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PREDOMINANCE OF *BACILLUS* SPP. DURING THE PRODUCTION OF MANTCHOUA, A TRADITIONAL KAPOK SEED FERMENTED CONDIMENT FROM BURKINA FASO

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ABSTRACT

Mantchoua is a fermented seed condiment produced from kapok tree (*Ceiba pentandra*) seeds in Burkina Faso. In this study, the microbiology of Mantchoua from raw material to final product was investigated in samples from two production sites (Pô and Bobo-Dioulasso). Four processing methods of Mantchoua production were characterized by determination of numbers of Aerobic Mesophilic Bacteria (AMB), *Bacillus* spp. and pH. A total of 251 *Bacillus* spp. from 619 AMB isolates were identified using M13-PCR and ITS-PCR typing, 16S rRNA and *gyrA* gene sequencing. AMB and *Bacillus* spp. counts in raw material ranged between 4.2-4.7 $\log_{10}$ CFU/g and 3.8-4.1 $\log_{10}$ CFU/g in kapok seeds and between 2.2-2.3 $\log_{10}$ CFU/g and 1.1-1.8 $\log_{10}$ CFU/g in ash lye solution, respectively. Microbial counts in seeds mash during fermentation ranged between 9-10.9 $\log_{10}$ CFU/g for AMB and between 8.6-10.5 $\log_{10}$ CFU/g for *Bacillus* spp. In dried Mantchoua, AMB counts ranged between 7.7-10.4 $\log_{10}$ CFU/g while *Bacillus* spp. counts ranged between 7.5-10.3 $\log_{10}$ CFU/g.
The fermentation of Mantchoua involved different species of *Bacillus* spp. At Bobo-Dioulasso pilot plant, *B. subtilis* subsp. *subtilis* dominated (50% of the *Bacillus* isolates) followed by *B. cereus sensu lato* (28% of the *Bacillus* isolates) while at Pô traditional production site, *B. cereus sensu lato* dominated (54% of the *Bacillus* isolates) followed also by *B. subtilis* subsp. *subtilis* (26% of the *Bacillus* isolates). For the Mantchoua processes including ash lye solution, pH were consistently higher during fermentation (pH 8.6-8.9), and the number of isolated *B. cereus sensu lato* were lower.

Keywords: *Bacillus* spp.; Fermentation; Kapok seeds; Mantchoua

INTRODUCTION

Mantchoua is a Kapok tree (*Ceiba pentadra*) seed condiment produced and consumed by some people in the Sahel region of Burkina Faso. Kapok tree seeds are of local interest as a valuable source of food raw material to be further explored in an African region of regular food shortage. Seed condiments are reported to be a valuable source of proteins, lipids, carbohydrates, essential amino acids, fatty acids and vitamins (**Achi, 2005**; **Yagoub and Mohammed, 2008**; **Parkouda et *al*., 2009**; **Dosumu et *al*., 2012**). The alkaline spontaneous fermentation during seed condiment production involves microorganisms able to degrade non-digestible carbohydrates in addition to proteolytic and lipolytic microorganisms, which play an active role in the physical, nutritional and organoleptic modifications of the seeds (**Parkouda et *al*., 2009**; **Olasupo et *al*., 2016**). Mantchoua is, like other seed condiments, used to enhance the flavor of soups and sauces eaten with the traditional staple foods of West Africa (**Achi, 2005**).
Mantchoua is traditionally produced at household level according to various processing methods. Generally, the processing of Mantchoua is very similar to the processing of Soumbala, in which the African locust beans are cooked twice. However, Mantchoua is fermented in two turns as opposed to Soumbala which is only fermented once (**Ouoba et *al*., 2008b**).
Aerobic endospore-forming bacteria (AEB) of the genus *Bacillus* have been reported to be the dominant microorganisms responsible for the fermentation of seed condiments (**Achi, 2005**; **Parkouda et *al*., 2009**, **Savadogo et *al*., 2011**). Previous studies showed that *B. subtilis, B. licheniformis, B. pumilus, B. cereus* and *B. amyloliquefaciens* were the dominant species occurring during the spontaneous fermentation of African locust bean (*Parkia biglobosa*) or soybean seeds for production of dawadawa, iru, afitin, sonru and soumbala (**Amoa-Awua et *al*., 2006**; **Azokpota et *al*., 2006a**, **2007**; **Ouoba et *al*., 2004**; **Sarkar et *al*., 2002**; **Savadogo et *al*., 2011**; **Olukunle et *al*., 2018**). Likewise, *Bacillus* species, notably *B. subtilis* are the dominant species involved in the fermentation of baobab seeds (*Adansonia digitata L.*) into maari (**Parkouda et *al*., 2009, 2010; Thorsen et *al*., 2015**). Previous studies revealed that the *Bacillus* spp. have the capability for secreting a wide range of enzymes (e.g. esterases, proteases, glucosidases and lipases) during seed fermentations, which leads to important biochemical changes, an increase in pH of up to 7.2-8.4 as well as aroma and flavor development (**Ouoba et *al*., 2003**; **Azokpota et *al*., 2006b**; **Oguntoyinbo et *al*., 2007b**; **Sarkar et *al*.**, 2002; **Parkouda et *al*., 2009**; **Olasupo et *al*., 2016**). Moreover, due to the spontaneous nature of the fermentation, the presence of potential spoilage and pathogenic microorganisms such as *B. cereus* has been reported (**Azokpota et *al*., 2006a**; **Ouoba et *al*., 2008b**; **Parkouda et *al*., 2010**; **Thorsen et *al*., 2015**).
To our knowledge, the processing and microbiota of Mantchoua have not been reported before. The aim of this study was to determine the AMB associated with four different processing methods of Mantchoua at two production sites in Burkina Faso. Furthermore, the profile of *Bacillus* spp. in the different steps of Mantchoua processing from raw materials to the final product was determined.

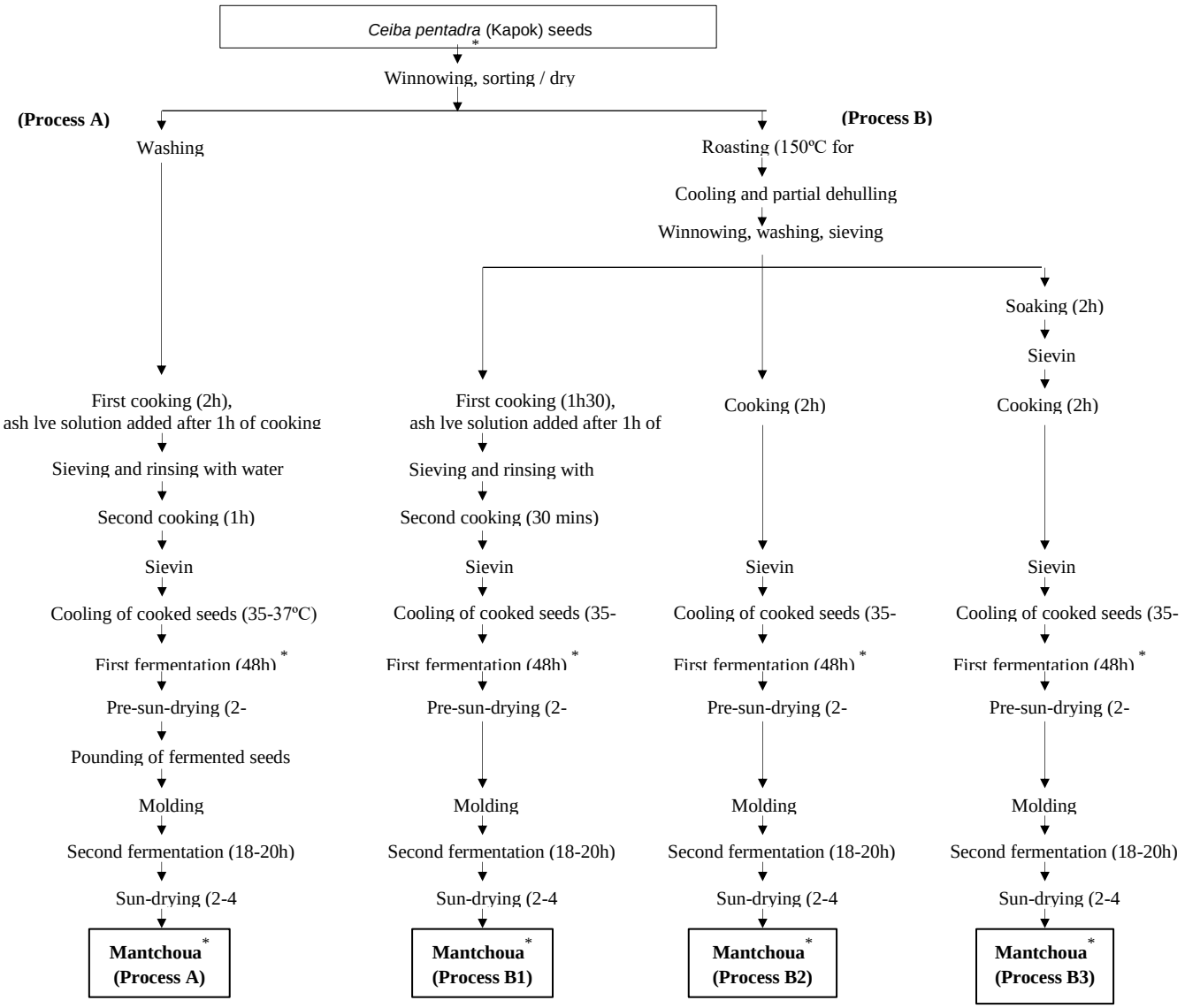

Figure 1 Flow diagram of traditional processing of kapok (*Ceiba pentadra)* seeds into Mantchoua using four different processing methods (A: unroasted seeds cooked with ash lye solution. B1: roasted and partially dehulled seeds, cooked with ash lye solution. B2: roasted and partially dehulled seeds, cooked without ash lye solution. B3: roasted and partially dehulled seeds, soaked and cooked without ash lye solution). *: sampling steps.

MATERIAL AND METHODS

Mantchoua processing and sampling

Four different processing methods of kapok seeds were studied at two different sites, in the city of Pô (Southern region of Burkina Faso, at 160 km from the capital Ouagadougou) at a traditional production site and at the pilot plant of Département Technologie Alimentaire (DTA/IRSAT/CNRST) in the city of Bobo Dioulasso (Western region of Burkina Faso, at 369 km from the capital Ouagadougou). The kapok seeds were purchased at local markets and the Mantchoua processing followed the flow diagram in Fig. 1, processes A, B1, B2 and B3 established after discussion and following of the technology with the producers. For process A, after dry cleaning (winnowing and sorting) and wet cleaning (washing), the seeds were cooked for 2 h with ash lye solution, sieved and rinsed with water before being cooked a second time for 1 h. After sieving and cooling (27-30°C), the cooked seeds were transferred into a polypropylene bag which was then tightly closed. A second polypropylene bag was wrapped around the first one, containing the seeds, before placing into a basket and left to ferment at ambient temperature (27-30ºC) for 48 h (first fermentation). The fermented seeds from the first fermentation were pre-sun-dried for 2-3 h, and thereafter pounded into a sticky cohesive mass, molded into balls and left to ferment for a second time for 18-20 h at ambient temperature (second fermentation). After the second fermentation, the balls were totally sun-dried for 2-4 days to stabilize the product. In process B, kapok seeds were roasted (150°C, 1 h) and partially dehulled before the cooking. For process B1, after partial dehulling, winnowing, washing and sieving, the seeds were cooked with ash lye solution for 1 h 30 min, sieved and rinsed with water. Then, they undergo a second cooking for 30 min. After the second cooking, the process followed the same steps as process A except that the seeds were not pounded after the pre-sun-drying. For process B2, after roasting, partial dehulling and washing, the seeds were cooked once for 2 h without ash lye solution. After sieving and cooling, the process followed the same steps as for process B1. The process B3 was similar to process B2 except that in process B3, after roasting, partial dehulling and cleaning, the seeds were soaked for 2 h and sieved before being cooked without ash lye solution. At Pô traditional production site, the Mantchoua was produced using the two processing methods both including ash lye solution, process A and B1 while at Bobo-Dioulasso pilot plant, the fermentation was followed for the four different processing methods (A, B1, B2 and B3, Fig. 1).

Sampling was performed in duplicate in the two production sites at the main steps of the flow diagram shown in Fig.1. At Pô production site, sampling included for each of both processes A and B1 the raw seeds (S), fermented seeds at the end of the first fermentation (F48) and at the end of the second fermentation (F72) as well as the sun-dried final Mantchoua product (P). A total of 16 samples were then taken. For the pilot Mantchoua fermentation at Bobo Dioulasso, sampling included for the four processes, raw materials (S), seeds at the onset of the fermentation (F0), fermenting seeds at the end of the first fermentation (F48) and second fermentation (F72), as well as the sun-dried final Mantchoua product (P). A total of 40 samples were then taken. In all cases 200 g of samples were aseptically collected using sterile spoons and sterile freezer bags with zip closing (Leader price, made in China imported by CeDoPalaiseau, France), kept in a thermo-cooler with ice blocs, transported to the microbiology laboratory of DTA and analyzed within 24 h. Samples of fermenting seeds were taken at the surface and in the center of the product.

pH determination

Ten grams of sample were homogenized with 20 mL of distilled water pH 7.0 in a stomacher bag (Masticor IUL, Barcelona, Spain) for 1 min at normal speed.

The pH of the homogenate was determined using a digital pH meter (Hanna, pH 211 Microprocessor pH meter, France) calibrated with standard buffer solutions pH 4.0 and pH 7.0 (Hanna, France). pH measurements were conducted in duplicate and means and standard deviation were calculated.

Enumeration, isolation and purification of Aerobic Mesophilic Bacteria (AMB)

Ten grams of each sample were aseptically homogenized with 90 mL of sterile diluent [0.1% (w/v) bactopeptone (Difco™, Detroit, Michigan, USA), 0.85% (w/v) NaCl (Merck, Germany), pH 7.0 ±0,2] by using a stomacher (Masticor IUL, Barcelona, Spain) at normal speed for 2 min to obtain 10^{-1} dilution. Serial dilutions were made from the homogenate of all samples, using 9 mL sterile diluent. Enumeration of AMB according to standard ISO (International Standard Organization) 4833 (2003) was obtained by pouring one milliliter from ten-fold dilutions in Plate Count Agar (PCA, Liofilchem S.R.L, Roseto degli Abruzzi TE, Italia), incubated aerobically at 30ºC for 72 h. After incubation, plates with 15-300 colony forming units (CFU) were counted and results expressed as Log_{10} CFU/g. Aiming at 20 isolates, all colonies from a random segment (> 15% of the area), of the highest dilution or suitable plates of PCA were picked and purified by successive streaking on nutrient agar (Merck, Darmstadt, Germany) as described by **Padonou et *al*. (2009)**. Pure cultures were maintained at -80ºC in nutrient broth (Merck, Darmstadt, Germany) containing 20% (v/v) glycerol (Merck). Working cultures were kept at 4°C on nutrient agar (Merck, Darmstadt, Germany). Microbial enumerations were conducted in duplicate and means and standard deviation were calculated.

Preliminary phenotypic characterization of isolates

For preliminary phenotypic characterization, colony morphologies were observed and the isolates were tested for catalase production using H_2O_2 solution (30%) (Laboratoire Gilbert, Hérouville Saint-Clair, France) and Gram reaction using 3% KOH (Fulkachimie GmBH, Switzerland) as described by **Gregersen (1978)**. Cell morphology was examined by phase-contrast microscopy (OLYMPUS BX40, Tokyo, Japan).

Identification of *B. cereus*

To identify *B. cereus* group species, presumptive *Bacillus* spp. identified following preliminary phenotypic characterization were spotted on *B. cereus* selective agar (Brilliance *Bacillus cereus* agar base (OXOID, Basingstoke, Hampshire, England) supplemented with Brilliance *Bacillus* selective supplement [SR0230E (OXOID, Basingstoke, Hampshire, England)], incubated at 30ºC for 24 h, as previously described by **Fricker et *al*. (2008)**. Colonies with a blue/green color, due to cleavage of 5-bromo-4-chloro-3-indolyl-ß-glucopyranoside by the enzyme ß-glucosidase usually present in *B. cereus*, were considered presumptive *B. cereus* species (**Fricker et *al*., 2008**). The affiliation to the *B. cereus* group was confirmed by inter transcribed spacer polymerase chain reaction (ITS-PCR) profiling as described by **Willumsen et *al*. (2005)**.

Genotypic characterization of *Bacillus* spp. isolates

Bacillus spp. isolates which were not considered presumptive *B. cereus* group species were grouped using M13-PCR with the M13 phage derived primer (5´-GAG GGT GGC GGC TCT-3´) (**Henderson et *al*., 1994**). DNA was extracted by using the InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction. The M13-PCR reaction mixture (25 µL) composition was: 12.5 µL Dream taq green (2X) PCR master mix, 2 µL 10 pM M13 primer, 1 µL template DNA, 9.5 µL sterile MilliQ water. All chemicals were purchased from Fermentas (St. Leon-Rot, Germany). PCR was performed in an Applied Biosystems thermal cycler 2720 (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.), under the following conditions: 35 cycles of denaturation at 94ºC for 60 s, annealing 40ºC for 60 s, extension 65ºC for 8 min; final elongation step at 65ºC for 16 min; holding at 4ºC. PCR products were separated by 1.5% agarose gel electrophoresis in 1.5 × TBE (5 h, 140 V) using a Generuler 1kb DNA ladder as reference (Fermentas, Vilnius, Lithuania). DNA fragments were stained with ethidium bromide solution (4 µg/L) and photographed (Alpha imager system, Alpha Innotech, San Francisco, USA). Cluster analysis of the M13-PCR profiles were performed using the BioNumerics 4.5 software (Applied Maths, Sint-Martens-Latem, Belgium), as described by **Nielsen et *al*. (2007)**.

Based on the M13-PCR clusters, a total of 45 representative isolates were selected for 16S rRNA gene sequencing using the universal primers 27f and 1510r, as previously described by **Satokari et *al*. (2001)**. Sequencing was performed by a commercial facility (Macrogen, Europe). 16S rRNA sequences were manually corrected and aligned using Chromas 2.33 (Technelysium) and CLC Genomics (CLC Bio, Aarhus, Denmark). Subsequently, the corrected nucleotide sequences were aligned to the 16S rRNA gene sequences in the GenBank database using the BLAST algorithm (**Altschul et *al*., 1997**) and in EzTaxon-e database as described by **Kim et *al*. (2012)**. Species and subspecies of the *B. subtilis* group as well as *B. pumilus* group species obtained by the GenBank and EzTaxon-e results were discriminated by *gyrA* sequencing, as described by **Agbobatinkpo et *al*. (2013)**. Sequencing was performed by a commercial facility (Macrogen Europe). The obtained nucleotide sequences were manually corrected using the CLC Main Workbench version 6.7 (CLC BIO, Aarhus, Denmark) and aligned to the *gyrA* sequences in GenBank database using the BLAST algorithm (**Altschul et *al*., 1997**).

Phenotypic discriminative tests

To discriminate between *B. amyloliquefaciens*, *B. methylotrophicus*, *B. atrophaeus* and *B. siamensis*, oxidase test were carried out as well as testing of their salt tolerance (0%, 7%, 10% (w/v) NaCl), and growth at different temperatures (45ºC, 50ºC and 55ºC) as described by **Madhaiyan et *al*. (2010)** and **Sumpavapol et *al*. (2010)**. The isolates, identified as *B. pumilus* group members (*B. pumilus*, *B. safensis*, *B. altitudinis*, *B. aerophilus* and *B. stratosphericus*) by 16S rRNA and *gyrA* gene sequencing, were differentiated by their ability to grow in nutrient broth pH 5.1 at 37ºC and growth at different temperatures (8ºC, 40ºC and 45ºC) (**Shivaji et *al*., 2006**).

Enzymatic profiles

Enzymatic profiles were determined for selected species [*B. altitudinis* (2P3a), *B. amyloliquefaciens* (K42.2), *B. licheniformis* (K31.1), *B. licheniformis* (K49.8), *B. safensis* (4P3), *B. safensis* (K2.4), *B. subtilis* (8P15)] using the API ZYM25 200 kit (BioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions, testing each strain twice.

Sequences

The nucleotide sequences determined in the present work have been assigned GenBank accession numbers: KJ882849-KJ882898.

RESULTS

Total aerobic mesophilic bacteria (AMB) counts, *Bacillus* spp. counts and pH

As shown in Table 1, total AMB counts at Bobo Dioulasso pilot plant were 4.7±0.01 log_{10} CFU/g and *Bacillus* spp. counts were 4.1±0.03 log_{10} CFU/g for the raw material (kapok seeds). At the initiation of fermentation (0 h) total AMB counts were between 1.8 and 5.3 log_{10} CFU/g while *Bacillus* spp. counts were between 1.3 and 4.3 log_{10} CFU/g, in the four processing methods. Total AMB counts and *Bacillus* spp. counts increased during the first fermentation and ranged between 9.0 and 10.8 log_{10} CFU/g and 8.8 and 10.4 log_{10} CFU/g, respectively, at the end of the first fermentation (48 h). At the end of the second fermentation (72 h), total AMB count were between 9.2 and 10.9 log_{10} CFU/g and *Bacillus* spp. counts were between 8,6 and 9,7 log_{10} CFU/g. The highest microbial counts were found for Mantchoua prepared with ash lye solution i.e. processes A and B1 (10.8-10.9 log_{10} CFU/g for total AMB and 10.2-10.4 log_{10} CFU/g for *Bacillus* spp.). In the final Mantchoua products, the microbial counts had decreased to between 7.9 and 10.0 log_{10} CFU/g and between 7.5 and 9.8 log_{10} CFU/g for total AMB and *Bacillus* spp., respectively. The processing methods applied during Mantchoua production affected the pH of the samples. Higher pH values were observed in samples processed with method A and B1 (cooked with ash lye solution), with a pH of 7.1±0.14 and 6.9±0.14, respectively, at the beginning of fermentation (0 h), which increased to the maximum pH of 8.9±0.14 and 8.6±0.14 at the end of the first fermentation (48 h), respectively. In the final products the pH decrease to 7.5±0.07 for samples of process A and 7.6±0.07 for samples of process B1. Lower pH values were observed in the processing samples B2 and B3 (without ash lye solution), which both had a pH of 6.5±0.07 at the beginning of the fermentation (0 h). An increase of pH was observed after the first fermentation (48 h) reaching 7.9±0.21 for samples processed with method B2 and 7.2±0.14 for samples processed with method B3. A slight decrease of pH was however observed at the end of the second fermentation with value of 7.1±0.14 for samples B2 and 6.8±0.14 for samples B3. In the final products of processes B2 and B3, pH increased again to reach 7.3±0.14 and 7.4±0.21 respectively.

Total AMB counts, *Bacillus* spp. counts and pH development for Pô production site are shown in Table 2. For the raw material, total AMB counts were 4.2±0.02 log_{10} CFU/g while *Bacillus* spp. counts were 3.8±0.01 log_{10} CFU/g. At the end of the first and second fermentation, the total AMB and *Bacillus* spp. counts ranged between 10.3 and 10.5 log_{10} CFU/g in samples processed with either method A or B1. In the final Mantchoua products, differences between the samples from the two processing methods were observed. Total AMB and *Bacillus* spp. counts were 10.4±0.1 and 10.3±0.08 log_{10} CFU/g, respectively, for samples processed with method A, while for samples processed with method B1 these counts were 7.7±0.06 and 7.6±0.02log_{10} CFU/g, respectively. The samples analyzed from Pô had, in general, a lower pH as compared to the samples from the pilot plant in Bobo-Dioulasso. During fermentation, samples processed with method A, had a

pH of 7.6±0.21 (48 h) and 7.7±0.07 (72 h), whereas samples processed with method B1 had a pH of 8.0±0.21 (48 h) and 7.5±0.14 (72 h). For both processing methods the pH dropped to 6.4±0.03 (A) and 6.7±0.07 (B1) in the final product.

Table 1 Total aerobic mesophilic counts (AMB), *Bacillus* spp. counts and pH during Mantchoua production at Bobo-Dioulasso using four processing methods (A, B1, B2 and B3)

		Processing method											
		A			B1			B2			B3		
Processing step	Sample	Total count (log_{10} CFU/g)	*Bacillus* spp. count (log_{10} CFU/g)	pH	Total count (log_{10} CFU/g)	*Bacillus* spp. count (log_{10} CFU/g)	pH	Total count (log_{10} CFU /g)	*Bacillus* spp. count (log_{10} CFU/g)	pH	Total count (log_{10} CFU/g)	*Bacillus* spp. count (log_{10} CFU/g)	pH
Raw material	S	4.7±0.01	4.1±0.03	6.2±0.14	4.7±0.01	4.1±0.03	6.2±0.14	4.7±0.01	4.1±0.03	6.2±0.14	4.7±0.01	4.1±0.03	6.2±0.14
	AL	2.3±0.02	1.1±0.01	12.3±0.03	2.3±0.02	1.1±0.01	12.3±0.03	-	-	-	-	-	-
1st fermentation	F0	5.3±0.05	4.3±0.02	7.1±0.14	2.0±0.02	1.6±0.00	6.9±0.14	1.8±0.01	1.6±0.07	6.5±0.07	1.8±0.04	1.3±0.06	6.5±0.07
	F48	9.0±0.02	10.2±0.07	8.9±0.14	10.8±0.05	10.4±0.01	8.6±0.14	9.1±0.08	nd	7.9±0.21	9.4±0.03	8.8±0.07	7.2±0.14
2nd fermentation	F72	10.9±0.04	nd	7.8±0.07	10.1±0.02	9.7±0.01	7.6±0.14	9.2±0.02	8.6±0.03	7.1±0.14	9.3±0.01	8.7±0.04	6.8±0.14
Final product	P	9.0±0.03	8.6±0.05	7.5±0.07	10.0±0.08	9.8±0.03	7.6±0.14	8.8±0.04	8.6±0.06	7.3±0.14	7.9±0.02	7.5±0.07	7.4±0.21

S: seeds; AL: Ash lye solution; F0: sample at the onset of the fermentation at 0 h; F48: sample after first fermentation at 48 h; F72: sample after second fermentation at 72 h; P: final product. nd: not determined; -: not included in the processing

Table 2 Total counts, *Bacillus* spp. counts and pH during Mantchoua production at Pô using two processing methods (A and B1)

Processing step	Sample	Processing method					
		A			B1		
		Total count (log_{10} CFU/g)	*Bacillus* spp. count (log_{10} CFU/g)	pH	Total count (log_{10} CFU/g)	*Bacillus* spp. count (log_{10} CFU/g)	pH
Raw material	S	4.2±0.02	3.8±0.01	6.1±0.14	4.2±0.02	3.8±0.01	6.1±0.14
	AL	2.2±0.01	1.8±0.02	12.0±0.07	2.2±0.01	1.8±0.02	12.0±0.07
1st fermentation	F48	10.5±0.07	10.4±0.03	7.6±0.21	10.5±0.01	10.5±0.04	8.0±0.21
2nd fermentation	F72	10.4±0.08	10.3±0.01	7.7±0.07	10.4±0.07	10.3±0.01	7.5±0.14
Final product	P	10.4±0.1	10.3±0.08	6.4±0.03	7.7±0.06	7.6±0.02	6.7±0.07

S: seeds; AL: Ash lye solution; F48: sample after first fermentation at 48 h; F72: sample after second fermentation at 72 h; P: final product.

Identification of *Bacillus* spp. isolated from Mantchoua

A total of 619 AMB were isolated from the samples taken throughout Mantchoua production at Bobo-Dioulasso (494 AMB isolates) and Pô (125 AMB isolates). From these, 251 isolates were characterized as *Bacillus* spp. (22% of all AMB isolates). Within the identified *Bacillus* spp. 99 isolates were identified as *B. cereus sensu lato* (39% of *Bacillus* spp.) based on their growth on *B. cereus* selective agar as well as by ITS-PCR profiling (results not shown). The remaining 152 isolates were identified as others species of *Bacillus* by M13-PCR following 16S rRNA gene sequencing, which allowed identification at group level combined with *gyrA* gene sequencing, which was used to identify at species and subspecies levels. The isolates clustered into 6 M13-PCR groups representing 5 different *Bacillus* species (Fig. 2).

The M13-PCR group 1 was closely related to *B. safensis* (1358 bp, 100% 16S rRNA gene homology in EzTaxon-e) and *B. pumilus* (99.8% 16S rRNA gene homology in Ez-taxon-e). Analysis of the partial *gyrA* sequences from M13-PCR group 1 identified the isolates as *B. safensis* (99.0-100% homology to sequences in Genbank). The isolate in M13-PCR group 2 showed 100% 16S rRNA gene (1358 bp) identity to *B. altitudinis*, *B. aerophilus* and *B. stratosphericus.* The *gyrA* gene sequence (445 bp) of the M13-PCR group 2 isolate showed, 99.3% identity to *B. stratosphericus* (GenBank APAS01000012.1) and 99.8% identity to *B. altitudinis* (Genbank). Combined with the ability to grow at 45ºC and at pH 5.1, the isolate of M13-PCR group 2 was finally identified as *B. altitudinis.* The M13-PCR group 3 was identified as *B. licheniformis* (1342bp, 2 sequences, 99.6% 16S rRNA gene homology in EzTaxon-e). Using partial *gyrA* gene sequencing (874 bp, 2 sequences) identification to *B. licheniformis* was confirmed (99.9% homology in Genbank). The M13-PCR groups 4 and 5 were initially identified as either *B. subtilis* or *B. tequilensis* (1359bp, 28 sequences, 99.7-100% 16S rRNA gene homology in EzTaxon-e). On the basis of the partial *gyrA* gene sequences (874bp, 15 sequences) both the M13-PCR group 4 and 5 were identified as *B. subtilis* subsp. *subtilis* with 99.5-100% homology to sequences in Genbank. The M13-PCR group 6 was identified as *B. amyloliquefaciens, B. methylotrophicus* and *B. siamensis* (1359bp, 14 sequences. 99.7-100% 16S rRNA gene homology in EzTaxon-e). By partial *gyrA* gene sequencing M13-PCR group 6 was identified as *B. amyloliquefaciens* subsp. *plantarum* (874 bp, 6 sequences, 98.7-100% homology in Genbank).

Distribution of *Bacillus* spp. in raw materials, during fermentation and in the final product

At the pilot plant site in Bobo Dioulasso, the seeds used as raw materials were dominated by *B. subtilis* subsp. *subtilis* and *B. cereus sensu lato* (each comprising 50% of the *Bacillus* isolates) whereas only *B. subtilis* subsp. *subtilis* was detected in the ash solution (100%). *B. subtilis* subsp. *subtilis* was the predominant *Bacillus* species during the fermentations (12-72 h) for two out of the four processing methods, i.e. accounting for 47% (A), 66% (B1), 40% (B2) and 30% (B3) of the *Bacillus* isolates. Likewise, *B. cereus sensu lato* was found to be the most abundant species during the fermentations for two out of the four processing methods, comprising 28% (A), 13% (B1), 48% (B2) and 50% (B3) of the isolates. *B. amyloliquefaciens* subsp. *plantarum* comprised 20% (A), 21% (B1), 12% (B2) and 20% (B3). For the final products from the Bobo-Dioulasso pilot plant, differences were seen between the processing methods. In the final product made by method A, *B. amyloliquefaciens* subsp. *plantarum* was found to be the dominant *Bacillus* species accounting for 75% of the *Bacillus* isolates. For the final products made by method B1 and B2, *B. subtilis* subsp. *subtilis* was found to be the dominant *Bacillus* species accounting for 66% and 70% of the isolates, respectively. The final product made by method B3, *B. subtilis* subsp. *subtilis* and *B. amyloliquefaciens* subsp. *plantarum* dominated each accounting for 43% of the *Bacillus* isolates. *B. licheniformis* additionally occur at the later stage of fermentation for method A accounting for 20% of the *Bacillus* isolates and in the final product accounting for 25%. Additionally, *B. licheniformis* was found in the final product for method B1 i.e. accounting for 17% of the *Bacillus* isolates. Lower amounts of *B. cereus sensu lato* were generally observed in the final products as compared to especially the early stages of the fermentation (0-12 h) where *B. cereus sensu lato* accounted for 54% (A), 23% (B1), 46% (B2) and 53% (B3) of the *Bacillus* isolates. In fact the presence of *B. cereus sensu lato* was only detected in the final Mantchoua products processed with roasted kapok seeds i.e. 17% (B1), 14% (B2) and 14% (B3).

At the traditional production site in Pô, the dominant *Bacillus* species identified in the seeds used as raw material were *B. cereus sensu lato* (72% of the *Bacillus* isolates), followed by *B. amyloliquefaciens* subsp. *plantarum* and *B. altitudinis* (each comprising 14% of the *Bacillus* isolates). In the ash lye the dominant species were *B. cereus sensu lato* (66% of the isolates) followed by *B. subtilis* subsp. *subtilis* and *B. safensis* (each comprising 17% of the isolates). The dominating species occurring during the fermentations (48-72 h) were the same for the two processing methods (A and B1), however their abundance varied. The fermentation in processing method A was dominated by *B. subtilis* subsp. *subtilis* (38% of the isolates), *B. cereus sensu lato* and *B. amyloliquefaciens* subsp. *plantarum* (each comprising 31% of the isolates). Contrary, processing method B1 was dominated by *B. cereus sensu lato* (55% of the isolates) followed by *B. subtilis* subsp. *subtilis* (39% of the isolates) with *B. amyloliquefaciens* subsp. *plantarum* only making up a minor part (6% of the isolates). The final products from Pô were dominated by *B. cereus sensu lato*, no matter the processing method i.e. 62% for method A and 88% for method B1. In addition to *B. cereus sensu lato*, *B. amyloliquefaciens* was detected in the final products i.e. 30% (A) and 12% (B), with only a minor amount of *B. subtilis* subsp. *subtilis* observed in the samples processed with method A (8% of the isolates).

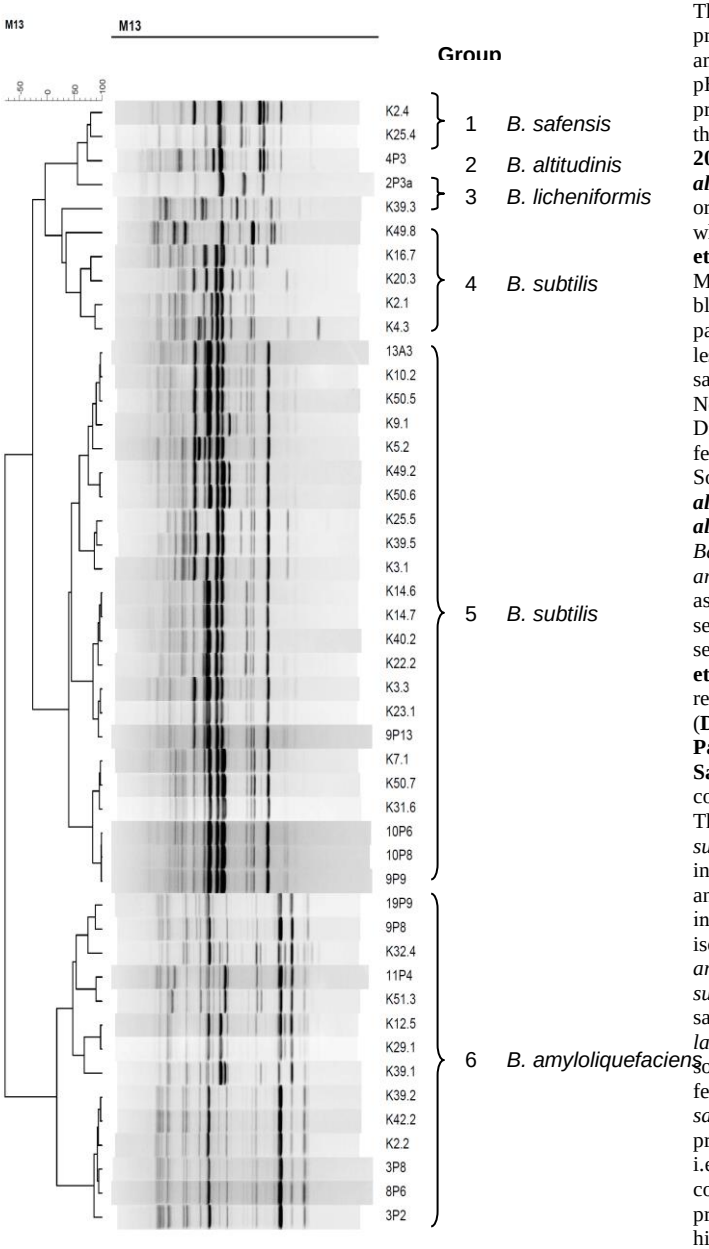

Figure 2 Dendogram obtained by cluster analysis of M13-PCR fingerprints for representative *Bacillus* spp. isolated during Mantchoua processing using the Pearson correlation coefficients between the densitometry traces and the clustering method of Ward. Only a sub-sample of representative sequenced isolates is shown. Identification was performed by 16S rRNA and *gyrA* gene sequencing.

Enzymatic profiles of *Bacillus* spp. from Mantchoua

The enzymatic profiles of selected *Bacillus* spp. isolates were determined using the API ZYM 25 kit (results not shown). All of the examined *Bacillus* spp. isolates were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), chymotrypsin, and α- and β-glucosidase. Except for the lack of β-galactosidase activity, the *B. amyloliquefaciens* subsp. *plantarum* isolate (K42.2) showed similar enzymatic profile as *B. licheniformis* (K49.8). Isolates of *B. altitudinis* (2P3a), *B. pumilus* (K2.4) and *B. safensis* (4P3) showed similar profiles, and produced higher β-glucosidase activity as compared to the other *Bacillus* spp. isolates tested.

DISCUSSION

The present work was the first to characterize *Bacillus* spp. associated with the production of Mantchoua in Burkina Faso, from raw materials, during fermentation and in the final product. The total aerobic mesophilic bacteria (AMB) counts and *Bacillus* spp. counts increased to a maximum of 10.9 $\log_{10}$ CFU/g and 10.5 $\log_{10}$ CFU/g, respectively, which is comparable to other high counts reported for different alkaline fermented seed condiments (**Terlabie et *al*., 2006**; **Parkouda et *al*., 2010**; **Ahaotu et *al*., 2013**).

The Mantchoua fermentation was alkaline, indicative of the secretion of proteolytic enzymes, followed by deamination of free amino acids and release of ammonia by *Bacillus* spp., as previously described by **Kada et *al*. (2008)**. The pH development in Mantchoua production differs from the pH development previously observed for the Ghanaian kapok seeds condiment, Kantong, where the pH typically decreased to 4.6 during fermentation (**Kpikpi et *al*., 2009**, **2010**). In Kantong processing *Bacillus* spp. also grew to high numbers (**Kpikpi et *al*., 2014**), although the main metabolic activity in Kantong is the production of organic acids by LAB, due to the addition of cassava flour to the kapok seeds, which serves as a source of carbohydrates supporting the growth of LAB (**Kpikpi et *al*., 2009, 2010**).

Mantchoua produced from unroasted and non dehulled seeds (process A) had a black color and a very strong odor as compared to Mantchoua made from roasted partially dehulled seeds (processes B1, B2, B3), which had a brown color and a less strong odor. AEB of the genus *Bacillus* were consistently isolated from all samples analyzed, from the beginning to the end of the Mantchoua processing. No other spore-forming genera were detected at the two production sites in Bobo-Dioulasso and Pô. The dominance of the genus *Bacillus* spp. in African alkaline fermented foods has been well documented in several studies on Dawadawa, Soumbala, Bikalga, Afitin, Maari and Okpehe (**Dakwa et *al*., 2005**; **Azokpota et *al*., 2007**; **Ouoba et *al*., 2004**, **2008a**; **Parkouda et *al*., 2010**; **Oguntoyinbo et *al*., 2010**; **Savadogo et *al*., 2011**; **Ahaotu et *al*., 2013**; **Olasupo et *al*., 2016**). The *Bacillus* spp. of Mantchoua included *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* subsp. *plantarum*, *B. licheniformis*, *B. safensis*, *B. altitudinis* as well as *B. cereus sensu lato*. These particular species have been reported by several authors to occur at variable levels in similar traditional alkaline fermented seeds condiments (**Parkouda et *al*., 2010**; **Ahaotu et *al*., 2013**; **Agbobatinkpo et *al*., 2013**; **Olukunle and Sanusi, 2018**). *B. subtilis* has previously been reported to be a predominant AEB in alkaline fermented foods and additives (**Dakwa et *al*., 2005**; **Azokpota et *al*., 2007**; **Oguntoyinbo et *al*., 2010**; **Parkouda et *al*., 2010**; **Savadogo et *al*., 2011**; **Agbobatinkpo et *al*., 2013**; **Sarkar et *al*., 1994**). Our results confirmed this with *B. subtilis* subsp. *subtilis* comprising 39% of the *Bacillus* spp. isolates from all analyzed samples.

The seeds at the pilot plant in Bobo-Dioulasso contained *B. subtilis* subsp. *subtilis* and *B. cereus sensu lato*, whereas only *B. subtilis* subsp. *subtilis* occurred in the ash lye solution. These two species persisted throughout the fermentation and in the final product from Bobo-Dioulasso. At the traditional production site in Pô, *B. cereus sensu lato* were predominating the seeds (71% of the *Bacillus* isolates) and ash lye solution (67% of the *Bacillus* isolates) with minor parts of *B. amyloliquefaciens* subsp. *plantarum* and *B. altitudinis* in the seeds and of *B. subtilis* subsp. *subtilis* and *B. safensis* in the ash lye solution. The fermentation samples and the final products from Pô were predominated by *B. cereus sensu lato*. The composition of *Bacillus* spp. in the raw materials (seeds and ash lye solution) hence seemed to influence the distribution of *Bacillus* spp. during the fermentation and in the final product. *B. licheniformis*, *B. altitudinis* and *B. safensis* occurred sporadically and only in some of the samples and do therefore probably not play a significant role in the fermentation. The processing methods, i.e. cooking with ash lye solution, additionally, seemed to have an effect on the composition of *Bacillus* spp. associated with Mantchoua. For the Mantchoua processes including ash lye solution (processing A and B1), pH were consistently higher during fermentation (pH 8.6-8.9 at the end of first fermentation), and the number of isolated *B. cereus sensu lato* were lower, suggesting that pH affected the growth of *B. cereus sensu lato*. This observation was in accordance with **Lindsay et *al*. (2002)** who reported that two different *B. cereus* strains had a slower growth rate at alkaline pH as compared to neutral pH. Additionally, an investigation on growth rates for different *Bacillus* spp. at varying pH values, showed that the species are influenced differently (**Lindsay et *al*., 2000**). Previous studies reported that bacteriocin producing strains of *B. subtilis* and *B. amyloliquefaciens* subsp. *plantarum* isolated from African fermented seeds

condiments inhibited the growth of *B. cereus* (**Kaboré et *al*., 2012**, **2013**; **Compaoré et *al*., 2013**). Similar inhibition of *B. cereus sensu lato* throughout the fermentation and in the final Mantchoua products by *B. subtilis* subsp. *subtilis* and *B. amyloliquefaciens* subsp. *plantarum* could possibly take place.

It is well known that the *Bacillus* spp. involved in fermentations, are using the nutritional components of the seeds converting them into products, which contribute to the chemical composition and the taste of the condiment (**Parkouda et *al*., 2009**). Kapok seeds consist of approximately 28% crude protein, 25% crude fiber, 7% starch, 5% sugars as well as fat, ash etc. (**Narahari and Rajini, 2003**). Several studies have previously reported that strains of *B. subtilis*, isolated from different alkaline fermented seeds, were able to produce degradative enzymes targeting proteins, carbohydrates and lipids (**Ouoba et *al*., 2003**; **Amoa-Awua et *al*., 2006**; **Terlabie et *al*., 2006**; **Oguntoyinbo et *al*., 2007a, 2007b**). The enzymatic profiles obtained in the present study suggested that most of the examined *Bacillus* spp. from Mantchoua have the capability for degrading seed proteins (trypsin, chymotrypsin activity), crude fiber (β-glucosidase) and fats (esterase and esterase-lipase activities) and are therefore likely to be involved in the biochemical transformation of the kapok seeds. The enzymatic profile of the *B. amyloliquefaciens* subsp. *plantarum* strains from Mantchoua were similar to those previously reported for *B. subtilis* strains isolated from Okpehe (**Oguntoyinbo et *al*., 2007a**), while the enzymatic profiles of the *B. licheniformis* isolate from Mantchoua was similar to the enzymatic profile reported for *B. licheniformis* isolated from cassava fermentation (**Amoa-Awua and Jakobsen, 1995**). Because of their high contents of protein and minerals, alkaline fermented food condiments are considered to be of relevance for improving the diet of the African people. However, indigenous fermented foods are still primarily produced without the use of starter cultures and under uncontrolled conditions (**Jespersen, 2003**). Often spoilage and pathogenic microorganisms associated with food poisoning can be isolated from these foods due to the lack of appropriate technology and production conditions (**Holzapfel, 2002**). Some strains of *B. cereus* are recognized as a foodborne pathogen capable of causing vomiting due to the production of cereulide, and diarrhea through the production of various enterotoxins (**Stenfors Arnesen et *al*., 2008**). Previous studies on alkaline fermented foods from Africa have revealed that *B. cereus* often occur in high numbers and harbor genes encoding the thermo-labile diarrheal toxins cytotoxin K, hemolysin BL and nonhemolytic enterotoxin as well as *cesB* part of the peptide synthesis complex producing the heat-stable toxin cereulide (**Ouoba et *al*., 2008b**; **Oguntoyinbo et *al*., 2010**; **Agbobatinkpo et *al*., 2013**; **Ahaotu et *al*., 2013**; **Thorsen et *al*., 2010, 2015**). The occurrence of *B. cereus sensu lato* at high rate (39.4% of AEB) in Mantchoua suggests that potential health risk for consumers and preventive measures to control their outgrowth in Mantchoua should be investigated. However, it is also worth noting that not all *B. cereus* spp. should be considered pathogenic and that some *B. cereus* strains are used as probiotics (**Cutting, 2011**).

CONCLUSION

The microbiota of Mantchoua was associated with the raw material and possibly the environment of production, utensils used and processing. The fermentation was alkaline indicating proteolysis and deamination processes by *Bacillus* spp. The predominant AMB in the Mantchoua production were *B. subtilis* subsp. *subtilis*, *B. cereus sensu lato* and *B. amyloliquefaciens* subsp. *plantarum*. Distribution of *Bacillus* spp. during the fermentation and in the final product seemed to be influenced by the *Bacillus* spp. occurring in the raw materials (seeds and ash lye solution) and especially the increase in pH during fermentation. The role of *B. cereus sensu lato*, although frequently present in alkaline fermented seeds condiments in Africa, was not elucidated and further research is required focusing on differences between strains of *B. cereus*. As reported in several studies, *B. subtilis* and *B. amyloliquefaciens* play an important role in seed fermentations and could be used as starter cultures for a controlled fermentation of kapok seeds. Additionally, including ash lye solution in all processing methods could be implemented in Mantchoua production to determine further the effect of high pH during fermentation on the amount of *B. cereus sensu lato* in the final products.

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