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Screening, isolation, characterization, and optimization of BSH activity from potential probiotic isolates from various sources

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ABSTRACT

Bile salt hydrolase (BSH)-producing probiotics can assimilate cholesterol from the body through de novo synthesis. The BSH enzyme was found in 23 of 513 isolates accessed from various sources. Five of the 23 BSH-positive strains have been selected for further study, based on their BSH activity, compared to two positive controls, *Lactobacillus acidophilus* and *Enterococcus lactis*. The Grams nature of the strains was determined and further examined for hemolytic activity, gelatinase, and catalase assay as per Indian Council for Medical Research—Department of Biotechnology recommendations. Two *Enterococcus faecalis* (CGz3 and CGz4) strains with γ-hemolytic, negative catalase, and gelatinase activity are selected for probiotic characterization, evaluating the organisms surface hydrophobicity, autoaggregation tests, tolerance to lysozyme, gastric acidity, bile salt and gastric juices (pepsin and pancreatin). The strain which withstands the harsh gastrointestinal conditions was considered for further experiments. To establish a standardized method to quantify the BSH activity of the potential probiotic isolate, substrate utilization was performed by screening sodium glycocholate (GCA) and taurocholic acid (TCA) at different concentrations. The optimal BSH activity was observed at the 16th hour and 0.1% (v/v) GCA. Based on the standardized protocol, factorial optimization of process parameters, such as pH, inoculum percentage, temperature, and revolutions per minute (RPM) was carried out for increased BSH activity. The optimal BSH activity was observed at pH 5.5 and 1% inoculum (v/v). The highest BSH activity was obtained at 40°C and 200 RPM. Among the other BSH-positive strains, *E. faecalis* CGz3 shows the best probiotic potential. The strain would be further studied for its ability to alleviate symptoms associated with non-alcoholic fatty liver disease (NAFLD), using a cell line-based study and associated gene regulation. In conclusion, *E. faecalis* CGz3 would have the potential to be used as a dietary supplement to treat metabolic disorders, such as hypercholesterolemia and NAFLD/metabolic-associated fatty liver disease.

1. INTRODUCTION

Probiotics are non-pathogenic bacteria, which when taken in sufficient quantities, benefit the health of their hosts. Numerous microorganisms, especially food-grade lactic acid bacteria (LAB), are used as additional cultures in various foods or pharmaceutical formulations [1]. Important selection criteria for potential probiotic strains include physiological properties and functional attributes linked to activity that supports or maintains health [2,3]. Bile salt hydrolase (BSH) activity in bacteria has been connected to cholesterol-lowering effects [4,5]. One of the most important properties of any probiotic strain for reducing blood cholesterol is its BSH activity. Free bile acids and amino acids are released from conjugated bile salts by the BSH enzymes that probiotics produce [5]. Inhibition of an increase in the immune response and a reduction in blood cholesterol levels are suggested functional effects of probiotics. Mammals' bile salt metabolism includes bile salt hydrolysis as a key metabolic process, which facilitates the excretion of bile salts. The N-terminal processing event of BSHs, which belong to the Ntn (N-terminal nucleophile) subfamily of enzymes, shows the main catalytic cysteine. The cysteine is found deep within the active site that forms in every Ntn enzyme's conserved αββα core. All BSHs have five more catalytically significant residues that are tightly conserved. Conjugated bile acids are broken down in the mammalian gut by bacterial BSH [6]. By releasing the glycine and/or taurine moiety from the side chain of the steroid core, the conjugated BSH enzyme deconjugates bile salts. The idea is that as free bile acids are more likely to be eliminated from the digestive system than conjugated bile salts, deconjugation of bile salts might help to reduce cholesterol levels. The demand for cholesterol as a precursor for the de novo synthesis of bile salts may rise if increased fecal loss of bile acids is brought on by bacterial BSH activity. This could lead to a decrease in cholesterol levels [7,8]. *Lactobacillus and Bifidobacterium,* native to the human gastrointestinal tract (GIT), are the first and most commonly used bacteria. *Leuconostoc*, *Pedicoccus*, *Lactococcus*, *Propionibacterium*, *Streptococcus* and *Enterococcus* species are other novel LAB [9–12]. *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, and *Peptostreptococcus*

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are among the Gram-positive intestinal bacteria that mediate BSH activity [13]. However, the need will be met as new strains with probiotic properties that are better than those currently available on the market are discovered. Thus, screening, isolating, and characterizing probiotic strains that are BSH-positive from diverse sources is the goal of the current study. Furthermore, standardization of BSH activity analysis, utilizing two substrates—taurocholic acid (TCA) and sodium thioglycocholate (GCA)—and optimization of process parameters such as pH, inoculum size, temperature, and revolutions per minute (RPM) were evaluated by one determinant at a time.

2. MATERIALS AND METHODS

2.1. Sample Collection

All samples were collected from various regions in Karnataka, India. Samples of cow and sheep milk are obtained from a nearby dairy and a sheep rarer located in Mathikere. The buffalo milk sample was collected from Gokak taluk in Belagavi district. Chicken intestine and gizzard samples were collected from a local butcher shop in Sadduguntepalya, Bengaluru district. The crab intestine sample was collected from crabs procured in seawater from Mulki, Dakshina Kannada district. The fish intestine sample was procured from a local meat shop at Sadduguntepalya, Bengaluru district. The duck feces sample was collected from Christ (Deemed to be University), Sadduguntepalya, Bengaluru district. All the samples were collected in autoclaved 50 ml centrifuge tubes under sterile conditions and were processed in laminar air flow aseptically.

2.2. Serial Dilution and Plating

The cow milk [14], sheep milk [15], and buffalo milk [16] samples, fresh duck feces [17], chicken gut [18], chicken gizzard [19], crab gut [20], and fish intestine [21] samples are macerated in a sterile mortar and pestle using a sterile saline solution (0.89%). The samples were serially diluted (10^{-1} to 10^{-9}) with sterile saline (0.89%) solution on De Man, Rogosa, and Sharpe agar (MRS) and incubated at 37˚C for 24 hours. Isolated colonies from different plates/dilutions were picked and subcultured on MRS agar by the grid plate method.

2.3. Screening of BSH Positive Strains by Thin Layer Chromatography (TLC)

The BSH activity of the isolated strains was assessed using TLC. Taurodeoxycholic acid (TDCA) was used as a substrate and cholic acid (CA) as a product standard. The freshly isolated colonies were inoculated in MRS broth containing 0.5% TDCA (10% standard solution; filter-sterilized) and incubated for 16 hours at 120 RPM and 37°C. After 16 hours, the samples were centrifuged for 10 minutes at 10,000 RPM and 4°C. The supernatant was preserved in a sterile centrifuge tube and subsequently utilized for the determination of BSH activity.

TLC: A droplet of 3 µl of supernatant was deposited on the TLC plates (4 × 4 cm, Silica Gel 60, Merck, Darmstadt, Germany). TDCA and CA solutions were used as standards. The mobile phase consists of a composition of 4:1.5:2:1 (v/v) isoamyl alcohol, acetic acid, propanol, and water. To visualize the bands (TDCA and CA), the TLC plate is subjected to derivatization with 10% $H_2SO_{4;}$ the TLC sheet is subsequently subjected to drying, utilizing a hot air gun [22].

2.4. BSH Assay

The total amount of CA released was estimated by measuring BSH activity following the procedure outlined by Irvin *et al.* (1944) and Kurzawa *et al.* (2006), with minor modifications. A 100 µl aliquot of supernatant (from the 0th and 16th hours) was mixed with 100 µl of 1% furfuraldehyde and the resulting mixture was incubated on ice for 10 minutes. The reaction mixture was mixed with 1.3 ml of H_2SO_4 (16 N) solution and incubated for 10 minutes at 70°C. The reaction was arrested by cooling the reaction mixture in an ice bath for 2 minutes. The concentration of total CA produced at the 16th hours was determined using the CA standard curve.

2.5. Gram Staining and 16s rRNA Sequencing

Strains were subjected to Grams staining according to the method prescribed by Coico [23], using a Hi-Media kit, crystal violet as the primary stain, and saffranine as the counter stain. The strain identification is done by 16s rRNA sequencing. The fragment of 16S rRNA gene is amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete polymerase chain reaction (PCR) amplicon band of 1,500 bp is observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon is carried out with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser. The consensus sequence of the 16S rRNA gene is generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence is used to carry out BLAST with the ‘nr” database of the NCBI GenBank database. Based on the maximum identity score, the first 10 sequences are selected and aligned using the multiple alignment software programme Clustal W. Distance matrix and phylogenetic tree are constructed using MEGA 10.

2.6. Endospore Staining

The test was conducted following the method outlined by Shuhadha *et al.* [24]. A grease-free slide with malachite green was prepared under boiling conditions and safranin was used as the counterstain to stain vegetative cells. The cells were then microscopically examined at 450X magnification for the presence of spores [24].

2.7. Catalase Activity

The test was conducted following the method described by Shuhadha *et al.* [24]. A drop of 3% hydrogen peroxide was applied to a loopful of microorganisms on a clean, grease-free slide and allowed to react. The presence of foam indicates a positive result for the catalase activity of the organism [24].

2.8. Gelatinase Activity

Gelatinase activity was assessed following the method described by Perin *et al.* [25]. A 5 µl aliquot of the 16 hours culture was spotted on the surface of nutrient gelatin (3% w/v) agar medium and incubated at 37˚C for 24 hours. Hydrolysis was confirmed by the appearance of an opaque halo surrounding the colonies [25].

2.9. Hemolysis Activity

The experiment was carried out according to the method described by Ruiz-Moyano *et al.* [26]. The isolated strains were cultured in MRS broth at 37°C for 16 hours, streaked onto 5% sheep blood agar plates, and incubated at 37°C for 48 hours. After 48 hours, results were recorded based on the observation of partial lysis of blood cells and a green zone (α-hemolysis), a clear zone around the bacterial growth (β-hemolysis), or no lysis (γ-hemolysis) surrounding the bacterial colonies [26].

2.10. Tolerance to Bile Salts

The experiment was carried out as described by Nueno-Palop and Narbad [27]. The isolates are tested for their ability to grow in the presence of bile salts (0.3%) supplemented in MRS broth. Samples were collected at the 0th and 3rd hours to determine viable colonies, which were counted by plating on MRS agar for 24 hours at 37°C [27].

2.11. Tolerance to Simulated Gastric Juice (Pepsin and Pancreatin)

Sterile MRS broth supplemented with 0.5% filter-sterilised pepsin was prepared and 1% (v/v) of the primary inoculum (OD_{600} = 1) was inoculated into the medium. Samples were taken at the 0th and 24th hours. Similarly, 10 ml MRS broth containing 1.0% pancreatin was cultured with 1% inoculation at 37°C. Samples were taken at regular intervals at the 0th and 3rd hours; viable bacteria were enumerated by plating on MRS agar for 24 hours at 37°C [28].

2.12. Tolerance to Gastric Acidity

The experimental strains were inoculated in MRS broth adjusted to different pH levels of 1.5, 2, 2.5, 3, and 6.5 (control). Samples were collected at the 0th and 3rd hours, plated on MRS agar, and incubated for 24 hours at 37°C to determine viability [29].

Table 1. BSH activity of the isolates in comparison with positive controls *E. lactis* and *L. acidophilus*.

Isolates	CA released (mg/ml)
Uninoculated control (MRS+TDCA)	55.2 ± 1.94
E. lactis (positive control)	**143.84 ± 3.43**
L. acidophilus (positive control)	**185.6 ± 2.86**
Milk sample isolate 4	98.24 ± 7.17
Milk sample isolate 8	**126.63 ± 3.97**
Chicken Gizzard 1	87.25 ± 5.77
Chicken Gizzard 3	**155.01 ± 2.77**
Chicken Gizzard 4	**145.12 ± 3.18**
Chicken Gizzard 6	87.43 ± 3.39
Chicken Gizzard 14	76.26 ± 2.82
Chicken Gizzard 15	94.76 ± 1.80
Chicken gut unmacerated 11	**179.56 ± 2.86**
Chicken gut unmacerated 13	**184.87 ± 2.34**

Bold values highlight the comparison of cholic acid liberated by the isolates and positive controls, an exclusion criterion for selection of BSH-positive isolates cholic acid producing equal to or more than positive controls.

2.13. Tolerance to Phenol

The phenol tolerance of the isolates was assessed by adding 0.4% (v/v) phenol to MRS broth, which was incubated for 24 hours. Samples from the 0th and 24th hours were then plated on MRS agar plates to confirm the survival of the isolates [3].

2.14. Tolerance to Iysozyme

Selected isolates were subjected to lysozyme tolerance, using the method described by Zago *et al.* (2011) with minor modifications. 1% (OD_{600} = 1) of bacterial cultures were inoculated in a sterile electrolyte solution for *in vivo* dilution by saliva (SES; 0.22 g/l $CaCl_2$, 6.2 g/l NaCl, 2.2 g/l KCl, 1.2 g/l $NaHCO_3$) in the presence of 100 mg/l of lysozyme (filter-sterilized); the samples were withdrawn at 3rd hours to determine the viability of the strains by plating the colonies on MRS agar. The bacterial suspensions in SES without lysozyme were considered as controls [30].

2.15. Surface Hydrophobicity Test

Bacterial adhesion was determined to assess the adhesion potential of microorganisms to surface hydrocarbons, which is a measure of adhesion to intestinal epithelial cells. The secondary culture was grown in MRS broth for 24 hours at 37°C. The samples are centrifuged at 10,000 RPM for 10 minutes. The cells were washed twice with 50 M K_2HPO_4 and the cells were suspended with xylene. The sample was vortexed for 2 minutes and incubated for 1 hour at 37°C. The aqueous solution was obtained and the absorbance of the aqueous solution was read at 560 nm [31].

2.16. Auto Aggregation Test

The procedure for auto aggregation follows Collado *et al.* [1] with slight modifications. Actively dividing cells were suspended in a phosphate buffer saline buffer to achieve an optical density (OD) of 0.5. After vortexing for 10 seconds, the 4 ml bacterial suspension was incubated at 30°C. After samples were taken out at two distinct times (0th and 24th hours), the auto-aggregation percentage was calculated using the formula $[1 - A_t/A_0] \times 100$, where "A_t" stands for absorbance at time "t" and A_0 for absorbance at 0th hour [31].

2.17. Cholesterol Assimilation

Zak's method was modified to evaluate the assimilation of cholesterol by *Enterococcus faecalis* CGz3. Water-soluble cholesterol (Sigma-Aldrich Pvt Ltd.) at concentrations of 0.2, 0.4, 0.45, and 0.5 mg/ml are added to the MRS broth, with 1% inoculum (OD_{600} = 1) and was incubated for 24 hours at 37°C and 120 RPM. Samples were taken at the 0th and 24th hours, which were then centrifuged for 10 minutes at 10,000 RPM and the supernatant was collected for further

Table 2. Preliminary characterization and safety evaluation of strains isolated from various sources.

Isolates from various sources	Gram's nature	Gelatinase activity	Catalase activity	Hemolytic activity	Endospore staining	16s rRNA identification
Milk sample 8 (MS8)	Gram-negative rod-shaped bacteria	Negative	Negative	γ-hemolysis	Negative	*E. coli*
Chicken gizzard 3 (CGz3)	Gram-positive cocci-shaped bacteria	Negative	Negative	γ-hemolysis	Negative	*E. faecalis*
Chicken gizzard 4 (CGz4)	Gram-positive cocci-shaped bacteria	Negative	Negative	γ-hemolysis	Negative	*E. faecalis*
Chicken gut Unmacerated isolated (CGUm)11	Gram-negative rod-shaped bacteria	Negative	**Positive**	**α-hemolysis**	Negative	*E. albertii*
Chicken gut Unmacerated isolated (CGUm)13	Gram-positive rod-shaped bacteria	Negative	**Positive**	**β-hemolysis**	Negative	*B. subtilis*

Bold values indicate the isolates are pathogenic and do not satisfy the selection criteria set by ICMR-DBT guidelines.

analysis. Percentage cholesterol reduction was calculated against the cholesterol standard.

2.18. Substrate Optimization

This method can be utilized to assess the specificity of cholates (GCA and taurocholic acid) in the presence of di- and monohydroxycholanates. Some of the crucial elements were identified to standardize the qualitative and quantitative assay of the BSH activity by modifying the techniques developed by Gregory and Poscoe [32]. *Enterococcus faecalis*' CGz3 BSH activity was evaluated using two distinct substrates—taurocholic acid (TCA) and GCA. Both a qualitative method (TLC) and a quantitative method (furfuraldehyde method), with minor modifications, are used to determine the BSH activity, .

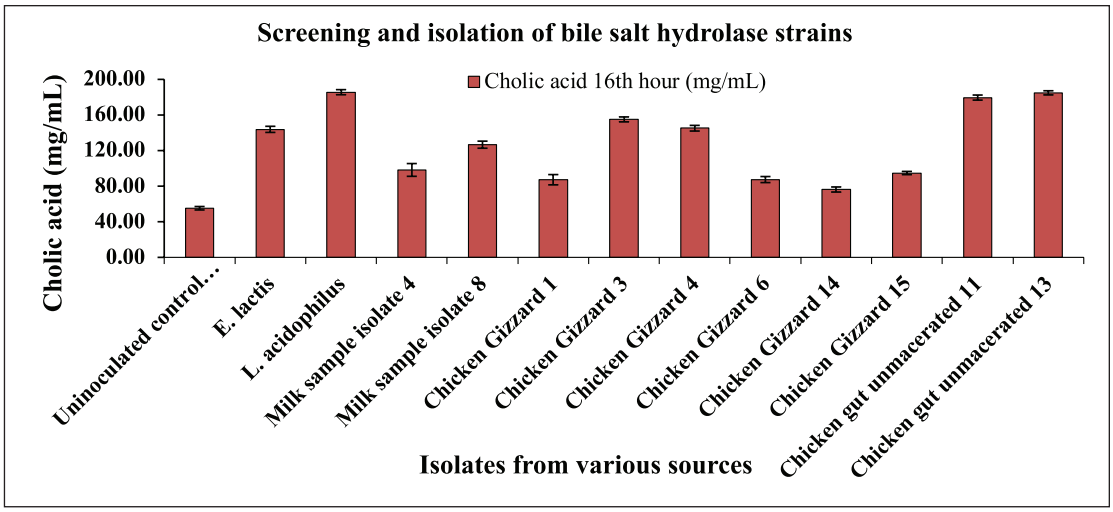

Figure 1. BSH activity of isolates from various sources in comparison with two positive controls *E. lactis* and *L. acidophilus*.

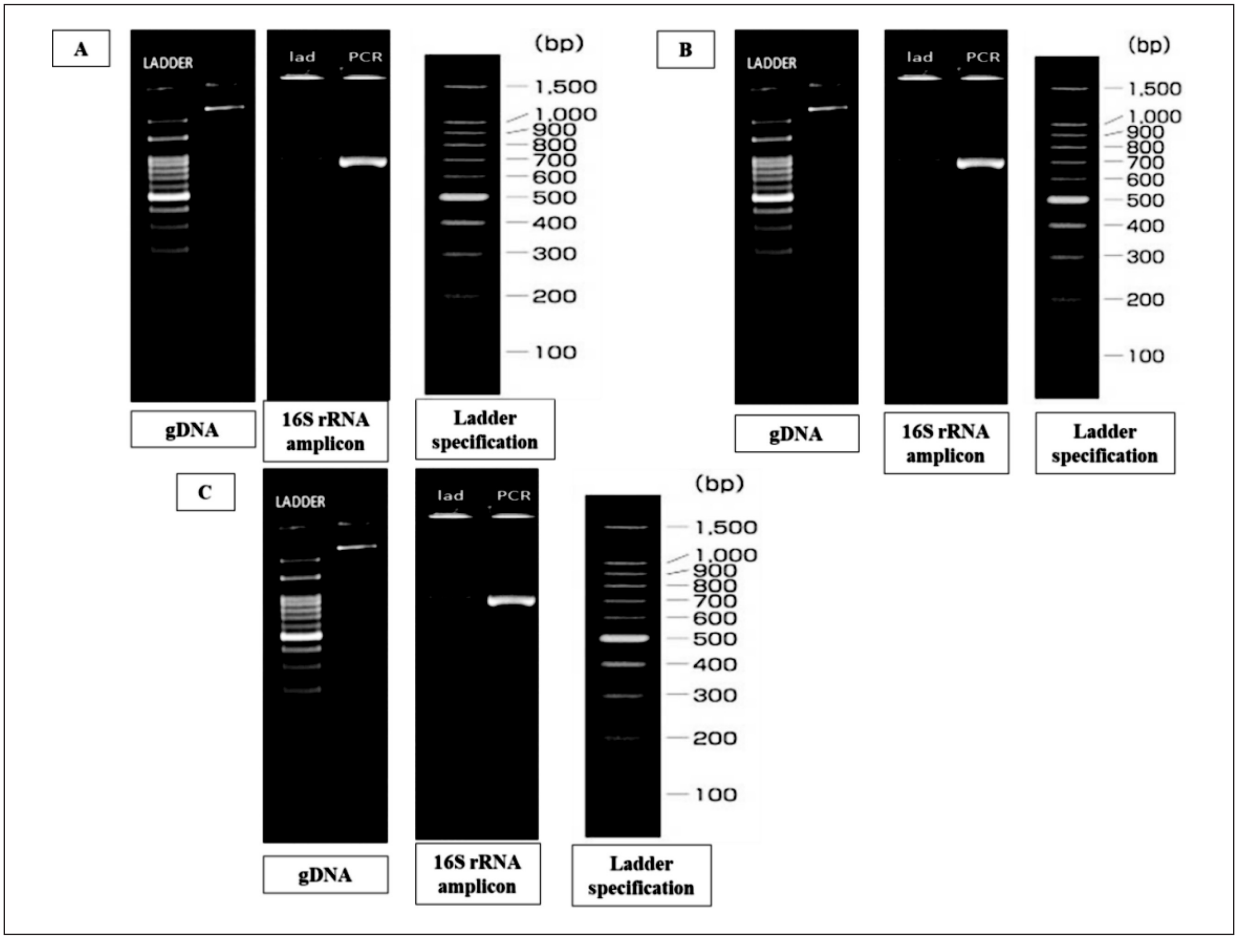

Figure 2. gDNA, 16S rRNA and ladder specification of the organisms. A) CH Gizz 3; B) CH Gizz 4; C) CG Um 13.

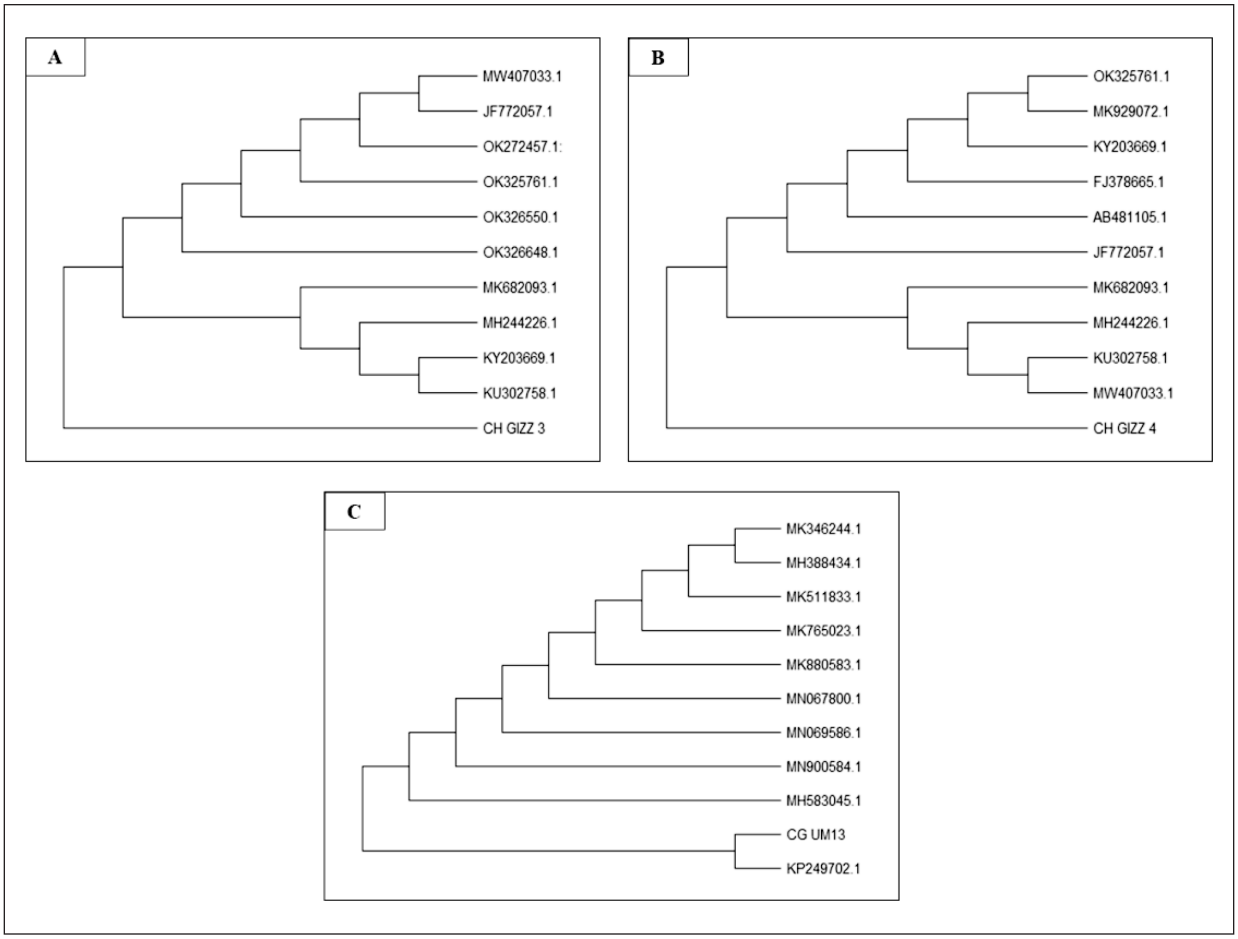

Figure 3. Phylogenetic tree of the isolates; A) CH Gizz 3; B) CH Gizz 4; C) CG Um 13.

Table 3. Probiotic characterization of the selected isolates.

Isolates	Percentage of survivability: simulated GIT conditions								
	Tolerance to gastric acidity					Other characterizations			
	pH: 1.5	pH: 2.0	pH: 2.5	pH: 3.0	pH: 6.5	Bile salt tolerance	Phenol tolerance	Pepsin tolerance	Pancreatin tolerance
E. faecalis CGz3	0	6.16 ± 1.06	8.69 ± 0.46	16.20 ± 0.42	76.76 ± 0.50	53.50 ± 2.06	96.68 ± 4.89	63.75 ± 1	91.61 ± 0.93
E. faecalis CGz4	Nil	Nil	Nil	Nil	Nil	42.49 ± 1.67	73.21 ± 6.96	Nil	64.24 ± 2.54

1% (OD_{600} = 1) of the *E. faecalis* CGz3 cells were inoculated into the MRS media at varying concentrations of the two substrates (0.05% to 0.4% v/v). The culture was incubated for 16 hours at 120 RPM and 37°C. During the 0th and 16th hours, samples are taken and after centrifuging for 10 minutes at 10,000 RPM and 4°C, the supernatant is used for BSH assay.

2.19. Standardization of UV-Spectrophotometric Method of BSH Assay

The supernatant was vortexed gently after being allowed to come to room temperature. The cell-free supernatant was mixed with 100 µl of 1% furfuraldehyde. The reaction mixture was incubated at 65°C for 15 minutes, followed by the reaction arrest step in a cold bath for 1 minute and 25 seconds. The absorbance was read at 605 nm. The presence of the BSH enzyme is shown by the reaction mixture turning from light brown to blue due to CA.

2.20. Substrate and Substrate Concentration Optimization

0.1% to 0.4% of the substrates, taurocholic acid and (TCA and GCA), were added to the MRS medium, to which 1% of (OD_{600} = 1) *E. faecalis* CGz3 was inoculated and incubated for 16 hours at 37°C, 120 RPM. Samples were taken at 0 and 16 hours, and they were centrifuged for 10 minutes at 10,000 RPM and 4°C. After collecting the supernatant, the method described above was employed to quantify BSH activity. Based on the results, the optimal substrate and substrate concentration were selected for all subsequent analysis.

2.21. BSH Assay Time-Point Optimization

Using the optimal substrate concentration, the assessment was carried out to determine the optimal time point for BSH activity. 0.1% sodium GCA was added to the MRS media and inoculated with 1% *E. faecalis* CGz3 (OD_{600} = 1); the sample was incubated for 0,

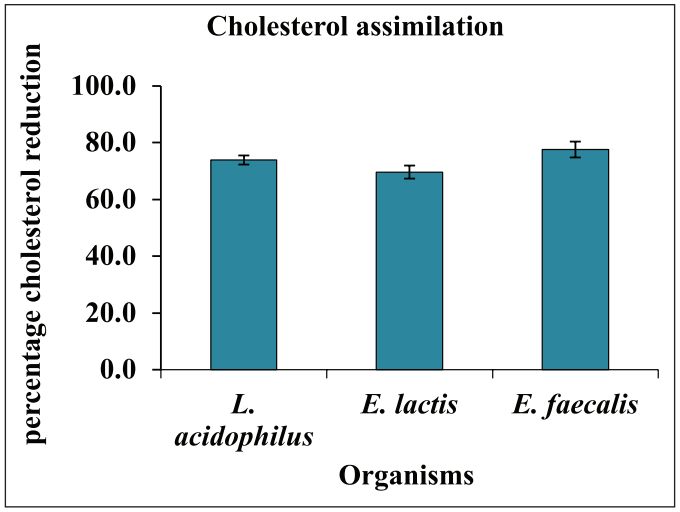

Figure 4. Cholesterol assimilation by *E. faecalis* CGz3 *in* comparison with two positive controls *L. acidophilus* and *E. lactis*.

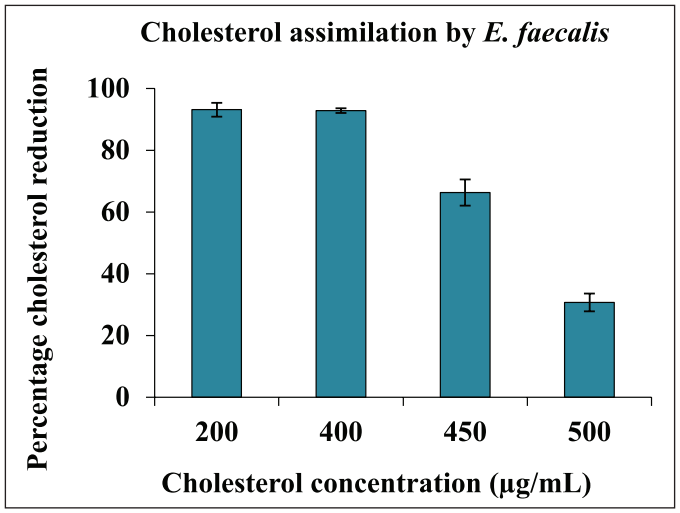

Figure 5. Percentage reduction in cholesterol by *E. faecalis* CGz3 in varying cholesterol concentrations.

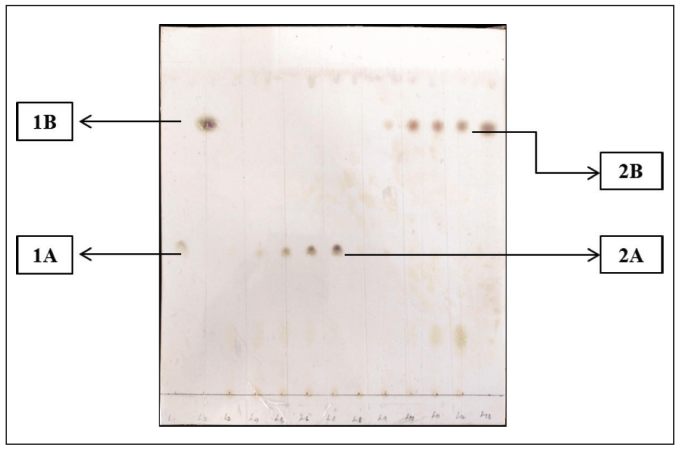

Figure 6. TLC; substrate concentration optimization; A: GCA (substrate); B: Standard CA (product). A: GCA band observed at 0th hour; B: CA band observed at 16th hours.

Table 4. Substrate concentration optimization for quantification of BSH activity using GCA as a substrate; 0.1% GCA was found to be the optimum concentration for quantification of BSH activity.

Concentrations of GCA (mg/ml)	CA 0th hour (mg/ml)	CA 16th hour (mg/ml)
0.05	210.1 ± 3.13[a]	236.5 ± 8.04[a]
0.1	1624.2 ± 8.67[b]	**2314.4 ± 11.37[e]**
0.2	1692.2 ± 8.45[c]	1011.7 ± 6.36[b]
0.3	1745 ± 8.67[d]	1147.8 ± 10.85[c]
0.4	1975.6 ± 13.25[e]	1314.4 ± 9.11[d]

Indicates the highest cholic acid liberated at 0.1% of GCA. The data are presented as mean ± SD (n = 3). Using IBM SPSS software, ver. 27.0. One way ANOVA revealed that values without a matching alphabet in the same column were substantially different ($p < 0.05$).

Table 5. Substrate concentration optimization for quantification of BSH activity using TCA as a substrate; 0.05% TCA was found to be the optimum concentration for quantification of BSH activity.

Concentrations of TCA (mg/ml)	CA 0th hour (mg/ml)	CA 16th hours (mg/ml)
0.05	402.9 ± 2.74[a]	**488.8 ± 1.27[a]**
0.1	1011.9 ± 7.35[b]	646.7 ± 23.2[b]
0.2	1295.3 ± 10.85[c]	1275.8 ± 31.46[c]
0.3	2455 ± 12.73[d]	2339.7 ± 22.95[d]
0.4	3659.2 ± 10.49[e]	3495.3 ± 25.12[e]

Indicates the highest cholic acid liberated at 0.05% of TCA. The data are presented as mean ± SD (n = 3). Using IBM SPSS software, ver. 27.0. One way ANOVA revealed that values without a matching alphabet in the same column were substantially different ($p < 0.05$).

Table 6. One-factor-at-a-time analysis for the optimization of BSH activity of *E. faecalis* CGz3. pH 5.5 and 1% inoculum (v/v) was found to be optimum for the quantification of BSH enzyme. Increased BSH activity was observed at 40°C and 200 RPM.

Process parameters		Total CA release (mg/ml)
	1	**111.1 ± 7.29[c]**
Inoculum %	2.5	9.2 ± 3.85[b]
	5	−25.8 ± 7.41[a]
	25	95.2 ± 7.643[a]
	30	87.2 ± 2.274[a]
Temperature	35	121.3 ± 7.011[b]
	40	**161.6 ± 9.87[c]**
	37 (Control)	139.7 ± 4.86[b]
	100	130 ± 6.67[a]
	150	161.1 ± 8.07[b]
RPM	**200**	**262.2 ± 7.22[c]**
	120 (Control)	127.7 ± 8.62[a]

The significance of the values has been mentioned in the caption of the table. The data are presented as mean ± SD (n = 3). Using IBM SPSS software, ver. 27.0, Duncan's Multiple Range Test (DMRT) shows that the mean values with similar alphabets are not statistically different at $p \leq 0.05$.

6, 8, 16, and 24 hours at 120 RPM and 37°C. The samples were taken at regular intervals and centrifuged for 10 minutes at 4°C and 10,000 RPM. The optimum BSH activity in the supernatants was determined by analyzing them at the time points of 0, 6, 8, 16, and 24 hours.

2.22. Design and Set up of Experiments for One Factor at a Time Analysis for Process Parameter Optimization

Five pH levels (5, 5.5, 6, 6.5, and 7), five temperature levels (25˚C, 30˚C, 35˚C, 37˚C, and 40˚C), three inoculum sizes (1%, 2.5%, and 5%), and four RPM levels (100, 120, 150, and 200 RPM) are examined to identify the optimal process parameters for BSH activity of *E. faecalis* CGz3.

2.23. Statistical Analysis

Values are presented as mean values and standard deviations of triplicate experiments. Significant analysis of variance was performed by Duncan's multiple range test and differences were considered statistically significant of p-value <0.05.

3. RESULTS

3.1. BSH Assay

A comparative analysis of BSH activity amongst 10 isolates in comparison with two BSH-positive controls, *Enterococcus lactis* and *Lactobacillus acidophilus,* was performed. Of the two positive controls, *L. acidophilus* produces 185.60 ± 2.86 mg/ml and *E. lactis* produces 143.85 ± 3.43 mg/ml of CA. Whereas chicken gizzard isolates (CGz3 and CGz4) produce 155.02 ± 2.77 and 145.13 ± 3.18 mg/ml, milk isolate 8 (MS8) produces 126.63 ± 3.97 mg/ml, chicken gut isolates (CGUm 11 and CGUm 13) produce 179.56 ± 2.86 mg/ml and 184.87 ± 2.34 mg/ml of CA. Isolates that produced equal to or more than the positive controls were selected for further characterization (Fig. 1).

3.2. Preliminary Characterization: Grams' Staining and 16s rRNA Sequencing; Hemolysis, Gelatinase, and Catalase Test

Various samples using a total of 513 isolates were screened for BSH activity, in which 23 predominant BSH colonies were isolated; 23 bacterial strains out of 513 isolates show the presence of CA bands on TLC, which confirms BSH activity. A quantitative assay was used for further screening of the BSH-positive strains to estimate the CA produced by these strains. A total of four isolates were

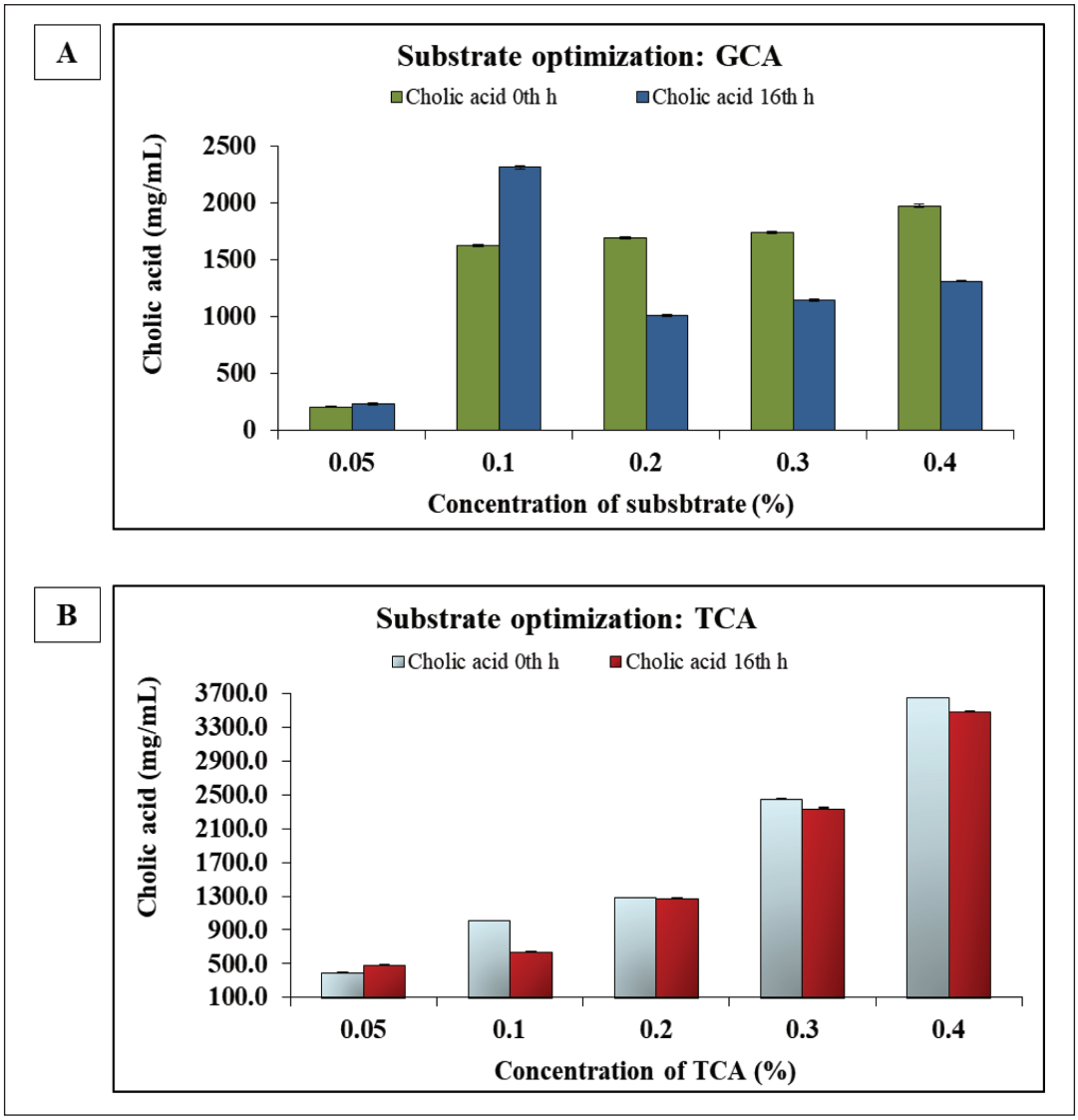

Figure 7. A: Substrate concentration optimization using GCA for optimum BSH activity of *E. faecalis* CG3; B: Substrate concentration optimization using TCA for optimum BSH activity of *E. faecalis* CGz3.

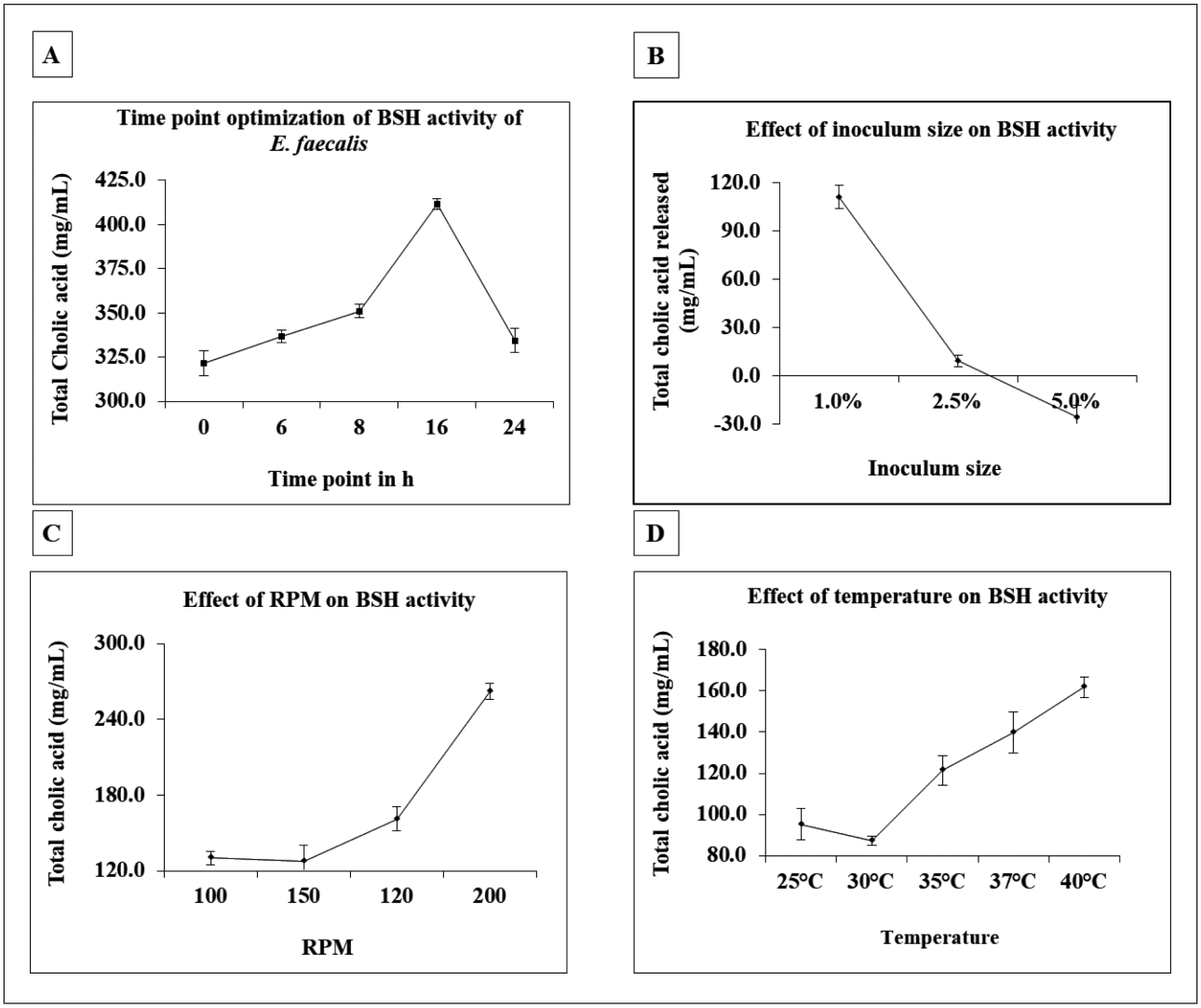

Figure 8. A: Time point optimization of BSH activity; B: Effect of inoculum size on the BSH activity; C: Effect of RPM on BSH activity; D: Effect of temperature on BSH activity of *E. faecalis* CGz3.

selected based on their ability to produce CA in comparison with *E. lactis* and *L. acidophilus* strains, which were used as the positive control (BSH-positive strains). The BSH activity estimated from isolates from the chicken gut isolate 11 (CG11) and chicken gut isolate 13 (CG13); chicken gizzard 3 (CGz3) and chicken gizzard 4 isolates (CGz4) in comparison with two positive controls *E. lactis* and *L. acidophilus,* show increased levels of CA produced (Table 1) at 16th hours expressed in mg/ml against the CA standard (1 mg/ml).

Preliminary screening of the isolates involves the Indian Council for Medical Research—Department of Biotechnology (ICMR-DBT) guidelines for the safety evaluation of probiotics. Based on the preliminary assessment, 16s rRNA sequencing, and the BSH activity in comparison with positive controls, two strains, *E. faecalis* CGz3 and *E. faecalis* CGz4 strains were chosen for further probiotic characterization. The results of the preliminary characterization are presented in Table 1- BSH activity of the isolates and Table 2- Probiotic characterization. Figure 1- BSH assay; Figures 2 and 3- 16s rRNA sequencing of the probiotic isolates.

3.3. Probiotic Characterization: Simulated GIT Conditions

The percentages of survivability of both *E. faecalis* (CGz3 and CGz4) strains were used to compare the probiotic characterization data. *E. faecalis* CGz3 was found to be resistant to the simulated gastric conditions; however, *E. faecalis* CGz4 was less resistant to simulated gastric conditions and was non-resistant to pepsin and gastric acidity (Table 3).

3.4. Tolerance to Iysozyme

When treated with 100 g/ml lysozyme, *E. faecalis* CGz3 shows 91.34% ± 5.57% survival at 0.5 hours, 97.75% ± 7.16% survivability at 1 hour, 97.27% ± 5.03% survivability at 1.5 hours, and 89.56% ± 1.96% survivability at 2 hours of incubation.

3.5. Surface Hydrophobicity Test

Enterococcus faecalis CGz3 was observed to have a surface hydrophobicity percentage of 20.39%.

3.6. Auto-Aggregation Test

The percentage of auto-aggregation in the *E. faecalis* CGz3 auto-aggregation test was 131.24%.

3.7. Cholesterol Assimilation in Comparison with *L. acidophilus*, *E. lactis*, and *E. faecalis* CGz3

After a 24-hour incubation period, *E. faecalis* CGz3 reduces cholesterol by 77.6% ± 2.8%, compared to 69.7% ± 2.3% and 73.9% ± 1.6% for the two positive controls, *E. lactis* and *L. acidophilus* (Fig. 4) and the percentage of cholesterol reduction at various concentrations of 200, 400, 450, and 500 µg/ml of cholesterol (v/v) was 93.11% ± 2.24^{c}%, 92.83% ± 0.75^{c}%, 66.3% ± 4.23^{b}%, and 30.71% ± 2.88^{a}%, respectively (Fig. 5).

3.8. Substrate Concentration Optimization

In comparison with GCA and TCA, 0.05% and 0.1% (v/v) of Sodium glycocholate/GCA were observed to have increased CA released at the end of 16 hours of incubation. An increase in CA was observed only at 0.05% (v/v) taurocholic acid. A gradual decrease in BSH activity was observed from concentrations 0.2%, 0.3%, and 0.4% of both substrates. Based on the outcome, 0.1% GCA was considered the optimal substrate concentration for the determination of BSH activity in all further experiments. All the trials were performed in triplicates (Fig. 5 – Graph) (Fig. 6 – TLC) (Tables 4 and 5).

3.9. Timepoint Optimization of BSH Activity

Based on the outcome of substrate concentration optimization, GCA was considered as the substrate for the determination of time point optimization of the BSH activity. Progressive increase in the release of CA 321.56 ± 6.9^{a}, 336.56 ± 3.6^{ab}, 351 ± 3.8^{b}, 411.56 ± 3.1^{c} and 334.33 ± 6.8^{a}, respectively, was observed throughout the time points 0th, 6th, 8th and 16th hours. However, a decrease in BSH activity was observed at 24 hours; therefore, 16th hours was considered to be optimum for quantification of BSH activity (Fig. 8A).

3.10. One Factor at a Time Analysis for Process Parameter Optimization

The optimal inoculum size and pH to quantify the BSH activity were determined to be 1% and pH 5.5, respectively. All five temperatures and RPM levels show increasing BSH activity, with 40°C and 200 RPM showing the highest BSH activity (Fig. 8B–D).

4. DISCUSSION

Five out of 23 BSH-positive strains were further characterized, including safety assessment and probiotic evaluation. The hemolytic, catalase, and gelatinase tests are conducted for the evaluation of virulence factors. When Enterococci and other LAB lack cytolysin coding genes is a beneficial trait, this characteristic property of Enterococci confirms that these can be used as food preparations [33]. However, Enterococci species are known to cause nosocomial infections; therefore, it is important for safety assessment of *Enterococcus* species to identify their viral traits. The most important virulence trait of *Enterococcus* spp. is cytolysin, which lyses the eukaryotic cells. Our results show *E. faecalis* CGz3 and CGz4 show γ-hemolysis on 5% blood agar plates, which predict the absence of cytolysin genes, such as *cylM*, *cylA,* and *cylB*. However, *Bacillus subtilis* exhibits β-hemolysis on 5% sheep blood agar, confirming the presence of pathogenic traits. Catalase activity facilitates the detection of catalase enzyme, which is essential in differentiating catalase-positive micrococcaceae from catalase-negative streptococcaceae. The test is also valuable in differentiating aerobic and obligate anaerobic bacteria, as anaerobes are known to lack the catalase enzyme [21,22]. *Bacillus subtilis* expressed catalase activity, upon adding H_2O_2 confirming the presence of catalase enzyme, which makes it incapable of further evaluations. Gelatinase, extracellular zinc metalloproteases, has been linked to virulence factors in certain strains. This enzyme is capable of hydrolyzing gelatin and collagen [34]. In the current assessment, neither of the *E. faecalis* strains produces a halo zone or zone of clearance on the nutrient-gelatin agar, which confirms the absence of gelatinase activity. Our findings align with the criteria set by the ICMR-DBT guidelines for the isolated *Enterococcus* strains, confirming their status as generally regarded as safe. Both enterococci strains show γ-hemolytic activity, negative catalase, and gelatinase activity, which provides evidence of non-pathogenicity of the strains, allowing for further characterization of probiotic potential (Table 2). The isolates, *E. faecalis* (CGz3 and CGz 4), were further subjected to probiotic characterization, which simulates the GIT. This enables the selection of strains likely to withstand harsh conditions in the stomach (gastric acidity) and gut environments (gastric juices), allowing for further exploration of their potential as probiotic cultures. Saliva contains lysozyme, which causes stress to bacteria in the mouth and the *E. faecalis* CGz3 strain shows tolerance to 100 mg/l of lysozyme for up to 2 hours. This stress then spreads to the stomach, which has a pH between 1.5 and 3.0, and the upper intestine, which includes bile salts. Both the Enterococci strains were tolerant to gastric acidity and bile salts. However, *E. faecalis* CGz4was intolerant to one of the gastric juices (Table 3), which was used as the exclusion criteria for further characterization. The important criteria for a probiotic strain are to withstand extreme conditions in the stomach and small intestine. Their resistance to bile and gastric acid is a crucial probiotic characteristic. Based on the tolerance to simulated gastric conditions, *E. faecalis* CGz3 was chosen as a potential probiotic strain and was considered for further characterization (Table 3).

Another key criterion for selecting probiotics is their ability to hydrolyze bile salts. The BSH enzyme is often associated with the ability to assimilate cholesterol [35]. In the current study, we observe that all the isolates were capable of deconjugating TDCA. (TDCA) at 16th hours (Fig. 1). The BSH enzyme hydrolyses bile salts to produce glycine and/or taurine, serving as a mechanism by which gut bacteria can reduce cholesterol levels in the human body. In the human colon, hydrolyzed bile salts are less absorbed, leading to increased excretion of free bile acids in feces. *De novo* synthesis can replenish the lost bile salts, thereby contributing to the reduction of serum cholesterol levels [36].

In a recent study by Malaviya *et al*. [37] , *E. faecalis* was proven to deconjugate GCA, and the maximum CA release was observed at 18th hours, whereas, in the current study, the maximum CA release was observed at 16th hours (Fig. 7A). Our strain, *E. faecalis* CGz3, was able to assimilate cholesterol *in vitro* (Figs. 4 and 5). In a simulated condition of severe hypercholesterolemia, carried out from low to high concentrations of cholesterol, it is observed that 93.11 ± 2.24^{c}, 92.83 ± 0.75^{c}, 66.3 ± 4.23^{b}, and 30.71 ± 2.88^{a} percentage cholesterol reduction was observed from 200 to 500 µg/ml of cholesterol, respectively, which proves that *E. faecalis* CGz3 can be a potential probiotic strain in the preparation of nutraceutical products with proven health benefits. In a recent study conducted by Zhu *et al.* [38], the beneficial effects of *E. faecalis* ATCC 19433, was demonstrated, where the strain contributes to the transportation of cholesterol potentially and modulates the composition of gut microbiota of hypercholesterolemic mice, by increasing the expression of ABCG5 and ABCG8. Their work highlights the decrease in total cholesterol in the serum and liver of mice and the increase of cholesterol in feces.

The result follows the previous studies, where strains show higher cholesterol assimilation. This provides insight into gene regulation studies involving metabolic-associated disorders, such as T2DM, non-alcoholic fatty liver disease (NAFLD)/metabolic-associated fatty liver disease, and MASH. In another study by Mishra *et al.* [37] the *E. faecalis* AG5 strain assimilates cholesterol in the presence of a bile salt mixture (0.3% GCA) [39].

For optimization of BSH activity, standardization is necessary. *Enterococcus faecalis* CGz3 is able to produce BSH in the presence of GCA (0.1%), which is transformed into CA in 16 hours (Fig. 7A and B) (Tables 4 and 5). The pH plays an important factor in the hydrolysis of GCA into CA, the intestine pH ranges from 5.5 to 7, and the BSH activity was analyzed similarly. It is assumed that the lactic acid production in the bacteria is directly proportional to pH and has a detrimental effect on metabolite production. Our study suggests that the BSH activity of *E. faecalis* CGz3 is active throughout the pH ranges selected, however, quantification is optimum at pH 5.5 (Table 6). This suggests that the BSH activity is inducible by GCA; whether BSH is synthesised constitutively or not, the enzyme could be considered active in the small intestine, where it is exposed to bile salts. The BSH activity was observed throughout the time and decreased during the 24-hour incubation time, suggesting that the highest BSH activity was achieved during the exponential log phase of the organisms' growth. Our study findings suggest that the other process parameters are also directly responsible for the BSH activity of *E. faecalis* CGz3. The inoculum size, RPM, and temperature have an impact on the BSH activity of *E. faecalis* CGz3. The optimal BSH activity is observed in 1% inoculum size at 16th hour. The decrease in BSH activity at 2.5% and 5% inoculum size may be due to quicker utilization of the substrate (GCA) in the presence of high bacterial count. An increase in BSH activity was observed in process parameters, such as higher temperature and higher RPM. The conditions optimized for the BSH activity might help scale up the process (Table 6). Overall, the strain was identified as a potential producer of the BSH enzyme, which plays a key role in cholesterol assimilation from blood serum. Enterococci are also frequently employed as starter cultures. Probiotic preparations and fermented meat, dairy, and vegetable products contain large populations of Enterococci, which aid in product ripening and flavor development. A variety of Enterococci formulations among the probiotics are some of the commercially available probiotics. ECOFLOR (Watlters Health Care, Den Haag, The Netherlands), Cylactin® (Hoffmann-La Roche, Basel, Switzerland), and Symbioflor 1 (SymbioPharm, Herborn, Germany) are a few examples of these [31,32].

5. CONCLUSION

The comprehensive results of the current study provide strong scientific evidence that *E. faecalis* CGz 3 could serve as a potential probiotic strain in the supportive management of metabolic-associated disorders, such as hypercholesterolemia, as well as other conditions like NAFLD and progressive non-alcoholic steatohepatitis.

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All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

9. CONFLICTS OF INTEREST

The author reports no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

REFERENCES

1. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains [Internet]. Eur Food Res Technol 2008; 226:1065–73. Available via https://api.semanticscholar.org/CorpusID:85423542
2. Kim DH, Park MH, Choi YJ, Chung KW, Park CH, Jang EJ, et al. Molecular study of dietary heptadecane for the anti-inflammatory modulation of NF-kB in the aged kidney. PLoS One 2013;8(3):e59316.
3. Riaz Rajoka MS, Mehwish HM, Siddiq M, Haobin Z, Zhu J, Yan L, et al. Identification, characterization, and probiotic potential of Lactobacillus rhamnosus isolated from human milk. Lwt 2017; 84:271–80.
4. Havenaar R, Huis In't Veld JHJ. Probiotics: a general view. Lact Acid Bact 1992;1:151–70.
5. Kimoto H, Ohmomo S, Okamoto T. Cholesterol removal from media by lactococci. J Dairy Sci 2002;85(12):3182–8.
6. Lechner K, Mckenzie AL, Kränkel N, Von Schacky C, Worm N, Nixdorff U, *et al.* High-risk atherosclerosis and metabolic phenotype: the roles of ectopic adiposity, atherogenic dyslipidemia, and inflammation. Metab Syndr Relat Disord 2020;18(4):176–85.
7. De Rodas BZ, Gilliland SE, Maxwell CV. Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC 43121 and calcium in swine with hypercholesterolemia induced by diet. J Dairy Sci 1996 Dec;79(12):2121–8.
8. Kneifel W, Jaros D, Erhard F. Microflora and acidification properties of yogurt and yogurt-related products fermented with commercially available starter cultures. Int J Food Microbiol 1993;18(3):179–89.

9. Mombelli B, Gismondo MR. The use of probiotics in medical practice. Int J Antimicrob Agents 2000;16(4):531–6.
10. Krasaekoopt W, Bhandari B, Deeth H. Evaluation of encapsulation techniques of probiotics for yoghurt. Int Dairy J [Internet] 2003;13(1):3–13. Available via https://www.sciencedirect.com/science/article/pii/S0958694602001553
11. Power DA, Burton J, Chilcott C, Dawes P, Tagg J. Preliminary investigations of the colonisation of upper respiratory tract tissues of infants using a paediatric formulation of the oral probiotic *Streptococcus salivarius* K12. Eur J Clin Microbiol Infect Dis 2008;27:1261–3.
12. Vinderola CG, Reinheimer JA. Lactic acid starter and probiotic bacteria: a comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. Food Res Int [Internet] 2003;36:895–904. Available via https://api.semanticscholar.org/CorpusID:83480721
13. Chikai T, Nakao H, Uchida K. Deconjugation of bile acids by human intestinal bacteria implanted in germ-free rats. Lipids [Internet] 1987;22(9):669–71. doi: 10.1007/BF02533948
14. Roy A, Panja AS, Maitra M, Bandopyadhyay B. Isolation and characterizations of probiotics from bovine (Cow) milk. Res Rev Biotechnol Biosci 2019;6(2):24–32.
15. Acurcio LB, Souza MR, Nunes AC, Oliveira DLS, Sandes SHC, Alvim LB. Isolation, enumeration, molecular identification and probiotic potential evaluation of lactic acid bacteria isolated from sheep milk. Arq Bras Med Vet Zootec 2014;66(3):940–8.
16. Shafakatullah N, Chandra M. Screening of raw Buffalo's milk from Karnataka for potential probiotic strains. Res J Recent Sci Res J Recent Sci 2014;3(9):73–8.
17. Kimprasit T, Sukontasing S, Amavisit P. *In vitro* selection of potential lactic acid bacteria isolated from ducks and geese in Thailand. Kasetsart J Nat Sci 2013;47(2):261–70.
18. Knarreborg A, Engberg RM, Jensen SK, Jensen BB. Quantitative determination of bile salt hydrolase activity in bacteria isolated from the small intestine of chickens. Appl Environ Microbiol. 2002;68(12):6425–8.
19. Idoui T. Probiotic properties of *Lactobacillus* strains isolated from gizzard of local poultry. Iran J Microbiol 2014;6(2):120–6.
20. Pramono_Screening and characterization of bacteria like producing lactic acid bacteria from mud cr.pdf.
21. Buntin N, Chanthachum S, Hongpattarakere T. Screening of lactic acid bacteria from gastrointestinal tracts of marine fish for their potential use as probiotics. Songklanakarin J Sci Technol 2008;30(SUPPL. 1):141–8.
22. Sedláčková P, Horáčková Š, Shi T, Kosová M, Plocková M. Two different methods for screening of bile salt hydrolase activity in *Lactobacillus* strains. Czech J Food Sci 2015;2015(1):13–8.
23. Coico R. Gram staining basic protocol commonly used techniques. Curr Protoc Microbiol 2005;3–4.
24. Shuhadha MFF, Panagoda GJ, Madhujith T, Jayawardana NWIA. Evaluation of probiotic attributes of *Lactobacillus* sp. isolated from cow and buffalo curd samples collected from Kandy. Ceylon Med J 2017;62(3):159.
25. Perin LM, Miranda RO, Todorov SD, de Franco BDGM, Nero LA. Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk. Int J Food Microbiol [Internet] 2014;185:121–6; doi: 10.1016/j.ijfoodmicro.2014.06.001
26. Ruiz-Moyano S, Martín A, Benito MJ, Casquete R, Serradilla MJ, de Córdoba MG. Safety and functional aspects of pre-selected lactobacilli for probiotic use in Iberian dry-fermented sausages. Meat Sci [Internet] 2009;83(3):460–7; doi: 10.1016/j.meatsci.2009.06.027
27. Nueno-Palop C, Narbad A. Probiotic assessment of *Enterococcus faecalis* CP58 isolated from human gut. Int J Food Microbiol 2011;145(2–3):390–4.
28. Liu Z, Jiang Z, Zhou K, Li P, Liu G, Zhang B. Screening of bifidobacteria with acquired tolerance to human gastrointestinal tract. Anaerobe 2007;13(5–6):215–9.
29. Balcázar JL, Vendrell D, de Blas I, Ruiz-Zarzuela I, Muzquiz JL, Girones O. Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. Aquaculture 2008;278(1–4):188–91.
30. Vizoso Pinto MG, Franz CMAP, Schillinger U, Holzapfel WH. *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. Int J Food Microbiol 2006;109(3):205–14.
31. Osmanagaoglu O, Kiran F, Ataoglu H. Evaluation of *in vitro* probiotic potential of *Pediococcus pentosaceus* OZF isolated from human breast milk. Probiotics Antimicrob Proteins 2010;2(3):162–74.
32. Gregory R, Pascoe TA. The quantitative determination of bile acids by means of a new color reaction and monochromatic light. J Biol Chem [Internet] 1929;83(1):35–42; doi: 10.1016/S0021-9258(20)70835-5
33. Ahmadova A, Todorov SD, Choiset Y, Rabesona H, Mirhadi Zadi T, Kuliyev A, *et al.* Evaluation of antimicrobial activity, probiotic properties and safety of wild strain *Enterococcus faecium* AQ71 isolated from Azerbaijani Motal cheese. Food Control [Internet] 2013;30(2):631–41; doi: 10.1016/j.foodcont.2012.08.009
34. Qin X, Singh KV, Weinstock GM, Murray BE. Effects of *Enterococcus faecalis* fsr genes on production of gelatinase and a serine protease and virulence. Infect Immun 2000;68(5):2579–86.
35. Liong MT, Shah NP. Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. Int Dairy J [Internet] 2005;15(4):391–8. Available via https://www.sciencedirect.com/science/article/pii/S0958694604002109
36. Begley M, Hill C, Gahan CGM. Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 2006;72(3):1729–38.
37. Malaviya A, Malviya S, Agarwal A, Mishra M, Dalmida V. Saccharomyces–eukaryotic probiotic for human applications. In: High value fermentation products, John Wiley & Sons, Hoboken, NJ, pp 211–29, 2019.
38. Zhu Y, Li T, Din AU, Hassan A, Wang Y, Wang G. Beneficial effects of *Enterococcus faecalis* in hypercholesterolemic mice on cholesterol transportation and gut microbiota. Appl Microbiol Biotechnol 2019;103(7):3181–91.
39. Asiva Noor Rachmayani. No Covariance structure analysis of health-related indicators in elderly people living at home, focusing on subjective health Title. 2015;6.

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