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Enterotoxin Production by *Bacillus cereus* Under Gastrointestinal Conditions and Their Immunological Detection by Commercially Available Kits

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Abstract

Currently, three commercial kits for *Bacillus cereus* enterotoxins Nhe and/or Hbl detection are available, namely, the *Bacillus* diarrheal enterotoxin visual immunoassay (BDE VIA™) kit (3M Tecra), *B. cereus* enterotoxin reversed passive latex agglutination (BCET-RPLA) kit (Oxoid), and the Duopath® Cereus Enterotoxins (Merck). The performance of the kits and their applicability to gastrointestinal simulation samples were evaluated. Then, the stability and production of enterotoxins Hbl and Nhe under gastrointestinal conditions were investigated. Enterotoxin production was absent or impaired at acidic pH, i.e., in gastric medium with pH 5.0 and lasagne verde with pH 5.5. *B. cereus* did produce enterotoxins Nhe and Hbl during anaerobic growth in intestinal medium at pH 7.0, but the toxins were instantly degraded by the enzymes in the host's digestive secretions. Preformed enterotoxins did not withstand gastrointestinal passage under the simulated conditions, which suggests that preformed enterotoxins in food do not contribute to the diarrheal food poisoning syndrome. In conclusion, diarrhea is probably caused by *de novo* enterotoxin production by *B. cereus* cells located closely to the host's intestinal epithelium.

Introduction

Bacillus cereus can cause emetic and diarrheal food poisoning by production of cereulide and several enterotoxins such as non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl, cytotoxin K (CytK) and enterotoxin FM and virulence factors such as hemolysins (HlyII and HlyIII), collagenases, phospholipases C and cereolysins, respectively (Ceuppens *et al.*, 2011).

B. cereus enterotoxins are unstable molecules, susceptible to heating (>55°C for ≥20 min) and protease activity (pronase, pepsin, trypsin, and chemotrypsin, 1–2 mg/mL, 1–24 h) (Granum *et al.*, 1993; Turnbull *et al.*, 1979). As a result, preformed enterotoxins in food are highly unlikely to retain their activity after food preparation and gastrointestinal passage. In contrast, the emetic toxin cereulide is highly resistant to heat (resistant to all normal food processing and food preparation temperatures), acid (resistant to pH values of 2–11), and protease activity (pepsin and trypsin) (Rajkovic *et al.*, 2008; Shinagawa *et al.*, 1996). Consequently, cereulide is not inactivated during gastrointestinal passage, and preformed cereulide in food plays an prominent role in emetic food poisoning.

Multiple detection methods for enterotoxins exist, including mass-spectrometry, immunological assays, and biological assays such as the vascular permeability reaction, rabbit ileal loop, and cytotoxicity assays. The biological assays are functional assays that determine the overall toxicity, resulting in the advantage of detecting all biologically active toxins with usually high sensitivity. On the other hand, the inherent pitfalls of biological assays are the dubious specificity, resulting in false-positive results for samples which contain other toxins. Moreover, enterotoxins Nhe, Hbl, and CytK are all toxic for Vero cells (Wehrle *et al.*, 2009), so a positive cytotoxicity assay requires further analysis to reveal which specific enterotoxins the *B. cereus* strain produces. Similar to the liquid chromatography mass spectrometry (LC-MS) assay for *B. cereus* emetic toxin cereulide (Delbrassinne *et al.*, 2011), all *B. cereus* enterotoxins can be detected by mass spectrometry assays (Gilois *et al.*, 2007). The strong points of these methods include their high specificity and sensitivity, while the main drawbacks are the extensive, labor-intensive sample preparation and high investment, running, and maintenance costs. The relatively fast, easy, and cheap immunological detection of enterotoxins make it suitable for research and routine

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Table 1. Overview of the Currently Available Commercial Kits for *Bacillus cereus* Diarrheal Toxin Detection

Characteristics of the kits	*BDE VIA™ (3M-Tecra)*	*BCET-RPLA (Oxoid)*	*Duopath® (Merck)*
Target toxin component	Nhe-AB	Hbl-L2	Nhe-B and Hbl-L2
Minimal sample volume (μL)	200	50	150
Sample type	food	food, isolate	food, isolate
Detection limit (ng/mL)			
according to the manufacturer	1	2	NA
according to scientific reports	2 to 5[a]	≥ 0.6[a]	6[b] and 20[b]
Price (€/sample)	12	19	14
Time until result (h)	5	20	0.5

[a]Beecher and Wong, 1994.
[b]Krause *et al.*, 2010.
NA, none available.

analysis. Their sensitivity and specificity for a particular toxin are high, and depend on the antibody quality (Beecher and Wong, 1994). Commercial detection methods for *B. cereus* enterotoxins are available in the form of immunological kits for components Nhe-A, Nhe-B, and Hbl-L2, but currently none exist for CytK or other virulence factors. An overview of the presently available kits, namely the BCET-RPLA kit (Oxoid, Lenexa, KS), the BDE VIA™ kit (3M-Tecra, St. Paul, MN), and the Duopath® kit (Merck, Whitehouse Station, NJ), is presented in Table 1.

The aim of this study was threefold: (i) to compare the currently available commercial kits for *B. cereus* enterotoxin detection in pure cultures and in gastrointestinal samples; (ii) use these kits to evaluate the enterotoxin stability under gastrointestinal conditions; and (iii) to investigate enterotoxin production by *B. cereus* under gastrointestinal conditions.

Materials and Methods

Enterotoxin detection

Samples of approximately 2 mL were taken. After pH adjustment of 7.0–8.0 (required by the BDE-VIA™ kit), the samples were sterilized by filtration over a 0.2-μm cellulose acetate membrane filter (Whatman, Springfield Mill, UK; required by the BCET-RPLA kit) and analyzed with the three different kits according to the manufacturer's instructions. The characteristics of the three commercial kits presently available for *B. cereus* enterotoxin detection are presented in Table 1.

Performance of the commercial kits for B. cereus *enterotoxin detection*

Antibody cross-reactivity to the medium components and other *B. cereus* enterotoxins was assessed for all kits by analyzing non-inoculated Tryptone Soya Broth (TSB), Brain-Heart Infusion (BHI), and gastrointestinal simulation media, of which the composition was based on an *in vitro* digestion model (Oomen *et al.*, 2003) with the addition of 8 g/L reactor feed for Simulator of the Human Intestinal Microbial Ecosystem (SHIME) (Molly *et al.*, 1993; Possemiers *et al.*, 2004) as the food source. The enterotoxin production of seven *B. cereus* strains (Table 2) was assessed after cultivation in BHI for 18 h at 36°C (according to the instruction of the BCET-RPLA kit) and in TSB for 24 h at 30°C (in-house protocol). The relative detection limits of the immunological kits were determined by serial dilution of the cell-free culture supernatant of *B. cereus* American Type Culture Collection (ATCC) 14579 (cultivation in TSB at 30°C for 24 h) in sterile TSB.

Stability of enterotoxins in TSB, gastric simulation medium, and intestinal simulation medium

A non-purified mixture of enterotoxins was obtained by filter sterilization (0.2 μm cellulose acetate membrane filter;

Table 2. Enterotoxin Production by *Bacillus cereus* Strains Cultivated in Tryptone Soya Broth (TSB) for 24 h at 30°C and in Brain-Heart Infusion (BHI) for 18 h at 36°C

			Haemolysin BL (Hbl)		*Non-haemolytic enterotoxin (Nhe)*	
B. cereus *strain*	*Origin*	*Minimal growth temperature (°C)*	*BCET-RPLA (Oxoid)*	*Duopath® (Merck)*	*Duopath® (Merck)*	*BDE VIA™ (3M-Tecra)*
ATCC 14579	reference type strain	> 10	+	+	+	+
NVH 1230-88	clinical (human faeces)	8	+	+	+	+
RIVM 9903295-4	clinical (human faeces)	7	±	−	+	+
FF 137	food (lasgane verde)	7	+	−	+	+
FF 73	food (lasgane verde)	10	+	+	+	+
LFMFP 381	food (dried potato flakes)	> 10	+	−	+	±
LFMFP 710	food (mashed potatoes)	7	±	±	+	±

ATCC, American Type Culture Collection; NVH, Norwegian School of Veterinary Science (Norwegian: Norges Veterinærhøgskole); RIVM, National Institute for Public Health and the Environment (Dutch: Rijksinstituut voor Volksgezondheid en Milieu); FF, Flanders' Food, Belgium; LFMFP, Laboratory of Food Microbiology and Food Preservation, Ghent University, Belgium.
+, always positive; −, always negative; ±, both + and − results obtained; ND, not determined.

Table 3. Relative Detection Limits of the Immunological Kits, Determined by Dilution of Cell-Free Culture Supernatant of *Bacillus cereus* American Type Culture Collection (ATCC) 14579 Cultivated in Tryptone Soya Broth (TSB) for 24 h at 30°C in Sterile TSB, and the Investigation of Cross-Reactions by Testing the Positive Controls of the Kits and Gastrointestinal Simulation Media and Lasagne Verde as Negative Controls

	Haemolysin BL (Hbl)		*Non-haemolytic enterotoxin (Nhe)*	
Sample	*BCET-RPLA (Oxoid)*	*Duopath® (Merck)*	*Duopath® (Merck)*	*BDE VIA™ (3M-Tecra)*
Cell-free culture supernatant of *B. cereus* ATCC 14579				
Undiluted	+	+	+	+
10× diluted	+	+	+	+
50× diluted	+	−	+	−
100× diluted	+	−	+	−
500× diluted	−	−	−	−
Positive control BDE VIA™ kit (3M-Tecra)	−	−	+	+
Positive control BCET-RPLA kit (Oxoid)	+	+	−	−
Tryptone Soya broth (TSB)	−	−	−	−
Brain Heart Infusion (BHI)	−	−	−	−
Gastric medium (undiluted)	+	−	−	−
Gastric medium (2× diluted)	+	−	−	−
Gastric medium (5× diluted)	−	−	−	−
Intestinal medium (undiluted)	+	−	−	−
Intestinal medium (2× diluted)	−	−	−	−
Intestinal medium (5× diluted)	−	−	−	−
Lasagne verde (10× diluted)	−	−	−	−

Whatman) of *B. cereus* NVH (Norwegian School of Veterinary Science; Norwegian: Norges Veterinærhøgskole) 1230-88 cultivated in TSB for 24 h at 30°C. One-milliliter samples of this enterotoxin mixture were incubated statically at 37°C in glass tubes containing 9 mL of sterile TSB with pH 7.2 for 24 h, in gastric simulation medium with pH 5.0 or pH 2.0 for 2 h, and in intestinal simulation medium with pH 6.5 for 2 h.

Enterotoxin production by B. cereus *during growth in gastric simulation medium, intestinal simulation medium, and lasagne verde*

B. cereus NVH 1230-88 vegetative cells (1 mL of a 24-h TSB culture at 30°C) were cultured in 59 mL gastric or intestinal medium for 6 h with or without competing bacteria in 120-mL glass bottles sealed with rubber caps at 37°C with gentle shaking (Yellow Line OS10 shaker [IKA] at 90 rpm) (Ceuppens *et al.*, 2010). The intestinal bacteria were obtained from the colon ascendens vessel of SHIME, in which a mixed bacterial community representative for the human colon is cultivated (Molly *et al.*, 1993; Possemiers *et al.*, 2004). The conditions were indicated as micro-aerobic if the headspace of bottles consisted of air, since the bottles contained 1–2 mM L-cystein (Sigma-Aldrich, St. Louis, MO) originating from the SHIME feed. Anaerobic conditions were obtained by flushing the headspace of the bottles with pure nitrogen gas for 30 cycles. By inserting a sterile syringe through the rubber cap, samples were taken for enterotoxin detection and for *B. cereus* enumeration by the spread plate method on Tryptone Soya Agar (TSA), except when intestinal bacteria were added. No replicate experiments were performed.

No significant differences were observed between *B. cereus* NVH 1230-88 and *B. cereus* ATCC 14579, so following experiment was performed with the latter, the type strain *B. cereus* ATCC 14579. Vegetative cells (100 μL of a 24-h TSB culture at 30°C) were inoculated in 30 g of lasagne verde (commercially obtained in a local supermarket) in stomacher bags at 30°C for 48 h in triplicate. The enterotoxin production was assessed with the Duopath® kit after filter sterilization (0.2 μm cellulose acetate filter, Whatman) of lasagne samples homogenized and 10-fold diluted in Physiological Peptone Salt solution (8.5 g/L NaCl (Sigma-Aldrich), 1 g/L peptone (neutralized bacteriological peptone; Oxoid). The total count was determined by plating on TSA and the *B. cereus* concentration by plating on Mannitol Egg-Yolk Polymyxin-B agar (MYP).

Enterotoxin production by B. cereus *under intestinal conditions*

The growth and enterotoxin production of *B. cereus* (strains 1–5 in Table 2) in 200 mL of intestinal simulation medium at 37°C at pH 7.0 (automatic pH controllers Fermac 260; Electrolab, Tewkesbury, UK) under anaerobiosis (30-min flushing of the headspace with nitrogen gas) was investigated in triplicate. The *B. cereus* inocula consisted of 10 mL of stationary culture in TSB (24 h at 30°C) and 60 g of lasagne verde (homogenized by stomachering for 3 min) as the food source. Every 2 h, *B. cereus* was enumerated by plating on TSA and the Duopath® kit tested for enterotoxins Hbl and Nhe. The intestinal simulation medium contained the following digestive host secretions: bile (bovine dehydrated fresh bile; Difco), pancreatin (from porcine pancreas; Sigma-Aldrich), lipase (porcine lipase; Sigma-Aldrich), and pepsin (from porcine stomach mucosa; Sigma-Aldrich), which were present in the vessels in the final concentrations of, respectively, 1.0, 1.0, 0.2, and 0.2 g/L. To determine the influence of digestive host secretions on enterotoxin detection and production, these enzymes were omitted during specific experiments. To investigate potential food matrix effects, 57 mL of sterile TSB

Table 4. Enterotoxin Stability in Tryptone Soya Broth (TSB), and Gastric and Intestinal Simulation Medium at 37°C

Medium	Time (h)	*Haemolysin BL (Hbl)*		*Non-haemolytic enterotoxin (Nhe)*	
		BCET-RPLA (Oxoid)	*Duopath® (Merck)*	*Duopath® (Merck)*	*BDE VIA™ (3M-Tecra)*
TSB (pH 7.2)	0.5	+	+	+	+
	6.0	+	+	+	+
	24.0	+	+	+	+
Gastric medium (pH 5.0)	0.5	+	+	+	+
	2.0	+	+	+	+
Gastric medium (pH 2.0)	0.5	−	−	−	−
	2.0	−	−	−	−
Intestinal medium (pH 6.5)	0.5	+	−	+	−
	1.0	+	−	−	−
	2.0	−	−	−	−

substituted the 60 g of lasagne verde as the food source. To enable detection of only newly produced toxins, the enterotoxins in the inoculum's culture supernatant were removed by centrifugation at 5000 *x g* for 30 min, followed by resuspension of the cells in 10 mL of sterile TSB prior to their inoculation in the vessels.

Results

Performance of the commercial kits for B. cereus *enterotoxin detection*

The three tested kits were specific for either Nhe or Hbl enterotoxin target components, since no cross-reactivity was revealed by the positive controls of the BCET-RPLA (Oxoid) and the BDE VIA™ (3M-Tecra) kits, and the negative medium controls (Table 3). Except for the BCET-RPLA kit, which required, respectively, fivefold and twofold dilution of gastric and intestinal medium to prevent false positive results. Supporting evidence for the BDE VIA™ kit having two target components Nhe-A and Nhe-B (Beecher and Wong, 1994) was found, since its positive Nhe-B control was also detected by the Duopath® kit (Merck). Nevertheless, the detection limit for Hbl-L2 was 10-fold lower with the BCET-RPLA kit than with the Duopath® kit, while the Duopath® kit was 10-fold more sensitive for Nhe than the BDE VIA™ kit (Table 3).

Enterotoxin Nhe and Hbl detection in the culture supernatant of different *B. cereus* strains with the different kits corresponded well with each other with minor differences (Table 2). The observed differences can be explained by the

Table 5. Production of Enterotoxins by *Bacillus cereus* During Growth in Gastric and Intestinal Simulation Media at 37°C and in Lasagne Verde at 30°C

Growth conditions	Time (h)	B. cereus *(log CFU/mL ± standard deviation)*	*Haemolysin BL (Hbl)*		*Non-haemolytic enterotoxin (Nhe)*	
			BCET-RPLA kit	*Duopath® kit*	*Duopath® kit*	*BDE VIA™ kit*
Gastric medium, micro-aerophilic,	0	5.8	+	−	+	−
pH 5.0, 37°C, *B. cereus* NVH 1230-88	2	6.0	+	−	+	−
	4	7.6	+	−	+	−
	6	7.9	−	−	+	−
Intestinal medium, micro-aerophilic,	0	5.8	+	−	−	−
pH 6.5, 37°C, *B. cereus* NVH 1230-88	2	6.0	+	−	−	−
	4	7.8	−	−	−	−
	6	7.9	−	−	−	−
Intestinal medium, anaerobic,	0	5.8	+	−	−	−
pH 6.5, 37°C, *B. cereus* NVH 1230-88	2	6.1	−	−	−	−
	4	7.5	−	−	−	−
	6	7.8	−	−	−	−
Intestinal medium, anaerobic, pH 6.5, 37°C,	0	ND	+	−	−	−
B. cereus NVH 1230-88, 6.5 log CFU/mL	2	ND	−	−	−	−
intestinal bacteria added initially	4	ND	−	−	−	−
	6	ND	−	−	−	−
Lasagne verde, aerobic, pH 5.5, 30°C,	0	4,5 ± 0,1	ND	−	−	ND
B. cereus ATCC 14579, 4.8 log CFU/mL	24	7,3 ± 0,3	ND	±	±	ND
bacteria naturally present	48	7,4 ± 0,4	ND	±	±	ND

+, positive; −, negative; ±, both + and − results obtained (one replicate was positive, while two were negative).
ND, not determined.

Table 6. Anaerobic Growth and Enterotoxin Production (Assessed with the Duopath® Kit) by *Bacillus cereus* in Intestinal Simulation Medium with and Without Digestive Secretions and Either Lasagne Verde or Tryptone Soya Broth (TSB) as the Food Source Kept at pH 7.0 and 37°C

Intestinal medium	*Food source*	B. cereus *inoculum*	*Parameter*	*Time* 0–1 h	2 h	4 h	6 h	8 h
Normal composition (final concentration of 1.0 g/L bile, 1.0 g/L pancreatin, 0.2 g/L lipase, 0.2 g/L pepsin)	Lasagne verde	*B. cereus* NVH 1230-88	log CFU/mL	5,35 ± 0,11	5,94 ± 0,66	6,98 ± 0,56	7,74 ± 0,54	8,00 ± 0,30
			Hbl	–	–	–	–	–
			Nhe	–	–	–	–	–
		B. cereus RIVM 9903295-4	log CFU/mL	4,83 ± 0,14	5,18 ± 0,00	5,90 ± 0,46	6,40 ± 0,14	7,24 ± 0,38
			Hbl	–	–	–	–	–
			Nhe	–	–	–	–	–
		B. cereus FF 137	log CFU/mL	4,21 ± 0,20	4,30 ± 0,03	5,35 ± 0,41	6,48 ± 0,40	7,22 ± 0,29
			Hbl	–	–	–	–	–
			Nhe	–	–	–	–	–
		B. cereus FF 73	log CFU/mL	5,32 ± 0,15	5,64 ± 0,16	7,01 ± 0,42	7,77 ± 0,35	8,04 ± 0,29
			Hbl	–	–	–	–	–
			Nhe	–	–	–	–	–
		B. cereus ATCC 14579	log CFU/mL	5,53 ± 0,10	5,71 ± 0,09	6,75 ± 0,32	7,44 ± 0,16	7,73 ± 0,16
			Hbl	–	–	–	–	–
			Nhe	–	–	–	–	–
		B. cereus ATCC 14579 (washed)	log CFU/mL	5,40 ± 0,16	5,67 ± 0,04	6,89 ± 0,48	7,49 ± 0,33	7,79 ± 0,29
			Hbl	–	–	–	–	–
			Nhe	–	–	–	–	–
	Tryptone Soya broth (TSB)	*B. cereus* ATCC 14579	log CFU/mL	5,69 ± 0,07	5,95 ± 0,07	7,17 ± 0,38	7,70 ± 0,18	7,66 ± 0,09
			Hbl	–	–	–	–	–
			Nhe	–	–	–	–	–
		B. cereus ATCC 14579 (washed)	log CFU/mL	5,78 ± 0,12	5,78 ± 0,22	6,80 ± 0,25	7,34 ± 0,23	ND
			Hbl	–	–	–	–	–
			Nhe	–	–	–	–	–
No digestive secretions	Tryptone Soya broth (TSB)	*B. cereus* ATCC 14579	log CFU/mL	5,62 ± 0,04	6,57 ± 0,19	7,74 ± 0,27	8,03 ± 0,30	8,04 ± 0,02
			Hbl	–	–	+	+	+
			Nhe	+	+	+	+	+
		B. cereus ATCC 14579 (washed)	log CFU/mL	5,75 ± 0,34	6,83 ± 0,04	7,92 ± 0,08	8,22 ± 0,32	8,11 ± 0,11
			Hbl	–	–	+	+	+
			Nhe	–	–	+	+	+
	Lasagne verde	*B. cereus* ATCC 14579	log CFU/mL	5,70 ± 0,06	6,19 ± 0,29	7,82 ± 0,16	8,35 ± 0,14	8,65 ± 0,25
			Hbl	+	+	+	+	+
			Nhe	+	+	+	+	+
		B. cereus ATCC 14579 (washed)	log CFU/mL	5,68 ± 0,11	6,19 ± 0,20	7,61 ± 0,20	8,29 ± 0,27	8,78 ± 0,18
			Hbl	–	–	+	+	+
			Nhe	–	–	+	+	+

+, positive; –, negative.
ND, not determined.

different detection limits of the kits, the varying levels of enterotoxin production among different *B. cereus* strains and the varying enterotoxin production by the same strain under different growth conditions. For example, the supernatant of *B. cereus* strain LFMFP 381 tested negative for Hbl-L2 with the Duopath® kit, but with the more sensitive BCET-RPLA kit a positive result was obtained. Some strains showed variable results for enterotoxin production after different culturing conditions. For example, the psychrotrophic strain LFMFP 710 tested negative for the Hbl target component after 18 h incubation in BHI at 36°C, but positive results were obtained after 18 h in BHI at 30°C and 24 h in TSB at 30°C.

Stability of enterotoxins in gastrointestinal simulation media

The enterotoxin Nhe and Hbl target components were relatively stable in TSB (> 24 h at 37°C) and in gastric simulation medium with pH 5.0 (> 2 h at 37°C; Table 4). However, in gastric medium with pH 2.0, no enterotoxin components were detected, indicating degradation within 30 min incubation at 37°C. Also in intestinal medium, the Nhe and Hbl target components were not stable, resulting in failure of Nhe detection after 0.5–1 h and after 0.5–2 h for the Hbl components, depending on the kit used.

Enterotoxin detection in gastrointestinal simulation media

Enterotoxins were detected immediately at the start of the experiments by the kits with the lowest detection limits (Table 5). These toxins originated from the inoculum supernatant, i.e., 1 mL of *B. cereus* culture in the stationary growth phase. After *B. cereus* NVH 1230-88 had grown in intestinal medium with pH 6.5 for 2–4 h, no enterotoxins were detected anymore, regardless of the presence of oxygen and competing intestinal microbiota. During growth in gastric medium with pH 5.0, Nhe was detected throughout the experiment with the

Duopath® kit, while Hbl was detected at 0–4 h with the BCET-RPLA kit.

During growth of *B. cereus* ATCC 14579 at 30°C in the food matrix lasagne verde (pH = 5.52, standard deviation of triplicate measurements ± 0.06), enterotoxins Hbl and Nhe were detected inconsistently after 24 and 48 h, namely, in only one out of three replicates.

Enterotoxin production in intestinal medium and lasagne verde

B. cereus strains grew anaerobically from approximately 5 log CFU/mL to approximately 8 log CFU/mL under intestinal conditions with lasagne verde as food source at pH 7.0 at 37°C for 8 h, but no enterotoxins were detected (Table 6). Substitution of lasagne by TSB as the food source gave similar results. To eliminate enzymatic degradation of enterotoxins in the intestinal simulation medium, the digestive host secretions (bile, pepsin, lipase, and pancreatin) were omitted. During these experiments, enterotoxins were detected throughout the experiment, indicating that negative results were obtained by enterotoxin degradation. Experiments were set up with washed inoculums to remove the enterotoxins present in the added inoculum cultures, which enabled detection of only newly produced enterotoxin components. During these experiments, enterotoxins Hbl and Nhe were detected after 4 h when the *B. cereus* population reached >7 log CFU/mL, but only if no digestive enzymes were added to the intestinal simulation medium.

Discussion

This study confirmed that diarrheal toxins Nhe and Hbl are rapidly degraded under intestinal conditions and under gastric conditions at low pH (Table 5), reinforcing the hypothesis that preformed enterotoxins do not contribute significantly to diarrheal food poisoning (Granum *et al.,* 1993; Turnbull *et al.,* 1979). Moreover, our experiments showed that *B. cereus* populations of >7 log CFU/mL produced enterotoxins Nhe and Hbl under intestinal conditions, if host secretions containing digestive enzymes were omitted to prevent enterotoxin degradation (Table 6). Rapid (<30 min) degradation of enterotoxins by pancreatic enzymes in intestinal simulation medium was also reported in another study (Wijnands *et al.,* 2005). The food matrix may offer protection against heat, acid, or enzymatic inactivation of enterotoxins and increase their stability during gastrointestinal passage, as indicated by the fourfold increased heat resistance of enterotoxin Nhe target components in milk in comparison with BHI (Baker and Griffiths, 1995). During our study, no protective effects of the composite food matrix lasagane verde on the stability and detection of enterotoxins were observed in comparison with TSB. In conclusion, the results of this study reinforce the hypothesis postulated by Wijnands *et al.* (2005) that enterotoxin production by *B. cereus* should occur in close proximity to the small intestinal epithelium in order to enable the unstable enterotoxins to reach and affect the host cells and cause diarrheal disease.

An important consequence of this hypothesis is that not all ingested *B. cereus* will contribute to diarrheal food poisoning, since only the ones producing enterotoxins in close proximity to the host epithelial cells are affecting the host. This means that the infective dose does not necessarily correspond with the ingested dose. Our experiments, mimicking the liquid and well-mixed conditions in the intestinal lumen, showed enterotoxin production when the population attained ≥7 log CFU/mL during the exponential growth phase. Since it was also observed that enterotoxins were rapidly degraded in the intestinal lumen, the question arises at which bacterial concentrations toxin production occurs if *B. cereus* is adhered to the semi-solid viscous mucus matrix and the intestinal host cells. The growth conditions in the mucus layer near the host cells differ tremendously from those in the intestinal lumen in many aspects, including the dissolved oxygen concentration, the viscosity, available nutrients, the density and diversity of the bacterial community, and the immunological and antimicrobial molecules secreted by the host (Van den Abbeele *et al.,* 2011). The growth and toxin production in the vicinity of the host should be investigated with simulation experiments which mimic this environment to elucidate which numbers of cells produce sufficient enterotoxins to initiate diarrheal disease.

B. cereus growth at low pH often results in delayed, decreased, or even abolished enterotoxin production (Ceuppens *et al.,* 2011). In agreement, enterotoxin production was absent during growth in gastric medium at pH 5.0 and severely impaired in lasagne verde with pH 5.5.

Three commercial kits for *B. cereus* enterotoxin detection were evaluated and found suitable to investigate enterotoxin production under gastrointestinal conditions. However, several observations were made that require attention for the correct data interpretation. Firstly, in the case of the BCET-RPLA kit (Oxoid), twofold and fivefold dilution of, respectively, intestinal and gastric samples was required to avoid false-positive results, but this was acceptable given its low detection limit. Secondly, the lower limit of Hbl component detection in a sample is 2 ng/mL for the BCET-RPLA kit (according to the manufacturers) and 20 ng/mL for the Duopath® kit (Krause *et al.,* 2010). This corresponds very well with our observations, in contrast to the detection limits for the Nhe components. In the latter case, either the detection limit of the Duopath® kit is lower than the suggested 6 ng/mL (Krause *et al.,* 2010) or the detection limit of the BDE VIA™ kit is higher than the limit of 1 ng/mL claimed by the manufacturer. Thirdly, false negative results were sometimes obtained for psychrotrophic strains due to their decreased growth rate at high (>30°C) temperatures, which was also observed during earlier studies (Moravek *et al.,* 2006). This problem originates from the kits' instructions to test the cell-free culture supernatant of the strains after incubation at a fixed time and temperature, and can be overcome by performing the test when a certain cultures density is reached instead of a certain incubation time, which may be insufficient for certain slow-growing strains. Fourthly, not all enterotoxin polymorphisms are detected by the kits, for example the NheB component produced by *B. cereus* NVH 391/98 was not recognized by the Duopath® kit (Krause *et al.,* 2010). It remains to be investigated whether these toxin variants have biological activity and thus whether they should be detected by the commercial kits. Moreover, Nhe and/or Hbl production was reported for other bacilli such as *B. circulans, B. licheniformis, B. pumilus, B. polymyxa, B. carotarum,* and *B. lentus,* using the BDE VIA™ and BCET-RPLA kits (Beattie and Williams, 1999; Griffiths, 1990). However, enterotoxin gene sequences were exclusively retrieved from *B. cereus* group strain genomes using NCBI megablast (Ceuppens *et al.,* 2011). Therefore, it remains to be

clarified whether other bacilli can produce similar enterotoxins or whether some cross-reactivity of the kits' antibodies exists. Fifthly, it must be noted that the immunological detection of enterotoxin target components not necessarily imply the presence of biologically active enterotoxins (Buchanan and Schultz, 1994). For example, thermal inactivation prevented Nhe detection with the BDE VIA™ kit, but not that of Hbl-L2 with the BCET-RPLA.

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