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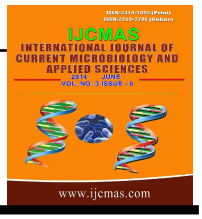

Original Research Article

Polyphasic approach to monitor the bacterial population dynamics in fermenting *Hibiscus sabdariffa* seeds to produce Mbuja

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Keywords

Mbuja, bacterial dynamics, PCR-SSCP, clone libraries, diversity

A B S T R A C T

This study describes the diversity and dynamics of bacterial communities in the fermentation of *Hibiscus sabdariffa* seeds to produce Mbuja, a traditional condiment from Cameroon, using culture-dependent and independent methods. The total aerobic mesophilic bacteria, lactic acid bacteria (LAB) and *Bacillus* spp. were numbered on specific culture media. The bacterial communities were characterized by PCR-SSCP, sequencing of 16S rRNA clone libraries and community-level physiological profiles. Viable cells counts evidenced the domination of *Bacillus* spp. during the process, and the continuous decrease of LAB. PCR-SSCP revealed three groups of bacteria succeeding with the first microorganism communities from day 2 to day 7 being similar. The two other groups were communities in day 8 and 9 and those from day 10. The sequencing of clone libraries showed that the first group communities was more diversified and consisted in *Bacillus* spp., *Lactobacillus, Streptomyces* and *Staphylococcus.* The last two groups of bacterial communities involved fewer genera but diverse *Bacillus* species different from those occurring at the first step. The early phase metabolism profile was exclusively oriented toward carbohydrates and the last targeted broad substrates with aminoacids and organic and carboxylic acids being the most important. This suggests a combination of species in starter formulation.

Introduction

The traditional African condiments, like Mbuja obtained by the fermentation of *Hibiscus sabdariffa* seeds, are very important to local people's diet.

The condiment is widely used for its nutritional value but also for its highly appreciated sensory and organoleptic properties. This product of alkaline

fermentation of *Hibiscus* seeds is normally used as flavoring agent in soup and sauces, especially in rural areas where people with low incomes and weak purchasing power cannot afford high technology processed foods and condiments. Fermented *Hibiscus sabdariffa* seeds are an appreciable source of proteins, lipids and minerals (Bengaly *et al.,* 2003; Mohamadou *et al.,* 2008; Parkouda *et al.,* 2008). In addition to their nutritional value, the health promoting potential of the condiments was shown to be of interest: their antioxidant content was determined at good levels (Mohamadou *et al.,* 2007).

However, as for other fermented condiments, important variations in the nutritional quality and the organoleptic properties of products from different origins are noted (Mohamadou *et al.,* 2009). This can be related to the environmental conditions during production and the use of uncontrolled bacterial inoculums. We initiated this research with the aim of mastering the production conditions in general, and selecting starter cultures in particular, to standardize the quality of the condiment.
In earlier studies we isolated *Bacillus* and lactic acid bacteria in samples of Mbuja from different producers and selected the most appreciated on the basis of sensory tests for use as inoculums in fermentation tests (Mohamadou *et al.,* 2009). Five *Bacillus* species (*B. subtilis, B. megaterium, B. pumilus, B. amyloliquefaciens* and *B. cereus*) and three lactic acid bacteria (*Lactobacillus brevis, Leuconostoc mesenteroides dextranicum* and *Pediococcus pentosaceus*) were isolated and identified (Mohamadou *et al.,* 2010; Mohamadou *et al.,* 2013).

It is well known that sensory and nutritional properties of fermented foods are the results of microbial activities in the fermentation process and are influenced by the various species involved. *Bacillus* spp., which are the most isolated bacteria from Mbuja by cultural methods, have been reported to be able to breakdown proteins and fats, thus improving the overall interest of the seeds (Ouoba *et al.,* 2003). However, these methods seem too limited to accurately describe the bacterial communities involved in the fermentation process as well as the interaction between the representatives of these communities.

The understanding of the bacterial communities' composition during the manufacture of Mbuja could be of great interest for the selection of starter culture to produce a condiment with generous organoleptic properties in a controlled process. Therefore, the assessment of the microbial dynamics and the insight of the different population' succession during the fermentation is required.
This work was carried out to assess the microbial dynamics and to picture the bacterial communities at different dates of manufacturing Mbuja and the main metabolic profiles of these populations at that time with a combined approach using culture dependent and independent methods.

Materials and Methods

Mbuja production and sampling procedure

Fermented seeds were prepared in the laboratory using traditional techniques and utensils (earthen-ware pots) following the flow diagram described by Mohamadou *et al*. (2009). Briefly, the *Hibiscus sabdariffa* seeds are cooked by water-boiling and inoculated with reference Mbuja from previous production, selected by tasters on the basis of its sensory characteristics

(taste, flavor, color and texture). The fermentation is traditionally realized in earthenware pots in 10 days and two steps. The first phase includes a continuous fermentation for 7 days in close pot. Upon the 7th day, seeds are removed from the pot, pounded and then re-introduced in the pot for 3 more days of fermentation (Mohamadou *et al.*, 2009). Five hundred g of *Hibiscus sabdariffa* seeds cooked by water-boiling were inoculated with 5g of Mbuja from Dzbam1, the most appreciated condiment by the panel of consumers in the sensory test carried out by Mohamadou *et al*. (2009). Triplicate samples of fermenting seeds were collected periodically and safely during the 10 days fermentation process for different analyses.

Assessment of bacterial dynamics by viable cells counts during the fermentation

Total aerobic mesophilic bacteria, lactic acid bacteria and spore forming *Bacillus* strains were screened at day 1, day 2, day 3, day 5, day 7, day 8, day 9 and day 10 during the fermentation of *Hibiscus sabdariffa* seeds, according to Mohamadou *et al.* (2009). Ten grams of seeds were safely sampled under a laminar flow hood, in a sterile stomacher bag and homogenized in 90 mL of tryptone salt buffer with a mix Stomacher (AES laboratoire, France). Then ten folds dilutions from the homogenates and plated on PCA (AES, France) for total aerobic mesophilic flora and on MRS (AES, France) for lactic acid bacteria numeration respectively. For spore forming *Bacillus*, samples were heat-treated (80°C for 10 minutes) before plating on glucose agar with bromocresol purple (BCP). Plates were incubated at 30°C for 24h under aerobic conditions (BCP and PCA plates) and under anaerobic condition using an anaerobic jar (Mitsubishi Gas Chemical Company, Japan) (MRS plates). For MRS and BCP plates, representative colonies were selected and tested for Gram-staining and catalase to confirm their potential belonging to lactic acid bacteria and to the *Bacillus* genus.

PCR-SSCP analyses

The PCR-SSCP analyses were realized to assess the dynamics of the bacterial communities in fermenting seeds with time. Analyses were carried out on the first, 2nd, 3rd, 5th, 7th, and 10th days of fermentation. The V3 region of bacterial 16S rDNA was amplified from total DNA with universal primers: w104 (5'-*6FAM*-TTACCGCGGCTGCTGGCAC-3', *Escherichia coli*, position R533) and w49 (5'-ACGGTCCAGACTCCTACGGG-3', *E. coli* position F331). The PCR-SSCP amplification mix consisted in 1.25 U of Pfu turbo (Stratagene, La Jolla, California), 5 µL of 10X buffer, 4 µL of dNTPs (2.5 mM), 200 ng of each primer, 1 µL of DNA extract and water to get a final volume of 50 µL. The amplification conditions were similar as those defined by Renault *et al.* (2012).

The amplicon size was checked by gel electrophoresis 2% (wt/vol) agarose stained with 1% ethidium bromide. PCR products were prepared for SSCP analysis according to Renault *et al.* (2012). The SSCP was realized with an ABI 310 Genetic Analyzer (Applied Biosystems, France). Peaks with the same migration time obtained by SSCP analysis represent a same operational taxonomic unit (OTU). Peaks sizes of each OTU were used as quantitative data for the multivariate analysis as described by Meot-Duros *et al.* (2011). As PCA is based on correlations

between variables, only OTUs with sufficient levels of variations were considered. Observing the distribution of our data, we selected OTUs for which variation coefficients (standard deviation divided by arithmetic mean) were higher than 45%. Considering that SSCP brings semi-quantitative measurements, we used Spearman correlation based on data ranks instead of Pearson correlation.

Clone library construction, screening and sequencing

The clone libraries were constructed on day 7 (which is the end of the first phase of fermentation) and day 10 (the end of the second phase of fermentation). Two libraries of about 30 clones were constructed from the DNA extracts of the bacterial communities in the fermenting *Hisbiscus sabdariffa* seeds. The PCR amplification of the bacterial 16S rDNA genes was carried out using the universal w18 forward primer (5'-GAGTTTGATCMTGGCTCAG-3') and w31 reverse primer (5'-TTACCGCGGCTGCTGGCAC-3'). The PCR was realized as described by Renault *et al.* (2012). PCR products were purified with QIAquick® PCR Purification Kit (Qiagen) according to the instructions by the manufacturer. Cloning and transformation of the PCR products were done with the pCR4-TOPO plasmid and TOP10 *Escherichia coli* competent cells from the Topo TA cloning kit as instructed by the manufacturer (Invitogen, Groningen, The Netherlands).

Recombinant cells were selected based on kanamycin resistance and *ccd* gene killer inactivation. The cells were then grown at 37°C for 24h in LB2X medium (20g/l tryptone, 10g/l yeast extract, 10g/l NaCl). Plasmid DNA was purified with a Montage SEQ 96 Sequencing Reaction Cleanup Kit (Millipore) and sequenced in a GenAmp® PCR System 9700 thermocycler (Applied Biosystem) under the conditions described by Renault *et al.* (2012). DNA sequences were obtained using an Applied Biosystems 3130xl Genetic Analyzer (Biogenouest, Roscoff, France) and were trimmed and cleaned with Phred (Ewing *et al.,* 1998) and SeqClean [TGIR, the Institute for Genomic Research, Rockville, MP, USA (http:// www. tigr.org/ tdb/ tgi/ software) softwares. These sequences were then compared to GenBank databases (www.ncbi.nlm.nih.gov/blast) with the BLAST program (Altschul *et al.,* 1990).

Assessment of bacterial communities' physiological profiles

The bacterial communities from fermenting seeds of *H. sabdariffa* were sampled at the beginning of the fermentation process (day1), at the end of the first step of fermentation (day7) and at the end of the whole process (day10). The bacterial mixtures and the control sterile water (150 µl/well) were distributed in 96-well Biolog® GN2 microplates (AES, France) (Garland and Mills, 1991). Plates were incubated at 30°C for 48 h and the rate of conversion of tetrazolium violet in the 96 wells determined by measurement of the optical density (OD) at 620 nm with a microplate reader (Titertek Multiscan® MCC1340). Data were standardized according to Renault *et al.* (2012) to fall within boundary limits (0 and 2) recommended by Grove *et al.* (2004) in order to minimize the effect of difference in bacterial mixture densities between plates. Correlations between individuals (days of fermentation) were determined based on the physiological profiles of bacterial communities. The most important

of the 95 variables, which were the carbon sources included in the Biolog®GN2 microplates, correlated with these individuals were described with StatBox 6.6 software (StatBox logiciels, Grimmersoft, Issy-les-Moulineaux, France).

Results and Discussion

Bacterial growth dynamics in the fermentation process

A total of 9 samples of fermenting *H. sabdariffa* seeds were collected over ten days period and analyzed at the beginning of the fermentation and every day to monitor the bacterial growth dynamics. Total aerobic mesophilic bacteria, *Bacillus* spp. and lactic bacteria, 3 sample each, were screened and numbered. The relative importance of these groups during the 10 days fermentation process is presented in Figure 1.

Initial microorganisms counts (day 0) were brought by the inocula. Initial total aerobic plate counts were 2.50×10^5 CFU/g. The total aerobic flora grew continuously throughout the fermentation process to reach 1.19×10^9 CFU/g at the seventh day (end of the first step of the process) and finally 1.31×10^{10} CFU/g at day 10.

The initial counts of lactic acid bacteria were relatively important compared to the two other groups. From 1.34×10^7 CFU/g at day 0, the lactic acid bacteria (LAB) grew to reach 1.63×10^7 CFU/g at day 3 before decreasing to 3.05×10^5 CFU/g at day 7. The second phase of fermentation began with a new growth phase observed between day 7 and day 8, with the LAB counts of 1.59×10^8 CFU/g. Then a new continuous decrease phase followed, and the final LAB counts were 1.42×10^7 CFU. Although the initial counts of LAB were the most important, their growth rate showed weaker and their final counts were the less important at the end of the fermentation process.

For the spore forming *Bacillus* spp., initial counts (2.4×10^5 CFU/g) were important but lesser than LAB initial counts. However, the growth rate was high and continuous from the first day to day 10. The spore forming *Bacillus* spp. counts at the end of the first step fermentation (day 7) were 1.14×10^9 CFU/g while the final counts were 7.72×10^9 CFU/g. After the two first days, the growth rate of *Bacillus* spp. was higher than that of LAB.

SSCP analysis of the bacterial communities during the fermentation

The SSCP profiles of the bacterial communities exhibited different features depending on the date of sampling during the fermentation process (Fig 2). These profiles were analyzed by PCA. The eigenvalues indicated that the first plane (F1 and F2) explained 66% of the total data variance and described comprehensively the succession of the bacterial communities during the fermentation of *H. sabdariffa* seeds (Fig 3). Thirty variables (OTU) are distributed in the first plane. The main observation is the separation on the axis F1 (PC1) of OTU associated with the first phase of fermentation (positive coordinates, close to the correlation circle) from those associated with the second phase of fermentation (negative coordinates, close to the correlation circle). Two OTU (OTU 21 and OTU 23), the third group, are located between the 2 other groups as those OTU characterize the 2 phases of fermentation.

With regard to individuals (days of fermentation) distribution on the first axis

(PC1), the SSCP profiles of bacterial communities were clearly separated into 2 groups after the first day of fermentation (Fig 4): communities from day 2 to day 7 and communities from day 8 to day 10.

Figure.1 Dynamics of bacterial populations during the fermentation. Three repetitions values of viable cells counts are represented and designated by *1*, *2*, and *3* following each of the three groups of bacteria investigated: *Bacillus* genera, Lactic Acid Bacteria (LAB) and Total Aerobic Mesophilic Flora (TAF).

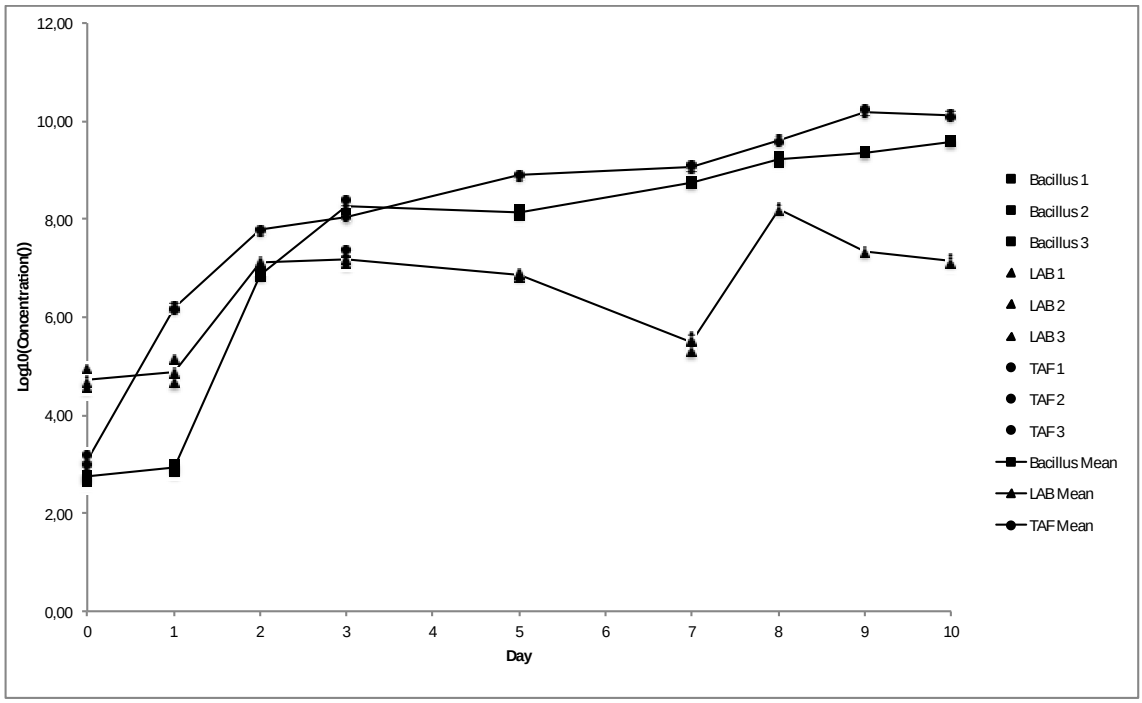

Figure.2 SSCP profiles of the bacterial communities at day 7 and at day 10

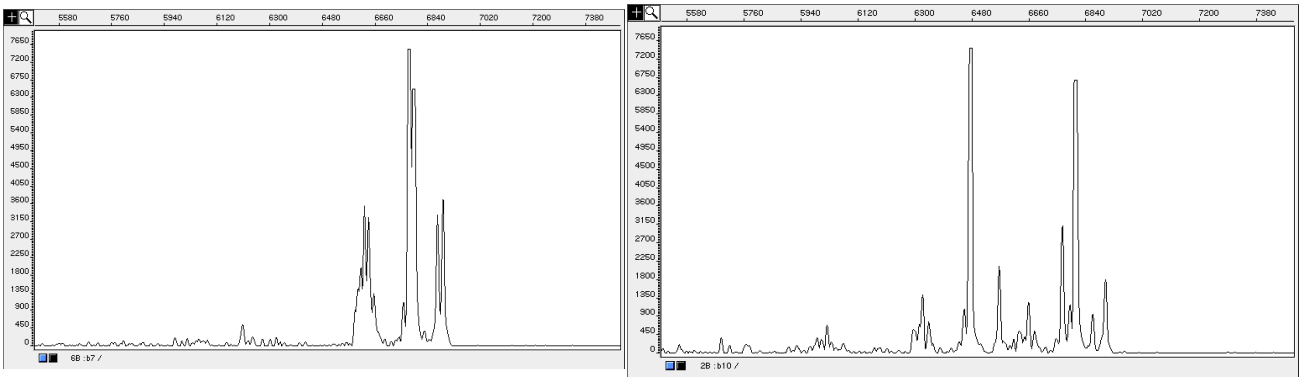

Figure.3 Distribution of the SSCP variables (Operational Taxonomic Units) in the first plane (PC1 and PC2). OTUs are SSCP peaks representing different phylotypes.
Three groups of OTUs are clearly separated.

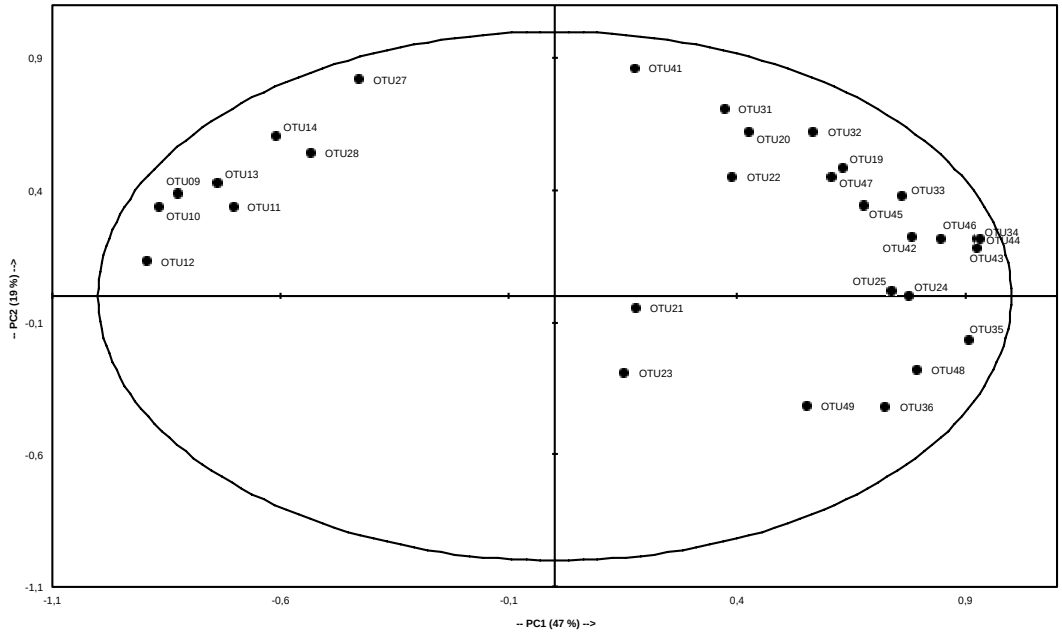

Figure.4 Distribution of the bacterial communities on the first plane (F1 and F2) obtained from Principal Component Analysis of PCR-SSCP peaks (OTU or phylotypes).
Analysis of the 16S rDNA clone libraries at day 7 and at day 10

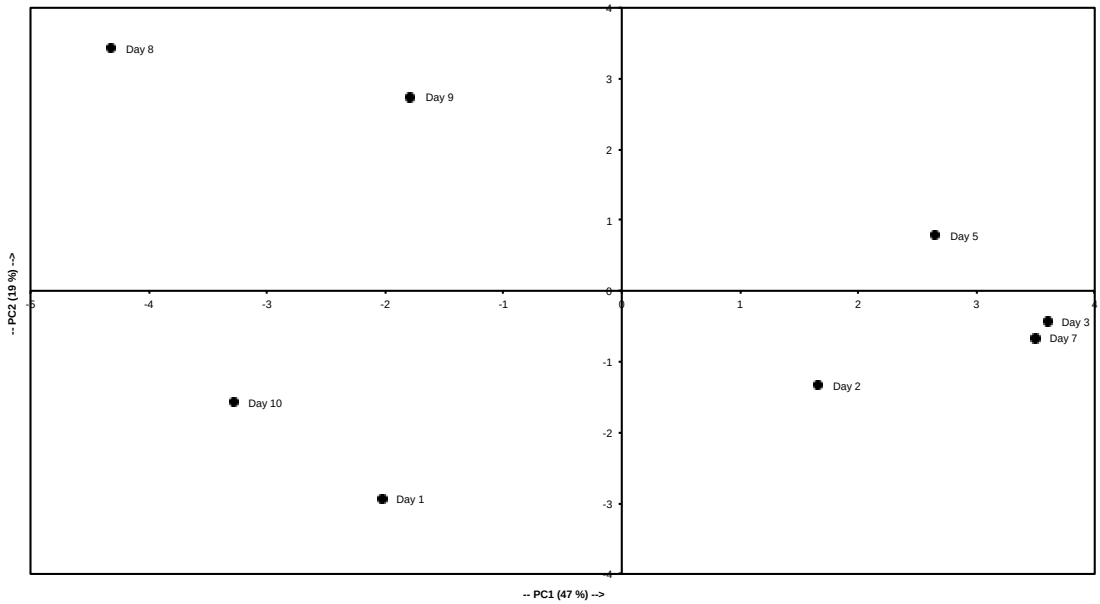

Table.1 Phylogenic diversity and affiliation of 16S rDNA genes at day 7 and day 10

N°	Clone abundance		Phylum affiliation	Closest microorganism or environmental clone	Accession N°	% Similarity
	Day 7	Day 10				
1	1		Firmicutes	*Bacillus* sp. NRS-967	AF169547.1	99
2	1		Firmicutes	*Bacillus sphaericus*	AB271742.1	98
3	1		Firmicutes	*Bacillus sphaericus* strain AU3	EF032669.1	97
4	1		Firmicutes	*Bacillus sphaericus* strain DSM 28	AJ310084.1	98
5	1		Firmicutes	*Lactobacillus sakei* strain 23K	CR936503.1	99
6	12		Firmicutes	*Staphylococcus saprophyticus* subsp. saprophyticus ATCC 15305	AP008934.1	98-99
7	12		Actinobacteria	*Streptomyces gougerotii* strain: NBRC 13043	AB249982.1	98-100
8		9	Firmicutes	*Bacillus pumilus*	DQ988522.1	98-99
9		2	Firmicutes	*Bacillus pumilus* isolate CECRIbio 02	DQ207559.1	99
10		12	Firmicutes	*Bacillus* sp. ARI8	DQ102370.1	96-99
11		1	Firmicutes	*Bacillus* sp. isolate 6:4, 659BP	AJ536427.1	99
12		1	Firmicutes	*Bacillus subtilis* strain Mali 52	AY211146.1	98
13		2		Uncultured bacterium clone JSC2-B8	DQ532171.1	99

The second axis (PC2) clearly dissociated the beginning and the end of the second phase of fermentation (day 8 to day 9 and day 10). The community observed on day 10 appears as the closer compared to that of day 1.

The bacterial diversity was assessed for day 7 and day 10 to picture the bacterial dynamics at two key steps within the fermentation process. At the whole, 60 16S rDNA genes were sequenced and analyzed for the two dates.

For day 7, the sequences were grouped into 7 phylotypes based on similarities with closest relatives (Table 1). All the phylotypes presented similarity between 97 and 100% with sequences identified earlier (Table 1). The sequences belonged to 4 bacterial genera: *Staphylococcus, Streptomyces, Bacillus* and *Lactobacillus.* The most abundant clones belonged to *Staphylococcus* (41% of the clones) and *Streptomyces* (41% of the sequences) genera. The sequences identified could be distributed in two phyla, with the first *Firmicutes* (58.62% of sequences) being more important than the second, *Actinobacteria* (41.38%).

Six phylotypes were identified according to similarities with the closest relatives at day 10 (Table 1). The similarities with identified sequences were between 96 and 99%. Almost all the sequences were assigned to *Bacillus* genus, except two associated with uncultured *Bacterium.* More than 90% of the sequences were affiliated to the phylum of *Firmicutes.*

Table.2 Description of the most metabolized substrates during the fermentation in decreasing order of importance

	Code	Name	Class
Substrates of day1	B11	D-mannitol	Carbohydrates
	B5	Gentiobiose	Carbohydrates
	C7	β-methyl-D-glucoside	Carbohydrates
	C6	D-trehalose	Carbohydrates
	C8	D-sorbitol	Carbohydrates
	C2	Sucrose	Carbohydrates
	A12	D-cellobiose	Carbohydrates
	A8	N-acetyl-D-glucosamine	Carbohydrates
	B10	Maltose	Carbohydrates
	B2	D-fructose	Carbohydrates
	B6	α-D-glucose	Carbohydrates
	D7	D-gluconic acid	carboxylic/organic acids
	B12	D-mannose	Carbohydrates
Substrates of days 7 and 10	G6	L-proline	Aminoacids
	F9	L-aspartic acid	Aminoacids
	G2	hydroxy L-proline	Aminoacids
	F10	L-glutamic acid	Aminoacids
	F5	D-alanine	Aminoacids
	H5	phenyl ethylamine	amide/amine
	G12	γ-amino butyric acid	Aminoacids
	F6	L-alanine	Aminoacids
	G7	L-pyroglutamic acid	Aminoacids
	D2	cis-aconitic acid	carboxylic/organic acids
	F8	L-asparagine	Aminoacids
	E1	p-hydroxyphenylacetic acid	carboxylic/organic acids
	E9	quinic acid	carboxylic/organic acids
	H9	Glycerol	diverse compounds
	F7	L-alanyl-glycine	Aminoacids
	D11	β-hydroxybutyric acid	carboxylic/organic acids
	A3	Dextrin	Polymers
	G9	L-serine	Aminoacids
	H2	D-glucose-6-phosphate	diverse compounds
	H6	Putrescine	amide/amine
	G8	D-serine	Aminoacids

The phylotypes appearing on day 10 of the fermentation are completely different from those identified for day 7 and the only phylum remaining at the end of the fermentation is that of *Firmicutes*.

The diversity and dynamics of bacterial communities during the fermentation of *H. sabdariffa* seeds to produce Mbuja, a protein-rich condiment from Cameroon and other African countries, was studied. Culture-dependant approach (viable cells counts), molecular methods (SSCP-PCR and clone libraries construction) and bacterial communities' physiological behavior were investigated to describe bacterial succession with fermentation time.

The viable cells counts targeted three groups of bacteria: the total aerobic mesophilic bacteria, lactic acid bacteria and *Bacillus* spp. The choice was based on previous studies by Mohamadou *et al.* (2009) who identified these groups in Mbuja samples from different origins. From the initial day, the total aerobic mesophilic bacteria grew rapidly, indicating a global and rapid increasing of bacterial counts. Hence, a continuous growth was noted from the first to the third day before a more steady growth until day 7, the end of the first step of fermentation. Another important phase of growth begins after the 7^{th} day until the end of the fermentation.

The two exponential growth phases could be explained by the availability of nutrients to bacteria during the production of Mbuja. In effect, the seeds are continuously fermented for 7 days (first step) and upon this first step, the fermenting seeds are pounded before a new fermentation step (2^{nd} step) for 3 more days. In this study, the increase in total aerobic mesophilic bacteria is due to the continuous growth of *Bacillus* spp. and lower growth of lactic acid bacteria. The proportion of the two last groups varied with fermentation time. From the beginning to the end of the process, lactic acid bacteria were dominated by *Bacillus* spp. and tended to progressively decrease. This poses the question of their role in fermenting *H. sabdariffa* seeds to Mbuja. If the relative importance of *Bacillus* spp. was already reported for the final products (Ouoba *et al.,* 2008; Mohamadou *et al.,* 2009), this study reveals that this importance is noted throughout the fermentation process, confirming that lactic acid bacteria plays a secondary role of accompanying flora to the *Bacillus* spp. From this point of view, our study complies fully with findings by other authors who studied the succession of bacteria during the fermentation of different proteinaceous seeds to various West African condiments. Indeed, *Bacillus* spp. were found to be the dominating bacteria with different secondary flora in the fermentation of Africa locust beans (*Parkia biglobosa*) (Azokpota *et al.,* 2006; Oguntonyinbo *et al.,* 2007; Adelekan and Nwadiuto, 2012).

Culture independent method, the PCR-SSCP, has also been used to monitor the succession of bacterial communities during the fermentation of *H. sabdariffa* seeds to Mbuja. The PCR-SSCP profiles analyses also showed a progressive structuration of the profiles with the fermentation time. With regard to PCR-SSCP profiles revealed by this work, two main observations could be made: the more important diversity than revealed by culture-based techniques and the evolving structure of bacterial communities with fermentation time.

As regards SSCP profiles, one of the main outcomes of this study is that there are three major clusters of fermentation days in which the bacterial communities are relatively homogeneous, during the process. This suggests that shifts in bacterial community occurred from day1 to group of days 2 to 7. Hence, from the beginning (day 2) to the end of the first step of fermentation (day 7), the bacterial communities seem to be of the same composition. Upon the first step (after pounding the fermenting seeds), a second bacterial community takes place and changes rapidly to the last bacterial community of day 10. At the whole, this study pointed out that three bacterial communities appeared during the fermentation with the most important being the two communities of day 7 and day 10.

The diversity of the OTUs (phylotypes) within these PCR-SSCP groups is probably more important than the two bacterial groups determined by culture on specific media. Apparently, this diversity decreases as the fermentation progresses toward day 10. Very few studies described with culture-independent methods the succession of bacterial communities during the fermentation of proteinaceous seeds to produce African condiments in general, and *H. sabdariffa*-made condiments in particular. We therefore investigated the diversity and relative abundance of bacterial phylotypes as well as their functional activity at the identified key dates of the *H. sabdariffa* seeds fermentation process.

The diversity of bacterial population at key dates as day 7 and day 10 and the difference within the phylotypes associated with each of these dates were confirmed by sequencing the 16S rRNA gene libraries. The bacterial communities at day 7 were more diversified with at least four genera identified: *Bacillus*, *Lactobacillus*, *Streptomyces* and *Staphylococcus*. While the former genera were already identified by culture-based methods, the two latter were isolated by Mohamadou *et al.* (2009). Incidently, 16S rRNA sequences of *Streptomyces* and *Staphylococcus* represented the most abundant clones (more than 80% of the clones).

The occurrence of staphylococci in fermenting proteinaceous seeds to produce *iru* and *sonru* have already been reported by Azokpota *et al.* (2006) and in final product of fermentation (*iru*) by Adewumi *et al.* (2013). However, *Staphylococcus* spp. are considered as potential food-borne pathogens and contaminants, which are probably due to poor hygienic conditions of operators in the traditional manufacturing of the condiments used as inoculum in this study. *Streptomyces* is a genus of Actinobacteria predominantly found in soil and may have contaminated *H. sabdariffa* seeds through earthenware pots in which the fermentation is realized. According to the PCR-SSCP profiles, microbial communities of day 2 to day 7 are similar. Probably, the bacteria appearing at this stage of the process consist in *Bacillus* spp., lactic acid bacteria and a number of contaminants from human pathogens and from the earthenware containers.

A different picture of the bacterial diversity is brought at day 10. Almost all sequences of 16S rRNA matched with *Bacillus* species belonging to the phylum of Firmicutes. After the 7^{th} day of fermentation, a new dynamics takes place with changes in bacterial structure. The contaminant flora (*Streptomyces* and

Staphylococcus spp.) was not detected and the diversity of genera is reduced to the sole *Bacillus* genus. However, the species appearing after day 7 were completely different from those identified before the end of the first step of fermentation. Indeed, while *B. sphaericus* was the most abundant clone at day 7, *B. pumilus* and *B. subtilis* the most important at the end of the fermentation process (day 10). Therefore the first step of the fermentation is associated with diversity of genera and the second with the diversity of *Bacillus* species. The predominance of *Bacillus* spp. may be due to their strong ability to compete with other genera. They are known to produce a wide range of broad spectrum antibacterial and antifungal molecules that inhibit the growth of Gram-positive and Gram-negative bacteria as well as yeasts and molds (Abriouel *et al.*, 2010).

The global physiology of bacterial communities is in favor of the microbial population structure highlighted by sequencing of 16S rRNA of the clones. In effect the metabolism evolved with fermentation time. However, the metabolism at the early stage of fermentation was opposed to the second phase. After the 7th day, the communities seem to use similar and more diversified substrates. The initial community (day 1) exclusively metabolized carbohydrates and not other substrates, indicating their relative physiological homogeneity. The change in the bacterial structure could be linked with the scarcity of carbohydrates which were used by sugar fermenting bacteria, probably lactic acid bacteria. From day 7 to the end of the fermentation (day 10), the bacterial tropism was more oriented towards aminoacids and carboxylic acids (metabolism of *Bacillus* spp.) while sugars were minor substrates certainly metabolized by the relatively low remaining lactic acid bacteria.

In conclusion, the use of different approaches in the assessment of bacterial dynamics gave complementary pictures of the evolution of the global communities in fermenting seeds. The cultural methods underestimated the diversity of bacterial communities while culture-independent techniques brought evidence of the implication of other microorganisms than *Bacillus* spp. and lactic acid bacteria at different stages of fermentation. The non-persistence of these organisms raised the ability of *Bacillus* spp. from Mbuja to compete and probably eliminate pathogens and other contaminants in the fermentation process. The succession of bacterial flora is in favor of the use of more than one species of *Bacillus* in formulating the standardized starter for a controlled fermentation process. Therefore the antimicrobial activities should be investigated and considered as a criterion for starter selection in further studies.

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