JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

COMPARATIVE CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM BOVINE AND NON-BOVINE MILK: FUNCTIONAL, ANTIMICROBIAL AND COSMETIC PERSPECTIVES

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https://doi.org/10.55251/jmbfs.13888

ARTICLE INFO

Received 28. 1. 2026
Revised 22. 3. 2026
Accepted 25. 3. 2026
Published 1. 4. 2026

Regular article

OPEN ACCESS

ABSTRACT

Lactic acid bacteria (LAB) are promising probiotic microorganisms with applications in food, health, and cosmetic industries. The objective of this laboratory-based experimental study was to isolate LAB from bovine and non-bovine milk and compare their functional, antimicrobial, and cosmetic potential. A total of 30 raw milk samples (camel, goat, cow, and buffalo) were collected from dairy farms in Mumbai and neighbouring areas. LAB isolates were evaluated for probiotic characteristics including tolerance to bile salt, phenol, acidic pH, and NaCl, along with aggregation ability, hydrophobicity, and antibiofilm activity. Safety assessment was performed using haemolysis assays, and antimicrobial activity was evaluated using resazurin-based minimum inhibitory concentration (MIC) assays against selected pathogens. Phenotypic identification was followed by molecular confirmation using 16S rRNA gene sequencing. Statistical analysis was performed using one-way ANOVA and results were expressed as mean ± standard deviation. The results demonstrated strong bile tolerance percentage by isolate GMI 1 (87.6±0.04 %), phenol tolerance by isolate GMI 1 (85.8±0.15 %), and NaCl tolerance by isolate CMI 3(88.71±0.06%). Furthermore, GMI 1 demonstrated best survival at pH 2 (80.18±0.22 %) and pH 3 (90.32±0.47 %). Strains BFI 2 (Buffalo milk), CMI 3 (Cow milk), and GMI 1 (Goat milk) exhibited the most promising probiotic properties including high hydrophobicity, auto-aggregation, and antibiofilm activity against pathogens such as *Acinetobacter baumannii*. All isolates were non-haemolytic and exhibited gamma haemolysis on blood agar plate. Molecular analysis identified the potential isolates as *Lactiplantibacillus plantarum* (BFI 2), *Ligilactobacillus salivarius* (CMI 3) and *Lactobacillus delbrueckii subsp. bulgaricus* (GMI 1). These findings highlight the potential of selected LAB strains as promising candidates for probiotic-based therapeutic and cosmetic applications, particularly for skin health.

Keywords: Lactic acid bacteria, probiotics, bile tolerance, acid tolerance, antimicrobial activity, biofilm inhibition, 16S rRNA sequencing

INTRODUCTION

Lactic acid bacteria (LAB) are a diverse group of Gram-positive, non-spore-forming microorganisms widely recognized for their beneficial roles in food fermentation and human health. These bacteria are commonly found in dairy products, fermented foods, and the gastrointestinal tract of humans and animals (**Voidarou *et al*., 2021**). LAB are known to produce organic acids, bacteriocins, exopolysaccharides that contribute to food preservation, confer antimicrobial activity, and modulate host microbiota **(Hernández-González *et al., 2021; Jadhav & Narayanaswamy, 2024*)**. Due to these beneficial properties, LAB have gained significant attention in biotechnology, functional food development, and probiotic research.

Probiotics are defined by the World Health Organization (WHO) and the International Scientific Association for Probiotics and Prebiotics (ISAPP) as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (***FAO/WHO*, 2001**). LAB represent one of the most widely studied probiotic groups due to their safety profile and functional benefits **(Ouili *et al*., 2025; Pristavu *et al*., 2025)**. These microorganisms contribute to improved gut health, immune modulation, and inhibition of pathogenic microorganisms through competitive exclusion and production of antimicrobial metabolites **(Dunne *et al*., 2001)**.

Antimicrobial resistance (AMR) among microbes is one of the major global threats to mankind. The rise of AMR has increased interest in among researchers to identify alternative strategies for eradication of pathogenic organisms. Probiotic bacteria such as LAB have demonstrated promising antimicrobial and antibiofilm activities against several clinically relevant pathogens **(Amelia, 2021; Fijan, 2016; Lopes *et al*., 2017; Yasmin *et al*., 2020).**

Recently, probiotics and their metabolites have gained attention in cosmetic and dermatological applications **(Blanchet-Réthoré *et al*., 2017; Dou *et al*., 2023; Hyseni & Glavas Dodov, 2023).** LAB-derived metabolites can regulate skin microbiota, enhance skin barrier function, and inhibit skin pathogens associated with dermatological disorders such as acne, eczema, and atopic dermatitis **(Chae *et al*., 2021; Cinque *et al*., 2017; Espinoza-Monje *et al*., 2021; Lopes *et al*., 2017)**. Additionally, the natural lactic acid produced by LAB contributes to maintaining skin pH, improving hydration, and promoting collagen synthesis, making these microorganisms valuable components in microbiome-friendly cosmetic formulations **(Dou *et al*., 2023; França, 2020).**

Although LAB have been extensively studied in bovine milk, limited research has explored the diversity and probiotic potential of LAB isolated from non-bovine milk sources such as camel and goat milk, particularly in the Indian context. These milk sources possess unique biochemical compositions that may support diverse microbial populations with novel probiotic properties **(Al Musa & Hussen Al-Garory, 2024)**. Camel milk is rich in immunoglobulins, antimicrobial peptides, and essential vitamins, with the ability to influence the diversity and functionality of microbial communities **(Behrouz *et al*., 2022)**. Alternatively, goat milk contains small fat globules, distinctive oligosaccharides, and bioavailable minerals **(Van Leeuwen *et al*., 2020)**. These attributes enhance beneficial microbial colonization and also lower allergenicity. Thus, non-bovine milk sources offer promising alternatives for isolating novel LAB strains with potential applications in skin-based formulations.

Therefore, the objective of this study was to isolate LAB from bovine and non-bovine milk samples and compare their functional and cosmetic-relevant assays (anti-biofilm and antimicrobial activity) through phenotypic characterization and molecular identification. The findings from our preliminary study provide valuable insights for the development of innovative, microbiome-friendly cosmetic products.

MATERIAL AND METHODS

Study Area

This study was conducted in Mumbai, Maharashtra, India. Raw milk from cow and buffalo were obtained from six local milk suppliers from Matunga, Chembur, Ghatkopar, Sion, Dharavi and Wadala region. Goat milk was procured from six

local milk suppliers from Dharavi, Sion, Bandra, Koliwada, Kurla (W), and Kurla (E), whereas camel milk was obtained from two different urban-dairy farms located in Goregaon and Kalyan. These areas were selected due to their wide spread geographical distribution and variety of feed provided to the animals, which could potentially be a source to obtain diverse LAB populations.

Study Design and Sample Collection:

This investigation was a laboratory-based experimental study conducted over a span of almost 17 months (March 2023 to August 2024). A total of 30 raw milk samples were collected from local milk suppliers and dairy farms in Mumbai and surrounding areas from diverse sources, including cow (n=11), buffalo (n=8), goat (n=5), and camel (n=6). To minimize external contamination, the first three streams of milk were discarded, and the fourth stream was collected into sterile, labelled glass containers and immediately taken to the lab in ice packs and stored at 4°C for further analysis. For LAB isolation, 10 mL of each milk sample was initially enriched in 100 mL of De Man Rogosa and Sharpe (MRS) broth and incubated at 37°C for 3 hr. Serial dilutions from the enriched sample were prepared ranging from 10^{-1} to 10^{-8} and 100 µL of each dilution was plated on MRS agar plates followed by anaerobic incubation at 37°C for 24–48 hr in vacuum desiccator. Only isolates that survived the subsequent purification stage were selected, maintained and propagated in MRS broth and preserved in 30 % glycerol. The selected isolates were stored at -20 ℃ until further analysis.

Media, reagents and equipment

De Man Rogosa and Sharpe (MRS) broth/agar, Brain Heart Infusion (BHI) broth, Lactobacillus Selection Base (LSB), Mueller–Hinton agar/broth, Tryptone soya broth (TSB), Luria–Bertani (LB) broth, peptone water base with 1 % sugar, Triple Sugar Iron agar was procured from HiMedia Laboratories (Mumbai, India). All chemicals were of analytical grade.Sterile PVDF syringe filters (Merck - Millex™ PVDF syringe filter pore size 0.22 µm, diam. 33 mm, sterile, hydrophilic) were used for separation of cells from cell-free supernatant. Anaerobic conditions for the growth of LAB were maintained in a vacuum desiccator.

Procurement of Standard Microbial Cultures

Lactic Acid Bacteria (LAB)

The reference LAB strain *Lactobacillus plantarum* MTCC 9495 was obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. The reference strain was maintained and propagated in MRS broth and preserved in 30 % glycerol, stored at -20 ℃ and sub cultured every 3 weeks.

Indicator Pathogens

Indicator pathogens for anti-biofilm activity were: *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus mutans, Serratia marcescens, and Vibrio cholerae* Clinically relevant skin pathogens used as target organisms for antimicrobial evaluation: *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli, and Bacillus subtilis.* All the cultures were grown and maintained in Muller Hinton broth and tryptic soy broth, preserved in 30 % glycerol and stored at -20 ℃. Cultures were sub-cultured every 3 weeks.

Morphological and Biochemical Characterization

Isolates were analyzed by Gram reaction, catalase activity, spore production, motility, and carbohydrate fermentation (glucose, sucrose, lactose, mannitol, maltose, dextrose) following established protocols **(Khushboo *et al*., 2023; Taye *et al*., 2021)**. Carbohydrate fermentation test was performed in peptone water base with 1% sugar with inverted Durham's tube and Andrade's indicator **(Gunkova *et al*., 2021).** Acid production is indicated by change in the color of broth to pink and gas production in inverted Durham's tube was checked. Furthermore, growth of the isolates at 30℃ and 45℃ on MRS broth, H_2S production on Triple Sugar Iron slant was studied. Only rod-shaped LAB were selected for detailed analysis as many well-known probiotic strains used in food and cosmetic applications belong to *Lactobacillus* and related genera **(Delanghe *et al*., 2021; Lebeer *et al*., 2022).**

Assessment of Probiotic Properties

The selected isolates were evaluated for their tolerance to bile salts (0.3%), phenol (0.4%), NaCl (6.5 %) and their ability to survive in simulated gastric pH (pH 2 and pH 3) using established methods **(Mojgani *et al*., 2015; Reuben *et al*., 2019).** The optical density (OD_{600}) was recorded at specific time intervals to check for turbidity as an indicator of growth (0–5 h for bile/phenol/NaCl and 0–6 h for gastric tolerance).

Aggregation and Hydrophobicity Assays

For autoaggregation, selected LAB were grown in LSB broth for 24 hr, centrifuged at 5000 rpm for 10 min and washed thrice in phosphate buffered saline pH 7.2 (PBS), resuspended in PBS (OD adjusted to 1.0) and incubated at room temperature. The optical density was measured at 600 nm at intervals from 0 to 24 h. Coaggregation between LAB isolates and indicator pathogens *Pseudomonas aeruginosa* and *Vibrio spps* was thoroughly evaluated using equal concentrations of bacterial suspensions, as per established protocols **(Datta *et al*., 2017; Wang *et al*., 2022)**. The hydrophobic characteristics of cell surfaces were evaluated by employing xylene, paraffin, or silicone oil to isolate LAB suspensions and by calculating the percentage decrease at OD_{600}.

Antibiofilm and Antimicrobial Assays

Cell-free supernatants (CFS) were obtained from 48 hr old cultures of LAB through centrifugation followed by filtration. The crude filtrate was passed through a 0.22 micron filter to separate cells from CFS. The crude CFS was extracted using equal volume of ethyl acetate and evaporated **(Caggianiello *et al*., 2016)**. The residue left behind after evaporation was dissolved in distilled water and used for antibiofilm and antimicrobial activity. The effectiveness of CFS against known skin pathogenic biofilms of *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus mutans, Serratia marcescens, and Vibrio cholerae* was evaluated using crystal violet assay in microtiter plates **(Saidi *et al*., 2023)**. The assessment of antimicrobial activity was carried out using resazurin-based minimum inhibitory concentration (MIC) assay against two Gram-positive and two Gram-negative organisms **(Hossain, 2024)**.

Safety Evaluation

Hemolytic activity was thoroughly analyzed by streaking isolates on blood agar and incubating them at 37°C for 24–48 hours. Isolates that showed γ-hemolysis (indicating no hemolysis) were classified as safe **(Halder *et al*., 2017).**

Molecular Identification

Molecular level analysis is preferred for genus and species level identification of LAB due to limitations in precise identification by phenotypic data alone **(Mohania *et al*., 2008)**. For molecular identification, genomic DNA was extracted using a CTAB-based method as described by Jha et al **(Jha *et al*., 2022)**. The amplification of the 16S rRNA gene was achieved with the primers 8F and 907R, and the resulting PCR products were sequenced. Phylogenetic analysis was done using MEGA X, using the Neighbour-Joining method **(Kumar *et al*., 2018; Saitou & Nei, 1987).**

Statistical Analysis

All experiments were conducted in triplicates and the results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine statistical significance among groups. Differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

Data quality assurance

All microbiological experiments were conducted under sterile conditions using standard aseptic techniques. Culture media were checked for sterility and expiry dates before use. Instruments including spectrophotometers and incubators were calibrated periodically to ensure accuracy and reproducibility of experimental results. Each experiment was performed in triplicate to minimize experimental error. All microbiological media was prepared fresh and used within the stated expiry date. Negative and positive control was maintained wherever applicable. Reference strains of known identity were included as positive control to validate experimental conditions.

RESULTS

Morphological and Biochemical identification of LAB isolates

A total of 30 raw milk samples collected from cows, buffaloes, goats, and camels were screened for the presence of LAB. Following enrichment and plating on MRS agar, distinct colonies showing typical LAB morphology were isolated and purified by repeated streaking. (Figure 1). Ten isolates from milk sources were selected for further characterization and designated according to their source: CAMI (camel milk), GMI (goat milk), CMI (cow milk), and BFI (buffalo milk). Selection of rods were preferred as literature study has well documented LAB, Gram-positive rods for cosmetic applications. Selected isolates were all Gram-positive, rods either singles or in chains, catalase-negative, non-motile, and non-spore formers, which are typical characteristics of lactic acid bacteria. None of the

isolates could produce H_2S on Triple Sugar Iron slant/butt (TSI), but could utilize all three sugars namely, lactose, sucrose and glucose with acidic end products within 24-48 hr. However, no gas production on TSI slant was observed. Furthermore, growth of all isolates was observed at both 30 °C and 45 °C. Carbohydrate fermentation profiles varied among isolates, indicating metabolic diversity among the LAB strains. The detailed morphological and biochemical characteristics of the isolates are summarized in Table 1.

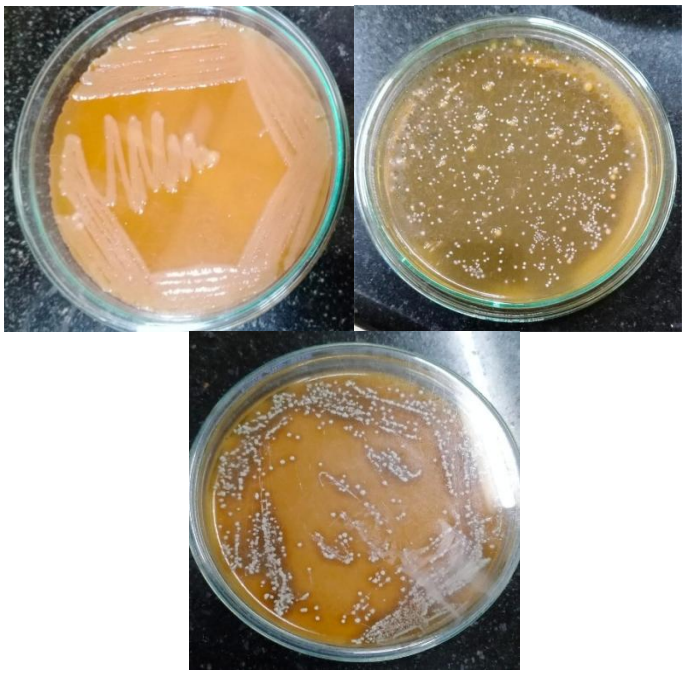


Figure 1 Representative images of purification and isolation of selected LAB isolates on MRS agar

Assessment of probiotic properties

The selected LAB isolates exhibited variable tolerance to bile salts (0.3%), phenol (0.4%), and NaCl (6.5%). Bile tolerance among the isolates ranged from 53.99 ± 0.34 % to 87.68 ± 0.04 % , with the highest tolerance observed in strain GMI 1 (87.68 ± 0.04 %), followed by CMI 3 (82.29 ± 0.37%), CAMI 3 (81.03 ± 0.56 %), and CAMI 2 (80.92 ± 0.56 %). The lowest bile tolerance was observed in CMI 1 (53.99 ± 0.34%). The mean bile tolerance among all isolates was 71.09 ± 12.89 % . Phenol tolerance ranged from 56.70 ± 0.32 % to 85.49 ± 0.19 %, with GMI 1 showing the highest tolerance. NaCl tolerance ranged from 66.54 ± 8.48 % to 88.71 ± 0.06 %, with CMI 3 showing the highest salt tolerance . These results demonstrate the ability of selected LAB strains to withstand intestinal stress conditions.

One way ANOVA was performed to check whether there are significant differences in the survival percentages among the isolates for all three parameters ($p<0.05$). The results are displayed in Table 2. Statistical analysis indicates that there is no significant difference among the tested parameters in the survival percentage of LAB ($p=0.09$, $p>0.05$). Further this is supported by the fact that $F = 2.60$ which is less than *Fcrit* =3.35 at 5 % significance. Thus, the variation seen among the survival percentage of the LAB isolates was not statistically significant. These findings sugegst the ability of LAB isolates to withstand intestinal stress conditions.

Acid Tolerance

All isolates showed survival under acidic conditions. At pH 2, survival ranged from 41.51 ± 0.60 % to 80.18 ± 0.22 %, whereas at pH 3, survival increased significantly, ranging from 62.01 ± 0.07 % to 90.32 ± 0.47 %. The highest survival under acidic stress was observed in GMI 1, indicating strong tolerance to simulated gastric conditions. These findings demonstrate the ability of selected LAB isolates to survive under gastrointestinal stress (Figure 3). Statistical analysis using Student's *t*-test revealed significant difference between survival at pH 3 and pH 2 ($t = -3.6$, $p = 0.00093$, $p<0.05$) (Table 3). Since the calculated *t* value exceeded *t* critical value ($t = 2.10$) we can say that there is significant difference in the survival of LAB isolates at pH 2 and pH 3.

Table 1 Preliminary screening and biochemical characterization of selected LAB isolates

Tests	CAMI 1	CAMI 2	CAMI 3	GMI 1	GMI 2	CMI 1	CMI 2	CMI 3	BFI 1	BFI 2
Gram staining	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive
Shape	Slender rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Arrangement	singles	singles	Singles and pairs	filamentous	Chains	chains and singles	singles	chains and singles	singles	pairs and singles
Biochemical tests										
Catalase	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-
Endospore	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-
Carbohydrate fermentation tests										
Glucose	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	-	+	-
sucrose	+	+	+/-	+	+	-	-	-	+	-
dextrose	+	+	+	+	+	-	-	+	+	-
mannitol	-	+	+	+	+	-	-	+	+	-
Growth at different temperatures										
30°C	+	+	+	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+	+	+	+

Legend : (+) Positive , (+/-) Weekly positive , (-) Negative

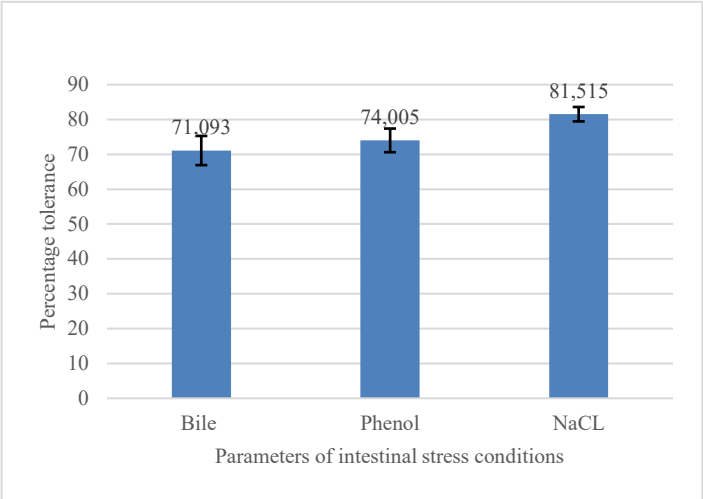


Figure 2 Mean percentage tolerance (±SE) of LAB isolates against intestinal stress conditions. LAB isolates showed mean tolerance values of 71.09 % for bile, 74.01% for phenol and 81.51% for NaCl. Error bars represent standard error of the mean. Statistical analysis using ne-way ANOVA showed no significant difference ($p > 0.05$) among the tested tolerance parameters.

Table 2 Summary statistics and one-way ANOVA comparing percentage tolerance of LAB isolates against intestinal stress conditions: bile, phenol and NaCl

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*	*SD*	*SE*
Bile	10	710.93	71.093	175.6260011	13.25239605	4.190775598
Phenol	10	740.05	74.005	115.0023389	10.72391435	3.391199476
NaCL	10	815.15	81.515	42.88320556	6.548526976	2.070826056

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	578.3264267	2	289.1632133	2.601078288	0.092675277	3.354130829
Within Groups	3001.60391	27	111.1705152			
Total	3579.930337	29				

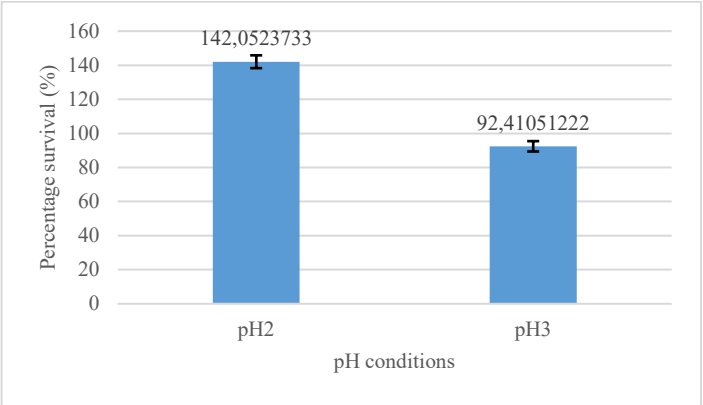


Figure 3 Comparison of survivability of LAB isolates at pH 2 and pH 3. Mean values (± variability) indicate higher survival at pH 3 than pH 2. Statistical analysis indicate demonstrate significant difference between the two conditions by Student's *t*-test ($p<0.05$).

Table 3 Statistical comparison of survival of LAB isolates at pH 2 and pH3 using students *t*-test. Mean survival rate at both pH indicate better survivability at pH 3 than pH 2 ($p<0.05$), indicating isolates are best adjusted to moderately acidic condition

t-Test: Two-Sample Assuming Equal Variances		
	pH2	***pH3***
Mean	61.202	78.827
Variance	142.0524	92.41051
Observations	10	10
Pooled Variance	117.2314	
Hypothesized Mean Difference	0	
df	18	
t Stat	-3.63992	
P(T<=t) one-tail	0.000937	
t Critical one-tail	1.734064	
P(T<=t) two-tail	0.001873	
t Critical two-tail	2.100922	

Hydrophobicity (Adhesion to Hydrocarbons)

Cell surface hydrophobicity of LAB isolates was evaluated using xylene, paraffin oil, and silicone oil. The highest adhesion to silicone oil was observed in CAMI 1 (74.46%), followed by GMI 1 (72.41%) and CMI 3 (60.72%). Adhesion to xylene ranged from 10.34 ± 0.70 % (CMI 1) to 46.82 ± 0.21 % (GMI 1), while adhesion to paraffin ranged from 37.74 ± 0.22 % (CMI 1) to 74.37 ± 0.62 % (CAMI 3). The mean average adhesion to various hydrocarbons demonstrates that adhesion to paraffin (53.15%) is higher, followed by silicone (48.27%) and the lowest was observed in xylene (23.35 %) (Figure 4). These results suggest that several isolates possess strong surface hydrophobicity, which is an important property for adhesion to host epithelial surfaces.

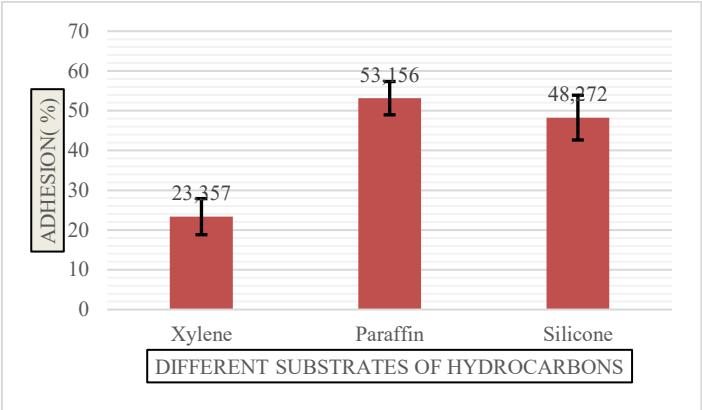


Figure 4 Mean percentage adhesion (±SE) of LAB isolates with different hydrocarbons (Xylene, Paraffin and silicone). LAB isolates showed mean percentage adhesion to xylene is 23.35%, 53.15 % for paraffin, and 48.27 % for silicone. Error bars represent standard error of the mean. Statistical analysis using one-way ANOVA showed that there is a significant difference ($p < 0.05$) among the adhesion capacity to hydrocarbons with respect to cell surface adhesion.

One-way ANOVA was performed in order to check whether there is any statistical difference in the adhesion capabilities of LAB isolates to different substrates (Table 4). Since the calculated *F* value exceeded the critical *F* value ($F = 10.97$, $F\ crit = 3.35$), we can reject the null hypothesis, and confirm that there is significant difference in the adhesion capabilities towards the hydrocarbons by the LAB isolates ($p<0.05$).

Table 4 Summary statistics and **(A)** One-way ANOVA comparing different adhesion substrates with respect to cell-surface adhesion of LAB isolates **(B)** Tukey HSD post-hoc multiple comparisons of cell surface hydrophobicity of LAB isolates across different substrates (xylene, paraffin and silicone). Here, A=Xylene, B=Paraffin and C=Silicone (p=0.01).

(A)

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*	*S.D*	*S.E*
Xylene	10	233.57	23.357	205.8907344	14.34889314	4.537518424
Paraffin	10	531.56	53.156	175.3887156	13.24344047	4.187943595
Silicone	10	482.72	48.272	317.1373956	17.80835185	5.631495321

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5108.63694	2	2554.31847	10.97189373	0.000325457	3.354130829
Within Groups	6285.75161	27	232.8056152			
Total	11394.38855	29				

(B)

treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
A vs B	6.1760	0.0010053	** p<0.01
A vs C	5.1637	0.0030668	** p<0.01
B vs C	1.0122	0.7416548	insignificant

Further comparison using Tukey's post hoc test was performed to reveal which hydrocarbon performed better than the other. Results are interpreted at 1 % significance level (Table 4 B). Result of xylene vs paraffin reveal statistically significant difference ($p<0.01$). Similar results were seen between xylene and silicone where p-value is 0.003($p<0.01$). However, there is no significant difference between paraffin and silicone ($p>0.01$). These findings suggest that adhesion to xylene is significantly different in adhering to epithelial surfaces than the other two hydrocarbon suggesting substrate specific adhesion of LAB isolates.

Autoaggregation and Coaggregation

Autoaggregation ability varied among isolates, ranging from 36.20 ± 1.03 % (CAMI 3) to 94.74 ± 0.40 %(CAMI 2) after 24 h followed by GMI 1 (94.43 ± 0.48 %) (Figure 5). Co-aggregation assay with *Pseudomonas aeruginosa* and *Vibrio cholerae* revealed strong interactions between LAB isolates and pathogenic bacteria. Notably, CAMI 1 demonstrated high co-aggregation ability with both pathogens (87.91±0.34 %) for *Pseudomonas aeruginosa* and (83.31 ± 0.32 %) for *Vibrio cholerae*) suggesting potential pathogen exclusion capability.

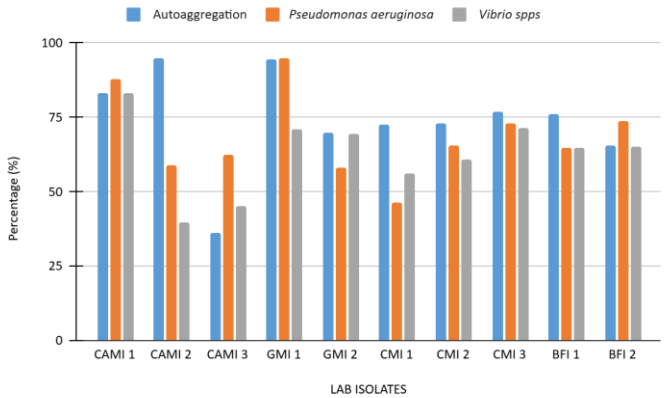


Figure 5 Percentage autoaggregation among LAB isolates and coaggregation of LAB isolates with indicator pathogens (*Pseudomonas aeruginosa* and *Vibrio spps)*

Biofilm Inhibition Activity

The CFS of the LAB isolates demonstrated a significant ability to inhibit biofilm development by pathogens, indicating notable variability. The strongest inhibition against the antibiotic-resistant pathogen *Acinetobacter baumannii* was noted in isolates CMI 1 (94.98 ± 1.22 %), CAMI 3 (92.14 ± 0.51 %), followed by BFI 1 (92.33 ± 1.41 %), and lowest was seen in BFI 2 (78.95 ± 0.55 %). Also, biofilm-inhibition against the well documented skin pathogen *Staphylococcus epidermidis* was given by CAMI 2 (79.37 ± 0.9 %), followed by CAMI 3 (77.22 ± 2.27 %) .

The percentage biofilm inhibition against the pathogen *Vibrio cholerae* showed the widest range of around 20 – 90 %, with the highest inhibition by BFI 2 (87.02 ± 0.28 %). Furthermore, the percent inhibition against *Escherichia coli* was in the range 45.61 ± 0.95 % (CMI 3) to 68.57 ± 2.03 % (CAMI 2), for *Pseudomonas aeruginosa* in the range of 35-60 %, *Proteus vulgaris* was about 20-35 % and *Proteus mirabilis* in the range of 50 – 90 %. However, the cell-free supernatants showed lowest inhibition against *Streptococcus mutans, Serratia marcescens* and *Staphylococcus aureus* ranging between 20% and 40% (Figure 6).

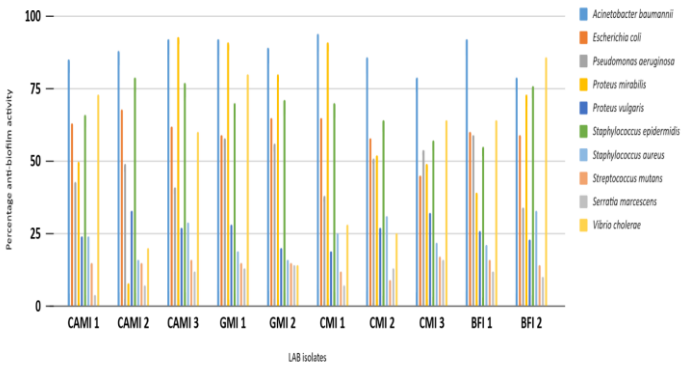


Figure 6 Antibiofilm activity of cell-free supernatant from LAB isolates against standard biofilm forming indicator pathogens : *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus mutans, Serratia marcescens, and Vibrio cholerae* by crystal violet assay in microtiter plates.

Antimicrobial Activity (MIC by Resazurin Assay)

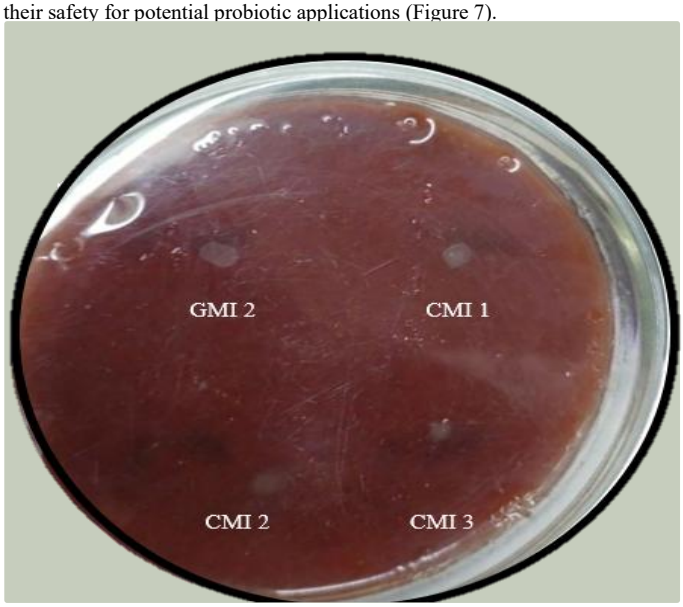
The antimicrobial activity of LAB isolates was evaluated using resazurin-based MIC assays against two Gram-positive and Gram-negative pathogens. Several isolates demonstrated strong inhibitory activity. Notably, CAMI 2, CAMI 3, GMI 1, CMI 1, CMI 2 and BFI 2 exhibited the strongest antimicrobial effects with MIC values as low as 0.03 mg/mL against certain pathogens. Among the tested bacteria, *Staphylococcus aureus* showed higher susceptibility compared with other pathogens (Table 5). The overall mean MIC against *Staphylococcus aureus* across all isolates was 0.108 ± 0.15 mg/mL, reflecting substantial variability. Among Gram-negative pathogen, *Pseudomonas aeruginosa*, the lowest MIC was 0.03 mg/mL (GMI 1 and BFI 2) and highest was 0.25 mg/mL (CMI 1). Against *Escherichia coli* , the values ranged from 0.03 mg/mL to 0.12 mg/mL. With *Bacillus subtilis* , the MIC values ranged from 0.03 mg/mL to 0.25 mg/mL. These findings strongly support CAMI 3, GMI 1, CMI 1 and BFI 2 as the most effective strains with significant antimicrobial potential alongside their high survival under stress conditions.

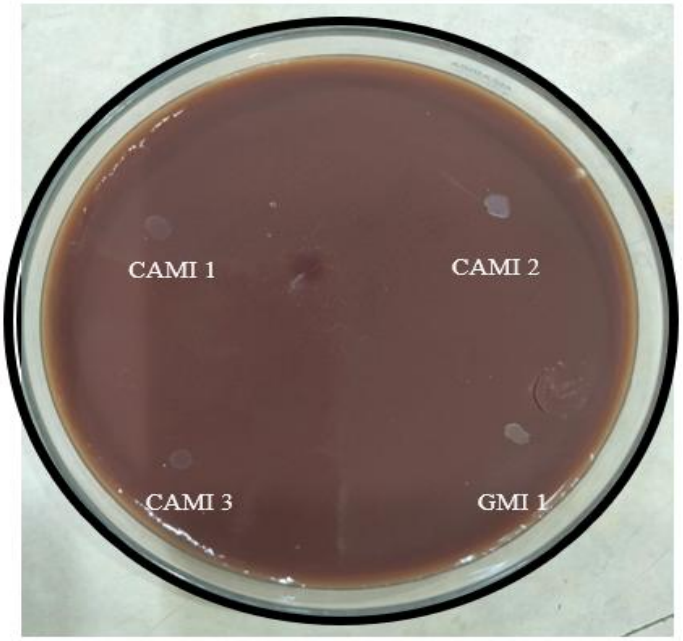
Table 5 Average MIC in mg/mL *(n=3)* of the CFS from LAB isolates against standard indicator pathogens (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis)*

Indicator pathogens → / LAB isolates ↓	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Bacillus subtilis*
	(mg/ml of CFS)			
CAMI 1	**0.06**	**0.06**	**0.25**	**0.06**
CAMI 2	**0.06**	**0.12**	**0.03**	**0.12**
CAMI 3	**0.06**	**0.12**	**0.03**	**0.03**
GMI 1	**0.12**	**0.03**	**0.03**	**0.12**
GMI 2	**0.12**	**0.06**	**0.06**	**0.25**
CMI 1	**0.03**	**0.25**	**0.03**	**0.12**
CMI 2	**0.03**	**0.12**	**0.5**	**0.12**
CMI 3	**0.06**	**0.06**	**0.06**	**0.12**
BFI 1	**0.06**	**0.06**	**0.06**	**0.25**
BFI 2	**0.12**	**0.03**	**0.03**	**0.12**

Safety Evaluation

Hemolytic activity was assessed using blood agar plates. All LAB isolates exhibited γ-hemolysis, indicating absence of hemolytic activity and confirming their safety for potential probiotic applications (Figure 7).





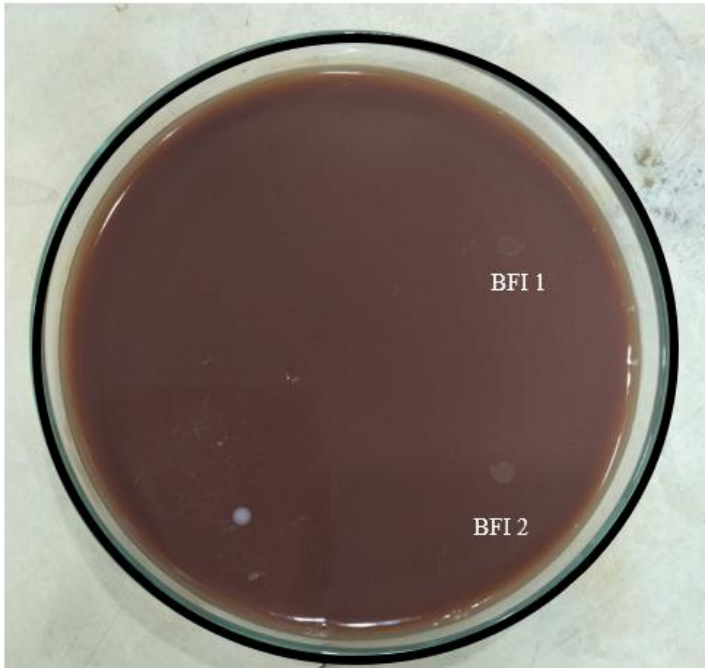


Figure 7 LAB isolates on blood agar plate after incubation for 24h showing gamma hemolysis **(**No zone of clearance)

Molecular Identification (16S rRNA Sequencing)

Genomic DNA was extracted from the selected ten LAB isolates, sequenced and 16S rRNA gene sequence was amplified. The amplified sequences were compared with sequences available at National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) . 16S rRNA sequencing confirmed the species identities, with sequences submitted to GenBank. Table 6 presents the summary of bacterial identification, including NCBI accession numbers.

Table 6 Source, molecular identification and NCBI accession number of identified LAB isolates

Sources	Isolates	NCBI Accession number	Scientific name
Camel milk	CAMI 1	PQ643355	*Lacticaseibacillus rhamnosus*
	CAMI 2	PQ643356	*Lactiplantibacillus paraplantarum*
	CAMI 3	PQ643357	*Lactiplantibacillus pentosus*
Goat milk	GMI 1	PQ643358	*Lactobacillus delbrueckii subsp. bulgaricus*
	GMI 2	PQ643359	*Limosilactobacillus fermentum*
Cow milk	CMI 1	PQ643360	*Lactobacillus helveticus*
	CMI 2	PQ643361	*Lacticaseibacillus paracasei*
	CMI 3	PQ643362	*Ligilactobacillus salivarius*
Buffalo milk	BFI 1	PQ643363	*Loigolactobacillus coryniformis*
	BFI 2	PQ643364	*Lactiplantibacillus plantarum*

Phylogenetic Analysis

The phylogenetic tree constructed via MEGA X (Neighbor-Joining method, 1000 bootstraps) confirmed accurate clustering of the isolates at the species level (Figure 8).

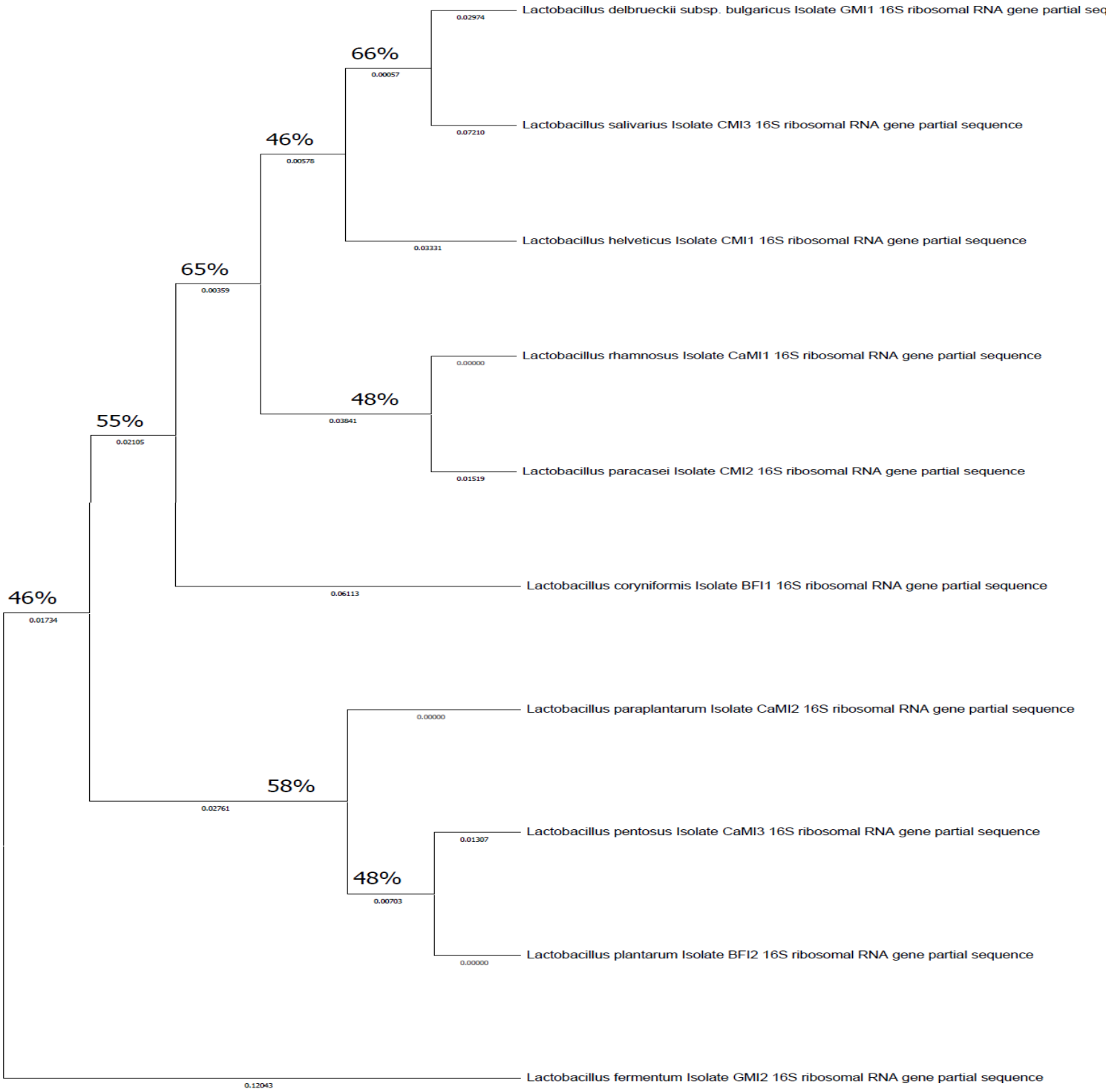


Figure 8 Phylogenetic tree of isolated LAB strains obtained by Neighbour-joining (NJ) method using MEGA 11 software. The branch node number shows percent bootstrap support. The accession numbers of the organisms are included in parentheses and the bar scale value 0.02 indicate the nucleotide substitutions per site.

DISCUSSION

In the present study, LAB were successfully isolated and characterized from raw milk obtained from bovine and non-bovine sources including camel, goat, cow, and buffalo. Molecular identification using 16S rRNA sequencing revealed ten distinct LAB species including *Lacticaseibacillus rhamnosus, Lactiplantibacillus paraplantarum, Lactiplantibacillus pentosus, Lactobacillus delbrueckii subsp. bulgaricus, Limosilactobacillus fermentum, Lactobacillus helveticus, Lacticaseibacillus paracasei, Ligilactobacillus salivarius, Loigolactobacillus coryniformis, and Lactiplantibacillus plantarum*. These findings confirm that raw milk represents an important natural reservoir of probiotic microorganisms. Similar LAB diversity has been reported in previous studies investigating milk-derived probiotics **(Reuben *et al*., 2019; Taye *et al*., 2021)**.

The probiotic potential of LAB isolates was evaluated through several functional assays including tolerance to bile salts, acidic pH, phenol, and NaCl. The isolates demonstrated strong tolerance to these stress conditions, which are essential characteristics for probiotic survival in the gastrointestinal environment. In particular, strain GMI 1 exhibited the highest bile tolerance (87.68 ± 0.04 %) and phenol tolerance (85.49 ± 0.19 %), indicating its strong resilience under intestinal conditions. One-way ANOVA revealed that there is no statistical variation between the parameters checked for survival of LAB isolates for bile, NaCl and phenol tolerance at 5 % significance. This report is further supported by F-value (2.60) and F critical (3.35). Thus, the variation observed between the survival percentage

of LAB isolates in bile, phenol and NaCl is not statistically significant. Overall, majority of the isolates demonstrates greater that 70% survival under atleast one survival condition. Comparable tolerance levels have been reported for LAB isolated from fermented dairy products, suggesting that milk-derived LAB possess strong adaptability to gastrointestinal stress **(Mojgani *et al*., 2015; Reuben *et al*., 2019).** Acid tolerance of the LAB isolates was checked by Student's *t*-test at 5% significance. LAB isolates exhibit significantly higher survival at pH 3 than pH 2, confirming improved tolerance to moderate acidic conditions. Among the tested isolates, GMI 1 showed better tolerance to pH 2 (80.18 ± 0.22%) and at pH 3 (90.32 ± 0.47 %) followed by CAMI 2 and CMI 3.

Adhesion-related properties such as hydrophobicity play an important role in the colonization of probiotic bacteria within host tissues. In the present study, isolates CAMI 1, GMI 1, and CMI 3 demonstrated strong cell surface hydrophobicity, which may enhance their ability to adhere to epithelial surfaces. Additionally, CAMI 2 and GMI 1 exhibited high auto-aggregation capacity (>94%), suggesting potential for biofilm formation and competitive exclusion of pathogens. One-way ANOVA for adhesion to hydrocarbon demonstrated that there are no significant differences in the adhesion capabilities to hydrocarbons among the LAB strains, which indicates strain specific diversity. Similar adhesion properties have been reported by Gonzalez *et al*., where it was observed that *Lactococcus lactis* showed > 90 % adhesion to hydrocarbons and exhibited superior cell surface adhesion potential **(Hernández-González *et al*., 2021).** Tukey HSD post-hoc analysis revealed that adhesion to xylene is significantly different than adhesion to paraffin and silicone.

Autoaggregation, and coaggregation plays an important role in determining pathogen exclusion from the gut region. Current study demonstrated that isolate GMI 1 showed superior autoaggregation and coaggregation capabilities with both tested pathogens, followed by CAMI 1. Our study showed comparable higher autoaggregation than that reported by Rao *et al* **(Rao *et al*., 2024).**

An important finding of the present study was the strong antibiofilm activity exhibited by several LAB isolates against clinically relevant pathogens. Notably, isolates CAMI 2, CAMI 3, BFI 2 and CMI 2 demonstrated significant inhibition of biofilm formation by *Staphylococcus epidermidis*. The ability of LAB to inhibit pathogenic biofilms is attributed to the production of antimicrobial metabolites such as organic acids, bacteriocins, and biosurfactants. Similar antibiofilm activity of LAB isolates against skin pathogens has been reported in previous studies **(Lopes *et al*., 2017; Melo *et al*., 2016).**

The antimicrobial activity of LAB isolates was further confirmed through MIC assays against both Gram-positive and Gram-negative pathogens. Among the isolates tested, CAMI 2, CAMI 3, GMI 1, CMI 1, and BFI 2 exhibited the strongest antimicrobial activity with MIC values as low as 0.03 mg/mL. Interestingly, the LAB isolates showed greater inhibitory activity against Gram-positive bacteria, particularly *Staphylococcus aureus*, compared with Gram-negative bacteria. This observation is consistent with earlier reports indicating that Gram-positive bacteria are generally more susceptible to LAB-derived antimicrobial compounds due to differences in cell wall structure **(Halder *et al*., 2017).**

Safety evaluation is an essential criterion in probiotic selection. All isolates in this study exhibited γ-hemolysis, indicating absence of hemolytic activity and confirming their safety for potential probiotic applications. Molecular identification and phylogenetic analysis further validated the taxonomic identity of the isolates and confirmed their close relationship with well-known probiotic species. Taken together, the results suggest that milk-derived LAB isolates possess multiple probiotic characteristics including stress tolerance, adhesion ability, antimicrobial activity, and safety profiles.

Overall, the findings of this study highlight the significant potential of LAB strains isolated from bovine and non-bovine milk as promising candidates for probiotic, therapeutic, and cosmetic applications. The multifunctional properties observed among selected isolates suggest their possible application in microbiome-friendly formulations aimed at improving both gastrointestinal and skin health.

CONCLUSION

This study successfully isolated and characterized lactic acid bacteria from bovine and non-bovine milk sources. The isolates demonstrated strong probiotic characteristics including tolerance to gastrointestinal stress, aggregation ability, antimicrobial activity, and safety profiles. Among the isolates, CAMI 2, CAMI 3, GMI 1, CMI 1, and BFI 2 emerged as the most promising strains based on their antimicrobial activity and functional properties. Among the tested pathogens, *Staphylococcus aureus* showed the highest susceptibility to LAB antimicrobial activity. These findings highlight the potential of milk-derived LAB strains for future applications in probiotic-based therapeutic and cosmetic formulations.

Acknowledgements: We are highly grateful to National facility of Biopharmaceuticals for kindly providing indicator bacteria used in this study. This study received no funding from any agency.

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