Scientific Research Publishing

Advances in Microbiology, 2019, 9, 917-930
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Giving More Benefits to Biosurfactants Secreted by Lactic Acid Bacteria Isolated from Plantain Wine by Using Multiplex PCR Identification

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How to cite this paper: Moukala, M.B., Kayath, C.A., Ahombo, G., Dangui, N.P.M., Kinavouidi, D.J.K., Mouélé, E.C.N. and Diatewa, M. (2019) Giving More Benefits to Biosurfactants Secreted by Lactic Acid Bacteria Isolated from Plantain Wine by Using Multiplex PCR Identification. *Advances in Microbiology*, **9**, 917-930.
https://doi.org/10.4236/aim.2019.911058

Received: October 3, 2019
Accepted: November 11, 2019
Published: November 14, 2019

Abstract

Fermented beverages have continued to give more surprises in terms of the presence of biomolecules and the diversity of microorganisms that may be contained. Republic of Congo is home to a panoply of fermented foods and beverages that are still not yet studied. This is the case of plantain wine fluently called banana wine. Within this framework, this work aims to study the role of Biosurfactant-like Biomolecules secreted during fermentation of plantain wine. Using MRS medium, 15 isolates bacteria have been found. 100% are able to secrete biosurfactant and 66.66% are extractible biosurfactants. 33% of isolates have been associated to *Lactobacillus plantarum* (Is2, Is9, Is12 and Is13) by using a one-step multiplex PCR that targets genes encoding for bacteriocins. Biosurfactants secreted by *L. plantarum* play an important role in the preservation of banana wine. The biosurfactants extracted with chloroform and ammonium sulphate are able to inhibit the growth of pathogenic bacteria including *Shigella flexneri*, *Salmonella* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Keywords

Plantain Wine, Fermented Beverage, *Lactobacillus* spp., Multiplexe PCR, Biosurfactants, Pathogenic Inhibition

1. Introduction

In the Republic of the Congo, there is a wide variety of traditional fermented

drinks, including sugar cane wine ("loungouila"), palm wine ("nsamba"), honey wine ("duma"). Other local wines basing on banana, orange, pineapple and grapefruit are not yet documented. The plantain wine commonly known as "banana wine" is known as "Mbamvu" in some Republic of Congo areas [1]. This fermented local beverage is produced according to traditional methods generally in areas where raw materials are grown. The rather crude fermentation technique consists in letting the juice extracted from the fruit turn to produce alcohol. Spontaneous fermentation is achieved by the presence of microorganisms from the plant material Physicochemical parameters are generally poorly controlled [1].

Also known as "Urwagwa" in Rwanda, banana wine is one of the oldest and most important beverages found in most East African countries. It is mainly consumed in three quarters of the Rwandan territory, where banana cultivation is a major agricultural activity [2].

Traditional practices show that Mbamvu is prepared from mature plantain slices suspended in water for one (1) to three (3) weeks. The microorganisms responsible for alcoholic fermentation are usually present on the surface of the raw materials that serve as their substrate [3]. In addition, the hygienic quality is based on the transformation conditions and the material used [4].

The choice of the fruit is justified by the fact that the plantain is the most consumed fresh fruit in the world [5] but beverage produced from plantains is little known.

So far, the techniques of making our local beverages such as nsamba "loungouila", orange wine and "Mbamvu" are poorly controlled and pose many difficulties from the hygienic point of view, in terms of preservation, rate of fermentation, alcohol, taste variation, which can lead to different health problems.

Some fermented foods and beverages have been studied in Republic of Congo and molecular identification of lactic acid bacteria, *Bacillus* spp., *Sacharomyces* spp. have been done as well [6]-[11]. Multiplex PCR techniques have been used to identify lactic acid bacteria [12] [13]. The concept "multiplex PCR" refers to a development of the PCR technique allowing the amplification, in a single reaction, of several distinct DNA segments. The pairs of primers corresponding to the different loci to be analyzed are introduced into the same reaction tube [12] [13]. PCR multiplexe amplicon using 16S RNA and 23S RNA genes were estimated on *Lactobacillus mali*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides* and *Oenococcus oeni* [14]. Virulence genes including *fsr*, *efm*, *esp*, *cylA*, *cad*1, *ace*, *gelE*, and *asa*1 have been used to identify *Enterococcus* spp. in multiplex technique [15]. Several lactic acid bacteria have been shown to be secreted biosurfactants which are amphiphilic compounds produced by either on the cell surface or secreted extracellularly [16] [17] [18]. Biosurfactant plays an important role in antimicrobial activities [16] [18] [19].

To our knowledge, few studies from the microbiological and biomolecular point of view have been carried out to be secreted directly biosurfactant in ba-

nana wine during fermentation. In the context of this work, this beverage seems to be interesting for the identification of biomolecules secreted during fermentation. To this end, in order to valorize that banana wine, some questions could be issued: What diversity of microorganisms can we encounter in banana wine? What are the secreted biomolecules in plantain wine? What is the role in preservation?

2. Methods

2.1. Manufacture of Plantain Wine and Isolation of Bacteria

The biological material used in this work is the plantain wine (Mbamvu) made in the laboratory by improving the traditional process. Dilutions were done, and bacterial suspension was streaked on PCA, MRS, LB, Chapman, SS Mossel, and TSN media as described by the manufacturers. Petri dishes were incubated at 37˚C for 24 h to 48 h. After the first isolation on Petri dishes, different colonies were obtained. Each colony from MRS of different appearance was separately isolated. Purification of the isolates was rigorously done by successive and alternating subcultures. Purity was estimated by using a microscope for morphological characterization. Gram status was determined by using 3% KOH.

2.2. Evaluation of Emulsion Index (E24/E48)

The emulsion index (E24 or E48) was calculated as an indicator for biosurfactants production. The medium was adjusted to pH 7.2 and supplemented with gasoline or diesel fuel (1 mL for 300 mL of medium). This experiment was done in triplicate. The E24 or E48 was investigated by adding crude oil with LB medium in 1:1 ratio (v/v). The solution was vortexed for 5 min and incubated for 24 h. The emulsion rate was calculated through the height of the emulsion layer. In addition, E24 was determined for gasoline and diesel fuel hydrocarbons. All the experiments were performed in triplicates, E24 or E48 = height of emulsion layer/total height of solution × 100.

2.3. Extraction of the Emulsifying Activity by Chloroform and Ammonium Sulfate

One volume of supernatant was added with an equal volume of chloroform (v/v). The mixture is strongly agitated by a vortex. After centrifugation at 6000 rpm for 10 min, the non-aqueous phase is recovered. The solvent was allowed to evaporate completely only without heating above 40˚C. The residue is dissolved in a PBS buffer. In terms of ammonium sulfate an overnight culture has been fuged at 13,000 rpm for 15 minutes to separate supernatant and pellet. Then 15 mL of supernatant were mixed with ammonium sulfate (80%) for 15 minutes. And finally this has been incubated in overnight. Mix has been fuged at 6000 rpm for 30 minutes. Pelet has been hommogenized by using PBS buffer. For both extractions (chloroform and ammonium sulfate) the emulsifying activity is tested in comparison with the supernatant at the start.

2.4. Inhibition Tests from Biosurfactant Extracts

The LB medium was prepared and poured on Petri dishes. After solidification of the medium, a volume of 0.1 ml of the bacterial suspension is seeded throughout the entire box. After drying, 20 µL of biosurfactant extract were deposited in three (3) different locations on a box. The dishes were incubated at 37˚C for 24 h and the diameters of the inhibition halos were measured.

2.5. Multiplex for Lactic Acid Bacteria Identification

In order to directly identify the isolates by using DNA technology, nine genes including *curA*, *sakP*, *sakQ*, *plnA*, *plnEF*, *entA*, *entB*, *entP* and *pedA* encoding for bactericins like molecules have been used. These have been selected in previous work [20] and this was checked in the NCBI (National Center for Biotechnology Information, https://blast.ncbi.nlm.nih.gov/Blast.cgi) genomic database of targeted strains. Microbesonline (http://www.microbesonline.org) have been also used for more checking list. Five lactic acid bacteria including *Lactobacillus curvatus*, *L. sakei*, *L. plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici* have been discriminated. The pDRAW32 software has been used for bioinformatic analysis. The primers were rigorously designed and selected (**Table 1**).

Table 1. Primers used in this work [21] [22] [23] [24] [25].

Multiplex groups	Targeted genes	Primers (5'-3')	Size (pb)	Targeted species
Group 1	*curA-F*	GTAAAAGAATTAAGTATGACA	180	*L. curvatus*
	curA-R	TTACATTCCAGCTAAACCACT		
	sakP-F	ATGGAAAAGTTTATTGAATTA	200	*L. sakei*
	sakP-R	TTATTTATTCCAGCCAGCGTTTC		
	entB-F	GAAAATGATCACAGAATGCCTA	162	*Enterococcus faecium*
	entB-R	GTTGCATTTAGAGTATACATTTG		
	entA-F	AAATATTATGGAAATGGAGTGTAT	126	*E. faecium*
	entA-R	GCACTTCCCTGGAATTGCTC		
	plnEF-F	GGCATAGTTAAAATTCCCCCC	428	*L. plantarum*
	plnEF-R	CAGGTTGCCGCAAAAAAAG		
Group 2	*entA-F*	AAATATTATGGAAATGGAGTGTAT	126	*E. faecium*
	entA-R	GCACTTCCCTGGAATTGCTC		
	entP-F	TATGGTAATGGTGTTTATTGTAAT	112	*E. faecium*
	entP-R	ATGTCCCATACCTGCCAAAC		
	pedA-F	AAAATATCTAACTAATACTTG	600	*Pediococcus acidilactici*
	pedA-R	TAAAAAGATATTTGACCAAAA		
	sakQ-F	ATGCAAAATACAAAAGAACTAA	200	*L. sakei*
	sakQ-R	CGCTTGTTTAGAGACACCCGTT		
	plnA-F	GTACAGTACTAATGGGAG	450	*L. plantarum*
	plnA-R	CTTACGCCAATCTATACG		

Extraction and purification of isolate genomic DNA were performed according to the NucleoSpin Microbial DNA (Macherey-NAGEL) kit. Briefly, isolates were grown in 5 mL LB broth for 24 h at 37˚C with stirring. The DNA purity was assessed by electrophoresis on agarose gel and by the ratio of optical densities 260/280nm. By using universal primers of 16S rRNA fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3'), bacterial confirmation has been done oriented.

Two groups of multiplex have been done (Table 1). 5 µL of each amplification product was mixed with 2 µL of loading buffer (BIOKE). Mixtures were subjected to electrophoresis on 1% agarose gel (w/v).

3. Results

3.1. Secretion of Biomolecules in Plantain Wine and the Role of Biosurfactants

3.1.1. Production of Biosurfactants

In order to carry out our research, we first sought to know if the biosurfactants are directly secreted in the plantain wine. The results of Figure 1 show emulsification percentages ranging between 15% to 30% of plantain wine emulsification index of randomly choosen samples after five (5) days fermentation (Figure 1).

The presence of biosurfactants secreted directly in the plantain wine led us to keep working by isolating and characterizing Lactic acid bacteria that can be able to do so. The isolation on MRS medium has been used to highlight the presence of bacteria of the genus *Lactobacillus*. Thus, 15 isolates were obtained, screened and purified from banana wine. These isolates have been the subject of various microbiological issues, biochemistry and molecular biology.

The purified isolates were macroscopically and microscopically characterized (data not shown). Fifteen (15) isolates were characterized. Among 15 isolates, 11 isolates are round, 4 oval. All isolates retained are Gram-positive bacteria. 13 isolates are sticks against 2 cocci. Of the 100% isolates, 20% are mobile and 80% are not. Only 7% of the isolates are catalase positive, 93% are catalase negative. 67% have a dry texture against 20% glutinous and 13% pasty. The fifteen isolates were used for various tests and molecular identification.

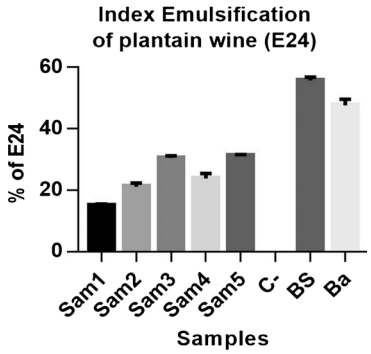

Figure 1. Percentage of emulsification of samples of Mbambu taken after 5 days of fermentation. Sam1, 2, 3, 4, 5: Samples from collected plantain wine. C-: *E. coli* used as negative control. BS: *Bacillus subtillis*, Ba: *B. amiloliquefascience* both are stored in the lab.

To highlight the production of biosurfactant, we performed the emulsification test from isolates. This study shows that isolates produced biosurfactants with an emulsification index (E24) ranged from 20% to 73% after 24 hours and from 20% to 90% corresponding to the emulsification index (E48) after 48 h (Figure 2). The emulsification index is concomitant to the bacterial growth.

Biosurfactants are extratables

We also investigated if biosurfactants were extractable after centrifugation of isolates cultures. Within acellular supernatant, 66% of isolates were biosurfactant extractable and these have E24 100% of ability to emulsify gasoline or diesel. This is included Is1, Is2, Is3, Is4, Is6, Is7, Is9, Is10, Is12 and Is15. Is5, Is8, Is11, Is12 and Is13 can produce biosurfactants in supernatant but these cannot be extracted using either chloroform or ammonium sulfate (Figure 3).

3.1.2. Inhibition of Pathogenic Bacteria by Biosurfactant Extracts

By using SS, Chapman, EMB, Cetrimide, TSN and Mossel no targeted pathgenic Bacteria can be isolated from 50 mL of plantain wine including *Shigella flexneri*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Staphyloccoccus aureus*, *Clostridium* spp and *Bacillus cereus*. To assess the absence of these bacteria, inhibition of pathogenic bacteria by using biosurfactant extracts from Is1, Is2, Is3, Is4, Is6, Is7, Is9, Is10, Is12 and Is15, have been done.

The biosurfactants extracts from the 10 isolates made it possible to carry out the inhibition tests on the pathogenic strains mentioned above. Figure 4 shows

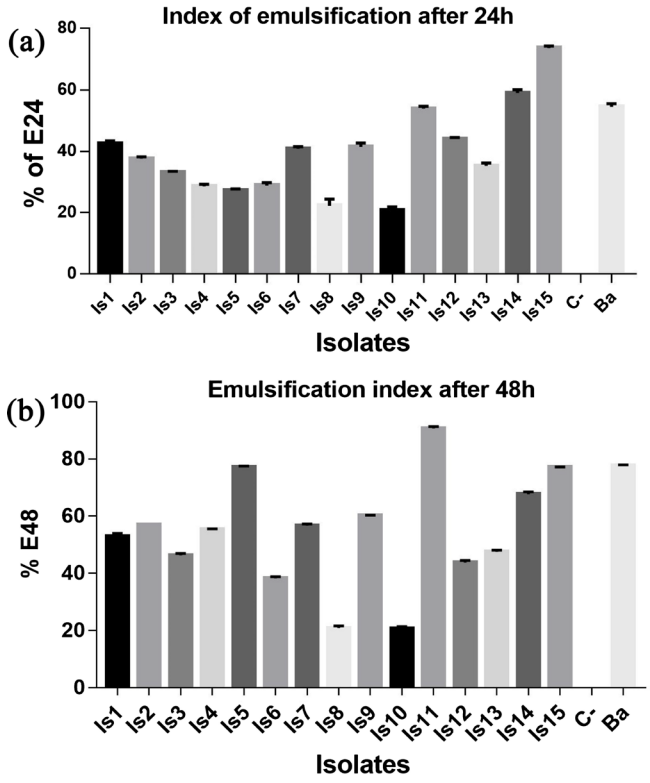

Figure 2. (a) Emulsification index after 24h (up), (b) Emulsification index after 48 hours (down) in the presence of gasoline.

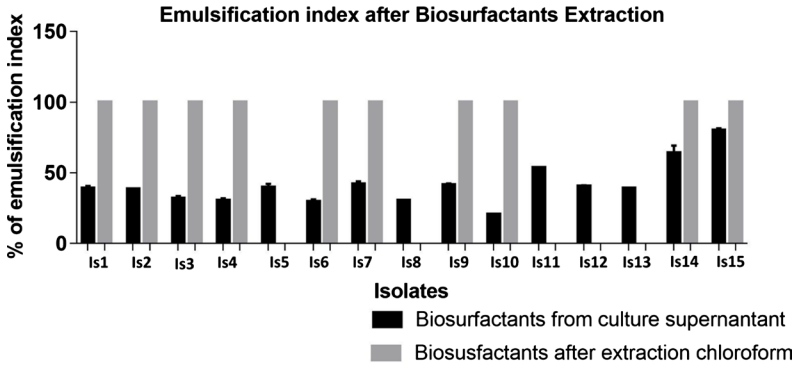

Figure 3. Extraction of biosurfactants from chrorloroform. Is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15: Isolates.

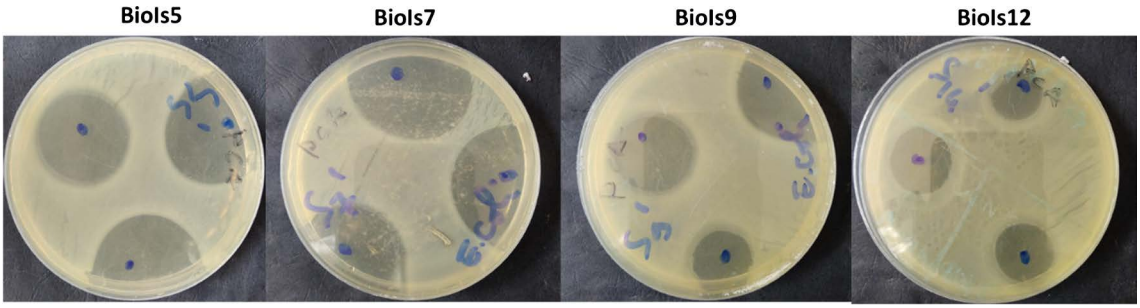

Figure 4. Inhibition halo appearance of biosurfactants isolated from Is5, Is7, Is9, Is12 against *E. coli* and *Shigella flexneri* M90T. BioIs5: Biosurfactant extracted from isolate 5; BioIs7: Biosurfactant extracted from isolate 7; BioIs9: Biosurfactant extracted from isolate 9; BioIs12: Biosurfactant extracted from isolate 12.

some exemples of the behavior of biosurfactant extracts (BioIs5, BioIs7, BioIs9 and BioIs12) against pathogenic bacteria.

In addition diameters of inhibition of all isolates have been measured. We find that BioIs3, Biosurfactant corresponding to isolate 3 (Is3), had developed a larger inhibition diameter on all selected strains, *i.e.* 3.3 cm ± 0.2 for *E. coli*, 4.2 cm ± 0.2 for *S. flexneri*, 3.3 cm ± 0.4 for *Salmonella* sp., 3.5 cm ± 0.4 for *P. aeruginosa* and 4.0 cm ± 0.2 for *S. aureus*. It should be noted that BioIs1 strongly inhibits the growth of *E. coli*. The diameters of inhibitions obtained made it possible to mount the graph below (Figure 5).

3.2. One Step PCR Multiplex Identification among *Lactoctocillus enterrococcus* and *Pediococcus* Strains

The purified isolates were the subject of genomic DNA extraction. Amplification using the 16S rRNA gene was performed on the genomic DNAs of the isolates in order to confirm the bacteria orientation. Nine (9) pairs of primers, targeting genes encoding for bacteriocins, have amplified and discriminated three (3) genera of lactic acid bacteria including *Lactobacillus*, *Enterococcus* and *Pediococcus*.

Direct identification have been done by targeting the 9 genes such as the *sakP* and *sakQ* genes corresponding to *Lactobacillus sakei*, *plnA*/*plnEF* to *Lactobacillus plantarum*, *curA* to *Lactobacillus curvatus*, *entA*/*entB*/*entP* to *Enterococcus faecalis*,

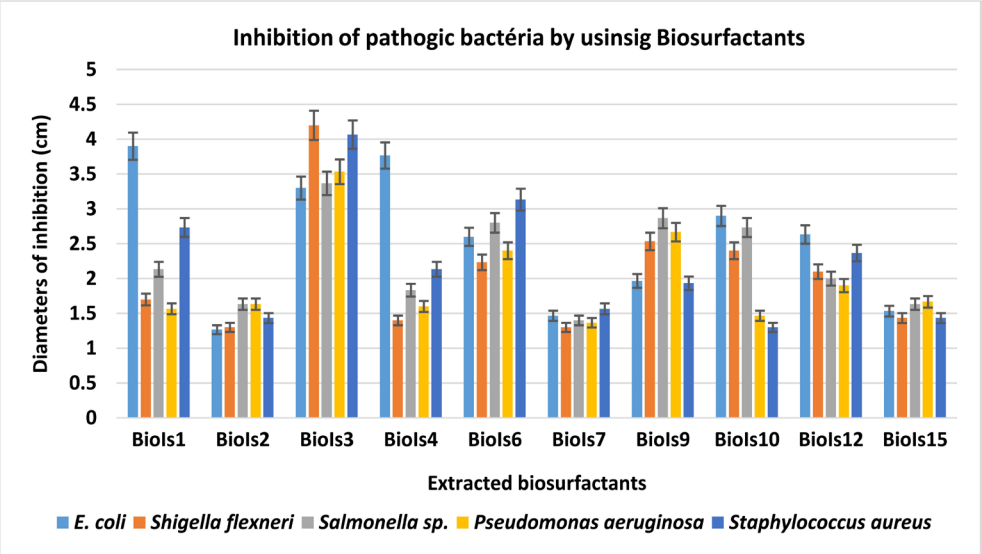

Figure 5. Effect of inhibition of biosurfactants on pathogenic bacteria by Biosurfactants. BioIs1: Biosurfactant extracted from isolate 1, BioIs2: Biosurfactant extracted from isolate 2, BioIs3: Biosurfactant extracted from isolate 3, BioIs4: Biosurfactant extracted from isolate 4, BioIs6: Biosurfactant extracted from isolate 3 isolate 6, BioIs7: Biosurfactant extracted from isolate 7, BioIs9: Biosurfactant extracted from isolate 9, BioIs10: Biosurfactant extracted from isolate 10, BioIs12: Biosurfactant extracted from isolate 12, BioIs 15: Biosurfactant extracted from isolate 15.

pedA to *Pediococcus acidilactici*. 18 PCR of two multiplex groups have been done and 135 single PCRs were performed with nine (9) primers pairs encoding bacteriocin genes. As results, amplifications allow to identify predominantly 33% of Lactic acid bacteria including Is2, Is9, Is12 and Is13 isolates as *L. plantarum*. Using the same multiplexe group *L. sakei*, *L. curvatus*, *E. faecalis* and *P. acidilactici* could not be identified (Table 2).

4. Discussion

In this work the main goal was to appreciate banana wine by its richness Biomolecules secreted during fermentation. Banana has been shown to widely contain bioactive molecules like polyphenol, flavonoids and carotenoids playing important roles in the well-being of humans [5]. The role of bacteriocins and biosurfactants should be considered again. Indeed, in many departments of the Republic of Congo, Congolese are consumers of fermented beverages and sometimes seek for new tastes, novel sensations and good protective beverage. Some molecule contained in banana including vitamin B, protein, amino acids and calories are also important. Plantain is mainly characterized by its sugar content and its density, a factor that conditions subsequent fermentation and ethanol production [5].

We have clearly shown that biosurfactants are highly secreted during fermentation in the extracellular medium. 15 isolates were found as part of this job. They are all capable of producing biosurfactants. 66.66% produce extractible biosurfactants. Until the writing of this paper, no study on fermented foods and

Table 2. Identification of microorganisms by amplification of the genes that encode bacteriocins. Is 2, 9, 12, 13: Isolates 2, 9, 12, 13. NI: unidentified.

Multiplex groups	Targeted genes	Size (pb)	Targeted species	Isolates identified
Group 1	*curA-F* *curA-R*	180	*Lactobacillus curvatus*	NI
	sakP-F *sakP-R*	200	*Lactobacillus sakei*	NI
	entB-F *entB-R*	162	*Enterococcus faecium*	NI
	entA-F *entA-R*	126	*Enterococcus faecium*	NI
	plnEF-F *plnEF-R*	428	*Lactobacillus plantarum*	Is2, Is9, Is12 and Is13
Group 2	*entA-F* *entA-R*	126	*Enterococcus faecium*	NI
	entP-F *entP-R*	112	*Enterococcus Faecium*	NI
	pedA-F *pedA-R*	600	*Pediococcus acidilactici*	NI
	sakQ-F *sakQ-R*	200	*Lactobacillus sakei*	NI
	plnA-F *plnA-R*	450	*Lactobacillus plantarum*	Is2, Is9, Is12 and Is13

drinks has shown the secretion of biosurfactants directly in the ferment. The biosurfactants extracted with chloroform and ammonium sulphate are able to inhibit the growth of pathogenic bacteria including *E. coli*, *S. flexneri* M90T, *Salmonella* spp, *P. aeruginosa* and *S. aureus*.

The tendency of lactic acid bactreria is to acidify the medium [4] [11] [26]. The capacity of microorganisms to secrete and biosurfactants in the extracellular medium could explain the absence of the pathogenic bacteria of the genus *Shigella*, *Salmonella*, *Staphylococcus* and although *Shigella* is able to live 2 hours inside of the stomach and cause bacillary dissentery [27] [28] [29]. Since the fermentation of the plantain wine lasts 96 hours, and the pH goes down very quickly, it is almost difficult for *Shigella* spp. and *Salmonella* spp. to resist in this condition. *Lactobacillus* spp. are also able to secrete other biomolecules such as bacteriocins [26] [30] which can also inhibit the growth of the bacterial pathogens mentioned above [31]. Biosurfactants have already been proposed as a preservative in the food industry [17] [32] [33]. The evaluation and the functional characterization of a biosurfactant produced by *L. plantarum* CFR 2194 have been done. Fourier transform infrared spectroscopy (FTIR) spectra demonstrated that

biosurfactants were constituted by protein and polysaccharide fractions by possessing a glycoprotein like structure [34]. We can postulate that our Biosurfactant could be a glycoprotein because this can be easily extracted from supernatent by using ammonium sulfate ($(NH_4)_2SO_4$) favourizing by the salting out mecanisms [35]. The probiotic effect and ability of bacteria of the *Lactobacillus* spp. to inhibit the growth of pathogenic bacteria has already been demonstrated [31] [36] [37] [38]. Many studies have focused on the inhibition of pathogens by targeting bacteriocins. A previous study on banana wine in Rwanda also showed the absence of these pathogenic bacteria [39].

By using specific culture media we have shown that the bacteria like *Shigella flexneri*, *Salmonella* spp., *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus*, *Clostridium* spp. are totally absent on the tested samples. MRS allow us to isolate 15 isolates from plantain wine. One step PCR multiplex have been used to identify isolates among five lactic acid bacteria species including *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactobacillus curvatus*.

The most representative being *L. plantarum* (33%) associated to Is2, Is9, Is12 and Is13. It should be interesting notified that no study has been focused on genes encoding for bacteriocins to discriminate bacilli and cocci from lactic acid bacteria in a one step multiplex-PCR. By the way several methods of screening, purification and antimicrobial potentialities of bacteriocin in Health Care for human being have been mentioned [40] [41] [42]. The development and validation of a one step Multiplex-PCR assay for the detection of ten *Lactobacillus* species, has been recently documented [12] [43]. In addition random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis was used to identify strains of *L. plantarum* [13] [44] [45].

5. Conclusion

This work allowed showing that biosurfactant can be secreted directly in the extracellular medium. *Lactobacillus* isolated from plantain wine is a part of this job. A novel one step multiplex PCR that targets genes encoding for bacterioscins allowed to identify *L. plantarum* (Is2, Is9, Is12 and Is13) that are able to secrete biosurfactants playing an important role in the preservation of banana wine. The biosurfactant extracts with chloroform and ammonium sulphate are able to inhibit the growth of pathogenic bacteria such as *Shigella flexneri*, *Salmonella* spp., *Pseudomonas aeruginosa* and *Staphilococcusn aureus*. This work also constitutes a scientific support, a reference tool and a source of information that may be of interest to future scientists and researchers who would like to deepen the analysis. The processes and methods we used, could serve as a basis and example for the manufacture of all other local wines under better conditions and standards for the health of the population. The control of local beverages production could be the very important input of nutritious beverages for the benefit of the people because contains very important bioactive compounds including polyphenol and flavonoïds and carotenoids.

Acknowledgements

We are grateful Prof. Clobite Bouka Biona (Université Marien Ngouabi), and Dr. Bienvenu Dinga (Université Marien Ngouabi) for their continuous encouragements and material support. This work was supported in part by Institut National de Recherche en Sciences Exactes et Naturelles (IRSEN).

Conflicts of Interest

The authors declare that the research was conducted in the absence of any intellectual commercial or financial relationships that could be construed as potential conflicts of interest.

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