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Safety and Functional Aspects of Selected Probiotic Lactobacilli Strains from Water of Cassava's Fermentation

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Authors' contributions

This work was carried out in collaboration between all authors. Author MEKF carried out the study, performed the statistical analysis and wrote the first draft of the manuscript. Authors FNZ and PMK designed the study and corrected the manuscript. Authors WRY, ZT and YL were involved in the sequencing of bile salt hydrolase genes. All authors read and approved the final manuscript.

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ABSTRACT

Many Lactobacilli isolated from foods present probiotic properties, but some of them have been linked to possible clinical cases. Therefore, it is necessary to verify their safety before probiotic use. Our previous works have demonstrated acid and bile tolerances of Lactobacilli strains from the water of cassava's fermentation, without regard to the molecular basis of these resistances or the safety and adhesive aspects of the isolates. This study aimed to investigate the genotypic basis of acid and bile tolerance, safety and adhesion properties of eight probiotic Lactobacilli. The functional properties of their bile salt hydrolases were also studied. All strains were screened for *gtf (acid and bile tolerance)*, *clpL (acid and bile tolerance)* and *hdc (biogenic amine production)* genes and

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investigated for hydrophobicity, co-aggregation and auto-aggregation. Moreover, hemolytic and gelatinase abilities, as well as antibiotic susceptibility, were examined for safety properties assessment. The eight selected strains were found to possess the *clpL* gene in their genome. They also showed excellent cell surface characteristics testifying their good adhesion and colonisation abilities. On the other hand, they were free from virulence factors (hemolytic and gelatinase activities), do not showed antibiotic resistance, and do not possess the *hdc* gene, a confirmation of their safe character for probiotic applications. The most promising probiotic candidates based on Principal Component Analysis were *L. paracasei* 62L (accession number: KU886178), *L. Plantarum* 84L (KU886185) and 86L (KU886187) with good auto and co-aggregation properties. The study of the predicted structure of *L. Plantarum* 86L bile salt hydrolase revealed that it has a structural similarity with previously identified bile salt hydrolases which substrate preference is glyco-conjugated bile. The Lactobacilli from the water of cassava's fermentation have exciting cell surface characteristics and are safe for probiotic applications.

Keywords: Lactobacilli; probiotics, safety properties, adhesion; bile salt hydrolase.

1. INTRODUCTION

Lactobacilli, microorganisms commonly found on foods, are also represented in the microbiota of human and many animals. They belong to the indigenous bacteria of the mammalian gastrointestinal tract and can contaminate or colonise raw food as well as multiply during fermentation [1]. Thus, in addition to the gastrointestinal tract, Lactobacilli are isolated from foods, including many fermented foods [2,3,4]. Many of these foods are obtained from a traditional fermentation, which does not use starters but relies on natural microflora (including Lactobacilli) of raw foods [5]. Many Lactobacilli strains from diverse origins and species display probiotic properties, which lead to their addition to human or animal feeding [6]. For some years now, probiotics have attracted increasing attention as potential vehicles of antibiotic resistance genes, which could be horizontally transferred to the gut microbiota including pathogenic microorganisms [7]. Therefore, it is necessary to check the susceptibility to antibiotics of Lactobacilli from these foods as well as other environments before probiotic use [8]. Beyond this property, others such as the absence of gelatinase and hemolytic activities are also required to verify the safety of probiotic strains [9]. Then, criteria for probiotic selection include lack of pathogenicity, tolerance to gastrointestinal conditions (acid and bile) and ability to adhere to the gastrointestinal mucosa [10]. Indeed, adherence of bacteria to intestinal epithelium is a prerequisite for the digestive tract colonisation. Adhesion is a complicated process involving non-specific (hydrophobicity) and specific ligand-receptor mechanisms. Adherence of bacterial cells is usually related to cell surface characteristics [11]. Our previous works have demonstrated *in vitro* the antimicrobial properties of Lactobacilli strains isolated from the water of cassava's fermentation to resist gastric acidity and bile salts. However, they were not interested in either the molecular basis of these resistances or the safety and adhesive aspects of the strains. These same studies reported an critical bile salt hydrolase (BSH) activity in these strains and the existence of the BSH-Lp1 gene in five of them [12]. But, the substrate specificity and mechanism of action of the BSH of these strains remains unexplored. The genotypic characterisation of the BSH of these strains would facilitate the identification of partially conserved amino acids responsible for the substrate preference and other regions that can be substituted for other amino acids. The identification of specific amino acids in BSH can also help theoretical and computational approaches derived from the structure [13]. Then, functional studies and comparisons with other characterised BSH proteins are useful [14]. This study aimed to investigate the genotypic basis of acid and bile tolerance, safety and adhesion properties of previously reported probiotic Lactobacilli strains. The functional properties of the BSH were also studied.

2. MATERIALS AND METHODS

2.1 Origin of Strains and Growth Conditions

The eighth Lactobacilli strains used in this study were previously isolated from the water of cassava's fermentation collected in in the Menoua divisionand preselected according to their high acid and bile tolerances as well as bile salt hydrolase using principal component analysis (PCA). They were also identifying by

16S RNA gene sequencing as follow: *L. paracasei* 62L (Accession number: KU886178); *L. plantarum* 63L (KU886179); *L. plantarum* 64L (KU886180); *L. plantarum 80L* (KU886184); *L. plantarum* 84L (KU886185); *L. plantarum* 85L (KU886186); *L. plantarum* 86L (KU886187); *L. plantarum* 106L (KU886189) [12]. All these strains were grown in Man Rogosa and Sharpe broth (Titan-Biotech®, India) and the strain *E. coli* ATCC 11775 in Mueller Hinton broth (Titan-Biotech®, India) at 37°C for 18-24 h. All the strains were conserved at -20°C in the suitable cultivation broth (MRS or Muller Hinton) containing 20% (v/v) glycerol.

2.2 PCR Detection of Acid and Bile Resistance Genes

The genetic screening was based on sets of genes involved in bile salt tolerance and pH survival. These genes are listed in Table 1 below. Direct colony PCR was performed in a total reaction volume of 50 µl containing 25 µl of 2x PCR master mix (Tsingke, China), 2µl (50 pmol) of primer and a speck of isolated bacterial colony. The amplification was performed as described by Stack et al. [15] and Vrancken et al. [16] and amplicons with Gold View™ were visualized after electrophoresis on 2% agarose gel.

2.3 Determination of Cell Surface Characteristics

2.3.1 Auto-aggregation

Auto-aggregation was assayed according to Solieri et al. [4] description with slight modification as follows. One ml of cell suspension (10^9 cfu.ml^{-1}) in phosphate buffer saline (PBS) was vortexed for 10 s and incubated at room temperature for 4 h and 5 h. After that, an aliquot (100 µl) of the upper suspension was taken and mixed with 500 µl PBS, to measure the absorbance at 600 nm. Auto-aggregation was expressed as the percent decrease in the absorbance after 4 h and 5 h relative to that of original suspension and determined using the equation:

$$\text{Auto-aggregation\%} = (1-A_t)/A_0 \times 100$$

Where A_t represents the absorbance at any time (4 or 5 h), and A_0 the absorbance at time t =0h.

2.3.2 Co-aggregation

Co-aggregation assays were performed according to the methodology described by Yadav et al. [17] with some modifications. The strain *E. coli* ATCC 11775 was taken as indicator microorganism. Overnight cultures of Lactobacilli in MRS broth and *E. coli* in Mueller Hinton broth were centrifuged (3500 rpm, 15 min) and the pellets obtained were washed twice with PBS solution (pH 6.0). They were re-suspended in the same buffer, vortexed and the absorbance at 600 nm was adjusted to 0.5± 0.05. Thereafter, equal volumes (2 ml) of Lactobacilli and *E. coli* suspensions were mixed and subsequently incubated at room temperature without agitation for 4 h. Control tubes contained 2 ml of the suspension of each bacterial species. After incubation, the upper phase of each suspension was carefully removed, and the absorbance (O.D 600 nm) was measured. The decrease in absorbance was considered as a measure of cell co-aggregation, which percentage was calculated as follow:

$$\text{Co-aggregation percentage} = [(OD_{Lac} + OD_{Eco}) - 2OD_{mix}/(OD_{Lac} + OD_{Eco})]X100$$

OD_{Lac}: optical density of Lactobacilli strain, OD_{Eco}: optical density of *E. coli*, OD_{mix}: optical density of mixture.

2.3.3 Hydrophobicity

The cell surface hydrophobicity of each strain was assessed by measuring microbial adhesion to hydrocarbons using the procedure described by Ekmekci et al. [18] with slight modifications. Briefly, cells at the stationary phase were centrifuged (3500 rpm, 15 min). The resulting pellet was washed twice with PBS, re-suspended in the same buffer and the O.D 600 nm was measured (A0). One milliliter of Chloroform or Hexane was then added to 5 ml of cell suspension, and mixed by vortexing for 2 min. Then, the water and Chloroform/Hexane phases were separated by incubation for one hour at 37°C. The aqueous phase was removed with care, and the O.D 600 nm was measured (A1). The percentage of the cell surface hydrophobicity was calculated using the formula:

$$\text{Hydrophobicity} = (1-A_1)/A_0X100$$

Table 1. Primers used for gene screening involve in pH and bile salt tolerance

General function	Gene	Predicted function	Primer Orientation Sequence (5' to 3')	Annealing temperature (°C)	Expected amplicons size (bp)	Relevant references
pH and bile salt survival	gtf	Glucan synthase	F: ACACGCAGGGCGTTATTTTG R : GCCACCTTCAACGCTTCGTA	58	374	19
	clpL	ATPase	F : GCTGCCTTYAAAACATCATCTGG R: AATACAATTTTGAARAACGCAGCTT	56	158	20

Table 2. Cell surface characteristics of the selected lactobacilli strains

Strains	Auto-aggregation		Co-aggregation	Hydrophobicity	
	t = 4h	t = 5h	t = 4h	Hexane	Chloroform
L. paracasei 62L	33.56±0.01^{ghiA}	63.36±3.65d^{efB}	32.10±1.85^{hi}	13.64±1.55^{eA}	18.50±1.16^{abB}
L. plantarum 63L	35.71±0.58^{jA}	67.23±1.82^{fghB}	21.95±0.92^{abc}	8.31±1.84^{bA}	61.26±0.46^{hB}
L. plantarum 64L	27.20±2.37^{cdA}	75.36±2.97^{iB}	24.61±1.50^{d}	29.25±0.18^{gA}	86.31±2.97^{iB}
L. plantarum 80L	28.15±0.36^{cdeA}	72.41±1.63^{fiB}	36.69±0.83^{j}	4.08±0.25^{aA}	15.89±0.19^{aB}
L. plantarum 84L	29.99±0.83^{efA}	60.35±2.52^{bcdB}	30.58±1.28^{fgh}	14.04±0.50^{eA}	18.40±0.83^{abB}
L. plantarum 85L	26.75±0.88^{cA}	56.99±0.51^{abB}	28.88±0.26^{ef}	9.95±0.25^{cA}	46.11±2.05^{fB}
L. plantarum 86L	31.99±0.72^{fghA}	64.11±1.97^{degA}	32.48±1.00^{hi}	10.51±0.39^{cdA}	42.65±2.03^{gB}
L. plantarum 106L	31.31±1.15^{fgA}	45.49±1.81^{jA}	23.05±0.92^{abcd}	5.20±0.59^{aA}	35.78±0.66^{cB}

[a] The values with different super script letters in a column differ significantly (P< 0.05)
[A] The values with different superscript letters in a line differ significantly (P< 0.05)

2.4 Safety Evaluation of the Lactobacilli Strains

2.4.1 Antibiotic susceptibility assay

Antibiotic sensitivity and resistance of the strains were analyzed according to the standardized broth microdilution method [19] using different antibiotic groups: β-lactams (penicillin G, ampicillin, and amoxicillin), tetracyclines (Tetracycline), Macrolides (Erythromycin) and Amphenicol (Chloramphenicol).

2.4.2 Haemolytic activity

Haemolytic activity was investigated as described by Gerhardt et al. [20].

2.4.3 Gelatinase activity

Gelatinase activity was carried out according to Harrigan and Mc Cance [21].

2.4.4 PCR detection of biogenic amines production genes

For the detection of histidine decarboxylase gene (*hdc*), tests were carried out by PCR using pure bacterial colonies and the following specific primers: *hdc* F (5′-AGATGGTATTGTTTCTTATG-3′) and *hdc* R (5′-AGACCATACACCATAACCTT -3′). Direct colony PCR was performed in a reaction mixture (25 µl final volume) containing 12.5 µl of 2x PCR master mix (Tsingke, China), 0.2 µM of each primer and a speck of isolated bacterial colony. The amplification was performed as described by Costantini et al. [22] and amplicons were analyzed as previously described.

2.5 Data Processing and Statistical Analysis

The assays were realized in triplicate, and statistical analysis of data obtained for the determination of cell surface characteristics was performed using the Graph Pad InStat software version 3.0.The differences were considered significant for P values <0.05. The set of variables was reduced by a factor extraction method using principal component analysis (PCA) in XLSTAT 2007.

2.6 Sequence Analysis of the BSH Gene

The amplicons of the BSH gene previously obtained were purified, and send to a commercial company for sequencing (http://www.ruibiotech.com). The possible chimeras were identified and trimmed using ChromasPro 1.7.7 software. These sequences were aligned with similar sequences present in the National Center for Biotechnology Information (NCBI) gene collection using BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein sequences were obtained after determination and selection of Open Reading Frame (OFR) on sequences using OFR finding. Finally, the reading nucleotide sequences were deposited in the GenBank database to obtain accession number.

2.7 Homology Modeling BSH

To determine the 3D structure of *L. plantarum* 86L BSH, the protein sequence was blasted against the protein data bank (PDB) database. Using the intensive mode, the Protein Homology/analogY Recognition Engine V 2.0 (Phyre2), was used to predict the homologous structures of *L. plantarum* 86L BSH [23]. The validation of the selected predicted protein structure was done using protein structure validation (PSVS) tool 5. The quality structure was analyzed using Ramachandran plot. Then, predicted residues were compared with the templates.

3. RESULTS

3.1 Cell Surface Characteristics of the Selected Lactobacilli Strains

The selected strains based on their pH and bile tolerance were examined *in vitro* for their surface properties to appraise their cell adherence capacity. These results showed that auto-aggregation of Lactobacilli strains increased with the increase of incubation time. Among the strains, *L. plantarum* 64L exhibited the significantly highest auto-aggregation percentage (75.36±2.97) not significantly different from 72.41±1.63% of *L. plantarum* 80L after 5 h of incubation. All the selected strains showed auto-aggregation percentages higher than 50% after 5 h of incubation except the lowest strain *L. plantarum* 106L (45.49±1.81).

Cell co-aggregation properties involve probiotic's interaction with surface components of pathogenic bacteria. The results showed that all the strains were able to co-aggregate with the pathogen *E. coli* ATCC 11775 tested, although the percentages varied significantly according to the Lactobacilli strain with ranged from

21.95±0.92 to 36.69±0.83% (Table 2). Strain *L. plantarum* 80L showed highest co-aggregation potential after a 5-h incubation period, followed by *L. plantarum* 86L (32.48±1%) and *L. plantarum* 62L (32.1±1.85) which were not significantly different (*P*> 0.05). All the Lactobacilli strains showed more cell surface hydrophobicity in the presence of chloroform than hexane. All strains when treated with chloroform showed cell surface hydrophobicity ranging from 15.89±0.19 to 86.31±2.97%. The strain *L. plantarum* 64L showed the significantly highest hydrophobicity percentage for both chloroform and hexane (86.31±2.97 and 28.69 ± 1.60, respectively).

3.2 Safety Properties and Molecular Bases of Ph/Bile Tolerance. of Selected Lactobacilli

All the selected Lactobacilli strains were subjected to safety properties such as antibiotic susceptibility, hemolytic and gelatinase activities as well as gene detection of biogenic amines production. The next table reports the MIC values of selected Lactobacilli strains to different antibiotic groups: β-Lactams which are cell wall inhibitors (penicillin G, ampicillin, and amoxicillin), tetracycline family (Tetracycline), Macrolide family (Erythromycin) and phenicoled family (Chloramphenicol) which are all protein synthesis inhibitors. None of the strains exhibited resistance to one of the antibiotics used (Table 3 Also, from all the strains tested, none exhibit ß-hemolytic (i.e.no hemolysis) when grown on blood agar or gelatinase activity. The PCR detection of genes involved in biogenic amines production shown that *hdc* gene was not presented in the genome of all strains screened. All the eight strains tested showed the *clpL* gene encoding ATPase and none for *gtf* gene (Table 4).

3.3 Principal Component Analysis of the Phenotypic Characteristics Related to Cell Adhesion Characteristic

The strains were submitted to PCA using, auto-aggregation, co-aggregation after 5 h, hydrophobicity in hexane and chloroform as variables. According to Kaiser's rules, the factors F1 and F2 were chosen as axes for representation. The contribution of variables to factors and correlation between variables were studied and presented in Table 5. It reveals that the hydrophobicity in the two hydrocarbons is correlated to axis F1 which explains 54.009% of the variability. However the co-aggregation and auto-aggregation are correlated to axis F2 (34.102% variability). About variables, Two hydrophobicities were the most correlated (Pearson ratio= 0.629), followed by hydrophobicity in hexane and auto-aggregation. Negative correlations were observed between co-aggregation and all hydrophobicities.

The projection of the variables onto the plane formed by the two selected axes F1 and F2 (Fig. 1) represents these relationships.

Table 3. Antibiotic susceptibility of the lab strains

Strains	MICs (µg/ml)					
	Peni	Ampi	Amox	Ery	Chloram	Tétra
L. paracasei 62L	4	2	1	0.5	2	1
L. plantarum 63L	2	0.5	0.125	0.5	2	2
L. plantarum 64L	4	2	1	0.5	2	2
L. plantarum 80L	2	0.125	0.125	0.5	2	2
L. plantarum 84L	4	2	0.5	0.5	2	2
L. plantarum 85L	4	0.5	0.5	0.5	2	2
L. plantarum 86L	0.5	0.125	0.125	0.5	1	1
L. plantarum 106L	4	0.125	0.25	0.5	1	1
Breakpoint*						
paracasei Sp	4	2	nd	1	4	4
plantarum Sp	4	2	nd	1	8	32

MICs: Minimal inhibitory concentrations, nd: not defined, Peni: penicillin, Ampi: ampicillin, Amox: amoxicillin Ery: erythromycin, Tetra: tetracycline, Chloram: chloramphenicol, Strepto: streptomycin

**according to the MIC breakpoints of Danielsen and Wind [24] for penicillin, and to the EFSA's MIC breakpoints for the remaining antibiotics [25]*

Table 4. Other safety properties and molecular bases of pH/bile tolerance of selected Lactobacilli

Strains	Hemolytic activity	Gelatinase activity	Biogenic amines gene production (hdc)	Ph and bile tolerance genes	
				ClpI	gtf
All the 8 Lactobacilli strains	-	-	-	+	-

- = Negative or absent; + = Present

Table 5. Pearson ratio of correlations between variables as well as variables and factors

Variables\Factors	F1	F2	Hydro. hexane	Hydro. chloroform	Auto-agg 5 h	Co-agg 5 h
Hydro. hexane	0.828	0.297	1			
Hydro. chloroform	0.941	-0.173	0.629	1		
Auto-aggregation 5 h	0.464	0.819	0.483	0.329	1	
Co-aggregation 5 h	-0.612	0.759	-0.235	-0.685	0.279	1

Thus, axis F1 is related to the hydrophobicities, while axis F2 is related to auto and co-aggregation. The same figure also shows the projection of the strains onto the plane formed by these axes. It appears that PCA allowed for the separation of 4 main groups. The first group, characterized by high values of all the parameters studied and positioned to the positive sides of F1 and F2, is composed of strain *L. plantarum* 64L. The second group characterized by high values of auto and co-aggregation, as they fall on the positive side of F2 and the negative side of F1 constituted of four strains: *L. paracasei* 62L, *L. plantarum* 80L, 84L, and 86L. The strain *L. plantarum* 63L constitutes the third group characterized by high values of hydrophobicities. The last group consists of strains *L. plantarum* 85L and 106L and characterized by low values of hydrophobicities, auto and co-aggregation.

3.4 BSH Genes Characterization

Among the eight strains segregated in four groups by PCA, five have been presented BSH lp1 gene in our previous study. These genes have been sequenced in this study. The sequences obtained have translated and deposited in NCBI GenBank under the following accession numbers (Table 6).

From these five strains, three of them namely *L. paracasei* 62L, *L. plantarum* 84L and 86L have presented good auto and co-aggregation properties and belonged to the second group based on PCA. Inside this group, *L. plantarum*86L have been previously recorded the highest significant BSH activity (93 U/mg in Oxgall presence). For these reasons, *L. plantarum*86L have been selected for BSH characterization.

3.4.1 Identification of the BSH gene from *L. plantarum* 86L

Based on the BLASTn results, putative BSH gene sequence of *L. plantarum* 86L displayed 100% identity with the BSH1 gene sequence of *L. plantarum* KLDS10344 (BSH gene accession number: KR075715.1), the conjugated bile acid hydrolase gene of *L. plantarum* (M96175.1) and

Table 6. Nucleotidic/amino lengths of Lactobacilli Bsh genes and their accession numbers

Strains	Sequence length after sequencing	Sequence length from selected OFR	Amino-acids	NCBI accesion number
L. paracasei **62L**	947 bp	840 bp	279	MF098535
L. plantarum **84L**	941 bp	864 bp	287	MF098536
L. plantarum **85L**	801 bp	801 bp	266	MF098537
L. plantarum **86L**	729 bp	726 bp	241	MF098538
L. plantarum **106L**	816 bp	642 bp	213	MF098539

OFR= Open Reading Frame

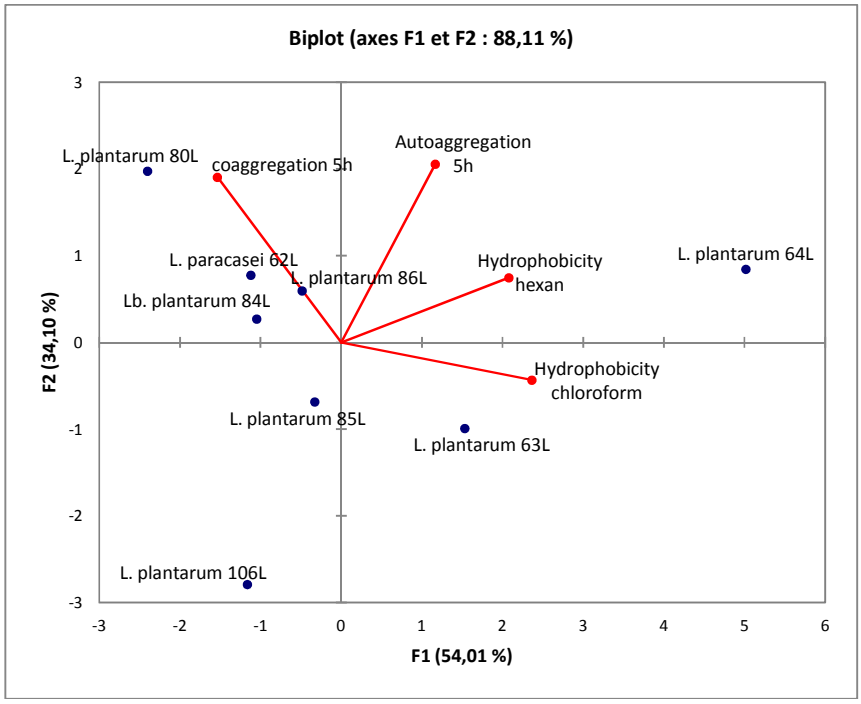

Fig. 1. Projection of the variables and strains onto the plane formed by the two factors (F1 and F2) obtained from PCA

99% with BSH1X4 gene of *L. plantarum* (KX530174.1). The BSH gene sequence of *L. plantarum* 86L (729 bp) encoding a 242 amino acid protein. The amino acid sequence of the *L. plantarum*86L BSH was aligned with amino acid sequences of available BSH crystal structure from bacterial of different species. *L. plantarum* 86L shared a sequence identity of 72% with *Enterococcus faecalis* BSH (EfBSH; 4WL3) a choloylglycine hydrolase family (EC 3.5.1.24), 57% with *L. salivarius* BSH (LsBSH; 5HKE), 40% with *Clostridium perfringens* BSH (2RLC), and 37% with *Bifidobacterium longum* BSH (2HEZ).*L. plantarum* 86L BSH also shared the lowest identity of 37% with penicillin V acylase (PVA) of *Lysinibacillus sphaericus* (2PVA) (Supplementary Fig. S1). The similarities of conserved amino acid residues such as Asn 79, Asn 171, and Arg 224, were revealed by the multiple sequence alignment results among all of the selected BSHs (Fig. 2).

3.4.2 Homology modelling of *L. plantarum* 86L BSH

Using Phyre 2, protein BLAST search of *L. plantarum* 86L BSH amino acid sequence was aligned with others amino acid sequences of BSH from different bacterial species with available BSH crystal structure in Protein Data Bank. This alignment showed that our amino acid sequence shared identity percentages of 72 with the BSH of *E. faecalis* (4WL3) and 52 with the BSH of *L. salivarius* (5HKE). *L. plantarum* 86L BSH also shared a sequence identity of 40 % with *Clostridium perfringens* BSH (2RLC), and 37% with both *Bifidobacterium longum* BSH (C2HEZ), and PVA of *Bacillus subtilis* (C2OQC). The same observations were revealed when SWISS-MODEL was used. Thus, the homology model of *L. plantarum* 86L BSH (Fig. 3) was predicted using SWISS-MODEL with *E. faecalis* BSH (4WL3) as a template, because it had a 100% confidence level and the highest identity percentage (Supplementary Fig. S1).

The secondary structure prediction of using Phyre 2 software revealed that predicted *L. plantarum* 86L BSH contains 26% of -helix, 24% of the beta strand, and 18% of disorder (Supplementary Fig. S2). This structure was similar to *E. faecalis* BSH. The Protein Structure Validation Suite (PSVS) analysis results of predicted *L. plantarum* 86L BSH structure suggested that the model was of good quality. The Ramachandran plot analysis showed that 88.1% of residues lie in the most favored regions; 11.4 and 0.5% of residues lie in allowed

and disallowed regions, respectively. The value of Procheck G-factor (all dihedral angles) was -0.1, and the VERIFY3D results showed that 78.1% of the residues had an averaged 3D-1D score ≥ 0.2, thus confirming that the predicted *L. plantarum* 86L BSH structure contained no conformational errors. The overall Z-score of the ProSA analysis was -7.01. Using ERRAT, the overall quality factor of the predicted86L BSH model was 96.095, which further confirmed its quality. The modeled *L. plantarum* 86L BSH protein structure was submitted to Protein Model Data Base (PMDB) with the model id PM0081288.

4. DISCUSSION

Our previous works reported the isolation of 12 Lactobacilli strains from the water of cassava's fermentation with high resistance to acid and bile tolerance. They also presented great bile salt hydrolase activities of strains in Oxgall and Taurodeoxycholate acid. But, their safety aspects, molecular bases of their reported acid and bile tolerances, as well as the characterization of the enzyme involved in recorded BSH activities were not investigated [12]. The present work was then undertaken to provide more information on the above raised

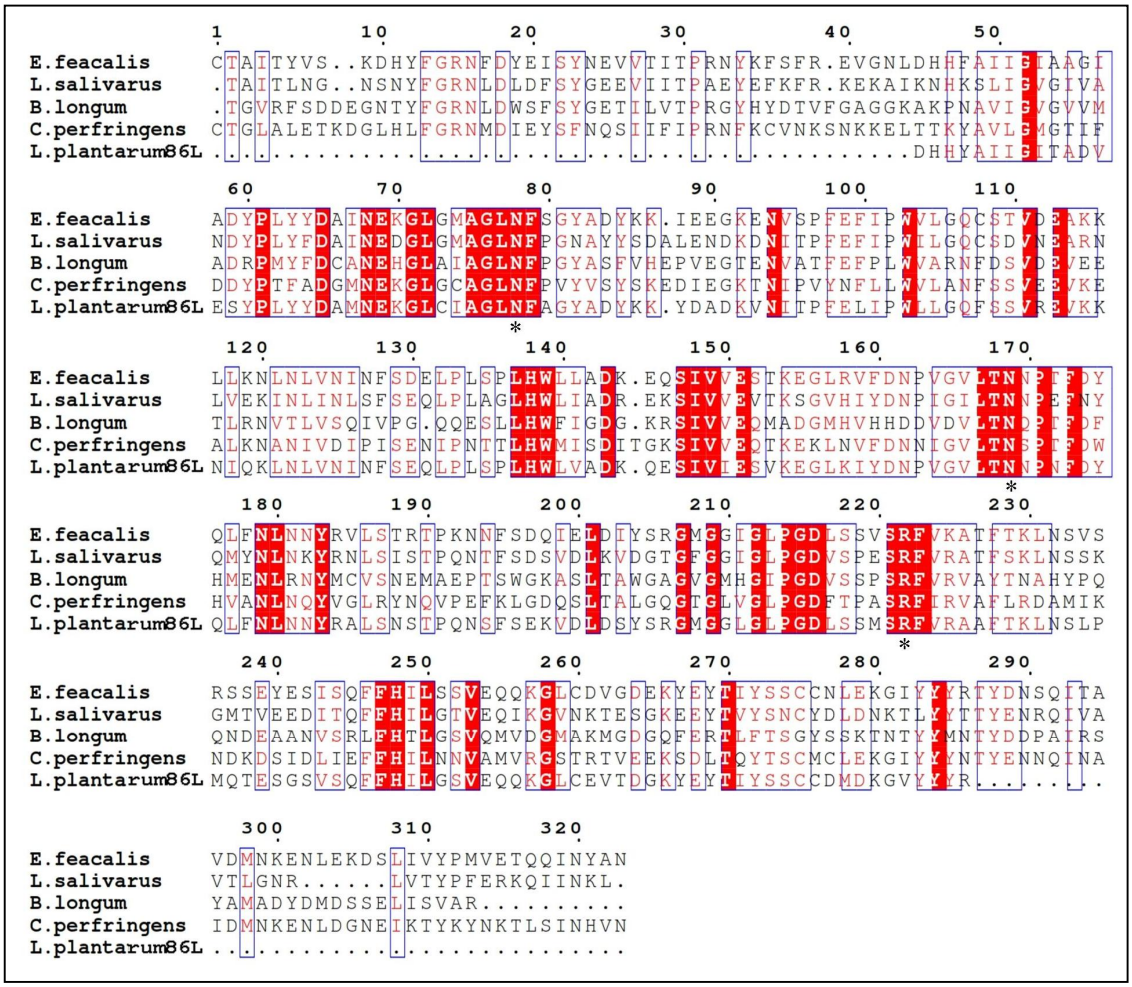

Fig. 2 Multiple sequence alignment of bile salt hydrolase (BSH) amino acid sequences from different bacterial species with *L. plantarum* 86L BSH.

The protein sequences were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) tools and identical residues were identified by ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/). The identical residues are shown in red background and the similar residues are shown as red color. The conserved residues here(Asn-79,Asn-171, and Arg-224) were marked as black asterisk

concerns. The results showed that none of all the eight strains screened showed *gtf* gene, meaning that their defense mechanism against acidity and bile stresses is not related to glycosyltransferase enzyme. This gene absence in the genome of strains can be explained by her phylogenetic distribution. Similarly, Turpin et al. showed that from the 38 isolates tested; none presented *gtf*gene [26]. The *clpL* gene was present in the genome of all the eight tested strains. This gene seems more distributed than *gtf* gene and could, therefore, play an important role in acid and bile tolerances. Indeed, studies have shown that *clpL* gene inactivation in *L. reuteri* ATCC 55730, resulted in a significant decrease of bacterial survival after incubation at pH 2.7 [27] or in medium containing 0.3% bile salts [28]. Another study reported the predominance of the *clpL* gene in 91% of the acidic tolerant isolates from their bacterial collection obtained from fermented pearl millet slurry and fermented starchy foods [26].

Fig. 3. Predicted three-dimensional structure of *L. plantarum* 86L BSH obtained from homology modeling with *Enterococcus faecalis* BSH (4WL3) as template using SWISS Model

Also, to resist hard conditions of gut through which it transits, a probiotic strain should colonize and adhere to the intestinal epithelial cells, where its exerts expected benefices. Hexane (apolar solvent) and chloroform (acidic solvent), were used to evaluate the hydrophobic/hydrophilic and electron acceptor (basic) characteristics of a bacterial surface, respectively [29,30]. All our probiotic Lactobacilli strains showed more affinity to chloroform than hexane. Zeraik and Nitschke in 2012 [31] reported the same higher affinity to chloroform than hexane with their bacterial strains. The higher affinity to chloroform was an indicative of the predominance of basic properties on the cell surface of the tested strains. The cells surface properties can permit to explicate the adhesion ability in some conditions, however the influence of other factors, like surface charge, exopolyssacharide production and the presence of flagella or fimbriae should also be taken into consideration. Thus, it becomes difficult to do generalizations concerning the adhesion process based only on some surface properties of the bacterial strains [31]. However, many studies suggested that strains with higher aggregation and hydrophobicity will have better chances to adhere to epithelial cells [32,33]. In this study, hydrophobicity in hexane seems to be taken with auto-aggregation (Pearson coefficient= 0.483). Auto-aggregation is an essential precondition for biofilm formation which also helps in adhesion and colonization of intestinal epithelial cells, whereas co-aggregation abilities with pathogens enable them to form an effective barrier that prevents adhesion of enteric pathogens on the intestinal epithelium [34]. In this study, except for strain *L. plantarum* 106L, all the strains tested showed high auto-aggregation abilities with percentages higher than 50% after 5 h of incubation. The highest recorded was 75.36± 2.97% from *L. plantarum* 80L. These results are similar to those of Nallala et al. [35] who reported strong auto-aggregation phenotype ranging from 60–80% with 13 Lactobacilli strains isolated from the crop, gizzard, intestine, and ceca of 7-week-old healthy broiler chickens in India. Our results suggest that our strains can colonize and adhere to epithelial cells. Contrary to auto-aggregation, co-aggregation implies the process of aggregation of bacterial cells of more than one type [36]. *In vitro* co-aggregation ability of strains with *E. coli* ATCC 11775 was studied and it was observed that all the strains tested showed varied co-aggregation abilities ranged from 21.95±0.92 to 36.69±0.83% after a 5-h incubation period. The strain *L. plantarum* 80L with 36.69±0.83% showed highest co-aggregation potential not significantly different ($p>0.05$) from 32.48±1% and 32.1±1.85 respectively from *L. plantarum* 86L and *L. paracasei* 62L. Similarly, Ramos et al. [37] reported that *L. plantarum* CH41 showed highest co-aggregation ability with *E. coli*. Furthermore, another recent study reported that three strains of *L. plantarum* isolated from Cameroonian fish

showed co-aggregation ability ranged from 9 to 25.83% with three common enteric pathogens [38]. Adding to barrier effect earlier mentioned, co-aggregation ability also ensures a close interaction of probiotic strain with a pathogen [39]. For the present strains with antimicrobial properties previously reported, it could have an added significance, as their antimicrobial mode of action against the co-aggregating pathogenic partner may allow its better elimination [40].

Many Lactobacilli usually used in foods as probiotics are recognized as safe (GRAS) and the qualified presumption of safety (QPS). Nevertheless, some recent reports have linked some Lactobacillus species to possible clinical cases [41]. Consequently, it is not useless to examine or verify the safety properties of new probiotic Lactobacilli candidates before their use in human or animal feeding. A probiotic strain with hemolytic activity could break down the epithelial layer while gelatinase activity could disturb the mucoid lining, and interfere with the normal functioning of these important linings. None of our strains has presented these two abilities. These absences of hemolytic and gelatinase activities are considered as none virulent evidence [9]. Many others authors have reported similar results, where different Lactobacilli strains from different African traditionally fermented foods were negative for hemolytic and gelatinase activities [2,3,42]. Besides, the screening *hdc* gene involved in the production of histamine (a biogenic amine) reveals that this gene was not found in the genome of all assayed strains. The absence of *hdc* gene is a positive or safety characteristic given the potential deleterious effect on the health of large amount of biogenic amines [26]. About, antibiotic susceptibility, the main threat associated with an antibiotic resistant probiotic is the risk of horizontal transfer of the corresponding acquired genetic determinants to commensal pathogenic bacteria, thus impairing successful antibiotic treatment of common microbial infections in human or animal [1]. Antibiotic-resistant microorganisms used as probiotics or not can, therefore, act as "reservoirs" of resistance genes, since they can colonize the human gastro intestinal tract through the food chain [43]. None of our assayed strains exhibited resistance to the antibiotics. Similar results were reported in Lactobacillus species. The strong susceptibility of our strains could be related to their origin ecosystem. Indeed, contrary to our results, Comunian et al., reported Lactobacilli strains of the same species than our owns, which have a high resistance to tetracycline and the erythromycin. However, the majority of their *L. paracasei* strains resistant to tetracycline and erythromycin originated from animal products in geographical zones where systematic use of antibiotics as growth promoters was carried out over the years in animal husbandry [1].

As result of BSH characterization, *L. plantarum* 86L BSH showed sequence identity percentages with BSH sequence of bacteria from different species, highest recorded (72%) with *E. feacalis*. It also shared the same identity percentage (37%) with *B. longum* BSH and PVA of *Bacillus subtilis* (C2OQC). Indeed, the two enzymes belong to the choloylglycine hydrolase family [44]. As BSH and PVA evolved from the same origin, it has reported that the two enzymes have the same important catalytic residues in the active site (Cys1, Arg16, Asp19, Asn79, Asn171, and Arg224) except for Asn79, which is replaced with tyrosine in PVA [45]. The identification of this Asn79, in our sequence, confirms its BSH membership [46]. Although the *L. plantarum* 86L BSHs have the same catalytic sites to other bacterial genera or Lactobacilli species, their substrate-hydrolyzing capabilities are not the same [12]. It has been suggested that most evolution occurs to broaden substrate specificity [47]. Thus, as well as BSHs from most bacterial species, our *L. plantarum*86L BSH seems to have substrate preferences toward glyco conjugated bile salts rather than tauro-conjugated ones [12]. The major reasons for these phenomena include an abundance of glyco-conjugated bile salts in nature and the steric encumbrance of taurine caused by its sulfur atom [48]. About the quality of the predicted 86L BSH model, her overall Z score of the ProSA analysis was -7.01 which is in the range of scores typically found for native proteins of similar sizes [49]. The overall quality factor of the predicted *L. plantarum* 86L BSH model calculated by ERRAT was 96.095, which further confirmed the quality of the predicted structure. This factor quality was higher than 90.033 of *L. gasseri* BSH model [44] and comparable to 96.349 of *L. plantarum*RPYR1which represent a stable structure [14]. Thus, the predicted 86L BSH model can be considered as good.

5. CONCLUSION

This study aimed to assess the genotypic basis of acid and bile tolerance, safety and adhesion properties of previously reported probiotic

Lactobacilli strains from the water of cassava's fermentation. The functional properties of BSH proteins were also studied. The eight selected strains assayed in this study presented good cell surface characteristics testifying their excellent adhesion and colonisation abilities. Furthermore, they do not possess virulence factors and antibiotic resistance, thus confirm their safe character for probiotic applications. Considering all variables studied here, the most promising probiotic candidates based on PCA were *L. paracasei* 62L, *L. Plantarum* 84L and 86L with good auto and co-aggregation properties. Phenotypically, the last strain was reported to exhibit the best BSH activity higher in glycoconjugate bile than taurocholate. The study of the predicted 3D structure of *L. Plantarum* 86L BSH revealed that it had strict conservation in its catalysis sites and had structural similarity with previously identified BSH enzymes with substrate preference found to be more inclined towards glyco -conjugated bile than other conjugated bile.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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