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Biodiversity of aerobic endospore-forming bacterial species occurring in Yanyanku and Ikpiru, fermented seeds of *Hibiscus sabdariffa* used to produce food condiments in Benin

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ABSTRACT

Yanyanku and Ikpiru made by the fermentation of Malcavene bean (*Hibiscus sabdariffa*) are used as functional additives for *Parkia biglobosa* seed fermentations in Benin. A total of 355 aerobic endospore-forming bacteria (AEFB) isolated from Yanyanku and Ikpiru produced in northern and southern Benin were identified using phenotypic and genotypic methods, including GTG_5-PCR, M13-PCR, 16S rRNA, *gyrA* and *gyrB* gene sequencing. Generally, the same 5–6 species of the genus *Bacillus* predominated: *Bacillus subtilis* (17–41% of isolates), *Bacillus cereus* (8–39%), *Bacillus amyloliquefaciens* (9–22%), *Bacillus licheniformis* (3–26%), *Bacillus safensis* (8–19%) and *Bacillus altitudinis* (0–19%). *Bacillus aryabhattai*, *Bacillus flexus*, and *Bacillus circulans* (0–2%), and species of the genera *Lysinibacillus* (0–14%), *Paenibacillus* (0–13%), *Brevibacillus* (0–4%), and *Aneurinibacillus* (0–3%) occurred sporadically. The diarrheal toxin encoding genes *cytK-1*, *cytK-2*, *hblA*, *hblC*, and *hblD* were present in 0%, 91% 15%, 34% and 35% of *B. cereus* isolates, respectively. 9% of them harbored the emetic toxin genetic determinant, *cesB*. This study is the first to identify the AEFB of Yanyanku and Ikpiru to species level and perform a safety evaluation based on toxin gene detections. We further suggest, that the *gyrA* gene can be used for differentiating the closely related species *Bacillus pumilus* and *B. safensis*.

1. Introduction

African locust bean (*Parkia biglobosa*) seeds are widely used for the production of fermented food condiments in West Africa (Oladunmoye, 2007). These condiments known as Dawadawa (Niger), Soumbala (Burkina Faso), Netetou (Senegal, Mali), Sonru, Iru and Afitin (Benin) are used to enhance the flavor of many dishes including soups and sauces, because of their sensory characteristics and high nutritional value (Azokpota et al., 2006). The fermentation of African locust bean is uncontrolled, leading to variations in stability, nutritional and hygienic quality of the final product. Recent studies on traditional processing of African locust beans have shown that indigenous practices have been developed to enhance control of the fermentation such as the addition of Yanyanku and Ikpiru. Yanyanku and Ikpiru are two fermented products based on *Hibiscus sabdariffa* seeds and are used as functional additives for African locust bean seed fermentation in Benin to produce Sonru and Iru, respectively (Azokpota et al., 2006; Agbobatinkpo et al., 2011).

Processing of *H. sabdariffa* seeds into Yanyanku and Ikpiru involves cooking, draining, alkaline fermentation, pounding/crushing, molding (if necessary) and sun drying (Fig. 1). However, variations are observed between and within these steps according to the know-how of the producers. The pH of the final product is generally above 8 (Agbobatinkpo et al., 2011). Various aerobic endosporeforming species, especially *Bacillus subtilis* group members, have been reported as predominant micro-organisms in alkaline fermented products from *H. sabdariffa* such as Bikalga (Ouoba et al., 2007). The sporeformers are usually reported to be the main micro-organisms responsible for the fermentation of high pH foods (Parkouda et al., 2009; Ouoba et al., 2008b). Occasionally, *Bacillus cereus* which is potentially able to cause two types of foodborne diseases, occur in high numbers in alkaline fermented foods giving rise to concern (Parkouda et al., 2010; Thorsen et al., 2010). *B. cereus* strains producing, at least, one of the three pore forming cytotoxins, hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe) and cytotoxin K (CytK) have been implicated as etiological agents of foodborne diarrhoeal disease (Beecher and MacMillan, 1991; Stenfors et al., 2008). This species is also responsible of emetic food

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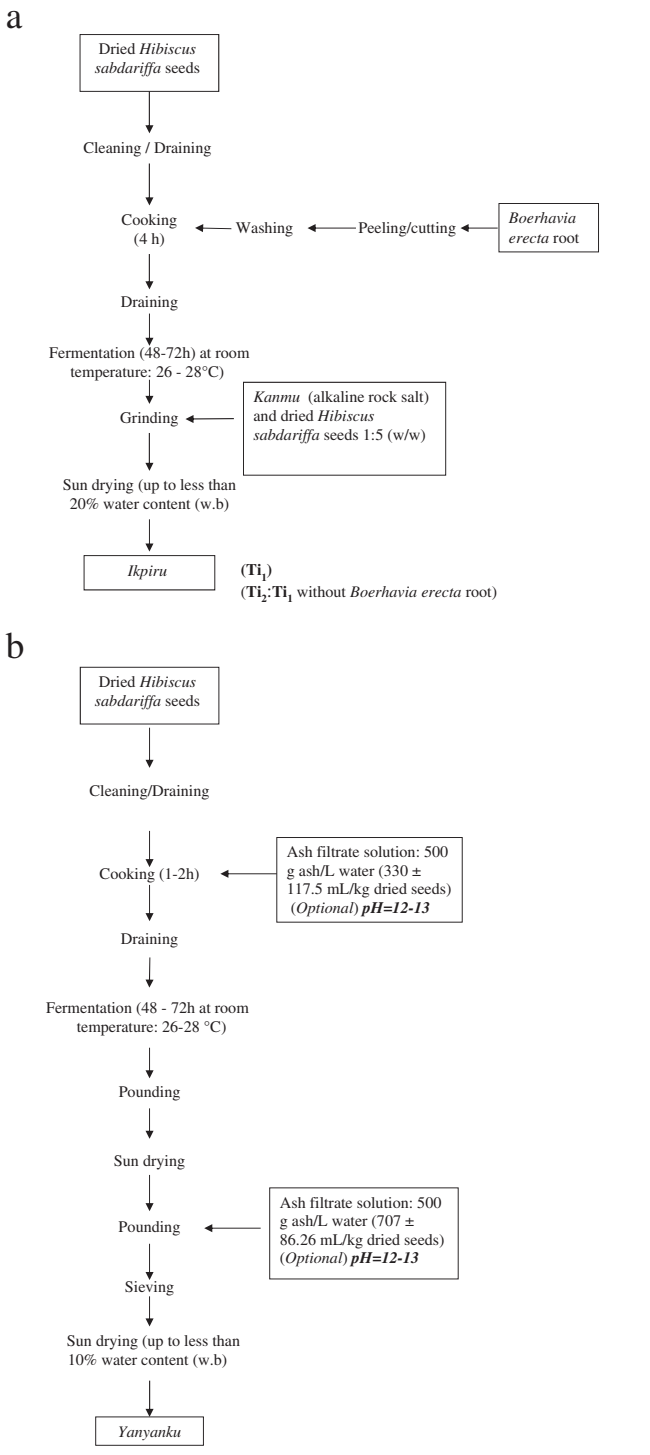

Fig. 1. a: Flow sheet of Ikpiru processing. Source: Agbobatinkpo et al. (2011). b: Flow sheet of Yanyanku processing. Source: Agbobatinkpo et al. (2011).

poisoning that occurs when the bacterium produces the toxin cereulide during growth in foods (Ehling-Schulz et al., 2004a). *Bacillus* spp., other than *B. cereus* are rarely a cause of foodborne intoxications (EFSA, 2008), and alkaline fermented foods are usually regarded as safe for consumption (Parkouda et al., 2009).

The biodiversity of *Bacillus* species isolated from Sonru and Iru has been studied (Azokpota et al., 2007) and it has also been found that Yanyanku and Ikpiru are dominated by aerobic endosporeforming bacteria (Agbobatinkpo et al., 2011), but no studies have been carried out to identify aerobic endosporeforming bacteria isolated from Yanyanku and Ikpiru used as functional additives during Sonru and Iru production, respectively. However, these additives may significantly affect the quality and microbiological status of the final product (Sonru and Iru) and there is thus a need to investigate the identity and the safety of micro-organisms from these fermented additives. Repetitive sequence-based PCR (Rep-PCR) (Parkouda et al., 2010), randomly amplified polymorphic DNA polymerase chain reaction typing (RAPD-PCR) (Thorsen et al., 2011b), and sequencing of the 16S rRNA gene combined with sequencing of core genes such as *gyrA* and *gyrB* and phenotypic tests (Chun and Bae, 2000; Nielsen et al., 2007; Parkouda et al., 2010; Thorsen et al., 2011b; Wang et al., 2007), are tools used to identify and differentiate between closely related aerobic endospore forming bacterial species.

The aim of the present study was to identify the aerobic endosporeforming bacteria isolated from additives Yanyanku and Ikpiru collected at different production sites in Benin, using phenotypic and genotypic methods. Furthermore, the safety of known pathogenic species has been assessed by PCR techniques.

2. Material and methods

2.1. Sampling

Samples of Yanyanku and Ikpiru from different technologies. Yanyanku from three technologies (Ty_1–Ty_3) and Ikpiru from two technologies (Ti_1 and Ti_2) according to Agbobatinkpo et al. (2011), (Fig. 1a, b) were collected in two municipalities of Benin, namely Parakou (Northern Benin) and Ketou (Southern Benin). Four samples were taken per technology. A total of 9 other samples of both additives were purchased from local markets in Ketou and Parakou. A total of twenty nine samples were used for this study (18 samples of Yanyanku and 11 samples of Ikpiru).

2.2. Isolation of aerobic endosporeforming bacteria from Yanyanku and Ikpiru

Ten grams of each sample was weighed aseptically and transferred into 90 mL sterile peptone-bacteriological salt solution (5 g peptone (Oxoid, LP 0037, Basingstoke, England), 8.5 g NaCl, 1000 mL distilled water, pH 7.0 ± 0.2) and homogenized for 1 min using a stomacher (Stomacher 400 circulator Seward, England). 5 mL of the primary dilution was heated at 80 °C for 10 min.

30 μL of each dilution (Herigstad et al., 2001; Chadare et al., 2010) was incubated on Nutrient Agar (NA) (Oxoid CM 0003, Basingstoke, England) at 37 °C for 48 h. Between 10 and 15 colonies were randomly picked from the highest dilution and purified by successive streaking on NA (Oxoid CM 0003, Basingstoke, England) until a complete pure culture was obtained. A total of 355 isolates were obtained. For long term storage isolates were stored in Nutrient Broth (Oxoid, CM 0001, Basingstoke, England) added 20% glycerol as cryoprotectant at −80 °C.

2.3. Identification of aerobic endosporeforming bacteria

2.3.1. Preliminary phenotypic characterization

Cell morphology, motility and presence of endospore were examined by microscopy (X1000 Zeiss Axiostar Plus, Germany). Gram determination was performed using the KOH method according to Gregersen (1978). Catalase production was determined by adding a drop of H_2O_2 solution (30%) to a colony on a glass slide.

2.3.2. Genotypic characterization

2.3.2.1. DNA extraction, Rep-PCR, M13-PCR fingerprint grouping. DNA was extracted from each isolate using the InstaGene Matrix (Bio-Rad

Laboratories, Hercules, CA USA) as described by the manufacturer. Genotypic grouping of the isolates was performed using Rep-PCR (GTG_5-primer) (Nielsen et al., 2007), followed by M13-PCR that was done for *B. cereus* and *Bacillus pumilus* group species as previously described (Henderson et al., 1994; Thorsen et al., 2010, 2011b). The Rep-PCR and M13-PCR profiles obtained were clustered using Bionumeric version 4.50 (Applied Maths, Sint-Martens-Latem, Belgium) as described by Nielsen et al. (2007). The dendogram was based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

2.3.2.2. 16S rRNA, gyrA and gyrB gene amplification and sequencing. Based on the Rep-PCR clustering of the isolates, representative isolates were chosen for sequencing of the 16S rRNA gene (102 isolates in total, i.e. at least the square root of number of isolates in one group). Amplification of the 16S rRNA gene was carried out as previously described (Satokari et al., 2001). PCR purification and sequencing were carried out by a commercial facility (Macrogen, South Korea). 16S rRNA sequences were manually corrected and aligned using Chromas 2.33 (Technelysium) and CLC Genomics (CLC Bio, Aarhus, Denmark) and compared against validated type strain sequences deposited in Eztaxon as described by Chun et al. (2007).

Sequence analysis of the *gyrA* and *gyrB* genes allows identification of members of the *B. subtilis* group to the species level on a genetic basis (Chun and Bae, 2000; Wang et al., 2007). Thus, representative isolates tentatively identified as belonging to the *B. subtilis* group (based on 16S rRNA gene sequencing) were selected for sequencing of the *gyrA* and *gyrB* genes as described by Roberts and Cohan (1995) and Yamamoto and Harayama (1995). In addition, representative isolates tentatively identified as belonging to the *B. pumilus* group (based on 16S rRNA gene sequencing) were also chosen for sequencing of the *gyrA* gene using the method described by Ouoba et al. (2008a). PCR purification and sequencing were carried out by a commercial facility (Macrogen, South Korea). Sequences were aligned with the GenBank database using the BLAST algorithm (Altschul et al., 1997).

2.3.2.3. Complementary tests. Different tests were carried out to identify the isolates to species level. Isolates identified as belonging to the *Bacillus cereus sensu lato* group were screened by PCR using previously described methods for the presence of the *Bacillus anthracis* specific virulence genes *lef*, *cya* (Hoffmaster et al., 2006), *pagA* and *capA* (Cheun et al., 2001), and the S-layer protein encoding gene (Cheun et al., 2001). The isolates were further screened by phase contrast microscopy for the presence of crystal inclusion bodies typical of *Bacillus thuringiensis* (Jensen et al., 2002), rhizoid colony morphology growth typical of *Bacillus mycoides* (di Franco et al., 2002), and growth at 7 °C, non-growth at 42 °C typical of *Bacillus weihenstephanensis* (Lechner et al., 1998). To discriminate between *Bacillus amyloliquefaciens*, *Bacillus methylotrophicus*, *Bacillus atrophaeus* and *Bacillus siamensis*, oxidase test, salt tolerance (0%, 7%, 10% (w/v) NaCl), and growth at different temperatures (45 °C, 50 °C and 55 °C) were carried out as described by Madhaiyan et al. (2010) and Sumpavapol et al. (2010). The *B. pumilus* group members (*B. pumilus*, *Bacillus safensis*, *Bacillus altitudinis*, *Bacillus aerophilus* and *Bacillus stratosphericus*) were differentiated by salt tolerance (17.4% (w/v) NaCl) and growth at 8 °C, 40 °C and 45 °C according to Shivaji et al. (2006). Further M13-PCR typing and grouping were performed as described above, including M13-PCR profiles of type strains of *B. altitudinis* (DSM 21631), *B. pumilus* (DSM 27) and *B. safensis* (DSM 19292), and DSM 354 designated as *B. pumilus* for comparison. *Lysinibacillus xylanilyticus* and *Lysinibacillus macroides* were differentiated by salt tolerance (0%, 5%, 6% and 7% (w/v) NaCl) and growth at 40 °C and 45 °C as described by Lee et al. (2010) and Coorevits et al. (2012). Growth in various salt concentrations was tested using tryptone soya agar (TSA) (Oxoid, CM0131, Basingstoke, England) and brain heart infusion agar (BHI-agar) (Fluka, 70138, Sigma Aldrich, India) supplemented with NaCl and incubated at 37 °C.

2.3.2.4. Detection of enterotoxin genes and hemolytic activity. The *B. cereus* isolates were screened for hemolytic activity on blood agar. Detection of the emetic specific gene fragment *cesb* was according to Ehling-Schulz et al. (2004b), and for the genes *hblA*, *hblD* and *hblC* encoding the enterotoxin complex hemolysin BL it was according to Hansen and Hendriksen (2001) and detection of *cytK-1* and *cytK-2* encoding cytotoxin K was according to Guinebretiere et al. (2006). DNA from cereulide producing *B. weihenstephanensis* MC67 (Thorsen et al., 2006), *B. cereus* NVH 391–98 and *B. cereus* ATCC 14579 (Fagerlund et al., 2004) were used as positive controls for *cesB*, *cytK-1* and *cytK-2* PCR, respectively. The DNA from strain ATCC 14579 was also used as positive control for detection of genes encoding hemolysin BL.

2.4. Nucleotide accession numbers

The sequences determined in the present study have been deposited in the GenBank database under the accession numbers JQ420803–JQ420900, JX535563–JX535566, and JX513907–JX513951.

Table 1
Microbiota and pH of Yanyanku and Ikpiru collected from different technologies.

Microorganisms	Southern Benin			Northern Benin			
	Ikpiru			Yanyanku			
	Ti_1	Ti_2	Market samples	Ty_1	Ty_2	Ty_3	Market samples
Aerobic endosporeforming bacteria (log_{10} CFU/g)	7.9	7.5	7.5	8.3	7.8	8.5	7.4
pH	9.9	9.9	8.2	8.6	7.4	7.5	7.7
Number of isolates	58	50	23	55	53	58	58
Number of isolates with 16S rRNA sequenced	21	10	5	12	14	16	21
Number of isolates with *gyrB* sequenced	2	0	1	4	5	1	2
Number of isolates with *gyrA* sequenced	4	2	2	3	6	2	4
Bacillus subtilis (%)	17	28	30	20	21	41	19
Bacillus amyloliquefaciens (%)	19	22	13	22	21	9	10
Bacillus licheniformis (%)	7	10	9	18	26	10	3
Bacillus cereus (%)	39	8	22	24	13	10	19
Bacillus safensis (%)	10	12	9	16	8	12	19
Bacillus altitudinis (%)	0	10	4	0	11	3	19
Bacillus circulans (%)	2	2	0	0	0	0	0
Bacillus flexus (%)	2	0	0	0	0	0	0
Bacillus aryabhattai (%)	2	0	0	0	0	0	0
Lysinibacillus spp. (%)	2	4	0	0	0	14	5
Brevibacillus laterosporus (%)	0	4	0	0	0	0	0
Paenibacillus dendritiformis (%)	0	0	13	0	0	0	2
Aneurinibacillus aneurinilyticus (%)	0	0	0	0	0	0	3

3. Results

3.1. pH and aerobic endosporeforming bacteria enumeration

The pH of Ikpiru and Yanyanku samples ranged from 8.2 to 9.9 and 7.4 to 8.6 with an average of 9.3 and 7.8, respectively. The AEFB count on NA ranged from 7.4 to 8.5 $\log_{10}$ CFU/g (Table 1).

3.2. Identification of aerobic endosporeforming bacteria

A total of 355 isolates were obtained. All isolates were characterized as Gram positive, catalase positive, endosporeforming and rod-shaped, except for six isolates which were characterized as Gram negative. The isolates were grouped by Rep-PCR fingerprinting using cluster analysis (Fig. S1). The 355 isolates investigated were grouped into 13 groups, while single isolates also occurred, following the cluster analysis of the Rep-PCR fingerprints. Representative isolates from the individual clusters were picked for 16S rRNA gene sequencing, which revealed that 93.8% of the isolates belonged to the genus *Bacillus*, 3.9% of the isolates to the genus *Lysinibacillus*, 1.1% to *Paenibacillus*, 0.6% to *Brevibacillus* and 0.6% of isolates to *Aneurinibacillus*.

The isolates belonging to Rep-PCR Groups 1A and B (Fig. S1, Fig. 2) were motile and were found to belong to the *B. subtilis* group (*B. subtilis*, *Bacillus tequilensis*) by 16S rRNA gene sequencing (99.8–100% similarity to EzTaxon deposited sequences). This group was the largest and comprised 88 isolates. Sequencing of the *gyrB* and *gyrA genes* (15 isolates) identified the isolates as *B. subtilis* subsp. *subtilis* (99–100% similarity to GenBank sequences).

Sequencing of the 16S rRNA gene showed that Rep-PCR Groups 2A and B (59 isolates) (Fig. S1, Fig. 2) also belonged to the *B. subtilis* group (*B. amyloliquefaciens*, *B. methylotrophicus*, *B. atrophaeus and*

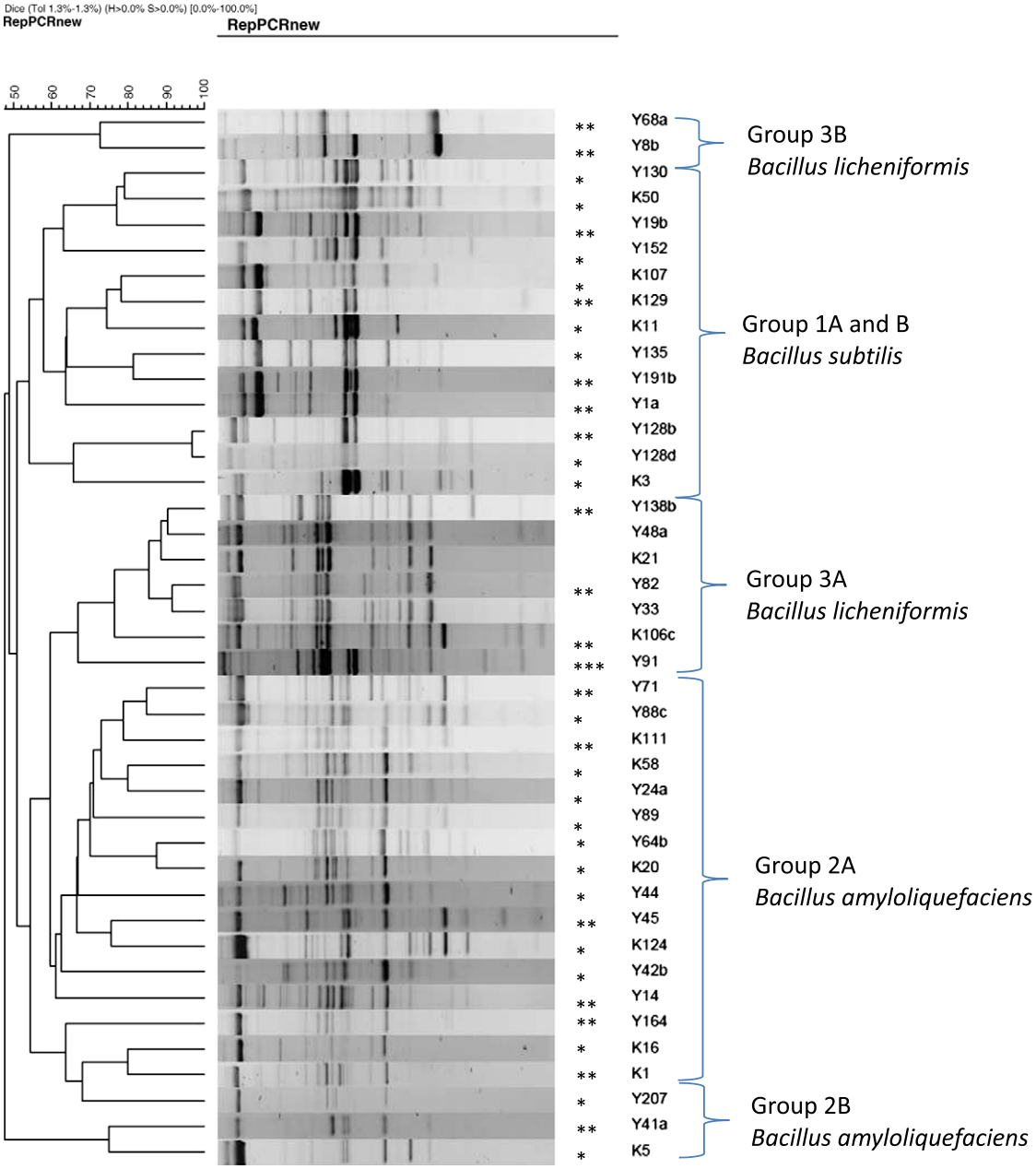

Fig. 2. Dendogram obtained by cluster analysis of Rep-PCR (GTG_5) fingerprints from *Bacillus subtilis* group (representative isolates) isolated from additives. The dendogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA). Sequenced (16S rRNA gene) isolated marked with *, Sequenced (16S rRNA, gyrA/gyrB genes) isolated marked with ** and Sequenced (*gyrB gene* only) isolated marked with ***.

B. siamensis) (99–100% similarity to EzTaxon sequences). The *gyrA* and *gyrB* gene sequences of 11 of the isolates showed 97–100% similarity to *B. amyloliquefaciens* subsp *plantarum*, 96–97% to *B. siamensis*, and up to 84% to *B. atrophaeus gyrA* and *gyrB* gene sequences deposited in GenBank. A single *gyrB* gene sequence was available for *B. methylotrophicus* showing 98–99% identity. Isolates from Groups 2A and B were however able to grow in tryptone soya agar (TSA) supplemented with 10% (w/v) NaCl. All the selected isolates (15 isolates) showed growth at 50 °C, while only 2 isolates grew at 55 °C. All the tested isolates were oxidase positive. Eventhough some of the isolates grew at 55 °C typical of *B. siamensis*, all other observed phenotypic features and the results for *gyrA* and *gyrB* gene sequencing altogether suggests that the isolates of Groups 2A and B belong to the species *B. amyloliquefaciens* (Sumpavapol et al., 2010; Madhaiyan et al., 2010).

The members of Rep-PCR Groups 3A and B (43 isolates) (Fig. S1, Fig. 2) were found to belong to the *B. subtilis* group (*Bacillus licheniformis*) according to 16S rRNA gene sequencing (98.9–99.2% similarity to EzTaxon). The sequencing of the *gyrA* and *gyrB genes* (7 isolates) identified this group as *B. licheniformis* (99–100% similarity to GenBank sequences) which was confirmed by the ability of the isolates from this group to grow under anaerobic conditions (Roberts et al., 1994).

The isolates belonging to Rep-PCR Groups 4A and B (69 isolates, Fig. S1) were found by 16S rRNA gene sequencing to belong to the *B. cereus* group (99.6–100% similarity to EzTaxon sequences). The macromorphology of the colonies proved that they were not *B. mycoides* as no isolates showed on agar plates the asymmetric hairy shape specific to *B. mycoides.* All isolates from this group showed growth at 43 °C in 24 h and none of them were able to grow at 7 °C for 14 days. The absence of crystalline inclusions characteristic for *B. thuringiensis* as observed by phase contrast microscopy, and the absence of the *B. anthracis* specific genes *cya*, *pagA*, *capA*, Edema Factor (*EF*) and the *B. anthracis* S-layer and Lethal factor (*lef*) protein encoding genes as determined by PCR ruled these species out, and based on the above mentioned characteristics, it was concluded that the Groups 4A and B isolates were all *B. cereus*.

The members of Rep-PCR Groups 5A and B (13 isolates, Fig. S1) were most closely related to *L. xylanilyticus* or *L. macroides* according to their 16S rRNA gene sequence (98–99% similarity to EzTaxon sequences). Six out of 13 isolates from this group were able to grow at 45 °C and in TSA supplemented with 5% (w/v) NaCl. The others were not able to grow at 45 °C or in TSA supplemented with 5% (w/v) NaCl. According to Coorevits et al. (2012) and Lee et al. (2010), *L. macroides* is not able to grow in the presence of 5% NaCl but grow at 45 °C and *L. xylanilyticus* is not able to grow at 45 °C but in the presence of 5% NaCl, respectively. Based on these results, it was concluded that this group may not be *L. xylanilyticus* or *L. macroides* but had similarities with both species and possibly represent a novel *Lysinibacillus* species. Fig. 3 shows the Rep-PCR profile for this group.

Sequencing of the 16S rRNA gene showed (99.6–100% similarity to EzTaxon sequences) that Groups 6A and B (Fig. S1, 25 isolates) belong to the *B. pumilus* group (*B. pumilus*, *B. aerophilus*, *B. altitudinis* or *B. stratosphericus*). Isolates (14 tested) from this group were not able to grow in TSA supplemented with 17.4% (w/v) NaCl typical of *B. stratosphericus*. However, all the tested isolates showed growth at 45 °C within 1–2 days typical of *B. altitudinis* (*B. aerophilus* and *B. stratosphericus* only grow at temperatures below 40 °C). Comparisons of the phylogenetic marker *gyrA* (3 isolates) with *gyrA* of the *B. altitudinis* type strain sequenced in this study and the *gyrA* gene of *B. aerophilus* (from a recently published whole genome shot gun contiqs, NZ_AJWW01000037), did not allow differentiation between the species (99–100% identity between the sequences). However, on the basis of phenotypic characteristics (Shivaji et al., 2006) and, M13 profile clustering with type strains (Fig. 4) the isolates were identified as *B. altitudinis.*

The members of Rep-PCR Group 7 (Fig. S1, 45 isolates) were found to be either *B. pumilus* or *B. safensis* based on their 16S rRNA gene sequence (99–100% similarity to EzTaxon sequences). Sequencing of the *gyrA gene* (5 isolates) strongly indicated (97–98% similarity, to GenBank sequences) that this group should be identified as *B. safensis*. The M13 profile clustering showed that isolates from Rep-PCR Group 7 were more closely related to *B. pumilus* DSM 354 than to the type strains *B. pumilus* DSM 27 and *B. safensis* DSM 19292 (Fig. 4). The *gyrA gene* sequences of the Rep-PCR Group 7 isolates showed between 96.7–99.8%, 97.8–98.0% and 90.2–90.4% identity with *gyrA* of

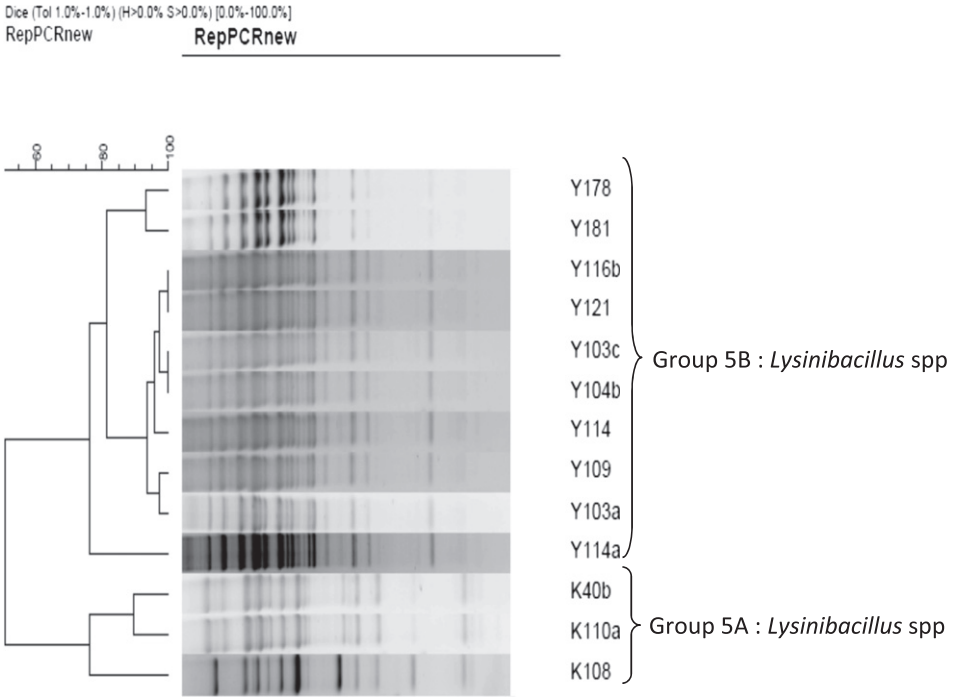

Fig. 3. Dendogram obtained by cluster analysis of Rep-PCR (GTG_5) fingerprints from group 5 isolates (see text). The dendogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

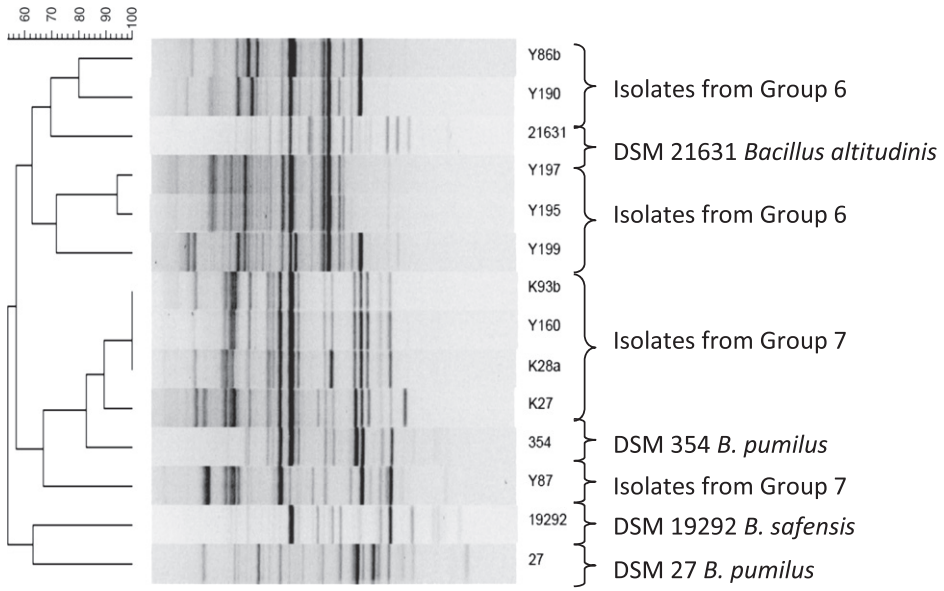

Fig. 4. Dendogram obtained by cluster analysis of M13-PCR (PM13) fingerprints from isolates representing isolates of Group 6 and Group 7 (see text). The dendogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

DSM 354, *B. safensis* DSM 19292 and *B. pumilus* DSM 27, respectively. *B. pumilus* DSM 354 showed 98% and 90.44% (*gyrA gene*) similarity with the type strain DSM 19292 (*B. safensis*) and DSM 27 (*B. pumilus*), respectively. These results strongly suggest that DSM 354 may be a *B. safensis* strain rather than a *B. pumilus* strain. All isolates of Rep-PCR Group 7 showed growth in TSA supplemented with 10% (w/v) NaCl and at 50 °C. Based on the observed features, it was concluded that the Rep-PCR Group 7 isolates were *B. safensis*.

Isolates occurring singularly or up to 3 isolates in a group were identified as *Bacillus flexus*, *Bacillus circulans*, *Paenibacillus dendritiformis*, *Brevibacillus laterosporus*, *Bacillus arayabatthai*, *Lysinibacillus* spp. and *Aneurinibacillus aneurinilyticus*, respectively, based on 16S rRNA sequencing (100%, 100%, 100%, 99%, 100%, 99.6% and 99.8% similarity to the respective sequences in EzTaxon).

3.3. Hemolytic activity and toxin gene profiles of *B. cereus*

Isolates identified as *B. cereus* (65 tested) showed hemolytic activity. The genes *hblA*, *hblC* and *hblD* encoding the enterotoxin protein complex, hemolysin BL were detected by PCR in 10, 22 and 23 isolates of the 65 isolates identified as *B. cereus*, respectively. All 65 isolates lacked the gene encoding the cytotoxin K-1 (*cytK-1*) while 59 of 65 isolates harbored the gene *cytK-2*. The emetic specific fragment *EM1* was also detected in 6 isolates. None of the 65 *B. cereus* isolates harbored all the toxin genes investigated.

4. Discussion

In the present study, the aerobic endosporeforming bacteria (AEFB) associated with Yanyanku and Ikpiru fermentation were identified using a combination of geno- and phenotypic methods. The AEFB species identified were similar in both additives and were composed mostly of *Bacillus* spp., with 54% of the isolates being members of the *B. subtilis* group (*B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens*). *Bacillus* spp. are usually reported to be the main micro-organisms responsible for the fermentation of high pH foods (Parkouda et al., 2009; Ouoba et al., 2008b). Several studies have reported the occurrence of *Bacillus subtilis sensu lato* species, with *Bacillus subtilis sensu stricto* often being dominant in similar alkaline fermented products such as Bikalga (Ouoba et al., 2007), Mbuja (Mohamadou et al., 2007) and Soumbala (Ouoba et al., 2004). *B. subtilis* was furthermore identified as the main *Bacillus* species in Sonru and Iru (Azokpota et al., 2007), condiments in which Yanyanku and Ikpiru are used as functional additives, respectively. This corresponds well with the results of the present study, where *B. subtilis* also was among the predominant species (25% of total number of isolates). Also *B. cereus* (19% of isolates) was in the present study identified as an important part of the microflora of Yanyanku and Ikpiru, confirming earlier studies, where *B. cereus* have been isolated from other alkaline fermented African foods based on fermented seed of *H. sabdariffa* such as Iru, Sonru, Afitin (Azokpota et al., 2007) and Mbuja (Mohamadou et al., 2007).

Furthermore, *Bacillus* species such as *B. safensis* (13%), *B. altitudinis* (7%), *B. aryabhattai* (<1%), *B. circulans* (<1%), and *B. flexus* (<1%) were isolated from Yanyanku and Ikpiru.

The genetic markers *gyrA* and *gyrB* have both been successfully used for differentiating the closely related *B. subtilis* group species (Chun and Bae, 2000; Wang et al., 2007). Similarly Satomi et al. (2006) reported that *B. safensis* share a mere 91.2% *gyrB* sequence similarity with *B. pumilus* suggesting that this marker is more useful for differentiation than the 16S rRNA gene. In the present study, the *B. safensis* type strain DSM 19292 shared 90.2% *gyrA* sequence similarity with the *B. pumilus* type strain DSM 27, which is similar to the results obtained by Satomi et al. (2006) for the *gyrB* gene. One strain deposited as *B. pumilus* (DSM 354) in the DSMZ culture collection was identified in 1952 (Smith et al., 1952) before the description of the species *B. safensis*. This strain showed 90.4% and 98% *gyrA* sequence similarity with *B. pumilus* DSM 27 and *B. safensis* DSM 19292, respectively, indicating that DSM 354 may be a *B. safensis* strain rather than a *B. pumilus* strain. Further, in the present study, we sequenced the *gyrA* gene of the *B. altitudinis* type strain. The sequence was 90%, 93% and 98% identical to those of the *B. pumilus*, and *B. safensis* type strains and *B. aerophilus* KACC 16563 (not a type strain), respectively (results not shown), and 99–100% identical to the strains identified as *B. altitudinis* by M13-PCR and phenotypic tests. The *gyrA* gene has not previously been used as marker for differentiating *B. pumilus* from *B. safenis* but based on the results obtained we suggest *gyrA* to be a useful marker for discriminating the above mentioned species; *B. pumilus* and *B. safensis* from each other, and *B. pumilus*, *B. safensis* from *B. altitudinis*/*B. aerophilus*.

Other genera (6.1% of isolates) were found in the additives: *B. laterosporus* (<1%), *A. aneurinilyticus* (<1%), *P. dendritiformis* (1%)

and *Lysinibacillus* (3.9%). *B. laterosporus*, *A. aneurinilyticus* and *P. dendritiformis* are aerobic endosporeforming bacteria but all of them were found to be Gram negative. This observation has previously been reported by Laubach (1916) (*B. laterosporus*), Raj et al. (2007) (*A. aneurinilyticus*) and Tcherpakov et al. (1999) (*P. dendritiformis*). The isolates of the genus *Lysinibacillus* were not clearly identified at species level in the present study. Differences in nucleotide numbers between sequences of isolates of Rep-PCR Groups 5A and B and the original GenBank sequences of *L. xylanilyticus* on one hand and *L. macroides* on the other hand were 7 and 11 to 14 base pairs, respectively. None of the phenotypic tests done on these isolates were able to confirm that they were either *L. macroides* or *L. xylanilyticus*. Thus, further studies will address the identification of these isolates possibly representing a novel species.

Several of the species identified in this study were also reported by Mohamadou et al. (2007), who identified in a comparable product (Mbuja, fermented product from *H. sabdariffa*) in Cameroon *B. licheniformis*, *B. cereus*, *B. pumilus*, *B. circulans*, *B. laterosporus*, and *B. brevis*. The different profiles of micro-organisms observed could be explained by variation of hygienic quality of the final product as reported by Sanni (1993) and Ouoba et al. (2007) for most traditional fermented foods.

B. cereus strains have been reported to produce non-hemolytic enterotoxin, hemolysin BL and cytoxin K causing diarrhea, and the heat stable emetic toxin cereulide causing vomiting (Stenfors et al., 2008). In the present study, the presence of genes encoding enterotoxins and *cesB* encoding a component of the cereulide synthetase complex varied according to the product (none of *B. cereus* strains from Ikpiru harbored the gene encoding the emetic specific gene fragment *EM1*) and to the isolate. None of the examined *B. cereus* strains harbored the *cytK-1* encoding gene, while *cytK-2* was present in 91% of *B. cereus* isolates from both additives. The result for *cytK* is consistent with previous reports showing that the *cytK-1* gene is only present in a limited number of strains, one of which has a reduced genome size compared to other *B. cereus* strains (Lapidus et al., 2008). The toxin *cytK-1* is highly toxic in the human intestine and represents a more severe hazard for consumers than *cytK-2* (Guinebretiere et al., 2006). The genes encoding hemolysin BL *hblA*, *hblC*, and *hblD* were present in 15%, 34% and 35% of *B. cereus* isolates from Yanyanku and Ikpiru, respectively; the percentages being much lower than for European *B. cereus* strains (59%, 72% and 64%) using the same primers (Hansen and Hendriksen, 2001). Only *B. cereus* strains (9%) from Yanyanku harbored the gene encoding the emetic specific gene fragment *EM1* encoding CesB. In previous studies, the presence of enterotoxin encoding genes and *cesB* has been detected in *B. cereus* strains isolated from several African alkaline fermented condiments such as Afitin, Iru and Sonru (Thorsen et al., 2010), and Soumbala and Bikalga (Ouoba et al., 2008b). Sonru and Iru processing involve the use of the functional additives Yanyanku and Ikpiru (Azokpota et al., 2006; Agbobatinkpo et al., 2011). Even though the additives are a source of contamination with *B. cereus*, the raw material African locust beans, has also been found to be contaminated with potentially toxin producing *B. cereus*, as enterotoxin and emetic toxin producing *B. cereus* were found in Afitin, a product produced without the use of functional additives (Thorsen et al., 2010, 2011a). There are no reports on foodborne illness due to the consumption of Afitin, Iru and Sonru (Azokpota et al., 2007; Thorsen et al., 2011a).

In conclusion, the present work identified the mixed-culture species involved in spontaneous *H. sabdariffa* seed fermentation with *B. subtilis* being the main species. Pathogenic spore forming bacteria were identified, emphasizing the need to identify and produce starter cultures for Yanyanku and Ikpiru fermentations able to prevent the growth of pathogenic bacteria and consistently produce fermented condiments of high quality.

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References

Agbobatinkpo, P.B., Azokpota, P., Akissoe, N., Kayodé, P., Da Gbadji, R., Hounhouigan, J.D., 2011. Indigenous perception and characterization of Yanyanku and Ikpiru two functional additives for the fermentation of African locust bean. Ecology of Food and Nutrition 50, 101–114.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhanz, J., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Azokpota, P., Hounhouigan, D.J., Nago, C.M., 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.

Azokpota, P., Moller, P., Hounhouigan, D.J., Jakobsen, M., 2007. Biodiversity of predominant *Bacillus* isolated from Afitin, Iru and Sonru at different fermentation time. International Journal of Biological and Chemical Sciences 1, 211–222.

Beecher, D.J., MacMillan, J.D., 1991. Characterization of the components of hemolysin BL from *Bacillus cereus*. Infection and Immunity 59, 1778–1784.

Chadare, F.J., Gayet, D.P., Azokpota, P., Nout, M.J.R., Linnemann, A.R., Hounhouigan, J.D., van Boekel, M.A.J.S., 2010. Three traditional fermented baobab foods from Benin, *Mutchayan, Dikouanyouri* and *Tayohounta*: preparation properties and consumption. Ecology of Food and Nutrition 49, 1–19.

Cheun, H.I., Makino, S.I., Watarai, M., Shirahata, T., Uchida, I., Takeshi, K., 2001. A simple and sensitive detection system for *Bacillus anthracis* in meat and tissue. Journal of Applied Microbiology 91, 421–426.

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.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B.K., Lim, Y.W., 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. International Journal of Systematic and Evolutionary Microbiology 57, 2259–2261.

Coorevits, A., Dinsdale, A.E., Heyrman, J., Schumann, P., Van Landschoot, A., Logan, N.A., De Vos, P., 2012. *Lysinibacillus macroides* sp. Nov., nom. Rev. International Journal of Systematic and Evolutionary Microbiology 62, 1121–1127.

Di Franco, C., Beccari, E., Santini, T., Pisaneschi, G., Tecce, G., 2002. Colony shape as a genetic trait in the pattern-forming *Bacillus mycoides*. BMC Microbiology 2, 33.

Ehling-Schulz, M., Fricker, M., Scherer, S., 2004a. *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. Molecular Nutrition & Food Research 48, 479–487.

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

European Food Safety Authority, 2008. Scientific opinion of the panel on biological hazards on a request from EFSA on the maintenance of the QPC list of microorganisms intentionally added to food or feed. The EFSA Journal 923, 1–48.

Fagerlund, A., Ween, O., Lund, T., Hardy, S.P., Granum, P.E., 2004. Genetic and functional analysis of the cytK family of genes in *Bacillus cereus*. Microbiology 150, 2689–2697.

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

Guinebretiere, M.H., Fagerlund, A., Granum, P.E., Nguyen-The, C., 2006. Rapid discrimination of *cytK-1* and *cytK-2 genes* in *Bacillus cereus* strains by a novel duplex PCR system. FEMS Microbiology Letters 259, 74–80.

Hansen, B.M., Hendriksen, N.B., 2001. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Applied and Environmental Microbiology 67, 185–189.

Henderson, I., Duggleby, C.J., Turnbull, P.C., 1994. Differentiation of *Bacillus anthracis* from other *Bacillus cereus* group bacteria with the PCR. International Journal of Systematic Bacteriology 44, 99–105.

Herigstad, B., Hamilton, M., Heersink, J., 2001. How to optimize the drop plate method for enumerating bacteria. Journal of Microbiological Methods 44 (2), 121–129.

Hoffmaster, R.A., Hill, K.K., Gee, E.J., Marston, K.C., De, K.B., Popovic, T., Sue, D., Wilkins, P.P., Avashia, B.S., Drumgoole, R., Helma, H.C., Ticknor, O.L., Okinaka, T.R., Jackson, J.P., 2006. Characterization of *Bacillus cereus* isolates associated with fatal pneumonias: strains are closely related to *Bacillus anthracis* and harbor *B. anthracis* virulence genes. Journal of Clinical Microbiology 3352–3360.

Jensen, G.B., Larsen, P., Jacobsen, B.L., 2002. Isolation and characterization of *Bacillus cereus*-like bacteria from faecal samples from greenhouse workers who are using *Bacillus thuringiensis*-based insecticides. International Archives of Occupational and Environmental Health 75, 191–196.

Lapidus, A., Goltsman, E., Auger, S., Galleron, N., Segurens, B., Dossat, C., Land, M.L., Broussolle, V., Brillard, J., Guinebretiere, M.H., Sanchis, V., Nguen-The, C., Lereclus, D., Richardson, P., Wincker, P., Weissenbach, J., Ehrlich, S.D., Sorokin, A., 2008. Extending the *Bacillus cereus* group genomics to putative food-borne pathogens of different toxicity. Chemico-Biological Interactions 171, 236–246.

Laubach, C.A., 1916. Studies on aerobic spore-bearing non pathogenic bacteria. Spore-bearing organisms in water. Journal of Bacteriology 1, 505–512.

Lechner, S., Mayr, R., Francis, K.P., Prüb, B.M., Kaplan, T., Wiebner-Gunkel, E., Stewart, G.S.A.B., Scherer, S., 1998. *Bacillus weihenstephanensis* sp. nov. is a

new psychrotolerant species of the *Bacillus cereus* group. International Journal of Systematic Bacteriology 48, 1373–1382.
Lee, C.S., Jung, Y., Park, T., Oh, T., Yoon, J., 2010. *Lysinibacillus xylanilyticus* sp. nov., a xylandegrading bacterium isolated from forest humus. International Journal of Systematic and Evolutionary Microbiology 60, 281–286.
Madhaiyan, M., Poonguzhali, S., Kwon, S., Sa, T., 2010. *Bacillus methylotrophicus* sp. nov., a methanol-utilizing, plant-growth-promoting bacterium isolated from rhizosphere soil. International Journal of Systematic and Evolutionary Microbiology 60, 2490–2495.
Mohamadou, B.A., Mbofung, C.M.F., Thouvenot, D., 2007. Functional potential of a product from traditional biotechnology antioxidant and probiotic potential of Mbuja, produced by fermentation of *Hibiscus sabdariffa* seeds in Cameroon. Journal of Food Technology 5, 164–168.
Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel, W.H., 2007. The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. International Journal of Food Microbiology 114, 168–186.
Oladunmoye, M.K., 2007. Effect of fermentation on nutrient enrichment of Locust beans (*Parkia biglobosa*, Robert bam). Research Journal of Microbiology 2, 185–189.
Ouoba, I.I.L., Diawara, B., Amoa-Awua, W.K., Traore, A.S., Moller, P.L., 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., 2007. Identification of *Bacillus spp*. from Bikalga, fermented seeds of *Hibiscus sabdariffa*: phenotypic and genotypic characterization. Journal of Applied Microbiology 104, 122–131.
Ouoba, I.I.L., Lei, V., Jensen, B.L., 2008a. Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. International Journal of Food Microbiology 121, 217–224.
Ouoba, I.I.L., Thorsen, L., Varnam, H.A., 2008b. Enterotoxins and emetic toxins production by *Bacillus cereus* and other species of *Bacillus* isolated from Soumbala and Bikalga, African alkaline fermented food condiments. International Journal of Food Microbiology 124, 224–230.
Parkouda, C., Nielsen, D.S., Azokpota, P., Ouoba, L.I.I., Amoa-Awua, W.K., Thorsen, L., Hounhouigan, J.D., Jensen, J.S., Tano-Debrah, K., Diawara, B., Jakobsen, M., 2009. The microbiology of alkaline-fermentation of indigenous seeds used as food condiments in Africa and Asia. Critical Reviews in Microbiology 35, 139–156.
Parkouda, C., Thorsen, L., Compaoré, S.C., Nielsen, D., Tano-Debrah, K., Jensen, S.J., Diawara, B., Jakobsen, M., 2010. Microorganisms associated with Maari, a Baobab seed fermented product. International Journal of Food Microbiology 142 (3), 292–301.
Raj, A., Chandra, R., Mohan, M., Reddy, K., Purohit, J.H., Kapley, A., 2007. Biodegradation of kraft lignin by a newly isolated bacterial strain, *Aneurinibacillus aneurinilyticus* from the sludge of a pulp paper mill. World Journal of Microbiology and Biotechnology 23, 793–799.
Roberts, M.S., Cohan, F.M., 1995. Recombination and migration rates in natural populations of *Bacillus subtilis* and *Bacillus mojavensis*. Evolution 49, 1081–1094.
Roberts, M.S., Nakamura, L.K., Cohan, F.M., 1994. *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acids composition. International Journal of Systematic Bacteriology 44, 256–264.
Sanni, A.I., 1993. The need for process optimization of African fermented foods and beverages. International Journal of Food Microbiology 18, 85–95.
Satokari, R.M., Vaughan, E.E., Akkermans-van, V.W.M., Saarela, M., de Vos, W.M., 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 67, 504–513.
Satomi, M., La Duc, T.M., Venkateswaran, K., 2006. *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. International Journal of Systematic and Evolutionary Microbiology 56, 1735–1740.
Shivaji, S., Chaturvedi, P., Suresh, K., Reddy, G.S.N., Dutt, C.B.S., Wainwright, M., Narlikar, J.V., Bhargava, P.M., 2006. *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. International Journal of Systematic and Evolutionary Microbiology 56, 1465–1473.
Smith, N.R., Gordon, R.E., Clark, F.E., 1952. Aerobic Spore Forming Bacteria. Monograph No 16. Department of Agriculture, Washington, U.S.
Stenfors, A.L.P., Fagerlund, A., Granum, P.E., 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Review 32, 579–606.
Sumpavapol, P., Tongyonk, L., Tanasupawat, S., Chokesajjawatee, N., Luxananil, P., Visessanguan, W., 2010. *Bacillus siamensis* sp. nov., isolated from salted crab (poo-khem) in Thailand. International Journal of Systematic and Evolutionary Microbiology 60, 2364–2370.
Tcherpakov, M., Ben-Jacob, E., Gutnick, D.L., 1999. *Paenibacillus dendritiformis* sp. nov., proposal for a new pattern-forming species and its localization within a phylogenetic cluster. International Journal of Systematic Bacteriology 49, 239–246.
Thorsen, L., Hansen, B.M., Nielsen, K.F., Hendriksen, N.B., Phipps, R.K., Budde, B.B., 2006. Characterization of emetic *Bacillus weihenstephanensis*, a new cereulide-producing bacterium. Applied and Environmental Microbiology 72, 5118–5121.
Thorsen, L., Azokpota, P., Hansen, B.M., Hounhouigan, D.J., Jakobsen, M., 2010. Identification, genetic diversity and cereulide producing ability of *Bacillus cereus* group strains isolated from Beninese traditional fermented food condiments. International Journal of Food Microbiology 142, 247–250.
Thorsen, L., Azokpota, P., Hansen, M.B., Ronsbo, H.M., Nielsen, F.K., Houinhouigan, D.J., Jakobsen, M., 2011a. Formation of cereulide and enterotoxins by *Bacillus cereus* in fermented African locust beans. Food Microbiology 28, 1441–1447.
Thorsen, L., Abdelgadir, S.W., Ronsbo, H.M., Abban, S., Hamad, H.S., Nielsen, S.D., Jakobsen, M., 2011b. Identification and safety evaluation of *Bacillus* species occurring in high numbers during spontaneous fermentations to produce Gergoush, a traditional Sudanese bread snack. International Journal of Food Microbiology 146, 244–252.
Wang, L.T., Lee, F.L., Tai, C.J., Kasai, H., 2007. Comparison of gyrB gene sequences, 16S rRNA gene sequences and DNA–DNA hybridization in the *Bacillus subtilis* group. International Journal of Systematic and Evolutionary Microbiology 57, 1846–1850.
Yamamoto, S., Harayama, S., 1995. PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of Pseudomonas-putida strains. Applied and Environmental Microbiology 61, 1104–1109.