Bile Salt Hydrolase Activity of Cholesterol-Lowering Probiotic Lactic Acid Bacteria Isolated from Indigenous Fermented Foods

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Abstract. The main risk factor for cardiovascular diseases is hypercholesterolaemia (elevated blood cholesterol levels). Many cholesterol-lowering drugs exist, but not without adverse side effects. Consequently, a natural dietary approach with little or no side effects is needed. This study aimed to isolate, characterise and identify probiotic lactic acid bacteria with cholesterol-lowering abilities *in vitro* and their bile salt hydrolase activity. Fifty-two isolates of lactic acid bacteria were isolated from indigenous fermented food products (ugba, ogi, ogiri, raw cow milk and yoghurt), of which 22 isolates were considered presumptive LAB after Gram staining and biochemical tests. The antimicrobial activity of the isolates was evaluated, and out of 22 LAB isolates, only six showed broad-spectrum antagonistic effects on test bacteria pathogens and good bile and acid tolerance. The six isolates had *in vitro* cholesterol assimilation between 19.34% and 53.60% and bile salt hydrolase activity between 2.52±0.21 and 6.67±0.21 activity/ml/min. The two most promising LAB isolates were selected based on cholesterol assimilation over 40.0% and bile salt hydrolase activity above 6.00 ml/min. The isolates were identified using API 50 CHL kits and medium and 16S rRNA gene sequencing. After genotypic identification, the two promising LAB isolates were identified as *Pediococcus acidilactici* MTA463550.1 and *Pediococcus acidilactici* MN994318.1. These data demonstrated the bile salt hydrolase activity and cholesterol-lowering effects of *Pediococcus acidilactici* MTA463550.1 and *Pediococcus acidilactici* MN994318.1. They can, therefore, be used as probiotic supplements in foods to help reduce the risks of cardiovascular diseases and improve heart health.

Keywords: Cholesterol; hypercholesterolaemia; cardiovascular diseases; Lactic Acid Bacteria; probiotic.

INTRODUCTION

Hypercholesterolemia is a lipid disorder marked by an excessive concentration of low-density lipoprotein (LDL) in the blood, often termed 'bad' cholesterol. This condition leads to the accumulation of fatty deposits in the arteries, known as atherosclerosis, which significantly increases the risk of cardiovascular diseases [1–3].

Cardiovascular diseases (CVDs) are the leading cause of death globally. The World Health Organization (WHO) reported that CVDs were responsible for an estimated 17.9 million deaths in 2019, that is 32% of deaths worldwide, with 85% of the deaths due to heart attack and stroke and over three-quarters taking place in low- and middle-income countries [4]. According to the latest WHO data published in 2020, coronary heart disease and stroke death rates in Nigeria reached 100.91 and 88.68 per 100,000 population, respectively [5].

Many cholesterol-lowering medications, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors and those that promote increased bile acid excretion, have been used to manage hypercholesterolemia [3, 6, 7]. Although pharmaceutical treatments for cholesterol reduction are available, their continuous use has been linked to several adverse effects, including liver damage, cognitive impairment, kidney damage, and muscle disorders [6, 8–10]. Consequently, a need exists to focus on the scien-

tific exploration of natural food products that can extensively lower serum cholesterol levels with few or no side effects.

Probiotics are live microorganisms that, when taken in sufficient quantities, provide health benefits to the host [11, 12]. Lactic Acid Bacteria (LAB) are regarded as a significant group of probiotic bacteria [13]. Cholesterol-lowering probiotics have become popular due to their safety and benefits for gut health. Several strains, including *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus casei*, *Bifidobacterium*, and *Enterococcus faecium*, have been found in healthy individuals' faeces, fermented dairy, and pickles. *Lactobacillus* and *Enterococcus* strains are particularly noted for their cholesterol-lowering effects [14, 15].

A Joint FAO/WHO working group [16] outlined that probiotics can be characterised by their resistance to gastric acid and bile salts, based on survival and growth studies, and their ability to produce antimicrobial compounds and reduce cholesterol [17, 18]. Other functional properties, such as Bile Salt Hydrolase (BSH) activity, are also considered when selecting probiotics. Certain probiotic lactic acid bacteria produce bile salt hydrolase, an enzyme that helps lower serum cholesterol by deconjugating bile salts and enhancing cholesterol assimilation [19, 20]. The BSH enzyme hydrolyses conjugated bile salts by cleaving their amide bonds. This process releases free cholic acid and forms deconjugated bile salts and amino acid residues [21]. Due to their lower solubility and decreased effectiveness in lipid absorption, deconjugated bile salts are expelled from the body via faecal excretion [22]. Therefore, this study aimed to isolate, characterise and identify potential probiotic Lactic Acid Bacteria with cholesterol-lowering ability and bile salt hydrolase activity.

METHODS

Samples Collection and preparation. A total of 25 fermented food samples were collected using sterile universal bottles and transported to the laboratory for microbiological analysis. One millilitre (ml) each of raw milk and yoghurt were homogenised respectively with 9 ml of sterile peptone physiological saline solution. Additionally, 10 g of ugba, ogiri and ogi were homogenised with separate 90 ml portions of sterile peptone physiological saline solution. Samples were transported to the laboratory in the ice box.

Isolation and Characterisation of Lactic Acid Bacteria. Raw milk samples were streaked on the agar surface of MRS agar after coagulation. 100 µL of the yoghurt (after serial dilution) was applied onto the MRS agar surface, fortified with 0.5% calcium carbonate ($CaCO_3$). The agar plates were then incubated at 37°C for 48 hours. After ten-fold serial dilution of the prepared samples of ogiri, ugba and ogi, 0.1 ml of each homogenate was plated on MRS agar [23], and all lined plates were incubated in an anaerobic jar at 35^0C for 48 hours. The obtained colonies were picked randomly and purified by three successive transfers on MRS agar.

The pure cultures of the isolates were characterised using Gram stain, cell morphology and catalase reaction according to standard procedures [24]. The resulting Gram-positive and catalase-negative isolates are considered LAB and were selected and stored by inoculation on MRS Agar slant and subsequently preserved as a stock culture. Furthermore, the cultures were transferred to MRS Broth media for subsequent examinations.

Screening of LAB for Antimicrobial activity. To prepare the cell-free culture supernatants for antimicrobial assay, the LAB isolates were cultivated in MRS broth at 37 °C and then centrifuged at 12,000 rpm for 10 minutes. The antimicrobial activity of these supernatants was evaluated using the agar well diffusion assay. In this assay, 100 µL aliquots of the supernatants were placed within 6 mm diameter wells that were cut into cooled soft nutrient agar plates containing 25 ml of medium previously seeded with the relevant indicator strains at a 1% v/v concentration. After 24 hours, the diameters of the zones where growth was inhibited were measured [25].

Acid and Bile salt tolerance. The acid and bile salt tolerance was assessed by observing the survival of LAB strains after exposure to pH 2.5 and bile salt. A 1 ml LAB culture in MRS Broth was centrifuged (10,000 rpm, 5 min) after 24 hours to create the inoculum. For the acid tolerance test, cell pellets were suspended in phosphate-buffered saline (PBS; Oxoid) that had been acidified to pH 2.5 using 0.1 N HCl. In the bile salt tolerance test, cell pellets were resuspended in PBS containing 0.3% (w/v) bile salt. Incubation was done at 37°C for 3 hours for acid tolerance and bile salt tolerance assessments. LAB counts were enumerated

3 hours after incubation on MRS agar at 37°C for 48 hours. The difference in colony numbers before and after incubation would indicate a reduction in LAB count due to acid or bile salt [26].

In vitro cholesterol-lowering effects

a) Growth and cholesterol assimilation. As described by [27], the pattern of growth and assimilation of cholesterol by the isolated LAB isolates were studied. Overnight cultures of the LAB isolates were inoculated (1% v/v) into freshly prepared MRS broth, which was supplemented with 0.3% oxgall (source of bile salts) and filter-sterilised water-soluble cholesterol (100 µg/ml) and incubated anaerobically at 37°C for 24 h. After the incubation period, the growths were followed by measuring the absorbance at 620 nm. The cells were harvested by centrifugation (9000 rpm, 15 min), and the remaining cholesterol in the spent broth was determined using O-phthalaldehyde colourimetric method [28].

b) Analysis of cholesterol removal from the medium. The amount of cholesterol in the medium after incubation was ascertained using the O-phthalaldehyde technique. This was done by adding 1 ml of KOH (33% w/v) and 2 ml of absolute (95%) ethanol with 1 ml of the cell-free broth and vortexed for a minute, followed by heating at 37°C for 15 min. After cooling, 2 ml of distilled water and 3 ml of hexane were forcefully added to mix with the lower layer, vortexed and evaporated in a water bath at 60°C. The residue was immediately dissolved in 2 ml of O-phthalaldehyde reagent. Ten minutes after adding the reagent, 1 ml of concentrated sulphuric acid was added and vortexed to mix. The solution's absorbance (at 550 nm) was determined after 10 min. All experiments were replicated twice. The ability of each isolate to reduce the amount of cholesterol in culture media was determined by the following equation:

$$\% \text{ of cholesterol reduction rate} = (C_O - C_T)/C_O \times 100$$

where CO is the medium's initial cholesterol; C_T is the final cholesterol in the medium.

Bile Salt Hydrolase (BSH) activity of probiotic LAB isolates

a) Qualitative determination of BSH activity. To determine bile salt hydrolase activity, taurodeoxycholic acid (TDCA) (Sigma-Aldrich, USA) was added to MRS as a detective ingredient in the experiment. LAB isolates were cultured on MRS agar containing 0.5% taurodeoxycholic acid (TDCA) and 0.037% $CaCl_2$, then incubated at 37°C for 48 h. Bile salt hydrolase activity was evaluated by halozone precipitation around the colony on the agar plate [29].

b) Quantitative determination of BSH activity. By measuring the amount of amino acid liberated from conjugated bile salts by the probiotic LAB isolates, the BSH activity can be determined [29]. From cultures grown for 20 h at 30°C, cells were harvested by centrifugation (9700 rpm, 15 min), washed twice with 0.1M sodium phosphate buffer containing 10 mM dithiothreitol (DDT), pH 6.8 and resuspended in the same buffer to obtain suspension with an optical absorbance (A600 nm) of 3.0. After sonication of the cell suspension and followed by centrifugation (9700 rpm, 15 min), the reaction mixture contained (sodium phosphate buffer, 10 ml of a 200 mM appropriate conjugated bile salt, DDT and 10 ml of cell-free extract). The reaction mixture was incubated at 37^0C for 30 min, then a sample (100 ul) was taken, and 200 ul of 15% (w/v) trichloroacetic acid was added to terminate the reaction. The sample was centrifuged (9700 rpm, 15 min), and 200 ul of the supernatant was added to 200 ul of distilled water and 1.9 ml of ninhydrin reagent. The mixture was mixed and boiled for 14 min. After cooling, the absorbance at 570 nm was determined using glycine as standard. One unit of BSH activity (U/ml) is defined as the amount of enzyme that liberated 1 mm of amino acid from the substrate per minute (30). All experiments were repeated twice.

Identification of the LAB isolates

a) Phenotypic characterisation. Cell morphology, growth at 15°, 37°, 45^0, and 65°C and growth in MRS containing 4% and 6.5% of NaCl were the tests that were done. The LAB isolates were phenotyped as described in Bergey's manual of systematic bacteriology [31]. According to the manufacturer's specifications, fermentation patterns were determined using API 50 CHL (Biomeriux, France).

b) Molecular identification based on 16S rRNA gene sequence

- Bacterial DNA extraction. DNA extraction was done according to the manufacturer's instruction using a Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo-Research Laboratory, California, USA).

- Agarose Gel Electrophoresis Genomic DNA. After separation, the gel was examined with a UV Trans-illuminator (TVD-1000R/FB) to view the DNA bands.

- DNA Quantification. The quality and quantity of the extracted DNA was measured using a nanodrop (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer).

- Polymerase Chain Reaction (PCR) - 16S rRNA Gene amplification. The samples were amplified with 27F (forward primer - 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (reverse primer – 5`- TACGGCTACCTTGTTACGAC – 3`) primers using the Peltier- Based Thermal Cycle, with serial number: 012-00014.

- Sequencing of the Bacteria DNA. After PCR amplification, 10µl of each PCR product was run on 1.5% agarose gel prepared with 1X TAE and stained with ethidium bromide(4µl). The electrophoresis was carried out at 100V for 45 min. The gel was examined using a UV Trans-illuminator (TVD-1000R/FB), and the resulting gel image was photographed with a camera.

The PCR products were sent to Inqaba Biotec West Africa Limited, Ibadan, Nigeria, for sequencing. According to the manufacturer's instructions, the fragments were sequenced using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000. The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053). The sequences were obtained in PDF and FASTA formats and then searched through the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information website. A phylogenetic tree based on 16S rDNA genes was constructed [32, 33].

Statistical analysis. Statistical data analysis was done using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) with post hoc Duncan's multiple tests and presented as mean ± standard deviation (SD). One-way analysis of variance was used to study the significant difference between the means with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Isolation and Characterisation of Lactic Acid Bacteria. 52 LAB isolates were isolated from different sources (raw cow milk, yoghurt, ogi ogiri, ugba), as shown in Table 1.

Table 1 – Isolation Sources of Lactic Acid Bacteria

No	Isolation Sources	Number of examined samples	Number of isolates	LAB isolates (colonies labelled with sample names and serial numbers)	Location
1	Ogi (OG)	5	12	OG1 to OG12	Ndi Oru local market, Oboro
2	Cow milk (CM)	5	16	CM1 to CM16	Animal Farm MOUAU
3	Yogurt (YG)	5	10	YG1 to YG10	Umuahia Market, Umuahia
4	Ugba (UG)	5	6	UG1 to UG6	Ndi Oru local market, Oboro
5	Ogiri (OR)	5	8	OG1 to OG8	Gate Six Market, Umudike
	Total	25	52		

Twenty-two isolates were considered presumptive LAB based on their Gram reaction, cell morphology and biochemical tests. The colonies appeared smooth, oval, and creamy white on the MRS agar plate. They were recorded as catalase-negative and Gram-positive cocci in pairs, long and short chains or tetrads, bacilli in pairs or chains and coccobacilli.

The antimicrobial activity of the selected LAB isolates. Out of the 22 presumptive LAB, only 6 (CM1, CM5, YG3, YG4, OG8 and OR7) isolates showed broad-spectrum antagonistic activity (at least 8mm zone of inhibition) against test bacteria pathogens (*Staphylococcus*, *Bacillus*, *Escherichia coli*, *Salmonella* and *Enterococcus*) and were selected for further probiotic evaluations.

Table 2 shows the results of antimicrobial activities of the LAB isolates against some bacteria pathogens. The results show significant variations in the activities of the various LAB species against the different pathogenic bacteria. Against *Staphylococcus aureus*, the inhibition zone diameters were between 10.33 mm (OR7) and 16.33 mm (CM1).

Table 2 – Antimicrobial activity of selected LAB isolates

Isolates	*Staphylococcus*	*Bacillus*	*E. coli*	*Salmonella*	*Enterococcus*
		Diameter zones of inhibition (mm)			
CM1	16.33 ± 0.58 b	12.67 ± 0.58 b	16.33 ± 0.58 b	17.33 ± 1.16 b	15.33 ± 0.58 b
CM5	13.33 ± 0.58 c	11.33 ± 1.16 bc	14.33 ± 1.16 c	14.33 ± 1.53 c	14.00 ± 1.00 bc
YG3	15.33 ± 1.16 b	12.33 ± 0.58 b	17.33 ± 1.16 b	17.67 ± 0.57 b	15.33 ± 0.58 b
OG8	12.67 ± 0.58 c	11.33 ± 0.58 bc	14.00 ± 1.00 c	14.67 ± 1.16 c	13.67 ± 0.58 c
YG4	12.33 ± 0.58	10.33 ± 0.58 c	12.67 ± 0.58 cd	14.33 ± 0.58 c	13.33 ± 0.58 c
OR7	10.33 ± 0.58 d	8.67 ± 0.58 d	11.33 ± 0.58 d	12.67 ± 1.53 d	10.67 ± 0.58 d
Chloramphenicol (standard)	24.00 ± 1.00 a	22.33 ± 1.16 a	27.00 ± 1.73 a	23.67 ± 0.58 a	24.33 ± 1.16 a

Notes: Values show means of triplicate analysis ± standard deviation. Figures with different superscripts in the column are significantly different (P<0.05).

Similar variations were recorded against *Bacillus*, another Gram-positive bacteria with inhibition zone diameter ranging from 8.67 mm (OR7) to 12.67 mm (CM1), while the zones of inhibition of *E. coli* were between 11.33 mm (OR7) and 17.33 mm (YG3). *Salmonella* and *Enterococcus* species were separately inhibited with diameters of 12.67 mm (OR7) to 17.67 mm (YG3) and 10.67 mm (OR7) to 15.33 mm (YG3 and CM1), respectively.

The level of inhibitions of the test pathogens by the LAB isolates cell-free supernatant (CFS) was relatively lower than those caused by the standard antimicrobial agent (Chloramphenicol), which inhibited the test pathogens at inhibition zone diameters of 22.33 mm (*Bacillus*) to 27.00 mm (*E. coli*). The above result indicated that *Bacillus* was the least susceptible, while *E. coli* was the most susceptible. Again, the LAB isolates varied significantly within themselves in their respective inhibitory actions against the bacteria pathogens. On comparative grounds, CM1 was the most effective, with a mean inhibitory diameter of 15.60 against the five bacteria pathogens. At the same time, the OR7 isolate recorded the most minor activity against the bacteria pathogens, with a mean inhibition diameter of 10.73.

Many lactic acid bacteria produce antimicrobial metabolites that can inhibit pathogen growth and help these bacteria compete for nutrients [34].

Figure 1 shows the relative potencies of the Lactic Acid Bacteria (CFS) extracts as antimicrobial agents compared to standard commercial antibiotics (chloramphenicol).

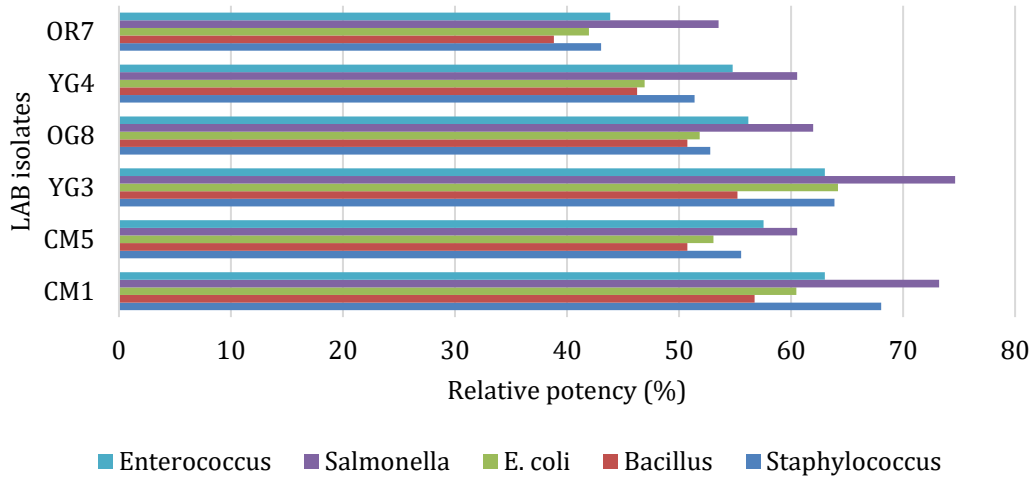


Figure 1 – Relative potencies of the LAB CFS extract against test pathogens

From the results, the range of potency of the LAB extracts varied with the LAB type (species) on one hand and with the test bacterial pathogens on the other. Against *Staphylococcus*, all but OR7 (43.04%) scored above the half mark (50%) with a range of 51.38% to 68.04%. YG3 and CM1 had higher potency scores of 63.88% and 68.04%, respectively, while YG4 recorded 51.38% and 55.54% by CM5 isolate.

In their actions against *E. coli*, the LAB CFS were relatively less potent, with an overall range of 41.96% to 60.48% compared to those recorded against *Staphylococcus*. However, Bacillus was relatively more resistant, with the LAB extract potency range of 38.33% to 56.74%. On the other hand, the relative potencies of the LAB CFS were much higher against Salmonella, with a range of 53.53% to 74.65%. However, the result did not show a distinctive trend as no marked differences existed between the potency pattern of the LAB isolates CFS against Gram-positive and Gram-negative bacteria.

Tolerance of selected LAB isolates to low acid and bile salt. An essential criterion for assessing probiotics is their resistance to stomach acid and bile in the small intestine. Since probiotics are usually taken orally, they must withstand these conditions to successfully colonise and function [35, 36].

The survival of the isolated LAB in low acid and bile salt conditions is shown in Table 3.

Table 3 – Tolerance of selected LAB isolates to low pH and bile

Isolates	pH								Bile
	2.5	3.5	4.0	4.5	5.0	5.5	6.0	6.5	0.3%
CM1	++	+	+	++	++	++	++	++	+
CM5	+	+	+	++	++	++	+	-	+
YG3	++	+	+	++	++	++	++	++	+
OG8	+	+	++	++	++	++	+	+	+
YG4	-	-	+	++	++	++	+	-	+
OR7	-	-	+	++	++	+	+	+	+

The isolates sustained growth at varying degrees when exposed to pH 2.5 (gastric acidic condition) for 3 hr. However, two isolates (YG4 and OR7) did not show growth at pH 2.5. OG8 isolate had sustained growth through the pH range of 4.0 to 5.5. At a pH of 4.0, YG4 isolated from yoghurt had slight growth, but the growth increased significantly at a pH range of 4.5 to 6.0. Other isolates like CM5 had slight growth between the pH of 3.5 and 4.0 but recorded sustained growth at the pH range of 4.5 to 5.5. The result showed that none of the LAB isolates could grow at a neutral pH of 7.0, indicating that they thrive well in an acidic and slightly acidic environment.

***In vitro* cholesterol-lowering effect of the selected probiotic LAB isolates.** Figure 2 shows the in vitro cholesterol assimilation test results on the LAB isolated from different fermented foods.

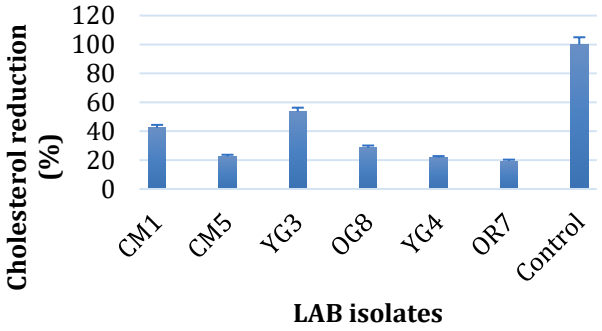


Figure 2 – *In vitro* cholesterol assimilation by LAB isolates

The results showed variations or significant differences ($P<0.05$) between the cholesterol assimilation of the different LAB isolates. Generally, the capacities of the different LAB isolates to reduce cholesterol varied between the ranges of 19.34% to 53.60%. YG3 recorded the highest cholesterol assimilation (53.60%), followed by CM1 (42.26%), while OR7 scored the lowest reduction (19.34%).

Since the assimilation of cholesterol in this context refers to the level of cholesterol reduction in the medium, the obtained result demonstrates the potential of the LAB isolates to reduce cholesterol in the culture medium (*in vitro*). Several studies have shown that certain strains of lactic acid bacteria can reduce cholesterol in culture media *in vitro* (37) through various mechanisms, such as binding to cellular surfaces and incorporating cholesterol into their cell membranes [21, 22, 26].

Bile Salt Hydrolase activity of the selected probiotic LAB isolates. All six selected LAB isolates showed halozone precipitation around the

colony when cultured on MRS agar containing 0.5% taurodeoxycholic acid (TDCA). Bile salt hydrolase activity was evaluated by halozone precipitation around the colony on an agar plate. Figure 3 shows the result of the quantitative determination of the Bile Salt Hydrolase (BSH) activity of the six LAB isolates from fermented foods.

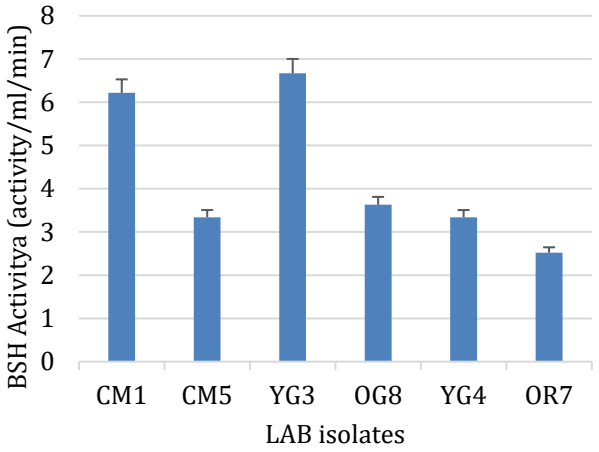


Figure 3 – Bile salt hydrolase activity of the selected LAB isolates on taurodeoxycholic acid (TDCA) sodium salt

The result showed slight but significant variations in the levels of the enzyme activity of the isolates. Generally, the activities ranged from 2.52 ± 0.021 U/ml/min to 6.67 ± 0.21 U/ml/min. CM1 and YG3 LAB isolates recorded high BSH activities of 6.22 U/ml/min and 6.67 U/ml/min respectively than the other LAB isolates which recorded 3.34U/ml/min (CM5), 3.63 U/ml/min (OG8), 3.34 U/ml/min (YG4) and 2.52 U/ml/min (OR7).

The result corroborated the positive result of the qualitative determination test, which indicated the presence of the enzyme (BSH) in all the selected probiotic LAB isolates through the production of halo precipitation zones on the inoculated MRS agar.

Some probiotic LAB species secrete bile salt hydrolase that reduces serum cholesterol [22], renders them tolerant to bile salts [38] and enhances bile excretion in the stool [19, 21, 22].

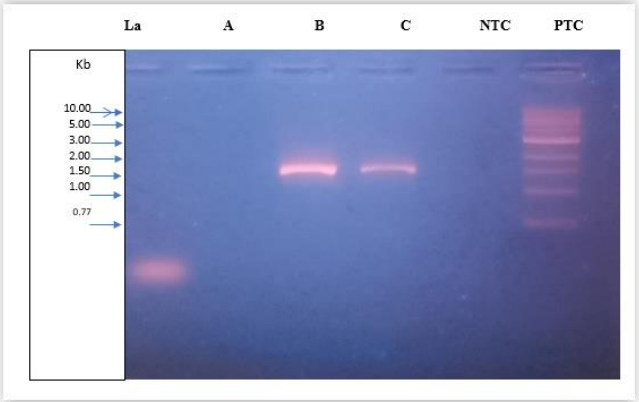
Table 4 – Performance of selected LAB isolates on carbohydrate metabolism tests using API 50 CH and API CHL medium

Test sample	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
Isolate code	Control	Glycerol	Erythritol	d-arabinose	l-arabinose	Ribose	d-xylose	l-xylose	Adonotol	B-methyl-d-xyloside	Galactose	Glucose	Fructose	Mannose	Sorbose	Rhamnose	Dulcitol	Inositol	Mannitol	Sorbitol	Methyl-d-mannoside	Methyl-d-glucoside	N-acetyl-glucosamine	Amygdalin	Arbutin	Esculine	
CM1	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	+	-	-	-	-	-	-	+	-	+	+	
CM5	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+	+	
YG3	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	
OG8	-	-	-	-	+	+	+	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	
YG4	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
OR7	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	+	
Test sample	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49			Identified species
Isolate code	Salicin	Celloboise	Maltose	Lactose	Melibiose	Sucrose	Trehalose	Insulin	Melezitose	Raffinose	Starch	Glycogen	Xylitol	B-gentiobiose	d-turranose	d-lyxose	d-tagatose	d-fucose	l-fucose	d-arabitol	l-arabitol	Gluconate	2-keto-gluconate	5-keto-gluconate			
CM1	+	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-			*Pediococcus acidilactici*
CM5	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-	-	+	+	+	+	+			*Lactobacillus brevis*
YG3	+	+	+	+	-	+	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-			*Pediococcus sp.*
OG8	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+	-	-	+	-	+	-	-			*Lactobacillus plantarum*
YG4	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-			*Lactobacillus delbrueckii spp bulgaricus*
OR7	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+			*Lactobacillus fermentum*

Table 5 – Morphological and biochemical characteristics of the selected LAB isolates

Isolates	Colonial morphology	Cell arrangement	Gram reaction	Spore formation	Motility	Catalase	Indole	Oxidase	Growth at 15°C	Growth at 37°C	Growth at 45°C	Growth at 65°C	4% NaCl	6.5% NaCl	Suspected bacteria
CM1	Creamy and mucoid	Cocci in pairs and tetrads	+	-	-	-	+	-	-	+	+	+	+	-	*Pediococcus sp.*
CM5	Convex, smooth and opaque	Short rods	+	-	-	-	-	-	+	+	-	-	+	+	*Lactobacillus brevis*
YG3	Creamy and mucoid	Cocci in pairs or tetrads	+	-	-	-	+	-	-	+	+	+	+	-	*Pediococcus sp.*
OG8	Circular, creamy and convex	Single rods and in pairs	+	-	-	-	-	-	+	+	-	-	+	+	*Lactobacillus plantarum*
YG4	Creamy grey, raised	Rods in chains	+	-	-	-	-	-	-	+	+	-	+	+	*Lactobacillus delbrueckii spp bulgaricus*
OR7	Circular, white and convex	Single rods and chains	+	-	-	-	-	-	-	+	+	-	+	+	*Lactobacillus fermentum*

Molecular identification based on 16Sr RNA gene sequence. The genotypic identification of the most promising LAB isolates (CM1 and YG3) based on their high *in vitro* cholesterol assimilation and bile salt hydrolase activity was done by 16S rRNA gene sequencing. The gel electrophoresis of the PCR amplicons of CM1 and YG3 indicated a 1500 base pair size, as shown in Figure 5.



Notes: La = Ladder, NTC = negative control, PTC = positive control); B = CM1; C = YG3.

Figure 5 – PCR gel image of amplicons from the 16S rRNA gene amplification of the isolates DNA

The CM1 and YG3 isolates were identified as *Pediococcus acidilactici* MTA463550.1 and *Pediococcus acidilactici* MN994318.1, respectively, as shown in Table 6.

Table 6 – Summary of BLAST prediction and Sequencing result

No	Sequence ID	Matched Organism	% identity	Accession number
1	CM1	*Pediococcus acidilactici*	98.86	MTA463550.1
2	YG3	*Pediococcus acidilactici*	97.60	MN994318.1

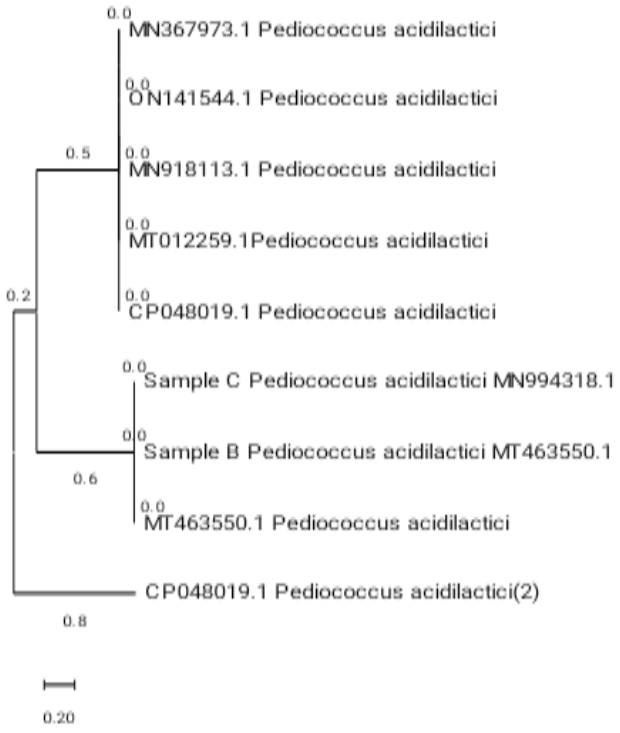


Figure 6 – Phylogenetic Analyses (Tree)

CONCLUSIONS

In conclusion, this study revealed that CM1 and YG3 isolates identified as *Pediococcus acidilactici* MTA463550.1 and *Pediococcus acidilactici* MN994318.1 isolated from fermented food products qualified as probiotics and showed vigorous bile salt hydrolase activity and assimilated cholesterol *in vitro*. The findings suggest that these Lactic Acid Bacteria strains can be used as probiotic supplements in foods to help reduce the risks of cardiovascular diseases and improve heart health.

However, *in vivo* animal models and human clinical trials to confirm these potentials are recommended as probiotic food supplements before full endorsement. In addition, an elaborate understanding of the mechanisms of hypocholesterolemic effects of probiotics is highly recommended, as well as extensive research for the production of probiotic-based therapeutic agents against hypercholesterolaemia.

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