Screening and molecular characterization of cholesterol-lowering lactic acid bacteria isolated from African oil palm wine (*Elaeis guineensis*) and corn beer.

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Abstract

Background: High serum cholesterol is a risk factor for cardiovascular disease (CVD), leading to many fatalities. Probiotic supplementation improves health benefits by lowering serum cholesterol. African oil palm (*Elaeis guineensis*) wine and corn beer have been described as potential sources of probiotic bacteria, but their cholesterol lowering activity has not been extensively investigated. Therefore in this study, we isolated and characterized lactic acid bacteria with probiotic and cholesterol lowering properties from these sources.

Methods: A total of fifty lactic acid bacteria (LAB) were isolated from palm wine and corn beer using de Man Rogosa and Sharpe (MRS) agar by pour plate method. Catalase negative and Gram positive colonies were considered as presumptive LAB. For probiotic properties and selection, acid and bile salt tolerance were evaluated based on viable colony count on MRS agar and cholesterol assimilation from culture media was performed by spectrophotometry. The data analysis was performed by One Way Analysis of Variance and p value< 0.05 was considered significant. The isolates with excellent cholesterol assimilation properties were selected and identified with species of morphology and biochemical analysis using API 50 CHL kit and genotypic identification by sequencing of 16 S rRNA gene.

Results: Fifty isolates had morphological features similar to LAB. They were catalase negative and Gram positive colonies. Among the fifty isolates, five were considered as acid and bile tolerant (viable count exceeded 6logCFU/ml), with two isolates having excellent cholesterol assimilation property (>80%). The result obtained using API 50 CHL kit did not agree with the genotypic identification which was used to confirm the identity of the isolates as most closely related to *Lactobacillus brevis* ATCC (Accession No.NR116238) and *Enterococcus faeccium* NBRC (Accession No. NR113904).

Conclusions: The two strains isolated from traditionally fermented drinks in Buea can be considered as potential probiotics. They can therefore be used to formulate food supplement needed to lower cholesterol in hypercholesterolemia patients.

Background

Cholesterol is a naturally occurring substance in the human body that plays vital roles in cell wall biosynthesis and hormone production. The liver and the intestinal mucosa are capable of synthesizing about 80% of *de novo* (endogenous) cholesterol needed in the body [1], 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) [2]. It has been predicted to remain the single leading cause of death for the next ten years, killing approximately 23.6 million people [3]. More than 80% of CDV deaths occur in low and

middle-income countries, affecting men and women at the same rates [4]. A recent study carried out in Cameroon showed that deaths related to CDV (in-hospital case-fatality) were 15.8% [5].

The use of low-fat diet so far is the most effective means of lowering elevated serum cholesterol [6]. This remedy however has limitations due to the unavailability of such diets and compliance of the consumer [7]. Probiotic bacteria appear as the most promising alternative. They are living organisms that confer a health benefit to the host when administered in adequate amount [8]. Lactic acid bacteria (LAB) remain the most common types of microbes used as probiotics, although certain yeasts are also used [9–10].

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. [11] and Parveens and Hafiz, [12] showed that LAB are among the most predominant microorganisms in locally 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 cognizance of the inherent health benefits of the microorganisms that are predominant in these beverages [13]. 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 hypercholesterolemia patients.

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 characterized LAB with probiotic properties and their ability to lower cholesterol *in vitro*.

Methods

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. [14]. 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 sterile 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^{o} 379 for African oil palm tree, recorded at the National Herbarium N^{o} 34163/HNC, while corn beer plant was identified through comparison with the botanic collection of D. Dang N^{o} 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^{o}C) to the University of Buea Life Science Laboratory and allowed for 2 days to undergo fermentation at ambient temperature (21–30 ^{o}C) before processing.

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^{o}C for 24 h. Repeated sub-culture by streaking on MRS agar was carried out to obtain pure colonies. Pure colonies were labeled 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.

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 in MRS agar plates was counted.

Tolerance to acid pH values

Acid tolerance was evaluated following the method described by Guan at al. [15] 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^{o}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 at 37^{o}C for 24 h. Aliquots were taken after 0 h and 3 h, serially diluted. Samples taken at 0 h were used as the control. Isolates that exhibited final counts $\geq 10^3$ CFU/ml or $\geq 10^6$ CFU/ml at low pH for 3 h, were considered to have moderate or good resistance, respectively. To perform enumeration, 1 ml of each of the suspensions was serially diluted up to the ten logarithmic fold and the viable microorganisms were counted in triplicates on MRS agar.

Bile tolerance

The bile salt resistance of selected isolates was determined by the method described by Argyri et al. [16] 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^{o}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 at 37^{o}C for 24 h. Aliquots were taken after 0 h and 3 h, serially diluted, and plated on MRA agar. Samples taken at 0 h were used as the control. Resistance to bile salt was evaluated based on viable colony counts on MRS agar in triplicates after incubation at 37 ^{o}C for 0 and 3 h, reflecting the average time spent by food in the small intestine.

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 [17]. **B**acteria

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^{o}C) and the unutilized 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 Ngongang et al. [18]. The percentage of cholesterol assimilation was determined by the equation established by Al-Sahel et al. [19]

$$A = \left(\frac{B}{C}\right) X100$$

Where A is the percentage of cholesterol that remained with the pellet, B is the absorbance of the sample containing the cells, and C is the absorbance of the sample without cells.

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

Identification of LAB isolates

Phenotypic identification of LAB isolates using API 50 CHL kit

Phenotypic identification of LAB isolates was performed by API 50 CHL (API kit, bioMérieux, 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 (bioMerieux®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 transferred into API 50 CHL strip wells and overlaid with paraffin oil to create an anaerobic condition. The strips were incubated at 37°C. The results were read after 24 h and confirmed after 48 h. Fermentation of carbohydrates was indicated by a yellow color except for the esculine test (black). Color reactions were scored against a chart provided by the manufacture [20]. The results were analyzed with API WEB (bioMerieux) database version 5.0.

Genotypic identification of LAB isolates using 16 S rRNA gene sequencing

Genomic extraction of the two strains of LAB was determined following the method described by Mulaw et al. [21] with some slight modifications.

DNA extraction of LAB isolates

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 ^{0} 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 ^{0} 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 seconds and centrifuged (Eppendorf centrifuge 5810 R, New York, USA) at 13,000 rpm for 10 min at 4^{o} 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^{o}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 ^{o} C for 10 min and finally dissolved in 30 μl TE buffer and stored at -20 ^{o} C for further study.

Amplification of DNA in polymerase chain reaction (PCR)

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

Gel electrophoresis

Two μl of each amplification mixture was subjected to electrophoresis in 1.5% (w/v) agarose gels in 0.5 x TAE buffer for 1 h at100 V. The DNA molecular mass marker (250 to 10000 bp) from inqaba biotech, South Africa was used as the standard. After electrophoresis, the gels were 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 for accession numbers [22]. A phylogenetic tree was constructed using MEGA 10 software to reduce all positions containing gaps and missing data in the trail sequence in order to evaluate the evolutionary relationship of Pw4 and Cb5 and their close relations.

Statistical analysis

All the tests were performed in triplicate, and the results were expressed as mean ± standard deviation. Data were analyzed 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).

Results

Isolation of lactic acid bacteria

A total of 60 LAB cultures were isolated on MRS agar. Forty-two of these cultures were isolated from palm wine and 18 from corn beer all sourced from Buea, South West Region of Cameroon. Preliminary identification of colonies carried out on the basis of cell morphology, microscopic examination and biochemical tests revealed smooth, oval, and cream white colonies on MRS agar plate. However, only ten colonies were catalase negative and Gram positive under microscopic examination and were considered as presumptive LAB. Table 1 shows the preliminary identification of the isolates.

Table 1.

Phenotypic characteristics of lactic acid bacteria

Isolates	Catalase test	Gram reaction	Shape	Origin of isolate
Pw1	-	+	Rods	Palm wine
Pw2	-	+	Shorts 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	-	+	Cocci	Corn beer
Cb5	-	+	Shorts rods	Corn beer
Cb6	-	+	Chain forming cocci	Corn beer

(+) Positive (-) Negative reactions

Probiotic properties of LAB isolates

Resistance to acid

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 figures 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.

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 ($p<0.05$). 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 figures 2a and 2b.

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.

Identification of LAB isolates

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 utilization of carbohydrates sources of the API CHL 50 kit by Pw4 and Cb5 isolates (Table 3).

Table 2:

Phenotypic Identification of lactic acid bacteria

Lab code	Source	Identification	Similarity index %
Pw4	Palm wine	*L. plantarum*	99.6%
Cb5	Corn beer	*L. pentosus*	99.5%

Table 3:
Carbohydrates fermentation by lactic acid bacteria isolates using API 50 CHL kit[a].

Test number	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	26	27
Strain code	control	Glycerol	Erythritol	d-	l-	Ribose	d-xylose	l-xylose	Adonotol	B methyl-	Galactose	Glucose	Fructose	Mannose	Sorbose	Rhamnose	Dulcitol	Inositol	Mannitol	Sorbitol	Methyi-d-mannoside	Methyl-d-glucoside	n-acetyl-glucosami	Amygdalin	Arbutin	esculine	Salicin	cellobiose
Pw4	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Cb5	-	-	-	-	+	+	+	'	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+

Test sample	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	Identified species
Strain code	Maltose	Lactose	Melibiose	Sucrose	Trehalose	Insulin	Melezitose	Raffinose	Starch	Gylcogen	Xylitol	B gentiobiose	d-turranose	d-lyxose	d-tagatose	d-fucose	l-fucose	d-arabitol	l-arabitol	Gluconate	2-keto-gluconate	5-keto-gluconate	
Pw4	+	+	+	+	+	-	+	-	?	?	-	+	+	-	+	-	-	-	-	?	-	-	*Lactobacillus plantarum*
Cb5	+	-	+	+	+	'	+	-	-	-	-	+	+	+	+	-	-	-	-	?	-	-	*Lactobacillus pentosus*

Genotypic identification of LAB isolates

The genotype identification of DNA using universal primer showed clear bands of isolates on agarose gel with approximate molecular weight 15000 bp. Results obtained using BLAST identification and MEGA 10 software revealed close similarity of 97.4% of Pw4 to *Lactobacillus brevis* strain ATC (Accession no. NR116238) and 99.54% of Cb5 to *Enteroccocus faecium* strain NBRC (Accession no.NR113904).

Phylogenetic trees of these selected isolates based on 16S RNA gene sequences are presented in Figures 4a and 4b.

Table 4:

Genotypic Identification of LAB Isolates.

Lab code	Source	Identification	Similarity index %	Accession no.
Pw4	Palm wine	*Lactobacillus. brevis* ATCC	97.4%	NR116238
Cb6	Corn beer	*Enterococcus faecium* NBRC	99.54%	NR113904

Discussion

Locally harvested palm wine and traditionally processed corn beer have been exploited as suitable sources for screening various LAB [23]. In Cameroon, palm wine and corn beer are widely consumed with little or no knowledge of the presence of a predominant LAB population [13]. Out of the fifty samples collected and grown on MRS agar plates, only ten isolates had morphological (microscopic and macroscopic), biochemical features similar to LAB (Table 1) as reported by Bennani et al. [24]. 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 [25]. 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%) (Table1). 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. [26] 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 Presumption of Safety) by the American Food and Drug Agency (FDA) and the European Food Safety Authority (EFSA), respectively [27]. One of such functional property proven to be more effective in hypercholesterolemia patients involves cholesterol assimilation in the liver and ileum. Recent studies have proven that probiotic LAB are able to assimilate cholesterol to a significant margin [28-29]. Gilliland et al. [30] 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. For efficient cholesterol assimilation, 0.4 % bile salt was added to the medium and grown under aerobiosis as described by Anandharaj et al. [31]. The results obtained (figure 3) showed two isolates (Pw4 and Cb5) with the best cholesterol assimilation ability (>80%). This implies that isolate Pw4 and Cb5 can efficiently be used to assimilate cholesterol *in vitro*. A similar study by Anila et al. [32] and Malakar et al. [33] revealed that *L. brevis* PLA 7, *L. brevis* PLA 14 and *Enteroccocus faecium* BASTUS 4 and 5 respectively isolated from fermented beverages were able to assimilate cholesterol significantly *in vitro*. A possible mechanism of cholesterol assimilation of Pw4 and Cb5 was the cell wall binding and incorporation of cholesterol within their phospholipid layer as described by Ooi and Lion [34]. However, Ramasamy, [35], highlighted that bile salt hydrolytic activity (BSH) of probiotics stands as one of the 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. A study carried out by Ngongang et al. [18] revealed alike trend during cholesterol assimilation in MRS culture medium as well as in albino rats.

In spite of the 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 [36]. The survival of LAB in the stomach is important because the pH of the stomach (HCl acid) 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 [37]. In this study, ten presumptive LAB were exposed to pH 2 and 3 for 3 h to assess their ability to withstand stressful conditions.

Among the ten isolates, seven had viable counts reaching 6log CFU/ml irrespective of the pH change. This implied that these isolates were able to tolerate acidic conditions at pH 2.0 and pH 3.0 and were therefore considered acid tolerant. Similar findings have been reported by Somashekaraiah et al. [38], who reported excellent acid tolerant properties of *Enterococcus faecium* (MH748610-MYSN) and *Lactobacillus brevis* (MH748630-MYSN) isolated from neera (a naturally fermenting coconut palm nectar). The increase in viability in pH 3.0 compared to pH 2.0 was similar to the results obtained by Vanniyasingam et al. [39].

Evaluating tolerance to bile is important because probiotic bacteria have to transit the duodenum (where bile acid is present) before initiating cholesterol assimilation in the ilium [40]. Bile salt is synthesized in the liver from cholesterol and stored in the gall bladder from where it is secreted into the duodenum in a conjugated form during fat metabolism. The physiological concentrations of human bile salts range from 0.3 to 0.5% [41]. In this study, the viable count of the isolates was determined after exposure to 0.2 and 0.4 % bile salt after 3 h of incubation. Seven strains were found to tolerate 0.2 % bile salt, while six strains were able to tolerate 0.4 % bile salt. This implies that high bile concentration affects the viability of isolates. This is because disruption of cellular homeostasis causes the dissociation of the lipid bilayer

and integral protein of their cell membranes, resulting in bacterial content leakage and cell death [42]. A similar result showed that *Enterococcus faecium* (MH748610-MYSN 18) and *Lactobacillus brevis* (MH748630-MYSN) isolated from some fermenting coconut palm nectar had bile salt tolerance of more than 80% [38]. Bile salt hydrolytic activity which is associated with the capacity to eliminate cholesterol from the intestinal environment has been shown to be an important mechanism for bile tolerance [43].

Conventional phenotypic methods alone are insufficient for the identification of LAB. Reliability can be achieved by the use of molecular techniques. The 16S rRNA gene is found to be a powerful tool for appreciating genetic variability among different species [44], which allows for the design and use of universal primers to discover and classify organisms into a wide range of taxa. Mulaw et al. [45] and Dowarah et al. [46] 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. In this study, the results obtained from the two identification methods did not agree (Tables 2 and 4). However, discrepancies between sequencing and phenotypic (API 50CH kit) methods have been reported for LAB and other bacteria [47]. The API 50 CHLassay is intended uniquely for the identification of those species included in the database. As a result, the strains not found in the identification table provided by bioMérieux SA, were successfully identified using the 16S rRNA gene sequencing. Moreover, a study by Bağder *et al.* [48] compared the results of the API 50 CHL test with 16 S rRNA results and found that the API test did not give reliable identification results, with only 71 out of 152 tested isolates in agreement.

In this study, a phylogenetic tree for each gene was constructed by the Maximum Likelihood method and minimum evolution method to access the evolutionary relationship between local samples (Pw4 and Cb5) and the highest query cover above 90% of samples [49]. The sequences of Pw4 and Cb5 showed similarity of 97.4% at 97% query coverage to *Lactobacillus brevis* strain ATCC 14869 (Accession no. NR116238) and 99.54% at 97% query coverage to *Enteroccocus faecium* strain NBRC 100486 (Accession no.NR113904) respectively. Taken into consideration these two identification methods in this study, the results obtained affirms the need of using molecular methods for typing newly isolated microorganisms.

Conclusion

Lactobacillus brevis strain ATCC 14869 (Accession no. NR116238) and *Enteroccocus faecium* strain NBRC 100486 (Accession no.NR113904) isolated from palm wine and corn beer in Buea, have good acid and bile tolerance and can be used in cholesterol assimilation especially for patients displaying symptoms of high serum cholesterol. The properties of these LABs identified, can permit them overcome the stressful acid and bile salt conditions in the stomach and duodenum respectively, to provide health benefits in the gut.

Abbreviations

16SrRNA: 16S ribosomal ribonucleic acid

API: Analytical Profile Index

CVD: Cardiovascular disease

CFU/ml: Colony forming unit per millilitre

LAB: Lactic acid bacteria

MRS: Man Rogosa and Sharpe agar

PCR: Polymerase Chain Reaction

WHO: World Health Organization

Declarations

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Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing Interest

We declare no competing interests

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Figures

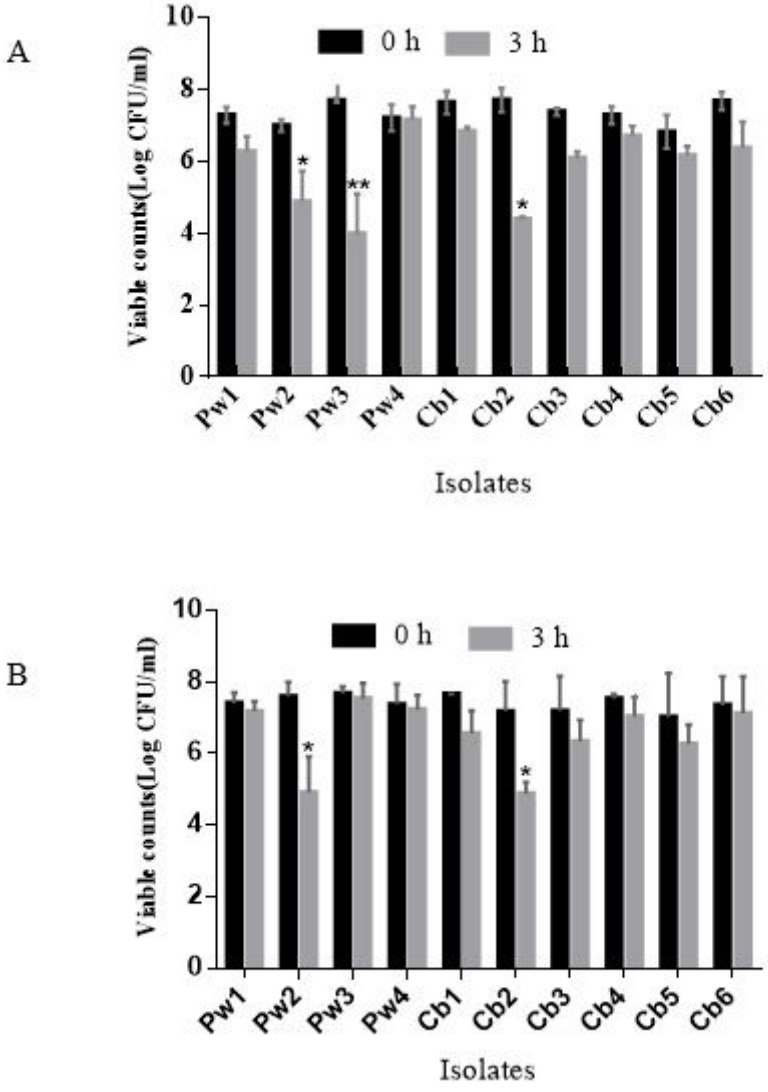

Figure 1

Effect of pH on the growth of lactic acid bacteria strains. Colony forming units were counted at 0 and 3 hours at (A) pH 2, and (B) pH 3. Data are expressed as mean ± standard deviation. * Significant differences at $p<0.05$, **$p<0.01$

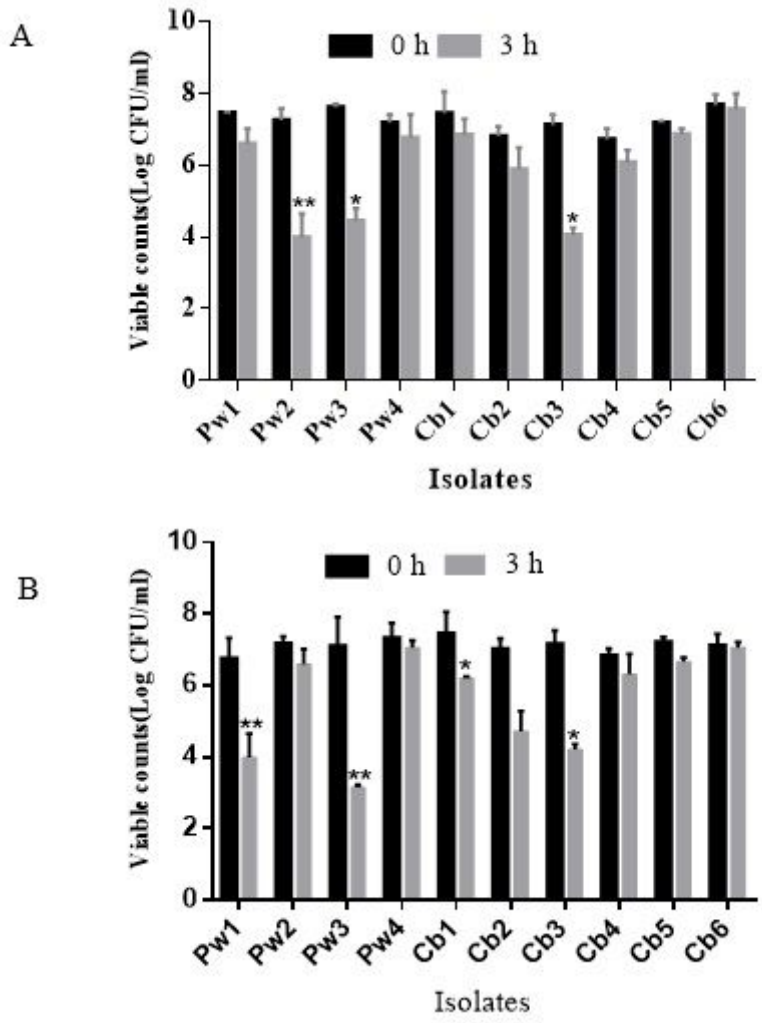

Figure 2

Effect of bile salt concentration on the growth of lactic acid bacteria strains. Colony forming units were counted at 0 and 3 hours at different bile salt concentration; (A) 0.2%, and (B) 0.4%. Data are expressed as mean ± standard deviation. * Significant differences at $p<0.05$, **$p<0.01$

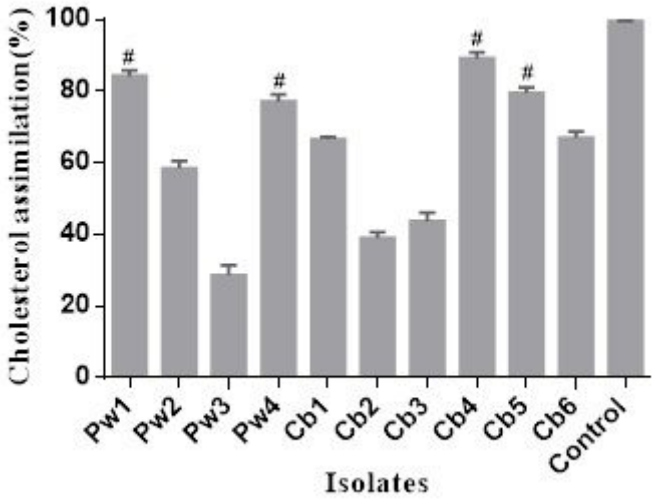

Figure 3

Cholesterol assimilation by LAB strains at 1% cholesterol and bile salt concentration of 0.4% for 24 h. Data are expressed as mean ± standard deviation. # assimilated cholesterol >75%

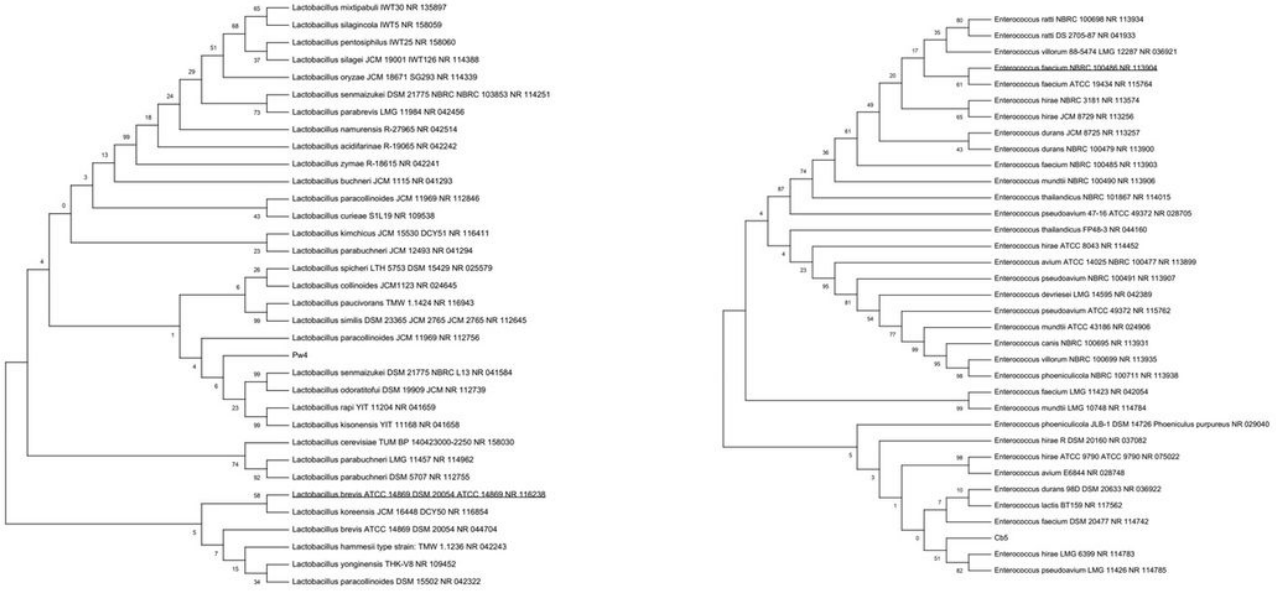

Figure 4

Phylogenetic tree constructed showing the position of isolate; (A) Pw4, (B) Cb5 and related Lactobacillus species.