Probiotic lactic acid bacteria isolated from traditional cameroonian palm wine and corn beer exhibiting cholesterol lowering activity

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ABSTRACT

High serum cholesterol is a risk factor for cardiovascular disease (CVD), leading to many fatalities. Traditional palm wine (Elaeis guineensis) and corn beer are noted as potential sources of probiotic bacteria with cholesterol lowering activity. In this study, we isolated and characterised lactic acid bacteria (LAB) with probiotic and cholesterol lowering properties from these sources. The isolates were identified using API 50 CHL kit and sequencing of 16 S rRNA gene. A hypercholesterolemia model involving three groups of eighteen diet-induced Wistar albino rats was established, and the serum lipid profile was analysed using biochemical kits. The genotypic identification of two best cholesterol lowering LAB revealed that they were strains of Levlactobacillus brevis and Enterococcus faecium. Their 16 S r RNA gene sequences were deposited in NCBI genbank with the accession numbers ON454506 and ON908682 respectively. These isolates effectively lowered LDL-c and increased HDL-c in rat sera, which are the main risk factors for CVD. These probiotics are potential candidates for functional foods formulation against hypercholesterolemia.

1. Introduction

Cholesterol is a naturally occurring substance in the human body that enhances cell wall biosynthesis and hormone production. The liver and the intestinal mucosa are capable of synthesising about 80% of de novo (endogenous) cholesterol needed in the body (Yang et al., 2020), requiring a small amount (20%) of dietary (exogenous) cholesterol. However, when dietary cholesterol becomes higher, it can lead to a condition known as hypercholesterolemia (elevated blood cholesterol). This causes the accumulation of cholesterol deposits in blood vessels, leading to blockage or narrowing of arteries which supply blood to vital organs like the heart and brain. Hypercholesterolemia is the major risk factor for cardiovascular diseases (CVD) (Zhong et al., 2019). With a prevailing death toll of nearly 23.6 million worldwide, CDV will most certainly become the prominent cause of death by 2030 (Ahire et al., 2012). More than 80% of CDV deaths occur in low and middle-income countries, affecting men and women at the same rates (Organization, 2017). A recent study carried out in Cameroon showed that deaths related to CDV (in-hospital case-fatality) were 15.8% (Nkoke et al., 2019).

The use of low-fat diet so far is the most effective means of lowering elevated serum cholesterol (Harcombe et al., 2016). This remedy however has limitations due to the unavailability of such diets and compliancy of the consumer (Janapala and Reddivari, 2020). Probiotic bacteria appear as one of the promising alternatives. They are living microorganisms that confer a health benefit to the host when administered in adequate amount (Gibson et al., 2017). Lactic acid bacteria (LAB) remain the most common types of microbes used as probiotics, although certain yeasts are also used (Lv et al., 2014; Mingmongkolchai and Panbangred, 2018).

Fermented drinks like palm wine and corn beer have been noted as potential niches that harbor a rich consortium of microorganisms due to the presence of simple sugars used as a substrate for growth. A study carried out by Chandrasekhar et al. (2012) and Parveens and Hafiz (2017).
(Parveen and Hafiz, 2003), showed that LAB are among the most predominant microorganisms in African traditionally fermented drinks. Palm wine and corn beer are widely consumed beverages in Africa and Cameroon in particular due to their low cost and ready availability. In addition to this extensive consumption, there is very limited cognisance of the inherent health benefits of the microorganisms that are predominant in these beverages (Ezeronye and Legras, 2009). The cholesterol assimilation property among others stands out as one of the most significant functional properties of LAB which can be used to lower cholesterol levels in hypercholesterolaemia patients. For probiotic LAB to efficiently carry out their health benefits including cholesterol assimilation it should be viable at a high concentration, at least \(10^6\) cfu/mL and supplementation with papaya juice is of vital importance (Priya et al., 2021). For the cultivation of probiotics bacteria, fruit juices serve as the best medium because the fruit juices contain potassium salt, bioflavonoids and vitamins etc, and they also have good results in prophylaxis and even in treating cardiovascular diseases (Mattila-Sandholm et al., 2002). The development and consumption of functional beverages has increased in recent years mainly due to the conscious importance of maintaining health and the over-demanding working schedules (Routray and Orsat, 2019).

However, little is known about the cholesterol lowering efficacy of probiotic LAB from locally harvested palm wine and traditionally processed corn beer in Cameroon. In this study, we isolated and characterised LAB with probiotic properties and their ability to lower cholesterol.

2. Materials and methods

2.1. Sample collection

Sap from African oil palm (Elaeis guineensis) was procured from palm wine tappers. Sap collection was done following the destructive method described by Onuche et al. (2012). Briefly, the tree was cut down and a cavity created by digging into the soft meristem of the tree trunk. A tube was inserted to make way for sap collection in a clean plastic bottle. Corn beer was collected with the use of a sterile plastic bottle from corn beer vendors. African oil palm and maize plant were identified at the National Herbarium in Yaounde. The identification was carried out through comparison with the botanic collection of M. Brut N° 379 for African oil palm tree, recorded at the National Herbarium N° 34163/HNC, while corn beer plant was identified through comparison with the botanic collection of D. Dang N° 81 also recorded at the National Herbarium under No 18625/SRF/Cam.

All samples were collected in Buea and immediately transported in ice-cool boxes (4°C) to the University of Buea Life Science Laboratory and allowed for 24 h to undergo fermentation at ambient temperature (21–30°C) before processing.

2.2. Isolation of lactic acid bacteria (LAB)

Media preparation was performed following the manufacturer’s instructions. Tenfold serial dilution was made by transferring 1 ml of each sample into 9 ml of peptone water. The pour plate method was used to enumerate bacteria cells (CFU/ml). Then, 0.1 ml from each dilution was transferred into sterile Petri dishes and covered with molten agar. The plates were incubated at 37°C for 24 h. Repeated sub-culture by streaking on MRS agar was carried out to obtain pure colonies. Pure colonies were labelled with codes, with Pw for palm wine LAB, and Cb for corn beer LAB and Arabic numbers attributed starting from 1. Preliminary identification involving colony morphology, Gram staining, and catalase tests were performed.

2.3. Probiotic properties of LAB

The major selection criteria used to determine the probiotic properties of LAB isolates were tolerance to low pH, bile salt tolerance, and in vitro cholesterol assimilation activity. All tests were performed in triplicates and the number of viable colonies on MRS agar plates was counted.

2.3.1. Tolerance to acid pH values

Acid tolerance was evaluated following the method described by Guan et al. (2017) with slight modifications. Overnight cultures of bacteria cells were washed three times with PBS (pH 7.0) to remove impurities and centrifuged (Eppendorf centrifuge 5810 R, New York, USA) for 10 min at 4°C at 5,000 rpm. The cell pellets were re-suspended in MRS broth adjusted to pH 2.0 and pH 3.0 (HI991001, Woonsocket, USA) using 3N HCl or NaOH. The cultures were then incubated anaerobically at 37°C for 24 h. Aliquots taken 3 h were enumerated by pour plate counts of all samples using 10-fold serial dilutions prepared in 0.1% peptone water. Viable microorganisms were enumerated in triplicates on MRS agar. Samples taken at 0 h were used as the control. Isolates that showed final counts \( \geq 10^7 \) CFU/ml or \( \geq 10^6 \) CFU/ml at low pH for 3 h, were considered to have moderate or good resistance.

2.3.2. Tolerance to bile salts

The bile salt tolerance of selected isolates was determined by the method described by Argyri et al. (2013) with minor modifications. Overnight cultures of bacteria cells were washed three times with PBS (pH 7.0) to remove impurities and centrifuged (5,000 rpm for 10 min at 4°C). The cell pellets were re-suspended in MRS broth containing 0.2 and 0.4 % oxgall bile salts (sigma Aldrich, Germany). The cultures were then incubated anaerobically at 37°C for 24 h. Aliquots were taken after 0 h and 3 h were enumerated by pour plate counts of all samples using 10-fold serial dilutions prepared in 0.1% peptone water. Samples taken at 0 h were used as the control. Tolerance to bile salts was assessed based on viable colony counts on MRS agar in triplicates after incubation at 37°C for 0 and 3 h, reflecting the average period spent by food in the small intestine.

2.3.3. Cholesterol assimilation from culture media

Based on the acid and bile tolerance of the selected strains, the ability of each strain to assimilate cholesterol in vitro was determined by a modified method described by Pereira and Gibson (Pereira and Gibson, 2002). Bacteria strains were inoculated into tubes, each containing 10 ml of MRS broth, 0.4% bile salts, and 1% acid solution of cholesterol (Sigma-Aldrich, cat #C3045-5G, Germany). The cultures were incubated at 37°C for 24 h. After incubation, the cultures were centrifuged (5,000 rpm for 10 min at 4°C) and the unutilised cholesterol estimated in the supernatant. This was carried out by spectrophotometry (Pharmacia LKB, England) at 540 nm and compared to the control as described by (Guan et al., 2017). The ability of each isolate to reduce the amount of cholesterol in culture media was determined by the following equation (Eq. 1):

\[
\% \text{ of cholesterol reducing rate} = \left( \frac{C_0 - C}{C_0} \right) \times 100
\]  

where \(C_0\) is the control broth and \(C\) is fermented broth.

Isolates having in vitro cholesterol assimilation properties were selected for biochemical identification using API 50 CHL assay.

2.4. Identification of LAB isolates

2.4.1. Phenotypic identification of LAB isolates using API 50 CHL kit

Phenotypic identification of LAB isolates was performed by API 50 CHL (API kit, bioMerieux, France) assay. Purified LAB cultures were cultivated in 20 ml MRS broth incubated at 37°C overnight, after which they were washed and re-suspended in API® 50 CHL medium (bio-Merieux®SA, 69280, France). The turbidity of the suspensions was determined by the McFarland method according to the instructions provided by the manufacturer. Cell suspensions were inoculated into API 50 CHL strip wells and overlaid with paraffin oil to create an
an anaerobic condition. The strips were incubated at 37 °C. The results were recorded after 24 h and confirmed after 48 h. A positive reaction was indicated by a yellow colour except for the esculine test (black). Colour reactions were scored against a chart provided by the manufacturer (De Angelis et al., 2001). The results were analysed with API WEB (bioMerieux) database version 5.0.

2.4.2. Genotypic identification of LAB isolates using 16 S rRNA gene sequencing

Genomic extraction of the two strains of LAB was determined following the method (Mulaw et al., 2019) with some slight modifications.

The genomic DNA was extracted from pure cultures of isolate Pw4 and Cb5. One ml of each pure liquid culture was centrifuged at 11,500 rpm for 10 min at 25 °C (Eppendorf centrifuge 5810 R, New York, USA). The supernatant was decanted and the cell pellets re-suspended into a tube containing 300 μl buffer (10mM Tris-HCl, pH 8.0; 50mM glucose, and 10mM EDTA) and 3 μl lysozyme (10 mg/ml). The pellets were lysed at 37 °C for 60 min and vortexed every 5 min, followed by placing in ice every 5 min. Threefold (300) μl of lysis buffer and 3 μl RNase were added to the mixture and incubated for 30 min and cooled on ice for 1 min. Then, 100 μl of 7.5 M solution of sodium acetate was added and vortexed for 25 s and centrifuged (Eppendorf centrifuge 5810 R, New York, USA) at 13,000 rpm for 10 min at 4 °C. The supernatant was transferred into a sterile tube, and 300 μl isopropanol was added and mixed gently. The resulting mixture was centrifuged at 13,000 rpm for 10 min at 4 °C (Eppendorf centrifuge 5810 R, New York, USA). Isopropanol was carefully removed by the use of a sterile Eppendorf pipette without dislocating the DNA pellets. The tubes were air-dried by inverting them on sterile filter paper. The DNA was washed by adding 400 μl of 70% ethanol and centrifuged at 5,000 rpm for 2 min at ambient temperature. The sediments were dried at 37 °C for 10 min and finally dissolved in 30 μl TE buffer and stored at -20 °C for further study.

The 16S rRNA coding region sequence was selected and amplified by PCR using the universal primers-forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-ACGGCTACCTTGTTAACGACTT-3') (STR CSF1P0F). The PCR conditions for the 30 cycles were as follows: 95 °C for 5 min (initial denaturation), 94 °C for min 30 s (denaturation), 42 °C for 1 min 30 s (annealing), 72 °C for 1 min 30 s (extension) 72 °C for 10 min (and final extension). The PCR amplicons were examined by gel electrophoresis (1%w/v).

Two μl of each amplification mixture was subjected to electrophoresis in 1.5% (w/v) agarose gels in 0.5 x TAE buffer and stained with ethidium bromide, washed, and photographed with UV transilluminator (Bio-Rad, Hercules, CA, USA). The partial 16S rRNA sequence analysis of the PCR products was sequenced by inqaba biotech, South Africa. The sequences obtained were compared using BLAST (basic local alignment search tool) and submitted to the GenBank sequence database to access their percentage similarity with other LAB sp. (Boubezari et al., 2018). Phylogenetic analysis was performed using MEGA 10 software in order to evaluate the evolutionary relationship of isolates Pw4 and Cb5 and their close relations. Isolate Pw4 which presented the closest relationship with other LAB sp. was used to check the stability of the isolate.

2.5. In vivo assessment of probiotic effect on the serum lipid profile

2.5.1. Animal feeding and experimental design

Eighteen Wistar albino rats (Rattus norvegicus), ranging between 10-12 weeks and weighing between 90-120 g were purchased from the animal house of the Department of Animal Biology, University of Dschang, Cameroon. All animals were handled according to the institutional guidelines defined by the University of Buea Institutional Animal Care and use Committee (UB-IACUC n°. 015/2019). Feed composition and animal grouping were carried out by the method described by Ngongang et al. [18]. The hyperlipidemic diet consisted of about 85% basal diet, 1% cholesterol and 10% lard (pig fat). The rats were randomly allocated into the following three groups (A) fed with hyperlipidemic diet and bacteria isolate (Pw4) (NC); the negative control group, fed with basal diet + oral gavage of deionized water and (PC); the positive control group, fed with hyperlipidemic diet and oral gavage of deionized water.

2.5.2. Animal studies

All animals were handled according to the institutional guidelines defined by the University of Buea Institutional Animal Care and use Committee (ethical clearance permit number: UB-IACUC n°. 015/2019). The animals were housed under standard conditions with a 12 h light and 12 h dark cycle. The temperature was maintained at 23 ± 2 °C and relative humidity at approximately 50 %. The rats were acclimatised for seven days before the start of the experiment. The animals were housed in metabolic cages and given a standard diet containing water daily throughout the study. The experiment was conducted over 4 weeks with oral gavages of 10^8 cfu/ml of bacteria isolate (Pw4) at a volume of 1.0 ml/kg body weight/day for each dose. The quantity of food consumed and the animal’s weight were monitored daily.

2.5.3. Blood collection and biochemical analysis

At the end of 30 days of feeding, the rats were allowed to fast overnight (12 h) and on the 31st day, they were anesthetised and blood was collected by cardiac puncture from the left ventricle by the use of a heparinised syringe into sterile non-anticoagulant tubes. The tubes were allowed to stand at room temperature for 1 h and then centrifuged at 3,000 rpm at 4 °C for 10 min to obtain serum. The sera were stored at -20 °C for further biochemical analysis.

Total cholesterol (Tc), high-density lipoprotein cholesterol (HDL-c), and triglyceric aldehyde (TG) levels in serum were measured using Tc, HDL-c, and TG assay kits (CHRONOLAB SYSTEMS in Barcelona, Spain) respectively, following manufacturer’s instruction. Very low density lipoprotein cholesterol (VLDL-c) and low density lipoprotein cholesterol (LDL-c) were measured using the method described by Friedewald et al. (1972) as follows (Eqs. (2) and (3)):}

\[ VLDL_c = \frac{TG}{5} \]  
\[ LDL_c = \frac{TC - HDL_c - \frac{TG}{5}}{ } \]  

2.6. Statistical analysis

All the tests were performed in triplicate, and the results were expressed as mean ± standard deviation. Data were analysed by the one-way ANOVA plus post hoc Duncan’s test by Statistical Package for Social Scientist (SPSS) version 20.0. Statistical significance was determined at p < 0.05. The phylogenetic trees were constructed using MEGA10 (version 10.0).

3. Results

3.1. Isolation of lactic acid bacteria

A total of 50 catalase negative and gram positive bacteria isolates were obtained on MRS agar. They were all considered as presumptive lactic LAB. Preliminary identification of colonies carried out the basis of cell morphology, microscopic examination and biochemical tests revealed smooth, oval, and cream white colonies on MRS agar plate.
Upon microscopic observation, rod-shaped isolates were predominant (Table 1).

### 3.2. Probiotic properties of LAB isolates

#### 3.2.1. Resistance to acid

Figure 1 (a) shows the results for the isolate the resistance to acid was important. The viable counts of all isolates ranged from 3.9-7 log CFU/ml after 3 h of exposure at pH 2.0. This ability of the strains to survive the acidic pH value after 3 h of incubation at 37 °C is presented in Figure 1a and 1b. Isolates Pw1, Pw4, Cb1, Cb3, Cb4, Cb5 and Cb6 had viable counts greater than 6 log CFU/ml, which was significantly different (p < 0.05) from the control (pH2.0 at 3 h). On the contrary, isolates Pw2, Pw3 and Cb2 had viability below 5 log CFU/ml which was not significantly different (p > 0.05) when compared to the control. However, when the pH value was increased to 3.0 for 3 h, the viability of all isolates increased in the range of 4.3–7 log CFU/ml. The viable counts of isolates Pw1, Pw3, Pw4, Cb1, Cb3, Cb4, Cb5, Cb6 exceeded 6 log CFU/ml (p < 0.05 at pH3.0 for 3 h). Contrariwise, the viability of isolates Pw2 and Cb2 fell below 5 log CFU/ml (p > 0.05). In total, isolate Pw1, Pw4, Cb1, Cb3, Cb4, Cb5 and Cb6 maintained a high viable count despite the change in pH values.

#### 3.2.2. Resistance to bile salt

The viability of all the isolates ranged from 3.9-7.5 log CFU/ml after 3 h of exposure to 0.2 % bile salt concentration. The viable count of isolate Pw1, Pw4, Cb1, Cb2, Cb4, Cb5 and Cb6 was higher than 6 log CFU/ml which was significantly different from the control (p < 0.05) Figure 1 (b). On the other hand, isolates Pw2, Pw3 and Cb3 had viability below 4.5 log CFU/ml. When the bile salt concentration was increased to 0.4 % after 3 h, the viability of all the isolates fell in the range of 3.1–7 log CFU/ml. The viability of Pw2, Pw4, Cb1, Cb4, Cb5 and Cb6 were greater than 6 log CFU/ml which were not significantly greater than the control (p > 0.5). On the contrary, isolates Pw1, Pw3, Cb2 and Cb3 had viable counts below 5 log CFU/ml which were significantly different from the control (p < 0.5).

In total, isolates Pw4, Cb1, Cb4, Cb5 and Cb6 maintained good viability irrespective of the change in bile salt concentration. This ability of the strains to survive the bile salt concentrations after 3 h of incubation at 37 °C is presented in Figure 2a and 2b.

#### 3.2.3. Cholesterol assimilation from culture media

The amount of cholesterol assimilated in-vitro ranged from 28 – 89 % after 24 h of incubation in the presence of bile salt (Figure 3). Seven strains assimilated cholesterol at a variable extent with Pw1, Pw4, Cb4 and Cb5 which displayed excellent assimilation property (>75%). Contrariwise, Pw3 and Cb3 had poor cholesterol (<45%) uptake and did not grow well in the medium. Strains Pw4 and Cb5 were selected for further studies.

![Figure 1](image1.png)

**Figure 1.** Effect of pH on the growth of ten lactic acid bacteria strains. Colony forming units were counted at 0 and 3 h at (A) pH 2, and (B) pH 3. Data are expressed as mean ± standard deviation. * Significant differences at p < 0.05.

### 3.3. Identification of LAB isolates

#### 3.3.1. Phenotypic identification of LAB isolates using API 50 CHL kit assay

Results from API 50 CH test kits and API web identified the two LAB isolates (Pw4) from palm wine *Lactobacillus plantarum* with similarity 99.6 % and (Cb5) from corn beer *Lactobacillus pentosus* 99.5% (Table 2). There was an insignificant variation in the utilisation of carbohydrates sources of the API CHL 50 kit by Pw4 and Cb5 isolates (Table 3).

#### 3.3.2. Genotypic identification of LAB isolates

The genotypic identification using universal primer showed clear bands of isolates on agarose gel with approximate molecular weight 1,500 bp, with the following nucleotide sequence:

```
Pw4
ARCCATATACATGCAAGTCAAGGGAAGGTTCCCGYG-
TRAAATTGACGTTCGACTGTAATTTAATTACAGAGGGAKTGG
CGAAGATTGATGAACACGGTTAGAAACATCGCAGAACAGCAG
GGGATACCACTTGGAAAGGTTGTTATATCTGGTTTGAAG
TCTTGATGATCAGCCGGCGGGGCTATAGCTAGTTGAGTGAAG
GCCGACCAGAGAGAAGTGATACGTAAGCCGAGCGAGAGTAAAT
CGGCACTATTGGAGCTAGACGAGCAGGCCAACACTCTACGGGA
GCCGAGCTAGAGCAACTTCCCAACATGAGCAAGAAAGTCTGAG
GACGAGCGCGCGACTGAAGGAACTTTGACGAAGAGAAGAGAG
CTCTGTTTATATAGGACAACTTTGACGACTTAGTGTTCAAG
GGTGTGACGGATTTATACGAGAGAAGCAGGCAGGCGGTAACG
CACGAGCGCGGTACTAAGCTTAGTGTTGCAAGCTGTTGGATT
TTTGGGACTAAAGCAGCAGCGGGGCTCTTTAGTGGTCAGT
GAAGATGGGTTCCGTTAAACCCGGAGAGAGCAGACGGTTGTAA
GACGGTATGCGAGAGACGAGAGACGAGACGAGACGAGACTG
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Results obtained using BLAST identification and MEGA 10 software revealed close similarity of 97.4% of Pw4 to Levl actobacillus brevis strain ATCC. The 16S rRNA sequence was deposited in NCBI genbank, and the accession number obtained was ON454506. For the isolate Cb5, similarity of 99.54% to Enteroccocus faecium strain NBRC was observed and after submission to NCBI genbank the accession was ON908682 (Table 4). Phylogenetic trees of these selected isolates based on 16S rRNA gene sequences are presented in Figures 4a and 4b.

3.4. Feed consumption and body weight of rats

After week 1 of the treatment trial, there was no significant difference in feed consumption in group A (fed with isolate Pw4), when compared to the positive control (PC) fed with hyperlipidemic diet. However, there was a surge in weekly weight gain in PC (17.64±0.04 g), when compared to the group A. After week 2 of the feeding trial, the quantity of food consumed by the PC group increased significantly (15.47±0.08 g), while group A remained at comparatively low level (12.94±0.05 g). This increase in feed consumption in the PC group was proportionate to the increase in weekly weight gain (22.83±0.02 g). Feed consumption and weekly weight gain were consistently higher in the PC group after weeks 3 and 4 of the feeding trial when compared to the group A and NC.

3.5. Assessment of serum lipid parameters after four weeks of feeding

The Tc levels in PC group was significantly higher (192.95±3.97 mg/dL) than that of the other groups (Table 5) after 4 weeks treatment. The test group (A) fed with isolate Pw4 recorded a Tc value of 159.66±7.49 mg/dL while 125.79±5.62 mg/dL was found in the serum of the NC group. The order of Tc values decreased from PC > A > B.

Table 2. Phenotypic Identification of lactic acid bacteria isolates.

| Isolate | Source            | Identification       | Similarity index % |
|---------|-------------------|----------------------|-------------------|
| Pw4     | Palm wine         | L. plantarum         | 99.6%             |
| Cb5     | Corn beer         | L. pentosus          | 99.5%             |

Isolates

GACATCTCTGCAATATTTAGATAGATGGGTCCTCCCTTTG
GACAGATGACAAATGATCAGTGGATTTGCTGAGCTGTTG
GTGARAGAATACGTTGTTTATAGTCCCGACCCAGACCCAGG
AATAACTAGTCAGCATATTGCTTGGGCGGCAATTACAYG

Cb5
CGGCAGGGGGGGCGCTTAATGCAATCCGAGTCAGMCT-
GGAGGGCGGAAACCCCATCAGATGCATATGGTCCCT
TGCGATGTTCTCAGCTCCATCTGCACTCAGCTCCATC
CACTGTGAGTGGTTGGTTAGTTCCCGCACCGAGCGCAC
AATAATCAGTGCAGCATTTCAGTTGGGGGCAATACAYG

Figure 2. Effect of bile salt concentration on the growth of ten lactic acid bacteria strains. Colony forming units were counted at 0 and 3 h at different bile salt concentration (A) 0.2%, and (B) 0.4%. Data are expressed as mean ± standard deviation. * Significant differences at p < 0.05.

Figure 3. Cholesterol assimilation by ten LAB strains at 1% cholesterol and bile salt concentration of 0.4% for 24 h. Data are expressed as mean ± standard deviation. Values with single letters assimilated cholesterol >75%.

Figure 4a.

Figure 4b.
Carbohydrates fermentation by lactic acid bacteria isolates using API 50 CHL kit.

| Strain code | xylose | xylose | Ribose | Salicin | control | Inositol | Arbutin | Sorbose | Dulcitol | Sorbitol | esculine | xyloside | Fructose | Glycerol | Adonitol | Erythritol | Ribose | Ribose | Ribose | Ribose | Mannose | Rhamnose | L-D-Xylitol | Xylitol | Xylitol | Xylitol | Gentiobiose | d-Mannose | d-Rhamnose | Pw4 | Pw4 | Pw4 | Pw4 | Pw4 | Pw4 |
|-------------|-------|-------|--------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|-----------|--------|--------|--------|--------|---------|---------|----------------|--------|--------|--------|--------------|----------|-----------|-----|-----|-----|-----|-----|-----|
| Test sample | +     | +     |        |         |         |         |         |         |         |         |         |         |         |         |         |           |          |          |          |          |          |          |                |        |        |        |              |          |           |     |     |     |     |     |     |
| Isolate     |        |        |        |         |         |         |         |         |         |         |         |         |         |         |         |           |          |          |          |          |          |          |                |        |        |        |              |          |           |     |     |     |     |     |     |

NC. The TG levels in serum were higher (p < 0.5) in the PC group when compared to the test groups A and the NC groups in the order of 210.42 ± 4.17 mg/dL, and 87.65 ± 15.74 mg/dL, respectively. The LDL-c level was significantly lower in groups A and NC (87.71 ± 6.49 mg/dL, and 73.74 ± 11.30 mg/dL) compared to PC (126.19 ± 7.76 mg/dL). There was no significant increase in VLDL-c levels in A and NC groups (28.60 ± 0.83 mg/dL, and 16.63 ± 3.96 mg/dL) when compared to the PC (42.08 ± 1.88 mg/dL). The HDL-c/LDL-c ratio in the A and NC groups were significantly higher than the PC group with the test group (A) which recorded the highest value (0.85 ± 0.09).

4. Discussion

Locally harvested palm wine and traditionally processed corn beer have been exploited as suitable sources for screening various LAB (Fossi et al., 2016). In Cameroon, palm wine and corn beer are widely consumed with little or no knowledge of the presence of a predominant LAB population (Ezeronye and Legras, 2009). The isolates obtained in this our study had morphological features similar to LAB as reported by Bennani et al. (2017). Four of these isolates were sampled from palm wine coded as Pw1, Pw2, Pw3, and Pw4 and six from corn beer coded Cb1, Cb2, Cb3, Cb4, Cb5 and Cb6. This implies that LAB cultures were predominant in corn beer when compared to palm wine. Fresh palm wine has been reported to have a rich population of microbial load than corn beer (Nkemnas & Lois). This causes rapid bioconversion of sugar present in palm wine to alcohol during fermentation. However, in this study, LAB cultures were isolated more (60%) from corn beer than from palm wine (40%). This indicated that a high microbial population is not synonymous to a high LAB presence. The finding in this study concurs with that of Nwachukwu et al. (2010) who successfully isolated LAB from fermented samples.

Probiotic LAB have been shown to have inherent health benefits and approved for human consumption primarily because they have acquired a status of GRAS (Generally Regarded as Safe) and QPS (Qualified Prescription of Safety) by the American Food and Drug Agency (FDA) and the European Food Safety Authority (EFSA), respectively (Hazards et al., 2020). Cholesterol assimilation is one of such property exploited over the years. Recent studies have proven that probiotic LAB are able to assimilate cholesterol to a significant margin (Mo et al., 2019; Wang et al., 2018). Gilliland et al. (1984) were the first to show that in-vitro efficiency of lactobacilli could be directly associated with their ability to assimilate cholesterol. As a result, in-vitro removal of cholesterol by lactobacilli has been consistently used as a screening tool for the selection of probiotic strain. In this study, in vitro cholesterol assimilation assay was performed to assess the cholesterol assimilation property of ten LAB.

The results obtained (Figure 3) showed that two isolates were able to assimilate cholesterol in the culture media to a significant margin. Similar studies have been carried out to assess the cholesterol lowering ability of LAB in culture media. Anila et al. (2016) and Malakar et al. (2017) revealed that L. brevis PLA 7, L. brevis PLA 14 and Enterococcus faecium BASTUS 4 and 5 respectively isolated from fermented beverages were able to assimilate cholesterol significantly in-vitro. Cell wall binding and incorporation of cholesterol within their phospholipid layer has been reported as a possible mechanism for cholesterol assimilation (Le and Liong, 2010). However, Ramasamy (Ramasamy et al., 2012), highlighted that bile salt hydrolytic activity (BSH) of probiotics stands as one of the

| Isolate | Source | Identification | Similarity index % | Accession no. |
|---------|--------|----------------|-------------------|---------------|
| Pw4     | Palm wine | Levilactobacillus brevis strain | ATCC | 97.4% | ON454506 |
| Cb5     | Corn beer | Enterococcus faecium strain | NBRC | 99.54% | ON908682 |

Table 4. Genotypic Identification of lactic acid bacteria.
Figure 4. Phylogenetic trees constructed showing the position of isolate Pw4 with related *Levilactobacillus* species (a) and Cb5 with related *Enterococcus* species (b).
most significant mechanisms for cholesterol removal and has been proposed by many researchers as a prerequisite for probiotic selection. Evaluating the in vitro cholesterol assimilation was significant to extrapolate a similar trend during in vivo studies.

In spite of a high cholesterol assimilation ability of LAB, they must overcome the stressful conditions of acid (stomach pH) and bile (duodenum) before initiating their health benefit (cholesterol lowering effect) in the ileum. Acid and bile tolerance is therefore regarded as a prerequisite for any LAB to be considered as a probiotic (Palachum et al., 2018). The stomach pH can be as low as 1.5 (without food), inhibiting metabolic activities thereby retarding the viability of *Lactobacillus* sp. The transition of this acidic medium (3 h) is vital for probiotics to initiate their beneficial effects on the distal part of the gut (Yavuzdurmaz, 2007). Similarly, the physiological concentrations of human bile salts range from 0.3 to 0.5% (Mainville et al., 2005). Beyond this range, cellular homeostasis is compromised causing the dissociation of the lipid bilayer and integral protein of their cell membranes, resulting in bacterial content leakage and eventual cell death (Mandal et al., 2006). In this study, the seven strains that tolerated the low pH and bile salt concentrations as seen in Figures 1 and 2 respectively were considered as acid and bile salt tolerant.

The isolates (Pw4 and Cb5) with the best cholesterol lowering ability were then subjected to biochemical identification using API 50 CHL kit and confirmed using molecular characterization. The result obtained showed that conventional phenotypic methods are insufficient as a standalone technique for the characterization of LAB. Reliability can be achieved by the use of molecular techniques since the 16S rRNA gene is found to be a powerful tool for appreciating genetic variability among different species (La Scola et al., 2003). This method allows for the design and use of universal primers to discover and classify organisms into a wide range of taxa. Mulaw et al. (2019) and Dowarah et al. (2018) have revealed the strain level identification of diverse LAB with potent probiotic properties isolated from traditionally fermented foods and other substrates using phylogenetic estimation of 16S rDNA genes.

Previous studies have revealed that in vitro studies alone are insufficient to draw any conclusion on the cholesterol reducing ability of most LAB. For this reason, isolate Pw4 (with the best cholesterol reducing ability) was subjected to in vivo studies to evaluate its effect on the serum cholesterol. Such in vivo studies have revealed that the micro biome has significant mechanisms for cholesterol removal and has been proposed by many researchers as a prerequisite for probiotic selection. Evaluating the in vitro cholesterol assimilation was significant to extrapolate a similar trend during in vivo studies.

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Previous studies have revealed that in vitro studies alone are insufficient to draw any conclusion on the cholesterol reducing ability of most LAB. For this reason, isolate Pw4 (with the best cholesterol reducing ability) was subjected to in vivo studies to evaluate its effect on the serum cholesterol. Such in vivo studies have revealed that the micro biome has an enlarged capacity to break down stored excess energy which when accumulated can cause weight gain (Turnbaugh et al., 2006). This explains the reason for the corresponding increase in weight gain observed after the fourth week of feeding trial. However, the presence of LDL-c in the rat sera was found below average indicating low risk for CVD. HDL-c and LDL-c are the two main lipoproteins whose concentration in blood influences serum cholesterol. The former reverses excess amount of cholesterol to the liver for degradation (Xenoulis and Steiner, 2010), thereby preventing the accumulation of cholesterol in blood. It is therefore considered as ‘good cholesterol’ (Kontush and Chapman, 2006). LDL-c on the other hand delivers cholesterol to cells, where it is used in membranes, and the synthesis of steroid hormones. However, if LDL-c amount exceeds 160 mg/dL, it can be associated with increased risk of CVD (Carmena et al., 2004), and stands as the main target for the treatment of lipid disorders (Liu et al., 2020; Zeitouni et al., 2021). The result obtained in this study indicated that oral administration of Pw4 lowered serum cholesterol caused by a high cholesterol diet. The LDL-s and HDL-c have a greater correlation with CVD, as a result, the LDL-c/HDL-c are therefore better predictors of CVDs than simple lipid parameters (Jeon et al., 2019). The highest HDL-c/LDL-c ratio observed in isolate Pw4 indicated low cholesterol concentration in the serum, which reduces plaque formation in the walls of the arteries, thereby reducing the risk of CVD. Jeon et al. (2019) also confirmed that probiotic LAB (*L. plantarum E.M*) showed a significant hypocholesterolemic effect and metabolism improvement in rats.

### Table 5. Assessment of serum lipid parameters after four weeks of feeding.

| Parameters | A (Pw4) | NC (Negative control) | PC (Positive control) |
|------------|---------|------------------------|------------------------|
| Tc (mg/dL) | 159.66 ± 7.49<sup>a</sup> | 125.79 ± 5.62<sup>b</sup> | 192.95 ± 3.97<sup>b</sup> |
| TG (mg/dL) | 147.28 ± 4.17<sup>b</sup> | 87.65 ± 15.74<sup>b</sup> | 210.42 ± 9.40<sup>b</sup> |
| HDL-c (mg/dL) | 43.40 ± 6.17<sup>b</sup> | 35.42 ± 9.60<sup>b</sup> | 24.68 ± 6.53<sup>b</sup> |
| LDL-c (mg/dL) | 87.71 ± 6.49<sup>b</sup> | 73.74 ± 11.30<sup>b</sup> | 126.19 ± 7.76<sup>b</sup> |
| VLDL-c (mg/dL) | 28.60 ± 0.83<sup>b</sup> | 16.63 ± 3.96<sup>b</sup> | 42.08 ± 1.88<sup>b</sup> |
| HDLC/LDLc | 0.85 ± 0.09<sup>b</sup> | 0.50 ± 0.19<sup>b</sup> | 0.19 ± 0.06<sup>b</sup> |

Values with different superscript in a row are significantly different (P < 0.05), n = 6 animals/group: Tc: Total cholesterol; TG: Triglyceride; HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol; VLDL-c: Very low density lipoprotein cholesterol; HDL-c/LDL-c: Ratio of High density lipoprotein cholesterol to Low density lipoprotein cholesterol. A, NC and PC are the different groups of rats fed with their respective diets.

### 5. Conclusion

Isolate Pw4 and Cb5 from palm wine and corn beer reduced the amount of LDL-c and increased HDL-c in rat sera. A change in the concentration of these lipoproteins in serum influences the lipid profile, since they are the main risk factors for CVD. Together with a low-cholesterol diet, isolate Pw4 and Cb5 can better lower serum cholesterol, thereby reducing the mortality rate due to CVD. For these isolates to function more even more efficiently, they can be supplemented in fruits. Probiotic supplementation increases it’s bioavailability in the gut, and protects them against harsh acidic conditions. However, more in vivo studies involving human subjects are recommended before they can be fully endorsed as food supplement in various industries.

### Declarations

**Author contribution statement**

Bertrand Tatsinkou Fossi: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dickson Ewelle Ekabe: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Liliane Laure Toukam, Henri Olivier Tatsilong Pambou: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Amandine Gagneux-Brunon, Céline Nkenfou Nguefeu, Bienvenu Bongue: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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**Data availability statement**

Data will be made available on request.

**Declaration of interest’s statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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