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Investigation of the diversity and safety of the predominant *Bacillus pumilus sensu lato* and other *Bacillus* species involved in the alkaline fermentation of cassava leaves for the production of Ntoba Mbodi

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Abstract

The objective of the study was to investigate the identity, diversity, and safety of the *Bacillus* population involved in the fermentation of cassava (*Manihot esculenta* Crantz) leaves for the production of Ntoba Mbodi, a Congolese food. Ninety bacteria were identified by phenotyping and genotyping using ITS-PCR, rep-PCR, and sequencing of the 16S rRNA, *gyrA, gyrB* and *rpoB* genes. Moreover, the isolates were screened for the presence of genes coding for haemolytic (*HblC*, *HblD*) and non-haemolytic enterotoxins (*NheA*, *NheB* and *NheC*), cytotoxin K (*CytK*) and emetic toxin (*EM1*) as well as their ability to produce haemolysin.

The investigations revealed the predominance (72.21 %) of species of the *Bacillus pumilus* group i.e. *B. safensis* (48), *B. pumilus* (7), and *B. pumilus sensu lato* (10). Other species of *Bacillus* including *B. cereus sensu lato* (11), *B. megaterium* (4), *B. subtilis* (4), *B. amyloliquefaciens* (2), *B. siamensis* (2), *B. licheniformis* (1) and *Lysinibacillus louembei* were also identified. Haemolytic, non-haemolytic and cytokin toxin genes were detected in the *B. cereus* strains which were also able to produce haemolysin. The emetic toxin gene was not detected in any isolates. The toxin genes screened were not detected in any of the non *B. cereus* species.

Key words: Cassava leaves (*Manihot esculenta* Crantz), fermentation, Ntoba Mbodi, *Bacillus*, identification, food safety

1. Introduction

Ntoba Mbodi is a popular fermented food in the Republic of the Congo where it constitutes a significant source of protein in the diet of the consumers. It is obtained by fermenting cassava leaves as follows: the leaves are harvested, allowed to wilt for 2-3 days, cleaned, cut into small pieces, washed with water, distributed into small portions and wrapped in large leaves such as those of the *Carica papaya* or *Cyrtosperma senegalense* plants, and allowed to ferment at ambient temperature for 2-4 days. It is essential to ferment cassava leaves, as the process eliminates or decreases significantly the presence of toxic components such cyanogenic compounds found in the raw material (Louembe et al., 2003; Kobawila, Louembé, Kéléké, Hounhouigan, & Gamba, 2005). Moreover, the fermentation process allows the release of nutritious elements such as essential amino and fatty acids as well as vitamins. During the process, a rise of pH to a value up to 10 is observed; thus the product is classified as an alkaline fermented food. In such types of product, the main microorganisms responsible for the fermentation are *Bacillus* species, such as *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. pumulis*, *B. sphaericus*, *B. cereus*, *B. xylanilyticus,* with *B. subtilis* commonly reported as the predominant species (Sanni, Ayermor, Sakyi-dawson, & Sefa-dedeh, 2000; Louembe et al., 2003; Azokpota, Hounhouigan, & Nago, 2006; Ouoba, Parkouda, Diawara, Scotti, & Varnam, 2000a; Mohamadou, Mbofung,& Thouvenot, 2009; Parkouda et al., 2009; Parkouda et al., 2010; Ahaotu et al., 2013). Bacteria belonging to the genus *Bacillus* are Gram positive, catalase positive endospore-forming, rod-shaped, aerobic and facultatively anaerobic. They are widely distributed in the environment and have been commonly isolated from acidic and

alcoholic, but mainly alkaline fermented foods, as well as various unprocessed and processed foods. An important characteristic of the genus is the ability of the isolates to sporulate and withstand adverse conditions. In alkaline fermented foods, secondary microorganisms such as lactic acid bacteria and *Staphylococcus* spp. have been also reported and play a lesser role than *Bacillus* species during the fermentation (Parkouda et al., 2009).

Unlike other alkaline fermented products such as Ugba, Natto, Soumbala, Maari, Bikalga, and Kinema that are obtained from seed based raw materials (Parkouda et al., 2009), Ntoba Mbodi is made of leaves and the production technology does not include a heating step. This may induce more differences in the microbial population as compared to the seed based alkaline fermented products. Similarly to most traditional fermented foods, the fermentation of cassava leaves is uncontrolled and this favours the occurrence of undesirable Gram-positive and Gram-negative pathogenic bacteria, leading to safety issues. Potential spore formers among pathogenic bacteria such as *B. cereus* are of great concern because of their capacity to survive different processing conditions, including heat treatment. The spores have high adhesion capability to various materials and may accumulate in the processing equipment, constituting thereby a serious hazard (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000). Also, uncontrolled fermentations induce variable nutritional and sensorial attributes. Thus, it is important to select well-defined starters and initiate controlled fermentations that will provide products with high nutritional quality and also good hygienic attributes and stability.

In the process of selecting starter cultures, identification of the microorganisms using well defined methods including both phenotypic and genotypic approaches is crucial (Holzapfel, 2002). It is also essential to use safe bacteria (e.g. absence of toxin production and antimicrobial resistance determinants) to protect the health of the consumers. Earlier studies (Louembé, Kobawila, Bouanga, & kéléké, 2003; Kobawila, Louembé, Kéléké, Hounhouigan, & Gamba, 2005) tried to characterize the microbial population of Ntoba Mbodi using only phenotypic methods, which are known to be of limited value when used for estimation of microbial diversity in an ecosystem (Sessitsch, Reiter, Pfeifer, & Wilhelm, 2002). Therefore, the aim of the study herein reported was to use both phenotypic and genotypic methods to screen the diversity and safety of the main microorganisms involved in the alkaline fermentation of cassava leaves. This constitutes an initial step in the selection process of multifunctional starter cultures for a controlled production Ntoba Mbodi.

2. Materials and methods

2.1. Identification of *Bacillus* isolates from Ntoba Mbodi

2.1.1. Enumeration, isolation and phenotypic characterization of the microorganisms

A total of 90 bacteria were isolated from unfermented cassava leaves, fermenting samples and Ntoba Mbodi collected at different markets and production places in two towns of the Republic of the Congo. The total aerobic mesophilic bacteria were enumerated on nutrient agar (NA; Oxoid CM0003, Basingstoke, UK) and characteristic *Bacillus* colonies isolated and purified. A sample (10 g) was aseptically transferred into a stomacher bag and homogenized in 90 ml sterile Maximum Recovery Diluent (MRD, Oxoid CM0733) for 2 min using a paddle-type blender (Colworth 400, AJ Seward,

London, UK). The suspension was serially diluted and each dilution spread on NA plates incubated at 37℃ for 24-48 h. After incubation, the bacteria were enumerated, and selected colonies streaked on NA and purified. The isolates were stored in nutrient broth (NB, Oxoid CM0001) containing 20 % (V/V) of glycerol and frozen (-20 ℃) until needed for further studies. For the phenotypic characterization, the isolates were streaked on NA and examined for colony and cell morphology, as well as tested for Gram, catalase, and oxidase reactions. Cell morphology was determined by light microscopy (Nikon Model Eclipse, E400, Japan) and the Gram reaction was evaluated using the KOH method (Gregersen, 1978).

2.1.2. Genotypic characterization and identification of the isolates

2.1.2.1. Extraction of DNA

Chromosomal DNA of a single colony of each isolate that had been grown on Tryptone Soya agar (TSA; Oxoid CM0131) at 37℃ for 48 h was extracted using InstaGene Matrix (Bio-Rad 732-6030, Hemel Hempstead, UK) according to the manufacturer's instructions. The extracts were stored at -20℃ until required.

2.1.2.2. Characterization of the isolates by 16S-23S rDNA ITS-PCR and rep-PCR

Amplification of the 16S-23S rDNA internal transcribed spacer (ITS) was carried out using methods previously described (Ouoba, Parkouda, Diawara, Scotti, & Varnam, 2000a; Anyogu, Awamaria, Sutherland, & Ouoba, 2014) and the primers depicted in Table 1. The PCR conditions were as follows: initial denaturation at 94℃ for 1 min followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min and elongation at 72℃ for 1 min. The PCR was ended with a final extension at 72℃ for 7 min and the amplified product cooled at 4℃. The characterization of the isolates by rep-

PCR was also done by applying previously described methodology (Ouoba, Parkouda, Diawara, Scotti, & Varnam, 2000a; Anyogu, Awamaria, Sutherland, & Ouoba, 2014) and using the GTG5 primer shown in Table 1. For the amplification, the following program was applied: initial denaturation at 94℃for 4 min followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 45℃ for 1 min and e longation at 65℃ for 8 min. The PCR ended with a final extension at 65℃ for 16 min, an d the amplified products cooled to 4℃. The DNA fragments generated from the ITS and r ep-PCRs were separated by applying 10 µl of each PCR product with 2 µl of loading buffer to 2 % (w/v) agarose gel (BioRad 4736). DNA molecular marker (Direct Load TM Wide Range DNA Marker; Sigma 7058) was used as a standard. The gel was run in Tris-Borate-EDTA buffer (1x TBE; Sigma T4415) for 1h 30 min at 120 V for ITS-PCR and 2 h 30 min at 140V for rep-PCR. Further, the gel was stained with ethidium bromide and photographed using a UV transilluminator. Bacteria showing the same ITS-PCR DNA profile were clustered in the same group and further differentiation by rep-PCR of the isolates of each ITS-PCR cluster was recorded. DNA profiles were grouped by visual screening and cluster analysis using the Bionumerics system (Dice's Coefficient of similarity, UPGMA; Applied Maths, Saint-Martens-Latem, Belgium).

2.1.2.3. Sequencing of the 16S rRNA, *gyrA*, *gyrB* and *rpoB* genes

The bacteria were first identified by the amplification and sequencing of the 16S rRNA gene as described by Ouoba, Parkouda, Diawara, Scotti, & Varnam (2008a). The primers used for the first amplification and sequencing are shown in Table 1. The following PCR conditions were used for the first amplification: initial denaturation at 95℃ for 5 min followed by 35 cycles at 94℃ for 1 min, 55℃ for 1 min and 72℃ for 1

min. The final extension was carried out at 72℃ fo r 5 min and the products cooled to 4℃. Positive PCR products were checked by electrop horesis, purified using the QIAquick PCR Purification kit (Qiagen, Crawley, UK) and sequenced. The PCR reaction was achieved by 35 PCR cycles with the following program: 95℃ for 2 min, then 35 cycles at 96℃ for 15 sec, 40℃ for 1 sec and 60℃ for 4 min.

In the case where closely related species could not be separated by 16S rRNA gene sequencing, sequencing of *gyrA*, *gyrB* and *rpoB* genes was carried out. For those genes, the same primers (Table1) were used for the first amplification and the sequencing. For the *gyrA* gene, the amplification was carried out by mixing 2 µl of each extracted DNA with a mixture containing 5 µl of 10 X PCR buffer containing 15 mM of MgCl2 (Applied Biosystems N8080160), 0.5 µl of dNTP (1.25 mM), 0.5 µl of each primer (21 pmol/µl, Table1), 0.2 µl of AmpliTaq polymerase (5 U; Applied Biosystems N808-0160, N808-0161) and 41.3 µl of sterile high purity water. The amplification was performed using the following conditions: Initial denaturation at 94℃ for 3 min, 35 cycles of 94℃ for 1 min, 50℃ for 1min, 72℃ for 1 min and a final extension step at 72℃ for 10 min. For the *gyrB* gene, the reaction mixture described by Thorsen et al. (2011a) was applied with the following PCR conditions: 94℃ for 2 min, then 30 cycles at 94℃ for 1 min, 66℃ for 1 min and 72℃ for 2 mi n. The final extension was carried out at 72℃ for 7 min and the product cooled to 4℃ . For the amplification of the *rpoB* gene, the method described by Anyogu, Awamaria, Sutherland, & Ouoba (2014) was used along with the following conditions: 94℃ for 2 min followed by 40 cycles of 94℃ for 30 s, 51℃ for 45 s, 68℃ for 50 s, and a final extension of 68℃ for 90 s. Electrophoresis was used to check the PCR products, and positive amplicons were

purified as described previously. For all genes, sequencing was carried out by electrophoresis on a 3730xl DNA Analyser-Titania (Applied Biosystems) and the isolates identified to genus and species level by analyzing the sequences in GenBank/EMBL/DDBJ Sequence database using the Basic Local Alignment Tool (BLAST) program (National Center for Biotechnology, MD, USA). Additionally, the 16S rDNA sequences were analysed using the EzTaxon server (Kim et al., 2012).

2.2. Investigation of the potential of the *Bacillus* isolates for production of enterotoxins, cytotoxin and emetic toxin

The isolates investigated included all *B. cereus* bacteria and representative isolates of each rep-PCR group for all other species (Table 2). An isolate of *B. cereus* from Soumbala (another alkaline fermented food) was used as a positive control (Ouoba, Thorsen, & Varnam, 2008b).

2.2.1. Haemolysis on blood agar

The *Bacillus* isolates were screened for their haemolytic activity on blood agar as follows: Columbia agar base (Oxoid CM003) was autoclaved at 121 ℃ for 15 min and horse blood (5%; Oxoid SR0050) added after cooling to 50 ℃, before distribution into Petri dishes. The *Bacillus* isolates were streaked on the agar and incubated at 37 ℃ for 48 h. Haemolysis was recorded by appearance of a zone of clearing around the colonies (Beta or complete haemolysis) or a dark and greenish coloration under the colonies (Alpha or partial haemolysis).

2.2.2. Detection of toxin genes

196 Chromosomal DNA of each isolate was extracted as described previously. All isolates
197 were tested for the presence of Hbl (*HblC*, *HblD*), Nhe (*NheA*, *NheB*, *NheC*), *CytK* and
198 *EM1* genes encoding respectively the production of haemolysin BL, non-haemolytic
199 enterotoxin complex, cytotoxin K and cereulide, using the method described by (Ouoba,
200 Thorsen, & Varnam (2008b). The primers used are depicted in Table 1. The cycling
201 program for all genes except for the EM1 gene was: initial denaturation at 94℃ for 2
202 min followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at the
203 corresponding temperature (Table 1) for 1 min and elongation at 72℃ for 2 min. The
204 PCR ended with a final extension at 72℃ for 5 min and the amplified products cooled to
205 4℃. The DNA fragments were separated by electrophoresis as described previously
206 and the gels were run at 120 V for 1 h. Gels were stained with ethidium bromide
207 solution and photographed using a UV transilluminator. For the detection of the emetic
208 specific gene fragment EM1, the following cycling program was applied: initial
209 denaturation at 95 ℃ for 15 min followed by 30 cycles of denaturation at 95 ℃ for 30 s,
210 annealing at 60 ℃ for 30 s and elongation at 72 ℃ for 1 min. The PCR ended with a
211 final extension at 72 ℃ for 5 min and the amplified products cooled to 4 ℃. The DNA
212 fragments were separated as described earlier.

213 2.2.3. Production of haemolytic enterotoxin by the *Bacillus* isolates

214 Production of haemolysin BL by the isolates was screened using the *B. cereus*
215 enterotoxin reversed passive latex agglutination (BCET-RPLA) toxin detection kit (Oxoid
216 D0950A). The BCET-RPLA kit detects, in particular, the L2 component (encoded by
217 *HblC*) of the Hbl enterotoxin complex (Beecher, schoeni, & Wong, 1995). All selected
218 isolates were included in the experiment, irrespective of the results obtained from the

screening of the presence of the *Hbl C* gene. This was to confirm the absence of the gene in those isolates where positive amplicons were not observed. The bacteria were screened for their ability to produce haemolysin in broth as follows: each isolate was grown overnight in 10 ml of Brain Heart Infusion (BHI; Oxoid CM225) and 1ml of the culture centrifuged at 5000 g for 5 min. The BCET-RPLA kit was used according to manufacturer's instructions to detect the presence of enterotoxin in the supernatant.

3. Results

During the fermentation, the total mesophile population increased from 10^6-10^7 to 10^{10}-10^{12} CFU/g) after 72 h. This was associated with an increase of pH from 5-6 to 8-10 during the same time. Phenotypically, the bacteria exhibited various macroscopic and microscopic features. As expected for *Bacillus* isolates, all bacteria were Gram and catalase positive, rod-shaped and endospore formers. For most isolates, the position of the spore was subterminal or terminal. However, a few isolates exhibited a centrally positioned spore. Depending on the isolate, the cells were arranged as single, pairs and chains. Most were motile, with however a reduced motility for cells that were in chains.

The identification experiments revealed that all bacteria belonged to the genus *Bacillus* except isolate NM73, which showed an affiliation with the genus *Lysinibacillus*. The latter genus has strong similarities with the genus *Bacillus*. Various species were identified (Table 2), principally those of the *B. pumilus* group (72.21 %) including *B. safensis* (53.33 %), *B. pumilus* (7.77 %), *B. altitudinis* and *B. pumilus sensu lato* (*B. pumilus* group of isolates that were not clearly differentiated, 11.11 %). Other species included *B. cereus sensu lato* (12.22 %), *B. megaterium* (4.44 %), *B. amyloliquefaciens*

(2.22 %), *B. siamensis* (2.22 %), *B. subtilis* (4.44 %), *B. licheniformis* (1.11%) and *Lysinibacillus louembei* (1.11%).

Characterization of the bacteria by ITS-PCR generated seven groups of isolates that were further subdivided into smaller clusters by rep-PCR. As seen in Table 2 and Fig.1., 8, 4, 3, 2 and 4 rep-PCR subgroups were obtained from ITS-PCR groups 1, 2, 3, 4 and 5 respectively. ITS-PCR group 1 included all species of the *B. pumilus* group; group 2, *B. cereus sensu lato*; group 3, *B. subtilis* and *B. siamensis*; group 4, *B. amyloliquefaciens*; group 5, *B. megaterium*; group 6, *B. licheniformis* and group 7, *Lysinibacillus louembei*. With the rep-PCR, all species from the *B. pumilus* group, as well as the *B. subtilis* and *B. siamensis* (formerly belonging to the species of *B. amyloliquefaciens),* were clearly differentiated (Table 2, Fig.1.). Furthermore, some of the species included different rep-PCR DNA profiles. *Bacillus megaterium* and *B. cereus sensu lato* exhibited four different rep-PCR profiles each, *Bacillus safensis*, *B. pumilus* and *B. subtilis* three patterns each, and *B. pumilus* sensu lato two patterns each (Table 2, Fig.1.).

Using 16S rDNA sequencing and EZtaxon analysis, all the *B. safensis* isolates were clearly identified. Furthermore, their identification was confirmed by sequencing of the *gyrB* or *gyrA* genes. For all other species, sequencing of the *gyrB*, or *gyrA* or *rpoB* genes was necessary to differentiate them from closely related species. The exception was with the isolates of *B. cereus sensu lato* that could not be differentiated. All genes sequencing identified the isolates as *B. cereus* or *B. anthracis* or *B. thuringiensis* equally, thus the isolates were referred to as *B. cereus sensu lato*. Also, 10 isolates of

the *B. pumilus* group could not be clearly identified irrespective of the gene sequenced and were referred to as *B. pumilus sensu lato*.

With regard to the safety of the isolates, it was noticed that most isolates (96.67 %) showed haemolytic activity on blood agar and the presence of toxin genes varied according to the isolate and the gene screened (Table 3). As expected, none of the genes investigated were detected in the non *B. cereus* species. The EM1 gene encoding the production of cereulide was not detected in any of the isolates screened. The three genes (*NheA*, *NheB* and *NheC*) encoding the production of the non-haemolytic complex enterotoxin were detected in all *B. cereus* screened, while 91.0 % of the isolates of that species showed the *HblC* gene, 72.70 % the *HblD* gene, 81.8 % both the *HblC* and *HblD* genes and 72.7 % the *CytK* gene. A portion of 63.3 % of the isolates showed all *NheA*, *NheB*, *NheC*, *HblC*, *HblD* and *CytK* genes. No *Hbl* genes screened for were detected in *B. cereus* NM 48. Out of the 11 *B. cereus* isolates from Ntoba Mbodi studied for toxin production, eight tested positive for the production of the L2 component of the haemolysin BL complex encoded by *HblC* gene (Table 3). Among the three isolates that did not produce the toxin, one (NM48) did not exhibit the *HblC* gene and the two other (NM78 and NM82) showed a weak amplification of the *HblC* gene. None of the non *B. cereus* species produced the L2 component of the haemolysin BL complex.

4. Discussion

Investigations into the microbial populations associated with the alkaline fermentation of cassava leaves for Ntoba Mbodi production revealed that different species and subspecies of bacteria are responsible for the fermentation. Surprisingly, species of the

286 *B. pumilus* group, mainly *B. safensis,* were dominant in most investigated samples. This
287 is unusual, as dominance of *B. subtilis* in alkaline fermented vegetables whether from
288 African or Asian origin is usually reported (Isu & Ofuya, 2000; Ouoba, Diawara, Amoa-
289 Awua, Traoré, & Lange Moller, 2004; Azokpota et al., 2006; Ouoba, Parkouda, Diawara,
290 Scotti, & Varnam, 2000a; Mohamadou, Mbofung, & Thouvenot, 2009; Parkouda et al.,
291 2009**).** The *B. pumilus* group of isolates includes *B. pumilus*, *B. safensis*, *B. altitudinis*,
292 *B. stratosphericus*, *B. aerophilus*, *B. xiamenensis* and *B. invictae*, which have close
293 phenotypic and genotypic features (Satomi, La Duc, & Venkateswaran, 2006; Liu et al.,
294 2013; Branquinho, Meirinhos-Soares, Carriço, Pintado, & Peixe, 2014a; Lai, Liu, &
295 Shao, 2014). They have been detected in numerous terrestrial and marine
296 environments as well as in the air at high altitudes (Satomi, La Duc, & Venkateswaran,
297 2006; Shivaji et al., 2006; Liu et al., 2013; Lai, Liu, & Shao, 2014).
298 The unexpected predominance of *B. safensis* in Ntoba Mbodi may be explained by the
299 absence of a heating step during the production and a raw material constituted of
300 leaves. In contrast with Ntoba Mbodi, most alkaline fermented food raw materials are
301 seeds that undergo a long cooking time before fermentation (Parkouda et al., 2009).
302 The origin of the bacteria found in the raw materials and carried through the
303 fermentation is mainly from the environment. A study by Liu et al. (2013) reported that
304 *B. safensis* is the dominant species of the *B. pumilus* group found in terrestrial
305 environments, whereas *B. altitudinis* is more widespread in marine settings. Thus, the
306 fact that *B. safensis* is the major species of the *B. pumilus* group in Ntoba Mbodi is not
307 surprising. The predominance of species of the *B. pumilus* group constitutes a safety
308 advantage, because of their ability to use cyanogenic compounds for their nutrition

(Meyers, Gokool, Rawlings, & Woods, 1991; Mekuto, Jackson, & Ntwampe, 2014). Cassava leaves contain cyanogenic compounds and the isolates probably contribute to reducing the toxicity of the leaves by decreasing the concentration of the toxic compounds. Louembé, Kobawila, Bouanga, & kéléké (2003) and Kobawila, Louembé, Kéléké, Hounhouigan, & Gamba (2005) reported a decrease of at least 70 % of the content of toxic compounds such as cyanide, cyanohydrin, and linamarine (cyanogenic glucoside) during the production of Ntoba Mbodi. Also, Lateef, Adelere, & Gueguim-Kana (2015) reported that *B. safensis* has promising biotechnological applications, especially in the production of enzymes (e.g. protease, amylase, lipase, inulase) and secondary metabolites. Additionally, some isolates of *B. pumilus* possess probiotic properties for humans and animals and the ability to eliminate plant insects (Hong, Duc, & Cutting, 2005; Molina, Cana-Roca, Osuna, & Vilchez, 2010; Perez-Garcia, Romero, & de Vicente, 2011).

Louembé, Kobawila, Bouanga, & kéléké (2003) and Kobawila, Louembé, Kéléké, Hounhouigan, & Gamba (2005) studied the microbiology of Ntoba Mbodi and reported the presence of *B. subtilis*, *B. amyloliquefaciens*, *B. megaterium*, *B. macerans*, *B. cereus*, *B. polymixa*, *B. brevis* and *B. pumilus* in the product. However, neither the presence of *B. safensis*, *B. siamensis* and *Lysinibacillus* spp, nor the predominance of *B. safensis* was described. The difference observed with our study may be related to the methodology used to investigate the isolates. The latter authors used phenotypic characteristics only to identify the bacteria tentatively, whereas in the current study, both phenotypic and genotypic methods were used for the first time to screen the microflora of Ntoba Mbodi. The exclusive use of phenotyping techniques often significantly

underestimates bacterial diversity in a particular ecosystem (Sessitsch, Reiter, Pfeifer, & Wilhelm, 2002). In the current research, a combination of genotypic methods that have been shown to be efficient in the identification of *Bacillus* species in other alkaline fermented foods (Thorsen et al., 2011a; Ahaotu et al., 2013; Anyogu, Awamaria, Sutherland, & Ouoba, 2014; Compaore et al., 2013) was used to provide an advanced insight of the diversity of the *Bacillus* population of Ntoba Mbodi at species and subspecies level. *Bacillus safensis*, *B. pumilus*, *B. subtilis*, *B. amyloliquefaciens*, *B. megaterium*, *B. cereus* and *Lysinibacillus* species have been demonstrated in other alkaline fermented products such as Soumbala, Mbuja/Bikalga, Maari, Ugba, Natto, Kinema and Thua-nao (Sanni, Ayermor, Sakyi-dawson, & Sefa-dedeh, 2000; Mohamadou, Mbofung,& Thouvenot, 2009; Parkouda et al., 2009; Parkouda et al., 2010; Ahaotu et al., 2013; Compaoré et al., 2013). The microbiological similarities of these types of foods are not surprising as they share some biochemical features, such as the high content of proteins (up to 40 %) whose degradation during the fermentation leads to the increase in pH. This constitutes a selection factor for particular microorganisms capable of withstanding alkaline conditions.

Except for *B. safensis,* which was identified by 16S rDNA sequencing/EZtaxon search, the sequencing of housekeeping genes *gyrB*, gyrA and *rpoB* was necessary to identify most isolates, as reported by earlier studies on *Bacillus* identification (Chun & Bae, 2000; La Duc, Satomi, Agata, & Venkateswaran, 2004; Wang, Lee, Tai, & Kasai, 2007; Thorsen et al., 2011a; Ahaotu et al., 2013; Liu et al., 2013; Anyogu, Awamaria, Sutherland, & Ouoba, 2014). For the isolates of *B. pumilus sensu lato* that could not be clearly identified, the use of other types of primers for the genes screened for may

assist the identification. Also, the new methodologies described by Branquinho et al. (2014b) for differentiating species of the *B. pumilus* group may be useful. For the *B. cereus sensu lato*, the differentiation has been often difficult and their real difference at species level is becoming more and more questionable. Isolate NM73 initially showed 98 % of 16S rDNA sequence similarity with *Lysinibacillus meyeri,* but differed by 14 base pairs, which is indicative of a potential new species of bacterium. Specific studies were performed to characterise the isolate as a new species and named it as *Lysinibacillus louembei* (Ouoba et al., 2015).

Ntoba Mbodi is produced using exclusively traditional methodologies and uncontrolled fermentation. Thus, the presence of potentially pathogenic bacteria such *B. cereus* is possible. Due to their ability to produce toxins that cause foodborne illnesses, the presence of *B. cereus* in food is of considerable concern for human health. Toxins produced by *B. cereus* include cereulide, cytotoxin (CytK), non-haemolytic enterotoxin (Nhe) and haemolysin BL (Hbl) (Agata et al., 1994; Granum & Lund, 1997; Stenfors, Fagerlund, & Granum, 2008). Such isolates can cause both food infections and intoxications resulting in e.g. vomiting and serious case of diarrhoea. All *B. cereus* investigated exhibited several toxin genes with isolates NM 54, NM59, NM79, NM80, NM81 and NM83 being the most potentially virulent and possible causes of foodborne disease as they exhibited all three Nhe genes (*NheA*, *NheB*, *NheC*) and Hbl genes (*HblC*, *HblD*) as well as the *CytK* gene (Guinebretiere, Broussolle, & Nguyen-The, 2002). The presence of Hbl genes and especially the *HblC* gene was confirmed by the production of haemolysin. There were no correlations between haemolytic activity on blood agar and presence of the Hbl genes and production of the haemolytic enterotoxin.

In fact, most non *B. cereus* isolates and one *B. cereus* strain showed haemolysis on blood agar but did not exhibit the Hbl genes and did not produce the haemolysin toxin. As reported by Lindback, Fagerlund, Rodland, & Granum (2004) and Ouoba, Thorsen, & Varnam (2008b), the presence all three Nhe genes is indicative of a potential ability of the bacteria to produce non-haemolytic enterotoxin. The absence of the *EM1* gene, encoding heat stable emetic toxin (cereulide) production, was also reported in previous studies on Soumbala, Bikalga, Gergoush and Ugba (Ouoba, Thorsen, & Varnam, 2008b; Thorsen et al., 2011a; Ahaotu et al., 2013). The production of cereulide by *B. cereus* isolates from alkaline fermented foods as reported by Thorsen et al. (2011b) for isolates from Afitin seems to be rather rare. Ntoba Mbobi is cooked before consumption, and most heat labile enterotoxins (Nhe and Hbl toxins) are likely to be destroyed before consumption if the product is well cooked. However, heat stable toxins such as cytotoxins (From, Pukall, Schumann, Hormazabal, & Granum, 2005) will not be destroyed. Furthermore, since *B. cereus* is a spore-former, spores ingested through the food may germinate in the large intestine and cause severe infections (Granum & Lund, 1997; From, Hormazabal, & Granum, 2007). Nevertheless, there is no official report of foodborne illnesses resulting from the consumption of alkaline traditional fermented foods such as Ntoba Mbodi. This may be related to non-availability of data and lack of statistics. None of the isolates belonging to the other species studied exhibited the toxin genes screened for, or were able to produce haemolysin. In general, non *B. cereus Bacillus* isolates are considered safe with regards to toxin production (Hosoi et al, 2003; Sanders, Morelli, & Tompkins, 2003), although specific strains of a few species such as *B. subtlis*, *B. licheniformis*, *B. pumilus* and *B. fusiformis* have been reported to contain

401 toxin genes and capable of producing cytotoxins, enterotoxins or ring-formed emetic
402 toxins (Salkinoja-Salonen et al.,1999; Rowan, Caldow, Gemmel, & Hunter, 2003; From,
403 Pukall, Schumann, Hormazabal, & Granum, 2005; From, Hormazabal, & Granum,
404 2007).

405 The research herein reported provides an extended understanding of the *Bacillus*
406 population of Ntoba Mbodi. This constitutes a new investigation into the microbial
407 dynamic of Ntoba Mbodi and is of great importance, as diseases originating from food
408 are a worrying and growing public health problem, whether in developed or developing
409 countries. The results of the study are important for the selection of potential
410 multifunctional starter cultures for controlled production of Ntoba Mbodi to deliver a
411 product with improved nutritional and hygienic quality. Further studies will address the
412 technological and probiotic properties of the bacteria as well another safety issues
413 related to transferable antimicrobial resistance determinants. It is advised that the
414 selection and use of multifunctional starter cultures is supported by training of the
415 producers in good hygienic and manufacturing practices to maximize positive food
416 safety outcomes.

417

References

Ahaotu, A., Anyogu, O.H., Njoku, N.N., Odu, J.P., Sutherland, & Ouoba, L.I.I. (2013). Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of Ugba. *International Journal of Food Microbiology,* 162, 95-104.

Anyogu, A., Awamaria, B., Sutherland, J.P. & Ouoba, L.I.I. (2014). Molecular characterisation and antimicrobial activity of bacteria associated with submerged lactic acid cassava fermentation. *Food Control*, 39, 119-127.

Azokpota, P., Hounhouigan, D.P, & Nago, M.C. 2006. Microbiological and chemical changes during the fermentation of African locust bean (*Parkia biglobosa*) to produce afitin, iru and sonru, three traditional condiments produced in Benin. *International Journal of Food Microbiology*, 107, 304-309.

Beecher, D.J., Schoeni, J.L, & Wong, A.C. (1995). Enterotoxic activity of hemolysin BL from *Bacillus cereus*. *Infection and Immunity,* 63, 4423-4428.

Branquinho, R., Meirinhos-Soares, L., Carriço, J., Pintado, M., & Peixe, L. (2014a). Phylogenetic and clonality analysis of *Bacillus pumilus* isolates uncovered a highly heterogeneous population of different closely related species and clones. *FEMS Microbiology Ecology*, 90, 689-698.

Branquinho, R., Sousa, C., Lopes, J., Pintado, M., Peixe, L., & Osório, H. (2014b). Differentiation of *Bacillus pumilus* and *Bacillus safensis* Using MALDI-TOF-MS. *Plos One*, 9, e110127.

Chun, J., & Bae, K.S. 2000. Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial gyrA gene sequences. *Antonie Van Leeuwenhoek,* 78, 123-127.

Compaoré, C., Nielsen, D., Ouoba, L., Berner, T., Nielsen, K., & Sawadogo-Lingani, H. et al. (2013). Co-production of surfactin and a novel bacteriocin by *Bacillus subtilis subsp. subtilis* H4 isolated from Bikalga, an African alkaline *Hibiscus sabdariffa* seed fermented condiment. *International Journal of Food Microbiology*, 162, 297-307.

Ehling-Schulz, M., Fricker, M. & Scherer, S. (2004). Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. *FEMS Microbiology Letters*, 232,189-195.

From, C., Pukall, R., Schumann, P., Hormazabal, V. & Granum, P.E. (2005). Toxin-producing ability among *Bacillus* spp. Outside the *Bacillus cereus* group. *Applied and environmental Microbiology* ,71,1178-1183.

From, C., Hormazabal, V. & Granum, P.E. (2007). Food poisoning associated with pumilacidin-producing *Bacillus pumilus* in rice. *International Journal of Food Microbiology*, 115, 319-324.

Gregersen, T. (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. *European Journal of Applied Microbiology and Biotechnology*, 5, 123-127.

Granum, P.E. & Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters*, 157, 223-228.

462 Guinebretiere, M., Broussolle, H.V, & Nguyen-The, C. (2002). Enterotoxigenic profiles of
463 food-poisoning and food-borne *Bacillus cereus* strains. *Journal of Clinical*
464 *Microbiology,* 40, 3053-3056.

465 Hansen, B.M. & Hendriksen, N.B. (2001). Detection ofenterotoxic *Bacillus cereus* and
466 *Bacillus thuringiensis* strains by PCR analysis. *Applied and Environmental*
467 *Microbiology*, 67, 185-189.

468 Holzapfel, W.H. (2002). Appropriate starter culture technologies for small-scale
469 fermentation in developing countries. *International Journal of Food Microbiology*,
470 75, 121-197.

471 Hong, H. A., Duc, L. H., & Cutting, S.M. (2005) The use of bacterial spore formers as
472 probiotics. *FEMS Microbiology Review*s, 29, 813-835.

473 Hosoi, T., Hirose, R., Saegusa, S., Ametani, A., Kiuchi, K., & Kaminogawa, S.(2003).
474 Cytokine responses of human intestinal epithelial-like Caco-2 cells to the
475 nonpathogenic bacterium *Bacillus subtilis* (natto). *International Journal of Food*
476 *Microbiology*, 82, 255-264.

477 Isu, N.R, & Ofuva, C.O. (2000). Improvement of the traditional processing and
478 fermentation of African oil bean (*Pentaclethra macrophylla* Bentham) into a food
479 smack 'ugba'. *International Journal of Food Microbiology,* 59, 235-239.

480 Kim, O., Cho, Y., Lee, K., Yoon, S., Kim, M., & Na, H. et al. (2012). Introducing
481 EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes
482 that represent uncultured species. *International Journal of Systematic and*
483 *Evolutionary Microbiology*, 62, 716-721.

Kobawila, S.C., Louembé, D., Kéléké, S., Hounhouigan, J., & Gamba, C. (2005). Reduction of the cyanide content during fermentation of cassava roots and leaves to produce Bikedi and Ntoba Mbodi, two food products from Congo. *African Journal of Biotechnology,* 4, 689-696.

La Duc, M.T., Satomi, M., Agata, N., & Venkateswaran, K. (2004). GyrB as a phylogenetic discriminator for members of the *Bacillus* anthracis-cereus-thuringiensis group. *Journal of Microbiology Methods,* 56, 383-394.

Lai, Q., Liu, Y., & Shao, Z. (2014). *Bacillus xiamenensis* sp. nov., isolated from intestinal tract contents of a flathead mullet (Mugil cephalus). *Antonie Van Leeuwenhoek*, 105, 99 -107.

Lateef, A., Adelere, I.A., & Gueguim-Kana, E.B. (2015). The biology and potential biotechnological applications of *Bacillus safensis. Biologia,* 70, 411-419.

Lindback, T., Fagerlund, A., Rodland, M.S., & Granum, P.E. (2004). Characterization of the Bacillus cereus Nhe enterotoxin. *Microbiology*, 150, 3959-3967.

Liu, Y., Lai, Q., Dong, C., Sun, F., Wang, L., Li, G., & Shao, Z. (2013). Phylogenetic Diversity of the Bacillus pumilus Group and the Marine Ecotype Revealed by Multilocus Sequence Analysis. Plos ONE, 8, e80097.

Louembé, D., Kobawila, S.C., Bouanga, G., & Kéléké, S. (2003). Etude microbiologique des feuilles fermentées de manioc: Ntoba mbodi. *Tropicultura*, 21, 106-111.

Mekuto, L., Jackson, V., & Ntwampe, S.K.O., (2014). Biodegradation of free cyanide using *Bacillus* Sp. consortium dominated by *Bacillus safensis*, *lichenformis* and

tequilensis strains: A bioprocess supported solely with whey. *Journal of Bioremediation and Biodegradation*, S18:004.

Meyers, P. R., Gokool, P., Rawlings, D. E., & Woods, D. R. (1991). An efficient cyanide-degrading *Bacillus pumilus* strain.]*ournal of General Microbiology*, 137, 1397-1400.

Mohamadou, B.A., Mbofung, C.M.F., & Thouvenot, D. (2009). Microbiological and organoleptic profiles of Mbuja: A traditional condiment produced by fermented *Hibiscus sabdariffa* seeds in Cameroun. *Journal of Food Technology*, 7, 84-91.

Molina, C., Cana-Roca, J., Osuna, A., & Vilchez, S. (2010). Selection of a *Bacillus pumilus* Strain Highly Active against Ceratitis capitata (Wiedemann) Larvae. *Applied and Environmental Microbiology*, 76, 1320-1327.

Nicholson, W.J., Munakata, N., Horneck, G., Melosh, H.J., & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology* Reviews, 64, 548-572.

Ouoba, L.I.I., Diawara, B., Amoa-Awua, W.K., Traoré, A.S, & Lange Moller, P. (2004). Genotyping of starter cultures of *Bacillus subtilis* and *Bacillus pumilus* for fermentation of African locust bean (*Parkia biglobosa*) to produce *Soumbala*. *International Journal of Food Microbiology,* 90,197-205.

Ouoba, L.I.I., Parkouda, C., Diawara, B., Scotti, C., & Varnam, A.H. (2008a). Identification of *Bacillus* spp from Bikalga, fermented seeds of *Hibiscus sabdariffa*: phenotypic and genotypic characterization. *Journal of Applied Microbiology*, 104, 122-131.

527 Ouoba, L.I.I., Thorsen, L., & Varnam, A.H. (2008b). Enterotoxins and emetic toxins
528 production by *Bacillus cereus* and other species of *Bacillus* isolated from
529 Soumbala and Bikalga, African alkaline fermented food condiments.
530 *International Journal of Food Microbiology,* 124, 224-230.

531 Ouoba, L.I.I., Vouidibio Mbozo, A.B., Thorsen, L., Anyogu, A., Nielsen, D.S., Kobawila,
532 S.C. & Sutherland, J.P. (2015). *Lysinibacillus louembei* sp. nov., a novel spore-
533 forming bacterium isolated from Ntoba Mbodi, alkaline fermented leaves of
534 cassava from The Republic of The Congo. *International Journal of Systematic*
535 *and Evolutionary Microbiology*, 65, 4256-4262

536 Parkouda, C., Nielsen, D.S., Azokpota, P., Ouoba, L.I.I., Amoa-Awua, W.K., Thorsen,
537 L., et al. (2009). The microbiology of alkaline-fermentation of indigenous seeds
538 used as food condiments in Africa and Asia. *Critical Review in Microbiology,*
539 35,139-156.

540 Parkouda, C., Thorsen, L., Compaoré, C., Nielsen, D., Tano-Debrah, K., & Jensen, J. et
541 al. (2010). Microorganisms associated with Maari, a Baobab seed fermented
542 product. *International Journal of Food Microbiology*, 142, 292-301.

543 Perez-Garcia, A., Romero, D., & de Vicente, A. (2011). Plant protection and growth
544 stimulation by microorganisms: biotechnological applications of Bacilli in
545 agriculture. *Current Opinion in Biotechnology*, 22, 187-193.

546 Rowan, N.J., Caldow, G., Gemmel, C.G., & Hunter, I.S. (2003). Production of diarrheal
547 enterotoxins and other potential virulence factors by veterinary isolates of

548 *Bacillus* species associated with non-gastrointestinal infections. *Applied and*
549 *Environmental Microbiology*. 69, 2372-2376

550 Salkinoja-Salonen, M.S., Vuorio, R., Andersson, M.A., Kamfer, P., Andersson, M.C.,
551 Honkanen-Buzalski,T., & Scoging, A.C. (1999).Toxigenicstrains of *Bacillus*
552 *licheniformis* related to food poisoning. *Applied and Environmental Microbiology*,
553 65, 4637- 4645.

554 Sanders, M.E., Morelli, L., & Tompkins, T.A. (2003). Sporeformers as human probiotics:
555 *Bacillus, Sporolactobacillus*, and *Brevibacillus*. *Comprehensive Review in Food*
556 *Science and Food Safety*, 2,101-110.

557 Sanni, A.I., Ayermor, G.S., Sakyi-dawson, E., & Sefa-dedeh, S. 2000. Aerobic spore-
558 forming bacteria and chemical composition of some Nigerian fermented soup
559 condiments. *Plant Foods for Human Nutrition,* 55, 111-118.

560 Satomi, M., La Duc, M.T., & Venkateswaran, K. (2006). *Bacillus safensis* sp. nov.,
561 isolated from spacecraft and assembly-facility surfaces. *International Journal of*
562 *Systematic and Evolutionary Microbiology*, 56, 1735- 1740.

563 Sessitsch, A., Reiter, B., Pfeifer, U., & Wilhelm, E. (2002). Cultivation-independent
564 population analysis of bacterial endophytes in three potato varieties based on
565 eubacterial and Actinomycetes-specific PCR of 16S rRNA genes. *FEMS*
566 *Microbiology Ecology, 39, 23-32.*

567 Shivaji, S., Chaturvedi, P., Suresh, K., Reddy, G.S., Dutt, C.B., Wainwright, M., Narlikar,
568 J.V., & Bhargava, P.M. (2006). *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp.
569 nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated

570 from cryogenic tubes used for collecting air samples from high altitudes.
571 *International Journal of Systematic and Evolutionary Microbiology*, 56, 1465-
572 1473.

573 Stenfors, A.L.P., Fagerlund, A., & Granum, P.E. (2008). From soil to gut: *Bacillus*
574 *cereus* and its food poisoning toxins. *FEMS Microbiology Reviews*, 32, 579-606.

575 Thorsen, L., Abdelgadir, W.S., Ronsbo, M.H., Abban, S., Hamad, S.H., Nielsen, D.S., &
576 Jakobsen, M. (2011) a. Identification and safety evaluation of Bacillus species
577 occurring in high numbers during spontenous fermentation to produce
578 Gergoush, a traditional Sudanese bread snack. *International Journal of Food*
579 *Microbiology*, 146, 244-252.

580 Thorsen, L., Azokpota, P., Munk Hansen, B., Hvillum Rønsbo, M., Fog Nielsen, K.,
581 Hounhouigan, D.J., & Jakobsen, M. (2011b). Formation of cereulide and
582 enterotoxins by *Bacillus cereus* in fermented African locust beans. *Food*
583 *Microbiology*, 28, 1441-1447.

584 Wang, L.T., Lee, F.L., Tai, C.J., & Kasai, H. (2007). Comparison of gyrB gene
585 sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the
586 *Bacillus subtilis* group. *International Journal of Systematic and Evolutionary*
587 *Microbiology*, 57, 1846-1850.

588 Yamamoto, S., & Harayama, S. (1995). PCR amplification and direct sequencing of
589 *gyrB* genes with universal primers and their application to the detection and
590 taxonomic analysis of *Pseudomonas putida* strains. *Applied and Environmental*
591 *Microbiology,* 61, 1104-1109.

592 **Figure caption**

593 Fig.1: Cluster analysis (Bionumerics: Dice's Coefficient of similarity, UPGMA) of the

594 different rep-PCR fingerprints of the *Bacillus* species isolated from Ntoba Mbodi.

595

596

597 Table **1:** Primers used in the current study

Primers	Sequence (5'-3')	Gene/Region	Annealing Temperature(℃)
S-D-Bact-1494-a-S-20-F	GTCGTAACAAGGTAGCCGTA	16-23S rRNA[a]	55
L-D-Bact-0035-a-A-15-R	CAAGGCATCCACCGT		
GTG_5	GTGGTGGTGGT GTG		45
pA-F	AGAGTTTGATCCTGGCT	16S rRNA[a]	55
pE-R	CCGTCAATTCCTTTGAGTTT		
pD	GTATTACCGCGGCTGCTG		40
rpoB-F	AGGTCAACTAGTTCAGTATGGAC	*rpoB*[b]	51
rpoB-R	AAGAACCGTAACCGGCAACTT		
UP1-F	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAA RTTYGA	*gyrB*[c]	66
UP2-R	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGC RTCNGTCAT		
gyrA-F	GAYTATGCWATGTCAGTTATTGT	gyrA	50
gyrA.R	GGAATRTTRGAYGTCATACCAAC		
HC F	GATAC(T,C)AATGTGGCAACTGC	*HblC*[d]	58
HC R	TTGAGACTGCTCG(T,C)TAGTTG		
HD F	ACCGGTAACACTATTCATGC	*HblD*[d]	58
HD R	GAGTCCATATGCTTAGATGC		
NA F	GTTAGGATCACAATCACCGC	*NheA*[d]	56
NA R	ACGAATGTAATTTGAGTCGC		
NB F	TTTAGTAGTGGATCTGTACGC	*NheB*[d]	54
NB R	TTAATGTTCGTTAATCCTGC		
NC F	TGGATTCCAAGATGTAACG	*NheC*[d]	54
NC R	ATTACGACTTCTGCTTGTGC		
CytK F	ACAGATATCGG(G,T)CAAAATGC	*cytK*[d]	54
CytK R	TCCAACCCAGTTWSCAGTTCD		
EM1 F	GACAAGAGAAATTTCTACGAGCAAGTACAAT	Unknown[f]	60
EM1 R	GCAGCCTTCCAATTACTCCCTTCTGCCACAGT		

598

599 [a] Ouoba, Parkouda, Diawara, Scotti, & Varnam (2008a); [b]Yamomoto and Harayama

600 (1995); [c] Thorsen et al (2011a) and Yamamoto & Harayama (1995); [d]Hansen &

601 Hendriksen (2001); [f]Gene of unknown function, Ehling-Schulz, Fricker, & Scherer

602 (2004).

603

604 **Table 2:** Origin and identity of the *Bacillus* isolates from Ntoba Mbodi

Samples	Origin/Fermen-tation time	Isolates	Groups ITS-PCR	Groups Rep-PCR	Identification 16S RNA/GyrB/GyrA/RpoB gene sequencing
A0	Site1/0h	NM1	1	1.2	*B. safensis*
		NM3	1	1.3	*B. safensis*
		NM4	1	1.2	*B. safensis*
		NM5	1	1.1	*B. safensis*
		NM6	1	1.1	*B. safensis*
		NM7	3	3.1	*B. subtilis*
A1	Site1/24h	NM8	1	1.2	*B. safensis*
		NM9	1	1.1	*B. safensis*
		NM10	1	1.1	*B. safensis*
		NM11	1	1.1	*B. safensis*
		NM12	1	1.2	*B. safensis*
		NM13	1	1.2	*B. safensis*
		NM14	1	1.1	*B. safensis*
		NM15	1	1.1	*B. safensis*
A2	Site1/48h	NM16	1	1.8	*B. pumilus sensu lato*
		NM17	1	1.8	*B. pumilus sensu lato*
		NM18	1	1.2	*B. safensis*
		NM19	1	1.2	*B. safensis*
		NM20	1	1.2	*B. safensis*
A3	Site1/72h	NM21	1	1.4	*B. pumilus*
		NM22	1	1.1	*B. safensis*
		NM23	1	1.1	*B. safensis*
		NM24	1	1.8	*B. pumilus sensu lato*
		NM25	1	1.8	*B. pumilus sensu lato*
		NM26	4	4.1	*B. amyloliquefaciens*
		NM27	1	1.1	*B. safensis*
		NM28	1	1.6	*B. pumilus*
		NM29	1	1.4	*B. pumilus*
		NM30	1	1.2	*B. safensis*
		NM31	1	1.1	*B. safensis*
		NM32	1	1.2	*B. safensis*
		NM33	1	1.5	*B. pumilus*
		NM34	1	1.4	*B. pumilus*
		NM35	3	3.2	*B. subtilis*
		NM36	1	1.6	*B. pumilus*
		NM37	1	1.1	*B. safensis*
		NM38	1	1.6	*B. pumilus*
		NM39	3	3.3	*B. subtilis*
		NM40	1	1.1	*B. safensis*
		NM41	3	3.2	*B. subtilis*
J0	Site 2/0h	NM42	1	1.2	*B. safensis*
		NM43	1	1.2	*B. safensis*
		NM44	1	1.3	*B. safensis*
		NM45	1	1.2	*B. safensis*
		NM47	1	1.2	*B. safensis*
		NM48	2	2.2	*B. cereus sensu lato*
J3	Site 2/72h	NM49	1	1.1	*B. safensis*
		NM50	1	1.8	*B. pumilus sensu lato*
		NM51	1	1.1	*B. safensis*
		NM52	1	1.8	*B. pumilus sensu lato*
		NM53	1	1.1	*B. safensis*

605

606 **Table 2 *continued*:** Origin and identity of the *Bacillus* isolates from Ntoba Mbodi

607

Samples	Origin/Fermentation time	Isolates	Groups ITS-PCR	Groups Rep-PCR	Identification 16S RNA/ GyrB/GyrA/RpoB gene sequencing
MB	Site 3/72h	NM54	2	2.1	*B. cereus sensu lato*
		NM55	1	1.1	*B. safensis*
		NM56	5	5.1	*B. megaterium*
		NM57	5	5.2	*B. megaterium*
		NM58	1	1.2	*B. safensis*
		NM59	2	2.1	*B. cereus sensu lato*
		NM60	1	1.1	*B. safensis*
MP	Site 4/72h	NM61	1	1.2	*B. safensis*
		NM62	1	1.2	*B. safensis*
		NM63	1	1.1	*B. safensis*
		NM64	1	1.2	*B. safensis*
		NM65	5	5.3	*B. megaterium*
		NM66	5	5.4	*B. megaterium*
		NM67	1	1.7	*B. pumilus sensu lato*
		NM68	1	1.2	*B. safensis*
		NM70	1	1.7	*B. pumilus sensu lato*
		NM71	1	1.1	*B. safensis*
		NM72	1	1.2	*B. safensis*
MBb	Site 5/72h	NM73	7	7.1	*Lysinibacillus louembei*
		NM74	6	6.1	*B. licheniformis*
		NM75	4	4.1	*B. amyloliquefaciens*
		NM76	3	3.4	*B. siamensis*
		NM77	3	3.4	*B.* siamensis
		NM86	1	1.1	*B. safensis*
		NM87	1	1.1	*B. safensis*
		NM88	1	1.1	*B. safensis*
		NM89	1	1.2	*B. safensis*
		NM90	1	1.2	*B. safensis*
		NM91	1	1.2	*B. safensis*
		NM92	1	1.7	*B. pumilus sensu lato*
		NM93	1	1.7	*B. pumilus sensu lato*
MPb	Site 6/72h	NM78	2	2.2	*B. cereus sensu lato*
		NM79	2	2.1	*B. cereus sensu lato*
		NM80	2	2.1	*B. cereus sensu lato*
		NM81	2	2.3	*B. cereus sensu lato*
		NM82	2	2.2	*B. cereus sensu lato*
		NM83	2	2.3	*B. cereus sensu lato*
		NM84	2	2.4	*B. cereus sensu lato*
		NM85	2	2.4	*B. cereus sensu lato*

608
609

610 **Table 3:** Detection of toxins genes and production of haemolysin by the *B. cereus*
611 isolates
612

Bacteria	Genes encoding the production of toxins							Haemolysis on blood Agar[b]	Production of haemolysin
	NheA	*NheB*	*NheC*	*HblC*	*HblD*	*CytK*	*EM1*		
B. cereus B 13 positive control	+[a]	+	+	+	+	+	-	+	+
B. cereus NM 48	+	+	+	-	-	-	-	+	-
B. cereus NM 54	+	+	+	+	+	+	-	+	+
B. cereus NM 59	+	+	+	+	+	+	-	+	+
B. cereus NM 78	+	+	+	+/-	-	+	-	+	-
B. cereus NM 79	+	+	+	+	+	+	-	+p	+
B. cereus NM 80	+	+	+	+	+	+	-	+p	+
B. cereus NM 81	+	+	+	+	+	+	-	+	+
B. cereus NM 82	+	+	+	+/-	-	-	-	+p	-
B. cereus NM 83	+	+	+	+	+	+	-	+p	+
B. cereus NM 84	+	+	+	+/-	+	+	-	+	+
B. cereus NM 85	+	+	+	+/-	+	-	-	+	+
B.safensis NM1	-	-	-	-	-	-	-	+	-
B.safensis NM 3	-	-	-	-	-	-	-	+	-
B.safensis NM 5	-	-	-	-	-	-	-	+	-
B.safensis NM 19	-	-	-	-	-	-	-	+	-
B. pumilus NM 21	-	-	-	-	-	-	-	+	-
B. pumilus NM 33	-	-	-	-	-	-	-	+	-
B. pumilus NM 34	-	-	-	-	-	-	-	+	-
B. pumilus NM 36	-	-	-	-	-	-	-	+	-
B. pumilus sensu lato NM 52	-	-	-	-	-	-	-	+	-
B. pumilus sensu lato NM 67	-	-	-	-	-	-	-	+	-
B. subtilis NM 7	-	-	-	-	-	-	-	+	-
B. subtilis NM 35	-	-	-	-	-	-	-	+	-
B. subtilis NM 39	-	-	-	-	-	-	-	+	-
B. amyloliquefaciens NM 75	-	-	-	-	-	-	-	+	-
B. siamensis NM 76	-	-	-	-	-	-	-	-	-
B. licheniformis NM 74	-	-	-	-	-	-	-	+	-
B. megaterium NM 56	-	-	-	-	-	-	-	-	-
B. megaterium NM 57	-	-	-	-	-	-	-	+	-
B. megaterium NM 65	-	-	-	-	-	-	-	+	-

613
614 [a]+: presence ; - : absence; p: partial;
615 [b] Beta hemolysis observed for all positive bacteria except for isolates NM79, NM80, NM82 and NM83
616 which exhibited an alpha hemolysis (p: partial)
617

618

Highlights:

- Various *Bacillus* species identified to interspecies and intraspecies by genotyping
- Main species*: Bacillus pumilus* group of species and mainly *B. safensis*
- *B. cereus* isolates: contain cytotoxin, haemolytic, non-haemolytic toxin genes
- *B. cereus* isolates: produce haemolytic enterotoxin
- Non - *B. cereus* isolates: do not contain toxin genes and do not produce haemolysin

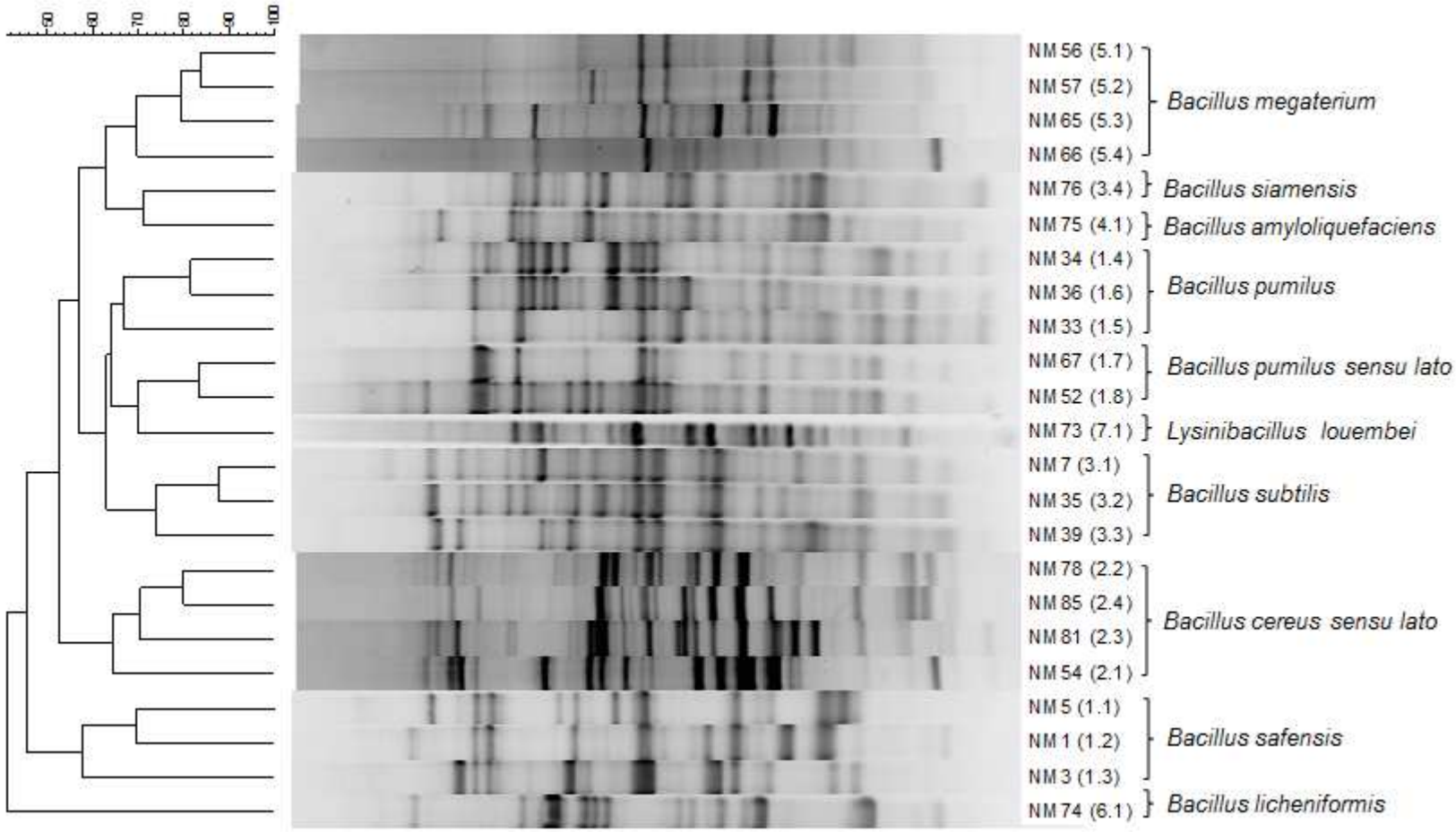
50
60
70
80
90
100
NM 56 (5.1)
NM 57 (5.2)
NM 65 (5.3)
NM 66 (5.4)
Bacillus megaterium
NM 76 (3.4)
Bacillus siamensis
NM 75 (4.1)
Bacillus amyloliquefaciens
NM 34 (1.4)
NM 36 (1.6)
NM 33 (1.5)
Bacillus pumilus
NM 67 (1.7)
NM 52 (1.8)
Bacillus pumilus sensu lato
NM 73 (7.1)
Lysinibacillus louembei
NM 7 (3.1)
NM 35 (3.2)
NM 39 (3.3)
Bacillus subtilis
NM 78 (2.2)
NM 85 (2.4)
NM 81 (2.3)
NM 54 (2.1)
Bacillus cereus sensu lato
NM 5 (1.1)
NM 1 (1.2)
NM 3 (1.3)
Bacillus safensis
NM 74 (6.1)
Bacillus licheniformis

Fig.1.