Original Research Article

Evaluation of Probiotic Potential of Lactic Acid Bacteria Isolated from Raphia Palm Wine (*Raffia mambillensis*)

ABSTRACT

Aim: The present study was undertaken to assess the probiotic potential of lactic acid bacteria strains isolated from raphia palm wine (*Raffia mambillensis* O.) of the South West region of Cameroon.

Study design: The study site and sample were randomly chosen and the responses assessed were submitted to analyses of variance.

Place and Duration of Study: The study was carried out in Ekona in the South West region of Cameroon, between February 2017 and October 2018.

Methodology: Physicochemical analyses were performed to characterize the Raphia palm wine samples. Lactic acid bacteria were isolated from these samples using pour plate method, and the isolates were tested for their antimicrobial activity, low pH and bile salts tolerance, antibiotic sensitivity, hemolytic activity and enzymes production. The active isolates were identified phenotypically using API 50 CHL.

Results: The results revealed that among the nine isolates, only three were active against *Escherichia coli* BL21, *Escherichia coli*, *Salmonella typhi*, *Salmonella enterica* and *Staphylococcus aureus* with inhibition diameters ranging from 10 to 25 mm. The three isolates which exhibited antimicrobial activity were able to survive at simulated stomach pH (pH 3.0) with survival rate above 80% and in the presence of bile salts at 1% with survival rates above 50%. All three isolates gave negative results for hemolytic activity and were resistant to most antibiotic treatments. These three isolates produced β-galactosidase, which is beneficial for lactose intolerance. They also produced some non-carcinogenic enzymes including leucine arylamidase, crystine arylamidase, acid phosphatase, α-galactosidase, α-glucosidase, β-glucosidase, and N-acetyl-β-glucosamidase. Identification of these three isolates with API kit 50 CHL reveals that they are *Lactobacillus lactis*, *Lactobacillus plantarum* and *Lactobacillus brevis*.

Conclusion: Our findings revealed that raphia palm wine can be an excellent source of probiotic lactic acid bacteria.

Keywords: Raphia Palm Wine, Lactic acid bacteria, Probiotics, Antimicrobial activity, Bile tolerance, Acid tolerance

1. INTRODUCTION

Raphia palm wine is a traditional alcoholic beverage widely distributed in the Cameroon. It is produced by natural fermentation of palm sap collected from various raphia palms such as *Raffia mambillensis*, *Raffia africanna* and *Raphia hookeri* [1]**.** The main microorganisms in raphia palm wine are yeast and bacteria mainly lactic acid bacteria (LAB) which is the dominant bacterial population responsible for the spontaneous fermentation of raphia palm sap [2]**.** Lactic acid bacteria comprise a large and diverse group of catalase negative, non-

spore forming, non-motile, gram positive cocci or rod shape bacteria, capable of producing lactic acid as the main end-product of the fermentation of carbohydrates. Lactic acid bacteria strains are important microorganisms used in food fermentations as they improve safety and stability of fermented products by extending the shelf life (Inhibit the growth of food spoilage microorganisms) and provide new organoleptic properties to fermented products as well as health benefits as a result of their probiotic properties [3,4]. Probiotic bacteria are live microorganisms which when administered in adequate amounts confer a health benefit on the host [5]. In order to exert their beneficial effect, probiotic bacteria should be capable of surviving passage through gastrointestinal tract the (GIT). Thus, it is essential for the bacteria to have protection systems to show high tolerance at the low pH in the stomach, digestive enzymes and bile salts of the small intestine [6]. Another important criterion for lactic acid bacteria to be considered as probiotic bacteria includes the ability to adhere to the intestinal epithelium cell and the ability to inhibit the pathogenic bacteria by producing antimicrobial substances such as lactic acid, hydrogen peroxide and bacteriocins [7]. For lactic acid bacteria present in raphia palm wine to provide health benefits, they need to be isolated and identified as potentially probiotic microorganisms. But this fermented raphia palm wine is one of the most neglected fermented alcoholic drink in Cameroon as prolong fermentation renders the drink unfit for consumption and thus most persons discard the drink few days after been tapped. Furthermore, despite the numerous strains of probiotic bacteria isolated from different sources including raw fruits and fresh vegetables [8], palm wine [9,10], milk and dairy products [11], “tarkhineh” [12] fermented table olives [13] and processed corn beer [14], but to the best of our knowledge, very few studies regarding the evaluation of probiotic properties and safety assessment of lactic acid bacteria isolated from raphia palm wine have been performed [10]. Therefore, the objective of this study was to evaluate the probiotic potential of lactic acid bacteria strains isolated from raphia palm wine (*Raffia mambillensis* O.) of the South West region of Cameroon.

2. MATERIAL AND METHODS

2.1 Sample collection

Freshly tapped raphia Palm Wine (*Raffia mambillensis* O.) were collected from local palm wine tappers in Ekona in the South West region of Cameroon. The samples were introduced into sterile bottles, kept in an icebox at 4°C and transported to the laboratory of Biotechnology of the University of Buea and allowed at room temperature to undergo fermentation for 7 days before analysis.

2.2 Pathogenic cultures

The pathogenic cultures used in this study were provided by the laboratory of Biotechnology of the University of Buea (Cameroon). They were *Staphylococcus aureus, Escherichia coli* BL21, *Salmonella enterica, Escherichia coli,* and *Salmonella typhimurium*. The strains were sub-cultured in Luria Bertani broth (LB) (LiofilChem, Italy) and kept at 4°C.

2.3 Physicochemical analyses of raphia palm wine samples

During fermentation, some physicochemical parameters of the raphia palm wine like pH and titratable acidity were measured every day. pH measurement was carried out in triplicates on 5 mL of sample diluted in 45 mL of distilled water using a digital pH meter (Oakion pH/MV/°C/°F meter). The titratable acidity of sample was determined according to the method described by [15]. 10 mL of sample was titrated against 0.1 N sodium hydroxide solution to a pink end-point using phenolphthalein as indicator. The titratable acidity was expressed as percentage of lactic acid using the formula:

$$\text{Titratable acidity (TA)} = (V1 \times N \times \text{Eq. Wt})/(V2 \times 10) \quad (1)$$

Where N = normality of NaOH, V1= volume of NaOH, V2 = volume of sample and Eq.Wt = equivalent weight of predominant acid (lactic acid).

2.4 Isolation and identification of lactic acid bacteria

LAB strains were isolated from fermented raphia palm wine using the dilution pour plate method. 25 mL of sample were added in 225 mL of sterile saline solution (NaCl, 0.85%, w/v). The obtained solution was serially diluted (10^{-1} to 10^{-5}) and 1 mL of each dilution was aseptically pipetted into sterile Petri dishes followed by addition of 10 mL of Man Rogosa and Sharpe agar (MRS) (LiofilChem, Italy) at 45°C. After homogenization, the inoculated plates were then allowed to cool at room temperature before being incubated at 30°C for 48 hours under anaerobic conditions. At the end of incubation, a preliminary catalase reaction test and gram staining was carried out. Catalase negative and Gram positive colonies which appeared on the plates were picked and sub-cultured three times on freshly prepared MRS agar to obtain single pure colonies. For long time storage, the isolates were kept at −80°C in MRS broths containing 15% (v/v) glycerol. These pure colonies were further identified based on their macroscopic and microscopic traits like colony elevation, shape, color, texture and Gram staining [16], their physiological characteristics such as growth at different temperatures (10, 15, 37 and 45°C), pH (1.5, 2, 2.5, 3, 4.4, 6 and 9.6) and NaCl concentrations (2.5, 6.5, 10, and 18% w/v) [17] and their biochemical traits like catalase, oxidase, indole production, methyl red test, Voges-Proskauer test, citrate utilization, and urease [18].

2.5 Evaluation of probiotic properties of isolates

In order to find out the ability of LAB isolates as probiotic agent, major selection criteria such as resistance to low pH, tolerance against inhibitory bile salt and the antimicrobial activity in this regard were chosen for the determination of probiotic properties of the isolates.

2.5.1 Antimicrobial activity of the isolates

Antimicrobial activity of the presumptive LAB isolated from raphia palm wine was determined by the disc diffusion method according to the protocol described by [19]. For this, one purified colony of each presumptive LAB isolate was cultured for 18 hours at 30°C in 10 mL of MRS broth. Later, culture was centrifuged (10 000g,10 min, 4°C) and cell-free supernatants were collected, filtered (0.45µm) and were used for antimicrobial test. 15 mL of sterile Mueller-Hinton agar (LiofilChem, Italy) were poured in Petri dishes. After solidification, the plates were seeded with 0.1 mL of 18 hours pathogenic culture at 5×10^5 cfu/mL (0.5 McFarland standard). Afterwards, sterile discs (6 mm of diameter) dipped into the cell free supernatants for impregnation were placed on the surface of inoculated MH plates. Disc dipped in sterile MRS broth was used as control. The plates were incubated at 37°C for 24 hours. After incubation, inhibition zones (clear zones around the discs) were measured. The LAB isolates with important antimicrobial activities were further evaluated to determine the other probiotic properties.

2.5.2 Tolerance to low pH

Survivial under acidic conditions of the LAB isolates which deserved antimicrobial activity was determined following the modified method of [10]. The LAB isolates were cultured in MRS broth at 37°C for 18h, followed by centrifugation at 5000g for 10 min at 4°C. Cells were washed twice with sterile phosphate bu□ered saline (PBS) and suspended in 10 mL of sterile PBS. 0.1 mL of cells suspension containing 10^7 cfu/mL was inoculated into 10 mL of 0.05 M sodium phosphate buffer with varying pH of 1.0, 2.0, 3.0 and 7.0. Cell counts, performed in triplicates, were calculated by counting the number of colonies on MRS agar after incubation at 37ºC for 24 hours and expressed as log 10 values of colony forming units per mL (cfu/mL). Survival rates were calculated using the following formula

$$\text{Survivial (\%)} = \frac{\text{Final viable counts after treatment (cfu/ml)}}{\text{Initial viable counts before treatment (cfu/ml)}} \times 100 \qquad (2)$$

2.5.3 Bile salts Tolerance

Tolerance to bile salt of the LAB isolates which deserved antimicrobial activity was determined by the method of [20]. The LAB isolates were cultured in MRS broth at 37°C for 18 hours. After incubation, bacteria cells were harvested by centrifugation for 10 min at 10000 rpm and 4°C using a Rotofix 32A centrifuge. Pellets were washed trice with sterile phosphate buffer saline and resuspended in PBS. A 100 µL of cells suspension was inoculated into 5 ml of sterile MRS broth containing 0.3, 0.5 and 1% (w/v) oxgall-bile (Sigma) and incubated at 37°C for 4 h. Cell counts were determined by plating on MRS agar as described previously.

2.6 Safety assessment

2.6.1 Antibiotic sensitivity

The antibiotic susceptibility was evaluated by the standard disc diffusion method as described [21]. Discs impregnated with the most important clinically antibiotics such as Ampicillin: 10 µg/disc, Tetracycline: 30 µg/disc, Kanamycin: 30 µg/disc, Streptomycin: 30 µg/disc, Vancomycin: 30 µg/disc, Gentamycin: 30 µg/disc, Chloramphenicol: 30 µg/disc, Erythromycin: 15 µg/disc, Cefataxime: 30 µg/disc, Ciprofloxacin:5 µg/disc were placed on the surface of MRS agar seeded with 24 hours cultures of the different LAB isolates (5×10^5 cfu/mL). Plates were incubated at 37◦C for 24 h. After incubation, plates were examined for the presence of inhibition zones around the antibiotic discs. The zone diameter of inhibition (ZDI) were measured. Isolates where categorized as sensitive (ZDI; ≥21 mm), intermediate (ZDI; 16-20 mm), or resistant (ZDI; ≤15 mm)

2.6.2 Analysis of Enzyme Activity of Lactic acid bacteria isolates

Enzyme activities of the LAB isolates were determined using the API -ZYM kit (Bio-Mérieux, France) following the manufacturer's instructions. Cupules of the API ZYM strips were inoculated with a 24 hours culture in MRS broth of the different LAB isolates which deserved antimicrobial activity and then incubated at 37°C for 4 hours. The reaction was carried out by the addition of the reagents (ZYM A and ZYM B). The enzymatic activity was graded from 1 to 5 according to the colour reaction chart. The approximate number of free nmol hydrolyzed substrate may be obtained from the colour intensity, 0: no activity; 1: liberation of 5 nmol; 2: liberation of 10 nmol; 3: liberation of 20 nmol; 4: liberation of 30 nmol and 5: [greater than or equal to] 40 nmol [22].

2.6.3 Hemolysis test

Hemolytic activities of the LAB isolates which deserved antimicrobial activity were evaluated using the method of [23]. The LAB isolates were cultured in MRS broth at 37°C for 15 hours and then transferred onto blood agar plates supplemented with 5% sheep's blood. The inoculated plates were incubated at 37°C for 24 hours. Hemolytic activity was detected as the presence of a clear zone around bacterial colonies (β-hemolysis).

2.7 Phenotypic identification of LAB isolates

Identification of the LAB isolates which deserved probiotic features at species level was done using the API 50CH kit (BioMérieux, France) following the manufacturer's instructions. The API 50 CH is a standardized system that associates the fermentation of 50 carbohydrates to bacteria species. It is used for the identification of *Lactobacillus* and related genera. Isolates grown for 24 h at 30^{o}C in MRS broth, were centrifuged (10000g for 5 min) and the collected cells were washed with sterile saline and resuspended in 5 mL API 50 CHL medium at a final concentration of 2 McFarland. That solution was used to fill the cupules of API 50 CHL followed by incubation at 35°C for 48 hours. The biochemical profile obtained for each LAB isolates was analyzed using the API identification software database (APILAB PLUS), Version 5.

2.8 Statistical Analysis

All analyses were carried out in triplicates. Analysis of variance was performed by one-way ANOVA procedures using SPSS 16.0 and Minitab 16.0. The least significant difference (LSD) was used to test for difference between means and significance was defined at $P<0.05$. Results are reported as mean values ± Standard Deviation.

3. RESULTS AND DISCUSSION

3.1 Physicochemical properties of raphia palm wine

The pH value of the freshly tapped raphia palm wine obtained in this study was 6.92 (Table 1). That value was close to pH of 7 reported in the literature for freshly tapped palm wine [24]. As time passes, the pH of raphia palm wine decreases until reach 3.48 at the 7th day of storage at room temperature. However, it comes from Table 1 that the titratable acidity of raphia palm wine increases as storage time progresses. These results indicate that palm wine undergoes spontaneous fermentation immediately after being tapped. As results of sugar's fermentation, organic acids are produced by lactic acid bacteria leading to decrease in pH and increase in titratable acidity. Similar observations were notified by [25] who found that, the pH of the palm wine ranged from 6.20 for freshly tapped palm wine to 2.86 by the 7th day of fermentation and titratable acidity of palm wine ranged from 1.96 for freshly tapped palm wine to 6.20 by the 7th day of fermentation.

Table 1. Evolution of pH and titratable acidity of raphia palm wine during fermentation

Time of fermentation (hours)	pH values	Titratable acidity (%)
0	6.92	1.99
24	5.39	2.31
48	4.99	2.66
72	4.45	3.01
96	4.23	3.34
120	4.12	4.68
144	3.92	5.79
168	3.48	6.51

3.2 Isolation and identification of lactic acid bacteria

Thirty-three isolates were obtained from fermented raphia palm wine. Table 2 presents the morphological, physiological and biochemical characteristics of the different isolates. After screening to eliminate catalase positive, oxidase positive and Gram-negative microorganisms, only nine isolates remained and were regarded as presumptive LAB (Table 2). The isolates where coded as follows SA, SB, SC, SD, SE, SF, SG, SH and SI. Among the 9 presumptive LAB isolates, 03 were rod-shaped and 06 were cocci-shaped. They were all Gram-positive, non-motile, oxidase negative and indole positive. They did not produce gas and ammonia and were positive to methyl red test and negative to Voges-Proskauer test and indole tests. These 9 isolates grew at temperatures of 10, 20, 25, and 37°C; cocci-shaped isolates grew at 45°C while rod-shaped isolates did not grow at 45°C (Table 2). Regarding NaCl and pH tolerance, all the 9 isolates did not grow at salinity of 18% and pH of 1.5 and 9.6. However, all the isolates grew well at pH 4.4 and pH 6; cocci-shaped cells did not grow at pH 2 and 2.5 but rod-shaped isolates grew at pH 2 and 2.5. Based on these results, the 3-rod isolates (SA, SF, SI) were identified as belonging to the genus *Lactobacillus*, 2 cocci-shaped isolates (SD, SE) belong to the genus *Pediococcus* and 4 remaining cocci-shaped isolates (SB, SC, SG, SH) belong to the genus *Leuconostoc*. LAB isolates belonging to the same genus as observed in this study were reported by [10], [14] and [26] among the 20, 14 and 35 LAB isolates that they were isolated from palm wine.

Table 2. General characteristics of isolates

Parameters		Isolates								
		SA	**SB**	**SC**	**SD**	**SE**	**SF**	**SG**	**SH**	**SI**
Colony morphology	Color	Whitish	Milky white	Milky white	Whitish	Whitish	Whitish	Milky white	Milky white	Whitish
	Texture	Coarse	Coarse	Coarse	Shinny	Coarse	Coarse	Coarse	Coarse	Coarse
	Edge	Smooth	Rough	Rough	Smooth	Smooth	Smooth	Rough	Rough	Smooth
	Elevation	Raised	Flat	Flat	Flat	Flat	Raised	Flat	Flat	Raised
Cellular morphology		Rods	Cocci	Cocci	Cocci	Cocci	Rods	Cocci	Cocci	Rods
Motility test		-	-	-	-	-	-	-	-	-
Gram staining		+	+	+	+	+	+	+	+	+
Catalase test		-	-	-	-	-	-	-	-	-
Oxidase test		-	-	-	-	-	-	-	-	-
methyl red test		+	+	+	+	+	+	+	+	+
Arginine test		-	-	-	-	-	-	-	-	-
Citrate utilization		-	+	+	+	+	-	+	+	-
Voges- Proskauer test		-	-	-	-	-	-	-	-	-
Urease test		-	-	-	-	-	-	-	-	-
Indole production		-	-	-	-	-	-	-	-	-
Growth at 10°C		+	+	+	+	+	+	+	+	+
Growth at 15 °C		+	+	+	+	+	+	+	+	+
Growth at 37°C		+	+	+	+	+	+	+	+	+
Growth at 45°C		-	-	-	-	-	-	-	-	-
Growth at pH 1.5		-	-	-	-	-	-	-	-	-
Growth at pH 2		+	-	-	-	-	+	-	-	+
Growth at pH 2.5		+	-	-	-	-	+	-	-	+
Growth at pH 3		+	-	-	-	-	+	-	-	+
Growth at pH 4.4		+	+	+	+	+	+	+	+	+
Growth at pH 6		+	+	+	+	+	+	+	+	+
Growth at pH 9.6		-	-	-	-	-	-	-	-	-
Growth in 2.5% NaCl		+	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl		+	+	+	+	+	+	+	+	+
Growth in 10 % NaCl		+	+	+	+	+	+	+	+	+
Growth in 18 % NaCl		-	-	-	-	-	-	-	-	-
Fermentation type		Hetero	Hetero	Hetero	Homo	Hetero	Homo	Hetero	Hetero	Homo
CO_2 production		+	-	+	-	-	-	-	+	+
Deduced Genus		*Lactobacillus*	*Leuconostoc*	*Leuconostoc*	*Pediococcus*	*Pediococcus*	*Lactobacillus*	*Leuconostoc*	*Leuconostoc*	*Lactobacillus*

3.3 Evaluation probiotics potential of LAB isolates

One of the criteria of a bacterium to be classified as a probiotic is its ability to inhibit the growth of harmful and pathogenic bacteria. LAB are well known producer of antimicrobial compounds which have high antimicrobial activity [27]. In this study, all nine LAB isolates from raphia palm wine were tested for antibacterial activity against some indicator microorganisms (*Staphylococcus aureus*, *Escherichia coli* BL21, *Salmonella enterica*, *Escherichia coli*, and *Salmonella typhimurium*). Only three isolates (SA, SF, SI) showed inhibitory activities against the pathogenic strains with *Salmonella typhi* being the most sensitive, followed by *Salmonella enterica, Staphylococcus aureus* and finally by *Escherichia coli* and *Escherichia coli* BL21. The antimicrobial activity against these pathogens was materialized by the formation of zones of inhibition around the disc impregnated with the cell free supernatants (CFS) from the three different LAB isolates. These zones of inhibition of the LAB isolates CFS are illustrated in Figure 1. The values of the diameter of the inhibition zone are summarized in Table 3. Among the test strains isolate SF showed the highest antibacterial activity against *Salmonella enterica* (25.29±0.92 mm), *Salmonella typhimurium* (25.34±1.36 mm), *Staphylococcus aureus* (24.82±0.41 mm), *Escherichia coli* BL21 (22.76±0.52 mm) and *Escherichia coli* (22.46±0.67 mm). Inhibition of *Salmonella enteric* subsp. *enterica* and *E. coli* by CFS of *Lactobacillus pentosus*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolated from palm wine was also reported in the literature [10]. The inhibitory activities of the three isolates SA, SF and SI observed in this study could be due to the ability of these isolates to produce bio-substances with antimicrobial properties such as bacteriocins, organic acids, hydrogen peroxide (H_2O_2) and diacetyl which could inhibit pathogenic microorganisms. [28] and [29] reported that lactic acid bacteria (LAB) produce substances that exert strong antagonistic activity against pathogenic bacteria, particularly those involved with food borne diseases. *Salmonella* sp. and *Escherichia coli* have been reported as leading cause of food borne illnesses and these pathogens have become very resistant to many antibiotics used in Cameroon [30]. Therefore, these antimicrobials substances produced by LAB isolated from raphia palm wine can be exploited for the treatment and prevention of food infections caused by *Salmonella* species*, Escherichia coli* and *Staphylococcus aureus*. Among all 9 isolates only 3 (SA, SF, SI) that showed antagonistic activities were selected for further probiotic study.

Table 3. Antimicrobial activity of isolate SA, SF and SI with inhibition zone diameter measured in mm

Pathogens	Diameter of Inhibition Zone (mm)		
	Isolate SA	Isolate SF	Isolate SI
Salmonella enterica	17.58±0.72^{a}	25.29±0.92^{a}	20.57±0.44^{b}
Salmonella typhi	17.66±0.70^{a}	25.34±1.36^{a}	21.33±0.37^{a}
Escherichia coli BL21	14.25±0.51^{c}	22.46±0.67^{c}	17.41±0.83^{e}
Staphylococcus aureus	17.19±0.85^{b}	24.82±0.41^{b}	20.32±0.42^{c}
Escherichia coli	14.55±0.36^{c}	22.76±0.52^{c}	18.59±0.50^{d}

Values are means ± standard deviation; Means with the different superscripts are significantly different ($p < 0.05$)

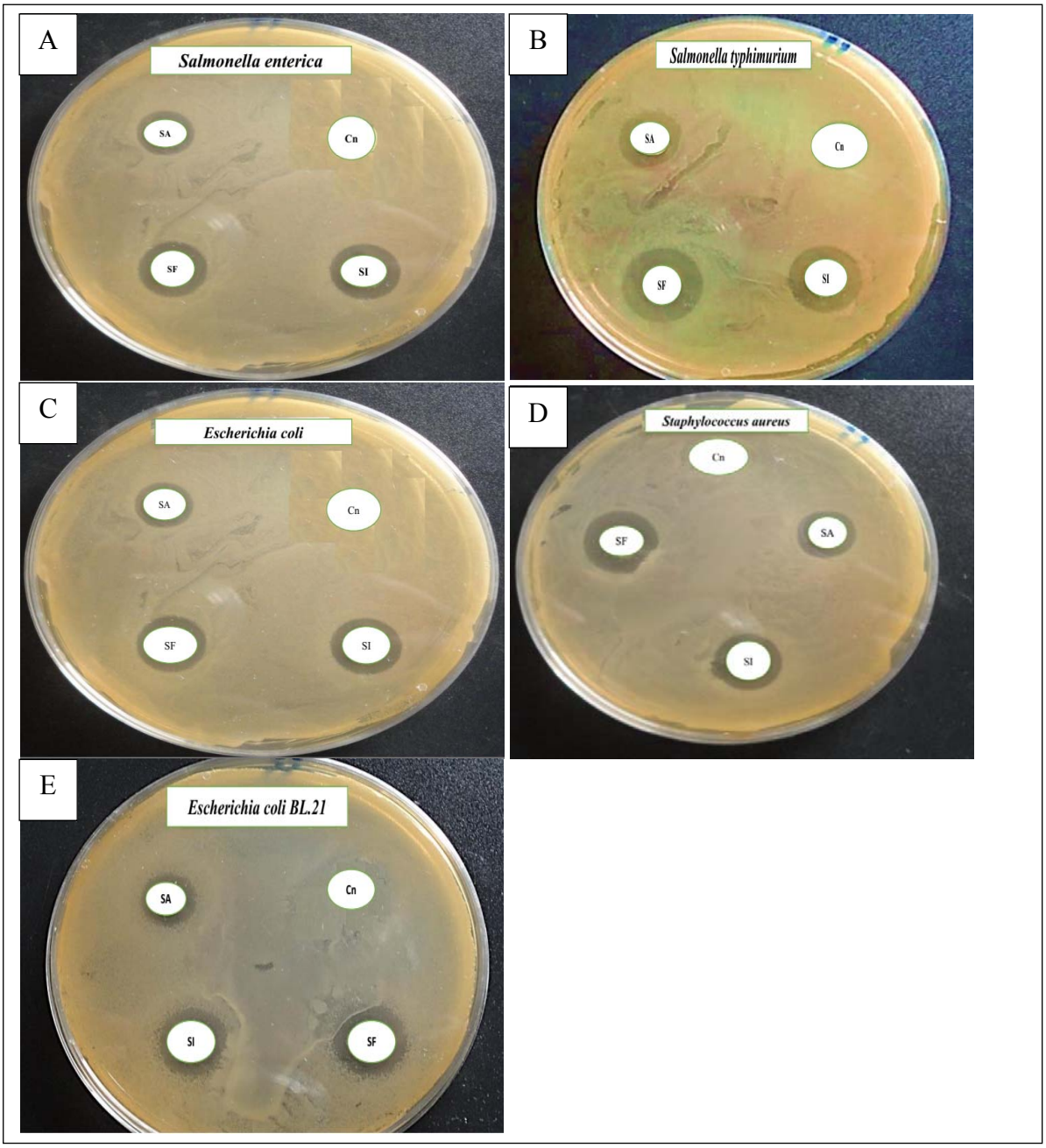

Figure 1. Antimicrobial activity of cell-free supernatants of isolate SA, SF and SI against *Salmonella typhi* (A), *Salmonella enterica* (B), *Escherichia coli* BL21 (C), *Escherichia coli* (D) and *Staphylococcus aureus* (E)

Another prerequisites of a bacterium to be classified as a probiotic is its ability to survive in the gut environment with acidic pH and inhibitory bile salts. Table 4 presents the effect of bile

salt concentration on survival of isolates SA, SF and SI. All the 3 isolates did not survive the acidic condition of pH 1.0 as survival rate remained zero throughout the experiment. Generally, as pH increases the survivial rate of the 3 LAB isolates also increases. [31] and [20] have also reported that the viability of LAB strains was significantly reduced at pH 2 compared with pH 3 and pH 7.

Table 4 Survival rate of isolate SA, SF and SI in acid and inhibitory bile salts

Strains	Acid resistant activity (%)				Growth in bile salt (%)		
	pH1	pH 2	pH3	pH7	0.3%	0.5%	1.0%
SA	-	53.12±0.23[a]	80.13±0.25[b]	99.21±0.33[c]	78.33±0.14[a]	60.22±0.14[b]	56.11±0.17[c]
SF	-	61.10±0.55[a]	88.85±0.67[b]	99.82±0.11[c]	79.89±0.74[a]	62.07±0.59[b]	56.67±0.33[c]
SI	-	55.17±0.38[a]	82.62±0.56[b]	99.36±0.36[c]	78.65±0.55[a]	61.33±0.21[b]	56.23±0.16[c]

It comes from table 4 that, at pH 2.0 all the 3 LAB isolates could survive approximately more than 50%. The isolate coded SF showed the highest resistance in this regard with survival rate of 61.10±0.55 % followed by isolate SI with survival rate of 55.17±0.38% and finally, isolate SA showed the lowest survival rate of 53.12±0.23%. When the pH was raised to 3.0, all three isolates exhibited a survival rate higher than 75%. Isolate SF showed the highest survival percentage of 88.85±0.67% and isolate SA showed the lowest survival rate of 80.13±0.25% incubation indicating that these isolates can be used as potential probiotic strains. The survivial rates at pH 2 and 3 observed in this study is comparable to the survival rates of 60.2% and higher than 75% respectively found at pH 2 and 3 by [12] in their study on probiotic LAB strains. When the pH raised up to 7.0, all isolates could survive with a survival percentage of approximately 100%.

To evaluate the potential of using LAB isolates as effective probiotics, it is generally necessary to evaluate their ability to resist the effects of inhibitory bile salt. All the 3 LAB isolates were tested for inhibitory bile salt tolerance and results are presented in Table 4. These results indicated that all 3 isolates can tolerate bile salts with survival rate being highest for all 3 isolates at 0.3 % bile salt concentration. The tolerance to bile salt could be explained by the fact that the protective effect of food matrix, may prevent the bacteria from bile exposure and hence, giving rise to the increased bile resistance [32]. Isolate SA SF and SI showed survival rates of 78.33±0.14%, 79.89±0.74% and 78.65±0.55% at 0.3% bile salt concentration respectively but when the concentration of inhibitory bile salt was increased up to 1.0%, a significant decrease ($p < 0.05$) of viability was observed with isolate SA, SF and SI showing survival rates of 56.11±0.17%, 56.67±0.33% and 56.23±0.16% respectively. The lower survival rates at higher bile concentration can be due to the fact that when bacteria are incubated with inhibitory bile salts, disruption of cellular homeostasis occurs which leads to the dissociation of lipid bilayer and integral protein of their cell membranes resulting in bacterial content leakage and finally death of the cell [33]. The results obtained in this study are in agreement with those reported by [34] who showed that Lactobacilli strains were viable after being exposed to bile salts ranging from 0.3-0.5% but showed diminished viability at higher bile salt concentrations.

3.4 Enzyme activity of LAB isolates

Another property that probiotics must deserved is the ability to produce useful enzymes for human well-being. In this study, the 3 LAB isolates which showed probiotic features were further screened for their enzymatic activity to detect any unfavorable enzyme like the carcinogenic enzyme such β-glucuronidase which are harmful to human health and presence of beneficial enzymes like α-galactosidase, β-galactosidase and β-glucosidase. Determination of enzyme production by isolates was an important criterion in conforming if our lactic acid bacteria strains are safe for human use. The enzymatic activities of the 3 LAB

isolates obtained from raphia palm wine, as evaluated by the API-ZYM system, are shown in Table 5. Results indicated that all isolates did not produce the β-glucuronidase, whereas beneficial enzymes were produced. These enzymes include alkaline phosphatase which catalyze and facilitate important physiological changes within cells, α-galactosidase which aids in digestion, may act as a treatment for both complex carbohydrate intolerance and regular gassiness, β-galactosidase which act as a key provider in the production of energy and a source of carbons through the breakdown of lactose to galactose and glucose and it is also important for the lactose intolerant communities as it is responsible for making lactose-free milk and other dairy products, acid phosphatase catalyze and facilitate important physiological changes within cells, Alkaline phosphatase commonly used in the dairy industry as an indicator of successful pasteurization, α-glucosidase which breaks down starch and disaccharides to glucose during digestion, and finally β-glucosidase which plays an important role in metabolism of glycolipids and dietary glucosides, and signaling functions. These results were in agreement with [35] who reported that LAB isolated from fermented dairy products produced enzymes including leucine arylamidase, crystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and N-acetyl-β-glucosamidase.

Table 5 Enzyme activity of the three selected LAB strains using API ZYM kit

Enzyme (nmol)	Enzymes activities		
	Isolate SA	Isolate SF	Isolate SI
Control	0	0	0
Acid phosphatase	4	4	4
Alkaline phosphatase	4	4	4
Cystine arylamidase	0	0	0
Esterase	0	0	0
Esterase lipase	0	0	0
Leucine arylamidase	3	3	3
Lipase	0	0	0
N-Acetyl-β-glucosaminidase	0	0	0
Naphthol-AS-BI-phosphohydrolase	0	0	0
Trypsin	0	0	0
Valinearylamidase	0	0	0
α-Chymotrypsin	0	0	0
α-Fucosidase	0	0	0
α-Galactosidase	5	5	5
α-Glucosidase	5	5	5
α-Mannosidase	0	0	0
β-Galactosidase	5	5	5
β-Glucosidase	5	5	5
β-Glucuronidase	0	0	0

Score 0 = 0 nmol, Score 1= 5 nmol, Score 2= 10 nmol, Score 3= 20 nmol, Score 4= 30 nmol, Score 5 ≥40 nmol.

3.5 Safety assessment of the LAB isolates

The antibiotic susceptibility test results for the isolated lactobacilli are represented in Table 6. It was observed that strains SA, SF, and SI were resistant to Kanamycin, Tetracycline, Chloramphenicol, Gentamicin, Ampicillin and Vancomycin. These 3 strains were intermediately susceptible to Streptomycin and Ciprofloxacin and sensitive to Erythromycin, and Ciprofloxacin. Some other reports have determined that LAB are resistant to the principal type of antibiotics such as Kanamycin, Streptomycin, Quinolone, Imidazole,

Nitrofurantoin and Fluoroquinolines [36]. The antibiotic resistance properties of lactic acid bacteria strains observed in the present study could be due to their ability to inactivate antibiotics through enzymatic reaction or to absence of cytochrome-mediated drug transport as reported in the literature [37].

Hemolytic activity was performed as one of the safety profiling methods in order to verify if LAB isolates possess a non-virulence nature. In our study, all three LAB strains showed a non-hemolytic activity (γ-hemolysis) which is an indicative of their non-virulence nature [38]. These three LAB strains exhibited no β-hemolytic activity, which are similar to the results reported [39]. [40] also noticed that none of the fifteen putative probiotics strains isolated in their study was found to be β-hemolytic.

Table 6 Results of susceptibility test and hemolytic activity of the three LAB isolates

Isolates	Antibiotic susceptibility test		Hemolytic activity
	Antibiotics	Antibiotic sensitivity	
SA	Vancomycin	R	γ hemolysis
	Streptomycin	I	
	Gentamicin	R	
	Kanamycin	R	
	Ampicillin	R	
	Erythromycin	S	
	Cefataxime	S	
	Ciprofloxacin	I	
	Tetracycline	R	
	Chloramphenicol	R	
SF	Vancomycin	R	γ hemolysis
	Streptomycin	I	
	Gentamicin	R	
	Kanamycin	R	
	Ampicillin	R	
	Erythromycin	S	
	Cefataxime	S	
	Ciprofloxacin	I	
	Tetracycline	R	
	Chloramphenicol	R	
SI	Vancomycin	R	γ hemolysis
	Streptomycin	I	
	Gentamicin	R	
	Kanamycin	R	
	Ampicillin	R	
	Erythromycin	S	
	Cefataxime	S	
	Ciprofloxacin	I	
	Tetracycline	R	
	Chloramphenicol	R	

S (ZDI: ≥ 21 mm) = Susceptible, R (ZDI: ≤15 mm) =resistant, IS (ZDI: 16 – 20 mm) = Intermediately Susceptible; Means with the different superscripts are significantly different ($p < 0.05$)

3.6 Phenotypic identification of *Lactobacillus* spp. strains isolated from raphia palm wines

After evaluation of probiotic properties, the 3 isolates of LAB (SA, SF, SF) belonging to the genus *Lactobacillus* showing good probiotics features were further characterized and identified using the API 50 CHL kit. Utilization of carbohydrates with the API 50 CHL kit is summarized in Table 7. Comparison with the API database revealed 99.6% homology of SA with *Lactobacillus brevis*, 99.9% of SF with *Lactobacillus plantarum* and 99.2% homology of SI with *Lactobacillus lactis*. This result is similar to the finding of [41] who isolated and identified from a fermented cereal product called Tarkhineh in Iran, LAB belonging to *Lactobacillus* spp. using the API 50 CHL kit. In the same way, [19] also used API 50 CHL system to identify *Lactobacillus* spp. strains isolated from palm wine.

Table 7. Fermentative profile of the different LAB isolates on API 50 CHL

Carbohydrate	Isolate SA	Isolate SF	Isolate SI
Glycerol	-	-	-
Erythritol	-	-	-
D - arabinose	-	-	-
L - arabinose	+	+	+
D - ribose	+	+	+
D - xylose	+	+	+
L - xylose	-	-	-
Adonitol	+	-	-
Xyloside	-	-	-
Galactose	+	+	+
Glucose	+	+	+
Fructose	+	+	+
Mannose	+	+	+
Sorbose	+	-	-
Rhamnose	-	-	-
Dulcitol	-	-	-
Inositol	-	-	-
Mannitol	+	+	+
Sorbitol	+	+	+
D - mannoside	-	+	-
D - glucoside	+	+	+
Glucosamine	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
Esculin	+	+	+
Salicin	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	+	+	+
Melibiose	+	+	+
Sucrose	+	+	+
Trehalose	+	+	+
Inulin	+	-	+
Melizitose	+	+	+
D - raffinose	+	+	+
Starch	-	-	-
Glycogen	-	-	-
Xylitol	-	-	-
Gentiobiose	+	+	+
D-Turanose	+	+	+
L - Lyxosis	-	-	-
D -Tagatosis	+	+	+
D - Fucose	-	-	-
L - Fucose	-	-	-
D - Arabitol	-	-	-
L - Arabitol	-	-	-
Gluconate	+	+	-
Control (No sugar)	-	-	-
Identified species	***L. brevis* (99.6%)**	***L. plantarum* (99.9%)**	***L. lactis* (99.2%)**

4. CONCLUSION

In summary, three strains of *Lactobacillus* spp. which deserved probiotic properties were isolated from a Cameroonian raphia palm wine and identified as *Lactobacillus lactis* SA, *Lactobacillus plantarum* SF and *Lactobacillus brevis* SI. Besides of their probiotic properties, these strains showed favorable enzymatic activity by producing some beneficial enzymes such as acid phosphatase, alkaline phosphatase, α-Galactosidase, α -Glucosidase, β-Galactosidase and β-Glucosidase, and they also showed no hemolytic activity. This study highlighted raphia palm wine as source of probiotic bacteria and suggested the potential utilization of the isolated *Lactobacillus* spp. as emerging tools in the prevention of human diseases and treatment of foodborne infections.

COMPETING INTERESTS

Authors declared that no competing interests exist.

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