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Enterotoxins and emetic toxins production by *Bacillus cereus* and other species of *Bacillus* isolated from Soumbala and Bikalga, African alkaline fermented food condiments

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ABSTRACT

The ability of various species of *Bacillus* from fermented seeds of *Parkia biglobosa* known as African locust bean (Soumbala) and fermented seeds of *Hibiscus sabdariffa* (Bikalga) was investigated. The study included screening of the isolates by haemolysis on blood agar, detection of toxins in broth and during the fermentation of African locust bean using the *Bacillus cereus* Enterotoxin Reverse Passive Latex Agglutination test kit (BCET-RPLA) and the *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (BDEVIA). Detection of genes encoding cytotoxin K (CytK), haemolysin BL (Hbl A, Hbl C, Hbl D), non-hemolytic enterotoxin (NheA, NheB, NheC) and EM1 specific of emetic toxin producers was also investigated using PCR with single pair and multiplex primers. Of 41 isolates, 29 *Bacillus* belonging to the species of *B. cereus*, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* showed haemolysis on blood agar. Using RPLA, enterotoxin production was detected for three isolates of *B. cereus* in broth and all *B. cereus* (9) in fermented seeds. Using BDEVIA, enterotoxin production was detected in broth as well as in fermented seeds for all *B. cereus* isolates. None of the isolates belonging to the other *Bacillus* species was able to produce enterotoxins either by RPLA or BDEVIA. Nhe genes were detected in all *B. cereus* while Hbl and CytK genes were detected respectively in five and six *B. cereus* strains. A weak presence of Hbl (A, D) and CytK genes was detected in two isolates of *B. subtilis* and one of *B. licheniformis* but results were inconsistent, especially for Hbl genes. The emetic specific gene fragment EM1 was not detected in any of the isolates studied.

1. Introduction

Among others, African fermented vegetable proteins include Soumbala and Bikalga. They are respectively fermented seeds of African locust bean also known as dawadawa (Nigeria) netetou (Senegal), kinda (Sierra Leone) and fermented seeds of *Hibiscus sabdariffa* also known as dawadawa botso (Niger), datou (Mali), furundu (Soudan). Soumbala and Bikalga are the most popular traditional fermented food condiments in Burkina Faso. They constitute to the African diet a valuable source of proteins (up to 30% for Bikalga and 47% for Soumbala), lipids, carbohydrates, essential amino, essential fatty acids and vitamins (Eka, 1980; Odunfa, 1985; Odunfa and Adesomoju, 1985; Abu-Tarboush et al., 1997; Bengaly, 2001; Yagoub et al., 2004; Ouoba et al., 2003a,b). Usually fermentation associated with vegetable proteins is alkaline, with *Bacillus* spp., mainly *Bacillus subtilis*, as the predominant bacteria responsible for the fermentation. Other species of *Bacillus* such as *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus badius*, *Bacillus sphaericus* and *Bacillus fusiformis* are also commonly found in the fermented seeds (Odunfa, 1981; Bengaly, 2001; Ouoba et al., 2004; Ouoba et al., 2008). The production of Soumbala and Bikalga is a traditional process and involves many steps, including cleaning, boiling (about 95 °C for up to 36 h), fermentation (48–72 h), steaming (Bikalga) and drying. The fermentation step is uncontrolled, leading to an instability and a variation of nutritional and hygienic quality of the final product. This problem has been observed in most traditional fermented foods in Africa (Sanni, 1993). Optimization of the production process is therefore important to increase acceptability and encourage consumption of a cheap source of protein. One major factor that contributes to an optimization of the production conditions is use of well-defined starter cultures for controlled fermentation (Odunfa, 1986; Sanni, 1993), leading to a product of consistent taste and quality, as well as improved marketability (Odunfa, 1986). Starter cultures may have other potential attributes (Holzapel, 1997) such as improved nutritional value by enrichment (e.g. through biosynthesis of vitamins, essential amino acids, and proteins), improved sensory quality, enhanced antimicrobial activity against foodborne pathogens, increased toxicological safety, reduction of preparation procedures, and demonstration of probiotic properties contributing to improvement of general health and well being.

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B. cereus causes both food poisoning and non-gastrointestinal infections (Drobniewski, 1993). Two principal types of food poisoning, emetic and diarrhoeal, have been described. The emetic type is caused by a small cyclic heat-stable peptide (cereulide), resulting in vomiting 0.5–6 h after ingestion (Agata et al., 1995; Ehling-Schulz et al., 2004a). The diarrhoeal type is attributed to enterotoxins, a group of heat-labile proteins including the three component toxins haemolysin BL (Hbl) (Beecher et al., 1995), non haemolytic enterotoxin (Nhe) (Granum et al., 1999), and the single protein, cytotoxin K (CytK) (Lund et al., 2000) causing abdominal pain and diarrhoea 8 to 16 h after consumption (Granum and Lund, 1997). Other species of *Bacillus* such as *B. subtilis*, *B. licheniformis*, *B pumilus*, *B. megaterium,* usually considered relatively safe, have been reported to produce enterotoxins and emetic toxins involved in foodborne illness (Rowan et al., 2003; From et al., 2005; Salkinoja-Salonen et al., 1999; From et al., 2007). From et al. (2007) identified a toxin, pumilacidin, produced by *B. pumilus,* as the probable cause of a food poisoning outbreak with acute symptoms developing during meals, followed few hours later by stomach cramps and diarrhoea of several days duration.

The aim of our study was to investigate ability of *Bacillus* isolates involved in fermentation of alkaline foods to harbour toxin genes and produce toxins under different conditions. This is part of an extended study to identify and select starter cultures, which may have probiotic capabilities, for controlled and safe fermentations, resulting in products free of toxins.

2. Material and methods

2.1. Microorganisms

Forty-one *Bacillus* isolates were investigated, including 16 isolates of *B. subtilis*, nine of *B. cereus*, seven of *B. licheniformis*, five of *B. pumilus*, one *B. badius*, one *B. sphaericus*, one *B. fusiformis* and one *Brevibacillus bortelensis* (Table 1). They were isolated from Bikalga (fermented seeds of *H. sabdariffa*) and Soumbala (fermented seeds of African locust beans, *Parkia biglobosa*) from different regions of Burkina Faso and identified by phenotyping and genotyping (Ouoba et al., 2004; Ouoba et al., 2008). Two isolates of *B. cereus* T1 (RIVM-BC307) and T2 (NC7401) (Agata et al., 1994) producing respectively enteroxins and emetic toxin were used as reference strains. Strain T1 was obtained from a survey in Dutch food commodities and kindly provided by Lucas Wijnands, Laboratory for Zoonoses and Environmental Microbiology, RIVM - Centre for Infectious Disease Control, Bilthoven, The Netherlands. Strain T2 was kindly provided by the Department of Food Science, Faculty of Life Science, University of Copenhagen, Frederiksberg, Denmark. During the study, the strains were maintained at 4 °C on Brain Heart Infusion (BHI) Agar prepared using BHI Broth (Oxoid CM129, Basingstoke, UK) with added 15 g/l of agar (Oxoid LP0013).

2.2. Haemolytic activity on blood agar

The initial investigation of enterotoxins production by the *Bacillus* isolates involved screening haemolytic activity on blood agar. Columbia agar base (Oxoid CM003) was autoclaved at 121 °C for 15 min and sheep blood (5%; Oxoid SR0051) added after cooling to 50 °C, before distribution into Petri dishes. The *Bacillus* isolates were streaked on the agar and incubated at 37 °C for 24 h. Haemolysis was recorded by appearance of a zone of clearing around the colonies.

2.3. Detection of Hbl enterotoxin production by RPLA

Each isolate was grown overnight in 10 ml of BHI (Oxoid CM225). One ml of the culture was centrifuged at 5000 g for 5 min and the pellet discarded. The presence of enterotoxin in the supernatant was detected using BCET-RPLA toxin detection kits (Oxoid TD0950A), following the instructions of the manufacturer. The BCET-RPLA kit detects in particular the L_2 component (encoded by *hblC*) of the Hbl enterotoxin complex (Beecher and Wong, 1994).

Table 1
Bacillus isolates studied and their origin

Bacillus isolates	Code	Origin
B. cereus	B1	Soumbala
	B2	Soumbala
	B8	Soumbala
	B13	Soumbala
	B17	Soumbala
	B18	Soumbala
	D2	Bikalga
	D3	Bikalga
	D7	Bikalga
	T1 (BC307)	Reference strain
	T2 (NC7401)	Reference strain
B. badius	I9	Bikalga
B. sphaericus	I4	Bikalga
B. fusiformis	D8	Bikalga
Brevibacillus bortelensis	H7	Bikalga
B. subtlis	A1	Bikalga
	A4	Bikalga
	B7	Soumbala
	B9	Soumbala
	B15	Soumbala
	C3	Bikalga
	C6	Bikalga
	E5	Bikalga
	F1	Bikalga
	F3	Bikalga
	G3	Bikalga
	G2	Bikalga
	H4	Bikalga
	I7	Bikalga
	I8	Bikalga
	J3	Bikalga
B. licheniformis	A3	Bikalga
	A6	Bikalga
	E2	Bikalga
	E3	Bikalga
	E4	Bikalga
	F9	Bikalga
	G5	Bikalga
B. pumilus	A2	Bikalga
	B6	Soumbala
	B10	Soumbala
	D5	Bikalga
	I10	Soumbala

2.4. Detection of Nhe enterotoxin production by BDEVI

Each isolate was grown overnight in 10 ml of BHI (Oxoid CM 225). The culture (10 ml) was centrifuged at 5000 g for 5 min and the pellet discarded. The presence of enterotoxin in 5 ml of supernatant was tested using the BDEVIA toxin detection kit (Tecra BDEVIA48, Biotrace International, Bridgend, UK) following the instruction of the manufacturer. The BDEVIA kit detects specifically the NheA, a 41 kDA component of the Nhe enterotoxin complex (Lund and Granum, 1996).

2.5. Detection of Hbl, Nhe and cytotoxin K genes by PCR

2.5.1. Extraction of DNA

Each isolate was streaked on BHI agar prepared using BHI Broth (Oxoid CM129) with an added 15 g/l of agar (Oxoid LP0013). The DNA of a pure colony was extracted using InstaGene (BioRad 732-6030, Hertfordshire, UK) according to the manufacturer's instructions. The extracted DNA was stored at −20 °C until required.

2.5.2. Detection of the genes Hbl (C, D, A), Nhe (A, B, C) and CytK

All isolates were tested for presence of the genes Hbl (A, C, D), Nhe (A,B,C) and Cyt k using two methods. The first involved use of a single pair of primers for detection of each gene Hbl A, Hbl C, Hbl D, Nhe A,

Nhe B, Nhe C, CytK and the second, use of multiplex primers for each group of Hbl and Nhe genes.

2.5.2.1. PCR using single pair of primers. The modified method of Guinebretiere et al. (2002) for detection of the Nhe, Hbl and CytK genes, was used. Amplification was carried out in 25 μl of reaction mixture containing 1 μl of DNA template, 2.5 μl of PCR buffer II (10×, Applied Biosystems N8080161, Foster City, Ca, USA), 2.5 μl of DNTP (2.5 mM; Promega U1511, Southampton, UK), 2.5 μl of $MgCl_2$ (25 mM, Applied Biosystems), 1 μl each primer (10 pmol/μl) (Table 2), 1 U of Taq DNA polymerase (Applied Biosystems) and 14.4 μl of autoclaved high purity water (Sigma, Gillingham, UK). Amplification consisted of 35 PCR cycles in a thermocycler (Applied Biosystems, Gene Amp PCR system 2700). The cycling program was: initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing temperature (Table 2) for 1 min and elongation at 72 °C for 2 min. The PCR ended with a final extension at 72 °C for 5 min and the amplified product cooled at 4 °C.

The DNA fragments were separated by applying 10 μl of each PCR product with 2 μl of loading buffer to 1.5% agarose gel (BioRad 4736). DNA molecular marker (Direct Load TM Wide Range DNA Marker; Sigma) was included as standard. The gel was run in tris-borate-EDTA (1x TBE; Sigma T4415) buffer for 30 min at 130 V, stained with ethidium bromide solution and photographed using an UV transilluminator.

The strain T1 was used as positive control for Hbl (A, C, D) and CytK and T2 for Nhe (A, B, C).

2.5.2.2. PCR using multiplex primers. For detection of Hbl genes using multiplex primers (Table 2), a modified method of Frederiksen et al. (2006) including the primers of Hansen and Hendriksen (2001) was used. Amplification was carried out in 25 μl of reaction mixture containing 5.0 μL of DNA, 2.5 μL of DNTP (2.5 mM), 1 μL of $MgCl_2$ (25 mM, Amplicon, Brighton, UK), primer amounts of 3 μL (30 pmol) for *hblA* and 0.5 μL (5 pmol) each for *hblD* and *hblC,* 0.5 μL of TEMPase Hot Start DNA Polymerase (Amplicon), 2.5 μL of 10xTEMPase bufferII ($KCl/(NH_4)2SO_4$, 15 mM MgCl2, 1%Tween20, Amplicon) and 5.5 μL of sterile high purity water. The PCR protocol of Frederiksen et al. (2006) was modified to an initial denaturation of 15 min at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 90 s. The final extension was at 72 °C for 5 min. Strain T1 was used as positive control.

For detection of Nhe genes using multiplex primers (Table 2) a modified method of Frederiksen et al. (2006), including the primers of Hansen and Hendriksen (2001), was also used. Amplification was carried out in 25 μl of reaction mixture containing 5.0 μL of DNA, 2.5 μL of DNTP (2.5 mM), 1 μL of MgCl2 (25 mM, Amplicon), 2 μL (20 pmol) of each primer (Table 2), 0.5μL of TEMPase Hot Start DNA Polymerase (Amplicon), 2.5 μL of 10xTEMPase buffer II (KCl/(NH4)2SO4, 15 mM MgCl2, 1%Tween20, Amplicon) and 5.5 μL of sterile high purity water (Sigma). The PCR protocol of Frederiksen et al. (2006) was modified to an initial denaturation of 15 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s. The final extension was at 72 °C for 5 min. Strain T2 was used as positive control. The DNA fragments were separated as previously described.

2.5.3. Detection of the emetic specific gene fragment EM1

This experiment was done according to the method of Ehling-Schulz et al. (2004b). The same PCR mixture as in 2.5.1.1 was applied, with the exception that TEMPase hot Start DNA Polymerase (Amplicon) was used instead of Taq DNA polymerase. The cycling program was: initial denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing 60 °C for 30 s and elongation at 72 °C for 1 min. The PCR was ended with a final extension at 72 °C for 5 min and the amplified product cooled at 4 °C. The DNA fragments were separated as previously described.

Table 2
Characteristics of primers

Primer name	Gene	Sequence (5′– 3′)	Primer position
HC F	*hblC*[a]	GATAC (T,C)AATGTGGCAACTGC	40–59
HC R		TTGAGACTGCTCG (T,C)TAGTTG	761–780
HD F	*hblD*[a]	ACCGGTAACACTATTCATGC	970–989
HD R		GAGTCCATATGCTTAGATGC	1780–1799
HA F	*hblA*[a]	AAGCAATGGAATACAATGGG	1951–1970
HA R		AGAATCTAAATCATGCCACTGC	3084–3105
NA F	*nheA*[a]	GTTAGGATCACAATCACCGC	430–449
NA R		ACGAATGTAATTTGAGTCGC	166–1185
NB F	*nheB*[a]	TTTAGTAGTGGATCTGTACGC	1682–1702
NB R		TTAATGTTCGTTAATCCTGC	2406–2425
NC F	*nheC*[a]	TGGATTCCAAGATGTAACG	2935–2953
NC R		ATTACGACTTCTGCTTGTGC	3599–3618
CK F	*cytk*[a]	ACAGATATCGG (G,T)CAAAATGC	1859–1878
Ck R		TCCAACCCAGTTWSCAGTTCD	2649–2668
Multiplex Hbl			
HblA1	*HblA*[b]	GTGCAGATGTTGATGCCGAT	671–690
HblA2		ATGCCACTGCGTGGACATAT	990–971
L_1A	*hblD*[b]	AATCAAGAGCTGTCACGAAT	2854–2873
L_1B		CACCAATTGACCATGCTAAT	3283–3264
L_2A	*hblC*[b]	AATGGTCATCGGAACTCTAT	1448–1467
L_2B		CTCGCTGTTCTGCTGTTAAT	2197–2178
Multiplex Nhe			
NheA 1	*nheA*[b]	TACGCTAAGGAGGGGCA	344–360
NheA 2		GTTTTTATTGCTTCATCGGCT	843–823
NheB 1	*nheB*[b]	CTATCAGCACTTATGGCAG	1500–1518
NheB 2		ACTCCTAGCGGTGTTCC	2269–2253
NheC 1	*nheC*[b]	CGGTAGTGATTGCTGGG	2820–2836
NheC 2		CAGCATTCGTACTTGCCAA	3401–3383
EM1F	Unknown[c]	GACAAGAGAAATTTCTACGAGCAAGTACAAT	–
EM1R		GCAGCCTTCCAATTACTCCTTCTGCCACAGT	–

[a]Guinebretiere et al., 2002. [b] Hansen and Hendriksen, 2001. [c] Gene of unknown function, Ehling-Schulz et al., 2004b.

2.6. Detection of enterotoxins using RPLA and BDEVIA during fermentation of African locust bean by B. cereus isolated from Soumbala and Bikalga

Dehulled seeds of African locust bean were boiled for 2 h, distributed in flasks (25 g) and autoclaved at 121 °C for 15 min. After cooling, separate flasks were inoculated with 5 ml (10^4 cfu/ml) of a pure culture of *B. cereus*. The inoculated seeds were incubated at 37 °C for 48 h to ferment. After fermentation, 10 ml of sterile distilled water was added to the beans. After mixing manually, 6 ml of liquid were removed and centrifuged at 10,000 g. Five ml and 4×25 μl of the supernatant were used to detect respectively the presence of toxin by BDEVIA and RPLA following the instructions of the manufacturers.

Unfermented seeds and fermented seeds from *B. subtilis* B7 that did not show any of the investigated genes were used as negative controls.

All experiments were repeated at least 3 times.

3. Results

Of 41 isolates studied, 29 showed hemolytic activity on blood agar (Table 3). This included 13 isolates of *B. subtilis*, eight of *B. cereus*, all isolates (5) of *B. pumilus*, and three of *B. licheniformis*. One isolate of *B. cereus* failed to show hemolytic activity. Using RPLA, pronounced presence of the L_2 component of the haemolysin BL complex encoded by *hblC* in broth was mainly detected for two isolates of *B. cereus* B13 and B17 from Soumbala (Table 3). Weak production of the L_2 component was also observed for three other isolates of *B. cereus* D2, D3, D7 from Bikalga. Using BDEVIA, production of the 41 kDa protein of the Nhe complex encoded by *nheA*, was detected for all *B. cereus* isolates with the highest production in broth for *B. cereus* strains D3 and D7 from Bikalga. Investigation of enterotoxin production during fermentation of African locust beans showed that *B. cereus* isolates produce Hbl (L_2) and Nhe (NheA) enterotoxins during

fermentation and production seems more pronounced in beans than in broth (Table 4). *B. cereus* strains B13 and B17 showed positive results for haemolysis, RPLA and BDEVIA. Enterotoxin production was not detected for any other species by either RPLA or BDEVIA.

As shown in Table 5, detection of genes encoding enterotoxins, cytotoxin K and the emetic specific gene fragment EM1 using PCR, showed that all *B. cereus* isolates contain the Nhe genes (A,B,C) encoding the production of the non-hemolytic enterotoxin complex. However, only *B. cereus* strains B13 and B17 contain all three Hbl genes (A, C, D) encoding haemolysin BL. A weak presence of Hbl A and C in *B. cereus* D2, D3 and D7, Hbl D in *B. cereus* D7 was also detected using the single pair PCR, but results were not reproducible. However, the latter results may be considered positive, since D2, D3, D7 were able to produce at least the L_2 component of the Hbl complex, as detected by RPLA. In general, none of the bacteria belonging to other species showed positive results for either Nhe or Hbl genes. Nevertheless, weakly positive results have been observed for *B. subtilis* A1 and *B. licheniformis* E3 for *Hbl A*, *B. subtilis* A1 and C3 for Hbl D, with a low degree of reproducibility. Results of detection of Hbl and Nhe genes respectively were largely in accordance with results of the RPLA and BDEVIA tests. Exceptions were *B. cereus* B1, B2, B8 and B18 from soumbala, which did not show any of the Hbl genes and did not produce toxins in broth (using the RPLA method) but did produce haemolysin BL (L_2 component) during fermentation of African locust bean. Differences in results were observed between the two methods used for detection of Nhe and Hbl genes. Using a single pair of primers with the specific sequences (Table 2), *B. cereus* B1, B2 were recorded as negative for Nhe A and Nhe B genes while *B. cereus* B8 was recorded negative for Nhe A. However, they were all positive for the same genes using the multiplex primers with different sequences described in Table 2. The same comment applies to *B. cereus* D2, D3 and D7, which were negative for Hbl genes on all occasions using the muliplex method but showed on some occasions positive results with the single pair method.

As seen in Table 5, *B. cereus* B8, B13, B17 from Soumbala and D2, D3 and D7 from Bikalga harbour the CytK gene encoding cytotoxin K production. A weakly positive, but relatively reproducible, result for the CytK gene was also observed for *B. licheniformis* E3. None of the *Bacillus* isolates investigated showed positive results for the emetic specific gene fragment EM1.

Of all 41 isolates studied, *B. cereus* B13 and B17 from soumbala harboured all the toxin genes investigated except the emetic specific gene fragment. All *B. cereus* isolates were also investigated for *B. thuringiensis* and *B. anthracis* toxin genes but were negative; furthermore, none of the isolates showed colonial morphology similar to *B. mycoides* (results not shown). It was observed that *B. subtilis* isolates B7, B9, B15 and *B. pumilus* B6 and B10 from soumbala that have been already selected as starter cultures and are in use for controlled fermentation of African locust bean at a production site in Burkina Faso, do not harbour or express any of the toxin genes investigated.

Table 3
Haemolysis on agar and enterotoxins production by RPLA and BDEVIA

Bacillus isolates		Haemolysis on blood agar	RPLA	BDEVIA
B. cereus	B1	+	−	4+
	B2	+	−	4+
	B8	+	−	3+
	B13	+	+++	3+
	B17	+	+++	3+
	B18	+	−	4+
	D2	+	±	4+
	D3	±	±	5+
	D7	−	±	5+
B. badius	I9	−	−	1−
B. sphaericus	I4	−	−	1−
B. fusiformis	D8	−	−	1−
Brevibacillus bortelensis	H7	−	−	1−
B. subtlis	A1	+	−	1−
	A4	+	−	1−
	B7	+	−	1−
	B9	+	−	1−
	B15	+	−	1−
	C3	+	−	1−
	C6	+	−	1−
	E5	−	−	1−
	F1	+	−	1−
	F3	−	−	1−
	G2	+	−	1−
	G3	+	−	1−
	H4	+	−	1−
	I7	+	−	1−
	I8	+	−	1−
	J3	−	−	1−
B. licheniformis	A3	−	−	1−
	A6	−	−	1−
	E2	+	−	1−
	E3	−	−	1−
	E4	+	−	1−
	F9	−	−	1−
	G5	+	−	1−
B. pumilus	A2	+	−	1−
	B6	+	−	1−
	B10	+	−	1−
	D5	+	−	1−
	I10	+	−	1−

Table 4
Production of enterotoxins during the fermentation of African locust beans

Bacillus isolates		RPLA	BDEVIA
B. cereus	B1	++	4+
	B2	++	4+
	B8	++	4+
	B13	+++	4+
	B17	+++	4+
	B18	++	4+
	D2	+	4+
	D3	++	4+
	D7	++	4+
B. subtilis	B7	−	−
Control		−	−

4. Discussion

Production of toxins investigated in this study varied according to isolate, species and type of toxin. As expected, all *B. cereus* isolates from either Soumbala or Bikalga harbour enterotoxin genes and are able to express the genes by producing enterotoxins, both in laboratory culture media and during fermentation of African locust bean. A high percentage (100%) of *B. cereus* harbour and express Nhe genes encoding non-haemolytic enterotoxin, as reported by Guinebretiere et al. (2002) for *B. cereus* isolates from vegetables. On the other hand, 66% harbour the CytK gene encoding cytotoxin K production, 55% the Hbl genes encoding haemolysin BL, and 0% the emetic specific gene fragment EM1.

Polymorphism among genes (differences in sequences) could cause inability to detect Hbl genes by PCR in some of the toxin-producing *B. cereus* strains, as reported by Hansen and Hendriksen (2006). Results obtained by Guinebretiere et al. (2002) indicated a high degree of polymorphism in the Hbl and Nhe genes (within the primer annealing sites) of food-associated isolates, as compared to strains responsible for food poisoning, leading to false negative PCR results, as observed in the present study for several *B. cereus* strains using the same primers. Similar false negative results were also observed by Hansen and Hendriksen (2001) for environmental isolates. *Bacillus cereus* B13 and B17 were positive in all PCR reactions for Nhe (A,B,C), Hbl (A,C,D) and Cyt K, showing a toxin gene profile typical of food poisoning strains, as observed by Guinebretiere et al. (2002). Production of all three components of the enterotoxin complexes Hbl and Nhe is necessary for biological activity (Lindback et al., 2004; Beecher and Wong, 1997).

Table 5
Detection of toxins genes in various species of *Bacillus*

Bacillus isolates		Nhe			Multiplex Nhe			Hbl genes			Multiplex Hbl			CytK	Emetic
		A	B	C	A	B	C	A	C	D	A	C	D		
B. cereus	B1	–[a]	–	+	(+)	+	+	–	–	–	–	–	–	–	–
	B2	–	–	+	(+)	+	+	–	–	–	–	–	–	–	–
	B8	+	+	+	+	+	+	–	–	–	–	–	–	+	–
	B13	+	+	+	+	+	+	+	+	+	+	+	+	+	–
	B17	+	+	+	+	+	+	+	+	+	+	+	+	+	–
	B18	–	+	+	+	+	+	–	–	–	–	–	–	–	–
	D2	+	+	+	+	+	+	–	V	–	–	–	–	+	–
	D3	+	+	+	+	+	+	–	V	–	–	–	–	+	–
	D7	+	+	+	+	+	+	–	V	V	–	–	–	+	–
	T1	nd	nd	nd	nd	nd	nd	+	+	+	+	+	+	+	–
	T2	+	+	+	+	+	+	nd	nd	nd	Nd	nd	nd	nd	+
B. badius	I9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. sphaericus	I4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. fusiformis	D8	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Brevibacillus bortelensis	H7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. subtlis	A1	–	–	–	–	–	–	V	–	V	–	–	–	–	–
	A4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B15	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	C3	–	–	–	–	–	–	–	–	V	–	–	–	–	–
	C6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E5	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Bacillus isolates		Nhe			Multiplex Nhe			Hbl genes			Multiplex Hbl			CytK	Emetic
		A	B	C	A	B	C	A	C	D	A	B	C		
B. subtlis	F1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	F3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	G2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	G3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	H4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	I7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	I8	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	J3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. licheniformis	A3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	A6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E3	–	–	–	–	–	–	V	–	–	–	–	–	(+)	–
	E4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	F9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	G5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. pumilus	A2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B10	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	I10	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a]: +: positive, (+): partial positive, V: variable, –: negative, nd: not detected.

As shown by Pruss et al. (1999), using immunological methods targeting each of the three components L_1, L_2 and B, 10 of 11 *B. cereus* strains able to produce the L_2 component also produced L_1 and B components of the Hbl complex, indicating a correlation between production of the L_2 component and the other two components.

The present study demonstrates that use of combined methods may be necessary to detect presence of toxin genes in bacteria. Differences observed between the single pair primers and the multiplex method can be attributable to differences in primer sequences. It has also been shown that ability of bacteria to produce toxins is not necessarily linked to their hemolytic activity. All the pre-cited toxins are involved in food poisoning, manifested by symptoms such as vomiting, diarrhoea and severe stomach pain. A concentration of 10^4–10^7 cfu/g of food enterotoxin-producing *Bacillus cereus* are required for diarrhoeal illness (Granum and Lund, 1997). This indicates that *B. cereus* isolates involved in fermentation of the traditional foods investigated potentially constitute a serious hazard in fermented foods, even though they are not usually the predominant bacterium; *B. subtilis* is predominant (Odunfa, 1981; Ouoba et al., 2004; Ouoba et al., 2008). Preparation of Soumbala and Bikalga usually involves a long cooking time that will destroy heat labile enterotoxins but not the heat stable cytotoxins (From et al., 2005) or the heat stable emetic toxin cereulide (Shinagawa et al., 1995). Furthermore, *B. cereus* is a spore-forming bacterium and there is a possibility that ingested spores may germinate and outgrow in the large intestine and cause diarrhoea (From et al., 2007). None of the *B. cereus* investigated harbour the emetic gene. Although Altayar and Sutherland (2005) reported that emetic toxin producing *B. cereus* is rare, many *B. cereus* and closely related species from many different origins has been reported as producers of the emetic toxin cereulide involved in food-borne illness (Ehling-Schulz et al., 2005; Thorsen et al., 2006). Our results showing that some *B. cereus* isolates do not produce toxins in broth but do so in fermented beans are in accordance with findings from a previous study, in which only four of 18 *Bacillus* isolates from clinical and food environments harbouring all three Hbl genes produced Hbl enterotoxin in BHI (Rowan et al., 2001). However, a further eight isolates belonging to various species of *Bacillus* were found to produce Hbl enterotoxin after growth in reconstituted baby food. This suggests that many *Bacillus* isolates that have the necessary diarrhoeagenic genes require specific environmental signal(s) for

transcriptional activation (Rowan et al., 2003). Environmental signals have been shown previously to modulate virulence factor expression in *B. cereus* and in other bacterial enteropathogens (Rowan and Anderson, 1997; Rowan, 1999).

Most of the isolates belonging to the other species of *Bacillus* studied have not been shown to harbour and express toxin genes and could be considered safe regarding these toxins. Exceptions include two isolates of *B. subtilis* and one of *B. licheniformis* that sometimes showed slight positive results for Hbl and CytK genes, with however low percentage of reproducibility for the Hbl genes. Although, those results should be considered as truly positive, the bacteria were not able to express the gene by producing toxin in either broth or fermenting seeds, using RPLA. Although *Bacillus* belonging to the *B. subtilis* group are usually considered safe (Hosoi et al., 2003; Sanders et al., 2003), *B. licheniformis* and *B. subtlis* can, in some circumstances, causes foodborne diseases (Kramer and Gilbert, 1989). In addition, investigation of toxins production by *Bacillus* species other than *B. cereus* showed that some strains of *B. subtilis*, *B. mojavensis*, *B. licheniformis*, *B. pumilus* and *B. fusiformis* harbour toxin genes and are capable of producing either cytotoxins, enterotoxins or ring-formed emetic toxins (Rowan et al., 2003; From et al., 2005; Salkinoja-Salonen et al., 1999; rom et al., 2007).

The results obtained in the present study demonstrate again the safety issue associated with foods prepared by traditional, uncontrolled fermentation and the necessity of selecting starter cultures for controlled fermentation, leading to a final product with increased nutritional and hygienic quality as well as improved stability (Odunfa, 1986; Sanni, 1993). *B. subtilis* B7, B9, B15 and *B. pumilus* B6, B10 have been selected as starter cultures for controlled production of Soumbala and are in use in a semi-industrial production site in Burkina Faso. This selection was based on their ability to degrade proteins, lipids and non-digestible oligosaccharides of the beans as well as their ability to inhibit and inactivate pathogenic bacteria and moulds (Ouoba et al., 2003a; Ouoba et al., 2003b; Ouoba et al., 2007a; Ouoba et al., 2007b). Controlled fermentations using the cited bacteria followed by sensory analysis by producers and consumers of Soumbala were conducted and Soumbala produced using starter cultures were clearly preferred to the traditional product (Ouoba et al., 2005). The study of their antimicrobial activity clearly showed the ability of mainly *B. subtilis* B7 and B15 to inactivate Gram positive (*B. cereus*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Salmonella enteritidis*, *Shigella flexneri*) as well as pathogenic moulds (as *Aspergillus ochraceus*) during controlled fermentation of African locust beans (Ouoba et al., 2007b). The use of starter culture preparations containing the two cited (B7 and B15) bacteria will prevent the occurrence of pathogenic and toxigenic bacteria as *B. cereus* during the fermentation of African locust beans leading then to a final safe product.

B. cereus B13 and B17 from Soumbala appeared to be the most toxigenic isolates. Most of the *Bacillus* species outside the *B. cereus* group investigated in the present study do not harbour toxin genes and can remain under consideration for use as starter cultures and probiotic bacteria.

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