucose utilization pattern. The majority (71 %) demonstrated detectable growth at 15 °C and 45 °C and tolerated NaCl concentrations up to 6.5 %. Regarding probiotic potential, the isolates were sensitive to widely used therapeutic antibiotics and exhibited strong antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Salmonella enteritidis*, and *Candida glabrata*. They also showed bile tolerance up to 0.3 % (w/v) and possessed milk coagulation ability. Our findings suggest these LAB strains have potential for use in fermented foods and livestock feed due to their probiotic properties, including antimicrobial activity, bile tolerance, and milk coagulation. They may serve as natural alternatives to antibiotics and additives, but further in vivo studies are needed to confirm their efficacy and stability.

1. Introduction

The majority of probiotic bacteria commonly used in food and feed are lactic acid bacteria (LAB), including *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus*, and *Leuconostoc*. In addition to LAB, other bacterial genera such as *Escherichia coli* and *Propionibacterium*, as well as yeast species like *Saccharomyces*, are frequently used as probiotics (Butel, 2014). While fermented dairy products and the animal intestine are the most common sources, probiotic bacteria can also be derived from various traditional fermented products made from diverse substrates (Rivera-Espinoza & Gallardo-Navarro, 2010). Additionally, non-dairy, plant-based foods such as fruits, vegetables, grains, and legumes have proven to be effective probiotic carriers (Rasika et al., 2021).

Fermented rice has the potential to serve as an alternative non-dairy probiotic carrier (Jeygowri et al., 2015). Traditional fermented rice-based foods, such as "*Diya Bath*" in Sri Lanka, offer several health benefits, including enhanced micronutrient bioavailability. The presence of LAB in fermented rice aids in breaking down anti-nutritional factors, further improving nutrient absorption (Karunaratne, 2018;

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Perera et al., 2010).

Various *Lactobacillus* species isolated from fermented rice have demonstrated probiotic properties, including antimicrobial activity, bile tolerance, and acid resistance (Victor-Aduloju et al., 2018). Recent studies on fermented rice from India identified functionally active *Lactobacillus fermentum* and *Lactobacillus plantarum* strains with strong gut-survival and health-promoting properties. Their key functional attributes, such as galactosidase activity and antibacterial effects, suggest potential applications in functional foods for lactose-intolerant individuals and immune support products (Bhatt et al., 2024). Therefore, assessing fermented rice as a traditional non-dairy probiotic source is essential.

The production of fermented dairy products relies on lactic acid, the primary metabolic end-product of LAB (Fernández et al., 2015). Probiotic microbes are widely incorporated into these products to enhance nutrient digestion and bioavailability, inhibit pathogenic bacteria in the gut, and improve sensory attributes like texture (Patrignani et al., 2020). Therefore, exploring unconventional sources of probiotic LAB is essential for identifying potential starter cultures and functional probiotics suitable for fermented milk products.

The use of probiotics as feed additives in livestock production has increased following the ban on Antibiotic Growth Promoters (AGPs) in the livestock and poultry industry (Mingmongkolchai & Panbangred, 2018). Probiotics offer numerous benefits, including improved growth, feed conversion efficiency, immune response, and protection against enteric pathogens (Yousaf et al., 2022). Further research is needed to identify promising probiotic strains and optimize their application in livestock, poultry, and fish as AGP alternatives. This study aims to isolate and characterize potential probiotic LAB species from fermented rice cultures, evaluating their physiological, biochemical, and probiotic properties. We hypothesize that fermented rice cultures harbor diverse LAB species with probiotic potential suitable for various applications.

2. Materials and methodology

2.1. Isolation of lactic acid bacteria

2.1.1. Sample preparation

Fermentation was conducted by soaking raw *Pachchaperumala* rice, a popular traditional heirloom variety in Sri Lanka, in sterile distilled water (1:3 rice-to-water ratio) overnight (12–16 h) at 27 °C with 70–80 % relative humidity. Sterilized cow milk and bee honey were then added as enrichment ingredients. After one week of fermentation, cultures were collected for analysis. The study included four replicates per sample, each prepared in separate batches.

2.1.2. Inoculation and incubation

The inoculation of bacteria from fermented rice cultures was performed according to the method described by Kowsaly et al., (2022) with modifications. Commercial de *Man, Rogosa, and Sharpe* (MRS) agar (HiMedia, India, M641I) was prepared following the manufacturer's instructions. A dilution series of fermented rice cultures was prepared up to 10^{-7} for four replicates. From each dilution, 100 μL was inoculated into prepared Petri dishes and incubated at 37 °C for in anaerobic jars (Oxoid Ltd, Hampshire, UK) with an anaerobic environment (<1 % O_2, 9–13 % CO_2) generated using AnaeroGen® sachets (AN0025A, Oxoid Ltd, Hampshire, UK).

2.1.3. Purification

Morphologically distinct and well-isolated colonies were selected based on colony morphology and suspended in MRS broth (HiMedia, India, GM369). Gram-positive, rod-shaped colonies were then streaked onto MRS agar plates and incubated at 37 °C under anaerobic conditions for 48 h. Distinct colonies were sub-cultured on fresh agar plates to obtain pure cultures.

2.2. Morphological identification of lactic acid bacteria isolates

The macroscopic characteristics of the isolates, including colony size, shape, color, and texture, were examined. Tests were conducted following the procedures outlined by the American Society for Microbiology (Hussey, 2016; Patricia Shields & Cathcart, 2011a; Reiner, 2013; Smith & Hussey, 2016).

2.2.1. Gram's staining

Air-dried and heat-fixed smears of pure cultures were sequentially stained with crystal violet, Gram iodine, a decolorizing agent, and safranin, with gentle washing after each step. The slides were then blot-dried with absorbent paper and examined under an oil immersion microscope (Optika, Italy B-190).

2.2.2. Catalase test

A small amount of a fresh, well-isolated colony was transferred onto a microscope slide using a sterile inoculating loop. A drop of 3 % H_2O_2 was then added. The rapid formation of air bubbles indicated a positive catalase test (Reiner, 2013).

2.2.3. Endospore test (spore staining)

A bacterial smear was prepared on a microscope slide and covered with 10 % aqueous malachite green, then steamed for 2 min. After washing, the smear was flooded with 0.5 % aqueous safranin, rinsed, dried, and examined under oil immersion for the presence or absence of green-stained spores (Hussey, 2016).

2.2.4. Motility test

The hanging drop method was used to assess the motility (Patricia Shields & Cathcart, 2011b). A loopful of fresh, overnight-grown broth culture was placed at the center of a coverslip. A cavity slide was then inverted and gently placed over the coverslip, creating a depression over the culture drop. The slide was carefully inverted, allowing the drop to hang freely in the cavity. The cells were observed under medium power (× 40) with reduced light for the presence or absence of movement.

2.3. Long-term preservation of isolates

Presumptive LAB isolates were preserved for future experiments by suspending bacterial cells in a cryoprotectant medium with 15 % (v/v) glycerol to minimize freezing damage. MRS broth was prepared according to the manufacturer's instructions, inoculated with pure culture colonies, and incubated at 37 °C for 24 h. After incubation, sterilized glycerol was added, and the samples were stored at -20 °C for further experiments.

2.4. Physiological and biochemical characterization of the isolates

2.4.1. Hydrogen sulfide production

Hydrogen sulfide (H_2S) production was assessed using Triple Sugar Iron (TSI) agar (HiMedia, India, M021). TSI agar slants were prepared according to the manufacturer's instructions. Fresh colonies from each isolate were separately inoculated into the butt and streaked on the slant. The tubes were incubated at 37 °C for 24 h, and H_2S production was indicated by the development of a black color in the butt due to ferrous sulfide formation.

2.4.2. Gas production from glucose fermentation

Fermentation patterns aid in bacterial classification. Each isolate was inoculated into test tubes containing MRS broth with glucose (20 g/L) and inverted Durham tubes, then incubated at 37 °C for 24 h. Gas accumulation in Durham tubes indicated CO_2 production from glucose fermentation.

2.4.3. Evaluating temperature tolerance

LAB temperature tolerance was assessed following Putri et al. (2020) with modifications. Test tubes containing MRS broth were inoculated with 1% (v/v) fresh overnight culture and incubated at 5, 15, 37, and 45 °C for 48 h. Growth was measured using a UV spectrophotometer (V-1100D, M.R.C LTD, Israel) at OD_{620}, with tolerance expressed as ΔOD_{620} from 0 to 48 h.

2.4.4. Evaluating NaCl tolerance

NaCl tolerance of LAB isolates was assessed following Putri et al. (2020), with few modifications. MRS broth was adjusted to 2, 4, and 6.5% NaCl and inoculated with 1 % (v/v) fresh overnight culture. After incubation at 37 °C for 48 h, growth was measured using a UV spectrophotometer at OD_{620}, with tolerance expressed as ΔOD_{620} from 0 to 48 h.

2.5. Molecular identification of lactic acid bacteria isolates

Molecular identification of selected isolates followed Adikari et al. (2021). Pure cultures were grown in MRS broth at 37 °C for 24 h, then centrifuged at 12,500 × g for 10 min. The supernatant was discarded, and pellets were stored at -20 °C for DNA extraction using a QIAamp® DNA mini kit (QIAGEN, USA). Extracted DNA was stored at -20 °C. The 16S rRNA gene was amplified using primers TH008 (forward 5′ AGRGTTYGATTMTGGCTCAG 3′) and PH1522 (reverse 5′ AAGGAGGTGATCCAGCCGCA 3′). PCR conditions included an initial denaturation (94 °C, 3 min), followed by 35 cycles of denaturation (94 °C, 45 s), annealing (50 °C, 60 s), elongation (72 °C, 90 s), and a final extension (72 °C, 10 min). PCR products (2 μL) were electrophoresed on a 1% agarose gel with ethidium bromide, using a 1 kb DNA ladder, and visualized with a Gel Documentation System (Vilber Lourmat®, France).

2.5.1. Sequencing of 16S rRNA gene

The total amplified product was electrophoresed on a 1 % agarose gel, excised, and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Purified products were sequenced via Sanger dideoxy sequencing by Genelabs® Medical Private Limited (Colombo, Sri Lanka). Resulting gene sequences were trimmed, cleaned, and compared using the BLAST algorithm in GenBank (NCBI, Bethesda, MD, USA). Identification was confirmed based on sequence similarity scores. The 16S rRNA gene sequences were deposited in the GenBank nucleotide database under the accession numbers: PV133518 (LAB-1), PV133524 (LAB-2), PV133521 (LAB-3), PV133523 (LAB-4), and PV133519 (LAB-5).

2.6. Determination of probiotic properties of lactic acid bacteria isolates

2.6.1. Determination of antibiotic sensitivity of the isolates

The disc diffusion method, standardized by Kirby and Bauer, was used to assess antibiotic sensitivity, following the American Society for Microbiology guidelines with minor modifications (Hudzicki, 2012). McFarland 0.5 standard solutions were prepared for each isolate. A sterile swab was dipped into the inoculum tube, and Mueller-Hinton (MH) agar (Oxoid, England, CM0337B) plates were inoculated by streaking the swab across the entire agar surface. The following antibiotics were tested: Ampicillin (25 μg/disc), Chloramphenicol (10 μg/disc), Ciprofloxacin (10 μg/disc), Cefotaxime (30 μg/disc), Erythromycin (15 μg/disc), Tetracycline (30 μg/disc), and Vancomycin (30 μg/disc) (MASTDISCS®, Mast Group Ltd.). Antibiotic-impregnated discs were placed on the agar and incubated at 37 °C for 16–18 h. After incubation, inhibition zones were measured and classified as sensitive or resistant based on American Society for Microbiology standards (Hudzicki, 2012).

2.6.2. Determination of antimicrobial properties of the isolates

The antimicrobial properties of the isolates were evaluated using the agar well diffusion assay, following the method described by Balouiri et al. (2016). McFarland 0.5 standard solutions were prepared for pathogenic species, including *Candida albicans, Candida glabrata, Staphylococcus aureus* (ATCC 29,213), *E. coli* (ATCC 25,922), and *Salmonella enteritidis*. Two milliliters of each prepared pathogen culture were flood-inoculated onto MHA plates (90 mm). Sterile cork borers (9 mm) were used to create wells on the MHA plates, which were then loaded with 180 μL of cell-free supernatants from overnight-grown broth cultures. Uninoculated MRS broth served as the negative control. Following incubation at 37 °C for 24 h, the inhibition zones around each well were measured. An inhibitory zone diameter exceeding 1 mm was considered indicative of significant antibacterial activity (Balouiri et al., 2016).

2.6.3. Determination of bile tolerance of the isolates

Bile tolerance of the isolates was assessed following the method of Hu et al. (2018). Each isolate was inoculated into two test tubes: one containing MRS broth with 0.3 % (w/v) bile salts (SRL, India, 50,362) and another without bile salts (control). Both tubes were incubated at 37 °C for 24 h. Bacterial growth was measured using a UV spectrophotometer, and bile tolerance was expressed as the percentage of OD620 in the presence of bile salts relative to the control.

2.6.4. Determination of milk coagulation ability of the isolates

McFarland 0.5 standard solutions were prepared for each isolate. One milliliter of the suspension was added separately to 10 mL of sterilized cow and buffalo milk and incubated at 45 °C for 16 h. Milk coagulation was assessed at the end of the incubation period. The pH of the milk was measured before inoculation and after incubation using a pH meter (Trans Instruments, Singapore, BP 3001, Range: 0 to 14 pH, Accuracy: ± 0.02 pH, Operating temperature: 5 to 40° C).

2.7. Statistical analysis

All data were analyzed using one-way ANOVA. A Two-Factor Factorial CRD model was applied to experiments such as temperature tolerance, NaCl tolerance, antibiotic sensitivity, and antimicrobial property testing. Statistical analysis was conducted using Minitab® version 21.2, with mean comparisons performed using Tukey's range test. Differences were considered statistically significant at $P < 0.05$.

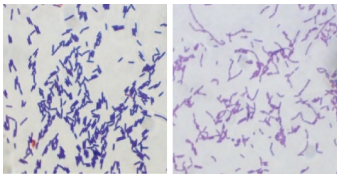
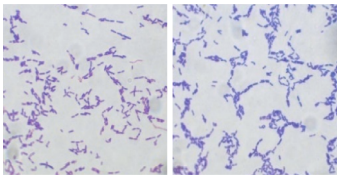
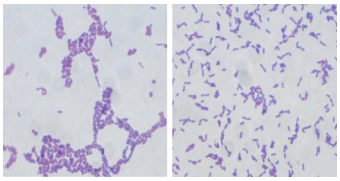
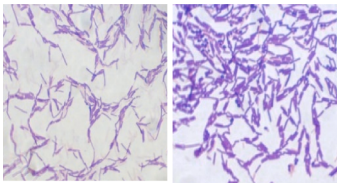
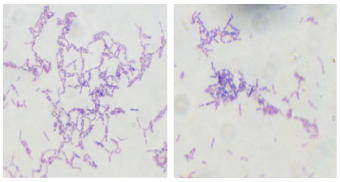
3. Results and discussion

3.1. Isolation and morphological characterization of lactic acid bacteria isolates

The primary isolation of LAB species was based on their growth on de MRS agar and colony morphology. Cell morphology was examined using an oil immersion objective (× 1000), revealing that most isolates from fermented rice cultures were rod-shaped bacilli. Five distinct morphological types were identified in this study, including short, medium, long, and very long rods, appearing as single cells, pairs, or short chains as illustrated in Table 1.

Morphologically, most LAB are coccobacilli or rod-shaped, with chain formation common in certain genera. They are Gram-positive, catalase-negative, non-spore-forming, and mostly non-motile species (Earnshaw, 1990). In this study, a subsequent purification process was carried out for catalase-negative and viable isolates. Gram-positive, rod-shaped bacteria were further tested for motility and endospore formation. A total of 48 isolates were classified as presumptive LAB based on their Gram-positive, catalase-negative, non-motile, and non-spore-forming characteristics. These isolates were preserved in MRS broth with 15 % (v/v) sterilized glycerol and stored at -20 °C for future investigations.

Table 1
Morphological characteristics of isolated lactic acid bacteria from fermented rice cultures.

Group	No. of Isolates	Morphological Description	Appearance under the Light Microscope (× 1000 Magnification)
LAB-1	15	Long size, Gram-positive, rod-shaped bacteria, arranged as single/pair or as chains	
LAB-2	15	Medium size, Gram-positive, rod-shaped bacteria, arranged as a pair/group or as chains	
LAB-3	13	Small, rectangular, Gram-positive, rod-shaped bacteria, arranged as a pair/group or as chains	
LAB-4	03	Very long size, Gram-positive, rod-shaped bacteria, arranged as single/pair or as a group	
LAB-5	03	Short chains of smaller cells, Gram-positive, rod-shaped bacteria, arranged as groups or chains	

3.2. Molecular characterization of lactic acid bacteria isolates

The identification of LAB isolates using 16S rRNA sequencing has emerged as a powerful molecular technique for exact identification of the isolates (Janda and Abbott, 2007). This method relies on sequencing the hypervariable regions of the bacterial 16S rRNA gene, which contain conserved regions suitable for primer binding as well as variable regions that provide species-specific sequence signatures. By comparing the obtained sequences with reference databases such as the National Center for Biotechnology Information (NCBI) GeneBank, LAB isolates can be accurately identified at the genus and species levels (Sun et al., 2015). The 16S rRNA gene sequencing identified the LAB isolates from fermented rice cultures, with sequence similarities ranging from 99.03 % to 99.56 % to reference strains in the GenBank database. The identified species included *Lacticaseibacillus casei, Lacticaseibacillus paracasei, Schleiferilactobacillus harbinensis, Liquorilactobacillus vini,* and *Lacticaseibacillus rhamnosus*. Detailed identification results, including sequence identities and accession numbers, are presented in Table 2.

The *Lactobacillus casei* group (recently known as *Lacticaseibacillus*), comprising *L. casei, L. paracasei*, and *L. rhamnosus*, sourced from fermented foods, and dairy. They are widely used in food fermentation, biopharmaceuticals, and health applications, promoting gut health and potentially treating various diseases. (Hill et al., 2018).

Das et al. (2019) identified and characterized *Lacticaseibacillus casei* from rice beer prepared in Assam, India, as part of a study on LAB.

Table 2
Identification of the lactic acid bacteria isolates from fermented rice cultures by sequence comparisons using NCBI BLAST algorithm.

Group of Isolates	Identified species	Identities (%)	GenBank ID of reference strain	Accession number
LAB-1	*Lacticaseibacillus casei*	99.46	JQ412731.1	PV133518
LAB-2	*Lacticaseibacillus paracasei*	99.56	CP104303.1	PV133524
LAB-3	*Schleiferilactobacillus harbinensis*	99.27	CP041364.1	PV133521
LAB-4	*Liquorilactobacillus vini*	99.03	AY681132.1	PV133523
LAB-5	*Lacticaseibacillus rhamnosus*	99.07	KM513647.1	PV133519

Identification was confirmed through 16S rRNA sequencing, highlighting its potential as a probiotic and starter culture. According to Jarocki et al. (2023) *L. casei* strains are notable for their beneficial effects on human health and their applications in industrial processes, including dietary supplements and probiotics. Additionally, their prophage sequences contribute to genetic diversity, facilitating colonization of new ecological niches and influencing bacterial metabolism. According to (Baliyan et al., 2021) *L. paracasei* was isolated from undistilled lugri (traditional fermented alcoholic beverage from the North-Western Himalayas, using wheat, rice, and barley as substrates),

demonstrating superior probiotic attributes, and antioxidant activity. Another study isolated *L. paracasei* from Tapuy, an indigenous alcoholic beverage made from fermented glutinous white rice. The study evaluated its probiotic properties, demonstrating its potential as a candidate probiotic strain due to various desirable attributes (Malilay et al., 2024a). Rice-based Lugri harboring the highest number of promising isolates. *Lacticaseibacillus paracasei* LUL:01 demonstrated superior antioxidant activity and successfully fermented milk while maintaining viable counts above legal requirements during 28 days of storage, making it a strong candidate for probiotic functional food applications (Baliyan et al., 2021).

L. rhamnosus was identified among the LAB isolated from fresh Chinese traditional rice wines. The study utilized a culture-dependent method alongside denaturing gradient gel electrophoresis to successfully monitor LAB diversity, including *L. rhamnosus* (Jiao et al., 2016). *Schleiferilactobacillus harbinensis* (previously known as *Lactobacillus harbinensis*) recently identified as a novel species from traditional Chinese fermented vegetables, "Suan cai," in Harbin, Northeastern China (Miyamoto et al., 2005). Taxonomic analysis revealed that these strains, initially classified alongside *Lactobacillus perolens* DSM 12,745, exhibited distinct genetic and phenotypic characteristics, including facultative heterofermentation, lactate and ethanol production, and a DNA G+C content of 53–54 mol %. However, there is currently no documented evidence of its isolation from fermented rice sources and this study is the first time that we document the presence of *Schleiferilactobacillus harbinensis* from fermented rice. *L. harbinensis* significantly enhances the organoleptic qualities and nutritional profile of fermented foods by converting carbohydrates into beneficial acids and hydrolyzing proteins into bioactive peptides, thereby increasing the overall nutritional value of the final product (Zheng et al., 2020).

Lactobacillus vini (recently known as *Liquorilactobacillus vini*) is a significant LAB spp. in the context of winemaking and ethanol fermentation, primarily due to its unique stress responses and metabolic capabilities. This bacterium not only survives in harsh industrial environments but also plays a role in the fermentation process, influencing both the quality and efficiency of production (Mendonça, 2018; Nogueira Da Silva et al., 2019) However, there is currently no documented evidence of its isolation from fermented rice sources.

3.3. Biochemical and physiological characterization of lactic acid bacteria isolates

3.3.1. H_2S production

In the current study, none of the isolates exhibited black precipitate formation in triple sugar iron (TSI) agar, indicating the absence of H_2S production (Table 3). In contrast, *E. coli* and *Salmonella*, used as positive controls, displayed gas production and H_2S precipitation in the anaerobic region of the medium (Fig. 1). TSI agar contains three carbohydrates: sucrose (1 %), lactose (1 %), and glucose (0.1 %). This method differentiates bacteria based on their ability to produce H_2S. Black precipitate formation in the anaerobic region of the medium indicates H_2S production from thiosulfate. This occurs due to the reaction between H_2S and ferrous ammonium sulfate, forming black ferrous sulfide. Gas production (CO_2 and O_2) was identified by agar displacement or splitting. In some cases, substantial gas production can cause the agar to be pushed upward within the tube (Lehman, 2000).

Lactobacillus spp. primarily ferment carbohydrates via the glycolytic pathway, in which sugars are metabolized to pyruvate and then converted to lactic acid. The absence of sulfur-reducing enzymes in LAB precludes the production of hydrogen sulfide as a fermentation by-product (LeBlanc et al., 2013). Furthermore, research has shown that the metabolic pathways of LAB are highly conserved across different species and strains. This metabolic uniformity suggests that LAB does not possess the biochemical pathways required for hydrogen sulfide production (Broadbent et al., 2012).

3.3.2. Gas production from glucose fermentation

Fermentation patterns help categorize bacteria into distinct groups (Table 3). Fig. 1 illustrates CO_2 production during glucose utilization. The results indicate that all isolates utilized glucose as a fermentation substrate. However, none produced CO_2, exhibiting a homofermentative sugar utilization pattern. This process primarily yields acid or, in some

Table 3
Physiological characteristics of isolated lactic acid bacteria from fermented rice cultures.

Group	Isolates	H_2S production	Gas production from glucose fermentation	Growth at different conditions						
				Temperatures (°C)				NaCl concentration (%)		
				5	15	37	45	2	4	6.5
LAB-1	1	–	–	–	+	+	+	+	+	+
	2	–	–	–	+	+	+	+	+	+
	3	–	–	–	–	+	+	+	+	+
	4	–	–	–	+	+	+	+	+	+
	5	–	–	–	+	+	+	+	+	+
	6	–	–	–	–	+	+	+	+	–
LAB-2	7	–	–	–	+	+	+	+	+	+
	8	–	–	–	+	+	+	+	+	–
	9	–	–	–	+	+	+	+	+	–
	10	–	–	–	+	+	+	+	+	+
	11	–	–	–	+	+	+	+	+	+
	12	–	–	–	+	+	+	+	+	+
LAB-3	13	–	–	–	+	+	+	+	+	+
	14	–	–	–	+	+	+	+	+	–
	15	–	–	–	+	+	–	+	+	+
	16	–	–	–	+	+	+	+	+	+
	17	–	–	–	–	+	+	+	+	+
	18	–	–	–	+	+	+	+	+	+
LAB-4	19	–	–	–	–	+	+	+	+	+
	20	–	–	–	+	+	+	+	+	+
	21	–	–	–	+	+	–	+	+	–
LAB-5	22	–	–	–	–	+	+	+	+	+
	23	–	–	–	+	+	+	+	+	–
	24	–	–	–	+	+	+	+	+	–

+ Positive reaction
- Negative reaction

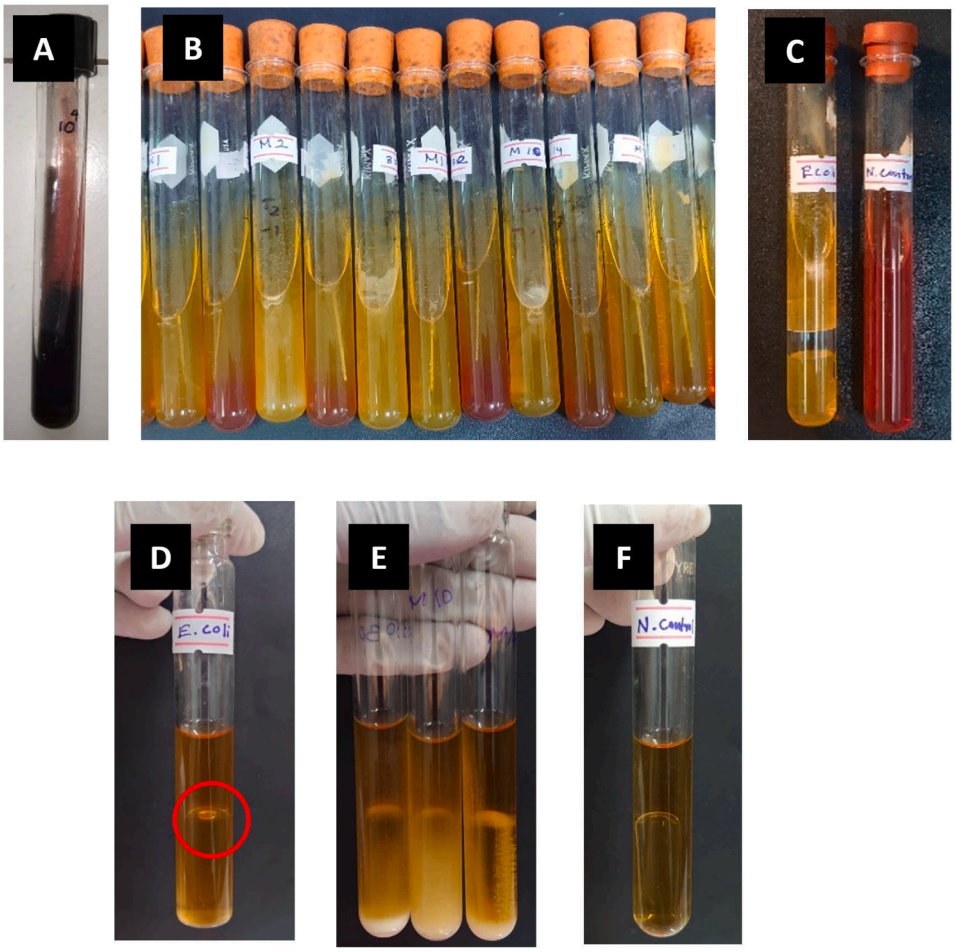


Fig. 1. H_2S and gas production from glucose fermentation by LAB isolates recovered from fermented rice cultures. The top row shows TSI slants inoculated with *Salmonella* (A), LAB isolates (B), *E. coli* (C), and a negative control (uninoculated tube). The bottom row illustrates carbon dioxide production patterns in *E. coli* as the positive control (D), LAB isolates from fermented rice cultures (E), and the negative control (F).

cases, acid with gas production (CO_2). The specific end-products vary based on the substrate, microbial species, enzymatic activity, and environmental conditions such as pH and temperature (Reiner, 2012).

According to Buron-Moles et al. (2019) the *Lacticaseibacillus casei* group exhibits homofermentative characteristics, primarily fermenting carbohydrates to lactic acid. It lacks key enzymes like 1-phosphofructokinase for D-mannose degradation and has limited metabolic diversity compared to heterofermentative species. *Schleiferilactobacillus harbinensis* also efficiently utilizes glucose via glycolysis, resulting in high lactic acid yields, a characteristic feature of homofermentative LAB (Buron-Moles et al., 2019). Similarly, *L. vini* is homofermentative, exclusively fermenting pentoses to produce DL-lactate as the sole end product. It does not generate gas from glucose and employs an inducible pentose phosphate pathway, distinguishing its metabolic pathway from heterofermentative lactobacilli (Rodas et al., 2006).

3.3.3. *Evaluating temperature tolerance of lactic acid bacteria isolates*

According to the temperature tolerance test, most isolates (71 %) exhibited detectable growth at both 15 and 45 °C, while 21 % grew only at 45 °C, and 8 % only at 15 °C. None of the isolates grew at 5 °C (Table 3). The effects of isolate groups and temperature on growth are shown in Fig. 2. LAB isolates exhibited optimal growth at 37 °C, with limited growth at 5 °C. However, no significant differences ($P > 0.05$) were observed between isolate groups and their growth at different temperatures.

According to Adu et al. (2018) *Lacticaseibacillus* strains endure prolonged heat stress (30–45 °C) by downregulating energy-intensive pathways and upregulating nitrogen and carbon transport systems. Heat shock proteins play a key role in their thermal stress response. Wang et al. (2015) highlighted that thermophiles like *L. harbinensis* adapt to extreme temperatures through genomic stability, protein disulfide bonding, heat shock responses, and thermostable protein expression. These mechanisms enhance their thermal tolerance and functional acclimatization. The temperature tolerance of Lactobacilli supports their viability during food processing, ensuring effective probiotic properties and stability for pathogen suppression in industrial probiotic foods (Fossi et al., 2017).

3.3.4. *Evaluating NaCl tolerance of lactic acid bacteria isolates*

The effect of different NaCl concentrations on isolate growth is shown in Fig. 2. Most isolates (71 %) tolerated NaCl concentrations up to 6.5 %, while the rest tolerated up to 4 % (Table 3). Growth decreased with increasing NaCl concentrations, but no significant differences ($P > 0.05$) were observed between isolate groups. *Lacticaseibacillus* spp. exhibit physiological and biochemical adaptations that enhance survival under osmotic stress. *L. casei* shows increased NaCl tolerance through enhanced biofilm formation and cation binding at high salt concentrations (0.8 M NaCl), with modifications in lipoteichoic acid aiding survival (Palomino et al., 2013). *L. rhamnosus* thrives at 4 % NaCl, indicating its potential for probiotic applications, particularly in vaginal microbiome restoration Silva et al. (2019). *L. paracasei* withstands up to 9 % NaCl in MRS broth, demonstrating its resilience in food environments and the gastrointestinal tract (Malilay et al., 2024b).

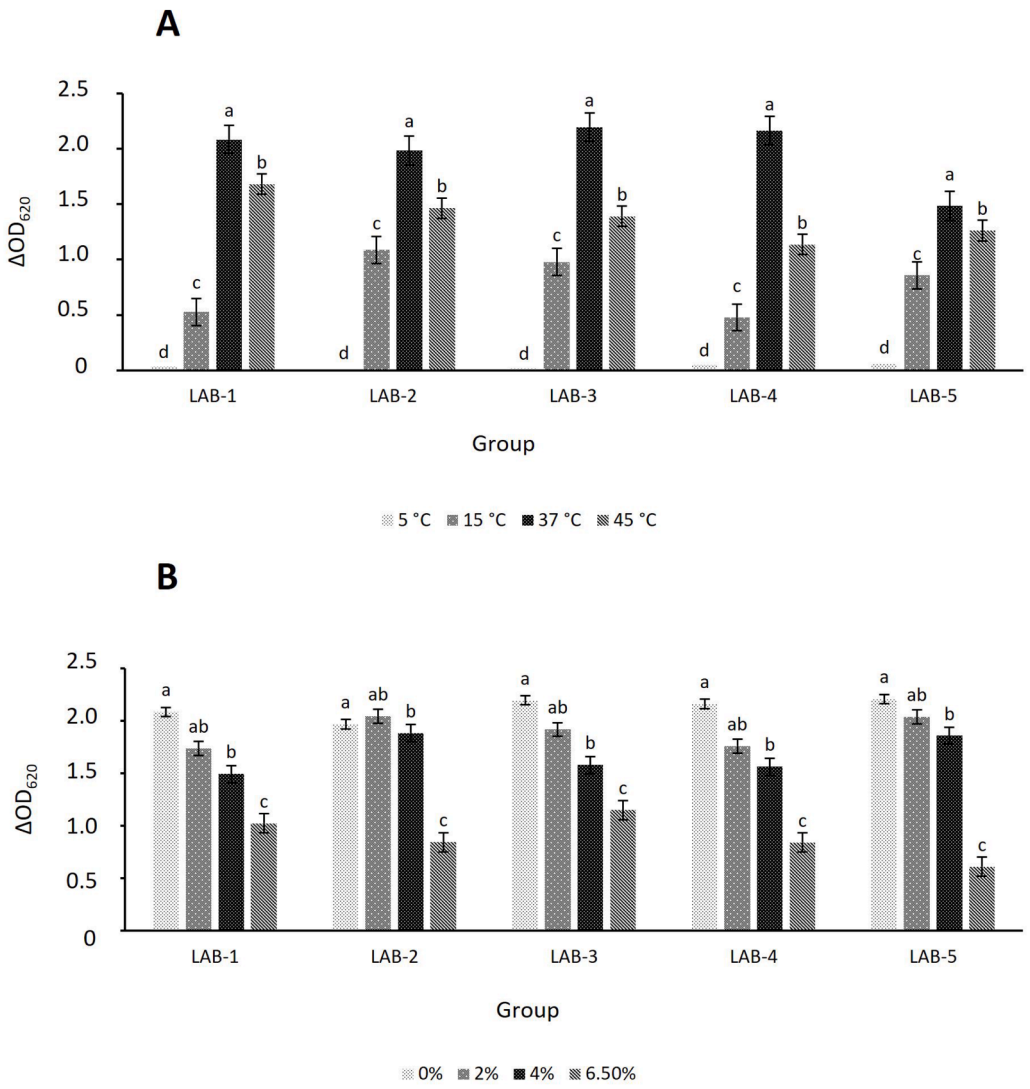


Fig. 2. Temperature (A) and sodium chloride (B) tolerance ability of different groups of isolates recovered from fermented rice cultures.

3.4. Determination of probiotic properties of lactic acid bacteria isolates

3.4.1. Antibiotic sensitivity of lactic acid bacteria isolates

The sensitivity of the isolates to commonly used antibiotics was tested by observing inhibition zones (Fig. 3). All five groups of isolated LABs were sensitive to all the antibiotics used (Table 4). According to the test results, the highest mean diameter inhibition zone was observed for erythromycin, and the lowest for chloramphenicol and ciprofloxacin. However, there were no significant differences ($P > 0.05$) among different groups of isolates and their inhibition zones.

Anisimova & Yarullina (2019) found that 90 % of *Lactobacillus* strains, including *Lacticaseibacillus* spp. demonstrated sensitivity to clindamycin, while 95 % were susceptible to tetracycline, erythromycin, and rifampicin. All strains were resistant to vancomycin, and aminoglycosides. According to Sharma et al. (2017) *L. casei* exhibited susceptibility to imipenem, meropenem, chloramphenicol, and erythromycin, with intermediate susceptibility to cefotaxime. Mangia et al. (2019) found that *L. paracasei* is susceptible to chloramphenicol, clindamycin, penicillin, amoxicillin, erythromycin, tetracycline, and ampicillin, as indicated by clear inhibition zones in antibiotic disc diffusion tests. *L. rhamnosus* also displayed sensitivity to ampicillin, tetracycline and erythromycin (Van Toi, Bao Toan Truong Quang Dang Khoa, & Ha Lien Phuong, 2023).

The assessment of antibiotic sensitivity in LAB is crucial for evaluating their safety and potential as probiotic candidates. LAB are Generally Regarded As Safe (GRAS) microorganisms. However, determining their susceptibility to antibiotics is essential to ensure that they do not harbor antibiotic-resistance genes or transfer resistance to pathogenic bacteria (Luerce et al., 2014). Strains that exhibit broad-spectrum sensitivity and minimal resistance to clinically relevant antibiotics are preferred for probiotic application. (Dicks & Botes, 2010).

Antibiotic resistance in probiotics can be beneficial if it is intrinsic, as it may help restore gut microbiota during antibiotic therapy. However, acquired resistance poses a significant risk due to the potential horizontal transfer of resistance genes to pathogens, which can lead to increased antibiotic resistance in harmful bacteria. Therefore, while intrinsic resistance may be advantageous, the presence of acquired resistance in probiotics raises safety concerns that necessitate careful evaluation of their antibiotic resistance profiles (Seyirt et al., 2023).

3.4.2. Determination of antimicrobial properties of lactic acid bacteria isolates

The antimicrobial properties of the LAB isolates against common pathogens were tested, and according to the size of the inhibition zones, all the isolates showed the ability to inhibit *E. coli* (ATCC 25,922), *S. enteritidis, S. aureus* (ATCC 29,213), and *C. glabrata* to different extents (Table 4). Cell-free supernatants of different LAB isolates gave the higher inhibition zones for *E. coli* and *C. glabrata* while the lower values were

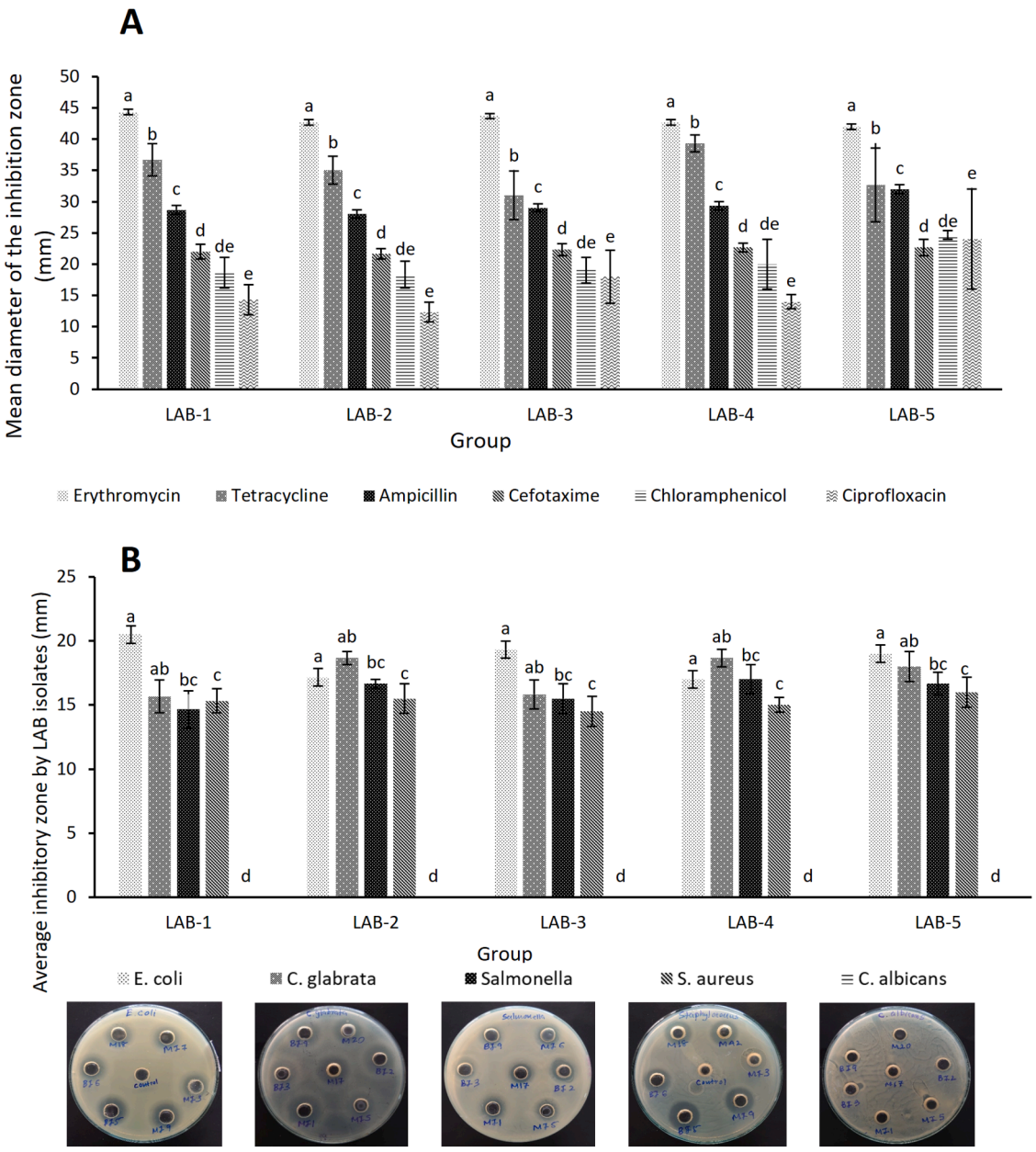


Fig. 3. Results of antibiotic sensitivity and antimicrobial properties of isolated lactic acid bacteria from fermented rice cultures. Inhibitory zones by LAB isolates against *E. coli, C. glabrata, Salmonella, S. aureus* and *C. albicans* are visualized in the bottom of the figure.

Table 4
Probiotic properties of isolated lactic acid bacteria from fermented rice-based cultures.

Group	Antibiotic sensitivity						Antimicrobial property (average inhibitory zone in mm)					Bile acid tolerance (% Reduction of ΔOD_{620})	pH reduction in cow milk	pH reduction in buffalo milk
	AP	C	CIP	CTX	E	TE	*E. coli*	*C. glabrata*	*Salmonella enteridica*	*S. aureus*	*C. albicans*			
LAB-1	S	S	S	S	S	S	P	P	P	P	N	49.60	1.26±0.08	1.26±0.05
LAB-2	S	S	S	S	S	S	P	P	P	P	N	42.70	1.23±0.05	1.28±0.04
LAB-3	S	S	S	S	S	S	P	P	P	P	N	19.26	1.35±0.08	1.59±0.07
LAB-4	S	S	S	S	S	S	P	P	P	P	N	47.90	1.47±0.09	1.31±0.07
LAB-5	S	S	S	S	S	S	P	P	P	P	N	17.39	1.55±0.09	1.39±0.09

(AP) Ampicillin, (C) Chloramphenicol, (CIP) Ciprofloxacin, (CTX) Cefotaxime, (E) Erythromycin, (TE) Tetracycline, S: Sensitive, P: Positive, N: Negative

for *Salmonella* and *Staphylococcus*. However, LAB isolates did not inhibit the growth of *C. albicans* (Fig. 3).

The key compounds responsible for the antimicrobial properties of probiotic bacteria include metabolites such as lactic acid, acetic acid, hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetone, carbon dioxide, and bacteriocins. These metabolites play a crucial role in inhibiting pathogenic microorganisms and serve as bio preservatives. (Monika et al., 2021). Bacteriocins secreting ability can indicate

potential probiotic bacteria by demonstrating selective inhibition of harmful gut microbiota species. (Elisa Heesemann Rosenkilde et al., 2024). Bacteriocins produced by bacteria can be accurately detected through microbiological assays, confocal microscopy, and gene expression analysis of biofilm formation-related genes (Kiousi et al., 2023a).

According to Fateh et al. (2024) *L. casei* demonstrated antimicrobial properties by inhibiting the growth of *Staphylococcus* isolates, *E. coli* and *Klebsiella pneumoniae*, suggesting its potential as a complementary or alternative therapy to antibiotics for bacterial infections. *L. paracasei* also demonstrated significant antimicrobial activity, particularly inhibiting biofilm formation of *Salmonella enterica*. It effectively limited the viability of planktonic cells of *Staphylococcus aureus* and *E. coli*, showcasing its potential as a probiotic with antimicrobial properties (Kiousi et al., 2023b).

L. rhamnosus exhibits significant antimicrobial properties through its cell-free supernatant and cell components, demonstrating growth-dependent inhibition against both Gram-positive and Gram-negative bacteria including *Streptococcus mutans* and *Fusobacterium nucleatum* through the production of organic acids and bacteriocins, demonstrating a broad spectrum of bacterial inhibition against various pathogenic strains (Guan et al., 2024; Zhang et al., 2023). Mosbah et al. (2018) found that *L. harbinensis* exhibits strong antifungal activity against various spoilage fungi in yoghurt, attributed to organic acids and newly identified compounds, including a spermine analogue and short cyclic polylactates, demonstrating potential applications in food and pharmaceutical industries.

3.4.3. Determination of the bile tolerance of lactic acid bacteria isolates

According to the test results, all five groups exhibited over 50 % resistance to bile acids, with the majority of isolates demonstrating the ability to survive in 0.3 % (w/v) bile salts (Table 4). Bile salts, particularly bile acids, are antimicrobial compounds produced by the liver and released into the small intestine to aid in lipid digestion and absorption (Merritt & Donaldson, 2009). The ability of probiotic LAB to withstand bile exposure is crucial for their survival, colonization, and persistence in the gastrointestinal tract, where they interact with the host immune system, modulate gut microbiota composition, and confer health benefits. Therefore, bile tolerance is a key criterion for selecting LAB strains for probiotic formulations, ensuring their viability and efficacy during transit through the digestive system (Marco et al., 2017).

Song et al. (2015) reported that *L. casei* demonstrated full tolerance to 0.3 % bile acid, demonstrating its ability to survive and grow in the presence of bile. This tolerance is attributed to specific proteins involved in membrane modification, cell protection, detoxification, and central metabolism (Hamon et al., 2012). According to de Oliveira Vogado et al. (2020) *L. paracasei* also demonstrates bile tolerance, as evidenced by its survival for over four hours in a bile salt solution. Its ability to hydrolyze bile salts further enhances its stability and growth in fermented milk. Similarly, *L. rhamnosus* displayed strong bile tolerance, with a survival rate exceeding 85 % after three hours of incubation in 0.3 % bile salt, highlighting its potential as a probiotic for gastrointestinal applications (Jiang et al., 2023).

In vitro assessments can identify potential probiotic strains; however, in vivo trials are necessary to confirm their efficacy and safety in a living organism. In vivo trials help evaluate the real-world effectiveness of probiotics in modifying bile acid metabolism (Neverovskyi & Polishchuk, 2023). For future use in vivo experiments, bacteria can be stored by freezing at -80 °C in a suitable cryoprotectant, such as glycerol or dimethyl sulfoxide (DMSO), to maintain viability. Alternatively, lyophilization (freeze-drying) can be employed for long-term storage, allowing for easier handling and reconstitution when needed for experimental purposes (Takanashi et al., 2014).

3.4.4. Determination of milk coagulation ability of LAB isolates

According to the test results, all five groups exhibited a decrease in milk pH after incubation, with most isolates demonstrating the ability to coagulate milk (Table 4). Milk coagulation is driven by LAB activity, particularly acid and exopolysaccharide production. Acidification serves as both a preservative and a flavour-enhancing process, while exopolysaccharides contribute to texture formation (Priyashantha et al., 2019). The milk coagulation ability of LAB is crucial for the production of fermented dairy products such as yoghurt, cheese, and kefir (Settanni & Moschetti, 2010).

According to Putranto et al. (2019), *L. casei* demonstrated significant milk coagulation activity (MCA) during a 12 h incubation, effectively coagulating casein and producing probiotic fresh cheese with excellent curd firmness. *L. paracasei* also exhibited MCA, indicating its potential as a starter culture for milk coagulation. However, MCA was not observed when grown in MRS broth, emphasizing the influence of growth conditions (Ahmad & Hassan, 2019). Sujaya et al., (2022) reported that *L. rhamnosus* effectively coagulates milk, making it a valuable strain for probiotic dairy production, contributing to both texture and health benefits. However, variations in enzyme activity among different *L. rhamnosus* strains suggest that not all isolates exhibit the same coagulation efficiency

Probiotics generally have an excellent safety profile, with mild, self-limiting side effects. However, caution is advised for immunocompromised individuals due to potential adverse events. Monitoring adverse effects and selecting well-studied strains can enhance safety and efficacy (Qasemi et al., 2023). Therefore, future research should focus on long-term effects and safety protocols, addressing gaps in understanding probiotic-host microbiota interactions and ensuring comprehensive coverage of probiotic regulations to maximize health benefits and ensure safety for human consumption. Safety assessments for probiotics intended for human consumption should include genetic characterization, antibiotic resistance analysis, pathogenicity and virulence gene evaluation, in vitro physiological tests, and in vivo toxicity studies (Chen et al., 2024).

4. Conclusions

Fermented rice cultures analyzed in this study were identified as a rich source of diverse LAB species. 16S rRNA sequencing revealed the presence of *Lacticaseibacillus casei, L. paracasei, L. rhamnosus, Schleiferilactobacillus harbinensis,* and *Liquorilactobacillus vini*. Biochemical and physiological characterization confirmed that none of the isolates produced H_2S, and all exhibited a homofermentative glucose metabolism. Most isolates (71%) demonstrated growth at both 15 °C and 45 °C, while a similar proportion tolerated NaCl concentrations up to 6.5 %, with the remaining isolates tolerating up to 4 % NaCl. The isolates exhibited promising probiotic properties, supporting their potential application in fermented dairy products and livestock production. They were susceptible to ampicillin, chloramphenicol, ciprofloxacin, cefotaxime, erythromycin, and tetracycline. The cell-free supernatants showed strong antimicrobial activity against Gram-negative pathogens (*S. enteritidis*) and Gram-positive pathogens (*Staphylococcus aureus*, ATCC 29,213). They also inhibited *E. coli* (ATCC 25,922) and *Candida glabrata* while demonstrating good bile tolerance in vitro. Most isolates, when added at 1 % (v/v) fresh culture, effectively coagulated cow and buffalo milk, reducing pH at 45 °C over 16 h. Given the strong market potential of fermented buffalo milk in Sri Lanka, where *Meekiri* holds cultural and economic significance, its commercialization is likely to be successful (Priyashantha et al, 2021). Further research is needed to assess additional probiotic characteristics, including acid tolerance, cell auto-aggregation, and surface hydrophobicity. Investigating the stability of probiotic traits during processing, storage, and delivery will help optimize their industrial application. Additionally, exploring their immune-modulating potential through in vivo studies and encapsulation techniques can enhance their functionality and efficacy as probiotics in dairy and livestock production systems.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability

Data will be made available on request.

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