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Assessment of dominant bacterial strains isolated from Ntoba mbodi, an indigenous African alkaline-fermented food, and their potential enzyme activities

Didine Priscilla Moutou-Tchitoula[1], Etienne Nguimbi[1], Stéphanie Giusti-Miller[2], Philippe Mora[2], Simon Charles Kobawila[1], Edouard Miambi[2*]

[1]Laboratoire de Biologie Cellulaire et Moléculaire, Faculté des Sciences et Techniques, Université Marien NGOUABI, BP 69 – Brazzaville, République du Congo.
[2]Faculté des Sciences – IEES Paris, Université Paris Est Créteil; 61 Avenue du Général de Gaulle, 94010 Créteil cedex, France.

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Ntoba mbodi is a stable and long-life alkaline-fermented food and the domestic-scale production depends upon microorganisms from the local environment. Previous studies on bacteria in Ntoba mbodi have reported the presence of *Bacillus* related species and other bacterial taxa. But the abundance of these bacteria in Ntoba mbodi still needs to be determined to assess their ecological importance in this particular environment. In the present study, we used an extinction dilution approach for isolation and taxonomic affiliation of the dominant isolates obtained from the highest positive dilution of Ntoba mbodi. We also tested the hypothesis that there is a convergence in the bacterial community profile in Ntoba mbodi, irrespective of the origins of the food, by assessing the set of dominant bacteria that was found in all the samples studied. The density of the bacteria ranged from 1.67×10^{10} to 2.8×10^{10} CFU g^{-1}. Eleven distinct morphotypes were isolated at the highest positive dilution of Ntoba mbodi and identified using 16S rRNA gene sequencing followed by phylogenetic analyses. Eight isolates were classified as *Bacillus* related species. A set of five strains was shared between all samples of Ntoba mbodi which were closest to *Bacillus altitudinis*, *Bacillus* sp. C-32, *Staphylococcus sciuri* and *Bacterium* NLAE-lz-H375. Of the eleven predominant isolates, eight showed cellulase activity and five showed xylanase and β-glucosidase activity.

Key words: Bacillus, cassava leaves, alkaline-fermentation, Ntoba mbodi, indigenous fermented foods.

INTRODUCTION

Fermentation is one of the most ancient forms of food preservation technologies in the world. It uses microorganisms to convert perishable and sometimes inedible raw materials into safe, shelf-stable and palatable foods or beverages (Ray and Montet, 2017).

Indigenous food fermentation represents an extremely valuable cultural heritage in most regions, and harbors a huge genetic potential of valuable, but hitherto unidentified,

*Corresponding author. E-mail: miambi@u-pec.fr.

microbial strains (Tamang et al., 2016).

Steinkraus (1996) classified fermentation processes into various categories including lactic acid, alcoholic, acetic acid and alkaline fermentations. Unlike lactic acid and alcoholic fermentations, alkaline fermentation is unusual in that it causes an increase in the pH of the food as a result of the release of ammonia from the breakdown of proteins in the food (Sarkar and Nout, 2014; Ray and Montet, 2017). Alkaline fermented foods are a group of lesser-known food products that are widely consumed almost exclusively in Asian and Africa countries (Streinkraus, 2002); Sarkar and Nout, 2014).

Ntoba mbodi is a fermented food from a household-scale alkaline-fermentation process which depends upon the microorganisms in the local environment (Louembe et al., 2003; Kobawila et al., 2005; Mokemiabeka et al., 2011). A pioneering study of the microflora of Ntoba mbodi, using phenotypic characterization of isolates, reported the presence of *Micrococcus varians, Bacillus macerans, Bacillus subtilis, Staphylococcus sciuri* and *Staphylococcus xylosus* (Louembe et al., 2003). Kobawila et al. (2005) using the same bacterial characterization approach reported on the presence of *Bacillus subtilis*, *Bacillus amyloliquefaciens, Bacillus megaterium, Bacillus macerans, Bacillus cereus, Bacillus polymixa, Bacillus brevis and Bacillus pumilus.* Recently, Vouidibio-Mbozo et al. (2017), by combining both phenotypic and genotypic methods identified *B. safensis, B. siamensis* and *Lysinibacillus* spp. in Ntoba mbodi. A novel species of the genus *Lysinibacillus* has even been isolated from Ntoba mbodi (Ouoba et al., 2015). Surprisingly, lactic acid bacteria (LAB), which are the microorganisms responsible for acidic indigenous food fermentations (Brauman et al., 1996; Lei and Jakobsen, 2004; Kostinek et al., 2007; Sawadogo-Lingani et al., 2007), were also found in Ntoba mbodi (Ouoba et al., 2010). The LAB isolates were closest to *Weissella confusa, Weissella cibaria, Lactobacillus plantarum, Pediococcus pentosaceus, Enterococcus casseliflavus, Enterococcus faecium, Enterococcus faecalis, Enterococcus avium* and *Enterococcus hirae* (Ouoba et al., 2010).

However, it is still unclear whether the isolates previously described were the predominant bacterial taxa in Ntoba mbodi. Indeed, isolation of colonies was based on phenotypic characteristics of *Bacillus* after cultivation of total mesophilic bacteria (Vouidibio-Mbozo et al., 2017) or LAB (Ouoba et al., 2010). Therefore the bacterial predominance in previous studies represents the fraction of each distinct species complete set of isolates or probably faster growing bacteria but does not reflect the abundance of each cultivable species in the initial samples of Ntoba mbodi. This raises the question of the ecological relevance of isolates obtained under these conditions. Are these bacteria species involved in the fermentation of Ntoba mbodi or simply transient or inactive forms? Knowing the abundance of different species can provide insight into how a community functions (Verberk, 2011). Likewise, understanding the bacterial diversity and determining the taxonomic classification of dominant species are necessary for determining the bacteria forming the “functional starter” (Leroy and De Vuyst, 2004).

Furthermore, the softening of the cassava leaves which is the main indicator for the end of the fermentation process in Ntoba mbodi has been ascribed to bacterial enzyme activity in Ntoba mbodi (Mokemiabeka et al., 2011). However, this conclusion was based on the results obtained by screening for enzyme activities in homogenates of fermented cassava leaves. This experimental approach could not establish whether the enzyme activities observed came from bacterial activity or from enzymes secreted by cassava leaves themselves. In addition, xylanase activity was not investigated while xylan is the second most abundant polysaccharide found in the plant kingdom after cellulose and is the major hemicellulose in plant cell walls (He et al., 2014).

The present study set out to assess the numerically dominant bacterial species in Ntoba mbodi using a dilution to extinction approach (Garland and Lehman, 1999; Gomez et al., 2004), focusing on colonies obtained from the highest positive dilution of the Ntoba mbodi. We also investigated the hypothesis that there was a convergence of bacterial community profiles in Ntoba mbodi irrespective of the origin of samples in Brazzaville (Congo) as has been demonstrated in other ecosystems (Jia et al., 2016). This hypothesis was tested by assessing the set of bacteria shared across samples of Ntoba mbodi purchased from different producers. Finally, we determined whether the numerically dominant bacteria taxa isolated had the enzyme activities necessary for breaking down the main polysaccharides that make up the plant material.

MATERIALS AND METHODS

Sample collection, culture and bacterial isolation

Samples of Ntoba mbodi were purchased from various markets in Brazzaville (Congo). They were produced in four different workshops. These samples were aseptically collected in containers and transported to the laboratory within 2 h using cooler boxes. The samples were immediately processed in the laboratory under sterile conditions for subsequent analyses. Samples from the same workshop were pooled to make a composite sample. Then a 10 g sub-sample was put into a sterile mortar with 90 mL of sterile 0.9% NaCl solution and homogenized using a pestle. A 1.5 mL-aliquot was centrifuged at 800 x g for 1 min to remove suspended solids. The supernatant was decimally diluted in 0.9% NaCl solution down to 10^{-8} for use as inocula. The pH was adjusted to 6.8 ± 0.2 and a 30 µL-aliquot of each dilution was plated onto sterile plate count agar (Merck, Germany). The plates were incubated at 37°C for 24 h. The colonies formed on the agar plates were then used to estimate the numbers of bacteria in the original sample. Colonies developing on plates from the last positive dilution were first grouped by their macroscopic and microscopic morphotypes. Then colonies representative of the most abundant morphotypes were

isolated by streaking three times. The isolates were kept on plate count agar (Merck, Germany) for few days at 4°C before use.

Identification of isolates using 16S rRNA gene sequencing and phylogenetic analyses

The bacteria were identified using 16S rRNA gene sequencing followed by phylogenetic affiliation analyses. Each isolate was grown in liquid nutrient broth (Merck, Germany) at 37°C for 24 h then a 1.5 mL subsample of the bacterial culture was transferred to a sterile Eppendorf tube and centrifuged at 10,000 g for 10 min. DNA was extracted from the pellets using Mobio kit PowerSoil, following the manufacturer's instructions. There were three replicates for each sample. The concentration of the extracted DNA was evaluated by UV absorption at 260 nm using a NanoDrop 2000C (Thermo Scientific).The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) as described by Miambi et al. (2003) with some modifications. PCR reaction was performed in a 25 µL reaction mixture (total volume) using Taq polymerase Ready-To-Go (Amersham Pharmacia Biotech, USA), 0.25 mM of primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.25 mM of primer 1390 R (5'-GAC GGG CGG TGT GTA CAA-3') and 20 ng of the template DNA. The PCR was carried out using a thermocycler (VWR, France) with an initial denaturation for 8 min at 94°C; 30 cycles of 30 s at 94°C, 1 min at 56°C and 30 s at 72°C, and a final extension for 15 min at 72°C. The PCR products were run on 1.5 % (wt/vol agarose-0.5X TAE) buffer gel stained with SYBR Safe stain (Invitrogen, France) at 80V for 30 min and the bands were recorded using a GelDoc 2000 transilluminator system (BioRad, Richmond, California, USA). The DNA sequence of both strands of the PCR products were determined by Beckman Coulter Genomics (Essex, United Kingdom) using an ABI 3730XL Sanger sequencer (Sanger et al., 1977) with the original primers 27f and 1390R. 16S rRNA gene sequences were aligned using Mega 6 (Tamura et al., 2013) and compared with those available in the NCBI GenBank database using the megaBLAST program (Zhang et al., 2000). Each sequence was aligned with the first 10 database sequences giving the highest scores of sequence similarity and the quality of the database sequences was assessed. Identification at genus level was defined as a 16S rRNA gene sequence similarity of ≥ 97% with that of the closest sequence in GenBank.

Enzyme assays

The predominant purified isolates obtained after enumeration were used for screening for extracellular enzyme activities. Each of the predominant isolates was transferred into 10 mL of sterile nutrient broth (Merck, Germany) and incubated at 30°C in a stirring incubator for 16 h. Then, the absorbance was measured using a spectrophometer (VWR, France) and adjusted to the same value for all cultures. Aliquots of these cultures were taken and placed in microplates for screening for cellulase, hemicellulase (xylanase), amylase and β-glucosidase activities. The protocol was based on methods described by Popova and Deng (2010); Shao and Lin (2018) with some modifications.

For cellulase, xylanase, amylase and β-glucosidase activities, the activities were assayed using carboxymethyl-cellulose, xylan, starch and p-nitrophenyl-β-D-glucopyranoside as substrates, respectively. All substrates were of analytical grade (from Sigma–Aldrich, St. Louis, MO, USA). For cellulase, hemicellulase and amylase assays, a 100 µL aliquot of culture suspension of each isolate was put into a well in a 96-well microplate with 50 µL of Mac Ilvain buffer (pH 7). Then 50 µL of the substrate was added to each well. The reaction solution was mixed by pipetting up and down several times with multi-channel pipette. Each microplate had a minimum of three replicates for each substrate and three control wells containing sterile water, buffer and substrate. The microplate was then incubated at 37°C for 30 min. Aliquots (25 µL) of this reaction solution were transferred to a 96-well microplate with 200 µL of Somogyi's reagent. The microplates were held at 100°C for 20 min and then cooled on ice. The microplate wells were covered to avoid evaporation. Finally, aliquots (50 µL) of the resulting reaction solution were transferred to a 96-well microplate with 25 µL of Nelson's reagent. Then, 200 µL of distilled water was added to each well and mixed by pipetting up and down several times using a multi-channel pipette. Absorbance was measured at 630 nm using a microplate reader (Bio-Tek EL800, Bio-Tek Instruments, Winooski, VT). The activities were expressed as pmol glucose $min^{-1}ml^{-1}$ for cellulase, xylanase and amylase. The glucose concentrations in the reaction solution were calculated using a calibration curve constructed using glucose solutions at various concentrations.

For β-Glucosidase activity, 50 µL of culture suspension from each isolate was put into a well in a 96-well microplate with 25 µL of Mac Ilvain buffer (pH 7). Then 50 µL of the substrate was added to each well and the contents were mixed by pipetting up and down several times. Controls were prepared similarly but without bacterial suspensions. After incubating the microplate at 37 °C for 1 h, they were centrifuged at 14,000 g for 5 min and 50 µL of the supernatant was transferred to a 96-well microplate with 250 µL of 2% Na_2CO_3 to stop the enzyme reaction. Absorbance was measured at 410 nm using the same microplate reader. The p-Nitrophenol concentrations in the reaction solutions were calculated using a calibration curve that was constructed using pNP standards. There were five replicates for each bacterial suspension and each microplate included a minimum of three replicates three controls. The enzyme activity was expressed as pmol of PNP min^{-1} mL^{-1}.

Statistical analysis

The correlations between of isolates obtained from Ntoba mbodi and their potential enzyme activities were tested by principal component analysis (PCA) using ADE 4 (Thioulouse et al., 1997). A permutation test was used to determine whether the differences were large enough to reject the null hypothesis. Significant differences between isolates on the first two axes were determined by one way ANOVA after verifying that the data distribution was normal. The Tukey post hoc test was used after ANOVA. Values were considered different at $p < 0.05$. Correlations were analyzed using Statgraphics, Centurion XVI (Sigma Plus, France) to establish statistical relationships.

RESULTS

Bacterial enumeration and isolation of predominant morphotypes in Ntoba mbodi

The density of total mesophilic bacteria in Ntoba mbodi varied with the samples (Figure 1). All four samples had a high number of mesophilic bacteria cultivable ranging from 1.67×10^{10} to 2.8×10^{10} CFU g^{-1} (Figure 1). Bacterial density for samples 1 and 2 were of the same order and lower than samples 3 and 4. Based on macroscopic and microscopic observations of the colonies at the highest dilution, eleven distinct morphotypes were isolated from the four samples of Ntoba mbodi. These isolates are listed in the Table 1. Of the eleven isolates, five (M1, M2, M3, M4 and M7) were found in the all samples and represented the core bacteria of Ntoba mbodi. Isolates

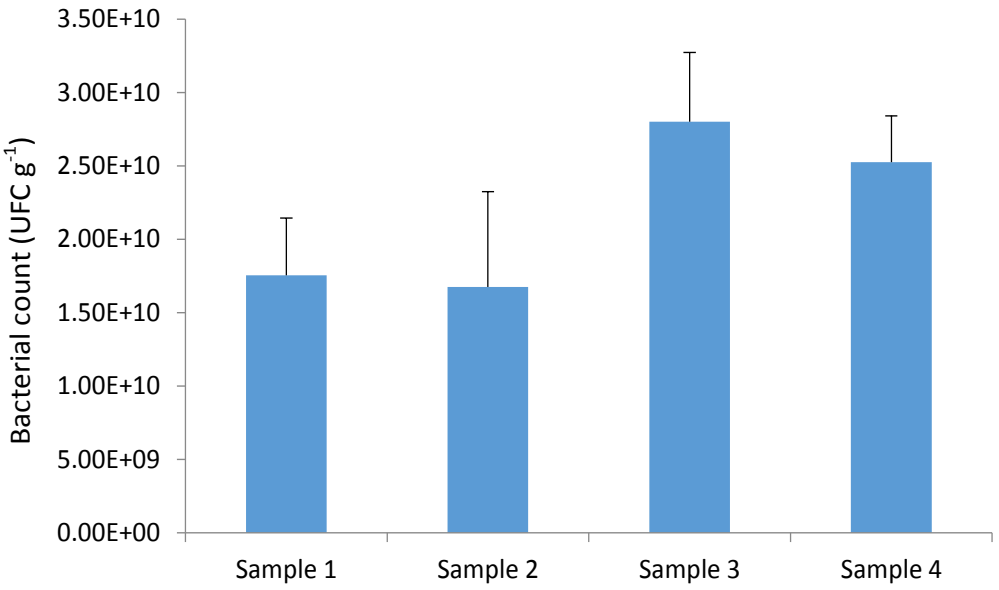


Figure 1. Enumeration of bacterial cells in the different samples of Ntoba-mbodi.

M10 and M11 were specific to samples 1 and 2 while M12 and M8 were only found in samples 4 and 2 respectively (Table 1).

Phylogenetic affiliation of predominant isolates

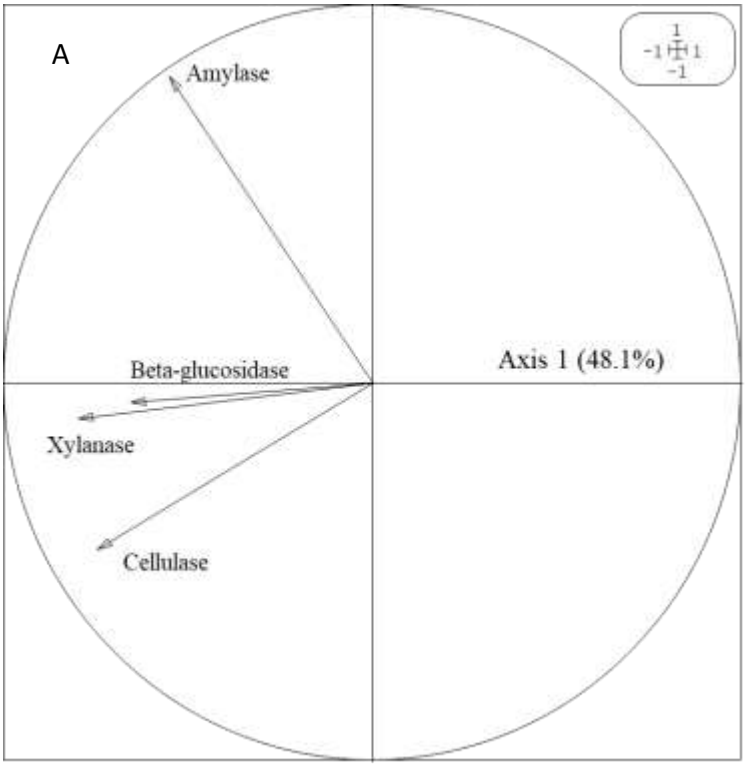
Verification of PCR products of the 16S rRNA genes of predominant isolates by electrophoresis in agarose gel indicated that the amplicons were about 1360 pb long (data not shown). The amplicons were nearly full-length and provided enough phylogenetic information for taxonomical identification of 55% of the isolates down to species level (Table 1). Most of the sequences showed 97% identity to the closest sequences already in the databases, and none was found to be apparently chimeric. About 73% of predominant were classified as *Bacillus* related species including a *Bacillus pumilus* strain, three strains of *Bacillus altitudinis*, two strains of *Bacillus* sp. C-32, two strains of *Bacillus* sp.01105 and *Bacillus aerophilus* strain. Non-Bacillus related species account for about 27% of the population and comprised *Staphylococcus sciuri*, *Kurthia* sp. B2 and *Bacterium* NLAE-ƶ-H375. The set of isolates found across all samples of Ntoba mbodi were closest to *Bacillus* related species (*Bacillus altitudinis* strain 126YG20, *Bacillus* sp. C-32) and *Staphylococcus sciuri*.

Screening the isolated bacterial strains for polysaccharide-degrading capacity

In the principal component analysis (PCA) of the four enzyme activities and the eleven isolates, axes 1 and 2 accounted for 48.1 and 21.6% of the variance respectively (Figure 2A). Together, these two axes explained about 70% of the variance. Xylanase, cellulase and β-glucosidase activities were opposed to amylase activity on axis 1. One group consisted of the strains M1, M4, M6, M10 and M12. Four of these strains were affiliated with the genus *Bacillus* and one with the genus *Kurthia*. They all showed higher xylanase and cellulase activities and, to a lesser extent β-glucosidase activity, than other isolates (Figure 3). A second group had two strains (M8 and M9), one affiliated with the genus *Bacillus* and one (M2) whose the closest relative was not well determined and classified as a relative of *Bacterium* NLAE-ƶ-H375 with 96% identity. Unlike the first group, the β-glucosidase, xylanase and cellulase activities of these strains were not significantly different from the other isolates (Figure 3). The strains M3 and M11, affiliated with the genus *Bacillus*, formed a third group. Their enzyme activities were the same order as those of the first group or second group. There were no significant differences in amylase activities between isolates and the amylase activity was lower than the cellulase, xylanase and β-glucosidase activities (data not shown).

DISCUSSION

This study set out to assess the variability in the bacterial populations across samples of Ntoba mbodi and the potential enzyme activities associated with the predominant strains isolated from Ntoba mbodi. Consistent with previous studies (Ouoba et al., 2010; Vouidibio-Mbozo et al., 2017), we showed that Ntoba mbodi contains high density of bacteria that varied with



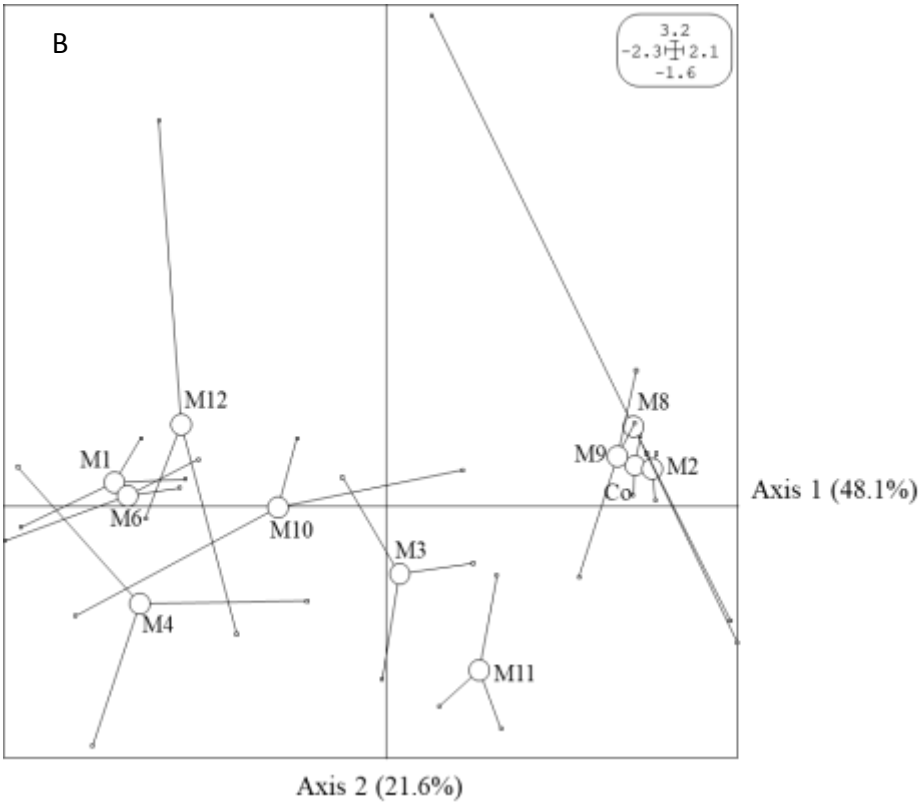


Figure 2. Principal component analysis (PCA) based on enzyme activities of the predominant strains. (A) Correlation circle. (B) Ordination of the strains in the plane defined by axes 1 and 2 of the PCA highlighting the profile of enzyme activity of the strains.

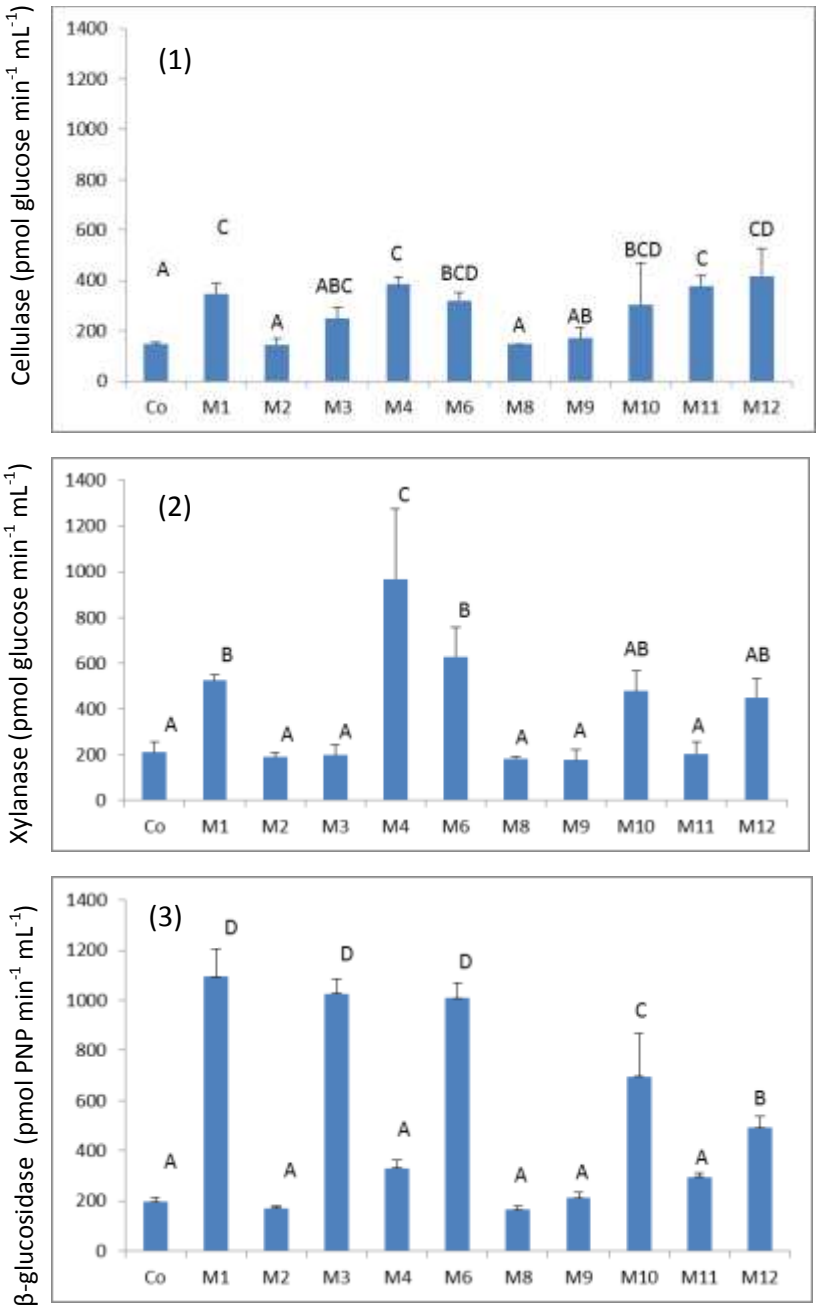


Figure 3. Enzyme activities of the predominant strains. (1) cellulase; (2) xylanase; (3) β-glucosidase. 1 and 2 are expressed as pmol glucose min^{-1} mL^{-1} and C as pmol PNP min^{-1} mL^{-1}. Co: controls. Different letters means that the results are significantly different ($p<0.05$) according to Fischer's PLSD-test. Mean values (n=5) ± standard error.

the samples from different producers. The variability of the bacterial community underlines that, like most indigenous food fermentation processes, the fermentation of Ntoba mbodi is driven by indigenous microorganisms originating in the raw materials, in autochthonous starter cultures, or in the processing environment itself (Bokulich and Mills, 2013).

The detection of predominant groups within the bacterial assemblages is the basis for determining the “functional starter” (Leroy and De Vuyst, 2004). Therefore we assessed the predominant taxa in samples of Ntoba mbodi from different producers. We found that Ntoba mbodi contain only a few predominant taxa, mainly *Bacillus* related species (72%), which were closest to

Bacillus pumilus, *Bacillus altitudinis* strain 126YG20, *Bacillus* sp. C-32, *Bacillus* sp.01105, *Bacillus altitudinis* strain SH156 and *Bacillus aerophilus* strain JF11. The presence of *Bacillus* related species is in agreement with previous studies in Ntoba mbodi (Louembe et al., 2003; Vouidibio-Mbozo et al., 2017) and in other alkaline food fermentation products (Steinkraus, 2002; Parkouda et al., 2009; Tamang et al., 2016). Comparison at species level revealed that the *Bacillus* related species previously reported in Ntoba mbodi, including *B. subtilis, B. amyloliquefaciens, B. megaterium, B. macerans, B. cereus, B. polymixa, B. brevis* and *B. pumilus* (Louembe et al., 2003; Kobawila et al., 2005), *B. safensis, B. siamensis* and *Lysinibacillus louembei* (Ouoba et al., 2015; Vouidibio Mbozo et al., 2017), were not found at the highest dilution of inoculum (Ntoba mbodi). The differences in the composition of bacteria assemblage in African alkaline-fermented foods can be explained by the variability in raw materials (Ouoba et al., 2010; Tamang et al., 2016). But the discrepancy between our results and those reported previously is more likely to be related to the different approaches used for determining the predominant strains using bacterial cultures. In the present study, only colonies obtained from the highest positive dilution of Ntoba mbodi were distinguished, on the basis of differences in macroscopic and microscopic observations, and isolated. These isolates were considered to be the predominant bacterial taxa in Ntoba mbodi. In previous studies, however, the various phenotypes of the Bacillus colonies were selected after enumeration of mesophilic bacteria (Vouidibio-Mbozo et al., 2017) and isolated at various dilution factors. The predominance in previous studies represents the percentage of distinct species among the total number of isolates but did not reflect the abundance of each species in initial sample of Ntoba mbodi. Knowing the abundance of different species can provide insight into how a community functions (Verberk, 2011). This raises the question of the relevance of isolates obtained in the present study. Do these bacterial species drive the fermentation of Ntoba mbodi or are they simply transient or inactive forms?

To address this question, we assessed whether there were predominant cultivable mesophilic bacteria found in all samples of Ntoba mbodi, irrespective of the producers. Although the household-scale fermentation process depends upon microorganisms from the local environment, we found a set of five isolates affiliated to *Bacillus altitudinis* strain 126YG20, *Bacillus* sp. C-32 (2 isolates), *Bacterium* NLAE-lz-H375 and *Staphylococcus sciuri* isolate YXY-5 that were found in all samples of Ntoba mbodi. These findings confirm our hypothesis that there is a set of bacteria common to all samples of Ntoba mbodi, irrespective of their origins, in Brazzaville (Congo) and suggest that the colonies isolated in the present study were not simply transient bacterial taxa. Determination of the set of bacteria common to all samples of Ntoba mbodi confirmed the convergence in taxonomic profiles of predominant bacteria regardless of the origin of the Ntoba mbodi. The convergence in taxonomic profiles of bacterial community has also been reported for other fermentation processes (Jia et al., 2016). Interestingly, the majority (60%) of the bacteria common to all samples of Ntoba mbodi were Bacillus-related species. Our results confirmed the importance of members of the genus *Bacillus* in vegetable fermentation processs (Steinkraus, 2002; Parkouda et al., 2009; Tamang et al., 2016). *Bacillus* spp. are also known for their ability to produce toxins that may cause food poisoning (Stenfors et al., 2008) and potentially pathogenic bacteria such *Bacillus cereus* have been reported in Ntoba mbodi (Vouidibio-Mbozo et al., 2017). Interestingly, these bacteria were not found among predominant isolates obtained in the present study.

Furthermore, the presence of strains affiliated with *Staphylococcus sciuri* in all samples of Ntoba mbodi in the present study raised the question of role of this bacteria group in fermentation of Ntoba mbodi. Members of this bacterial group are widespread in nature and have been isolated from various food products of animal origin (Garcia et al., 2002; Papamanoli et al., 2002). They are commonly present on skin and mucus membranes of a wide range of pets and farm animals (Kloos, 1980). It is, therefore, possible that their presence in Ntoba mbodi is from contamination during Ntoba mbodi processing due to handling practices. Recently, however, Amao et al. (2018) demonstrated that *Staphylococcus* spp. played important role by increasing free amino acids in iru (fermented locust beans) and by providing lipolytic enzyme activity during the fermentation. The role of *Staphylococcus* spp. in the fermentation of Ntoba mbodi still needs to be determined.

The softening of cassava leaves in Ntoba mbodi has been ascribed to bacterial enzyme activities (Mokemiabeka et al., 2011). But, unlike the extensively studied role of bacterial enzyme activities in softening cassava roots (Brauman et al., 1996; Kouhoundé et al., 2014), this has not previously been confirmed. Since it was still unclear whether the enzymes were from the bacteria or from the cassava leaves in Ntoba mbodi, the predominant isolates obtained were screened for some polysaccharide-degrading enzyme activities. Cellulase, xylanase, amylase and β-glucosidase were targeted because plant biomass is made of complex polysaccharides, primarily cellulose and hemicelluloses (Cragg et al., 2015). Out of eleven predominant isolates, eight showed cellulase activity and five showed xylanase and β-glucosidase activities. These findings demonstrate for the first time the bacterial origin of enzyme activities in Ntoba mbodi and confirm that microorganisms change the chemical composition of the raw materials during fermentation (Tamang et al., 2016). It is noteworthy to underline that 18% of predominant isolates including Bacillus related species did not have any of the enzyme

activities targeted. This finding suggests that despite the abundance and the role of *Bacillus* related species in alkaline fermentation processes (Tamang et al., 2016), not all members of this bacterial group are involved in the degradation of polysaccharides in Ntoba mbodi. It is possible that, in this alkaline fermentation, these bacteria may be involved in the production of bioactive and nutritive compounds during the process (Marco et al., 2017). The absence of amylase activity in all isolates was not expected. These results can be explained by the low starch content in cassava leaves compared to the roots.

Conclusions

Our study has shown that the density of bacteria in Ntoba mbodi was high and varied with the origin of the samples. Predominant bacteria in Ntoba mbodi comprised a few bacterial taxa dominated by Bacillus related species. Although the fermentation of Ntoba mbodi depends on microorganisms from the environment, there is a core set of bacteria that was found in all final products. More than 50% of predominant strains had enzyme activities for breaking down cellulose and xylan, which are the main components of plant materials. Further studies are needed to investigate the diversity of bacteria in Ntoba mbodi, without using cultures, to gain a better insight into the role played by bacteria in the functioning of this particular ecosystem.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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