

Screening for bacteriocins producing probiotic bacteria from fermented sap of palm trees (*Elaeis guineensis* and *Raffia sudanica*): production and partial characterization of bacteriocins

Abstract

The spread of food spoilage microflora and food borne pathogenic bacteria are threat for food security and human health. The prolonged use of antibiotics or chemical preservatives to eradicate this microflora is disadvantageous because of the occurrence of resistant microbial strains. Bacteriocins from probiotic lactic acid bacteria currently appear as one of the best alternatives. Palm wine has been reported as rich source of lactic acid bacteria (LAB), however very few studies have been carried out on bacteriocin from palm wines isolates. This work aims at screening for bacteriocins producing lactic acid bacteria isolated from palm wines. LAB was isolated on de Man Rogosa and Sharpe agar using pour plating method. Bacteriocin activity was determined using cell free supernatant pretreated with 0.1M sodium hydroxide and catalase on Mueller Hinton Agar. LAB isolates were selected based on the capacity of their CFS to inhibit the growth of indicator microorganisms. The selected strains were identified phenotypically using API 50 CHL bioMerieux Kit and colony PCR was used to confirm the identity of the selected isolates. Three LAB isolates (IS6, IS9, IS13) were selected for their ability to produce bacteriocins with high antimicrobial activity (diameter of inhibition ≥ 20 mm) against *Escherichia coli*, *Salmonella enterica*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. Inactivation of antimicrobial activity of CFS by proteolytic enzymes allowed confirming that they are bacteriocins. The selected LAB was identified as strain of *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Lactobacillus brevis*. The ability to significantly inhibit *Listeria monocytogenes* main cause of food spoilage at low temperature gives them the status of technological strains. In terms of their GRAS status and their probiotic potential, these strains may have interesting applications in agro-industries.

Keywords: probiotics, bacteriocins, lactic acid bacteria, colony PCR, food borne pathogens, food spoilage, antibiotic resistance, bio preservation

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Abbreviations: LAB, lactic acid bacteria; CFS, cell free supernatant; GRAS, generally regarded as safe

Introduction

In recent years, the intense use of antibiotic in breeding has led to the increase in number of antibiotic resistant food borne pathogens. The development or isolation of a new generation of antimicrobial agents is increasingly important. The use of bacteriocins from Lactic acid bacteria (LAB) or probiotic bacteria have received significant attention as a novel approach to the control of pathogenic microorganisms and especially food borne pathogenic bacteria.¹⁻³ Bacteriocins are ribosomally synthesized antimicrobial proteins⁴ that are produced by some bacteria species, including member of the LAB.⁵ They are lethal to bacteria other than the producing strain.⁶ Some bacteriocins produced by LAB inhibit not only closely related species but are also effective against food-borne pathogens such as *Listeria monocytogenes*, *Clostridium botulinum*, *Staphylococcus aureus*, *Salmonella sp* and *Escherichia coli*, *Campylobacter sp*.⁷ Bacteriocins generally exert their antibacterial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in

death of microorganisms.⁴ Nisin was the first antimicrobial polypeptide found in LAB⁸ at the time of discovery, the producing strains were identified as *Streptococcus lactis* (later classified as *Lactococcus lactis*).⁹ Today Nisin is an authorized preservative in at least 48 countries, in which it is used in a variety of food products, including cheese, canned food and cured meat.⁷ Another commercially produced bacteriocin is pediocin PA-1 produced by *Pediococcus acidilactici* and marketed as ATTA TM2431 (Kerry Bioscience, Carrigaline, Co, Cork, Ireland). Bacteriocin from LAB is advantageous for their use against food borne pathogens because there is Generally Regarded as Safe (GRAS).

Previous studies were able to characterize bacteriocin from LAB species isolated from different ecological niches such as milk, meat, vegetables, fermented foods as well as mouth and intestine of mammals. However, very few studies dealt with the LAB from palm and wines. These beverages have been reported as rich sources of LAB with probiotic potential.⁸ Palm wines are fermented beverages produced from the sap of various palm tree species. The drink is particularly common in parts of Africa, south India and the Philippines. In Africa, the sap is most often taken from wild date palms such as *Phoenix sylvestris* (the palmyra) and *Caryota urens*, from oil palms such as

Elaeis guineensis or from Raphia, kithul or nipa palms.^{9,18} Very few studies have been carried out on bacteriocins from lactobacilli of palm wines. Therefore the purpose of this study was:

- i. Isolation, selection and identification of bacteriocin producing LAB from palm wines harvested in the South west Region of Cameroon.
- ii. Determination of antimicrobial activity of bacteriocin against most important foodborne pathogens and partial characterization of bacteriocins from selected LAB.

Materials and methods

Collection of samples

The palm wine samples were collected in different localities of the South West region of Cameroon (Muea, Bonakanda, Ekona, Ndongo and Mile 16). Two types of palm wine were collected. The palm wine produced by natural fermentation of the sap of palm oil tree (*Elaeis guineensis*) and that obtained by natural fermentation of the sap from raffia (*Raffia sudanica*). Twenty samples were collected by type of wine. The samples were introduced into sterile test tubes, placed in a cooler at temperature of 4°C and transported to the laboratory for the isolation of bacteriocin producing lactic acid bacteria.

Isolation and phenotypic identification of lactic acid bacteria

LAB was isolated from wine sample by pour plate method using de Man Rogosa and Sharpe (MRS) agar. For this purpose ten-fold serial dilution was realized with saline solution (NaCl, 0.85% w/v). One mL aliquot of the 10⁻⁴ and 10⁻⁵ dilutions were aseptically disposed on sterile plates. MRS agar was poured onto it and allowed to set. All plates were incubated at 30°C for 48hr under anaerobic conditions. After the incubation, a preliminary catalase test was carried out. Catalase negative discrete colonies which appeared on the plates with distinct morphological differences such as color, shape and size were picked and purified 2-3 times by re-streaking on fresh MRS agar. The pure cultures were further characterized using Gram staining test and cell morphology examinations. Catalase negative and Gram positive isolates were preserved in 15%(v/v) glycerol agar at -80°C until identification. The bacteriocin activity was expressed as the diameter of the zone of inhibition caused by microbial test strain and expressed in mm. Carbohydrate fermentation patterns of LAB were determined using API 50 CHL kit (bioMerieux, France). The APILAB PLUS database software was used to interpret the results.

Molecular characterization of bacteriocin producing lactic acid bacteria

Extraction template DNA

A Single colony from each pure culture was picked by a transfer loop and suspended in 100µl of sterile distilled water in eppendorf tubes and each tube was vortex for 30 seconds. Each suspension was boiled using thermomixer comfort at 100°C for 10mins to lyse the cells and inactivate nucleases. The suspensions were centrifuged using Biofuge fresco centrifuge at 9500g for 5mins. Each supernatant was carefully collected and put in clean PCR tubes and used as templates for PCR.

Amplification of 16S rDNA region of the selected strains by PCR Reaction

16S rDNA of selected strains was amplified by PCR using the

primers 16S1fw: 5'AGAGTTTGATCCTGGCTCAG 3' and 16S2r: 5'ACGGCTACCTTGTTAACGACTT 3'. The forward primer is complementary to the 5' end of 16S rDNA and the reverse primer is complementary to the 3' end of 16S rDNA region. 5µl of each template was used. Amplification was performed in a 25µl total reaction volume containing 12.5µl of Master Mix, 2.5µl of each primer, 2.5µl of sterile distilled water and 5µl of template DNA. PCR reactions were performed in a Peltier thermal cycler.

Separation of amplified Fragments

After the completion of PCR reaction, amplified products were separated in a 1.5%(w/v) agarose gel. For this purpose, 1.5g agarose was dissolved in 100ml 1xTAE buffer and the agarose solution was boiled. Agarose solution was cooled to nearly 40°C. After cooling, 1.5µl ethidium bromide solution (10mg/ml) was added. The agarose gel was poured into the gel casting stand and the combs were placed. When the gel was solidified, the combs were removed.

For loading, 10µl of amplification were loaded into wells. After the loading of samples, 5µl of DNA molecular weight marker (Gene Ruler, Fermentas) were loaded into the first well. Finally, electrophoresis was performed using instrument H5 Horizontal gel electrophoretic system, at 100mA. Amplification products were visualized in a gel imager documentation system called BIO-RAD molecular imager Gel Doc™ XR+with image laboratory™ software. The presence of DNA fragments sized between 1500-2000bp indicated that targeted amplification was achieved.

Screening of lactic acid bacteria for their bacteriocinogenic activity

The ability of each LAB isolate to exert an antibacterial effect against indicators microorganisms (*Escherichia coli* B121, *Escherichia coli*, *Salmonella enterica* subsp. serovar, *Salmonella tenesse*, *Salmonella typhimurium*, *Listeria monocytogens*, *Staphylococcus aureus*, *Bacillus cereus*) was examined by disc diffusion method. The isolated LAB strains were inoculated in 5ml MRS broth and incubated under anaerobic condition at 30°C for 18-24 hr. Cell-free supernatant (CFS) was obtained by centrifugation of this culture using Biofuge fresco centrifuge at 10.000xg for 10min at 4°C. To clarify whether the antimicrobial activity detected derived from an organic acid or hydrogen peroxide (H₂O₂), the CSF was adjusted to pH 7.0 by adding 1N NaOH to eliminate the inhibitory effect of organic acids and 3000U/ml of catalase was added to eliminate the potential inhibitory effect of hydrogen peroxide produced by the isolates. The so treated CFS was filtered through 0.45µm filter and used as crude bacteriocin solution. The discs for antimicrobial testing were prepared from Whatman filter paper Number 1 and autoclaved at 121°C for 15min. Aliquots of 200µl from each cell-free supernatant or crude bacteriocin were placed on the sterile discs. Lactic acid bacteria free MRS broth impregnated discs were used as negative control. The discs were allowed to dry in a clean oven at 35°C for 2hours. The impregnated discs were placed on Mueller-Hinton agar seeded with 18hr culture of indicator microorganisms. Plates were incubated at 37°C for 24hr. Bacteriocin activities were determined by measuring the diameter (in mm) of inhibition zone around the discs.

Determination of the biological nature of the antimicrobial activity of bacteria

Further characterization in order to determine the biological nature of the antimicrobial activity of the selected LAB was carried out. They were cultured in MRS broth at 30°C for 24hr and the CFS of selected LAB isolates were tested for their sensitivity to proteolytic and

amylolytic enzymes. 5mL of cell free supernatant was treated for 2hr at 30°C with 1mg/ml final concentration of papain, trypsin, proteinase K, protease, α -amylase, β amylase. The remaining bacteriocin activity was determined using disc diffusion method.

Bacteriocin production

Strains were cultured in MRS broth at 30°C for 24hr without agitation and optical density was measured at 600 nm recorded at 4hr intervals. The bacteriocin activity was expressed as the diameter of the zone of inhibition caused by microbial test strain in mm.

Partial purification of bacteriocin

To partially purify bacteriocins, the CFS was concentrated with ammonium sulphate precipitation at 80% (w/v) saturation. Then, each mixture was centrifuged at 15500xg, 4°C for 30min (Biofuge fresco centrifuge) and the pellets obtained containing proteins were re-suspended in 2ml of potassium phosphate buffer and dialyzed against the same buffer for 24hours at 4°C in the dialysis tubing. The partially purified bacteriocins were collected in sterile containers and stored at -20°C.

Determination of the molecular weight of partially purified bacteriocins

To estimate the molecular mass of the bioactive peptides, the Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% discontinuous gel was performed. Briefly a 15% resolving gel was prepared by combining 5.0ml of 30%(w/v) acrylamide, 2.3ml of distilled water, 2.5ml of 1.5M Tris (pH 8.8), 0.1ml of 10% SDS. Finally, 0.004ml of TEMED and 0.1ml of 10%w/v ammonium persulphate were added and the solution was carefully mixed and quickly poured into the glass plate cassette. Once the gel had set, the distilled water was carefully tipped off and 5% stacking gel solution was added to the top of the resolving gel. The 5% stacking gel solution was prepared by combining 0.83ml of 30%w/v acrylamide mix, 3.4ml of distilled water, 0.63ml of 1.0M Tris (pH 6.8) and 0.05ml 10% SDS. Finally 0.005ml of TEMED and 0.05ml of 10% ammonium persulphate were added and the solution was carefully mixed and quickly poured on top of the resolving gel. A Telfon comb was quickly inserted into the spacer gel solution and the gel allowed polymerizing for 20min. Following polymerization, the Telfon comb was removed and the cassette placed in an electrophoretic tank. The tank was filled with 1X loading buffer electrode buffer and protein samples in non-reducing SDS loading buffer were loaded into the wells using capillary pipette tips. The gel was run in a BIO-RAD power pac basic mini-PROTEAN® Tetra system at 100V until the dye front reached the bottom of the resolving gel. After electrophoresis, the gel was placed into Coomassie stain for a minimum of 2hours. The gel was destained using multiple changes de-stain solution (45% v/v methanol, 10% v/v acetic acid).

Results

Isolation, selection and identification of bacteriocigenic lactic acid bacteria

Eighteen catalase negative and gram positive isolates rod-shaped and non-motile were obtained from various samples of wines. The codes were assigned to these isolates presenting the preliminary characteristics of lactic acid bacteria; (catalase-negative, gram positive and non-motile). Nine codified IS1, IS2, IS3, IS4, IS5, IS6,

IS7, IS8, IS9 were isolated from fermented sap of palm oil tree while nine other codified IS10, IS11, IS12, IS13, IS14, IS15, IS16, IS17 and IS18 were isolated from fermented raffia sap. Only three isolates (IS6, IS9, IS13) showed important antimicrobial activities and high potential bacteriocin production materialized by the formation of inhibition zone (diameter \geq 20mm) around the disc impregnated with the cell free supernatant (CFS) pre-treated with catalase and sodium hydroxide. The Figure 1 presents the plate assays for inhibitory activity of some isolates against food borne pathogenic bacteria. The values of the diameter of the inhibition zone are summarized in Table 1. These isolates were selected for further studies. The biochemical characteristics of the selected LAB are reported in Table 2. The Table 1 shows the antimicrobial activity of pretreated supernatant by catalase and sodium hydroxide solution. The indicator strains used in this work were Gram positive and Gram negative. The results in Table 1 show that the indicator *E. coli* was the most sensitive, followed by *Listeria monocytogenes* and *Salmonella enterica*.

Table 1 Antimicrobial activity of crude bacteriocins (mm) from selected lactic acid bacteria isolates (Isolate IS6, IS9, IS13) against some bacteria, determined by disc diffusion method on Müller-Hinton agar. The data shown are averages of triplicate assays with SD within 10% of mean value

Isolate code	Indicator strains	Diameter of inhibition zone (mm)
IS6	<i>Escherichia coli</i> BL21	29.5 \pm 0.7
	<i>Escherichia coli</i>	27.3 \pm 0.7
	<i>Salmonella enterica</i>	15.2 \pm 0.5
	<i>Salmonella typhimurium</i>	11.3 \pm 1.2
	<i>Listeria monocytogenes</i>	18.3 \pm 0.8
	<i>Staphylococcus aureus</i>	14.5 \pm 0.3
	<i>Bacillus sp</i>	8.3 \pm 0.5
	<i>Escherichia coli</i> BL21	28.5 \pm 0.2
	<i>Escherichia coli</i>	25.3 \pm 1.3
IS9	<i>Salmonella enterica</i>	13.4 \pm 0.8
	<i>Salmonella typhimurium</i>	10.7 \pm 0.5
	<i>Listeria monocytogenes</i>	17.3 \pm 0.4
	<i>Staphylococcus aureus</i>	15.5 \pm 0.5
	<i>Bacillus sp</i>	8.3 \pm 0.1
	<i>Escherichia coli</i> BL21	26.5 \pm 0.4
	<i>Escherichia coli</i>	25.3 \pm 1.3
	<i>Salmonella enterica</i>	13.4 \pm 0.8
	<i>Salmonella typhimurium</i>	10.7 \pm 0.5
IS13	<i>Listeria monocytogenes</i>	17.3 \pm 0.4
	<i>Staphylococcus aureus</i>	15.5 \pm 0.5
	<i>Enterococcus faecalis</i>	12.5 \pm 0.3
	<i>Bacillus sp</i>	8.3 \pm 0.1

Phenotypic and molecular identification of selected lactic acid bacteria strains

The three bacteriocigenic strains IS6, IS9 and IS13 were biochemically characterized using API 50 CHL BioMerieux Kit. The

carbohydrate fermentation profile is summarized in Table 2. Based on the fermentation profile, Ribose, Galactose, Glucose, Fructose, Mannose, Mannitol, Sorbitol, Methyl-D-mannoside, N-Acetyl-Glucosamine, Amygdalin, Arbutin, Esculin, Salicin, Cellobiose, Maltose, Lactose, Melibiose, Sucrose, Trehalose, Melezitose, β Gentiobiose and D-turanose were utilized by *Lactobacillus* sp (IS6). IS9 showed higher utilization rates to these same carbohydrates. IS13 showed higher utilization rates with, L-arabinose, Ribose, Adonitol, Galactose, Glucose, Fructose, Mannose, Sorbose, Mannitol, Sorbitol, Methyl-D-glucoside, N-Acetyl Glucosamine, Amygdalin, Arbutin, Esculin, Salicin, Maltose, Lactose, Melibiose, Sucrose, Trehalose, Melezitose, Raffinose, β Gentiobiose, D-tagatose, D-arabitol, Gluconate and 2-Keto-Gluconate. All the three strain did not .did not

utilize Glycerol, Erythritol, D-arabinose, Starch, D-tagatose, D-arabitol and Gluconate. Comparison with the API database revealed 99.2% homology of IS6 with *Lactobacillus plantarum*, 99.9% homology of IS9 with *Lactobacillus rhamnosus* and 98.05% homology of IS13 with *Lactobacillus brevis*. Based on their carbohydrate fermentation profile these isolates were phenol typically identified as being strains of *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus brevis*. Amplification of DNA and electrophoresis on agarose gel allowed observing the amplicons. The sizes of the amplicons are substantially equal to 1.5kbp, which corresponds to the size of the lactobacilli amplicon. This molecular identification showed that all the selected bacteriocinogenic strains in our study belong to the genus *Lactobacillus*.

Table 2 Biochemical characteristics of *Lactobacillus* isolated from palm wines

Test number	Strains cod	IS6	IS9	IS13
1	Heterofermentative	+	+	+
2	Gaz production	+	+	+
3	Optimum Temper	37	37	37
4	Growth at 10°C	-	-	-
5	Dextran	-	-	-
6	Ammonia from Arginine	+	-	-
7	Nitrate reduction	-	-	-
8	Glycerol	-	-	-
9	Erythritol	-	-	-
10	D-arabinose	-	-	-
11	L-arabinose	+	+	+
12	Ribose	+	+	+
13	D-xylose	+	-	+
14	L-xylose	-	-	-
15	Adonitol	-	-	+
16	β methyl-D-Xyloside	-	-	-
17	Galactose	+	+	+
18	Glucose	+	+	+
19	Fructose	+	+	+
20	Mannose	+	+	+
21	Sorbose	-	-	+
22	Rhamnose	-	-	-
23	Dulcitol	-	+	-
24	Inositol	-	-	-
25	Mannitol	+	+	+
26	Sorbitol	-	+	+
27	α -Methyl-D-mannoside	+	-	nc
28	α -Methyl-D-glucoside	+	+	+
29	N-Acetyl-Glucosamine	+	+	+
30	Amygdalin	+	+	+
31	Arbutin	+	+	+

Table Continued..

Test number	Strains cod	IS6	IS9	IS13
32	Aesculin	+	+	+
33	Salicin	+	+	+
34	Cellobiose	+	+	+
35	Maltose	+	+	+
36	Lactose	+	+	+
37	Melibiose	+	+	+
38	Sucrose	+	+	+
39	Trehalose	+	+	+
40	Inulin	-	+	+
41	Melezitose	+	+	+
42	Raffinose	+	+	+
43	Starch	-	-	-
44	Glycogen	-	-	-
45	Xylitol	-	-	-
46	β Gentiobiose	+	+	+
47	D-turanose	+	+	+
48	D-lyxose	-	-	nc
49	D-tagatose	+	+	+
50	D-fucose	-	-	-
51	L-fucose	-	-	-
52	D-arabitol	nc	-	-
53	L-arabitol	-	-	-
54	Gluconate	nc	nc	nc
55	2-Keto-Gluconate	nc	-	-
56	5-Keto-Gluconate	nc	-	-

Lactobacillus plantarum *Lactobacillus rhamnosus* *Lactobacillus brevis*

+: positive reaction, -: negative reaction, nc, non-conclusive

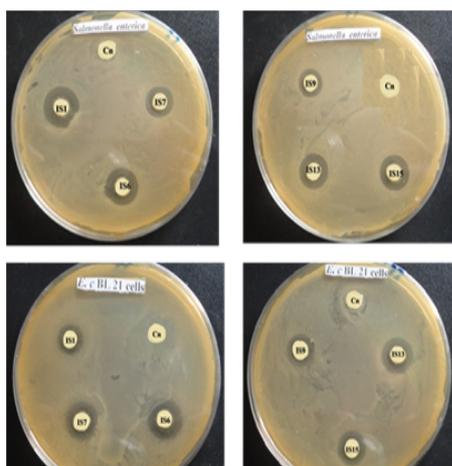


Figure 1 Plate assays for inhibitory activity of crude bacteriocin solutions of the selected lactic acid bacteria isolated from palm and raffia wines.

Effect of enzymes on bacteriocin activity of lactic acid bacteria selected

The action of enzymes on the supernatant obtained from the cultures of the various selected strains is summarized in Table 3. No bacteriocin activity was observed after treatment with proteinase K, protease, trypsin and papain.

Determination of molecular mass of partially purified bacteriocins

Three randomly chosen partially purified bacteriocins from the 6 obtained during antimicrobial detection test were analyzed on SDS-PAGE (15%w/v) and stained with Coomassie brilliant blue. On sodium dodecyl sulfate polyacrylamide gel electrophoresis, these 3 partially purified bacteriocins showed single bands each. The molecular mass of the 3 partially purified bacteriocins IS13, IS6 and IS9 were determined as ~6kDa, ~10kDa and ~3kDa (Figure 5).

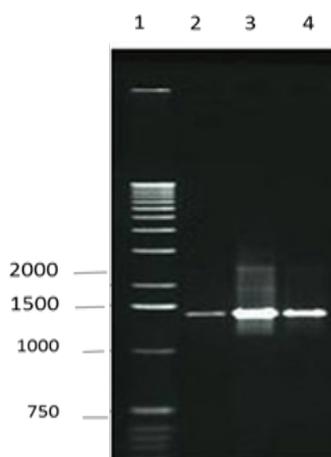


Figure 2 PCR amplification pattern of 16S rDNA of the 3 bacteriocin producing lactic acid bacteria strains on 1.5% agarose gel.

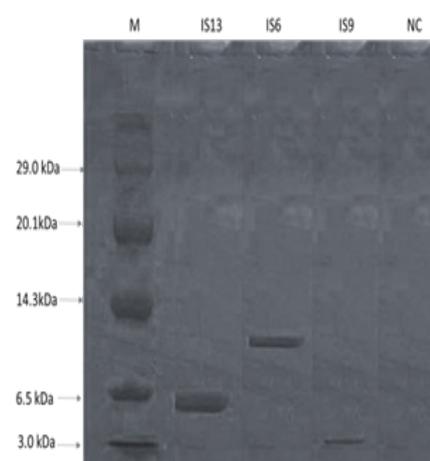


Figure 3 Electrophoregram of crude and partially purified bacteriocin produced by strains IS6, IS9 and IS13.

Table 3 Effect of enzymes and papain on bacteriocin activity of lactic acid bacteria selected

Isolates	Cell free supernatant treatment	Bacteriocin activity
Lactobacillus plantarum (IS6)	Papain	-
	Trypsin	-
	Proteinase k	-
	Protease	-
	α -amylase	+
	B-amylase	+
Lactobacillus rhamnosus (IS9)	Papain	-
	Trypsin	-
	Proteinase k	-
	Protease	-
	α -amylase	+
	B-amylase	+
Lactobacillus brevis (IS13)	Papain	-
	Trypsin	-
	Proteinase k	-
	Protease	-
	α -amylase	+
	B-amylase	+

Discussion

This study highlights the evidence of lactic acid bacteria in the palm wine samples collected in the south-western region of Cameroon. The existence of this lactic microflora in these traditional drinks is in accordance with data previously obtained by Okafor,¹⁰ which shows that palm wines harvested in Nigeria were potential niches of lactic acid bacteria strains. Phenotypic identification based especially on the morphology of cells and their fermentation profile reveals that our palm wine samples were rich in lactic acid bacteria belonging to the genus *Lactobacillus*, (short rods and are catalase negative). According to Okafor¹⁰ and Awua et al.,¹¹ the dominant bacterial population of palm wine was described as lactic acid bacteria, especially strains of

Lactobacillus plantarum, *Leuconostoc mesenteroides*, *Lactobacillus lactis* and *Lactobacillus mesenteroides subsp. dextranicum*.

As stated by Felis et al.¹² phenotypic methods alone are inadequate for identification of lactic acid bacteria and should be confirmed by molecular methods to achieve a reliable identification. Theoretically, the amplicon size of 16S rDNA region on the genomic DNA of lactic acid bacteria is 1500bp. In the present study bacteriocin producing strains were identify by PCR technique to confirm if they were actually LAB. The 16S rDNA region of each bacteriocin producing strain was amplified. The length of amplification products varied between 1000 to 2000bp. These results shows that the three bacteriocin producing strains isolated in this study were lactic acid bacteria because the

amplicon sizes of their 16S rDNA region amplified was approximately 1500bp. Many research works have focused on the application of molecular biology techniques for the rapid detection and differentiation of LAB. The use of primers and probes that target genes encoding ribosomal ribonucleic acid (rRNA) is promising. Due to the high interspecies variability of this region, rDNA sequences coding for the 16S and 23S rRNA has been validated as a means of identification. However, the data in the literature concern the identification of strains known to be lactobacilli to the species level Dubernet et al.¹³ The approaches in preliminary identification of isolated bacterial strains of various bacterial species were morphological characterization like positive growth on MRS agar medium, colony color and outcomes of microscopic analysis and all were to suggest the presence of lactic acid bacteria in the palm wine samples under consideration. These identification approaches were in agreement with almost each and every study on lactic acid bacteria¹⁴ in that all the isolated strains could grow on MRS agar, bacteria colonies were whitish, circular and convex when observed with eye and the bacteria cells were gram positive when observed under the microscope after gram staining and catalase negative.

The CFS of the strains *Lactobacillus plantarum* (IS6), *Lactobacillus rhamnosus* (IS9), *Lactobacillus brevis* (IS13) showed significant antimicrobial activity after treatment with normal sodium hydroxide solution and catalase. The persistence of this activity in these strains after treatment shows that this activity is not associated with the presence of an organic acid or hydrogen peroxide but rather a substance of another nature. Inactivation of antimicrobial agents by proteolytic enzymes shows that they are of protein nature, so there are well bacteriocins. This finding is in accordance with that obtained by Todorov et al.¹⁵ These authors observed the persistence of bacteriocin activity after the treatment of CFS of lactic acid bacteria isolated from boza a traditional cereal beverage from Bulgaria. Our selected isolates exhibited high inhibitory activity against indicator organisms, the inhibition halo was beyond 20mm whereas according to Schillinger et al.⁹ inhibition was scored positive if the width of the clear zone around the colonies of the culture strains was 0.5 mm or larger. The selected strains showed antimicrobial activity against pathogenic strains such as *Salmonella typhimurium* and *Staphylococcus aureus*. It appears from these results biomedical interest of LAB isolated in this work. Furthermore these selected strains have shown their ability to inhibit the growth of the microflora responsible of spoilage of foodstuffs; including *Listeria monocytogenes* renowned for its ability to deteriorate the quality of food products at low temperatures. These results are comparable to those of Itoh.¹⁶ These authors reported the inhibition of food borne pathogenic bacteria by bacteriocins from *Lactobacillus gasseri*.

Lactic acid bacteria are generally regarded as safe (GRAS),^{17,18} the ability of the isolates obtained in this study to inhibit foodborne pathogens is one of the most important probiotic properties. Most of the probiotic are lactic bacteria from gastrointestinal tract. According to the current FAO/WHO definition: “probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host”, such as inhibition of the proliferation of pathogenic bacteria.¹⁹ The partially purified bacteriocins obtained during antimicrobial test were analyzed on SDS-PAGE (15%w/v) and stained Coomassie brilliant blue. These three partially purified bacteriocins appeared as single bands in SDS-PAGE with molecular weight approximately ~ 6kDa for IS13 bacteriocin, ~10kDa for IS6 bacteriocin and ~3kDa for IS9 bacteriocin. The bacteriocins of lactic acid bacteria belonging to class-I and II have molecular weight

(<5kDa) and (<10kDa) respectively e.g., *Pediococcus acidolactici* (3.5kDa), *L. cinC-TA33a* (4.6kDa) and *L. curvatus* SB13 (10kDa).²⁰ The lower molecular mass of IS13 bacteriocin (6kDa) and IS9 bacteriocin (3.5kDa) suggested that they might belong to class I bacteriocin group. The molecular mass of IS6 bacteriocin (10kDa) suggested that it might belong to class II bacteriocin group.

Conclusion

The palm wine commonly consumed in traditional environment in Cameroon is a potential source of lactic acid bacteria. *Lactobacillus* genus appears as the dominant flora of these traditional drinks. These lactic strains produce palm wine for some, bacteriocins having a significant inhibitory activity against foodborne pathogens and spoilage flora against the food. The remarkable fact in this work is the ability of these bacteriocins to also inhibit Gram negative strains suggesting their potential application in the biomedical field and their used as probiotic bacteria. The ability to significantly inhibit *Listeria monocytogenes* which is the main cause of food spoilage at low temperature gives them the status of technological strains. In terms of their safety and their GRAS status, these strains may have interesting applications in agro-industries.

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Conflict of interest

The author declares no conflict of interest.

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