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Characterization of Cholesterol Lowering Lactic Acid Bacteria Isolated from Palm Wine and Maize Beer and Assessment of Their Use in the Production of Probiotic Papaya Juice

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Elevated serum cholesterol is a main risk factor for heart disorders. Most probiotic products administered to lower cholesterol are dairy products which are not suitable for lactose-intolerant individuals. In this study, we assessed the cholesterol-lowering efficacy of LAB isolated from traditionally fermented drinks in diet-induced rats and determine their efficacy in the production of non-dairy, probiotic formulations using papaya juice. LAB were isolated from palm wine and corn beer on MRS agar using a pour-plate technique. Identification was carried out using 16S rRNA gene sequencing. A hypercholesterolemia model in which diet-induced Wistar albino rats were assigned into four groups was established. Oral gavage was carried out for 30 days. On the 31st day, the rats were dissected and the serum lipid profile was analyzed using biochemical kits. A 10^6 cfu/ml of a 24-h-old culture of selected lactobacilli was used to inoculate papaya juice and incubated at 37℃. Microbial and chemical changes were assessed during papaya fermentation and after four weeks of cold storage. Two selected isolates (Pw1 and Cb4) had in vitro cholesterol reduction of > 80%. These two isolates lowered lipid profile (triglyceride, total cholesterol, LDL-c) significantly, and increased HDL-c levels ($p < 0.5$) in the rat sera. Phylogenetic analysis showed that Pw1 was 98.86% similar to *Limosilactobacillus fermentum*, while Cb4 was 99.54% similar to *Enteroccocus faecium*. Both strains fermented papaya juice with cell viability reaching 8.92×10^8 cfu/ml and 25.3×10^8 cfu/ml respectively, and were still viable after 4 weeks of cold storage.

Keywords: **Hypercholesterolemia, probiotic, lactic acid bacteria, papaya juice, serum cholesterol**

Introduction

Hypercholesterolemia (high blood cholesterol concentration) is a significant cardiovascular disease (CVD) risk factor [1] and a major public health concern. According to the World Health Organization (WHO), cardiovascular disease is the leading cause of mortality worldwide, claiming an estimated of 17,9 million lives annually [2]. Due to poor consumer compliance, lifestyle modifications and therapeutic agents such as lipid-lowering medications have had limited efficacy in reducing serum cholesterol [3].

Probiotic bacteria have been utilized as a more cost-effective treatment for patients with elevated serum cho-

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lesterol levels. The hypocholesterolemic effects of certain probiotics have been reported in both animal models [4] and human clinical trials [5], as they are regarded as generally safe [6]. Probiotics are live microorganisms that impart a health benefit on the host when administered in adequate quantities [7]. Lactic acid bacteria (LAB) and bifidobacteria are the most common probiotic organisms, but yeasts and bacilli may also be used [8, 9].

Due to the presence of simple sugars utilized as a substrate for development, fermented beverages such as palm wine and corn beer have been identified as potential sources that harbor a viable and active consortium of LAB. The consumption rates of traditionally fermented drinks are high in West Africa and Cameroon in particular. This is because of their low cost and easy accessibility. In spite of this consumption, the potential benefits of the microorganisms that ferment these products have not been fully exploited.

Fruits and dairy products have been utilized as probiotic bacteria delivery systems in numerous dietary media [11, 12]. The primary source of probiotic consumption has been dairy products. Reportedly, the presence of milk lipids and milk allergies can have a variety of negative health effects on individuals, particularly those who are lactose intolerant [13]. Fruits have been shown to be more beneficial due to their high content of dietary fiber, vitamins, minerals, phytochemicals, and anti-oxidants that prevent cell oxidation.

The species *Carica papaya* Linn contains very little saturated fat, cholesterol, and sodium. This makes it a preferred fruit for obese individuals on weight-loss programs [13]. It is grown on all five continents, but the majority is produced in Asia, Central America, and Africa. India, Brazil, Nigeria, Indonesia, and Mexico are some of the world's leading papaya-producing nations [14]. In several ecological zones throughout the year in Cameroon, pawpaw is one of the fruits that is accessible in a variety of forms [15]. The combined health benefits of probiotics and fruit juice are systemic, digestive, and nutritional [16]. Moreover, fruit juice serves as a medium for protecting probiotics from hazardous gastrointestinal conditions, making them a more effective and less expensive mean for lowering serum cholesterol. The purpose of this study was to examine the cholesterol-lowering efficacy of LAB isolated from traditionally fermented beverages and fermenting papaya juice with selected LAB strains in rats on a diet.

Materials and Methods

Sample collection

Forty samples of palm toddy (*Elaeis guineensis*) were acquired from collectors of palm wine. In brief, the tree was cut down, a hole was drilled into the tender meristem of the tree trunk, and a tube was inserted to enable sap to be collected in a clean plastic container. In addition, forty corn beer (maize beer) samples were collected from corn beer vendors using a sterile plastic bottle. The National Herbarium in Yaoundé identified the African oil palm and the maize plant. African oil palm tree was identified through comparison with the botanic collection of M. Brut No. 379, which was recorded at the National Herbarium under No. 34163/HNC. Maize plant was identified through comparison with the botanic collection of D. Dang No. 81, which was also recorded at the National Herbarium under No. 18625/SRF/Cam.

All samples were collected in Buea and promptly transported in ice-cool boxes (4℃) to the Life Science Laboratory at the University of Buea, where the palm wine sap was fermented for 24 h under ambient conditions (25℃) before the isolation of lactic acid bacteria.

Isolation of lactic acid bacteria

Preparation of media was carried out using the instructional manual. Lactic acid bacteria (LAB) were isolated on de Man Rogosa and Sharpe (MRS) agar by pour plating technique. Briefly, one ml from 10^{-5} and 10^{-7} dilution was transferred into sterile petri dishes and covered with MRS agar. The plates were incubated at 37℃ for 24 h. To have pure colonies, repeated streaking on MRS agar was done, and they were labeled with codes such as Pw for palm wine LAB and Cb for corn beer LAB. LAB were distinguished from other microorganisms through colony morphology, Gram staining, and catalase testing. Pure Catalase-negative and Gram-positive isolates were presumed to be LAB and tested for their tolerance to acid and bile salt. These colonies were preserved for future use in 15% glycerol agar at -80℃.

Probiotic properties

Acid tolerance and bile tolerance. Following a previous method [18] with minor modifications, acid and bile tol-

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erance assessments were conducted. To eliminate contaminants, overnight Pw and Cb isolates (cultures) were three times washed with PBS (pH 7.0) and centrifuged at 5,000 ×*g* for 10 min at 4℃. The cell pellets were added to MRS broth with a pH meter (HI991001, Romania) and 3N HCl or NaOH to adjust the pH to 2.5.

The cell pellets were resuspended in MRS broth adjusted to pH 7 and containing 0.3% oxgall bile salts (Sigma Aldrich, Germany) to determine bile tolerance. The cultures were then incubated for 24 h at 37℃. After 0 and 3 h, aliquots were serially diluted, plates on MRS agar, and quantified as colony-forming units per milliliter (cfu/ml). The samples collected at 0 h served as the control.

In vitro cholesterol assimilation

Prior to assessing cholesterol assimilation in culture media, acid and bile tolerance tests were conducted on Pw and Cb isolates to identify those with higher resistance to gastrointestinal tract conditions. Pw1 and Cb4 were tested for their ability to assimilate cholesterol *in-vitro* using a modified technique of Pereira [19]. Isolates were inoculated into test tubes containing 10 ml of MRS broth, 0.3% bile salts, and 1% acid solution of cholesterol (catalog number C3045-5G, Sigma-Aldrich) followed by incubation at 37℃ for 24 h. The isolates were then centrifuged (5,000 ×*g* for 10 min at 4℃), and the supernatant was analyzed with a spectrophotometer (Pharmacia bioteck, England) at 540 nm and compared to the control experiment. The cholesterol absorption rate was computed using the formula

$$A = \left(\frac{B}{C}\right) \times 100$$

where A represents the percentage of cholesterol remaining in the pellet, B represents the absorbance of the sample containing cells, and C represents the absorbance of the sample without cells.

Isolates with cholesterol assimilation properties *in-vitro* were selected for further molecular characterization.

Identification of isolates

Pure cultures of isolates Pw1 and Cb4 were used to extract their genomic DNA at 25℃. One ml of each purified liquid culture was centrifuged at 11,500 rpm for 10 min. The cell fragments were resuspended in a tube containing 300 µl buffer (10 mM Tris-HCl, pH 8.0; 50 mM glucose, and 10 mM EDTA) and 3 µl lysozyme (10 mg/ml). The pellets were lysed at 37℃ for 60 min and vortexed every 5 min, then placed on ice every 5 min. An aliquot of 300 µl of lysing buffer and 3 µl of RNase were added to the mixture, which was then incubated for 30 min and chilled for 1 minute on ice. Then, 100 µl of a 7.5 M sodium acetate solution was added, vortexed for 25 seconds, and centrifuged for 10 min at 13,000 rpm and 4℃. Transferring the supernatant to a sterile tube, 300 µl of isopropanol was added and delicately mixed. The resulting mixture was centrifuged for 10 min at 13,000 ×*g* and 4℃. Using a sterile Eppendorf pipette, the isopropanol was removed while preserving the DNA particles. The tubes were desiccated in 25℃ air by inverting them on sterile filter paper and exposing them to the open air. The DNA pellets were purified in 400 µl of 70% ethanol and centrifuged for 2 min at 5,000 rpm and 25℃. The residues were dried at 37℃ for 10 min before being solvated in 30 µl of TE buffer and stored at -20℃ for future use.

DNA amplification by polymerase chain reaction (PCR)

PCR was used to amplify the sequence of the 16S r RNA coding region using forward (5'-AGAGTTTGATCC-TGGCTCAG-3) and -reserve (5'-ACGGCTAC-CTTGT-TAACGACTT-3) universal primers. Conditions for the 30-cycle PCR: 95℃ for 5 min (initial denaturation), 94℃ for 1 min 30 s (denaturation), 55℃ for 1 min 30 s (annealing), 72℃ for 1 min 30 s (extension), and 72℃ for 10 min (final extension).

Gel electrophoresis

After allowing the amplicons to settle, 2 µl of each amplification mixture was electrophoresed for 1 h at 100 V on 1.5% (w/w) agarose gels in 0.5 × TAE buffer. The standard was DNA molecular weight marker (250 to 10000 bp) molecular ladders from Inquaba biotech, South Africa. After electrophoresis, ethidium bromide was used to stain the gels. washed and photographed with a Bio-Rad UV transilluminator (Hercules, USA). South African company Inquaba Biotech determined the fragmentary 16S r RNA sequence analysis of the PCR products. BLAST was used to determine the extent of similarity between the obtained sequences and those of

other species [23]. The sequences were deposited in the NCBI Genebank to obtain an accession number.

Animal feeding and experimental design

Twenty-four Wistar albino rats (Rattus norvegicus), 10 to 12 weeks old and weighing between 90 and 120 g were purchased from the animal house of the University of Dschang, Cameroon's Department of Animal Biology. All animals were managed in accordance with the University of Buea Institutional Animal Care and Use Committee's (UB-IACUC no. 015/2019) institutional guidelines. The method described by Ngongang et al. [20] was used to classify feed composition and animal groups. The hyperlipidemic diet consisted of approximately 89% basic diet, 1% cholesterol, and 10% pork fat (lard). The rats were randomly divided into four groups: (1) fed with hyperlipidemic diet and bacteria isolate (Pw1), (2) fed with a high lipid diet and isolate (Cb4); (3) the negative control group, fed with basic diet + oral doses of deionized water; and (4) the positive control group, fed with a high lipid diet and oral doses of deionized water.

Animal studies

The rats were housed in normal 24-h cycles ggcomprised of 12 h of day and 12 h of night. The temperature remained constant at 25℃, and the relative humidity remained close to 50%. Acclimatization of the rats was done for Seven days prior to the commencement of the experiment. Throughout the experiment, the animals were housed in plastic enclosures and fed with a basic diet including a daily supply of water. The experiment was conducted for four weeks with oral gavages of 10^8 cfu/ml of bacteria isolates (Pw1 or Cb4) administered at a volume of 1.0 ml/kg body weight/day for each dose. The daily monitoring of the quantity of food consumed and the animal's weight were recorded.

Blood collection and biochemical analysis

At the end of 30 days of feeding, the rats were allowed to fast for 12 h, and on the 31st day, the rats were anesthetized their blood was collected by cardiac puncture in Eppendorf tubes. After 60 seconds at room temperature, the blood was centrifuged at 3,000 rpm and 4℃ for ten min to collect serum, which was stored at -20℃ for further analysis.

Serum levels of total cholesterol (Tc), high-density lipoprotein cholesterol (HDL-c), and triglyceraldehyde (TG) were measured using assay kits (CHRONOLAB SYSTEMS, Spain) according to the manufacturer's instructions. Using the following formula, very low density lipoprotein cholesterol (VLDL-c) and low density lipoprotein cholesterol (LDL-c) were calculated [26].

$$VLDLc = \frac{TG}{5}$$

$$LDLc\ (\text{mg/dl}) = Tc - \left(HDLc + \frac{TG}{5}\right)$$

Papaya processing and fermentation

Ripened pawpaw (*Carica papaya* Linn, solo variety) fruits with 85–90% maturity and free from visual blemishes were purchased from Njombe (Littoral Region, Cameroon). They were washed with potable water, peeled manually, and sliced into small sizes of about 10–15 mm. Using a sterile fruit juice mixer (Royal line, Germany), the pulp was crushed to form a homogenous solution. The solution was then diluted with distilled water forming a 1:1 ratio of fruit juice to water. After blending, the juice was filtered with a clean sieve (hot water wash) to get a smooth composition free from the pulp. The resulting solution was then pasteurized (heated for 5 min at 80℃) and refrigerated after cooling at 5 ± 1℃.

The fermentation of papaya juice was carried out by the previous method [27]. Briefly, 24 h cultures of isolate Pw1 and Cb4 in MRS broth were washed three times in PBS buffers and centrifuged at 4,000 rpm for 10 min. A final concentration of approximately 10^6 cfu/ml was obtained by comparing the turbidity of the cell pellets to a Mac Farland standard No. 0.5. Fermentation in two replicates was carried out in sterilized 90 ml glass bottles. Each of these glass bottle was inoculated with 0.5% of the $>10^5$ cfu/ml of the 24 h cultures and incubated at 37℃ for 72 h. Samples were collected after every 24 h for chemical and microbiological analysis.

Effect of cold storage on cell viability and probiotic papaya juice

The effect of cold storage was measured after 72 h of fermentation at 37℃. Fermented samples were stored at 4℃ for four weeks. Weekly samples were collected to

determine the viability of probiotic bacteria in papaya juice expressed in cfu/ml.

Statistical analysis

Using the Statistical Package for the Social Scientist (SPSS) version 20.0 and one-way ANOVA, we determined the efficacy of the two isolates in lowering various lipid parameters in albino rats based on data presented as mean standard deviation. Each test was conducted in triplicate. p-values < 0.05 were regarded as significant. A post hoc Tukey test was utilized to compare the means of each treatment.

Results

Isolation of lactic acid bacteria

On MRS agar, 80 different bacterial cultures were isolated, with 42 being isolated from palm wine and 38 from corn beer. On the MRS agar plate, identification based on cell morphology, microscopic inspection, and biochemical assays revealed smooth, oval, and cream-colored colonies. Only ten colonies (cocci and rods) were examined under a microscope and classified as presumptive LAB since they were Gram positive and catalase negative. The tentative identification of the isolates from palm wine and corn beer is displayed in Table 1.

Tolerance to gastro-intestinal conditions

When inoculated in a medium of pH 2.5 after 3 h, the viability of the isolates ranged from 3.9 to 7 log cfu/ml. Fig. 1A displays the isolates' ability to survive following incubation. After 3 h at pH 2.5, the viable counts for isolates Pw1, Pw4, Cb1, Cb3, Cb4, Cb5, and Cb6 exceeded 6 log cfu/ml, but this difference was not statistically significant from the control. In contrast to the control (0 h), isolates Pw2, Pw3, and Cb2 showed viable counts less than 5 log cfu/ml. After 3 h of exposure, isolate Pw4 had the highest viable count (7.15 log cfu/ml) of any isolate.

After 3 h incubation in 0.3% bile salt concentration, the viable count for each isolate ranged from 3.9 to 7.5 log cfu/ml (Fig. 1B). After 3 h at 0.3% bile salt, the vitality of isolates Pw1, Pw4, Cb1, Cb2, Cb4, Cb5, and Cb6

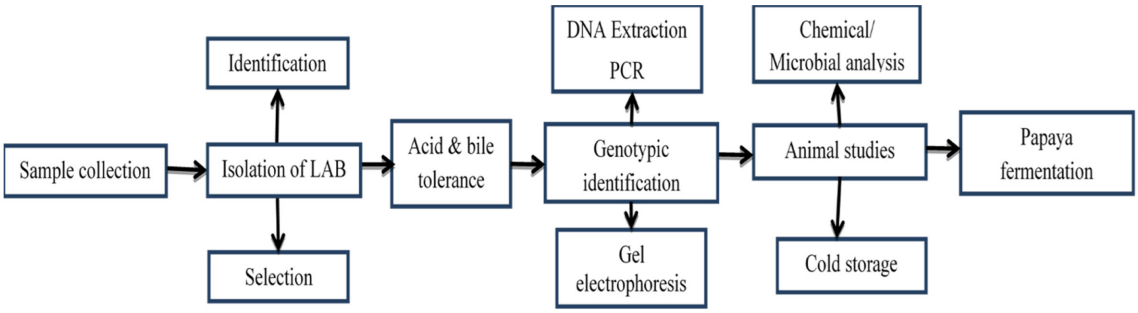

Table 1. Phenotypic characteristics of LAB isolates change codes.

Isolates	Catalase test	Gram reaction	Shape	Origin of isolate
Pw1	-	+	Rods	Palm wine
Pw2	-	+	Rods forming clusters	Palm wine
Pw3	-	+	Shorts rods	Palm wine
Pw4	-	+	Rods	Palm wine
Cb1	-	+	Rods	Corn beer
Cb2	-	+	Chain forming cocci	Corn beer
Cb3	-	+	Short rods	Corn beer
Cb4	-	+	Shorts rods	Corn beer
Cb5	-	+	Chain forming cocci	Corn beer
Cb6	-	+	Chain forming cocci	Corn beer

(+) Positive (-) Negative reactions

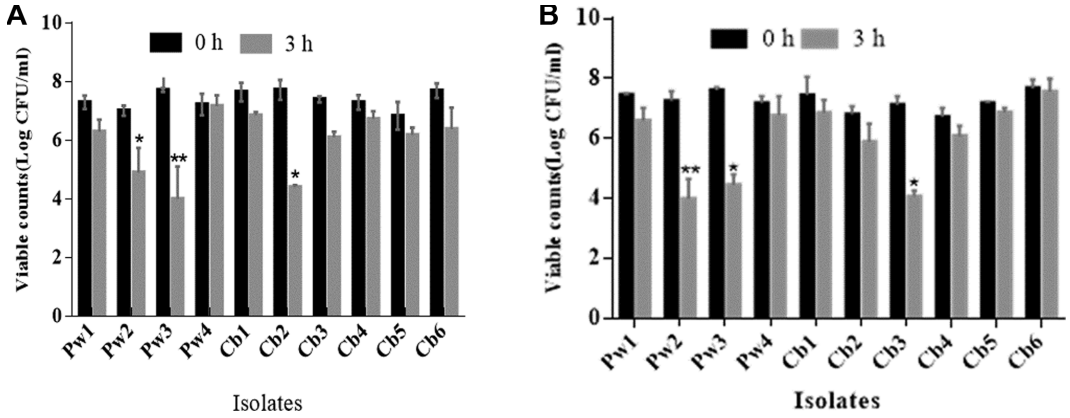

Fig. 1. Survival of LAB isolate at pH 2.5 (A) and in bile salt (B). Viable LAB isolates were counted (log cfu/ml) at 0 h and after 3 h. Bars show mean ± standard deviation. Significant differences *$p < 0.05$, **$p < 0.01$.

exceeded 6 log cfu/ml which was comparable to the control (0 h). After 3 h at 0.3% bile salt, the viability of isolates Pw2, Pw3, and Cb3 dropped below 4.5 log cfu/ml. The most viable count was found in isolate Cb6 (7.5 log cfu/ml). Fig. 1B shows the survivability of these strains in bile salt condition after 3 h at 37℃.

In vitro cholesterol reduction

After 24 h of incubation in the presence of bile salt, the percentage of cholesterol absorbed in vitro ranged from 18 to 89% (Fig. 2). Six strains of bacteria digested cholesterol to varying degrees, although Pw1 and Cb4 had the best assimilation rates (>80%). Pw3, Cb2, and Cb3 on the other hand, absorbed cholesterol poorly (45%) and did not thrive in the medium. The strains Pw1 and Cb4 were chosen for additional research.

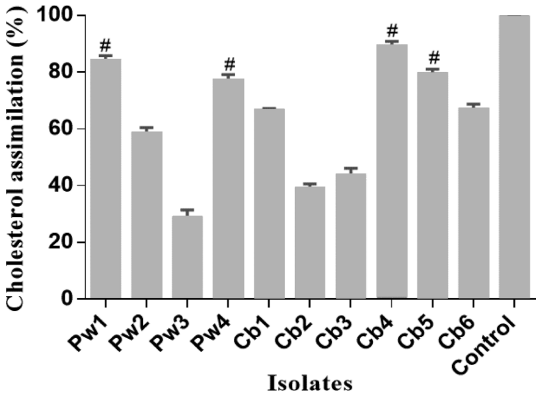

Fig. 2. Cholesterol assimilation by LAB strains. LAB strains were cultured in 1 % cholesterol and bile salt concentration of 0.4 % for 24 h. Data are expressed as mean ± standard deviation. Bars with # represent isolate with cholesterol assimilation >75%.

Identification of LAB isolates

According to the phylogenetic analysis based on the 16S r RNA gene sequence (Table 2), Pw1 strain had a 99.8% relationship with *Enterococcus faecium* strain and Cb4 strain had a 98.86% relationship with *Limosilactobacillus fermentum* strain. Both strains were deposited into the NCBI database.

In-vivo measurement of cholesterol assimilated by bacterial strains

Rat feed consumption and body weight. As shown in Table 3, at the end of the first week of the experiment, the difference in food intake between groups A, B, the negative control (NC) and the positive control (PC) was insignificant. However, when compared to the other test groups, the weight gain in the PC (17.64 ± 0.04 g) rose substantially (Table 4). The PC group had significantly

Table 2. The 16S rRNA sequencing identification.

Isolate	Blast identification	Accession n°	% identity between query and subject
Pw1	*Limosilactobacillus fermentum* strain	OQ363311	98.86%
Cb4	*Enteroccocus faecium* strain OZB1-4	OQ376366	99.54%

Table 3. Weekly Food Consumed (g) by Rats.

Weeks	A (Pw1)	B (Cb4)	NC (Negative control)	PC (Positive control)
1	12.55 ± 0.76[a]	12.27 ± 0.02[a]	12.37 ± 0.06[a]	13.59 ± 0.24[a]
2	13.52 ± 0.05[a]	12.55 ± 0.12[a]	11.92 ± 0.06[a]	15.47 ± 0.08[b]
3	13.68 ± 0.11[a]	11.81 ± 0.05 [a]	11.91 ± 0.01[a]	18.82 ± 0.01[b]
4	13.07 ± 0.02[a]	10.52 ± 0.04[a]	10.78 ± 0.16[a]	19.72 ± 0.13[b]

Table 4. Weekly weight gain by rat (g).

Weeks	A (Pw1)	B (Cb4)	NC (Negative control)	PC (Positive control)
1	13.87 ± 1.02[a]	15.29 ± 0.23[a]	13.13 ± 0.03[a]	17.64 ± 0.04[b]
2	17.07 ± 0.04 [a]	21.28 ± 0.03 [a]	14.22 ± 0.15 [a]	22.83 ± 0.02[b]
3	17.44 ± 0.01 [a]	16.80 ± 0.00 [a]	14.08 ± 0.03 [a]	23.93 ± 0.04 [b]
4	18.23 ± 0.03 [a]	23.68 ± 0.04 [a]	17.03 ± 0.03 [a]	25.55±0.093 [b]

more food consumed by the end of the second week (15.47 ± 0.08 g), whereas the other test groups had continued to consume food at relatively low levels (13.52 ± 0.05 g). According to the rise in weekly weight growth (22.83 ± 0.02 g), the PC group's increase in feed consumption was proportional. Weekly weight increase and feed consumption were consistently greater in the PC. $P < 0.05$ indicates that values with various superscripts in a row are statistically distinct. A; fed with rich lipid diet and bacteria isolate (Pw1). B; fed a high lipid diet and isolate (Cb4), whereas NC was served a basic diet plus oral doses of deionized water. PC; The PC group was fed a hyperlipidemic diet (approximately 85% basal diet, 1% cholesterol, 10% lard (pig fat), and deionized water via oral gavage). A, B, NC, and PC represent the various treatment groups and their respective food consumption.

At the end of the feeding trial, the quantity of total cholesterol in the positive control group was greater than in the other groups (Table 5). The test groups had Tc values of 147.99 ± 10.72 mg/dl with Pw1 and 175.90 ± 4.27 mg/dl with Cb4, whereas the negative control group had a Tc value of 125.79 ± 5.69 mg/dl. The TC values were as follows: PC > A > B > NC. The serum TG levels were higher ($p < 0.05$) in the PC group compared to the test groups (A and B) and the NC group, in the following order: 210.42 ± 9.40, 157.17 ± 20.86, 122.50 ± 14.50, and 87.65 ± 15.74 mg/dl. LDLc levels were markedly lower in groups A, B, and NC (66.91 ± 17.97, 81.37 ± 6.53, and 73.74 ± 11.30 mg/dl, respectively) than in group PC (126.19 ± 7.76 mg/dl).

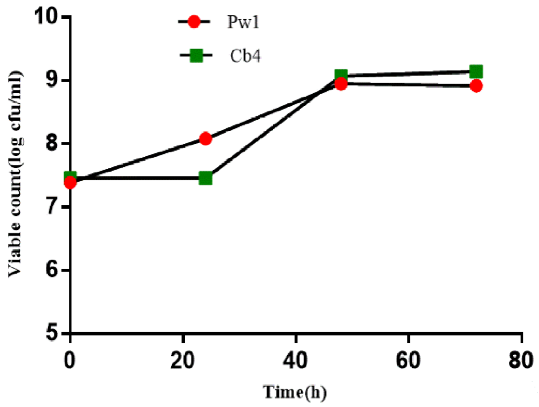

Fig. 3. Change in cell viability during papaya fermentation.

Growth of lactic acid bacteria during papaya juice fermentation

Fig. 3 depicts the proliferation of two lactobacilli strains in papaya fruit juice during fermentation. During the latency phase of fermentation, Pw1's viability steadily increased, whereas Cb4's viability remained unchanged. After 24 h of fermentation, Pw1's viability increased steadily to a maximum of 8.54 log cfu/ml, while Cb4's viability increased abruptly to a maximum of 9.15 log cfu/ml. Pw1 and Cb4 reached 8.93 log cfu/ml and 9.23 log cfu/ml, respectively, when the fermentation time was extended to 72 h.

Change in pH and acidity

The pH values provided a fair summary of the fermentation's development. During 72 h of fermentation, the pH of Pw4 and Cb4 fluctuated. The pH values of Cb4

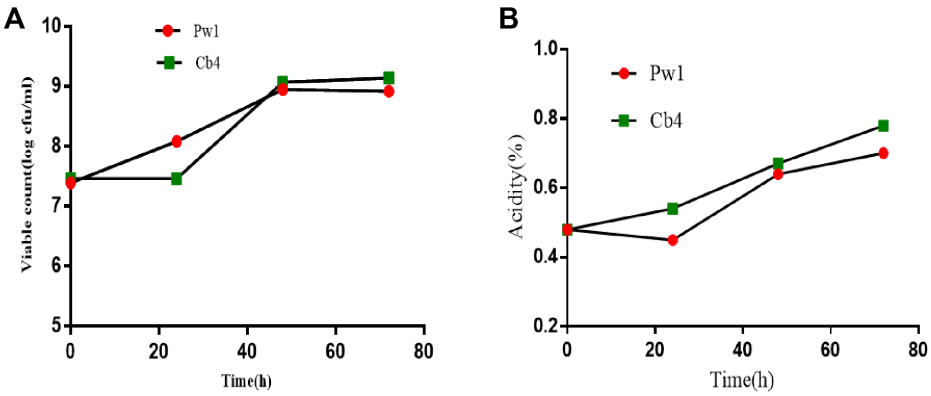

Fig. 4. Change in pH and acidity during papaya fermentation.

decreased from 5.9 after 0 h to approximately 4.3–4.25 after 48 h and then to 4 after 72 h (Fig. 4A). However, papaya fruit juice fermented by Pw1 displayed a different trend of pH changes, with the pH value decreasing significantly from 5.9 (after 0 h) to 4.50 (after 24 h) and then marginally decreasing to 3.8 (after 48 h) to 3.6 (after 72 h), which was significantly lower than Cb4 (Fig. 4A). After 0 to 72 h of fermentation, the change in pH observed in the two strains was inversely proportional to the change in viability (Fig. 4B).

Effect of cold storage

The effect of cold storage on the viability of Pw1 and Cb4 in papaya juice after 72 h of fermentation at 4℃ is depicted in Table 6. After four weeks of cold storage, the viability of both cultures in the fermented papaya fluids decreased, but all cultures remained between 106 and 108 cfu/ml. Pw1's viability was greater after 4 weeks (1.1107 cfu/ml) than Cb4's (1.490.06107 cfu/ml), despite Pw1's lower initial viability.

Table 5. Effect of storage at 4℃ for four weeks of two LAB Strains.

Time (weeks)	Survival (cfu/ml)	
	Pw1	Cb4
0	$2.3 \pm 0.35 \times 10^{9a}$	$1.65 \pm 0.21 \times 10^{9a}$
1	$6.65 \pm 0.21 \times 10^{8a}$	$6.3 \pm 0.14 \times 10^{8a}$
2	$5.53 \pm 0.07 \times 10^{7a}$	$4.8 \pm 0.03 \times 10^{8a}$
3	$2.5 \pm 0.14 \times 10^{7a}$	$3.1 \pm 0.02 \times 10^{8a}$
4	$1.25 \pm 0.21 \times 10^{7a}$	$1.49 \pm 0.06 \times 10^{7b}$

Values with different superscript in a row are significantly different ($p < 0.05$).

Discussion

In this study, LAB cultures were evaluated for their ability to reduce cholesterol and ferment papaya juice. Bennani et al. [28] reported that these isolates shared close similarities (colony morphology and Gram staining) with Lactic acid bacteria, as shown in Table 1. This indicates that of the eighty isolates on MRS agar, only ten (rods and cocci) were presumed to be LAB. Four of these ten isolates derived from oil palm sap and were designated Pw1, Pw2, Pw3, and Pw4, while the isolates derived from maize beer were designated Cb1, Cb2, Cb3, Cb4, Cb5, and Cb6. Traditionally fermented beverages such as palm wine and maize beer have been identified as suitable niches for numerous LAB [29, 30].

An essential functional characteristic of probiotics is cholesterol-lowering activity [31]. However, only two of the ten isolated strains, Pw1 from palm wine and Cb4 from maize beer, were able to assimilate more than 80 percent of the cholesterol in MRS medium. During growth, the primary mechanism involved in this assimilation is the absorption and co-precipitation of cholesterol into bacterial exopolysaccharides (EPSs). These EPSs can promote intestinal colonization by adhering to intestinal epithelial cells or by forming a protective occlusion [32, 33].

According to Gilliland et al. [34], only colonies grown in the presence of bile salts under aerobic conditions assimilated cholesterol. The degree of assimilation was found to be proportional to the oxgall concentration (0.1% to 0.3%). In this study, 0.3% bile salt and 1% cholesterol in MRS medium with a pH of 7.0 were utilized to

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determine the level of cholesterol assimilation by Pw1 and Cb4. The results indicated that both isolates are able to reduce cholesterol in vivo when pH values range from 6.5 to 7.0. Ishimwe et al. [35] demonstrated a substantial correlation between cholesterol absorption and bile salt concentration. Phylogenetic analysis revealed that these isolates were closely related to LAB species. Numerous investigations have identified these LAB from fermented foods. Tilahun et al. [36] assert that various species of fermenting bacteria isolated from fermented foods exhibit evolutionary relationships. For these isolates to be effective, they must be delivered to the intestines, where they are optimally adapted to reduce cholesterol.

For any *Lactobacillus* species to be considered a probiotic, it must be administered as a food supplement at a minimum dose of 10^6–10^9 cfu/ml per 100 ml of probiotic product at the time of consumption [37]. To determine the viability of Pw1 and Cb4 isolates, they were exposed to a low pH and bile salt concentration for three hours. These two isolates were able to withstand and surmount the gastric condition while maintaining a minimum viability of > 10^6 cfu/ml (Fig. 1), according to the obtained results.

Fossi et al. [38] also confirmed that LAB cultures isolated from palm wine can survive in the gut's stressful environment. The majority of scientific investigations [39, 40] have demonstrated that LAB cultures are sensitive to low pH and high bile salt concentrations. This could be the result of a high influx of H+ causing physiological stress, resulting in dissociation of the lipid bilayer, bacterial content leakage, and ultimately cell demise [41, 42]. Nonetheless, the increase in viability observed at pH 2.5 and 0.3% bile salt concentration (Fig. 2) suggests that these strains possessed a mechanism that allowed them to tolerate optimal growth in these severe conditions.

After four weeks of oral administration of Pw1 and Cb4 to rodents, a progressive weight gain was observed, indicating that despite the high cholesterol diet, the weight gain in groups A and B was negligible (Table 3). It has been discovered that feeding with lactobacillus isolates reduces body weight. Park et al. observed a comparable trend in both the test and control animals as a result of higher probiotic doses administered [43]. Low levels of lipids in the serum of rats confirmed the insignificant increase in weight gain. Lipids are organic molecules that are insoluble in water and therefore cannot be transported in aqueous solutions such as plasma. They are conveyed as lipoprotein macromolecular complexes. HDL-c and LDL-c are the two indicators that regulate the serum cholesterol level. HDL-c serves a crucial role in the RCT pathway by removing excess cholesterol from the serum [44], thereby reducing the CVD risk factor. LDL-c transports cholesterol to cells in the body, where it is utilized to synthesize hormones and is an integral component of the cell membrane.

It is therefore associated with an increased risk of atherosclerosis and coronary heart disease [45] and is the primary treatment target for lipid disorders [46]. This study found that oral administration of probiotics could ameliorate hypercholesterolemia brought on by a high-cholesterol diet. LDL-s and HDL-c have a greater correlation with CVD than basic lipid parameters; consequently, LDL-c/HDL-c are better predictors of CVDs than simple lipid parameters [47]. The LDL-c/HDL-c ratio was used to determine the treatment group with the most effective cholesterol-lowering properties. The higher HDL-c/LDL-c ratio observed with the Pw1 strain in comparison to the Cb4 strain revealed a greater capacity for plaque regression, thereby reducing the risk of CVDs across a broad range of cholesterol concentrations. In a previous study evaluating the cholesterol absorption properties of *L. plantar*um and L. pentosus in Wistar albino rats [20], comparable results were obtained. A similar study concluded that probiotic LAB (*L. plantarum* E.M.) enhanced metabolic activities and significantly decreased serum cholesterol in rodents [48]. To accomplish a significant level of cholesterol assimilation, Pw1 and Cb4 strains were able to hydrolyze conjugated bile acids, excrete them more quickly, and reduce the level to which they can be absorbed, thereby facilitating the excretion of cholesterol via feces [49]. In order to compensate for the loss of bile salts, this procedure increases the demand for cholesterol in de novo synthesis.

To improve the delivery of these probiotics to the recipient, they can be incorporated into various food matrices, such as dairy products, fruits, and vegetables [11]. Due to their high content of carbohydrates, polyphenols, vitamins, minerals, and dietary fibers, fruits have been shown to be excellent matrices for lactic acid fermenta-

tion [50]. In addition, they are abundant in aroma-enhancing precursors, such as phenolic compounds, amino acids, carbohydrates, and glycosides [51].

The ability of Pw1 and Cb4 strains to ferment papaya juice without nutrient supplementation or pH adjustment was examined during this investigation. The survival of both strains under varied conditions indicated that papaya juice served as a medium for the selected strains' sugar utilization. In a previous study [52], the trend of probiotic viability increased after fermenting papaya juice with *L. plantarum* and *L. acidophilus*. In addition, a previous study [53] also demonstrated similar outcomes during the fermentation of papaya juice with strains of lactobacilli, including *E. faecium*.

Since fermented papaya juice is perishable, it must be stored at low temperatures to prolong its expiration life. Nonetheless, studies have demonstrated that, similar to low pH, low temperatures can impair the viability of probiotic microbes in fermented papaya juice [54]. In this investigation, fermented papaya juice was stored at 4 degrees Celsius for four weeks in order to assess the viability of the strains for maximum health benefits. The obtained result confirmed the claim that low temperatures influence the viability of probiotics. Despite a slight decline in the viability of Pw1 and Cb4 strains, acceptable levels were determined (Table 6) for optimal health benefits. This study's results are comparable to those of previous studies [55, 56] that found viable cell proliferation to be between 10^6 and 10^8 cfu/ml after four weeks of cold storage.

The isolate Pw1 was closely related to the *Limosilactobacillus fermentum* strain, and the isolate Cb4 was closely related to the *Enterococcus faecium* species. strain significantly decreased serum cholesterol in rodent sera. Incorporating them in a food matrix to enhance protection against stressful gastric conditions prior to oral administration can increase their efficacy in the gut, despite the fact that they were effective at lowering cholesterol. After 72 h of fermentation in papaya juice and four weeks of refrigerated storage, they remained viable. Before it can be approved for human clinical trials, however, it must be determined whether papaya juice fermented with these two strains can improve strain viability to substantially lower serum cholesterol when administered orally to albino rats.

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Author's Contribution

BTF conceived the study, oversaw the investigation, and edited the manuscript. DEE was responsible for the isolation, probiotic evaluation assays, molecular characterization, statistical analysis, in vivo investigations, and manuscript writing. LLTT and RBA contributed to the sample collection, manuscript editing, and discussion. FT and PMJ supervised the project. All authors perused and approved the submission of the final manuscript.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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