

***In vitro* characterization of five indigenous lactic acid bacteria for their potential probiotic-rich semi-solid milk production**

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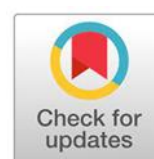
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Abstract

The main objective of the present study is to characterize five lactic acid bacteria (LAB) isolated from traditional natural fermented foods (viz. Chhurpi, Janr, Kinema, Whey, Gundruk, and Sinki) of Darjeeling hills, West Bengal, India, for their use as potential probiotic starter cultures in homemade fermented semi-solid milk recipes. The isolated strains were characterized morpho-biochemically, with whole-cell protein profiles and 16S rRNA gene sequence analysis. The strains were also tested for antibiosis, hemolysis, and antibiotic sensitivity. The *in vitro* probiotic traits were assessed for gastric acid tolerance, bile salt tolerance, cholesterol-lowering activity, cell wall hydrophobicity, and the efficiency of folate and niacin production. The fermented milk-producing efficiency of the strains was assessed by measuring exopolysaccharide production, β -galactosidase activity, fermented milk quality, and residual lactose content. The biochemical and 16S rRNA gene sequence analysis identified the strains as *Lactobacillus* sp. LAB-04 (GenBank Ac. No.: HQ141911), *Lactobacillus* sp. LAB-06 (GenBank AC. No.: MG066499), *Pediococcus* sp. LAB-11 (GenBank Ac. No.: HQ141913), *Lactobacillus* sp. LAB-39 (GenBank Ac. No.: MG066500), and *Lactobacillus* sp. LAB-40 (GenBank Ac. No.: HQ141915). The *in vitro* probiotic parameters revealed that all five strains exhibited significant tolerance to acidic gastric conditions and bile salts (ox-bile, 5% w/v), as well as cell wall hydrophobicity and cholesterol-binding ability. The strains produced higher levels of exopolysaccharides in lactose- and glucose-amended media and showed good β -galactosidase activity. The semi-solid milk produced by the strains had negligible lactose content. The experimental evidence showed that these five LAB strains could serve as potent probiotic starter cultures in homemade fermented semi-solid milk recipes, and that the preparation could be beneficial for malnourished and lactose-intolerant individuals.

Keywords: β -galactosidase, Exopolysaccharide; Indigenous lactic acid bacteria; Potential probiotics; Semi-solid milk

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Introduction

Animal milk, a highly nutritious source of carbohydrates, proteins, fats, and vital minerals such as calcium, is used conventionally in various forms by all human races. Lactose is the primary carbohydrate in milk, with approximately 4.0 to

5.0% (cow milk). However, approximately 20% of Asians, 50% in South America and Africa, and 15% in the United States, including infants, young children, adults, and elderly people, suffer from milk intake due to the inability to absorb milk lactose due to the deficiency of "lactase-phlorizin hydrolase" or lactase (β -galactosidase, EC 3.2.1.23/26) localized

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to the tips of the villi of the small intestine (Heyman 2006), commonly called '**lactose intolerance**'. This, in turn, limits the intake of raw milk by these groups. Recent evidence indicates that dietary lactose enhances calcium absorption (Jackson and Savaiano, 2001). Thus, lactose intolerance may also predispose to inadequate bone mineralization, resulting in osteoporosis (Jackson and Savaiano, 2001). It is established that fermented milk products, such as curd, cheese, and yogurt, have lower lactose content and are thus associated with fewer milk-related problems (Saavedra 2001; Heyman 2006). Homemade fermented milk products are a crucial source of nutrition for malnourished people in developing countries. Fermented semi-solid milk has been reported for its numerous beneficial and therapeutic applications, including the maintenance of intestinal microflora, treatment of inflammatory bowel disease (IBD), protection against diarrhoea and colon cancer, and enhancement of the immune system (Mattila-Sandholm et al., 2002; Karna et al., 2007; Gille et al., 2018).

Many studies have shown that frequent consumption of fermented milk reduces the risk of type 2 Diabetes (Roberfroid, 2000; Gille et al., 2018). A cohort study has also established that fermented milk consumption may lead to weight management in obese people. Therefore, microbial supplementation with selected, potent β -galactosidase-positive, probiotics-enriched fermented milk products might be an alternative option for these nutritionally deprived individuals and others worldwide who require a nutritionally rich diet. A large fraction of the rural and urban population of the Darjeeling Hills and other parts of India suffer from malnutrition due to their poverty level below the poverty line. However, they have traditionally maintained a good practice of domesticating animals such as cows, buffaloes, goats, camels, and yaks, as well as processing and consuming fermented milk products such as Chhurpi, Kefir, Dahi, and Whey in their daily diet (Dewan and Tamang 2006; Rai et al. 2016). Most of these fermented foods are dominated by indigenous lactic acid bacteria and other lactose fermenters of unknown health benefits (Dewan and Tamang 2006; Rai et al. 2016). Therefore, these indigenous LABs can be exploited to assess probiotic strains and starter cultures in milk fermentation/yogurt production.

Autochthonous starter cultures are key resources for targeted fermented food production. (Margaralho et al., 2021; Munekata et al., 2023). Preparing fermented semi-solid milk on a household scale is easy. It can be used as a solid or semi-solid.

Therefore, there is no need to add artificial solidifying agents during processing. Thus, the fermented, semi-solid milk-based application of probiotic strains could be an effective and viable option for supplementing lactose-intolerant and malnourished individuals worldwide (Mattila-Sandholm et al., 2002; Gille et al., 2018). Among the probiotic LABs, *Lactobacillus* and *Pediococcus* are widely being trialled for various applications (Tuomola et al. 2001; Lee et al. 2007). Barigela and Bhukya (2021) characterized the potential probiotic properties of the *Pediococcus acidilactici* TMAB26 strain isolated from traditional Indian tomato pickle. They observed that the strain exhibited significant cytotoxicity against HT-29 ($94.91\% \pm 1.27$) and Caco-2 ($92.63\% \pm 0.63$) cancer cells, compared to peripheral blood mononuclear cells (PBMCs) alone. The culture supernatant reduced the mRNA levels of the proinflammatory cytokine tumour necrosis factor-alpha (TNF- α) and Interleukin-6 (IL-6) and increased the mRNA levels of the anti-inflammatory cytokine Interleukin-10 (IL-10). To be used as probiotic strains, bacteria should, in addition to their health benefits, be tolerant of the stresses of the gastrointestinal environment, such as acid, bile salts, and anaerobic conditions, and colonize the intestine via cell wall hydrophobic interactions. Therefore, these parameters should be assessed against the isolates to be claimed as probiotics. Additionally, hypercholesterolemia is another primary concern that poses a significant health risk. Due to the intricate relationship between bile salts and serum cholesterol levels, bile salt hydrolase (EC 3.5.1.24)-positive probiotic strains are now considered a potential biological approach for treating hypercholesterolemia (Mandal et al. 2009).

Folic acid and niacin are two essential water-soluble vitamins of the vitamin B complex family. The significant roles of folic acid include producing red blood cells and preventing anaemia, facilitating rapid cell division and growth, and supporting DNA synthesis and protein metabolism. During pregnancy, women are advised to consume ~ 800 μg of folic acid daily. At the same time, niacin is responsible for various physiological and enzymatic functions, including proper metabolism of fats, carbohydrates, and proteins, proper functioning of the nervous system, and maintenance of skin complexion. Therefore, regular intake of folic acid and niacin, as well as their supplementation, is essential for everyone's life, especially during pregnancy for women's health. In India, folate (vitamin B11), niacin (B3), and iron deficiencies are predominant public health hazards (Wilson and

O'Connor, 2021). Epidemiological studies indicated that folate deficiency is often associated with an increased risk of breast cancer and colorectal cancer (Sellers et al., 2001; Terry et al., 2002). Therefore, microbial supplementation of these vital vitamins through probiotic-enriched fermented food products might be an alternative for malnourished people and others.

The people of the Darjeeling Hills have traditional fermentation practices for producing various fermented food products, including Chhurpi, Janr, Kinema, Whey, Gundruk, and Sinki, as well as homemade green vegetables, pulses, milk, and fish products. Most fermented foods are dominated by lactic acid bacteria (Dewan & Tamang, 2007; Tamang, 2009; Tamang et al., 2016, 2020). Therefore, microbial supplementation of these vital vitamins through probiotic-enriched fermented food products may be an alternative for this target group of malnourished individuals, as well as for other groups. The main objective of the present study is to explore the suitability of five indigenous lactic acid bacteria (LAB) strains isolated from naturally fermented foods in the Darjeeling Hills for use as superior probiotic starter bacteria in homemade fermented semi-solid milk preparations to combat malnutrition.

Materials and methods

Chemicals, media, and strains used in this study

D-Dextrose, Dipotassium hydrogen phosphate, D-Lactose, Hydrochloric acid, Ox-bile, Phosphoric acid, Resazurin, Sodium acetate, Sodium citrate,

Whole-cell protein profiles

The strains were grown in MRS broth for 15 hours at 30°C in a BOD incubator. The cells were harvested by centrifugation at 7168 x g for 10 min at 4 °C (R-24 BL, Remi Cooling Centrifuge, Mumbai, India). The pellet was washed twice with sterile water, and the cell density was adjusted to 1.5 (O.D. at 620 nm). It was then kept on ice until use. The cells were centrifuged again and resuspended in 100 mM Tris-HCl buffer with 8% SDS (w/v) (pH 6.5). Lysis was performed by sonication at 100 Amplitude for 3x10 sec cycles with a probe sonicator (Labsonic M, Sartorius, Germany). The cell-free culture supernatant (CFS) was also collected for a whole-cell protein profile assay. The protein content was estimated using the Lowry method with BSA as the standard (Lowry et al., 1951). The strains' whole-cell protein profiles were analysed by 12% glycine SDS-PAGE using the Laemmli protocol (Laemmli, 1970) and stained with Coomassie blue G-250. The

Sodium hydroxide, and Tween 80 were of analytical reagent grade and purchased from SRL Chemicals Pvt. Ltd., India. Lactobacillus MRS broth, bromocresol purple broth, and yeast extract were purchased from Hi-Media, Mumbai, India. The Gram stain kit, endospore staining kit, and carbohydrate fermentation and biochemical tests kit were purchased from HiMedia, Mumbai, India. The Genomic DNA purification kit was purchased from GeNei, India; the primers and DNA ladder were purchased from Sigma, USA. *Enterococcus faecalis* MB1, *Leuconostoc mesenteroides* Ly, and *Pediococcus acidilactici* LAB-05 were kindly provided by Professor Narayan C. Mandal, Department of Botany, Visva-Bharati, India; *Listeria monocytogenes* MTCC 657, *Pseudomonas aeruginosa* MTCC 741, *Salmonella enterica* serovar Typhimurium MTCC 98, and *Staphylococcus aureus* MTCC 96 were purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India.

Isolation and characterization of the LAB isolates

Isolation of lactic acid bacteria was done from locally available traditional fermented foods (viz. Chhurpi, Janr, Kinema, Whey, Gundruk, and Sinki) of Darjeeling Hills, W.B., India (Avg. Altitude 2590 meters) through dilution plating on De Man, Rogosa, and Sharpe plates (Mandal et al. 2011). Strains were selected randomly based on their whitish, circular, elevated, non-motile colonies and texture, and were characterized morphologically and biochemically following the standard protocol (Mandal et al., 2011).

image was captured with a Canon DSLR camera (Japan). The original image of the gel was converted to a 32-bit grayscale image, then the image was colour inverted, and other image aesthetics (brightness, contrast, etc.) were adjusted to enhance the clarity of the bands in the lane and convert that to numerical data for detecting banding patterns in each lane using the “ImageJ” image processing software. Finally, depending on the band pattern, a dendrogram was generated in R 3.6.3.

16S rRNA sequence analysis

Molecular characterization of the strains was performed by 16S rRNA gene sequence analysis using the standard protocol (Sambrook and Russel, 2001). The 16S rRNA gene was amplified using a 50 µl volume containing 2 µl of crude DNA preparation, 50 pMol universal eubacterial primers 8f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'GGTTACCTTGTTACGACTT3') primers

(Sigma Aldrich, USA), 10 µl of 10X buffer (Sigma Aldrich, USA), 200 µM each deoxyribonucleoside triphosphate, and 5U/ul of Super *Taq* polymerase (Sigma Aldrich, USA). PCR cycling conditions were 94 °C for 1 min, annealing temperature at 53 °C for 1 min, and 72 °C for 1 min, and for the last 35 cycles with post-run extension at 72 °C for 1 min by the Bio-Rad MJ-Mini Thermal cycler system (USA). The PCR product was sequenced using 704f (5'-GTAGCGGTGAAATGCGTAGA-3') and 907r (5'-CCGTCAATTCMTTTRAGTTT-3') primers. The sequencing study was done by Chromous Biotech Pvt. Ltd, Bangalore (<http://www.chromous.com/>). Sequences were edited by BioEdit software (v7.0.5), and BLAST searched (Program BLASTN 2.2.24+) from the NCBI database for strain identification and submitted to GenBank for accession numbers. Working cultures were maintained on MRS agar plates and on slants at 4 °C, and subcultured every 15 days. Long-term storage was performed in a 10% glycerol-skim milk solution at -10 °C. The strains were deposited in the University repository center under the code UGB-MCC_VM_ *Lactobacillus* sp. LAB-04; UGB-MCC_VM_ *Lactobacillus* sp. LAB-06; UGB-MCC_VM_ *Pediococcus* sp. LAB-11; UGB-MCC_VM_ *Lactobacillus* sp. LAB-39; and UGB-MCC_VM_ *Lactobacillus* sp. LAB-40.

Antibiosis, haemolytic activity, and antibiotic sensitivity study

The isolated strains were checked for antimicrobial compound production against the food-associated microbes like *Enterococcus faecalis* MB1, *Leuconostoc mesenteroides* Ly, *Listeria monocytogenes* MTCC 657, *Pediococcus acidilactici* LAB 5, *Pseudomonas aeruginosa* MTCC 741, *Salmonella typhimurium* MTCC 98, and *Staphylococcus aureus* MTCC 96, and also cross-antibiosis among the LAB strains by the spot-on-lawn method and agar well diffusion method (Mandal et al., 2011; Dey et al., 2019). The isolates were streaked on ready-made Sheep Blood Agar Plate (HiMedia, MP1301, Mumbai, India) (comprising Casein enzymic hydrolysate 14.00; a Peptic digest of animal tissue 4.50; Yeast extract 4.50; Sodium chloride 5.00; Agar 12.50; Sheep Blood 5.00; g/Litre, Final pH 7.3±0.2 at 25 °C and observed for the clear, colourless zone (beta haemolysis) or greenish zone around the colonies (alpha haemolysis) or no haemolysis, i.e., no change in the medium (Gamma hemolysis).

The strains' antibiotic sensitivity was determined using 24 preloaded Dodeca discs (7mm, HiMedia, Mumbai, India). The antibiotics used were

comprised of Amoxyclav (AMC), 30 µg; Cefazolin (CFZ), 30 µg; Cefoxitin (FOX), 30 µg; Cefuroxime (CXM), 30 µg; Ciprofloxacin (CIP), 5 µg; Co – Trimoxazole (COT), 25 µg; Erythromycin (ERY), 15 µg; Levofloxacin (LVX), 5 µg; Linezolid (LZD), 30 µg; Penicilin-G (PEN), 10 Unit; Teicoplanin (TEI), 30 µg; and Vancomycin (VAN), 30 µg (Dodeca G – IX Plus); and Himedia Dodeca Universal X comprising of Imipenem (IPM), 10 µg; Piperacilin (PIP), 100 µg; Co-Trimoxazole (COT), 25 µg; Aztreonam (ATM), 30 µg; Ceftazidime (CAZ), 30 µg; Chloramphenicol (CHL), 30 µg; Kanamycin (KAN), 30 µg; Netillin (NET), 30 µg; Tetracyclin (TET), 30 µg; Tobramycin (TOB), 10 µg; Piperacillin/Tazobactam (PIT), 100/10 µg; and Ticarcillin/Clavulanic acid (TCC), 75/10 µg; (HiMedia, MP1301, Mumbai, India). The fresh 8 h culture of the isolates was spread over the MRS plates, and the discs containing the antibiotics were placed on the lawn of culture, incubated at 30 °C for 24 h, and observed for growth inhibition; the diameter of growth inhibition was measured and tallied with the reference chart of antibiotic sensitivity provided by the manufacturer.

Assay for in vitro probiotic properties and fermented milk production

The strains' efficacy was assessed in two significant heads: (A) Assessment of in vitro Probiotic traits and (B) Fermented semi-solid milk production efficiency. The detailed methodologies are mentioned below:

A. Assessment of Probiotic Traits

The probiotic traits, including tolerance to gastric acidic pH, bile salt tolerance, cholesterol removal, cell wall hydrophobicity, and β-galactosidase activity, were assessed to evaluate the suitability of these strains as potential probiotic bacteria. The detailed methodology is mentioned below.

Gastric acid tolerance, Bile salt hydrolase, and Bile salt tolerance assay

Gastric acid pH tolerance and bile salt hydrolase studies were performed using the standard method (Mandal et al., 2009). Acidic pH tolerance was tested by treating the strains at pH 2.0 in MRS broth adjusted to that pH with 5 (N) HCl and 5 (N) NaOH. MRS broth at pH 6.5 was used as a positive control. Freshly prepared 18-h-grown cells were harvested by centrifugation, washed in sterile water, and resuspended to an optical density (600 nm) of 0.5. 100 µl of cells were inoculated into the treatment broths, which were incubated at 37 °C for 3 h. Cell viability was assessed using the plate dilution

method at 1-hour intervals. Acid tolerance was compared with that of the untreated sample. For bile salt tolerance, MRS broth containing a different concentration of ox-bile (0 to 5.0%, w/v) was prepared, and the pH was adjusted to 7.3 using 1(N) NaOH / HCl. The overnight (~15 h) cultures grown in MRS broth were inoculated at 1% (v/v) and incubated at 37 °C for 24 h. After incubation, growth was monitored by measuring optical density (OD) at 620 nm, and the final pH was recorded to assess bile salt tolerance of the respective strains.

Bile salt deconjugation potentiality of the strains and isolation and detection of bile salt hydrolase enzyme

The effect of oxygen limitation on bile salt deconjugation potential was compared in both aerobic and anaerobic conditions. The anaerobicity was created using sodium thioglycollate (0.2%, w/v) in freshly prepared media in screw-cap tubes with resazurin as a redox indicator. The bile salt deconjugation potential of the strains was monitored in two phases. In one phase, the overnight-grown cultures were inoculated (1%, v/v) into MRS + bile salt (0.3% ox bile, w/v) at pH 7.3 and incubated for 72 h at 37 °C under both aerobic and anaerobic conditions. A 5 mL culture aliquot was taken at 24 h, and growth, final pH, and bile salt deconjugation were measured to assess their extended activity. In another phase, overnight-grown cultures were inoculated (1%, v/v) into MRS + bile salt (0.3% ox bile, w/v) at pH 7.3 and incubated for 24 h at 37 °C. A 5 mL culture aliquot was taken at 3 h intervals, and growth, final pH, and bile salt deconjugation were measured (Mandal et al. 2009). MRS without bile salt was treated as a control. Extraction and estimation of free bile acid (cholic acid) were done using cholic acid as a standard (µg/mL) (Mandal et al. 2009).

Harvesting of the bile salt hydrolase enzyme was performed according to the protocol of Corzo and Gilliland (1998). Overnight-grown strains in MRS + 0.3% ox-bile (50 mL each) were centrifuged at 7168 x g for 10 min at 4 °C (R-24 BL, Remi Cooling Centrifuge, Mumbai, India). The supernatant was taken for enzyme harvesting. Chilled methanol was added to the supernatant at a 2:1 (v/v) ratio, and the mixture was incubated for 8 h at 4 °C. The mixture was then centrifuged at 7168 x g for 10 min at 4 °C. The bile salt hydrolase precipitate was dissolved in 1 mL of 50 mM sodium acetate buffer + 4 mM EDTA (pH 5.4). The bile salt hydrolase enzyme protein content was determined according to the protocol of Lowry et al. (1951) using BSA as the

standard. The enzyme concentration was set at 1 mg/mL. The bile salt deconjugation efficacy of the BSHs was evaluated in a 5 mL reaction mixture as described above, with the cell suspension replaced by the enzyme preparation (100 µl). BSH purity and zymogram assay were evaluated by subjecting aliquots in 10% polyacrylamide native gel electrophoresis, followed by Coomassie blue stain. The enzyme's zymogram was determined by incubating the gel in an ox-bile solution (0.3% ox-bile in 25 mM phosphate buffer, pH 7.3) for 3 h, then observing for the formation of a white precipitate.

Cholesterol-lowering activity

Filter-sterilized cholesterol (Water-insoluble, HiMedia, India) dissolved in a small volume of ethanol was added to MRS medium (without Tween 80) at a concentration of 100 µg/mL. Overnight-grown strains were inoculated at 1% culture (v/v, approx. 8×10^7 CFU/mL) into cholesterol-amended media under aerobic and anaerobic conditions and incubated at 37 °C for 48 h. The cholesterol content in the cell-free spent broth was measured by the o-phthalaldehyde method (Mandal et al., 2009).

Cell wall hydrophobicity test

The cell-surface hydrophobicity of the strains was determined using the bacterial adherence to hydrocarbons (BATH) test (Zita and Hermansson 1997). The bacteria were harvested during the exponential growth phase (~15 h) by centrifugation at 7168 x g for 10 min, washed twice with PBS (20.0 mM; pH 7.2), and resuspended in the same buffer. The suspension was adjusted to 1.5 OD (O.D._{600 nm} (A_1)) (approximately 10^8 CFU/mL cell density). Three mL bacterial suspension was mixed with 2 mL of organic solvents, viz. n-Hexene (polarity index, PI, 0.0), Carbon tetrachloride (CCl₄, PI 1.56), Toluene (PI 2.4), Xylene (PI 2.5), and n-Butanol (PI 3.9) of different polarity indices. The mixtures were vortexed for 90 sec, the two phases were allowed to separate for 2 h, and the O.D. at 600 nm (A_2) of the aqueous phase was measured in a spectrophotometer (UV-Vis 1700, Shimadzu, Japan). The percentage of cell adhesion to hydrocarbons was calculated using the formula:

$$\text{Percent Hydrophobicity (\%)} = \frac{A_1 - A_2}{A_1} \times 100$$

The experiment was repeated three times, and the results were presented as mean ± standard error. The degree of hydrophobicity of a strain was assigned as strongly hydrophobic, moderately hydrophobic, weakly hydrophobic, and hydrophobic with

percentage adhesion values on the scale of >20%, 10–20%, 1–10%, and <1%, respectively.

Auto aggregation and coaggregation assay

For the auto aggregation and coaggregation assay, cells from an overnight culture were harvested by centrifugation at 7168 x g for 10 min, washed 3 times, and resuspended in 20 mM PBS (pH 7.2). Auto aggregation was measured as described by Kos et al. (2003).

$$\text{Auto aggregation (\%)} = \frac{1 - A_t}{A_0} \times 100$$

where 'A₀' represents absorbance at 0 h and 'A_t' represents absorbance at 1, 3, 6, and 24 h.

A coaggregation assay was performed using the method described by Basson et al. (2008) with the non-LAB pathogenic strains, viz. *Listeria monocytogenes* MTCC 657 and *Salmonella enterica* serovar Typhimurium MTCC 98 and LAB strains in an equal proportion (v/v) following the same procedure. The optical density of the bacterial cell suspension was adjusted to 0.5 at 600 nm. A suspension of 2 mL of each LAB strain was mixed with an equal volume (2 mL) of each of the pathogenic strains. Absorbance was again recorded immediately after mixing, and 1 and 3 h at 600 nm, and coaggregation was calculated using the following formula.

$$\text{Co - aggregation (\%)} = \frac{A_{(\text{mixing})} - A_t}{A_0} \times 100$$

Where A_(mixing) is the absorbance taken immediately after mixing.

A_t is the absorbance taken at intervals of 1 and 3 h

A₀ is the initially adjusted absorbance of 0.5 at 600 nm.

Folate and niacin production efficiency

The strains were screened for folate and niacin production using folic acid assay medium (HiMedia, Mumbai, India) and niacin assay medium (Hi-Media, Mumbai, India), respectively. The 15 h-grown cultures in MRS broth were inoculated into 5 mL of folic acid and niacin acid assay media (pH 6.8±0.2) at 1% (v/v), incubated for 24 h at 37 °C in the dark. The growth was compared with that in the regular culture medium, MRS. The turbidity observed after incubation indicates that the culture produces folate and niacin. The growth rates of the strains were compared in the same culture medium amended with folic acid or niacin (2 µg/mL). Bacterial growth is calculated from turbidity measured at 546 nm and 595 nm using UV-Vis spectrophotometry (UV-1800, Shimadzu, Japan) in

the folic acid and niacin acid assay media. The change in growth was calculated using the formula:

$$\% \text{ growth} = \frac{O.D_{(V-SM)} - O.D_{(V-DM)}}{O.D_{(VDM)}} \times 100$$

V= vitamin; SM= supplemented media; DM= deficient media; VDM= vitamin deficient media

B. Fermented semi-solid milk-producing efficiency of the strains

Semi-solid milk is an essential fermented milk product used worldwide. The quality of fermented milk is assessed by parameters such as starter culture viability, acidity, and solidification. The solidification of semi-solid milk relies on the exopolysaccharide-producing efficiency of the strains. The methodologies of these assessments are described below:

Exopolysaccharides production efficiency

Exopolysaccharide (EPS) production efficiency of the strains was evaluated under three sugar compositions in MRS media: dextrose and lactose (5% each, w/v) and a combined dextrose-lactose (2.5% each, w/v). For EPS production, the strains were grown in EPS-producing MRS broth (amended with the respective sugar) overnight at 37 °C. The active culture was inoculated into EPS-MRS media (50 mL each) at 2% (v/v) and incubated at 37 °C for 24 h. The supernatant was collected after centrifugation at 7168 x g for 10 min. The pellet was washed with 0.9% saline solution and dried in a hot-air oven (~60 °C) until cell powder formed for dry mass calculation. To the supernatant, 10% TCA (final concentration, v/v) was added, and the mixture was kept at 4 °C for 12 h. The protein precipitates were removed by centrifugation at 7168 x g for 10 min. The exopolysaccharide was precipitated from the supernatant with 30% (v/v) ethanol and harvested by centrifugation. The precipitated exopolysaccharide was washed with acetone and quantified using the phenol-sulphuric acid method (Dubois et al., 1956), with dextrose as the standard. The exopolysaccharide production efficiency is expressed as mg EPS/g dry mass.

β-galactosidase activity

The β-galactosidase activity of the strains was determined using p-nitrophenyl galactopyranoside (PNPG) as the substrate. For β-galactosidase activity, the strains were inoculated into MRS-L (MRS amended with an equal proportion of D-lactose in place of D-dextrose) broth at 1% (v/v) from an overnight culture and incubated for 12 h at 37 °C. Cells were then harvested by centrifugation at

7168 x g for 10 min at 4 °C. The pellet was washed twice with sterile water, resuspended in 100 mM PEM buffer (100 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM magnesium chloride, pH 7.0), and the cell density was adjusted to 1.5 (O.D. at 620 nm). The suspension was kept on ice until use. The cell-free culture supernatant (CFS) was also collected for assay. The β -galactosidase assay mixture was prepared by mixing 2 mL CFS with 0.2 mL of 0.01 M PNPG (in 100 mM PEM buffer, pH 7.0), and incubating at 37 °C for 30 min in a digital water bath. The reaction was stopped by thoroughly mixing 0.5 mL of 2 M sodium carbonate. The mixture was then centrifuged at 7168 x g for 15 min, and the supernatant was measured at 420 nm using a spectrophotometer (UV-Vis 1800, Shimadzu, Japan). The residual PNP concentration was determined from the PNP standard curve. One unit of β -galactosidase is defined as the amount of enzyme required to hydrolyze one mM of PNPG/min under the assay conditions.

Assessment of fermented semi-solid milk quality

Following the standard protocol (Nagaoka 2019), semi-solid milk was produced using monoculture and mixed culture strains. In brief, the strains were grown in MRS broth overnight at 37 °C, cells were harvested by centrifugation at 7168 x g for 10 min, washed with sterile water, resuspended in sterile water, and kept on ice until use. 50 mL of homogenized milk {Amul Taaza, India, which comprises total fat 3 g; saturated fat 1.9 g; Cholesterol 8 mg; total carbohydrate (including milk lactose) 4.7 g; added sugar 0 g; Protein 3.1 g; Calcium 150 mg; Phosphorous 130 mg; Sodium 50 mg; Thiamine 42.5 μ g; riboflavin 120 μ g; Niacin 100 μ g; Folic acid 7.5 μ g; Vitamin A 75 μ g; Vitamin D 0.5 μ g; Approx. values per 100 mL were taken in a sterilized container, heated to 85–90 °C for 10 min, and then cooled to ~40 °C. The cell suspension ($\sim 2 \times 10^8$ CFU/mL for monoculture and 1/5 of each strain for mix culture) was inoculated into the respective milk preparation at 2% (v/v, either mono- or mix culture), mixed thoroughly, and incubated at 30 °C for 15 h. The preparation was cooled to refrigerated temperature (5 °C) for 12 hrs and observed for gelling. All the preparations were made under normal environmental conditions. The semi-solid milk quality was assessed by microbiological analysis, such as cell viability, and by physical characterization, including pH, the degree of whey syneresis, β -galactosidase retention activity, and residual lactose in the fermented milk.

a) Microbiological analysis

The viable cells of fermented milk bacteria were counted by aseptically taking 1 g of the fermented semi-solid milk sample after 1 day of storage at 5 °C. The sample was then vortexed in 10 mL of sterile distilled water for 2 min and diluted 10^{-7} . One mL of suspension was poured onto the plates of sterile bromocresol purple plate {comprising of Peptic digest of animal tissue 10.00 g; Meat extract 3.00 g; Sodium chloride 5.00 g; Lactose 10.00 g; Bromo cresol purple 0.020 g per 1000 mL; Final pH 7.2 ± 0.2 at 25 °C} count agar (HiMedia, India), incubated at 37 °C for 72 h and the number of viable colonies were counted and calculated as CFU/mL/g of fermented milk mass. The final pH was also checked with a pH meter (pH Tutor, Eutech, Germany).

b) Physical characterization of semi-solid milk

The physical properties, such as whey syneresis, of fermented milk samples were measured after 1 day of storage at 5 °C. The degree of whey syneresis of semi-solid milk (30–40 g) was defined as the ratio (%) of the volume of supernatant to precipitate after centrifugation (2150 x g, 10 min, 4 °C) to the semi-solid milk sample (Keogh and O'Kennedy 1998).

c) Residual lactose content estimation

The β -galactosidase retention activity assay of the starter culture in semi-solid milk was performed by taking 2 mL of the whey sample and measuring it as described earlier. The lactose content in the spent whey was determined using the Lane-Eynon titration method (Kim et al., 1997).

$$\text{Lactose (\%)} = \frac{[(x - y) \times 0.05]}{2.5}$$

where x = Volume of lactose solution used in the control sample; and y = Volume of lactose solution used in the whey sample (Sharma et al., 2013).

Statistical analysis

All experiments were performed in triplicate. The results were expressed as the mean $\pm$ SE of three replicates. Image processing of the whole-cell SDS-PAGE was done using the “ImageJ” image processing software, and the dendrogram was created using R 3.6.3 statistical software.

Results and Discussion

Characterization of the isolates

Forty LAB strains were isolated from traditional fermented foods, including Chhurpi, Gundruk, Janr, Kinema, Sinki, and Whey. Five isolates were selected for further studies based on the fundamental

in vitro probiotic assessment (Bile salt hydrolysis, bile salt tolerance, and tolerance to acidic pH). The isolates were designated as LAB-04, LAB-06, LAB-11, LAB-39, and LAB-40 (Figure 1). The morphological, biochemical, and sugar utilization profiles of the LAB strains are presented in Table 1. It was found that all strains were aerotolerant, microaerophilic, catalase-negative, Gram-positive, homo- or heterofermentative, and non-spore-forming. There were significant variations in sugar utilization among the strains. All the strains could ferment D-arabinose, Dextrose, Dulcitol, Esculin hydrolysis, Fructose, Galactose, Glycerol, Inositol,

Inulin, Maltose, Mannitol, Mannose, Melibiose, Raffinose, Salicin, Sodium gluconate, Sorbitol, Sucrose, Trehalose, Xylitol, Xylose; while all of them failed to ferment Arabitol, α -Methyl-D-glucoside, and α -Methyl-D-mannoside, Sorbitol, and Sorbose; failed to utilize Citrate, Lysine, Ornithine, Phenylalanine, and did not produce H₂S and Urease. There were no significant differences in final pH among the sugars tested, with values ranging from approximately 4.3 to 4.8. All strains showed luxuriant growth at 4% NaCl (w/v) and tolerated up to 6.5% NaCl. The strains had pH tolerance ranging from 4.5 to 9.6.

Table 1. Morphological and biochemical characteristics of the strains

Morphological Characteristics	LAB-04	LAB-06	LAB-11	LAB-39	LAB-40
Colony colour	Whitish	Whitish cream	Whitish cream	Whitish	Whitish
Cell shape	<i>Bacillus</i> 2-3 cells in a chain	<i>Bacillus</i> 2-3 cells in a chain	<i>Coccus</i> , 2 cells in a chain	<i>Bacillus</i> , 2 cells in a chain	<i>Bacillus</i> , 2-3 cells in a chain
Cell size	Small, 3x1.07 μm^2	Small, 3.21x1.2 μm^2	Small, 2.8 μm	Large, 2.5x1.0 μm^2	Large, 3x1.07 μm^2
Sugar fermentation					
Adonitol	++	++	–	–	++
Cellobiose	++	+++	+	–	+++
D-Mannose	++	+++	++	+++	+++
Erythritol	+	+	–	–	+
Galactose	++	+++	+	+++	++
Inositol	+	++	–	++	+++
Lactose	++	++	++	++	++
L-Arabinose	++	++	+	–	++
Mannitol	+++	++	–	+++	++
Melezitose	++	++	+	–	+++
ONPG	++	++	++	–	++
Rhamnose	+++	+++	+	–	+++
Ribose	++	++	+	+	++
Sorbitol	+++	++	–	++	+++
Xylose	++	++	–	+	++
Biochemical Tests					
Citrate utilization	–	–	–	–	–
Malonate utilization	++	+/-	–	+++	+
Nitrate	++	+/-	–	+++	+
Gas production	–	–	–	–	–
NaCl tolerance					
4%	+++	++	++	+++	+++
6.5%	++	+	++	+	++
10%	+	+	+	–	+
15%	+	–	–	–	–

Here, ‘+++’, indicates positive response with OD_{620nm} values > 1.8; ‘++’, indicates positive response with OD_{620nm} values > 1.0; ‘+’, indicates positive response with OD_{620nm} values $\geq 0 - 1.0$; and ‘–’, indicates negative response after 24 hrs incubation at 37°C. ND means not determined.

Here, all the strains were positive for D-arabinose, Dextrose, Dulcitol, Esculin hydrolysis, Fructose, Galactose, Glycerol, Inositol, Inulin, Maltose, Mannitol, Mannose, Melibiose, Raffinose, Salicin, Sodium gluconate, Sorbitol, Sucrose, Trehalose,

Xylitol, Xylose; and were negative for Arabitol, Citrate utilization, H₂S production, Lysine utilization, Ornithine utilization, Phynylalanine, Sorbitol, Sorbose, Urease, α -Methyly-D-glucoside, and α -Methyly-D-mannoside.

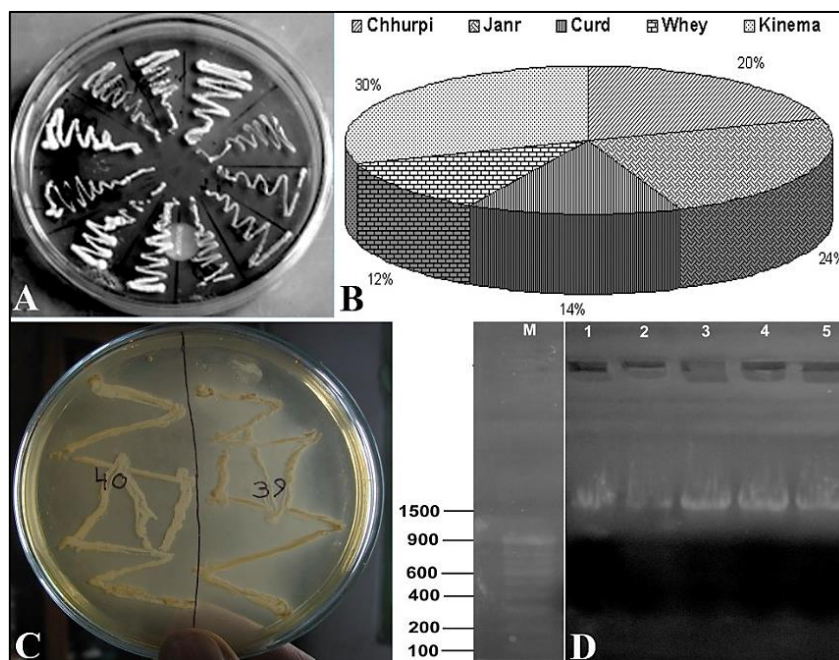


Figure 1. Isolation and assays of lactic acid bacteria for probiotic properties. (A) Pure culture maintenance on MRS agar plates; (B) Proportions of LAB isolates from the traditional fermented foods; (C) Assay for bile salt hydrolase; (D) Detection of PCR amplification of the selected strains in 1% agarose gel. Here, the numbers in Figure C indicate the strains LAB-39 and LAB-40, and the numbers in Figure D indicate the lane number. Lane 1: LAB-04; lane 2: LAB-06; lane 3: LAB-11; lane 4: LAB-39; and lane 5: LAB-40.

The whole-cell protein profiles and dendrogram analysis showed that the protein banding of the strains LAB-05 (a standard strain, *Pediococcus acidilactici* LAB-05, GenBank Ac. No.: GQ240304), LAB-06, and LAB-11 had some similarity, forming a clade, while the strains LAB-04, LAB-39, and LAB-40 formed another clade (Figure 2A-C). The 16S rRNA sequence analysis identified the strains as *Lactobacillus* sp. LAB-04 (GenBank Ac. No.: HQ141911), *Lactobacillus* sp. LAB-06 (GenBank Ac. No.: MG066499), *Pediococcus* sp. LAB-11 (GenBank Ac. No.: HQ141913), *Lactobacillus* sp. LAB-39 (GenBank Ac. No.: MG066500), and *Lactobacillus* sp. LAB-40 (GenBank Ac. No.: HQ141915). The phylogenetic positions of the strains are shown in Figure 2. The Blast search showed that *Lactobacillus* sp. LAB-04, *Lactobacillus* sp. LAB-06 and *Lactobacillus* sp. LAB-39 had 98%, 99%, and 99% similarity matches with *Lactobacillus brevis* ATCC 14869, respectively, a human isolate from the faecal

source, while the strain *Pediococcus* sp. LAB-11 showed 98% similarity to *Pediococcus acidilactici* NGRI 0510Q, an isolate from Ryegrass silage on Ishigaki Island, Okinawa, Japan, and *Lactobacillus* sp. LAB-40 is very closely related to the species *Lactobacillus plantarum* JCM 1149 (an isolate from Pickled cabbage), with a 99% match. The whole-cell protein profiles also show some similarities with the 16S rRNA phylogeny of the strains. The antibiosis assay revealed that none of the isolates exhibited antimicrobial activity against the food-associated pathogens or the LAB isolates. None of the isolates was alpha- or beta-hemolytic (figure not shown). All isolates were Vancomycin-resistant in antibiotic sensitivity testing at 30 μ g/mL, while the rest showed larger growth inhibition zones, indicating their sensitivity to these antibiotics at the respective doses. These safety assessment studies indicate that they are neither antagonistic nor pathogenic, negating their probiotic efficacy studies.

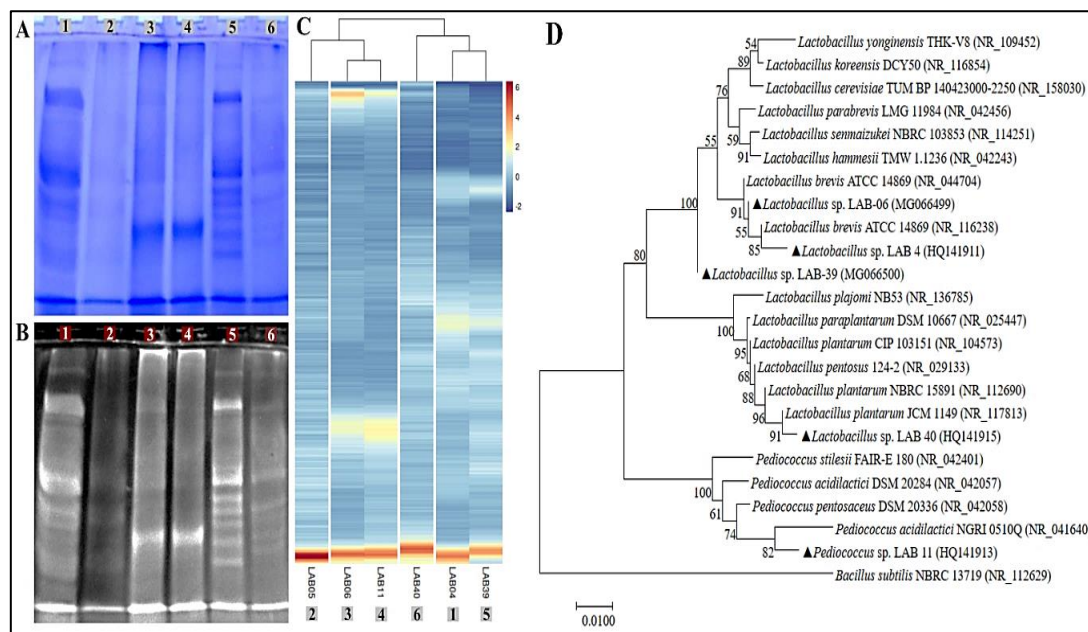


Figure 2. Whole-cell protein profile in 12% glycine SDS-PAGE and 16S rRNA phylogenetic tree of the strains as inferred by the neighbor-joining method. (A) Original image; (B) Processed image; (C) Dendrogram of clustered strains based on similarity in banding pattern in lanes; and (D) Phylogenetic tree. Here are the numbers in the figure. A and B show the lanes. Lane 1: LAB-04; lane 2: *Pediococcus acidilactici* LAB-05; lane 3: LAB-06; lane 4: LAB-11; lane 5: LAB-39; and lane 6: LAB-40. Here, the bar in Figure D indicates the bootstrap distances, based on 1000 repetitions. The triangular symbol represents the strains in the present study.

A. Probiotic traits

Acid tolerance

Gastric pH tolerance is a fundamental challenge for probiotic strains. To survive and colonize the GI tract in appreciable numbers, the strain must tolerate and survive the hostile acidic pH (~2.0) of the stomach for at least 1 hour. The acidic tolerance study showed a moderate decrease in CFU counts (from approximately log 9.0 to log 6.0) up to 90 minutes after the treatment; cell survival decreased to log 4.0 (Figure S1). Acidic pH stress tolerance varied among strains, including *Pediococcus* sp. LAB-11 was more acid-tolerant, and *Lactobacillus* sp. LAB-04 was very sensitive to acidic pH. The rapid decrease in CFU in prolonged incubation time might also be due to starvation, as the treatment solution contained only mineral salts. Fernandez et al. (2003) found that acidic gastric salt treatment reduced the CFU number to approximately 4.0 log CFU/mL from 8 log CFU/mL in *Lactobacillus acidophilus* UO 001 and *Lactobacillus gasseri* UO 002 (Fernandez et al. 2003). Liong and Shah studied the acid tolerance of 11 *Lactobacillus* strains in MRS-ox gall (0.3%, w/v, pH 2.0) broth for 2 h at 37 °C and found significant variation in viability (Liong and Shah 2005). Margalho et al. (2021) reported that among the 220 wild LAB isolates from Brazilian artisanal cheeses (BAC), 92 (41.8%) were resistant to low pH (2 and 3). Thus, compared to the other

reported strains, the present five strains were more tolerant of the acidic gastric condition.

Bile salt tolerance and deconjugation potential of the strains

Bile salt tolerance and bile salt deconjugation efficiency are crucial factors in selecting a probiotic strain. It has been hypothesized that higher bile salt deconjugation efficiency leads to greater cholesterol removal from serum (Mandal et al., 2009). Therefore, identifying more potent bile salt-deconjugating strains is a pressing need to combat hypercholesterolemia through probiotics. The present study revealed that on MRS-ox bile agar plates, the strains exhibited a foggy halo around the colonies (Figure 1C), indicating their ability to deconjugate bile salts by releasing bile salt hydrolase (BSH). All five strains showed acceptable bile salt tolerance up to 5% (w/v) ox-bile at 37 °C, which is a much higher bile tolerance than the human gut content (13–46 meq/L). With increasing ox-bile concentration, all strains showed reduced growth compared to the control (Figure S2). Among these, *Pediococcus* sp. LAB-11 showed greater bile salt tolerance than the others. However, bile salts significantly affected the growth of all strains (Figure 3a-e). It was estimated that LAB-06 and LAB-11 had the highest bile salt deconjugation (4.89 μ M/mL, 5.87 μ M/mL, respectively) in the anaerobic condition, and that this increased with the incubation time (Figure 3f-j).

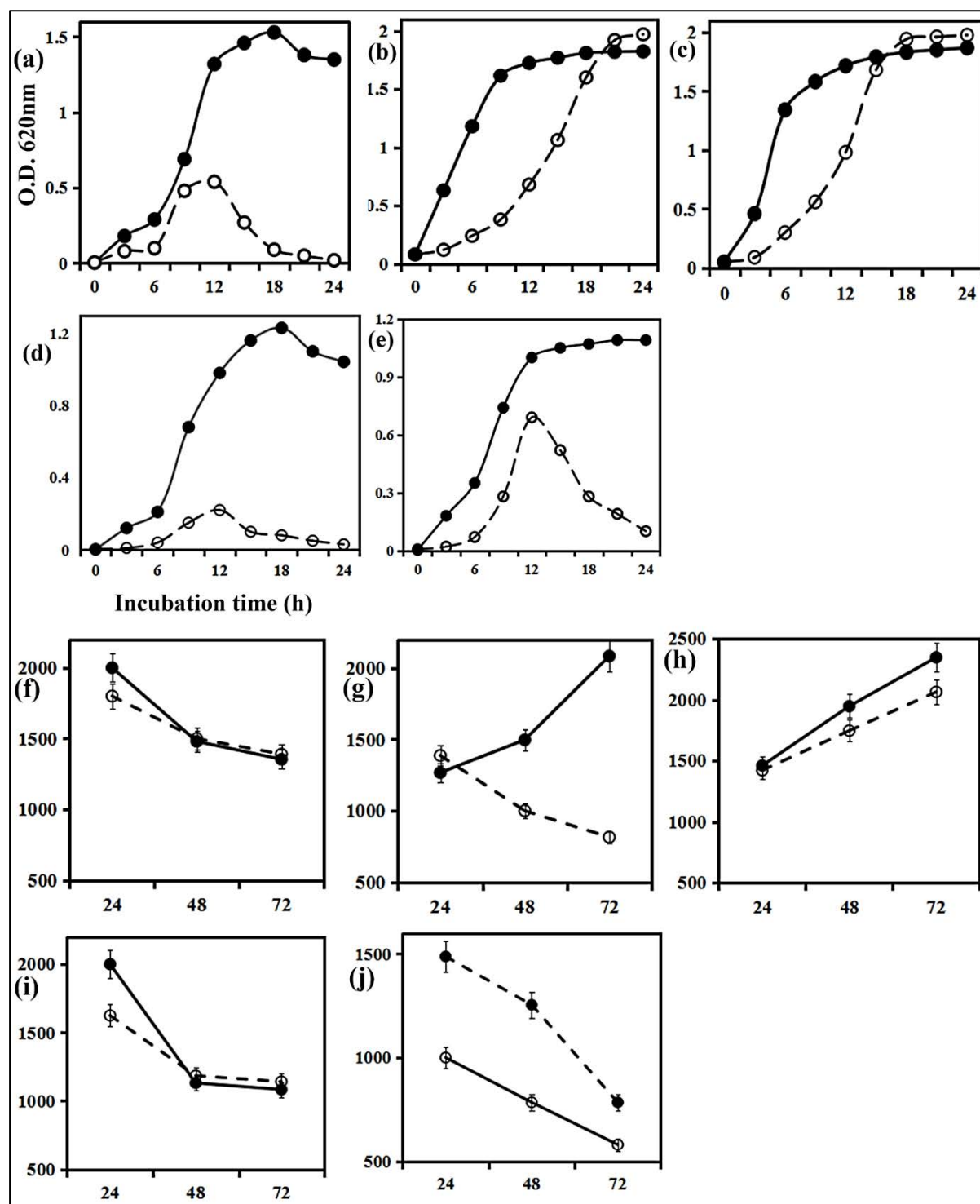


Figure 3. Effect of bile salt and dissolved oxygen availability on growth and bile salt deconjugation by the strains at 37 °C. (a & f) LAB-04; (b & g) LAB-06; (c & h) LAB-11; (d & i) LAB-39; and (e & j) LAB-40. The results shown here were the average of triplicate trials $\pm$ standard error. Here, the solid lines in figures 'a-e' indicate MRS w/o ox-bile, and dotted lines indicate MRS+ ox-bile, and the solid lines in figures 'f-j' indicate aerobic conditions, and the dotted line indicates anaerobic conditions.

In contrast, LAB-04 and LAB-39 exhibited significantly higher bile salt deconjugation (4.89 $\mu\text{M/mL}$ and 2.44 $\mu\text{M/mL}$, respectively) at 24 h of incubation. Moreover, it is noticeable that the anaerobic condition significantly affected LAB-06 and LAB-40 compared to the other strains (Figure 3f-j). The bile salt deconjugation efficiency was

much higher in all strains than in the reported strains *Lactobacillus gasseri* SBT 0270 and *L. gasseri* SBT 0274 (1.88 $\mu\text{M/mL}$ and 1.94 $\mu\text{M/mL}$, respectively). Corzo and Gilliland (1999) reported a BSH activity of 25.5 nmol/min per mL for *Lactobacillus acidophilus* O16, a human isolate.

It was found that the present strains had much more potent bile salt hydrolase activity (Figure 4a). It was also observed that with the gradual increase in cell density, there was an increase in the deconjugation rate, and it reached a maximum during the exponential phase of growth, and after that, it

decreased gradually. Among the strains, LAB-39 showed the highest bile salt deconjugation (7.63 $\mu\text{M/mL}$) at 9 h of incubation, followed by LAB-04 and LAB-40 (6.07 $\mu\text{M/mL}$ and 6.46 $\mu\text{M/mL}$, respectively).

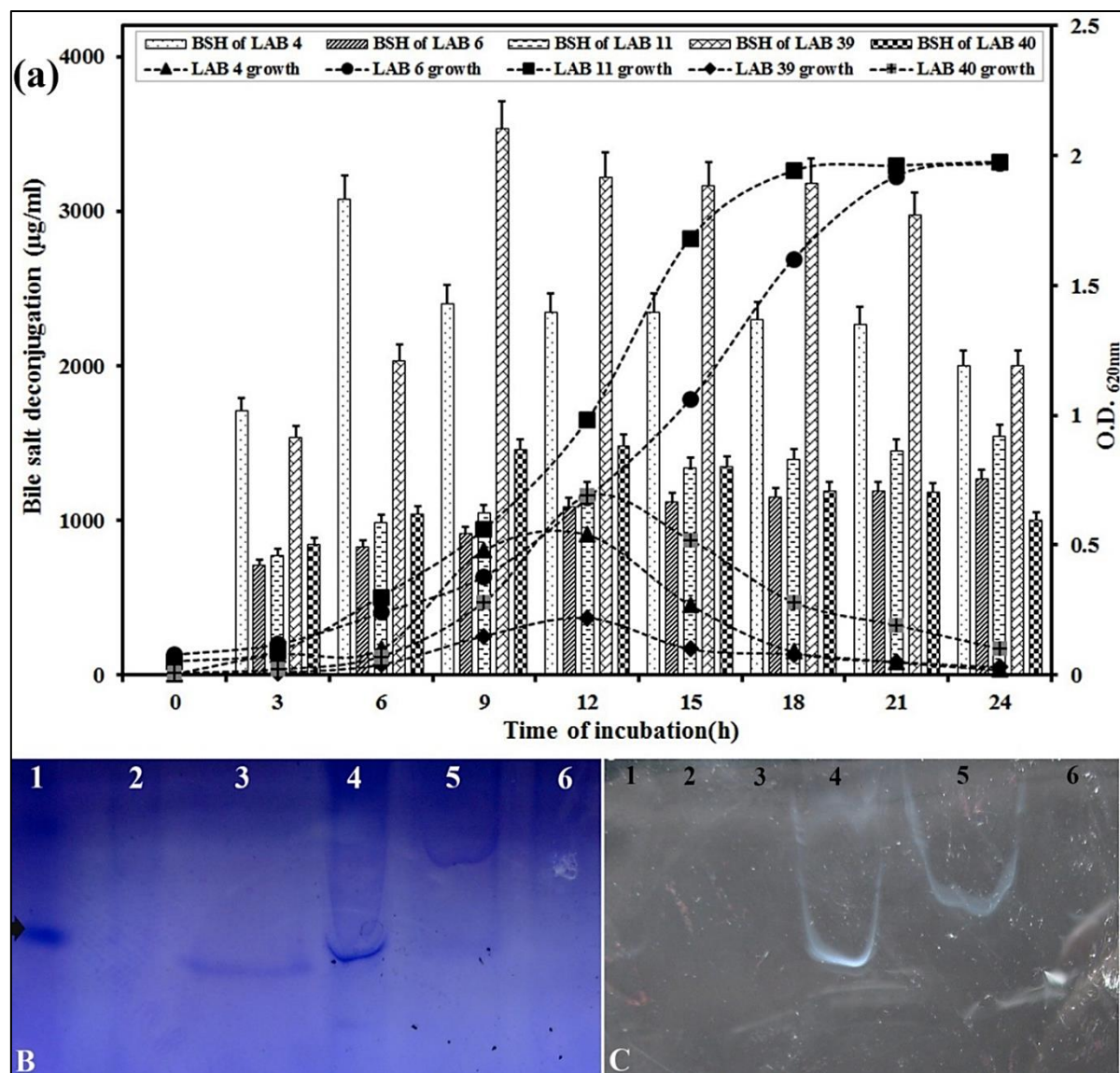


Figure 4. BSH activity of the strains during growth at 37 °C and analysis in 10% native PAGE and zymogram assay. (a) BSH activity of the strains during growth; (b) Coomassie-stained gel; and (c) BSH zymogram assay. BSH activity was estimated as cholic acid released ($\mu\text{g/mL}$). In Figure A, the bar represents the bile deconjugation, and the line represents the growth. Results shown here were the average of triplicate trials $\pm$ standard error. Here, the numbers in Figures b and c indicate the lanes. Lane 1: BSA (66.0 kDa); lanes 2-6 indicate the strain names, viz. Lane 2: LAB-04; lane 3: LAB-06; lane 4: LAB-11; lane 5: LAB-39; and lane 6: LAB-40, respectively. The arrow indicates the standard MW of BSA.

Interestingly, the strains with significantly lower growth in bile salt-amended media (LAB-04 and LAB-39) had higher bile salt deconjugation potential. Thus, the slow-growing strains have higher enzyme titers than the fast-growing ones. Usman and Hosono reported that the most rapid deconjugation by *Lactobacillus gasseri* SBT 0274

and *L. gasseri* SBT 0270 was achieved during 6 to 9 h of incubation at 37 °C, with the full cholic acid release (2.03 and 1.58 $\mu\text{M/mL}$, respectively) at 12 h of incubation (Usman and Hosono 1999). Thus, compared with other published results, the present strains had much higher bile salt-hydrolyzing efficiency. The BSH purification and subsequent

zymogram analysis in native PAGE showed that the MW was 50-100 kDa, using BSA as a standard (Figure 4b, c).

Cholesterol-lowering efficiency of the strains in vitro

High serum cholesterol is a major concern for good health. The use of probiotic strains to reduce serum cholesterol is now a new vista in combating hypercholesterolemia. Therefore, potent probiotic strains with higher cholesterol removal efficiency might contribute to this field. The present study shows that the strains LAB-04, LAB-11, and LAB-39 had greater cholesterol removal efficiency in anaerobic conditions at 48 h incubation than at 24 h (Figure 5A-E). However, the cholesterol removal capability of the strain LAB-06 was higher in aerobic conditions than in anaerobic conditions, while anaerobicity did not cause any drastic change in the cholesterol removal capacity of LAB-40. The cholesterol-removal ability of all strains decreased gradually over 72 h of incubation. The cholesterol-removal capability of LAB-39 at 48 h was slightly higher (88.67 µg/mL) than that of the reported

Lactobacillus gasseri SBT 10241 (86.5 µg/mL) strain under anaerobic conditions (Usman and Hosono 1999). Usman and Hosono and Kimoto et al. opined that *in vitro* removal of cholesterol from media aliquot by the *Lactobacillus* strains was either due to binding of cholesterol to that organism or due to co-precipitation with cholic acid in bile salt amended media, as opined by Usman and Hosono (1999); Kimoto et al. (2002); Zhang et al. (2008). Taranto et al. (2000) also demonstrated that the removal of cholesterol by *Lactobacillus reuteri* CRL 1098 was closely related to the presence of bile salts in the culture medium. However, in this experiment, no bile salt was added to the cholesterol-MRS medium. Therefore, the strains' cholesterol-removal efficiency was not due to co-precipitation but rather to binding to the cells. The daily faecal loss of bile acids is only about 5%, replenished by *de novo* bile acid synthesis from cholesterol in the liver. Therefore, these strains' higher bile salt deconjugation efficiency could reduce serum cholesterol by releasing free cholic acid in faeces more than others.

Table 2. Auto-aggregation and coaggregation of the LAB strains

	Auto-aggregation (%), Mean ± SD				
Strain	1 h	3 h	6 h	24 h	
LAB4	25.1 ± 1.07	55.1 ± 1.67	73.7 ± 1.89	94.6 ± 2.22	
LAB6	22.3 ±1.42	46.7 ± 1.2	56.5 ± 1.81	94.8 ± 1.83	
LAB11	48.7 ±1.30	63.7 ± 1.89	87.7 ± 1.89	96.7 ± 1.92	
LAB39	42.2 ±1.71	66.7 ± 1.39	80.2 ± 1.36	83.7 ± 1.19	
LAB40	23.3 ± 1.22	53.3 ± 2.20	73.7 ± 1.89	85.2 ± 1.50	
	Co-aggregation (%), Mean ± SD				
Strains	1 h	3 h	Strains	1 h	3 h
LAB4 x Lm	12.1 ±1.30	25.8 ±0.80	LAB4 x St	7.5 ± 2.05	17.1 ±0.92
LAB6 x Lm	38.5 ±1.40	52.3 ±1.42	LAB6 x St	11.9 ±2.00	25.6 ±2.23
LAB11 x Lm	42.5 ±1.47	48.7 ±1.30	LAB11 x St	11.3 ±3.78	22.3 ±1.90
LAB39 x Lm	28.0 ±1.51	42.2 ±1.71	LAB39 x St	13.9 ±2.00	42.5 ±1.47
LAB40 x Lm	21.0 ±1.31	52.2 ±1.71	LAB40 x St	12.3 ±1.62	17.5 ±1.70

Data represented as Mean ± SD, each in triplicate. Here, Lm and St denote *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium, respectively.

Hydrophobicity test, auto aggregation, and coaggregation of the strains

A probiotic bacterium must adhere to the gut epithelial cells to colonize the host tissues. This

colonization helps the organism exert beneficial health effects over a long time and outcompete pathogenic bacterial colonization. It has been hypothesized that cell-surface hydrophobicity may

mediate the hydrophobic interactions between probiotic strains and the hydrophobic surfaces of intestinal membranes (Greene and Klaenhammer 1994; Reid et al. 2003). Cell-surface lectin-like proteins have been suggested to play an essential role in the defense, adhesion, and recognition of bacterial cells (Isberg and Barnes, 2002). The broad range of hydrophobicity in Lactobacilli was observed to be 6-73% (Greene and Klaenhammer, 1994; Kos et al., 2003; Dhewa et al., 2008). The higher hydrophobicity value indicates a greater

ability of Lactobacilli to adhere to epithelial cells. The present study shows that *Lactobacillus* sp. LAB-04, *Pediococcus* sp. LAB-11 and *Lactobacillus* sp. LAB-40 had good adhesive properties with an average HI of approximately 30%, while *Lactobacillus* sp. LAB-06 and *Lactobacillus* sp. LAB-39 had low hydrophobicity (Avg. HI approx. 2–5) (Figure 5f). Thus, in vitro hydrophobicity tests indicate that these strains could adhere to the host epithelium and exhibit better probiotic potential through colonization of host tissue.

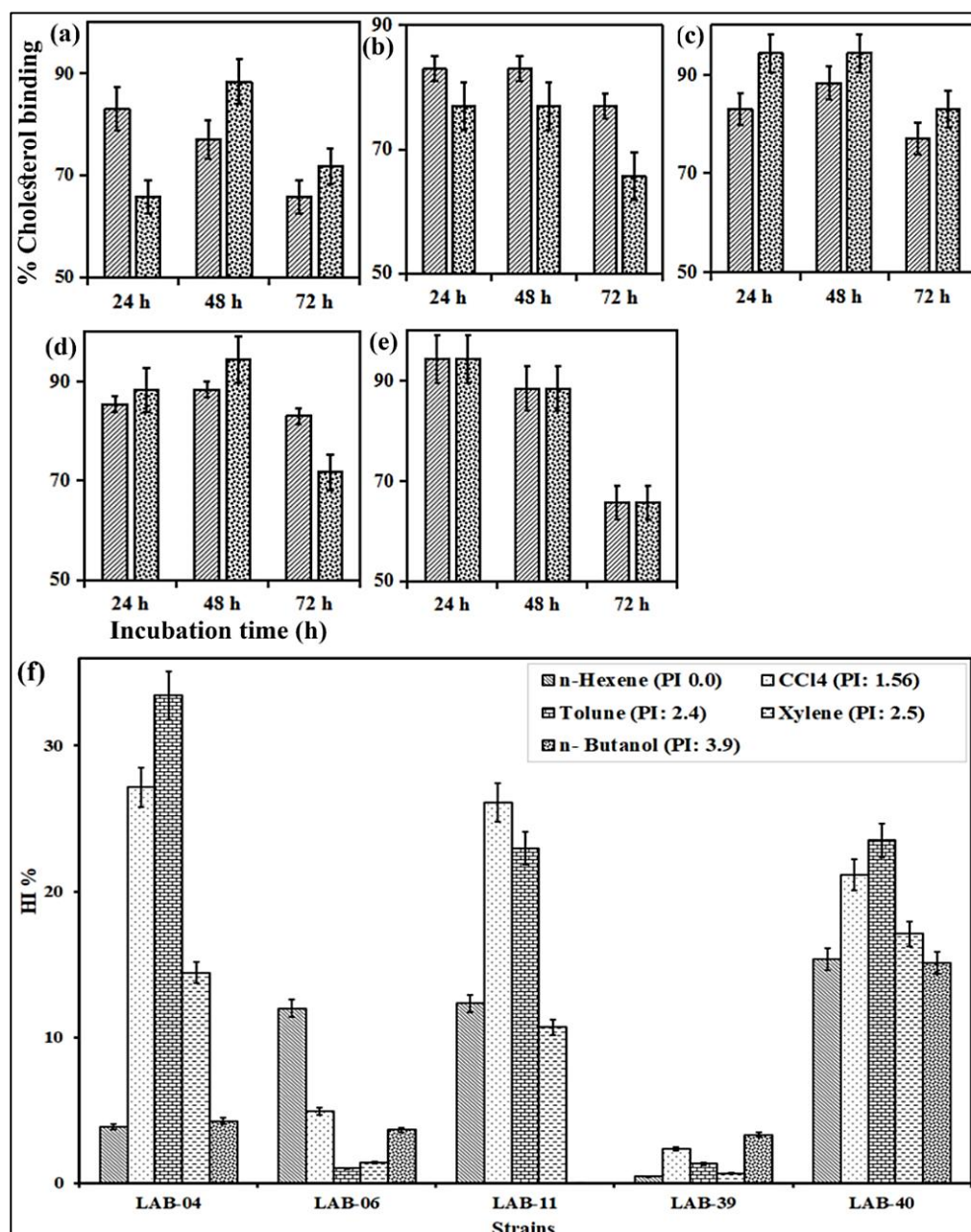


Figure 5. Cholesterol binding and cell-wall hydrophobicity of the strains. (A-E): Cholesterol binding of the strains; (F) Cell-wall hydrophobicity. Here, the figures (A) LAB-04; (B) LAB-06; (C) LAB-11; (D) LAB-39; and (E) LAB-40. The results shown here are the averages of triplicate trials ($\pm$ standard error). The initial cholesterol concentration in the medium was 100 μ g/mL. The crossbars indicate aerobic, and the dotted bar indicates anaerobic conditions. (F) Here, the solvents used are Carbon tetrachloride (CCl₄, PI 1.56), Toluene (PI 2.4), n- Butanol (PI 3.9), Xylene (PI 2.5), and n-Hexane (PI 0.0). PI indicates the polarity index with its values.

Auto-aggregation and coaggregation are important characteristics in the preliminary screening of probiotic bacteria for potential use. The percent auto-aggregation of various LAB strains ranges between 22.3 ± 1.42 – 48.7 ± 1.30 , 46.7 ± 1.2 – 66.7 ± 1.39 , 56.5 ± 1.81 – 87.7 ± 1.89 , and 83.7 ± 1.19 – 96.7 ± 1.92 for the period of 1, 3, 6, and 24 h, respectively (Table 2). The percent auto-aggregation value increases over time, and comparatively higher values ($>90\%$) were observed in the LAB-04, LAB-06, and LAB-11 strains. All the LAB strains coaggregated with pathogenic strains to varying degrees. Higher coaggregation percentages were observed after 3 h than after 1 h (Table 2). Similar percentages of auto-aggregation and coaggregation were observed in other *Lactobacillus* strains with *S. aureus* and *S. typhimurium*, respectively (Lang et al., 2010; Tuo et al., 2013; Ahmed et al., 2018).

B. Fermented semi-solid milk production traits

Exopolysaccharides production efficiency

The exopolysaccharide (EPS) production efficiency of the strains is shown in Figure 6A. It was found that there were significant variations in EPS production among the strains when specific carbon (sugars) and nitrogen (yeast extract) sources were

used. It was found that lactose was a better sugar source than dextrose or a dextrose-lactose mix for LAB-04, LAB-06, and LAB-40, whereas dextrose alone or a dextrose-lactose mix was more productive for LAB-39 and LAB-11, respectively. It was found that yeast extract produced EPS levels similar to those of dextrose and/or lactose in strains LAB-11 and LAB-40, whereas it failed to surpass the carbon sources in strains LAB-04, LAB-06, and LAB-39.

This indicates that the strains could produce a higher EPS titer without additional nitrogen requirements for large-scale production or during milk fermentation. Moreover, a small amount of glucose amendment may enhance EPS production during milk fermentation. The EPS production efficiency was promisingly high compared to other reported strains, such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus sake* 0-1 (Thapa and Zhang, 2009). The exopolysaccharides produced by the strains were not ropy or slimy in texture. Upon harvest, it produced a powdery appearance, indicating its non-sticky nature and ability to form hydrocolloids. So, the EPS produced by the strains could significantly impact the viscosity of the fermented milk products.

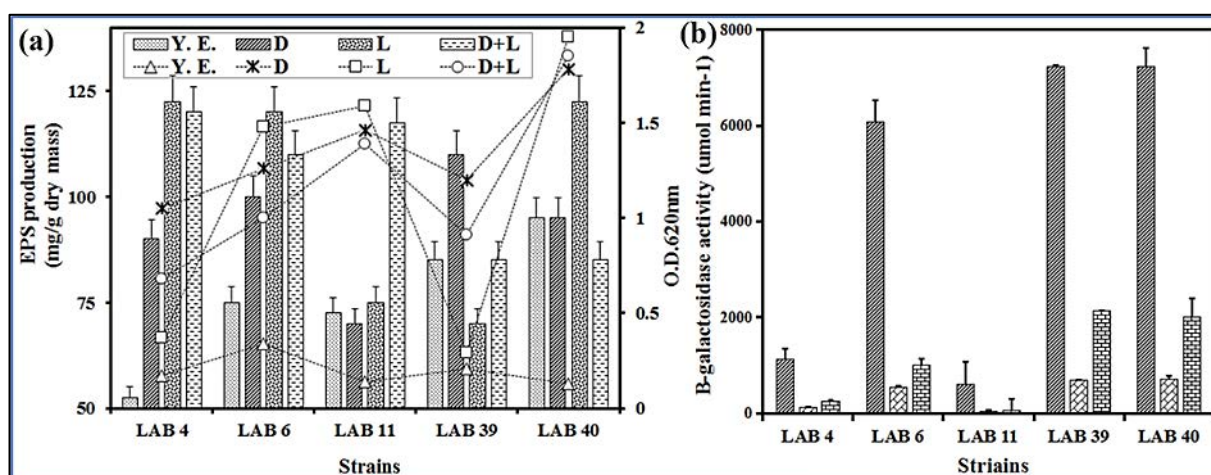


Figure 6. Exopolysaccharides and β -galactosidase production efficiency of the strains. (A) Exopolysaccharides production; (B) β -galactosidase activity. In Figure A, the bars indicate exopolysaccharide production, while the dotted lines indicate the respective media's growth (O.D._{620nm}). The abbreviations YE, D, L, and D+L indicate yeast extract, dextrose, lactose, dextrose mix, and lactose. In Figure B, the striped bars indicate the β -galactosidase activity of whole-cell strains, cross-striped bars indicate cell-free broth, and check bars indicate semi-solid milk preparation. The results shown here are the averages of triplicate trials ($\pm$ standard error).

Folate and niacin production efficiency

The study revealed that all strains exhibit significantly reduced growth in folic acid- and niacin-deficient media compared with the traditional MRS medium. They showed much-reduced growth

in the vitamin-deficient media, except for LAB-40 in the folic acid medium. However, vitamin fortification in the respective media has slightly enhanced the growth of both niacin and folic acid, as shown in Table 3. This indicates that the strains could compensate for the vitamin requirement by synthesizing folic acid and niacin.

Table 3. The growth of the strains in folic acid- and niacin-deficient media compared with the traditional MRS medium

Name of the vitamin	OD _{620 nm}				
	LAB-04	LAB-06	LAB-11	LAB-39	LAB-40
Folic acid	0	12%	8%	22%	10%
Niacin	16%	0	13%	3.5%	0
MRS	100%	100%	100%	100%	100%

The study shows that different LAB strains like *Bifidobacterium* spp., *Lactococcus lactis*, *Leuconostoc* spp., and *Streptococcus thermophilus* were able to produce a high amount of folate, while, among the *Lactobacillus* species, only *Lactobacillus plantarum* could produce folate, whereas other species were shown to consume it (Sybesma et al. 2003; LeBlanc et al., 2011; Capozzi et al., 2012; Mosso et al., 2018; Zhang et al., 2020). B-group vitamins are promising in preventing and treating disorders such as inflammation, colitis, mucositis, and colon cancer. Therefore, vitamin-producing lactic acid bacteria, especially strains that produce folate and niacin, could be used as adjunctive treatments for this wide range of diseases.

Fermented semi-solid milk production efficiency

The monoculture fermented milk preparations had higher whey content (less solidified) than the mixed-culture preparations. The degree of whey syneresis was higher (20-30%) in monoculture than in mixed-culture (~12%) fermented milk samples. This value was significantly lower than the reported fermented milk preparation (Vareltzis et al., 2016). The average CFU/mL/g of viable cells was log 7.477 for monoculture and log 9.641 for mixed culture. This indicates that fermented semi-solid milk may serve as a suitable substrate for probiotic use by the strains. The final pH of semi-solid milk was 4.5-5.5 for monoculture and 4.5-4.9 for mixed culture. The β -galactosidase activity in broth culture and fermented milk preparation was high in whole cells. However, it is significantly lower in the culture aliquot (Figure 6B). This indicates that the enzyme is cell-bound and maintains its galactosidase activity in fermented milk preparations. The probiotic strains also significantly reduced the residual lactose in the fermented milk preparation, either in monoculture (avg. 0.125% lactose) or in mixed culture (avg. 0.086% lactose). So, the strains could be promising starter cultures for the preparation of probiotic fermented milk (Heller 2001). Vareltzis et al. reported that the addition of whey protein

concentrate (WPC), albumin, sodium caseinate, or a mixture of these in the yogurt fermentation leads to a significantly lower syneresis (7.5%) and higher consistency (2000 cp) than untreated control sets (25% and 1500 cp, respectively) (Vareltzis et al. 2016). The use of probiotic fermented milk has been reported to combat different non-communicable diseases and improve personal health (Mattila-Sandholm et al. 2002; Gille et al. 2018). Also, the use of different prebiotics in this fermented milk preparation could help relieve constipation, suppress diarrhoea, and reduce the risk of osteoporosis, atherosclerotic cardiovascular disease, insulin resistance, obesity, and type-2 diabetes. The synbiotic, i.e., a combination of probiotics and prebiotics, might also improve the survival of bacteria as they cross the upper gastrointestinal tract, thereby enhancing their effects in the large bowel and making their effects additive or synergistic.

Conclusion

The assessment of five lactic acid bacteria strains isolated from naturally fermented foods of the Darjeeling hills for in vitro probiotic potential shows that the strains exhibit good gastric pH tolerance, bile salt deconjugation, cholesterol removal, and high exopolysaccharide production efficiency, and can produce semi-solid fermented milk. These indigenous strains exhibit greater efficacy in bile salt deconjugation and thus have a higher cholesterol-removal capacity than the reported strains. The high β -galactosidase activity of the strains also enables their use as dairy inoculants in the preparation of lactose-free cheese or semi-solid milk. Thus, it can be concluded that these five strains have some promise for use as a consortium in the production of homemade fermented semi-solid milk to combat lactose intolerance and malnutrition. Further, an in vivo probiotics study would validate the strains for their safe use as a dairy starter.

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Compliance with ethical standards

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Availability of data and material: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest: The authors declare that they have no conflict of interest.

Authors' contributions

All authors contributed to the study's conception and design. Biplab Bandyopadhyay, Amit K. Chakraborty, Preetika Chhetri, Rajsekhar Adhikary, Subhajit Mandal, Rupam Saha: performed the experimentation, methodology, investigation, data curation, validation, and writing of the original manuscript draft. Prashanta Kumar Mitra did the whole-cell protein profile analysis and data visualization; Vivekananda Mandal made the conceptualization, design of experimentation, formal analysis, validation, fund acquisition, supervision, visualization, writing – review, and editing of the final manuscript.

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