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A Meta-Advance of *Bacillus*-Mediated Biosurfactant Augmentation in the Chikwangue Composition

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Abstract

Cassava is the most widely distributed food crop in Central Africa. Chikwangue, also known as kwanga in the Republic of Congo, is a starchy fermented cassava product that is a staple food in the country. This work aims to determine the composition of bioactive compounds in chikwangue, including biosurfactant-like molecules and proteins content. Antibacterial activities were investigated through the preliminary emulsification index of chikwangue and fermented paste. Antibacterial assay, 16S rRNA, cytK, hblD, nheB and entFM PCR amplifications, DNA sequence analysis, NCBI homology analysis, and phylogenic tree were performed using NGPhylogeny. fr and iTOL (interactive of live). Fermented cassava paste and chikwangue contain biosurfactants with an emulsification index of 50%. The total protein concentration in fermented cassava paste was 4 g/ml and the chikwangue was 2.5 g/mL Further sequence analysis showed that isolates shared a homology of up to 99.9% with *Bacillus cereus* PQ432941.1, *B. licheniformis* PQ432758.1, *B. altitudinis* PQ432754.1, *B. subtilis* PQ432759.1, *B. mojavensis* PQ432755.1, *B. tequilensis* MT994788.1, *B. subtilis* MT994789.1, *Paenibacillus polymyxa* PQ452544.1, *B. velezensis* PQ452545.1, *B. thuringiensis* PQ432763.1, *B. pumilus* PQ432762.1, *B. subtilis* MT994787.1, *B. mycoides* PQ432890.1, *B. thuringiensis* PQ432766.1, *B. subtilis* PQ432757.1 and *B. amyloliquefaciens* PQ432756.1. Importantly, the emulsification index (E24) ranged from 60 to 100% and the crude biosurfactant for the *Bacillus* strains mentioned above could easily inhibit the growth for pathogen Gram-negative bacteria (*S. enterica, S. flexneri*, *E. coli*, *Klebsiella* sp. and *P. aeruginosa*) with diameters ranging from 2.3 ± 0.1 cm to 5.5 ± 0.4 cm. On the other hand, the diameters of Gram-positive pathogenic bacteria (*B. cereus* and *S. aureus*) varied between 1.5 ± 0.5 cm and 4.0 ± 0.2 cm. These findings involve the promise purpose of

Bacillus isolated from retted cassava, and this study systematically uncovered the biodiversity and distribution characteristics of retted paste cassava and chikwangue.

Keywords

Bacillus, Augmentation, Biosurfactant, Proteins

1. Introduction

Bacillus species are a cosmopolitan genus, present in various environments, capable of forming spores under conditions hostile to life [1]. They are recognised for their biotechnological importance [2], due to their ability to rapidly produce biomolecules involved in biocatalysis [3] [4], and biopreservatives such as biosurfactants [2] [5]-[9]. Fermented foods still occupy an important place in the diet of populations (reference). Cassava (*Manihot esculenta* Crantz) is a staple food crop grown in tropical and subtropical areas [10]. It is best known for its daily use as a starch in various dishes. Dishes based on fermented tubers vary according to countries and regions [10]. The nutritional values of the cassava plant in roots and leaves were centralised around glucosides, lipids, proteins, dietary fibre and some essential minerals [11]. Multiple pharmacological activities of cassava, including anti-inflammatory, analgesic, anti-cancer, antibacterial, anti-diabetic, antihypercholesterolemic, anti-diarrhoeal, and antihelmintic activity, have been demonstrated [11].

It is important to remember that the antibacterial and antioxidant effect is due to the presence of phenolic compounds. Traditionally, foods made from fermented cassava and derivatives remain widely consumed foods throughout the world, reaching up to 90% consumption in the Republic of Congo.

Reviews postulated that cassava fermentation could enhance the content of bioactive compounds such as phenolic compounds. Bacteria of the genus *Bacillus* are producers of biosurfactants such as surfactins and other lipopeptides [12]. Several studies have shown that these bacteria constitute most of the flora during the fermentation of cassava tubers (reference). *B. subtilis* has been widely used in the production of biosurfactants using cassava fermentation [7] [13].

The involvement of bacteria of the genus *Bacillus* in the increase of phenolic compounds has been clearly demonstrated in our laboratory using ginger juice fermentation of ginger juice [13]. The phenolic compounds found in cassava tubers have not yet been the subject of a detailed study. Most of the research has focused on cassava leaves and stems in terms of the composition of phenolic compounds [11].

In this work, we are interested in chikwangue made using a fermentation process. Chikwangue is a traditional Congolese fermented and retted cassava tuber food with promises of prebiotic and probiotic *Bacillus* species. The composition of chikwangue is highly dependent on the cassava species. The protein composition is not the same in all species of *Manihot esculenta* [14]. In addition to lipids,

carbohydrates, and cyanogenic compounds [15], no studies have directly demonstrated the presence of biosurfactants in chikwangue. Particular attention will be paid to the nutritional interest of this food in terms of protein content and amino acids. This work primarily aims to determine the composition of chikwangue in terms of biosurfactant compounds. Many studies claim that cassava has an antibacterial effect. Cassava contains cynogenic compounds that could play this role [16] [17]. However, no studies have investigated chikwangue for its action against pathogenic pathogens. In this work, we highlight and discuss the origin of biosurfactants as well as their biochemical nature.

2. Methods

2.1. Biological Materials and Biosurfactant Production

Ten cassava tubers were harvested from two different markets located in the Bacongo and Makélékélé districts of the Brazzaville, Republic of Congo. Each sample was processed to test the presence or name of biosurfactants.

To control the fermentation of cassava tubers, we peeled and washed 500 g of cassava tubers, which we then left to ferment for 4 days. After fermentation, we proceeded to defibration to recover the retted dough. Once drained, this dough was traditionally kneaded to begin the transformation process into chikwangue, which was heated to boiling for 2 to 4 hours. Chikwangue was left at room temperature to cool. The emulsification test was carried out considering all key stages of fermentation (Batch 1: Solution containing the liquid of freshly harvested and crushed cassava tubers. A 5 ml solution was obtained after filtration. Batch 2: Solution whose fermented and retted tubers were mixed with physiological saline. A 5 ml solution was obtained after filtration. Batch 3: 10 g of Chikwangue were mixed with physiological saline. The mixture was vortexed. All batches of samples were subjected to a protein assay using the Bradford method. A 5 mL solution was obtained after filtration. Each batch was carried out three times in a row to optimise reproducibility. In this experiment, a physiological saline control was used. Each batch was mixed with 5 ml of essence (v/v). The mixture was shaken vigorously for 5 minutes with a vortex mixer (VELP Scientifica, Italy). The cassava tubers were then incubated at room temperature at room temperature for 24 hours. Subsequently, the height of the emulsion layer and the total height of the mixture were measured (all experiments were carried out in triplicate), and the emulsification index (E24%) was calculated using the standard formula E24% = (He/Ht) × 100, where He represents the height of the emulsion, Ht the total height of the mixture and E24% the percentage of emulsification after 24 hours.

2.2. Isolation and Characterization of Strains

To explain the presence of biosurfactant in batches 1 and 2, 10 g were aseptically collected in a sterile tube at different stages of chikwangue preparation. Samples were homogenized with physiological sterile water and distributed in sterile microbiological tubes. Then successive dilutions were performed, and the bacterial

suspensions were inoculated in a Mossel agar medium (10.0 g of peptone, 1.0 g of meat extract, 10.0 g of mannitol, 10% of egg yolk, 0.01 g of polymyxin B sulphate, 0.025 g of phenol red, 10.0 g of sodium chloride, 14.0 g of agar and pH 7.2) to promote the growth of *Bacillus*. The plates were incubated at 37˚C for 24 hours and the colony count was carried out in triplicate. The purification of the isolates was carried out rigorously by successive subcultures using Luria-Bertani (10 g of peptone, 5 g of yeast extract, 10 g of NaCl). The morphological characteristics of the colonies, such as shape, size, and color, were recorded. Morphological characterisation was performed using a light microscope (OPTIKA, Italy), and Gram-staining was performed with a solution of 3% potassium hydroxide (KOH). For future experiments, all purified isolated cultures were stored at −20˚C in Luria-Bertani (LB) broth containing 20% glycerol (v/v).

2.3. Genomic DNA Extraction, Molecular Identification and Bioinformatics Analysis

Isolates with a good percentage of the emulsification index were subjected to genomic DNA extraction and purification. This experiment was carried out using the NucleoSpin Microbial DNA Kit (Macherey-NAGEL, Germany). Briefly, isolates were cultured in 5 ml of LB broth for 24 hours at 37˚C with shaking. DNA purity was assessed by the UV absorbance ratio (260/280 nm). 1 µL of template DNA with concentrations equalling 10 - 20 ng/µL. Universal 16S rRNA primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') were used to amplify the 16S RNA gene (Eurogentec). 5 µl of each amplification product was mixed with 2 µL of loading buffer (BIOKE, The Netherlands). The mixtures were then electrophoresed on a 1% (w/v) agarose gel. The molecular weight marker used was the 10-kb 2-Log DNA sample (BIOKE, The Netherlands).

The confirmation of the identification of *Bacillus* species was carried out according to the method proposed by Kaya Ongoto *et al.* [18]. The species *B. amyloliquefaciens, B. subtilis, B. pumilus, B. licheniformis, B. altitudinis, B. mojavensis, B. safensis* and *B. atrophaeus* were identified through the amplification of the *fibE* gene, responsible to produce the fibrinolytic enzyme. PCR products were purified using the Gel Extraction Kit (Omega Biotek), the purified products were subjected to Sanger sequencing (3130xl Genetic Analyser, Applied Biosystems). The sequences obtained were aligned with Bio Numerics 7.5 software (Applied Maths, Belgium) and manually corrected to resolve the discrepancies between the sense and antisense strands. The sequences were compared with homologous sequences contained in sequence databases through the NCBI portal using the BLASTn programme (https://www.ncbi.nlm.nih.gov/). A multiplex endpoint PCR assay was used to identify *B. cereus* enterotoxins: hemolysin BL (*hblD*) with Eurogentec primers (*hblD*-F (5'-ATGAAAAAATTTCCATTCAAAGTACTAAC-3') and *hblD*-R (5'-GAATATCATTCCAACTTTCTTTAGCGGC-3'), non-haemolytic enterotoxin (nhe) with primers nheB-F (5'-ATGGCTCTATCAGCACTTATGGCAG-3') and nheB-R (5'-TTAAGCTTTTTTCGTATCTACTACTTTA-

ATAC-3') and enterotoxin FM toxin genes (entFM) with primers entFM-F (5'-GTTGCAGTTCCAGGTATGGATTCTGC-3') and entFM-R (5'TTCTGCAC-TAATGAACTGACCGTTTCC-3'). Amplification of the cytotoxin K gene (CytK) was detected in isolates classified as *B. mycoides* CytK-F1 (5′-CAAAACTCA(T/C)-CTATGCAATTATGCAT-3′), CytK-F2 (5′-AAAATGTTTAGCATTATCCG-CTGT-3′) and CytK-R (5′-ACCAGTTGTATTAATAACGGCAATC-3′). The phylogenic tree has been designed to facilitate the execution of phylogenetic workflows. The new generation phylogenetic services of NGPhylogeny.fr have been used to perform iTOL (interactive of live) [19].

2.4. Crude Biosurfactant Extraction and Antagonistic Effect

Isolates identified with a higher percentage of biosurfactant production were extracted as previously done [20]. Briefly, after 24 hours of incubation, the bacterial suspension of each isolate was centrifuged at 11,000 rpm for 15 minutes and the obtained supernatant was treated with HCl until a pH of 2.0 was obtained. The mixture obtained was then incubated at 4˚C for 24 hours. After incubation, a deposit was observed at the bottom of the tube, indicating the presence of the biosurfactant extract. This mixture was centrifuged again at 11,000 rpm for 15 minutes and once the supernatant was removed, the precipitate was collected by elution in 500 µL of 1X phosphate buffer saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 1.76 mM KH_2PO_4) and kept cool to perform the inhibition test.

The antibacterial activity of the biosurfactant extract was evaluated against seven laboratory pathogenic strains (*E. coli*, *S. aureus*, *S. flexneri*, *S. enterica*, *B. cereus*, *P. aeruginosa* and *Klebsiella* sp), according to the method described previously [21]. The wells were prepared aseptically in plates containing counting agar. The microorganism to be tested was inoculated in the gel, then a volume of 75 µl of the biosurfactant extract was deposited in the wells. After an incubation period of 24 hours at 37˚C, the diameter of the inhibition zones was measured. The average of the three measurements was taken to ensure the reproducibility of the results.

2.5. Hydrolase Production Assay: Proteolytic, Amylolytic, and Cellulolytic Activities

To explain the increase in total crude secreted protein concentration in fermented and retted cassava paste (RCP) and diluted chikwangue (DC), we first resuspended 10 g of RCP and 10 g of chikwangue in 1X phosphate buffer saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4). The solutions (RCP and DC) were vortexed and filtered using filters with a diameter of 0.22 µm to exclude bacteria and fibres. After filtration, the solutions were centrifuged at 10,000 xg and concentrated 10 times using concentrators (PierceTM Protein Concentrators PES, 5K MWCO, 0.5 to 100 ml). The protein assay had been performed using the Bradford method (Bio-rad, USA). The proteolytic, amylolytic, and cellulolytic activities of the solution had been tested to degrade casein in skim milk, cassava starch, and

cellulose, on the one hand. On the other hand, the ability of *B. amyloliquefaciens, B. subtilis, B. pumilus, B. licheniformis, B. altitudinis, B. mojavensis, B. safensis and B. atrophaeus, B. mycoides, B. cereus, B. thuringensis, B. mycoides, Paenibacillus polymixa* and *B. tequilensis* strains to produce proteases, amylases, cellulases was based on their ability to degrade casein in skim milk, cassava starch, and cellulose as substrates. The methodology carried out was previously described [7] [20]. In terms of proteolytic activities, an overnight culture of *Bacillus* strains was prepared in Luria broth (LB) at 37˚C. The 10 ml of culture was then centrifuged at 12,000 × g for 10 min and the supernatant was concentrated 10 times and 75 µL of each culture supernatant was transferred to a well containing 1% agarose and skim milk (10%). Regarding the amylolytic and cellulolytic activities, 1 g of starch and 0.5 g of cellulose were added separately in 100 ml of LB agar. The Petri dishes were incubated at 37˚C for 24 h. Positive activity was revealed by the presence of a halo around the well; the diameters of the hydrolysed halo were measured. A Lugol solution (Merck, Germany) was used to reveal positive amylolytic and cellulolytic activities. The percentage of lysis for each isolated colony was evaluated using the formula% L = ((DT DC)/DT), where% L is the percentage of lysis, DT is the total diameter (colony and halo) and DC is the colony diameter. The average of the three measurements was determined.

2.6. Statistical Analysis

GraphPad Prism version 8.0 software was used to determine significance thresholds. Principal component analysis (PCA) was used to investigate the correlation between pathogenic bacteria in antagonistic activities. Before ordination, strains abundance data was transformed to better meet the assumptions of normality [22] using ln (x + 1). All analyses were performed using CANOCO (Canonical Community Ordination, version 4.5) [23].

3. Results

3.1. Biochemical and Microbiological Composition of Chikwangue

To demonstrate the presence of biosurfactants in fresh cassava, fermented tubers, and chikwangue, we tested the emulsifying activity of samples from batches number 1, 2 and 3. The results show that batch number 1, the emulsification index was zero (E24: 0%), which shows that fresh cassava does not contain any biosurfactants. However, batches 2 and 3 allowed to obtain emulsification indices (E24) ranged from 50% to 100% (**Figure 1(A)**, **Figure 1(E)**). *Bacillus* spp. is a remarkably diverse bacterial species that is capable of growth within many environments. In parallel, the protein concentrations of the samples were measured. The concentration of the sample of tubers without fermentation was 450 mg/mL. On the other hand, the sample of fermented cassava paste was 4 g/ml and the chikwangue was 2.5 g/mL. The extraction of biosurfactants in the presence of HCl allowed one to show that the total biosurfactants retained their emulsifying activity (**Figure 1(B)**, **Figure 1(F)**). The total crude extract of biosurfactants was able to inhibit the growing of

S. flexneri, Klebsiella sp., Salmonella sp., E. coli, Pseudomonas, Staphylococcus aureus and Bacillus cereus (Figure 1(C), Figure 1(D)). The crude biosurfactant sample directly from fermented cassava tuber paste had diameters ranging from 2.4 ± 0.4 (cm) to the chikwangue 3.8 ± 0.4 (cm) compared to biosurfactant samples whose diameters varied between 1.9 ± 0.7 (cm) to 3.4 ± 0.4 (cm) except for antagonism with the strain of *Staphylococcus strain,* which had a large diameter of the order of 4.7 ± 0.4 (cm) (Figure 1(C), Figure 1(D)).

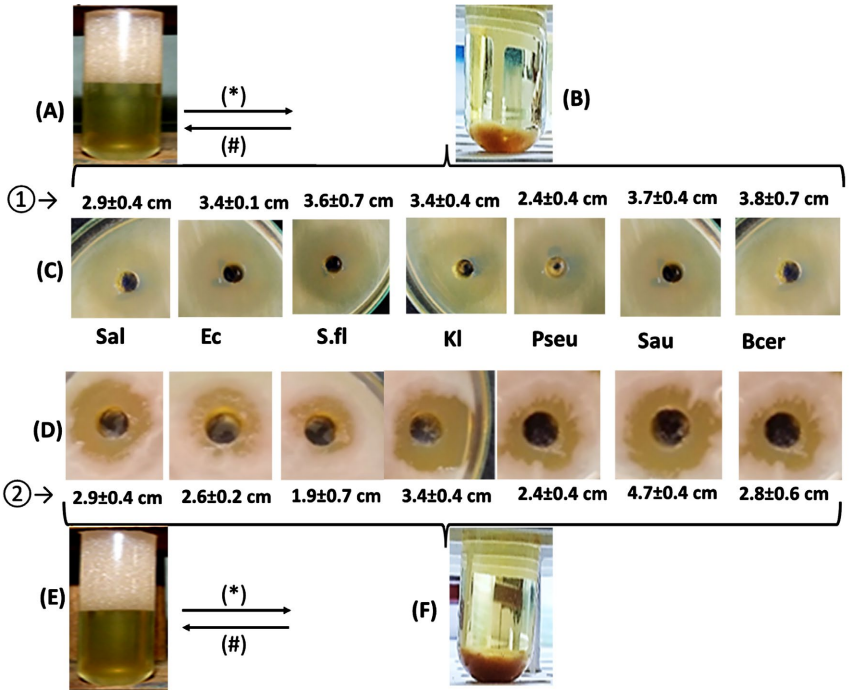


Figure 1. From the production of biosurfactants to the antagonism test of fresh samples of fermented paste from cassava tubers and chikwangue. A: Emulsification index for biosurfactant isolated from retted cassava pasta, B: Crude extract of biosurfactant from retted cassava pasta, C: Antibacterial effects of the the biosurfactant isolated from the retted cassava pasta. D: Antibacterial effects of biosurfactant isolated from chikwangue. E: Biosurfactants isolated from chikwangue suspension. F: Crude extract of chikwangue biosurfactant. (*): Biosurfactant extraction process using HCl, F: crude extract of biosurfactant (#): Emulsification index showing the activity of biosurfactants extracted from retted cassava pasta, ①: Diameters (cm) of chikwangue the crude extract of biosurfactants isolated from the retted cassava paste before cooking, ②: Diameters (cm) of the crude extract of biosurfactants isolated. S.fl: *Shigella flexneri*, Sal: *Salmonella* spp., Ec: *Escherichia col,i*, Kl: Klebsiella spp., Psau: *Pseudomonas* spp., Bcer: *B. cereus*,.

3.2. Bacillus Isolates Molecular Identification

After serial dilutions, isolation, and culture of the unfamiliar bacteria, the culture characteristics, morphology observation, biochemical test, and precursory antibacterial test had been done. A total of 100 isolates were obtained. An emulsification test was performed on the 100 isolates. 16% of isolates including Mon33, Mon7liche, Mon7alt, Mon10sub, Mon12moj, Mon25, Mon31, Mon55, Mon77, Mon26, Mon30, Mon24, Mon36, Mon40, mon67sub, and mon67amy were the

subject of molecular identification using 16S RNA, cytK, hblD, nheB and entFM genes. The sequences were submitted to the NCBI portal with the following accession numbers *B. cereus* (Genbank: PQ432941.1), *B. licheniformis* (Genbank: PQ432758.1), *B. altitudinis* (Genbank: PQ432754.1), *B. subtilis* (Genbank: PQ432759.1), *B. mojavensis* (Genbank: PQ432755.1), *B. tequilensis* (Genbank: MT994788.1), *B. subtilis* (Genbank: MT994789.1)

Paenibacillus polymyxa (Genbank: PQ452544.1), *B. velezensis* (Genbank: PQ452545.1), *B. thuringiensis* (Genbank: PQ432763.1), *B. pumilus* (Genbank: PQ432762.1)*B. subtilis*, (Genbank: MT994787.1), *B.mycoides* (Genbank: PQ432890.1), *B.thuringiensis* (Genbank: PQ432766.1), *B. subtilis* (Genbank: PQ432757.1) and *B. amyloliquefaciens* (Genbank: PQ432756.1). *B.* species belonging to group II were amplified with the *fibE* gene encoding subtilisin. This concern *B. subtilis*, *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. altitudinis, B. mojavensis, B. safensis*, *B. atrophaeus* and B. *mojavensis*. The detected DNA fragments at around 800 bp were sequenced, which confirmed the bacterial species. *The B. cereus* species had haemolytic activity. PCR amplification from the nicked genes of strain *B. cereus* with primers hblD-F/hblD-R, nheB-F/nheB-R, entFM-F/entFM-R allowed us to make the difference between *B. thuringensis* and *B. cereus*. PCR amplification of the cytK gene from strain *B. mycoides* with the set CytK_F1 and CytK_R allowed the production of a 369-bp amplicon, while the usage of the primer pair F2-CytK and R-CytK resulted in the formation of a 238-bp amplicon.

We established the phylogenetic tree of the strains that were obtained using live iTOL interactive of live (Figure 2). As a result, *Bacillus* spp. Respect their phylogenic relationship.

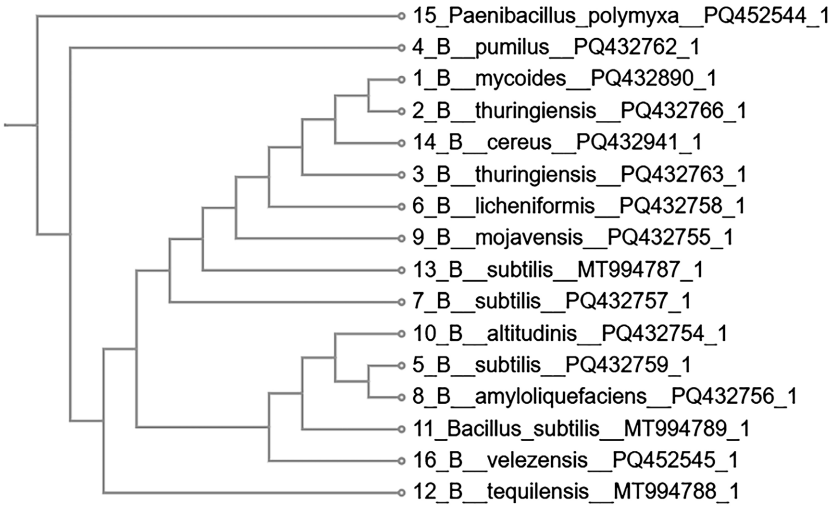


Figure 2. Phylogenetic tree of the 16S rRNA sequences of some *Bacillus* strain. The tree was built using iTOL interactive of live. The name of species had been associated with sequence accession numbers in GenBank. The neighbour-joining consensus tree used 1000 bootstrap replicates. Numbers represent the distance at each branch.

3.3. Comparison of Hydrolase Production and Biosurfactant Increase the Biomolecules Concentrations of Retted Cassava Paste (RCP) and Diluted Chikwangue (DC)

The amylolytic, proteolytic and cellulase activities of fresh tubers, fermented cassava tuber paste and chikwangue were compared with the capacity of the different strains identified. The percentages of activities range between 60 and 100%. However, it should be noted that chikwangue presents a percentage of 0% in its proteolytic, amylolytoic and cellulosic activity. Chikwangue could emulsify up to 47% (Figure 3). The suspension was concentrated 10 times and emulsified to 100%.

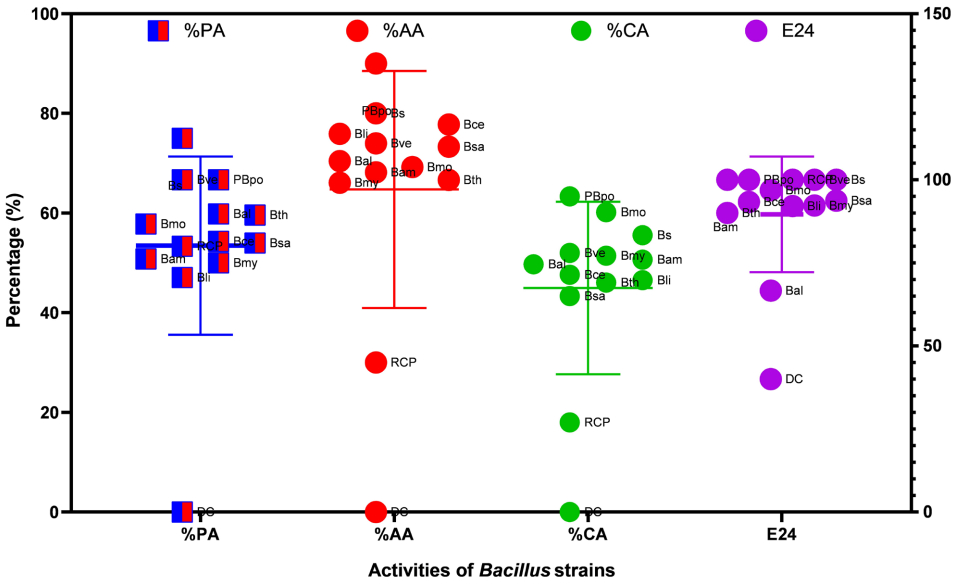


Figure 3. Comparison of hydrolase production and biosurfactant. RCP: retted cassava paste, DC: diluted chikwangue, Bs: *B. subtilis*, Bam: *B. amylolechefasciens*, Bli, *B. licheniformis*, Bmy: *B. mycoides*, Bal: *B. altitudinis*, Bce: *B. cereus*, Bth: *B. thuringensis*, Bmo: *B. mojavensis*, Bsa: *B. safensis*, Ppp: *Pb. Polymyxa*, Be: *B. velezensis*. %PA: percentage of proteolytic activity, the small squares are marked in red and blue. %AA: percentage of amylolytic activity, the small circle is marked in red. % CA: percentage of cellulolytic activity, the small circle is marked in green. E24: Emulsification index after 24 hours, the small circle is marked in violet.

3.4. Bacillus Isolated from Retted Cassava Paste Have Antimicrobial Properties

Biosurfactant extracts of *Bacillus* strains including *B. subtilis*, *B. amylolechefasciens*, *B. licheniformis, B. mycoides, B. altitudinis, B. cereus, B. thuringensis, B. mojavensis, B. safensis Pb. polymyxa, B. velezensis,* and *B. subtilis* strain 48 (used as a positive control) were obtained. Antibacterial tests on pathogenic bacteria (*E. coli, S. flexneri, S. enterica, Klebsiella* sp., *P. aeruginosa, S. aureus,* and *B. cereus*) were performed. This work revealed that all biosurfactant extracts of *Bacillus* strains could inhibit the growth of pathogenic bacteria. The diameters of the Gram-negative pathogen bacteria (*S. enterica*, *S. flexneri*, *E. coli*, *Klebsiella* sp., *P.*

aeruginosa) ranged from 2.3 ± 01 cm to 5.5 ± 0.4 cm. On the other hand, the diameters of Gram-positive pathogenic bacteria (B. cereus and S. aureus) varied between 1.5 ± 0.5 cm and 4.0 ± 0.2 cm. These values appear to be lower than those obtained with Gram-negative bacteria. The diameters of *S. enterica* ranged from 2.6 ± 0.3 cm to 4.8 ± 0.2 cm. The biosurfactant of *B. subtillis* was the most significant inhibition compared to *Salmonell*a. The diameters of *E. coli* ranged from 2.3 ± 0.1 cm to 5.5 ± 0.4 cm. The biosurfactant of *B. licheniformis* was the most significant inhibition compared to *E. coli*. (Table 1). The bacterium of the genus *Shigella* were more sensitive to biosurfactants isolated from Bacillus whose diameters varied between 3.1 ± 0.1 cm and 4.7 ± 0.4 cm. The bacteria of the genus *Klebsiella* were also sensitive to biosurfactants isolated from *Bacillus* whose diameters varied between 3.1 ± 0.1 cm and 4.1 ± 0.5 cm (Table 1).

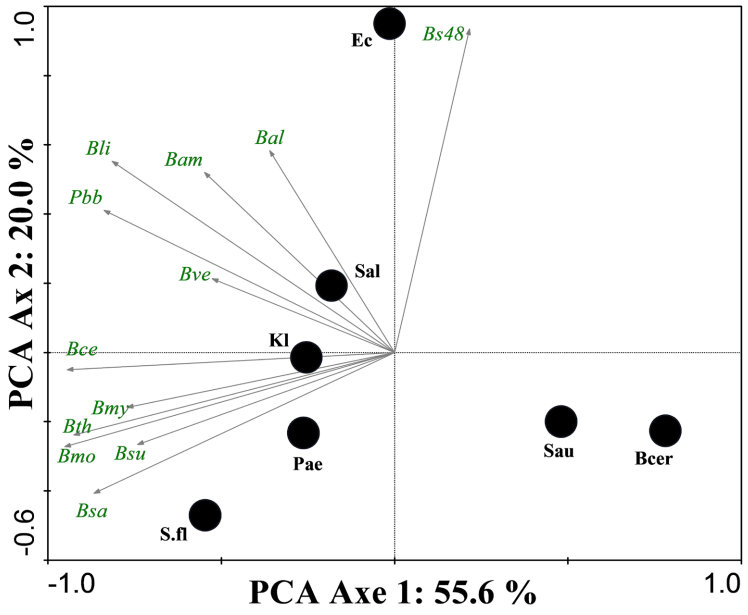
Table 1. Antibacterial effect of Bacillus strains on pathogenic bacteria.

		Pathogenic bacteria codes						
Codes	**Strains**	**Sal**	**Ec**	**Bcer**	**Sau**	**S.fl**	**Kl**	**Pae**
Bsu	***B. subtilis***	4.8 ± 0.2	2.3 ± 01	2.0 ± 0.4	3.0 ± 0.0	4.1 ± 0.1	4.1 ± 0.5	3.3 ± 0.1
Bam	***B. amylolechefasciens***	3.7 ± 0.5	3.3 ± 0.2	2.3 ± 0.1	2.5 ± 0.3	3.1 ± 0.1	3.1 ± 0.5	2.3 ± 0.1
Bli	***B. licheniformis***	4.7 ± 0.7	5.5 ± 0.4	2.0 ± 0.1	2.6 ± 0.4	4.1 ± 0.0	4.1 ± 0.0	4.5 ± 0.3
Bmy	***B. mycoides***	3.7 ± 0.5	3.1 ± 0.0	2.2 ± 0.1	2.8 ± 0.6	4.5 ± 0.1	3.5 ± 0.5	4.1 ± 0.1
Bal	***B. altitudinis***	2.6 ± 0.3	3.8 ± 0.5	2.1 ± 0.1	3.1 ± 0.1	3.7 ± 0.5	3.7 ± 0.1	2.8 ± 0.4
Bce	***B. cereus***	2.7 ± 0.2	2.9 ± 0.4	1.5 ± 0.5	2.1 ± 0.1	4.6 ± 0.4	3.6 ± 0.2	2.9 ± 0.3
Bth	***B. thuringensis***	2.9 ± 0.4	2.8 ± 0.3	1.7 ± 0.1	2.7 ± 0.2	4.7 ± 0.4	3.7 ± 0.3	3.8 ± 0.3
Bmo	***B. mojavensis***	3.1 ± 0.7	2.3 ± 0.2	1.6 ± 0.5	2.0 ± 0.0	4.7 ± 0.4	3.7 ± 0.2	3.3 ± 0.1
Bsa	***B. safensis***	2.9 ± 0.6	2.3 ± 0.3	2.2 ± 0.2	2.0 ± 0.0	4.5 ± 0.2	3.5 ± 0.1	3.3 ± 0.1
Pbb	***Pb. polymyxa***	3.3 ± 0.3	3.8 ± 0.5	2.1 ± 0.4	2.0 ± 0.0	3.3 ± 0.1	3.3 ± 0.1	3.8 ± 0.4
Bve	***B. velezensis***	3.3 ± 0.1	3.8 ± 0.5	3.1 ± 0.4	2.0 ± 0.0	3.3 ± 0.4	3.3 ± 0.2	4.8 ± 0.3
Bs48	***B. subtilis st 48***	4.3 ± 0.4	4.8 ± 0.7	4.1 ± 0.1	4.0 ± 0.2	3.8 ± 0.4	3.9 ± 0.4	3.8 ± 0.6

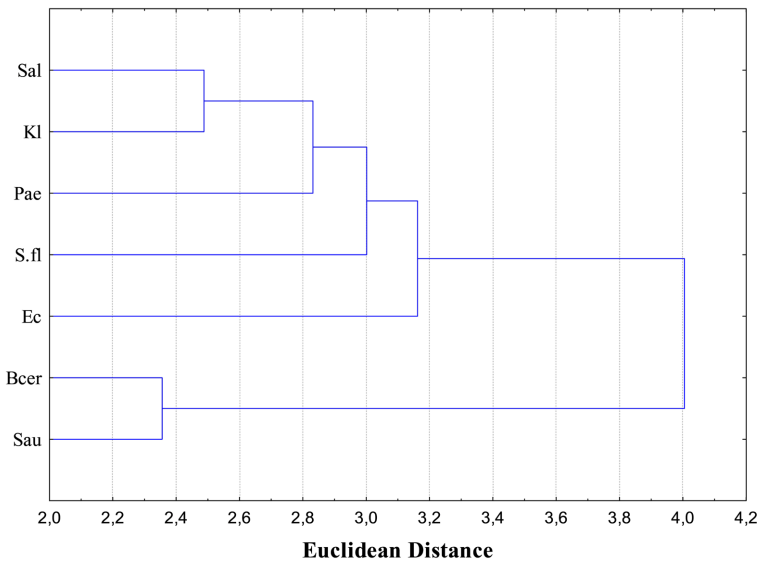
Distances and correlations were determined based on PCA analyses and dindogram construction based on inhibition diameters (Figure 4(A)-(C)). Synergy

of the antibacterial effect was determined. Based on the Euclidean distances, the antibacterial effect was grouped. Note the groups whose antibacterial effect is correlated with group 1 consisting of B. cereus and S. aureus (Figure 4(B)). Both bacteria are Gram-positive bacteria. However, group 2 is larger. It consists of two subgroups. The inhibition values of group 2a, including S. flexneri and E. coli are close and those of group 2b correlate with S. enterica, P. aeruginosa and Klebsiella bacteria (Figure 4(B)).

In the Euclidean distance analysis, the longest distance was observed between *B. cereus* and *S. flexneri* whose value was around 7.08. The lowest value was observed between *B. cereus* and *S. aureus* whose distance was 2.36 (Figure 4(A)-(C)).



(A)



(B)

Euclidean Distance							
	Sal	**Ec**	**Bcer**	**Sau**	**S.fl**	**Kl**	**Pae**
Sal	0,00						
Ec	3,26	0,00					
Bcer	5,17	4,96	0,00				
Sau	4,00	4,25	**2,36**	0,00			
S.fl	3,75	5,20	**7,08**	5,84	0,00		
Kl	2,49	3,41	5,30	4,01	3,00	0,00	
Pae	2,87	3,16	5,33	4,68	3,35	2,83	0,00

(C)

Figure 4. A: PCA of the antibacterial effect of Bacillus strains on pathogenic bacteria. Bsu: *B. subtilis*, Bam: *B. amylolechefasciens*, Bli: *B. licheniformis*, Bmy: *B. mycoides*, Bal: *B. altitudinis*, Bce: *B. cereus*, Bth: *B. thuringensis*, Bmo: *B. mojavensis*, Bsa: *B. safensis*, Pbb: *P. polymyxa*, Bve: *B. velezensis*, Bs48: *B. subtilis* strain 48 (MK099888.1). S.fl: *S. flexneri,* Sal: *S. enterica.,* Ec: *E. coli*, Kl: *K. pneumoniae*, Psau: *P. aeruginosa*, Bcer: *B. cereus.*

4. Discussion

In this work, amylolytic, cellulosic, and proteolytic activities and biosurfactant production ranged from 60 to 100%. The biosurfactants were extractable and retained their respective activities. The use of biosurfactants on the pathogenic bacteria *S. flexneri*, *Klebsiella* sp., *S. enterica*, *E. coli*, *P. aeruginosa*, *S. aureus,* and *B. cereus*, has shown that the *Bacillus* isolated from fermented cassava paste all have an antibacterial effect. The nature and structure of many biosurfactants isolated by *Bacillus* have been identified and characterised. This is the case for soponin, whose instability varies between 30˚C and 70˚C [24].

Microorganisms, including *Bacillus* spp., can produce a variety of bioactive cyclic lipopeptide molecules such as surfactin and iturin [25]-[28]. Surfactin, iturin, lichenysin, and fengycin of *Bacillus* spp. are among the most popular lipopeptides [29] [30]. Natural *Bacillus* belonging to the 9 group, including *B. subtilis, B. amyloliquefaciens, B. subtilis, B. pumilus, B. licheniformis, B. altitudinis, B. mojavensis, B. safensis, B. atrophaeus and B. mojavensis,* generate limited amounts of surfactin (<10% of their biomass), which functions as an antibiotic [31]. It is well known that surfactin is one of the most important biosurfactants, synthesised by nonribosome peptide synthases (NRPS) encoded by the srfA operon (srfAA, srfAB, srfAC, and srfAD) [32]-[36]. The srfA operon is found in all 9 groups belonging to the genus *Bacillus* by containing conserved motifs in the SrfAA, SrfAB, and SrfAC Genes [37]-[39]. Genes encoding the synthesis and production of biosurfactants, such as iturin and fingencin, in *Bacillus* are known. These genes are conserved in the 9 Bacillus groups [40] [41]. Therefore, the biodiversity in bacteria of the genus *Bacillus* allows them to increase the added value of cassava as a lipopeptide biosurfactant. It has been demonstrated that when biosurfactants are

secreted in large amounts, bacteria of the genus *Bacillus* can secrete enzymes that degrade the same biosurfactants. Furthermore, the production of biosurfactants has been optimised [42]. During the fermentation process, the secretion of biosurfactant is controlled. The lipopeptide isolated from *B. velezensis* exhibits good production at pH (2 - 10), antimicrobial properties against drug resistant foodborn *B. cereus* and human pathogen *S. aureus*. Studies show that surfactin isoforms are stable at temperatures up to 80˚C, iturin is between 30˚C and 70˚C [26]. The observed antibacterial activity is not only solely attributable to *Bacillus* strains. Other microbial species could contribute to this effect [43].

This work has highlighted the presence of biosurfactants in the final product of chikwangue. These biosurfactants are extractable and remain active even though the external cooking temperature of the chikwangue is around 200˚C. Cooking in water is perfectly suitable for chikwangue. This method is also the most widely used in the process of obtaining the cooking of chikwangue. Cooked this way, chikwangue would retain most of its nutritional values if it does not overflow too much during the indicated cooking time.

We were unable to identify the free amino acids contained in chikwangue. We are not afraid to postulate that the chikwangue could contain free non-essential and essential amino acids in cassava such as tryptophan (W), lysine (K), methionine (M), phenylalanine (F), threonine (T), valine (V), leucine (L), isoleucine (I) and histidine (H). We have shown that *Bacillus* can secret several hydrolases, including cellulases, amylases, and pectinases. For example, the percentage of essential amino acids in subtilisin (AprE) is 38.1%, in amylase (AmyE) 40% and in cellulase 42% (https://web.expasy.org/cgi-bin/protparam/protparam). This is done without counting the biofilm formation matrix proteins including TasA and TapA [7]. During fermentation stage, some bacterial species die to replace others [43]. All of these aspects justify the diversity in total proteins. Proteases secreted in the fermented cassava paste could easily digest proteins and would allow the release of free amino acids in the final product, which is chikwangue. It should also be added that the Maillard reaction that occurs during cooking of food is a non-negligible aspect [44]. It is partly responsible for the browning and the development of aromas [45]. Although cooking denatures certain proteins, new molecules are formed from amino acids and certain simple sugars. The amino acids could be found in chikwangue in a free state.

Based on the activities obtained in the different variants resulting from endogenous innovations that have appeared in the chikwangue manufacturing processes, we believe that the stability of biosurfactants has been underestimated, contrary to the different information obtained in scientific databases. We do not claim to conclude that this work is complete because we were unable to isolate, purify, and characterise the isoforms of biosurfactants (fengycin, iturin, surfactin, and soponin) from freshly cooked chikwangue.

Our study focused on bacteria of the genus *Bacillus*. However, lactic acid bacteria produce many metabolites with antimicrobial properties, such as organic

acids, hydrogen peroxide, carbon dioxide, reuterin, diacetyl, and bacteriocins [46]-[49]. The heat stability of the bacteriocins produced by lactic acid bacteria was demonstrated, at a temperature below 95˚C [50] [51]. We can postulate that the presence of bacteriocins, biosurfactants in chikwangue, and its derivatives could explain the increase in bioactive compounds, including essential amino acids. Chikwangue should contain biosurfactants and bacteriocins, thus providing an antibacterial effect.

In addition to the bacteria of the genus *Bacillus* found, the fermented retted paste of cassava tubers contains significant biodiversity that contributes not only to the organoleptic characteristics of chikwangue but also to the richness in quantity of proteins. The genera *Staphylococcus*, *Lactococcus, Lactobacillus, Leuconostoc*, *Enterococcus,* and *Pediococcus* have been identified and characterised [43]. They are an important source of proteins. *Bacillus* spp. is widely used to produce enzymes for the biocatalysis industries. These organisms not only generate a suitable range of enzymes but also can secrete them into culture medium at high concentrations [52]. High concentration values of fermented and retted dough were obtained in this work. Consuming chikwangue would be beneficial to health, especially since this bioactive molecule has anticancer, antibacterial, antifungal, and antiviral activities.

Many authors have shown the antagonistic effect of biosurfactants on the pathogens used in this work [21] [53]-[55]. However, this first work is of an important nature because it has shown the correlations of the antibacterial effect that exist between pathogenic strains. No other study has shown this before. However, it is important to note that these inhibition values are very variable.

In relation to the phylogenetic tree established in this work, the bacterial species belong to three groups (group I and II). This bacterial diversity explains the diversity of biosurfactants secreted mainly in fermented cassava paste and chikwangue. This is the first time an antibacterial effect has been observed in so many species of *Bacillus* at the same time. The pathogenic bacteria used belong to two large groups: two from Gram-positive and five from Gram-negative. This study demonstrates that most *Bacillus bacteria* contain bioactive molecules that inhibit the growth of other pathogens.

The correlation between the antibacterial activity of the biosurfactants of the *Bacillus* strain and the Gram negative and Gram-positive pathogens analysed by CANOCO indicates that the biosurfactants of *Bacillus* strains have shown significant antagonistic activities against Gram-negative pathogenic strains compared to Gram-positive bacteria. The low inhibitory potency observed in *B. cereus* could be explained within the framework of the genetic polymorphism that *B. cereus* shares with other groups of *Bacillus* spp. found in the work.

5. Conclusion

This work gives us the opportunity to contribute to the identity card of the inclusive composition of crude biosurfactants and proteins in the chikwangue.

Previous studies give less information on the real composition of biomolecules constituting chikwangue of which this final product undergoes an important process of fermentation by microorganisms, including *Bacillus*, lactic acid bacteria, yeasts and molds. Approaches that include mass spectrometry could open a better perspective on the biochemical advantages offered by Chikwangue. Although important and scientifically significant, many studies have been carried out on cassava with a focus only on cyanogenic compounds. This vision had systematically disoriented the culinary aspects of cassava tubers and their final product, which is chikwangue. The antibacterial role of chikwangue is not a dream. Products derived from cassava fermentation should receive more attention. The cargo of microorganisms present during fermentation explains the added value of this food. It is very difficult to accept that the leaves and stems contain phenolic compounds and that nothing is found in the tubers. This work opens other avenues for studies of chikwangue. The chemical structure of the biosurfactant is composed of thermally stable fatty acids or sugars, it might resist breakdown at 200˚C. Lipopeptides with disulfide bonds or other stabilizing features may also show heat resistance. These studies underscore the potential of Bacillus-mediated biosurfactant production in enhancing the fermentation process and safety of chikwangue. By leveraging the antimicrobial and emulsifying properties of biosurfactants, it is possible to improve the quality and shelf life of this traditional food, contributing to food security and public health in regions where chikwangue is a dietary staple. In summary, integrating Bacillus species into chikwangue fermentation presents a promising avenue for augmenting its composition and safety, warranting further research and application in food biotechnology.

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Conflicts of Interest

The authors have declared that no competing interests exist.

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