Original Research Article

Screening and Characterization of Putative Probiotic *Lactobacillus* Strains from Honey Bee Gut (*Apis mellifera*)

ABSTRACT

The objective of this work was to isolate, identify and characterize lactobacilli strains from the intestinal tract of honey bees as putative probiotics. We obtained eighty-five isolates. At the end of screening based on physiological properties, 17 isolates were pre-selected and their resistance to gastrointestinal stress was evaluated. Twelve (12) with good resistance after 3 h exposure to low pH values (pH2, pH3) were subjected to determination of their *in vitro* BSH activity. The research of the *bsh*-A, *bsh*-B, *Bsh*1 and *Bsh*-*Lp*1 genes encoding the BSH enzyme was also conducted. Four isolates (H46, H82, H21 and H28) were resistant, seven others tolerant (H6, H15, H47, H24, H67, H44, H80) and only one was sensitive (H63) to oxgall bile salt. Determination of BSH activity revealed that all strains hydrolyze bile salts, with a preference for oxgall as opposed to Taurodeoxycholate. H15 and H47 isolates showed the highest BSH activities, which were 103.82 ± 12.93 U/mg and 98.53 ± 2.86 U/mg, respectively, with no significant difference (p>0.05). Only the *bsh*-*Lp*1 gene was amplified in isolate H24 and H28. None of the strains showed the *bsh*-1, *bsh*-A or *bsh*-B genes. After sequencing *bsh*-*Lp*1 gene of H24 and H28, the BSH proteins deduced from the complete ORF showed high similarity with those of GenBank database. Antimicrobial activity revealed the inhibition zone against pathogenic and food spoilage bacteria. Isolates were identified based on the sequencing of 16S rRNA encoding gene as *Lactobacillus plantarum* (75%) and *Lactobacillus paraplantarum* (25%).

Keywords: *Honey bees; Lactobacilli; Bile salt hydrolase; Antimicrobial Activity; 16S rRNA; Gene sequencing.*

1. INTRODUCTION

Over the last few decades, probiotic lactic acid bacteria (LAB) have become increasingly popular in fighting diseases that impair human health [1]. Factors contributing to this enthusiasm include the emergence of scientific and clinical evidence demonstrating the efficacy of certain probiotic strains and the increasing consumer demand for natural drug substitutes. Probiotics are "live microorganisms which, when administered in sufficient quantities, confer benefits to the host's health" [2].

Various studies indicated that probiotics might improve lactose intolerance, have a positive influence on the intestinal flora of the host, stimulate/modulate mucosal immunity, reduce inflammatory or allergic reactions, reduce diarrhea, constipation, candidiasis, blood cholesterol and competitively exclude pathogens [3].

Cholesterol is a vital substance in the human body. Long-standing highblood cholesterol levels may lead to atherosclerosis and therefore, may cause a major risk of developing cardiovascular diseases (CVDs). By the year 2030, CVDs will affect approximately 23.3 million people around the world [4]. Although a drug is used, it is often suboptimal, expensive and can cause adverse side effects [5].These pharmaceuticals are mostly based on the interruption of the enterohepatic circulation (EHC) of bile salts [6]. In recent years, LAB identified as probiotics are increasingly popular in challenging these diseases.

Bile salt metabolism and cholesterol metabolism are closely linked. Bile is a digestive secretion that plays a key role in lipid solubilization, as it behaves like biological detergents [6]**.** The deconjugation, one of the mechanisms that the lactobacilli use to counteract the action of bile salts, is catalyzed by bile salt hydrolases (BSH) which release glycines/taurines from the steroid nucleus, thus reducing the solubility of bile at low pH and reduces its detergent activity [7]**.** The hydrolysis of bile salts by LAB breaks the enterohepatic cycle of bile salts and may contribute to lower the blood cholesterol level (cholesterol-lowering effect). Oral consumption of probiotic LAB has been shown to considerably decrease cholesterol levels by as much as 22 to 33%. This cholesterol-lowering property can be in part attributed to BSH activity [8,9].

But, once ingested, probiotic LAB come into contact with the stressful conditions of the gastrointestinal tract. They must survive and remain metabolically active under these circumstances. Gastric acidity and the disaggregation properties of bacterial membranes by bile salts are the primary challenges. It is also important for these LABs to exhibit resistance against the autochthonous microflora to improve the ability to colonize the digestive tract and express their probiotic functions [10]. This resistance to the microflora can occur particularly by the production and secretion of antimicrobial compounds. Some strains of lactic bacteria can synthesize bactericidal/bacteriostatic molecules such as organic acids, hydrogen peroxide, carbon dioxide, diacetyl and especially bacteriocins [11]. The selection of LAB strains that are able to withstand the stressful conditions into the gastrointestinal tract of humans and improve their probiotics benefits is a challenge.

Several microorganisms live in symbiosis with insects that have special diets. The bees are an example. Their stomach is filled with nutrients and nectar, and hence constitutes a micro-aerobic environment which, at an optimal temperature of 35°C in the hive serves as a useful ecological niche for LAB [12]. In fact, LAB and predominantly Lactobacilli has been found in the gastrointestinal tract of bees [13]. Previous studies have reported the isolation from honey bee of LAB with probiotic potential and their applicability in controlling infections in bees. However, none to the best of our knowledge have addressed the selection of potential probiotic LAB from honey bees for their use in Human. Moreover, the microbiota of the honey bees in Cameroon has not yet been explored, whereas it may possess LAB that can provide beneficial effects in humans. The selection of probiotic LAB has been based on *in vitro* physiological tests to different stress factors such as low pH, and bile salts [14]. In addition to these physiological tests, the use of molecular markers is an approach that would lead to improved screening in order to obtain the strains presenting the most wanted potentials.

In the present study, LAB isolated from honey bee digestive tract were screened for properties such as pH and bile salts tolerances, bile salts hydrolysis as well as antimicrobial activity using phenotypic criteria as well as molecular markers.

2. MATERIAL AND METHODS

2.1 Isolation and purification of lactic acid bacteria

One hundred and twenty honey bees (*Apis mellifera*) were collected from hives and honey vendors in five localities of the Menoua Division (West-Cameroon): Fossong Wentcheng (5°24'N; 9°56'E), Penka-Michel centre (5°27'N; 10°18'E), Dschang (''Marché B'') (5°27'N; 10°02'E), Bamendou ''Qt Nguim'' (5°26'N; 10°12'E), Balessing (''King Place'') (5°30'N; 10°15'E). The samples were collected by trapping in sterilized bottles. Once in the laboratory, the bottles were stored at + 4°C for 3-5 min to stop or decrease the mobility of the bees. Using the method described by Mahesh et al. [15], the stomach contents of the bees were collected and introduced into 5 ml of MRS broth supplemented with 5% (w/v) L-cysteine-HCl for 48 h activation at 37°C. Each culture was subsequently streaked onto MRS Agar medium supplemented with 5% (w/v) L-cysteine-HCl and incubated at 37°C for 48 h. At the end of the incubation period, colonies of different appearance were isolated and cultured in MRS broth medium. The purity of isolates was assessed by re-streaking on a fresh MRS agar medium. Gram staining was carried out, and

Gram-positive rod-shaped bacteria were selected and evaluated for their physiological parameters such as catalase activity, CO_2 production from glucose, growth at 10°C and 45°C.

2.2 Phenotypic and genotypic tests related to acid and bile salts tolerance

2.2.1 Evaluation of the ability to tolerate acidity

The ability of the LAB isolates to tolerate acidity was assessed using the method of Verdenelli et al. [16]. Resting cell suspensions were prepared by harvesting (10,000 g; 10 min at 4 °C) exponentially grown (16-18 h) lactobacilli cultures. Resting cell suspension (10^8 CFU/ml) was introduced in different citrate buffers (pH 2, pH 3, and pH 6.5) for 3 h. The suspensions were then centrifuged at 5,000 g for 5 min at 4 °C twice and washed in sterile saline solution to eliminate the citrate buffer. Cell pellets were suspended in physiological solution, and a series of tenfold dilution (10^{-2} to 10^{-10}) were prepared. 50 µl of each dilution was plated on to MRS-Cys-HCl agar and incubated at 37°C for 24-48 h. Percentage of viable bacteria was expressed as the ratio between the counts after 3 h and at 0 h incubation time.

2.2.2 Evaluation of the ability to survive in the presence of bile salts

The capacity to grow in the presence of bile (bile salt tolerance) was also evaluated following the method of Verdenelli et al. [16]. with slight modifications. MRS broth containing 0, 0.3, 0.5 or 1% w/v oxgall (a mixture of conjugated and unconjugated bile salts, a natural dried bovine bile component; DIFCO) were used. The absorbance at 560 nm (A_{560nm}) was measured at hour intervals up to 8 h. The results were expressed as the time difference of growth in the control (MRS without oxgall) and the test media (MRS containing 0.3, 0.5 or 1% oxgall) measured by a 0.3 unit increase in A_{560nm} as described by Gilliland et al. [17].

The difference between the time required to increase the A_{560nm} of 0.3 units for a given bile salt concentration and that needed for the control is the stunted growth retardation. This time was calculated at 0.3% of bile salts and the isolates classified according to their sensitivity to bile salts based on their growth retardation (d) according to the criteria described by Château et al. [18]. All experiments were carried out in triplicate.

2.2.3 Screening for the presence of genes involved in resistance to acidity and bile salts.

The genes involved in pH and bile salt tolerances that were screened are shown in Table 1. The genomes of the LAB isolates were screened by direct colony PCR. The primers used for each PCR reaction were designed based on the literature (references in Table 1).The following conditions were used for PCR: initial denaturation at 95°C for 5 min, then 40 cycles of the denaturation set at 95°C for 1 min, hybridization (at the annealing temperature of each gene) for 1 min, polymerization at 72°C for 1 min and a final step of additional elongation at 72°C for 10 min. Then, 10 µl of PCR product was analyzed on 1% agarose gel with GoldView™ for DNA staining in Tris-acetate-EDTA buffer 0.5X (TAE, pH 8.5) for 20 min at 130 V and the reading done by UV trans-illumination.

Table 1. List of primers used to screen genes involved in acid and bile salts tolerances in the collection of bacteria

General function	Gene	Predicted Function	Primer (5 ' to 3' sequence)	An.T (°C)	Size (bp)	References
Survival to acidity	*Hdc*	Histidine decarboxylase	Fd-AGATGGTATTGTTTCTTATG R-AGACCATACACCATAACCTT	52.0	367	[19]
pH and bile salt survival	*gtf*	Glucan synthase	Fd-ACACGCAGGGCGTTATTTG R-GCCACCTTCAACGCTTCGTA	58.0	374	[20]
	clpL	ATPase	Fd-GCTGCCTTYAAAACATCATCTGG R-AATACAATTTTGAARAACGCAGCTT	56.0	158	[21]

An.T = Annealing temperature, Size = Expected amplicon size, bp = base pair

2.3 Phenotypic and genotypic tests related to Bile Salt Hydrolase activity

2.3.1 In vitro evaluation of Bile Salt Hydrolase activity

The BSH activity was measured by determining the concentration of amino acids released from conjugated bile salts (oxgall and taurodeoxycholate, DIFCO) as described by [9]. One unit of BSH activity (U/mg) was defined as the amount of enzyme that releases 1 µmol of amino acids from the substrate per minute.

2.3.2 Screening for the presence of genes involved in the Bile Salt Hydrolase activity

The primers used are shown in Table 2 and the PCR reaction was carried out in a reaction mixture consisting of 25 µl of 2 x Master Mix, 2 µl of primer (1µM) and a bacterial colony of the pure isolate in a final volume of 50 µl. A heating step was performed at 94 °C for 2 min, and the PCR program consisted of 30 cycles composed of 3 steps as follows: denaturation at 94 °C for 1 min, hybridization at 58°C for 20 s, elongation at 72°C for 2 min. After these 30 cycles, a final extension step at 72°C for 10 min was performed. Then, the amplicons were analyzed as previously described.

Table 2 List of primers used to amplify the genes responsible for the expression of the BSH enzyme

Genes	Primer (5 ' to 3' sequence)	An.T	Reference
bsh-A	**F**: TACAACTATTCATTTAGACGCAATATCC		
(*Lb acidophilus* BshA)	**R**: CACTCTGCCAACACTCCATAACG		
bsh-B	**F**:CAAAAGCCATTTATTCCGACTGA		
(*Lb acidophilus* BshB)	**R**:CATAATTTATTACTTCCTTTGTTAGACAGC	58°C	[22]
bsh-Lp1	**F**:TGTATTTTAGTAGGTATTTCAAGCATCTC		
(*Lb plantarum* Bsh1)	**R**: CAATGAAATGGTTACGATTACGC		
bsh-1	**F:** GCCATTAAGCAATTCGGGTTATA		
(*Lb casei* Bsh)	**R:**CCAATGATTGGTCTCTCGTTCA		

An.T = Annealing temperature

2.3.3 Sequence Analysis of bsh

The purified amplicons of the *bsh* gene of the isolates were sequenced by an automated DNA sequencer using the services of a commercial company (http://www.ruibiotech.com). The sequences were aligned with similar sequences present in the National Center for Biotechnology Information (NCBI) gene collection (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST2 program from the NCBI was used for nucleotide sequence analysis and amino acid sequence deduction. Protein sequences were aligned using ClustalW software package. The nucleotide sequences were deposited in the GenBank database to obtain Accession Number.

2.4 Antimicrobial activity

The direct antimicrobial activity of the LAB strains was evaluated as described by [23]. Indicator bacteria were selected based on their involvement in gastrointestinal infections and food spoilage: *Listeria innocua* ATCC 33090, *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* DSM 20523, *Bacillus cereus* 11778, *Proteus mirabilis* (Clinical isolate), *Escherichia coli* ATCC 13706, *Salmonella enterica* serovar Typhi ATCC 6539, *Pseudomonas aeruginosa* ATCC 20027. *Lactobacillus plantarum* 5S is a bacteriocin's sensitive strain obtained from our laboratory collection and used as positive control.

2.5 Molecular Identification of selected LAB isolates by 16S rRNA gene sequencing

The primers (Forward: 5′-AGAGTTTGATCCTGGCTCAG-3′ and Reverse: 5′-CTACGGCTACCTTGTTACGA- 3′) previously designed by Weisburg et al. [24] were used to amplify the nearly completed 16S rRNA encoding gene. Direct colony PCR reaction was carried out in a reaction mixture consisting of 25 µl of 2X Master Mix, 4 µl of primers (1µM) and a bacterial colony of the pure isolate in a final volume of 50 µl. A step of heating was carried out at 94°C for 2 min. The PCR program of 30 cycles consisting of 3 steps was done: denaturation at 94°C for 1 min, hybridization at 42°C for 20 s, elongation at 72°C for 2 min. After these 30 cycles, a final extension step at 72°C for 10 min was performed. The amplicons were analyzed as described earlier. After amplification of the 16S rRNA encoding gene, DNA fragments of about 1400bp were observed in the agarose gel. The amplicons were then purified and sent to a commercial facility for sequencing (http://www.ruibiotech.com, China, Beijing). After sequencing, the chimeras within the sequences were identified and trimmed using ChromasPro 1.7.7 software. The sequences were aligned with similar sequences retrieved from the NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). CLUSTAL multiple sequence alignment was performed and the 16S rRNA gene sequences of the strains were deposited in the NCBI GenBank to obtain their nucleotide sequences accession numbers.

2.6 Statistical analysis

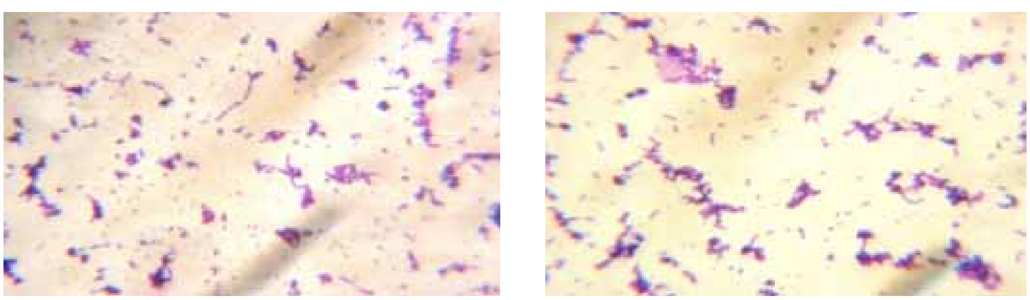
The results were expressed as the mean ± standard deviation and then analyzed by the analysis of variance (ANOVA) using the Graph Pad InStat software (GraphPad Software Inc., www.graphpad.com, V3). When differences existed, means were compared between them by the Student-Newmann-Keuls test at the probability threshold 0.05.

Principal Component Analysis (PCA) was applied to acid survival (pH2 and pH3), 1% bile salt survival rate, time to increase absorbance (ΔDO_{600nm}) to 0.3 units at 1% bile salt, as well as the BSH activity on the Oxgall. The XLSTAT 2007.8.04 software (Addinsoft, Paris, France, http://www.slstat.com) was used and a normalized Pearson (n) PCA was applied.

3. RESULTS

3.1 Isolation of LAB

A total of eighty-five pure and Gram-positive isolates were obtained. Microscopic observation revealed that they were rod-shaped and arranged in pairs or chains of varying length (Fig. 1). Among these isolates, 17 do not produce CO_2 from glucose and were preselected for future testing.



Isolate H15 Isolate H80

Fig. 1. Microscopic observation of two isolates under an optical microscope (x1000)

3.2 Tolerance to acidity by the pure isolates

The 17 preselected isolates were tested for resistance to low pH and the results are presented in Table 3. In general, there was a significant decrease ($p<0.05$) in the survival percentage of the isolates when the pH decreases from 6.5 to 3 and then to 2. At pH2, 8 isolates out of 17 (47.06%), namely H21, H32, H45, H48, H51, H51, H55, and H63 showed survival percentages below 50%, while the significantly ($p<0.05$) highest survival percentage was 93.00 ± 1.73% with isolate H15. At pH3, only the isolate H55 has a survival percentage below 50%, while the other isolates had survival percentages greater than or equal to

65%. The significantly ($p<0.05$) highest values were those of the isolates H15 and H47, respectively 95.67 ± 2.08% and 96.17 ± 1.26%. The 12 isolates with survival percentages greater than 45% at pH 2 have been selected for the further assays.

Table 3. Percentage of survival of the isolates after incubation for 5 h in citrate buffer at different pH values

	Survival (%)		
Isolates	**pH2**	**pH3**	**pH6.5**
H6	$61.67±2.89^{Ab}$	$81.67±1.53^{Bbejk}$	$96.00±2.00^{Cbdeghi}$
H15	$93.00±1.73^{Ac}$	$95.67±2.08^{Bci}$	$98.00±2.00^{Cbeghi}$
H21	$45.67±2.08^{Ad}$	$73.00±2.65^{Bdg}$	$98.67±1.15^{Cbegh}$
H24	$62.67±2.52^{Ab}$	$83.33±2.89^{Bekj}$	$95.00±0.00^{Ca}$
H28	$75.67±2.08^{Ae}$	$91.00±3.61^{Bcfhi}$	$98.67±1.15^{Cbef}$
H32	$30.00±5.00^{Af}$	$70.67±3.06^{Bdg}$	$90.33±2.52^{Ca}$
H44	$50.00±5.00^{Ad}$	$72.33±2.52^{Bdg}$	$91.33±1.15^{Cad}$
H45	$13.67±3.21^{Aa}$	$77.33±6.43^{Bbg}$	$94.67±4.16^{Cae}$
H46	$72.33±2.52^{Ag}$	$92.33±2.52^{Bhic}$	$97.67±2.08^{Cbeghi}$
H47	$88.67±1.15^{Ah}$	$96.17±1.26^{Bi}$	$98.33±0.58^{Cbeghi}$
H48	$8.33±2.89^{Aa}$	$65.00±5.00^{Bd}$	$90.67±1.15^{Cac}$
H51	$8.33±2.89^{Aa}$	$87.67±2.52^{Bfhjk}$	$97.17±1.04^{Cbeghi}$
H55	$11.67±2.89^{Aa}$	$13.33±2.89^{Ba}$	$95.50±1.80^{Cafh}$
H63	$48.33±7.64^{Ad}$	$70.00±5.00^{Bdg}$	$97.00±1.73^{Cbeghi}$
H67	$58.33±2.89^{Ab}$	$87.67±2.52^{Bfk}$	$95.67±2.08^{Cbcdeghi}$
H80	$63.33±2.89^{Ab}$	$72.67±2.52^{Bd}$	$95.00±3.00^{Cafg}$
H82	$61.67±2.89^{Ab}$	$71.67±2.89^{Bdg}$	$95.50±1.80^{Cafi}$

^{A,B,C}*: *On the same row, values with identical letters do not differ significantly (p>0.05) compared to the MRS-Cys control.

$^{a, b,c,d,e,f,g,h,i,j,k}$*:*On the same column, values with identical letters do not differ significantly (p>0.05). Values represent the mean±SD of three trials (n=3).

3.3 Tolerance to bile salts

The survival percentages of isolates at different oxgall concentrations (0.3, 0.5 and 1%), vary between 79.64 ± 0.78 and 93.71 ± 0.92 after exposure to 0.3% oxgall (Table 4). At 0.5% oxgall, only isolate H44 had a survival rate lesser than 72%, i.e., 59.23 ± 3.99. The isolate H46 showed a survival rate greater than 91% for any concentration. This isolate has a higher survival ($p<0.05$) at 1% oxgall compared to all other isolates. However, only the isolate H44 has a survival of less than 50% with 1% of bile salts.

At all oxgall concentrations, the time (min) required to increase the absorbance by 0.3 units for each isolate (Table 4) didn't increase significantly for isolates H46, H82, H6, H15, H47, H21, and H24 compared to the control (MRS-Cys). For the isolate H67, this time was significantly different from the control ($p<0.05$) at 0.5% oxgall. It differed significantly ($p<0.05$) from the control at 1% oxgall with the isolates H44, H80 and H63.

The difference between the time required to increase the OD of 0.3 units for a given bile salt concentration and that required for the control represents the accrued growth delay (in minutes). This value was calculated (Table 4). This growth delay varies between 10 ± 0.0 min and 50 ±17.32 min for the concentration of 0.3% of bile salts.

Table 4. Parameters indicating the behavior of the isolates in Bile Salt

	Parameters							
	Survival rate after 24 h			Time (min) required to increase the absorbance by 0.3 unit				GD *
Isolates	0.3% BS	0.5% BS	1% BS	MRS-Cys	0.3% BS	0.5% BS	1% BS	0.3% BS
H6	92.48±0.98^{Abcd}	84.28±1.30^{Bbcehi}	82.06±1.80^{Bchi}	140±17.32^{Aac}	170±17.32^{Aac}	170±17.32^{Aac}	180±00.00^{Aa}	II
H15	92.20±0.41^{Abcde}	75.48±0.87^{Bdfkl}	71.19±2.39^{Cbde}	130±17.32^{Aac}	160±17.32^{Aac}	160±17.32^{Aac}	160±17.32^{Aa}	II
H21	89.22±1.25^{Abcdefgh}	75.52±1.06^{Bfkl}	67.08±1.28^{Cbdefgk}	160±17.32^{Aac}	170±17.32^{Aac}	170±17.32^{Aac}	170±17.32^{Aa}	I
H24	81.47±1.06^{Aa}	72.19±0.30^{Bdfgk}	69.60±0.45^{Cbdeg}	120±00.00^{Aa}	140±17.32^{Aac}	140±17.32^{Aa}	140±17.32^{Aa}	II
H28	93.57±1.95^{Abc}	87.65±1.82^{Bh}	85.69±1.47^{Bh}	140±17.32^{Aac}	170±17.32^{Aac}	170±17.32^{Aac}	180±00.00^{Ba}	I
H44	83.10±4.38^{Aa}	59.23±3.99^{Bj}	48.67±1.76^{Cj}	170±17.32^{Abc}	200±34.64^{Abc}	200±34.64^{Aac}	260±34.64^{Bb}	II
H46	93.71±0.92^{Ab}	92.10±1.08^{Aa}	91.84±1.15^{Aa}	130±17.00^{Aac}	140±17.00^{Aac}	140±17.00^{Aa}	140±17.00^{Aa}	I
H47	81.52±0.08^{Aah}	84.82±0.49^{Bbehi}	74.25±1.09^{Cbcei}	130±17.32^{Aac}	160±17.32^{Aac}	160±17.32^{Aac}	160±17.32^{Aa}	II
H63	79.64±0.78^{Aa}	77.06±0.54^{Al}	62.59±13.56^{Adfgk}	170±17.32^{Abc}	220±34.64^{Abc}	220±34.64^{Abc}	260±34.64^{Bb}	III
H67	90.12±0.87^{Abcdefg}	84.96±1.35^{Bbhi}	82.96±0.72^{Bhi}	120±00.00^{Aa}	130±17.32^{Aa}	160±17.32^{Bac}	170±17.32^{Ba}	II
H80	84.28±2.14^{Aa}	76.15±1.49^{Bkl}	69.46±1.70^{Cbdegk}	170±17.32^{Abc}	200±34.64^{Abc}	200±34.64^{Aac}	260±34.64^{Bb}	II
H82	90.52±3.89^{Abcdef}	85.66±4.30^{Abh}	75.53±3.53^{Bbci}	130±17.32^{Aac}	140±17.32^{Aac}	140±17.32^{Aa}	140±17.32^{Aa}	I

*^{A,B,C}: On the same row, values with identical letters do not differ significantly (p>0.05) compared to the MRS-Cys control. ^{a,b,c,d,e,f,g,h,i,j,k}: On the same column, values with identical letters do not differ significantly (p>0.05). Values represent the mean±SD of three trials (n=3). BS: Bile Salts (oxgall); *GD=Growth delay: Distribution of isolates according to the growth delay and classification designed by* Château et al. [18]. *I= Resistant (d≤15min), II= Tolerant (15<d≤40 min), III= Poorly tolerant (40<d≤60 min), IV= Sensitive (d>60min).*

3.4 Genes involved in acid and bile salts resistance

Genes have been sought to provide an explanation for the mechanism used by the isolates to tolerate acid and bile salts. The results showed that only the *clpL* gene (encoding ATPase) was found in the genome of all the 12 isolates tested (Table 5). Fig. 2 shows the electrophoresis gel of the amplicons.

Table 5. Genes responsible for acid and bile salts survival in different isolates

	Genes responsible for acid and bile salts survival			Bile salt hydrolase encoding genes			
	Acid resistance	Acid and bile salts resistance					
Isolates	*Hdc*	*clpL*	*gtf*	*Bsh-Lp1*	*Bsh-1*	*Bsh-A*	*Bsh-B*
H6	-	+	-	-	-	-	-

H15	-	+	-	-	-	-	-
H21	-	+	-	-	-	-	-
H24	-	+	-	+	-	-	-
H28	-	+	-	+	-	-	-
H44	-	+	-	-	-	-	-
H46	-	+	-	-	-	-	-
H47	-	+	-	-	-	-	-
H63	-	+	-	-	-	-	-
H67	-	+	-	-	-	-	-
H80	-	+	-	-	-	-	-
H82	-	+	-	-	-	-	-

+ = Presence of the gene, - = Absence of the gene

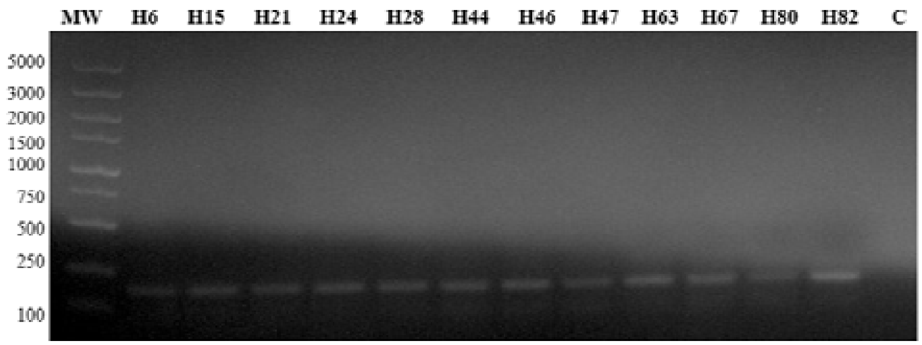


Fig. 2. Electrophoresis gel of PCR amplification of *clpL* gene encoding ATPase
MW: Molecular weight marker in base pair, C: negative control.

3.5 *In vitro* activity of Bile Salt Hydrolase of the isolates

Fig. 3 shows the BSH activity of the isolates in the presence of 0.3% each of oxgall and taurodeoxycholate. This experiment showed that isolates exhibited a different level of hydrolysis activity on oxgall and taurodeoxycholate. As we can notice, the BSH activity of the isolates is higher in the presence of oxgall than Taurodeoxycholate. Isolates H47 and H15 showed the highest activity on oxgall (98.53 ± 2.86 U/mg and 103.82 ± 12.93 U/mg respectively), whilst the lower value was observed with isolate H63 (15.10 ± 4.74 U/mg). On the other hand, BSH activity in the presence of taurodeoxycholate, was relatively low but still detectable. Compared to oxgall, the high value was 7.1 ± 1.9 U/mg (isolate H24) and the lower value at 0.03 ± 0.028 U/mg (isolate H24).

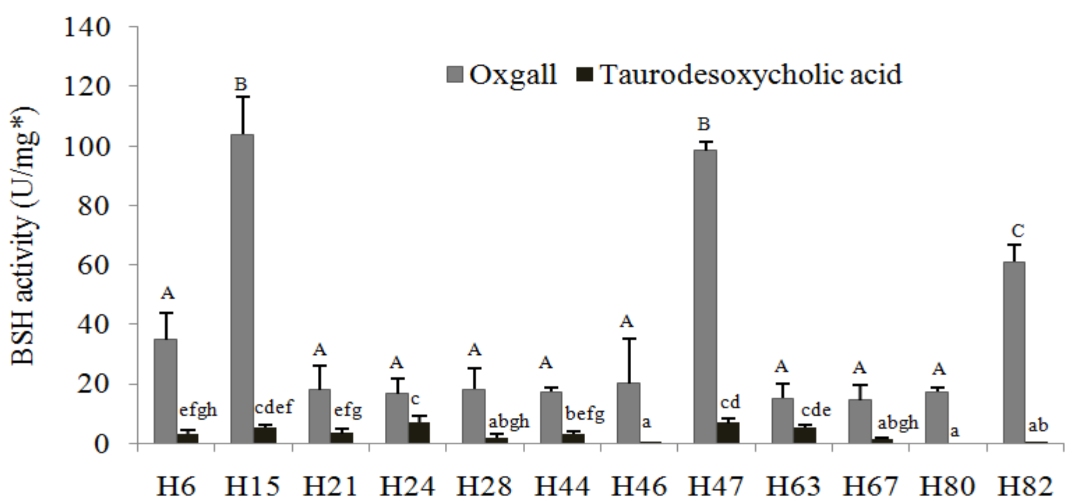


Fig. 3. Bile Salts Hydrolase activity of different isolates

*[A,B,C;a,b,c,d,e,f,g,h]: For the same type of bile salt, values with identical letters do not differ significantly (p>0.05). Values represent the mean±SD (n=3), Error bars represent standard deviation. * 1 U/mg represents the amount of enzyme which releases 1 µmol of amino acid from the substrate per minute. TDC: sodium Taurodeoxycholate.*

3.6 Genes involved in the Bile salts hydrolysis

For specific genes responsible for the hydrolysis of the bile salts, the *bsh-Lp1* (*L. plantarum* Bsh1) gene was amplified only in isolate H24 and H28 (Figure 4). While, none of the *bsh*-1, *bsh*-A and *bsh*-B genes were amplified on any of the isolates (Table 5).

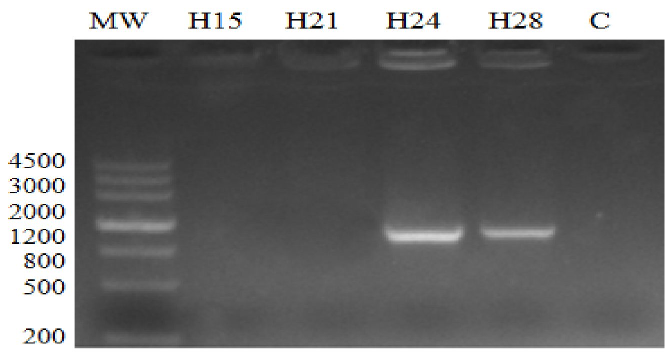


Fig. 4. Electrophoresis gel of PCR amplification of *bsh-Lp1 gene* amplified in isolate H24 and H28

MW: Molecular weight marker in base pair, C: negative control.

After sequencing the *bsh-Lp1*, DNA sequences of BSH were obtained. They were designated Bsh_H24 and Bsh_H28 respectively for the two isolates H24 and H28. The fragment contained single ORF 705 nucleotides encoding a 234 amino acids protein with Bsh_H24, and ORF 726 nucleotides encoded 241 amino acids protein with Bsh_H28 (Fig. 5). Both nucleotides are flanked by an alanine start codon (GCT) and a translational termination codon (TAA). The complete sequence has been deposited in GenBank database under the accession number of MF098540 and MF098541 respectively for the Bsh_H24 and Bsh_H28. Using the ClustalW program, these BSH sequences were aligned with other from GeneBank database. In general, the deduced amino acid sequence of the Bsh_H24 and Bsh_H28 display 100% identity with BSH-related proteins from *Lactobacillus plantarum* subsp. Plantarum P-8 (Accession number: AGL65610.2). They also exhibit 99% identity with BSH-related proteins from *Lactobacillus* sp. DPP8 (Accession number: ALT14558.1) and *Lactobacillus plantarum* (Accession number: ACA49878.1).

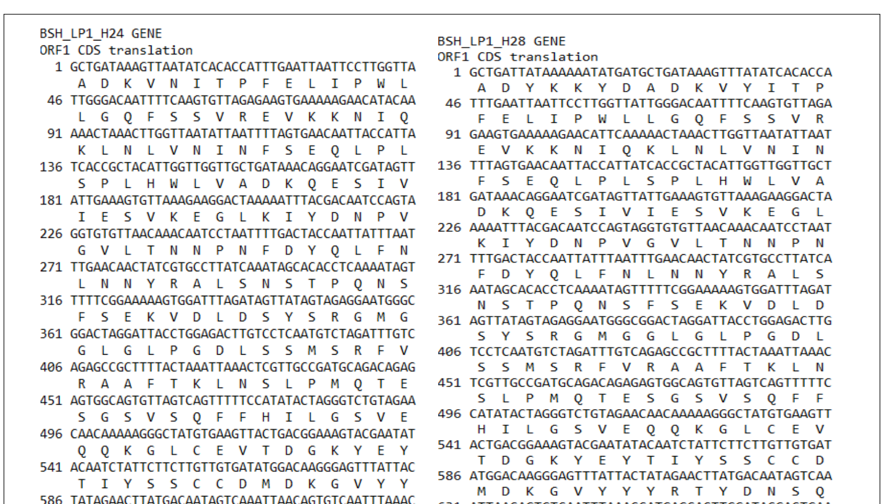


Fig. 5. Nucleotide sequence and deduced amino acid sequence of the BSH_H24 and BSH_H28 gene of the respectively H24 and H28 Isolate (further identified as *Lactobacillus plantarum* sp.)
On the left, Bsh_H24 gene (accession number MF098540) and on the right Bsh_H28 gene (accession number MF098541) deposited in NCBI GenBank data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.7 Principal Component Analysis (PCA)

With PCA, we noticed that the first axis (F1) makes it possible to explain 60.54% of the total variance, and separates the isolates into two groups: those that survive acidity and bile salts (right) and those presenting excellent growth time performance (low time required to increase the absorbance by 0.3 unit and growth delay) (left). The second axis (F2) that opposes survival to the acidity (top) and survival to the bile salts (bottom) explains 23.93% at its level (correlation Biplot, Fig. 6). The variables BSH activities on oxgall and survival are almost orthogonal represented, indicating that they are significantly uncorrelated. The isolates well represented on the F1 axis are H44, H63 and H47, while on the second Principal Component F2 has a high contribution of isolates H15 and H47.

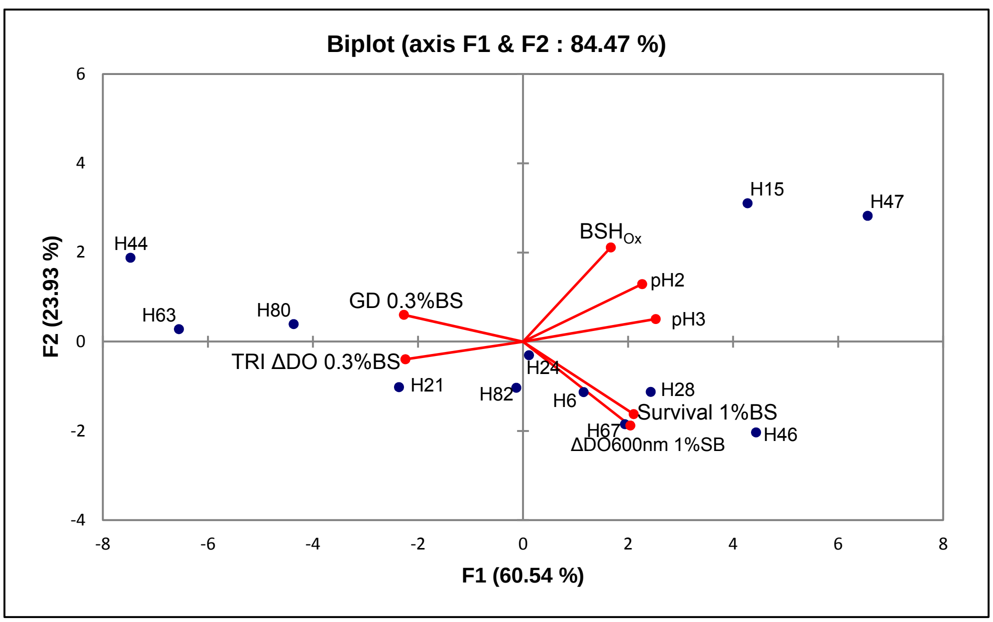


Fig. 6. Graphical representation of the correlation Biplot from the Principal Component Analysis
pH3, pH2: Survival rate to pH2 and pH3. ΔDO0.3%BS: Variation of absorbance A600nm after 24h at 0.3% bile salt. TRI ΔDO 0.3%BS: Time Required to Increase A600nm by 0.3 units (min). Survival 1%BS: Survival rate in 1% Bile Salt after 24h. GD 0.3%BS: Delay of growth during the exponential phase at 0.3% bile salt (min). BSH_{Ox}: Bile Salt Hydrolase Activity on the Oxgall (U/mg).

3.8 Antimicrobial activity

Table 6 indicates results of preliminary antimicrobial activity against a range of indicator bacteria such as *L. plantarum* 5S (bacteriocin's sensitive strain), food spoilage or pathogenic bacteria on the agar medium by the spot technique. It appears that all our isolates exhibit the antimicrobial effect against *L. plantarum* 5S strain. Furthermore, isolates showed antibacterial activities against the indicator bacteria with different levels. The isolates H15 and H24 showed higher antagonistic activity.

Table 6. Inhibitory activity of the isolates against *L. plantarum* 5S and the indicator spoilage or pathogenic bacteria

Strains	H6	H15	H21	H24	H28	H44	H46	H47	H63	H67	H80	H82
***Lb.p* 5S**	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SA	-	++++	-	+++	+++	-	+	-	-	-	+++	-
ST	-	+++	++	++++	-	-	-	-	++	++	-	-
BC	-	+++	+	+++	-	-	-	-	+++	+	+	-
EC	-	+++	-	+++	-	+	-	-	-	-	-	+
SM	-	++	-	+	-	-	+	-	-	-	-	-
LI	-	++	-	-	-	-	-	-	++	++	++	-
PA	-	-	-	+	+++	+++	+++	-	+	-	+	-
PM	+++	++++	-	++++	++	++	-	+++	-	+	-	++

– no inhibition; + 1.0–3.0 mm (weak); ++ 3.1–6.0 mm (good); +++ 6.1–14.0 mm (very good); ++++ >14.0 mm (strong). The diameter of inhibition was calculated as the difference between the total of inhibition zone and the diameter of growth spot of selected strains (n=3). Lb.p 5S: L. plantarum 5S. LI: Listeria innocua ATCC 33090. SA: Staphylococcus aureus ATCC 25923. SM: Streptococcus mutans DSM 20523. BC: Bacillus cereus 11778. PM: Proteus mirabilis (Clinical isolate). EC: Escherichia coli ATCC 13706. ST: Salmonella enterica serovarTyphi ATCC 6539. PA: Pseudomonas aeruginosa ATCC 20027.

3.9 Molecular Identification of Lactic Bacteria

A step of characterization of the isolates based on the PCR amplification and the sequencing of the gene encoding the 16S rRNA was carried out to identify the 12 pre-selected isolates and the results are presented in Table 7. The gene targeted in all these isolates has been amplified (Fig. 7). Therein, we can find the code of each isolate and its origin, the accession number provided by NCBI (from KU886166 to KU886177), the genus and species name of the corresponding lactic bacteria strain. All the 12 isolates were categorized as the genus *Lactobacillus* which showing more than 99% identity to *L. plantarum* and *L. paraplantarum* already present in the NCBI GenBank.

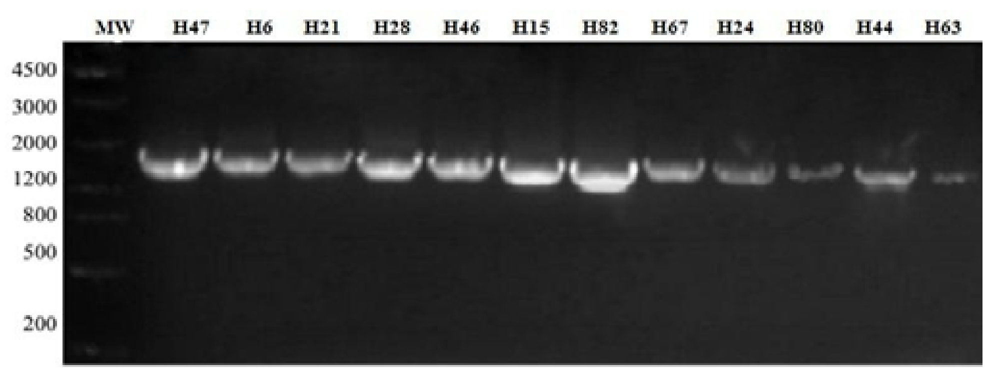


Fig. 7. Electrophoresis gel of PCR amplification of the gene encoding 16S rRNA
MW: Molecular weight marker in base pair

Table 7. The16S rRNA gene sequencing identification of LAB isolates

Strains	Origin	16S rRNA sequencing identification	Sequence length (bp)	% Query coverage	% Max identity	Accession number
H6	Fossong Wentcheng	*Lactobacillus plantarum*	816	100	99	KU886167
H15	Penka-Michel centre	*Lactobacillus plantarum*	720	100	100	KU886171
H21	Penka-Michel centre	*Lactobacillus plantarum*	748	100	100	KU886168
H24	Penka-Michel centre	*Lactobacillus plantarum*	777	100	99	KU886174
H28	Penka-Michel centre	*Lactobacillus plantarum*	708	100	99	KU886169
H44	Bamendou, *QtNguim*	*Lactobacillus plantarum*	597	100	99	KU886176
H46	Bamendou, *QtNguim*	*Lactobacillus plantarum*	708	100	99	KU886170
H47	Bamendou, *QtNguim*	*Lactobacillus plantarum*	939	100	100	KU886166
H63	Balessing, *King Place*	*Lactobacillus paraplantarum*	729	100	100	KU886177
H67	Balessing, *King Place*	*Lactobacillus paraplantarum*	726	100	99	KU886173
H80	Balessing, *King Place*	*Lactobacillus paraplantarum*	1147	100	99	KU886175
H82	Balessing, *King Place*	*Lactobacillus plantarum*	588	100	100	KU886172

4. DISCUSSION

The sensitivity of the isolates to low pH was carried out to predict their behavior during gastrointestinal transit in human. We found that 52.94% and 94.11% could survive respectively to pH 2 and pH3. It has been shown that species of the genus *Lactobacillus* are tolerant to gastric acid conditions [25]. Our results are in agreement with those of Prasad et al. [26] who obtained acid-tolerant strains from 200 isolates, following their survival of nearly 80% after exposure to pH3 for 5 h. Several mechanisms have been elucidated to explain the resistance of lactobacilli to acid stress. Among them are the protomotor force F1F0 ATPase, DNA repair mechanisms, modification of the composition, architecture and stability of the plasma membrane, production of alkaline compounds by the action of urease or Arginine deiminase (ADI), and the management of denatured proteins [27].

Bile salts are the second important factor faced by probiotic LAB in the digestive tract. The growth delay at 0.3% bile salt obtained with our isolates is significantly better than that of strains of commercial lactic acid bacteria isolated from faeces by Mirlohi et al. [28]. They showed that strain *L. plantarum* A7 exhibited growth delay greater than 1 hour at 0.3% bile salts. For the use as a probiotic in humans, LAB must survive at a concentration of 0.3% bile salts [29]. According to the classification of Château et al. [18], none of our isolates was classified as sensitive to bile salts. Thus, all the isolates with survival of more than 79%, at 0.3% bile salt concentration, can probably overcome the bile stress in the intestine if subject to *in vivo* assay.

Known mechanisms can explain this resistance. We can mention the extrusion of the bile, achieved by efflux systems including the multidrug resistance (MDR) family [30]. Another mechanism is the deconjugation of bile acids. It is catalyzed by bile salts hydrolases (BSH), enzymes that release glycines/taurins from the steroid nucleus, which lowers the solubility of bile at low pH and reduces their detergent activity on bacterial membranes [7].

All the 12 isolates tested were found to possess the *clpL* gene coding for ATPase. This gene could have played an important role in the resistance to acid and biliary stress found during *in vitro* phenotypic tests. Turpin et al. [31] reported the presence of *clpL* gene allowing low pH resistance in 91% to 100% of the isolates from their collection. In *Lactobacillus reuteri* ATCC 55730, studies have shown that inactivation of *clpL* has resulted in a significant decrease in bacterial survival after incubation at pH 2.7 [32] or medium containing 0.3% bile salts [33].

Genes have been sought to provide an explanation for the mechanism used by these isolates tolerate the gastrointestinal stress conditions. None amplification of *gtf* and *hdc* genes can be explained by their phylogenetic distribution. Indeed, the primers used would be restricted to a set of species or subspecies absent from the collection of lactic bacteria that we have isolated; or it is possible that the tolerance to acidity and resistance to bile salts found *in vitro* is due to a mechanism controlled by other genes. Similar results were obtained by Turpin et al. [31]. They showed that none out of 38 isolates tested harbored the genes *gtf* and *hdc*. Non-expression of the *hdc* gene is an advantage because; ingestion of a large amount of histamine can cause serious problems into the body. This biogenic amine is formed after decarboxylation of histidine by the enzyme histidine decarboxylase encoded by the *hdc* gene.

In this study, we also evaluated the ability of isolates to perform BSH activity. We observed that they possessed this activity. According to Tanaka et al. [34], in an analogous manner, all the lactobacilli isolated from the gastrointestinal media possess the BSH activity. In all of our isolates, BSH activity was higher with oxgall than with Taurodeoxycholate. Therefore, they have a substrate preference for oxgall. It has been demonstrated that most strains of LAB exhibit high activity with conjugated bile salt mixtures than with a particular type of conjugated bile acid [9]. Also, Kumar et al. [35] showed that the majority of lactobacilli tested had more valuable BSH activity on glycocholate than on taurocholate or taurodeoxycholate. However, since glycocholate is the most abundant of the bile salts found in humans, it would be advantageous to have isolates exhibiting a preference for glycocholate. According to Brashears et al. [36], these isolates could be candidate for the *in vivo* reduction of serum cholesterol levels. Lately, it has been revealed that the BSH take part in a most important role in cholesterol metabolism, thus influencing the serum cholesterol levels [35]. It has also been suggested that BSH activity must be essential in the choice of probiotic organisms with cholesterol-lowering properties, given that microorganisms that do not deconjugate bile salts cannot reduce cholesterol from a medium to a significant level [9, 35].

Several authors have suggested that the resistance of lactobacilli to the toxicity of bile salts in the duodenum could be attributed to the activity of the BSH enzyme [37]. Although BSH activity is widespread in lactobacilli, there is not always a direct relationship with their ability to resist bile [38]. This situation is supported by the numerous functional studies carried out on lactobacilli strains whose genome is sequenced but for which the deletion of a gene coding for BSH does not necessarily have any consequence on the strain survival in the presence of bile salts [39,40]. This enzyme is encoded by the *bsh* gene. Our results showed only the *bsh*-Lp1 gene (*L. plantarum* Bsh1) in H24 and H28. Studies have shown that the presence and genetic organization of *bsh* genes in lactobacilli are very variable [41]. There are four alleles of the *bsh* (*bsh*-Lp1, *bsh*-Lp 2, *bsh*-Lp 3 and *bsh*-Lp 4) in *L. plantarum*, but the highest BSH activity is correlated with the *bsh*-Lp 1 [39].

Some of our isolates have very high percentages of survival at high bile salt concentrations without having such a high BSH activity. The case of the isolate H46 which exhibited a very low BSH activity of 20.20 ± 14.83 U/mg compared to that of the H15 isolate (103.82 ± 12.93) on oxgall. The same observation was also noted by the principal component analysis (PCA), where the variables such as the activity of BSH on oxgall and bile salt survival were shown to be significantly uncorrelated. Recent studies have shown that the resistance of lactobacilli can not necessarily be associated with the presence of BSH [42]. According to the PCA, it can be concluded that the strains H15 and H47, based on their representation, are strongly resistant to acidity; the strains H28, H67 and H46 are more tolerant to bile salts.

The satisfying probiotics are supposed to exhibit their antimicrobial activities generally against pathogens in the gastrointestinal system [43]. In this study, we used pathogenic bacteria (such as *L. innocua*, *S. aureus*, *S.* Typhi, *E. coli* and *B. cereus*) because they are often found as food-borne pathogens that might cause gastroenteritis. The presence of the inhibition zones indicated the antibacterial activity of our isolates on the indicator bacteria. In fact, LABs are recognized for their production of various antimicrobial substances (organic acid, hydrogen peroxide, diacetyl, reuterin and bacteriocins) [44]. The production of these substances responsible for the antagonistic phenomenon by our isolates is important to their antimicrobial property, and they could more expect to be used as probiotic.

According to the joint FAO/WHO [2] expert report on the presence of probiotics in food, it is necessary to know the genus and species of a probiotic strain**.** All the 12 isolates were found to belong to the genus *Lactobacillus*. Indeed, as reported by Tannock [25], the genus *Lactobacillus* occurs in a variety of habitats including plants, the gastrointestinal tract of animals such as bees, and is the most dominant LAB found in the intestinal tract of bees [45]. The high presence of the *plantarum* species was noted by [46] who found that the *L. plantarum* strain was the most abundant (51.02%) of the 5 *Lactobacillus* phylotypes identified in the honey bee *Apis dorsata* in Malaysia. The high presence of *plantarum* and *paraplantarum* species in this collection could be explained by the origin of the type of bee collected during sampling. We collected foraging bees, which are frequently in contact with plants (including pollen and nectar of the flowers); those are habitats of *plantarum* species. This species may well be found in the intestinal tract of bees because most lactic acid bacteria that exist in the intestinal tract of bees are also isolated from pollen [47]**.**

5. CONCLUSION

The results obtained in this study show that Lactobacilli isolated from honeybee gut in the Menoua division (West-Cameroon) can survive low pH and bile salts. Their BSH activity may contribute to lower the blood cholesterol levels. They also possess antibacterial activity on pathogenic bacteria. Thus, the cultures obtained in this work could be presumed as potential probiotic bacteria. Complementary investigation of certain strain will attest their safety, probiotics properties and more.

CONFLICT OF INTEREST

The authors declare no competing interests
Ethical approval and consent is not applicable.

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