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Full Length Research Paper

Microbiological and biochemical assessment of crushed red pepper from *Capsicum frutescens* preserved in jars and manufactured in local markets in Republic of Congo

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Microorganisms in fermented crushed red pepper from the plant of the genus *Capsicum frutescens* are involved in organoleptic changes and food product alterations. This work aimed to study the sanitary quality and the impact of microorganisms and additional salt in the preservation of crushed red pepper sold in the local market. It appears from this work that crushed red pepper sold at the market has a tolerable nutritious quality. The presence of enteric bacteria for several samples decreases with time and disappear after the 10th day of fermentation. No fecal and pathogenic bacteria of the genus *Salmonella*, *Shigella*, *Staphylococcus aureus* or *Legionella* sp. were found in crushed red pepper. This food is characterized by a pH which around 4.2±0.01. The concentration of salt is ranged between 12 g/L and 124 g/L. Five potential beneficial bacteria (PBB) like *Paenibacillus* sp., *Bacillus marisflavi, Bacillus pseudomycoides, Bacillus pumilus, Bacillus megaterium*were isolated and identified using the PCR-amplification and sequencing of 16S RNA gene. These bacteria have been shown the ability to secrete biomolecules and to inhibit the growth of other bacteria on dishes.

Key words: crushed red pepper, preservation, potential beneficial bacteria, biomolecule.

INTRODUCTION

Red pepper is crushed and sold in local markets in Republic of Congo. Nearly 99.99% of restaurants buy or manufacture red pepper sauce. This sauce is stored in glass containers and used as an additional condiment at every meal. Many studies have been conducted on the particular dried chili on Biochemical quality (Yuliana et al. 2011; Omer et al. 2015) and microbiological quality. Bacteria of genera *Cronobacter* spp (Garbowska et al., 2015), *Pediococcus* sp., *Weissella* sp, *W. confusa*, *W. cibaria*, *W. paramesenteroides*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus* sp., *Leuconostoc* spp. and *Lactobacillus* sp. were found in dry chili. Among the LAB identified, *Leuconostoc citreum*, *Lc. Mesenteroides* and *W. confusa* are the

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more representatives (Sagoo et al., 2009; Sade et al., 2016). Red pepper contains different elements such as vitamin C, β-carotene and phenolic compounds which are responsible for the pungent principle (Clergé Tchieganga 1999). In reasonable quantities, chili stimulates digestion and increases food intake and this contribute to the increase of basal metabolic rate (Gonzalez et al. 1998; Lejeune et al. 2003). It has also been shown that capsaicin may increase satiety and reduce food intake. Red peppers contain several types of antioxidants throughout ripening. Capsaicin, flavonoids and alpha-tocopherol are the best known with antioxidants activity in chili (Luqman and Rizvi, 2006). At high doses, it can be irritating stomach and causes ulcers (Sambaiah and Satyanarayana, 1980). It was shown that capsaicin containing in crushed red pepper is involved in the cholesterol removal (Yuliana et al., 2011).

The crushed red pepper sold in the market is usually eaten fresh or stored for many days without worrying about quality and preservation. No published study has been previously focused on the physicochemical and microbiological quality of fresh crushed red pepper manufactured at the market. In this work, we evaluated the microbiological and organoleptic quality of crushed red pepper preserved in glass jars and sold in the local markets of Pointe-Noire and Brazzaville.

MATERIALS AND METHODS

Sampling

Ninety (90) jars containing crushed and fermented red pepper were purchased in six (6) different markets. Three (3) groups of samples in Brazzaville (Moungali (MM), Bakongo (BG), Poto-poto (PP) and three (3) other groups in Pointe Noire (Grand Marché (GM), Tié-Tié (TT) and Fond Tié-Tié (FT)) markets. Samples were transported to the laboratory and kept in the laboratory at 4°C. Jars have been opened at the time of the taking of samples for analysis. pH determination of the samples was obtained using pH meter (HI98129 HANNA).

Microbiological analysis

Total count of microorganisms

Enumeration of microorganisms of each crushed chili stored in jars was made by the method of inoculation on petri dishes. It consists in aseptically mixing 10 g of each sample of crushed chili in vials containing 10 ml of sterile physiological saline (0.85% w/v). The saline was used to shoot successive decimal dilutions (10^{-1} to 10^{-8}).

0.1 mL of each dilution is applied to the appropriate media PCA (Plate Count Agar) for the enumeration of total microorganisms after 24, 48 and 72 h depending on the type of growth.

Characterization of isolates to the stage of the genus was carried out using plating on specific medium as EMB for enterobacteriaceae, Mossel for *Bacillus* sp., TSN for *Clostridium*, SS and DCL for *Salmonella* and *Shigella*, Chapman for *Staphiloccocus*, MRS and M17 for lactic acid bacteria (LAB).

The isolates were confirmed using microscopic examination and Gram staining using 3% KOH in order to differentiate the Gram-positive bacteria and Gram-negative bacteria. The detection and enumeration of spore-forming bacteria was made after incubation of at 80 ° C for 15 min in a water bath.

The pure cultures are maintained in tubes containing suitable broth supplemented with 20% glycerol (v/v) at -20°C. Before use the stored isolates were retested by transferring an inoculum in broth and on adequate agar incubated at 30, 37 or 40, 44°C, for 24 h. The evolution of the enteric bacteria according to the variation of the pH was followed for 15 days of fermentation. The pH was collected; the number of enteric bacteria was calculated.

Molecular identification

Extraction and purification of genomic DNA was performed according to the kit NucleoSpin Microbiol DNA (Macherey-NAGEL).

Briefly, the targeted isolate is grown in 5 mL LB broth for 16 to 24 h at 37 ° C with stirring. The DNA purity was assessed by electrophoresis on agarose gel and by the ratio of optical densities 260/280 nm. Using the oligonucleotides (Appendix 1), 16S RNA gene was amplified by PCR (Thermalcycler, Biorad).

At the end of amplification, an aliquot of 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) in Tris-Acetate-EDTA buffer (TAE 0.5x) prepared from a concentrated stock solution 10X (ROCH) for half -time between 100-160V.

The 10 kb 2-Log (BIOKE) is used as a molecular weight marker. Gel was then photographed (Panasonic DMC-FZ62).

The PCR products were purified using the solution for Gel Extraction kit (Omega Bio-tek), the purified products were subjected to sequencing by the Sanger technique (3130xl Genetic Analyser (Applied Biosystems) using the oligonucleotides of (Appendix 1). The sequences obtained were aligned with the software Bio Numerics 7.5 (Applied Maths, Belgium) and corrected manually to resolve discrepancies between the sense and antisense strands.

Sequences were compared with homologous sequences contained in the sequence databanks through the portal NCBI (National Center for Biotechnology Information (http://www.ncbi.gov/Blast.cgi) using the BLAST program (Basic Local Search Alignment Tools) based on the identification criterion published by Drancourt (Drancourt et al., 2000).

Table 1. Total count of microorganisms of freshly harvested samples. GM, Grand Marché de Pointe noire, TT, market of Tié-Tié, FT, market of Fond-Tié-Tié, MG, Market of Moungali district, PP, market of Poto-poto district and BG, market of Bacongo district.

Total microorganisms (CFUx10^4/g)											
GM1	43±1,7	TT1	32±3,6	FT1	78±8	MG1	58,3±4,1	PP1	55±5	BG1	59,3±4,0
GM2	112±2,6	TT2	77,6±11,0	FT2	91±4,5	MG2	60,3±5,6	PP2	46,6±4,7	BG2	53,6±10,5
GM3	84,6±5,8	TT3	185,6±3,2	FT3	155,6±4,0	MG3	59,3±5,8	PP3	49,3±10,4	BG3	71±5,2
GM4	94,6±6,6	TT4	179±5,2	FT4	50,3±5,6	MG4	47,6±7,2	PP4	50,3±4,9	BG4	47,3±6,8
GM5	190±4,3	TT5	94,3±5,6	FT5	79±9,5	MG5	82,3±9,8	PP5	86,3±10,5	BG5	63,3±6,4
GM6	89,6±5,6	TT6	80,6±4,6	FT6	84±5,5	MG6	89±6,2	PP6	80,6±7,3	BG6	52,6±8,9
GM7	136,3±8,5	TT7	88±8	FT7	81±4	MG7	61,6±4,1	PP7	98±2	BG7	62,6±5,8
GM8	109±3,6	TT8	90,6±5,0	FT8	170,6±5,5	MG8	75,6±10,0	PP8	90,6±6,65	BG8	62±2,6
GM9	95,6±14,0	TT9	111,6±10,0	FT9	56,3±6,0	MG9	30,6±9,2	PP9	84,3±11,8	BG9	55±5,2
GM10	163,6±10,2	TT10	70,6±6,8	FT10	68,3±7,0	MG10	43,6±1,5	PP10	88±1	BG10	26,6±7,3
GM11	89,6±8,0	TT11	89, ±5,8	FT11	80,3±6,6	MG11	47,3±3,5	PP11	30±7,5	BG11	52±6,0
GM12	139±5,5	TT12	65±4,3	FT12	89±6,2	MG12	77±10	PP12	61,3±9,07	BG12	76,6±1,5
GM13	151,3±8,9	TT13	95,6±4,5	FT13	84,6±5,8	MG13	86,6±9,0	PP13	38±1	BG13	85,6±1,1
GM14	203,3±3,5	TT14	91±5,2	FT14	77±10,1	MG14	90,6±7,2	PP14	38±1	BG14	80,3±7,5
GM15	168,3±2,08	TT15	98±2	FT15	75,3±9,6	MG15	68±6,2	PP15	36,3±6,6	BG15	71,3±5,8

Determination of chloride content

Dilution series are produced from samples (1/2 to 1/100) to enable reading. A chloride test has been used. The chloride concentration is measured semi-quantitatively by visual comparison of the reaction zones of the test strip with the colored series of a color scale. The procedure consists of immersing all reaction zones of the test strip about one second in the solution and after one minute and to identify the colored series of color scale that is closest.

RESULTS

Counting of total microbiota

To assess the total number of microorganisms 90 samples of crushed red peppers freshly harvested in different markets were tested. The total number of microorganisms is between 3.2×10^5 and 2.10^7 CFU/g (Table 1).

Determination of pH of freshly harvested crushed chili samples

To test the acidity of different samples, the pH was measured on all freshly taken samples at random from the markets of Brazzaville and Pointe Noire. pH measurements on samples from all markets showed that pH values vary between 4.2±0.01 and 5.5±0.02. In many cases, the pH was around 4.5±0.01 (Figure 1).

Evolution of enterobacteria in fermented crushed red pepper

To assess the sanitation status of preserved crushed chili in jars and sold at the market, a total count has been conducted to characterize microbiota in terms of enterobacteria basing on GM samples, TT and FT in specific media such as VRBD and EMB (Figure 2).

After incubation at 37°C, the following figure summarizes the results obtained. Some contain enteric bacteria and others not. The GM3 and GM4 jars contain more entericbacteria (7 ± 3) 10^6 CFU/g. Other samples are ranging between 10^5 and 10^6 CFU/g.

The presence of enteric bacteria, which are capable of growing at 44 ° C and corresponding to the fecal coliforms, has been also tested. No culture has been revealed positive.

The influence of pH on enterobacteria was assessed basing on samples produced and harvested from Moungali market (Figure 3). The number of enterobacteriaceae decreases depending on the fermentation time. After the 10th day pH stabilizes around 4.2 ± 0.01. Analysis of isolates from the M17 and MRS was used to confirm the presence of LAB that was between $4.10^4$and 7.10^5 CFU/g. the more representative LAB were *Lactocillus* sp.

Pathogenic bacteria assessment

Pathogens were searched by culturing samples on specific media, Chapman for *Staphylococcus*, Mossel for *Bacillus cereus*, TSN for *Clostridium*, realizing the decimal dilution technique. No culture has revealed the presence of such pathogens (data no shown).

In order to confirm the presence or absence of genus *Shigella flexneri*, successive dilutions were performed on the SS and DCL media basing on samples GM3, GM4, GM9, GM10, GM11, GM12, GM13, GM14, GM15, TT2, TT3, TT4, TT5, TT6, TT9, TT10, FT1, FT2, FT6, FT7, FT8, FT9, FT10, FT11, FT12, FT13, FT14, FT15, FT6, FT9 and FT10. 67% of these isolates showed

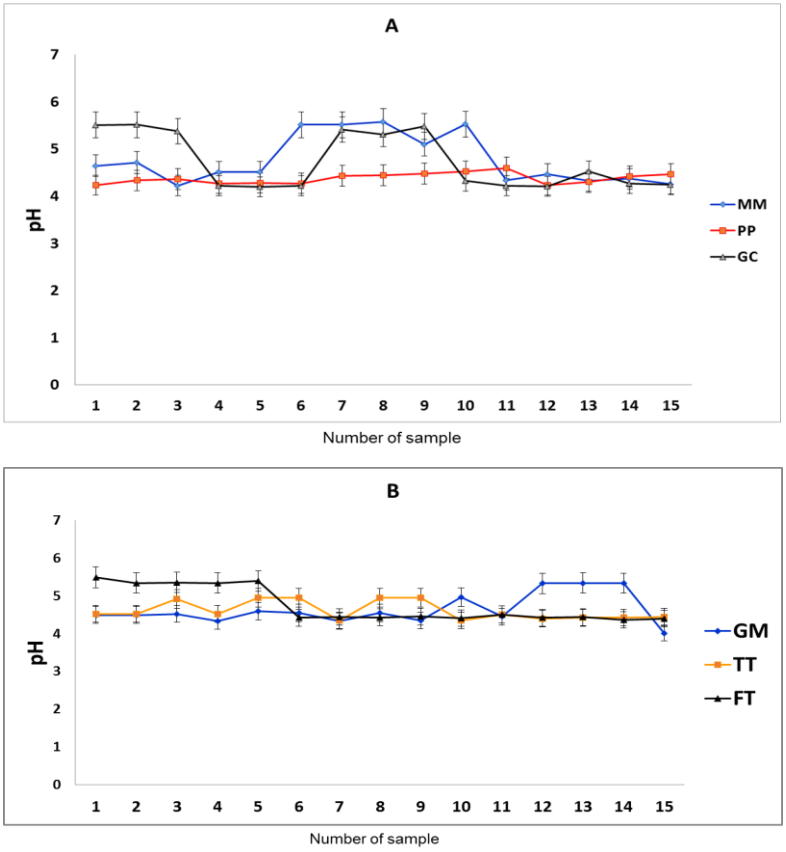

Figure 1. Measurement of pH according to the different samples freshly harvested from local markets. A: Jars of crushed red pepper harvested in Brazzaville from different market (MM: Moungali, GC: Bacongo and PP: Potopoto). B: Jars of crushed red pepper harvested in Pointe Noire (Grand Market (GM), Tié-Tié(TT) and the Fond Tié-Tié (FT)).

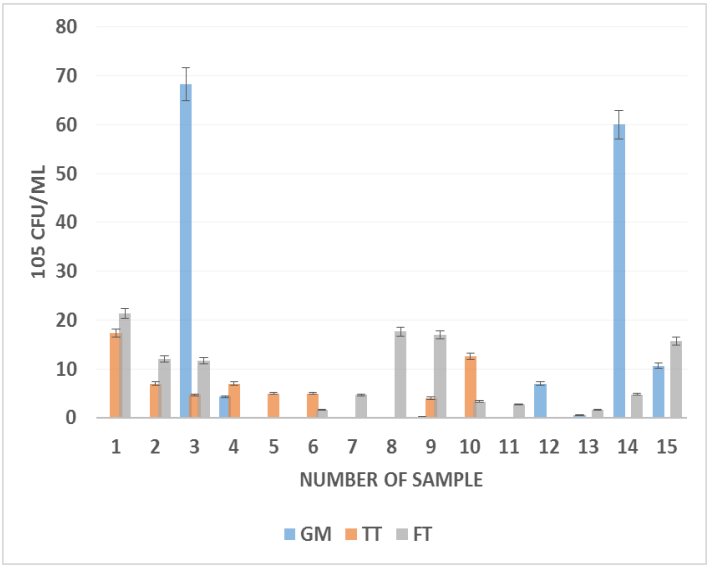

Figure 2. Enumeration of enteric bacteria from Pointe Noire markets. Each sample of crushed red pepper stored in jars was made by the method of inoculation on petri dish. Sample from Pointe Noire ((Grand Market (GM), Tié-Tié (TT) and the Fond Tié-Tié (FT)). was harvested using EMB, DCL or VRBD.

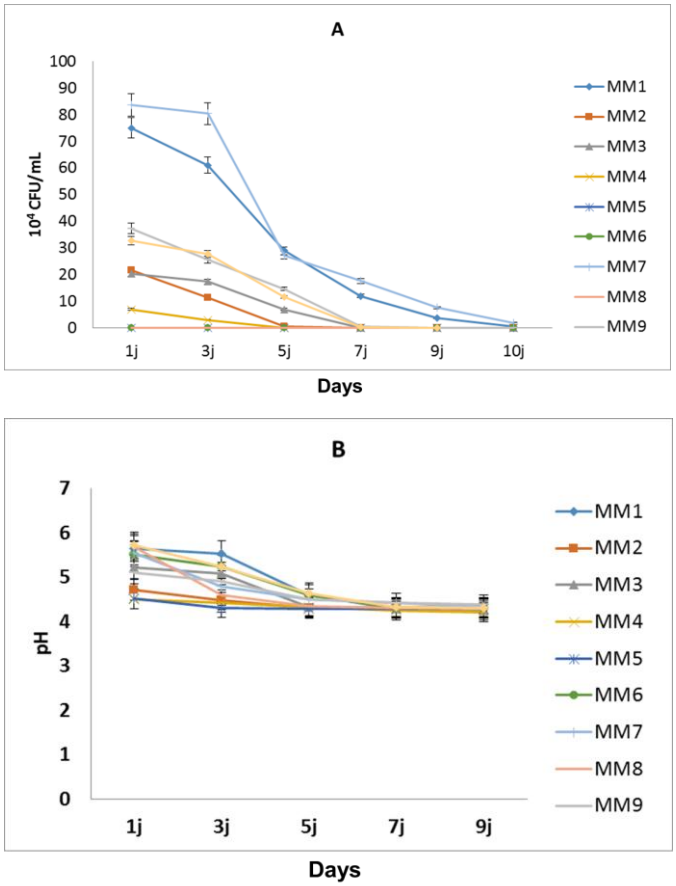

Figure 3. Correlation between the acidification of the crushed red pepper and enteric bacteria. A: evolution of the number of enteric bacteria as a function of the fermentation time, MM 1 to 10, sample from Moungali market. B: evolution of pH as a function of fermentation time.

no phenotype corresponding to the genera *Shigella* sp. As against the isolates (GM4, GM9, TT10, FT12, FT13, FT14, FT15, FT6, FT9 and FT10), corresponding to profiles of bacteria of the genus *Shigella*. Samples were plated on TSB medium supplemented with Congo Red and were tested by PCR on colony using oligo nucleotides IcsBsf/IpgAXho1, IpgAs/IpgAas which are respectively specific of *icsB* and *ipgA* genes (Kayath et al. 2010) of *Shigella flexneri* strains M90T (serotype 5) wild-type and carried by the pathogenicity island of the virulence plasmid, the pWR100 (Buchrieser et al. 2000). As results no bacteria were red on dishes and no gene was amplified in these samples, which may reflect the absence of this type of pathogen in the fermented crushed red pepper (data no shown).

Determination of chloride content

To study the nutritional and organoleptic quality of crushed chili, the salt concentration has been determined of each sample collecting in Pointe noire. The results showed that the concentration of salt in crushed red pepper samples is ranged from 12.8 to 179.2 g/L (Figure 4).

Molecular identification

In order to achieve molecular identification, potential beneficial bacteria (PBB) was investigated in crushed red pepper preserved in jars.

The isolates were plated on either LB medium or the PCA medium at 37°C for 24 to 96 h. Isolates with a halo around the colony were isolated and purified. This halo testifies phenotypically a zone of inhibition on petri dishes that could match a Biomolecule secretion profile (Figure 5).

Isolates were purified and genomic DNA was isolated, PCR using oligos FD1 and rP2 were performed (Figure 6). The DNA fragments of the 16S RNA gene obtained were sequenced. The bioinformatics analysis was performed. The Table 2 shows results obtained basing on the study of Drancourt et al. (2000). No amplification was obtained with isolates B and D (data no shown) using same primers.

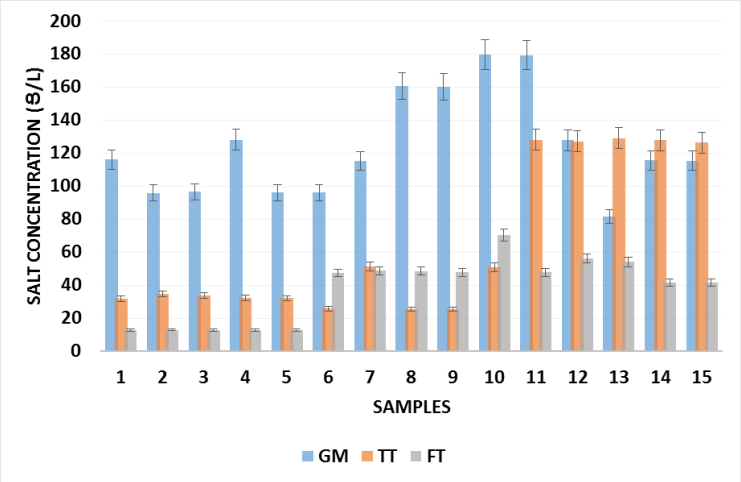

Figure 4. Determination of Salt concentration from samples of Pointe Noire. Forty five samples were assessed, fifteen samples for each market, Grand Market (GM), Tié-Tié (TT) and Fond Tié-Tié (FT)). Salt content was measured using Chloride test.

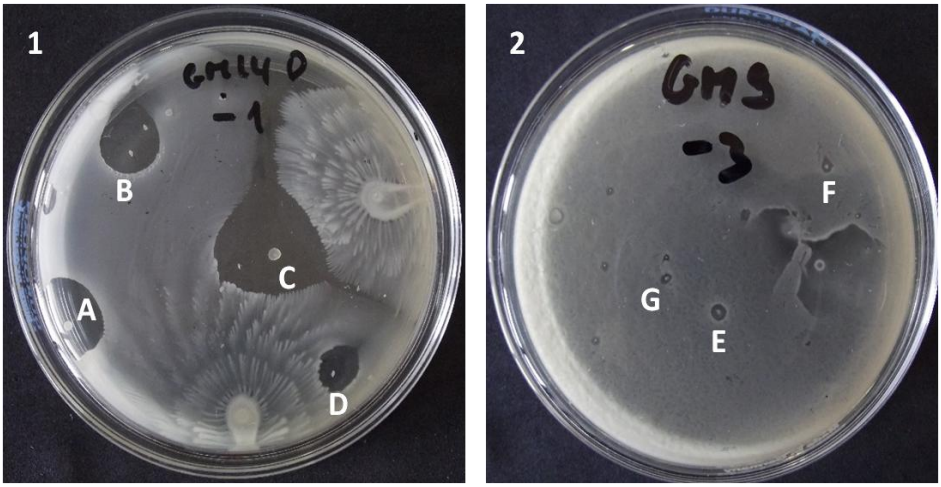

Figure 5. Different isolates obtained after culture on LB (1) and PCA (2) media. A, B, C, D, E, F and G and E are letters given to targeted isolates on dishes. GM 14 and 9 are samples from Pointe Noire market.

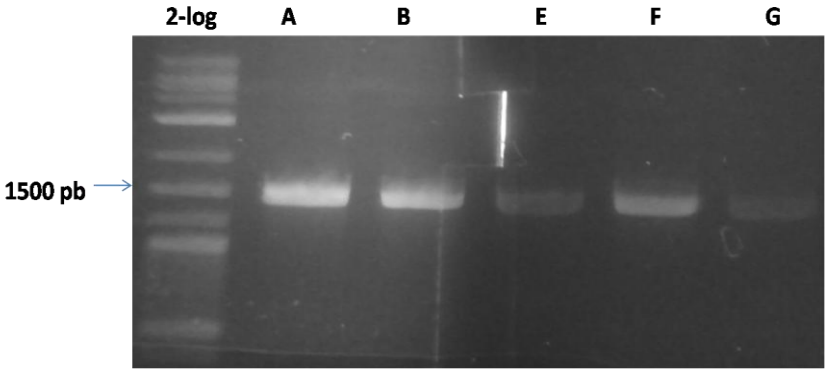

Figure 6. Amplification of 16S RNA gene strains obtained from petri dishes culture. A, B, E, F and G, corresponding letters on petri dishes (see Figure 5), 2-Log is molecular weight (Bioké). The targeted fragment is 1.5 kb of size.

Table 2. Strains obtained after bioinformatics analysis. GM14 and GM9, samples number 14 and 9 from Grand Market (GM). A, B, E, F and G: given names of isolates. Id%: proportion of identity.

Samples	Isolates	Strains	Id %
GM14	A	*Paenibacillus*sp.	99%
GM14	B	*Bacillus marisflavi*	100%
GM9	E	*Bacillus pseudomycoides*	99%
GM9	F	*Bacillus pumilus*	99%
GM9	G	*Bacillus megaterium*	99%

DISCUSSION

Crushed red pepper preserved in jars is consumed about 99% during meals in Republic of Congo. This crushed chili undergoes fermentation and contains important microorganisms in the context of preservation. This work aimed to assess the sanitation status of the crushed pepper and to contribute to the knowledge of microbiological and Biochemical factors that play an important role in preserving the crushed pepper sold at market. This study assessed the number of microorganism which is between $3.2x10^5$ and 2.10^7 CFU/g. Among microorganisms, Gram-positive bacteria are predominantly the more representative (96%) especially the genus Bacillus and lactic acid bacteria (LAB). Other microorganisms were found like Yeast including the genus *Saccharomyces* sp (4%).

As previously shown in fermented chili sauces (Omer et al., 2015), this study has shown that in most crushed chili sold in the markets, enteric bacteria and fecal coliforms are absent except for samples in which production was made in unsanitary conditions. The pH of crushed red pepper is ranged from 4.2 ± 0.01 to 5.5 ± 0.01. We also showed that the acidification of the medium is accompanied by the onset of LAB on MRS and M17 whose number varies between 4.10^4 and 7.10^5 CFU/g. As previously shown LAB community are the more predominant taxonomic units in acidic fermentation. The number increases after acidification of the crushed red pepper (Nakayama et al., 2007; Assohoun-Djenia et al., 2016).

Gram-negative bacteria are generally less concern in brewing microbiology, and they serve as indicator microorganisms to predict hygiene and sanitation conditions in fermentation (Paradh, 2015). In this work we first showed that enteric bacteria disappear after the tenth day of crushed red pepper fermentation and we studied the correlation between the number of enteric bacteria and the decreasing of pH. This study showed that enteric bacteria decrease in function of the fermentation time. This could be due when the acidification of the medium is decreasing. This decrease could be due with the increase and the onset of lactic acid bacteria after 10th day.

Amplification and sequencing of the gene which encodes the 16S RNA gene allowed identifying of five bacteria capable of secreting biomolecules. The id% showed bacteria closed to *Paenibacillus* sp., *Bacillus marisflavi*, *Bacillus pseudomycoides*, *Bacillus pumilus*, *and Bacillus megaterium*. All five strains are capable of inhibiting growth of other non-identifying bacteria on the PCA and LB medium by secreting biomolecules.The presence of PBB supplemented with LAB could play an important role in the preservation of crushed red pepper stored in jars. The genus of *Bacillus* have been showed to secrete biomolecules including proteases (Nguimbi et al. 2014), biosurfactant (Mnif et al. 2016) and bacteriocins-like substances (Barboza-Corona et al., 2009; Pacheco-Cano et al., 2014; Hanafy et al., 2016; Leite et al., 2016) and other biomolecules (Rafigh et al., 2014). This study demonstrates that the 5 strains are potential to secrete bacteriocins and other biomolecules. Bacteriocins-like molecules would be interesting to more characterize.

To keep the crushed red pepper stored in jars, traders mix the mash with salt (NaCl) with unknown concentration. In this work, we showed that the salt concentration is between 12.8 g / L and 179.2 g / l. NaCl is known to play an important role in preserving; furthermore the amount of salt could be reduced. The salt quantity and the relationship between salt concentrations with PBB should be optimized. In this work, we showed that PBB were specially found in GM9 and GM14 in which salt concentration is respectively 115,5 ± 0,2 g/L and 160 ± 0 g/l. Deep studies should be interesting to assess in order to more understand the proliferation of bacteria and the secretion of biomolecules in such salt concentration.

Conclusion

The assessment of crushed red pepper particularly showed the interest of achieving this work. In Republic of Congo, the fermented crushed red pepper is made in non-hygienic conditions. In this work we showed that fermented crushed red pepper sold in local markets has a tolerable nutritional quality. Enteric bacteria decrease considerably after the tenth day of fermentation. In this work, we identified 5 potential beneficial bacteria that are

able to secrete biomolecules. The chemical composition of pepper, organoleptic quality and the presence of PBB play an important role in the preservation of crushed red pepper.Different concentrations of salt were found and PBB could be grown in high salt concentration. More studies should be interesting for the characterization of biomolecules and the determination of relationship between PBB and salt concentration.

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Appendix 1. Oligonucleotides used in this work.

16S NF1	5'- TWA CAC ATG CAA GTC GAR CG- 3'	~1450	amplification and sequencing
16S NF2	5'- CCA ACA TCT CAC GAC ACG AG-3'		
fD1	5'-AGAGTTTGATCCTGGCTCAG-3'	~1450	amplification and sequencing
rP2	5'-ACGGCTACCTTGTTACGACTT-3'		
PCR 16S MOTT			
MB-UZ1	5'GAC GAA CGC TGG CGG CGT GCT TAA C3'	~1450	amplification and sequencing
MB-UZ2	5'-CGT CCC AAT CGC CGA TC-3'		
PCR 16S universelle			
16SUNIV1_SSU 536_SeqF	5'-GTGCCAGCMGCCGCGGTAATAC-3'		sequencing
16SUNIV2_SSU926_SeqF	5'-AAACTYAAAKGAATTGACGG-3'		sequencing
16SUNIV3_SSU685_SeqF	5'-TCTACGCATTTCACYGCTAC-3'		sequencing
16SUNIV4_SSU27_préSeqF	5'-AGAGTTTGATCMTGGCTCAG-3'	~1450	amplification
16SUNIV5_SSU1492_préSeqR	5'-TACGGYTACCTTGTTACGACTT-3'		