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Hydrolytic and Antimicrobial Activities of Bacteria Isolated from Fermented Peppers Sold in Markets at Brazzaville, Republic of the Congo

Faly Armel Soloka Mabika[1,2*], Elgie Viennechie Gatsé[1,2], Ngo Itsouhou[1,2], Tarcisse Baloki Ngoulou[1,2], Mérite Berthelie Mouanda Nzoussi[1,2], Nguimbi Etienne[1,2]

[1]Laboratory of Cell and Molecular Biology, Faculty of Sciences and Techniques, Marien Ngouabi University, Brazzaville, Republic of the Congo
[2]Unit of Molecular Microbiology and Bioinformatics, Faculty of Sciences and Techniques, Marien Ngouabi University, Brazzaville, Republic of the Congo
Email: *armelmabika@gmail.com

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Abstract

Microorganisms are omnipresent in all environments and play mainly the role of transformers, thanks to the multiple enzymes they are able to produce. In order to valorize fermented foods in the Republic of the Congo, this work aimed to characterize and study some properties of microorganisms isolated from samples of peppers sold in three markets of Brazzaville. A numeration of the total aerobic mesophilic flora (TAMF) was made in a solid medium, allowing the evaluation of each sample's microbial concentration. The microbial mass varied from 2.8×10^5 CFU/g for the Ouénzé sample to 1.8×10^4 CFU/g for the Total sample and 2×10^4 CFU/g for the Moungali market sample. The evaluation of the enzymatic properties of the Bacillus isolates showed that 68.42% were capable of producing cellulases and 78.94% were capable of producing amylases and proteases. Antimicrobial activities revealed that 63.15% of the isolates were able to secrete inhibitory substances against *E. coli* and *Staphylococcus aureus*. Molecular analysis by PCR amplification, sequencing of the 16S rRNA gene and BLAST bioinformatics analysis provides newly identified bacteria strains with new accession numbers in GenBank: *Bacillus thuringiensis MBCBR*322 (OP474008), *Bacillus megaterium MBCBJ*1822 (OP476493), *Bacillus thuringiensis MBCBR*222 (OP476494), *Priestia megaterium MBCBJ*2022 (OP476495) and *Lactobacillus paraplantarum MBCBR*1522 (OP476496). Multiple sequences alignment of identified sequences with their homologs of GenBank has shown high similarities. The phylogenetic inference assay has provided the two groups of strains observed in this study, and the two groups are very coherent with the phylogeny of the reference.

Keywords

Antimicrobial Activity, Hydrolytic Activity, Sequencing, Pepper, Brazzaville

1. Introduction

The chilli pepper, native to Central and Latin America, is a dicotyledonous plant belonging to the family Solanaceae, of the genus Capsicum of which five species are known: *Capsium annum*, *Capsicum baccatum*, *Capsicum frustescens*, *Capcicum* chinense and *Capsicum* [1]. Indeed, pepper is consumed worldwide, especially appreciated for its fruits consumed as a vegetable, in fresh condiments or in powder form. In recent years, world pepper production has increased steadily, from 10,769,000 tonnes in 1991 to 22,168,000 tonnes in 2002, *i.e.* doubling in the space of a decade, making pepper the most widely cooked spice in the world, particularly in hot countries [2]. In the Republic of the Congo, the red pepper is ground and sold on local markets. This sauce is stored in glass containers and used as an additional condiment at every meal. Chilli, especially the red one, contains various elements such as vitamin C, β-carotene and phenolic compounds that are responsible for the pungent principle [3]. In reasonable amounts, chilli stimulates digestion and increases food intake and this contributes to the increase of the basal metabolic rate [4] [5]. It has also been shown that capsaicin can increase satiety and reduce food intake. Red chillies contain several types of antioxidants throughout the ripening process. Capsaicin, flavonoids and alpha tocopherol are the best known for antioxidant activity in chilli [6]. Capsaicin was originally of great importance from a dietary and medicinal point of view. It has antibacterial, antiseptic, diuretic, sudorific, digestive and other properties in the fight against diabetes (American of Clinical Nutrition, 2006) [7]. Numerous studies have been conducted on dried chilli, mostly based on biochemical and microbiological quality [8].

Bacteria of the genera *Cronobacter* spp. [9], *Pediococcus* sp., *Weissella* sp., *W. confusa*, *W. cibaria*, *W. paramesenteroides*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus* sp., *Leuconostoc* spp. and *Lactobacillus* sp. were found in dried chilli. Among the lactic acid bacteria identified, *Leuconostoc citreum*, *L. Mesenteroides* and *W. confusa* are the most representatives [10] [11]. In Congo, two studies are known on chillies sold in markets based on the literature we had access to. The first is the one carried out by [12], whose study was essentially on the microbiological quality of crushed chilli fruits put in jars for sale in the markets of Pointe Noire. This study shows the presence of bacteria of the genus Bacillus and also Enterobacteria, whose presence decreases with time, and also lactic acid bacteria testifying to an acid fermentation. The second study [13] analysed the diversity of bacteria in the *Bacillus cereus* group through the analysis of the 16S rRNA gene and the phylogenetic relationships of the bacteria of this group.

Microorganisms are omnipresent in nature and are likely to contaminate

foodstuffs and cause several pathologies. Studies on the microbiological analysis of dry chilli [14] reported the presence of the following bacteria: *Cronobacter* spp., *Pediococcus* sp., *Weissella* sp., *W. confusa*, *W. cibaria*, *W. paramesenteroides*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus* sp. and *Leuconostoc* spp., some of which can cause human infection. This is why it is important to control the microbiological quality of the products we consume. Chilli is consumed in different forms, and in this fermented chilli, there are bacteria that can have several properties.

In this work, jars of crushed and fermenting chilli fruits were bought in three markets in the city of Brazzaville, namely: the Total market, the Moungali market and the Ouenzé market, with the aim of carrying out a characteristic study of the microorganisms contained in the chilli. Molecular identification by analyzing the 16SrRNA gene has been held, and sequencing and phylogenetic classification of isolated strains was done.

2. Materials and Methods

2.1. Collection of Chilli Pots and Making Composites

Nine pots of chillies crushed on the same day were bought in three markets in Brazzaville, three pots per market: Moungali market, Ouenzé market and Total market. The three pots from each market were mixed to obtain a single pot (Composite) in order to have a more representative microbial biodiversity.

2.2. Quantification of Microorganisms and Standards

Quantification was done according to the fermentation time of the composites, 10 g of chillies from each composite were taken and then added to 90ml of sterile physiological water in an Erlenmeyer flask for a stock solution (SM). With the pipette, 1 ml was taken from the stock solution and then transferred to the test tube mark which already contained 9 ml of physiological liquid, it was mixed to obtain a homogeneous 10^{-1} solution. So on with 10^{-2}, 10^{-3}, up to 10^{-5}. 100 µl of the inoculum from a micropipette was placed in the center of the agar of each of the five chosen media, previously poured onto the Petri dishes. The Petri dishes in the inverted position were placed in the oven at 37˚C for 24 hours [12].

Colony counts were performed to estimate the number of viable bacteria present in the volume of the sample inoculated on the culture medium. The values obtained were expressed in CFU/g using the following relationship:

$$\mathbf{UFC/ml = N/Vd}$$

where N = number of colonies, V = seeded volume (ml), d = dilution considered, this quantity must be reduced to 1 g for UFC/g.

2.3. Phenotypic Characterization of the Pepper Composite Isolates

The study of the characteristic colonies of the different bacterial groups was done using classical microbiology techniques in search of a certain number of morphological, physiological and biochemical characters as described by [15] [16].

2.4. Testing the Biological Activities of the Isolates

2.4.1. Culture and Optical Density Measurement

The biological activities were determined using the supernatant after the concentration (optical density) of the inoculum was measured with a spectrophotometer at 600 nm [17]-[19]. Isolates were grown in LB medium at 37˚C for 24 hours. For enzymatic and antibacterial activities. The culture was centrifuged for 10 minutes at 1000 RPM.

2.4.2. Enzymatic Assay of Isolates

1) Cellulolytic activity

The cellulose plate is prepared with the following composition: 0.5 g of cellulose and 1.5 g of agar dissolved in 100 ml of distilled water. This plate is used to demonstrate the production of cellulolytic enzymes, present in the supernatant after culture. The cellulose agar was poured and cooled, wells were made and 100 µl of the supernatant was placed in each well using a micropipette. The activity was read after 48 hours in Lugol's with halos appearing around the wells [18].

2) Amylolytic activity

The starch plate was prepared with the following composition: 1 g of starch and 1.5 g of agar dissolved in 100 ml of distilled water. Amylolytic activity was performed to test for amylolytic enzymes, 100 µl of the supernatant was placed in the wells made in the starch agar and incubated. The reading was taken after 48 h, the revelation is done with Lugol's with the appearance of halos around the wells [18].

3) Proteolytic activity

1 g of agarose and 10 ml of skimmed milk dissolved in 100 ml of distilled water. In a 250 ml Erlenmeyer flask containing 100 ml of 0.1 N PBS, we dissolved 1 g of agarose, heated until complete dissolution, after cooling to 55~60˚C, added 10 ml of skimmed milk, homogenised the mixture. Then poured into Petri dishes; after solidification, prepare wells in the gel and place in each well 50 µl of supernatant from the culture centrifugation. The dishes are placed in an oven at 37˚C for approximately 12 hours. The observation of a clear translucent zone indicates that the strain produces a proteolytic enzyme with a caseinolytic effect [18] [20].

2.4.3. Antimicrobial Assay

Antibacterial activity was performed in Bacillus in order to look for isolates capable of inhibiting the growth of other pathogenic microorganisms by bacteriostatic or bactericidal effect. Three pathogens available in the Moleculary laboratory of the Faculty of Sciences and Techniques were tested: *E coli*, *Staphylococcus*, and *Pseudomonas aeruginosa*.

1) Characteristics of the pathogenic strains used

The pathogenic strains used in this study were the same used by [21], they are presented in **Table 1**.

2) Culture conditions and pathogen inoculum

Strains of the pathogenic bacteria: *E. coli*, *Staphylococcus* and *Pseudomonas aeruginosa*, were plated onto nutrient agar. One colony of each strain was taken

and diluted in 1 ml of sterile physiological fluid, and the Optical Density balanced at (0.08 to 0.1 corresponding to 0.5 Mc Farland) was measured using the spectrophotometer at 625 nm; the suspensions were plated on the agar plate and incubated for 15 - 30 min, and wells were made in the agar plate.

Table 1. Characteristics of the pathogenic strains used in this study.

Pathogenic strains	Origin	Characteristics
Escherichia coli MN40	Urine	Resistant to penicillin, kanamycin, and tetracycline
Staphylococcus aureus MN47	Pus	Resistant to meticillin
Pseudomonas aeruginosa MN41	Waste water	Imipenem and tetracycline resistant

3) Evaluation of antibacterial activity of bacterial isolates

100 ul of the supernatant of each bacillus isolate to be tested for antibacterial activity was dispensed with a micropipette in the wells made on the pathogenic plates and the plates were incubated in the oven at the temperature of 37˚C for 48 h [21]-[23]. The activity reading is reflected by the appearance of inhibition zones around the wells.

2.5. Molecular Analysis of the 16S rRNA Gene

Conventional identification methods based on the study of phenotypic characteristics do not allow reliable identification of species. Therefore, a molecular study on 16S rRNA analysis was undertaken as follows: extraction of genomic DNA, amplification of 16S ribosomal DNA with universal primers specific to prokaryotes [24], sequencing and bioinformatics analysis.

2.5.1. DNA Extraction

The extraction of genomic DNA by the NucleoSpin kit and was carried out as indicated by the manufacturer. It was the same as used by [19].

2.5.2. PCR Amplification of Extracted DNA

1) Primer design

Universal primers which are well known, were used in this study they are listed in Table 2. They were synthesized by the company Macogene France.

Table 2. Universal primers for 16S rRNA gene amplification used in this study.

Primer	Nucleotide sequence	Reference
fD 1	5'-AGAGTTTGATCCTGGCTCAG-3'	Weisburg *et al.* (1991) [24]
rP 2	5'-ACGGCTACCTTGTTACGACTT-3'	

2) Mix and PCR conditions

The PCR mix was prepared as [25] with a modification in the number of PCR

cycles. A final volume was 50 µL, in a 200 µL Eppendorf tube, containing 30 µL of sterile distilled water, 2 µL of DNA, 3 µL of each sense and antisense primer, 1 µl of dNTP, 10 µL of the buffer. and 1µL of the Taq polymerase enzyme. The PCR amplification was carried out in a GenAmp PCR system 2400 thermocycler (Perkin Elmer) according to the following steps:

initial denaturation at 95˚C for 5 min; 35 cycles each of the cycle comprising: denaturation at 95˚C for 40 seconds, hybridization at 56˚C for 40 seconds, elongation at 72˚C for 1 minute 20 seconds; a final elongation at 72˚C for 5 minutes.

2.5.3. Electrophoresis of DNA

Genomic DNA and PCR DNA fragments were assayed in 1% Agarose Gel Electrophoresis.

The TBE buffer (Tris-Borate-EDTA) pH 8.3 was used. The migration at 100 volts lasted one hour and staining with BEwas hold for 20 minutes. A size marker was used to allow the revelation to distinguish the size of the bands of the obtained amplicons.

2.5.4. Sequencing

Sequencing was done as [19], by Macrogene France, briefly the PCR products for the 16S rRNA genes were purified using the NucleoFast 96 PCR plate (Macherey-Nagel EURL, France) and sequenced using terminator chemistry BigDye on an ABI3730 sequencer (Applied Biosystems, Foster City, California, United States). The sequencing was carried out by electrophoresis on a DNA analyzer 3730xl-Titania (Applied Biosystems) using the same primers. The assembly was carried out by Codon Code.

2.6. Results Analysis

Statistical results for all microbiological classic techniques and also for enzyme activities were processed by Microsoft Excel.

The *in silico* analysis of the biological sequences was performed first by using BLASTn. BLASTn is one of the modules of the BLAST family, it uses a nucleotide query sequence submitted to it, querying a nucleic acid sequence database. BioEdith has been used to generate multiple sequences alignment. The phylogenetic tree was inferred by MEGA

3. Results

3.1. Enumeration

The enumeration was done from Petri dishes on six different media: the PCA, Mossel, EMB, Sabouraud, MRS and Chapman.

Table 3 provides information on the total aerobic mesophilic flora (TAFM) count. All the samples studied show microorganisms in different proportions; over the three sites as a whole, the Ouénzé market shows a higher number with a concentration of microorganisms of 2.8.105 CFU/g, followed by the Total market with a concentration of 1.8.104 CFU/g and the Moungali market with 1.2.104

CFU/g.

Table 3. Average values and standard deviations of microorganisms on PCA in CFU/g.

Sites	Total market	Moungali market	Ouénzé market
UFC/g	$1.8 \times 10^4 \pm 0.075 \times 10^4$	$1.2 \times 10^4 \pm 0.45 \times 10^4$	$2.8 \times 10^5 \pm 0.55 \times 10^5$

Figure 1 shows the CFU/g on Mossel medium as a function of fermentation time. The count of the Bacillus genus shows that this genus (Bacillus) is more abundant in the Ouénzé market sample with a load of 94×10^4 CFU/g; followed by the total market with a load of 1.5×10^4 CFU/g and the Moungali market with 1.3×10^4 CFU/g. It can be seen that in all three sites, the bacterial load is high on the first day; it decreases considerably on the third day and until the seventh day.

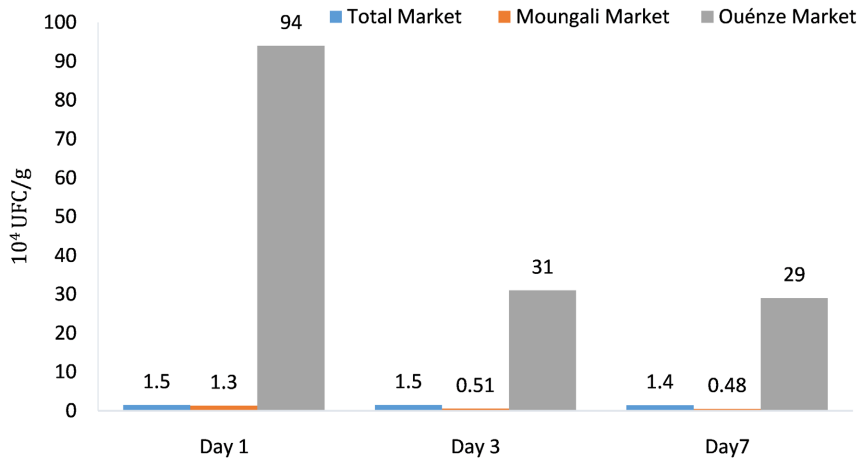


Figure 1. Enumeration of bacteria of the genus Bacillus in the different chilli samples on Mossel medium.

The enumeration carried out, represented in Figure 2, shows the load of Enterobacteriaceae in the three samples. The sample from the Ouénzé market has a high load estimated at 140×10^3 CFU/g, followed by the Moungali market with a load of 9.5×10^3 CFU/g and finally the total market with a load of 0.05×10^3 CFU/g. It can be seen that the bacterial load decreases on the third day for the samples purchased from the Ouénzé and Moungali markets and is cancelled out on the seventh day. On the other hand, on the sample from the total market, there was an absence of colonies on the first day, the colonies appear on the third day and decrease considerably on the seventh day.

- **On Sabouraud medium**

Figure 3 shows the yeast counts on our samples. This figure shows that the load of the Ouénzé market sample is high, with a value of 170×10^4 CFU/g; followed by the Moungali market with a load of 0.72×10^4 CFU/g and the total market with a load of 0.023×10^4 CFU/g. It can be seen that the bacterial load decreases on the third day for the Ouénzé and Moungali market samples, and then disappears on

the seventh day. On the other hand, in the total market sample, a low bacterial concentration was observed on the first day, which increased on the third day and decreased considerably on the seventh day.

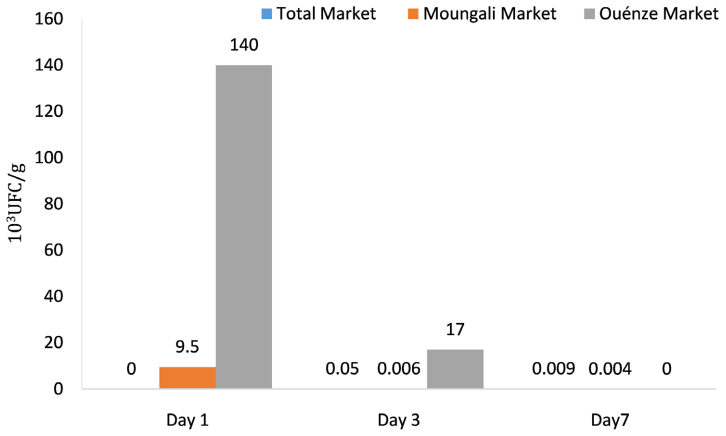


Figure 2. Enumeration of *Enterobacteriaceae* in the different samples on EMB medium.

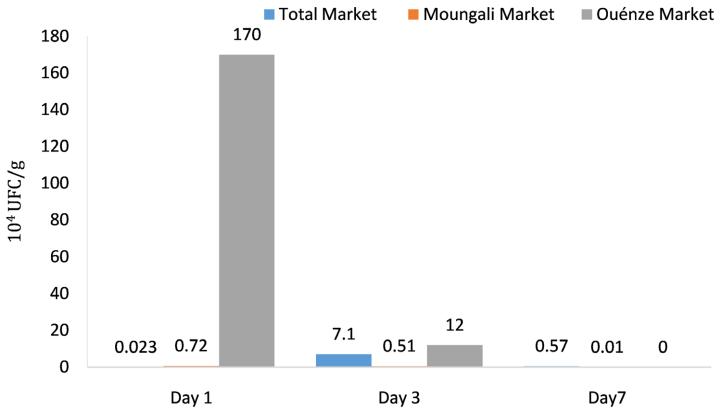


Figure 3. Fungi count in the different samples on Sabouraud medium.

- **On MRS medium**

Figure 4 shows the CFU/g on MRS medium after fourteen days. The Lactobacillus load is higher with 12×10^2 CFU/g on the Moungali market sample followed by 9.4×10^2 CFU/g and there is an absence of colonies in the total market sample after 14 days.

- **Isolated**

The different morphotypes obtained after purification of the colonies on different media are shown in **Figure 5**. A total of 58 isolates were characterised and preserved for phenotypic characterisation and biological activities.

3.2. Phenotypic Characterization

Fifty-eight isolates were obtained after isolation and purification, distributed as

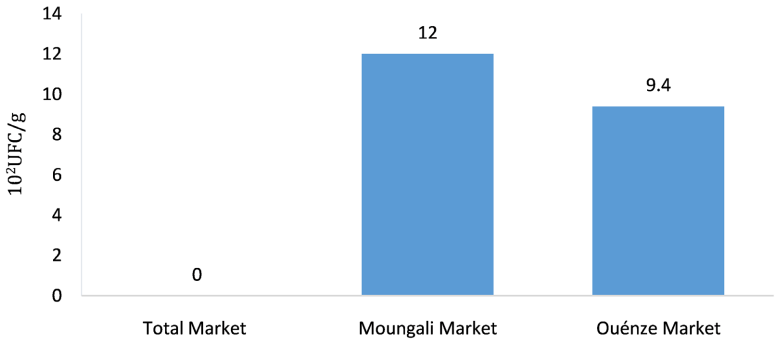


Figure 4. CFU/g of samples on MRS medium after day 14.

Figure 5. The main morphotypes.

follows (**Figure 6**): 22 isolates from peppers from the total market; 19 isolates from peppers from the Moungali market and 17 isolates from peppers from the Ouenzé market. Cultural, morphological and biochemical characteristics of the isolates Out of a total of 58 isolated strains, the majority of isolates were Gram-positive strains compared to Gram-negative strains, *i.e.* moderately mobile and immobile respectively, and mostly catalase-positive. These cells are cocci, rod and coccobacilli. These isolates are dominated by the presence of pink colonies followed by yellow and white; these isolates are creamy, dry and slimy in appearance.

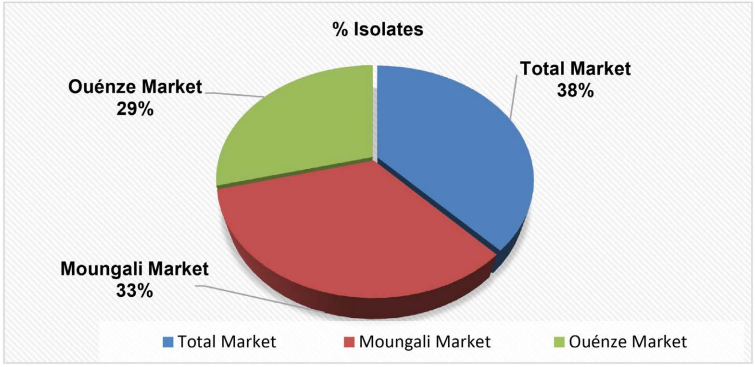


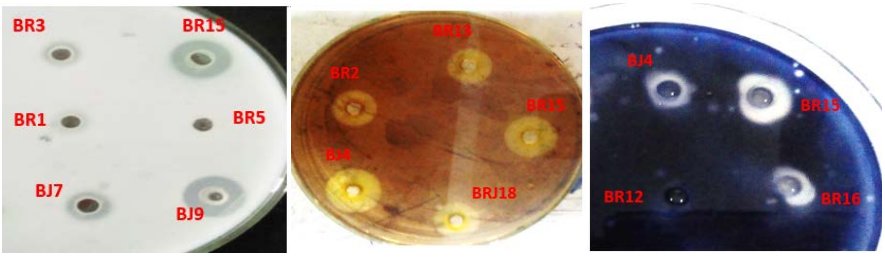
Figure 6. Distribution of isolates by market.

3.3. Biological Activities

3.3.1. Cellulase Production

Figure 7 shows the evidence of cellulase production by the isolates used, this

production is illustrated by the presence of light halos around the wells that received the supernatant of the culture of each isolate. The presence of a halo around each of the wells indicates that each isolate produces amylase, cellulase and protease.



(a) (b) (c)

Figure 7. Halos showing the degradation of cellulose, starch and casein by the isolates. (a) Casein degradation halo; (b) Cellulase degradation halo; (c) Starch degradation halo.

Figure 8 shows the cellulase production profiles of different isolates, with cellulase production varying numerically with each isolate. The most productive isolates are: BJ9, BJ7, BR1 and BJ17. The least productive isolates are: BR13 and BR16.

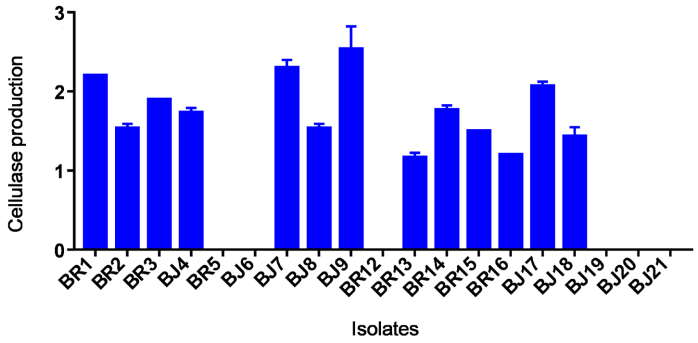


Figure 8. Cellulase production profiles of the isolates used.

Figure 9 shows the different amylase production profiles of the isolates used, which differ from isolate to isolate. The most quantitatively productive isolate is BJ7. While BJ8 and BR14 produce quantitatively less amylases.

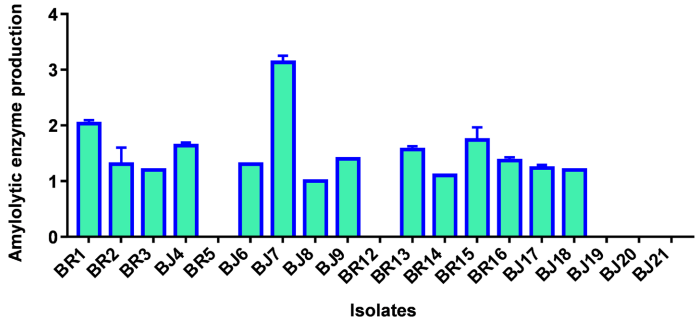


Figure 9. Amylase production profiles of the isolates used.

Figure 10 shows the different protease production profiles of the isolates. Each isolate produced quantitatively different proteases. The highest producers are isolates BR15, BJ7, BJ9 and BR1. The lowest producing isolates are: BR2, BR13, and BR14.

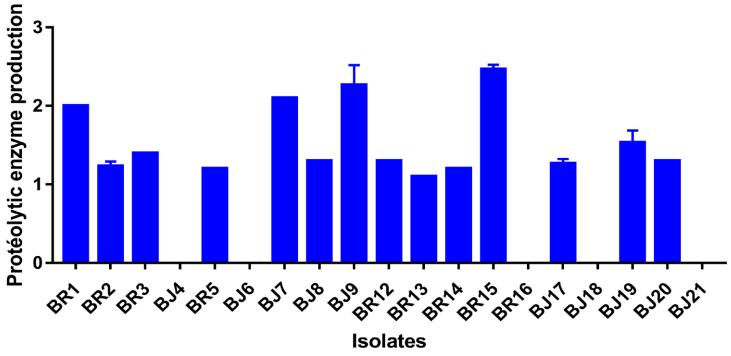


Figure 10. Protease production profiles of the isolates used in this study.

Figure 11 shows the principal component analysis of the simultaneous production of three types of enzymes by the different isolates in this study. Axes 1 and 2 represent 64.5% and 28.7% of the variance respectively. It can be seen that isolates producing all three types of enzymes studied form a group and are positively correlated. Those producing only two or one of the three types of enzymes are also correlated and form two groups according to the number of enzymes produced. The eccentricity of the BJ21 strain can be seen, which is not correlated, reflecting the fact that it does not produce all three types of enzymes.

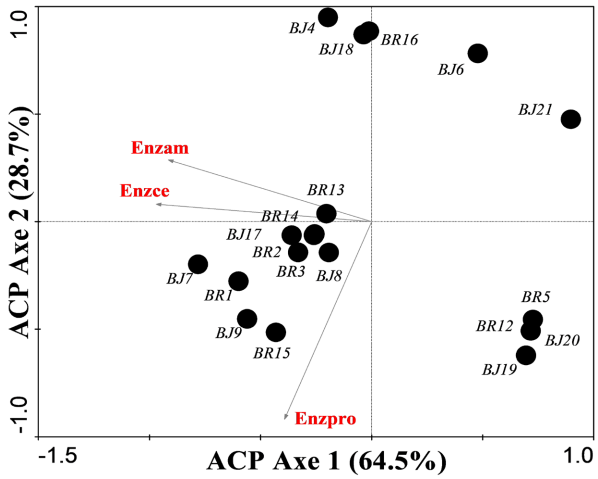
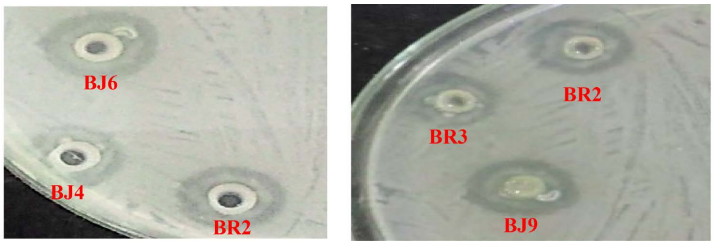


Figure 11. Evaluation of the three-enzyme production.

3.3.2. Antibacterial Activity

Figure 12 shows clear halos around each well on the Gel, these halos reflect the growth inhibition of the pathogen used. Figure 12(a) is the inhibition of *E. coli* and Figure 12(b) is the inhibition of *Stapylococcus aureus*.



(a) (b)

Figure 12. Halos showing growth inhibition of pathogen *E. coli* and *Staphylococcus aureus*. (a) Halo growth inhibition of *E. coli*; (b) Halo growth inhibition of *Staphylococcus aureus*.

Figure 13 shows the component analysis of the variation in pathogen growth inhibition diameters by the isolates used. These isolates mainly inhibit two pathogens. The diameters of inhibition varied according to the isolates used. Isolates BR2, BJ6, BJ9, BR3 and BJ17 strongly inhibited the growth of both pathogens, in particular *E. coli* and *Staphylococcus aureus*, and were positively correlated on axis 1 of the PCA, which represents 70.2% of the variance. On the other hand, isolates without antimicrobial activity against three pathogens are on the opposite side, negatively correlated. However, none of the isolates inhibited the growth of *Pseudomonas aeruginosa*.

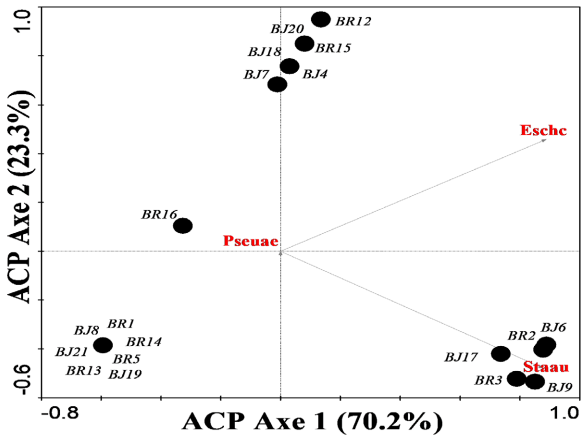


Figure 13. Illustration of antibacterial activities against three pathogen strains.

3.4. Electrophoresis of Genomic DNA on 1% Agarose Gel

Figure 14 shows the genomic DNA fragments of the isolates used after agarose gel extraction. No molecular marker is used, but the genomic DNA fragments are visible. These fragments are aligned at the same level.

3.5. 1% Agarose Gel Electrophoresis of Amplicons

Figure 15 shows the post-PCR fragments (amplicons) of the isolates used on a 1% agarose gel. Taking into account the molecular weight marker the gDNA amplicons

are visible for five isolates and have an approximate size of 1500 bp.

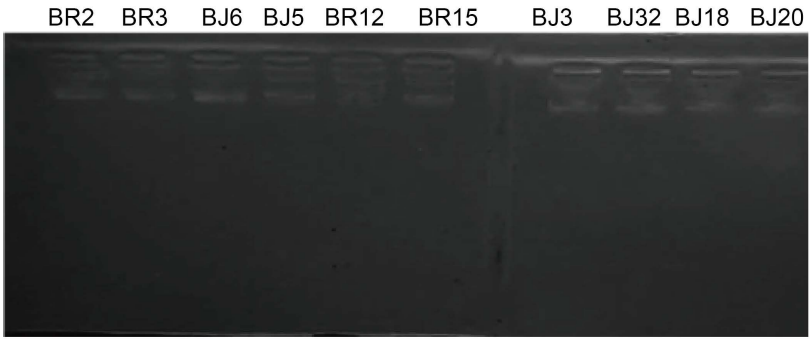


Figure 14. 1% agarose gel electrophoresis of genomic DNA fragments from the isolates used in this study.

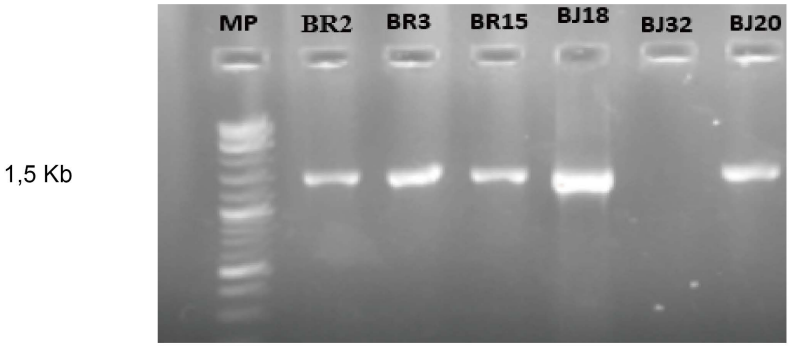


Figure 15. Electrophoresis of DNA amplicons on 1% agarose gel for some isolates used.

3.6. *In Silico* Analysis of Nucleic Acid Sequences of Some Isolates

BLASTn Analysis and Submission of Sequences to GenBank and Accession Numbers

The BLASTn parameters are well specified, the percentage of identity illustrates well the similarity rate between sequences. The E. value is borderline. The homologous sequences from GenBank allowed the identification of some isolates. The sequences were submitted to GenBank and accession numbers were assigned to the sequences of the isolates studied. Table 4 shows new identified bacterial strains by analyzed 16SrRNA sequences and new GenBank accession numbers.

Table 4. The correspondence between identified strains and accession numbers.

Identified bacterial strains	Accession numbers
*Bacillus thuringiensis strain MBCBR*322	OP474008
*Bacillus megaterium strain MBCBJ*1822	OP476493
*Bacillus thuringiensis strain MBCBR*222	OP476494
*Priestia megaterium strain MBCBJ*2022	OP476495
*Lactobacillus paraplantarum strain MBCBR*1522	OP476496

3.7. Multiple Sequence Alignment of Identified Strains and Some GenBank Homologous Sequences

The sequences of newly identified strains were aligned in order to see not only the distance and similarity rate, but also to confirm the events that will have passed in the course of evolution between strains, and to test the efficiency of the 16S rRNA gene to discriminate between neighbouring strains of the same genus or bacteria of different genera. Figure 16 shows a portion of the multiple alignment of the studied sequences and their homologues, the tool used is: BioEdit, the similarity of the sequences is stronger between the two *Bacillus thuringiensis*, then between the three *Bacillus megaterium*, while the two lactobacillus strains are also very close.

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CLUSTAL O(1.2.4) multiple sequence alignment


OP476496        -----------TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGARCG 49
OP928160.1      -----------TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGARCG 49
B.              ----------------------------------------------------GTCGA 5
MBCBJ2022       ------------------------------------TGCCTAATACATGCAAGTCGAGCG 24
MBCBJ1822       --------TCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG 52
MN447140.1      AGTTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG 60
MBCBR222        ----TTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG 56
MBCBR322        -------ATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG 53
HQ917121.1      ----------------------------------------------------GTCGAGCG 8
                                                                       *

OP476496        AACTCKGGTAKATGATTGGTGCTTGCAYCATGTTASATTTGAGTGAGCGGCGGACGGGTG 109
OP928160.1      AACTCKGGTAKATGATTGGTGCTTGCAYCATGTTASATTTGAGTGAGCGGCGGACGGGTG 109
B.              GCGAACTGA---------------AGAAGCTTGCTTCTTGACGTTAGCGGCGGACGGGTG 50
MBCBJ2022       AACTGATTA---------------GAAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTG 69
MBCBJ1822       AACTGATTA---------------GAAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTG 97
MN447140.1      AACTGATTA---------------GAAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTG 105
MBCBR222        AATGGATTG---------------AGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTG 101
MBCBR322        AATGGATTG---------------AGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTG 98
HQ917121.1      AATGGAAA-----------------GAGCTTGCTCTTTGAAGTTAGCGGCGGACGGGTG 50
                                        *           ** ***************

OP476496        AGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACWCCKGGAAACMGATGCTAATA 169
OP928160.1      AGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACWCCKGGAAACMGATGCTAATA 169
B.              AGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATA 110
MBCBJ2022       AGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATA 129
MBCBJ1822       AGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATA 157
MN447140.1      AGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATA 165
MBCBR222        AGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATA 161
MBCBR322        AGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATA 158
HQ917121.1      AGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATA 110
                ************* ********  ***************  * ****** *  *******

OP476496        CCGGATAACAACCYTGGACGCATGGTTCGAGKTTGA-AAGAYGGTTCGGCTRTCACTTWT 228
OP928160.1      CCGGATAACAACCYTGGACGCATGGTTCGAGKTTGA-AAGAYGGTTCGGCTRTCACTTWT 228
B.              CCGGAAAGGATCTTCTCCTTC-TGGGAGATGATTGA-AAGTGGTTTCGGCTATCACTTAC 168
MBCBJ2022       CCGGATAGGATCTTCTCCTTCATGG---GAGATTGAAAGATGGTTTCGGCTATCACTTAC 186
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MBCBJ1822       CCGGATARGATCTTCCCTT-CATAGGGAGAGATTGAAAGATGGTTTCGGCTATCACTTAC 216
MN447140.1      CCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTAC 225
MBCBR222        CCGGATAACATTTTGAACYGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTAT 221
MBCBR322        CCGGATAACATTTTGAACYGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTAT 218
HQ917121.1      CCGGATAACATTTTGAACTGCATGGTTCGAAATTGAA-AGCGGCTTCGGCTGTCACTTAT 169
                ***** *  *          * * *       ****      * ******* ******

OP476496        GGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC 288
OP928160.1      GGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC 288
B.              AGATGGGCCCGCGGTGCATTAACTATTTGG-GAGGTAACGGCTCACCAAGGCAACGATGT 227
MBCBJ2022       AGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC 246
MBCBJ1822       AGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC 276
MN447140.1      AGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC 285
MBCBR222        GGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC 281
MBCBR322        GGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC 278
HQ917121.1      GGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGT 229
                 ***** ******  ****** *** **** ****************************
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Figure 16. Part of multiple alignment of the five identified isolate sequences with their homologues.

3.8. Phylogenetic Tree

In **Figure 17**, the strains identified from the sequences were studied to assess the degree of relatedness between them, the figure shows the phylogenetic tree. The two *Bacillus thuringiensis* strains are close to each other as indicated by the Bootstraps (0.0), therefore are of the same taxonomic round. The same is true for the two strains: *Priestia megaterium* and *Bacillus megaterium*, all these strains have their last common ancestor close to them.

The *Lactobacillus* paraplantarum strain is also closed to his homologs as testified by the Bootsraps. If we consider the last common ancestor in this tree, it is obviously seen that two different groups are emerging in this tree, all the strains in one way and the two strains of *Bacillus thuringiensis* in the other way.

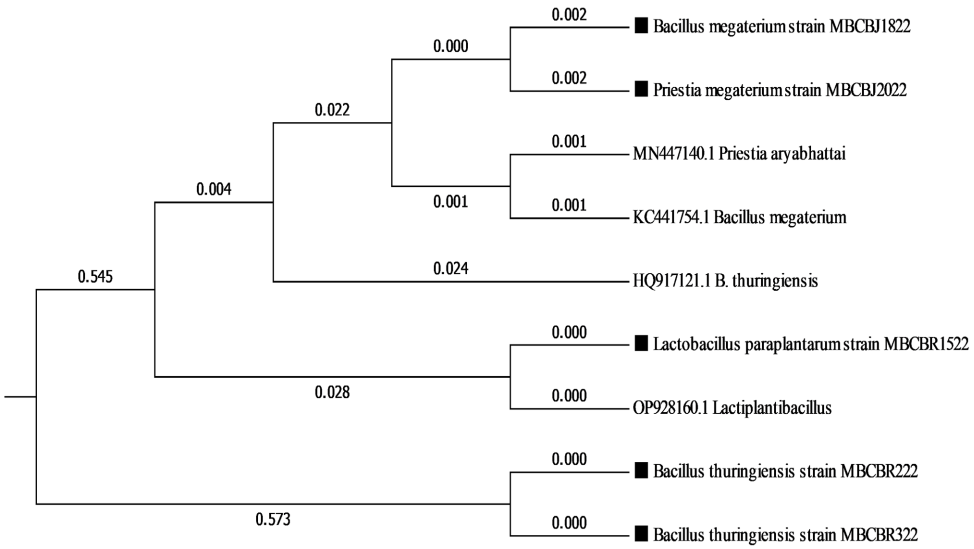

Figure 17. Phylogenetic tree of nine sequences in this study, among them five new identified sequences and four homologs.

4. Discussion

The enumeration of total aerobic mesophilic flora (TAMF) showed that for the three market sites, the number of microorganisms estimated in CFU varies from one sample to another with a predominance of the Ouénzé market site (2.8.105 CFU/g) compared to the other two sites, the Total market (1.8.104 CFU/g) and the Moungali market (2.104 CFU/). Several previous studies have reported the presence of different bacterial groups in fermented foods, e.g. the work of [26], on fermented cassava leaves (Ntoba mbodi) [12], who worked on pepper samples from the markets of Pointe Noire. Recent studies by Onyankouang *et al*. [13], also confirmed previous work on the presence of microorganisms in the samples studied. Among the bacteria counted were the genus *Bacillus*, results that are in agreement with those found by Onyankouang *et al*. [13], but also with those of Nguimbi *et al*. [25], working on crushed and packed pumpkin seeds. These results also show that for bacteria of the genus Bacillus, the proportions are different depending on the site of purchase of the sample, similar results have already been obtained by Armel SolokaMabika *et al*. [19].

This work also shows that the proportions of enterobacteria in the samples of peppers studied decreased progressively with time, as illustrated in **Figure 7**, a finding already made by [12], who also showed that the proportions in CFU/g of enterobacteria decreased with time until they disappeared after seven days. This can be explained by changes in the conditions of the culture medium, which are becoming increasingly unfavourable for these bacteria [19]. Lactic acid bacteria were also isolated in the present study, testifying to the diversity of microorganisms contained in the fermented chilli. These lactic acid bacteria having the ability to elaborate bacteriocins (REF), are therefore important for antibacterial control and also preservation of organoleptic quality of fermented chilli. The diversity of lactic acid bacteria has also been shown by the work of [27] who have demonstrated the diverse presence of lactic acid bacteria in fermented chillies by using galleries.

On Mossel, the phenotypic characterization results show a diversity of colonies but with two main trends in terms of yellow or pink colour, and in terms of rod shape and finally dry and creamy aspects are predominant. The cells are predominantly gram-positive and catalase-positive and mostly produce a hydrolase [28].

The 19 isolates studied were able to produce three types of hydrolases: proteases, cellulases and amylases. The production of hydrolases is different from one isolate to another. This reflects the specificity of each isolate. This ability to produce various enzymes has been reported in studies by Ngô *et al*. and Kaya-Ongoto *et al*. [21] [28], who also studied isolates obtained from Mossel and capable of producing all three types of hydrolases in different proportions. The antimicrobial activities illustrated in **Figure 11** on *E. coli* and *Staphylococcus aureus* strains show one more property of bacteria of the genus Bacillus to produce substances remarkably capable of inhibiting the growth of other bacterial groups. These results are in line with those published by Ngô *et al*. [21].

The molecular genotyping study of the 16S gene of the isolates yielded usable results. Visualisation of the 1% Agarose Gel electrophoresis profile revealed visible bands with a size of 1500 Pb (1.5 kb) as shown in **Figure 13**. Bioinformatics analysis after sequencing identified five strains consisting mainly of the genus. The same size has been already demonstrated by Nguimbi *et al*. (2020) [25], which used Extracted DNA fragments and has got the same size.

The PCR amplicons of 16rDNA PCR was also visualized amplified in 1% Agarose Gel electrophoresis as it was done from the Bacillus strains isolated from squash seed in the Republic of the Congo by Nguimbi *et al*. [25]. These results were consistently comparable.

New strains of Bacillus in GenBank are a starting of ongoing for selecting producing-bioactives compounds from fermented food. New pathways will be explored.

The phylogenic tree has revealed the diversity of bacillus strains in the fermented pepper, and these results are constantly comparable with [12], which worked on fermented pepper bought in the market of Pointe Noire. These two studies may play a key role when going for the establishment of rulers in security and food safety in industry in Congo Brazzaville.

5. Conclusion

At the end of this study, it was found that chillies sold in the markets of Brazzaville contain bacteria and yeast. The concentrations of microorganisms vary according to the markets and the duration of fermentation. Bacteria of the genus Bacillus isolated in these peppers are likely to produce hydrolases (amylases, cellulases, and proteases) and antimicrobial substances against *E. coli and Staphylococcus aureus*. The progressive disappearance of enterobacteria after seven days suggests that chillies can be consumed after seven days without risk of intoxication. After amplification and sequencing of the 16S rRNA gene, isolates BR3, BJ18, BR2 and BJ20 were identified and put in GenBank, and new accession numbers were assigned. *Bacillus thuringiensis MBCBR*322 (OP474008), *Bacillus megaterium MBCBJ*1822 (OP476493), *Bacillus thuringiensis MBCBR*222 (OP476494), *Priestia megaterium MBCBJ*2022 (OP476495) and *Lactobacillus paraplantarum MBCBR*1522 (OP476496). The isolate BR15 was identified as a strain of *Lactobacillus paraplantarum MBCBR*1522 (OP476496).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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