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Probiotic properties and safety assessment of *Enterococcus durans* DMGUD5 and its suitability as a dairy starter culture to manufacture fermented dairy product

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This study aimed to isolate and characterize exopolysaccharide (EPS)-producing lactic acid bacteria (LAB) from goat milk and evaluate their probiotic potential and suitability as starter cultures for fermented dairy products. Of the 60 LAB isolates, 19 demonstrated EPS production ability, of which six strains exhibited the highest EPS production. These isolates were identified by 16S rDNA sequencing and assessed for their probiotic properties, including acid and bile tolerance, survivability in simulated gastric and pancreatic juices, auto-aggregation, cell surface hydrophobicity, lysozyme and phenol tolerance, antibacterial and antifungal activities, antioxidative capacity, and safety tests. *Enterococcus durans* DMGUD5 exhibited the most promising probiotic characteristics and was used to prepare fermented curd. The curd was analyzed for its proximate composition and shelf life, considering its sensory attributes, pH, probiotic viability, and microbial safety. DMGUD5 demonstrated high EPS production, superior acid and bile tolerance, strong survival under simulated gastrointestinal conditions, and significant antibacterial, antifungal, and antioxidative activities. The curd prepared using DMGUD5 had acceptable sensory properties and maintained probiotic viability above 6 $\log_{10}$ CFU/g for eight days under refrigerated storage (4±1°C). *E. durans* DMGUD5, exhibits promising probiotic properties and can be used as a starter culture for the production of functional fermented dairy products.

Keywords: Exopolysaccharide, Lactic acid bacteria, Probiotic viability, Antibiotic sensitivity, Curd, Goat milk

Goats are highly adaptable animals found in all continents except Antarctica, with 93.4% of the global population residing in Asia and Africa. Asia, where goats were first domesticated around 10,000 years ago, accounted for 57.7%, while Africa held 35.7%, mainly in sub-Saharan regions, such as Chad and Ethiopia. The global goat population is approximately 1.1 billion, with India leading with approximately 150 million goats, followed closely by China with 132.4 million[1]. Goat milk, which closely resembles cow's milk in nutritional content, offers numerous health benefits, easy digestibility, and a rich nutrient profile[2]. Goat milk has emerged as a significant contributor to the global demand for non-bovine milk, accounting for 13.5% of the processed non-bovine milk[3]. The digestibility, nutritional richness, and hypoallergenic properties of goat milk make it an attractive alternative for consumers. Moreover, its native microbiota positions it as a key resource for developing fermented products. Lactic acid bacteria (LAB) are essential for spontaneous fermentation and significantly influence food product transformation[4,5]. The nutritional superiority, digestibility, and hypoallergenic nature of goat milk make it an intriguing alternative. The intricate autochthonous microbiota further underscores the potential of fermented products. LAB are prominent players in spontaneous fermentation and drive the transformation of food products[6]. The potential of goat milk to prevent colorectal and colon cancers has garnered attention, and the demand for functional dairy alternatives is on the rise, driven by concerns over lactose intolerance and saturated fats[3]. LAB, which naturally occurs in dairy products, enhances immunity, supports lactose absorption, and reduce diarrhea symptoms[7]. Probiotic organisms, such as LAB, have gained prominence in food processing, bolstering immunity, lactose absorption, and combating diarrhea.

LAB synthesizes a diverse array of exopolysaccharides (EPSs), the structures of which vary in terms of side chains, glycosidic linkages, substitutions, and charges. These EPSs consist of sugars, such as glucose, galactose, rhamnose, and their derivatives, and their composition is influenced by the growth medium, bacterial strain, and gene-regulated enzyme activity. EPSs are broadly classified as homopolysaccharides (HoPS) and heteropolysaccharides (HePS)[8]. LAB-derived HoPSs,

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such as dextran, mutan, reuteran, levan, and β-D-glucans, are composed of repeating units of a single sugar type and are synthesized via glycosyl hydrolase enzymes using energy from glycosidic bonds. EPS are natural, non-toxic bioproducts with diverse structures and biological functions[9]. Although LAB produce lower quantities are produced by LAB than other bacteria, they exhibit superior functional properties and stress resistance[10]. Owing to their unique rheological and water-binding capabilities, these EPSs are widely used in the food industry as emulsifiers, thickeners, gelling agents, encapsulants, moisture retainers, and health fortifiers. EPS-producing LAB often exhibit probiotic characteristics, potentially offering health benefits and improved survival of gut bacteria. In goat milk products, EPS-producing LAB are particularly significant due to the growing interest in alternatives for consumers with cow milk allergies[8].

Several studies have isolated and characterized putative probiotic LAB strains from goat milk across various breeds and regions[2,3,11,12]; however, EPS-producing LAB from goat milk remain largely unexplored. Therefore, this study aimed to isolate EPS-producing LAB from goat milk, assess their probiotic potential and safety, and explore the application of the most promising strain, *Enterococcus durans* DMGUD5, in producing fermented dairy products. By utilizing the unique properties of this strain, this study aimed to promote functional dairy innovations, reduce reliance on synthetic additives, and enhance the health benefits of dairy foods. This study highlights the potential for expanding EPS-producing LAB applications in the dairy industry to produce healthier and more sustainable products.

Material and Methods

Materials and sample collection

All chemicals and biochemical reagents were sourced from reputable laboratory chemical suppliers in India. The microbial cultures were acquired from the "National Collection of Industrial Microbes (NCIM), Pune, India." Milk samples were collected from goat herds in the Aravali hills of the Mewar region, Rajasthan, India, with a total of 36 samples obtained from 36 different herders. Each sample (200 mL) consisted of milk pooled from five healthy goats within individual herds, with lactation periods ranging from 15 to 120 days, to minimize the influence of lactation duration, feeding practices, and genetic differences. The samples were aseptically collected in sterilized collection bottles from various locations (spaced at least 25 km apart) within the study area. They were transported to the laboratory in an icebox and stored in a refrigerator at temperatures below 5 °C until subsequent analysis.

Isolation, confirmation and purification

Milk samples (1 mL) were serially diluted with 10 mL autoclaved distilled water. From suitable dilutions, 0.1 mL was spread plated on MRS and M17 agar. The plates were incubated at 37 °C and 42 °C for 48–72 h to allow colony development. Distinct colonies were randomly selected and transferred to 5 mL of MRS and M17 broths, followed by purification through repeated streaking on MRS/M17 agar. The isolates were confirmed as LAB by Gram staining, catalase testing, and capsule staining. Pure cultures were preserved on agar slants under refrigeration (below 5 °C) and stored at −18°C in glycerol for future use[13].

Screening of exopolysaccharides-producing LAB (ELAB*)*

MRS/M17 agar plates were supplemented with 20 g/L lactose as a carbon source to promote EPS production in the cells. Selected LAB isolates were streaked onto sugar-supplemented MRS/M17 agar plates and kept in incubator at 37±1°C for 36–48 h. The plates were observed for the development of slimy or mucoid colonies, which indicated EPS production. Then, evaluated the "ropy" characteristics of the colonies by gently extending them with an inoculation loop; ropy colonies formed long, viscous strands[14].

The quantification of exopolysaccharides (EPS) was quantified using standard working protocol with slight modifications. To remove proteins and other insoluble components, 40% (w/v) trichloroacetic acid (TCA) was supplemented to the culture broth at a ratio of 1:3 (TCA: broth) and centrifugation in a refrigerated centrifuge (rpm-10,000,time-30 min, and temperature-4 °C). The resulting supernatant was combined with three volumes of chilled 95% ethanol and incubated at 4 °C for 24 h to precipitate the EPS. The precipitate was collected via centrifugation at 10,000 rpm for 30 min at room temperature. The total EPS concentration was determined using the phenol-sulfuric acid method, with glucose as the standard[15]. Glucose was selected because the phenol–sulfuric acid assay quantifies the total carbohydrate content

based on the formation of a colored complex after acid-mediated dehydration of sugars. As crude EPS primarily comprises carbohydrate monomers, glucose serves as an appropriate reference compound for expressing results in glucose equivalents, a practice widely adopted in EPS quantification studies.

Biochemical and physiological characterization of EPS-producing isolates

The isolates were subjected to standard tests for the identification of LAB, including Gram staining, spore staining, catalase activity, oxidase test, urease test, nitrate reduction test, citrate utilization, and growth assessment under different physiological conditions (temperature, pH, and NaCl concentrations), followed by evaluation of carbohydrate fermentation patterns to confirm their LAB characteristics.Gram staining, spore staining, catalase activity, and oxidase test was performed as described by Mulaw *et al*[16].

Citrate utilization was assessed on Simmons citrate agar using the method described by Meena, Joshi *et al*.[17]. Carbohydrate fermentation pattern in different sugars was performed according the method described by Mulaw *et al*[16]. Physiological characterization, such as temperature tolerance (MRS broth tubes incubated at 15, 37, and 45 °C), salt tolerance (at 2%, 4%, and 6.5% NaCl in MRS broth), and pH tolerance (pH of MRS broth adjusted to 4.5, 6.5, and 8.5) was performed to measure the growth based on turbidity. The growth of the cultures was measured after 24 h and rated from no growth (−) to heavy growth (++++) across all tests[16].

Genotypic characterization

Species-level identification of isolates was conducted using 16S ribosomal DNA (rDNA) gene sequencing. Genomic DNA was extracted using a commercial kit (Hi-Media, Mumbai), and its purity was confirmed via agarose gel electrophoresis. PCR amplifications were conducted using an Eppendorf Vapo Protect Thermal Cycler. The resulting amplicons were outsourced for sequencing. Sequence homologies were analyzed via comparative analysis using the "National Centre for Biotechnology Information (NCBI)" and the "Basic Alignment Search Tool (BLAST)" at (http://www.ncbi.nlm.nih.gov/) Multiple sequence alignment was performed using CLUSTALW, and a phylogenetic tree was constructed via the maximum-likelihood method based on Kimura's two-parameter model with Mega software version 11.0, and bootstrap analysis with 1000 replicates was performed to assess the robustness of the tree[18].

Assessment of probiotic properties of EPS-producing LAB strains

Acid and bile tolerance

Acid and bile tolerance of isolates was tested to check survival in the gut environment, as previously described by Meena, Joshi *et al*.[17]. The cultures were incubated in M17 broth for 24 h at 37 °C in an anaerobic environment and then centrifuged at 5000 rpm for 10 min at 4 °C. After washing in phosphate-buffered saline (pH 7.2), the pellets were reconstituted in M17 broth and adjusted to pH 2.0, 3.0, and 1 N HCl (pH 6.5 as the control). The culture tubes were kept in incubator at 37±1°C for 0, 2, and 3 h, serially diluted, and plated on M17 agar for viable LAB count ($\log_{10}$ CFU/mL).

For bile tolerance test, harvested culture pellets were added in a test tube containing 5 mL sterilized M17 broth. Then, Oxgall bile salt (HiMedia, Mumbai, India) at the concentration of 0.3% (w/v) was added and a control sample without bile salts was prepared. The culture test tubes were kept in incubator at 37±1°C for 0, 2, or 3 h. After serial dilution, 100 µL of each sample was plated on MRS agar for viability count and expressed as $\log_{10}$ CFU/mL[17].

Culture viability in simulated gastric and pancreatic conditions

To assess the probiotic viability of isolates under gastrointestinal conditions, we simulated gastric and pancreatic juices. Simulated gastric juice was freshly prepared using pepsin (3 g/L; HiMedia, Mumbai, India) in sterile saline and adjusted to pH 2.0 with 1 N HCl. Simulated pancreatic juice was prepared using pancreatin (1 g/L; HiMedia, Mumbai, India) and bile salts (0.3% w/v, Oxgall; HiMedia, India) dissolved in a sterile phosphate buffer (pH 8.0). Both solutions were prepared immediately before use to maintain the enzyme stability and activity throughout the assay. Isolates were first exposed to gastric juice for 3 h, followed by pancreatic juice for 24 h. Viability was measured at the start and end of each exposure period to determine the survival rate of the isolates using the protocol used by Meena *et al*.[18]. Fresh overnight grown isolates were harvested by centrifugation at 8000 rpm for 8 min at 4°C, washed with PBS, and added in gastric juice to an absorbance of 1.2 at 500 nm. The cultures were shaken peristaltically at 200 rpm for 3 h at 37±1ºC. After 0 and 3 h, the samples were serially diluted and plated on MRS agar

to determine their viability. The survival (%) under simulated gastric-juice conditions was calculated using Equation 1.

$$\text{Gastric juice survival (\%)} = \left[\frac{\log_{3h}}{\log_{0h}}\right] \times 100 \quad (\ldots 1)$$

where $\log_{0h}$ represents the culture viability at 0 h and $\log_{3h}$ represents the culture viability after 3 h of incubation.

To simulate the sequential conditions of the human gastrointestinal tract, the gastric juice-treated pellets were subsequently exposed to pancreatic juice, allowing the assessment of the cumulative survival of the isolates under conditions mimicking passage through the stomach and small intestine. After 3 h of incubation with gastric juice, the culture pellet was harvested using refrigerated centrifuge (8000 rpm/ 8 min/4 °C). The culture pellets were rinsed using phosphate-buffered-saline and resuspended in pancreatic juice. The cultures were incubated for 24 h at 37±1°C in an orbital shaker at 200 rpm to simulate gastrointestinal conditions. The samples were serially diluted and plated on M17 agar to assess survival after 0 and 24 h of pancreatic digestion[18]. Equation (2) was used to determine the survival % of the culture samples.

$$\text{Pancreatic juice ssurvival (\%)} = \left[\frac{\log_{24h}}{\log_{0h}}\right] \times 100 \quad (\ldots 2)$$

where $\log_{0h}$ represents the culture viability at initial stage (0 h), and $\log_{24h}$ represents the culture viability after 24 h of incubation.

Auto-aggregation

Auto-aggregation was performed to assess the ability of LAB cells to adhere to each other, which reflects their potential to colonize and persist in the gut of the host. Overnight-grown cultures were harvested by centrifugation (11,000 rpm, 4 °C, 10 min), and the cell pellets were washed twice with phosphate-buffered saline (PBS) and resuspended in PBS to an initial optical density (OD_{600}) of 0.5. A 5 mL aliquot of the suspension was transferred to a 15 mL falcon tube, gently vortexed for 10 s and incubated at 37 °C for 1 h. After incubation, 1 mL of the upper suspension was carefully removed, and its absorbance was measured at 600 nm[18]. Equation 3 was used to record and compute the auto-aggregation:

$$\text{Auto-aggregation (\%)} = \left[\frac{(1-OD_{time})}{OD_0}\right] \times 100 \quad (\ldots 3)$$

Where OD_0 is the initial optical density of the cell suspension and OD_{time} is the final optical density of the cell suspension (upper-layer) after an hour of incubation, respectively.

Cell surface hydrophobicity

Cell surface hydrophobicity (CSH), an indicator of LAB adherence to the gut epithelial cells, was evaluated using standard microbial adhesion assays. LAB cultures were centrifuged at 8,000 rpm for 10 min at 4 °C, washed twice with PBS (pH 7.3), and resuspended in sterile 0.1M KNO_3 (pH 6.2). The initial absorbance (I_a) was adjusted to 0.55–0.66 at 600 nm. A 3:1 mixture of cell suspension (3 mL) and xylene was incubated at 37 °C for 10 min, vortexed for 1 min, and further incubated for 30 min. The aqueous phase was carefully removed and its absorbance (I_b) was measured at 600 nm[18]. The reduction in absorbance was used to determine the % hydrophobicity, which was then computed using Equation 4.

$$\text{Cell surface hydrophobicity (\%)} = \left[\frac{(Ia-Ib)}{Ia}\right] \times 100 \quad (\ldots 4)$$

Lysozyme and phenol tolerance

Lysozyme and phenol tolerances of EPS-producing LAB strains were evaluated to assess their ability to survive in conditions mimicking the human oral cavity and gastrointestinal tract.

Overnight-grown LAB cultures were assessed for their phenol and lysozyme tolerances. For phenol resistance, cultures were inoculated into sterilized MRS broth containing 0.4% (v/v) phenol and incubated at 37 °C for 24 h. Viability was determined using the standard plate count method on MRS agar and expressed as CFU/mL[18]. For lysozyme tolerance, cultures were harvested by centrifugation, washed twice with PBS, and resuspended in the same buffer. Ten microliters of the cell suspension were added to a simulated saliva solution (containing $CaCl_2$ 0.22 g/L, NaCl 6.2 g/L, KCl 2.2 g/L, and $NaHCO_3$ 1.2 g/L) containing 100 mg/L lysozyme (HiMedia, India) and incubated at 37 °C for 2 h, alongside a control without lysozyme. Survival was determined by serial dilution and plating on MRS agar, and results were expressed as a percentage of CFU/mL[18].

Antibacterial activity

Foodborne pathogenic bacteria (*Staphylococcus aureus* NCIM 2079, *Listeria monocytogenes* NCIM 5260, *Bacillus cereus,* NCIM 2217, and

Escherichia coli NCIM 20265) were used as test organisms to check the antibacterial activities of the isolates in the experiment. An active 24-h culture of isolated strains was subjected to centrifugation in refrigerated centrifuge at 5000 rpm for 10 min at 4 °C to a get cell-free-supernatant (CFS). Subsequently, indicator pathogens were cultured overnight on Nutrient Agar (HiMedia, Mumbai, India), and 100 µL of each pathogen suspension was evenly spread on pre-prepared and solidified NA plates, which were then allowed to air-dry for 30–45 min. Wells with a 6 mm diameter were created on the agar using a sterile borer, and 100 µL of CFS from each LAB isolate was added to the wells. After proper absorption of CFS in agar, the petriplates were kept in BOD incubator 37±1 °C for 24–36 h. An inhibitory zone with at diameter greater than 1 mm surrounding the wells was considered a positive zone of inhibition (ZOI)[17].

Anti-oxidative activity

The antioxidant potential of the isolates was assessed based on their ability to scavenge 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals. Overnight-grown cultures were harvested by centrifugation (5000 rpm, 4 °C, 15 min), washed twice, and resuspended in PBS. The ABTS working solution was prepared by mixing 5 mL of ABTS (HiMedia, Mumbai, India) with $K_2S_2O_8$ and incubating it in the dark for 24 h to generate the ABTS radicals. A reaction mixture was prepared by combining 200 µL of ABTS solution with 15 mL of PBS. Ten microliters of the EPS-producing isolate cell suspension was added, mixed by gentle shaking for 30 s, and the absorbance was measured at 750 nm after 5 min. The percentage inhibition of ABTS radicals was calculated based on the reduction in the absorbance relative to that of the control[18].

Antifungal activity

The antifungal properties of potential EPS-producing probiotic LAB isolates against fungal pathogens (*Aspergillus niger* NCIM-1004, *Alternaria solani* MTCC-2101, and *Candida albicans* NCIM 3268) were evaluated using the well diffusion method. LAB cultures were grown overnight in MRS broth at 37 °C, and the cell-free supernatant (CFS) was obtained by centrifugation (5000 rpm, 10 min, 4 °C) and sterile filtration. Fungal pathogens were cultured on Potato Dextrose Agar (PDA) plates and a spore suspension was prepared in sterile saline. PDA plates were uniformly inoculated with 20 µL fungal spores (10^6 spores/mL), and wells (6 mm diameter) were punched into the agar plates. Each well was filled with 100 µL of LAB CFS, and the plates were incubated at 25±1 °C for 2 and 7 days. The presence of inhibition zones surrounding the LAB colonies was assessed as an indicator of antifungal activity[17].

Sugar fermentation ability

The suitability of the isolated strains as a dairy starter culture was assessed by their ability to ferment skim milk media. A solution of skim milk with a total solids content of 10% (w/v) was prepared using skimmed milk powder (SMP) and double-distilled water. This solution was added to test tubes (10 ml in each) and sterilized using an autoclave. The test tubes were then cooled to ambient temperature and inoculated with the isolates at a concentration of 1% (v/v), resulting in a cell count of approximately 10^4 CFU/mL. The test tubes were then placed in an incubator (37±1 °C) for 24 h. Subsequently, samples of the fermented products in test tubes were kept in the refrigerated (4±1 °C) conditions. The survival of the isolated cultures (ELAB) was evaluated at various time points, including 0, 12, and 24 h during the fermentation phase, as well as at 7, 14, and, 21 days during storage in a refrigerator (4±1 °C)[18].

Amylolytic activity

EPS-producing strains were cultivated in M17 broth overnight before being streaked on modified M17 agar media. The M17 media was modified by supplementing with 0.2% starch. Then, petriplates were kept in incubator at 37±1 °C for 48–72 h. As a detecting agent, the plates were completely saturated with Gram iodine solution. Plates with distinct halo zones support the findings of α-amylase activity, according to Meena *et al.*[18].

Safety assessment of EPS-producing LAB strains

Safety assessment of the isolates was performed using the antibiotic susceptibility test and nuclease and hemolytic activities. The antibiotic susceptibility of the isolates was evaluated using the disc diffusion method. In this study, antibiotic discs such as Tetracycline (TE), Amikacin (AK), Ciprofloxacin (CIP), Vancomycin (VA), Ofloxacin (OF), Cefotaxime (CTX), Ampicillin (AMP), Erythromycin (E), Clindamycin (CD), and Cotrimoxazole was used. Susceptibility profiles were determined using the guidelines formulated by the "Clinical and Laboratory Standards Institute"[19], with isolates classified as

susceptible (S) when zones of inhibition (ZOI) were ≥ 20 mm, intermediate (I) when the ZOI was ≥ 15 mm, and resistant (R) when the ZOI was ≤ 14 mm[17].

Deoxyribonuclease (DNase) agar medium was used to assess DNase enzyme synthesis following the method described by Sangprapai *et al*.[20]. The hemolytic activity of the isolates was assessed by examining the plates for β-hemolysis (clear zones around colonies), α-hemolysis (green zones around colonies), and γ-hemolysis (non-clear zones) using sheep blood agar[18]. Gelatine liquefaction was performed to assess the non-pathogenicity of the strains, according to the method described by Kang *et al*.[21].

Preparation of curd using potential EPS- producing probiotic strain

The probiotic strain *Enterococcus durans* DMGUD5, identified as the most promising candidate based on its probiotic characteristics, was selected for curd preparation to evaluate its potential as a starter culture. The strain was cultivated in M17 broth at 37 °C for 21 h to attain a cell density of approximately 6 log CFU/mL. After incubation, the culture was centrifuged at 8,000 rpm for 10 min at 4 °C, and the harvested cells were washed with phosphate-buffered saline[17]. These cells (biomass) were used as inoculum for curd production.

For curd preparation, one liter of cow milk was blended with skim milk powder and standardized to obtain toned milk containing 3.05 ± 0.05% fat and 9.05 ± 0.05% solids-not-fat. The milk was heated at 95 °C for 20 min to inactivate pathogenic and spoilage microorganisms and then cooled to 37 ± 1 °C. The milk was subsequently inoculated with 2% (v/v) of *Enterococcus durans* DMGUD5 biomass, containing at least 8 $\log_{10}$ CFU/g. The inoculated milk was aseptically dispensed into 100 mL sterile polyethylene terephthalate (PET) containers in a laminar flow chamber and incubated at 37 ± 1 °C for 18 h[22]. The resulting curd was stored at 4 ± 1 °C for further shelf-life and quality evaluation.

Proximate analysis and shelf-life evaluation of curd

The developed product was analyzed for moisture, fat, protein, ash, and solid-not-fat (SNF) contents following the standardized procedures outlined by Bunkar *et al*.[23]. Shelf-life evaluation was carried out every two days at 4 ± 1 °C using parameters such as pH, sensory evaluation, probiotic viability, yeast and mold counts, and coliform presence. Sensory evaluation of curd samples was conducted using a 9-point hedonic scale, where 1 indicated "extremely disliked" and 9 indicated "extremely liked." A panel of 30 members (17 men and 13 women, aged 18–53 years) comprising trained and semi-trained individuals participated in the assessment. Prior to evaluation, the panelists were oriented about the product characteristics and trained using a reference curd prepared with similar ingredients through traditional fermentation. Samples were served in coded, odor-free cups at 7 ± 1 °C under controlled laboratory conditions. The panelists evaluated the attributes, including appearance, texture, flavor, taste, and overall acceptability. The sensory scores were recorded and statistically analyzed to determine the differences among the treatments[24]. Microbial counts, such as probiotic viability, coliform and fungal counts, were enumerated according to the method adopted by Meena, Taneja, *et al*.[7]. Microbial counts are expressed as $\log_{10}$ CFU/g.

Statistical analysis

The data obtained are expressed as the mean ± standard deviation. To rigorously evaluate the results, one-way analysis of variance (ANOVA) was employed, followed by Duncan's post-hoc test for further statistical differentiation. These analyses were performed using the IBM SPSS Statistics 22 software, with statistical significance set at $P< 0.05$. Graphical representations of the data were meticulously generated using the Origin Pro 2018 software.

Results and Discussion

Isolation and screening of EPS producing LAB isolates

Presumptive LAB were isolated from goat milk samples by serial dilution and plating on MRS and M17 agar, followed by incubation at 37 °C for 48 h. Sixty morphologically distinct colonies were identified in this study. Isolated colonies were streaked onto MRS and M17 agar supplemented with 4% sucrose to assess their morphological features. The colony texture was evaluated by gently touching the surface with a sterile inoculation loop. Colonies exhibiting a ropy texture formed visible filaments upon loop extension, while mucoid colonies appeared slimy but did not form strands, consistent with the criteria described by Yadav *et al*.[14].

Of the 60 isolates, 19 showed mucilaginous colonies, indicating EPS production. The colonies showed elongated, pliable filaments when extended with loops, suggesting the potential for further EPS

development in the subsequent product formulation stages. In the current investigation, the phenol sulfuric approach was used to evaluate the exopolysaccharide production of 19 isolates that passed the qualitative test[15]. Six of the 19 isolates, DMGUD5, DMGUD10, DMGUD17, DMGUD21, DMGUD36, and DMGUD41, produced the highest amounts of EPS at 4.29±0.12, 3.27±0.11, 2.4±0.06, 1.16±0.09, 2.17±0.11, and 0.49±0.08 mg/ml, respectively. Earlier, Joshi & Koijam[25] isolated *Lactococcus lactis* from a traditionally fermented beverage and observed EPS production of 3.340 mg/mL on MRS-sucrose medium. Similarly, Abdellah *et al.*[26] reported EPS yields ranging from 1.60 to 7.40 mg/mL for various *Lactobacillus* strains, 1.24 to 1.70 mg/mL for *Enterococcus*, 1.32 to 1.34 mg/mL for *Pediococcus*, and 1.26 to 3.19 mg/mL for *Streptococcus* strains. However, Pintado *et al.*[27] reported a high yield of EPS production by different LAB strains in the range of 194–1187 mg/g of dry biomass, but the extraction and purification methods were different.

Phenotypic, biochemical and genotyping characterization

The five most EPS-producing LAB isolates were preliminarily identified by assessing their morphological, physiological, and biochemical traits according to Bergey's Manual. Morphological examination of isolates DMGUD5, DMGUD10, DMGUD17, DMGUD36, and DMGUD41 revealed Gram-positive, non-spore-forming cocci occurring singly, in pairs, or in short chains, forming circular, smooth, cream-colored colonies, occasionally with a mucoid appearance, whereas isolate DMGUD21 lacked a mucoid layer. Biochemical characterization showed that all EPS-producing isolates exhibited typical characteristics of lactic acid bacteria, being Gram-positive, non-spore-forming, and negative for catalase, oxidase, urease, nitrate reduction, and citrate utilization tests, confirming their fermentative metabolic nature. The carbohydrate fermentation patterns varied among the isolates, indicating strain-specific metabolic diversity. All isolates efficiently fermented fructose, lactose, and trehalose, whereas variable utilization was observed for sucrose, maltose, mannitol, and cellobiose (Suppl. Table 1). Such diversity in sugar utilization reflects their adaptability to different carbon sources and potential for exopolysaccharide synthesis under varied substrates.

Physiological characterization demonstrated optimal growth at 37 °C and pH 6.5, moderate growth at 45 °C, 6.5% NaCl, and pH 3.5–8.5, and limited growth at 15 °C, indicating mesophilic nature, thermotolerance, osmotolerance, and pH resilience (Suppl. Tables 2–4). Strains DMGUD5, DMGUD10, and DMGUD36 exhibited higher growth across the tested conditions, highlighting their broader robustness. Collectively, these morphological, biochemical, and physiological characteristics suggest that the isolates may be members of the genus *Enterococcus* or *Lactococcus* according to Bergey's Manual.

Further, these six LAB isolates obtained from goat milk were identified through 16S rRNA gene sequencing, showing high sequence similarity (≥99.7%) to reference strains in the NCBI GenBank database (Suppl. Table 5). The isolated DMGUD5 exhibited 100% identity with *Enterococcus durans* strain FMA8 (accession No.OR230521), DMGUD10, and DMGUD41 showed 100% similarity with *Enterococcus hirae* strains 708 and LVP13 (accession Nos.OR251373 and OR346346), while DMGUD17 matched *Enterococcus mundtii* strain 69HN6I3 (accession no. OR230522). Similarly, DMGUD36 displayed 100% identity with *Enterococcus faecium* strain WS322 (accession No. OR230523), and DMGUD21 showed 99.73% similarity to *Lactococcus lactis* strain 897 (accession no. OR346345). These results confirmed that the isolates primarily belonged to the genera *Enterococcus* and *Lactococcus*, indicating their close phylogenetic relationship with well-characterized lactic acid bacteria. Fig. 1 illustrates the phylogenetic tree of the different isolated LAB species, showing their evolutionary relationships based on genetic data. It highlights the genetic diversity and relatedness among strains, aiding in understanding their taxonomy, functional traits, and potential applications.

Evaluation of probiotic properties of EPS-producing isolates

Acid and bile tolerance

Acid and bile tolerance characteristics are crucial for the oral administration of probiotics as they ensure their viability and functionality upon reaching the intestines. The acid tolerance of the six EPS-producing LAB isolates was assessed by measuring their viability ($\log_{10}$ CFU/mL) after exposure to pH 2.0, 3.0 and 3 h (Fig 2A and B). Among the isolates, DMGUD5 exhibited the highest acid tolerance, maintaining a viability above 6.5 $\log_{10}$ CFU/mL at both pH levels after 3 h. In contrast, DMGUD41 showed the lowest survival, with viability dropping to 5.88 $\log_{10}$ CFU/mL at pH 2.0, after 3 h.

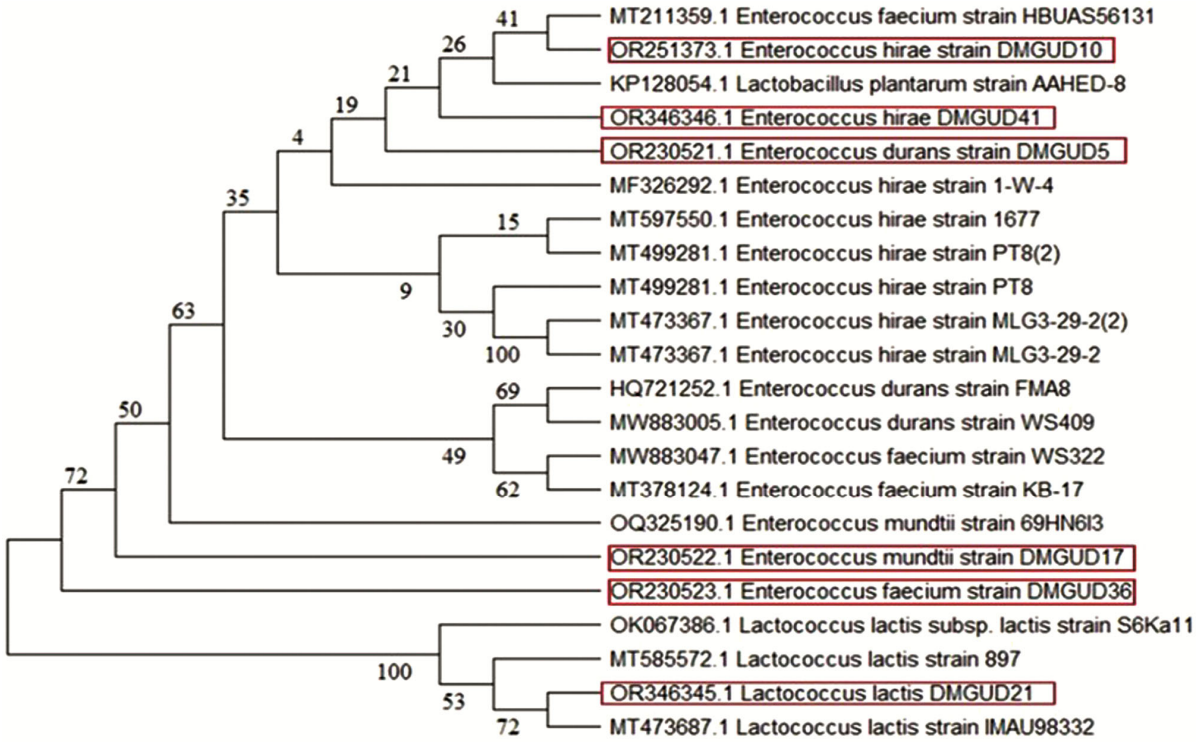

Figure 1 — Neighbor-joining tree in MEGA 11.0 with 1000 bootstrap values illustrating the phylogenetic connection of an isolated strain based on a 16S rRNA distance matrix study.

These findings align with those of recent studies that highlight variability in acid tolerance among LAB strains. Earlier work by Ghatani *et al.*[28] highlighted that *Enterococcus* strains isolated from churpi, particularly, *Enterococcus lactis* YY1 and *E. durans* HS03 showed satisfactory cell viability at pH 2.0 after 3-h incubation. Similarly, Sharma *et al.*[29] reported that LAB strains isolated from camel milk exhibited good acid tolerance, surviving at pH 3.0, with *E. lactis* (cam 14) and *L. plantarum* (cam 15) maintaining growth even at pH 2.0. Among Enterococcus species, *E. lactis* (cam 14) demonstrated the highest acid tolerance, indicating strain-dependent variation in acid resistance. Such differences underscore the importance of strain-specific assessments when evaluating probiotic potential. The superior acid tolerance observed in DMGUD5 suggests its potential as a robust probiotic candidate capable of surviving the acidic environment of the human stomach.

Bile tolerance is pivotal for gastrointestinal survival of probiotic strains. In this study, activated cells were exposed to 0.3% bile salt for 3 h to simulate stomach conditions (Fig. 2C). Viability declined with prolonged exposure across the isolates. Survivability decreased for up to 3 h, with the exception of DMGUD5 and DMGUD10. Premasiri *et al.*[2] cited that the transit time of food through the small intestine is 1 to 4 h, and bile concentration is 0.3 percent, posing a significant challenge to probiotic survival. Previous studies have demonstrated that certain LAB strains isolated from the milk of different species exhibit resilience to bile concentrations, maintaining viability in environments containing 0.3% bile salts[2,3,17,30]. The production of exopolysaccharides (EPS), particularly β-glucans, by LAB has been linked to enhanced stress tolerance. For instance, *Lactobacillus paracasei* NFBC 338, a β-glucan-producing strain, showed a 5.5-fold increase in survival under bile stress compared with its non-EPS-producing counterpart[31]. This protective effect is attributed to the EPS forming a barrier that mitigates the detrimental effects of bile salts on the bacterial cells. However, not all LAB strains exhibit uniform bile tolerance. In a study evaluating 70 LAB isolates from different cereal-based fermented products, exhibited variable acid tolerance (pH- 2.0 and 3.0), and only a subset showed resilience to 0.3% bile concentrations. This variability highlights the importance of strain-specific assessment when selecting probiotics for functional food applications[3].

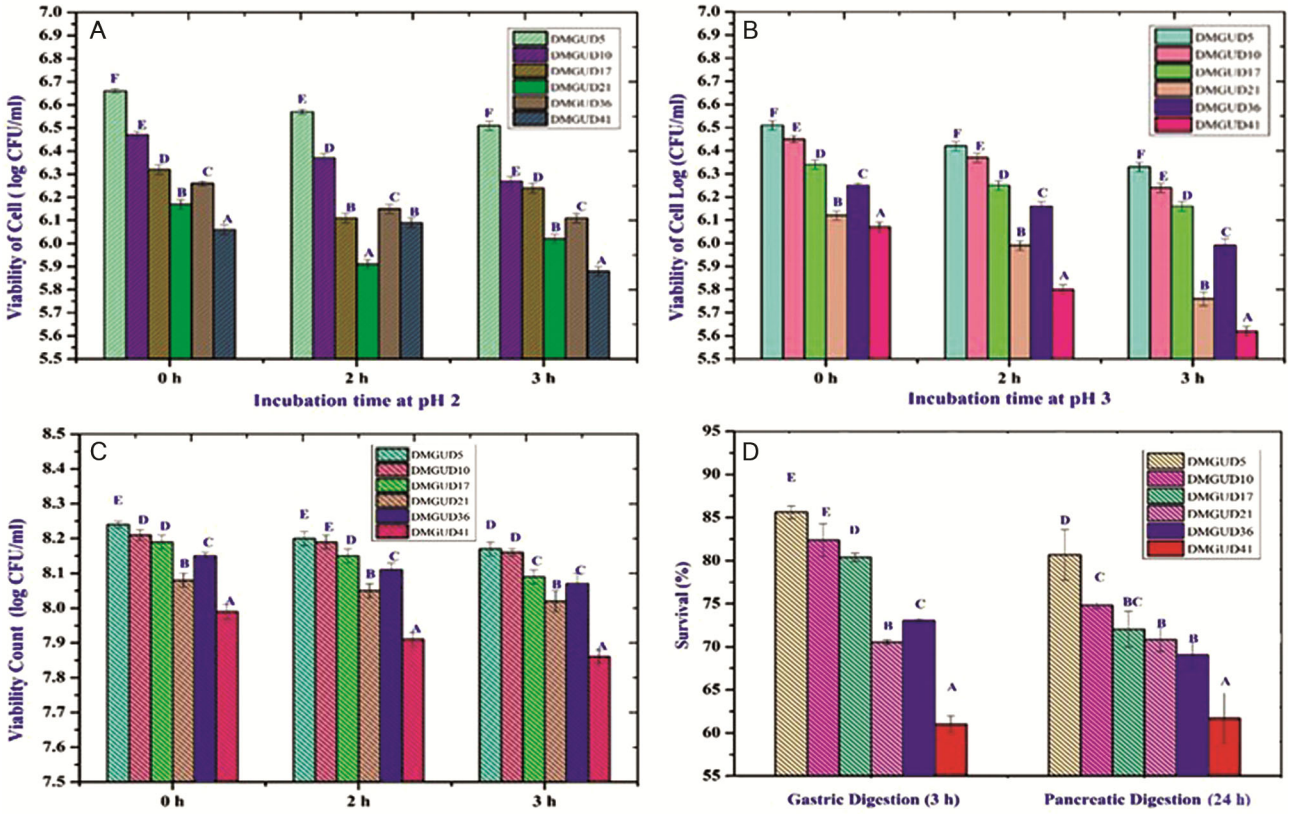

Figure 2 — (A) Acid tolerance at pH 2.0 (B) Acid tolerance at pH 3.0 (C) Bile salt tolerance (D) Gastric and pancreatic juice digestion. All values are presented as mean±SD. Different letters (A, B, C, D, and E) above the bars denote statistically significant differences (P<0.05), as noted by Duncan's test. DMGUD5, 10, 17, 21, 36, and 41 denote *Enterococcus duran* DMGUD5, *Enterococcus hirae* DMGUD10, *Enterococcus mundtii* DMGUD17, *Lactococcus lactis* DMGUD21, *Enterococcus faecium* DMGUD36, and *Enterococcus hirae* DMGUD41, respectively.

Survivability in gastric and pancreatic juices

The survival of the LAB strains under simulated gastric and pancreatic conditions was assessed to evaluate their potential as probiotics. After 3 h of incubation in simulated gastric juice, all LAB isolates showed survival rates exceeding 50% (Fig. 2D). Notably, isolate *Enterococcus duran* DMGUD5 exhibited the highest survival rate at 85.62 ± 0.74%**,** significantly outperforming the other isolates (P < 0.05). After 24 h of exposure to simulated pancreatic conditions, DMGUD5 again demonstrated superior resilience, with a survival rate of 80.67 ± 2.92%**,** which was significantly higher than that of the other isolates (P < 0.05) (Fig. 2D).

The potential of the isolates to colonize the gastrointestinal tract was assessed by examining their survival under simulated gastric and intestinal digestion conditions. The ability to withstand pancreatic enzymes secreted into the small intestine to facilitate the digestion of proteins, carbohydrates, and lipidsis considered a key functional trait of probiotics[17]. All tested isolates demonstrated resilience in both simulated gastric and pancreatic environments, indicating their suitability for intestinal colonization. These findings are consistent with previous research on lactic acid bacteria isolated from milk of different species[3,30].

Auto-aggregation and cell-surface hydrophobicity

The interaction between bacterial cells and the gastrointestinal mucosa is a key functional attribute of cell surfaces that significantly influences their role within the gut environment. An important parameter associated with this interaction is the self-aggregation (auto-aggregation) capacity of bacterial isolates, which was assessed via sedimentation in this study. The results indicated that the adhesion potential of the isolates improved with increasing auto-aggregation over time. Among the tested strains, *Enterococcusduran* DMGUD5 exhibited the highest auto-aggregation percentage after 3 h of incubation (Fig. 3A). According to Nath *et al.*[32], potential

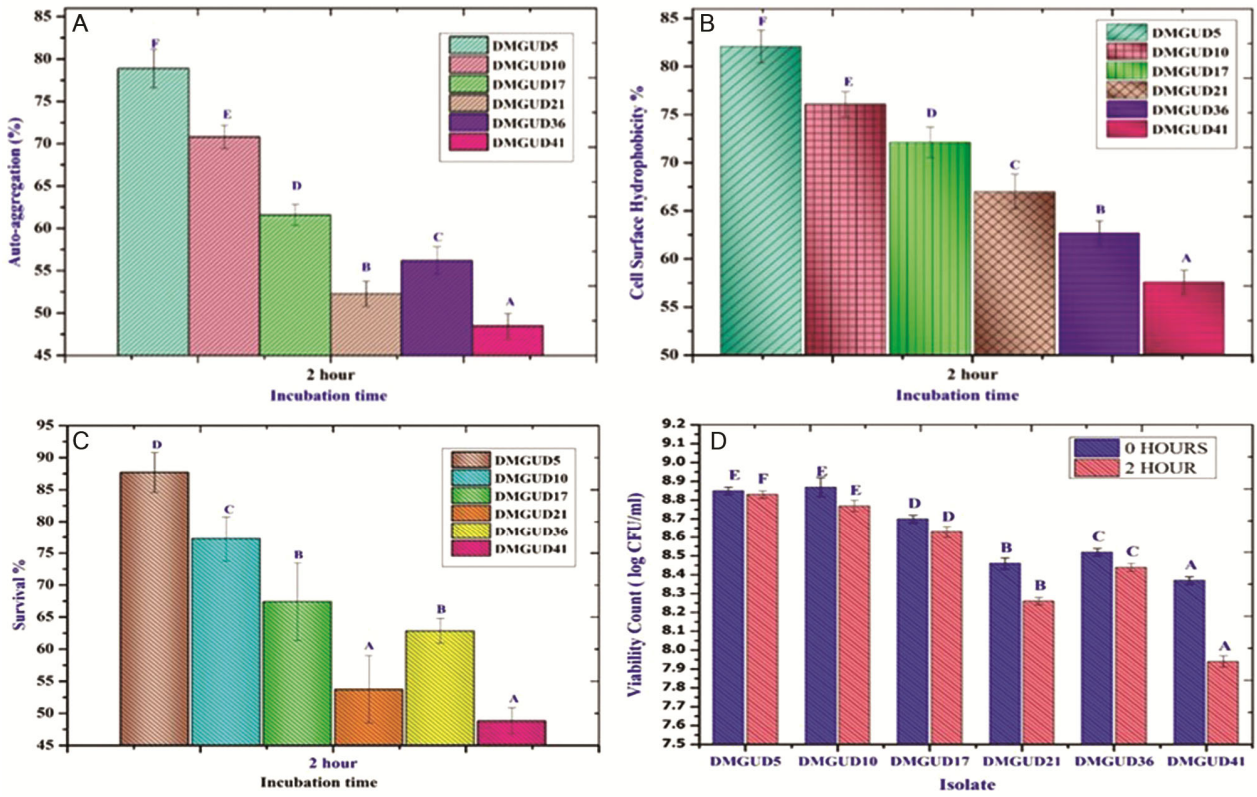

Figure 3 — (A) Cell auto-aggregation (B) Cell surface hydrophobicity (C) Lysozyme tolerance (D) Phenol tolerance. All values are presented as mean±SD. Different letters (A, B, C, D, and E) above the bars denote statistically significant differences (P<0.05). DMGUD5, 10, 17, 21, 36, and 41 denote *Enterococcus duran* DMGUD5, *Enterococcus hirae* DMGUD10, *Enterococcus mundtii* DMGUD17, *Lactococcus lactis* DMGUD21, *Enterococcus faecium* DMGUD36, and *Enterococcus hirae* DMGUD41, respectively.

probiotic strains isolated from sour rice demonstrated a broad range of auto-aggregation capacities (16–50%), contributing to the inhibition of pathogenic microorganism colonization and attachment. The DMGUD5 strain isolated in the present study displayed an auto-aggregation value of 37%, which is consistent with the findings (38-56%) reported by Meena et *al*.[17]. In contrast, Azhar *et al*.[3] documented lower auto-aggregation values (approximately 28.45%) for coccus-shaped bacterial species.

Cell surface hydrophobicity (CSH), assessed using the nonpolar solvent xylene, revealed CSH values ranging from 57.60% to 82.1% (Fig. 3B). Among the isolates, DMGUD5 showed the highest hydrophobicity (82.1%). Similar results were observed for isolated strains, such as *Lactiplantibacillus plantarum* P2[17], *Enterococcus faecium*[3], and *Lb. plantarum*[29] from milk of different species. Cell surface hydrophobicity (CSH) plays a significant role in bacterial auto-aggregation and adhesion to various surfaces[17]. Elevated CSH values are typically associated with the presence of glycoproteins, whereas lower values are linked to surface polysaccharides. This hydrophobicity can differ not only between species and strains but also due to factors such as cell age and interactions between cell surface chemistry and growth medium components. CSH values exceeding 40% generally indicate a hydrophobic cell surface. A positive correlation was observed between CSH and auto-aggregation, suggesting that hydrophobic interactions play a pivotal role in enhancing bacterial adhesion to intestinal epithelial cells. Higher hydrophobicity facilitates stronger interactions with mucosal surfaces due to reduced interfacial tension and increased affinity for host tissues[33]. Therefore, isolates with elevated hydrophobicity, such as DMGUD5, are likely to exhibit superior adhesion efficacy, which is a desirable trait for probiotic function and persistence in the gastrointestinal tract[18].

Lysozyme resistant

Assessment of lysozyme resistance revealed that all tested LAB isolates maintained survival rates above

48% following a 2 h incubation period (Fig. 3C). Among these, isolate DMGUD5 exhibited a significantly higher survival rate (P<0.05), reaching 87.70 ± 3.09%, indicating a superior tolerance to lysozyme-induced stress. This level of resilience surpassed that of the other strains analyzed. Lysozyme tolerance, which reflects bacterial survival under conditions mimicking salivary stress, has previously been documented in certain LAB strains, with some maintaining viability above 52% after 2 h of exposure. Among them, *Lactiplantibacillus plantarum* P2 exhibited a survival rate of 83.59 ± 3.49% [17]. Similar studies involving LAB from cereal-based fermented foods have reported diverse levels of resistance (53-96%); *Lactiplantibacillus plantarum* KMUDR7 showed survival rates as high as 96.75% [18]. The high survivability observed for DMGUD5 in this study aligns well with earlier findings demonstrating robust lysozyme tolerance in selected LAB strains.

Phenol tolerance

Phenol resistance of EPS-producing strains was tested using 0.4% phenol for 24 h at 37°C (Fig. 3D). Notably, no inhibition was observed for DMGUD5, DMGUD10, or DMGUD17, indicating strong resistance. In contrast, DMGUD21, DMGUD36, and DMGUD41 were found to be sensitive to phenol exposure. After 24-h incubation, viability counts ranged from 7.94 to 8.83 log CFU/mL, showing the overall robustness of the isolates. DMGUD5 exhibited remarkable performance under phenol stress, further emphasizing its potential probiotic properties. Furthermore, the ability to tolerate phenol at a concentration of 0.4% is a significant trait of potential probiotics. Phenol, a byproduct of amino acid deamination in the gastrointestinal tract, hinders microbial growth. DMGUD5, DMGUD10, and DMGUD17 demonstrated phenol tolerance, while the other strains displayed decreasing trends post-24-h phenol exposure. Recently, similar observations were reported for *Lactiplantibacillus plantarum* P2 isolated from sheep milk with 0.4% phenol tolerance[17] and variable phenol tolerance among different LAB isolates[18].

Antibacterial activity

The antimicrobial efficacy of LAB strains was evaluated against a panel of food-borne pathogens using cell-free supernatants. The diameters of the observed inhibition zones ranged from 0 to 24 mm, indicating varying degrees of antagonistic activity among the isolates (Table 1). The strain DMGUD5 demonstrated the highest antimicrobial effect, producing inhibition zones of 20.33 ± 0.58 mm against *Staphylococcus aureus* and 19.33 ± 0.58 mm against *Staphylococcus epidermidis*, along with marked inhibition against other tested bacterial strains. The antimicrobial properties of probiotic LAB are pivotal for their selection as functional strains, particularly owing to their ability to suppress pathogenic and spoilage microorganisms. This inhibitory effect is commonly attributed to the production of various antimicrobial metabolites, including organic acids (lactic, acetic, and propionic acids), hydrogen peroxide (H_2O_2), diacetyl, surfactants, and bacteriocins[34]. The strong inhibitory performance of DMGUD5 underscores its potential application in food preservation and safety, as its natural antimicrobial capabilities can reduce reliance on synthetic preservatives and enhance the microbial quality of fermented food products.

To ensure a rigorous comparison, the antibacterial efficacy of the isolates was assessed alongside

Table 1 — Antibacterial activity of LAB strains (ZOI in mm) and percentage inhibition of ABTS radicals (Values are mean±SD, n=3 for every sample)

Isolate	*Staphylococcus aureus* NCIM 2079	*Listeria monocytogenes* NCIM 5260	*Bacillus cereus* NCIM 2217	*Escherichia coli* NCIM 20265	% Inhibition of ABTS radicals
DMGUD5	20.33±0.58^{e*}	19.33±0.58^{d}	15.67±0.58^{a}	8.33±1.53^{c}	69.53±1.98^{f*}
DMGUD10	17.33±1.15^{d}	16.67±0.58^{c}	14.33±0.58^{a}	6.33±0.58^{b}	51.79±1.38^{e}
DMGUD17	15.33±0.58^{c}	16.33±0.58^{c}	11.67±1.15^{b}	0±0^{a}	40.67±1.37^{d}
DMGUD21	11.67±1.15^{b}	11.67±0.58^{b}	10.33±0.58^{b}	0±0^{a}	20.04±1.53^{b}
DMGUD36	13.67±0.58^{c}	12.67±1.53^{b}	10.67±1.53^{b}	5.67±1.15^{b}	23.89±2.37^{c}
DMGUD41	9.33±1.53^{a}	10.33±1.53^{a}	8.33±1.53^{a}	0±0^{a}	14.05±1.60^{a}

*Values with different superscript letters differ significantly (P <0.05) along the rows. DMGUD5, 10, 17, 21, 36, and 41 denote *Enterococcus duran* DMGUD5, *Enterococcus hirae* DMGUD10, *Enterococcus mundtii* DMGUD17, *Lactococcus lactis* DMGUD21, *Enterococcus faecium* DMGUD36, and *Enterococcus hirae* DMGUD41, respectively.

standard antibiotic discs at known concentrations, as presented in Table 3. The data demonstrated that certain isolates (e.g. DMGUD5 and DMGUD10) produced inhibition zones comparable to or moderately lower than those of standard antibiotics, such as tetracycline, amikacin, and ciprofloxacin, confirming the functional potential of these strains. This comparative approach validates the antimicrobial activity of EPS-producing LAB in relation to clinically relevant antibiotic standards, providing a meaningful benchmark for their biopreservative and therapeutic potential. The variability in sensitivity among the isolates reflects strain-specific antimicrobial metabolite production, which aligns with previous studies highlighting the diverse bioactive profiles of LAB[35].

Anti-oxidative activity

The current study elucidates the antioxidative potential of lactic acid bacteria (LAB) isolates, quantified using an ABTS radical cation decolorization assay. The results reveal a wide range of antioxidative activities among the isolates, spanning from 14.05 ± 1.60% to 69.53 ± 1.98%. Notably, isolate DMGUD5 exhibited the highest radical scavenging activity at 69.53 ± 1.98%, a statistically significant value ($P< 0.05$) compared to the other strains.

Oxidative stress, primarily induced by the overproduction of reactive oxygen species (ROS), is a pathological hallmark of various chronic disease in humans. Antioxidants play a pivotal role in mitigating oxidative damage by neutralizing free radicals. The antioxidative mechanisms of LAB are multifaceted, encompassing the biosynthesis of bioactive compounds such as glutathione, folate, and short-chain fatty acids (e.g. butyrate), regulation of host antioxidative genes, inhibition of ROS generation, and modulation of gut microbiota composition[36]. Furthermore, specific cellular components, such as cell envelope-associated proteins and exopolysaccharides (EPS), contribute substantially to the antioxidant functions of LAB[37]. The results of the isolated strains are consistent with those of previous studies on the phenol tolerance of isolated LAB[16–18].

Antifungal activity

Antifungal activity is a key functional trait of probiotic strains, particularly in the context of competitive exclusion and inhibition of pathogenic fungi within the gastrointestinal tract. These strains exhibited varying degrees of antifungal activity (Table 2). Among the LAB strains tested, DMGUD5 demonstrated the most potent antifungal efficacy by effectively suppressing fungal growth. This antagonistic potential can be attributed to a combination of competitive exclusion mechanisms and secretion of antifungal metabolites, which interfere with fungal colonization and survival[6]. For a LAB strain to be suitable as a starter culture, it must not only promote host health but also contribute to food preservation by extending shelf life through the inhibition of spoilage organisms, including fungi[6]. A wide array of LAB strains isolated from diverse fermented and natural food sources have been shown to possess antifungal properties. In particular, strains such as *Lactiplantibacillus plantarum* P2 and *Lactobacillus paracasei* P1 have been identified as promising candidates because of their consistent inhibitory effects against mycotoxigenic fungi[17]. Similarly, *Enterococcus faecium* strains derived from raw goat milk exhibited significant antifungal activity, further supporting the antifungal potential of food-associated LAB strains[3].

Table 2 — Antifungalactivity of LAB strains(ZOI in mm) (Values are mean±SD, n=3 for every sample)

Isolate	*Aspergillus niger* NCIM-1004 2 days	*Aspergillus niger* NCIM-1004 7 days	*Alternaria solani* MTCC-2101 2 days	*Alternaria solani* MTCC-2101 7 days	*Candida albicans* NCIM 3268 2 days	*Candida albicans* NCIM3268 7 days
DMGUD5	15.33±1.25^{d}*	14±0.82^{c}	12.33±1.25^{d}	11.33±1.2^{c}	16.67±0.94^{d}	15.33±0.47^{d}
DMGUD10	12.33±0.47^{c}	11.33±0.47^{b}	9.33±0.47^{c}	7.67±0.47^{b}	16.33±0.47^{d}	14.67±0.47^{d}
DMGUD17	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	11.67±0.47^{c}	10.33±1.25^{c}
DMGUD21	10.33±0.94^{b}	9.33±1.7^{b}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
DMGUD36	12.33±1.25^{c}	11±1.41^{b}	7.33±0.47^{b}	6.67±0.47^{b}	12.67±1.25^{c}	11.33±0.94^{c}
DMGUD41	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	7.67±0.94^{b}	6.67±0.94^{b}

*Values with different letters in the superscript differsignificantly (P <0.05) row-wise (significance determined using Duncan'stest). DMGUD5, 10, 17, 21, 36, and 41 denote *Enterococcus duran* DMGUD5, *Enterococcus hirae* DMGUD10, *Enterococcus mundtii* DMGUD17, *Lactococcus lactis* DMGUD21, *Enterococcus faecium* DMGUD36, and *Enterococcus hirae* DMGUD41, respectively.

Sugar fermentation ability

The ability of EPS-producing strains to endure fermentation (for a duration of 24 h) and storage (over a span of 21 days) in a refrigerated environment was evaluated. The results are shown in Fig. 4. In the context of fermentation, all examined isolates exhibited significant growth ($p< 0.05$), with growth rates ranging from 2.87±0.06-8.90±0.06 $\log_{10}$ CFU/g. Notably, DMGUD5 (8.90±0.06 $\log_{10}$ CFU/mL) displayed the most noteworthy growth rate, surpassing the other LAB isolates examined. During the storage period at refrigeration temperature (4°C), a distinctive trend was observed. With the exception of DMGUD5 and DMGUD10, which demonstrated substantial increases in viability at the 14-day mark compared to that at 7 days, the viability of all isolates exhibited a pronounced reduction. Among these, DMGUD5 stood out, recording the highest viability count (5.18±0.06 $\log_{10}$ CFU/mL) after 21 days, followed closely by DMGUD10. This assessment underscores the varying abilities of LAB isolates to withstand the rigors of fermentation and storage, contributing to a comprehensive understanding of their behavior under these conditions.

For probiotic viability, the EPS-producing strains were cultured in skimmed milk media and displayed growth during 24-h of fermentation and varying trends during storage. DMGUD5 and DMGUD10 maintained acceptable cell viability for up to 14 days, whereas all isolates exceeded the recommended limit of 10^6 CFU/g for health benefits during fermentation. Previous studies[17,18] have reported similar trends for potential probiotic strains.

Amylolytic activity

Amylolytic activity of candidate probiotic LAB is essential for breaking down dietary starches and enhancing nutrient availability and gut health. Isolates DMGUD5, 10 and 17 showed mild amylolytic activity. The strains showed no activity or zones around the colonies. Amylolytic LAB harboring the amyA gene for starch-to-lactic acid conversion via α-amylase were observed in DMGUD5, 10 and 17, with variable activity. The production of exopolysaccharides (EPSs) safely enhances food texture. EPS provide benefits, including protection, biofilm formation, and cell recognition, although structure-function relationships remain strain-specific[37].

Safety of isolated strains

Antibiotic susceptibility

The antibiotic susceptibility profiles of six EPS-producing LAB isolates (DMGUD5, DMGUD10, DMGUD17, DMGUD21, DMGUD36 and DMGUD41) were evaluated against ten commonly used antibiotics using the disc diffusion method. The isolates demonstrated varied resistance patterns, reflecting strain-specific responses in their intrinsic or acquired resistance to different antibiotics (Table 3). Following CLSI interpretive standards, inhibition

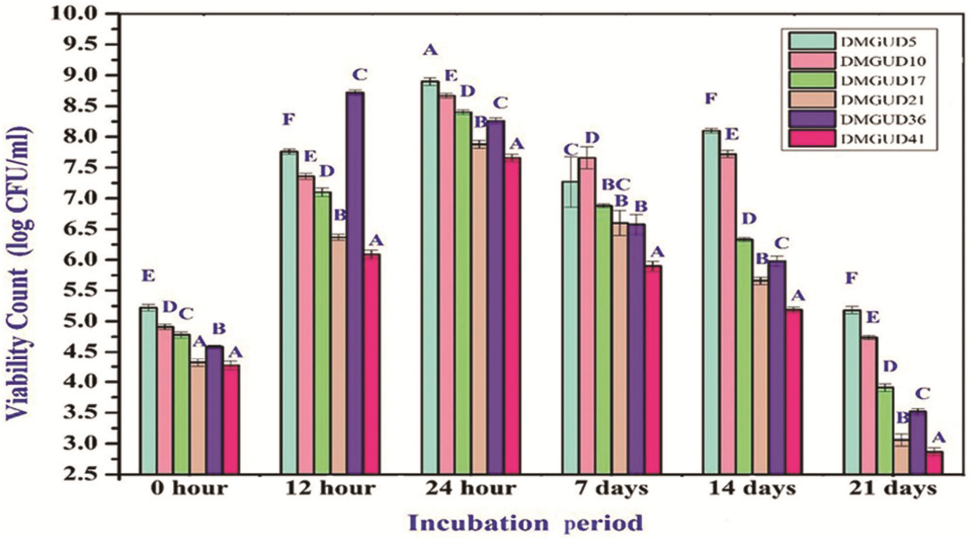

Figure 4 — Fermentation ability of EPS-producing strains in skim milk media (lactose sugar) at different storage periods (survival of bacterial cells after storage – $\log_{10}$ CFU/ml). Values are the mean ± SD (n = 3 for every tested sample), and different capital case letters above the bars denote significant differences (P <0.05). DMGUD5, 10, 17, 21, 36, and 41 denote *Enterococcus duran* DMGUD5, *Enterococcus hirae* DMGUD10, *Enterococcus mundtii* DMGUD17, *Lactococcus lactis DMGUD21, Enterococcus faecium* DMGUD36, and *Enterococcus hirae* DMGUD41, respectively.

Table 3 — Antibiotic susceptibility (diameter, in mm, of inhibition zone) test of exopolysaccharide-producing (EPS) strains

Antibiotics Disc - Concentration	Isolates					
	DMGUD5	DMGUD10	DMGUD17	DMGUD21	DMGUD36	DMGUD41
Tetracycline (TE)- (30 µg/disc)	S	R*	R	MS	MS	R
Amikacin (AK) - (30 µg/disc)	S	MS	MS	S	S	MS
Ciprofloxacin (CIP) - (5 µg/disc)	S	R	MS	S	MS	MS
Vancomycin (VA) - (30 µg/disc)	MS	R	R	R	R	S
Ofloxacin (OF) - (5 µg/disc)	S	MS	MS	MS	R	MS
Cefotaxime (CTX) - (30µg/disc)	S	R	MS	MS	S	R
Ampicillin (AMP) - (10 µg/disc)	MS	R	R	MS	R	MS
Erythromycin (E) - (15 µg/disc)	R	R	R	R	MS	S
Clindamycin (CD) - (2 µg/disc)	MS	R	S	MS	R	MS
Cotrimaxazole - (25 µg/disc)	MS	MS	R	S	S	MS

*R - Resistant (Zones ≤ 14 mm), MS- Moderately Sensitive (15–19 mm), S - Sensitive (≥ 20 mm). DMGUD5, 10, 17, 21, 36, and 41 denote *Enterococcus duran* DMGUD5, *Enterococcus hirae* DMGUD10, *Enterococcus mundtii* DMGUD17, *Lactococcus lactis* DMGUD21, *Enterococcus faecium* DMGUD36, and *Enterococcus hirae* DMGUD41, respectively.

zone diameters ≥20 mm were considered sensitive (S), 15–19 mm were considered moderately sensitive (MS), and ≤14 mm were considered resistant (R).The isolates DMGUD5, DMGUD36, and DMGUD41 displayed wider inhibition zones (≥20 mm) for amikacin, ciprofloxacin, ofloxacin, and cefotaxime, indicating a strong susceptibility to aminoglycosides, fluoroquinolones, and β-lactam antibiotics. Among these, DMGUD5 (*Enterococcus durans*) exhibited broad-spectrum sensitivity, except to erythromycin, highlighting its potential safety for probiotic use. This susceptibility is encouraging from a probiotic safety perspective as it reduces the risk of resistance gene transfer[38].

In contrast, DMGUD10 (*E. hirae*) and DMGUD17 (*E. mundtii*) showed narrow inhibition zones (≤14 mm) against tetracycline, vancomycin, and ampicillin, confirming resistance that corresponds to the intrinsic and plasmid-mediated resistance mechanisms commonly reported in *Enterococcus* species. Moderate inhibition zones (15–19 mm) observed for cotrimoxazole and clindamycin across several isolates indicated an intermediate sensitivity. Vancomycin resistance detected in DMGUD10, DMGUD17, DMGUD21 and DMGUD36 is consistent with the presence of altered cell wall peptidoglycan precursors, a known mechanism in vancomycin-resistant enterococci[39]. The isolate DMGUD21 (*Lactococcus lactis*) demonstrated moderate sensitivity to most antibiotics, which is consistent with its generally non-pathogenic and low-resistance nature. Overall, DMGUD5 and DMGUD36 showed the highest antibiotic susceptibility, whereas the other isolates exhibited strain-specific resistance profiles.

These findings are consistent with those of previous studies reporting widespread resistance to these antibiotics among enterococcal and lactic acid bacterial strains, likely due to their overuse in medical and veterinary settings[39]. Although some resistance in probiotics is considered intrinsic and non-transferable, particularly among LAB, any acquired resistance genes on mobile elements can pose serious health risks if transferred to pathogens[40]. However, it is crucial to support these findings through molecular characterization to ensure that no mobile antibiotic resistance genes are present.

DNase activity, hemolytic behavior, and gelatinase production

A fundamental criterion for evaluating the safety of probiotic candidates is the absence of virulence-associated traits, such as DNase activity, hemolytic behavior, and gelatinase production. None of the tested LAB strains exhibited DNase activity, aligning with the general expectation that probiotic strains, being nonpathogenic, should not produce deoxyribonuclease enzymes capable of degrading host DNA[17]. Similarly, hemolytic activity, a marker often linked to pathogenic potential, was absent in all isolates tested. The lack of clear or green zones on the blood agar plates indicated γ-hemolysis (non-hemolytic activity), confirming the non-pathogenic nature of the strains[21]. The safety assessment also included evaluation of gelatinase activity**,** an indicator of the ability of the strain to hydrolyze gelatin, a protein substrate. Gelatinase contributes to tissue degradation and pathogenesis in certain bacterial species. The absence of these virulence factors, DNase activity, hemolysis, and gelatinase production,

Table 4 — Shelf-life evaluation of curd during storage at 4±1 ˚C (Values are mean±Standard deviation, n=3)

Storage (days)	Sensory score (1-9 scale)	pH	Probiotic viability count (log10 CFU/g)	Fungal count (log10 CFU/g)	Coliform count (log10 CFU/g)
0	8.31±0.2^{f}*	4.33±0.02^{f}	7.59±0.31^{e}	NC**	NC
2	8.18±0.17^{ef}	4.26±0.02^{ef}	7.54±0.31^{e}	NC	NC
4	8.09±0.15^{e}	4.21±0.02^{e}	7.48±0.29^{e}	NC	NC
6	7.87±0.2^{d}	3.99±0.02^{d}	7.14±0.22^{d}	NC	NC
8	6.34±0.14^{c}	3.51±0.02^{c}	6.41±0.29^{c}	NC	NC
10	5.87±0.16b	2.98±0.03^{b}	5.83±0.2^{b}	NC	NC
12	4.72±0.17^{a}	2.56±0.03^{a}	4.41±0.35^{a}	NC	NC

*Values with different letters in small case differ significantly (P <0.05) row-wise; NC**means no colony formation.

suggests that the LAB isolates under investigation, particularly DMGUD5, possess a favorable safety profile suitable for potential probiotic and food-grade applications. These findings were consistent with the safety criteria for food-grade probiotic strains[18,21,29].

Proximate and shelf-life analysis of curd

The most efficient and safe probiotic strain, *Enterococcus durans* DMGUD5, was chosen for curd preparation.The final curd comprised fat, protein, lactose, solid-not-fat (SNF), ash, and moisture, with concentrations of 2.97 ± 0.03 %, 3.27 ± 0.03 %, 5.08 ± 0.07 %, 9.07 ± 0.03 %, 0.72 ± 0.03 %, and 87.97 ± 0.03 %, respectively.

Table 4 presents the shelf-life evaluation of curd stored at 4±1 °C over a period of 12 days, assessing parameters such as sensory score, pH, probiotic viability, and microbial safety (fungal and coliform counts).The sensory scores gradually declined during storage. On day 0, the curd had high acceptability, with a mean score of 8.31±0.2, which significantly (P < 0.05) decreased to 4.72±0.17 by day 12. This decline corresponds to visible changes in the texture, flavor, and overall appearance of the product, which are key factors for consumer acceptability. Parallel to the sensory decline, the pH of the curd dropped progressively from 4.33±0.02 at the start to 2.56±0.03 on day 12. This decrease in pH indicates increased acidification during storage, likely due to the continued metabolic activity of lactic acid bacteria. A significant pH reduction correlates with the observed decline in sensory quality, as excessive acidity can adversely affect taste and texture. The probiotic viability count (Log_{10} CFU/g) also decreased significantly over the storage period, from 7.59±0.31 on day 0 to 4.41±0.35 on day 12. Although a decrease was expected over time due to storage stress and acid accumulation, the product retained an acceptable level of probiotic viability (>6 Log_{10} CFU/g) until day 8, suggesting that the curd maintained its functional probiotic benefits for at least one week under refrigerated conditions. The viability of probiotics is influenced by factors such as pH, fat content, protein concentration, and type and amount of sugar present[24]. Importantly, fungal and coliform counts remained undetectable (no colony formation) throughout the 12-day storage period, indicating a good microbiological quality and hygienic preparation. The absence of spoilage organisms suggested that the curd remained microbiologically safe during the study period. The sensory score of the product remained acceptable for up to 8 days. These characteristics are comparable to those of market curds prepared indigenously[22]. Therefore, the shelf life of the curd under refrigerated conditions was determined to be eight days. In conclusion, the probiotic strain used in curd production is suitable as a starter culture for milk production.

Conclusion

This study explores exopolysaccharide (EPS)-producing lactic acid bacteria (LAB) isolated from goat milk, focusing on their probiotic potential land their suitability as starter cultures for fermented dairy products. Sixty LAB isolates were obtained and six were selected based on EPS production. *Enterococcus durans* DMGUD5 exhibited the most promising probiotic characteristics, including high EPS production; superior acid and bile tolerance; strong survivability under simulated gastrointestinal conditions; and significant antibacterial, antifungal, and antioxidative activities. The absence of virulence factors and broad antibiotic susceptibility of DMGUD5 further support its safety for potential

probiotic applications. Based on excellent probiotic traits, safety profiling, and highest EPS production, DMGUD5, was chosen as starter culture for preparation of fermented curd. The curd maintained acceptable nutritional, sensory, and microbial quality with a shelf life of 8 days under refrigerated storage and probiotic viability of 6.41±0.29 $\log_{10}$ CFU/mL up to 8 days, exceeding the recommended threshold of 10^6 CFU/mL. These findings support the strain's potential as a functional probiotic starter culture. Future research should focus on scaling up the production of *Enterococcus durans* DMGUD5 for industrial applications, optimizing fermentation conditions to maximize EPS yield and probiotic viability, and conducting *in vivo* trials to confirm its health benefits and safety in human subjects. Additionally, exploring the strain's functionality in different dairy and non-dairy matrices can broaden its application in developing diverse functional foods. From an industrial perspective, DMGUD5 presents a promising candidate for commercial probiotic formulations and functional dairy products due to its robust technological properties, health-promoting potential, and ability to enhance texture and shelf life through EPS production.

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Conflict of interest statement

The authors declare no conflicts of interest to disclose.

Ethical Considerations

This article does not involve any studies involving animal subjects.

Consent statement

Prior approval and informed consent has been taken as per the institute policy to conduct the sensory evaluation of the curd.

Author contribution

All authors have read and agreed to the published version of the manuscript.

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References

1 World Population Review. *Goat Population by Country* 2025.

2 Premasiri DLDAS, Rajawardana DU, Mudannayake DC & Hewajulige IGN. Isolation, characterization and identification of industrially beneficial probiotic lactic acid bacteria from goat milk. *J Agric Sci - Sri Lanka*, 16 (2021)369.

3 Azhar FUA, Mehnaz S, Akbar A & Sadiq MB. Probiotic characterization of *Enterococcus* spp. isolated from raw goat milk. *Brazilian Arch Biol Technol,* 65 (2022) 1.

4 Meena KK, Taneja NK, Jain D & Ojha A. Bioactive components and health benefits of maize-based fermented foods : A review. *Biointerface Res Appl Chem,* 13 (2023) 1.

5 Sionek, B, Szydłowska, Küçükgöz, K, & Krajewska, DK. Traditional and new microorganisms in lactic acid fermentation of food. *Fermentation*, 9 (2023) 12.

6 Meena KK, Taneja NK, Jain D & Ojha A. Spontaneously fermented cereal based products : An ancient health promoting formulae for consumption of probiotic lactic acid bacteria. *Biointerface Res Appl Chem,*13 (2023) 465.

7 Meena KK, Taneja NK, Jain D, Ojha A, Badgujar P & Raigar RK. Physicochemical, morphological, and microbial characterization of spray-dried dairy–cereal grain-based probiotic beverage. *Futur Postharvest Food,* 2 (2025) 372.

8 Meghwal D, Meena KK, Singhal R, Gupta L & Panwar NL. Exopolysaccharides producing lactic acid bacteria from goat milk : Probiotic potential, challenges, and opportunities for the food industry. *Ann Phytomedicine*, 12 (2023) 200.

9 Bibi A, Xiong Y, Shahid M, Rajoka, R, Mehwish HM, Radicetti E, Umair M, Shoukat M, Kashif M, Khan I & Aadil RM. Recent advances in the production of exopolysaccharide (EPS) from *Lactobacillus* spp . and its application in the food industry : A review. *Sustainability,* 13 (2021) 12429.

10 Jurášková D, Ribeiro SC & Silva CCG. Exopolysaccharides produced by lactic acid bacteria: From biosynthesis to health-promoting properties. *Foods,* 11 (2022) 156.

11 Veettil VN & Chitra AV. Probiotic lactic acid bacteria from goat's milk potential producer of bacteriocin: Evidence from liquid chromatography-mass spectrometry. *J Pure Appl Microbiol*, 16 (2022) 305.

12 Islam MZ, Uddin ME, Rahman MT, Islam MA & Harun-ur-Rashid M. Isolation and characterization of dominant lactic acid bacteria from raw goat milk: Assessment of probiotic potential and technological properties. *Small Rumin Res,* 205 (2021) 106532.

13 Padmavathi T, Bhargavi R, Priyanka PR, Niranjan NR & Pavitra PV. Screening of potential probiotic lactic acid bacteria and production of amylase and its partial purification. *J Genet Eng Biotechnol*, 16 (2018) 357.

14 Yadav MK, Song JH, Vasquez R, Lee JS, Kim IH & Kang DK. Methods for detection, extraction, purification, and characterization of exopolysaccharides of lactic acid bacteria - A systematic review. *Foods,* 13 (2024) 3687.

15 Dubois M, Gilles KA, Hamilton JK, Rebers PA & Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem*, 28 (1956) 350.

16 Mulaw G, Sisay Tessema T, Muleta D & Tesfaye A. In vitro evaluation of probiotic properties of lactic acid bacteria isolated from some traditionally fermented Ethiopian food products. *Int J Microbiol,* 2019 (2019) 7179514.

17 Meena KK, Joshi M, Rao P, Lal N, Gupta L & Meena S. Evaluation of probiotic properties of lactic acid bacteria isolated from sheep milk and its suitability as a starter in a milk-cereal-based beverage. *Food Biomacromolecules*, 2 (2025) 69.

18 Meena KK, Taneja NK, Jain D, Ojha A, Kumawat D & Mishra V. In vitro assessment of probiotic and technological properties of lactic acid bacteria isolated from indigenously fermented cereal-based food products. *Fermentation* 8 (2022) 529.

19 CLSI. Performance Standards for Antimicrobial Susceptibility Testing. *30th ed.CLSI M100 ED30:2020.* 40 (2020).

20 Sangprapai W, Prasitpuriprecha C, Jantama K & Jantama SS. Probiotics isolated from Thai fermented foods for potential uses against foodborne pathogens. *Asia-Pacific J Sci Technol,* 27 (2022) 11.

21 Kang MS, Yeu JE & Hong SP. Safety evaluation of oral care probiotics *Weissella cibaria* CMU and CMS1 by phenotypic and genotypic analysis. *Int J Mol Sci,* 20 (2019) 2693.

22 Kumari V, Kumar N & Mandal S. Evaluation of technological attributes of *Lactococcus lactis* cultures for preparation of dahi and misti dahi (sweetened dahi). *Asian J Dairy Food Res,* 40 (2021) 358.

23 Bunkar DS, Bharti P, Meena KK, Goyal SK & Paswan VK. Studies on the optimization and development of functional instant kodo millet based porridge mix. *Int J Curr Microbiol Appl Sci, 9* (2020) 1462.

24 Joshi M, Meena KK, Kumar A & Meena S. Optimization of a novel probiotic-fermented pearl millet-based strained yoghurt-like functional dessert: Physicochemical, microbial and sensory characterization. *Food Sustain,* 3 (2025) 4.

25 Joshi SR & Koijam K. Exopolysaccharide production by a lactic acid bacteria, *Leuconostoc lactis* isolated from ethnically fermented beverage. *Natl Acad Sci Lett,* 37 (2014) 59.

26 Abdellah M, Ahcne H, Benalia Y, Saad B & Abdelmalek B. Screening for exopolysaccharide-producing strains of thermophilic lactic acid bacteria isolated from Algerian raw camel milk. *African J Microbiol Res*, 8 (2014) 2208.

27 Pintado IE, Barbosa C, Pintado ME, Malcata FX & Gomes MP. Efficient screening and enhanced exopolysaccharide production by functional lactic acid bacteria (LAB) in lactose supplemented media. *Appl Microbiol Theory & Technol,* 5 (2024) 1.

28 Ghatani K, Thapa S, Sha SP, Sarkar S, Modak D & Bhattacharjee S. Revealing probiotic potential of *Enterococcus* strains isolated from traditionally fermented chhurpi and healthy human gut. *Front Microbiol,* 13 (2022) 909987.

29 Sharma A, Lavania M, Singh R & Lal B. Identification and probiotic potential of lactic acid bacteria from camel milk. *Saudi J Biol Sci, 28* (2021) 1622.

30 Sheikh A, Mohamed IHI, Almathen F, Alfattah M & Khalifa A. Beneficial probiotic bacteria prevalence in different lactating dromedary camel milk of Saudi Arabia. *Saudi J Biol Sci,* 31 (2024) 103879.

31 Stack HM, Kearney N, Stanton C, Fitzgerald GF & Ross RP. Association of beta-glucan endogenous production with increased stress tolerance of intestinal Lactobacilli. *Appl Environ Microbiol,* 76 (2010) 500.

32 Nath S, Roy M, Sikidar J, Deb B, Sharma I & Guha A. Characterization and in-vitro screening of probiotic potential of novel *Weissella confusa* strain GCC_19R1 isolated from fermented sour rice. *Curr Res Biotechnol,* 3 (2021) 99.

33 Danchik C & Casadevall A. Role of cell surface hydrophobicity in the pathogenesis of medically-significant fungi. *Front Cell Infect Microbiol*, 25 (2021) 10.

34 Singhal R, Meena KK, Meghwal D, Gupta L, Panwar NL & Meena S. Bacteriocin producing lactic acid bacteria from camel milk and its fermented products: A review. *Ann Phytomedicine*, 12 (2023) 208.

35 Mgomi FC, Yang Y ran, Cheng G & Yang ZQ. Lactic acid bacteria biofilms and their antimicrobial potential against pathogenic microorganisms. *Biofilm,* 5 (2023) 100118.

36 Bryukhanov AL, Klimko AI & Netrusov AI. Antioxidant properties of lactic acid bacteria. *Microbiol,* 91 (2022) 463.

37 Korcz E & Varga L. Exopolysaccharides from lactic acid bacteria: Techno-functional application in the food industry. *Trends Food Sci Technol*, 110 (2021) 375.

38 Duche RT, Singh A, Wandhare AG, Sangwan V, Sihag MK, Nwagu TNT, Panwar H & Ezeogu LI. Antibiotic resistance in potential probiotic lactic acid bacteria of fermented foods and human origin from Nigeria. *BMC Microbiol*, 23 (2023)142.

39 Soares R, Miranda C, Cunha S, Ferreira L, Martins A, Igrejas G & Poeta P. Antibiotic resistance of Enterococcus species in ornamental animal feed. *Animals,* 13 (2023) 1761.

40 Im EJ, Lee HHY, Kim M & Kim MK. Evaluation of enterococcal probiotic usage and review of potential health benefits, safety, and risk of antibiotic-resistant strain emergence. *Antibiotics,* 12 (2023) 1327.