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Exploring Dairy-Derived Probiotics: Microbiological, Biochemical and Functional Profiling of Indigenous Lactic Acid Bacteria Isolates

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Abstract

Lactic acid bacteria (LAB) derived from indigenous dairy sources represent a valuable and largely untapped reservoir for the development of safe, effective, and cost-efficient probiotic formulations. The present study aimed to isolate, characterize, and functionally evaluate LAB from fresh and fermented milk samples obtained from local dairy producers in Lucknow, Uttar Pradesh, India. Using selective media — de Man, Rogosa and Sharpe (MRS) agar, milk agar, and M17 agar — five phenotypically distinct isolates designated M1 through M5 were recovered and subjected to a comprehensive battery of morphological, physiological, biochemical, and functional tests. All five isolates were confirmed as Gram-positive, catalase-negative, non-spore-forming organisms consistent with the defining characteristics of LAB. Bacilli-shaped isolates (M1–M3) were provisionally identified as belonging to the Lactobacillus group, while cocci-shaped isolates (M4–M5) were consistent with Lactococcus or Leuconostoc genera. Biochemical profiling revealed differential patterns of carbohydrate fermentation, citrate utilization, and exopolysaccharide (EPS) production ranging from 95.3 mg/L (M5) to 117.3 mg/L (M2). Probiotic functional assessment demonstrated that M1, M2, and M3 exhibited robust tolerance to simulated gastric acid stress (pH 3.0, 2 h) and bile salt concentrations of 0.3%, both critical determinants of gastrointestinal survivability. Haemolytic activity testing confirmed the absence of beta-haemolysis in all isolates, indicating a favourable safety profile. Antibiotic susceptibility profiling showed general sensitivity to erythromycin, clindamycin, cephalothin, and oxytetracycline, with intrinsic resistance to ofloxacin and co-trimoxazole — a resistance pattern characteristic of and acceptable in probiotic LAB. Collectively, isolates M1, M2, and M3, with M3 being most promising overall, meet the key criteria for probiotic candidacy. These findings advocate for systematic exploitation of indigenous dairy microbiota as sustainable platforms for next-generation probiotic innovations.

Keywords: Dairy probiotics; exopolysaccharides; antibiotic susceptibility; Lactobacillus; functional food; gastrointestinal health

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1. Introduction

In contemporary food science, clinical microbiology, and translational medicine, probiotics have become one of the most studied topics. Probiotics are defined by the World Health Organization and the Food and Agriculture Organization of the United Nations as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (https://www.fao.org/food/food-safety-quality/a-z-index/probiotics/en/). Over the past three decades, probiotics have gained both commercial and scientific support (Hill et al., 2014). The International Scientific Association for Probiotics and Prebiotics (ISAPP) later improved this basic definition, which emphasizes the necessity of viability, efficacy, and dose-specificity as crucial requirements for a microorganism to be classified as probiotic (Sanders et al., 2019; Vinderola et al., 2022).

Lactic acid bacteria (LAB) are the largest and most clinically verified category of microorganisms investigated for probiotic potential. Lactic acid is the main metabolic end product of LAB, a phylogenetically varied group of Gram-positive, catalase-negative, non-spore-forming, and acid-tolerant bacteria. *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus,* and *Enterococcus* are important genera, many of which have been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority or Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration (EFSA, 2017; Rastogi et al., 2022).

The most historically significant ecological niche for LAB is found in dairy products, especially fermented milks such as yogurt, kefir, dahi, and traditional artisanal cheeses (figure 1) (Ağagündüz et al., 2025). Strong selective pressure is exerted by the complex biochemical environment of fermented dairy substrates, which is marked by varying pH, osmotic stress, and carbohydrate availability. This environment encourages the enrichment of stress-tolerant, metabolically versatile LAB strains with innate probiotic-like characteristics (Mugampoza et al., 2020). Indigenous dairy microbiota from developing nations, especially those with long-standing artisanal fermentation traditions, constitute a particularly rich and genetically diverse source of novel probiotic candidates that have not yet been fully characterized (Nataraj et al., 2020).

It is crucial from a scientific and strategic standpoint to identify and characterize probiotic LAB from native dairy sources. Scientifically speaking, native strains may differ from commercially recognized strains like *Lactobacillus rhamnosus* GG or *Bifidobacterium animalis* subsp. lactis BB-12 due to their distinct metabolic capacities, stress-resistance mechanisms, and bioactive compound production profiles (Tripathi & Giri, 2014). From a strategic perspective, locally sourced strains present the possibility of culturally suitable, reasonably priced probiotic solutions customized to dietary patterns and gut microbiome compositions specific to a given region, especially in low- and middle-income nations where access to commercial probiotics (figure 2) is restricted (Nair & Nair, 2020).

A microorganism's probiotic potential is assessed using a number of interrelated factors. A basic taxonomic framework is provided by morphological and phenotypic characterization (Bjorkroth & Holzapfel, 2006). Exopolysaccharide (EPS) generation, citrate utilization, and carbohydrate fermentation patterns are examples of biochemical profiling that aids in defining metabolic adaptability and functional contribution to fermented food matrices (Ruas-Madiedo & de los Reyes-Gavilán, 2005). Importantly, worldwide regulatory and scientific organizations consider functional safety characteristics such as antibiotic susceptibility, hemolytic activity, and acid and bile salt tolerance to be the major screening criteria (EFSA, 2012; Sliti et al., 2026).

Figure 1: Various types of Probiotic Food used across world; From Left to Right Cheese, Kefir, Curd, Sourdough, Pickel, Kombucha, Yogurt, Kimchi, Fermented Rice

Figure 2: Various types of Probiotic supplements used in day-to-day life

A gap in the systematic characterization of dairy-derived LAB from the northern Indian region of Lucknow was intended to be filled by the current investigation. We describe the complete microbiological, biochemical, and functional profile of five LAB isolates that were isolated from fresh and fermented milk samples. The findings offer a solid basis for selecting probiotic candidates that can be added to functional food items and nutraceutical formulations meant to promote systemic and gastrointestinal health.

2. Probiotics and Gut Health: A Mechanistic Overview

A complex web of biochemical, immunological, and ecological interactions mediates the link between probiotic microbes and human gastrointestinal health. In order to contextualize the probiotic potential of recently discovered LAB strains and to provide the scientific basis for their development as functional health products, it is imperative to comprehend these mechanisms.

2.1 The Gut Microbiome and Its Health Significance

Approximately $10^{31\text{-}32}$ microbial cells, representing over 1,000 bacterial species, are found in the human gastrointestinal system. The microbiome, or collective genome, encodes roughly 3 million genes (Thursby & Juge, 2017). The gut microbiome is now understood to be a key factor in human physiology, impacting immune system development, hormone regulation, nutrient metabolism, epithelial barrier integrity, and neurological function via the gut-brain axis (O'Riordan et al., 2025). Anxiety, depression, autoimmune disorders, metabolic syndrome, and inflammatory bowel disease have all been linked to dysbiosis, a pathological disturbance of microbiome composition and function (Marchesi et al., 2016; Dwivedi et al., 2021).

2.2 Mechanisms of Probiotic Action

Probiotics affect host health in a number of ways (figure 3). One of the most well-studied processes is competitive exclusion, in which probiotic LAB reduce pathogen colonization density by competing with enteropathogens for intestinal adhesion sites and nutritional substrates (Alshatari & Ziarno, 2026). Furthermore, a number of LAB strains generate bacteriocins, which are ribosomally synthesized antimicrobial peptides that have specific inhibitory effects on rival bacteria such as *Clostridium perfringens, Listeria monocytogenes*, and *Staphylococcus aureus* (Reuben & Torres, 2024).

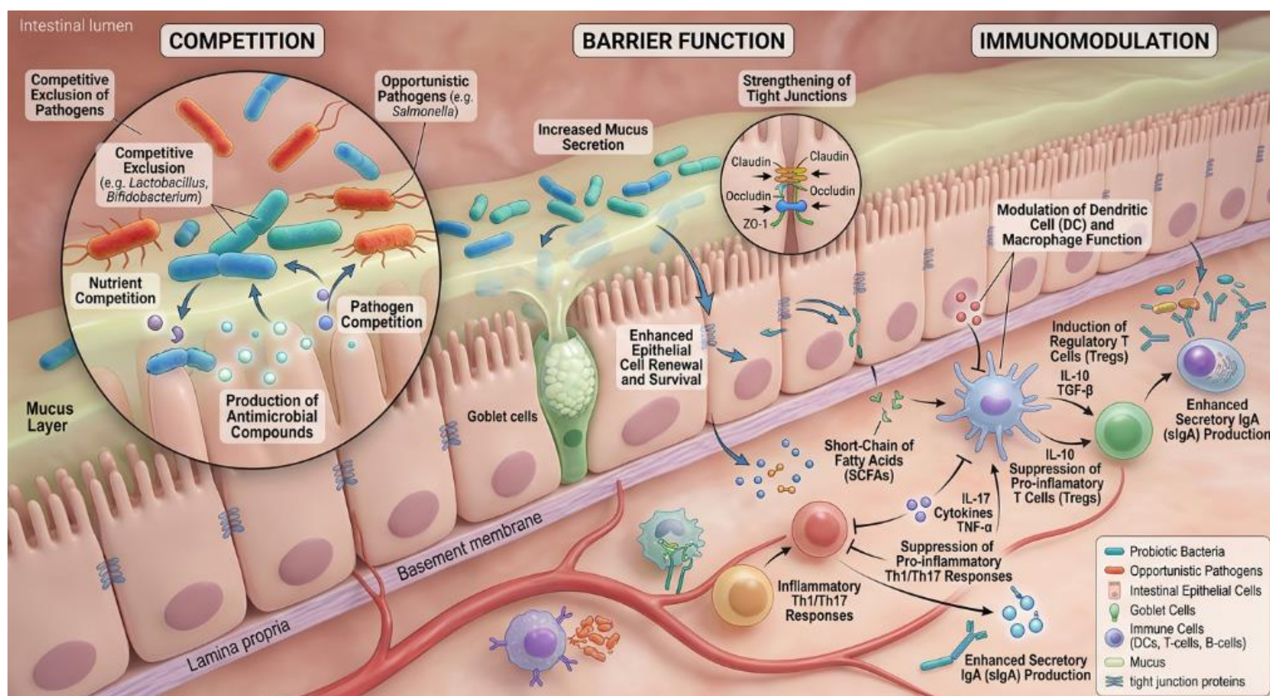


Figure 3: Mechanisms of Action of various Probiotic bacteria in gut

Another important mechanism is immunomodulation. Through pattern recognition receptors such Toll-like receptors (TLRs) and NOD-like receptors (NLRs), probiotic LAB directly interact with intestinal epithelial cells and underlying immune cells, especially dendritic cells and macrophages (Abraham et al., 2022). These interactions can strengthen mucosal immune homeostasis by increasing secretory IgA (sIgA) production, modulating T-regulatory cell activity, and promoting the production of anti-inflammatory cytokines (IL-10, TGF-β) (Mazziotta et al., 2023).

Another important way that probiotic-enriched microbiomes improve host metabolism is through the

fermentation of dietary fibers and undigested carbohydrates, which produce short-chain fatty acids (SCFA) (Baba et al., 2025). Colonic fermenters produce butyrate, acetate, and propionate, which act as energy substrates for colonocytes, control the permeability of the epithelial barrier, and reduce colonic inflammation by controlling immune gene expression through epigenetic regulation (Louis et al., 2014; Koh et al., 2016).

2.3 Exopolysaccharides: Multifunctional Probiotic Effectors

The production of exopolysaccharides (EPS) by LAB is becoming more widely acknowledged as a crucial factor in determining probiotic activity (Jurášková et al., 2022). High-molecular-weight carbohydrate polymers, or EPS, are secreted extracellularly and can be discharged into the environment or capsular (attached to the cell surface). EPS has a variety of biological functions, such as promoting intestinal colonization by improving adherence to mucosal surfaces, immunostimulatory effects through interaction with macrophage TLR receptors, prebiotic activity by selectively stimulating beneficial commensal bacteria, and protection of probiotic cells from gastric acid and bile stress (Shukla & Tangney, 2025). Additionally, EPS enhances the viscosity, mouthfeel, and consumer acceptance of fermented foods like yogurt and cheese by increasing their rheological and textural qualities (Korcz & Varga, 2021).

2.4 Stress Tolerance as a Prerequisite for Probiotic Efficacy

A probiotic microbe must make it through the harsh conditions of the stomach and small intestine in order to have its beneficial impact on health at the level of the colon. Pepsin, pancreatic proteases, bile salts, and gastric acid (pH 1.5–3.5) are all powerful physiological barriers that lower viable cell numbers by multiple logarithmic orders (Han et al., 2021). According to Papadimitriou et al. (2016), probiotics are only deemed therapeutically viable if they retain adequate viability (usually ≥10◦–10^2 CFU/mL at the intestinal target region) during exposure to various stresses.

2.5 Safety Considerations for Probiotic Selection

A non-negotiable requirement for probiotic candidacy is safety. Because beta-haemolytic bacteria have virulence characteristics that can lyse erythrocytes and are linked to pathogenic potential, the lack of haemolytic activity, especially beta-haemolysis, is a crucial safety indication (Pot et al., 2014). Because probiotic organisms must not carry transmissible antibiotic resistance genes, which could spread to pathogenic bacteria through horizontal gene transfer and contribute to the global antimicrobial resistance crisis, antibiotic susceptibility profiling is equally important (Darbandi et al., 2021).

3. Methodology

3.1 Sample Collection and Processing

Samples of fresh and fermented milk were gathered aseptically from nearby artisanal producers and dairy farms in Lucknow, Uttar Pradesh, India. To reduce microbial community shifts, samples were processed within two hours of collection and placed in sterile containers that had been previously refrigerated on ice. Each sample (1 mL) was serially diluted in sterile phosphate-buffered saline (PBS, pH 7.2) from 10^{-1} to 10^{-6} and 0.1 mL aliquots of each dilution were plated in duplicate onto selective and differential medium.

3.2 Isolation of LAB on Selective Media

To increase the variety of isolate recovery, three selective media systems were used. The standardized protocol as described elsewhere was used in the study (Rastogi et al., 2020). Lactobacilli were selectively cultivated on De Man, Rogosa, and Sharpe (MRS) agar (Hi Media, India), which was prepared in accordance with the manufacturer's instructions and supplemented with either fresh milk or fermented milk. Similar supplements were added to M17 agar, which specifically promotes the growth of lactococci and streptococci. Clear lytic halos surrounding colonies were seen to indicate proteolytic activity in milk agar, which was made by autoclaving 10% reconstituted skim milk with nutrient agar base. To simulate gut-like conditions, all plates were incubated anaerobically at 37°C for 24 hours using an anaerobic jar with Anaerogen sachets (Oxoid).

3.3 Morphological and Physiological Characterization

Different colony types were chosen after incubation according to morphological traits such as size, shape, color, surface roughness, opacity, and regularity of the margin. Repeated single-colony isolation was used to cultivate representative colonies in order to ensure their purity.

3.3.1 Gram staining

To evaluate cell wall composition and cellular shape under light microscopy at 40× magnification, Gram staining was carried out using the conventional four-step process.

3.3.2 Catalase Test

Freshly produced colonies on glass slides were treated with 3% hydrogen peroxide to measure catalase activity; if no bubbles formed, the colonies were considered catalase-negative.

3.3.3 Endospore staining

The Schaeffer-Fulton spore staining method was used to assess endospore generation.

3.3.4 Temperature Survibility Test

By inoculating broth cultures and observing turbidity after 24 hours of incubation, growth at cardinal temperatures (15°C and 45°C) was assessed, providing initial genus-level differentiation.

3.4 Biochemical Characterization

3.4.1 Carbohydrate Fermentation Test

Using phenol red broth supplemented with glucose, lactose, and maltose (1% w/v each), carbohydrate fermentation profiling was carried out. Durham tubes were inserted for gas detection. Gas buildup in the Durham tube signified gas-producing fermentation, while a color shift from red to yellow indicated acid production from fermentation.

3.4.2 Citrate Utilization Test

Simmons citrate agar was used to measure citrate utilization; the use of citrate as the only carbon source was shown by a color shift from green to blue.

3.4.3 Exopolysaccharide Synthesis Test

The phenol-sulphuric acid colorimetric method was used to measure the synthesis of exopolysaccharide (EPS) using glucose as the standard. After growing bacterial cultures in MRS broth for 24 hours at 37°C and centrifuging them for 15 minutes at 10,000 rpm, the supernatant was combined with cold ethanol (3:1 v/v) and left to incubate at 4°C overnight. Centrifugation was used to extract the precipitated EPS, which was then dissolved in distilled water and measured for absorbance at 490 nm (Rastogi et al., 2021).

3.5 Probiotic Functional Assessment

3.5.1 Haemolytic Activity

After streaking all five isolates on Columbia blood agar supplemented with 5% (v/v) defibrinated sheep blood, the cultures were aerobically incubated for 24 to 48 hours at 37°C. The presence of clear (beta-haemolysis), greenish (alpha-haemolysis), or no (gamma-haemolysis) zones surrounding the colonies was assessed on the plates.

3.5.2 Acid Tolerance

Logarithmic-phase cultures in MRS broth were exposed to simulated gastric fluid (pH adjusted to 3.0 using 1 N HCl, with 3 g/L pepsin) for two hours at 37°C with constant shaking (150 rpm) in order to assess acid tolerance. Using serial plating on MRS agar and 48 hours of incubation at 37°C, viable cell counts were measured at 0 and 2 hours. The percentage decrease in viable count was used to depict survival.

3.5.3 Bile Salt Tolerance

By inoculating cultures in MRS broth enriched with 0.3% (w/v) ox-gall bile salts (Sigma-Aldrich), a physiologically relevant concentration that approximates circumstances in the proximal small intestine, tolerance to bile salts was evaluated. Viable counts were calculated at 0 and 4 hours after cultures were cultured for 4 hours at 37°C. The percentage survival in comparison to the untreated control was used to express the results.

3.5.4 Antibiotic Susceptibility Testing

All isolates' antibiotic susceptibility patterns were ascertained using the Kirby-Bauer disc diffusion method in compliance with the Clinical and Laboratory Standards Institute's guidelines (CLSI, 2021). To support picky LAB development, overnight cultures were swabbed equally onto Mueller-Hinton agar supplemented with 5% defibrinated sheep blood after being adjusted to 0.5 McFarland standard turbidity (≈1.5 x 10^2 CFU/mL) in sterile saline. After applying antibiotic discs, the plates were incubated for 18 to 24 hours at 37°C. Using CLSI/EUCAST breakpoints, inhibition zones were measured in millimeters and classified as Susceptible (S), Intermediate (I), or Resistant (R).

4. Results

4.1 Isolation and Colony Morphology

Five representative isolates, designated M1 through M5, were chosen from the primary plates based on colony shape (figure 4). Isolate M1 developed small (1–3 mm), creamy white, circular, convex colonies with complete edges and a smooth, glossy surface when it was recovered from MRS agar supplemented with fresh milk. Isolate M2, which was developed on MRS agar with fermented milk, produced mid-sized, opaque, grayish-white colonies with a distinctive "sour" smell and a border that was somewhat uneven or lens-shaped. Large, white-to-off-white colonies encircled by conspicuous, clear halos of proteolysis, signifying extracellular protease activity, were the distinguishing feature of isolate M3, which was obtained from milk agar. Compared to the bacillus-type isolates, isolate M4, which was isolated from M17 agar supplemented with fresh milk, formed very small (pin-point), translucent-to-white, smooth, regular-edged colonies. Recovered from M17 agar with fermented milk, isolate M5 developed tiny, slimy or mucoid, glistening, elevated, whitish-grey colonies that were consistent with *Leuconostoc*-type organisms that overproduce EPS. Table 1 provides specifics with all details.

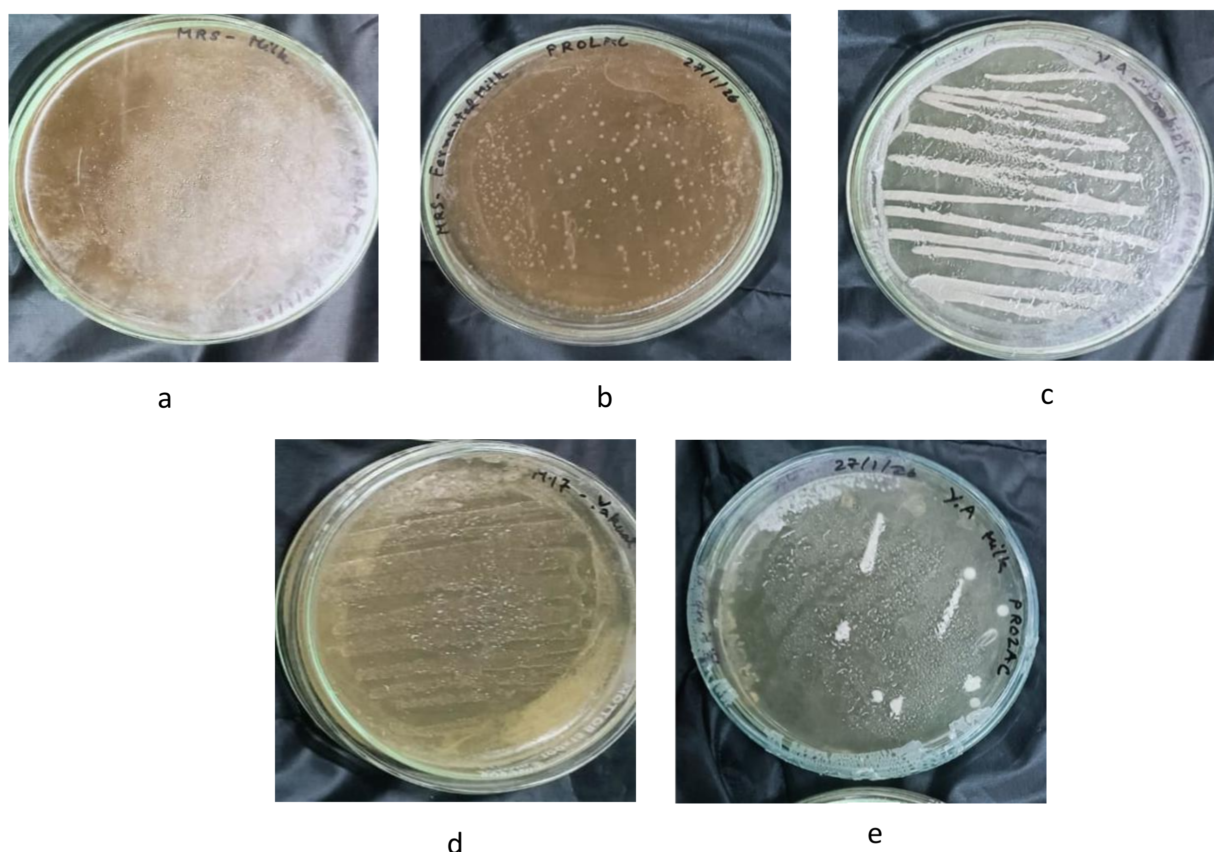


Figure 4: The five Probiotic Isolates which were obtained from samples, denoted by a. M1, b. M2, c. M3, d. M4, e. M5

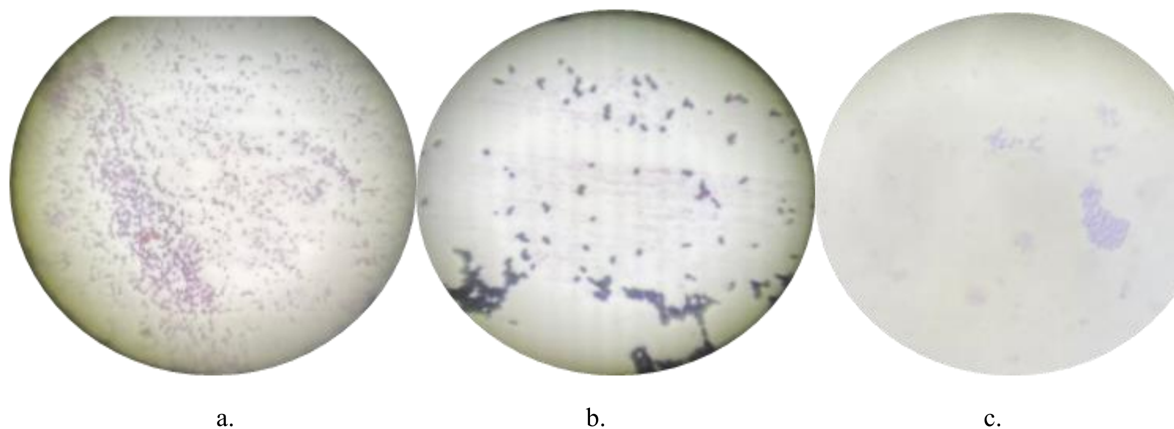
Table 1. Isolation Source and Colony Characteristics of LAB Isolates

Isolate	Source	Medium Used	Colony Characteristics
M1	Fresh Milk	MRS + Milk	Small (1–3 mm), creamy white, circular, convex, entire margins, smooth/glistening surface
M2	Fermented Milk	MRS + Fermented Milk	Mid-sized, grayish-white, opaque, slightly irregular or lens-shaped, distinct 'sour' odor
M3	Fresh Milk	Milk Agar	Large, white to off-white, surrounded by clear zone of proteolysis (halo) on milk agar
M4	Fresh Milk	M17 + Milk	Very small (pin-point), translucent to white, smooth, regular edges; smaller than Lactobacillus-type colonies
M5	Fermented Milk	M17 + Fermented Milk	Small, slimy or mucoid (Leuconostoc-type), glistening, raised, whitish-grey colonies

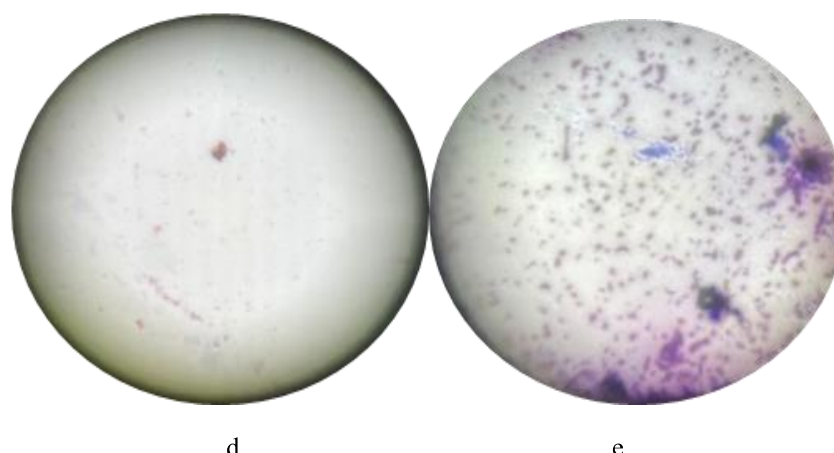
4.2 Morphological and Physiological Profile

The conventional Gram staining method verified that all five isolates were Gram-positive (figure 5). M1 had a moderate bacillus morphology, whereas M2 and M3 had little bacilli, according to microscopic analysis at 40× magnification. Coccal cell morphologies were seen in isolates M4 and M5. All isolates had negative catalase activity, which is consistent with LAB's distinctive incapacity to break down hydrogen peroxide because it lacks haem-containing catalase enzymes, a crucial trait that distinguishes LAB. All isolates lacked endospore production, indicating that they were not sporulating and setting them apart from Bacillus and Clostridium species. (table 2).

Growth at cardinal temperatures allowed for significant differentiation at the genus level. The ability of isolates M1, M2, M3, and M4 to grow at 15°C is consistent with mesophilic or psychrotrophic lactobacilli and lactococci. M5, on the other hand, flourished at 45°C, a temperature profile more characteristic of thermophilic streptococci or some *Leuconostoc* strains, but failed to develop at 15°C. On the other hand, M1 and M2 were able to grow at 45°C, whereas M3 and M4 could not, indicating a wider heat tolerance that may be connected to *Lactobacillus thermotolerans* or related species. M5 only grew at 45°C, which is consistent with some thermophilic coccal LAB or the genus *Leuconostoc mesenteroides*.



a. b. c.



d. e.

Figure 5: Gram Staining of the five probiotic isolates. a. M1, b. M2, c. M3, d. M4, e. M5

Table 2. Morphological and Physiological Characteristics of LAB Isolates

Test / Parameter	M1	M2	M3	M4	M5
Gram Reaction	Positive	Positive	Positive	Positive	Positive
Cell Shape	Moderate Bacilli	Small Bacilli	Small Bacilli	Coccus	Coccus
Catalase Activity	Negative	Negative	Negative	Negative	Negative
Spore Formation	Negative	Negative	Negative	Negative	Negative
Growth at 15°C	Positive	Positive	Positive	Positive	Negative
Growth at 45°C	Positive	Positive	Negative	Negative	Positive

4.3 Biochemical Characterization

The discoloration of phenol red broth and the buildup of gas in Durham tubes showed that all five isolates fermented glucose with acid and gas generation. This demonstrated either homofermentative or heterofermentative glucose metabolism, resulting in the formation of CO_2 and lactic acid, respectively. In M1, M2, and M3, lactose fermentation produced acid but no gas; in M4 and M5, lactose fermentation produced acid but also changed color from red to yellow instead of keeping the intermediate red tone. The lack of gas and color change in phenol red maltose broth in all five isolates indicates that none of the isolates fermented maltose. This is in line with reports that many dairy LAB species have a limited capacity to ferment maltose (Khalid, 2011).

In M1, M3, and M4, citrate utilization was positive; in M2 and M5, it was negative (figure 6). By using citrate lyase, citrate-utilizing LAB can create flavor compounds like diacetyl and acetoin, which are crucial to the flavor and aroma of dairy products, especially butter and buttermilk (Al-Kharousi, 2025).

All isolates produced EPS, with M2 producing the greatest concentration (117.3 mg/L), followed by M3 (107.3 mg/L), M1 (105.6 mg/L), M4 (103.6 mg/L), and M5 (95.3 mg/L). As a result, all five isolates meet the criteria for being EPS-producing strains, which is an important characteristic for food fermentation technology as well as probiotic activity (Table 3).

Table 3. Biochemical Profile of LAB Isolates

Biochemical Test	M1	M2	M3	M4	M5
Glucose Fermentation	Yellow / Acid / Gas	Yellow / Acid / Gas	Yellow / Acid / Gas	Yellow / Acid / Gas	Yellow / Acid / Gas
Lactose Fermentation	Red / Acid / No Gas	Red / Acid / No Gas	Red / Acid / No Gas	Yellow / Acid / No Gas	Yellow / Acid / No Gas
Maltose Fermentation	Red / No Change / No Gas	Red / No Change / No Gas	Red / No Change / No Gas	Red / No Change / No Gas	Red / No Change / No Gas
Citrate Utilization	Positive	Negative	Positive	Positive	Negative
EPS Production (mg/L)	105.6	117.3	107.3	103.6	95.3

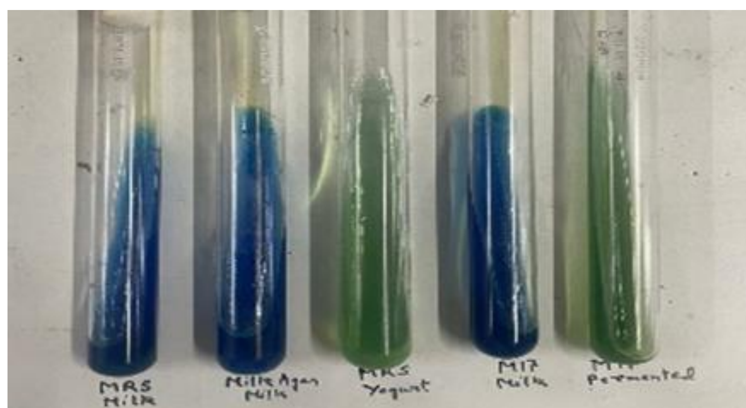


Figure 6: Result of Citrate Utilization Test of the probiotic isolates

4.4 Haemolytic Activity

None of the five isolates showed beta-haemolysis (full lysis of red blood cells) or alpha-haemolysis (partial lysis), indicating gamma-haemolytic or non-haemolytic phenotypes, according to an evaluation of haemolytic activity on 5% sheep blood agar (figure 7). Since hemolysis is linked to virulence factors and pathogenic potential, the lack of hemolytic activity is a crucial safety requirement for the certification of a microbe as a probiotic candidate (Pot et al., 2014; Rastogi et al., 2020). The biosafety of all five isolates is strongly supported by this finding.

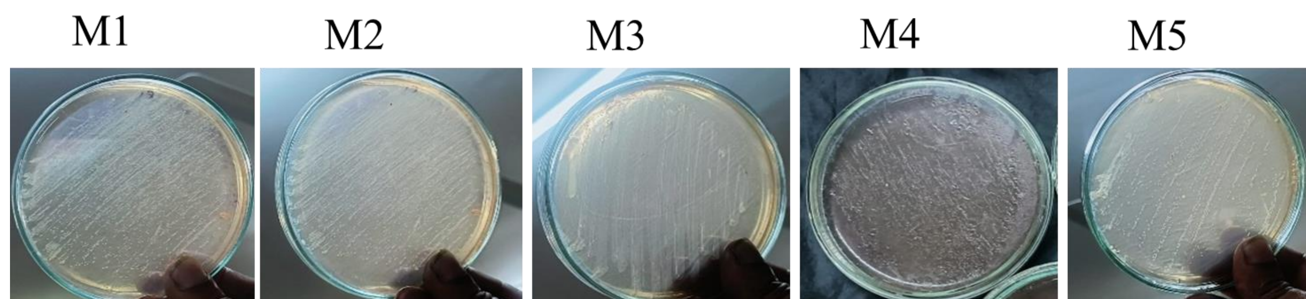


Figure 7: Results of the non-hemolytic nature of the probiotic isolates: M1 to M5 respectively on 5% sheep blood agar

4.5 Acid and Bile Salt Tolerance

The ability of isolates to tolerate simulated stomach conditions varied significantly over a 2-hour exposure period in the acid tolerance assay at pH 3.0. Strong resistance to acidic stress was demonstrated by isolates M1, M2, and M3, which had excellent survival rates and maintained viability with no decline in viable cell counts after two hours. Out of the five isolates, isolate M5 exhibited the least amount of acid resistance, whereas isolate M4 showed a moderate level of acid tolerance (figure 8). According to these findings, the bacillus-type isolates (M1–M3) appear to have more intrinsic acid stress mechanisms, which is in line with the established acid tolerance response (ATR) systems found in Lactobacillus species (Papadimitriou et al., 2016).

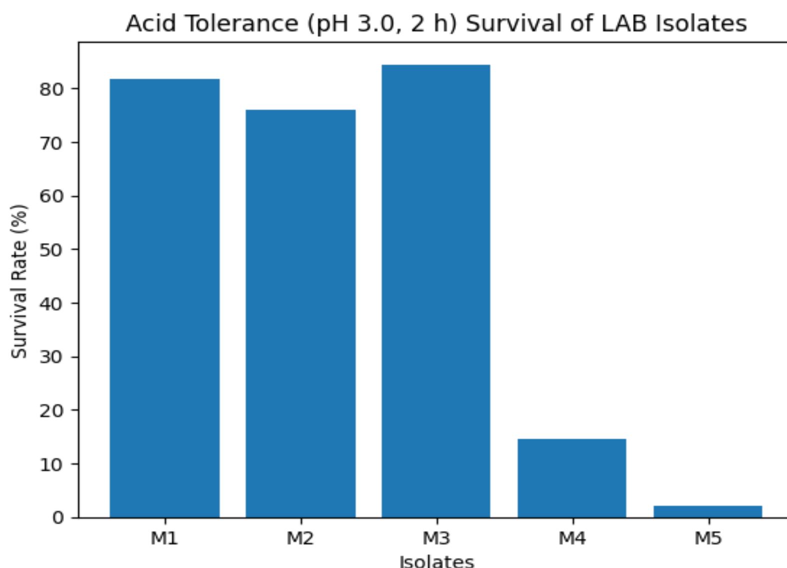


Figure 8: The Acid tolerance assay demonstrated the ability of isolates to survive at pH 3.0

The evaluation of bile salt tolerance at 0.3% ox-gall similarly distinguished the isolates' probiotic potential. Strong bile resistance was shown by M1, M2, and M3, and their survival rates were much higher than those of M4 and M5 (figure 9). Because conjugated bile salt concentrations in the proximal intestine can reach 0.3–0.5% during and after meals, the capacity to withstand bile stress is essential for effective colonization and functional activity in the small intestine (Jin et al., 2026). The probiotic usefulness of isolates M4 and M5 in the context of gastrointestinal applications was further limited by their poor tolerance to bile salt.

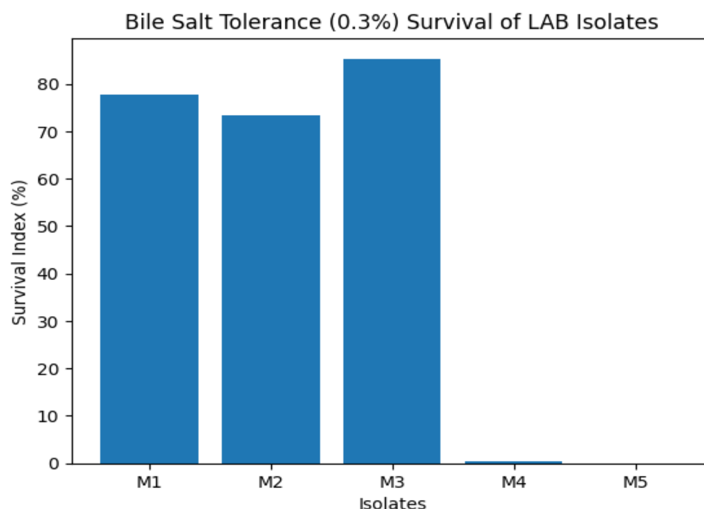


Figure 9: The Bile Salt Tolerance of the isolates was measured for their ability to endure bile stress

4.6 Antibiotic Susceptibility Profile

Using the Kirby-Bauer disc diffusion method for antibiotic susceptibility testing, useful patterns were found for each of the eight antibiotics assessed. With inhibition zones ranging from 17–28 mm, 19–24 mm, 17–22 mm, and 18–24 mm, respectively—values significantly above typical susceptibility thresholds—all isolates were susceptible to erythromycin (E), clindamycin (CD), cephalothin (CEP), and oxytetracycline (OT). These sensitivities show that the isolates would not be a danger of resistance transmission in therapeutic settings and do not carry resistance determinants for these clinically significant antibiotic classes (EFSA, 2012).

M1–M3 were sensitive to avilamycin (AV), but M4 and M5 displayed intermediate and resistance, respectively. M1, M2, M3, and M5 (R) all showed intrinsic resistance to the fluoroquinolone ofloxacin (OF), with M4 exhibiting intermediate resistance. In the same vein, every isolate exhibited co-trimoxazole (COT) resistance. M2, M4, and M5 were responsive to gentamicin (GEN), but M1 and M3 exhibited intermediate resistance. Since LAB lacks the particular transport mechanisms and target sites for these antibiotic classes, ofloxacin and co-trimoxazole resistance in LAB is well-documented and thought to be intrinsic rather than horizontally acquired; this intrinsic resistance does not pose a safety risk when using probiotics (Darbandi et al., 2021). Table 4 presents complete antibiotic susceptibility data.

Table 4. Antibiotic Susceptibility Profile of LAB Isolates (Kirby-Bauer Disc Diffusion Method)

Antibiotic (Code)	M1	M2	M3	M4	M5
Erythromycin (E)	26 mm (S)	28 mm (S)	25 mm (S)	22 mm (S)	21 mm (S)
Clindamycin (CD)	22 mm (S)	24 mm (S)	21 mm (S)	20 mm (S)	19 mm (S)
Cephalothin (CEP)	18 mm (S)	19 mm (S)	17 mm (S)	22 mm (S)	20 mm (S)
Avilamycin (AV)	15 mm (S)	14 mm (S)	16 mm (S)	12 mm (I)	11 mm (R)
Oxytetracycline (OT)	24 mm (S)	22 mm (S)	20 mm (S)	18 mm (S)	19 mm (S)
Ofloxacin (OF)	8 mm (R)	7 mm (R)	9 mm (R)	11 mm (I)	10 mm (R)
Gentamicin (GEN)	12 mm (I)	14 mm (S)	11 mm (I)	15 mm (S)	16 mm (S)
Co-trimoxazole (COT)	6 mm (R)	6 mm (R)	8 mm (R)	10 mm (R)	9 mm (R)

S = Susceptible; I = Intermediate; R = Resistant. Values represent mean inhibition zone diameters (mm). Interpretations per CLSI/EUCAST guidelines.

A comprehensive evaluation of each isolate's probiotic potential is made possible by the integrated interpretation of morphological, physiological, biochemical, and functional data. *Lactobacillus casei, Lactobacillus paracasei*, or *Lactobacillus fermentum* are examples of facultatively heterofermentative Lactobacillus species that are well-represented in the dairy environment and have been shown to have strong probiotic qualities (Rastogi et al., 2023). Isolates M1 and M2, both MRS-recovered bacillus-type organisms that can grow at both 15°C and 45°C. Both M1 and M2 are excellent probiotic candidates due to their combination of acid tolerance, bile resistance, EPS generation, and antibiotic sensitivity.

Isolate M3 is a proteolytically active LAB with noteworthy probiotic functional characteristics that was recovered from milk agar with evident proteolytic halos. Its small-bacillus morphology, strong EPS production, high acid and bile tolerance, and incapacity to grow at 45°C all point to membership in a psychrotolerant

Lactobacillus group, such as *Lactobacillus acidophilus* or *Lactobacillus plantarum*, species that have been thoroughly investigated for mucosal adhesion, immunomodulation, and clinical probiotic efficacy (Tripathi & Giri, 2014). From the standpoint of dairy technology, M3's proteolytic ability provides even more value by aiding in the hydrolysis of proteins and the creation of flavor in cheese and fermented milk products.

Coccal-type organisms found on M17 agar, isolates M4 and M5, correspond to the *Lactococcus* or *Leuconostoc* genera, respectively. *Lactococcus lactis* subsp. diacetylactis, a recognized dairy starter organism, is consistent with M4's temperature profile (15°C growth, no 45°C growth), coccal shape, and citrate positive. However, the direct gastrointestinal probiotic efficacy of M4 and M5 is diminished by their weak tolerance to acid and bile, indicating that their functional utility is predominantly in dairy fermentation technology rather than gut-health-directed applications (Saleem et al., 2024). The biosafety of all isolates for food consumption is confirmed by their consistent gamma-haemolysis and typically favorable antibiotic susceptibility profile.

6. Discussion

The current study produced a comprehensive functional dataset with obvious implications for probiotic creation by methodically evaluating five LAB isolates from native dairy sources against a multi-parameter probiotic candidacy framework. The results add to the increasing amount of research showing the diversity of functionally different, safety-validated LAB strains found in traditional and indigenous dairy ecosystems (Mugampoza et al., 2020; Rastogi et al., 2023).

In accordance with accepted best methods for dairy LAB isolation, the combination of MRS, M17, and milk agar were effective in capturing phenotypic diversity across the *Lactobacillus* and *Lactococcus* genera. From a technological and nutritional standpoint, the discovery of proteolytic halos on milk agar for M3 is significant because proteolytic LAB aids in the ripening and curd formation of cheese as well as the release of bioactive peptides with antihypertensive, antimicrobial, and immunomodulatory qualities (Rastogi et al., 2020b).

The study's EPS production characteristics align with the larger body of research on LAB EPS. According to research showing that fermented milk-adapted strains frequently exhibit increased EPS synthesis as a stress adaptation mechanism (Niu et al., 2025), M2 produced the highest EPS yield (117.3 mg/L). EPS-producing strains have shown improved adhesion to intestinal mucosa, increased survivability during food processing and storage, and immunostimulatory activity (Uhegwu & Anumudu, 2025). When it comes to the development of functional foods and the durability of probiotic formulations, EPS generation is particularly useful due to these combined characteristics.

The results of this study on acid and bile tolerance are consistent with a stratification found in dairy LAB in several similar studies. Because of their evolutionary adaption to the acidic conditions of fermented dairy products, lactobacillus-type strains often show better acid tolerance than coccal LAB (Collet et al., 2026). The results of Argyri et al. (2013), who found comparable tolerance thresholds in probiotic LAB isolated from fermented plant and dairy matrices, are consistent with the high survival of M1, M2, and M3 at pH 3.0 for two hours.

Results on bile salt resistance support M1–M3's superiority as probiotic options. The main enzymatic mechanism of bile detoxification in intestinal LAB, bile salt hydrolase (BSH) activity, is broadly distributed among *Lactobacillus* species. It lessens the cytotoxicity caused by detergents by enabling conjugated bile acid deconjugation (Nair & Nair, 2020). According to their biological habitat, M4 and M5 may have low bile tolerance since M17-recovered genera are more frequently used in dairy starting than probiotic applications (Terzic-Vidojevic et al., 2020).

The anticipated phenotypic resistance profiles of dairy LAB and the interpretive standards used in probiotic safety evaluations by EFSA and CLSI are in line with the antibiotic susceptibility patterns found in this investigation. Due to the lack of fluoroquinolone uptake transporters and sulphonamide-sensitive dihydropteroate synthase isoforms, respectively, many LAB species have inherent resistance to ofloxacin and co-trimoxazole, which has been shown in multiple molecular studies to be non-transferable (EFSA, 2012; Darbandi et al., 2021). Since transferable aminoglycoside resistance genes like aac(6')-aph(2" have occasionally been found on mobile genetic elements in lactobacilli, the gentamicin intermediate resistance seen in M1 and M3 calls for additional molecular research to verify their absence (Dec et al., 2025).

With its strong acid and bile tolerance, notable EPS production (107.3 mg/L), proteolytic activity, non-

haemolytic phenotype, and largely favorable antibiotic susceptibility profile, M3 is the most all-around potential probiotic candidate, according to the results. M1 and M2 have similar functional characteristics and are close seconds. This hierarchy of probiotic potential is in line with our previous research that has shown *Lactobacillus*-type bacteria have better colonization effectiveness and gastrointestinal durability (Rastogi et al., 2020; Rastogi et al., 2021).

The lack of molecular identification of isolates to the species level, which would offer conclusive taxonomic placement and allow literature-based comparison with defined reference strains, is one of the study's limitations. Furthermore, the intricacy of gastrointestinal dynamics, mucosal adhesion, and host immunological interaction in vivo cannot be adequately replicated by the in vitro nature of probiotic functional assays, despite their widespread use in primary screening. To enable thorough genomic investigation of bacteriocin gene clusters, EPS biosynthesis operons, stress response genes, and antibiotic resistance determinants, future research should incorporate 16S rRNA gene sequencing and whole-genome sequencing. Prior to clinical assessment, in vitro adhesion tests employing human intestinal epithelial cell lines (such as Caco-2 or HT-29) and eventually animal model investigations would confirm the probiotic effectiveness of lead isolates.

In light of India's growing functional food market, the results of this study also have significance for the value-adding of native dairy microbiota. India offers an unmatched opportunity for the development of culturally appropriate probiotic dairy products incorporating locally characterized LAB strains, with a per capita dairy consumption of about 427 grams per day and a dairy sector valued at over USD 150 billion (Nataraj et al., 2020; NDDB, 2023). Hundreds of millions of Indians eat fermented foods like dahi, lassi, and chaas on a daily basis. These foods are perfect for delivering probiotic LAB, which have been specifically designed to survive in conditions relevant to the composition of the Indian gut microbiome.

7. Conclusion

Five LAB isolates from native dairy sources in northern India have been successfully isolated, morphologically described, biochemically profiled, and functionally assessed in this study. All isolates meet the basic safety requirements for probiotic candidacy, which include being gram-positive, catalase-negative, non-spore-forming, and non-haemolytic. Strong acid and bile salt tolerance, significant EPS generation, and a favorable antibiotic susceptibility profile predominantly reflecting inherent resistance patterns were among the robust probiotic functional features exhibited by isolates M1, M2, and especially M3. The poor gastrointestinal stress tolerance of isolates M4 and M5 limited their application to starter culture functions in dairy fermentation rather than direct probiotic use. The indigenous dairy microbiota is confirmed by this study to be a biologically accessible, diverse, and durable reservoir for the identification of new probiotic LAB strains. To translate these findings into commercially viable probiotic functional food items, more molecular characterization, genomic analysis, adhesion studies, and in vivo validation are necessary.

Author's contribution: AA: Data curation, Literature study, Analysis, Writing original draft. AN: Literature study, Analysis, Writing original draft, Illustrations. AS: Conceptualization, Supervision, Reviewing & editing the original draft. All authors approved the final version.

Author Declaration Statements

Declaration: The authors hereby declare that the manuscript submitted for consideration is an original work and has not been published or submitted elsewhere for publication. The authors take full responsibility for the integrity, accuracy, and ethical compliance of the work presented in the manuscript.

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Conflict of Interest: All authors confirm that:

- Any potential conflicts of interest, whether financial or non-financial, have been fully disclosed. – ~~Yes~~ / Not Applicable√
- All sources of funding and financial support received for the conduct of the study have been appropriately acknowledged. – ~~Yes~~ / Not Applicable√
- Necessary ethical approvals have been obtained from the relevant institutional or regulatory bodies for studies involving human participants, animals, or sensitive data, wherever applicable. – ~~Yes~~ / Not Applicable√

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