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Evaluation of Bile salt hydrolase activity and cholesterol-binding ability of lactic acid bacteria isolated from milk and meconium

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Abstract:

Lactic acid bacteria (LAB) have long been recognised as probiotics that support the digestive system and help maintain host health. Beyond their typical features, such as tolerance to low pH, high salt concentrations, adherence to host tissues, and diverse metabolic capabilities that produce a range of beneficial compounds, LAB strains with Bile salt hydrolase (BSH) activity have been implicated in cholesterol reduction. This study isolated eight strains of LAB from human milk, cow's milk, and meconium. The eight LAB strains were then investigated for BSH production, BSH activity, and cholesterol-binding properties. Notably, all eight strains demonstrated robust bile salt tolerance to ox gall and bile salts (including cholic acid and taurocholic acid) after 20 hours of cultivation. BSH enzyme activity varied, ranging from 0.28 to 18.04 U/ml, while cholesterol binding ranged from 34.38 to 104.02 µg/ml. Among the strains evaluated, SMB68, SMB33, and LS13 stood out as superior candidates; these strains were identified as *Ligilactobacillus salivarius (L. salivarius), Enterococcus faecalis (E. faecalis),* and *Lactiplantibacillus plantarum (L. plantarum)*, respectively. Cholesterol-binding values reached 108.02, 107.33, and 81.94 µg/ml, respectively. These strains hold potential for application in products aimed at preventing lipid-related diseases.

Keywords: **bile salt hydrolase, bile salt tolerance, cholesterol-binding ability, lactic acid bacteria, probiotics.**

***Classification numbers*: 2.2, 3.5**

1. Introduction

Probiotics and prebiotics have emerged as functional food components that enhance health beyond basic nutrition. LAB are industrially important probiotic microorganisms widely used in dairy products and food fermentation. They have attracted significant interest in recent decades due to their therapeutic potential. Their health-promoting effects have

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been well documented, including the regulation of gut microbiota and the alleviation of obesity, allergies, irritable bowel syndrome, and immune modulation. Additionally, LAB exhibit hypocholesterolaemic effects through various mechanisms, such as the deconjugation of bile salts via the enzyme BSH, production of short-chain fatty acids, assimilation of cholesterol into bacterial cell membranes, and conversion of cholesterol to coprostanol, a non-absorbable sterol that is excreted in the faeces [1].

BSH activity is prevalent among Gram-positive bacteria in the mammalian intestinal microbiota, including *Bifidobacterium* spp., *Lactobacillus acidophilus (L. acidophilus), Lactobacillus gasseri (L. gasseri), Lactobacillus johnsonii (L. johnsonii)* and *Lactobacillus plantarum (L. plantarum)* [2-3]. BSH catalyses the hydrolysis of glycine- or taurine-conjugated bile acids, releasing amino acid residues and free bile acids. Since cholesterol is the precursor for bile salt synthesis in the liver, the degradation of bile salts by BSH increases the demand for cholesterol. This process underlies the cholesterol-lowering mechanism mediated by the BSH enzyme. BSH also contributes to the ability of bacteria to bind cholesterol to their cell membranes, thereby affecting membrane potential, fluidity, and tensile strength [4].

This study aimed to evaluate the BSH activity and cholesterol-binding ability of the cell membranes of various LAB strains, with the objective of identifying potential probiotic candidates for cholesterol-lowering applications.

2. Materials and methods

2.1. Materials

Fifteen samples of meconium and breast milk were collected at the Department of Neonatology, Tu Du Hospital, Ho Chi Minh city. Five freshly collected cow's milk samples were obtained from a dairy farm in Cu Chi district, Ho Chi Minh city. All samples were kept cold in iceboxes and transported to the Food Biotechnology Department on the same day.

The following chemicals were purchased from Sigma, USA: ox gall (B8631), taurodeoxycholic acid (580221), glycodeoxycholic acid (361311), and cholesterol (C3045). De Man-Rogosa-Sharpe (MRS) medium (M001) was obtained from Himedia, India. Solvents included n-hexane (110-54-3), ethyl acetate (141-78-6), methanol (XL1100024), acetic acid (64-19-7), and ethanol (64-17-5), purchased from Xilong, China.

2.2. Methods

2.2.1. Isolation of lactic acid bacteria

LAB were isolated following the method described by M.A.S. Abubakr (2018) [5]. Serial dilutions were prepared by adding 1 g of meconium or 1 ml of milk to 9 ml of

0.9% NaCl solution, generating dilutions from 10^{-1} to 10^{-6}. Then, 0.1 ml of the last three dilution concentrations was pipetted and spread onto MRS agar plates containing 1% $CaCO_3$, followed by incubation at 37°C under anaerobic conditions for 24-48 hours.

Colonies that appeared white or creamy, smooth, circular with entire margins, and exhibited clear zones of $CaCO_3$ degradation were selected for purification. The purified isolates were subjected to morphological analysis using Gram staining (Gram Stain Kit, K001-1KIT, Himedia, India) and biochemical identification, including the catalase test with 3% H_2O_2 and the oxidase test (oxidase reagent, R026, Himedia, India). Isolates that were Gram-positive, catalase-negative, and oxidase-negative were selected and stored at -80°C in MRS broth containing 30% glycerol, as described by M.A.S. Abubakr (2018) [5].

2.2.2. Assessment of bile salt tolerance

Bile salt tolerance was investigated according to the method of M.T. Liong, et al. (2005) [6]. Ox gall, cholic acid, and glycocholic acid were used as different bile sources. Bile salt media were prepared by adding 0.3% (w/v) ox gall, cholic acid, or glycocholic acid to MRS broth and autoclaving at 121°C for 15 minutes.

LAB were cultured in MRS broth overnight at 37°C with shaking at 200 rpm under anaerobic conditions (GasPak anaerobic system and Anaerobic bag MGC, C-1D04, Japan). The optical density was adjusted to 1.0 to ensure uniformity. Subsequently, 0.1 ml of the bacterial suspension was inoculated into 10 ml of bile salt medium and incubated at 37°C. MRS broth without bile salts was used as a control.

After 20 hours of incubation, 100 µl of each bacterial suspension was serially diluted (10-fold) and spread-plated into MRS agar. Viable cell counts (CFU/ml) were determined after 48 hours of incubation at 37°C under anaerobic condition. The experiment was repeated three times.

2.2.3. Quantification of BSH enzyme activity

Determination of BSH enzyme activity by thin layer chromatography (TLC)

The quantitative TLC method described by C.F. Guo, et al. (2011) [7], with minor modifications, was used to determine BSH activity. LAB strains were cultured at 37°C for 24 hours under anaerobic conditions in MRS broth with 0.3% taurodeoxycholic acid (TDCA) and 0.3% glycodeoxycholic acid (GDCA).

After incubation, the cultures were centrifuged at 5000 rpm for 10 minutes at 4°C. Then, 5 µl samples were spotted onto TLC plates (20 cmx20 cm silica gel 60 F_{254}, Merck). The TLC plates were developed in a mobile phase consisting of *n*-hexane, ethyl acetate, methanol, and acetic acid at a ratio of 20:20:5:2 (v/v). When the solvent front

had migrated to within approximately 0.5-1 cm of the upper edge, the TLC plates were removed from the development chambers and oven-dried at 110°C for 1 min.

The TLC plates were sprayed with 10% H_2SO_4 solution and heated until spots appeared. BSH activity was considered positive if spots appeared on the TLC plates corresponding to cholic acid (CA), TDCA, and GDCA spots (control). The experiment was repeated three times.

Determination of BSH activity: Quantitative BSH activity was determined according to the method described by H. Tanaka et al. [8], with some modifications.

Preparation of BSH enzyme: Selected LAB strains were cultured in MRS broth for 24 hours at 37°C under anaerobic conditions. Cultures were centrifuged at 7000 rpm for 10 minutes at 4°C, washed twice with phosphate buffer (pH 7.2) and resuspended in 3 ml of the same buffer. To prevent enzyme oxidation, 10 µl of 10 mM dithiothreitol was added. The suspensions were sonicated (Q700, Qsonica, USA) for 10 minutes at 30 kHz and 40 W, in cycles of 10 seconds on and 10 seconds off, while kept in ice water. Supernatants were centrifuged at 7000 rpm for 10 minutes at 4°C and stored as cell-free extracts at -20°C until analysis.

BSH activity assay: Fifty microlitres of cell-free extract were mixed with 100 µl of sodium phosphate buffer (pH 6) and 10 µl of 10 mM GDCA or TDCA. The mixture was incubated at 37°C for 30 minutes, and the reaction was terminated by adding 50 µl of 15% (w/v) trichloroacetic acid. The mixture was centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatant was collected for amino acid quantification.

The ninhydrin assay was used to determine amino acid content. A 100 µl aliquot of supernatant was mixed with 1.9 ml of ninhydrin reagent. The mixture was boiled for 14 minutes and cooled under running water. Absorbance was measured at 570 nm using an ELISA microplate reader (VersaMax, Molecular Devices, USA). Glycine concentration was determined using a standard curve. One unit (U) of BSH activity was defined as the amount of enzyme required to liberate 1 µmol of amino acid per minute [9]. All experiments were performed in triplicate.

2.2.4. Observation of cholesterol absorption by FTIR analysis

FTIR analysis was performed according to the method of O. Kleiner, et al. (2002) [10]. LAB strains were cultured in MRS medium supplemented with 300 µg/ml cholesterol and in non-supplemented MRS medium as a control. Cultures were incubated with shaking at 200 rpm for 48 hours at 37°C under anaerobic conditions.

Cell biomass was collected by centrifugation at 5000 rpm for 15 minutes and washed three times with sterile 0.9% saline. Biomass samples were compressed with dehumidified KBr and analysed using an FTIR spectrometer (FTIR-4700, Jasco, Japan)

in the range of 400-4000 cm^{-1} at a resolution of 4 cm^{-1}. Functional groups were identified by comparing spectral peaks with those of a cholesterol standard.

2.2.5. Observation of cholesterol absorption by scanning electron microscopy (SEM)

The ability of LAB to absorb cholesterol was evaluated using SEM. LAB strains were cultured in MRS medium with or without 100 mg/l cholesterol and incubated with shaking at 200 rpm for 48 hours at 37°C under anaerobic conditions.

Cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C, followed by two washes with 0.9% NaCl solution. Sample preparation for SEM followed the protocol described by W.A. Hassanein, et al. (2013) [11]. Briefly, LAB cells were treated with 100 µl of 20% glutaraldehyde, vortexed, and incubated at room temperature for 1 hour, followed by dehydration in 50% ethanol. Subsequently, 200 µl of 1% osmium tetroxide was added, and the samples were incubated for 1 h at room temperature followed by a rinse with distilled water. Sequential dehydration was carried out using a graded ethanol series (30%, 50%, 70%, and 100%). The prepared samples were mounted on carbon tape and coated for imaging. After processing, the samples were examined under a scanning electron microscope (Zeiss EVO 300VP, Oberkochen, Germany) to observe the presence or absence of cholesterol on the bacterial surface. All experiments were conducted in duplicate.

2.2.6. Determination of LAB-absorbed cholesterol content by HPLC-UV

Cholesterol removal by LAB was evaluated following a modified protocol based on A.M.P. José et al. [12]. Briefly, 100 mg of cholesterol was dissolved in 10 ml of absolute ethanol and sterilised by filtration through a 0.22 µm membrane filter. Then, 150 µL of the cholesterol solution was added to 4.85 ml of MRS medium to obtain a final cholesterol concentration of 300 µg/ml. One percent (%) of overnight-grown LAB was inoculated into the cholesterol-supplemented medium. The control consisted of LAB cultured in MRS broth without cholesterol. Both test and control cultures were incubated with shaking at 200×g for 36 hours at 37°C under anaerobic conditions. After incubation, the cultures were centrifuged at 5000 rpm for 15 min at 4°C. The resulting bacterial pellets were washed three times with 0.9% NaCl and centrifuged again under the same conditions. The final biomass was used for cholesterol quantification by HPLC (Alliance e2695, Waters, USA). The stationary phase was a silica gel column (4.6x250 mm, particle size 5 µm) and the mobile phase consisted of 1% (v/v) isopropanol in *n*-hexane. Cholesterol was detected using a UV-VIS detector at 202 nm. The total run time was 17 minutes with a flow rate of 1 ml/min at 20°C. Each experiment was performed in duplicate.

The samples were prepared by adding 0.1 g of dried biomass to a mixture containing 0.2 g of ascorbic acid and 5.5 ml of 12% KOH in 90% ethanol. The mixture was vortexed and saponified at 80°C for 15 min, followed by cooling for 2 min. Cholesterol was extracted three times by adding 1.5 ml of distilled water and 3 ml of hexane containing 0.025 g/l butylated hydroxytoluene (BHT) (Sigma, USA). The organic phases were collected, evaporated, and the residues were reconstituted to a final volume of 1 ml with the mobile phase. Cholesterol absorption was calculated as the cholesterol content detected in the bacterial biomass.

2.2.7. Bacterial classification by analysis of 16S rRNA sequence

Three strains exhibiting optimal bile salt tolerance, BSH enzyme production, and cholesterol binding were selected for gene sequencing based on the 16S region according to the method of S. Itoi, et al. (2009) [13]. The 8F and 1492R primers were used to amplify the 16S rRNA. Positive PCR products were sequenced using the 16S RNA sequencing method. BLAST analysis was performed to compare the obtained nucleotide sequences with those on the NCBI GenBank.

2.2.8. Statistical analysis

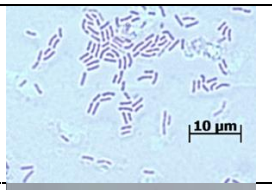
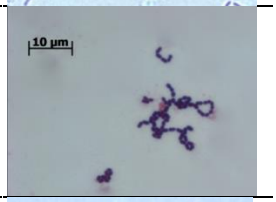
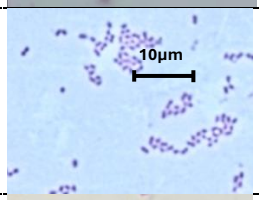
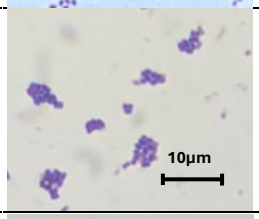
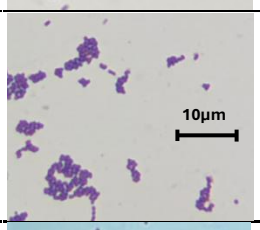
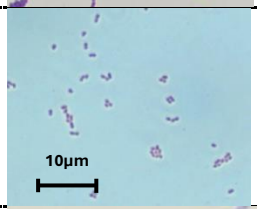
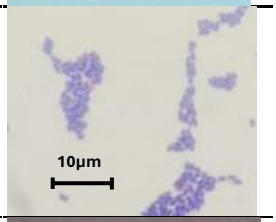
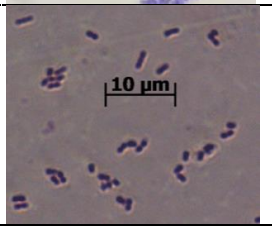
Experimental data were analysed using Excel 2019. Analysis of variance (ANOVA) was performed using Minitab 16.2. Phylogenetic trees were constructed using MEGA7 software.

3. Results and discussion

3.1. Isolation of LAB

A total of eight LAB strains were isolated from various sources, including cow's milk, meconium, and breast milk, based on their typical morphological characteristics (white or creamy colonies, coccus or rod-shaped, Gram-positive, catalase-negative and oxidase-negative). Detailed characteristics of the isolated strains are presented in Table 1.

Table 1. Morphological characteristics of isolated LAB strains.

Strain	*Source isolate*	*Catalase test*	*Oxidase test*	*Gram stain*	*Cell morphology*	
LS13	cow's milk	-	-	+	single rod	
PS19	meconium	-	-	+	long-chained rod	
PS22	meconium	-	-	+	cocci	
PS24	meconium	-	-	+	cocci	
PS36	meconium	-	-	+	rod	
SMB33	breast milk	-	-	+	single, diploid, cocci	
SMB62	breast milk	-	-	+	cocci	
SMB68	breast milk	-	-	+	paired rods	

3.2. Assessment of bile salt tolerance

Bile acids are specific and quantitatively important organic components of bile. They include primary bile acids (cholic acid and chenodeoxycholic acid), secondary bile

acids (deoxycholic acid), and conjugated bile salts (taurocholic acid and glycocholic acid) [14]. These compounds play a crucial role in fat digestion and facilitate the absorption of fat-soluble vitamins, including vitamins A, D, K, and E. Bile salts also exhibit antibacterial properties; therefore, the ability of bacteria to colonise the intestine depends on their tolerance to bile in order to survive in the digestive system, such as through the synthesis of the BSH enzyme [15]. The results of bile salt tolerance of the LAB strains are shown in Fig. 1.

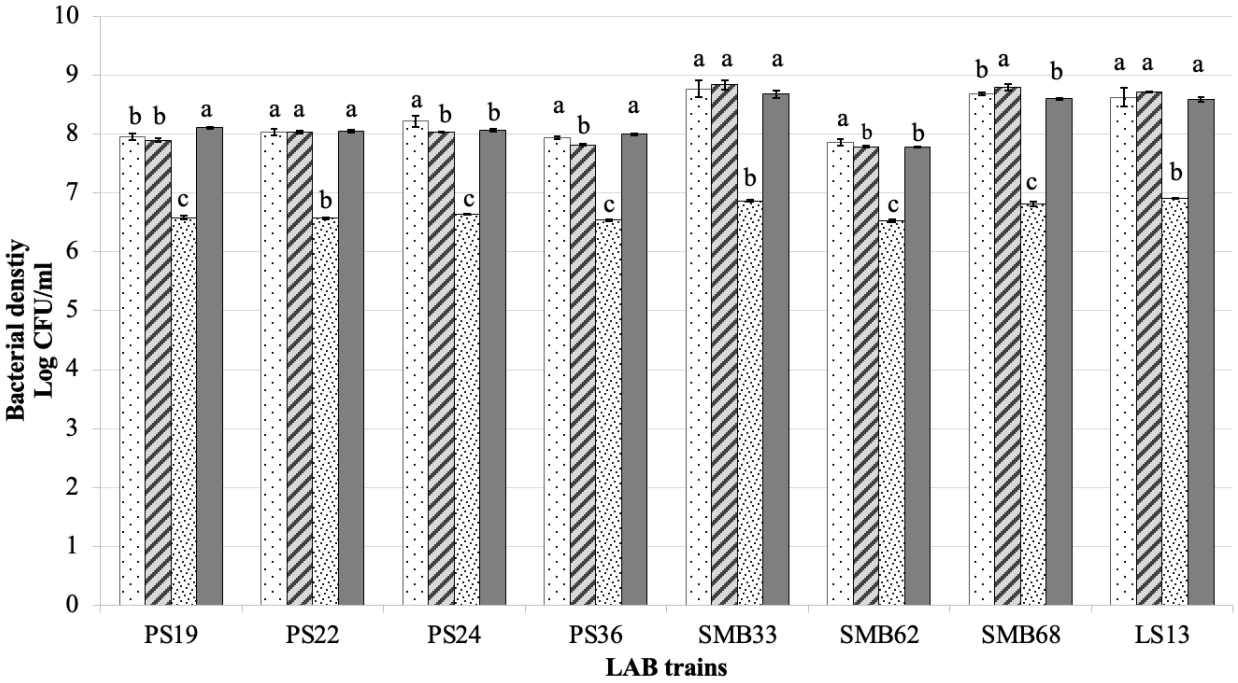


Fig. 1. Bacterial density of LAB strains after 20 hours of culture in MRS medium supplemented with bile salts (ox gall, cholic acid taurocholic acid). *Different lowercase letters (a, b, c, d) indicate statistically significant differences ($p<0.05$).

All eight LAB strains exhibited bile salt tolerance, with superior tolerance to ox gall and taurocholic acid compared with cholic acid. At 0 hour of culture, bacterial density was adjusted to 5.0 log CFU/ml, and no statistically significant differences were observed among the strains. After 20 hours, all eight LAB strains demonstrated the ability to survive and grow in environments supplemented with different bile salts. As shown in Fig. 1, the bacterial density of all LAB strains exceeded 8.0 log CFU/ml in MRS medium supplemented with ox gall and taurocholic acid, which was comparable to the control. Strains SMB33, SMB68, and LS13 showed a remarkable ability to tolerate taurocholic acid and ox gall, with bacterial densities reaching approximately 8.5 log CFU/ml; among them, SMB33 achieved the highest density of 8.84 log CFU/ml. However, the viable counts of LAB strains cultured in the presence of cholic acid were lower than those in the control cultures without supplementation, suggesting that cholic acid may negatively affect LAB growth.

Bile salt tolerance in LAB is also associated with the ability to synthesise the BSH enzyme. M.M.C. Alba, et al. (2017) [16] demonstrated that, to enhance survival in bile salt-rich environments, bacteria produce BSH enzymes, which are more effective against taurocholic acid than cholic acid. The BSH enzyme hydrolyses conjugated bile salts into steroid nuclei and amino acids (glycine or taurine), which can be utilised as carbon and nitrogen sources [17].

3.3. Observation of cholic acid production by BSH-producing LAB strains using thin layer chromatography

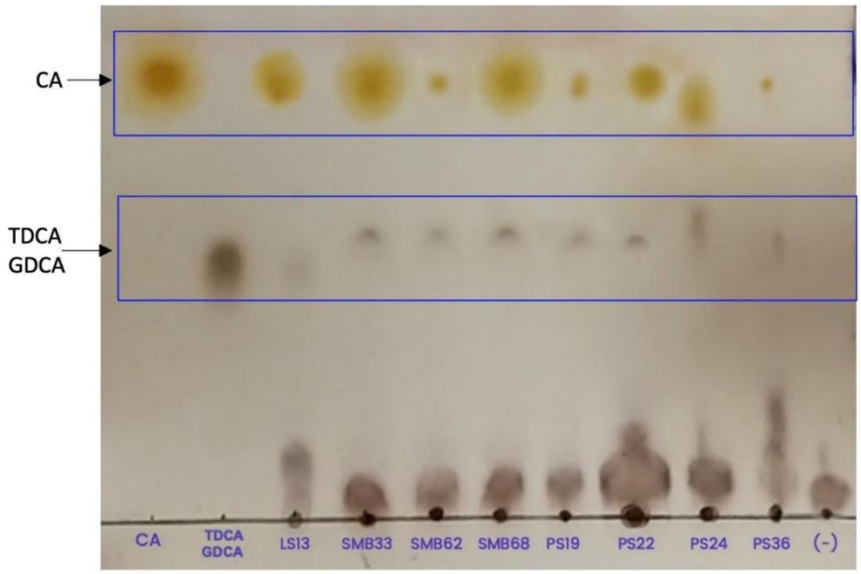


Fig. 2. Thin layer chromatography results of the BSH enzyme production ability of LAB strains, negative control is MRS medium. CA: cholic acid; TDCA: taurodeoxycholic acid.

The BSH-positive strains were identified by the appearance of distinct spots corresponding to CA, TDCA, and GDCA on the thin-layer chromatography (TLC) plates. In this experiment, TDCA and GDCA were used as conjugated bile salt substrates to evaluate deconjugation activity. This reaction, catalysed by BSH, results in the release of free CA, which is a key indicator of BSH activity. Notably, although glycocholic acid (GCA) and taurocholic acid (TCA) are primary bile acids and are more abundant in human bile than TDCA, a secondary bile acid, they were not included in this assay. However, GCA and TCA are frequently used in other studies as representative substrates for assessing BSH activity due to their physiological relevance [17-18]. The presence of clear CA spots in all tested strains, as shown in Fig. 2, confirms BSH-mediated deconjugation of TDCA or GDCA.

3.4. Quantification of bile salt hydrolase enzyme activity

The activity of the BSH enzyme influences the adaptability of bacteria in the gut. F. Miremadi, et al. (2014) [19], Z. Dong, et al. (2025) [20] demonstrated a relationship

between bile salt hydrolysis, bile tolerance, and cholesterol incorporation into bacterial cell membranes. In this study, the BSH enzyme activity produced by LAB strains was quantified, and the results are shown in Fig. 3.

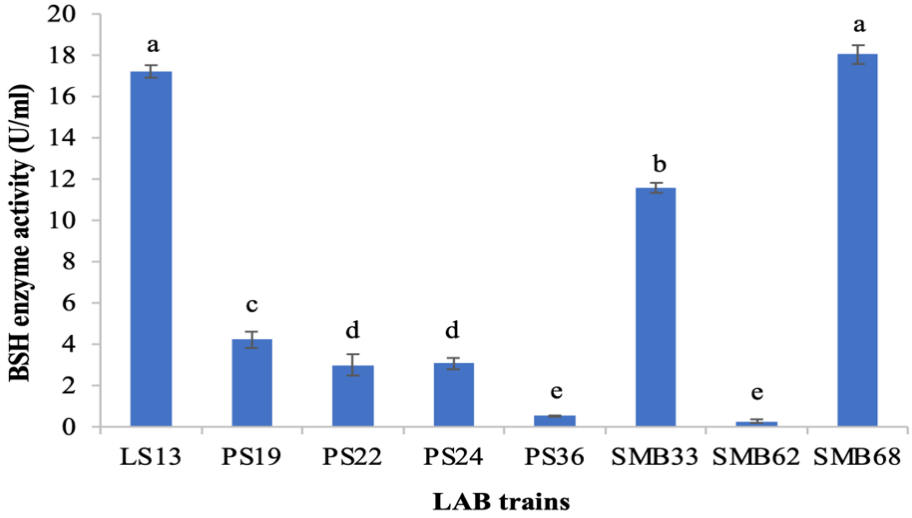


Fig. 3. BSH enzyme activity (U/ml) in LAB strains by colorimetric method. One enzyme unit (U) is defined as the amount of enzyme required to release one µM of amino acid from the substrate in 1 minute. *Different lowercase letters (a, b, c, d) indicate statistically significant differences ($p<0.05$).

Among the eight LAB strains evaluated, SMB68, LS13, and SMB33 demonstrated the highest BSH activity when GDCA was used as the substrate, with values of 18.04, 17.22, and 11.56 U/ml, respectively (Fig. 3). In contrast, PS19, PS22, and PS24 exhibited moderate BSH activity, ranging from 3.00 to 4.24 U/ml, while PS36 and SMB62 showed minimal activity, at 0.54 and 0.28 U/ml, respectively. These findings indicate substantial strain-dependent variation in bile salt deconjugation capacity under the tested conditions.

Comparable variations in BSH activity have been reported in previous studies. For example, M.G. Shehata, et al. (2016) [18] observed BSH activity levels ranging from 0.25 to 3.09 U/ml in LAB strains isolated from milk. Among them, *Lactococcus lactis* subsp. *lactis* (L. *lactis*) was identified as the most promising strain, capable of removing 43.7% of cholesterol from the cell surface (equivalent to 43.7 µg/ml) and exhibiting a BSH activity of 2.47 U/ml. Similarly, J.G. Hernández-Gómez, et al. (2021) [21] reported high deconjugation rates for *L. plantarum* DGIA1 isolated from cheese: 100% for glycodeoxycholate (GDC), 81% for taurocholate, and 92% for taurodeoxycholate. Although BSH activity was not quantified in U/ml in that study, the extent of deconjugation suggests an estimated activity of approximately 1-3 U/ml, based on standard enzymatic equivalence.

In the present study, BSH activity was determined through a colourimetric assay based on the release of free amino acids, a widely accepted method for assessing glycodeoxycholic acid hydrolysis. Similar methodologies have been employed in previous studies to evaluate BSH activity in LAB strains such as *L. casei*, *L. acidophilus*, *L. delbruecki* subsp. *bulgaricus*, and *L. lactis.* [8, 17, 22]. Given their notably high BSH activity, LS13, SMB68, and SMB33 emerge as promising candidates for further development as cholesterol-lowering probiotic strains.

3.5. Determination of cholesterol absorption by FTIR analysis

FTIR spectroscopy was employed to investigate the interaction between LAB cells and cholesterol by comparing spectra of bacterial biomass grown with and without cholesterol supplementation. In the absence of cholesterol, characteristic absorption bands were observed in all LAB strains at the following positions: O-H/N-H stretching (~3434 cm^{-1}), C-H (~2961 cm^{-1}), P–H (~1653 cm^{-1}), C≡C (~1540 cm^{-1}), ester COOR (~1409 cm^{-1}), amide I (C=O, ~1238 cm^{-1}), amide II (N-H, ~1069 cm^{-1}), and sulfamide S=O (~623 cm^{-1}). Upon cholesterol supplementation, distinct shifts in these peaks were noted. Notably, the O-H/N-H stretching band shifted to ~3296 cm^{-1}, the C-H group to ~2960 and ~1171 cm^{-1}, and CH_2/CH_3 peaks emerged at ~2362 and ~2340 cm^{-1}, indicating altered membrane composition. Minor but consistent changes were also observed in the amide I/II and S=O regions. These spectral differences indicate that cholesterol was incorporated into or bound to the bacterial cell surface, leading to rearrangement of membrane lipids and proteins.

The results were shown in Fig. 4. FTIR spectra of LAB strains cultured in media with (+CHL) and without (-CHL) cholesterol supplementation, compared with standard cholesterol. Each panel (A-BH) represents a different LAB strain: (A) LS13, (B) PS19, (C) PS22, (D) PS24, (E) PS36, (F) SMB33, (G) SMB62, and (H) SMB68. The green line indicates the spectrum of LAB biomass grown without cholesterol (-CHL), the red line represents biomass grown in cholesterol-supplemented medium (+CHL), and the blue line corresponds to pure cholesterol.

Therefore, the FTIR results confirmed that all eight LAB strains exhibited cholesterol-binding capacity, although the degree of interaction, as reflected by the intensity and position of peak shifts, varied among strains. These findings align with previous reports that membrane incorporation of cholesterol results in conformational and compositional changes detectable by FTIR [23-24].

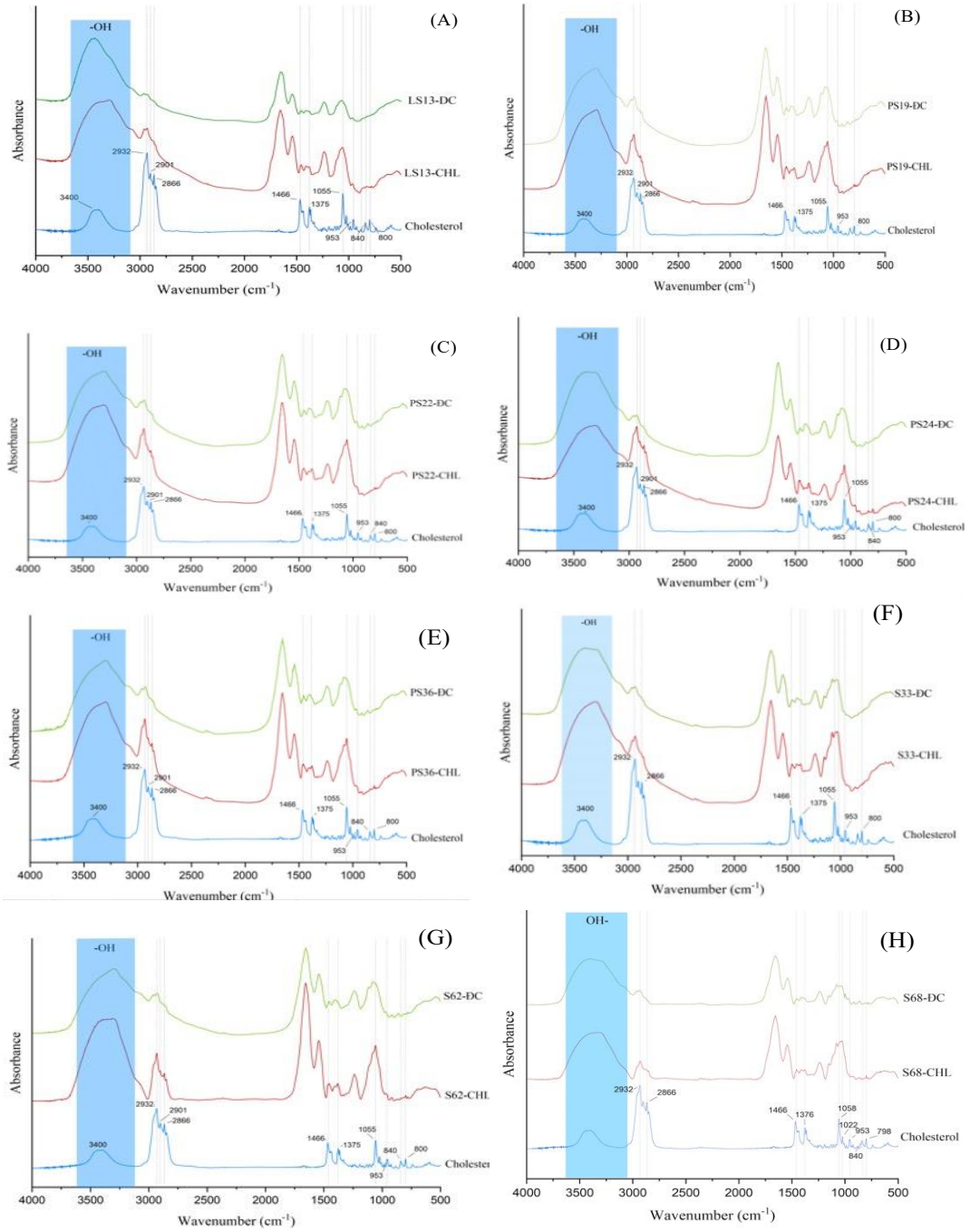


Fig. 4. The FTIR spectra of bacterial strains cultured in media with and without cholesterol supplementation comparing to standard cholesterol: (A) LS13; **(B)** PS19; **(C)** PS22; **(D)** PS24; **(E)** PS36; **(F)** SMB33; **(G)** SMB62; **(H)** SMB68.

3.6. Determination of cholesterol absorption by scanning electron microscopy (SEM)

The chemical and structural properties of cell wall peptidoglycans enable them to bind cholesterol [25]. All eight LAB strains in the study exhibited light spots on the cell surface, indicating cholesterol attachment during fermentation. This process leads to a reduction in environmental cholesterol. The results in Fig. 5 demonstrate cholesterol adhesion on the cell surfaces of the SMB68 strain. Cholesterol was not observed on the

cell surface of the control strain, *L. brevis* ATCC 14869. This result is consistent with previous FTIR analysis.

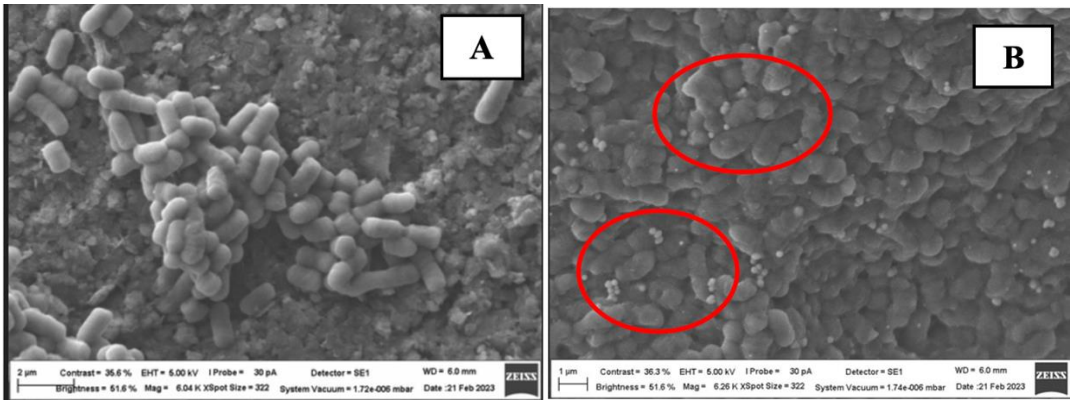


Fig. 5. SEM imaging results of strains grown in cholesterol-supplemented medium. *L. brevis* ATCC 14869 (control) does not appear to bind cholesterol to the cell surface **(A)** while strain SMB68 does bind cholesterol to the cell surface in the circled areas **(B)**.

H. Kimoto, et al. (2002) [26] observed morphological changes in *Lactococcus* cells grown with cholesterol. M.T. Liong, et al. (2005) [6] confirmed that both live and heat-killed LAB cells could remove cholesterol, supporting a dual mechanism. H.S. Lye, et al. (2010) [27] further demonstrated cholesterol accumulation in the phospholipid bilayer, confirming physical incorporation into the cell membrane.

Compared with previous studies, our findings not only reaffirm the surface-binding mechanism but also provide direct visual evidence using SEM, which is less commonly reported in earlier LAB studies. The consistency between SEM and FTIR results strengthens the claim that cholesterol removal by LAB occurs via multiple complementary mechanisms, including surface adsorption, membrane incorporation, and possibly intracellular assimilation during growth.

3.7. Determination of LAB-absorbed cholesterol content by HPLC-UV-Vis

The cholesterol-binding ability of LAB is closely associated with the structural and chemical composition of their cell envelopes. Key components such as charged amino acid residues, peptidoglycan layers, teichoic acids, and surface-associated proteins provide multiple sites for interaction with cholesterol molecules. These interactions are typically mediated by hydrophobic forces and electrostatic attractions, enabling cholesterol to associate with the bacterial cell surface. Notably, several studies have reported that even non-viable or heat-inactivated LAB cells retain their cholesterol-binding capability due to the structural stability of their cell wall and membrane components [24]. This suggests that both live and inactivated LAB cells may play a role in reducing intestinal cholesterol absorption.

As shown in Fig. 6, the cholesterol-binding capacities of the eight LAB strains varied significantly after 48 hours of incubation in cholesterol-enriched medium.

Among them, strains SMB68 and SMB33 exhibited the highest cholesterol removal efficiencies, with values of 108.02 and 107.33 µg/ml, respectively. In contrast, strains PS36, PS24, and PS19 demonstrated the lowest binding capacities, ranging from 34 to 42 µg/ml. HPLC chromatograms showing the cholesterol content of the strains are presented in Fig. 7. These strain-specific differences may reflect variations in cell wall architecture, surface hydrophobicity, growth performance under bile salt conditions, as well as differences in BSH activity.

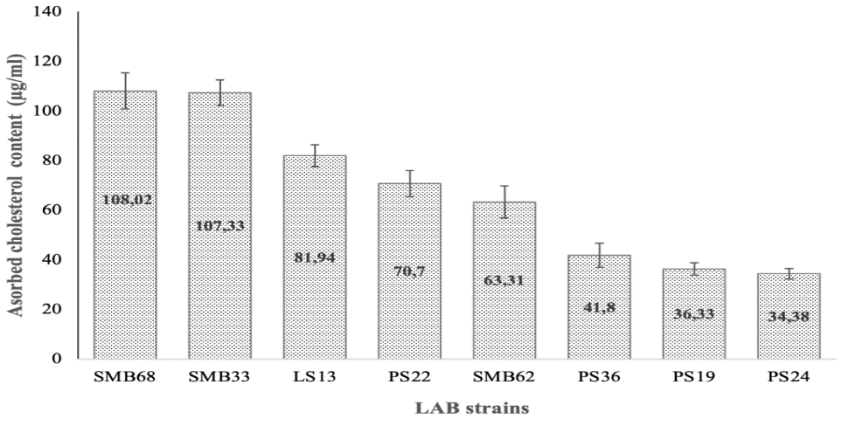
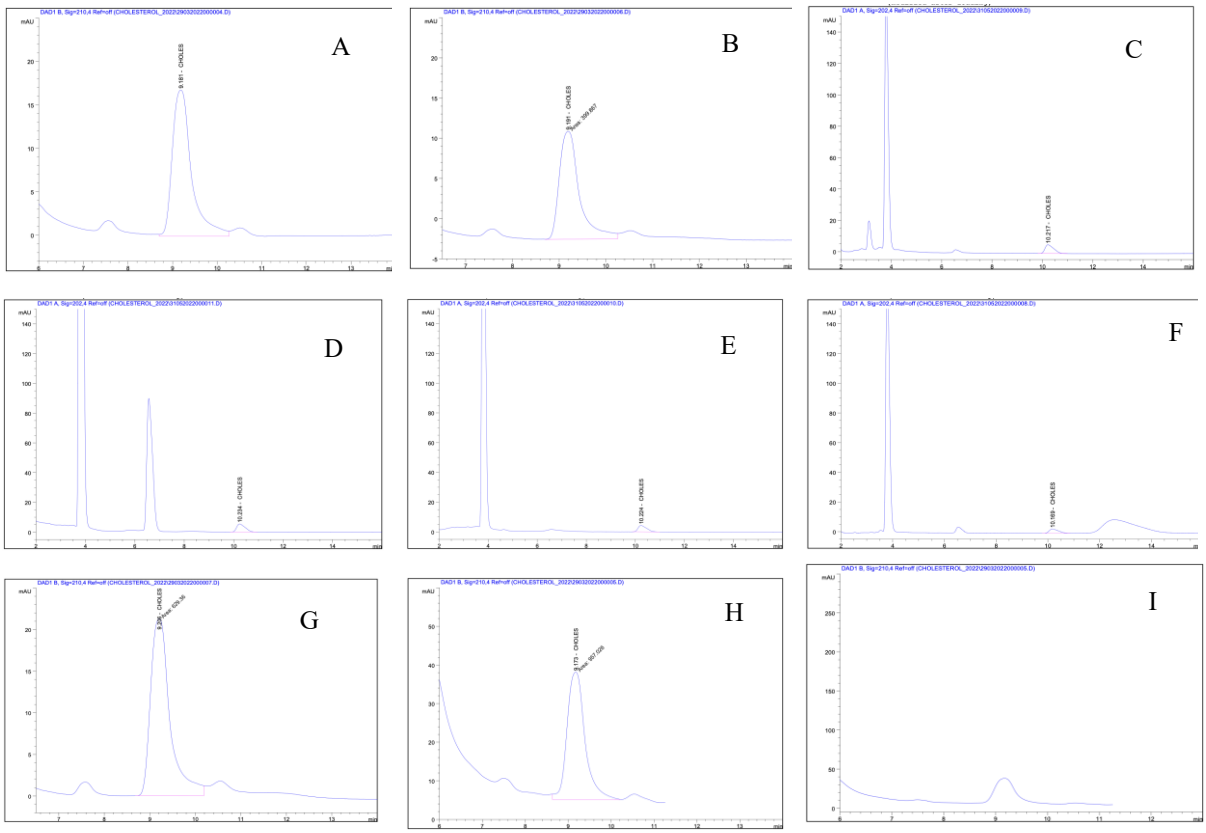


Fig. 6. Cholesterol assimilation potential of LAB isolates.



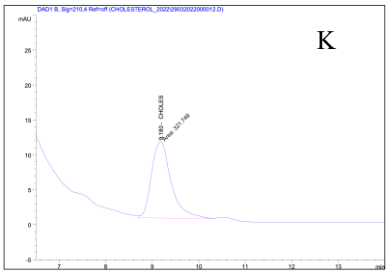


Fig. 7. HPLC chromatograms showing the cholesterol content of SMB33: (A), LS13 **(B)**, PS22 **(C)**, SMB62 **(D)**, PS36 **(E)**, PS19 **(F)**, PS24 **(G)**, SMB68 **(H)**, *L. brevis* strain - strain control **(I)**, and cholesterol standard **(K)**.

It has been well established that the cholesterol-binding potential of LAB strains is influenced by multiple factors, including growth rate, metabolic activity, and cell surface properties [6, 23]. Among these, BSH activity plays a particularly important role in cholesterol-lowering mechanisms. The enzyme catalyses the deconjugation of conjugated bile acids, producing free bile acids and amino acids. This reaction reduces the reabsorption efficiency of bile acids in the intestine, thereby enhancing faecal excretion of bile salts and diverting more endogenous cholesterol towards bile acid synthesis. Moreover, the deconjugation process facilitated by BSH may lead to the co-precipitation of cholesterol with free bile acids, or promote its incorporation into the bacterial cell membrane, especially under conditions favouring lipid affinity and membrane fluidity [28]. Therefore, the combination of passive cholesterol binding through surface interactions and active enzymatic bile salt deconjugation contributes synergistically to the reduction of cholesterol availability in the host intestine.

In the present study, strains SMB68, SMB33, and LS13 demonstrated both high BSH activity and strong cholesterol-binding capacity, along with notable tolerance to bile salts. These characteristics underscore their potential as promising probiotic candidates for cholesterol-lowering applications.

3.8. Molecular identification by 16S rRNA sequencing

The molecular identification of three selected LAB strains, LS13, SMB33, and SMB68, was performed using 16S rRNA gene sequencing, followed by phylogenetic analysis. As shown in Fig. 8, the phylogenetic tree revealed that the three isolates clustered with their respective reference strains, supporting their taxonomic identification at the species level.

The 16S rRNA sequence of strain LS13 showed 100% identity with *L. plantarum* SN13T (Accession No. MT545068.1), which is consistent with its placement in the phylogenetic tree, where it clustered tightly with *L. plantarum* with a bootstrap value of 100%. This confirms a high degree of confidence in its species-level classification. Strain SMB33 was found to share 100% sequence identity with *Enterococcus faecalis*

(E. faecalis) strain FMAMOB6 (Accession No. OR018313.1) and 99% identity with *E. faecalis* ATCC 29212 (Accession No. NR040789.1). In the phylogenetic tree, SMB33 grouped within the *E. faecalis* cluster alongside multiple reference strains, supported by a high bootstrap value of 99%, affirming its identity as *E. faecalis*. Strain SMB68 demonstrated 98% sequence identity with *L. salivarius* (Accession No. MT611731.1). Phylogenetically, SMB68 clustered with both MT611731.1 and the type strain DSM 103789 of *L. salivarius*, supported by a moderate bootstrap value (85%). Although the bootstrap value was slightly lower than ideal, the close genetic relationship and consistent functional traits, such as bile salt tolerance, BSH activity, and cholesterol-binding, support its classification within the *L. salivarius* species.

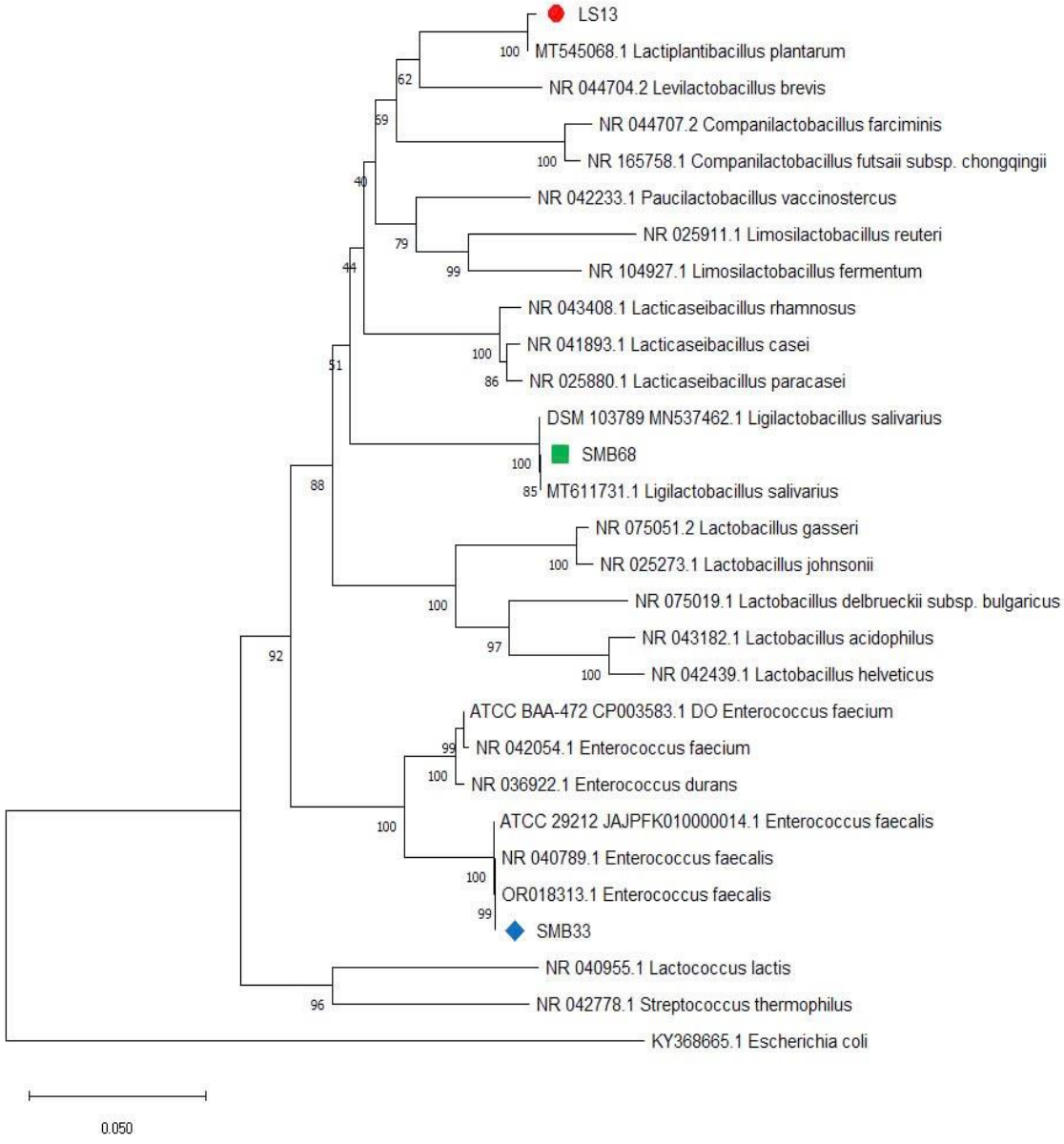


Fig. 8. Phylogenetic tree based on the 16S rDNA gene sequences of the isolates and the related standard strains.

4. Conclusions

This study demonstrated that selected LAB strains possess key probiotic characteristics related to cholesterol-lowering potential, including bile salt tolerance, BSH activity, and cholesterol-binding capacity. Among the eight strains evaluated, SMB68 (*L. salivarius*), SMB33 (*E. faecalis*), and LS13 (*L. plantarum*) exhibited the most promising results. These strains showed high BSH activity (18.04, 11.56, and 17.22 U/ml, respectively) and strong cholesterol-binding ability (108.02, 107.33, and 81.94 µg/ml, respectively). These results suggest potential applications in probiotic products that support human cholesterol reduction. Bile-hydrolysing strains, through the activity of the BSH enzyme, show considerable promise for controlling serum cholesterol levels. Furthermore, optimisation of BSH enzyme activity and culture conditions for LAB biomass production may contribute to the development of probiotic or enzyme-based products with potential benefits for cholesterol regulation.

CRediT author statement

Dung Nguyen Thi: Writing original draft, Data curation, Validation; Supervision; Trang Nguyen Thi Thuy: Writing original draft, Methodology, Data gathering, Formal analysis; Toan Nguyen Van: Writing original draft, Formal analysis, Methodology; Hong Dao Nu Dieu: Methodology, Formal analysis, Data gathering; Tu Bui Le Kha: Data gathering, Formal analysis; Lieu Nguyen Thi: Methodology, Formal analysis.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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