

Article

In Vitro Assessment of Probiotic and Technological Properties of Lactic Acid Bacteria Isolated from Indigenously Fermented Cereal-Based Food Products

Kamalesh Kumar Meena^{1,2,*}, Neetu Kumra Taneja², Devendra Jain³, Ankur Ojha⁴, Dinesh Kumawat¹ and Vijendra Mishra²

- ¹ Department of Dairy and Food Microbiology, Maharana Pratap University of Agriculture and Technology, Udaipur 313001, India
- ² Department of Basic and Applied Sciences, National Institute of Food Technology and Entrepreneurship Management, Sonipat 131028, India
- ³ Department of Molecular Biology and Biotechnology, Maharana Pratap University of Agriculture and Technology, Udaipur 313001, India
- ⁴ Department of Food Science and Technology, National Institute of Food Technology and Entrepreneurship Management, Sonipat 131028, India
- * Correspondence: kamleshjr14@gmail.com



Citation: Meena, K.K.; Taneja, N.K.; 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* **2022**, *8*, 529. <https://doi.org/10.3390/fermentation8100529>

Academic Editors: Farhad Garavand and Eoin Byrne

Received: 11 September 2022

Accepted: 8 October 2022

Published: 11 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: The present study concerns the isolation and characterization of potential probiotic bacteria isolated from indigenously fermented cereal-based products commonly produced by tribal people of the Aravali hills region of India and the documentation of their unexplored probiotic attributes. The isolated strains were evaluated for probiotic attributes, such as bile salt and acid tolerance, lysozyme and phenol tolerance, antagonistic and antifungal activity, cell autoaggregation, cell-surface hydrophobicity, simulated gastric and pancreatic digestion, antioxidative potential, bile salt hydrolase activity, and H₂O₂ production. The safety of isolates was assessed by antibiotic sensitivity, hemolytic activity, DNase activity, and biogenic amine production assays, while technological properties, such as fermenting ability, amylolytic activity, and EPS production, were also evaluated. A total of 70 LAB isolates were screened initially, and 6 strains showed good potential as probiotic candidates in in vitro assessments. The efficient strains were identified using phenotyping and biochemical characterization, which results were further confirmed and recognized at the strain level using phylogenetic analysis and 16S rDNA sequencing. The current study has shown that *Lactiplantibacillus plantarum* KMUDR7 isolated from “Makka ki Raab” has excellent probiotic attributes and could be a potential probiotic for product preparation. However, other strains, *Lactobacillus delbrueckii* subsp. *bulgaricus* KMUDR1 and *Lacticaseibacillus rhamnosus* KMUDR9, showed good properties, while KMUDR14, -17, and -20 also have comparable probiotic attributes.

Keywords: fermented foods; probiotic attributes; lactic acid bacteria; antagonistic activity; antioxidative potential; antibiotic susceptibility; amylolytic activity; fermentation ability

1. Introduction

Lactic acid bacteria are generally considered safe and have been used in food preservation for several centuries. In the fermented dairy and food industries, LAB make a significant contribution and have an economic impact. There are various studies that have focused on the health benefits of LAB in the modern fermented food processing sector [1]. The predominant and most commonly found lactic acid bacterial genera are “*Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Vagococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* 1” [2]. LAB are used to ferment sugar into lactic acid as the major end-product, which further leads to the enhancement of the sensorial, textural, nutritional, and functional attributes of fermented food products [3,4]. Lactobacilli are a major and diverse group among all LAB. The main characteristics of the group are that they are

Gram-positive, do not form endospores, are catalase-negative, and non-pathogenic, the bacteria belonging to the GRAS category of organisms [5]. Lactic acid bacteria have reportedly been isolated from a variety of sources, such as raw biotic matrices (grains, crops, plant surfaces, and silages), fermented food products (fermented milk, cereals, fruits, and vegetables), and gastrointestinal and vaginal tracts [6–9]. The major application of LAB is in food fermentation to further reduce food spoilage and inhibit pathogenic microbes by virtue of their antimicrobial activities, such as bacteriocin production. Furthermore, their immunostimulatory function and benefits as live probiotic microbes have led to an increasing demand for new indigenous strains with wider benefits [10–12]. Each fermented product species provides a distinct environment in terms of competing microorganisms, natural vegetative antagonists, as well as substrate accessibility, type, and concentration of probiotics under various physical factors. Despite these similarities, there are significant differences in substrates and products, as well as the types of microbes used in the production of fermented foods and beverages around the world. Therefore, a number of studies have been conducted to isolate and characterize LAB from diverse and unique natural food matrices [6].

LAB are used as probiotics due to their various beneficial health effects, i.e., suppression of cancer [13], antidiabetic activity [14], allergy prevention [15], cholesterol-lowering effects [16], protection from pathogens [17], immunomodulation [18,19], risk-reduction of diseases [12], and improvement of microflora of the gastrointestinal tract [20]. Currently, most of the LAB strains used as probiotics belong to the *Lactobacillus* and *Bifidobacterium* genera [21] and are used as biocontrol agents due to their mycotoxin-reducing abilities and enhancement of bioavailability [3]. Probiotic microbes are selected on the basis of various criteria, such as technological attributes (cell growth, stability, and viability in raw food substrates), their effects on the sensorial attributes of products, gastrointestinal tract survivability, and functionality (cell autoaggregation, cell-surface hydrophobicity, bacteriocin production, immunomodulation, antimicrobial activity, and safety) [22]. However, in vivo studies and clinical trials are required to confirm such properties.

Several fermented foods and beverages are manufactured throughout the world using cereals, with area-specific local variations in terms of composition and processes of manufacturing [23–26]. Although India has a wide variety of fermented foods, indigenous fermented product preparation and consumption practices are mostly limited to particular locations and specific communities. Traditional fermented food preparation is one of the world's oldest biotechnological processes, in which microorganisms play a crucial role in sensory enhancement, enrichment, health-promoting properties, and food preservation [3,27,28]. *Raabadi* or *Rabadi* is a popular traditionally fermented cereal-based beverage consumed daily in northwestern India, mainly in rural parts of Haryana, Rajasthan, and Punjab [29,30]. It is a very important staple food and provides nutrition and energy to millions of people with low and average incomes in India. A Raabadi-like milk-cereal-based fermented product, "Makka Ki Raab," is traditionally prepared in Southern Rajasthan, India, which is a very popular drink for people of the region, especially tribals. It is prepared by mixing and fermenting maize flours with "khatti chhaschh" or buttermilk, followed by boiling with continuous stirring for 20–30 min and adding salt as per need. Then, the prepared product is cooled and consumed directly or after mixing with buttermilk as a breakfast beverage. *Olia* is a curd-rice-like product prepared occasionally in households using cooked rice and Dahi. Rice cooked the previous night is mixed with dahi and khand (raw sugar) in the morning and consumed as a breakfast meal. A *Jalebi* is a sweet spiral dish widely consumed in the region, prepared using cereal flour, gram flour, and sugar syrup. *Kadhi* is another cereal-based spiced curd sauce that is prepared using gram flour, buttermilk, and spices. It can be consumed with steamed rice or roti, or pulao.

The probiotic attributes of LAB are strain-specific, and strain characteristics vary from one product to another [31]. Cereal-based fermented products are recognized as a good source of probiotic strains of LAB. These products contain high counts of LAB, which may be a potential source for exploring beneficial probiotic strains. Although various

researchers have isolated, screened, and characterized lactic acid bacterial probiotic strains from cereal-based fermented products in different parts of the world [28,32–37], none of these researchers has studied the cereal-based fermented food products of the Udaipur region (tribal-population-dominant part of Southern Rajasthan, India). Therefore, our investigation aimed to describe, isolate, and evaluate the potential probiotic lactic acid bacterial strains from the cereal-based fermented foods of the studied area.

2. Materials and Methods

2.1. Sample Collection

The samples of traditionally fermented cereal-based fermented food products (Makka ki Raab, Olia, Jalebi batter, Raabadi, and Kadhi) were obtained from Udaipur and surrounding districts of Southern Rajasthan, India. The product samples (200 g each) were aseptically collected from the households in sterilized containers and immediately transferred to an ice box. Then, the samples were transported to the laboratory on the same day and kept in a refrigerator (5 ± 1 °C) until further examinations were performed.

2.2. Isolation and Purification of LAB Isolates

A quantity of 25 gm of each collected sample was diluted with 25 mL of sterile phosphate solution and the required serial dilution was prepared. Then, appropriate dilutions (0.1 mL of each sample) were spread-plated on pre-solidified MRS agar plates in duplicate and kept in anaerobic jars at 37 °C for 36–48 h. Thereafter, MRS plates with countable and separate colonies were taken for the random selection of colonies, which were further transferred into 5 mL MRS broth. The cultures were purified by repeated streaking on MRS agar plates and confirmed by Gram staining, catalase testing, and spore staining. The pure cultures were preserved in MRS agar slants and kept in a refrigerator below 5 °C, while stock culture was stored at -20 °C in glycerol for further use [21].

2.3. Evaluation of Probiotic Properties (In Vitro)

2.3.1. Acid Tolerance

The tolerance of isolates towards low-pH or acidic conditions was performed using the protocol used in [33]. The isolated cultures were grown overnight (24 h) in MRS broth by incubation in anaerobic conditions at 37 °C before use. Then, the activated cultures were centrifuged for harvesting of the cells at 5000 rpm at 4 °C for 10 min. After centrifugation, the pellets were subjected to washing in phosphate-buffered saline solution (pH 7.2) and then resuspended in test tubes containing 5 mL sterile MRS broth. The pHs of the test tubes containing active cultures were adjusted to different pH values (2.0, 3.0) by adding 1 N HCL to create gastric-like environments. A control sample was prepared by adjusting the pH of the MRS broth to 6.5. After pH adjustment, the test tubes were incubated at 37 °C for different time intervals (0, 2, and 3 h) and serially diluted with sterile PBS. The appropriate homologous serial dilutions were plated in MRS agar media. The viable LAB counts were enumerated in \log_{10} CFU/mL using the plate count method.

2.3.2. Bile Tolerance

The bile tolerance of LAB isolates was assessed using bile salts, as described in [33]. The pellets of culture isolates were obtained as described above and resuspended in 5 mL MRS broth containing 0.3% (*w/w*) bile salts (Sigma Aldrich, St. Louis, MO, USA). One control sample was prepared without adding bile salts. The samples were incubated at 37 °C and withdrawn after intervals of 0, 2, and 3 h. Samples of 100 μ L were taken on MRS agar plates for viability counts after the performance of appropriate serial dilutions. The cell viability counts were determined and expressed as \log_{10} CFU/mL.

2.3.3. Antagonistic Activity

The antagonistic activity of isolates was determined according to [21], with some modifications. The test organisms in the study were obtained from NCIM, Pune, India.

The most acid-bile-resistant culture isolates were used for further analysis and activated in sterile MRS broth supplemented with 1% glucose. The overnight-grown cells of LAB isolates were subjected to centrifugation at 5000 rpm for 10 min at 4 °C.

The cell-free supernatant (CFS) of each isolate was collected and used for testing antimicrobial activity against different pathogens. The pathogenic indicator organisms were grown overnight in Nutrient Agar (HiMedia, Mumbai, India). A 100 µL volume of each active pathogenic culture was evenly spread on the surface of an already prepared and solidified nutrient agar (NA) plate. The plates were kept for 30–45 min at room temperature for drying. Thereafter, wells were prepared on dried Nutrient Agar plates using a sterile borer (6 mm diameter). A volume of 100 µL CFS of each selected LAB isolate was placed in the wells and incubated at 37 °C for 24–48 h. An inhibitory zone of more than 1 mm was considered a positive zone of inhibition (ZOI) surrounding the wells, and ZOIs were measured with a caliper in millimeters. The test was performed in triplicate for each isolate against each pathogenic strain.

2.3.4. Antifungal Activity

The potential probiotic LAB isolates were tested for antifungal activity by the agar overlay method adopted in [38], with slight modifications. The antifungal activity of the isolates was tested against *Aspergillus brasiliensis* NCIM 1196, *Alternaria solani* MTCC 2101, and *Candida albicans* NCIM 3471. The overnight-grown active cultures were streaked on two separate equidistant spots on the MRS agar plates and incubated at 37 °C for 24 h for antifungal spectrum assay. The culture-spotted MRS agar plates were overlaid with 0.7% soft potato dextrose agar evenly mixed with 20 µL spore suspension of each tested fungal pathogenic species containing 10⁶ spores/mL. The prepared plates were incubated aerobically at 25 ± 2 °C for 2 and 7 days and observed for a clear zone of inhibition around the spotted areas of the LAB colonies.

2.3.5. Resistance to Phenol

Resistance to phenol in the selected cultures was assessed according to the protocol used in [33]. The ability to resist phenol is considered an important evaluation criterion for putative probiotic strains because phenol may inhibit LAB in the gastrointestinal tract. Sterilized MRS broth test tubes supplemented with 0.4% (*v/v*) phenol were prepared. Then, overnight active cultures were inoculated in the test tubes and incubated at 37 °C for 24 hrs. After incubation, cell viability counts were enumerated in MRS agar using the standard plate count method.

2.3.6. Tolerance to Lysozyme

The tolerance of selected LAB cultures to lysozyme was defined according to the method given in [39], with minor modifications. The cultures were grown overnight in MRS broth and centrifuged, and the pellets were resuspended in PBS solution, having been washing twice with the same buffer. A solution was prepared in vitro to mimic in vivo saliva conditions using sterile electrolyte solution (CaCl₂ 0.22 g/L; NaCl 6.2 g/L; KCl 2.2 g/L; NaHCO₃ 1.2 g/L) supplemented with 100 mg/L lysozyme (HiMedia). A 10 µL cell suspension was inoculated in SES solution. A control indicator was also prepared using SES without adding lysozyme. The culture samples were incubated at 37 °C for 2 h. The viable cell counts were determined as percentages of CFU/mL after serial dilutions and plating in MRS agar.

2.3.7. Cell Autoaggregation

The autoaggregation abilities (cell-adhesion properties) of LAB cells in the gut are important properties for maintaining LAB populations in the gut. Cell autoaggregation abilities were measured according to the method of [32], with slight modifications. The overnight-grown cultures of LAB isolates were harvested by centrifugation (11,000 rpm, 4 °C, 10 min). The cell pellets were washed 2 times with PBS and resuspended with the

same buffer (PBS) to adjust absorbance (initial optical density of culture suspension— x) at 0.5 at 600 nm. Then, 5 mL of diluted mixture was placed in a 15 mL falcon tube and incubated at 37 °C for 1 h after gentle vortexing for 10 s. The upper phase (1 mL) of the incubated suspension was removed carefully, and the absorbance (final OD of the upper-layer suspension) was measured at 600 nm. The autoaggregation was recorded and calculated using the following formula:

$$\text{Percentage autoaggregation} = (x - y/x) \times 100 \quad (1)$$

where x represents the initial optical density of the culture suspension and y represents the final OD of the upper-layer suspension after 1 h incubation.

2.3.8. Cell-Surface Hydrophobicity

The cell-surface hydrophobicity of bacterial cells is the ability to adhere to the surface of hydrocarbons, which is an important indicator of the ability of putative probiotic LAB to adhere to the epithelial cells of the gut. The adhesion of LAB isolates to xylene was measured according to [32], with slight modifications. The overnight-grown cell culture was harvested (8000 rpm, 4 °C for 10 min), washed twice with PBS (pH 7.3), and resuspended in sterile 0.1 M KNO₃ (pH 6.2). The absorbance (initial OD— A) of the suspended culture was adjusted to 0.55–0.66 at 600 nm. The cell suspension (3 mL) of the above and a hydrocarbon solvent (xylene, 1 mL) were mixed at a ratio of 3:1 and incubated at 37 °C for 10 min, followed by vortexing for 1 min. The aqueous phase (1 mL) was removed carefully after incubation at 37 °C for 30 min. The absorbance (final OD after incubation— B) of the aqueous phase was enumerated at 600 nm. The percentage hydrophobicity was measured by a decrease in absorbance and calculated using the following formula:

$$\text{Percentage cell-surface hydrophobicity} = (1 - B/A) \times 100 \quad (2)$$

2.3.9. Survivability in Gastric and Pancreatic Juices

The survivability of cultures under gastric and pancreatic conditions was evaluated as per the method adopted in [38], with slight modifications, in gastric juice and pancreatic juice under simulated in vitro conditions. Both simulated juices (gastric and pancreatic) were prepared as described in [40]. The fresh overnight (24 h)-grown isolated samples were centrifuged (6000 rpm, 4 °C, 10 min); the cell pellets were washed with PBS, resuspended with gastric juice, and adjusted to obtain a final absorbance of 1.2 at 500 nm. The culture samples were subjected to peristaltic movement by being kept in an orbital shaker (LABTOP Instrument Pvt Ltd., Palghar, India) at 200 rpm at 37 °C for 3 h. The samples were plated in MRS agar in appropriate serial dilutions after 0 h (immediately after adding gastric juice) and 3 h. Then percentage survivability in the simulated gastric-juice conditions was determined using the following formulae:

$$\text{Percentage survival in gastric juice} = (\log\text{TPGJ}_3/\log\text{TPGJ}_0) \times 100 \quad (3)$$

where $\log\text{TPGJ}_0$ is the log cell count at 0 h and $\log\text{TPGJ}_3$ is the count after 3 h incubation

The culture (after incubation with gastric juice for 3 h) was centrifuged (6000 rpm at 4 °C for 10 min) and the cell pellets were washed in PBS, then mixed with the same volume of pancreatic juice (PJ). The culture samples were incubated in an orbital shaker at 200 rpm for continuous shaking to simulate gut conditions at 37 °C for 24 h. The samples were plated in MRS agar after 0 h (immediately after adding pancreatic juice) and 24 h after appropriate serial dilution to enumerate the simulated pancreatic-digestion survival count. The percentage survivability of the culture samples was calculated as per the following formula:

$$\text{Percentage survival in pancreatic juice} = (\log\text{TPPJ}_{24}/\log\text{TPPJ}_0) \times 100 \quad (4)$$

where $\log\text{TPPJ}_0$ is the cell count at 0 h and $\log\text{TPPJ}_{24}$ is the count after 24 h incubation

2.3.10. Antioxidative Potential of LAB Isolates

The method was based on the ability of LAB cells to scavenge ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radicals and performed as per the (slightly modified) procedure given in [33]. Briefly, fresh, overnight-grown LAB isolates were harvested by centrifugation (5000 rpm, 4 °C, 15 min) and the pellets were washed twice and resuspended in PBS. The working solution was prepared using ABTS (HiMedia) solution (5 mL) with $\text{K}_2\text{S}_2\text{O}_8$ and incubated for 24 h in dark bottles to form ABTS radicals. A quantity of 15 mL of PBS was mixed with 200 μL of the ABTS solution, and absorbance was determined at 750 nm. An aliquot of suspended cell cultures (10 μL) was mixed in the above solution and shaken for 30 s. Then, absorbance was measured after 5 min, and the percentage of inhibition was determined in terms of absorbance.

2.3.11. Hydrogen Peroxide Production

Determination of H_2O_2 production by the LAB isolates was performed as per [33]. The overnight-grown culture was streaked on MRS agar plates supplemented with 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2 mg/L peroxide horseradish (SRL Chemicals). The ABTS and horseradish peroxide were supplemented with sterilized MRS agar (molten condition) before being poured onto Petri plates. The plates were incubated at 37 °C for 2–3 days under anaerobic conditions. The observance of blue halos surrounding the colonies was an indication of the formation of H_2O_2 (positive test).

2.3.12. Bile Salt Hydrolase (BSH) Activity

The BSH activity of cell culture isolates was performed according to the method adopted in [32], with slight modifications. For this, 10 μL samples of the overnight-grown cultures were streaked on a dry surface of MRS agar medium containing CaCl_2 (0.37 g/L) and 0.5% (*w/v*) different bile salts (sodium taurodeoxycholate, sodium taurocholate) (Hi-Media). The plates were incubated at 37 °C for 2–3 days in an anaerobic jar. Positive BSH activity was considered if precipitation zones were observed surrounding the colonies.

2.4. Safety Assessment

2.4.1. Antibiotic Sensitivity Test

The antibiotic sensitivity test for the culture isolates was carried out according to the method adopted in [32], with minor modifications. Soft MRS agar (0.7% *w/v*) was poured onto sterile Petri plates and kept at ambient temperature for solidification. The overnight-grown cells of LAB isolates (100 μL) were spread on MRS agar plates and kept for drying. The different antibiotic disks were put on the dried plates and incubated in a refrigerator (4 °C) for 30 min for smooth and rapid diffusion of antibiotics. These plates were incubated at 37 ± 2 °C for 48 h. The diameters of clear zones surrounding the antibiotic disks were measured with calipers (in mm). The data obtained were interpreted as per “Performance Standards for Antimicrobial Disc Susceptibility Testing”, described by “Clinical and Laboratory Standards Institute” [41] and EUCAST (European Committee on Antimicrobial Susceptibility Testing) [42].

2.4.2. Hemolytic Activity

The hemolytic activity of the putative probiotic isolates was determined by the protocol adopted in [40], with slight modifications, as it is considered an important criterion for the safety assessment of newly isolated strains. Solidified blood agar plates were prepared after proper mixing with sheep blood 5% (*v/v*). Then, overnight-grown cell cultures were streaked on the plates and incubated at 37 ± 2 °C for 2–3 days. The results were assessed based on the formation of zones of hemolysis (α , β , or γ) surrounding the bacterial colonies. The microbial culture of *Staphylococcus aureus* ATCC 2079 was taken as the positive control.

2.4.3. DNase Activity

The deoxyribonuclease (DNase) activity of the LAB isolates was measured by the method adopted in [43], with slight modifications. The isolates were grown overnight in MRS broth, and the cell populations were adjusted to 10^8 CFU/mL. The LAB cultures were streaked on DNase test agar and incubated at 37 °C for 24 h. Clear zones of DNase activity were observed after flooding the plates with 1 N HCl. *Staphylococcus aureus* ATCC 2079 was taken as a positive control to compare the deoxyribonuclease (DNase) activities of the isolates.

2.4.4. Qualitative Evaluation for Biogenic Amine (BA) Formation

The qualitative screening of BA formation was detected using the method of [44], with minor modifications. The overnight-grown cultures were inoculated at 1% *w/v* in 5 test tubes of sterile decarboxylase broth. One test tube was used as a control (without any amino acids), and the remaining four tubes were supplemented with 1% *w/v* of one of the amino acids (L-Lysine, L-Histidine, L-Arginine, and L-Tyrosine; Sigma, Igea Marina, Italy). The final pH levels of the broths in all the test tubes were adjusted to 5.3, and the tubes were incubated at 37 °C after 48 h. The appearance of purple coloring in the broth media corresponding to the control indicated a positive reaction for decarboxylase activity in the tested cultures.

2.5. Technological Properties of the LAB Isolates

2.5.1. Amylolytic Activity

The previously stocked LAB isolates were grown overnight in MRS broth and streaked on modified MRS agar plates (MRS media prepared without glucose but with 0.25% starch). The plates were incubated at 37 °C for 48–72 h. Then, Gram's iodine solution was flooded on plates as a detecting agent. The clear halo zones in the plates were examined, these being indications of α -amylase activity [44].

2.5.2. Screening of Exopolysaccharides

The screening of exopolysaccharide production by LAB isolates was performed as described in [45]. The isolated LAB strains were streaked on plates containing 20 g/L of sugar (glucose, fructose, sucrose, or lactose) and MRS agar, followed by incubation of the plates at 37 °C for 36 to 48 h. The development of slimy colonies by LAB isolates on the sugar-containing plates confirmed the production of exopolysaccharides. "Ropy" characters were also assessed by extending colonies with an inoculation loop.

2.5.3. Milk Fermenting Ability

The six LAB isolates were tested for fermenting ability to produce fermented lactic acid products using skim milk (TS—10% *w/w*) prepared using skim milk powder (Amul Brand, GCMMFL, Anand) and double-distilled water, followed by sterilization in an autoclave (121 °C/15 min) [46]. The LAB isolates inoculated at 1% (*v/v*) had cell counts of about 10^4 CFU/mL in the sterilized skim-milk test tubes. After inoculation, the test tubes were incubated at 37 °C for 24 h. The prepared fermented product samples were stored in a refrigerator for 21 days. The viability of LAB strains was determined at 0, 12, and 24 h during the fermentation process and at 7, 14, and 21 days during storage in refrigerated conditions.

2.6. Phenotypic and Biochemical Characterization

The potential probiotic LAB isolates were identified on the basis of their morphological, physiological, and biochemical characteristics, as recommended in Bergey's Manual of Determinative Bacteriology. Cell morphology was examined by Gram staining, and catalase and endospore tests were also performed. Only Gram-positive, catalase-negative, and non-spore-forming cells were further evaluated and identified. The growth of the selected isolates was checked at different temperatures (4 °C, 10 °C, 37 °C, and 45 °C) and

salt concentrations (4% and 6.5%), while fermentation type was adjudged based on the formation of CO₂ from glucose, as per the method used in [21]. Citrate and carbohydrate utilization tests were performed, as per the methods recommended in Bergey's Manual of Determinative Bacteriology.

2.7. Molecular Identification of Probiotic LAB Isolates Using 16s rDNA Gene Sequencing

The 16S rDNA sequencing technique was used as a molecular tool to identify the isolates. DNA from the six selected isolates was purified using a DNA isolation kit (Hi-media, Mumbai), and the quality of the isolated DNA was assessed on agarose gel. PCR amplification of the 16s rDNA gene fragments of the potential LAB isolates was performed using the universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-CTACGGCTACCTTGTACGA-3'). The amplified samples were outsourced to Mr-Biologist LLP, Pune, India, for 16S rDNA sequencing. The obtained sequences for each strain were examined by comparing them with those of reference bacteria available in the NCBI (National Centre for Biotechnological Information) Genbank database (available at: www.ncbi.nlm.nih.gov, accessed on 10 September 2022), using the BlastN search program and the closest match considered for the species, which showed high levels of homology (>95%). A phylogenetic tree was prepared after pairwise alignment using CLUSTAL W [47], using sequences obtained from NCBI Genbank. Kimura's two-parameter model [48] was used for the construction of a maximum-likelihood tree, which was further tested by bootstrap analysis with 1000 replicates [49] using Mega 10.0 software.

2.8. Statistical Analysis

All the experiments were conducted in triplicate (n = 3), and the results were shown as means ± standard deviations. Statistical significance was determined by one-way and two-way ANOVAs accompanied by Duncan's post hoc test using IBM SPSS Statistics 22 at $p < 0.05$. The graphs were plotted using OriginPro 2018 software.

3. Results

3.1. Acid and Bile Tolerance

A total of 70 LAB isolates were initially screened from different products by morphological and cultural characteristics. Out of 70 isolates, 50 isolates were found to be Gram-positive, catalase-negative, and non-spore-forming, and these were screened and selected under acidic conditions using a fast selective procedure, resulting in four groups. Out of the 50 isolates studied, 23 exhibited poor tolerance to acidic conditions, 13 showed fair tolerance, 8 isolates showed very good tolerance, and 6 isolates showed exceptional tolerance. Furthermore, 14 acid-tolerant LAB isolates were tested for bile salt tolerance after being exposed to acidic conditions, followed by phenol tolerance, antagonistic and antifungal activity, lysozyme resistance, and cell-surface properties. In this study, based on their probiotic properties, 6 efficient isolates among 14 were chosen for further analysis. The current paper majorly focuses on these six isolates, which were found to be efficient for all the tested probiotic criteria. The acid tolerances (pH 2.0 or 3.0) of the candidate probiotic LAB strains were evaluated to determine their abilities to survive in the acidic conditions of the stomach [28,32]. Humans have a digestive fluid known as bile which enables the emulsification and solubilization of lipids and lipid-soluble vitamins in the body for proper digestibility [28]. A higher bile concentration is injurious to the micro-flora and cell membranes of the host. A 0.3% *w/v* bile concentration is critical and sufficient for checking the bile tolerance of strains. The selected cell cultures were temporally evaluated for their viability counts at different pH levels (2.0 and 3.0) at 0, 2, and 3 h (Table 1). The tolerances of the isolates to bile salts at a 0.3% concentration at 0, 2, and 3 h at 37 °C were found to be more than 50% (Table 2). The LAB isolates KMUDR7 and KMUDR1 showed the highest acid and bile tolerances.

Table 1. Acid tolerance of LAB strains (viability of cells after exposure—log CFU/mL).

Isolate	pH (2.0)			pH (3.0)		
	0 h	2 h	3 h	0 h	2 h	3 h
KMUDR1	9.35 ± 0.03 ^{dC} *	9.07 ± 0.04 ^{cB}	8.93 ± 0.04 ^{cA}	9.53 ± 0.11 ^{cD}	9.24 ± 0.09 ^{cE}	9.1 ± 0.02 ^{aF}
KMUDR7	9.41 ± 0.02 ^{eC}	9.33 ± 0.01 ^{dB}	8.99 ± 0.03 ^{dA}	9.62 ± 0.06 ^{cD}	9.33 ± 0.07 ^{aE}	9.36 ± 0.03 ^{aF}
KMUDR9	9.22 ± 0.03 ^{cC}	8.8 ± 0.03 ^{bB}	8.63 ± 0.03 ^{bA}	9.38 ± 0.04 ^{bD}	8.87 ± 0.05 ^{bcE}	8.82 ± 0.11 ^{aF}
KMUDR14	9.15 ± 0.03 ^{abC}	8.79 ± 0.08 ^{bB}	8.6 ± 0.03 ^{bA}	9.27 ± 0.06 ^{abD}	8.84 ± 0.06 ^{bE}	8.67 ± 0.13 ^{bF}
KMUDR17	9.12 ± 0.04 ^{aC}	8.72 ± 0.08 ^{bB}	8.55 ± 0.04 ^{aA}	9.23 ± 0.05 ^{aD}	8.72 ± 0.1 ^{cE}	8.62 ± 0.08 ^{cF}
KMUDR20	9.19 ± 0.02 ^{bcC}	8.67 ± 0.04 ^{aB}	8.5 ± 0.03 ^{aA}	9.17 ± 0.05 ^{aD}	8.88 ± 0.08 ^{cE}	8.66 ± 0.05 ^{dF}

* Values are means ± SDs (n = 3 for each tested sample); values with different letters (superscripts—a, b, c, d, e) in lower case differ significantly (p < 0.05) row-wise, whereas values with different letters (superscripts—A, B, C, D, E, F) in upper case differ significantly (p < 0.05) column-wise.

Table 2. Bile salt tolerance (0.3% salt concentration) of LAB strains (viability of cells after exposure—log CFU/mL).

Isolate	0 h	2 h	3 h
KMUDR1	7.32 ± 0.07 ^{cC} *	6.41 ± 0.04 ^{eB}	6.13 ± 0.06 ^{fA}
KMUDR7	7.88 ± 0.04 ^{dC}	6.68 ± 0.13 ^{fB}	6.28 ± 0.04 ^{eA}
KMUDR9	7.28 ± 0.04 ^{cC}	6.08 ± 0.05 ^{dB}	5.93 ± 0.05 ^{dA}
KMUDR14	7.23 ± 0.05 ^{bcC}	5.94 ± 0.04 ^{cB}	5.38 ± 0.04 ^{cA}
KMUDR17	7.05 ± 0.03 ^{aC}	5.74 ± 0.03 ^{bB}	5.27 ± 0.06 ^{bA}
KMUDR20	7.16 ± 0.07 ^{bcC}	5.3 ± 0.06 ^{aC}	5.05 ± 0.04 ^{aA}

* Values are mean ± SDs (n = 3 for each tested sample); values with different letters (superscripts—a, b, c, d, e, f) in lower case differ significantly (p < 0.05) row-wise, whereas values with different letters (superscripts—A, B, C) in capital case differ significantly (p < 0.05) column-wise.

3.2. Antagonistic and Antifungal Activity

A typical potential probiotic LAB isolate needs to exert antimicrobial activity against several pathogenic microbes by producing antimicrobial compounds, such as bacteriocin, organic acids (lactic acid, acetic acid, propionic acid), H₂O₂, diacetyl, and surfactants [50,51]. The LAB isolates were evaluated for their antagonistic activities against common food-borne pathogens. The zones of inhibition of the cell-free supernatants of different isolates were found to be in the range of 0 to 24 mm. The isolate KMUDR7 showed the largest diameters in its zones of inhibition against *Staphylococcus aureus* NCIM 5345 (23.33 ± 1.15 mm), *Staph. epidermis* NCIM 2493 (21.33 ± 1.53 mm), and other tested pathogens, while KMUDR1 showed a good ZOI (18 ± 1 mm) against *Staph. epidermis* NCIM 2493 (Table 3).

Table 3. Antibacterial activities (diameters, in mm, of inhibition zones) of the LAB strains.

Isolate	Tested Pathogens					
	<i>Staphylococcus aureus</i> NCIM-5345	<i>Staph epidermis</i> NCIM 2493	<i>Bacillus subtilis</i> NCIM 2063	<i>E. coli</i> NCIM 2065	<i>Enterococcus aerogenes</i> NCIM 5139	<i>Pseudomonas auruginosa</i> NCIM 3471
KMUDR1	15 ± 1 ^b *	18 ± 1 ^b	14.67 ± 0.58 ^c	8.67 ± 0.58 ^b	7.67 ± 0.58 ^b	11 ± 1 ^c
KMUDR7	23.33 ± 1.15 ^c	21.33 ± 1.53 ^c	17 ± 1 ^d	10.67 ± 0.58 ^c	9 ± 1 ^d	13.33 ± 1.53 ^d
KMUDR9	15.67 ± 1.15 ^b	17 ± 1 ^b	11.33 ± 0.58 ^b	0 ± 0 ^a	0 ± 0 ^a	9 ± 1 ^b
KMUDR14	15 ± 1 ^b	13.33 ± 1.53 ^a	10.67 ± 1.15 ^b	0 ± 0 ^a	7.67 ± 0.58 ^b	0 ± 0 ^a
KMUDR17	17 ± 1 ^b	13.33 ± 1.53 ^a	11.67 ± 1.15 ^b	8.33 ± 0.58 ^b	8 ± 1 ^b	10 ± 1 ^{bc}
KMUDR20	12.33 ± 1.15 ^a	12.33 ± 1.53 ^a	9.67 ± 0.58 ^a	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a

* Values (a, b, c, and d) are means ± SDs (n = 3 for each tested sample); values with different letters differ significantly (p < 0.05) along the rows.

The LAB isolate KMUDR7 showed the largest zones of inhibition after 2 days against *Aspergillus brasiliensis* NCIM 1196 (22.67 ± 1.25), *Alternaria solani* MTCC 2101 (22.33 ± 1.25), and *Candida albicans* NCIM 3471 (21.33 ± 2.49). The isolate KMUDR14 showed no inhibitory activity against *Candida albicans* after 2 and 7 days, whereas KMUDR17 did not show a zone of inhibition against *Aspergillus brasiliensis* after 7 days. The other isolates were observed to have varying degrees of antifungal activities, as presented in Table 4.

Table 4. Antifungal activities (diameters, in mm, of inhibition zones) of the LAB strains.

Isolate	Tested Fungal Strain					
	<i>Aspergillus brasiliensis</i> NCIM 1196		<i>Alternaria solani</i> MTCC 2101		<i>Candida albicans</i> NCIM 3471	
	2 Days Incubation	7 Days Incubation	2 Days Incubation	7 Days Incubation	2 Days Incubation	7 Days Incubation
KMUDR1	$18.67 \pm 1.7^d *$	16.33 ± 1.25^d	16.17 ± 0.85^d	13.5 ± 1.08^e	14 ± 0.82^c	11.33 ± 0.47^c
KMUDR7	22.67 ± 1.25^e	20.67 ± 1.7^e	22.33 ± 1.25^e	18.17 ± 1.03^d	21.33 ± 2.49^d	18.67 ± 2.05^d
KMUDR9	15.33 ± 0.94^c	11.33 ± 0.94^c	13.17 ± 0.62^{bc}	12.5 ± 1.08^{cd}	14 ± 0.82^c	13 ± 0.82^c
KMUDR14	8.33 ± 0.47^a	7.33 ± 0.47^b	15.17 ± 0.85^{cd}	10.83 ± 0.62^{bc}	0 ± 0^a	0 ± 0^a
KMUDR17	9.33 ± 1.25^{ab}	0 ± 0^a	11.33 ± 0.62^{ab}	10.5 ± 0.41^b	12.67 ± 1.7^c	10.67 ± 1.25^{bc}
KMUDR20	11 ± 0.82^b	9 ± 0.82^b	10 ± 1.78^a	0 ± 0^a	9.33 ± 1.25^b	8.33 ± 1.25^b

* Values are means \pm SDs (n = 3 for each tested sample); values with different letters (a, b, c, d, e) differ significantly ($p < 0.05$) along the rows.

3.3. Resistance to Phenol

The phenol tolerance of all six isolates was evaluated using a 0.4% concentration of phenol for 24 h at 37 °C. The isolates KMUDR1, KMUDR7, and KMUDR17 were not inhibited by the phenol concentration, but the other three isolates (i.e., KMUDR9, KMUDR14, and KMUDR20) were found to be sensitive. The viability counts for all isolates after incubation (24 h) were found to be in the range of 7.56 to 8.97 log CFU/mL. The isolates KMUDR7 and KMUDR17 were significantly ($p < 0.05$) increased during incubation (Figure 1).

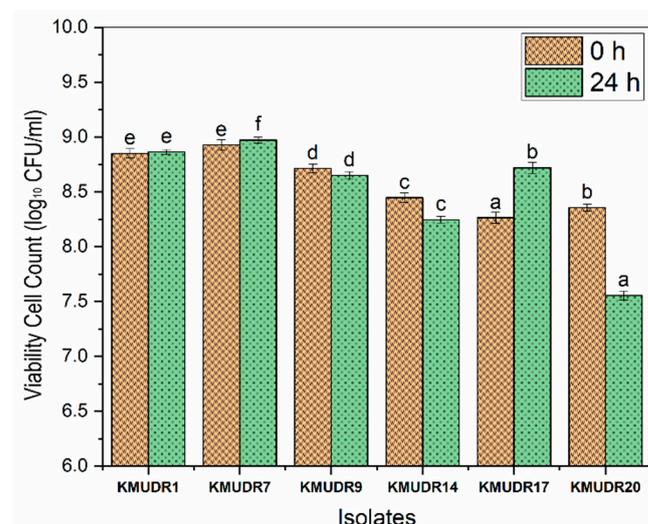


Figure 1. Survival of the tested LAB isolates using a 0.4% phenol concentration. Different letters above the bars denote statistically significant differences.

3.4. Lysozyme Tolerance and Cell-Surface Properties

All the tested LAB isolates showed more than 53% survival against lysozyme exposure for 2 h, whereas significantly ($p < 0.05$) high survivability was observed for KMUDR7 ($96.75 \pm 1.96\%$) in comparison to the other isolates.

The cell-surface hydrophobicity of the bacterial cells was evaluated using hydrocarbon xylene to check their abilities to adhere to the surfaces of hydrocarbons. The tested isolates showed good and varied surface hydrophobicities, which were found to be in the range of 55.93 ± 2.69 to $86.77 \pm 2.98\%$. The isolate KMUDR7 ($86.77 \pm 2.98\%$) showed the highest hydrophobicity, followed by KMUDR1 ($75.3 \pm 2.76\%$), whereas the lowest was shown by KMUDR17 ($55.93 \pm 2.69\%$). The isolate KMUDR7 had significantly ($p < 0.05$) higher cell-surface hydrophobicity compared to the other tested isolates. Cell autoaggregation was evaluated to check the abilities of LAB cells to form clumps due to the interaction of different cell-surface substances (e.g., proteins, peptidoglycans, and lipoteichoic acid). All the tested LAB isolates showed cell autoaggregation in the range of 50.29 ± 1.79 to $82.47 \pm 2.58\%$. Further, there was significantly ($p < 0.05$) higher cell autoaggregation recorded for the isolates KMUDR7 ($82.47 \pm 2.58\%$) and KMUDR1 ($72.37 \pm 2.45\%$) in comparison to the other isolates (Figure 2).

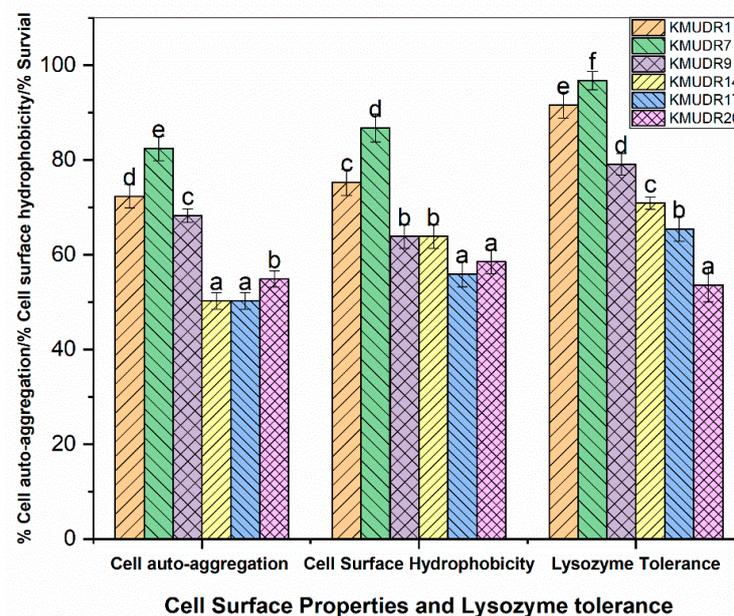


Figure 2. Cell-surface properties (cell autoaggregation and cell-surface hydrophobicity) and lysozyme tolerance. Different letters above the bars denote statistically significant differences ($p < 0.05$).

3.5. Survivability in Gastric and Pancreatic Juices

The survival of LAB isolates under simulated gastric-juice digestion with digestive enzymes is depicted in Figure 3. The isolate KMUDR7 showed the highest survival ($85.54 \pm 1.01\%$) in *in vitro* conditions after 3 h incubation, while the other isolates showed more than 50% survival, except for KMUDR20 ($48.58 \pm 1.73\%$). KMUDR7 had a significantly higher ($p < 0.05$) survival in comparison with the other isolates. The isolates were also evaluated in simulated pancreatic-digestion environments, and their viability percentages were estimated after 24 h incubation. All the tested isolates showed survivability, which was an indication of their ability to colonize in the intestine (Figure 3). A significantly higher ($p < 0.05$) viability was recorded for KMUDR7 ($78.71 \pm 1.59\%$) in comparison to the other LAB isolates. The lowest survival was observed for KMUDR17 ($42.91 \pm 2.85\%$).

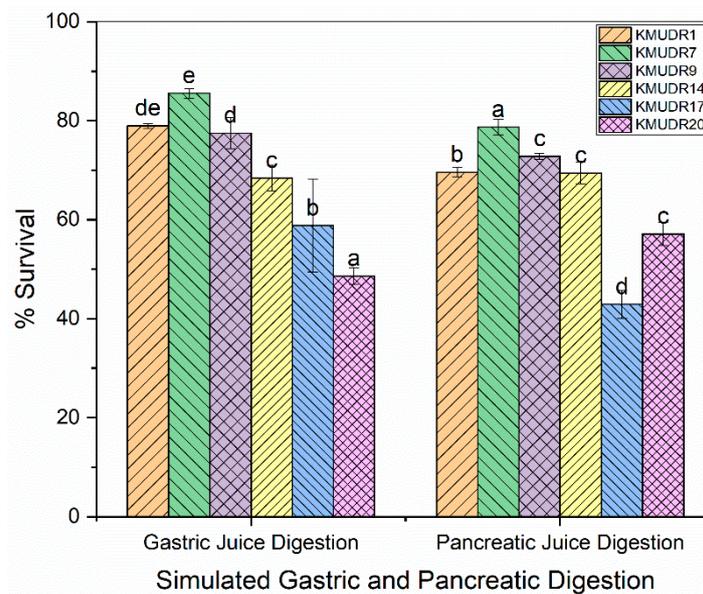


Figure 3. Simulated gastric- and pancreatic-juice digestion survival (values means \pm SDs). Different letters above the bars denote statistically significant differences ($p < 0.05$).

3.6. Antioxidative Potential

The antioxidative ability of LAB isolates was determined using ABTS, and the results are depicted in Figure 4. All the isolates were observed to have antioxidative potentials ranging from $15.04 \pm 1.42\%$ to $75.11 \pm 1.9\%$. The isolate KMUDR7 ($75.11 \pm 1.9\%$) was recorded as having a significantly higher ($p < 0.05$) inhibition, followed by KMUDR1 ($69.59 \pm 1.48\%$) in comparison to the others.

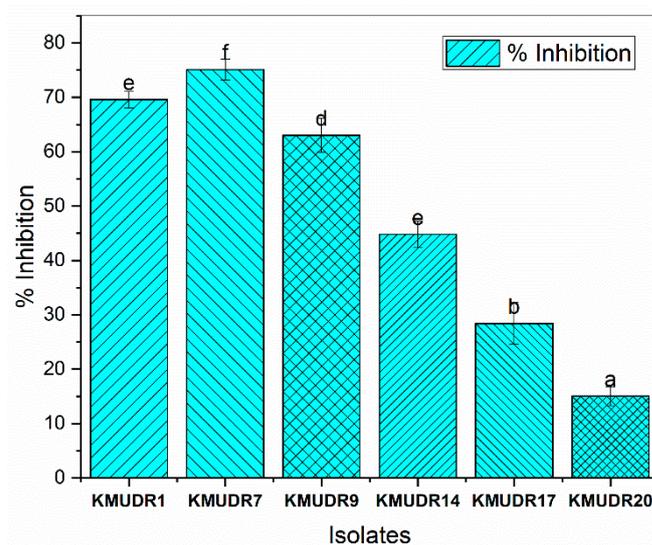


Figure 4. Percentage inhibition of ABTS radicals by LAB isolates (values means \pm SDs). Different letters above the bars denote statistically significant differences ($p < 0.05$).

3.7. H₂O₂ Production and BSH Activity

H₂O₂ exhibits bactericidal activity against microbial cells, thus enhancing the shelf life and food-safety of products. The production of H₂O₂ by different isolates was assessed by assessing the growth of cultures on MRS media plates containing ABTS. The intensity of color was used as a parameter for evaluating the level of H₂O₂ production. The highest H₂O₂ production was observed for KMUDR7, followed by KMUDR1. There were no blue

color halos found in the plates for the isolates KMUDR14, KMUDR17, and KMUDR20, which was an indication of no production of H₂O₂.

The BSH activity of the LAB isolates was assessed by exposing them to a high concentration of bile salts. BSH-positive strains hydrolyze and deconjugate bile salts, which is a desirable property for a probiotic strain with respect to surviving toxicity and colonizing in the intestine. The MRS plates were examined for the formation of a zone of precipitation surrounding the isolates showing positive BSH activity (Table 5). All the tested isolates were observed to have positive BSH activity, except KMUDR17, while the largest zone of precipitation was recorded for KMUDR7.

Table 5. Amylolytic and BSH activities as well as H₂O₂ production of the LAB strains.

Isolate	α-Amylase Activity	BSH Activity		H ₂ O ₂ Production
		Sodium Taurodeoxycholate	Sodium Taurocholate	
KMUDR1	+ ^a	+ ^b	++ ^b	+ ^c
KMUDR7	+ ^a	++ ^b	++ ^b	++ ^c
KMUDR9	+ ^a	+ ^b	+ ^b	- ^c
KMUDR14	- ^a	+ ^b	+ ^b	- ^c
KMUDR17	- ^a	- ^b	- ^b	- ^c
KMUDR20	- ^a	+ ^b	+ ^b	- ^c

-^a No zones surrounding colonies, +^a clear zones surrounding colonies (diameter of zone < 3 mm), -^b no precipitation zones surrounding colonies, +^b slight precipitation zones surrounding colonies, ++^b intense precipitation zones surrounding colonies, -^c no blue halo surrounding colonies, +^c slight blue halo surrounding colonies, ++^c intense blue halo surrounding colonies.

3.8. Safety Assessment

3.8.1. Antibiotic Sensitivity Test

The antibiotic sensitivity test was performed for the selected six LAB isolates against different antibiotics, as per EFSA guidelines. All the tested LAB isolates showed resistant phenotypes as per the breakpoint scale (ZOI ≤ 14) against tetracycline and ciprofloxacin. The isolate KMUDR7 showed resistance to all the tested antibiotics, except Chloramphenicol and Erythromycin. The isolates KMUDR17 and -20 were observed to have medium sensitivity (15–19 mm) and non-resistance (sensitive ≥ 20 mm) against most antibiotics. There was variable sensitivity observed against different antibiotics, which results are presented in Table 6.

Table 6. Antibiotic susceptibilities (diameters, in mm, of inhibition zones) of the isolated LAB strains.

Antibiotics	Concentration (µg/disc)	Isolates					
		KMUDR1	KMUDR7	KMUDR9	KMUDR14	KMUDR17	KMUDR20
Gentamicin	(10 µg/disc)	MS*	R	S	MS	MS	S
Penicillin G	(10 µg/disc)	S	R	S	MS	MS	MS
Chloramphenicol	(30 µg/disc)	S	MS	S	R	MS	MS
Vancomycin	(15 µg/disc)	R	R	S	S	S	MS
Kanamycin	(30 µg/disc)	R	R	MS	S	MS	MS
Streptomycin	(30 µg/disc)	MS	R	MS	S	MS	MS
Tetracycline	(30 µg/disc)	R	R	R	R	R	R
Erythromycin	(15 µg/disc)	S	MS	MS	R	S	MS
Ciprofloxacin	(5 µg/disc)	R	R	R	R	R	R
Cefadroxil	(30 µg/disc)	R	R	S	S	MS	S

* R—resistant (zones ≤ 14 mm), MS—medium-sensitive (15–19 mm), S—sensitive (≥ 20 mm).

3.8.2. Hemolytic Activity, DNase Activity, and Biogenic Amine Production

Probiotic bacteria are considered non-pathogenic; therefore, they should not exhibit any zone of hemolysis (i.e., be non-hemolytic) or DNase activity. There was no zone of

hemolysis observed for any of the LAB isolates, which was an indication of γ -hemolytic activity. In this study, none of the tested isolates showed DNase activity during the experiment. The screening of biogenic amine production is also one of the important safety criteria for evaluating the safety of putative probiotic LAB isolates. All the tested LAB isolates were found to be negative for biogenic amine production, as none of them changed the color of decarboxylase broth to purple. Therefore, all tested LAB isolates were considered non-pathogenic and safe for human consumption.

3.9. Technological Properties

3.9.1. Amylolytic Activity

Amylolytic activity is a desirable property for LAB intended for use as starter cultures in the preparation of fermented foods. The isolates KMUDR1, KMUDR7, and KMUDR9 showed weak amylolytic activity, whereas no activity (no zones surrounding the colonies) was observed for the other tested strains.

3.9.2. EPS Production

EPS production is a desirable property for starter cultures and probiotic strains due to its ability to improve the body and texture, firmness, consistency, and mouthfeel of foods. EPS production was identified by the development of slimy colonies on sucrose-containing agar media. All the LAB isolates were able to form characteristic slimy colonies and were considered EPS-producing isolates. The isolates KMUDR1 and KMUDR7 showed long, stretchy filaments when stretched with loops, and this EPS production may further develop during product development.

3.9.3. Milk Fermenting Ability

All selected LAB isolates were tested for their ability to survive during fermentation (24 h) and storage (21 days) in refrigerated conditions, and the data are presented in Table 7. All the isolates showed significant growth ($p < 0.05$) during fermentation ranging from 4.17 ± 0.05 to 8.94 ± 0.04 log CFU/mL. Among all the isolates, KMUDR7 (8.94 ± 0.04 log CFU/mL), followed by KMUDR1 (8.81 ± 0.04 log CFU/mL), showed higher significant growth compared to the other tested LAB isolates. During storage under refrigeration (4 °C), all isolates decreased significantly, except KMUDR1 and KMUDR7, which showed significant increases at 14 days as compared to the viability counts at 7 days. The isolate KMUDR7 (4.82 ± 0.06 log CFU/mL) was recorded as having the highest viability count after 21 days, followed by KMUDR1, whereas the minimum count was recorded for KMUDR17 (2.68 ± 0.19 log CFU/mL).

Table 7. Cell viabilities (log₁₀ CFU/mL) of LAB strains during skim-milk fermentation (24 h at 37 °C) and storage (21 days at 4 °C).

Isolate	Viability Count during Fermentation			Viability Count during Storage		
	0 h	12 h	24 h	7 d	14 d	21 d
KMUDR1	4.81 ± 0.09 ^{dA} *	7.28 ± 0.07 ^{eB}	8.81 ± 0.04 ^{eC}	7.04 ± 0.08 ^{dD}	7.34 ± 0.11 ^{dE}	4.28 ± 0.16 ^{dF}
KMUDR7	5.1 ± 0.03 ^{eA}	7.79 ± 0.1 ^{fB}	8.94 ± 0.04 ^{eC}	7.74 ± 0.07 ^{eD}	7.99 ± 0.1 ^{eE}	4.82 ± 0.06 ^{eF}
KMUDR9	4.67 ± 0.08 ^{cA}	7.1 ± 0.05 ^{dB}	8.55 ± 0.09 ^{dC}	6.9 ± 0.05 ^{cdD}	5.04 ± 0.08 ^{cE}	3.56 ± 0.11 ^{cF}
KMUDR14	4.37 ± 0.1 ^{bA}	6.81 ± 0.06 ^{cB}	8.16 ± 0.12 ^{cC}	6.72 ± 0.05 ^{cD}	4.84 ± 0.12 ^{bE}	2.78 ± 0.18 ^{abF}
KMUDR17	4.29 ± 0.07 ^{abA}	8.72 ± 0.08 ^{bB}	7.87 ± 0.12 ^{bC}	6.49 ± 0.25 ^{bD}	4.65 ± 0.11 ^{aE}	2.68 ± 0.19 ^{aF}
KMUDR20	4.17 ± 0.05 ^{aA}	5.91 ± 0.07 ^{aB}	7.3 ± 0.1 ^{aC}	5.9 ± 0.08 ^{aD}	4.57 ± 0.12 ^{aE}	2.96 ± 0.06 ^{bF}

* Values are mean \pm SDs (n = 3 for each tested sample); values with different letters (a, b, c, d, e, f) differ significantly ($p < 0.05$) along the rows, whereas different letters (A, B, C, D, E, F) denote significant differences down the columns.

3.10. Phenotyping and Biochemical and Molecular Characterizations

The six potent LAB isolates possessing efficient probiotic attributes were subjected to various phenotyping and biochemical tests and were found to be Gram-positive, catalase-

negative, and non-spore-forming. Genus-level identifications were performed on the basis of biochemical tests, according to Bergey’s Manual of Bacteriology. These isolates were further confirmed and identified by partial 16S rDNA sequencing, and the phylogenetic positions of the LAB species are shown in Figure 5. The isolate KMUDR7, identified as *Lactiplantibacillus plantarum*, showed 96.51% similarity to the closest match for the reference sequence in NCBI Genbank and was found to be the best among all the tested LAB isolates, with excellent probiotic and technological attributes. The identification and accession numbers of all the tested isolates are presented in Table 8. The other LAB isolates were identified as *Lactobacillus delbrueckii* subsp. *bulgaricus* (KMUDR1), *Lacticaseibacillus rhamnosus* (KMUDR14), *Lactobacillus helveticus* (KMUDR17), *Limosilactobacillus fermentum* (KMUDR17), and *Limosilactobacillus reuteri* (KMUDR20).

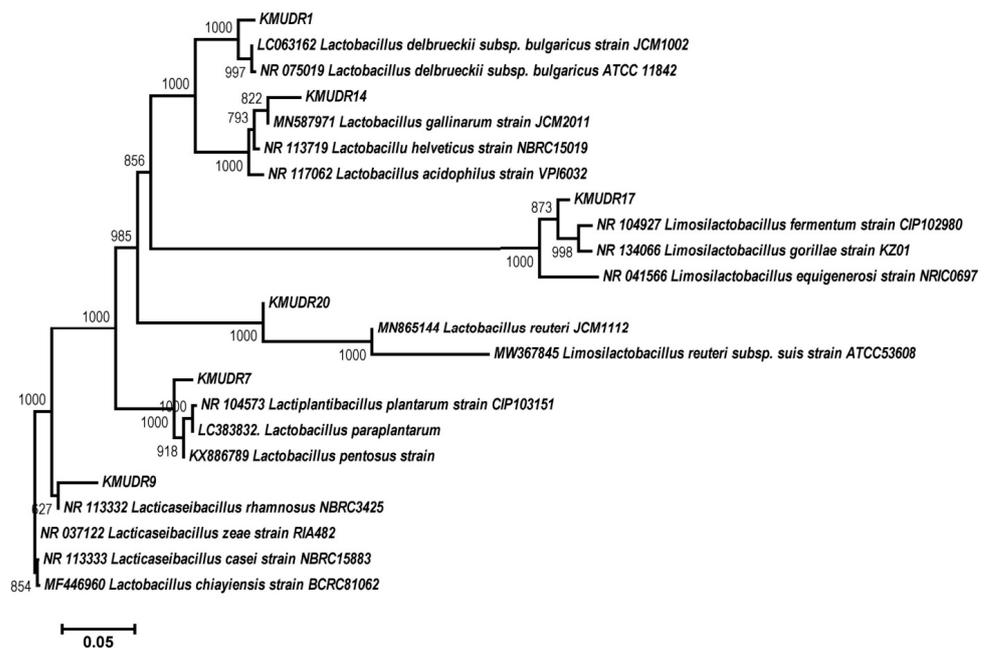


Figure 5. Phylogenetic analysis of the potent LAB strains based on partial 16S rDNA sequencing.

Table 8. 16s rDNA identification of strains.

Strain Designation	Strain Organism	Type of Cereal-Based Fermented Food	Closest Homolog	Similarity (%)	GenBank Accession Number (NCBI)
KMUDR1	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	Raabadi	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842 = JCM 1002	99.87	ON954521
KMUDR7	<i>Lactiplantibacillus plantarum</i>	Makka Ki Raab	<i>Lactiplantibacillus plantarum</i> strain CIP 103151	96.51	ON954522
KMUDR9	<i>Lacticaseibacillus rhamnosus</i>	Raabadi	<i>Lacticaseibacillus rhamnosus</i> strain NBRC 3425	99.49	ON954523
KMUDR14	<i>Lactobacillus helveticus</i>	Oliya	<i>Lactobacillus helveticus</i> strain NBRC 15019	97.22	ON954524
KMUDR17	<i>Limosilactobacillus fermentum</i>	Makka ki Raab	<i>Limosilactobacillus fermentum</i> strain CIP 102980	98.77	ON954525
KMUDR20	<i>Limosilactobacillus reuteri</i>	Jalebi batter	<i>Limosilactobacillus reuteri</i> strain NBRC 15892	98.63	ON954526

4. Discussion

The acid tolerances (pH 2.0 or 3.0) of the candidate probiotic LAB strains were evaluated to determine their abilities to survive in the acidic conditions of the stomach [28,32]. Ahire et al. 2021 reported that *Lactobacillus plantarum* UBLP40 had 73% cell viability at a low pH (2.0) when incubated for 3 h [32]. *Weissella confusa* strain GCC_19R1, *Lactobacillus plantarum* RPY1, *L. helveticus* K14, *Lactobacillus plantarum* KJ722784, *Lb. plantarum* DM5, *Lb. plantarum* LD1, *Lb. plantarum* K90, and *Lb. fermentum* K75 strains, isolated from different traditionally fermented cereal-based products, also showed good cell viability at low pHs [28,33,52–55]. Humans have a digestive fluid known as bile which enables the emulsification and solubilization of lipids and lipid-soluble vitamins in the body for proper digestibility [28,56]. A higher bile concentration is injurious to the microflora and cell membranes of the host. *Lactobacillus plantarum* UBLP40, isolated from traditional Indian fermented products, was exposed to bile (0.3% *w/v* concentration) and cell viability was checked [32]. Ref. [57] demonstrated that *Lactobacillus plantarum* strain growth decreased with increasing concentrations of bile, which might be due to the detrimental effects of bile. Furthermore, bile tolerance of potential probiotic strains is considered critical for bacterial survival in the gastrointestinal (GI) tract. In this study, at low pHs (2.0 and 3.0), decreasing trends were observed for viability counts with increased exposure times for all isolates. The survivability of isolates was also found to decrease with bile salt concentration (0.3%) up to 3 h exposure. However, the isolates KMUDR7 and KMUDR1 showed minimum decreases in cell counts during the studied period (0 to 3 h). The resistance of many lactic acid bacterial cells might be due to their ability to develop mechanisms to survive in acidic conditions, including stimulation of H⁺-ATPases, alteration of cell envelopes, ingestion of protons inside cells, and the formation of alkalis [58]. A typical potential probiotic LAB isolate needs to exert antimicrobial activity against several pathogenic microbes by producing antimicrobial compounds, such as bacteriocin, organic acids (lactic acid, acetic acid, propionic acid), H₂O₂, diacetyl, and surfactants [50,51]. Some of the bacteria produce biologically active proteinaceous substances with antimicrobial attributes that are known as bacteriocins. The majority of bacteriocins are effective against Gram-positive bacteria [59], but some of them also exhibit antimicrobial activity against other spoilage-causing microflora, as well as pathogens [60]. Recently, [32] reported the inhibitory potential of *Lactobacillus plantarum* UBLP40 against various food pathogens, viz., *Micrococcus luteus*, *Staphylococcus aureus* subsp. *aureus*, *Pseudomonas aeruginosa*, and *E. coli*. Some other recent reports are also available regarding the antimicrobial potentials of isolated LAB strains from products of Indian origin [28,33,54,55,61]. Some strains, such as *Lactobacillus plantarum* (BBC32A, BBC33, BIF43) [40], *L. helveticus* K14 [54], and *Lactobacillus plantarum* KJ722784 [62], from other cereal-based products have also shown good colonization properties in *in vivo* tests. Antimicrobial activity is essential for the prevention of pathogens by competitive expulsion in the gut. KMUDR7 showed the highest antifungal activity among all the tested LAB isolates against fungi. Potential probiotic isolates bind to the GI tract and avoid the colonization of fungal species by competitive expulsion and by producing antifungal metabolites [63]. It is important for a probiotic strain used as a starter culture to provide beneficial effects to the host and extend the shelf life of food [64]. Recently, various LAB strains isolated from different food matrices were observed with antifungal activities against mycotoxigenic fungi, and efficient strains were identified as *Lb. pentosus* and *Lb. sanfranciscensis* [65]. Another study also reported the isolation of the efficient antifungal isolate MYSN 106 from Neera samples [38].

Further, phenol tolerance at a 0.4% concentration is considered an important attribute for a potential probiotic strain. Phenol is produced in the gastrointestinal tract as a result of deamination of some amino acids and is considered a toxic metabolite for microbial growth. In this study, KMUDR1, -7, and -17 showed phenolic tolerance; however, other strains showed decreasing trends after 24 h exposure to phenol. Previously, other researchers also reported phenol tolerance at 0.4% for the *Lb. plantarum* RYPR1 and RYPR9 [33] and variable tolerance for different LAB isolates [38,66]. Lysozyme tolerance *in vitro* is de-

terminated to assess the survivability of strains under stressful conditions of lysozyme exposure, as lysozyme is present in saliva. Selected lactobacilli strains showed high tolerance to lysozyme even after 3 h exposure to lysozyme, with survivabilities of more than 69% [46]. Other researchers also reported varying degrees of resistance for LAB isolated from fermented foods, with maximum survival values of up to 96.69% [33,62]. In our study, KMUDR7 was observed to have the maximum survivability ($96.75 \pm 1.96\%$), whereas others showed $>53\%$ survivabilities after 60 min exposure, which is in agreement with other studies [33,62].

The adhesion properties of bacteria with respect to solvents are used to estimate their abilities to colonize on the gastrointestinal wall. According to [32], a strain (*Lactobacillus plantarum* UBLP40) isolated from fermented foods showed good adhesion to xylene (higher cell-surface hydrophobicity), which was an indication of its good adhesion ability for colonization in the intestine. The adhesion ability of the potential strain to xylene at 30 min was higher in comparison to four *Lb. plantarum* strains [40]. In the current study, KMUDR7 showed the highest cell-surface hydrophobicity (CSH), while the other isolates were observed to have CSHs $>55\%$. Similar trends were also reported for isolated strains, such as *Lb. plantarum* RPY1 [33], *Lb. helveticus* K14 [54], and *Lb. plantarum* KJ722784 [62], from different traditionally fermented cereal-based food products. A potential probiotic strain should have the ability to colonize in the GIT and attach to the intestinal epithelium [28]. According to [28], the candidate probiotic strains had varied autoaggregation activities ranging from 16 to 50%. This property helps to prevent the colonization and adhesion of pathogenic microbes. Recently, [28] evaluated the cell autoaggregation properties of the *Weissella confusa* strain GCC_19R1 isolated from sour rice, and the activity was observed to be 38.7%. In the present study, variable degrees of cell autoaggregation (54.89 to 82.47%) were observed for all the tested LAB isolates. The results were in agreement with those for other strains, such as *Lactobacillus plantarum* UBLP40 isolated from idli batter [32] and *Lb. plantarum* RPY1 isolated from Raabadi [33], and strains such as *Lactobacillus plantarum* (BBC32A, BBC33, BIF43) [40], *Lb. helveticus* K14 [54], and *Lb. plantarum* KJ722784 [62] isolated from other cereal-based products also showed good colonization properties in vivo tests.

The simulated gastric- and pancreatic-digestion experiments were performed to check the survival of LAB isolates in the adverse environment of the gastrointestinal tract. All the tested LAB isolates in the present study showed more than 45% survival, which is in accordance with results for previously reported strains of *Lactobacilli* of cereal-based food origin [32,33,40,62]. Various reports support the claim that *Lactobacilli* isolated from cereal-based foods are less resistant to pancreatic juice than gastric juice [32]. The isolate KMUDR7 showed good survivability ($85.54 \pm 1.01\%$) and can be considered a potential probiotic candidate. Using the ABTS method, the relative abilities of LAB isolates to scavenge ABTS were observed to check their antioxidative potentials. The isolates KMUDR1, -7, and -9 showed more than 60% inhibition of ABTS radicals, which indicates their ability to produce radical cations for antioxidant suppression. The antioxidative potential of food-borne probiotic bacteria is supported by various mechanisms, such as the formation of antioxidative compounds (i.e., folate, butyrate, and glutathione), stimulation of antioxidative host genes, suppression of genes associated with reactive oxygen species (ROS) formation, and modulation of gut microflora [67]. The results of the current study are in agreement with results for LAB isolated from Raabadi [33].

H₂O₂ production by LAB cultures is also considered beneficial, due to its ability to exert antimicrobial activities against a range of spoilage and pathogenic microbes. H₂O₂ cytotoxicity is due to the formation of certain bactericidal reactive oxides (e.g., hydroxyl radicals) by the action of reducing agents and peroxidases [68]. Ref. [33] reported that out of six potential isolates, three strains (RYPR1, RYPR9, and RYPC7) were able to produce H₂O₂. In the present study, two isolates, KMUDR1 and -7, were observed to produce H₂O₂.

BSH activity has also recently been identified as a desirable probiotic feature for strain selection, and the production of BSH enzymes by LAB facilitates the survival of bacteria

under high bile acid toxicity conditions. The deconjugation of bile salts by LAB is an indication of host–microbe interaction in the gut, which facilitates the functional regulation of cholesterol metabolism [67]. Recently, [57] reported that a *Lactobacillus plantarum* strain isolated from foods showed the selective ability to deconjugate bile salts, in contrast to earlier research reports that human-origin strains showed BSH activity. Moreover, this activity was strain-dependent, as a varying degree of bile salt deconjugation was observed among the strains. In the present study, all the isolates, except KMUDR17, precipitated the tested bile salts, which is in agreement with earlier studies [32,33].

A probiotic should not produce any toxic substances and should improve safety for consumers by inhibiting pathogens and preventing the production of biogenic amines. These strains should not show hemolytic or DNase activities, these being considered important criteria for the non-virulence of strains. Strains should be resistant to different antibiotics and must not have any transferable antibiotic-resistance genes [28,69]. However, highly antibiotic-sensitive probiotic strains with non-transferable resistance genes are considered safe and have been observed to have beneficial effects after antibiotic therapy in terms of eliminating gut pathogens [32,70]. Several strains of *Lactobacillus* sp. were reported to be safe according to the parameters of antibiotic susceptibility and hemolytic activity [20,32,38,40]. The results of the present study are in accordance with earlier reports for LAB isolates from cereal-based foods [32,33,54]. Hence, all the tested LAB strains are considered safe potential probiotics based on antibiotic susceptibility profiling, hemolytic activity, and biogenic amine production under in vitro conditions.

Amylolytic LAB are very rare in nature; they contain the amyA gene, which facilitates the conversion of starch into lactic acid by producing α -amylase enzymes. The most proficient starch-fermenting LAB are associated with the genera *Lactobacillus* and *Lactococcus* [71]. Three strains of *Lactobacillus acidophilus* showing high amylolytic activities (A4, L9, and L23) were isolated from pigs and characterized as potential probiotic candidates [72]. Recently, [73] reported three strains of *Lactobacillus plantarum* with probiotic attributes possessing high amylase activities isolated from Chinese cereal-based fermented foods. In our study, KMUDR1, -7, and -9 showed weak amylolytic activities. However, amylolytic activities are strain-dependent [74]. The production of exopolysaccharides (EPSs) is an important desirable property for improving the textural attributes of cereal-based fermented foods without any safety concerns [75]. Several reports have presented and explored exopolysaccharide-producing bacteria and yeasts isolated from cereal-based products [36,44]. The EPS production of *Lactobacillus plantarum* isolated from “Nigerian traditional fermented cereal gruel ogi” was investigated by Fourier-transform infrared spectroscopy, and the EPS production for the tested strains was found to be in the range of 1.36 g/L to 2.18 g/L [44]. In a similar study, thirteen LAB strains isolated from Boza were evaluated for EPS production and potential use as adjunct cultures, and most of the strains showed significant production of EPS [76]. The production of EPS in foods provides various benefits to living beneficial bacteria, such as protection from adverse conditions, toxic molecules, phagocytosis apart from biofilm formation, and cell recognition [77]. Various structures of EPSs perform different roles in EPS-producing cells, which are still unknown and mostly strain-dependent [78]. The six selected isolates were grown in skim-milk media to check their suitability as probiotics and viability during fermentation and storage. All isolates showed growth during fermentation up to 24 h but decreasing trends were observed during storage. The cell populations of all six tested cultures were found to be above the recommended limit of 10^6 CFU/mL for exerting beneficial effects on human health during fermentation. The isolates KMUDR1 and -7 showed acceptable concentrations up to 14 days of storage. Previously, some other researchers [46,79] also reported the same trend for a potential probiotic strain.

Morphological, phenotypic, and biochemical characterizations were performed for all six potential probiotic stains to identify the genera of the isolates. Researchers widely use these parameters for the identification of LAB from different indigenously fermented foods products, i.e., Neera samples [38], Raabadi [33], yoghurt [80], and sour rice [28].

Ref. [21] reported that the LAB isolates E031, T035, and K011 showed growth at 4% and 6.5% NaCl concentrations, whereas E052 did not grow at a 6.5% NaCl concentration. All the six LAB isolates were further confirmed using phylogenetic tree construction and the 16S rDNA sequencing technique. The four different potential probiotic lactobacilli strains (*Lactobacillus plantarum* JCM1149, *Lactobacillus paracasei* NBRC15889, *Lactobacillus plantarum* CIP103151, and *Lactobacillus paracasei* subsp. *tolerans* NBRC15906) were identified from indigenously fermented foods products of Ethiopia (Teff dough, Ergo, and Kocho) using 16S rDNA sequencing [21]. According to [54], a 16S rDNA gene sequence analysis of the LAB isolates from different fermented foods showed that all efficient probiotic isolates belonged to the *Lactobacilli* genus. Similarly, some other researchers have also used this technique for the strain-level identification of potential probiotic strains isolated from various food matrices [28,38,46,80,81].

5. Conclusions

The current study aimed at the isolation and characterization in vitro of probiotic bacteria from traditional cereal-based fermented products from the Aravali hills region of Southern Rajasthan, India. Given the favorable probiotic attributes of the LAB isolates, particularly *Lactiplantibacillus plantarum* KMUDR7 and *Lactobacillus delbrueckii* subsp. *bulgaricus* KMUDR1, it can be concluded that cereal-based fermented products are good sources for the isolation of probiotic bacteria. The potential probiotic strains could be further used as starter cultures to prepare indigenous cereal-based products at a commercial level as well as in other futuristic functional foods for wider public benefit. However, further in vivo trials may be carried out to validate their potential health-promoting attributes and applications at an industrial scale.

Author Contributions: Conceptualization, K.K.M., A.O. and V.M.; Data curation, N.K.T., A.O. and D.K.; Formal analysis, K.K.M.; Investigation, K.K.M.; Methodology, K.K.M. and V.M.; Project administration, N.K.T.; Resources, D.J., A.O. and D.K.; Software, D.J. and D.K.; Supervision, D.J. and V.M., Validation, D.J. and A.O.; Visualization, N.K.T. and D.K.; Writing—original draft, K.K.M.; Writing—review and editing, N.K.T. and V.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank the College of Dairy and Food Technology, MPUAT, Udaipur, India, and the National Institute of Food Technology Entrepreneurship and Management (NIFTEM), Kundli, India, for financial assistance and provision of facilities.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mallappa, R.H.; Balasubramaniam, C.; Nataraj, B.H.; Ramesh, C.; Kadyan, S.; Pradhan, D.; Muniyappa, S.K.; Grover, S. Microbial Diversity and Functionality of Traditional Fermented Milk Products of India: Current Scenario and Future Perspectives. *Int. Dairy J.* **2021**, *114*, 104941. [CrossRef]
2. Saeed, Z.K.; Abbas, B.A.; Othman, R.M. Molecular identification and phylogenetic analysis of lactic acid bacteria isolated from goat raw milk. *Iraqi J. Vet. Sci.* **2020**, *34*, 259–263. [CrossRef]
3. Setta, M.C.; Matemu, A.; Mbega, E.R. Potential of probiotics from fermented cereal-based beverages in improving health of poor people in Africa. *J. Food Sci. Technol.* **2020**, *57*, 3935–3946. [CrossRef]
4. Cunningham, M.; Azcarate-Peril, M.A.; Barnard, A.; Benoit, V.; Grimaldi, R.; Guyonnet, D.; Holscher, H.D.; Hunter, K.; Manurung, S.; Obis, D.; et al. Shaping the Future of Probiotics and Prebiotics. *Trends Microbiol.* **2021**, *29*, 667–685. [CrossRef]
5. Abarquero, D.; Renes, E.; Fresno, J.M.; Tornadijo, M.E. Study of Exopolysaccharides from Lactic Acid Bacteria and Their Industrial Applications: A Review. *Int. J. Food Sci. Technol.* **2022**, *57*, 16–26. [CrossRef]
6. Endo, A.; Tanizawa, Y.; Arita, M. Isolation and Identification of Lactic Acid Bacteria from Environmental Samples. In *Methods in Molecular Biology*; Humana Press: New York, NY, USA, 2019; Volume 1887.

7. Verni, M.; Rizzello, C.G.; Coda, R. Fermentation Biotechnology Applied to Cereal Industry By-Products: Nutritional and Functional Insights. *Front. Nutr.* **2019**, *6*, 42. [[CrossRef](#)]
8. Rezac, S.; Kok, C.R.; Heermann, M.; Hutkins, R. Fermented Foods as a Dietary Source of Live Organisms. *Front. Microbiol.* **2018**, *9*, 1785. [[CrossRef](#)]
9. Sethi, S.; Anurag, R.K. Probiotic and Prebiotic Plant Milk Dairy Foods. In *Probiotics and Prebiotics in Foods*; Academic Press: Cambridge, MA, USA, 2021. [[CrossRef](#)]
10. Vinderola, G.; Reinheimer, J.; Salminen, S. The Enumeration of Probiotic Issues: From Unavailable Standardised Culture Media to a Recommended Procedure? *Int. Dairy J.* **2019**, *96*, 58–65. [[CrossRef](#)]
11. Rodríguez, L.G.R.; Mohamed, F.; Bleckwedel, J.; Medina, R.B.; De Vuyst, L.; Hebert, E.M.; Mozzi, F. Diversity and Functional Properties of Lactic Acid Bacteria Isolated From Wild Fruits and Flowers Present in Northern Argentina. *Front. Microbiol.* **2019**, *10*, 1091. [[CrossRef](#)]
12. Paiva, I.H.R.; Duarte-Silva, E.; Peixoto, C.A. The role of prebiotics in cognition, anxiety, and depression. *Eur. Neuropsychopharmacol.* **2020**, *34*, 1–18. [[CrossRef](#)]
13. Górska, A.; Przystupski, D.; Niemczura, M.J.; Kulbacka, J. Probiotic Bacteria: A Promising Tool in Cancer Prevention and Therapy. *Curr. Microbiol.* **2019**, *76*, 939–949. [[CrossRef](#)]
14. Oforu, F.K.; Elahi, F.; Daliri, E.B.-M.; Han, S.-I.; Oh, D.-H. Impact of thermal treatment and fermentation by lactic acid bacteria on sorghum metabolite changes, their antioxidant and antidiabetic activities. *Food Biosci.* **2021**, *45*, 101502. [[CrossRef](#)]
15. Lei, W.; Liu, C.; Pan, L.; Peng, C.; Wang, J.; Zhou, H. Screening of probiotic Lactobacilli with potential anti-allergic activity based on hyaluronidase inhibition and degranulation of RBL-2H3 cells in vitro. *LWT* **2020**, *140*, 110707. [[CrossRef](#)]
16. Gil-Rodríguez, A.M.; Beresford, T. Bile Salt Hydrolase and Lipase Inhibitory Activity in Reconstituted Skim Milk Fermented with Lactic Acid Bacteria. *J. Funct. Foods* **2021**, *77*, 104342. [[CrossRef](#)]
17. Barcenilla, C.; Ducic, M.; López, M.; Prieto, M.; Álvarez-Ordóñez, A. Application of lactic acid bacteria for the biopreservation of meat products: A systematic review. *Meat Sci.* **2021**, *183*, 108661. [[CrossRef](#)]
18. Shahbazi, R.; Sharifzad, F.; Bagheri, R.; Alsadi, N.; Yasavoli-Sharahi, H.; Matar, C. Anti-Inflammatory and Immunomodulatory Properties of Fermented Plant Foods. *Nutrients* **2021**, *13*, 1516. [[CrossRef](#)]
19. Meena, K.K.; Kapila, S.; Chand, R.; Kumawat, D.K.; Verma, G. Specific Immune Response of Oral Administration of Dahi and Milk Fermented with *Lactobacillus helveticus* against *Salmonella enteritidis* in Mice. *Indian J. Dairy Sci.* **2017**, *70*, 404–410.
20. Minj, J.; Chandra, P.; Paul, C.; Sharma, R.K. Bio-functional properties of probiotic *Lactobacillus*: Current applications and research perspectives. *Crit. Rev. Food Sci. Nutr.* **2020**, *61*, 2207–2224. [[CrossRef](#)]
21. Mulaw, G.; Tessema, T.S.; 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*, 1–11. [[CrossRef](#)] [[PubMed](#)]
22. Ilango, S.; Antony, U. Probiotic microorganisms from non-dairy traditional fermented foods. *Trends Food Sci. Technol.* **2021**, *118*, 617–638. [[CrossRef](#)]
23. Tamang, J.P.; Cotter, P.D.; Endo, A.; Han, N.S.; Kort, R.; Liu, S.Q.; Mayo, B.; Westerik, N.; Hutkins, R. Fermented Foods in a Global Age: East meets West. *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 184–217. [[CrossRef](#)]
24. Kohajdová, Z. *Fermented Cereal Products*; Elsevier, B.V.: Amsterdam, The Netherlands, 2017; ISBN 9780444636775.
25. Aspri, M.; Papademas, P.; Tsaltas, D. Review on Non-Dairy Probiotics and Their Use in Non-Dairy Based Products. *Fermentation* **2020**, *6*, 30. [[CrossRef](#)]
26. Chaudhari, A.; Dwivedi, M.K. The Concept of Probiotics, Prebiotics, Postbiotics, Synbiotics, Nutribiotics, and Pharmabiotics. In *Probiotics in the Prevention and Management of Human Diseases*; Academic Press: Cambridge, MA, USA, 2022.
27. Ray, M.; Ghosh, K.; Singh, S.; Mondal, K.C. Folk to functional: An explorative overview of rice-based fermented foods and beverages in India. *J. Ethn. Foods* **2016**, *3*, 5–18. [[CrossRef](#)]
28. 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.* **2021**, *3*, 99–108. [[CrossRef](#)]
29. Sreeja, V.; Prajapati, J.B. Ethnic Fermented Foods and Beverages of Gujarat and Rajasthan. In *Ethnic Fermented Foods and Beverages of India: Science History and Culture*; Springer: New Delhi, India, 2020.
30. Pintu, R.; Verma, B. Optimization of Rabadi-like Sorghum Based Fermented Milk Beverage. *J. Agrisearch* **2019**, *6*, 194–198. [[CrossRef](#)]
31. McFarland, L.V.; Evans, C.T.; Goldstein, E.J.C. Strain-Specificity and Disease-Specificity of Probiotic Efficacy: A Systematic Review and Meta-Analysis. *Front. Med.* **2018**, *5*, 124. [[CrossRef](#)]
32. Ahire, J.J.; Jakkamsetty, C.; Kashikar, M.S.; Lakshmi, S.G.; Madempudi, R.S. In Vitro Evaluation of Probiotic Properties of *Lactobacillus plantarum* UBLP40 Isolated from Traditional Indigenous Fermented Food. *Probiotics Antimicrob. Proteins* **2021**, *13*, 1413–1424. [[CrossRef](#)]
33. Yadav, R.; Puniya, A.K.; Shukla, P. Probiotic Properties of *Lactobacillus Plantarum* RYPR1 from an Indigenous Fermented Beverage Raabadi. *Front. Microbiol.* **2016**, *7*, 1683. [[CrossRef](#)]
34. Aladeboyeje, O.T.; Sanli, N.O.; Buyuk, U. Evaluation of the Antimicrobial Efficacy of Some Fermented Traditional Turkish Beverages with Probiotic Potentials. *Johns. Matthey Technol. Rev.* **2021**, *66*, 337–350. [[CrossRef](#)]
35. Heperkan, Z.D.; Bolluk, M.; Bülbül, S.S. Structural analysis and properties of dextran produced by *Weissella confusa* and the effect of different cereals on its rheological characteristics. *Int. J. Biol. Macromol.* **2019**, *143*, 305–313. [[CrossRef](#)] [[PubMed](#)]

36. Şentürk, D.Z.; Dertli, E.; Erten, H.; Şimşek, Ö. Structural and technological characterization of ropy exopolysaccharides produced by *Lactobacillus plantarum* strains isolated from Tarhana. *Food Sci. Biotechnol.* **2019**, *29*, 121–129. [[CrossRef](#)] [[PubMed](#)]
37. Fekri, A.; Torbati, M.; Khosrowshahi, A.Y.; Shamloo, H.B.; Azadmard-Damirchi, S. Functional effects of phytate-degrading, probiotic lactic acid bacteria and yeast strains isolated from Iranian traditional sourdough on the technological and nutritional properties of whole wheat bread. *Food Chem.* **2019**, *306*, 125620. [[CrossRef](#)]
38. Somashekaraiah, R.; Shruthi, B.; Deepthi, B.V.; Sreenivasa, M.Y. Probiotic Properties of Lactic Acid Bacteria Isolated From Neera: A Naturally Fermenting Coconut Palm Nectar. *Front. Microbiol.* **2019**, *10*, 1382. [[CrossRef](#)]
39. Zago, M.; Fornasari, M.E.; Carminati, D.; Burns, P.; Suárez, V.; Vinderola, G.; Reinheimer, J.; Giraffa, G. Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiol.* **2011**, *28*, 1033–1040. [[CrossRef](#)]
40. Bhushan, B.; Sakhare, S.M.; Narayan, K.S.; Kumari, M.; Mishra, V.; Dicks, L.M.T. Characterization of Riboflavin-Producing Strains of *Lactobacillus plantarum* as Potential Probiotic Candidate through in vitro Assessment and Principal Component Analysis. *Probiotics Antimicrob. Proteins* **2020**, *13*, 453–467. [[CrossRef](#)]
41. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing*. 30th Ed. CLSI M100 ED30:2020; CLSI: Wayne, PA, USA, 2020.
42. Matuschek, E.; Brown, D.F.J.; Kahlmeter, G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin. Microbiol. Infect.* **2014**, *20*, O255–O266. [[CrossRef](#)]
43. Sangprapai, W.; Prasitpuriprecha, C.; Jantama, K.; Jantama, S.S. Probiotics Isolated from Thai Fermented Foods for Potential Uses against Foodborne Pathogens. *Asia-Pac. J. Sci. Technol.* **2022**, *27*, 1–11. [[CrossRef](#)]
44. Adesulu-Dahunsi, A.T.; Jeyaram, K.; Sanni, A.I.; Banwo, K. Production of exopolysaccharide by strains of *Lactobacillus plantarum* YO175 and OF101 isolated from traditional fermented cereal beverage. *PeerJ* **2018**, *6*, e5326. [[CrossRef](#)]
45. Ruas-Madiedo, P.; Reyes-Gavilán, C.D.L. Invited Review: Methods for the Screening, Isolation, and Characterization of Exopolysaccharides Produced by Lactic Acid Bacteria. *J. Dairy Sci.* **2005**, *88*, 843–856. [[CrossRef](#)]
46. Samedí, L.; Charles, A.L. Isolation and characterization of potential probiotic *Lactobacilli* from leaves of food plants for possible additives in pellet feeding. *Ann. Agric. Sci.* **2019**, *64*, 55–62. [[CrossRef](#)]
47. Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F.; Higgins, D.G. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **1997**, *25*, 4876–4882. [[CrossRef](#)] [[PubMed](#)]
48. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [[CrossRef](#)] [[PubMed](#)]
49. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [[CrossRef](#)] [[PubMed](#)]
50. Silva, D.R.; Sardi, J.D.C.O.; Pitangui, N.D.S.; Roque, S.M.; da Silva, A.C.B.; Rosalen, P.L. Probiotics as an alternative antimicrobial therapy: Current reality and future directions. *J. Funct. Foods* **2020**, *73*, 104080. [[CrossRef](#)]
51. Jung, J.; Kim, S.; Lee, J.Y.; Yoon, S.; You, S.; Kim, S.H. Multifunctional properties of *Lactobacillus plantarum* strains WiKim83 and WiKim87 as a starter culture for fermented food. *Food Sci. Nutr.* **2019**, *7*, 2505–2516. [[CrossRef](#)]
52. Gupta, A.; Tiwari, S.K. Probiotic Potential of *Lactobacillus plantarum* LD1 Isolated from Batter of Dosa, a South Indian Fermented Food. *Probiotics Antimicrob. Proteins* **2014**, *6*, 73–81. [[CrossRef](#)]
53. Das, D.; Goyal, A. Potential Probiotic Attributes and Antagonistic Activity of an Indigenous Isolate *Lactobacillus plantarum* DM5 from an Ethnic Fermented Beverage “Marcha” of North Eastern Himalayas. *Int. J. Food Sci. Nutr.* **2014**, *65*, 335–344. [[CrossRef](#)]
54. Mishra, B.K.; Hati, S.; Das, S.; Kumari, R. Evaluation of Probiotic Potentials of *Lactobacillus* Isolated from Traditional Fermented Foods of Garo Hills, Meghalaya, India. *Rev. Med. Microbiol.* **2018**, *29*, 120–128. [[CrossRef](#)]
55. Sharma, K.; Attri, S.; Goel, G. Selection and Evaluation of Probiotic and Functional Characteristics of Autochthonous Lactic Acid Bacteria Isolated from Fermented Wheat Flour Dough Babroo. *Probiotics Antimicrob. Proteins* **2019**, *11*, 774–784. [[CrossRef](#)]
56. Urdaneta, V.; Casadesús, J. Interactions between Bacteria and Bile Salts in the Gastrointestinal and Hepatobiliary Tracts. *Front. Med.* **2017**, *4*, 163. [[CrossRef](#)]
57. Prete, R.; Long, S.L.; Gallardo, A.L.; Gahan, C.G.; Corsetti, A.; Joyce, S.A. Beneficial bile acid metabolism from *Lactobacillus plantarum* of food origin. *Sci. Rep.* **2020**, *10*, 1–11. [[CrossRef](#)] [[PubMed](#)]
58. Ayyash, M.M.; Abdalla, A.K.; AlKalbani, N.S.; Baig, M.A.; Turner, M.S.; Liu, S.Q.; Shah, N.P. Invited Review: Characterization of New Probiotics from Dairy and Nondairy Products—Insights into Acid Tolerance, Bile Metabolism and Tolerance, and Adhesion Capability. *J. Dairy Sci.* **2021**, *104*, 8363–8379. [[CrossRef](#)] [[PubMed](#)]
59. Pisoschi, A.M.; Pop, A.; Georgescu, C.; Turcuş, V.; Olah, N.K.; Mathe, E. An overview of natural antimicrobials role in food. *Eur. J. Med. Chem.* **2018**, *143*, 922–935. [[CrossRef](#)] [[PubMed](#)]
60. Kumariya, R.; Garsa, A.K.; Rajput, Y.; Sood, S.; Akhtar, N.; Patel, S. Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microb. Pathog.* **2019**, *128*, 171–177. [[CrossRef](#)] [[PubMed](#)]
61. Kumari, A.; Angmo, K.; Monika; Bhalla, T.C. Probiotic Attributes of Indigenous *Lactobacillus* Spp. Isolated from Traditional Fermented Foods and Beverages of North-Western Himalayas Using in Vitro Screening and Principal Component Analysis. *J. Food Sci. Technol.* **2016**, *53*, 2463–2475. [[CrossRef](#)]
62. Angmo, K.; Kumari, A.; Savitri; Bhalla, T.C. Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. *LWT* **2016**, *66*, 428–435. [[CrossRef](#)]
63. Dopazo, V.; Luz, C.; Quiles, J.M.; Calpe, J.; Romano, R.; Mañes, J.; Meca, G. Potential application of lactic acid bacteria in the biopreservation of red grape from mycotoxigenic fungi. *J. Sci. Food Agric.* **2021**, *102*, 898–907. [[CrossRef](#)]

64. Schettino, R.; Pontonio, E.; Gobbetti, M.; Rizzello, C.G. Extension of the Shelf-Life of Fresh Pasta Using Chickpea Flour Fermented with Selected Lactic Acid Bacteria. *Microorganisms* **2020**, *8*, 1322. [[CrossRef](#)]
65. Panebianco, F.; Caridi, A. New insights into the antifungal activity of lactic acid bacteria isolated from different food matrices. *Grasas Aceites* **2021**, *72*, e400. [[CrossRef](#)]
66. Reuben, R.; Roy, P.C.; Sarkar, S.L.; Alam, R.-U.; Jahid, I.K. Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. *BMC Microbiol.* **2019**, *19*, 253. [[CrossRef](#)]
67. Garcia-Gonzalez, N.; Battista, N.; Prete, R.; Corsetti, A. Health-Promoting Role of *Lactiplantibacillus plantarum* Isolated from Fermented Foods. *Microorganisms* **2021**, *9*, 349. [[CrossRef](#)] [[PubMed](#)]
68. Erttmann, S.F.; Gekara, N.O. Hydrogen peroxide release by bacteria suppresses inflammasome-dependent innate immunity. *Nat. Commun.* **2019**, *10*, 1–13. [[CrossRef](#)]
69. Mokoena, M.P.; Mutanda, T.; Olaniran, A.O. Perspectives on the probiotic potential of lactic acid bacteria from African traditional fermented foods and beverages. *Food Nutr. Res.* **2016**, *60*, 29630. [[CrossRef](#)] [[PubMed](#)]
70. Riane, K.; Sifour, M.; Ouled-Haddar, H.; Idoui, T.; Bounar, S.; Boussebt, S. Probiotic properties and antioxidant efficiency of *Lactobacillus plantarum* 15 isolated from milk. *J. Microbiol. Biotechnol. Food Sci.* **2019**, *9*, 516–520. [[CrossRef](#)]
71. Tsafrakidou, P.; Michaelidou, A.M.; Biliaderis, C.G. Fermented Cereal-based Products: Nutritional Aspects, Possible Impact on Gut Microbiota and Health Implications. *Foods* **2020**, *9*, 734. [[CrossRef](#)]
72. Lee, H.; Gilliland, S.; Carter, S. Amyolytic Cultures of *Lactobacillus acidophilus* : Potential Probiotics to Improve Dietary Starch Utilization. *J. Food Sci.* **2001**, *66*, 338–344. [[CrossRef](#)]
73. Xu, Y.; Zhou, T.; Tang, H.; Li, X.; Chen, Y.; Zhang, L.; Zhang, J. Probiotic potential and amyolytic properties of lactic acid bacteria isolated from Chinese fermented cereal foods. *Food Control* **2019**, *111*, 107057. [[CrossRef](#)]
74. Chaves-López, C.; Rossi, C.; Maggio, F.; Paparella, A.; Serio, A. Changes Occurring in Spontaneous Maize Fermentation: An Overview. *Fermentation* **2020**, *6*, 36. [[CrossRef](#)]
75. Angelin, J.; Kavitha, M. Exopolysaccharides from probiotic bacteria and their health potential. *Int. J. Biol. Macromol.* **2020**, *162*, 853–865. [[CrossRef](#)] [[PubMed](#)]
76. Heperkan, D.; Daskaya-Dikmen, C.; Bayram, B. Evaluation of lactic acid bacterial strains of boza for their exopolysaccharide and enzyme production as a potential adjunct culture. *Process Biochem.* **2014**, *49*, 1587–1594. [[CrossRef](#)]
77. Karygianni, L.; Ren, Z.; Koo, H.; Thurnheer, T. Biofilm Matrixome: Extracellular Components in Structured Microbial Communities. *Trends Microbiol.* **2020**, *28*, 668–681. [[CrossRef](#)]
78. Korcz, E.; Varga, L. Exopolysaccharides from lactic acid bacteria: Techno-functional application in the food industry. *Trends Food Sci. Technol.* **2021**, *110*, 375–384. [[CrossRef](#)]
79. Yerlikaya, O.; Ender, G.; Torunoglu, F.A.; Akbulut, N. Production of Probiotic Milk Drink Containing: *Lactobacillus acidophilus*, *Bifidobacterium animalis* Subsp. *lactis* and *Lactobacillus casei*. *Agro Food Ind. Hi. Tech* **2013**, *24*, 49–52.
80. Heidari, Z.; Ghasemi, M.F.; Modiri, L. Antimicrobial activity of bacteriocin produced by a new *Latilactobacillus curvatus* sp. LAB-3H isolated from traditional yogurt. *Arch. Microbiol.* **2021**, *204*, 1–12. [[CrossRef](#)]
81. Afshari, A.; Hashemi, M.; Tavassoli, M.; Eraghi, V.; Noori, S.M.A. Probiotic bacteria from 10 different traditional Iranian cheeses: Isolation, characterization, and investigation of probiotic potential. *Food Sci. Nutr.* **2022**, *10*, 2009–2020. [[CrossRef](#)]