

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

4
5 Alain B. Voudibio Mbozo^a, Simon C. Kobawila^a, Amarachukwu Anyogu^b, Brigitte
6 Awamaria^b, Delphin Louembe^a, Jane P. Sutherland^b, L. Irene I. Ouoba^{b,c,*}

7
8 ^a Faculté des Sciences, Université Marien N'Gouabi, Brazzaville, Congo

9 ^b Microbiology Research Unit, School of Human Sciences, London Metropolitan
10 University London N7 8DB, UK

11 ^c Ouoba-Consulting, London, UK

12
13 *Corresponding author

14 Dr L. Irene I. Ouoba

15 Tel.: +44 (0) 20 71334154

16 Email: ouobairene@hotmail.com

17 **Abstract**

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

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

34

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

37

38 1. Introduction

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

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

67 Unlike other alkaline fermented products such as Ugba, Natto, Soumbala, Maari,
68 Bikalga, and Kinema that are obtained from seed based raw materials (Parkouda et al.,
69 2009), Ntoba Mbodi is made of leaves and the production technology does not include a
70 heating step. This may induce more differences in the microbial population as compared
71 to the seed based alkaline fermented products. Similarly to most traditional fermented
72 foods, the fermentation of cassava leaves is uncontrolled and this favours the
73 occurrence of undesirable Gram-positive and Gram-negative pathogenic bacteria,
74 leading to safety issues. Potential spore formers among pathogenic bacteria such as *B.*
75 *cereus* are of great concern because of their capacity to survive different processing
76 conditions, including heat treatment. The spores have high adhesion capability to
77 various materials and may accumulate in the processing equipment, constituting
78 thereby a serious hazard (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000).
79 Also, uncontrolled fermentations induce variable nutritional and sensorial attributes.
80 Thus, it is important to select well-defined starters and initiate controlled fermentations
81 that will provide products with high nutritional quality and also good hygienic attributes
82 and stability.

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

96 **2. Materials and methods**

97 2.1. Identification of *Bacillus* isolates from Ntoba Mbodi

98 2.1.1. Enumeration, isolation and phenotypic characterization of the microorganisms

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

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

115 2.1.2. Genotypic characterization and identification of the isolates

116 2.1.2.1. Extraction of DNA

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

121 2.1.2.2. Characterization of the isolates by 16S-23S rDNA ITS-PCR and rep-PCR

122 Amplification of the 16S-23S rDNA internal transcribed spacer (ITS) was carried out
123 using methods previously described (Ouoba, Parkouda, Diawara, Scotti, & Varnam,
124 2000a; Anyogu, Awamaria, Sutherland, & Ouoba, 2014) and the primers depicted in
125 Table 1. The PCR conditions were as follows: initial denaturation at 94°C for 1 min
126 followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and
127 elongation at 72°C for 1 min. The PCR was ended with a final extension at 72°C for 7
128 min and the amplified product cooled at 4°C. The characterization of the isolates by rep-

129 PCR was also done by applying previously described methodology (Ouoba, Parkouda,
130 Diawara, Scotti, & Varnam, 2000a; Anyogu, Awamaria, Sutherland, & Ouoba, 2014) and
131 using the GTG5 primer shown in Table 1. For the amplification, the following program
132 was applied: initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation
133 at 94°C for 30 s, annealing at 45°C for 1 min and elongation at 65°C for 8 min. The PCR
134 ended with a final extension at 65°C for 16 min, and the amplified products cooled to
135 4°C. The DNA fragments generated from the ITS and rep-PCRs were separated by
136 applying 10 µl of each PCR product with 2 µl of loading buffer to 2 % (w/v) agarose gel
137 (BioRad 4736). DNA molecular marker (Direct Load™ Wide Range DNA Marker;
138 Sigma 7058) was used as a standard. The gel was run in Tris-Borate-EDTA buffer (1x
139 TBE; Sigma T4415) for 1h 30 min at 120 V for ITS-PCR and 2 h 30 min at 140V for
140 rep-PCR. Further, the gel was stained with ethidium bromide and photographed using a
141 UV transilluminator. Bacteria showing the same ITS-PCR DNA profile were clustered in
142 the same group and further differentiation by rep-PCR of the isolates of each ITS-PCR
143 cluster was recorded. DNA profiles were grouped by visual screening and cluster
144 analysis using the Bionumerics system (Dice's Coefficient of similarity, UPGMA; Applied
145 Maths, Saint-Martens-Latem, Belgium).

146 2.1.2.3. Sequencing of the 16S rRNA, *gyrA*, *gyrB* and *rpoB* genes

147 The bacteria were first identified by the amplification and sequencing of the 16S
148 rRNA gene as described by Ouoba, Parkouda, Diawara, Scotti, & Varnam (2008a). The
149 primers used for the first amplification and sequencing are shown in Table 1. The
150 following PCR conditions were used for the first amplification: initial denaturation at
151 95°C for 5 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1

152 min. The final extension was carried out at 72°C for 5 min and the products cooled to
153 4°C. Positive PCR products were checked by electrophoresis, purified using the
154 QIAquick PCR Purification kit (Qiagen, Crawley, UK) and sequenced. The PCR reaction
155 was achieved by 35 PCR cycles with the following program: 95°C for 2 min, then 35
156 cycles at 96°C for 15 sec, 40°C for 1 sec and 60°C for 4 min.

157 In the case where closely related species could not be separated by 16S rRNA
158 gene sequencing, sequencing of *gyrA*, *gyrB* and *rpoB* genes was carried out. For those
159 genes, the same primers (Table1) were used for the first amplification and the
160 sequencing. For the *gyrA* gene, the amplification was carried out by mixing 2 µl of each
161 extracted DNA with a mixture containing 5 µl of 10 X PCR buffer containing 15 mM of
162 MgCl₂ (Applied Biosystems N8080160), 0.5 µl of dNTP (1.25 mM), 0.5 µl of each primer
163 (21 pmol/µl, Table1), 0.2 µl of AmpliTaq polymerase (5 U; Applied Biosystems N808-
164 0160, N808-0161) and 41.3 µl of sterile high purity water. The amplification was
165 performed using the following conditions: Initial denaturation at 94°C for 3 min, 35
166 cycles of 94°C for 1 min, 50°C for 1min, 72°C for 1 min and a final extension step at
167 72°C for 10 min. For the *gyrB* gene, the reaction mixture described by Thorsen et al.
168 (2011a) was applied with the following PCR conditions: 94°C for 2 min, then 30 cycles
169 at 94°C for 1 min, 66°C for 1 min and 72°C for 2 min. The final extension was carried
170 out at 72°C for 7 min and the product cooled to 4°C. For the amplification of the *rpoB*
171 gene, the method described by Anyogu, Awamaria, Sutherland, & Ouoba (2014) was
172 used along with the following conditions: 94°C for 2 min followed by 40 cycles of 94°C
173 for 30 s, 51°C for 45 s, 68°C for 50 s, and a final extension of 68°C for 90 s.
174 Electrophoresis was used to check the PCR products, and positive amplicons were

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

181 2.2. Investigation of the potential of the *Bacillus* isolates for production of enterotoxins,
182 cytotoxin and emetic toxin

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

187 2.2.1. Haemolysis on blood agar

188 The *Bacillus* isolates were screened for their haemolytic activity on blood agar as
189 follows: Columbia agar base (Oxoid CM003) was autoclaved at 121 °C for 15 min and
190 horse blood (5%; Oxoid SR0050) added after cooling to 50 °C, before distribution into
191 Petri dishes. The *Bacillus* isolates were streaked on the agar and incubated at 37 °C for
192 48 h. Haemolysis was recorded by appearance of a zone of clearing around the
193 colonies (Beta or complete haemolysis) or a dark and greenish coloration under the
194 colonies (Alpha or partial haemolysis).

195 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°C for 2
202 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at the
203 corresponding temperature (Table 1) for 1 min and elongation at 72°C for 2 min. The
204 PCR ended with a final extension at 72°C for 5 min and the amplified products cooled to
205 4°C. 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 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 30 s,
210 annealing at 60 °C for 30 s and elongation at 72 °C for 1 min. The PCR ended with a
211 final extension at 72 °C for 5 min and the amplified products cooled to 4 °C. 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

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

225 3. Results

226 During the fermentation, the total mesophile population increased from 10^6 - 10^7 to 10^{10} -
227 10^{12} CFU/g after 72 h. This was associated with an increase of pH from 5-6 to 8-10
228 during the same time. Phenotypically, the bacteria exhibited various macroscopic and
229 microscopic features. As expected for *Bacillus* isolates, all bacteria were Gram and
230 catalase positive, rod-shaped and endospore formers. For most isolates, the position of
231 the spore was subterminal or terminal. However, a few isolates exhibited a centrally
232 positioned spore. Depending on the isolate, the cells were arranged as single, pairs and
233 chains. Most were motile, with however a reduced motility for cells that were in chains.
234 The identification experiments revealed that all bacteria belonged to the genus *Bacillus*
235 except isolate NM73, which showed an affiliation with the genus *Lysinibacillus*. The
236 latter genus has strong similarities with the genus *Bacillus*. Various species were
237 identified (Table 2), principally those of the *B. pumilus* group (72.21 %) including *B.*
238 *safensis* (53.33 %), *B. pumilus* (7.77 %), *B. altitudinis* and *B. pumilus sensu lato* (*B.*
239 *pumilus* group of isolates that were not clearly differentiated, 11.11 %). Other species
240 included *B. cereus sensu lato* (12.22 %), *B. megaterium* (4.44 %), *B. amyloliquefaciens*

241 (2.22 %), *B. siamensis* (2.22 %), *B. subtilis* (4.44 %), *B. licheniformis* (1.11%) and
242 *Lysinibacillus louembei* (1.11%).

243 Characterization of the bacteria by ITS-PCR generated seven groups of isolates that
244 were further subdivided into smaller clusters by rep-PCR. As seen in Table 2 and Fig.1.,
245 8, 4, 3, 2 and 4 rep-PCR subgroups were obtained from ITS-PCR groups 1, 2, 3, 4 and
246 5 respectively. ITS-PCR group 1 included all species of the *B. pumilus* group; group 2,
247 *B. cereus sensu lato*; group 3, *B. subtilis* and *B. siamensis*; group 4, *B.*
248 *amyloliquefaciens*; group 5, *B. megaterium*; group 6, *B. licheniformis* and group 7,
249 *Lysinibacillus louembei*. With the rep-PCR, all species from the *B. pumilus* group, as
250 well as the *B. subtilis* and *B. siamensis* (formerly belonging to the species of *B.*
251 *amyloliquefaciens*), were clearly differentiated (Table 2, Fig.1.). Furthermore, some of
252 the species included different rep-PCR DNA profiles. *Bacillus megaterium* and *B.*
253 *cereus sensu lato* exhibited four different rep-PCR profiles each, *Bacillus safensis*, *B.*
254 *pumilus* and *B. subtilis* three patterns each, and *B. pumilus sensu lato* two patterns
255 each (Table 2, Fig.1.).

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

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

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

282 **4. Discussion**

283 Investigations into the microbial populations associated with the alkaline fermentation of
284 cassava leaves for Ntoba Mbodi production revealed that different species and
285 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

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

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

332 underestimates bacterial diversity in a particular ecosystem (Sessitsch, Reiter, Pfeifer, &
333 Wilhelm, 2002). In the current research, a combination of genotypic methods that have
334 been shown to be efficient in the identification of *Bacillus* species in other alkaline
335 fermented foods (Thorsen et al., 2011a; Ahaotu et al., 2013; Anyogu, Awamaria,
336 Sutherland, & Ouoba, 2014; Compaore et al., 2013) was used to provide an advanced
337 insight of the diversity of the *Bacillus* population of Ntoba Mbodi at species and
338 subspecies level. *Bacillus safensis*, *B. pumilus*, *B. subtilis*, *B. amyloliquefaciens*, *B.*
339 *megaterium*, *B. cereus* and *Lysinibacillus* species have been demonstrated in other
340 alkaline fermented products such as Soumbala, Mbuja/Bikalga, Maari, Ugba, Natto,
341 Kinema and Thua-nao (Sanni, Ayermor, Sakyi-dawson, & Sefa-dedeh, 2000;
342 Mohamadou, Mbofung, & Thouvenot, 2009; Parkouda et al., 2009; Parkouda et al.,
343 2010; Ahaotu et al., 2013; Compaoré et al., 2013). The microbiological similarities of
344 these types of foods are not surprising as they share some biochemical features, such
345 as the high content of proteins (up to 40 %) whose degradation during the fermentation
346 leads to the increase in pH. This constitutes a selection factor for particular
347 microorganisms capable of withstanding alkaline conditions.

348 Except for *B. safensis*, which was identified by 16S rDNA sequencing/EZtaxon search,
349 the sequencing of housekeeping genes *gyrB*, *gyrA* and *rpoB* was necessary to identify
350 most isolates, as reported by earlier studies on *Bacillus* identification (Chun & Bae,
351 2000; La Duc, Satomi, Agata, & Venkateswaran, 2004; Wang, Lee, Tai, & Kasai, 2007;
352 Thorsen et al., 2011a; Ahaotu et al., 2013; Liu et al., 2013; Anyogu, Awamaria,
353 Sutherland, & Ouoba, 2014). For the isolates of *B. pumilus sensu lato* that could not be
354 clearly identified, the use of other types of primers for the genes screened for may

355 assist the identification. Also, the new methodologies described by Branquinho et al.
356 (2014b) for differentiating species of the *B. pumilus* group may be useful. For the *B.*
357 *cereus sensu lato*, the differentiation has been often difficult and their real difference at
358 species level is becoming more and more questionable. Isolate NM73 initially showed
359 98 % of 16S rDNA sequence similarity with *Lysinibacillus meyeri*, but differed by 14
360 base pairs, which is indicative of a potential new species of bacterium. Specific studies
361 were performed to characterise the isolate as a new species and named it as
362 *Lysinibacillus louembei* (Ouoba et al., 2015).

363 Ntoba Mbodi is produced using exclusively traditional methodologies and uncontrolled
364 fermentation. Thus, the presence of potentially pathogenic bacteria such *B. cereus* is
365 possible. Due to their ability to produce toxins that cause foodborne illnesses, the
366 presence of *B. cereus* in food is of considerable concern for human health. Toxins
367 produced by *B. cereus* include cereulide, cytotoxin (CytK), non-haemolytic enterotoxin
368 (Nhe) and haemolysin BL (Hbl) (Agata et al., 1994; Granum & Lund, 1997; Stenfors,
369 Fagerlund, & Granum, 2008). Such isolates can cause both food infections and
370 intoxications resulting in e.g. vomiting and serious case of diarrhoea. All *B. cereus*
371 investigated exhibited several toxin genes with isolates NM 54, NM59, NM79, NM80,
372 NM81 and NM83 being the most potentially virulent and possible causes of foodborne
373 disease as they exhibited all three Nhe genes (*NheA*, *NheB*, *NheC*) and Hbl genes
374 (*HblC*, *HblD*) as well as the *CytK* gene (Guinebretiere, Broussolle, & Nguyen-The,
375 2002). The presence of Hbl genes and especially the *HblC* gene was confirmed by the
376 production of haemolysin. There were no correlations between haemolytic activity on
377 blood agar and presence of the Hbl genes and production of the haemolytic enterotoxin.

378 In fact, most non *B. cereus* isolates and one *B. cereus* strain showed haemolysis on
379 blood agar but did not exhibit the Hbl genes and did not produce the haemolysin toxin.
380 As reported by Lindback, Fagerlund, Rodland, & Granum (2004) and Ouoba, Thorsen,
381 & Varnam (2008b), the presence all three Nhe genes is indicative of a potential ability of
382 the bacteria to produce non-haemolytic enterotoxin. The absence of the *EM1* gene,
383 encoding heat stable emetic toxin (cereulide) production, was also reported in previous
384 studies on Soumbala, Bikalga, Gergoush and Ugba (Ouoba, Thorsen, & Varnam,
385 2008b; Thorsen et al., 2011a; Ahaotu et al., 2013). The production of cereulide by *B.*
386 *cereus* isolates from alkaline fermented foods as reported by Thorsen et al. (2011b) for
387 isolates from Afitin seems to be rather rare. Ntoba Mbobi is cooked before consumption,
388 and most heat labile enterotoxins (Nhe and Hbl toxins) are likely to be destroyed before
389 consumption if the product is well cooked. However, heat stable toxins such as
390 cytotoxins (From, Pukall, Schumann, Hormazabal, & Granum, 2005) will not be
391 destroyed. Furthermore, since *B. cereus* is a spore-former, spores ingested through the
392 food may germinate in the large intestine and cause severe infections (Granum & Lund,
393 1997; From, Hormazabal, & Granum, 2007). Nevertheless, there is no official report of
394 foodborne illnesses resulting from the consumption of alkaline traditional fermented
395 foods such as Ntoba Mbodi. This may be related to non-availability of data and lack of
396 statistics. None of the isolates belonging to the other species studied exhibited the toxin
397 genes screened for, or were able to produce haemolysin. In general, non *B. cereus*
398 *Bacillus* isolates are considered safe with regards to toxin production (Hosoi et al, 2003;
399 Sanders, Morelli, & Tompkins, 2003), although specific strains of a few species such as
400 *B. subtilis*, *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

418 **References**

- 419 Ahaotu, A., Anyogu, O.H., Njoku, N.N., Odu, J.P., Sutherland, & Ouoba, L.I.I. (2013).
420 Molecular identification and safety of *Bacillus* species involved in the
421 fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of
422 Ugba. *International Journal of Food Microbiology*, 162, 95-104.
- 423 Anyogu, A., Awamaria, B., Sutherland, J.P. & Ouoba, L.I.I. (2014). Molecular
424 characterisation and antimicrobial activity of bacteria associated with submerged
425 lactic acid cassava fermentation. *Food Control*, 39, 119-127.
- 426 Azokpota, P., Hounhouigan, D.P, & Nago, M.C. 2006. Microbiological and chemical
427 changes during the fermentation of African locust bean (*Parkia biglobosa*) to
428 produce afitin, iru and sonru, three traditional condiments produced in Benin.
429 *International Journal of Food Microbiology*, 107, 304-309.
- 430 Beecher, D.J., Schoeni, J.L, & Wong, A.C. (1995). Enterotoxic activity of hemolysin BL
431 from *Bacillus cereus*. *Infection and Immunity*, 63, 4423-4428.
- 432 Branquinho, R., Meirinhos-Soares, L., Carriço, J., Pintado, M., & Peixe, L. (2014a).
433 Phylogenetic and clonality analysis of *Bacillus pumilus* isolates uncovered a
434 highly heterogeneous population of different closely related species and clones.
435 *FEMS Microbiology Ecology*, 90, 689-698.
- 436 Branquinho, R., Sousa, C., Lopes, J., Pintado, M., Peixe, L., & Osório, H. (2014b).
437 Differentiation of *Bacillus pumilus* and *Bacillus safensis* Using MALDI-TOF-MS.
438 *Plos One*, 9, e110127.

439

- 440 Chun, J., & Bae, K.S. 2000. Phylogenetic analysis of *Bacillus subtilis* and related taxa
441 based on partial *gyrA* gene sequences. *Antonie Van Leeuwenhoek*, 78, 123-
442 127.
- 443 Compaoré, C., Nielsen, D., Ouoba, L., Berner, T., Nielsen, K., & Sawadogo-Lingani, H.
444 et al. (2013). Co-production of surfactin and a novel bacteriocin by *Bacillus*
445 *subtilis* *subsp. subtilis* H4 isolated from Bikalga, an African alkaline *Hibiscus*
446 *sabdariffa* seed fermented condiment. *International Journal of Food*
447 *Microbiology*, 162, 297-307.
- 448 Ehling-Schulz, M., Fricker, M. & Scherer, S. (2004). Identification of emetic toxin
449 producing *Bacillus cereus* strains by a novel molecular assay. *FEMS*
450 *Microbiology Letters*, 232, 189-195.
- 451 From, C., Pukall, R., Schumann, P., Hormazabal, V. & Granum, P.E. (2005). Toxin-
452 producing ability among *Bacillus* spp. Outside the *Bacillus cereus* group. *Applied*
453 *and environmental Microbiology*, 71, 1178-1183.
- 454 From, C., Hormazabal, V. & Granum, P.E. (2007). Food poisoning associated with
455 pumilacidin-producing *Bacillus pumilus* in rice. *International Journal of Food*
456 *Microbiology*, 115, 319-324.
- 457 Gregersen, T. (1978). Rapid method for distinction of Gram-negative from Gram-
458 positive bacteria. *European Journal of Applied Microbiology and Biotechnology*,
459 5, 123-127.
- 460 Granum, P.E. & Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS*
461 *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 of enterotoxic *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 Reviews*, 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.

- 484 Kobawila, S.C., Louembé, D., Kéléké, S., Hounhouigan, J., & Gamba, C. (2005).
485 Reduction of the cyanide content during fermentation of cassava roots and
486 leaves to produce Bikedì and Ntoba Mbodi, two food products from Congo.
487 *African Journal of Biotechnology*, 4, 689-696.
- 488 La Duc, M.T., Satomi, M., Agata, N., & Venkateswaran, K. (2004). GyrB as a
489 phylogenetic discriminator for members of the *Bacillus anthracis-cereus-*
490 *thuringiensis* group. *Journal of Microbiology Methods*, 56, 383-394.
- 491 Lai, Q., Liu, Y., & Shao, Z. (2014). *Bacillus xiamenensis* sp. nov., isolated from intestinal
492 tract contents of a flathead mullet (*Mugil cephalus*). *Antonie Van Leeuwenhoek*,
493 105, 99 -107.
- 494 Lateef, A., Adelere, I.A., & Gueguim-Kana, E.B. (2015). The biology and potential
495 biotechnological applications of *Bacillus safensis*. *Biologia*, 70, 411-419.
- 496 Lindback, T., Fagerlund, A., Rodland, M.S., & Granum, P.E. (2004). Characterization of
497 the *Bacillus cereus* Nhe enterotoxin. *Microbiology*, 150, 3959-3967.
- 498 Liu, Y., Lai, Q., Dong, C., Sun, F., Wang, L., Li, G., & Shao, Z. (2013). Phylogenetic
499 Diversity of the *Bacillus pumilus* Group and the Marine Ecotype Revealed by
500 Multilocus Sequence Analysis. *Plos ONE*, 8, e80097.
- 501 Louembé, D., Kobawila, S.C., Bouanga, G., & Kéléké, S. (2003). Etude microbiologique
502 des feuilles fermentées de manioc: Ntoba mbodi. *Tropicultura*, 21, 106-111.
- 503 Mekuto, L., Jackson, V., & Ntwampe, S.K.O., (2014). Biodegradation of free cyanide
504 using *Bacillus* Sp. consortium dominated by *Bacillus safensis*, *lichenformis* and

- 505 *tequilensis* strains: A bioprocess supported solely with whey. *Journal of*
506 *Bioremediation and Biodegradation*, S18:004.
- 507 Meyers, P. R., Gokool, P., Rawlings, D. E., & Woods, D. R. (1991). An efficient cyanide-
508 degrading *Bacillus pumilus* strain. *Journal of General Microbiology*, 137, 1397-
509 1400.
- 510 Mohamadou, B.A., Mbofung, C.M.F., & Thouvenot, D. (2009). Microbiological and
511 organoleptic profiles of Mbuja: A traditional condiment produced by fermented
512 *Hibiscus sabdariffa* seeds in Cameroun. *Journal of Food Technology*, 7, 84-91.
- 513 Molina, C., Cana-Roca, J., Osuna, A., & Vilchez, S. (2010). Selection of a *Bacillus*
514 *pumilus* Strain Highly Active against *Ceratitidis capitata* (Wiedemann) Larvae.
515 *Applied and Environmental Microbiology*, 76, 1320-1327.
- 516 Nicholson, W.J., Munakata, N., Horneck, G., Melosh, H.J., & Setlow, P. (2000).
517 Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial
518 environments. *Microbiology and Molecular Biology Reviews*, 64, 548-572.
- 519 Ouoba, L.I.I., Diawara, B., Amoa-Awua, W.K., Traoré, A.S, & Lange Moller, P. (2004).
520 Genotyping of starter cultures of *Bacillus subtilis* and *Bacillus pumilus* for
521 fermentation of African locust bean (*Parkia biglobosa*) to produce *Soumbala*.
522 *International Journal of Food Microbiology*, 90,197-205.
- 523 Ouoba, L.I.I., Parkouda, C., Diawara, B., Scotti, C., & Varnam, A.H. (2008a).
524 Identification of *Bacillus* spp from Bikalga, fermented seeds of *Hibiscus*
525 *sabdariffa*: phenotypic and genotypic characterization. *Journal of Applied*
526 *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., Voudibio 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). Toxigenic strains 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-dede, 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 spontaneous 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

ACCEPTED MANUSCRIPT

597 Table 1: Primers used in the current study

Primers	Sequence (5'-3')	Gene/Region	Annealing Temperature(°C)
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	AGGTCAACTAGTTTCAGTATGGAC	<i>rpoB</i> ^b	51
rpoB-R	AAGAACCGTAACCGGCAACTT		
UP1-F	GAAGTCATCATGACCGTTCTGCAYGCNNGGNGNAA RTTYGA	<i>gyrB</i> ^c	66
UP2-R	AGCAGGGTACGGATGTGCGAGCCRTCACRTCNGC RTCNGTCAT		
gyrA-F	GAYTATGCWATGTCAGTTATTGT	<i>gyrA</i>	50
gyrA.R	GGAATRTTRGAYGTCATACCAAC		
HC F	GATAC(T,C)AATGTGGCAACTGC	<i>HblC</i> ^d	58
HC R	TTGAGACTGCTCG(T,C)TAGTTG		
HD F	ACCGGTAACACTATTCATGC	<i>HblD</i> ^d	58
HD R	GAGTCCATATGCTTAGATGC		
NA F	GTTAGGATCACAATCACCGC	<i>NheA</i> ^d	56
NA R	ACGAATGTAATTTGAGTCGC		
NB F	TTTAGTAGTGGATCTGTACGC	<i>NheB</i> ^d	54
NB R	TTAATGTTCGTTAATCCTGC		
NC F	TGGATTCCAAGATGTAACG	<i>NheC</i> ^d	54
NC R	ATTACGACTTCTGCTTGTGC		
CytK F	ACAGATATCGG(G,T)CAAAATGC	<i>cytK</i> ^d	54
CytK R	TCCAACCCAGTTWSCAGTTCD		
EM1 F	GACAAGAGAAATTTCTACGAGCAAGTACAAT	Unknown ^f	60
EM1 R	GCAGCCTTCCAATTACTCCCTTCTGCCACAGT		

598

599 ^a Ouoba, Parkouda, Diawara, Scotti, & Varnam (2008a); ^bYamamoto and Harayama
600 (1995); ^c Thorsen et al (2011a) and Yamamoto & Harayama (1995); ^dHansen &
601 Hendriksen (2001); ^fGene 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/Fermentation time	Isolates	Groups ITS-PCR	Groups Rep-PCR	Identification 16S RNA/GyrB/GyrA/RpoB gene sequencing
A0	Site1/0h	NM1	1	1.2	<i>B. safensis</i>
		NM3	1	1.3	<i>B. safensis</i>
		NM4	1	1.2	<i>B. safensis</i>
		NM5	1	1.1	<i>B. safensis</i>
		NM6	1	1.1	<i>B. safensis</i>
		NM7	3	3.1	<i>B. subtilis</i>
		A1	Site1/24h	NM8	1
NM9	1			1.1	<i>B. safensis</i>
NM10	1			1.1	<i>B. safensis</i>
NM11	1			1.1	<i>B. safensis</i>
NM12	1			1.2	<i>B. safensis</i>
NM13	1			1.2	<i>B. safensis</i>
NM14	1			1.1	<i>B. safensis</i>
NM15	1			1.1	<i>B. safensis</i>
A2	Site1/48h	NM16	1	1.8	<i>B. pumilus sensu lato</i>
		NM17	1	1.8	<i>B. pumilus sensu lato</i>
		NM18	1	1.2	<i>B. safensis</i>
		NM19	1	1.2	<i>B. safensis</i>
		NM20	1	1.2	<i>B. safensis</i>
A3	Site1/72h	NM21	1	1.4	<i>B. pumilus</i>
		NM22	1	1.1	<i>B. safensis</i>
		NM23	1	1.1	<i>B. safensis</i>
		NM24	1	1.8	<i>B. pumilus sensu lato</i>
		NM25	1	1.8	<i>B. pumilus sensu lato</i>
		NM26	4	4.1	<i>B. amyloliquefaciens</i>
		NM27	1	1.1	<i>B. safensis</i>
		NM28	1	1.6	<i>B. pumilus</i>
		NM29	1	1.4	<i>B. pumilus</i>
		NM30	1	1.2	<i>B. safensis</i>
		NM31	1	1.1	<i>B. safensis</i>
		NM32	1	1.2	<i>B. safensis</i>
		NM33	1	1.5	<i>B. pumilus</i>
		NM34	1	1.4	<i>B. pumilus</i>
		NM35	3	3.2	<i>B. subtilis</i>
		NM36	1	1.6	<i>B. pumilus</i>
		NM37	1	1.1	<i>B. safensis</i>
		NM38	1	1.6	<i>B. pumilus</i>
		NM39	3	3.3	<i>B. subtilis</i>
		NM40	1	1.1	<i>B. safensis</i>
J0	Site 2/0h	NM41	3	3.2	<i>B. subtilis</i>
		NM42	1	1.2	<i>B. safensis</i>
		NM43	1	1.2	<i>B. safensis</i>
		NM44	1	1.3	<i>B. safensis</i>
		NM45	1	1.2	<i>B. safensis</i>
		NM47	1	1.2	<i>B. safensis</i>
		NM48	2	2.2	<i>B. cereus sensu lato</i>
		J3	Site 2/72h	NM49	1
NM50	1			1.8	<i>B. pumilus sensu lato</i>
NM51	1			1.1	<i>B. safensis</i>
NM52	1			1.8	<i>B. pumilus sensu lato</i>
NM53	1			1.1	<i>B. safensis</i>

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	<i>B. cereus sensu lato</i>		
		NM55	1	1.1	<i>B. safensis</i>		
		NM56	5	5.1	<i>B. megaterium</i>		
		NM57	5	5.2	<i>B. megaterium</i>		
		NM58	1	1.2	<i>B. safensis</i>		
		NM59	2	2.1	<i>B. cereus sensu lato</i>		
		NM60	1	1.1	<i>B. safensis</i>		
		MP	Site 4/72h	NM61	1	1.2	<i>B. safensis</i>
				NM62	1	1.2	<i>B. safensis</i>
				NM63	1	1.1	<i>B. safensis</i>
NM64	1			1.2	<i>B. safensis</i>		
NM65	5			5.3	<i>B. megaterium</i>		
NM66	5			5.4	<i>B. megaterium</i>		
NM67	1			1.7	<i>B. pumilus sensu lato</i>		
NM68	1			1.2	<i>B. safensis</i>		
NM70	1			1.7	<i>B. pumilus sensu lato</i>		
NM71	1			1.1	<i>B. safensis</i>		
MBb	Site 5/72h	NM72	1	1.2	<i>B. safensis</i>		
		NM73	7	7.1	<i>Lysinibacillus louembei</i>		
		NM74	6	6.1	<i>B. licheniformis</i>		
		NM75	4	4.1	<i>B. amyloliquefaciens</i>		
		NM76	3	3.4	<i>B. siamensis</i>		
		NM77	3	3.4	<i>B. siamensis</i>		
		NM86	1	1.1	<i>B. safensis</i>		
		NM87	1	1.1	<i>B. safensis</i>		
		NM88	1	1.1	<i>B. safensis</i>		
		NM89	1	1.2	<i>B. safensis</i>		
		NM90	1	1.2	<i>B. safensis</i>		
		NM91	1	1.2	<i>B. safensis</i>		
		NM92	1	1.7	<i>B. pumilus sensu lato</i>		
MPb	Site 6/72h	NM93	1	1.7	<i>B. pumilus sensu lato</i>		
		NM78	2	2.2	<i>B. cereus sensu lato</i>		
		NM79	2	2.1	<i>B. cereus sensu lato</i>		
		NM80	2	2.1	<i>B. cereus sensu lato</i>		
		NM81	2	2.3	<i>B. cereus sensu lato</i>		
		NM82	2	2.2	<i>B. cereus sensu lato</i>		
		NM83	2	2.3	<i>B. cereus sensu lato</i>		
NM84	2	2.4	<i>B. cereus sensu lato</i>				
NM85	2	2.4	<i>B. cereus sensu lato</i>				

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
	<i>NheA</i>	<i>NheB</i>	<i>NheC</i>	<i>HblC</i>	<i>HblD</i>	<i>CytK</i>	<i>EM1</i>		
<i>B. cereus</i> B 13 positive control	+ ^a	+	+	+	+	+	-	+	+
<i>B. cereus</i> NM 48	+	+	+	-	-	-	-	+	-
<i>B. cereus</i> NM 54	+	+	+	+	+	+	-	+	+
<i>B. cereus</i> NM 59	+	+	+	+	+	+	-	+	+
<i>B. cereus</i> NM 78	+	+	+	+/-	-	+	-	+	-
<i>B. cereus</i> NM 79	+	+	+	+	+	+	-	+p	+
<i>B. cereus</i> NM 80	+	+	+	+	+	+	-	+p	+
<i>B. cereus</i> NM 81	+	+	+	+	+	+	-	+	+
<i>B. cereus</i> NM 82	+	+	+	+/-	-	-	-	+p	-
<i>B. cereus</i> NM 83	+	+	+	+	+	+	-	+p	+
<i>B. cereus</i> NM 84	+	+	+	+/-	+	+	-	+	+
<i>B. cereus</i> NM 85	+	+	+	+/-	+	-	-	+	+
<i>B. safensis</i> NM1	-	-	-	-	-	-	-	+	-
<i>B. safensis</i> NM 3	-	-	-	-	-	-	-	+	-
<i>B. safensis</i> NM 5	-	-	-	-	-	-	-	+	-
<i>B. safensis</i> NM 19	-	-	-	-	-	-	-	+	-
<i>B. pumilus</i> NM 21	-	-	-	-	-	-	-	+	-
<i>B. pumilus</i> NM 33	-	-	-	-	-	-	-	+	-
<i>B. pumilus</i> NM 34	-	-	-	-	-	-	-	+	-
<i>B. pumilus</i> NM 36	-	-	-	-	-	-	-	+	-
<i>B. pumilus sensu lato</i> NM 52	-	-	-	-	-	-	-	+	-
<i>B. pumilus sensu lato</i> NM 67	-	-	-	-	-	-	-	+	-
<i>B. subtilis</i> NM 7	-	-	-	-	-	-	-	+	-
<i>B. subtilis</i> NM 35	-	-	-	-	-	-	-	+	-
<i>B. subtilis</i> NM 39	-	-	-	-	-	-	-	+	-
<i>B. amyloliquefaciens</i> NM 75	-	-	-	-	-	-	-	+	-
<i>B. siamensis</i> NM 76	-	-	-	-	-	-	-	-	-
<i>B. licheniformis</i> NM 74	-	-	-	-	-	-	-	+	-
<i>B. megaterium</i> NM 56	-	-	-	-	-	-	-	-	-
<i>B. megaterium</i> NM 57	-	-	-	-	-	-	-	+	-
<i>B. megaterium</i> 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

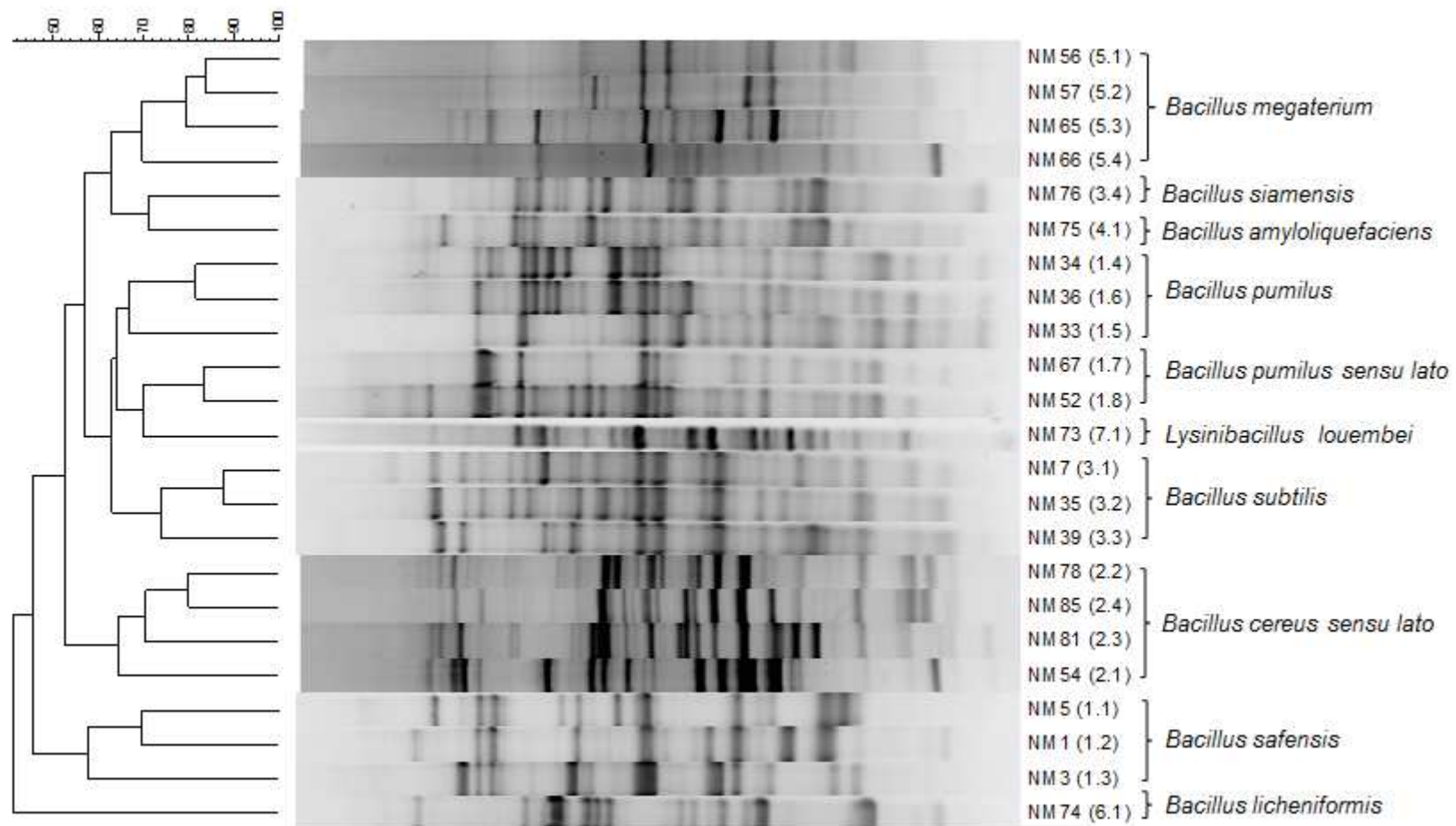
616 which exhibited an alpha hemolysis (p: partial)

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

**Fig.1.**