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Isolation of Cholesterol-Reducing Potential Probiotic Bacterial Strains from Home-Made Fermented Foods

Dr. Suchitra Naidu[1], Dr. Y. Sabitha[2]

[1]Assistant Professor, Loyola academy, Hyderabad
[2]Research Head, Ciencia Labs, Hyderabad

Abstract: Fermented foods are widely acknowledged as a potential primary source of probiotics. The primary objective of the present study was to investigate the cholesterol assimilation and probiotic characteristics of bacteria isolated from Dosa batter and Chana Dal. The essential characteristics of non-haemolytic fermenting bacteria, which are relevant to probiotics, were evaluated through in vitro testing. These features encompass acid tolerance (at a concentration of 4%), tolerance to saline conditions (at a concentration of 2.5%), and bile salt tolerance (up to a concentration of 2.5%). The results of these tests yielded six bacterial isolates with promising probiotic potential, namely CDS4, CDS5, CDS12, DBS6, DBS10, and DBS12. Among these isolates, strains CDS5 and DBS12 exhibited notable cholesterol action. The 16S rDNA gene sequencing analysis of CDS5 and DBS12 revealed that they corresponded to *B. subtilis* strain TBC025 and JC43, respectively. This study presents evidence supporting the selection of a certain probiotic strain as an effective option, particularly in the context of reducing cholesterol levels in diet.

Keywords: Probiotics, Fermented foods, cholesterol lowering activity, *Bacillus subtilis*

1. Introduction

When taken in adequate amounts, probiotics are non-pathogenic microorganisms that benefit the host's health (FAO/WHO, 2006). Microbial flora has always been an element of the human diet for a very long time, mostly coming from fermented foods. Since prehistoric times, curd and pickles have been essential components of the human diet. The recent discovery of the function of microorganisms in health for humans and animals has resulted in the manufacturing of foods altered with microbial flora, such as probiotics, whether it be through innate immunity and intestinal development or food digestion and illness prevention. Probiotics are a crucial class of functional foods that are commonly taken up as microbial dietary supplements. Taking up probiotics in normal diet can be crucial for preserving a gut flora with a high percentage of healthy bacteria to harmful bacteria. By maintaining this equilibrium, individuals can improve their immunity, digestion, and infection resistance. Probiotics have been shown to have positive benefits in the treatment of a number of disorders, including IBS (irritable bowel syndrome), acute diarrhea, and Crohn's disease. Bloating and gas are the two adverse effects that probiotics most usually cause yet are normally safe and easily tolerated.

Humans have been living in close proximity to various types of living organisms that have been beneficial and also harmful for the health. The epidermis of the skin, the mouth, and the gastrointestinal system (GI tract) are all places where these organisms can be found. This flora has also been identified to be aiding digestion of the individuals (McGhee *et al.* 1999; Mcfarlane *et al.* 1997). These microbes of the gut are acquired rapidly since the birth of the person and stay relatively stable during his/her lifespan, maintaining homeostasis. During the growth of the individual, the micro flora interacts with the host and leads to the development of a very unique and different intestinal immune system. As a result, it is a matter of great challenge for this host immunity to differentiate the virulent and non-virulent organisms and stimulate protective immunity to the host avoiding a protracted inflammatory response that can compromise the integrity of the GI tract's mucosa (Mcfarlane *et al.* 1997).

Probiotics have been included in the diet since very long in many types of foods and beverages (Saarela *et al.* 2000), the probiotic microorganism that is mostly used as probiotics in foods and beverages majorly belong to *Bifidobacterium* and *Lactobacillus,* these two genera are both recognized as generally safe (GRAS) and are both present in the human gut (Parvez *et al.* 2006). Although it is crucial that the organisms survive their passage via the GI tract in order to reach the colon, prior studies have shown low probiotic microbes' survival in the finished food product as well as a substantial decrease in their ability to survive under conditions involving elevated stomach acidity as well as elevated bile levels in the small intestine (Sanchez *et al.* 2005; Bron *et al.* 2004; Sanchez

et al. 2007). The probiotics are usually available as powders that are mostly prepared using freeze drying method and regularly employed in the food industry and might harm the cells (Saarela *et al.* 2005). Therefore, cutting-edge technologies are needed to increase the lifespan of health-promoting microorganisms during food manufacturing as well as via the storage and ingesting processes in order to ensure the delivery of probiotics to people.

Consuming probiotics in the diet has been shown to help with a vast range of medical conditions, including carcinoma and female uro-genital infections and also infantile diarrhea, antibiotic-associated diarrhea, recurrent Clostridium difficile colitis (CDI), infections due to H-bacter pylori, inflammatory bowel disease, and recurrent colitis (Reid *et al.* 2003). Other positive benefits of probiotics include enhancing lactose intolerance, lowering blood cholesterol, promoting nutritional utilization, and lowering the usage of antibiotics (Guo *et al.* 2010). Probiotic bacteria in food have frequently been advised for the demonstration of health benefits and improving one's immunity (Lahteinen *et al.* 2010). In addition to playing a crucial part in the manufacture of membranes, steroid hormones, vitamin D, and other substances, cholesterol is an essential part of all biological cells. Another crucial role for cholesterol in the human body is the formation of bile acids, which are required for the emulsification of lipids and their ingestion and assimilation (Kumar *et al.* 2012). According to Aloglu and Oner (2006), elevated blood cholesterol is a well-known significant risk factor for ischemic heart disease and is thought to be three times more common in individuals with high LDL levels compared to those with a normal profile of lipid.

In consideration of the above facts, numerous bacterial strains have been found to have hypocholesterolemic action, which lowers gut cholesterol levels and protects against coronary heart disease. By binding to the cell membrane of both deceased and active probiotic bacteria cells, bile acids can be assimilated or de conjugated to remove cholesterol (Saraniya and Jeevaratnam, 2015). Due to their capacity to maintain a healthy balance of microflora in the human gastro-intestinal tract, the probiotic microbe *Bacillus subtilis*, a non-virulent, catalase positive and gram-positive bacterium, has received a lot of attention (Cutting *et al.* 2011; Rengpipat *et al.* 1998). The spores of *B. subtilis* have been found to survive extreme pH and low oxygen conditions and can remain in the lower intestine as dormant but also in viable state and possibly induce some beneficial effects (Barbosa *et al.* 2005; La Ragione *et al.* 2003). Research has also found that *B. subtilis* can enhance the viability as well as growth of *Lactobacillus* (Hosoi *et al.* 2000). Furthermore, it has been argued that the extracellular matrix component - polyglutamic acid, which is generated by *B. subtilis*, can be utilized to increase survivability of probiotics while freeze-drying and storing (Bhat *et al.* 2013; Bhat *et al.* 2015). Probiotic microbes could also be shielded by the extracellular matrix from artificial gastric fluid, which is comparable to the stomach's acids (Bhat *et al.* 2015).

This presented study aims to isolate probiotic bacteria from naturally and traditionally fermented foods native to south India that can lower the cholesterol levels and additionally, look at their numerous phenotypic traits, which are frequently employed in their selection and screening processes and the ability to decrease cholesterol levels.

2. Materials and methods

Collection of the Samples

Two naturally fermented food samples were taken i.e., dosa batter prepared from rice and black gram (*Oryza sativa* and *Phaseolus mungo*) in ratio of 1: 2, washed, soaked for 6 hrs, then grinded and allowed to ferment overnight and the second sample chana daal batter prepared from chana (*Chickpea lentils*) were collected aseptically with sterile tubes & stored at 4ºC in the laboratory (Agaliya & Jeevaratnam, 2013).

Isolation and Phenotypic Characterization of Bacteria

Concisely, 0.5g of sample was taken and mixed into 4.5ml of sterile saline (1:10 ratio), the mixture was homogenized for about a minute or two. A range of diluents 10^{-1} to 10^{-8} were prepared and then plated on De Man, Rogosa & Sharpe (MRS Agar). The plates were then incubated at 37ºC for 24-48 hrs anaerobically as Dosa batter strains (DBS) and Chana daal strains (CDS). After incubation, colonies were counted, characterized and selected based on their morphological characteristics following Bergey's manual and then sub-cultured to check purity.

$$\text{CFU/mL} = \frac{\text{No. of colonies counted} \times \text{Dilution factor}}{\text{volume of sample plated(mL)}}$$

Biochemical characterization of the strains was done using various tests such as Gram's staining, catalase, carbohydrate fermentation & gas production, hydrogen sulfide test, arginine hydrolysis test, MRVP, citrate utilization test etc. (Hameed *et al.*, 2022; Agaliya & Jeevaratnam, 2013).

Primary Screening for LAB:
Catalase Test:
In order to neutralise harmful types of oxygen metabolites like H_2O_2, bacteria that thrive in oxygen-rich environments develop the enzyme catalase. Hydrogen peroxide's bactericidal effects are counteracted by the enzyme catalase, which also offers protection. In general, anaerobic bacteria lack the catalase enzyme. Hydrogen peroxide, H_2O_2, is converted into oxygen and water by the action of catalase (Reiner 2010).
A small amount of isolated individual bacterial colony was transferred on to a surface of a clean glass slide, and the glass slide was dried using a loop or sterile wooden stick. A drop of 3% H_20_2 was placed on to the slide and mixed. Rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling gives a positive result, no bubbles or only a few scattered bubbles give a negative one (C. Abiola and V.O. Oyetayo 2016).

Gram Staining:
Gram staining is a method of staining used to distinguish and classify bacterial species into two large groups: gram-positive and gram-negative bacteria. Using a sterilized inoculating loop and a clean glass slide, the isolated colony was transferred from the Petri plates and fixed with heat. Gently, the smear was flooded with crystal violet and air dried for 1 minute. The slides were gently rinsed with distilled water followed by fixation with Gram's iodine for 1 minute. The slides were again rinsed with distilled water and decolourised using 95% ethyl alcohol drop by drop for 5-10 sec until the alcohol ran almost clear immediately after slides were rinsed again and stained with safranin to counter-stain and air dried for 45 seconds. The slides were then examined under oil immersion (100x magnification) in a compound microscope and differentiated as Gram positive (blue or purple) and Gram negative (pink) (C. Abiola and V.O. Oyetayo 2016).

Secondary Screening for LAB:
Biochemical and Physiological characterization Tests:
Carbohydrate Fermentation and Gas Production:
Carbohydrate or sugar fermentation is a test to determine if a bacterium could ferment a particular carbohydrate. The fermentation patterns are useful in differentiating among bacterial groups or species. It detects the presence of gas or acid that results from the fermentation of carbohydrates. Broth media was prepared by mixing all ingredients in distilled water and heated gently to dissolve it. Two types of sugars one hexose-glucose and one pentose-ribose were mixed in it depending on the requirement. Test tubes of 13 x 100 mm were filled with 4-5 ml of phenol red carbohydrate broth with Durham tube without air bubbles. The medium was sterilized by autoclaving at 121°C for 15 min. The final pH should be 7.4 + 0.2. Each test tube was aseptically inoculated with the test strain using 0.1 % (100 µl) of overnight culture and incubated at 35-37°C for 18-24 hours. A yellow colour of media indicates production of acid, while media displacement in the Durham tubes indicates gas production. Longer incubation periods may be required to confirm a negative result. (C. Abiola and V.O. Oyetayo 2016).

Hydrogen Sulphide Test:
The capacity of some bacteria to break down sulphur-containing substances into hydrogen sulphide during metabolism is frequently used as a test measure for their identification in labs. SIM is more sensitive in the detection of H_2S, as this medium contains ferrous ammonium sulphate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulphide. Production of hydrogen sulphide can be detected when ferrous sulphide, a black precipitate, is produced as a result of ferrous ammonium sulphate reacting with H2S gas. Labelled tubes with SIM agar were inoculated by stab inoculation and incubated at 37°C for 24-48 h and observed for formation of black precipitate on the medium (Bergey 1994).

Arginine Hydrolysis Test:
The arginine hydrolysis test analyses if the microbe can use the amino acid arginine as a source of carbon and energy for growth. The arginine dihydrolase enzyme makes use of arginine. Tubes with arginine hydrolysis broth were inoculated with inoculum from a pure overnight culture of the strains and incubated at 35-37°C for 24 hours and the preliminary results were determined. The microbe must first use the glucose present to cause the pH to drop indicated by a change to light yellow colour. The arginine dihydrolase enzyme is triggered by the medium's acidification. To allow the microorganism to utilize the arginine presently, the culture was incubated for a further 24 hours at 35–37°C. The final results were then obtained by observing the tube for 48 hours (Barile 2012).

MRVP Test:
When given glucose, the Methyl Red (MR) test detects if the bacterium ferments mixed acids. One of the fundamental taxonomic traits that aids in differentiating distinct genera of enteric bacteria is the types and quantity of fermentation products generated by anaerobic fermentation of glucose.
Acetoin can be discovered using the Voges-Proskauer (VP) test in a bacterial broth culture. The test is conducted by adding alpha-naphthol as well as potassium hydroxide to the bacteria-inoculated Voges-Proskauer broth. Acetylmethylcarbinol is produced when glucose is digested for the test. Acetylmethylcarbinol is converted to diacetyl in the presence of oxygen and a strong base, where it subsequently combines with guanidine substances often present in the broth's peptone medium. Alpha-naphthol enhances colour, however the colour shift from blue to red can happen without it.
Both examinations are focused on the search for certain carbohydrate metabolism breakdown products.
The prepared medium (pH 6.9) was allowed to equilibrate to room temperature and then inoculated to with 18-24 h overnight culture and incubated aerobically at 35°C for 24-48 hours, after which 1ml of the broth was taken in a clean test tube for MR and VP test respectively. For MR test, after 48 hours 5ml of the broth was tested with few drops of Methyl Red reagent and then immediately observed for a red colour indicating positive reaction. For VP test, 0.6ml of 5% alpha-naphthol and 0.2ml of 40% KOH was added and then gently shaken o expose he medium to atmospheric oxygen and left undisturbed for 10-15 minutes. It was then observed for a pink-red colour development and can be read up to, but no beyond, an hour (C. Abiola and V.O. Oyetayo 2016).

Citrate Utilization Test
Simmons Citrate Agar is used for this test. The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source (Difco, 1998; MacFaddin 2000). The production of alkaline waste products from citrate metabolism is the basis for a positive diagnostic test. The colour of a pH indicator changes to show how the medium's pH rises as a result. Required amount of the media was weighed and dissolved in distilled water and sterilized by autoclave at 121°C for 15 min. The media was then poured in sterilized test tubes and kept in slant position at room temperature and after solidification, the media was stab inoculated with inoculum of overnight cultures of the strains. Finally, the tubes were incubated for 24 h at 35-37^{o}C

Identification of Bacteria Using 16S rDNA Sequencing
The approach by Naeem et al., was used for extracting the bacterial strain's DNA (Naeem *et al.* 2018). Following being suspended in 20 µL of TE buffer (Tris EDTA), a single-strain colony of bacteria was subjected to 95 °C for 10 minutes in a thermal cycler followed by centrifugation at a speed of 6000 rpm for 2–3 min to get a DNA template from the supernatant. A single band of high-molecular weight DNA was seen when its quality was assessed on a 1.0% agarose gel. The 16S rRNA-F and 16S rRNA-R primers amplified a fragment of the 16S rRNA gene. When resolved on an agarose gel, a single distinct 1500 bp PCR amplicon band was seen. To get rid of impurities, the PCR amplicon underwent purification. The 16S rRNA-F and 16S rRNA-R primers were used in forward and reverse DNA sequencing reaction of the PCR amplicon on an ABI 3730xl Genetic Analyzer. The 16S rRNA gene consensus sequence was created using aligner software using forward and reverse sequencing data.

Phylogenetic Analysis
The NCBI GenBank database's 'nr' database was searched using the 16S rRNA gene sequence. The first 10 sequences were chosen and aligned using the multiple alignment programme Clustal W based on the maximum identity score. Using MEGA 10, a distance matrix and phylogenetic tree were created.
Using the Tamura-Nei model and the Maximum Likelihood approach, the course of evolution was determined (Kimura *et al.* 1980). Automatically generated initial tree(s) for the heuristic search were created by first applying the BioNJ and Neighbor-Join algorithms to a matrix of pairwise distances which has been calculated with the help of Tamura-Nei model, and then choosing the topology that had the highest log likelihood value. Eleven nucleotide sequences were subject to this investigation in which 1st+2nd+3rd+Non coding codon locations were included. In MEGA X, evolutionary studies were carried out (Kumar *et al.* 2018).

Analysis of Potential Probiotic Strains via Their Properties
Tolerance to Bile, NaCl and Acid
Putative probiotic bacterial isolates were grown in MRS broth to examine the tolerance potential. Then, 1 mL of the bacterial culture was centrifuged for 5 minutes at 4 °C and 12,000 g to obtain the cell pellet. MRS broth was individually adjusted to 2% and 4% in several test tubes. Cell pellets from earlier were then added to adjusted

MRS broths, and the mixture was then incubated at 37 °C for 3 hours. A pH 7 MRS broth was utilized for the control measurements.
Utilizing three different MRS broth media containing 1%, 2.5%, and 5% bile salts, tolerance to bile salts was calculated. Fresh bacterial cultures (18-hour growth) were centrifuged, collected, and resuspended in 1 mL of 1% bile salt-enhanced MRS broth before being incubated at 37 °C. The broth was transferred onto MRS agar plates followed by incubation for 3 and then 5 hours at 37 °C. Later, colonies were observed.
The same process was used to prepare the MRS broth variation that had 2.5% and 5% bile salts added. The bacterial strains' tolerance to NaCl was tested using the same methodology as was used to investigate bile salt tolerance (Lee *et al.* 2012).

Proteolytic Activity Screening
By combining 10 g of skim milk powder with 100 mL of bacteriological agar media, skim milk agar plates were prepared. The next step was to add fresh cultures of suspected probiotic bacterial strains to skim milk agar plates, which were then incubated for 24-48 hours at 37 °C. Proteolytic activity was revealed by the colonies' surrounding translucent halos (Pailin *et al.*, 2001).

Amylolytic Activity Screening
100 mL of distilled water were mixed with 2.5 g of nutrient agar (NA) and 1 g of starch, and the mixture was autoclaved at 121 °C for 15 minutes. Presumptive probiotic bacterial strains were smeared on the Petri-plates having starch agar followed by incubation at 37 °C for 24 hours and flooded with a 1% iodine solution. Clear zones around the streaked lines indicated the presence of amylase enzyme, but their absence suggested a negative result. (Bernfeld *et al*. 1955).

Hemolytic Activity Screening
Overnight grown *Lactobacillus* cultures were plated on nutrient agar containing 4% of sheep blood agar base followed by incubating them for 48 hours at 37 °C. Zones of hydrolysis around the colonies were observed (Naeem *et al.* 2018). The findings were divided into three groups: α-haemolysis (slight hydrolysis with the appearance of green zones around the colonies), β-haemolysis (clear zones of hydrolysis around colonies), and γ-haemolysis (without any alteration in the medium).

Assay for Analyzing Reduction of Cholesterol
Potential strains of probiotic bacterial were grown in MRS broth with 0.4% bile at 37 °C (HiMedia Laboratories, Mumbai, India). Then, 10 mg/mL of polyoxyethanyl-cholesteryl sebacate, a water-soluble cholesterol that has undergone filter sterilization, was added to the broth. Then overnight culture of the strains was used as inoculum and were grown at 37 °C for 24h anaerobically after being inoculated. Cholesterol estimation was done using 1ml of each sample in eppendorfs followed by centrifugation at 5000 rpm for 6min. Then 100µl of 33% KOH and 100µl ethanol was added to 500 µl of supernatant. After this, 250 µl of deionized water and hexane, 1ml, was added to the eppendorf. Following a minute of vortexing, the phases in the eppendorfs were left to separate at room temperature. Following this about 200 µl of hexane layer is taken in new tubes and evaporated, then 200 µl of reagent ($FeCl_3$ dissolved in CH_3COOH) is added and evaporated. Finally, 125 µl of H_2SO_4 is added to the tubes and vortexed for a minute and maintained for 15 minutes at room temperature. Absorbance is read at 570 nm using a spectrophotometer against cholesterol powder as the standard (Shobharani *et al.* 2016).

Antagonistic activity of the potential probiotic strain
The probiotic microorganisms under investigation were cultivated in MRS broth at a temperature of 37 ^{o}C. Subsequently, their antagonistic activity against *Escherichia coli* (*E. coli*) and *Bacillus* was assessed using the well diffusion method. The bacterial cultures (50 µl and 100 µl) were evenly distributed on the surface of MH agar. Wells were subsequently made on the agar surface, into which potential probiotic strains were introduced. The plates were incubated at 37^{o}C for 24 hrs, and the zone surrounding the cultures was evaluated and measured to assess the antagonistic activity of the possible strains (Lee *et al*. 2012).

Optimization for the utilization of cholesterol at various parameters
The strain that exhibited more favourable results in contrast to other strains was selected to maximise cholesterol utilisation across multiple variables, including cholesterol content, pH levels, and incubation duration. The strain was cultured in MRS broth supplemented with varying quantities of cholesterol (0.2, 0.4, 0.6, 0.8, and 1 µl). The procedure described above was employed to ascertain the proportion of assimilated cholesterol (Shobharani *et al.* 2016). After determining the best concentration of cholesterol that demonstrates the highest assimilation by the

strains, this concentration is next utilised to explore the optimal pH and incubation period. To ascertain the optimal growth and pH conditions of the selected strain, overnight cultures were introduced into MRS broth with varying pH levels (2, 4, 5, 7, and 8). The pH was modified by employing concentrated acetic acid and 5N sodium hydroxide. The broths that had been inoculated were incubated under anaerobic conditions for 24 hours at a temperature of 37°C. Absorbance measurement is performed at a specific wavelength of 570 nm using a spectrophotometer. The standard for this measurement is the uninoculated broth. The same methodology was employed to ascertain the most favourable duration of incubation (Hoque et al. 2010). The experiment involved determining the optimal concentration of cholesterol and pH, followed by incubation for several durations (1, 6, 12, 24, and 48 hours) and subsequent measurement of growth.

3. Result and Discussion

Probiotic Bacteria Isolation

A total of two fermented food samples were analyzed for isolation of probiotic bacteria using MRS agar media. The samples were serially diluted and plated in MRS agar plates by pour plate technique and incubated for 24-48 hrs. The seventh diluent and eighth diluent plates of chana dal (3.45 X 1010CFU/ml) and dosa batter (2.6 X 1011 CFU/ml) respectively (Table 1) had good isolated individual colonies (Figure 1), that were selected at random and screened based on gram staining and catalase test. All the isolates were stored and used for further testing.

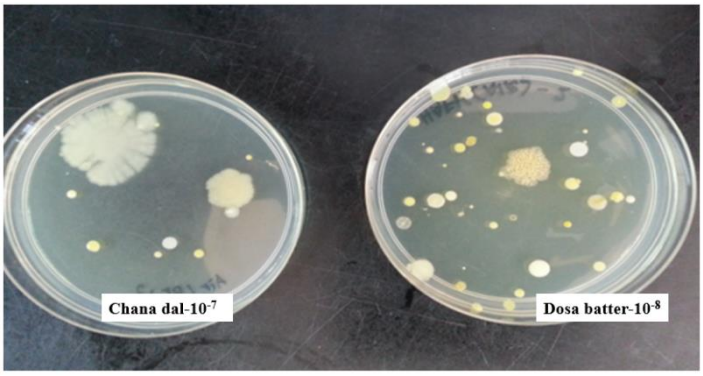


Figure 1: MRS agar plates inoculated with chana dal and dosa batter sample respectively

Table 1: Colony forming units/ml

Petri-plate code	No. of colonies counted	Volume of the sample plated (ml)	Dilution Factor	CFU/ml
CDS-7	345	0.1 ml	10-7	3.45 X 1010
DBS-8	260	0.1 ml	10-8	2.6 X 1011

Initially based on morphological characteristics (Table 2) the colonies were chosen and sub-cultured, a total of twenty-three bacterial isolates, eleven from chana dal sample and twelve from dosa batter sample were isolated and streaked on MRS agar plates for checking purity, the obtained pure cultures of the strains were kept for additional research by being suspended with glycerol (Agaliya & Jeevaratnam 2013) and further screened through biochemical characterization. These isolates were classified as Gram-positive rod shaped as the Gram staining test revealed a purple-blue colour. Additionally, when hydrogen peroxide was poured on the bacterial cultures, they failed to generate any gas bubbles, indicating that they were catalase negative with the exception of CDS5 and DBS12.

Table 2: Colony and cell morphology characteristics

Colony No.	Form	Colour	Elevation	Margin	Surface
DBS-1	Irregular	Cream white	Raised	Undulate	Wrinkled
DBS-2	Irregular	Light yellowish	Raised	Undulate	Wrinkled
DBS-3	Irregular	Light yellowish	Raised	Undulate	Wrinkled
DBS-4	Irregular	Cream white	Flat	Serrated	Wrinkled
DBS-5	Round	Cream white	Flat	Entire	Wrinkled
DBS-6	Irregular	Cream white	Flat	Undulate	Wrinkled
DBS-7	Irregular	Cream white	Raised	Serrated	Wrinkled

DBS-8	Punctiform	Orange	Raised	Undulate	Smooth
DBS-9	Irregular	Cream white	Flat	Serrated	Dull
DBS-10	Irregular	Cream white	Raised	Undulate	Wrinkled
DBS-11	Irregular	Cream white	Raised	Undulate	Wrinkled
DBS-12	Punctiform	Cream white	Umbonate	Undulated	Wrinkled
CDS-1	Irregular	Cream white	Raised	Entire	Wrinkled
CDS-3	Punctiform	Cream white	Raised	Entire	Smooth
CDS-4	Round	Cream white	Raised	Undulate	Wrinkled
CDS-5	Irregular	Cream white	Raised	Entire	Wrinkle
CDS-6	Irregular	Cream white	Raised	Undulate	Smooth
CDS-7	Irregular	Cream white	Flat	Serrated	Wrinkle
CDS-8	Round	Cream white	Raised	Entire	Smooth
CDS-9	Round	Cream white	Raised	Entire	Smooth
CDS-10	Round	Cream White	Raised	Entire	Smooth
CDS-11	Round	Cream white	Raised	Entire	Smooth
CDS-12	Punctiform	Cream white	Flat	Entire	Wrinkled

All the putative probiotic strains were subjected to biochemical characterization using various test (Figure 2). On the basis of which the biochemical characteristics of the isolates, all of them were found to utilize glucose as their carbon source with the production of gas. Based on the bacteria's capacity to generate gas from glucose, homo-fermentative and hetero-fermentative isolates are identified (Wu et al. 2009). The isolates were observed to reduce sulfur-containing compounds to sulfide as H2S gas indicated by the blackening on the media except the strains CDS-8, CDS-9, DBS-6. Majority of the strains were found to give a negative result for arginine hydrolysis except CDS-10 only. For MRVP test, CDS-1, CDS-2, CDS-3, CDS-4, CDS-6, CDS-7, CDS-8, CDS-10, DBS-6, DBS-7, DBS-8, DBS-9 gave a positive result for MR test whereas the rest gave negative. However, for VP test only CDS-5 and DBS-12 gave a positive result. For citrate utilization tests, all the strains were found to give a positive result (Table 3).

Table 3: Characterization of specific bacterial isolates in terms of their morphology and biochemistry

Strain ID	Gram Staining	Catalase	Morphology	Carbohydrate Fermentation				Hydrogen sulfide (H2S) production test	Arginine dihydrolase test	MRVP		Citrate utilization Test
				Glucose		Ribose				MR Test	VP Test	
				Acid Production	Gas Production	Acid Production	Gas Production					
CDS-1	+	-	Rod	+	+	+	+	+	-	+	-	+
CDS-2	+	-	Rod	+	+	+	+	+	-	+	-	+
sCDS-3	+	-	Rod	+	+	+	+	+	-	+	-	+
CDS-4	+	-	Rod	+	+	+	+	+	-	+	-	+
CDS-5	+	+	Rod	+	+	+	+	+	-	-	+	+
CDS-6	+	-	Rod	+	+	+	+	+	-	+	-	+

CD S-7	+	-	Rod	+	+	+	+	+	-	+	-	+
CD S-8	+	-	Rod	+	+	+	+	-	-	+	-	+
CD S-9	+	-	Rod	+	+	+	+	-	-	-	-	+
CD S-10	+	-	Rod	+	+	+	+	+	+	+	-	+
CD S-11	+	-	Rod	+	+	+	+	+	-	-	-	+
CD S-12	+	-	Rod	+	+	+	+	+	-	-	-	+
DB S-1	+	-	Rod	+	+	+	+	+	-	-	-	+
DB S-2	+	-	Rod	+	+	+	+	+	-	-	-	+
DB S-3	+	-	Rod	+	+	+	+	+	-	-	-	+
DB S-4	+	-	Rod	+	+	+	+	+	-	-	-	+
DB S-5	+	-	Rod	+	+	+	+	+	-	-	-	+
DB S-6	+	-	Rod	+	+	+	+	-	-	+	-	+
DB S-7	+	-	Rod	+	+	+	+	+	-	+	-	+
DB S-8	+	-	Rod	+	+	+	+	+	-	+	-	+
DB S-9	+	-	Rod	+	+	+	+	+	-	+	-	+
DB S-10	+	-	Rod	+	+	+	+	+	-	-	-	+
DB S-11	+	-	Rod	+	+	+	+	+	-	-	-	+
DB S-12	+	+	Rod	+	+	+	+	+	-	-	+	+

* '+' indicates positive and '-' indicates negative

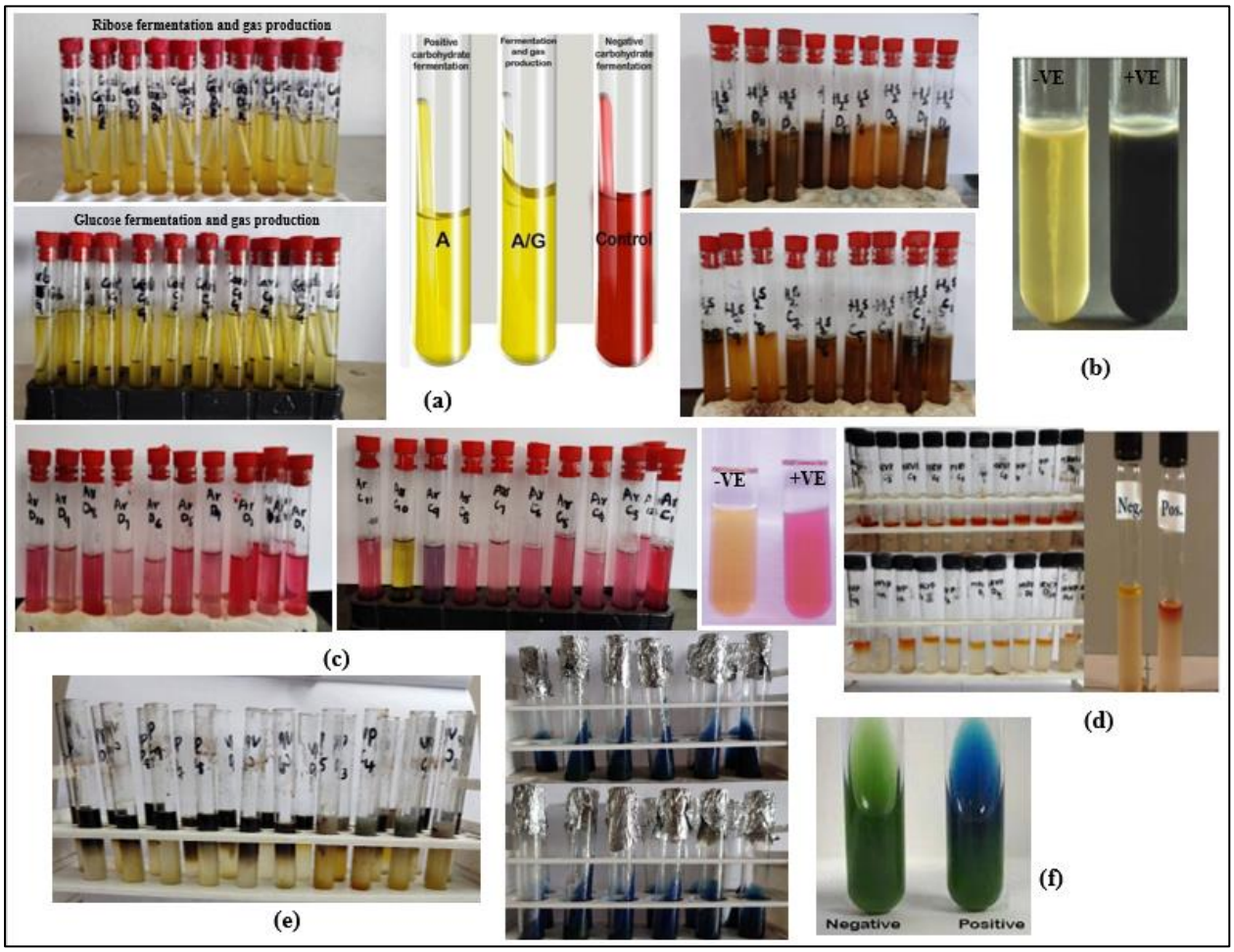


Figure 2: Biochemical Tests (a) Carbohydrate utilization Result after 48 h, (b) H2S test results after 48 h incubation, (c) Arginine test results after 48 h of incubation, MRVP test (d) MR test, (e) VP test after 48 h of incubation, (f) Citrate utilization Test results after 24 hours of Incubation.

The putative probiotic bacterial isolates were found to thrive most effectively at 37°C, that were then narrowed down on the basis of tolerance profiles for acid (HCl), NaCl and bile salts. Under a basic microscope, these bacterial isolates were seen as bacillus. A total of 6 isolates were selected on the basis of these probiotic properties i.e., CDS4, CDS5, CDS12, DBS6, DBS10, DBS12.

Table 4: Effect of different Bile salt concentrations (1%, 2.5%, 5%) on the survivability of the candidate probiotics strains

Strain ID	1% Bile	2.5% Bile	5% Bile
CDS1	+	+	-
CDS3	+	+	-
CDS4	+	+	-
CDS5	+	+	-
CDS6	+	+	-
CDS7	+	+	-
CDS8	+	+	-
CDS9	+	+	-
CDS10	+	+	-
CDS11	+	+	-
CDS12	+	+	-
DBS1	+	+	-
DBS2	+	+	-

DBS3	+	+	-
DBS4	+	+	-
DBS5	+	+	-
DBS6	+	+	-
DBS7	+	+	-
DBS8	+	+	-
DBS9	+	+	-
DBS10	+	+	-
DBS11	+	+	-
DBS12	+	+	-

* '+' indicates positive and '-' indicates negative

According to the findings, bile salt resistance reduced as bile salt concentration increased. The six selected strains CDS4, CDS5, CDS12, DBS6, DBS10 and DBS12 showed good growth in 1% and 2.5% Bile salt containing media, however no growth was observed in 5% bile salt containing media (Table 4).

Table 5: Effect of different NaCl concentrations (1%, 2.5%, 5%) on the survivability of the candidate probiotics strains

Strain ID	1% NaCl	2.5% NaCl	5% NaCl
CDS1	+	+	-
CDS3	+	+	-
CDS4	+	+	-
CDS5	+	+	-
CDS6	+	+	-
CDS7	+	+	-
CDS8	+	+	-
CDS9	+	+	-
CDS10	+	+	-
CDS11	+	+	-
CDS12	+	+	-
DBS1	+	+	-
DBS2	+	+	-
DBS3	+	+	-
DBS4	+	+	-
DBS5	+	+	-
DBS6	+	+	-
DBS7	+	+	-
DBS8	+	+	-
DBS9	+	+	-
DBS10	+	+	-
DBS11	+	+	-
DBS12	+	+	-

* '+' indicates positive and '-' indicates negative

The findings demonstrate that when content of NaCl increased, the ability to withstand the same reduced. The six selected strains CDS4, CDS5, CDS12, DBS6, DBS10 and DBS12 demonstrated good growth in 1% as well as 2.5% NaCl containing media, however no growth was observed in 5% NaCl containing media (Table 5).

Table 6: Effect of HCl concentrations (2%, 4%) on the survival capacity of the potential probiotic strains.

Strain ID	2% HCl	4% HCl
CDS1	-	-
CDS3	+	+
CDS4	+	+
CDS5	+	+
CDS6	+	+

CDS7	+	+
CDS8	+	+
CDS9	+	+
CDS10	+	+
CDS11	+	+
CDS12	+	+
DBS1	+	+
DBS2	+	+
DBS3	+	+
DBS4	+	+
DBS5	-	-
DBS6	+	+
DBS7	+	+
DBS8	+	+
DBS9	+	+
DBS10	-	+
DBS11	+	+
DBS12	+	+

The findings demonstrate the possibility for resistance to HCl in a number of strains and showed good growth in 2% and 4% HCl acid containing media (Table 6).
A variety of in vitro enzymatic potential assays were performed on the six potential probiotic bacterial strains chosen based on their probiotic properties. Most of the strains showed varied degrees of enzymatic capacity in our investigation while accessing the enzymatic potential of the strains of bacteria, however, CDS4, CDS5, DBS6, DBS10 and DBS12 showed good amylolytic activity whereas only DBS12 showed good proteolytic activity. All the strains showed γ-haemolysis (no change in the media) for haemolytic activity test (Table 7).

Table 7: Enzymatic potential of the presumed probiotic bacterial isolates

Strain ID	Haemolytic Activity	Amylolytic Activity	Proteolytic Activity
CDS4	-	+	-
CDS5	-	+	-
CDS12	-	-	-
DBS6	-	+	-
DBS10	-	++	-
DBS12	-	+	+

Legend: (+) weakly positive; (++) strongly positive; (-) zero activity.

Estimation of Cholesterol Assimilation
Our research demonstrates that the quantity of cholesterol that is left over after assimilation changes with the variance of cholesterol content in the medium; as a result, cholesterol concentration is an important element in cholesterol absorption. CDS4, CDS5, CDS12, DBS6, DBS10 and DBS12 were tested to be assimilating good amount of cholesterol with the remnant percentage of cholesterol found being 12.1% 23.2%, 18%, 17.4%, 16% and 19.4% respectively. Out of these probiotic strains CDS5 and DBS12 showed high cholesterol lowering activity due to which these two strains were selected for further testing using 16S rDNA Sequencing (Figure 3).

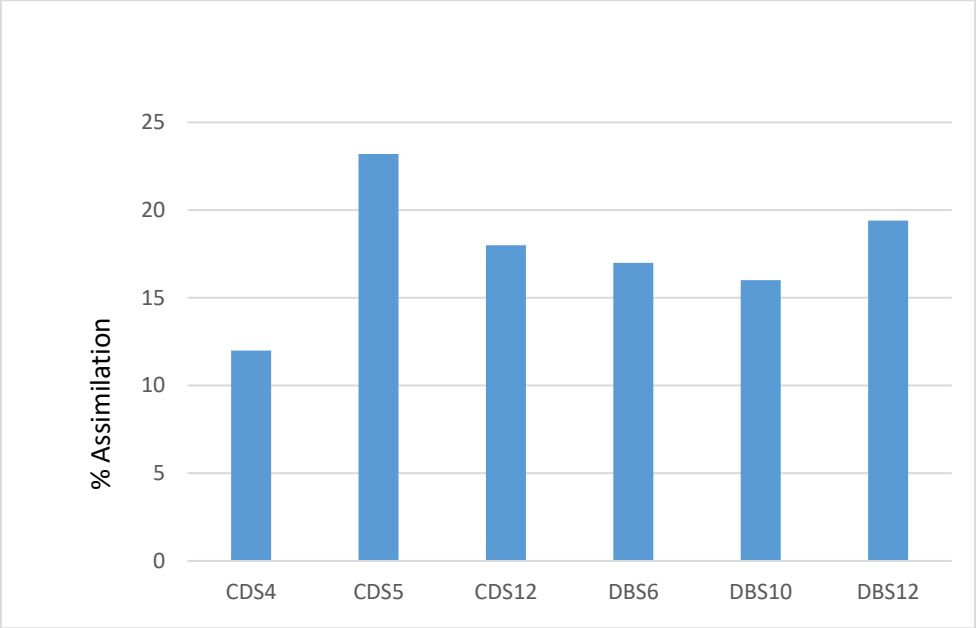


Figure 3: Cholesterol lowering activity of selected probiotic isolates in percentage

On the basis of nucleotide homology & phylogenetic analyses, 16S rDNA sequencing proved that the strains CDS5 and DBS12 were *Bacillus subtilis*.

Bacterial identification based upon 16S rRNA sequencing

The CDS5 strain was molecularly identified by sequencing of 16S rRNA gene region. The molecular identification of CDS5 strain 16S rRNA partial sequence has confirmed the strain as *Bacillus subtilis* TBCO25 and DBS12 as *Bacillus subtilis* JC43.

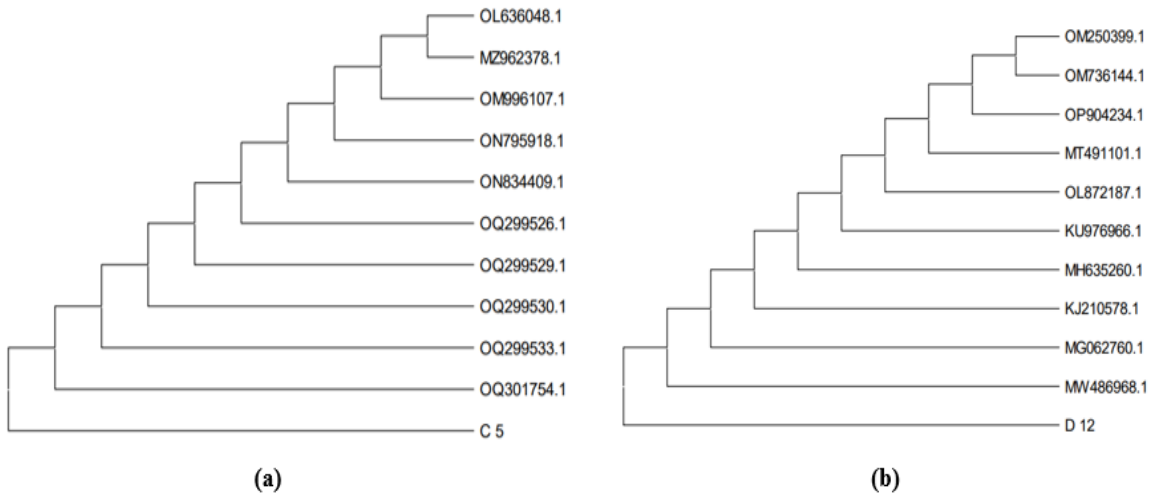


Figure 4: Phylogenetic tree of the strain CDS5 and DBS12 showing the relationships between the type of species that are most closely related, as determined by 16S rRNA sequencing

Morphological And Biochemical Characteristics of Strains (TBCO25, D5, JC43)

The three strains (TBC025, D5, JC43) were identified based on morphological and biochemical characteristics. The morphological and biochemical characters of strains TBCO25, D5 and JC43 are presented in tables below (Table 8). The morphological observations revealed that TBC025, D5 and JC43 strains are Gram positive rod shaped, non-endospore forming bacteria. These three strains showed negative for catalase test as no bubbles were

observed indicating that these strains lack the catalase enzyme. The biochemical characteristics revealed that for Methyl red test the 3 strains were found to be positive as they showed red colour on the surface of the medium after addition of methyl red indicator indicating acid production. For Vogues Proskauer test after addition of reagents there was no change in colour and remained brown colour on the surface of the medium indicating negative for Voges Proskauer test indicating that the strains do not have the ability to produce neutral end products as a result of glucose fermentation. For Hydrogen sulfide production test, the 3 strains were found to be positive as blackening on the medium was observed and for the Arginine hydrolysis Test, the tubes turned from orange to pink after 24 hours and remained pink after 48 hours and did not turn to yellow, indicating 3 strains are negative for Arginine hydrolysis test. For Proteolytic Test there was no formation of a clear zone around the colony. The 3 strains were found to be negative for Proteolytic test. For the Hemolytic Test there was no appearance of zone of clearing around the colonies. The 3 strains were found to be negative for Hemolytic test. Amylolytic Test there was no zone around the colonies after the addition of iodine. The 3 strains were found to be negative for the Amylolytic Test.

Table 8: Morphological and Biochemical Characteristics of Strains (TBCO25, D5, JC43)

S.NO	Test	*Bacillus subtilis* TBC025	*Bacillus subtilis* D5	*Bacillus subtilis* JC43
1.	Gram staining	Gram positive rods	Gram positive rods	Gram Positive rods
2.	Endospore staining	Non spore forming	Non spore forming	Non spore forming
3.	Catalase Test	Negative	Negative	Negative
4.	Methyl red (MR) Test	Positive	Positive	Positive
5.	Voges Proskauer (VP) Test	Negative	Negative	Negative
6.	Hydrogen sulfide (H2S) Production Test	Positive	Positive	Positive
7.	Arginine hydrolysis Test	Negative	Negative	Negative
8.	Proteolytic Test	Negative	Negative	Negative
9.	Hemolytic Test	Negative	Negative	Negative
10.	Amylolytic Test	Negative	Negative	Negative

Characterization of probiotic properties of *Bacillus subtilis* strains

The probiotic characteristics of the chosen strains of *Bacillus subtilis* (TBCO25, D5, JC43) were assessed by examining their performance under diverse conditions, including varying concentrations of NaCl (1%, 2%, 3%, 4%), pH levels (1, 3, 5), and bile salt concentrations (1% and 2%). With the exception of *Bacillus subtilis* TBCO25, all other stains yielded negative results when tested on a 2% NaCl solution. All of the strains tested exhibited positive results for the remaining variables. *Bacillus subtilis* JC43 had favourable outcomes across all pH levels, while *Bacillus subtilis* D5 yielded negative results under all pH conditions. Conversely, *Bacillus subtilis* TBCO25 demonstrated positive outcomes exclusively at pH 5. All of the strains exhibited positive results when subjected to different concentrations of bile (Table 9).

Table 9: Characterization of probiotic properties of *Bacillus subtilis* strains

S.NO	Test	*Bacillus subtilis* TBCO25	*Bacillus subtilis* D5	*Bacillus subtilis* JC43
1.	1% Nacl	Positive	Positive	Positive
2.	2% Nacl	Negative	Positive	Positive
3.	3% Nacl	Positive	Positive	Positive
4.	4% Nacl	Positive	Positive	Positive
5.	pH 1	Negative	Negative	Positive
6.	pH 3	Negative	Negative	Positive
7.	pH 5	Positive	Negative	Positive
8.	1% Bile salt	Positive	Positive	Positive
9.	2% Bile salt	Positive	Positive	Positive

Quantitative Test

Quantitative assays were conducted on three distinct strains of *Bacillus subtilis* (TBC025, D5, JC43) encompassing various evaluations, namely the NaCl tolerance test, Acid tolerance test, Bile salt tolerance test, Adherence test, Temperature assessment, and Antagonistic activity analysis.

NaCl Tolerant Test: Various concentrations of NaCl were employed to assess the viability of the strains. *Bacillus subtilis* D5 exhibited the greatest degree of viability compared to other stains across various NaCl concentrations, with a maximum survival rate of 99.29% seen at a 1% NaCl concentration. *Bacillus subtilis* TBCO25 exhibited the highest percentage, reaching 61.52%, when exposed to a 1% NaCl concentration. The experimental findings indicate *that Bacillus subtilis* JC43 did not yield any discernible outcomes (Table 10) (Figure 5).

Table 10: NaCl Tolerant Test

S.NO	NaCl concentration	Percentage of *Bacillus subtilis* TBCO25	Percentage of *Bacillus subtilis* D5	Percentage of *Bacillus subtilis* JC43
1.	1% NaCl	61.52%	99.29%	87.15%
2.	2% NaCl	41.78%	95.29%	84.96%
3.	3% NaCl	39.57%	90.82%	78.78%
4.	4% NaCl	19.23%	70.11%	61.69%

Formula: Survival %= (NaCl containing broth medium at 0D 600nm)/ (Blank culture medium at OD 600nm) × 100

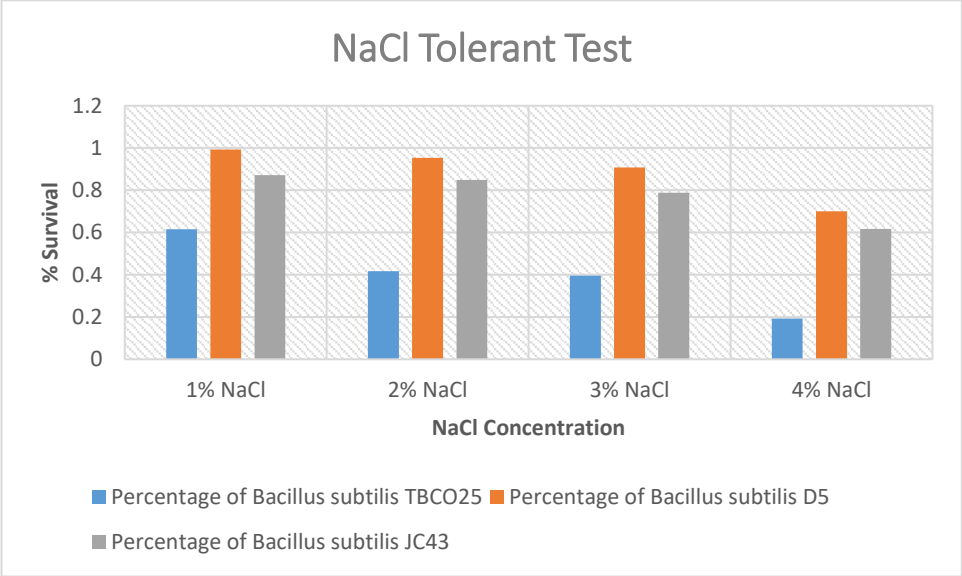


Figure 5: Graphical representation of the NaCl Tolerance Test for the *Bacillus* species

Acid Tolerant Test: Different pH values were employed to assess the viability of the stains. *Bacillus subtilis* TBCO25 exhibited the largest percentage, 48.89%, under pH 5 conditions. *Bacillus subtilis* D5 demonstrated a percentage of 14.35% under the same pH conditions. Conversely, *Bacillus subtilis* JC43 did not yield any discernible findings (Table 11) (Figure 6).

Table 11: Acid Tolerant Test

S.NO	Acid concentration	Percentage of Bacillus subtilis TBCO25	Percentage of Bacillus subtilis D5	Percentage of Bacillus subtilis JC43
1.	pH 1	4.60%	9.88%	3.69%
2.	pH 3	8.71%	13.64%	5.93%
3.	pH 5	48.89%	14.35%	10.78%

Formula: Survival % = (log CFU at 3 hours) / (log CFU at 0 hours) x 100

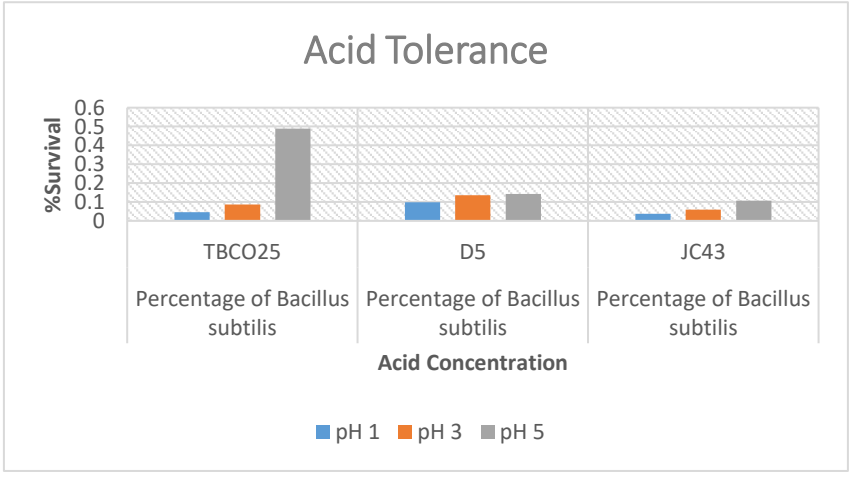


Figure 6: Graphical representation of the Acid Tolerance Test for the *Bacillus* species

Bile Salt Tolerant Test: The study involved the evaluation of three distinct strains of *Bacillus subtilis* (TBC025, D5, JC43) under varying concentrations of bile salts. The study involving *Bacillus subtilis* JC43 did not produce any significant findings. Both cultures, *Bacillus subtilis* TBCO25 and *Bacillus subtilis* D5, exhibited favourable outcomes, with the former achieving the greatest result of 100.80% and the latter achieving 95.05%, specifically at a bile content of 1% (Table 12) (Figure 7).

Table 12: Bile Salt Tolerant Test

S.NO	Bile salt concentration	Percentage of *Bacillus subtilis* TBCO25	Percentage of *Bacillus subtilis* D5	Percentage of *Bacillus subtilis* JC43
1.	1% Bile salt	100.80%	95.05%	85.09%
2	2% Bile salt	66.73%	85.41%	62.06%

Formula: Survival % = (Bile salt containing culture medium at OD 600nm) / Blank culture medium at OD 600nm) x 100

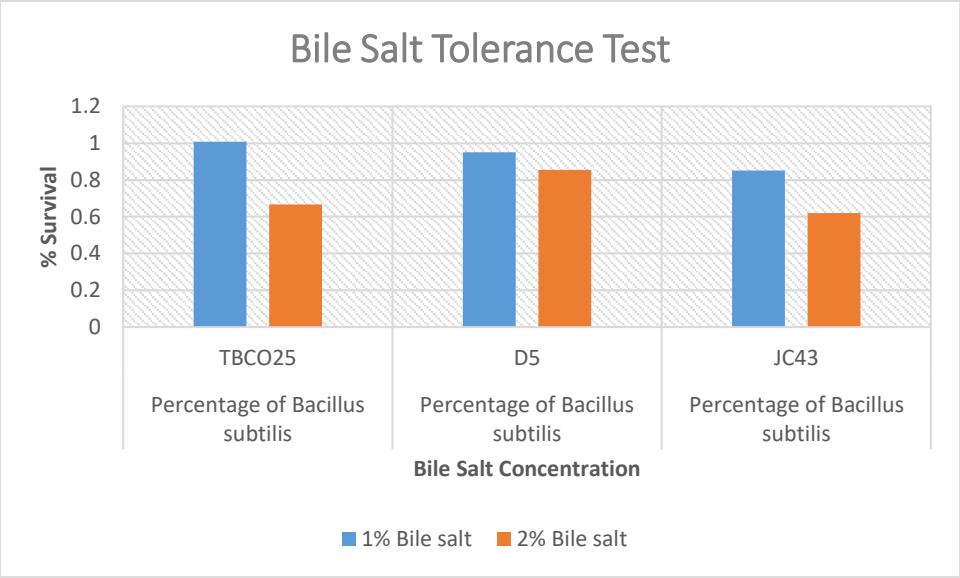


Figure 7: Graphical representation of the Bile Salt Tolerance Test for the *Bacillus* species

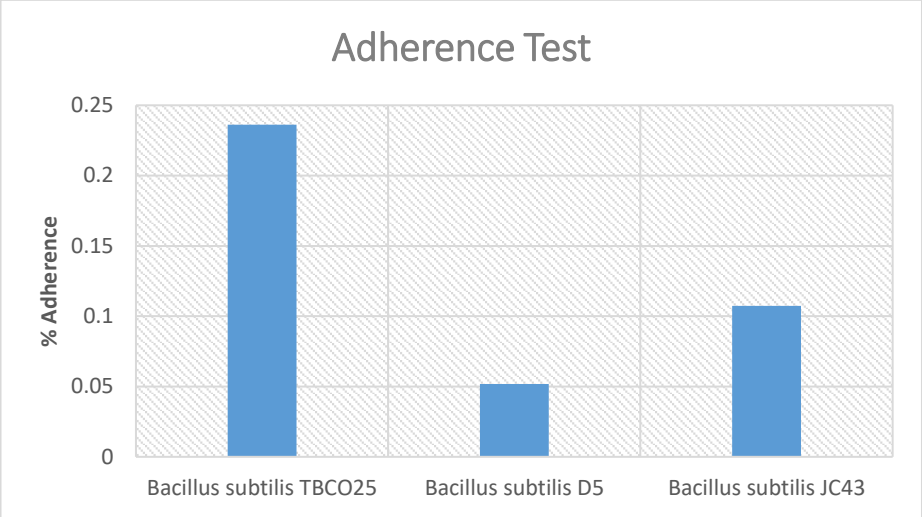
Adherence Test: A test was conducted to assess the adherence of *Bacillus subtilis* strains (TBC025, D5, JC43). The strain *Bacillus subtilis* TBCO25 exhibited a percentage of 23.62%, while *Bacillus subtilis* D5 demonstrated a percentage of 5.19%. The experiment involving *Bacillus subtilis* JC43 did not yield any significant findings (Table13) (Figure 8).

Table 13: Adherence Test

S.NO	*Bacillus subtilis* strains	Percentage (%)
1.	*Bacillus subtilis* TBCO25	23.63%
2.	*Bacillus subtilis* D5	5.19%
3.	*Bacillus subtilis* JC43	10.73%

Formula: % = (AO – At) / (AO) x 100
AO and at are absorbance before and after contact with n- hexane after 30 minutes

Figure 8: Graphical representation of the Bile Salt Tolerance Test for the *Bacillus* species



Temperature: The growth patterns of three separate strains of *Bacillus subtilis* (TBC025, D5, JC43) were assessed at temperatures of 37ºC and 60ºC. *Bacillus subtilis* TBCO25 exhibited a growth rate of 77.15% at a temperature of 37°C, while *Bacillus subtilis* D5 demonstrated a growth rate of 82.86% under the same conditions. The experimental findings indicate that *Bacillus subtilis* JC43 did not yield any discernible outcomes (Table 14) (Figure 9).

Table 14: Temperature

S.NO	*Bacillus subtilis* strains	Percentage of Temperature at 37OC	Percentage of Temperature at 60OC
1.	*Bacillus subtilis* TBCO25	77.15%	23.04%
2.	*Bacillus subtilis* D5	82.86%	13.22%
3.	*Bacillus subtilis* JC43	69.63%	19.03%

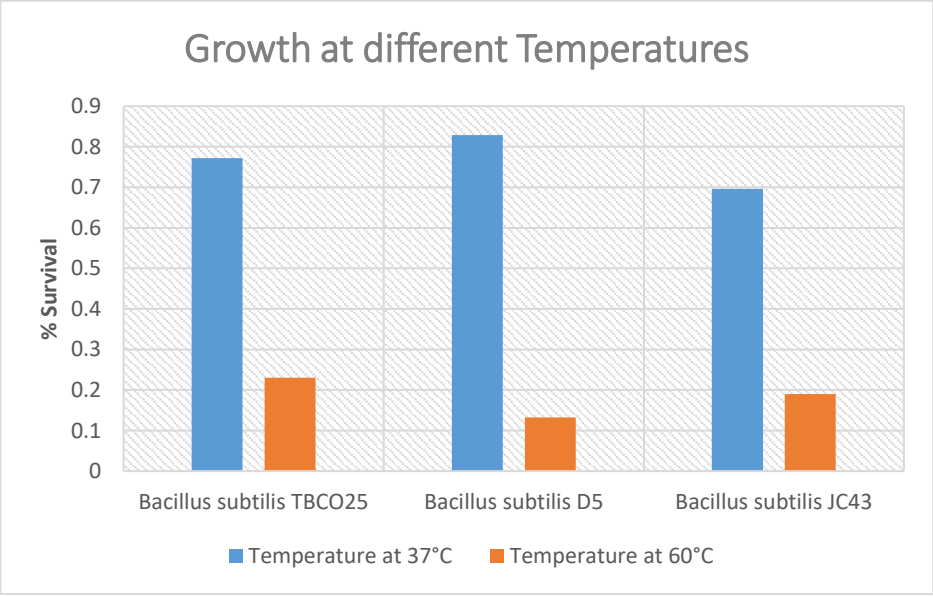


Figure 9: Graphical representation for growth at different Temperatures for the *Bacillus* species

Antagonistic Activity

The present study aimed to assess the antagonistic activity of three strains of *Bacillus subtilis* (TBC025, D5, JC43) against two bacterial species, namely *E. coli* and *Bacillus*. The evaluation was conducted against 50 µl and 100 µl of the cultures. All of the strains exhibited antagonistic activity against the aforementioned microorganisms (Table 15) (Figure 10).

Table 15: Antagonistic Activity

S.NO	*Bacillus subtilis* strains	*E. coli*		*Bacillus*	
		50 µl	100 µl	50 µl	100 µl
1.	TBCO25	1.2cm	1.1cm	0.9cm	0.8cm
2.	D5	1.2cm	1cm	0.7cm	0.9cm
3.	JC43	1.1cm	1.2cm	1cm	0.9cm

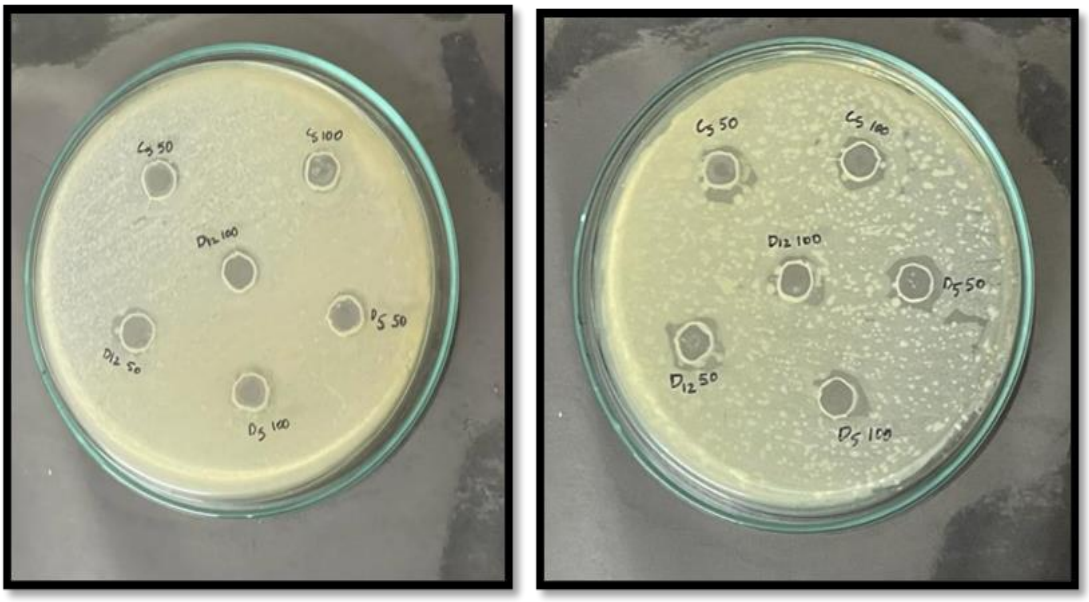


Figure 10: Aantimicrobial potential of Bacillus strains

Determination of cholesterol reducing ability in *Bacillus subtilis* strains

The capacity of the strains to assimilate cholesterol was assessed and shown in Table 16. The cholesterol assimilated by probiotic strains was determined as follows:

Cholesterol assimilated (ug/ml) = [Cholesterol at 0 hours] (ug/ml) – [Cholesterol at 24 hours] (ug/ml)

Cholesterol assimilated by each strain was also calculated in terms of percent cholesterol assimilation:

% of Cholesterol assimilated = [Cholesterol assimilated] (ug/ml) / [Cholesterol at 0 hours] × 100

Among the tested strains, *Bacillus subtilis* TBCO25 exhibited the highest percentage of 81.18%, followed by *Bacillus subtilis* D5 with 81.05%, and *Bacillus subtilis* JC43 with 72.22%.

Figure 11: Cholesterol Standard curve

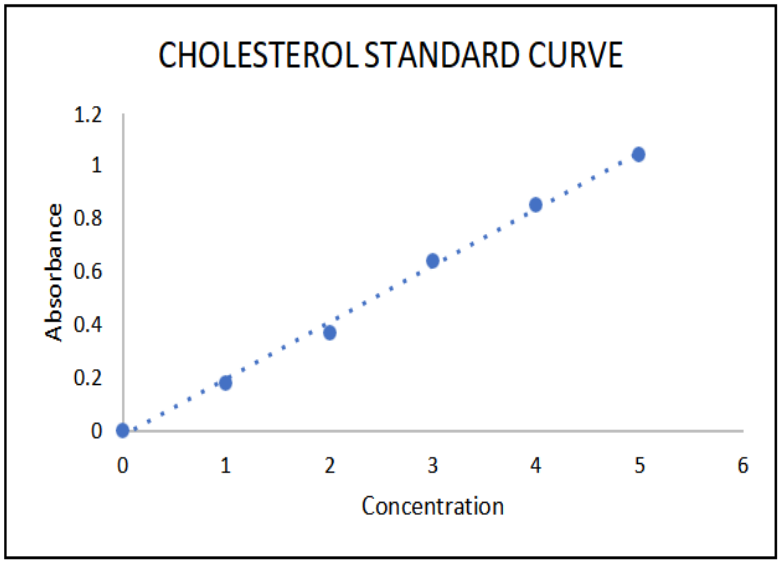


Table 16: Cholesterol Assimilated by Probiotic Strains

S.NO	*Bacillus subtilis* strains	Values	Cholesterol assimilated	Percentage of cholesterol assimilated
1.	*Bacillus subtilis* TBCO25	1.411	6.089	81.18%
2.	*Bacillus subtilis* D5	1.421	6.079	81.05%
3.	*Bacillus subtilis* JC43	2.083	5.417	72.22%

Optimization for the utilization of cholesterol at various parameters

Since *Bacillus subtilis* TBCO25 exhibited the highest percentage of cholesterol assimilated, it was selected for Optimisation in the utilisation of cholesterol at several parameters, including cholesterol concentration, pH, and incubation period. This strain demonstrated favourable outcomes compared to other stains. The investigation demonstrated that when the cholesterol concentration reached 0.2 µl, the maximum percentage of cholesterol assimilation was seen, with a value of 90.92% (Figure 12a). The investigation revealed that the assimilation of cholesterol exhibited a notable increase across several pH values (2, 4, 5, 7, 8), reaching a peak value of 97.10% at pH 2, while maintaining a cholesterol concentration of 0.2 µl (Figure 12b). During a 1 hr incubation period, the percentage of assimilated cholesterol was found to be 95.42%, which represents the highest observed value (Figure 12c).

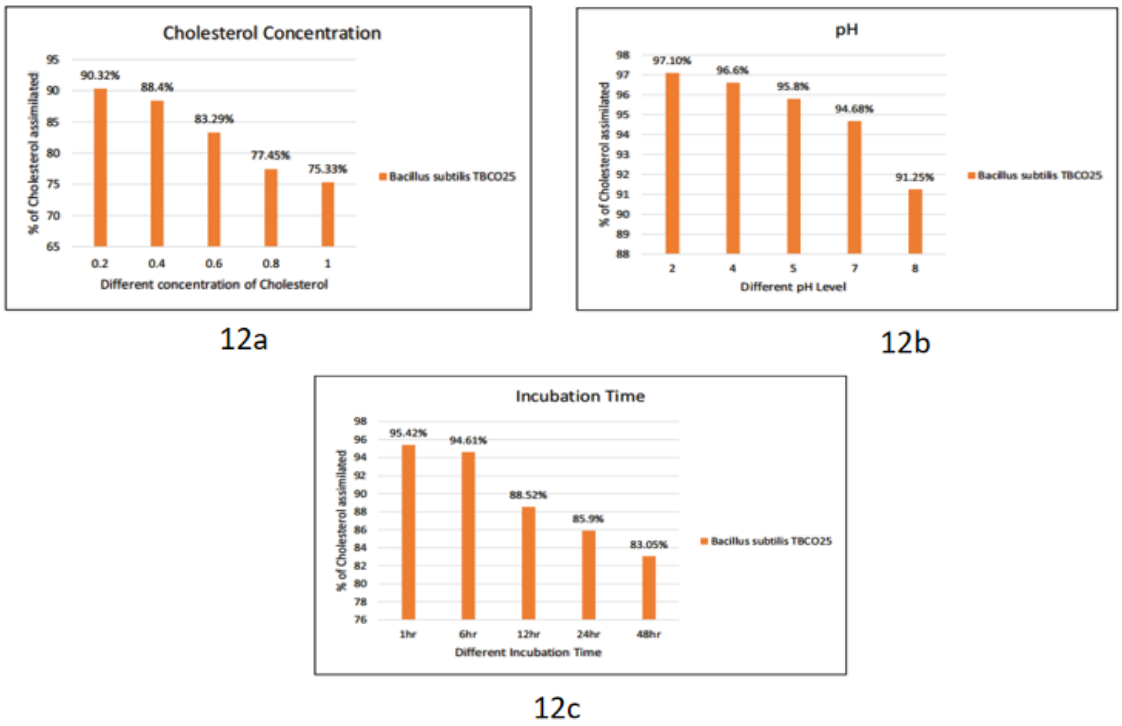


Figure 12: Optimization for the utilization of cholesterol at different a) concentration of cholesterol; b) pH level; and c) incubation time

4. Discussions

The strains TBCO25, D5 and JC43 were determined for cholesterol reducing ability and the strains showed different percentage of cholesterol assimilation. The percentage of cholesterol assimilated by TBCO25 strain is 81.18%. The percentage of cholesterol assimilated by D5 strain is 81.05% and JC43 strain showed cholesterol assimilation of about 72.22%. Among all the 3 strains, the TBCO25 strain showed more percentage of cholesterol assimilation compared to D5 and JC43. The TBCO25 strain was further assessed for cholesterol reducing property at various parameters such as different cholesterol concentration, pH and incubation time. For different cholesterol concentrations like 0.2, 0.4, 0.6, 0.8 and 1mg/ml, the TBCO25 strain at 0.2mg/ml concentration showed the maximum percentage of cholesterol assimilation (90.32%). At different pH levels like pH 2, pH 4, pH 5, pH 7 and pH 8, the TBCO25 strain showed a maximum percentage of cholesterol assimilation at pH 2 (97.10%). At different incubation times, like 1hr, 6hrs, 12hrs, 24hrs and 48hrs, the TBCO25 strain showed a maximum percentage of cholesterol assimilation at 1hr (95.42%). Hence, the strain TBCO25 showed more cholesterol reducing ability compared to D5 and JC43 strains.

Due to the pattern of the biochemical characteristics of all the strains and also probiotic properties, enzymatic assays and cholesterol assimilation assay 16S rDNA sequencing was performed. Based on 16S rDNA sequencing, the bacterial samples CDS5 and DBS12 were tentatively molecularly identified and found to be from a single probiotic group: Bacillaceae and were further described as TBC025 and JC43 respectively. Through phylogenetic research, the sequence homologies were discovered with CDS5 (TBC025) demonstrating 100% identity to *Bacillus velezensis* strain FZB42 and DBS12 (JC43) demonstrating 99.41% identity to the same strain. To protect the cell membrane from the extreme stomach acidity, LAB eliminates protons and lactic acid from the cell (Vijayakumar et al. 2015). Our results have been found to be in agreement with other research where *B. subtilis* showed its capacity to survive highly acidic and saline conditions (Wang et al. 2010; Ikeuchi et al. 2003). The property to survive highly acidic nature is considered very important as remaining viable in the stomach of humans at pH of about 2.0 is essential to impart the probiotic properties. The bacterial isolates were also subjected 1%, 2.5% & 5% (w/v) bile salt concentrations. Due to the presence of bile salts in the upper gut, probiotic bacteria like *Lactobacillus* and *Bacillus subtilis* should possess the potential to thrive there and impart its benefits to the host.

The liver secretes bile salts, which are then stored in the gallbladder and transported to the intestinal tract. Because these substances may potentially harm living cells, it is thought that a potential probiotic isolate's ability to resist them is important because it is necessary for their survival under these circumstances (Succi et al. 2005). Our study revealed that the capacity to tolerate bile salts varied between the tested isolates and this capacity reduced with the increase in bile salts concentration. All of the tested strains showed good growth in 1% and 2.5% (w/v) concentration of bile salt, however, no growth was observed in media with 5% (w/v) of bile salt concentration.
Due to the fact that many organisms have the capacity to produce exotoxins that induce partial or total lysis of human or animal cells, hemolytic activity study is a significant prerequisite for the selection of probiotic strains. All the six strains with presumptive probiotic ability demonstrated γ-hydrolysis, as no blood cell hydrolysis was seen. The enzymatic potency of the chosen probiotic bacterial strains was examined in vitro. In our presented work, all of the strains demonstrated different levels of enzymatic potential, showing good amylolytic activity except CDS12, however no proteolytic activity was seen for the strains except DBS12 on skimmed milk agar plates. Our study was found in agreement with the findings of Dash et al. (2015) where they found a strain of *B. subtilis* that could be used for production of amylase, whereas the study conducted by Pant et al. (2015) suggested better protease production on gelatin than on skimmed milk in nutrient agar medium.
In one of the study conducted by Zanotti et al. (2015), they explored the ability of Bifidobacteria to lower the amount of cholesterol in a synthetic media used for growth of colonies and found *Bifidobacterium bifidum* PRL2010 strain to be assimilating cholesterol, the transcriptomic study demonstrated substantially higher transcription level of putative transporter and reductase genes, indicating specialized mechanisms for cholesterol assimilation, additionally, in vivo research showed that the mice's fecal microbiota had changed to include bacteria that aid in cholesterol metabolism (Zanotti et al. 2015). Recent research by Aswani et al. (2021), demonstrated the first incidence of isolation of a cholesterol-lowering probiotic yeast. They found CP-I, a yeast culture isolated from frass of *Pyrrharctia isabella* caterpillar, interestingly found to reduce cholesterol levels by about 9.16% in in-vitro conditions, CP-I strain was found 97% similar to *Saccharomyces cerevisiae* (Aswani et al. 2021). Similarly, strains of *L. acidophilus*, *E. faecalis*, a probiotic *Lactococcus lactic* subsp. *Lactis* (Shehata et al. 2016) have also been found to show strong cholesterol lowering property (Gao & Li et al. 2018). In the following study we observed that six strains to be having the most cholesterol-lowering property out of which two strains, identified as *B. subtilis* i.e., TBC025 and JC43 were found to show highest cholesterol-lowering activity.
Future work could also investigate the potential of using these probiotics in animal studies to determine their efficacy in reducing cholesterol levels in vivo. Long-term studies could be conducted to assess the safety and effectiveness of these probiotics in humans, including those with existing medical conditions such as hypercholesterolemia. Additionally, research could explore the potential of using these probiotics in conjunction with dietary interventions to optimize their effects on cholesterol levels.

5. Conclusion

In the present study, 23 LAB strains were isolated from different sources and only six isolates were showing cholesterol assimilating property. Among the promising LAB isolates, CDS5 which isolate from fermented chana dal and identified as were *B. subtilis* strain TBC025 and cholesterol removal ability (23.8%), DBS12 which isolate from Dosa batter and identified as were *B. subtilis* strain JC43 the cholesterol removal ability was 19.4%. This study needs to evaluate in vivo to understand possible underlying mechanism of cholesterol-lowering effects of these strains.

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Author contributions
Dr. Suchitra Naidu: Conceptualization, Methodology, Investigation, Validation.
Dr. Y. Sabitha: Conceptualization, Data-Curation, Project administration, Writing – review.
Data availability
All data generated or analyzed during this study are included in this published article.

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