Bacillus cereus associated food borne disease

quantitative aspects of exposure assessment and hazard characterization

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Voorwoord

Voor u ligt het resultaat van een uitdaging die ik met mezelf ben aangegaan. Een uitdaging in de zin van “hoever kan ik zelf gaan, waar liggen mijn mogelijkheden, mijn grenzen”. Juist op het moment dat besloten was het onderzoek naar de gezondheids effecten veroorzaakt door mycotoxinen in voedsel te staken diende het *Bacillus* onderzoek zich aan. En kon een al langer levend en uitgesproken verlangen om “de uitdaging” aan te gaan omgezet worden in daden.

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Abstract

Bacillus cereus is a versatile, spore-forming micro-organism capable of invoking two different types of food borne disease: a diarrhoeal syndrome, caused by enterotoxins produced during growth in the small intestine and an emetic syndrome, caused by the emetic toxin cereulide, produced in food prior to consumption. To enable a better risk assessment for the two syndromes, we focused investigations on exposure assessment and hazard characterization.

B. cereus exposure assessment includes investigations to obtain detailed information on the prevalence of potentially pathogenic *B. cereus* strains in Dutch food commodities. The data revealed that almost all of the strains found in the investigated food commodities posses the potential to produce one or more virulence factors, i.e. enterotoxins or cereulide. Also the concentration of *B. cereus* in the samples has been determined.

The hazard characterization focused on processes in the gastro-intestinal tract that lead to the diarrhoeal syndrome using model systems. Spores of *B. cereus* pass the gastric barrier, the first line of defence in humans, unaffected, while the number of vegetative cells able to survive simulated gastric conditions depends on the strain type, food type and the type of food acidification capacity of the stomach. It was shown that up to 26% of the vegetative cells can survive the gastric passage, indicating that they may contribute to the onset of diarrhoeal disease.

The enterotoxins, responsible for the onset of disease through the destruction of the small intestinal epithelium, were shown to be highly instable molecules in simulated intestinal conditions. We have shown that adhesion and subsequent production of enterotoxins in very close proximity of the epithelium are a prerequisite to circumvent degradation of the enterotoxins by proteolytic activity in the small intestine. The potential for adhesion was demonstrated for spores and vegetative cells at an efficiency rate of approximately 1%. In a model system the correlation between adhered *B. cereus* cells and the measure of destruction of differentiated Caco-2 cells, mimicking small intestinal epithelial cells, was proven.

B. cereus is also a micro-organism with a broad growth temperature range. The growth temperature profile of the strains is of importance for both their ability to grow in foods and to cause disease. We revealed that strains able to grow at temperatures below 10ºC

(psychrotrophic strains) seem to be less pathogenic than strains lacking this ability (mesophilic strains). This is not only due to their lower growth potential at 37ºC, but also due to their lower growth potential in conditions simulating the small intestine.

B. cereus spores may be very important for the onset of disease, but before they can grow and produce enterotoxins they first have to germinate. They contain receptors that need triggering by certain low molecular weight substances to set of germination. Such inducers may not only be present in the food, also differentiated Caco-2 cells were found to induce spores of some strains to germinate.

Through integration of the results of the investigations described in this thesis the annual number of cases of diarrhoeal disease in the Netherlands is estimated to range from 17,000 to 310,000. Data on the prevalence of *B. cereus* in Dutch food commodities together with literature and outbreak data were used to estimate the number of cases of emetic disease. These range from 630,000 to 3 million per year.

1

Introduction

General

In 1955 Steinar Hauge inoculated sterilized vanilla powder with 10^4 cells *Bacillus cereus* per gram and prepared a vanilla sauce from it. He left the sauce at room temperature for 24 hours, and subsequently consumed the sauce. Around 13 hours later he experienced severe abdominal pain and diarrhoea that lasted for 8 hours. It was the ultimate proof that *Bacillus cereus* was able to cause food borne disease (Hauge, 1955). The reason for this voluntary experiment were four outbreaks of food borne disease in Norway between 1947 and 1949, involving around 600 individuals. The food vehicle in these outbreaks was vanilla sauce, prepared from corn starch, which appeared to be contaminated with 10^4 *B. cereus* per gram. After preparation the vanilla sauce was stored at room temperature until it was served and eaten the next day. Before that date, the disorder in the taxonomy of *B. cereus* was the cause of slow recognition of the micro-organism as being able to lead to food borne disease. After the first description of the micro-organism (Frankland and Frankland, 1887), several cases of food borne disease caused by *Bacillus*-like organisms have been described (Lubenau, 1906; Seitz, 1913; Brekenfeld, 1926). Since the experiment of Hauge numerous food borne outbreaks attributed to *B. cereus* and associated with watery diarrhoea have been reported (Table 1.1).

In 1976 a novel toxigenic activity by *B. cereus* was described, based on monkey feeding studies (Melling et al. 1976). This was the first report on emetic properties of *B. cereus*, caused by an extra cellular heat stable compound with a molecular weight < 10,000 Dalton (Melling and Capel, 1978)

Bacillus cereus and gastro-intestinal disease

Although recognized as an agent causing food borne disease after the experiments by Hauge the mechanism of pathogenesis remained unknown for more than 20 years. The first description of a diarrhoeal toxin came in 1972 after partial purification by gel chromatography (Spira and Goepfert, 1972). It lasted until 1979 before clearly two toxic entities both produced by *B. cereus* were distinguished, each causing different symptoms (Turnbull et al. 1979). From that time on, *B. cereus* was recognized to be the cause of two types of disease, a diarrhoeal syndrome and an emetic syndrome.

Table 1.1 A selection of food borne outbreaks caused by *Bacillus cereus*

Year	# persons affected	Implicated food	Reference
Diarrhoeal			
1990	4	cod fish	(Van Netten et al. 1990)
1995	17	stew	(Granum et al. 1995)
2000	300	chicken and rice meal	(Ripabelli et al. 2000)
2002	95 and 78	cake at 2 banquets	(Ghelardi et al. 2002)
2002	25	mayonnaise	(Gaulin et al. 2002)
Emetic			
1997	2	spaghetti with pesto	(Mahler et al. 1997)
2000	116	vegetarian rice dish	(Essen et al. 2000)
2000	7	vegetarian dish	(Ripabelli et al. 2000)
2001	4	fried rice	(Grein, 2001)
2003	2	pasta dish	(Jääskeläinen et al. 2003b)
2005	6	pasta salad	(Dierick et al. 2005)

The diarrhoeal syndrome and its virulence factors

The diarrhoeal syndrome is a typical example of a toxico-infection, caused by enterotoxins after the ingestion of food contaminated with *Bacillus cereus*. After passage of the stomach the vegetative cells and/or spores reach the small intestine. Spores germinate; vegetative cells grow and produce enterotoxins. Vegetative cells are believed to be of minor importance in the onset of the disease due to their inactivation in the stomach.

The enterotoxins affect the epithelial lining of the small intestine causing disturbance of the water – solute transport. Eventually this may lead to diarrhoea. The syndrome is characterized by abdominal cramps and watery diarrhoea within 8 – 16 hours after consumption of contaminated food. The symptoms generally last no more than 24 hours, although longer

duration times have been described (Kramer and Gilbert, 1989; Granum, 2007).

In the classification of Granum et al. (1995) *B. cereus* belongs to the group of micro-organisms causing disease through enterotoxin production without any direct interaction with the small intestinal epithelium. The disease symptoms are comparable to food borne disease caused by *Clostridium perfringens,* although the pathogenic mechanism is different. After all, the enterotoxin produced by *C. perfringens* is released during sporulation in the small intestine, whereas the enterotoxins of *B. cereus* are produced during growth in the small intestine (McClane, 1997; Granum, 2007).

The various steps in this pathogenic mechanism have not all been fully elucidated. According to earlier research intraluminal multiplication of micro-organisms was not involved in fluid accumulation in the ileum, and therefore *B. cereus*-induced diarrhoea was probably the result of intoxication rather than infection (Spira and Goepfert, 1972). After all, feeding of cell free preparations of *B. cereus* cultures induced diarrhoea in monkeys treated with 10% sodium bicarbonate. Also, a direct correlation was observed between the ability to cause fluid accumulation in rabbit ileal loops, altered skin capillary permeability and diarrhoea in the monkeys (Goepfert, 1974). Later however, it was found that crude or purified material was not able to elicit diarrhoea. This suggested that human diarrhoea was caused by toxins developed after growth in the intestine following ingestion of large amounts of organisms.

During the 1980's and 1990's research concerning the diarrhoeal syndrome increased due to the discovery and identification of enterotoxins. Improvement of molecular biological techniques has increased knowledge on the production and regulation of enterotoxins largely.

However, despite all the research dedicated to understand the diarrhoeal syndrome, hardly any data are known concerning the dose response relationship. Based on epidemiological data using numbers of *B. cereus* detected in food related to (possible) diarrhoeal outbreaks, the number of cells necessary to induce diarrhoeal symptoms has been estimated to be 10^3 – 10^8 cells per gram food (Granum and Lund, 1997). Apart from the experiment by Hauge (1955) one study with human volunteers has been described linking exposure level to disease complaints. In this study volunteers consumed naturally contaminated milk. The number of complaints by the volunteers was related to the number of *B. cereus* per consumption: the higher the number of *B. cereus* the more

complaints (see Table 1.2) (Langeveld et al. 1996). The results of the experiment, however, may be biased due to a concurrent flu epidemic.

Enterotoxins are responsible for the onset of the diarrhoeal syndrome. They damage the epithelial barrier causing fluid accumulation in ligated rabbit ileal loops and they alter the vascular permeability of guinea-pig skin. Also they show cytotoxicity towards VERO cells (African Green Monkey kidney cells) (Glatz et al. 1974; Gilbert and Kramer, 1984).

Table 1.2 Number of consumptions and number of complaints classified according to number of *Bacillus cereus* cells ingested per consumption of contaminated milk (Langeveld et al. 1996)

	Number of consumptions		
		Complaints	
Number of *B. cereus* cells ingested	Total	Yes (%)	No (%)
$< 10^6$	132	5 (4)	127 (96)
10^6 - 10^7	32	2 (6)	30 (94)
10^7 - 10^8	26	2 (8)	24 (92)
$> 10^8$	69	9 (13)	60 (87)

Several enterotoxins produced by *B. cereus* have been described, namely haemolysin BL (HBL), non haemolytic enterotoxin (NHE), cytotoxin K, and enterotoxin FM. Of these HBL, NHE and cytotoxin K have been related to food borne disease (Table 1.3).

Haemolysin BL (HBL)

The first described enterotoxin of *B. cereus* is now called haemolysin BL (HBL). Previous names are diarrhoeagenic factor, fluid accumulation factor and vascular permeability factor (Shinagawa et al. 1991a; Shinagawa et al. 1991b; Sutherland and Limond, 1993). All names are somehow related to the effect of HBL observed in an *in vivo* and *in vitro* experiment (Kramer and Gilbert, 1989). HBL is a three component protein toxin, encoded by three polycistronic genes *hbl*A, *hbl*C and *hbl*D.

Table 1.3 Short descriptions of the *Bacillus cereus* (entero)-toxins

(Entero)toxin	# subunits	mol. weight (kDa) of each subunit	genes	detection methods	role in food borne disease	reference
HBL[1]	3	38	*hblA*	EIA[a], cytotoxicity test	proven	*(Beecher et al. 1995)*
		40	*hblC*	BCET-RPLA[b]		
		45	*hblD*			
NHE[2]	3	39	*nheA*	EIA, cytotoxicity test	proven	*(Lund and Granum, 1996)*
		45	*nheB*	BCET-RPLA		
		36	*nheC*			
Cytotoxin K[3]	1	34	*cytK*	cytotoxicity test	proven	*(Lund et al. 2000)*
Enterotoxin FM[4]	1	45			unknown	*(Shinagawa, 1990b)*
Cereulide		1.2	NRPS[c] complex	cytotoxicity test, boar sperm test, LC-MS[d]	proven	*(Mahler et al. 1997)* *(Dierick et al. 2005)* *(Essen et al. 2000)*

[a] *EIA = enzyme immuno assay*

[b] *BCET-RPLA = Bacillus cereus enterotoxin – reverse passive latex agglutination*

[c] *NRPS complex = non ribosomal peptide synthetase complex*

[d] *LC-MS = liquid chromatography – mass spectrometry*

[1] *(Beecher and Lee Wong, 1994)*

[2] *(Lund and Granum, 1996)*

[3] *(Lund et al. 2000)*

[4] *(Asano et al. 1997)*

The genes encode the B, L_1, and L_2 components, respectively. The function of a fourth gene in the operon, *hbl*B, is unknown. The molecular weight of the individual proteins is 38 kDa[1] (B-component), 40 kDa (L_1-component), and 45 kDa (L_2-component). The primers, described initially for the detection of the individual enterotoxin genes by Heinrichs et al. (1993) and Ryan et al. (1997), have been optimized to cope with polymorphisms in the gene (Guinebretiere et al. 2002).

Non haemolytic enterotoxin (NHE)

The first indication of another enterotoxigenic complex of *B. cereus* was found in 1996. Investigation of over 300 strains from various sources including strains from a number of outbreaks revealed that HBL could not have been the causative agent in some of the outbreak cases. The cytotoxic effects had to be assigned to a hitherto unknown enterotoxin (Granum et al. 1996). The new three component enterotoxin was discovered after a food borne outbreak in Norway (Lund and Granum, 1996). It appeared to lead to symptoms similar to those caused by HBL, but it lacked the haemolytic activity. Even though they contain several structural resemblances, the cytotoxic potential of the two three component enterotoxins, HBL and NHE, were different (Lund and Granum, 1997). Elucidation of the sequences of the genes provided sequences for primers for each of the three genes (Granum et al. 1999). Later comparison of gene sequences of various strains resulted in improved primers for the detection of the separate genes (Guinebretiere et al. 2002). Like HBL, translation of the NHE operon is regulated by the PlcR regulator (Granum, 2007).

Cytotoxin K

An outbreak of severe food poisoning in France, killing three individuals, led to the discovery of cytotoxin K (Lund et al. 2000). The toxin is a single protein with a molecular weight of approximately 34 kDa. Cytotoxin K causes more severe diarrhoea including necrotic enteritis (Lund et al. 2000). In potency the cytotoxin K, as discovered in this outbreak, appears to be the strongest form discovered so far. Later research revealed that the less potent cytotoxin K variants, also named cytotoxin K like enterotoxin or cytK-2, were approximately 89% amino acid homologous to the original cytotoxin K (cytK-1) and were 20% less toxic towards human intestinal Caco-2 cells and Vero-cells. This suggests that

[1] kDa = kiloDalton

cytK-2 may be less harmful than cytK-1 (Fagerlund et al. 2004). Typically, the outbreak reported by Lund *et al*. (2000) is the only one involving cytotoxin K. Recently, a PCR-method was developed to discriminate between the two CytK varieties (Guinebretiere et al. 2006). Two other strains were found to produce the cytK-1 during these investigations.

Enterotoxin FM

Enterotoxin FM was discovered after cloning and expression of a gene. Toxicity of this gene product was compared to a previously described enterotoxin with approximately the same molecular weight (Shinagawa, 1990a). Enterotoxin FM has, up to now, not been related with outbreaks of diarrhoeal *B. cereus* syndrome.

Regulation of enterotoxin expression

Translation of the operons of HBL, NHE and CytK is regulated by a gene that also regulates phospholipase C expression, called the phospholipase C regulator (PlcR) gene (Agaisse et al. 1999). More detailed knowledge on the regulation by PlcR and the control of PlcR has been gained since (Lereclus et al. 2000; Okstad et al. 1999; Slamti and Lereclus, 2002; Slamti et al. 2004; Slamti and Lereclus, 2005).

Mode of action of the enterotoxins

The mode of action of the two three-component enterotoxins HBL and NHE is more or less similar (Lund and Granum, 1997). All three components of the HBL-complex are necessary for maximal enterotoxic activity, according to cytotoxicity experiments with VERO-cells (Powell, 1987; Rousset and Dubreuil, 2000; Belaiche, 2000; Black et al. 2005). The optimal ratio for HBL activity is 1:1:1 (Beecher et al. 1995). The latest model for the action of HBL suggests that all three components bind to the target cells and constitute a membrane attacking complex leading to lysis of the target cells (Beecher and Lee Wong, 1997).

Although all three components of the NHE-complex are necessary for maximal cytotoxic activity as well, the optimal ratio is 10:10:1 (NHE-A : NHE-B : NHE-C) (Lindback et al. 2004).

The mode of action of cytotoxin K is different. The amino-acid sequence of cytotoxin K suggests the toxin to be a β-barrel channel-forming toxin such as β-toxin from *Clostridium perfringens* and γ- and α-haemolysin of *Staphylococcus aureus* (Hardy et al. 2001). Its symptoms include more severe epithelial lesions and bloody diarrhoea.

Detection of enterotoxins and enterotoxic activity

The definition for enterotoxic activity is: *a bacterial protein that, following the release into the intestine, causes cramps, diarrhoea and nausea* (Zaid et al. 1999). The oldest methods for the detection of enterotoxic activity are the vascular permeability assay and the rabbit ileal loop test (Glatz et al. 1974; Spira and Goepfert, 1972). Due to reduction in the use of experimental animals and the complexity of these tests they are no longer used routinely. These tests can be considered the standard for the detection of enterotoxins, including those produced by *B. cereus.*

Current methods employ cell lines; the most commonly used for the detection of cytotoxicity of diarrhoeal toxins are CHO (Chinese hamster ovary) cells and VERO (monkey kidney) cells. The CHO cell test is more sensitive than the commercially available immunological tests (Buchanan and Schultz, 1994). Yet, comparison of various cell lines for their efficacy on the detection of enterotoxic activity showed that VERO cells appeared to be the most suitable for detecting enterotoxic activity (Wegscheider, 2004). The specificity of the cell test cannot be guaranteed since other extracellular proteins produced by *B. cereus*, such as haemolysins and proteolytic enzymes, may also react in the test.

Two commercial kits are available for determining the presence of enterotoxin (components), the TECRA kit and the BCET-RPLA kit (Oxoid). The TECRA kit detects the 45kDa component from the NHE-complex. The BCET-RPLA (*Bacillus cereus* enterotoxin reverse passive latex agglutination) assay detects the L_2 component of the HBL-complex.

Though not commercially available, monoclonal antibodies have been developed against each of the three components of the HBL enterotoxin (Dietrich et al. 1999). The L_1 component is detected by a sandwich ELISA, using two different monoclonal antibodies. The other two components, B and L2, are detected by an inhibition ELISA, each using one monoclonal antibody. The L2-test cross-reacts with the B component of the NHE enterotoxin. Recently monoclonal antibodies against each of the three components of the NHE-enterotoxin were described (Dietrich et al. 2005). No immunological detection method for detecting cytotoxin K has been developed yet, although the development and production of monoclonal antibodies against cytotoxin K was one of the aims of a European project on the prevention of *Bacillus cereus* (EU-Project QLK1-2001-00854).

The emetic syndrome and its virulence factor

The pathogenic mechanism of the emetic syndrome is different from that of the diarrhoeal syndrome. *B. cereus* can grow in food prior to consumption and may produce emetic toxin (cereulide) either before or after preparation of the food. Shortly after ingestion, and when sufficient toxin has been ingested, the symptoms arise. The emetic syndrome is a typical example of food intoxication. The main symptoms are nausea and vomiting, similar to the symptoms caused by *Staphylococcus aureus* enterotoxin (Granum and Lund, 1997).

Cereulide is a heat and pH stable circular dodecadepsipeptide consisting of three repeating units of four amino acids, each consisting of D-O-leucine, D-alanine, L-O-valine and L-valine (Agata et al. 1994; Agata et al. 1995b). The structure resembles the structure of the known potassium ionophore valinomycin (see Figure 1.1), which suggested an ionophoric nature for cereulide as well. Such ionophoric properties were indeed detected (Mikkola et al. 1999). Moreover, cereulide proved to possess even stronger ionophoric properties than valinomycin at physiological concentrations of K+ (Teplova et al. 2006). Synthetically assembled cereulide proved to have the same pathological effects (vacuolation of Hep-2 cells and emetic toxicity) as the natural compound (Isobe et al. 1995).

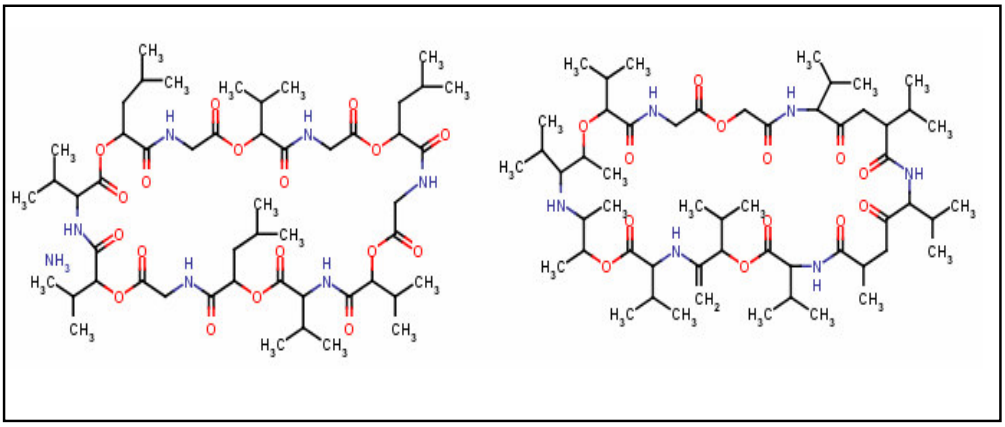

Figure 1.1 Comparison of the amino acid compositions of cereulide [D-Ala – D-O-Leu – L-Val – L-O-Val]$_3$ (left) and of valinomycin [D-Val – L-O-Ala – L-Val – D-O-Val]$_3$ (right) (Teplova et al. 2006)

The production of cereulide by *B. cereus* is a multifaceted process. Recently, two research groups showed that cereulide was produced by a non ribosomal peptide synthetase (NRPS) complex (Toh et al. 2004; Horwood et al. 2004). After initial partial characterization of the complex, resulting in a PCR method highly specific for cereulide producing *B. cereus* strains (Ehling-Schultz et al. 2004), the entire cluster was identified (Ehling-Schulz et al. 2006).

Determination and confirmation of cereulide (-like) activity and identity

Cereulide not only induces vomiting, but also vacuolization of cells. For this it passes the mitochondrial membrane and disrupts the energy production by mitochondria (Mikkola et al. 1999). This last property is used for the detection of cereulide-like activity of *B. cereus* strains and in food commodities, suspected for the presence of cereulide-like toxin (Andersson et al. 1998). Two major tests are known, the Hep-2 cell test (Finlay et al. 1999) and the boar sperm test (Andersson et al. 1998). None of the tests is specific for cereulide only: the former detects any molecule that can inhibit growth of Hep-2 cells, the latter detects any molecule that can inhibit sperm motility. Since its first description the boar sperm test has not only been transformed into a rapid test (Andersson et al. 2004), also the read-out of the test has been automated to provide more objective results (Rajkovic et al. 2006). The identity of the cereulide-like compound can be confirmed by a LC-MS method (Häggblom et al. 2002).

Enterotoxins responsible for the diarrhoeal syndrome can be detected by serological/immunological methods (Dietrich et al. 1999; Dietrich et al. 2005; Shinagawa et al. 1991b). However, cereulide, can not detected by such methods. The molecule is too small in size to function as an immunogen itself. Nor can it be conjugated to known immunogens such as keyhole limpet hemocyanin, ovalbumin, bovine serum albumin or tetanus toxoid to initiate the production of antibodies in test animals.

Bacillus cereus and non-gastro-intestinal disease

Besides food borne diseases *B. cereus* may cause local and systemic non food related infections including endocarditis and wound infections (Drobniewski, 1993). Also ocular infections, such as keratitis, endophthalmitis and panophthalmitis are reported but are not common (Kotiranta et al. 1999). Although the enterotoxin HBL was suggested to be involved as a virulence factor in these ocular infections, no increased

inflammatory responses in endophthalmitis could be found when comparing a HBL^+ strain with an HBL^- strain (Callegan et al. 1999).

Systemic non food related infections, such as endocarditis and post-operative meningitis, may occur in immunologically compromised patients (Steen et al. 1992; Barrie et al. 1992; Berner et al. 1997). Drobniewski (1993) described the involvement of *Bacillus cereus* in ocular infections and in cases of systemic infections, mainly pneumonia, endocarditis and meningitis cases.

Microbiology of *Bacillus cereus*

Bacillus cereus is a Gram-positive, spore-forming, motile, facultative aerobic rod. The cell width and length are 1.0 – 1.2 μm and 3 – 6 μm, respectively. The spores are ellipsoidal to cylindrical in shape, and are produced centrally to terminally in the vegetative cell. Motility is lost during the early stages of sporulation, which may take place after 2 to 3 days on most media. *B. cereus* belongs to the *B. cereus* group, i.e. a group of *Bacillus* species which are closely related morphologically and molecularly. The other members of this group are *B. thuringiensis, B. anthracis, B, mycoides, B. pseudomycoides* (Nakamura, 1998) and *B. weihenstephanensis* (Lechner et al. 1998).

Based on genetic similarities some investigators state that *B. thuringiensis, B. anthracis* and *B. cereus* should be regarded as one species (Helgason et al. 2000b). This indicates the complexity of the taxonomy of the genus *Bacillus*. Even within the species *B. cereus*, a subgroup can be discerned: investigation of 100 strains from different origin (food commodities, outbreaks of *B. cereus* induced food borne disease and environment) learned that emetic strains showed distinct characteristics within the *B. cereus* group (Carlin et al. 2006). Moreover, emetic *B. cereus* strains appear to be more closely related to *B. anthracis* than not-emetic *B. cereus* strains (Apetroaie et al. 2005).The close relationship between *B. cereus* and *B. anthracis* has been underlined by the discovery that the cereulide synthetase cluster is located on a pXO1-like virulence plasmid, similar to the plasmid containing the toxin genes in *B. anthracis* (Ehling-Schulz et al. 2006).

Most species from the *B. cereus* group can be differentiated on the following criteria: colony morphology, haemolysis, motility, susceptibility for penicillin, parasporal crystal inclusion, and on various biochemical characteristics (see Table 1.4).

Table 1.4 Criteria for differentiating between four closely related *Bacillus cereus* group members

Species	Colony morphology	Haemolysis	Motility	Susceptibility to penicillin	Parasporal crystal inclusion
B. cereus	white	+	+	-	-
B. anthracis	white	-	-	+	-
B. thuringiensis	white/grey	+	+	-	+
B. mycoides	rhizoid	(+)	-	-	-

From: (Granum, 2007)

Three of the species from the *B. cereus* group are involved in human and animal disease. *B. cereus* is known to cause food-borne disease as described earlier. *B. anthracis* is a mammalian pathogen through plasmid coded toxins. Crystal toxins produced by *B. thuringiensis* are pathogenic to species of insects. Little is known about the pathogenicity of *B. mycoides* and *B. pseudomycoides*. The recently discovered *B. weihenstephanensis* is a true psychrotrophic micro-organism (Lechner et al. 1998). Although it may possess the ability to produce toxins involved in food borne disease, it is mainly associated with food spoilage since, like other psychrotrophic strains, it growth potential at 37ºC is very poor (Larsen and Jorgensen, 1999).

Toxicity of related organisms

As stated before the members of the *B. cereus* group, are closely related. DNA-DNA hybridization studies on *B. cereus*, *B. anthracis* and *B. thuringiensis* have shown that these organisms share relatively high levels of chromosomal base sequence identity. Based on the 16S rRNA sequences, similarities of more than 99% were observed (Ash et al. 1991).

The genes encoding the virulence factors of *B. anthracis* and *B. thuringiensis* are plasmid based. The genes encoding the *B. cereus* toxins that cause diarrhoea are located on the chromosome, and the genes encoding the cereulide synthetase complex are plasmid based. Since DNA-DNA hybridization and 16S rRNA sequence comparisons deal with the chromosomal DNA, it is highly likely that besides their "normal" virulence, *B. thuringiensis* and *B. anthracis* are potentially enterotoxigenic.

Indeed, enterotoxin encoding genes have been found in both *B. thuringiensis* and *B. anthracis*. The PlcR gene in *B. anthracis* is, however, not intact and PlcR is not produced. Therefore *B. anthracis* is not able to produce enterotoxins (Agaisse et al. 1999). *B. thuringiensis* on the other hand has been proven to produce enterotoxins (Damgaard, 1995; Lereclus et al. 2000), and such strains have even been isolated from food (Damgaard et al. 1996). No confirmed food borne illness cases caused by *B. thuringiensis* have been described yet. This, however, may also be due to the difficulties encountered in the discrimination between *B. cereus* and *B. thuringiensis*. The most reliable method for distinguishing the two species is the detection of the parasporal inclusions in *B. thuringiensis*. Other methods, such as DNA-based methods (Manzano et al. 2003; Chen and Tsen. H.Y., 2002; Te Giffel et al. 1997b; McMinn et al. 1996; Johnson, 1984) have been suggested, but have so far not proven adequate enough for distinguishing the two species. PCR detection of the toxin-gene carrying plasmid of *B. thuringiensis* can be used for discrimination between the two species (Ben-Dov et al. 1997).

Prevalence of *Bacillus cereus* in food commodities

Bacillus cereus is commonly isolated from soil, meat, and vegetable products. Similar to the distribution of food borne disease (diarrhoeal form associated with carbohydrate poor commodities and emetic form associated with carbohydrate rich commodities), there seems to be a distribution of types of strains found in soil and agricultural products. In rice paddies the prevalence of emetic strains is around 44% (Ueda and Kuwabara, 1993), whereas only three strains out of 177 (1.7 %) isolated from soil in Scotland used for the production of vegetables produced emetic toxin (Altayar and Sutherland, 2006).

Milk is regularly investigated for the presence and enterotoxin production abilities of *B. cereus* (Christiansson et al. 1989; Van Netten et al. 1990). Te Giffel et al. (1997a) investigated pasteurized low fat milk from refrigerators in Dutch households, and found *B. cereus* at low levels in 40% of the samples. Despite the worldwide prevalence of *B. cereus* in milk, surprisingly few food poisoning cases caused by *B. cereus* from milk have been reported.

The increasing popularity of cooked chilled foods may lead to problems with sporeforming bacteria such as *B. cereus*. The mild heat treatment used for REPFEDs (REfrigerated Processed Foods of Extended

Durability) may eliminate vegetative forms of micro-organisms, but not spores. Choma et al. (2000) found 20% of the vegetable purées pasteurized in their final package to be contaminated with low levels of *B. cereus*, < 10 CFU g^{-1}. After 20 days at 10ºC 50% of the samples were positive for *B. cereus* at levels between 10^4 and 10^6 CFU g^{-1}. Del Torre et al. (2001) found 33% of the REPFED samples they investigated to be contaminated with *B. cereus* at levels < 10^2 CFU g^{-1}. With increasing storage temperatures and storage times the counts increased.

Rusul and Yaacob (1995) investigated a variety of foods, including noodles, spices, grains, vegetables and cooked foods, for the presence of *B. cereus*. Most samples were found to contain *B. cereus* at levels between 10^2 – 10^6 CFU g^{-1}. The toxin-producing capacity of the strains was not investigated.

Incidence of *B. cereus* food borne disease

European literature contains numerous accounts of food poisoning caused by *B. cereus*-like spore-forming organisms since the beginning of the 20th century. Lubenau (1906) described an outbreak involving patients and staff in a sanatorium: 300 of the 400 people suffered from profuse diarrhoea, stomach cramps and vomiting. Although he called the spore forming organism, found in meatballs from the incriminated meal, *Bacillus peptonificans*, the description resembles that of *Bacillus cereus*. In 1913 Seitz reported the isolation of a *cereus*-like bacillus from the faeces of a man suffering from enteritis and profuse diarrhoea (Seitz, 1913).

Nowadays many countries register food borne outbreaks and investigate the causative agents. In principle this should lead to better knowledge of the magnitude of the problem of food borne disease. However, since not all food borne diseases are notifiable, the magnitude of the problem is still hard to estimate including the role of *B. cereus*. Moreover, due to the relatively mild symptoms of *B. cereus* food borne disease, patients do not generally seek medical attention, thus no test is performed to identify the causative organism. From the available data it can be concluded that *B. cereus* is a micro-organism frequently causing food borne outbreaks. In The Netherlands it causes the highest number of bacterial food borne outbreaks (Table 1.5). Also in Norway, in 1999 and 2000, *B. cereus* was responsible for the highest number of bacterial food borne outbreaks (Table 1.6).

Table 1.5 Incidence of *Bacillus cereus* food borne outbreaks in the Netherlands

Investigated period	% outbreaks of total	Ranking in bacterial agents number of food borne outbreaks	Reference
1991 – 1994	19	1st	(Simone et al. 1997)
1993 – 1998	2.4	1st	(Anonymous, 2000b)
1999 – 2000	3.1	1st	(Anonymous, 2003a)
2001	3.9	1st	(Duynhoven et al. 2002)
2003	4.1	1st	(Duynhoven et al. 2004)
2004	2.8	1st	(Duynhoven et al. 2005)
2005	3.0	1st	(Doorduyn et al. 2006)
2006	5.4	1st	(Doorduyn et al. 2007)

Table 1.6 Incidence of *Bacillus cereus* food borne outbreaks in countries other than The Netherlands

Country	Investigated period	% outbreaks of total number of food borne outbreaks	Ranking in bacterial agents	Reference
Finland	1993 – 1998	7.9	3rd	(Anonymous,2000f)
	1999	1.2	4th	(Anonymous,2003e)
	2000	1.4	3rd	(Anonymous, 2003e)
France	1993 – 1998	0.8	6th	(Anonymous, 2000c)
	1999	0.73	6th	(Anonymous, 2003b)
	2000	5.08	3rd	(Anonymous, 2003b)
Germany	1993 – 1998	1.1	7th	(Anonymous, 2000g)
	1999	1.5	4th	(Anonymous, 2003f)
	2000	2.2	3rd	(Anonymous, 2003f)
Hungary	1993 – 1998	0.8	4th	(Anonymous, 2000d)
	1999	1.5	5th	(Anonymous, 2003c)
	2000	0.9	5th	(Anonymous, 2003c)
Norway	1993 – 1998	22	2nd	(Anonymous, 2000e)
	1999	35	1st	(Anonymous, 2003d)
	2000	32	1st	(Anonymous, 2003d)
Spain	1993 – 1998	0.3	11th	(Anonymous, 2000h)
Sweden	1993 – 1998	4.8	3rd	(Anonymous, 2000i)
	1999	-	-	(Anonymous, 2003g)
	2000	2.7	5th	(Anonymous, 2003g)
UK	1993 – 1998	2.0	7th	(Anonymous, 2000j)
	1999	1.1	9th	(Anonymous, 2003h)
	2000	-	-	(Anonymous, 2003h)
United States	1988 – 1992	1.1	5th	(Anonymous, 1996)
	1993 – 1997	0.5	7th	(Anonymous, 2000a)

Objective of this thesis

Bacillus cereus, a spore forming micro-organism with ubiquitous distribution, is known to cause food borne disease. The short-lasting and relatively mild symptoms do not urge patients to seek medical assistance. Therefore, the real extent of the number of disease cases is hard to determine. Another factor is the lack of knowledge on the mechanisms by which disease is caused. It is known that for the diarrhoeal type *B. cereus* has to be ingested, has to grow in the small intestine and has to produce enterotoxins. For the emetic type of disease, the emetic toxin, cereulide, has to be produced in food prior to consumption The main objectives of this thesis were the closer investigation of processes in the gastro-intestinal tract that may contribute to the onset of or the protection against *B. cereus* diarrhoeal disease, and the closer investigation of factors involved in the production of emetic toxin. In combination with prevalence data the outcomes of these investigations should contribute to a better estimate on the incidence of disease.

The investigations can be categorised within the framework of risk assessment, with data on prevalence of the micro-organism falling within the scope of exposure assessment, and data on the pathogenic mechanism falling within the scope of hazard characterization.

The work described in this thesis was carried out within a project named: *Quantitative research concerning* Bacillus cereus *within the framework of risk assessment, with an emphasis on exposure assessment and hazard characterization* on the account of the former Food and Commodities Inspectorate, the present Food and Consumer Products Safety Authority.

Outline of this thesis

This thesis deals with two important issues in risk analysis, namely exposure assessment and hazard characterization.

Exposure assessment was addressed by studying the prevalence of potentially pathogenic *Bacillus cereus* strains in various food commodities. The strains, isolated from randomly sampled food commodities in the Netherlands, were investigated for the presence of genes encoding enterotoxins haemolysin BL (HBL), non haemolytic enterotoxin (NHE) and cytotoxin K (CytK), for the growth temperature profile of the strains (psychrotrophic and mesophilic) by discrimination of the 16S rDNA signature, and for the ability to produce cereulide-like toxin. The results of these characterizations are described in Chapter 2.

Investigations regarding the mechanisms leading to disease, in order to deal with the hazard characterization, focused on part 2 and 3 from the pathogenic mechanism of the diarrhoeal syndrome, presented as the current working model in Figure 1.2.

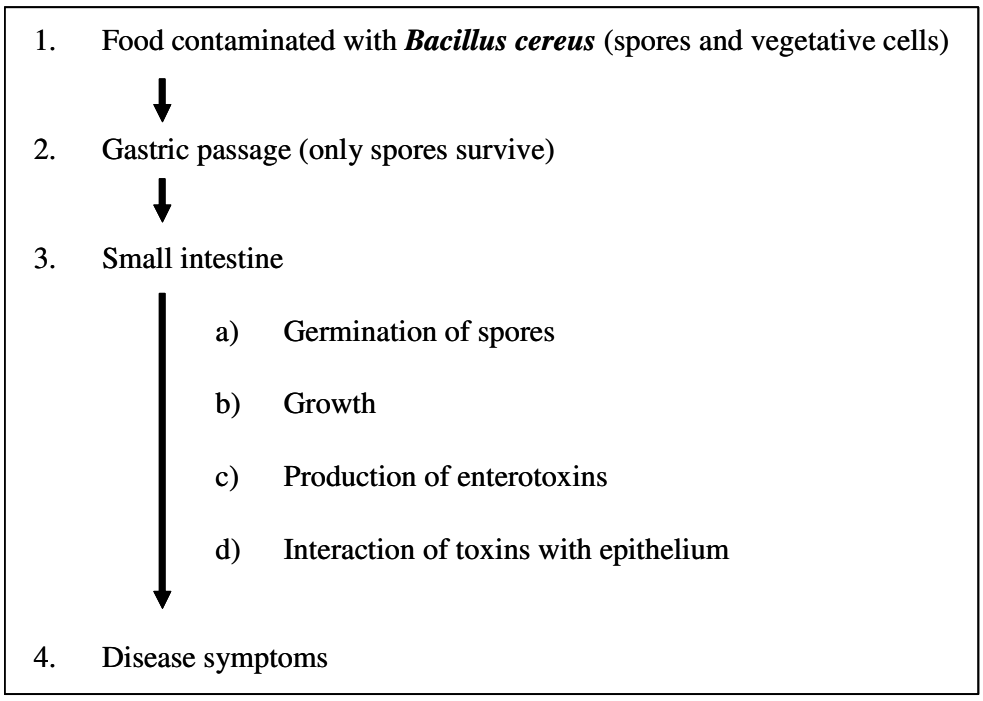

Figure 1.2 General pathogenesis of the diarrhoeal syndrome.

The investigations were carried out using simulations of the gastro-intestinal conditions namely simulated gastric and intestinal fluids to mimic conditions in the stomach and small intestine, and differentiated Caco-2 cells to mimic the epithelial cells of the small intestine.

In Chapter 3 the influence of simulated gastro-intestinal fluids on the survival of spores, their germination and growth in simulated intestinal fluid has been investigated. Spores of a selection of strains were exposed to simulated gastric fluid to record their survival capacity, and were exposed to simulated intestinal fluid to investigate their germination and growth potential.

Spores of *B. cereus* are well equipped to withstand unfavourable conditions such as low pH and elevated temperatures, and can therefore take part in the pathogenesis of the diarrhoeal syndrome. Since the pH in the stomach may vary due to the buffering capacity of the food that functions as a carrier and because of reduced acidification capacity of the stomach such as generally found in elderly people, vegetative cells of *B. cereus* may survive the gastric passage. Their contribution to the pathogenesis of the diarrhoeal syndrome was determined. The results of these investigations are described in Chapter 4. Exposure to simulated gastric fluid of different acidic pH-values resulted in pH dependent decimal reduction values (D_{pH}). We used these D_{pH}-values and models for the course of pH in the stomach during the consumption of food to quantify the number of vegetative cells able to pass the stomach, which after arrival in the small intestine can take part in the pathogenic mechanism.

Upon arrival in the small intestine, spores and vegetative cells come into contact with intestinal fluid and the small intestinal epithelium. Investigations on the interaction between the epithelium and spores are described in Chapter 5. Based on the effect of Caco-2 cells on spore germination, it is suggested that small intestinal epithelial cells may initiate germination of spores. Based on the diversity in Caco-2 induced germination of the *B. cereus* strains included in this study, the in situ germination capacity may be an important determinant of *B. cereus* pathogenicity.

The actual diarrhoeal symptoms are caused by enterotoxins produced by *B. cereus* growing in the small intestine. The behaviour of NHE in simulated intestinal fluid and the interaction of *B. cereus* cells and

the enterotoxin with epithelial cells were studied more closely and the results are described in Chapter 6.

In Chapter 7, finally, the risks for diarrhoeal and emetic disease are estimated and discussed

2

Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in the Netherlands

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Abstract

Randomly selected food commodities, categorized in product groups, were investigated for the presence and number of *B. cereus*. If positive, and when possible, five separate colonies were isolated and investigated for the presence of four virulence factors: presence of genes encoding three enterotoxins [haemolysin BL (HBL), non haemolytic enterotoxin (NHE), and cytotoxin K], and the ability to produce cereulide. In addition, the presence of psychrotrophic and mesophilic signatures was determined.

The genes for NHE are found in more than 97% of the isolates, those for HBL in approximately 66% of the isolates, and the gene for cytotoxin K in nearly 50% of the isolates. Significant associations between product groups and (combinations of) virulence factors were: the relatively low percentage of isolates from the "flavourings"-group containing genes encoding NHE and the higher than average occurrence of both the genes encoding HBL and NHE in the "pastry"-group. Cereulide was produced by 8.2 % of the isolates, but only in combination with the presence of genes for one or more other virulence factors.

Most isolates (89.9 %) were mesophilic, minorities of the isolates were psychrotrophic (4.4 %) or of intermediate signature (5.7 %). In the product group "milk and milk products" the incidence of strains with psychrotrophic or intermediate signatures is significantly higher than in the other product groups.

In the product groups "flavourings", "milk and milk products", "vegetable(s) and vegetable products", "pastry", and "ready-to-eat foods" a relatively high number of samples contain high numbers of *B. cereus*. Within the product group "ready-to-eat foods", the products containing rice and pasta show a relatively high incidence of high numbers of *B. cereus*.

Introduction

Bacillus cereus is a ubiquitous spore forming Gram positive micro-organism, capable of causing two types of food borne disease. The emetic syndrome (an intoxication) is caused by cereulide, a pH and temperature stable cyclic peptide toxin (Agata et al. 1995b), and disease may arise after the ingestion of food contaminated with cereulide. The diarrhoeal syndrome (a toxico-infection) is caused by enterotoxins, which are produced by vegetative cells growing in the small intestine after consumption of food contaminated with spores and/or vegetative cells (Kramer and Gilbert, 1989). To date three enterotoxins, capable of inducing the diarrhoeal symptoms of the toxico-infection have been described (Granum, 2007). These are the three-component enterotoxin haemolysin BL (HBL), the three-component enterotoxin non haemolytic enterotoxin (NHE), and the single-component enterotoxin cytotoxin K. The symptoms caused by cytotoxin K are more severe than those caused by the other two enterotoxins: cytotoxin K not only causes watery diarrhoea but also necrosis. Based on the mode of action the (entero)-toxins ought to be regarded as virulence factors.

Another important feature of *B. cereus* is the ability to grow at a wide range of temperatures. However, grouping of strains according to growth temperature is confusing. Where one definition divides strains in those with a high temperature growth range (10-42ºC) and those with a low temperature growth range (4-37ºC), another definition divides strains upon their ability to grow below 7ºC. Psychrotrophs can grow below 7ºC; mesophiles cannot (Meer et al. 1991; von Stetten et al. 1998). In food microbiology mesophiles are characterized as strains with a temperature optimum around 37ºC, while the optimum for psychrotrophs lies around 25-30ºC (Adams and Moss, 2000).

Although the potential to cause disease is known, little to no details are known about the number of *B. cereus* cells necessary to cause either of the syndromes, let alone about the dose of (entero)-toxin necessary to induce disease. From outbreak data, the number of *B. cereus*, able to cause disease, has been estimated to be higher than 10^3 cells per gram food (Granum, 2007).

In order to improve the knowledge on exposure assessment with respect to both syndromes, more data on the prevalence of *B. cereus* in daily food commodities should be available. Previous investigations yielded data on *B. cereus* in milk (Larsen and Jorgensen, 1997; Te Giffel et

al. 1996; Te Giffel et al. 1996) and data on *B. cereus* in other food commodities such as refrigerated processed foods of extended durability (REPFED's) (Choma et al. 2000; Del Torre et al. 2001) and chicken products (Smith et al. 2004). Choma et al. (2000) investigated REPFED samples for the presence of *B. cereus* and found low initial numbers (< 10 $CFU \cdot g^{-1}$). The growth characteristics of the isolates were determined during storage of the products at various temperatures, and cytotoxicity of supernatants of the isolates was investigated. Instead of investigating the cereulide producing potential of the isolates, Choma et al. (2000) determined their starch degrading properties. This feature is linked to but not qualifying for the production of cereulide. Del Torre et al. (2001) found that 33% of REPFED samples were contaminated with low initial levels (< 10^2 $CFU \cdot g^{-1}$) of *B. cereus* but did not investigate the isolates for virulence factors. Smith et al. (2004) detected *B. cereus* in 27 of 60 samples of chicken products after enrichment. Testing the isolates for the presence of enterotoxin coding genes by PCR, resulted in finding no isolates carrying the cytotoxin K gene and a high prevalence of the genes coding for the non haemolytic enterotoxin. Smith et al. (2004) did not study the cereulide producing ability of their isolates.

Since (entero-)toxins cause the symptoms of the emetic and diarrhoeal syndrome, an inventory on the prevalence of these virulence factors in food commodities would be preferable over an inventory on *B. cereus* itself. However, direct detection methods are not (yet) available for all enterotoxins and the methods available to detect cereulide have not yet been optimized for the direct detection [boar sperm test (Andersson et al. 1998) and HEp-2 cell test (Finlay et al. 1999)], or are too expensive to use for large scale investigations [LC-MS method (Häggblom et al. 2002)].

The aim of the investigations described in this paper is to make an inventory of the occurrence of potentially pathogenic *B. cereus* strains in retail food commodities in The Netherlands. Strains are considered potentially pathogenic when possessing the genes encoding one or more (entero-) toxins, and/or when able to produce cereulide *in vitro*. Also the growth temperature profile of each strain was determined, as earlier research has shown a relation between this trait and the importance for the onset of diarrhoeal disease.

Materials and Methods

Collection of samples and isolates

The Food and Consumer Product Safety Authority (VWA) collected food samples at retail level. The food samples were investigated for the presence and, in most cases, also for the level of *Bacillus cereus* by plating decimal dilutions on MEYP[2]-agar. Occasionally only the 10^{-2}, 10^{-3}, or the 10^{-4}-dilution (the Dutch legal limit being 10^5 *B. cereus* ml^{-1} or g^{-1} for most foods) was investigated, and presence or absence was recorded. When *B. cereus* was present in a food sample, and if available, five typical colonies were selected and sent to the National Institute for Public Health and the Environment (RIVM) for further characterization of the isolates. Viable counts were available from most samples.

The isolates sent for further investigation did not cover all positive *B. cereus* samples: only some of the samples were used for extensive characterization of their isolates.

Classification of the samples

The VWA uses a classification for the food commodities which is based on the former "Kokswarenbesluit" (Anonymous, 1979). Accordingly, the collected food samples were classified in the following product groups which comprise food commodities used for regular meals: pastry, vegetable(s) and vegetable products, ready-to-eat foods, milk and milk products, oil(s) / fat(s) and oil and fat products, flavourings, fish and fish products, and meat and meat products. All samples from the milk and milk products group were from commodities that need storage at refrigerator temperatures according to the manufacturers.

Preparation and storage of isolates

All isolates were grown on Columbia agar with sheep blood (overnight at 30°C) for purity assessment. If pure, the isolate was subcultured on Tryptone Soy agar (TSA) in a 9 cm Petri dish for ten days at 30°C for cereulide production, and in 10 ml Brain Heart Infusion broth (BHI) for 1 day at 30°C for DNA extraction. Also, the isolate was stored at -70°C by adding 200 µl BHI culture to a tube containing glass beads and 200 µl glycerol.

[2] MEYP = mannitol egg yolk phenol red (Mossel et al. 1967)

Determination of ability to produce cereulide

The colonies were collected from the TSA plates with a cell scraper and suspended in 1 ml methanol in 1.5 ml micro centrifuge tubes (Eppendorf AG, Hamburg, Germany). After vigorous mixing on a vortex-mixer the tubes were placed on a blood tube rotator for 30 minutes for homogenization of the suspension. This suspension was centrifuged (5 min, 13,000xg), and the supernatant transferred to a glass tube. The supernatant was dried at ca. 60°C with a flow of nitrogen. The residue was resuspended in 1 ml cell culture medium and stored at 4°C until further investigation. Further investigation consisted of screening 5 two-fold dilutions (1:2 to 1: 32) of the extracts for the presence of cereulide using the previously described HEp-2 cell test (Finlay et al. 1999). The optical densities (OD's) of the dilutions of the isolates were compared to OD's of positive and negative controls (extracts from NCTC 11143 and NCTC11145, respectively). Resemblance of the sample OD's with the OD's of the negative or positive control, determined the sample to be either negative or positive. If no conclusion could be drawn from this test investigation of the extract was extended to dilutions starting at 1: 8 and ranging to 1: 16,392. Optical densities were plotted against the dilutions and compared with the positive and negative control. If showing the same trend in optical density as the positive control an extract was recorded as positive. In all other cases extracts were designated as negative. As positive results were not confirmed by LC-MS, all positive isolates are referred to as producing the cereulide-like compound.

Detection of genes for enterotoxins

After the overnight cultivation in BHI, DNA was collected from the *B. cereus* strain by using the Wizard Genomic DNA Purification Kit (Promega, Madison WI, USA). Purified DNA was stored at -20°C until further use.

The presence of enterotoxin genes was determined by various PCR protocols. For detection of the haemolysin BL (HBL)–genes, haemolytic enterotoxin (NHE)-genes and the cytotoxin K-gene previously described protocols were used (Guinebretiere et al. 2002; Heinrichs et al. 1993; Ryan et al. 1997). The reverse primer for the cytotoxin K PCR, as described by Guinebretiere et al. (2002), was replaced by a primer with the following sequence: TCC AAC CCA GTT (A T) (G,C), as the wrong sequence for this primer was mentioned in the publication (M.H.

Guinebretiére, personal communication). The three components of HBL are encoded by a single operon. Therefore, if any of the three genes encoding HBL was detected we assumed all three HBL genes were present. The same strategy was use for the detection of NHE genes.

Determination of growth temperature profile

The distinction between psychrotrophy and mesophily was determined by using a previously described PCR-method (von Stetten et al. 1998), detecting a difference in the 16S rDNA gene. After electrophoresis of the PCR products, mesophilic strains are recognized by a 250 base pair (bp) fragment and psychrotrophic strains by a 180 bp fragment. Intermediate strains possess both fragments upon electrophoresis. Earlier results show a good correlation between assessing growth temperature profile by culture at various temperatures and the PCR method (Prüss et al. 1999)

Results

Table 2.1 Distribution of samples and isolates of *Bacillus cereus* as determined on MEYP-agar over the product groups which were investigated for virulence factors

Product group	Samples	Isolates	Isolates per sample
Oil(s) and fat(s) and oil and fat products	1	5	5
Fish and fish products	6	15	2.5
Meat and meat products	6	24	4
Flavourings	22	92	4.2
Milk and milk products	17	80	4.7
Ready-to-eat foods	81	384	4.7
Vegetable(s) and vegetable products	30	115	3.8
Pastry	19	81	4.3

For this study 796 isolates, originating from 182 samples, were investigated for the following factors: presence of genes encoding the enterotoxins, ability to produce cereulide, and growth temperature signature. The distribution of the samples and isolates over the product groups as defined in the “Kokswarenbesluit” (Anonymous, 1979) and employed by the VWA is shown in Table 2.1. These isolates, sent to the RIVM for further investigation, originated from approximately 10% of all the samples investigated by the VWA (the selection was random).

Table 2.2 Distribution of growth temperature signatures in numbers (and percentages) of isolates of *Bacillus cereus* in various food product groups. The total number of isolates per product group is shown in the bottom line. The total number of isolates (and percentages) per growth temperature signature is shown in the last column

Growth temperature signature	Oil(s) and fat(s) and oil and fat products [# isolates (%)]	Fish and fish products [# isolates (%)]	Meat and meat products [# isolates (%)]	Flavourings [# isolates (%)]	Milk and milk products [# isolates (%)]	Ready-to-eat foods [# isolates (%)]	Vegetable(s) and vegetable products [# isolates (%)]	Pastry [# isolates (%)]	Total # isolates (%) per signature
Mesophilic	5 (100)[a]	9 (60)	19 (79.2)	91 (98.9)	58 (72.5)	356 (92.7)	105 (91.4)	73 (90.1)	716 (89.9)
Psychrotrophic	0 (0)	6 (40)	5 (20.8)	0 (0)	5 (6.3)	12 (3.1)	5 (4.3)	2 (2.5)	35 (4.4)
Intermediate	0 (0)	0	0	1 (1.1)	17 (21.2)	16 (4.2)	5 (4.3)	6 (7.4)	45 (5.7)
Total # isolates	5	15	24	92	80	384	115	81	796

[a] *Percentages were calculated by dividing the number of isolates by the total number of isolates in each product group*

The distribution of the growth temperature signatures over the various product groups is shown in Table 2.2. The distribution is given in absolute numbers of isolates per profile per product group and in percentages of isolates per product group. In the last column the overall numbers of isolates per profile are shown with their corresponding percentages. The majority of the isolates is mesophilic (89.9%), and only minorities are either psychrotrophic or of intermediate signature (4.4% and 5.7% respectively). Remarkably, none of the strains from the "flavourings"-group have a psychrotrophic signature, although a large group of samples originates from this product group. Apart from one isolate (1.1%) which is of intermediate nature, all other isolates (98.9%) have mesophilic signatures and presumably do not grow at temperatures below 10°C (Adams and Moss, 2000).

In order to determine whether a significant number of samples from a certain product group is different from the overall results with respect to growth temperature profile, the mean percentages per growth temperature profile with the 95% confidence intervals were determined. This was accomplished by adding up the percentages of the different groups, dividing the total by the number of groups and determining the confidence interval of the mean value. Taking all product groups into account, the results are shown in Table 2.3, in columns "mean" and "interval", respectively. The product groups that do not fall within the confidence interval, and that are therefore significantly different, are shown in Table 2.3 in columns "below interval" and "above interval", respectively. As the product groups with small numbers of isolates might bias the result, the same calculations were performed with product groups with > 80 isolates only. Also similar calculations with product groups with >80 isolates were carried out excluding the "milk and milk products" group. Both calculations indicated that a significant number of isolates from the "milk and milk products" group has a psychrotrophic signature (data of the last calculations not shown).

Notably, all isolates able to produce the cereulide-like toxin are of mesophilic nature. This is in concurrence with results previously described by (Pielaat et al. 2005).

Table 2.3 Overall distribution (in percentages of all isolates) of growth temperature signatures of *Bacillus cereus* as determined by PCR (von Stetten et al. 1998) and the product groups scoring outside the 95% confidence interval

Growth temperature signature	Mean[a]	95% confidence interval	Product group scoring below interval	Product group scoring above interval
Mesophilic	82.6	74.0 – 97.2	fish and fish prod. milk and milk prod.	flavourings
Psychrotrophic	9.6	-2.1 – 21.3		fish and fish prod. meat and meat prod.
Intermediate	4.8	-1.2 – 10.8		milk and milk prod.

[a]) *Mean percentages were determined by dividing the added sum of the percentages per product group by the number of product groups (= 8)*

Table 2.4 gives a summary of the analyses of potential to produce cereulide-like toxin and presence of enterotoxin genes. For each product group the occurrence of each (combination of) virulence factor(s) (genes coding for enterotoxins and ability to produce cereulide) is described. Also the percentage of isolates containing a certain combination of virulence factors is mentioned. The virulence factor "ability to produce cereulide-like toxin" is never found as a single factor, but always in combination with the presence of the gene(s) for at least one of the enterotoxins. Some combinations of virulence factors have not been found, namely production of cereulide-like toxin together with only genes encoding HBL, production of cereulide-like toxin together with only genes encoding cytotoxin K, and production of cereulide-like toxin together with genes encoding HBL and cytotoxin K but not NHE. Some combinations of virulence factors are found only very scarcely, namely genes encoding HBL in the absence of other toxin genes, the gene encoding cytotoxin K in the absence of other toxin genes, production of cereulide-like toxin in the presence of genes encoding NHE and cytotoxin K, production of cereulide-like toxin in the presence of genes encoding HBL, NHE and cytotoxin K, genes encoding HBL and cytotoxin K, and genes encoding NHE and cytotoxin K. Of all isolates 95.4% contain the genes for NHE, either as a single virulence factor or in combination with other factors. Approximately 66% of the isolates contain the genes for HBL, and nearly 50% of the isolates contain

Table 2.4 Distribution in numbers and percentages of isolates of *Bacillus cereus* per product group over the (combinations of) virulence factors. The total number of isolates per group is shown in the bottom line, the overall number of isolates (and percentages) for each (combination of) virulence factor is shown in the last column

Toxin and genes	Oil(s) and fat(s) and oil and fat products [# isolates (%)]	Fish and fish products [# isolates (%)]	Meat and meat products [# isolates (%)]	Flavourings [# isolates (%)]	Milk and milk products [# isolates (%)]	Ready-to-eat foods [# isolates (%)]	Vegetable(s) and vegetable products [# isolates (%)]	Pastry [# isolates (%)]	Total # samples (%) per (combination of) virulence factor
none[a]	0 (0)	0 (0)	3 (12.5)	1 (1.1)	0 (0)	1 (0.26)	0 (0)	0 (0)	5 (0.6)
cer[b] (alone)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
nhe[c] (alone)	5 (100)	4 (26.7)	3 (12.5)	5 (5.4)	24 (30)	90 (23.4)	18 (15.7)	14 (17.2)	163 (20.5)
hbl[d] (alone)	0 (0)	0 (0)	2 (8.3)	5 (5.4)	1 (1.3)	5 (1.3)	8 (7.0)	0 (0)	21 (2.6)
cytK[e] (alone)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (1.3)	0 (0)	0 (0)	5 (0.6)
cer-nhe	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.3)	26 (6.8)	0 (0)	2 (2.5)	29 (3.6)
cer-hbl	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
cer-cytK	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
cer-hbl-nhe	0 (0)	2 (13.3)	0 (0)	1 (1.1)	11 (13.8)	15 (3.9)	2 (1.7)	1 (1.2)	32 (4.0)
cer-hbl-cytK	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
cer-nhe-cytK	0 (0)	0 (0)	0 (0)	1 (1.1)	0 (0)	0 (0)	0 (0)	1 (1.2)	2 (0.3)
cer-hbl-nhe-cytK	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)	1 (0.9)	0 (0)	2 (0.3)
hbl-nhe	0 (0)	4 (26.7)	0 (0)	16 (17.4)	19 (23.8)	70 (18.2)	21 (18.3)	30 (37.0)	160 (20.1)
hbl-cytK	0 (0)	0 (0)	0 (0)	0 (0)	2 (2.5)	4 (1.0)	0 (0)	0 (0)	6 (0.8)
nhe-cytK	0 (0)	4 (26.7)	1 (4.2)	7 (7.7)	6 (7.5)	36 (9.4)	2 (1.7)	6 (7.4)	62 (7.8)
hbl-nhe-cytK	0 (0)	1 (6.7)	15 (62.5)	56 (60.9)	16 (20.0)	131 (34.1)	63 (54.8)	27 (33.3)	309 (38.8)
total # isolates/group	5	15	24	92	80	384	115	81	

[a] *none = no cereulide-like toxin production nor genes for enterotoxins*
[b] *cer = cereulide-like toxin production*
[c] *nhe = genes encoding non haemolytic enterotoxin*
[d] *hbl = genes encoding haemolysin BL*
[e] *cytK = gene encoding cytotoxin K*

the gene for cytotoxin K. Of all isolates approximately 8% are positive in the HEp-2 cell test, i.e. producing cereulide-like toxin.

Although no significant correlations could be found between the occurrence of specific (combination of) virulence factors and specific product groups (data not shown), two combinations attract attention: the relatively high percentage of isolates from the "pastry"-group containing the combination HBL-NHE, and the relatively high percentage of isolates from the "flavourings"-group containing solely NHE.

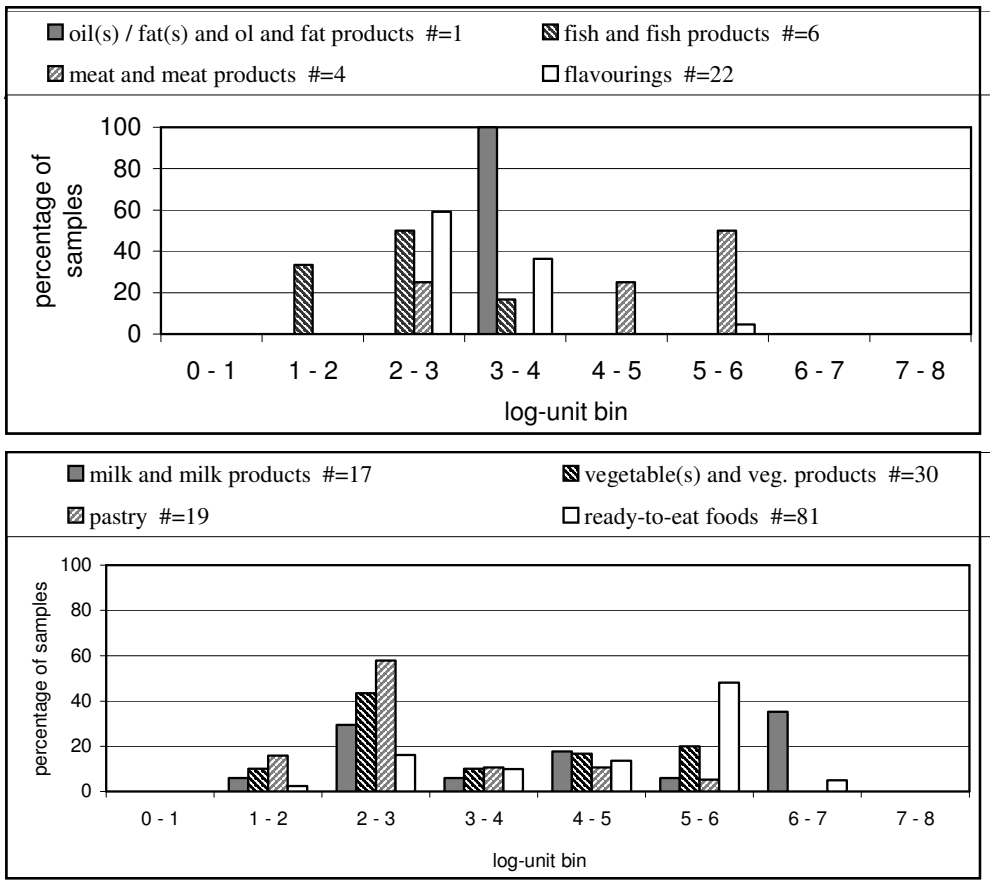

Figure 2.1 Distribution of numbers of *Bacillus cereus* per gram food (CFU/g), determined on MEYP-agar, within the product groups. Numbers of CFU/g are presented in log-unit bins. The percentages of samples from the total number of samples per product group, as shown in the legend, falling within a certain log unit bin of CFU counts are shown. Each sub-figure depicts two product groups as indicated.

Determination of the number of *Bacillus cereus* per gram food (CFU/g) using MEYP-agar was carried out for most samples. The counts were divided in log-unit bins, and all counts have been categorized accordingly for each product group. The results are shown in Figure 2.1,

each graph showing the results of four product groups. Per product group, the number of samples falling into a certain log-unit bin is shown as a percentage of the total number of samples in each product-group. The distributions in the "meat and meat products" group, the "flavourings" group, the "ready-to-eat foods" group and the "pastry" group show little variation: for the "pastry"-group mostly low numbers of *B. cereus* are found, for the "flavourings" and "ready-to-eat foods" groups mostly the high numbers of *B. cereus* are found.
Although they appear to be more evenly distributed, the numbers of *B. cereus* in the "milk and milk products" and "vegetable(s) and vegetable products" groups also show high peaks at the high and low numbers respectively.
The "ready-to-eat foods" group consists of a large number of samples, originating from a large variety of products. When taking a closer look, especially the samples that were taken from rice- and pasta-containing dishes (each amounting to nearly 20 samples) showed remarkably high numbers of *B. cereus*, mostly 10^5 or more per gram (data not shown).

The characterization as described here was carried out with a random selection of isolates collected between March 2001 and October 2004. Therefore, it is possible that the proportion of isolates with a relative high *B. cereus* count is not representative for what might be found during a whole year, taking all samples into account. To put the number of samples with high *B. cereus* counts into perspective with the actual situation, the database from the Food and Consumer Product Safety Authority (VWA) was consulted.

A survey was made for a 12-month period, May 2002 – May 2003, regarding the analyses of food commodities for numbers of *B. cereus* per gram food. The numbers are expressed in various manners: exact numbers of *B. cereus* per gram and relative numbers such as < 10, < 100, or > 100,000 *B. cereus* per gram. The numbers of *B. cereus* of all samples are categorized per product group in being < 10^5 CFU/g and > 10^5 CFU/g. Five samples could not be included in this categorization. The results are shown in Table 2.5.

Table 2.5 Numbers of *Bacillus cereus* determined on MEYP-agar in samples of various food product groups subdivided in the two categories of $<10^5$ CFU/g and $\geq 10^5$ CFU/g, determined in period May 2002 – May 2003 by the Food and Consumer Product Safety Authority

Product group	Total # samples	# samples (%) with CFU/g < 105	# samples (%) with CFU/g > 105
Oil(s) and fat(s) and oil and fat products	60	60 (100)	0 (0)
Fish and fish products	102	102 (100)	0 (0)
Meat and meat products	280	280 (100)	0 (0)
Flavourings	1384	1382 (99.86)	2 (0.14)
Milk and milk products	5943	5932 (99.81)	11 (0.19)
Ready-to-eat foods	22744	22677 (99.71)	67 (0.29)
Vegetable(s) and vegetable products	637	636 (99.84)	1 (0.16)
Pastry	2637	2636 (99.96)	1 (0.04)

The exact *B. cereus* counts have been categorized per product group in log-unit bins, and the results of this categorization are shown in Figure 2.2. The percentages of samples per log-unit bin are depicted in each sub-figure. For each product group the number of samples with an exact *B. cereus* count is mentioned in the legend of each sub-figure.

Figures 2.1 and 2.2 vary for the numbers of investigated samples and the percentages per product groups, but from both figures it is obvious that the same product groups, namely “flavourings”, ‘milk and milk products”, “vegetable(s) and vegetable products”, “pastry”, and “ready-to-eat foods”, show a certain percentage of samples with high counts of *B. cereus*.

Discussion

Assessing the exposure to *Bacillus cereus* is an important issue in estimating the risk for food borne disease by this micro-organism. The actual disease symptoms, however, are caused by (entero-) toxins, either produced during growth in the gut (enterotoxins), or produced during growth in food (emetic toxin). Assessing the ability of strains to produce such virulence factors is therefore just as important, if not even more so. Besides these virulence factors the growth temperature profile of strains is also of importance for exposure assessment, as mesophilic strains appear to be more important for diarrhoeal disease due to their growth potential at higher temperatures (Adams and Moss, 2000).

We chose for determining the potential for production of (entero-) toxins, instead of direct detection of (entero-) toxins for three reasons. Firstly, the food samples were collected by the Inspectorate and transport

of food samples to the RIVM would have caused a logistic problem. Secondly, as enterotoxins would not survive the gastric passage due to low pH and the presence of proteolytic enzymes, determining their presence in food commodities prior to consumption would be irrelevant (Shinagawa et al. 1991c).

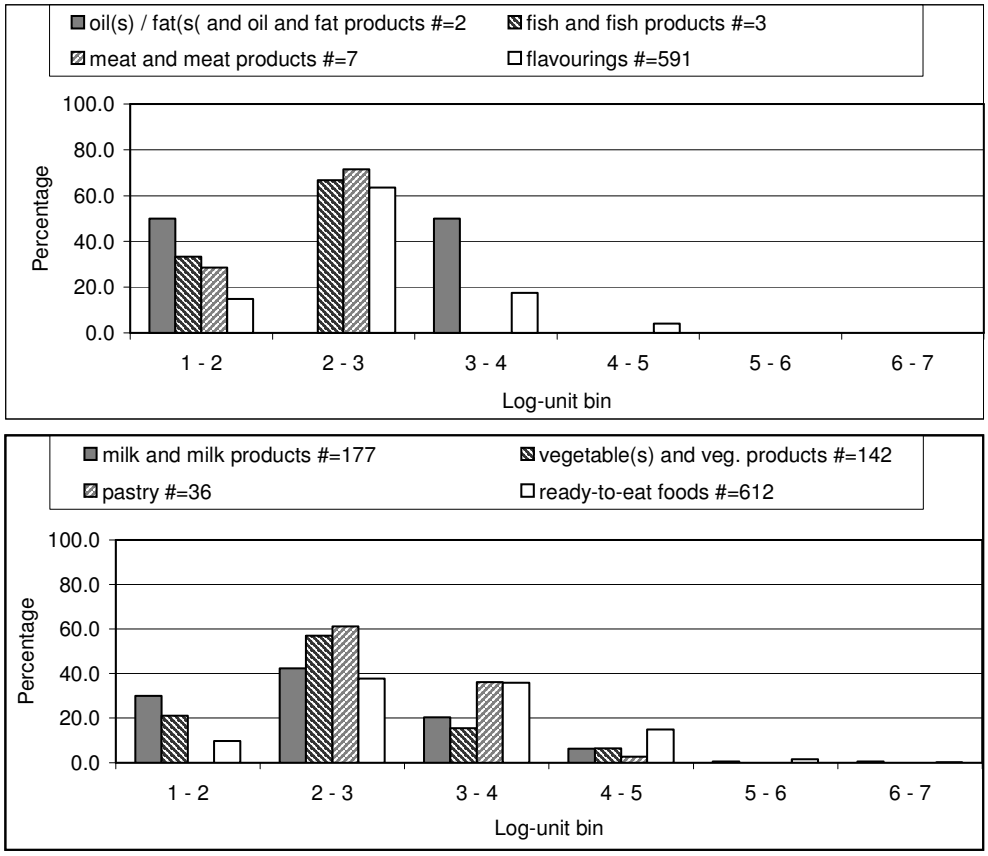

Figure 2.2 *Distribution of numbers of* Bacillus cereus *per gram food (CFU/g), determined on MEYP-agar, within the product groups as extracted from the database from the Food and Consumer Product Safety Authority for the period May 2002 – May 2003. Numbers of CFU/g are presented in log-unit bins. The percentages of samples falling within a certain log unit bin of CFU counts are shown. Each sub-figure depicts two product groups as indicated.*

Thirdly, as direct detection of cereulide in foods is a procedure that has not yet been optimized for all food categories tested in this study, it could not be used. Whether the (entero-) toxins are really produced by all isolates carrying the genes was not established. This means that over-estimation of the eventual risk is possible.

From the results it can be concluded that the genes encoding the enterotoxin NHE are detectable in nearly all isolates. Similar results have been found earlier (Anderson Borge et al. 2001; Hansen and Hendriksen, 2001).

Some (combinations of) virulence factors, such as cereulide production in the absence of any other toxin gene(s), have not been found in the isolates, other (combinations of) virulence factors are very rarely found.

No apparent associations between food commodities and the presence of virulence factors can be detected from our results. The only associations we detected, which might be more relevant, are the low percentage of samples in the "flavourings" group containing solely the genes encoding NHE, and the relatively high occurrence of the combined genes encoding HBL and NHE in the "pastry" group. The absence of apparent associations is in contradiction with some epidemiological information. For example, rice and pasta dishes (as representatives for carbohydrate-rich food commodities) are very often involved in emetic outbreaks. However, in these food groups no elevated prevalence of isolates able to produce cereulide is seen. This may mean that other factors influence the expression of certain traits.

A remarkably large proportion of strains from the "milk and milk products" group has a psychrotrophic signature. The 72.5% of the isolates from this group having a mesophilic signature is significantly below the 95% confidence interval of the mean prevalence of isolates possessing a mesophilic signature, and the 21.3% of the strains having an intermediate signature is far above the 95% confidence interval of the mean prevalence of isolates with an intermediate signature. Therefore, we conclude that in the "milk and milk products" group the occurrence of strains with a psychrotrophic signature (27.5% in total) is above average. This is comparable with earlier investigations, which found that 29 of 115 strains (25%) isolated from Danish milk were of psychrotrophic signature (Larsen and Jorgensen, 1999). Also in the product groups "fish and fish products" and "meat and meat products" remarkably high percentages of psychrotrophic strains are found. However, the numbers of strains in these groups is low compared to the "milk and milk products" group. These findings suggest that strains with a psychrotrophic signature are more prevalent in products stored at refrigerator temperature. However, since only milk products have been investigated, further research is necessary to validate this suggestion.

Quite a number of samples investigated for *B. cereus* have colony counts on MEYP-agar exceeding the Dutch tolerance level of 10^5 CFU·g^{-1} (see Figure 2.1). To investigate a possible bias due to analysing only a limited number of samples taken over a three year period, the overall data form the Food and Consumer Product Safety Authority were considered. The number of samples investigated for *B. cereus* and the outcome of these investigations over a one-year period were investigated. This evaluation, referred to in Table 2.5 and Figure 2.2, shows that the actual numbers of samples with a *B. cereus* colony count above 10^5 g^{-1} are less than suggested by Figure 2.1. However, both evaluations (Figures 2.1 and 2.2) point in the same direction where product groups with elevated *B. cereus* counts are concerned. Therefore, the conclusion must be that, although the numbers of samples exceeding the Dutch tolerance level as shown in Figure 2.1 are an over-estimation of the numbers averagely found per year, certain product groups are more prone to higher colony counts. These product groups are: "flavourings", "milk and milk products", "vegetable(s) and vegetable products", "pastry", and "ready-to-eat foods".
This fact must be taken into account for assessing the risk of disease due to *B. cereus* in food.

In general, this work yields three major results. Firstly, all isolates appear to have at least one enterotoxin gene, which means that all these *B. cereus* strains are potentially pathogenic. Secondly, in general the incidence of psychrotrophic strains is low. However, in products that need storage at low temperatures the incidence is significantly higher. Thirdly, the incidence of strains producing a cereulide-like toxin is low, but not different from results from other parts of the world.
The PCR results on the incidence of enterotoxin genes have to be regarded with caution. On the one hand the occurrence of genes cannot be translated directly into the ability to produce the enterotoxin(s). Other genetic regulation mechanisms influence the actual transcription and translation. As these mechanisms were not taken into account here, this may lead to an over-estimation when these results are translated into human risk. On the other hand, the results as shown here may be incomplete due to diversity in the enterotoxin genes for HBL and NHE as described by Guinebretiere et al. (2002). As this was not taken into consideration, some genes might have been missed. This leads to under-estimation of the occurrence of these genes.

Only about 10% of the samples investigated for *B. cereus* by the Food and Consumer Product Safety Authority was characterized in detail.

Still, these data can be used, together with the observation that some product groups contain relatively high numbers of *B. cereus* for estimating exposure to *B. cereus*, and evaluating the consequences for a risk assessment concerning *B. cereus* derived food borne illness.

3

Spores from mesophilic *Bacillus cereus* strains germinate better and grow faster in simulated gastro-intestinal conditions than spores from psychrotrophic strains

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Abstract

The species *Bacillus cereus*, known for its ability to cause food borne disease, consists of a large variety of strains. An important property for discrimination of strains is their growth temperature range. Psychrotrophic strains can grow well at refrigerator temperatures but grow at 37°C with difficulty. Mesophilic strains on the other hand are unable to grow below 10°C, but grow well at 37°C.

Spores of six psychrotrophic and six mesophilic strains were investigated for their ability to survive and grow in simulated gastro-intestinal fluids, mimicking the conditions in the gastro-intestinal tract.

The germination potential of psychrotrophic and mesophilic spores in simulated intestinal fluid appeared to be similar. Under these conditions, 5 out of 6 mesophilic strains showed growth, and only 2 out of 6 psychrotrophic strains. Temperature (37°C) and simulated gastro-intestinal conditions together influenced germination and growth.

Introduction

Bacillus cereus is a ubiquitous, Gram-positive, spore-forming micro-organism capable of causing food borne disease (Kramer and Gilbert, 1989). Two syndromes can be distinguished, an emetic syndrome and a diarrhoeal syndrome (Fricker et al. 2007).

The emetic syndrome is caused by cereulide, a heat- and pH-stable peptide toxin (Agata et al. 1994). Consumption of food contaminated with this toxin may lead to emesis between 30 minutes and 5 hours after ingestion (Drobniewski, 1993).

The diarrhoeal syndrome is caused by enterotoxins that are produced during growth of *B. cereus* in the small intestine. At present three enterotoxins, able to cause the diarrhoeal syndrome, have been described: haemolysin BL (HBL), non haemolytic enterotoxin (NHE) and cytotoxin K. HBL and NHE are three component proteins, whereas cytotoxin K is a single protein toxin (Beecher and Lee Wong, 1994; Granum et al. 1999; Lund et al. 2000). Symptoms caused by the latter toxin are more severe and may even involve necrosis. In general, the onset of symptoms is within 6 to 24 hours after consumption of the incriminated food.

Among the strains of *B. cereus* a great diversity exists, for instance with respect to the presence of enterotoxin genes, with respect to the ability to produce emetic toxin and with respect to the ability to grow at various temperatures. Although many *B. cereus* strains are able to form enterotoxins, not all of those strains appear to be able to cause disease. The so-called psychrotrophic strains are able to grow at low temperatures as can be encountered in refrigerators. Generally, these do not grow very well at temperatures around 37ºC, like in the human body. Mesophilic strains, on the other hand, easily grow at 37°C and higher, and may survive refrigerator temperatures without growth (Montville, 1997; Adams and Moss, 2000).

The mechanism of the diarrhoeal syndrome is believed to be as follows: food contaminated with *B. cereus* spores and/or vegetative cells is consumed. Spores pass through the stomach, reach the small intestine, germinate, grow, and thereby produce enterotoxins. Vegetative cells are believed to be very susceptible to stomach conditions, and will therefore hardly reach the small intestine. However, during food consumption the pH in the stomach may reach values of around 4.5 – 5 (Dressman et al. 1990; Russell et al. 1993). At such values also vegetative cells may survive the conditions in the stomach, and subsequently reach the small intestine

intact. Also under certain circumstances short residence times in the stomach or protective compounds may help vegetative cells to pass the gastric barrier. In the small intestine these vegetative cells may grow and thus also contribute to the onset of disease.

The aim of the research described in this paper was to investigate how germination of spores and subsequent growth of a variety of *B. cereus* strains are influenced by gastro-intestinal conditions. In the framework of risk assessment these investigations fall within the scope of hazard characterization. Because of ethical problems such research cannot be performed *in vivo*; therefore simulated conditions were used. Several formulations for simulated gastric and intestinal fluids have been described (Rotard et al. 1995; Astwood et al. 1996; Cooper et al. 1995). Spores of several strains were exposed to simulated gastric and intestinal fluids separately. Also experiments were carried out in which spores were exposed to simulated intestinal fluid after exposure to simulated gastric fluid, thereby mimicking gastro-intestinal transfer.

Materials and Methods

Strains.

The twelve *B. cereus* strains used for these experiments are described in Table 3.1. Strains were grown on MEYP[3]-agar (Mossel et al. 1967) and subsequently tested for glucose fermentation, acetylmethylcarbinol production according to Voges-Proskauer, nitrate-reduction and gelatine turn-over. Purity was checked by plating the strains on Columbia agar with sheep blood and overnight incubation at 30°C. All culture media were produced by the Dutch Vaccine Institute (NVI, the Netherlands).

The temperature profile of the strains was established by a previously described method based on polymerase chain reaction (von Stetten et al. 1998). Of the psychrotrophic strains only strain 20 was previously identified as *B. weihenstephanensis* (M.H.Guinebretière, personal communication). The other psychrotrophic strains were not investigated with the specific methods described by Lechner et al. (1998) to assess *B. weihenstephanensis* identity.

[3] MEYP = mannitol egg yolk phenol red

Table 3.1 Origin of *Bacillus cereus* strains

Strain	Original number	Origin	Source	Growth temp. profile[a]
2	9901672	RIVM[b]	Human faeces	m
3	9901909-1	RIVM	Human faeces	p
5	9902169-2	RIVM	Human faeces	m
7	9902187-1	RIVM	Human faeces	p
17	9903043-1	RIVM	Human faeces	p
18	9903295-4	RIVM	Human faeces	p
20	TZ 415	INRA	Cooked chilled food[c]	p
22	Z 4222	INRA	Cooked chilled food[c]	p
25	11143	NCTC	Food poisoning (emetic)	m
26	11145	NCTC	Food poisoning (diarrhoeal)	m
27	4384	DSMZ	Food poisoning (diarrhoeal)	m
28	1230-88	NVH	Human faeces	m

NCTC = National Collection of Type Cultures
DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen
(German Collection of Micro-organisms and Cell cultures)
NVH = Norwegian School of Veterinary Sciences
[a] = temperature profile: m = mesophilic, p = psychrotrophic
[b] = strains from a RIVM project on the incidence of pathogenesis of human gastroenteritis
[c] = reference (Choma et al. 2000)

Spore production.

Pure *B. cereus* strains were grown on PEMBA[4]-agar (Holbrook and Anderson, 1980) for 48 hours at 30°C. Spores were collected by scraping colonies from the plates, using distilled water, and centrifugation (10 minutes 1800xg). Washings and centrifugation were continued until the suspension was milky white (generally 4 times). After the final centrifugation step, spores were suspended in physiological salt solution, heated 10 minutes at 80ºC, and stored in 1.5 ml vials (1 ml per vial) at -70ºC. There might be a risk that spores from some of the psychrotrophic strains are partly inactivated, since the D_{80}-value of the psychrotrophic strains were 14, 28, > 100, 75, 29 and 21 minutes for strains 3, 7, 17, 18, 20 and 22, respectively (unpublished data). However, within the set up of the experiments we chose to standardize inactivation at the set time and temperature. Spore counts were performed after the final suspension of spores in physiological salt solution (the final suspensions were not adjusted to equal spore concentrations before storage and experiments).

[4] PEMBA = polymyxin pyruvate egg yolk mannitol bromothymol blue

Simulated gastric and intestinal fluids

The simulated gastric and intestinal fluids, SGF and SIF respectively, are based upon previously described formulations (Rotard et al. 1995). SGF consisted of sodium chloride (NaCl) (2.75 g/l), sodium dihydrogen phosphate (NaH_2PO_4) (0.27 g/l), potassium chloride (KCl) (0.82 g/l), calcium chloride ($CaCl_2$) (0.4 g/l), ammonium chloride (NH_4Cl) (0.05 g/l), hydrochloric acid (HCl) 37% (15 ml/l), glucose (0.65 g/l), glucuronic acid (0.02 g/l), urea (0.085 g/l), glucosamine (0.33 g/l), bovine serum albumin Fraktion V(1 g/l), pepsin (1 g/l) and mucin (Type II) (3 g/l). Using HCl the pH was set at 1.0, 2.5, or 4.5, dependant on the experiment. SGF was sterilized by filtration through a 0.22 µm filter (Nalgene, USA).

SIF consisted of NaCl (6.58 g/l), mono sodium carbonate ($NaHCO_3$) (3.98 g/l), potassium dihydrogen phosphate (KH_2PO_4) (0.66 g/l), KCl (0.043 g/l), magnesium chloride ($MgCl_2$) (0.17 g/l), urea (0.075 g/l), bovine serum albumin (Fraktion V) (1.2 g/l), lipase (Type II from porcine pancreas, 100-400 units/mg using olive oil) (0.375 g/l), pancreatin (from porcine pancreas, 350 FIP-U/g activated protease) (2.25 g/l), $CaCl_2$ (60 mg/l), and bile salts (from ox gall, dried and unfractionated) (1.5 g/l). Lipase (L3126), bile salts (B3883), mucine (M2378) and $CaCl_2$ (C5080) were from Sigma (St. Louis, USA). Glucuronic acid (art. nr. 49335) was from Fluka (Buchs, Switzerland): glucosamine (art. no. 346299) was from Calbiochem (EMD Biosciences Inc., San Diego, USA). Sodium chloride (art. no. 106404), NaH_2PO_4 (art. no. 106345), KCl (art. no. 104933), NH_4Cl (art. no. 101145), HCl (art. no. 100317), glucose (art. no. 108337), bovine serum albumin Fraktion V (art. no. 112018), pepsin (art. no. 107190), KH_2PO_4 (art. no. 104877), $MgCl_2$ (art. no. 105833), urea (art. no. 108487) and pancreatin (art. no. 107130) were from Merck (Darmstadt, Germany). Before the addition of lipase, pancreatin, and bile salts the basic SIF-solution was sterilized by filtration through a 0.22 µm filter (Nalgene, USA). The absence of micro-organisms in the lipase, pancreatin, and bile salts was verified by plating the powders from the container directly to Columbia agar with sheep blood (Oxoid, Basingstoke, UK), and incubating the plates overnight at 30°C and 37°C. The pH of SIF was 7.8 ± 0.3.

Evaluation of survival and growth

Samples (1 ml), taken during the experiments, were investigated for the total number of colony forming units (vegetative cells and spores, i.e. total count) and for the number of spores (i.e. spore count) after heating

the 10x diluted original samples for 10 minutes at 80°C. Ten-fold dilutions of the samples in physiological salt solution containing 0.1% (w/v) peptone were plated in duplicate on Trypton Soy agar [Dutch Vaccine Institute (NVI), the Netherlands]. Agar plates were incubated overnight (i.e. 18 – 20 hours) at 30°C, and numbers of colonies were recorded from countable plates ($15 < x < 150$ per plate). The means of the duplicates of total and spore counts were calculated and used for further evaluations.

Identical experiments were carried out with all twelve strains, unless mentioned otherwise.

Reproducibility of experiments

Both the simulated gastric and intestinal fluids had to be produced freshly for each experiment. In addition, only a limited number of strains could be tested on one day. Therefore, a variety of batches of simulated fluids were used. To assess the reproducibility of results between tests, spores of strain 2 were subjected to the tests in each experiment, and served therefore as an internal control. Considering the complexity of the experiments, this method of assessing reproducibility was preferred over twice or thrice repeating the experiments with all strains.

Behaviour of Bacillus cereus *spores in gastric conditions*

After thawing, spores were suspended in 50 ml simulated gastric fluid (SGF) at pH 2.5 for 60 minutes at 37°C in a water bath. Samples were taken at the start of the incubation (t=0), after 30 minutes (t=30) and at the end of the incubation period (t=60).

Behaviour of Bacillus cereus *spores in intestinal conditions*

After thawing, spores were suspended in 50 ml simulated intestinal fluid (SIF) at pH 7.8 +/- 0.3 in a water bath at 37ºC for 4 hours. Samples were taken at the start of the experiment (t=0) and at each hour afterwards, expressed in minutes (t= 60, 120, 180, and 240).

Behaviour of spores in simulated gastro-intestinal transfer

After thawing, spores were suspended in 50 ml SGF at pH 2.5. After one hour incubation at 37ºC, 1 ml SGF suspension was transferred to 50 ml SIF and incubated further for 4 hours at 37ºC.

Samples were collected at the start of the experiment in SGF (t=0 SGF), after 60 minutes incubation in SGF (t=60 SGF), immediately after

transfer to SIF (t=0 SIF) and every hour during incubation in SIF, expressed in minutes (t=60 SIF, 120 SIF, 180 SIF, and 240 SIF).
Similar experiments, with SGF of pH 1.0 and of pH 4.0, were carried out with one psychrotrophic strain (22) and one mesophilic strain (27).
To compensate for the 50-fold dilution upon transfer of one ml SGF to 50 ml SIF the total and spore counts in SIF have been recalculated.

Influence of low temperature storage on behaviour under gastro-intestinal circumstances

Since food commodities are often stored in refrigerators, the influence of such treatment on the behaviour of spores in simulated gastro-intestinal conditions was investigated. Following incubation at 10ºC overnight, spores were exposed to SGF at pH 2.5 and subsequently to SIF. Samples were collected and investigated as described under "Behaviour of spores in simulated gastro-intestinal transfer". These experiments were carried out with psychrotrophic strains 7, 18, and 22 and mesophilic strains 2, 5, and 27.

Comparison of growth in brain heart infusion broth and SIF

The experimental temperature of 37°C is less favourable for psychrotrophic strains, in comparison to mesophilic strains. To exclude the possibility that differences in growth rate are to be attributed to this experimental temperature only, the following experiments were carried out. Stationary phase cells of all strains, obtained after overnight growth in Luria Bertani Glucose broth[5] at pH 7.0 were grown simultaneously in Brain Heart Infusion broth (BHI) and SIF (without the addition of lipase, pancreatin, and bile) at 37°C. At hourly intervals total counts were performed in duplicate as described under "Evaluation of survival and growth". From these data generation times were calculated as previously described (Anonymous 1997).

[5] Luria Bertani Glucose broth consists of 10 g Trypton (Oxoid L42, Oxoid Ltd., Basingstoke, UK), 5 g yeast extract (Oxoid L21), 10 g sodiumchloride (Merck 106404, Merck GmbH, Darnstadt, Germany), and 4 g glucose (Merck 108337) per liter. To set the pH at 7.0 MOPS (morpholinepropanesulfonic acid, Roche 1124684, Mannheim, Germany) was added (20.9 g l^{-1})

Results

Behaviour of spores in simulated gastric conditions at pH 2.5

In Figure 3.1 the spore and total counts of psychrotrophic organisms (Figure 3.1A) and mesophilic organisms (Figure 3.1B) in SGF at pH 2.5 are shown. The spore counts from strains 3, 7, 20 and 22 were about one log unit lower than the total counts, the changes in spore counts for all other strains were comparable to the changes in total counts.

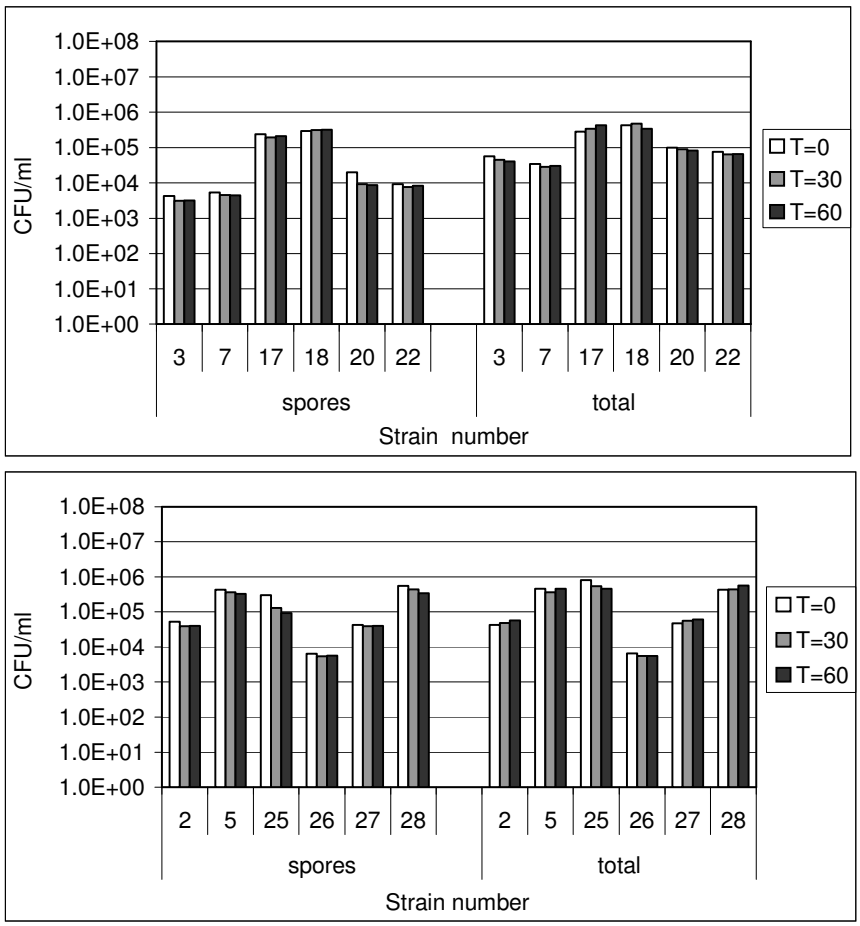

Figure 3.1 Effect of incubation of spores of psychrotrophic (top) and mesophilic (bottom) *Bacillus cereus* strains in simulated gastric fluid at pH 2.5 at 0, 30 and 60 minutes at 37°C.

During the incubation period the total counts and the spore counts did not show dramatic changes, neither for the psychrotrophic strains nor for the mesophilic strains. The changes in numbers of spores that took place were within one log unit. So, even with a residence time of 60 minutes, no relevant inactivation takes place in stomach.

Behaviour of spores in simulated intestinal conditions

In Figures 3.2 and 3.3 the behaviour of spores in SIF is shown in total counts and spore counts: in Figure 3.2 the results from the psychrotrophic strains are shown, Figure 3.3 represents the mesophilic strains.

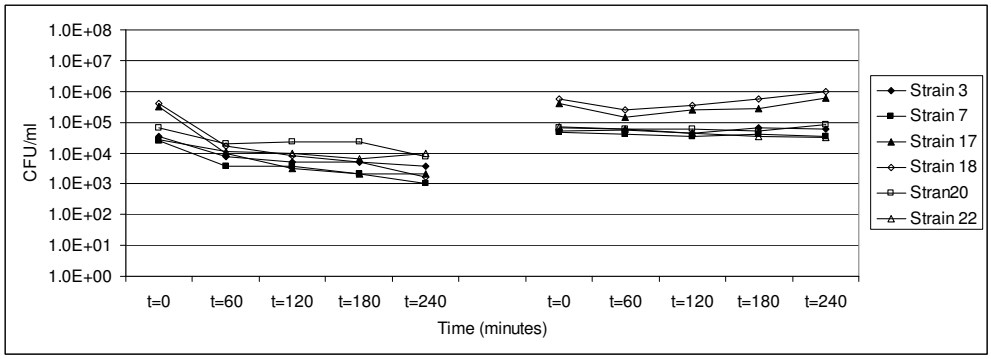

Figure 3.2 Spore counts (left) and total CFU (right) after the addition of spores of psychrotrophic *Bacillus cereus* strains to simulated intestinal fluid at 37°C.

In both figures the results for the spore counts are shown on the left, and the results for the total counts are shown on the right. All 6 psychrotrophic strains show a decrease in spore count during the incubation period. For two strains, 17 and 18, this decrease in spore count amounts to more than one log unit, while for the other four strains the decrease is approximately one log unit or less. The decrease in spore counts indicates germination of spores.

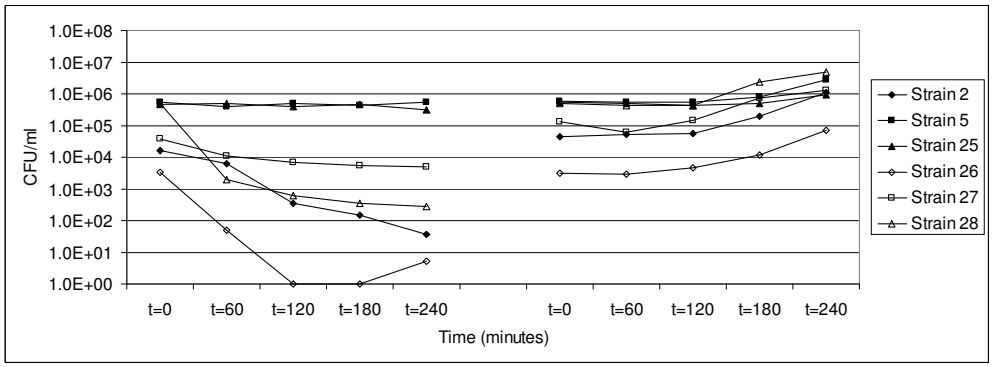

Figure 3.3 Spore counts (left) and total CFU (right) after the addition of spores of mesophilic *Bacillus cereus* strains to simulated intestinal fluid at 37°C.

Strains 17 and 18 are the only two strains that show a slight increase in total counts, after a sort of adaptation phase during the first hour of incubation. This may indicate growth of vegetative cells after germination.

The mesophilic strains, 5 and 25, show no decrease in spore count, indicating no germination. This is also expressed by the slight increase in total counts, indicating minor growth of vegetative cells. The other four strains show germination and growth of vegetative cells, as indicated by the decrease in spore count and the increase in total counts by one or more log units within 4 hours.

Behaviour of spores in the simulated gastro-intestinal transfer

The behaviour of spores in SIF, after pre-incubation during 60 minutes in SGF at pH 2.5, was investigated. In Figures 3.4 and 3.5 results are shown for the psychrotrophic and mesophilic strains, respectively. In both figures the (calculated) spore counts are shown on the left, and the (calculated) total counts on the right.

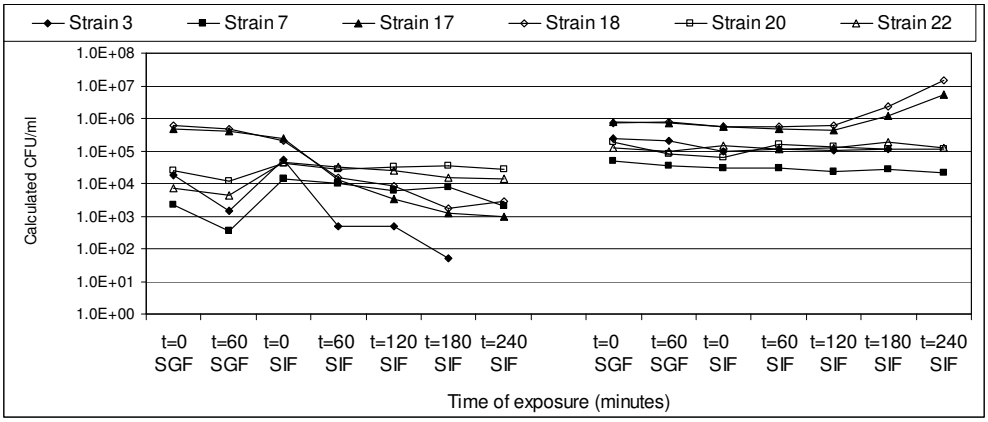

Figure 3.4 Behaviour of spores of psychrotrophic strains in simulated gastric fluid and subsequent simulated intestinal fluid, spore counts (left) and total counts (right) at 37°C.

The psychrotrophic strains 17 and 18 (Figure 3.4) not only show a marked decrease in spore counts, indicating germination, but also a clear increase of at least 0.8 log units in total counts, indicating growth of vegetative cells. Although strain 3 shows a marked decrease in spore count, there is no increase in total count for this strain. This may indicate that the spores germinate, but subsequently do not grow. The differences between initial spore counts and initial total counts in Figure 3.4, and the "dip" in spore counts at t=60 will be discussed elsewhere (see Discussion).

The mesophilic strain 5 shows no germination and growth, as indicated by the lack of decrease in spore count and increase in total count. Strain 25, which showed hardly any growth in SIF (see Figure 3.3) now shows marked growth, although the numbers of spores do not seem to

change over time. In most cases clear decreases in spore counts were seen. Absence of visible decrease in the charts does not mean that no spores have germinated. Only a small percentage of germinating spores can result in relevant vegetative growth. Exposure to low pH in SGF appears to trigger germination in SIF.

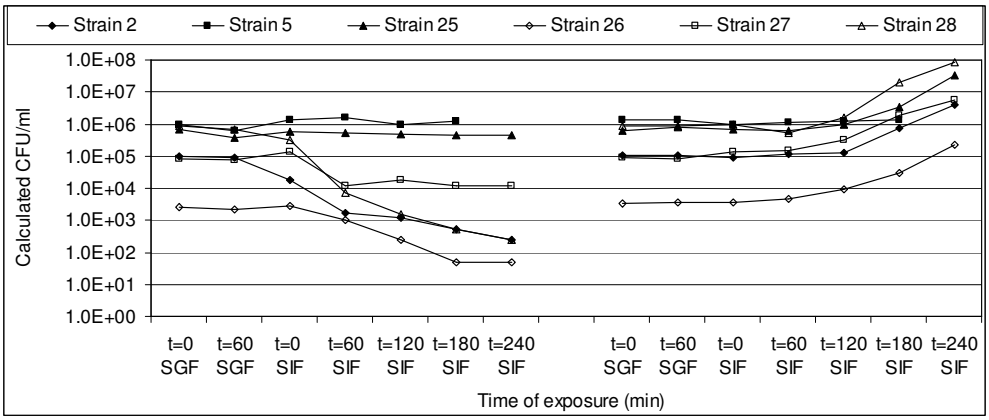

Figure 3.5 Behaviour of spores of mesophilic strains in simulated gastric fluid and subsequent simulated intestinal fluid, total counts (left) and spore counts (right) at 37°C.

The other four mesophilic strains show both marked decrease in spore count and marked increase in total count, indicating germination and growth in SIF after exposure to low pH in SGF. The increase in total counts for two of these four strains (27 and 28) appears to be more dramatic than without pre-incubation in SGF. These findings indicate that the low pH may have a triggering effect on germination, resulting in subsequent growth of vegetative cells.

Influence of low temperature incubation prior to exposure to gastric fluid and subsequent transfer to intestinal fluid

The low temperature pre-incubation of spores as carried out here does not affect the total and spore counts significantly (data not shown).

Influence of pH of gastric fluid on the behaviour of spores in intestinal fluid

These experiments were carried out with one psychrotrophic strain (22) and one mesophilic strain (27), both randomly selected; the results are shown in Figure 3.6. For both strains, SGF at pH 1.0 appears to slightly decrease the number of organisms that are able to germinate and grow in SGF, as indicated by the lower total counts. Like in the other

experiments the spore counts for strain 22 do not vary much over time, but after exposure to SGF at pH 1.0 the total counts remain lower than after exposure to the other two pH-values. Even after exposure to pH 1.0 strain 27 grows in SIF, but to a lower level than at higher pH.

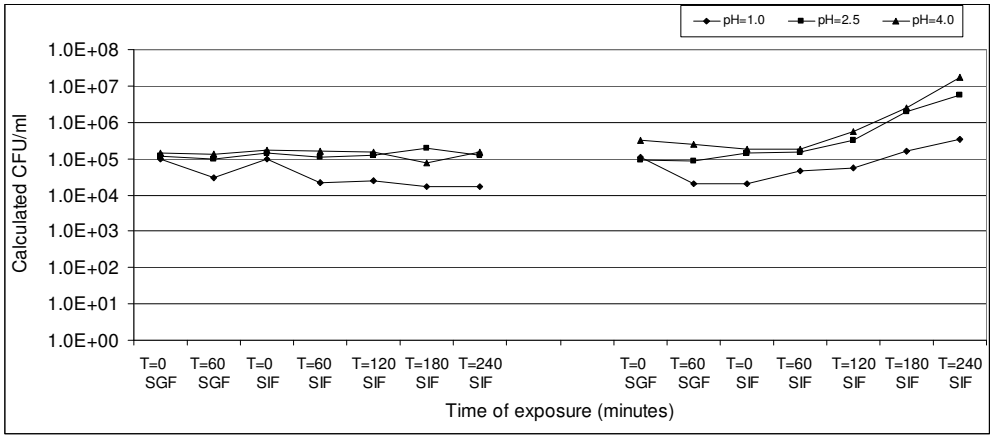

Figure 3.6 Comparison of exposure of spores of psychrotrophic strain 22 (left) and mesophilic strain 27 (right) to various pH values in simulated gastric fluid on the subsequent behaviour in simulated intestinal fluid. Only total counts are shown.

Comparison of growth in BHI and SIF

In Table 3.2 the generation times (in minutes) of all strains in BHI and in SIF are shown. The generation times of the psychrotrophic strains in SIF are on average 58% higher than the generation times in BHI. For the mesophilic strains the difference is on average 32%. The mean generation time of psychrotrophic strains in BHI is 32 minutes, being nearly 50% higher than the mean generation time of the mesophilic strains in BHI (21 minutes).

The mean generation time of the psychrotrophic strains in SIF (49 minutes) is approximately 75% higher than the mean generation time of the mesophilic strains in SIF (28 minutes). As all these experiments have been carried out at 37°C, this indicates that the differences in generation times between psychrotrophic and mesophilic strains are not only attributable to the temperature, but also to the environment.

Table 3.2 Comparison of generation times (in minutes) of psychrotrophic and mesophilic strains in Brain Heart Infusion broth (BHI) and in simulated intestinal fluid at 37°C

	BHI	SIF	Ratio of SIF/BHI
Psychrotrophic strains			
3	49	74	1.51
7	31	44	1.42
17	22	43	1.95
18	24	42	1.75
20	32	49	1.53
22	32	43	1.34
Mean ratio			1.58
Average	32	49	1.55
Mesophilic strains			
2	22	46	2.09
5	27	26	0.96
25	19	27	1.42
26	19	23	1.21
27	19	24	1.26
28	21	21	1.00
Mean ratio			1.32
Average	21	28	1.31

Reproducibility of experiments

To assess the reproducibility of the experiments the total counts and the spore counts of the repeated experiments with strain 2 (Figure 3.7) involving the exposure of spores to gastric fluid followed by incubation in intestinal fluid, were log transformed. Per sample point in time the average and standard deviation were calculated. The results were considered to be reproducible when the standard deviation per sampling point in time was within 0.25 log unit (95% confidence interval ± 0.5 log unit). At all time points the standard deviation was ≤ 0.25 (the maximum standard deviation found in the total counts was 0.21, in the spore counts 0.25). Therefore, the reproducibility of the experiments is considered to be satisfactory.

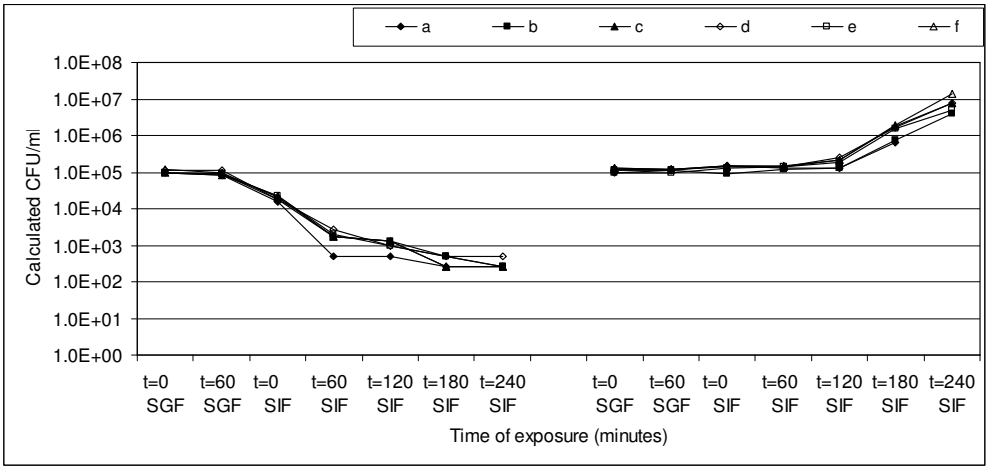

Figure 3.7 Reproducibility of experiments. Development of spore count (left) and total count (right) for strain 2: a to f represent different experiments.

Discussion

Mimicking what happens in the stomach and the small intestine after the ingestion of food and recording the fate of *Bacillus cereus* spores during these events is difficult, since many different variables are involved. Various systems to investigate these phenomena have been described, each method with its own shortcomings and advantages (Clavel et al. 2004). We have chosen for a static system, mimicking the gastric and intestinal fluids in a state resembling feeding, i.e. including pepsin, bile salts and pancreatic secretion, but without food (components) present. Thus, possible protection of micro-organisms by food or food components, possible induction of germination of spores by food or food components, and possible influences of the small intestinal epithelium are not taken into account. Yet, even under these conditions no complete inactivation of spores took place during the one hour exposure of spores to pH 2.5 in SGF (see Figure 3.1). For some strains (3, 7, 20, 22) the spore count dropped immediately after addition to SGF by about one log-unit, but after this initial drop spore counts did not change significantly. This phenomenon was also observed during the experiments in which spores were first incubated in SGF and subsequently transferred to SIF. Several explanations could be given for this phenomenon. Firstly, this initial drop in spore count could be due to the production procedure: after production and before storage at -70°C the spore suspension was heated at 80°C for 10 minutes. This combination of heating and freezing of spores may have caused part of them to become “activated”. This activation caused these spores to die in the heat treatment needed to assess the numbers of spores

during the exposure to simulated gastric fluid. A second explanation could be that aggregation of spores as a protection against adverse conditions, resulted in an under-estimation of the spore content. When subsequently transferred to SIF, the change of conditions can then result in dissolving the aggregates, and hence better estimates of spore counts. And thirdly, the phenomenon may be explained by the method of investigation of the samples. For the determination of the total count dilutions were made from the sample taken from SGF or SIF, but for determination of the spore count the first 10x dilution of the original sample was heat treated. Therefore the composition of the heat treated sample was different from the original sample, which may have caused different behaviour of the various strains. No further investigations were started to assess the cause of the drop in spore count. Apart from the initial drop in spore count for the four given strains, very limited inactivation of spores was observed, even after exposure during one hour at pH 1.0. These results largely correspond with the observations of Clavel et al. (2004) that, apart from a slight decrease at pH 1.0 – 1.4, the spore counts were hardly affected by low pH media. The media Clavel et al. (2004) used consisted of a basic gastric electrolyte solution supplemented with various food derived media. Possible protection of the food-derived additives, however, was not investigated in their experiments.

Both psychrotrophic and mesophilic strains were used in the experiments because of their different behaviour at the temperatures of importance, namely chilled storage at abusive temperature and the human body temperature. The experiments were performed with twelve strains from different origin as representation of the diversity within the genus *B. cereus*. From the results it can be concluded that 4 of 6 psychrotrophic strains (3, 7, 20, and 22) and 2 of 6 mesophilic strains (25 and 27) do practically not grow in simulated intestinal fluid, as illustrated by an increase of less than 1 log unit in 240 minutes, i.e. the duration of the experiments (see Figures 3.2 and 3.3). Pre-exposure to gastric fluid at pH 2.5 alters this finding favourably for the mesophilic strains: with pre-exposure the number of mesophilic strains that germinate and grow in simulated intestinal fluid changes from 4 to 5. Strains 25, 27 and 28 appear to be positively affected by the pre-exposure at pH 2.5 in SGF: their total numbers during the incubation in SIF rise more than without pre-exposure to low pH. Strain 5, on the contrary, appears to be negatively affected by the pre-exposure: less growth results in simulated intestinal fluid with than

without pre-exposure to low pH. More detailed research should provide an explanation for these observations.

The difference in behaviour between psychrotrophic and mesophilic strains is not only attributable to the poorer growth of psychrotrophic strains at 37°C, but the environment plays a role as well. From Table 3.2 it can be derived that generation times of the mesophilic strains are less influenced by the different growth media than the psychrotrophic strains. For the mesophilic strains the differences in generation times between BHI and simulated intestinal fluid are less than for the psychrotrophic strains.

Even though SGF and SIF had to be prepared freshly each day, the results can be compared to each other. Figure 3.7 and the accompanying statistical calculations make clear that the experiments can be reproduced well with strain 2. The differences in numbers of viable cells at each time point were ≤ 0.5 log unit and well within the order of the measuring error of the duplicate plate counts. Therefore, we conclude that the experiments can be compared to each other, and that differences between strains are not to be attributed to differences in daily batches of SGF and SIF.

Overnight pre-incubation of spores at 10ºC was meant to mimic storage of refrigerated food products at abuse temperature, and to investigate the influence on germination and growth potential of spores. In our experiments no changes in total counts and spore counts were observed. Two remarks have to be made. First, long term storage of spores at 4ºC leads to a decrease of viable spores (Dufrenne et al. 1994). This phenomenon has also been observed in other experiments conducted in our lab (results not published). The duration of storage (overnight) as described in this paper may be too short to be able to observe any effects. Secondly, the temperature of 10ºC is high for a standard refrigerator. Proper refrigerated storage should take place at 4ºC, but in general higher temperatures are found (Notermans et al. 1997; Johnson et al. 1998). The experiments, as performed here, merely mimic the overnight storage of food in abuse conditions.

From the results of the combination experiments (Figures 3.4 and 3.5) can be concluded that approximately 3 hours of intestinal conditions are needed to obtain an increase in CFU count of at least 1 log unit. In other words, the lag time in these experiments is about 4 hours, i.e. 1 hour exposure to gastric conditions and 3 hours to intestinal condition. To evaluate the impact of these findings, several issues have to be taken into

consideration. Firstly, *in vitro* production of enterotoxins, the actual perpetrators of diarrhoeal disease, is observed to start when the total count is about 10^7 cfu ml^{-1}. Such counts are normally encountered in the (late) exponential growth phase. Comparatively, for routine production of enterotoxins, a growth period of 5 to 6 hours is used (Beecher and Lee Wong, 1994; Dietrich et al. 1999). Within this time-span the total count increases from approximately 10^7 to 10^9 cfu ml^{-1}. Secondly, the time of onset of symptoms lies between 6 and 24 hours after consumption of contaminated food (Kramer and Gilbert, 1989). And thirdly, the production of enterotoxin in micro-aerobic conditions like the small intestine is comparable with aerobic conditions but growth is slower (Granum et al. 1993). Therefore, relatively fast adapting strains producing high amounts of enterotoxins would therefore be most important to set off the diarrhoeal syndrome. Since the minimal incubation period for the diarrhoeal syndrome is approximately 6 hours, relatively fast adapting strains producing high amounts of enterotoxins are required for onset of the symptoms.

Overall, we conclude that spores of mesophilic strains of *B. cereus* germinate better and grow faster in simulated gastro-intestinal conditions than psychrotrophic strains. Therefore, we conclude that mesophilic strains, when ingested in equal amounts as psychrotrophic strains, may be more important for the onset of diarrhoeal symptoms caused by *B. cereus*.

4

Modelling the number of viable vegetative cells of *Bacillus cereus* passing through the stomach

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Submitted for publication

Abstract

The diarrhoeal syndrome caused by *Bacillus cereus* starts with the consumption of food contaminated with spores and/or vegetative cells. The cells must pass the stomach, germinate and grow in the small intestine, and produce enterotoxins that invoke the diarrhoeal symptoms. Vegetative cells are believed to play a minor role due to their inactivation by the low pH of the stomach. However, during the consumption of food the pH in the stomach may reach values up to pH 5, allowing survival of vegetative cells.

The number of vegetative cells passing the stomach was modelled using the decimal reduction times at low pH (D_{pH}). From the D_{pH} values inactivation rates were calculated and incorporated in a model describing the course of the gastric pH during the consumption of food and a model describing the transfer of food from the stomach to the small intestine. Stationary and exponential phase cells from psychrotrophic and mesophilic *B. cereus* strains were included in this study, all isolated from food and faeces of healthy and ill individuals. All experimental work was carried out using simulated gastric conditions.

The model shows that 3 – 26 % of the original numbers of viable vegetative cells survive the gastric passage, dependent on growth phase and gastric pH.

This study shows that a substantial number of *B. cereus* vegetative cells may play a role in the onset of diarrhoeal disease.

Introduction

Bacillus cereus, a Gram-positive rod shaped spore-forming micro-organism, is the etiological agent of two types of food-borne disease, an intoxication leading to emesis and a toxico-infection leading to diarrhoea (Kramer and Gilbert, 1989). The intoxication, or emetic syndrome, is caused by a single peptide toxin produced in the food prior to consumption (Agata et al. 1995b). The toxico-infection, or diarrhoeal syndrome, is caused by enterotoxins produced in the small intestine, including two three-*component* toxins (haemolysin BL and non haemolytic enterotoxin), and a single protein toxin (cytotoxin K) (Beecher et al. 1995; Granum et al. 1999; Lund et al. 2000). As *B. cereus* is a ubiquitous organism, it can be found in/on a variety of food commodities. Vegetables are very prone for *B. cereus* contamination, but also milk and meat are often contaminated with this organism (Drobniewski, 1993).

B. cereus food and human isolates can be subdivided based on growth temperature profile, in either mesophilic or psychrotrophic strains. Mesophilic strains do not grow below 10°C but grow well at 37°C; psychrotrophic strains can grow at temperatures below 10°C but not, or only poorly, at 37°C (Adams and Moss, 2000). Food may, therefore, contain mesophilic and/or psychrotrophic vegetative cells of *B. cereus* in different phases of growth, and their spores.

In general, the pathogenic mechanism of the toxico-infection is described starting with the consumption of food contaminated with *B. cereus* cells (vegetative cells and spores). The spores pass the stomach, reach the small intestine where they germinate and grow. Also potentially some vegetative cells survive the stomach and grow in the small intestine. During growth in the small intestine, enterotoxins are produced that disturb the electrolyte – water transport over the membrane of the epithelial cells of the small intestine, resulting in diarrhoea (Belaiche 2000). As a consequence of the low pH in the stomach, vegetative cells of *B. cereus* are believed to be inactivated upon gastric passage, and consequently take no part in the pathogenesis. However, during the consumption of food the pH in the stomach varies and may even reach values up to pH 5, a condition allowing the survival of *B. cereus* vegetative cells (Kramer and Gilbert, 1989; Dressman et al. 1990). However, with time progressing, the gastric pH decreases, and reaches values detrimental to vegetative cells. In the mean time, portions of the food are transferred from the stomach to the small intestine, a process that has been investigated and described in detail

by Clarkston et al. (1997). Therefore, despite their sensitivity to low pH, dependent on the amount and type of food and, moreover, age of the consumer, a significant number of vegetative cells may survive gastric passage. This number will depend on the course of the gastric pH, the inactivation at this pH, and the speed with which food is transferred through the stomach. Two of these processes, namely the course of the gastric pH and the transfer of food through the stomach, have been modelled by Takumi et al. (2000).

In this study, survival of stationary and exponential phase vegetative cells of both mesophilic and psychrotrophic *B. cereus* strains was determined in simulated gastric fluid at various pH-values, and expressed as decimal reduction times at the various pH-values (D_{pH}-values). In combination with the models from Takumi et al. (2000), these data were used to quantify the number of viable vegetative cells passing the stomach of healthy young and elderly adults after the consumption of a solid meal. The implications of the results for the onset of diarrhoeal disease caused by *B. cereus* are discussed.

Materials and Methods

Strains

The strains used for these investigations are listed in Table 3.1.

Simulated gastric fluid

The composition and preparation of simulated gastric fluid (SGF) was, based on a formulation by Rotard et al. (1995), previously described by Oomen et al. (2003). SGF consisted of sodium chloride (2.75 g l^{-1}), sodium dihydrogen phosphate (0.27 g l^{-1}), potassium chloride (0.82 g l^{-1}), calcium chloride (0.4 g l^{-1}), ammonium chloride (0.05 g l^{-1}), glucose (0.65 g l^{-1}), glucuronic acid (0.02 g l^{-1}), urea (0.085 g l^{-1}), glucosamine (0.33 g l^{-1}), bovine serum albumin fraction V (1.0 g l^{-1}), pepsin (from porcine gastric mucosa) at 1.0 g l^{-1} and mucine (Type II from porcine stomach) at 3.0 g l^{-1}. Depending on the experiment, the pH was set with hydrochloric acid (1.0 mol l^{-1}). All reagents, except mucine, were from Merck (Darmstadt, Germany). Mucine was from Sigma (St. Louis, MO, USA).

Determination of total counts and spore counts

The total number of viable cells in the samples taken during the experiments was determined by the plate count method on Tryptone Soy agar using decimal dilutions of the samples in physiological salt solution containing 0.1% (w/v) peptone. Numbers of spores were determined by the same plate count method using decimal dilutions of the heated (10 minutes at 80°C) samples in physiological salt solution containing 0.1% (w/v) peptone.

Production of vegetative cells

Stationary phase vegetative cells of *B. cereus* were produced by transferring a glass bead from the -70°C spore-stock to 10 ml Brain Heart Infusion (BHI) broth (NVI, The Netherlands), incubation at 37°C (30°C for the psychrotrophic strains) during 5 hours, and subsequently transferring 0.2 ml BHI-culture to 25 ml LBG-MOPS [i.e. Luria Bertani broth containing 0.4% glucose (w/v) buffered with morpholine-propanesulfonic acid (Boehringer, Mannheim, Germany)] at pH 7.0. The LBG-MOPS culture was incubated 18 – 20 hours at 37°C (30°C for the psychrotrophic strains).

Exponential phase vegetative cells of *B. cereus* were produced by transfer of a glass bead from the -70°C stock to 10 ml Brain Heart Infusion (BHI) broth and 18 – 20 hours incubation at 37°C for mesophilic strains and 30°C for the psychrotrophic strains. Subsequently, a loop-full of BHI-culture was transferred to LBG-MOPS (pH 7.0). The LBG-MOPS culture was incubated at 37°C (mesophilic strains) or 30°C (psychrotrophic strains) during 5 hours.

Exposure of Bacillus cereus *to simulated gastric conditions*

B. cereus cell suspension (200 µl stationary or exponential phase culture) was added to 50 ml simulated gastric fluid in a 60 ml infusion bottle preheated at 37°C in a water bath. The mixture was further incubated in the water bath at 37°C. Samples were taken at the start of the incubation (t=0), and at regular intervals thereafter. For stationary phase cells the pH of the simulated gastric fluid was 4.0, 3.5, or 3.0; for exponential phase cells pH was 4.5, 4.0, or 3.5. The sampling intervals decreased with decreasing pH of the simulated gastric fluid. All determinations were carried out in duplicate at separate days with different inocula.

Determination of D_{pH} -values

The total counts were Log [10] transformed. The Log-values were plotted against time and the first and last values of the initial steepest linear part of the plot were used to determine the D_{pH}-values. In case more than two values fell in the initial steepest part of the plot linearity was determined by linear regression analysis: in such cases the correlation coefficients (R^2) were 0.95 or higher.

The D_{pH}-value, the time necessary to reduce the number of *B. cereus* with one log-unit at a certain pH, was determined by dividing the time interval of the steepest linear part by the change in log numbers of this part.

In formula:

$$D_{pH} = (t_2 - t_1)/(\log B_1 - \log B_2) \quad \text{(min)} \qquad \text{Eq. 1}$$

where
t_1 and t_2 = time point t_1 and t_2 (min) of the steepest linear part
B_1 and B_2 = total counts of *B. cereus* at t_1 and t_2

Modelling the overall passage of Bacillus cereus *through the stomach*

The main principles used to model the food transport and passage of *B. cereus* are: 1) the time dependent inactivation of *B. cereus* due to gastric pH described as fraction of the initial number (N_0) entering the stomach and 2) a meal entering the stomach in bulk and, from thereon, transported to the small intestine in fractions. Both principles have been described by Takumi et al. (2000).

In brief, the following equations have been used to model the overall passage of *B. cereus* vegetative cells through the stomach.

Basic principle 1 is formulated in Equation 2 which describes the course of gastric pH after food consumption:

$$pH(t) = (pH_{max} - pH_{min})\, e^{-kt} + pH_{min} \qquad \text{Eq. 2}$$

where, according to Takumi et al. (2000),
t = time (min)

k = acidification rate: 1.6×10^{-2} min^{-1} for young adults (from 23 to 50 years), 3.9×10^{-3} min^{-1} for elderly adults (from 70 to 84 years)

pH_{min} = the minimal pH in the stomach [2.0 for young adults and 0.0 for elderly]

pH_{max} = maximal pH in the stomach [5.0 for young adults and 5.1 for elderly]

This means that for t=0, $pH(0) = pH_{max}$ and that the pH decreases exponentially as time increases.

To be able to use the bacterial inactivation model described by Takumi et al. (2000) for further analysis, inactivation rates (δ) were calculated from the D_{pH}-values obtained in the experiments with constant pH using the following formula:

$$\delta = Ln(10)/D_{pH} \quad (min^{-1}) \qquad \text{Eq. 3}$$

Plotting of the δ-values as a function of pH showed a linear relation to be most effective, as described in Equation 4:

$$\delta(pH) = \alpha * pH + \beta \qquad \text{Eq. 4}$$

where

δ = inactivation rate, (min^{-1})

α = the slope of the curve and

β = the intersection with the Y-axis at pH=0 (= extrapolated inactivation rate at pH = 0).

Basic principle 2 is formulated in Equation 5 which describes the transport of a meal from the stomach into the small intestine in time [f(t)]:

$$f(t) = \frac{b^{-a} e^{-t/b} t^{a-1}}{\Gamma[a]} \qquad \text{Eq. 5}$$

where
a = the number of steps in which a meal is transported through the stomach (1.7 for young adults and 1.9 for elderly people),
b = the average time for each step (81 minutes for young adults and 83 minutes for elderly people) and
Γ[a] is the gamma distribution.

Equation 6 describes the overall passage of food containing micro-organisms. The first integral depicts the sum of the individual passages from food portions from stomach to small intestine during a certain time period; the second integral depicts the inactivation over the same time period, as described in detail by Takumi et al. (2000).

$$N_{total} = N_0 \int_0^{time} f(t)\, e^{-\int_0^t \delta(\tau)\, d\tau}\, dt \qquad \text{Eq. 6}$$

where
N_{total} = the total number of vegetative cells surviving the passage of the stomach, N_0 = the initial number of cells entering the stomach,
f(t) describes the meal transport (= equation 5) and
the exponential function $e^{-\int \delta(\tau) d\tau}$ describes the inactivation of micro-organisms during residence of food in the stomach (which includes equations 2 and 4).

Parameter estimation and determination of surviving number of Bacillus cereus *cells*

Parameters α and β in Eq. 4 were estimated using linear regression analysis on the inactivation rates $\delta = \ln(10)/D_{pH}$ obtained for each strain in each growth phase at each pH.

The number of vegetative cells (N_{total}) surviving transport through the stomach was determined as follows. The total ingested number of *B. cereus* (N_0) was set at 10^5. Subsequently, the model was run for each strain

in exponential and stationary phase using the appropriate parameter values and a total time of 250 minutes, which is the maximal emptying time of food from the stomach as modelled by Takumi et al. (2000).

Results

D_{pH}-values, calculated using Equation 1, and their corresponding calculated inactivation rates (δ) were determined for all strains in stationary and exponential phase and are shown in Table 4.2.

Figure 4.1 serves as an example for determination of the D_{pH}-values. It shows the numbers of surviving exponential phase cells at the indicated incubation times from strain 7 in simulated gastric fluid at pH 3.5, 4.0, and 4.5. At pH 3.5 the numbers of colony forming units per millilitre at 0 and 5 minutes incubation time were used to determine the D-value, at pH 4 the values between 0 and 30 minutes incubation, and at pH 4.5 the data from time-points between 0 and 90 minutes. The latter time point was selected since the number of CFU/ml at 120 minutes resulted from spores (total count = spore count), and this value could therefore not be used for the calculation of the D-value for inactivation of vegetative cells at pH 4.5. The spore concentrations at the latter time points corresponded with the concentrations in the starting LBG-cultures indicating that the spores were present from the start of the experiment onwards (data not shown).

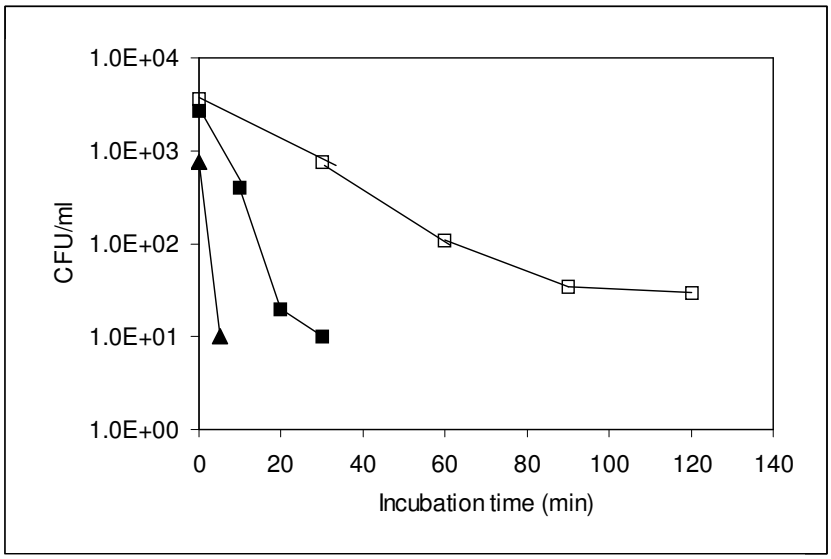

Figure 4.1 Course of total counts (CFU/ml) after addition of exponential phase vegetative cells from *Bacillus cereus* strain 7 to simulated gastric fluid of pH 3.5 (▲), 4.0 (■), and 4.5 (□) respectively.

Table 4.2 D_{pH}-values (min) of stationary phase and exponential phase cells of *Bacillus cereus* at various pH-values, and their corresponding calculated inactivation rates (δ, min^{-1})

Stationary phase mesophilic strains

Strain	pH 4.0		pH 3.5		pH 3.0	
	D_{pH}	δ	D_{pH}	δ	D_{pH}	δ
2	20	0.1	6	0.4	5	0.5
5	38	0.1	20	0.1	4	0.6
25	31	0.1	3	0.8	3	0.8
26	9	0.3	3	0.8	< 2*	1.2
27	19	0.1	3	0.8	5	0.5
28	37	0.1	10	0.2	7	0.3

Stationary phase psychrotrophic strains

Strain	pH 4.0		pH 3.5		pH 3.0	
	D_{pH}	δ	D_{pH}	δ	D_{pH}	δ
3	30	0.1	2	1.2	<1*	2.3
7	12	0.2	3	0.8	<1*	2.3
17	20	0.1	3	0.8	<1*	2.3
18	18	0.1	4	0.6	3	0.8
20	10	0.2	6	0.4	<1*	2.3
22	11	0.2	5	0.5	<1*	2.3

Exponential phase mesophilic strains

Strain	pH 4.5		pH 4.0		pH 3.5	
	D_{pH}	δ	D_{pH}	δ	D_{pH}	δ
2	104	0.02	5	0.5	<1*	2.3
5	95	0.02	5	0.5	<2*	1.2
25	64	0.04	10	0.2	<2*	1.2
26	33	0.1	9	0.3	<2*	1.2
27	44	0.1	3	0.8	<2*	1.2
28	77	0.03	5	0.5	<2*	1.2

Exponential phase psychrotrophic strains

Strain	pH 4.5		pH 4.0		pH 3.5	
	D_{pH}	δ	D_{pH}	δ	D_{pH}	δ
3	43	0.1	3	0.8	<2*	1.2
7	43	0.1	7	0.3	<2*	1.2
17	57	0.04	4	0.6	<2*	1.2
18	62	0.04	3	0.8	<2*	1.2
20	40	0.1	3	0.8	<2*	1.2
22	42	0.1	6	0.4	<2*	1.2

*: *for values <1 and <2 the exact values 1 and 2 were used for calculating the inactivation rates δ.*

At most pH-values the average Log D_{pH}-values of the mesophilic strains seem to exceed those of the psychrotrophic strains (Figure 4.2), suggesting that mesophilic strains are more acid-resistant than psychrotrophic strains at the incubation temperature of 37ºC. The use of more strains is necessary for further investigation of this observation.

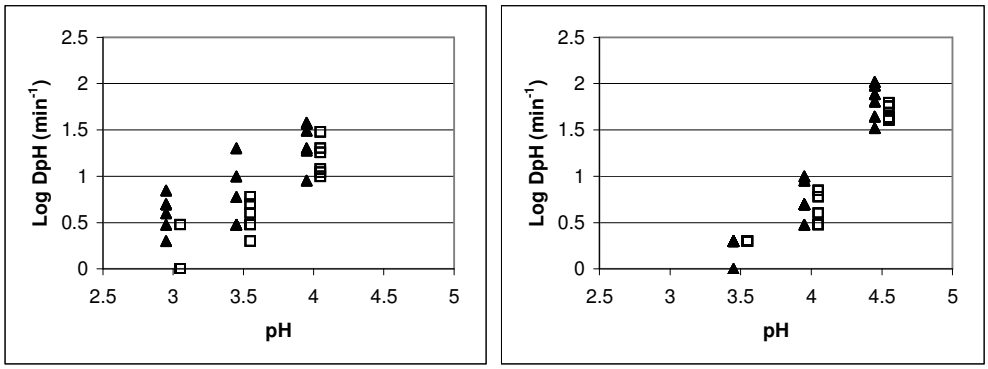

Figure 4.2 Average Log D_{pH} (min) values of mesophilic (▲) and psychrotrophic (□) *Bacillus cereus* strains in stationary (left) and exponential (right) phase cells.

Table 4.3 lists the parameter estimates of α and β and correlation coefficients for the linear equations describing the inactivation as a function of pH per strain per growth phase (Eq. 4). Examples of the linear regression analyses are shown in Figures 4.3A and 4.3B for strains 28 and 20 respectively, in stationary phase. Overall, a linear relationship between pH and inactivation rates appeared to be appropriate as indicated by correlation coefficients > 0.9 for 16 out of 24 strain types. The largest exceptions were strains 25 and 27 in stationary phase with correlation coefficients of 0.75 and 0.28 respectively.

Notably, inactivation of the vegetative cells of *B. cereus* did not start at the pH_{max} but at lower values: approximately pH 4.5 for exponential phase cells and approximately pH 4.0 for stationary phase cells. The linear character of the inactivation (Eq. 4) suggests potential growth between pH_{max} and the pH value where the inactivation rate is 0 (i.e. around 4 – 4.5). We assumed the growth rate between pH_{max} and the pH value where inactivation started to be zero. This means that in that pH-interval food portions containing *B. cereus* were transported to the small intestine without any inactivation or growth of *B. cereus*.

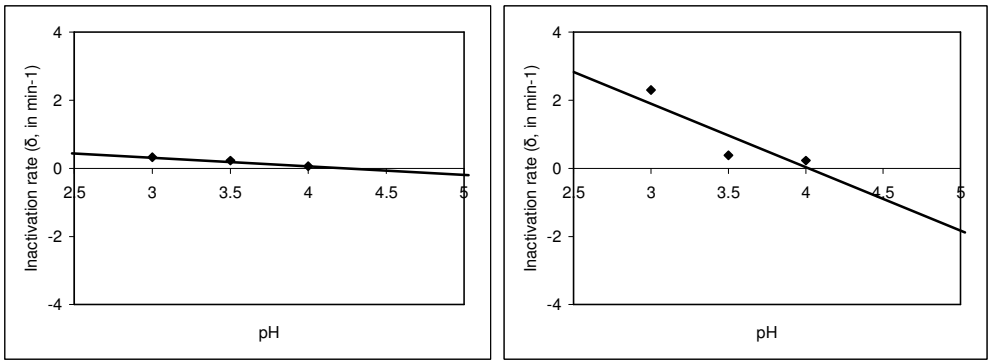

Figure 4.3 Plots of inactivation rates (δ, min^{-1}) versus pH for *Bacillus cereus* strains 28 (left) and 20 (right) in stationary phase. The lines represent the estimated regression lines: δ(pH) = -0.267*pH + 1.1415 (strain 28) and δ(pH) = -2.073*pH + 8.2273 (strain 20).

Also in Table 4.3, the numbers of surviving cells for each strain in each growth phase are listed for both young adults and elderly people. The numbers of surviving cells were determined in a time interval of 250 minutes starting at t=0 and pH_{max}. However, as explained previously, the inactivation rate was set at 0 between pH_{max} and the pH value where the inactivation rate crosses the X-axis. These pH-values are also shown in Table 4.3. As a consequence, all vegetative cells survived the gastric passage in the time interval in which the pH dropped from pH_{max} to the value where the inactivation rate became positive.

The course of equations 2, 5, and 6, describing the dynamics of the gastric pH, the transport of food from the stomach to the small intestine and the logarithm of the total number of surviving vegetative cells in the stomach is visualized in Figure 4.4. For these calculations the inactivation data for strain 26 in stationary phase were applied to the model describing the course of the pH for both a young adult (Figure 4.4 left) and an elderly individual (Figure 4.4 right).The parameters α and β are -0.9 and 3.9 respectively, as can be read from Table 4.3. From the line describing the surviving number of vegetative cells for young adults a shoulder (i.e. the time during which no inactivation of vegetative cells occurs) of approximately 15 minutes can be derived; for elderly individuals this shoulder is approximately 40 minutes. During this shoulder the pH drops from 5 to 4.3 at which inactivation of vegetative cells starts.

Table 4.3 Estimated parameters α and β for the inactivation equation, correlation coefficient (R^2) for the estimated relation, the calculated number of vegetative cells passing the stomach in young adults [N(t) young] and elderly people [N(t) elderly] after ingestion of 10^5 cells, and the $pH_{inact.=0}$

Stationary phase mesophilic strains

Strain	α	β	R^2	N(t) young	N(t) elderly	$pH_{inact.=0}$*
2	-0.3	1.5	0.91	$6.9x10^3$	$1.5x10^4$	4.4
5	-0.5	2.1	0.83	$1.2x10^4$	$2.6x10^4$	4.0
25	-0.7	3.0	0.75	$7.3x10^3$	$1.7x10^4$	4.3
26	-0.9	3.9	0.99	$6.4x10^3$	$1.6x10^4$	4.3
27	-0.3	1.6	0.28	$3.0x10^3$	$6.3x10^3$	4.8
28	-0.3	1.1	0.98	$9.7x10^3$	$2.0x10^4$	4.3

Stationary phase psychrotrophic strains

Strain	α	β	R^2	N(t) young	N(t) elderly	$pH_{inact.=0}$*
3	-2.2	9.0	0.99	$8.9x10^3$	$2.2x10^4$	4.0
7	-2.1	8.5	0.94	$9.2x10^3$	$2.2x10^4$	4.0
17	-2.2	8.7	0.95	$9.6x10^3$	$2.3x10^4$	4.0
18	-0.6	2.7	0.95	$7.5x10^3$	$1.8x10^4$	4.3
20	-2.1	8.2	0.81	$9.9x10^3$	$2.4x10^4$	4.0
22	-2.1	8.3	0.84	$9.8x10^3$	$2.4x10^4$	4.0

Exponential phase mesophilic strains

Strain	α	β	R^2	N(t) young	N(t) elderly	$pH_{inact.=0}$*
2	-2.3	10	0.89	$4.2x10^3$	$1.1x10^4$	4.4
5	-1.1	5.1	0.98	$4.2x10^3$	$1.1x10^4$	4.5
25	-1.1	4.9	0.88	$4.8x10^3$	$1.2x10^4$	4.4
26	-1.1	4.8	0.88	$4.5x10^3$	$1.1x10^4$	4.5
27	-1.1	5.1	0.97	$3.2x10^3$	$8.2x10^3$	4.6
28	-1.1	5.0	0.98	$4.1x10^3$	$1.1x10^4$	4.5

Exponential phase psychrotrophic strains

Strain	α	β	R^2	N(t) young	N(t) elderly	$pH_{inact.=0}$*
3	-1.1	5.0	0.97	$3.2x10^3$	$8.2x10^3$	4.6
7	-1.1	4.9	0.92	$4.4x10^3$	$1.1x10^4$	4.5
17	-1.1	5.0	0.99	$3.7x10^3$	$9.6x10^3$	4.5
18	-1.1	5.1	0.97	$3.2x10^3$	$8.4x10^3$	4.6
20	-1.1	5.0	0.97	$3.1x10^3$	$8.1x10^3$	4.6
22	-1.1	4.9	0.95	$4.2x10^3$	$1.1x10^4$	4.5

**: $pH_{inact.} = 0$ indicates the pH at which the inactivation curve crosses the X-axis.*

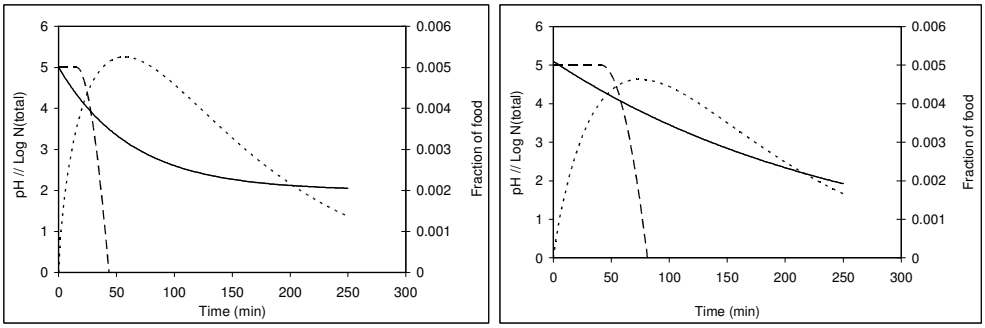

Figure 4.4 Comparison of model outcome for young adults (4A) and elderly individuals (4B) with respect to gastric pH (solid line), logarithm of the concentration of vegetative cells per each food fraction from *Bacillus cereus* strain 26 in stationary phase in the stomach (broken line), and fraction of food transported from the stomach to the small intestine (dotted line) as function of time.

The transfer of stationary phase vegetative cells of strain 26 is demonstrated in Figure 4.5 at pH conditions mimicking those encountered during gastric passage of *B. cereus* in young adults (Figure 4.5 left) and elderly individuals (Figure 4.5 right), respectively.

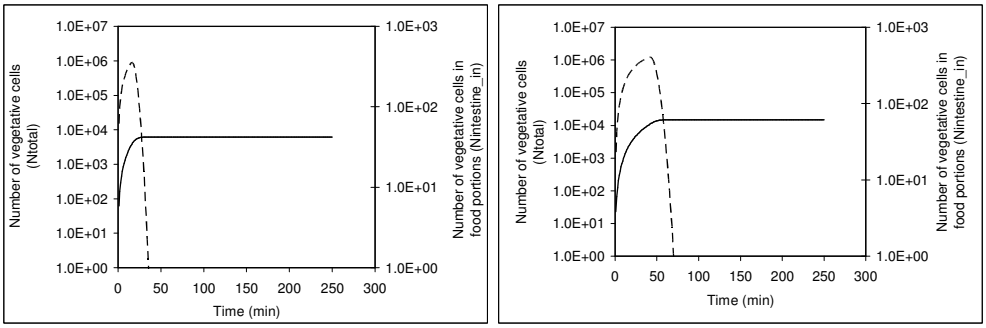

Figure 4.5 Cumulative number of vegetative cells from *Bacillus cereus* strain 26 in stationary phase transported to the small intestine (N_{total}, solid line) and the number of vegetative cells from strain 26 in stationary phase transported per time unit from the stomach to the small intestine ($N_{intestine\_in}$, broken line) as function of time. Comparison between young adults (left) and elderly individuals (right).

Both the total number of cells entering (cumulatively) the small intestine [N_{total}] and the number of cells entering the small intestine per time period [$N_{intestine\_in}$] are shown. From Figures 4.5A and 4.5B can be read that the overall time during which vegetative cells are transported from the stomach to the small intestine is approximately 40 minutes for young adults and approximately 70 minutes for elderly individuals, respectively. After this time all cells present in the stomach are inactivated due to the decreasing pH with proceeding of time.

Discussion

Up to now, spores have been assumed to be the main vehicles in the onset of diarrhoeal disease caused by *B. cereus*, because of their ability to pass the gastric barrier, to germinate, grow and produce enterotoxins in the small intestine (Clavel et al. 2004; see also Chapter 3 of this thesis). This study, however presents evidence that also vegetative cells of *B. cereus*, contained in food, can survive low pH conditions as encountered during gastric passage in humans, and thus could contribute to the onset of diarrhoeal disease upon arrival and subsequent growth and toxin production in the small intestine.

Here, we calculated the numbers of vegetative cells from *B. cereus*, both in stationary and exponential phase that can pass the stomach of young and elderly healthy adults after the consumption of a solid meal. The numbers of surviving cells are putatively higher in healthy elderly people than in healthy young adults. The decrease in numbers is 1 to 2 log-units, for both exponential and stationary phase vegetative cells of *B. cereus*.

The highest number of viable vegetative cells that reach the small intestine in healthy young adults starting with an initial total number of 10^5 cells is 1.2×10^4 cells, i.e. with mesophilic strain 5 in stationary phase, which is around one tenth of the original number. In healthy elderly people this percentage is even higher, namely 26% with mesophilic strain 5 in stationary phase. Overall, with the *B. cereus* strains used, the percentage of surviving cells lies between 3 and 12 % and between 6 and 26 % for young adults and elderly people, respectively, based on the presumed stomach pH values encountered in these two consumer groups.

The survival of viable vegetative cells, expressed as time needed for a decimal reduction at a certain pH (D_{pH} -value), rapidly decreased with lowering pH in the simulated gastric fluid without the inclusion of food components. Decreasing the pH with half a unit lowered the mean D_{pH}-values of both mesophilic and psychrotrophic strains remarkably. We noted a difference in low-pH survival capacity between *B. cereus* stationary phase cells and exponential phase cells, a phenomenon that has been described for other micro-organisms such as *Listeria monocytogenes* and *Salmonella* T*yphimurium* as well (Lee et al. 1994; Jobin et al. 2002; O'Driscoll et al. 1996). Moreover, the average Log D_{pH}-values of the mesophilic stains are at most pH-values higher than those of the

psychrotrophic strains, suggesting a higher acid resistance of the mesophilic strains at the conditions tested.

In our study we used vegetative cells that, before exposure to simulated gastric fluid, had been grown at pH 7.0. Since acid tolerance is inducible in *B. cereus* (Jobin et al. 2002), the number of surviving vegetative cells may even increase, when acid tolerance has been activated such as may happen in mildly acidic foods and drinks. This means that the pH of the food commodity in which the vegetative cells have grown might also play a role in determining the level of survival of vegetative cells.

The quantification as described here is the result of exposure experiments with vegetative cells in simulated gastric fluid without the addition of food components. Addition of protective food components during exposure to the simulated gastric fluid might have resulted in even higher D-values i.e. lower inactivation rates and with that, in even higher numbers of surviving vegetative cells of *B. cereus*. This suggests that the number of surviving vegetative cells as mentioned here will thus be probably an under-estimation of the number of surviving cells.

In addition to stomach pH-values, gastric emptying is an important but complicated process regulated by food dependent stimuli, which also may affect the efficiency of *B. cereus* gastric passage. Here, characteristics of the individual such as gender and age, and intrinsic characteristics such as neural, hormonal and intestinal feedback stimuli are important determinants (Hasler, 2003; Lin et al. 2005). The influence of food type (fluid or solid) on gastric emptying is reflected by the lag time between gastric filling and emptying: liquids pass almost immediately after entering the stomach while solid foods experience a lag period (Weisbrodt, 2001a). Such food type dependent lag period is not included in our model. What is included in the model is a lag phase, caused by a pH-dependent lack of inactivation of *B. cereus*. After starting the model at pH_{max} (5 for young adults and 5.1 for elderly people) the number of *B. cereus* in the food portions remains at the highest level (broken lines in Figures 4.4A and 4.4B). The time period during which food portions are transported from the stomach to the small intestine with the highest number of *B. cereus* per portion depends on the individual (young adults and elderly people in Figures 4.4A and 4.4B respectively) and on the pH at which inactivation starts.

Like Dressman et al. (1990) we showed age-dependent stomach pH-values to be of importance. Not only the mean numbers of surviving cells in elderly people exceeded the number for young adults (Table 4.3),

but also the lag period in inactivation of the vegetative cells and the total time during which vegetative cells are transported from stomach to small intestine were longer in elderly people than in young adults (compare Figures 4.4 and 4.5).

Current models describing gastric emptying are based on data from different sources (Takumi et al. 2000; Minekus 1998). Each model describes the course of the gastric pH slightly different, since each model uses different food types, different assumptions for missing data and different estimations for immeasurable data, leading to variations in the outcomes. We have shown the impact of the course of pH on the number of surviving vegetative cells due to the use of different model parameters for young adults and elderly people. The most important finding of the modelling study described in this paper is, however, the demonstration that large fractions, up to 26 % of the ingested vegetative cells, may survive the gastric passage and may thus contribute to the onset of the diarrhoeal syndrome of *B. cereus*.

5

Germination of *Bacillus cereus* spores is induced by germinants from differentiated Caco-2 cells, a human cell line mimicking the epithelial cells of the small intestine

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Abstract

Spores of eleven enterotoxigenic strains of *Bacillus cereus* isolated from foods and humans, adhered with similar efficiencies to Caco-2 cells, whereas subsequent germination triggering was only observed with eight of these strains. Notably, Hep-2 cells did not trigger germination, while spores of all strains displayed similar germination efficiencies in BHI.

Introduction

The food borne *Bacillus cereus* diarrhoeal syndrome is caused by enterotoxins produced during growth of the micro-organism in the small intestine. When *B. cereus* spores are ingested, germination and subsequent outgrowth are essential steps in the onset of the diarrhoeal syndrome. Germination is a complicated process involving the action of germination receptors in the dormant spores and specific germinants. Spores lacking receptors are strongly impaired in their response to germinants (Barlass et al. 2002; Hornstra et al. 2006; Setlow, 2003).

We aimed to investigate adhesion of *B. cereus* spores to differentiated Caco-2 cells and assessed the germination-inducing capacity of these cells and/or their released compounds.

Materials and Methods

The *B. cereus* food and human isolates, except strain 25, used for these investigations are described in Table 3.1 (page 55). Production and storage of spores were described previously (see Chapter 3 of this thesis). Culturing and differentiation of Caco-2 cells in 12-well plates using Dulbecco's Modified Eagle Medium (DMEM, Gibco 42430) supplemented with fetal calf serum (FCS) and antibiotics, as well as culturing of Hep-2 cells using Minimal Essential Medium with Hank's salts (MEM, Gibco 21575) supplemented with FCS and antibiotics were carried out according to prescribed procedures (Pinto et al. 1983; Finlay et al. 1999). Adhesion/invasion experiments with Caco-2 cells and Hep-2 cells were carried out in 12-well plates. Spores, washed with DMEM or MEM (dependent on the cell type), were added to the cells in the plates and incubated. After 1 hour the plates were washed with DMEM/MEM and either treated with 1% Triton X-100 in PBS or further incubated with DMEM/MEM for 1 hour and subsequently treated with Triton. The 1-hour procedure was used to establish adhesion/invasion; the 2-hour procedure method was used to investigate germination. After treatment with Triton total and spore counts were determined as previously described in Chapter 3. Similar adhesion/invasion and germination experiments were carried out in cell-free plates. Confocal Laser Scanning Microscopy (CLSM) was used to assess invasion after staining with propidium iodide (Molecular Probes) according to the manufacturer's instructions.

Results and discussion

Spores from all eleven strains were found to adhere to differentiated Caco-2 cells (Figure. 5.1). The numbers of adhered *B. cereus* cells were plotted against the number of added *B. cereus* cells, and as the correlation coefficient (R^2) of the trend line is 0.947, we concluded that adhesion is of a non-specific nature instead of through the action of specific adhesins like p104, that is involved in adhesion of *Listeria monocytogenes* to Caco-2 cells (Pandiripally et al. 1999).

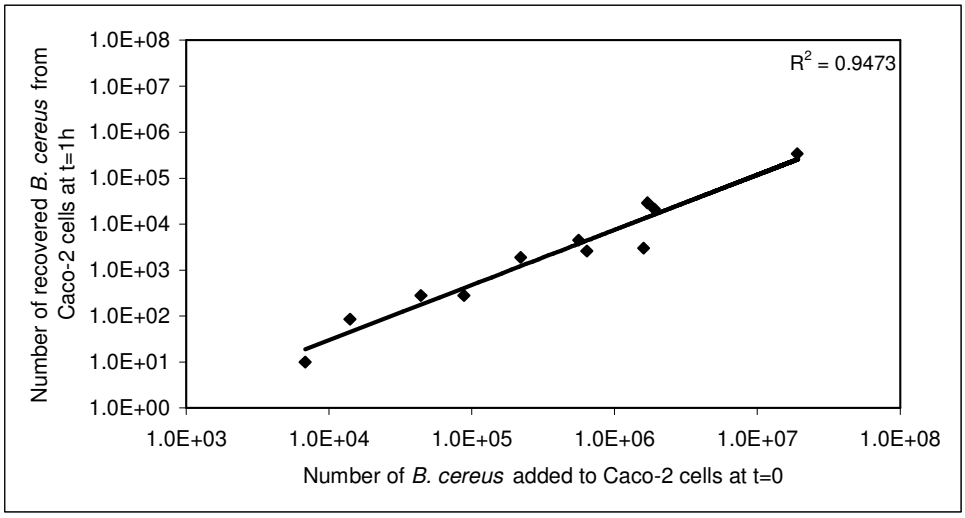

Figure 5.1 Correlation between numbers of *Bacillus cereus* added to Caco-2 cells at t=0 and recovered total numbers of B. cereus adhered to Caco-2 cells after 1 h incubation. Each dot represents results from one strain.

The adhesion efficiency was approximately 1% for spores of all eleven strains. Earlier, Andersson et al. (1998) found 4 out of ten strains to adhere to differentiated Caco-2 cells. The differences in adhesion efficiency between their and our investigations may be due to differences in detection technique: where Andersson et al. (1998) used microscopy to assess adhesion, we used the more sensitive culture method. In experiments with the selected strains 2 and 28, adhesion of spores to Hep-2 cells was observed as well (Figure 5.2).

Subsequently, we investigated germination induction upon adhesion to Caco-2 cells with all eleven *B. cereus* strains. In these experiments, total and spore counts were assessed both in the starting spore suspension as well as after the two hour incubation with Caco-2 cells. After incubation with differentiated Caco-2 cells differences in germination capacity become apparent between the strains, as reflected in

the ratios between total and spore counts (Table 1, column 4). Control experiments were carried out in the absence of Caco-2 cells, and these revealed a lack of germination capacity.

For all strains germination indices were calculated, i.e. the germination ratio in the absence of Caco-2 cells divided by the ratio in the presence of Caco-2 cells. A cut-off value of 2.5 was used for germination: this value is clearly higher than the average 1.5 ratio found upon determining total and spore counts of spore suspension. Eight of the eleven strains have a germination index higher than 2.5 (ranging from 3.0 to 90) indicating Caco-2 induced germination.

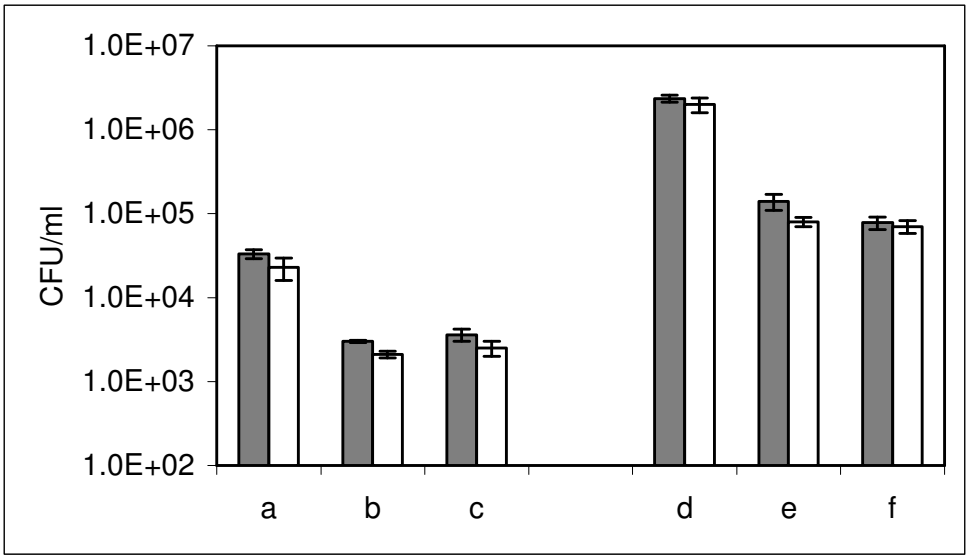

Figure 5.2 Impact of Hep-2 cells on germination of adhered spores from *Bacillus cereus* strain 2 (Fig. 3a-c) and strain 28 (Fig. 3d-f). Grey bars indicate total counts, white bars indicate spore counts. Fig. 3a and 3d: initial spore and total counts of B. cereus spore suspensions used in the experiments; Fig. 3b and 3e: spore and total counts after 1 incubation with Hep-2 cells. Fig 3c and 3f: spore and total counts after 1 hr incubation in plates without Hep-2 cells.

Such results were not obtained when the experiments were carried out with Hep-2 cells: total and spore counts remained approximately the same after the one hour incubation, indicating that no Hep-2 induced germination had occurred. This shows that germination triggering is not a common trait of epithelial cell lines. Notably, spores from all eleven strains show similar germination efficiency in Brain Heart Infusion broth (data not shown). No statistical analyses were carried out to investigate the correlation between germination induction and origin of the strains, as the

numbers of strains per subgroup (healthy individual, food borne disease, and food) were too small to justify such analyses.

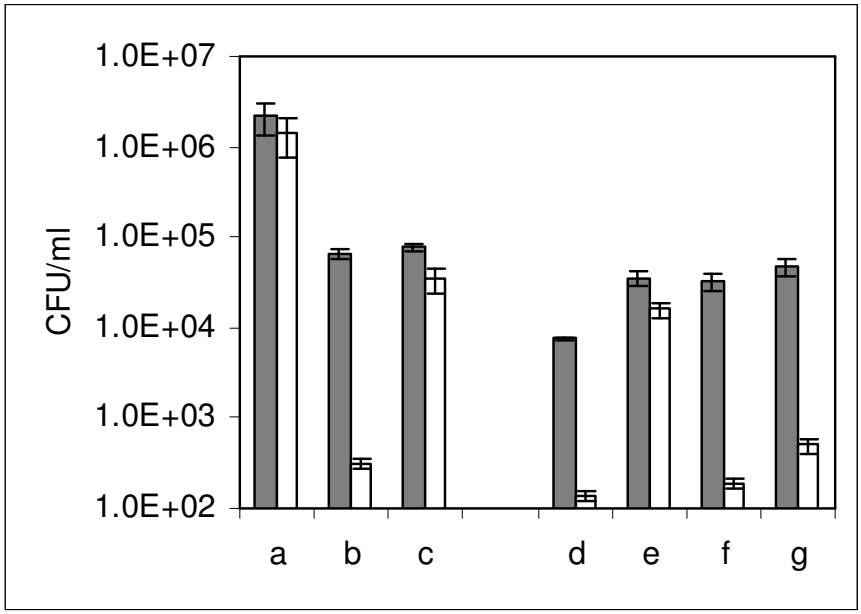

Figure 5.3 Influence of various factors on *Bacillus cereus* strain 28 spore germination inducing capacity of Caco-2 culture supernatant. Grey bars represent total counts, white bars represent spore counts. Total counts and spore counts of strain 28 used in the experiments at time zero (a), counts of adhered *B. cereus* after 1 h incubation with (b) and without (c) Caco-2 cells; total and spore counts of adhered *B. cereus* after 1 h incubation with Caco-2 cell culture supernatant (d), and after 1 h incubation with supernatant pre-incubated with spores from B. cereus strain 11 (e); total and spore counts after incubation for 1 h with heat-treated (f) and pancreatin-treated (g) Caco-2 culture supernatant.

Using spores from strain 28 we started characterization of the Caco-2 produced germination inducing compound(s). Supernatant from Caco-2 cells induced germination efficiently as indicated by the low spore counts compared to total counts, while supernatant from Caco-2 cells that had been pre-incubated for 1 h with *B. cereus* spores, did not. This indicates that the germinant released by Caco-2 cells can be bound (absorbed) and/or inactivated (degraded) by spores. In addition, heating for 5 minutes at 100ºC and addition of proteolytic enzymes as present in porcine pancreatin (Merck) had no effect on the germination inducing capacity of the Caco-2 produced compound(s) (Figure 5.3).

The differences in Caco-2 induced germination efficiency between the strains may be due to differences in germination receptor

profiles and/or differences in expression levels of the receptors (Hornstra et al. 2005). In *B. cereus* ATCC 14579 seven putative ger-operons have been identified; these may equip the spore with seven functional germination receptors, although this number may vary for other *B. cereus* strains (Ivanova et al. 2003). A range of germinants have been identified including L-alanine, L-phenyl-alanine, L-glutamine, inosine, and mixtures of L-asparagine, glucose, fructose and K^+ (Barlass et al. 2002; Hornstra et al. 2006; Preston and Douthit, 1988). All these compounds are, like the Caco-2 cell produced germinant(s), insensitive to heat and protease activity. The precise identity of the Caco-2 cell-produced germinant(s) remains to be elucidated, together with the role of specific germination receptors in the pathogenicity of enterotoxic *B. cereus*.

Table 5.1 Spore counts, total counts and ratios spore count / total count at t=2 of adhered *Bacillus cereus* spores in the presence and absence of Caco-2 cells (with Caco-2 and without Caco-2, respectively). In the last column the germination indices are shown

	With Caco-2			Without Caco-2			
Strain	Total count	Spore count	Spore percentage[#]	Total count	Spore count	Spore percentage[#]	Germination index*
2	1.3E+02	5.0E+00	3.8	5.6E+02	2.7E+02	48.2	12.7
3	3.2E+03	7.0E+02	21.9	1.1E+04	2.3E+03	20.9	1.0
5	1.5E+05	1.7E+05	113.3	3.2E+04	2.3E+04	71.9	0.6
7	5.4E+03	8.0E+02	14.8	6.0E+03	3.0E+03	50.0	3.4
17	2.8E+04	5.1E+03	18.2	8.5E+04	9.3E+04	109.4	6.0
18	5.0E+04	5.3E+03	10.6	1.3E+05	1.2E+05	92.3	8.7
20	8.6E+03	4.0E+02	5.7	2.9E+04	1.5E+04	51.7	9.1
22	9.6E+03	9.5E+02	9.9	2.6E+04	8.7E+03	33.5	3.4
26	1.7E+02	9.0E+01	52.9	3.0E+01	3.0E+01	100.0	1.9
27	5.4E+03	1.9E+02	3.5	4.5E+03	4.7E+02	10.4	3.0
28	6.4E+04	3.1E+02	0.5	7.8E+04	3.4E+04	43.6	90.0

[#] spore percentage = spore count divided by total count * 100

* germination index = percentage in experiment without Caco-2 cells divided by percentage in experiment with Caco-2 cells

6

Caco-2 cell surface-associated growth of enterotoxic *Bacillus cereus* is a prerequisite for initiation of cytotoxic effects in simulated intestinal fluid

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To be published

Abstract

Bacillus cereus can cause diarrhoea due to the production of enterotoxins in the small intestine, an environment known to contain many stress factors including large amounts of proteases.

To assess the enterotoxic capacity of *B. cereus* under conditions mimicking the *in vivo* situation, spore adhesion, outgrowth and enterotoxin activity were determined in a 1:1 mixture of simulated intestinal fluid (SIF) and cell culture fluid with differentiated Caco-2 cells as a target. Notably, enterotoxins added to this mixture were found to be inactivated within seconds, whereas addition of protease inhibitors reduced the loss of enterotoxin activity. This fast inactivation suggests that only enterotoxins produced in close proximity of epithelial cells can interact with their target. Indeed, we found *B. cereus* spores to adhere to Caco-2 cells in the 1:1 mixture of SIF and cell culture fluid, followed by their germination and surface-associated outgrowth resulting in disruption of the Caco-2 cell layer.

Based on our results it can be suggested that growth of enterotoxic *B. cereus* at the small intestinal epithelial cell surface, with concomitant production of enterotoxins, is a key factor in the pathogenesis of this food-borne human pathogen.

Introduction

Bacillus cereus has been reported to be involved in various diseases including periodontitis (Helgason et al. 2000a), endophtalmitis (Callegan et al. 1999; Essex et al. 2004), wound infection (Brett et al. 2005; Darbar et al. 2005), cutaneous infection (Anonymous, 2005), and food borne gastro-intestinal disease (Kramer and Gilbert, 1989). The latter type of disease caused by *B. cereus* is divided into two types: an emetic syndrome and a diarrhoeal syndrome. The latter syndrome is caused by enterotoxins produced by *B. cereus* during growth in the small intestine (Kramer and Gilbert, 1989). Three enterotoxins have been reported to be associated with diarrhoeal disease: haemolysin BL (HBL), non haemolytic enterotoxin (NHE) and cytotoxin K (Granum and Lund, 1997; Granum et al. 1999; Lund et al. 2000). Although it is generally accepted that enterotoxins are unstable at low pH, little is known about the stability of *B. cereus* enterotoxins in gastro-intestinal conditions. Shinagawa *et al.* (1991) studied the stability and antigenicity of *B. cereus* vascular permeability factor (VPF), later known to be enterotoxin HBL (Shinagawa et al. 1991c). Partially purified VPF, subjected to pH values ranging from pH 3 to pH 12 at 35°C for 30 minutes, was only found to be fully stable at pH values between 6 and 8. Under conditions exceeding this pH-range the activity was found to decrease at least two-fold. Recently, acid sensitivity of HBL was confirmed using extracts of a *B. cereus* strain known to produce specifically this enterotoxin (Oxford, 2005).

Moreover, Shinagawa et al. (1991) found complete loss of VPF activity after exposure during 30 minutes to 400 µg/ml trypsin or 100 µg/ml pepsin, indicating the sensitivity of HBL to proteolytic activity (Shinagawa et al. 1991c). Another indication for the protease sensitivity of enterotoxins is the necessity to use EDTA to block activity of proteases produced by *B. cereus* itself during the production of enterotoxin extracts (Beecher and Wong, 2000; Wegscheider, 2004).

Obviously the main question to be answered is how enterotoxins are able to cause damage to the epithelium of the small intestine, an environment known to contain extensive proteolytic activity. Therefore, the production and activity of enterotoxins was investigated under conditions mimicking those in the human gastro-intestinal tract and with differentiated Caco-2 cells as a target. These cells possess most of the characteristics of human small intestinal epithelial cells (Bolton et al. 2000; Jaradat and Bhunia, 2003; Pinto et al. 1983). The use of Caco-2 cells for

closer investigation of the interactions between the small intestinal epithelium and *B. cereus* cells has been described before (Andersson et al. 1998; Minnaard et al. 2001; Minnaard et al. 2004; Rowan et al. 2001). However, these interactions have only been studied in cell culture media, not in conditions mimicking the small intestine including the presence of bile salts and pancreatic excretions. In previously described investigations, a 1/1 mixture composed of simulated intestinal fluid (SIF) and experimental cell culture fluid has been described that maintained differentiated Caco-2 cells functional for at least 8 hours (Ingels et al. 2002; Oomen et al. 2003).

Here we report the use of this model system to assess adhesion of *B. cereus* spores, their germination, outgrowth and enterotoxic capacity of vegetative cells. Evidence is provided that growth of enterotoxic *B. cereus* at epithelial cell surfaces, with concomitant production of enterotoxins, is a key factor in the pathogenesis of this food-borne human pathogen.

Materials and Methods

Strains and extracts

Culture extracts containing either enterotoxin NHE or HBL, for use in EIA, cytotoxicity tests and enterotoxin stability tests were prepared as previously described (Dietrich et al. 1999; Wegscheider, 2004). To assess interaction of enterotoxic *B. cereus* with host cells in simulated intestinal fluid (see below), two human isolates were selected: *B. cereus* strain 9901672, isolated from a healthy individual (de Wit et al. 2001) and producing NHE, and *B. cereus* 1230-88, an isolate from an individual fallen ill in an outbreak of *B. cereus*-induced diarrhoea, that produces enterotoxins HBL and NHE (described as strains 2 and 28 in Table 3.1). The latter strain was kindly provided by Dr. P. E. Granum of the Norwegian School of Veterinary Science in Oslo, Norway.

Spore production and evaluation of survival and growth

Spores of strains 9901672 and 1230-88 were produced as described in Chapter 3. Growth of *B. cereus* was determined by the spread plate method on Trypton Soy Agar as described in Chapter 3.

Culturing of Caco-2 cells and Vero cells

Caco-2 cells, human colorectal adenocarcinoma cells, were obtained from the American Type Culture Collection (HTB-37, ATCC,

USA) and were cultured in DMEM % [i.e. Dulbecco's Modified Eagle Medium (Gibco, 42430-025) supplemented with heat-inactivated foetal calf serum (10% v/v, Integro B.V, the Netherlands), 100x non-essential amino acids (1% v/v, Gibco, 11140-035), 2 mM glutamine (Gibco 25030-024), and 0.05 g/l gentamycine (Gibco, 15750-037)]. Cells were collected every 7^{th} day by washing the monolayer twice with 0.022% (w/v) disodium-ethylenediamine tetra acetic acid (di-Na-EDTA, Acros, 14785) in phosphate buffered physiological salt solution (PBS, 0.07M, pH 7.2) and trypsinising the cells using 0.05 g/l trypsin (Gibco, 25050-014), in 0.022% di-Na-EDTA in PBS. Cells were seeded at a concentration of $1x10^6$ cells in 10 ml DMEM 10% in 75 cm^2 culture bottles (Costar 3376). The bottles were incubated at 37ºC in a gas atmosphere with 5% carbon dioxide. The culture medium was refreshed every 4^{th} day after passage of the cells.

Differentiated Caco-2 cells were prepared by seeding cells from passage 25 to 45 in 12-well plates at a concentration of $1.6x10^5$ cells/ml in DMEM 10%. In each well one ml of this suspension was pipetted. Plates were incubated at 37ºC in a gas atmosphere with 5% carbon dioxide for 14-16 days before use in adhesion experiments to allow the Caco-2 cells to differentiate. The medium in the wells was replaced by fresh medium three times a week.

Cytotoxicity experiments with Caco-2 cells were performed with cells differentiated in 96 wells plates at a concentration of $1x10^5$/ml (100 µl/well). Vero cells, kidney cells of the African green monkey (*Cercopithecus aethiops),* were cultured in M 199 1% [Medium 199 (Gibco 22340) supplemented with 1% v/v foetal calf serum (Integro B.V, the Netherlands) and penicillin/streptomycin (Gibco 15070) at 50 units/ml and 0.05 g/ml respectively]. Twice a week the cells were collected by washing and trypsinising the monolayer as described before. Cells were seeded at a concentration of $1x10^6$ cells in 15 ml M 199 1% in 75 cm^2 culture bottles (Costar 3376). The bottles were incubated at 37ºC with 5% carbon dioxide. Cytotoxicity experiments with VERO-cells were carried out in 96-wells plates at a concentration of $1x10^5$ cells/ml (100 µl/well).

Experimental intestinal fluid

The experiments were carried out in a 1/1 mixture of simulated intestinal fluid (SIF) and experimental culture medium (ECM). SIF was prepared as described previously (Rotard et al. 1995) and contained sodium chloride (NaCl) at 6.58 g/l, mono sodium carbonate ($NaHCO_3$) at 3.98 g/l, potassium dihydrogen phosphate (KH_2PO_4) at 0.66 g/l, potassium

chloride (KCl) (0.043 g/l), magnesium chloride ($MgCl_2$) at 0.17 g/l, urea at 0.075 g/l, bovine serum albumin (Fraktion V) at 1.2 g/l, lipase (Type II from porcine pancreas, 100-400 units/mg using olive oil) at 0.375 g/l, pancreatin (from porcine pancreas, 350 FIP-U/g activated protease, containing trypsin, chymotrypsin, etc.) at 2.25 g/l, calcium chloride ($CaCl_2$) at 60 mg/l, and bile salts (from ox gall, dried and unfractionated) at 1.5 g/l. The pH of SIF was 7.8 ± 0.3.
All reagents, except lipase and bile salts (Sigma, St. Louis, USA) were from Merck (Darmstadt, Germany). Before the addition of lipase, pancreatin, and bile salts the basic SIF-solution was sterilized by filtration through a 0.22 µm filter.
ECM consisted of Dulbecco's Modified Eagle Medium (Gibco, 42430-025) supplemented with 1% (v/v) 100x non-essential amino acids (Gibco, 11140-035) and 2 mM glutamine (Gibco 25030-024).

Cytotoxicity tests

Tests were carried out essentially as described by Wegscheider (2004). Twofold dilutions of extracts and appropriate controls were pipetted into 96-wells plates (100 µl per well) containing differentiated Caco-2 cells or Vero-cells. The plates were incubated overnight at 37°C and a gas atmosphere with 5% carbon dioxide (CO_2). Supernatant was removed from the wells and a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Acros Organics, Belgium) [5 mg/ml in Minimal Essential Medium with Earle's salts, without L-glutamine and phenol red (Gibco 51200)] was added (50 µl per well). After 3 hours incubation at 37ºC in a gas atmosphere with 5% carbon dioxide the MTT solution was replaced with 50 µl dimethyl sulphoxide (DMSO, Acros Organics, Belgium) per well. Optical densities were determined with an Anthos II plate-reader (Anthos Labtec Instruments GmbH, Austria) at 570 nm after briefly shaking the plates.

Stability of enterotoxin activity in ECM/SIF 1:1

SIF was mixed with ECM in a 1:1 ratio, and pipetted in quantities of 4.5 ml in reagent tubes. 100 µl CGY-extract containing enterotoxin NHE was added to each tube, and incubated at 37°C. At specific time points, samples were taken from the incubation mixture and stored at -20°C until further research. All extracts were investigated for their cytotoxic effect on Vero-cells. Similar experiments were carried out in the presence of the protease inhibitors di-Na-EDTA (1 µg/ml, final

concentration) and Pefa-Bloc (Roche Applied Science, Mannheim, Germany) used at concentrations recommended by the manufacturer.

Adhesion of B. cereus *to Caco-2 cells*

B. cereus spores (strain 1230-88) were added to differentiated Caco-2 cells in cell culture medium and incubated for 1 hour. Previous investigations have shown that spores and vegetative cells of this strain adhere to Caco-2 cells and that the spores readily germinate upon exposure to Caco-2 cells (see Chapter 5). Subsequently, the wells were washed three times with ECM to remove non-adhered *B. cereus* vegetative cells. After each washing, two wells were treated with Triton-solution as described above. The Triton-extract was used to assess the number of *B. cereus* vegetative cells adhered to Caco-2 cells, by plating decimal dilutions of the extract in 1% peptone (w/v) in physiological salt solution in duplicate on Trypton Soy Agar [TSA, The Dutch Vaccine Institute (NVI), the Netherlands]. After incubation of the plates for 18 – 22 hours at 30°C the number of colony forming units (CFU) in the Triton extracts was determined.

EIA detection of enterotoxin

EIA was used to detect either enterotoxins HBL or NHE as described previously (9). CGY-extract containing enterotoxin HBL served as positive control in both the L_1 and the L_2-EIA. CGY-extract containing enterotoxin NHE served as negative control in the L_2-EIA. CGY-extract served as negative control in both EIA's.

Caco-2 experiments

Differentiated Caco-2 cells in 12-well plates were washed three times with a 1/1 mixture of ECM and SIF before *B. cereus* spores were added. Differentiated Caco-2 control cells were washed three times with ECM or ECM/SIF. These control cells were not exposed to *B. cereus* spores but treated the same as Caco-2 cells that were exposed to spores.

Spores from -70ºC stock solutions from *B. cereus* strains 9901672 or 1230-88 were washed twice with and suspended in ECM/SIF. Per vial of spores 1 ml ECM/SIF was used for preparing the spore suspension. To each well with differentiated Caco-2 cells 40 µl spore suspension was added, the plate was centrifuged briefly (1 minute, 175xg) and incubated for 1 hour at 37°C in a gas atmosphere with 5% carbon dioxide.

Subsequently, the wells were washed three times with ECM/SIF to remove the non adhered *B. cereus* vegetative cells/spores. 1 ml ECM/SIF was added to each well (defined as *t=0*) and the plates were further incubated at 37ºC in a gas atmosphere with 5% carbon dioxide. Every hour thereafter, *B. cereus* adhesion and growth, and Caco-2 cell destruction in ECM/SIF were assessed. Cell destruction was estimated macroscopically and microscopically. Adhesion and growth were determined after removing at the different time points the ECM/SIF supernatant from two wells with exposed Caco-2 cells and adding 1 ml 1% (v/v) Triton X-100 (Merck, Darmstadt) in phosphate buffered physiological salt solution (0.07M, pH 7.2) per well to disintegrate the monolayer and release *B. cereus* cells from the Caco-2 cells. The Triton-extracts were used to determine the number of *B. cereus* adhered to the Caco-2 cells after plating decimal dilutions on Trypton Soy Agar and incubating these overnight at 30ºC.

Results

Stability of enterotoxin activity in SIF/ECM 1/1

The cytotoxic effect of culture extract containing enterotoxin NHE against Vero-cells was determined in SIF/ECM (mixed in 1/1 ratio) in the absence and presence of protease inhibitors (Figure 6.1).

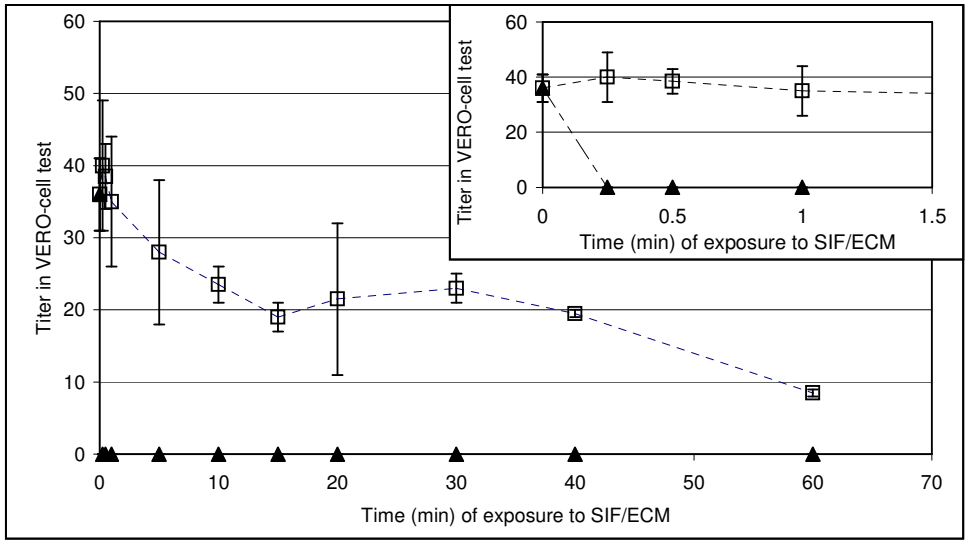


Figure 6.1 Cytotoxic activity of *Bacillus cereus* culture extracts containing enterotoxin NHE in SIF/ECM 1:1 against Vero-cells, in the absence (▲) and presence (□) of protease inhibitors; the insert shows the enterotoxin activity in the first minute after addition to the incubation mixture.

Notably, enterotoxin activity in SIF/ECM appeared to be reduced to zero within 15 seconds exposure time, whereas in the presence of protease inhibitors enterotoxin activity only gradually decreased in time, with approximately 25% of the activity still remaining after 60 minutes exposure. These results clearly show a high sensitivity of enterotoxin NHE to degradation by proteases present in SIF/ECM including trypsin and chymotrypsin. Since NHE and HBL show great resemblance in structure and function (Lund and Granum, 1997) and since proteolytic degradation of HBL by trypsin has been shown before (Shinagawa et al. 1991c), it is highly conceivable that this enterotoxin will also be inactivated in SIF/ECM. These results suggest that only enterotoxins produced in close proximity of epithelial cells can interact with their target. Therefore, a set of experiments was designed to assess the interaction of *B. cereus* spores with human cell lines including adhesion capacity, surface-associated outgrowth and enterotoxic activity.

Adhesion of B. cereus *to Caco-2 cells*

Spores from *B. cereus* strain 1230-88 were used to investigate adhesion to differentiated Caco-2 cells. After the one hour incubation period, the non-adhered *B. cereus* spores and/or cells are washed away. Subsequently, the number of adhered *B. cereus* cells was assessed by determining the number of *B. cereus* cells in the Triton extracts of the Caco-2 cells. The numbers of *B. cereus* recovered from the Triton extracts after each consecutive washing step are shown in Figure 6.2.

In each washing step supernatant and washing fluids are aspirated nearly completely, leaving at a maximum approximately 1% of the original volume in the well. In theory, if *B. cereus* cells (spores and/or vegetative cells) would not adhere to Caco-2 cells, each consecutive washing step would result in *B. cereus* counts decreasing 2 log units with each step as shown in Figure 2. However, *B. cereus* spores and cells appear to adhere firmly to the differentiated Caco-2 cells since the recovered numbers of *B. cereus* decrease with a factor 2.5 to 5 per washing step where a factor 100 is expected theoretically.

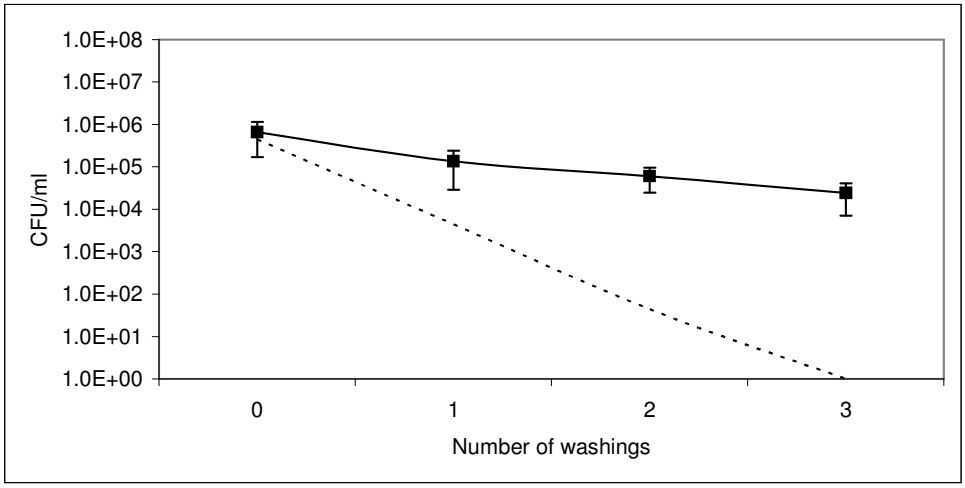


Figure 6.2 Determination of the number of *Bacillus cereus* cells from strain 1230-88 adhered to differentiated Caco-2 cells in cell culture medium as present in the Triton extracts after each consecutive washing of the Caco-2 monolayer. The solid line represents the measured number of adhered *B. cereus* after each washing (■); the dotted line represents the theoretical numbers of *B. cereus* adhering to Caco-2 cells assuming that each washing step removes approximately 99% of the *B. cereus* cells.

Activity of enterotoxin extracts

To establish the relationship between enterotoxin levels and the cytotoxic effects, the amount of NHE in culture extract was assessed with an Enzyme Immuno Assay (EIA) and the cytotoxicity against Vero-cells was determined concomitantly. For this, ten-fold diluted CGY-extract containing enterotoxin NHE and consecutive two-fold dilutions from this extract were analyzed using EIA detecting the NheB component of NHE enterotoxin, and the cytotoxic impact on Vero-cells was assessed (Figure 6.3).

The concentrations of enterotoxin are presented at the X-axis as dilutions of the CGY-extract. The reaction in the EIA and the cytotoxicity of the initial ten-fold dilution were each set at 100%. The EIA-reactions and cytotoxicity of the following two-fold dilutions are expressed as percentages of the EIA-reaction and cytotoxicity of this initial ten-fold dilution of the extract. The results clearly show that the lines representing the cytotoxic effect and the concentration of enterotoxin display identical trends, which indicates the involvement of enterotoxin NHE in the cytotoxic effect.

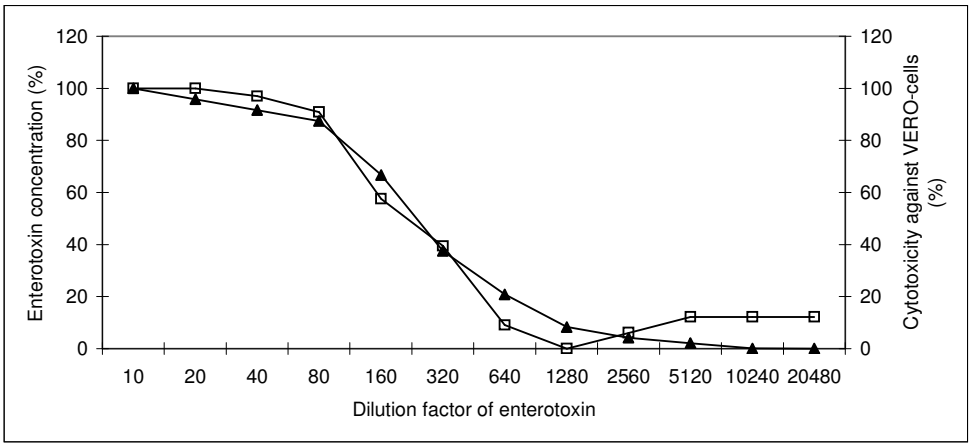
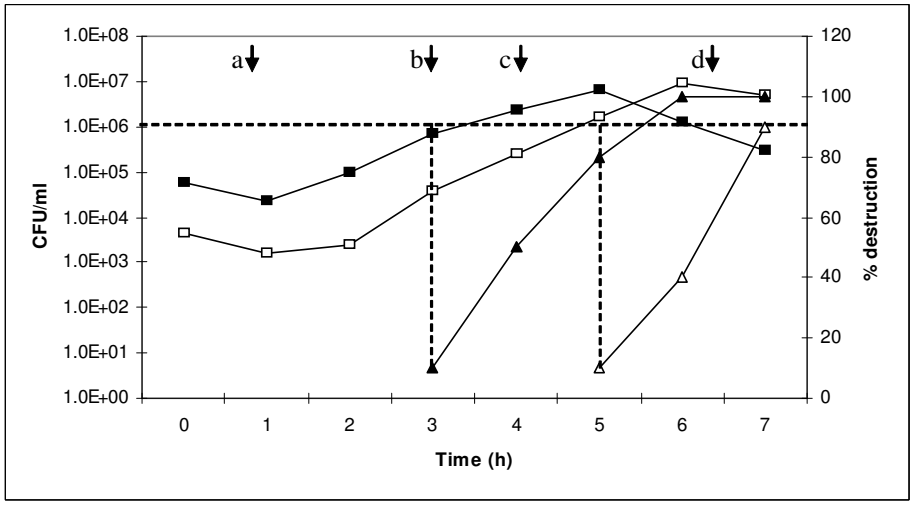


Figure 6.3 Correlation between enterotoxin concentration determined in EIA (▲) and cytotoxicity against Vero-cells (□) of dilutions of a CGY-extract containing enterotoxin NHE. The enterotoxin concentration on the X-axis is expressed as dilution factor of the enterotoxin preparation. Enterotoxin concentration and toxicity against Vero-cells are expressed in percentages, with the concentration and cytotoxicity of the 10-fold diluted CGY-extract set at 100%.

Model system for the diarrhoeal syndrome

The interaction of *B. cereus* spores with human cell lines including adhesion capacity, surface-associated outgrowth and enterotoxic activity were subsequently analyzed in conditions mimicking those in the human small intestine. Therefore, differentiated Caco-2 cells in 1/1 SIF/ECM were exposed to *B. cereus* spores from strains 9901672 or 1230-88 during 1 hour; after washing away the non-adhered *B. cereus* cells, surface-associated growth of *B. cereus* cells was monitored and their cytotoxic effects were assessed analyzing Caco-2 cell layers macroscopically and microscopically. For both strains, the numbers of Caco-2 adhered *B. cereus* cells at each time point and the corresponding percentages of destruction of the Caco-2 monolayer are shown in Figure 6.4A and visualized in Figure 6.4B. For both *B. cereus* strains at cell counts of approximately 10^6 per ml i.e. approximately 10^6 cells of *B. cereus* per 10^6 Caco-2 cells, the first signs of destruction of the Caco-2 monolayer were observed (indicated by the dotted lines in Figure 6.4A), finally resulting in complete destruction at high numbers of *B. cereus* as shown by the cytotoxic effects in Figure 6.4B.

A.



B.

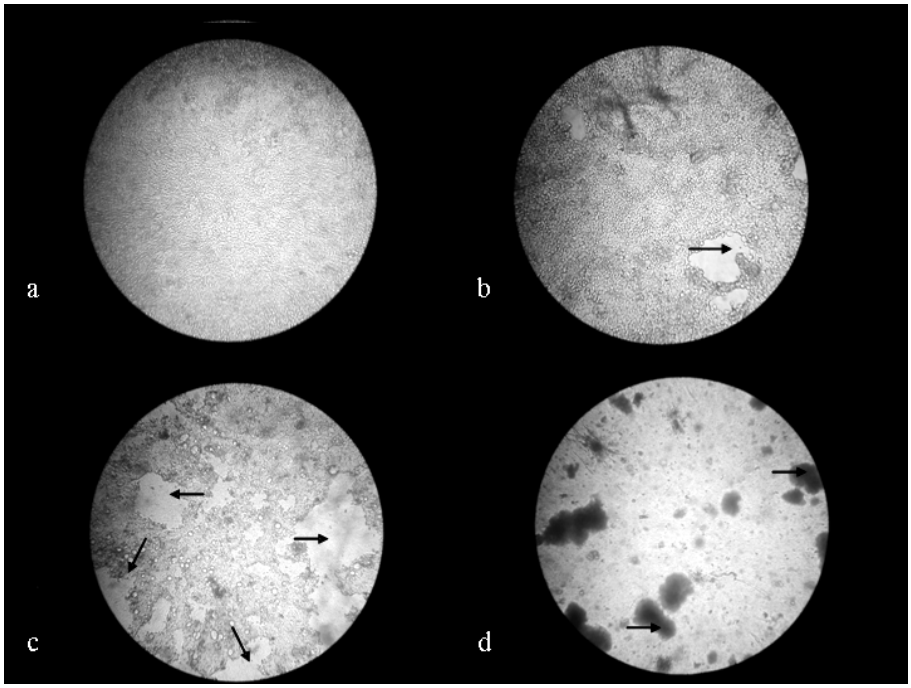


Figure 6.4 Surface-associated growth of *Bacillus cereus* strains 9901672 (producing NHE) and 1230-88 (producing NHE and HBL) adhered to differentiated Caco-2 cells in SIF/ECM 1:1 (□ for strain 9901672, ■ for strain 1230-88) and measure of destruction of monolayer during growth (Δ for strain 9901672, ▲ for strain 1230-88) (A) as estimated from microscopic and macroscopic observations (B). The characters a, b, c and d in Figure 4A represent the various stages of destruction as shown in Figure 4B; a = no destruction, b = ca. 10% destruction (some patches without Caco-2 cells, indicated by black arrow), c = ca. 50% destruction (large patches without Caco-2 cells, indicated by black arrows) and d = complete destruction of the monolayer (remnants of Caco-2 cells floating in the medium, indicated by black arrows).

Discussion

The *B. cereus* diarrhoeal syndrome is caused by enterotoxins produced in the small intestine. Our results show that enterotoxins are very rapidly inactivated in conditions simulating the small intestinal lumen fluid. Evidence is presented that this rapid inactivation of enterotoxins is caused by proteolytic degradation by intestinal enzymes including trypsin and chymotrypsin, present in the pancreatic extract used to produce the simulated intestinal fluid (SIF) for these investigations. Shinagawa *et al.* (1991) did previously show that enterotoxins could be degraded by trypsin and pepsin causing loss of activity (Shinagawa et al. 1991c), but their experiments were not carried out in conditions mimicking the small intestinal lumen fluid. Based on our observations we concluded that enterotoxins have to be produced in close proximity to the epithelial host cells in order to reach their target. Considering all the contractile movements of the small intestine, causing the contents to be mixed continuously (Weisbrodt, 2001b), such close proximity can only be achieved through adhesion of *B. cereus* cells (spores and/or vegetative cells) to the small intestinal epithelium and subsequent growth at and/or in the very close vicinity of the epithelial surface.

Adhesion of *B. cereus* to differentiated Caco-2 cells, serving as a model for small intestinal epithelium, has been described earlier (Andersson et al. 1998; Minnaard et al. 2004; see also Chapter 5), and our studies show indeed that the removal of *B. cereus* cells by subsequent washing steps was not proportional to what could be expected theoretically. Therefore, it was concluded that *B. cereus* cells indeed adhere to differentiated Caco-2 cells. This finding was supported by confocal laser scanning microscopy showing adhesion of strain 1230-88 to differentiated Caco-2 cells but no invasion (data not shown).

Adhesion and surface-associated growth of *B. cereus* to Caco-2 cells indicates that enterotoxins can be produced within close proximity of the epithelial cells, which enables efficient targeting of the enterotoxins. Indeed, using a model system composed of differentiated Caco-2 cells and a mixture of ECM and SIF in 1:1 ratio, surface-associated growth of *B. cereus* strains 9901672 (producing NHE) and 1230-88 (producing HBL and NHE) was shown to be associated with cytotoxic effects on Caco-2 cells. Notably, the onset of the cytotoxic effect is observed at comparable numbers of adhered *B. cereus* cells i.e. at approximately 10^6 CFU per 10^6

Caco-2 cells. The fact that the strain producing NHE and the strain producing NHE and HBL displayed similar cytotoxic efficiency points to an important role for NHE in pathogenicity. This result is supported by earlier observations by Moravek et al. (2006) who showed that the level of NHE was highly correlated with most of the cytotoxic activity of *B. cereus* isolates, which indicated NHE to have a higher diarrhoeagenic potential than HBL.

Based on our results it can be suggested that growth of enterotoxic *B. cereus* at the small intestinal epithelial cell surface, with concomitant production of enterotoxins, is a vital step in the pathogenesis of this food-borne human pathogen. These results contribute to clarification of the pathogenic mechanism of the diarrhoeal syndrome caused by *B. cereus* and contribute to a better risk assessment of this syndrome.

7

Integration of results and conclusive remarks

Bacillus cereus is known for two different types of food borne disease, a diarrhoeal and an emetic syndrome. The two syndromes differ greatly; the diarrhoeal form, a toxico-infection, is caused by enterotoxins produced during growth of the organism in the small intestine, the emetic form, an intoxication, is caused by emetic toxin or cereulide produced in food prior to consumption (Figure 7.1) (Kramer and Gilbert, 1989). Compared to Figure 1.2 new insights (bold) from this study, have been added in this figure.

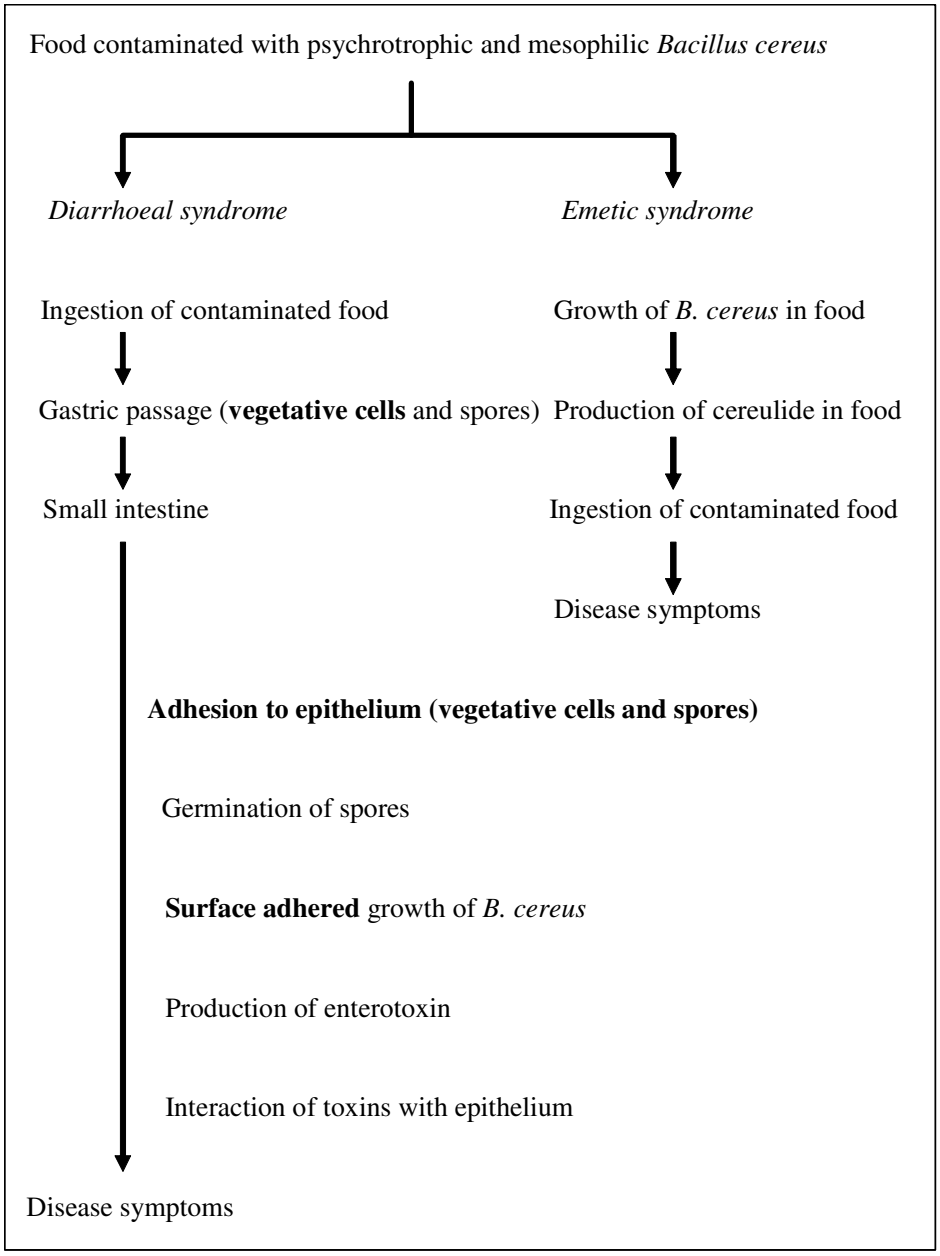


Figure 7.1 General pathogenesis of the diarrhoeal and the emetic syndrome.

B. cereus is a versatile micro-organism with various forms of appearance in morphology and growth characteristics. It can grow over a broad temperature and pH range. Moreover, the spore forming capacity allows the micro-organism to withstand adverse environmental conditions. Investigations carried out within the context of this thesis have learned that nearly each strain is potentially pathogenic (chapter 2), i.e. nearly each strain either carries the genes for one or more of the enterotoxins or is capable of producing the emetic toxin. Combined with the great variety in niches where the micro-organism can be found or where it can survive, raises the question why we are not affected by *B. cereus* every day.

To answer this question the results described in this thesis will be discussed and integrated into a risk for disease ($P_{disease}$) for both syndromes separately. The risk for diarrhoeal disease ($P_{diarrhoeal\ disease}$) is built up of two parts, namely the risk that a meal is contaminated with a potentially pathogenic diarrhoeal strain ($P_{diarrhoeal\ exposure}$), and the risk that such exposure leads to disease symptoms ($P_{diarrhoeal\ hazard}$). The risk for emetic disease ($P_{emetic\ disease}$) is built up of two parts as well, namely that a meal is contaminated with cereulide ($P_{emetic\ exposure}$) and the risk that such exposure leads to emetic symptoms $P_{emetic\ hazard}$). In the following paragraphs the risks for disease will be estimated for each of the syndromes.

The diarrhoeal syndrome

The diarrhoeal syndrome is caused by enterotoxins produced by *B. cereus* cells growing in the small intestine. Details about the various stages between the ingestion of *B. cereus* cells with contaminated food and the onset of disease (see Figure 7.1) are scarce, if not lacking at all. Within the context of this thesis a number of investigations have been carried out resulting in more detailed knowledge on several of these stages.

We discovered a difference in growth potential in simulated small intestinal conditions between psychrotrophic and mesophilic stains of *B. cereus* (Chapter 3). Of the 6 mesophilic strains used in the study, 5 strains showed growth and 1 strain did not. Of the 6 psychrotrophic strains only 2 of 6 strains showed growth. The poorer growth characteristics of the psychrotrophic strains were due to temperature (37ºC) in combination with the environmental conditions as set by the simulated intestinal fluid. In contrast, germination and subsequent growth in Brain Heart Infusion broth at 37ºC was similar for all strains.

We found that vegetative cells may contribute substantially to the disease

invoking process (Chapter 4). Modelling of the gastric passage showed that after ingestion of 10^5 *B. cereus* vegetative cells with a solid meal, 3 – 26% of the vegetative cells survived gastric conditions. This is caused by a raise in gastric pH to values of up to 5 during the consumption of food.
Small intestinal cells, the target cells for the enterotoxins, are not just passive targets for *B. cereus* cells, spores or vegetative cells. They are able to induce germination of *B. cereus* spores, and with that growth of the micro-organism (chapter 5). Spores from eight of the eleven strains germinated, the other three did not.

The diarrhoeal symptoms are caused by enterotoxins that are produced by *B. cereus* cells. We have shown that enterotoxins are highly instable molecules in conditions simulating small intestinal conditions, i.e. including bile and proteolytic enzymes as present in pancreatic juice (chapter 6). This instability requires production of enterotoxins close to the small intestinal epithelium, their target cells. This can be achieved by adhesion of *B. cereus* cells to the small intestinal epithelium, a property *B. cereus* cells indeed possess as shown in chapter 6. We found that *B. cereus* spores and vegetative cells adhered to differentiated Caco-2 cells, mimicking human small intestinal epithelial cells, with an efficiency of approximately 1%, Moreover, we demonstrated the validity of this hypothesis by showing that the onset of degradation of Caco-2 cells, mimicking the small intestinal epithelial cells, was proportional to the concentration of adhered *B. cereus* cells.

All these findings have their influence on the risk for diarrhoeal disease by *B. cereus* ($P_{disease}$), which is built up from a risk for exposure ($P_{exposure}$) and a risk for hazard (P_{hazard}). Overall, $P_{disease} = P_{exposure} * P_{hazard}$. In the following discussion, carried out following the scheme in Figure 7.1, this influence will be shown and discussed, assuming that one spore or vegetative cell is sufficient to cause disease (single hit theory).

The P_{hazard} is set at 1 at the moment of ingestion of food contaminated with *B. cereus*.

After ingestion of contaminated food, the stomach, with its low pH and presence of pepsin, is the first line of defence against food borne pathogens. Spores pass the stomach unaffected, as shown in Chapter 3. This means that the risk, when dealing with food contaminated with spores only, is not reduced and remains one ($P_{hazard} = 1$).
In case food is contaminated with vegetative cells only, the change in risk depends on the number of vegetative cells that is able to survive stomach

conditions. When food enters the stomach the pH is raised to values where *B. cereus* survives. With ongoing transfer of gastric contents to the small intestine the pH decreases again, with less surviving micro-organisms as a consequence. This is not unique for *B. cereus*, but goes for other low-pH-sensitive bacteria such as *Campylobacter jejuni* and *Salmonella* T*yphimurium* (Waterman and Small, 1998). We estimated the highest numbers of *B. cereus* vegetative cells, surviving simulated gastric conditions during the consumption of a solid meal, as follows: 12% for mesophilic cells in young adults (m/y), 26% for mesophilic cells in elderly people (m/o), 9.9% for psychrotrophic cells in young adults (p/y), and 24% for psychrotrophic cells in elderly people (p/o) (see Table 4.3). These numbers lead to the following maximal risks: 1.2×10^{-1}, 2.6×10^{-1}, 9.9×10^{-2}, and 2.4×10^{-1} respectively (Figure 7.2).

Our finding, that vegetative cells may well contribute to the onset of disease, are contradicted by Wilcks et al. (2005). After oral administration of vegetative cells and spores to human-flora-associated rats, they could not retrieve *B. cereus* cells from the faeces of the animals. The reason for this is probably that Wilcks et al. (2005) administered the vegetative cells to the rats without any food. Therefore, the vegetative cells were exposed directly to the gastric conditions of the rats [pH of rat stomach reaches values as low as 3.3 (Zwart et al. 1999)], leading to a very low survival. In comparison, we found in simulated human gastric fluid at pH 3.5 a decimal reduction time (D_{pH}) for *B. cereus* vegetative cells of less than 2 minutes, depending on the strain, growth temperature signature and growth phase of the vegetative cells (Chapter 4).

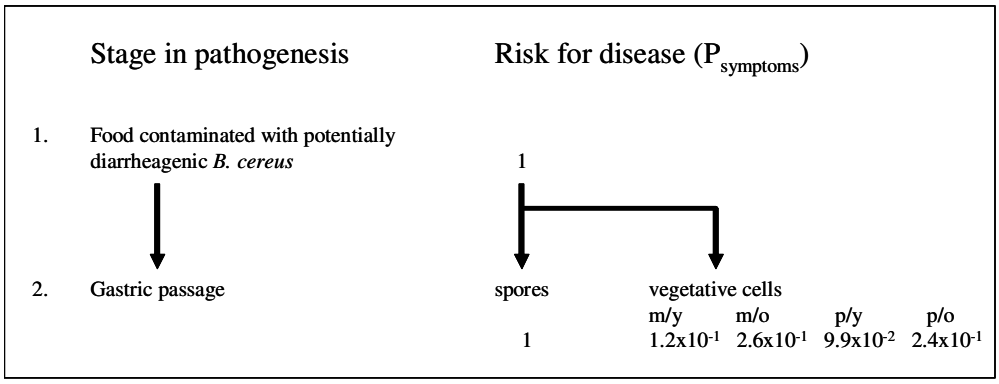


Figure 7.2 Influence of gastric passage on risk for symptom with m/y = mesophilic veg. cells in young adults, m/o = mesophilic veg. cells in elderly people, p/y = psychrotrophic veg. cells in young adults, p/o = psychrotrophic veg. cells in elderly people.

The actual size of the risk after ingestion of vegetative cells is influenced by the type of *B. cereus* strain, the composition and "liquidity" of the food, and the age of the individual. Clavel et al. (2004) examined the influence of food composition on the survival of vegetative cells using simulated gastric medium enriched with various food constituents. They concluded that milk had a protective effect: vegetative cells were inactivated slower in simulated gastric medium enriched with milk than in simulated gastric medium enriched with either pea soup or chicken broth. Lipids in milk might be responsible for this protective effect. Such effect was also demonstrated by D'aoust (1985), who found a very low infective dose of 1 – 6 cells of *Salmonella Typhimurium* when ingested with cheddar cheese. In comparison, the infective dose for *Salmonella anatum* in eggnog, as determined in human volunteers, was 10^6 – 10^7 cells (Teunis et al. 1996), although from outbreak data a considerable lower infective dose has been determined (Blaser and Newman, 1982).

The influence of liquidity of food was studied by Camilleri et al. (1985). As shown in Figure 7.3 liquid food is transported faster to the small intestine than solid food. With that vegetative cells in liquid food are transported faster and therefore exposed to unfavourable gastric conditions for a shorter period of time.

The risk (P_{hazard}) increases with the increasing ratio spores/vegetative cells. This means that the risks, as estimated before for vegetative cells alone, become higher when combinations of vegetative cells and spores are present in the ingested food.

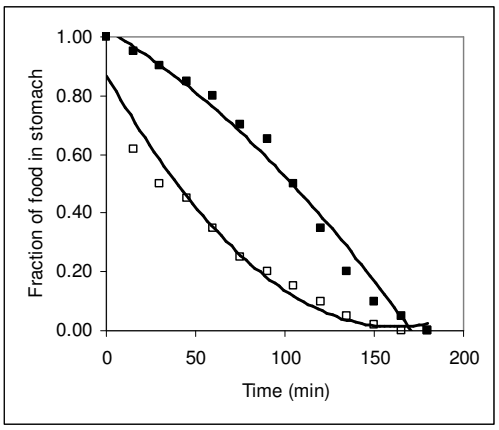


Figure 7.3 Relation between gastric emptying and liquidity of food (□ = liquid food, ■ = solid food; Camilleri et al. 1985).

After passing the stomach spores and vegetative cells reach the small intestine, where we found four strain-dependent characteristics to be of importance for the onset of diarrhoeal disease: adhesion potential of *B. cereus* to epithelial cells, germination efficiency of spores, growth temperature signature (mesophily – psychrotrophy), and potential to produce enterotoxins.

Adhesion appears to be of vital importance for the onset of diarrhoeal symptoms, since enterotoxin NHE is rapidly degraded in simulated small intestinal conditions (Chapter 6). As NHE and HBL are highly similar molecules, in composition as well as in working mechanism (Lund and Granum, 1997), we assume that HBL is equally instable in simulated intestinal conditions. We have shown that this degradation is due to the proteolytic activity as present in the pancreatic juice, a substance that (Clavel et al. 2007) did not add to their simulated intestinal juice when they proved the enterotoxin production to be dependent on bile concentration and food composition. Our investigations show the influence of proteolytic activity in simulated intestinal fluid for the pathogenic mechanism: to ensure that enterotoxins can reach their target cells, the small intestinal epithelium, they have to be produced in very close proximity of their target cells, a condition that can only be achieved through adhesion of *B. cereus* cells to the small intestinal epithelium. Earlier, Andersson et al. (1998) had suggested that adhesion might be an additional virulence factor. Our findings go beyond this suggestion: no adhesion means no diarrhoeal symptoms.

Adhesion to small intestinal epithelial cells was mimicked by using differentiated Caco-2 cells. It might be an intrinsic capacity of all *B. cereus* cells since both spores and vegetative cells of all twelve strains we used for these investigations show adhesion. The adhesion efficiency of *B. cereus* spores and vegetative cells is approximately 1% (Chapter 5). This means that the P_{hazard} is reduced by a factor 100 upon the entrance of *B. cereus* cells in the small intestine, and becomes as follows: 0.01 (= $1x10^{-2}$) for spores, and 1.2x10-3, 2.6x10-3, 9.9x10-4 and 2.4x10-3 for mesophilic veg. cells in young adults, mesophilic veg. cells in elderly people, psychrotrophic veg. cells in young adults and psychrotrophic veg. cells in elderly people respectively (Figure 7.4).

In determining the adhesion efficiency two factors have not been taken into account: the influence of small intestinal commensal micro-flora and differences between individuals. With respect to the influence of commensal flora the number of data is limited. The upper two thirds of the

small intestine (duodenum and jejunum) contain only low numbers of micro-organisms, which range from 10^3 – 10^4 bacteria/ml, such as acid tolerant lactobacilli and streptococci. In the distal part of the ileum the numbers of micro-organisms are around 10^7 – 10^8 bacteria/ml (Hao and Lee, 2004).

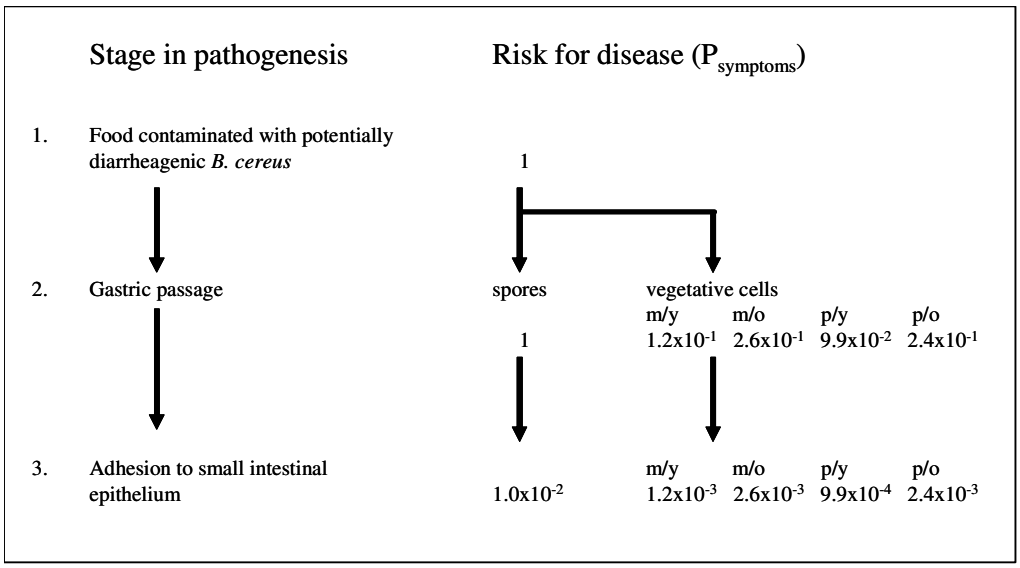


Figure 7.4 Influence of adhesion of *Bacillus cereus* cells to small intestinal epithelium on risk for symptoms. Explanation of abbreviations see Figure 7.2.

Lactobacillus species are known for their ability to inhibit adhesion of pathogenic bacteria such as *Salmonella* T*yphimurium* to Caco-2 cells or intestinal mucus (Coconnier et al. 2000; Tuomola and Ouwehand, 1999; Collado and Salminen, 2007). Studying the intestinal flora requires special methods. Bernhardt and Knoke (1989) developed a continuous flow culture to study the anaerobic micro-flora of the duodenum. Wilcks et al. (2005) used human-flora-associated rats to study the influence of intestinal flora on the behaviour of *B. cereus* in the intestinal tract. Extra handicap in the study of the interaction of pathogens with the small intestinal micro-flora is the individuality of the commensal micro-flora: each individual has his/her own micro-flora (Kalser et al. 1965; Thadepalli et al. 1979)

Without germination in the small intestine spores lose their pathogenicity and, as a consequence, no disease symptoms will occur. Germination is a complicated process in which a number of factors play a role. *B. cereus* spores are equipped with germination receptors, seven of which have been identified in *B. cereus* ATCC 14579 (Hornstra et al.

2006). These receptors may be triggered by specific germinants that usually consist of small heat stable, protease resistant molecules such as L-alanine, L-phenyl-alanine, L-glutamine, inosine, and mixtures of L-asparagine, glucose, fructose and K^+. (Barlass et al. 2002; Hornstra et al. 2005; Preston and Douthit, 1988).

We found, in experiments carried out in cell culture media that in the absence of other germinants differentiated Caco-2 cells, mimicking small intestinal epithelial cells, produce one or more substances capable of inducing germination of *B. cereus* spores. Spores from eight of the eleven strains used for these experiments were induced to germinate. This decreases P_{hazard} with a factor 0.73 when starting with spores. With this the P_{hazard} for spores changes from $1.0x10^{-2}$ to $7.3x10^{-3}$ for spores (Figure 5). P_{hazard} for vegetative cells is not affected by the germination inducing potential of the differentiated Caco-2 cells and remains between $3.0x10^{-4}$ and $2.6x10^{-3}$ (Figure 7.5). Since it cannot be excluded that germinants in the consumed food are able to induce germination of spores of the remaining three strains, the reduction factor of 0.73 may well be closer to one.

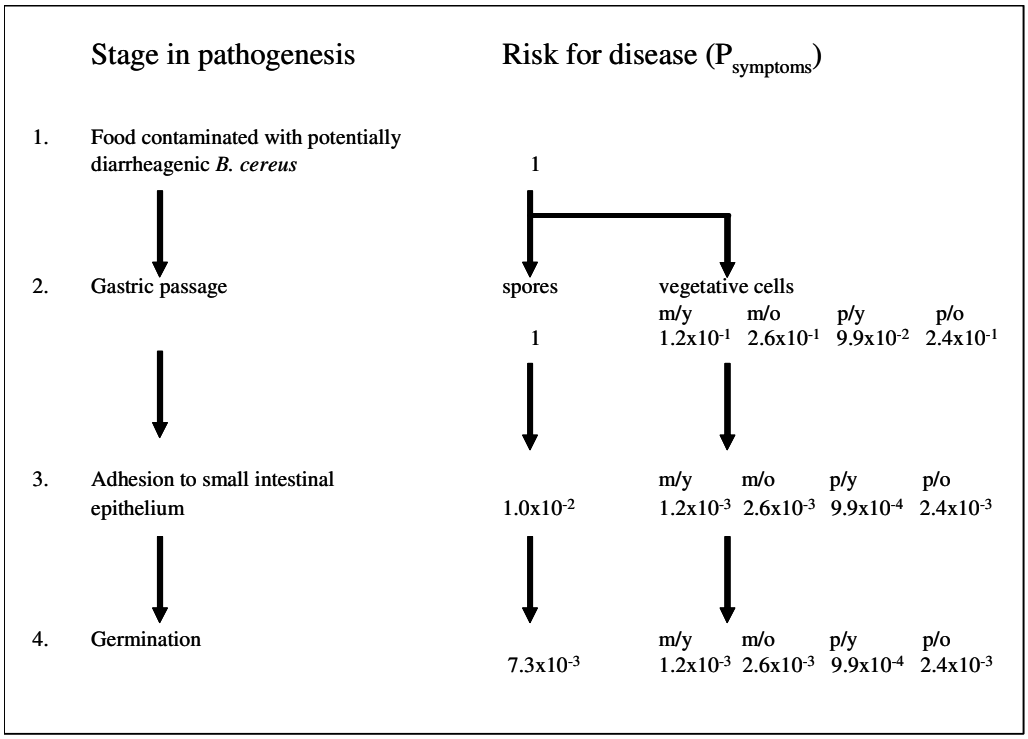


Figure 7.5 Influence of germination potential of *Bacillus cereus* spores in presence of differentiated Caco-2 cells on risk for symptoms. Explanation of abbreviations see Figure 7.2.

The growth temperature signature of the individual strains appears to play a role in growth of *B. cereus* in simulated small intestinal conditions. When food is contaminated with mesophilic *B. cereus*, the risk for disease is higher than when the contaminating strain is psychrotrophic. We found that 5 of the 6 mesophilic strains and 2 of the 6 psychrotrophic strains were able to germinate and grow in simulated intestinal conditions (chapter 3). Previously, Clavel et al. (2007) found that psychrotrophic strain TZ415 was more susceptible to bile than the mesophilic strains they used. This indicates that psychrotrophic strains are more susceptible for conditions in the small intestine. Our findings affect the risks for disease as follows: P_{hazard} for mesophilic spores goes from $7.3x10^{-3}$ to $6.1x10^{-3}$, P_{hazard} for psychrotrophic spores goes from $7.3x10^{-3}$ to $2.4x10^{-3}$. The P_{hazard} for vegetative cells become as follows: 1.9x10-3, 2.2x10-3, 3.3x10-4 and 8.0x10-4 for mesophilic cells in young adults, mesophilic cells in elderly people, psychrotrophic cells in young adults, and psychrotrophic cells in elderly people respectively. (Figure 7.6).

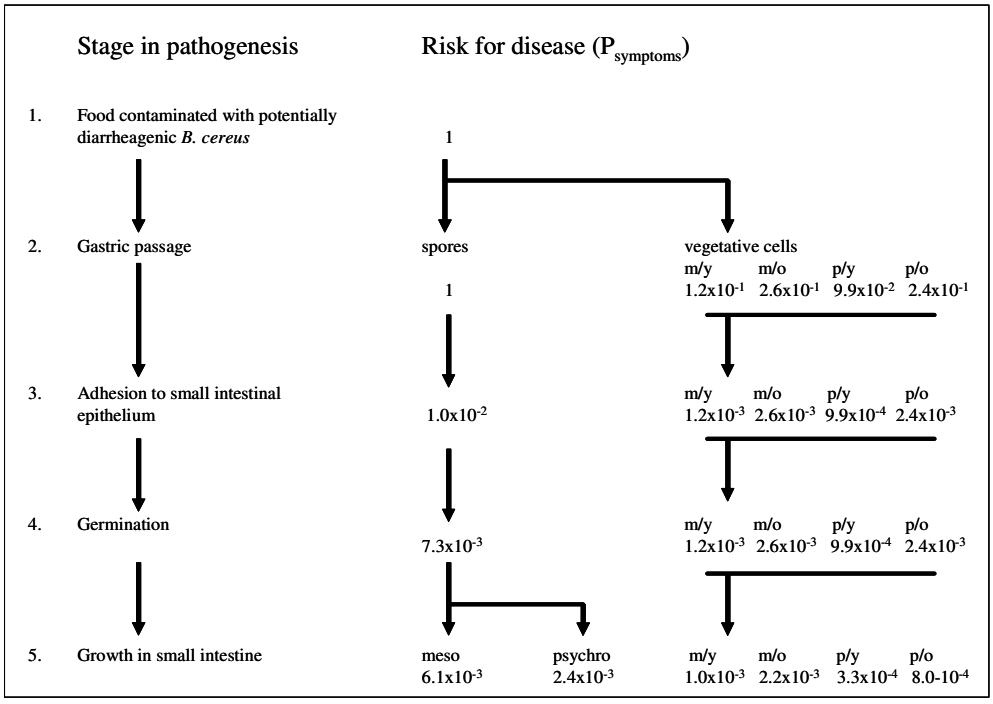


Figure 7.6 Influence of growth potential of *Bacillus cereus* in simulated intestinal fluid on risk for symptoms. Explanation of abbreviations see Figure 7.2.

The enterotoxin producing potential, qualitatively and quantitatively, has not been taken into account for calculating P_{hazard}, Hardly any data are available on the differences in toxicity between the various enterotoxins. Recently a comparison of the toxicity of strains

producing NHE or NHE and HBL made clear that the toxicity of strains containing both enterotoxin complexes was not significantly different from strains containing only the NHE complex (Moravek et al. 2006).

With this one part of the equation for the risk of diarrhoeal disease, namely P_{hazard}, is known. This risk falls apart in several different determinants of risk, dependent on *B. cereus* cell type, age of the individual, and growth temperature signature of the contaminating strain.

Three observations have to be taken into account for calculating the risk of exposure to a meal containing potentially pathogenic *B. cereus.,* $P_{diarrhoeal\ exposure}$. Firstly, our survey regarding the prevalence of potentially pathogenic *B. cereus* strains (chapter 2) derive from positive samples only, and all calculations sofar have been based on this survey. However, in 2001 the Food and Consumer Product Safety Authority investigated 1635 ready-to-eat meals and found that 39 (= 2.4%) meals contained *B. cereus* (Jansen and in 't Veld, 2002). For further calculations we assume that the prevalence of 2.4% in ready-to-eat meals is representative for all food commodities and not only for ready-to-eat meals, this means that $P_{exposure}$ = 0.024.

Secondly, in the Netherlands a tolerance level of 105 CFU/g is in force for B. cereus. The survey over a 12-month period from May 2002 – May 2003 showed that from ready-to-eat samples 67 of 22,744 contained > 10^5 CFU/g (see Table 2.5). This leads to a risk of exposure of $P_{exposure\ >10^5}$ = 0.0029.

The third observation concerns the pathogenic potential of *B. cereus* strains. The inventory as described in Chapter 2 made clear that 97% of all strains have the potential to produce NHE, 66% the potential to produce HBL, and nearly 50% the potential to produce cytotoxin K-like (CytK-like) enterotoxin. Thus, 97% of all strains are potentially pathogenic. However, the presence of genes encoding the enterotoxins was determined, not the actual production. The actual production of enterotoxin may not only depend on the presence of the genes, but also on other factors such as expression regulation by the PlcR-regulon (Salamitou et al. 2000; Slamti et al. 2004), food ingredients such as glucose that is able to repress HBL production (Ouib et al. 2006; Garcia Arribas and Kramer, 1990), protection or sensitization to bile by food constituents (Clavel et al. 2007). Therefore, the percentage of pathogenic strains may only be smaller than the 97% as indicated by the presence of enterotoxin encoding genes.

Moreover, for our calculations we assume that the toxicity of all strains is equal, but intragenic differences may lead to differences in toxicity. Such

differences have been demonstrated for cytotoxin K. The original cytotoxin K, as produced by the strain in which the toxin was first discovered (Lund et al. 2000), is the most potent form of the toxin found as yet. Genes encoding cytotoxin K-like enterotoxins produced by other strains have a similar nucleic acid composition of up to 89%, but the toxicity against Vero-cells of the cytotoxin K-like variants is less than that of the original cytotoxin K (Fagerlund et al. 2004).
Also we assume that the toxicity of the different enterotoxins is equal. However, Moravek et al. (2006) found no significant differences in toxicity between strains producing either NHE and HBL or NHE only, not only indicating that HBL apparently plays a minor role in the toxicity of *B. cereus*, but also that toxicity of the different enterotoxins is not equal. Although it may be an overestimation, we assume that 97% of all isolates are potentially pathogenic with respect to the diarrhoeal syndrome. Consequently, when assuming that even one CFU will lead to disease, $P_{exposure}$ becomes: 0.97* 0.024 = $2.3x10^{-2}$. When assuming that > 10^5 CFU will lead to disease $P_{exposure >10^5}$ becomes: 0.97*0.0029 = 0.0028.

The overall risks for diarrhoeal disease, $P_{disease}$ ($P_{disease} = P_{exposure} * P_{hazard}$), depending on the type of *B. cereus* present in the food, are shown in Table 7.1.

Table 7.1 Risks for disease assuming that even one CFU will lead to disease (Pdisease) and assuming that only > 10^5 CFU/g will lead to disease

	Meso spores	Psychro spores	Meso/young	Meso/old	Psychro young	Psychro/old
$P_{exposure}$			$2.3x10^{-2}$			
P_{hazard}	$6.1x10^{-3}$	$2.4x10^{-3}$	$1.0x10^{-3}$	$2.2x10^{-3}$	$3.3x10^{-4}$	$8.0x10^{-4}$
$P_{disease}$	$1.4x10^{-4}$	$5.6x10^{-5}$	$2.3x10^{-5}$	$5.0x10^{-5}$	$7.6x10^{-6}$	$1.8x10^{-5}$
$P_{exposure > 1E5}$			$2.8x10^{-3}$			
P_{hazard}	$6.1x10^{-3}$	$2.4x10^{-3}$	$1.0x10^{-3}$	$2.2x10^{-3}$	$3.3x10^{-4}$	$8.0x10^{-4}$
$P_{disease > 1E5}$	$1.8x10^{-5}$	$7.1x10^{-6}$	$2.9x10^{-6}$	$6.3x10^{-6}$	$9.6x10^{-7}$	$2.3x10^{-6}$

#: meso = mesophilic, psychro = psychrotrophic, meso/young = mesophilic veg. cells in young adults, psychro/young = psychrotrophic veg. cells in elderly people, meso/old = psychrotrophic veg. cells in young adults, psychro/old = psychrotrophic veg. cells elderly people.

To clarify what stage(s) in the pathogenesis has(have) the largest impact in reducing the risk for disease, the various stages have been plotted against the risk for symptoms (Figure 7.7).

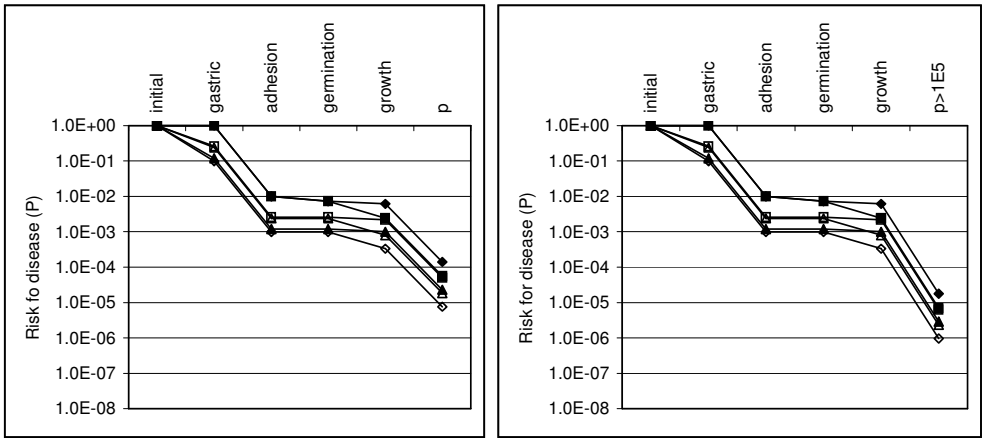


Figure 7.7 Decrease in risk for diarrhoeal disease ($P_{symptoms}$) per stage in pathogenesis, (black diamond = mesophilic spores, black squares = psychrotrophic spores, black triangles = mesophilic veg. cells in healthy young adults, open triangles = psychrotrophic veg. cells in healthy young adults, open squares = mesophilic veg. cells in elderly people, open triangles = psychrotrophic vegetative cells in elderly people). Left calculated with prevalence of 2.4%, = overall prevalence of *B. cereus*. Right calculated with prevalence of 0.29%, = prevalence of *B. cereus* at > 10^5 CFU/g.

From Figure 7.7 it becomes clear that the necessity for *B. cereus* to adhere to epithelial cells causes and the prevalence of *B. cereus* strains result in the largest reduction in risk for disease for all types of *B. cereus* cells (spores and vegetative cells, mesophiles and psychrotrophs). For psychrotrophic vegetative cells also the gastric passage decreases the risk for symptoms significantly.

Knowing the risk for disease per meal, the total number of meals leading to disease can be calculated. Assuming that each meal contains at least one ingredient contaminated with *B. cereus*, and that each day of the year 3 meals are consumed per individual (i.e. per year 365 * 3 = 1095 meals per individual), and that the Dutch population number is 16 million, the total number of meals becomes 1095*16 million = 17.5 billion (= 1.75×10^{10}). Multiplying the different risks for disease with this total numbers of meals gives for each cell type and with that a number of disease cases per year.

Multiplying the different risks for disease with these total numbers of meals gives for each cell type a number of disease invoking meals, and with that the number of disease cases, assuming that even one CFU will

lead to disease and a number of disease invoking meals, and thus disease cases, assuming that for disease $>10^5$ CFU/g are necessary (Table 7.2).

Table 7.2 Numbers of disease cases, assuming that for disease even one CFU is necessary (#cases) and assuming that for disease > 105 CFU/g are necessary (#cases $>10^5$)

	Meso spores	Psychro spores	Meso/young	Meso/old	Psychro/young	Psychro/old
$P_{disease}$	$1.4x10^{-4}$	$5.6x10^{-5}$	$2.3x10^{-5}$	$5.0x10^{-5}$	$7.6x10^{-6}$	$1.8x10^{-5}$
$P_{disease > 1E5}$	$1.8x10^{-5}$	$7.1x10^{-6}$	$2.9x10^{-6}$	$6.3x10^{-6}$	$9.6x10^{-7}$	$2.3x10^{-6}$
# meals			$1.75x10^{10}$			
# cases	$2.4x10^6$	$9.8x10^5$	$4.0x10^5$	$8.7x10^5$	$1.3x10^5$	$3.2x10^5$
# cases > 1E5	$3.1x10^5$	$1.2x10^5$	$5.1x10^4$	$1.1x10^5$	$1.7x10^4$	$4.1x10^4$

This gives two results. Assuming that one CFU can cause diarrhoeal disease the number of disease cases range from 130,000 to 2.4 million. Assuming that only concentrations of *B. cereus* $\geq 10^5$ CFU/g can cause diarrhoeal disease, the number of disease cases ranges from 17,000 to 310,000.

The first result, assuming that even one CFU can cause disease, is an over-estimation. It deals only with *B. cereus* cells and not with the types and levels of the enterotoxin produced by these cells. As indicated by Moravek et al. (2006) the importance of the different enterotoxins is not equal. The role in toxicity of HBL in the investigated strain appeared to be of minor significance compared to the contribution of NHE. Similarly, Fagerlund et al. (2004) found differences in toxicity of cytotoxinK-like enterotoxin.

The numbers of cases assuming that $> 10^5$ CFU/g will lead to disease, however, is an under-estimation. The tolerance level holds for food commodities at the time the products are samples in retail. This, however, is not the time of consumption. What happens between the time of purchase in retail and the time of consumption may influence the level of contamination. If the food commodities are treated properly the risk that the number of contaminating cells increases is minimal. If, however, in the period between purchase and storage at home the time-temperature conditions are abused, by for example leaving food unrefridgerated for a longer period of time, the number of contaminating cells may increase, and with that the risk of disease may increase.

In conclusion, the risk of falling ill with the diarrhoeal syndrome after the ingestion of food contaminated with *B. cereus* depends on more factors than exposure and the behaviour in the gastro-intestinal tract, as studied in this thesis. Toxicity of enterotoxins, dose response relations of the enterotoxins and other not yet mentioned factors may play a role as well. However, the lack of data on the latter subjects prevents their inclusion in the determination of the risk for diarrhoeal disease at this moment. In this thesis exposure to food borne *B. cereus* and some stages in the pathogenic mechanism of the diarrhoeal syndrome were investigated for their influence on the risk for disease. The results indicate a number of disease cases ranging from 40,000 to 2.3 million per year, assuming that any number of CFU's of *B. cereus* is able to cause disease. Assuming that only numbers of *B. cereus* $\geq 10^5$ CFU/g food will be able to cause disease, the number of cases ranges from 17,000 to 310,000 per year.

The emetic syndrome

For calculations involving the emetic syndrome we assume that strains capable of producing cereulide are not able to produce enterotoxins, and are thus not capable of inducing diarrhoea.

The emetic syndrome is a pure intoxication, as shown by the pathogenic mechanism in Figure 7.1. The risk for disease, ($P_{disease}$), can be determined similar to the risk for diarrhoeal disease, namely $P_{disease} = P_{exposure} * P_{hazard}$.

$P_{exposure}$ depends on the prevalence of *B. cereus* strains in food commodities and on the prevalence of emetic strains in food commodities. For the prevalence of *B. cereus* strains in food commodities we use the same data as for the diarrhoeal syndrome from Jansen and in 't Veld (2002). They determined the prevalence in ready-to-eat meals to be 2.4%, meaning a $P_{exposure}$ of 0.024. For the following calculations we assume, like in the calculations for the diarrhoeal syndrome, that this prevalence percentage is representative for all food commodities. In the survey described in Chapter 2 of this thesis 8.2% of the strains were found to be able to produce cereulide-like toxin. This leads to a $P_{exposure}$ of 0.082 * 0.024 = 0.002 (2.0×10^{-3}) assuming that all emetic strains are equally pathogenic.

The second factor in the determination of the chance for emetic disease ($P_{disease}$) is the chance that the contaminating strain leads to emetic disease symptoms, $P_{emetic\ hazard}$, Since the emetic syndrome is an intoxication $P_{emetic\ hazard}$ has to do with the dose leading to disease. No data

are available enabling a dose response relation for humans. For shrewd mice one research group determined a dose response relation (Agata et al. 1995b). In 2000 a large outbreak of *B. cereus* emetic disease occurred in Almere, the Netherlands (Essen et al. 2000). In this outbreak 116 students of 120 that consumed a vegetarian rice dish fell ill (attack rate = 116/120*100% = 97%). During the investigation of this outbreak two *B. cereus* strains were recovered from food remnants and vomit. The production potential of these strains was determined with the Hep-2 test as described by Finlay et al. (1999). Based on its titre in the Hep-2 cell test, the least concentration of cereulide in the Hep-2 cell test, the estimated amount of consumed food and the estimated weight of an average patient, the dose of cereulide leading to disease in the Almere outbreak was calculated to be approximately 9.5 µg/kg bodyweight. This cereulide dose was compared to the doses that 46 strains isolated in the survey described in Chapter 2 would have produced under the same conditions.

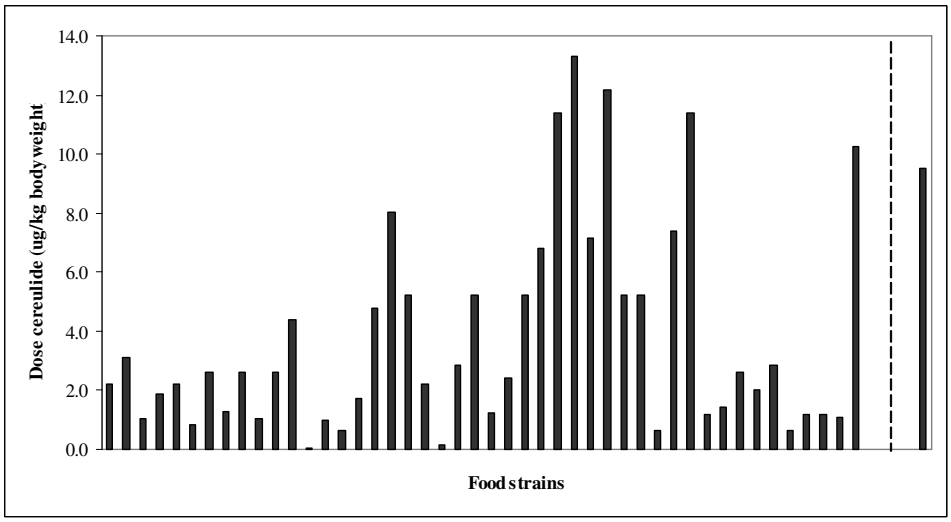


Figure 7.8 Disease invoking dose of cereulide from one of the Almere strains (bar right of the broken line) compared to the doses emetic strains, as found in the survey described in Chapter 2, would have produced in the same conditions.

All these strains, including the Almere strain, were grown to similar numbers before determining their cytotoxic potential in the Hep-2 cell test. The Hep-e cell test titres were recalculated to doses of cereulide and plotted in Figure 7.8., where each of the 46 strains is represented by a bar, and where the bar right of the dashed line represents the Almere strain. Seven of these 46 strains (= 15%) came to doses equal to or higher than the Almere strains (Figure 7.8). This means that P_{hazard} is 0.15, and with that

$P_{disease}$ becomes: 0.002 ($_{Pexposure}$) * 0.15 (P_{hazard}) = 0.0003 (= $3x10^{-4}$).
The dose of 9.5 µg/kg, however, led to an attack rate of nearly 100%. What the effect of lower doses is, and to what attack rates they lead, is not known due to the lack of data. One experiment by Agata et al. (1995b) was carried out in which shrewd mice in groups of 5 were given various doses of cereulide, ranging from 4 to 32 µg/kg. The result of this experiment is shown in Figure 7.9, where the black squares represent the results by Agata et al. (1995), and where the open square represents the dose in the Almere outbreak (previous page).

The results of this dose response experiment were fitted using Mathematica® in a dose response curve (Figure 7.10.).

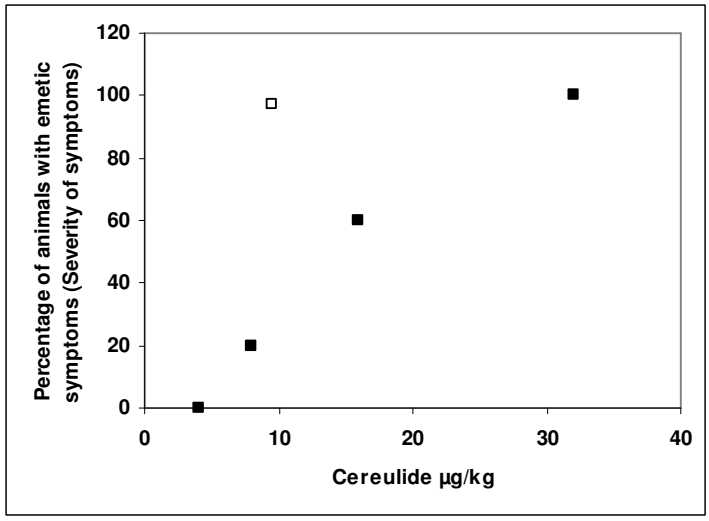


Figure 7.9 Results of the dose response experiment with shrewd mice (black squares) as carried out by (Agata et al. 1995b). . The open square is the dose determined in the Almere outbreak.

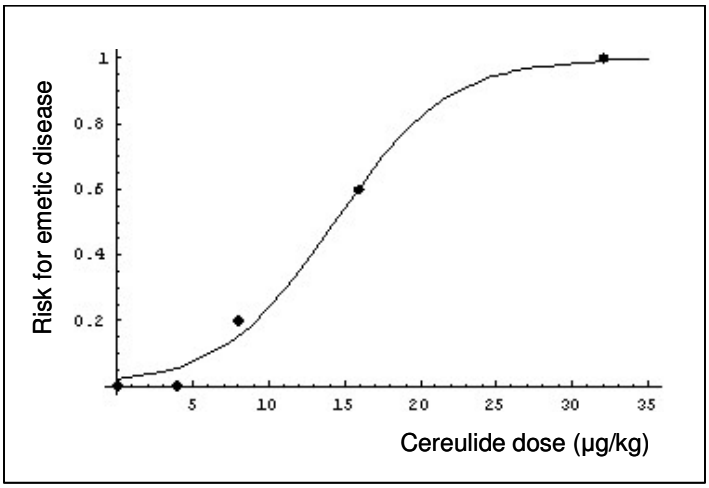


Figure 7.10 Dose response curve calculated with Mathematica® from the results by (Agata et al. 1995b).Equation: $y = 1/1+46.6166*e^{-0.257984*x}$

Based on the one point representing the dose in the Almere outbreak a dose response relation might be calculated. The uncertainty of the result, however, would be very high. Therefore, we did not attempt to calculate such dose response relation.

The dose giving a 0% attack rate in mice was 4 µg/kg, which is eight times less than the dose leading to 100% attack rate (32 µg/kg). For humans no dose leading to a 0% attack rate is known, although earlier (Jääskeläinen et al. 2003b) reported a toxic dose for humans of ≤ 8 µg/kg. Assuming a linear relation in the doses would mean that 9.5/8 = 1.2 µg/kg would be the dose for a 0% attack rate in humans. Applying this dose to the 46 emetic strains would mean that 32 of the 46 strains (= 70%) would produce higher doses than this 1.2 µg/kg, and would therefore lead to disease in humans. This means that P_{hazard} would be 0.7. Introduction of this P_{hazard} into the equation for the risk for emetic disease leads to a $P_{disease}$ = 0.002 ($P_{exposure}$) * 0.7 (P_{hazard}) = 0.0014 (=$1.4x10^{-3}$).

Assuming the consumption of $1.75x10^{10}$ meals per year in the Netherlands leads to the following numbers meals able to induce emetic disease: $3x10^{-4}$ * $1.75x10^{10}$ to $1.4x10^{-3}$ * $1.75x10^{10}$ = $5.3x10^{6}$ to $2.5x10^{7}$ meals, and with that to 5.3 to 25 million cases of emetic disease.

Introducing the tolerance level of 10^5 CFU/g into these calculations means that the number of meals leading to emetic disease is reduced, since 0.29% of all investigated food commodities contains ≥ 10^5 CFU/g. The risk for exposure becomes 0.082*0.0029 = $2.4x10^{-4}$. And consequently, the risks for disease become: $2.4x10^{-4}$* 0.15 = $3.6x10^{-5}$ and $2.4x10^{-4}$*0.7 = $1.7x10^{-4}$, and the numbers of meals leading to disease become $3.6x10^{-5}$*$1.75x10^{10}$ = $6.3x10^{5}$ (630,000) to$1.7x10^{-4}$*$1.75x10^{10}$ = $3.0x10^{6}$ (3 million).

These calculations are based on a number of assumptions. Firstly, we assumed that cereulide production is independent of the substrate the emetic strains grow in/on. However, there are large differences in cereulide production when emetic *B. cereus* strains are grown on different substrates. In general, emetic strains prefer carbohydrate rich food commodities for growth as reflected by the reports on outbreaks where often rice dishes or pasta dishes are involved (Mahler et al. 1997; Jääskeläinen et al. 2003b; Agata et al. 2002; Ripabelli et al. 2000; Dierick et al. 2005). Various investigations have described the growth potential and toxin production of emetic strains in relation to food constituents. Agata et al. (2002) found the highest toxin titres in boiled and fried rice and in spaghetti. Jääskeläinen et al. (2003a) described that rice-containing pastries accumulated high cereulide contents. Another food component that may be of influence on

the availability of cereulide is fat. After inoculating milk with different fat percentages, from full fat milk to skimmed milk, we found comparable numbers of *B. cereus* per ml milk. But the cereulide titre decreased with increasing fat percentage (Wijnands et al., unpublished results).

Secondly, we assumed that cereulide production is independent of environmental factors, such as aeration and temperature. However, aeration of a milk culture resulted in higher toxin production than in a stationary culture (Agata et al. 2002; Jääskeläinen et al. 2004; Häggblom et al. 2002). Finlay et al. (2000) found that toxin levels after growth at 12 to 15ºC were significantly higher than after growth at 30ºC, whereas experiments by (Häggblom et al. 2002) showed that no cereulide is produced at temperatures below 8ºC and above 40ºC.

Thirdly, we assumed that a dose of cereulide leads to similar symptoms in all individuals. Even though in the Almere outbreak the attack rate was 97%, the symptoms varied over the individuals. A small number of patients had to be hospitalized, whereas the others experienced symptoms ranging from nausea to severe vomiting leading to hospitalization. The susceptibility per individual is variable. Another example of varying susceptibility is shown in the outbreak in Switzerland, in which one of the affected individuals died, while the other experienced severe abdominal pain and diarrhoea (Mahler et al. 1997). According to Agata et al. (1995b) cereulide uses 5-HT_3 receptors, which can be found in the stomach as well as in the proximal part of the small intestine. Whether individual response differences are caused by differences in numbers of receptors or otherwise remains to be investigated.

An important issue that has not yet been discussed is the role of the consumer in the onset of disease. When studying outbreaks of emetic disease by *B. cereus* such as the Almere outbreak (Essen et al. 2000), the Swiss outbreak (Mahler et al. 1997) and the Belgian outbreak (Dierick et al. 2005), it is striking that in all cases abuse of time-temperature combinations occurred. Food was either stored in a refrigerator set at a temperature of 12ºC, food was left to cool down at room temperature after heating and before storage in a refrigerator, or food was left to cool down at room temperature in too large quantities. In each of these cases *B. cereus* must have been present beforehand and was given the opportunity to grow and produce cereulide-like toxin.

In The Netherlands a tolerance level for *B. cereus* in food is set at 10^5 CFU/g. This is the maximum level of *B. cereus* that is allowed in an end product at retail level. Cereulide is formed during growth of the

micro-organism in food. Preliminary investigations show that during growth of the micro-organism no detectable cereulide-like toxin is produced at number $\leq 10^5$ CFU/ml (data not shown). In case an end-product, before bringing it to the market, is heated to reduce the number of micro-organisms, the number of *B. cereus* cells need not reflect the amount of cereulide-like toxin, since the toxin is not affected by the heating process. Therefore, to prevent the onset of emetic disease by *B. cereus* a tolerance level for cereulide-like toxin might be more appropriate. Two major problems still form an obstacle: the lack of a dose-response relation indicating at what concentrations it is safe to consume food containing cereulide, and the lack of a rapid method to determine the concentration of cereulide in food commodities. The latter problem may be overcome by using the method described by Rajkovic et al. (2007), who designed an automated test based on the boar sperm test described by Andersson et al. (1998). To address the former problem, outbreaks should be investigated and documented in much more detail. Not only data on the occurrence and level of the incriminating *B. cereus* strain(s) should be recorded but also data on the amount of cereulide in food remnants and preferably in vomit of the patients. This asks for new protocols on how to approach outbreaks in which *B. cereus* is suspected. In emetic outbreaks attention should be focused on the detection of cereulide while in diarrhoeal outbreaks attention should be focused on detection of *B. cereus* strains.

In conclusion, based on the assumptions made and considering the lack of data enabling a proper dose response determination for humans, the number of disease cases due to emetic strains of *B. cereus* ranges from 630,000 to 3 million per year in the Netherlands.

Falling ill with the emetic syndrome by *B. cereus* not only depends on the initial load of *B. cereus*, but also on the way the food is treated prior to consumption. Various aspects, such as the conditions favourable for cereulide-like toxin production and determination of a dose response relation, should be further investigated in order to be able to estimate the number of emetic disease cases per year in The Netherlands. Meanwhile, to prevent disease, several measures may be appropriate: firstly, a tolerance level for the amount of cereulide or a tolerance level for the numbers of *B. cereus* that may be reached in whole chain from raw material to end-product, and secondly, repeatedly pointing out the dangers of maltreatment of food to consumers and retailers.

Conclusive remarks

Bacillus cereus is recognized as an important cause of food borne disease, not only in the Netherlands but elsewhere as well. The incidence of *B. cereus* food borne disease, however, is hard to estimate due to a vast underreporting. An important reason for this is the relatively short lasting and mild symptoms, withholding people to seek medical assistance.

By combining data on exposure and hazard as described in this thesis, the incidence of the diarrhoeal syndrome has been calculated in two ways: assuming that only one CFU of *B. cereus* may already lead to disease, and assuming that only $\geq 10^5$ CFU/g will lead to disease. When assuming that one CFU of *B. cereus* can lead to disease, the number of diarrhoeal disease cases ranges from 130,000 to 2.4 million per year; assuming that $\geq 10^5$ CFU/g are necessary to invoke disease, the number of cases ranges from 17,000 to 310,000 per year.

Similarly the risk for emetic disease was determined. By combining data on exposure and published and unpublished data on dose and dose response of cereulide, the numbers of emetic cases were calculated to range from 5.3 – 25 million per year, assuming that one CFU of *B. cereus* produces the amount of cereulide that can lead to disease. When assuming that $> 10^5$ CFU/g are needed to produce an amount of cereulide leading to disease, the numbers of cases range from 630,000 to 3 million per year.

The figures calculated above differ from the estimates by the Dutch Health Council (Gezondheidsraad, 2000) and the National Institute for Public Health and the Environment (Van Kreijl et al. 2004), both based on epidemiological data. They estimated the number of *B. cereus* disease cases at approximately 50,000 and 37,500 respectively, much lower than the numbers of cases we calculated. What remains is the question whether the numbers of disease cases due to food borne *B. cereus* are acceptable or not. This question was addressed earlier by Pielaat et al. (2005), and they suggested changing the tolerance level for *B. cereus* in food commodities from 10^5 CFU/g to 10^3 CFU/g. Their calculations were based mainly on the growth potential of *B. cereus* strains in simulated gastro-intestinal fluids, and were thus only applicable for strains causing the diarrhoeal syndrome.

The calculations made in this thesis indicate that the majority of the cases of food borne disease by *B. cereus* are emetic cases. In our

opinion this type of disease should be treated as an intoxication, as disease is due to cereulide. For setting a tolerance level for emetic disease a different approach should be considered. Three approaches can be considered to achieve a reduction in numbers of food commodities contaminated with emetic strains of *B. cereus*.

The first approach would be to set a limit to the number of *B. cereus* that may be present during the entire food chain. Preliminary (non-published) experiments have shown that during growth of emetic strains of *B. cereus* no cereulide is detected at concentrations of cells $\leq 10^5$ CFU/g. This, however, needs further investigation with respect to growth and cereulide production in a large number of substrates, as the results are very preliminary and based on growth in one medium only.

The second approach would be to monitor food for strains able to produce cereulide. This has become a realistic method since the unravelling of the mechanism behind cereulide production through a non ribosomal peptide synthetase complex (Ehling-Schulz et al. 2005; Ehling-Schulz et al. 2006). Recent findings that all strains positive for the presence of this NRPS-complex were also able to produce cereulide sustain the applicability of methods detecting (parts of) the NRPS-complex. Also the set-up of a real time PCR assay for the detection of cereulide producing potential has made rapid screening of strains a realistic method (Fricker et al. 2007).

The third approach is detection of cereulide in food commodities. Various methods are available for the detection of cereulide or cereulide-derived effects. A disadvantage sofar is that these methods are not (yet) readily applicable to the investigation of food, but require isolation and culture of the contaminating strain first. Cereulide-derived effects can be detected are vacuolation or inhibition of sperm motility. Vacuolation can be detected by the Hep-2 cell test according to Finlay et al. (1999). Sperm inhibition can be detected by the rapid boar sperm test according to Andersson et al. (2004) or by the automated version of this test by Rajkovic et al. (2007). Detection of cereulide can only be achieved by liquid chromatography coupled to a mass spectrometer (Häggblom et al. 2002). This last method can be employed for the direct detection of cereulide, after extraction of the compound from food or other matrices. For all these approaches more knowledge on a dose response relation for humans is of importance. This can be achieved by gathering more data from outbreaks or by translation of data from animal experiments to the human situation. When studying outbreak data, investigators should not

only investigate the presence and numbers of *B. cereus*, but also the presence and level of cereulide should be recorded. Moreover, details on the incriminating food, the preparation of this food and the conditions during the preparation and storage of the food should be recorded in order to obtain more data on the conditions for cereulide production. The latter information can also be obtained by elaborate laboratory research.

An important issue is the role of the consumer/food preparer in the onset of *B. cereus* food borne disease. Most cases and outbreaks of *B. cereus* food borne disease derive from maltreatment of food commodities with respect to time-temperature combinations. Although such mistreatment may have its effect on other food borne micro-organisms as well, it is especially important with respect to *B. cereus* because of its capacity to form spores, which may survive heating processes. During cooling processes they may germinate and grow to high numbers or enabling the production of high levels of cereulide. Consumers should be informed repeatedly on the risks that are inherent to food preparation.

Conclusively, food borne disease by *B. cereus* will not be banished. But the number of cases/outbreaks can be reduced by various measures. Tolerance levels may be of assistance to keep the number of food borne disease cases in control, but it is advisable to make a distinction between the two syndromes. For the diarrhoeal syndrome a tolerance level based on a number of colony forming units is useful. For the emetic syndrome it could be considered to set a tolerance level based on an amount of cereulide.

References

1. Adams, M.R. and Moss, M.O. (2000) Food Microbiology. Cambridge, The Royal Society of Chemistry.

2. Agaisse, H., Gominet, M., Okstad, O.A., Kolstø, A.B. and Lereclus, D. (1999) PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. *Molecular Microbiology* **32,** 1043-1053.

3. Agata, N., Mori, M., Ohta, M., Suwan, S., Ohtani, I. and Isobe, M. (1994) A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. *FEMS Microbiology Letters* **121**, 31-34.

4. Agata, N., Ohta, M., Mori, M. and Isobe, M. (1995b) A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus. FEMS Microbiology Letters* **129**, 17-20.

5. Agata, N., Ohta, M. and Yokoyama, K. (2002) Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. *International Journal of Food Microbiology* **73**, 23-27.

6. Altayar, M. and Sutherland, A.D. (2006) *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. *Journal of Applied Microbiology* **100**, 7-14.

7. Anderson Borge, G.I., Skeie, M., Sorhaug, T., Langsrud, T. and Granum, P.E. (2001) Growth and toxin production of *Bacillus cereus* isolated from different food sources. *International Journal of Food Microbiology* **69**, 237-246.

8. Andersson, A., Granum, P.E. and Rønner, U. (1998) The adhesion of *Bacillus cereus* spores to epithelial cells might be an additional virulence mechanism. *International Journal of Food Microbiology* **39,** 93-99.

9. Andersson, M.A., Jääskeläinen, E.J., Shaheen, R., Pirhonen, T., Wijnands, L.M. and Salkinoja-Salonen, M.S. (2004) Sperm bioassay for rapid detection of cereulide-producing *Bacillus cereus* in food and related environments. *International Journal of Food Microbiology* **94**, 175-183.

10. Andersson, M.A., Mikkola, R., Helin, J., Andersson, M.C. and Salkinoja-Salonen, M. (1998) A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. *Applied and Environmental Microbiology* **64,** 1338-1343.

11. Anonymous (1979) Kokswarenbesluit. Warenwet. Koninklijke Vermande B.V. Utrecht (In Dutch)

12. Anonymous (1996) Surveillance for foodborne-disease outbreaks - United States. *Morbidity and Mortality Weekly Report* **45**, 1-55.

13. Anonymous (2000a) Surveillance for foodborne-disease outbreaks - United States. *Morbidity and Mortality Weekly Report* **49**, 1-51.

14. Anonymous (2000b) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: the Netherlands.

15. Anonymous (2000c) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: France.

16. Anonymous (2000d) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: Hungary.

17. Anonymous (2000e) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: Norway.

18. Anonymous (2000f) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: Finland.

19. Anonymous (2000g) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: Germany.

20. Anonymous (2000h) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: Spain.

21. Anonymous (2000i) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: Sweden.

22. Anonymous (2000j) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 7th report 1993-1998: United Kingdom, England and Wales.

23. Anonymous (2003a) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 8th report 1999-2000: the Netherlands.

24. Anonymous (2003b) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 8th report 1999-2000: France.

25. Anonymous (2003c) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 8th report 1999-2000: Hungary.

26. Anonymous (2003d) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 8th report 1999-2000: Norway.

27. Anonymous (2003e) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 8th report 1999-2000: Finland.

28. Anonymous (2003f) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 8th report 1999-2000: Germany.

29. Anonymous (2003g) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 8th report 1999-2000: Sweden.

30. Anonymous (2003h) WHO surveillance programme for control of foodborne infections and intoxications in Europe. 8th report 1999-2000: United Kingdom, England and Wales.

31. Anonymous (2005) Outbreak of cutaneous *Bacillus cereus* infections among cadets in a university military program - Georgia, August 2004. *Morbidity and Mortality Weekly Report* **54**, 1233-1235.

32. Apetroaie, C., Andersson, M.A., Spröer, C., Tsitko, I., Shaheen, R., Jääskeläinen, E.L., Wijnands, L.M., Heikkilä, R. and Salkinoja-Salonen, M.S. (2005) Cereulide-producing strains of *Bacillus cereus* show diversity. *Archives of Microbiology* **184**, 141-151.

33. Asano, S.I., Nukumizu, Y., Bando, H., Iizuka, T. and Yamamoto, T. (1997) Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis*. *Applied and Environmental Microbiology* **63,** 1054-1057.

34. Ash, C., Farrow, J.A., Dorsch, M., Stackebrandt, E. and Collins, M.D. (1991) Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *International Journal of Systematic Bacteriology* **41,** 343-346.

35. Astwood, J.D., Leach, J.N. and Fuchs, R.L. (1996) Stability of food allergens to digestion in vitro. *Nature Biotechnology* **14**, 1269-1273.

36. Barlass, P.J., Houston, C.W., Clements, M.O. and Moir, A. (2002) Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of gerL and gerQ operons. *Microbiology* **148**, 2089-2095.

37. Barrie, D., Wilson, J.A., Hoffman, P.N. and Kramer, J.M. (1992) *Bacillus cereus* meningitis in two neurological patients: an investigation into the source of the organisms. *Journal of Infection* **25**, 291-297.

38. Beecher, D.J. and Lee Wong, A.C. (1994) Improved purification and characterization of hemolysin BL, a hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. *Infection and Immunity* **62,** 980-986.

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

40. Beecher, D.J. and Lee Wong, A.C. (1997) Tripartite hemolysin BL from *Bacillus cereus.* Hemolytic analysis of component interactions and a model for its characteristic paradoxical zone phenomenon. *Journal of Biological Chemistry* **272,** 233-239.

41. Beecher, D.J. and Wong, A.C.L. (2000) Tripartite haemolysin bl: isolation and characterization of two distinct homologous sets of components from a single *Bacillus cereus* isolate. *Microbiology* **146,** 1371-1380.

42. Belaiche, J. (2000) Pathophysiology of acute infectious diarrhea. *Acta Endoscopica* **30,** 177-184.

43. Ben-Dov, E., Zaritsky, A., Dahan, E., Barak, Z., Sinai, R., Mansherob, R., Khamraevv, A., Troistkaya, E., Dubitsky, A., Berezina, N. and Margalith, Y. (1997) Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. *Applied and Environmental Microbiology* **63**, 4883-4890.

44. Berner, R., Heinen, F., Pelz, K., van Velthoven, V., Sauer, M. and Korinthenberg, R. (1997) Ventricular shunt infection and meningitis due to *Bacillus cereus*. *Neuropediatrics* **28**, 333-334.

45. Bernhardt, H. and Knoke, M. (1989) Recent studies on the microbial ecology of the upper gastro-intestinal tract. *Infection* **17**, 259-263.

46. Black, E.P., Koziol-Dube, K., Guan, D., Wei, J., Setlow, B., Cortezzo, D.E., Hoover, D.G. and Setlow, P. (2005) Factors influencing germination of *Bacillus subtilis* spores via activation of nutrient receptors by high pressure. *Applied and Environmental Microbiology* **71**, 5879-5887.

47. Blaser, M.J. and Newman, L.S. (1982) A review of human Salmonellosis: I. Infective dose. *Reviews of Infectious Diseases* **4**, 1096-1106.

48. Bolton, A.J. , Osborne, M.P. and Stephen, J. (2000) Comparative study of the invasiveness of *Salmonella* serotypes *Typhimurium*, *Choleraesuis* and *Dublin* for Caco-2 cells, HEp-2 cells and rabbit ileal epithelia. *Journal of Medical Microbiology* **49**, 503-511.

49. Brekenfeld, H. (1926) Lebensmittelbakterien und Vergiftungen. *Zentralblatt für Bakteriologie, I Originale* **99**, 353-385.

50. Brett, M.M., Hood, J., Brazier, J.S.D.B.I. and Hahne, S.J. (2005) Soft tissue infections caused by spore-forming bacteria in injecting drug users in the United Kingdom. *Epidemiology and Infection* **133**, 575-582.

51. Buchanan, R.L. and Schultz, F.J. (1994) Comparison of the Tecra VIA kit, Oxoid BCET-RPLA kit and CHO cell culture assay for the detection of *Bacillus cereus* diarrhoeal enterotoxin. *Letters in Applied Microbiology* **19,** 353-356.

52. Callegan, M.C., Jett, B.D., Hancock, L.E. and Gilmore, M.S. (1999) Role of hemolysin bl in the pathogenesis of extraintestinal *Bacillus cereus* infection assessed in an endophthalmitis model. *Infection and Immunity* **67,** 3357-3366.

53. Camilleri, M., Malagelada, J.R., Brown, M.L., Becker, G. and Zinsmeister, A.R. (1985) Relation between antral motility and gastric emptying of solids and liquids in humans. *American Journal of Physiology* **249**, G580-G585.

54. Carlin, F., Fricker, M., Pielaat, A., Heisterkamp, S., Shaheen, R., Salkinoja-Salonen, M.S., Svensson, B., Nguyen-the, C. and Ehling-Schulz, M. (2006) Emetic toxin-producing strains of *Bacillus cereus* show distinct characteristics within the *Bacillus cereus* group. *International Journal of Food Microbiology* **109**, 132-138.

55. Chen, M.L. and Tsen. H.Y. (2002) Discrimination of *Bacillus cereus* and *Bacillus thuringiensis* with 16S rRNA and gyrB gene based PCR primers and sequencing of their annealing sites. *Journal of Applied Microbiology* **92**, 912-919.

56. Choma, C. and Granum, P.E. (2002) The enterotoxin T (bceT) from *Bacillus cereus* can probably not contribute to food poisoning. *FEMS Microbiology Letters* **217**, 115-119.

57. Choma, C., Guinebretiëre, M.H., Carlin, F., Schmitt, P., Velge, P., Granum, P.E. and Nguyen the, C. (2000) Prevalence, characterization and growth of *Bacillus cereus* in commercial cooked chilled foods containing vegetables. *Journal of Applied Microbiology* **88**, 617-625.

58. Christiansson, A., Naidu, A.S., Nilsson, I., Wadstroem, T. and Pettersson, H.E. (1989) Toxin production by *Bacillus cereus* dairy isolates in milk at low temperatures. *Applied and Environmental Microbiology* **55,** 2595-2600.

59. Clarkston, W.K., Pantano, M.M., Morley, J.E., Horowitz, M., Littlefield, J.M. and Burton, F.R. (1997) Evidence for the anorexia of aging: gastro-intestinal transit and hunger in healthy elderly vs. young adults. *American Journal of Physiology* **272**, R243-R248.

60. Clavel, T., Carlin, F., DArgaignaratz, C., Lairon, D., Nguyen-the, C. and Schmitt, P. (2007) Effects of porcine bile on survival of *Bacillus cereus* vegetative cells and Haemolysin BL enterotoxin production in reconstituted human small intestinal media. *Journal of Applied Microbiology* **103**, 1568-1575.

61. Clavel, T., Carlin, F., Lairon, D., Nguyen-The, C. and Schmitt, P. (2004) Survival of *Bacillus cereus* spores and vegetative cells in acid media simulating human stomach. *Journal of Applied Microbiology* **97**, 214-219.

62. Coconnier, M.H., Liévin, V., Lorrot, M. and Servin, A.L. (2000) Antagonistic activity of *Lactobacillus acidophilus* LB against intracellular *Salmonella enterica* serovar *typhimurium* infecting human enterocyte-like Caco-2/TC-7 cells. *Applied and Environmental Microbiology* **66**, 1152-1157.

63. Collado, M.C.M.J. and Salminen, S. (2007) Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Letters in Applied Microbiology* **45**, 454-460.

64. Cooper, I., Lord, T. and Tice, P. (1995) Hydrolysis studies in simulated gastro-intestinal fluids. *Food Additives and Contaminants* **12**, 769-777.

65. D'aoust, J.Y. (1985) Infective dose of *Salmonella typhimurium* in cheddar cheese. *American Journal of Epidemiology* **122**, 717-720.

66. Damgaard, P.H. (1995) Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides. *FEMS Immunology and Medical Microbiology* **12,** 245-250.

67. Damgaard, P.H., Larsen, H.D., Hansen, B.M., Bresciani, J. and Jorgensen, K. (1996) Enterotoxin-producing strains of *Bacillus thuringiensis* isolated from food. *Letters in Applied Microbiology* **23,** 146-150.

68. Darbar, A., Harris, I.A. and Gosbell, I.B. (2005) Necrotizing infection due to *Bacillus cereus* mimicking gas gangrene following penetration trauma. *Journal of Orthopaedic Trauma* **19**, 353-355.

69. De Wit, M.A.S., Koopmans, M.P.G., Kortbeek, L.M., Wannet, W.J.B., Vinjé, J., van Leusden, F., Bartelds, A.I.M. and van Duynhoven, Y.T.H.P. (2001) Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. *American Journal of Epidemiology* **154**, 666-674.

70. Del Torre, M., Della Corte, M. and Stecchini, M.L. (2001) Prevalence and behaviour of *Bacillus cereus* in a REPFED of Italian origin. *International Journal of Food Microbiology* **63**, 199-207.

71. Dierick, K., Van Coillie, E., Swiecicka, I., Meyfroidt, G., DeVlieger, H., Meulemans, A., Hoedemaekers, G., Fourie, L., Heyndrickx, M. and Mahillon, J. (2005) Fatal family outbreak of *Bacillus cereus*-associated food poisoning. *Journal of Clinical Microbiology* **43**, 4277-4279.

72. Dietrich, R., Fella, C., Strich, S. and Märtlbauer, E. (1999) Production and characterization of monoclonal antibodies against the hemolysin bl enterotoxin complex produced by *Bacillus cereus. Applied and Environmental Microbiology* **65,** 4470-4474.

73. Dietrich, R. , Moravek, M., Burk, C., Granum, P.E. and Märtlbauer, E. (2005) Production and characterization of antibodies against each of three subunits of the *Bacillus cereus* non haemolytic enterotoxin complex. *Applied and Environmental Microbiology* **71**, 8214-8220.

74. Doorduyn, Y. , Broek, M.J.M.v.d. and Duynhoven, Y.T.H.P.v. (2007) Registratie van voedselinfecties en -vergiftigingen bij de Inspectie voor de Gezondheidszorg en Voedsel en Waren Autoriteit, 2006. RIVM report 300103 001, Bilthoven, The Netherlands: National Institute for Public Health and the Environment (RIVM).

75. Doorduyn, Y. , Broek, M.J.M.v.d. and Duynhoven, Y.T.H.P.v. (2006) Registratie van voedselinfecties en -vergiftigingen bij de Inspectie voor de Gezondheidszorg en Voedsel en Waren Autoriteit, 2005. RIVM report 330010004, Bilthoven, The Netherlands.: National Institute for Public Health and the Environment (RIVM) .

76. Dressman, J.B., Berardi, R.R., Dermentzoglou, L.C., Russell, T.L., Schmaltz, S.P., Barnett, J.L. and Jarvenpaa, K.M. (1990) Upper gastro-intestinal (GI) pH in young, healthy men and women. *Pharmaceutical Research* **7,** 756-761.

77. Drobniewski, F.A. (1993) *Bacillus cereus* and related species. *Clinical Microbiology Reviews* **6,** 324-338.

78. Dufrenne, J., Tatini, S. and Notermans, S. (1994) Stability of spores of *Bacillus cereus* stored on silica gel. *International Journal of Food Microbiology* **23,** 111-116.

79. Duynhoven, Y.T.H.P.v., Boer, I.M.d. and Broek, M.J.M.v.d. (2005) Registratie voedselinfecties en -vergiftigingen bij de Inspectie voor de Gezondheidszorg en Voedsel en Waren Autoriteit, 2004. RIVM report 330010003, Bilthoven, The Netherlands: National Institute for Public Health and the Environment (RIVM).

80. Duynhoven, Y.T.H.P.v., Brandsema, P., Chardon, J.E., Evers, E.G., Leusden, F.v. and Broek, M.J.M.v.d. (2004) Registratie voedselinfecties en -vergiftigingen bij de Inspectie voor de Gezondheidszorg en Voedsel en Waren Autoriteit, 2003. RIVM report 330010003, Bilthoven, The Netherlands: National Institute for Public Health and the Environment (RIVM).

81. Duynhoven, Y.T.H.P.v., Eerden, L.J.M.v. and Broek, M.J.M.v.d. (2002) Registratie voedselinfecties en -vergiftigingen bij de Inspectie voor de Gezondheidszorg en Voedsel en Waren Autoriteit, 2001. RIVM report 330010003, Bilthoven, The Netherlands: National Institute for Public Health and the Environment (RIVM).

82. Ehling-Schultz, M., Fricker, M. and Scherer, S. (2004) *Bacillus cereus*, the causative agent of an emetic type of food-borne illnesses. *Molecular Nutrition and Food Research* **48**, 479-487.

83. Ehling-Schulz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M. and Scherer, S. (2006) Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pX01. *BMC Microbiology* **6**, 20-31.

84. Ehling-Schulz, M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M., Märtlbauer, E. and Scherer, S. (2005) Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. *Applied and Environmental Microbiology* **71**, 105-113.

85. Essen, R., de Ruiter, C. and de Wit, M. (2000) Massale voedselvergiftiging in het Kotterbos te Almere (in Dutch). *Infectieziekten Bulletin* **11**, 205-207.

86. Essex, R.W., Charles, P.G. and Allen, P.J. (2004) Three cases of post-traumatic endophthalmitis caused by unusual bacteria. *Clinical and Experimental Ophhtalmology* **32**, 445-447.

87. EU-Project QLK1-2001-00854. Preventing *Bacillus cereus* foodborne poisoning in Europe: Detecting hazardous strains, tracing contamination routes and proposing criteria for foods.

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

89. Finlay, W.J.J., Logan, N.A. and Sutherland, A.D. (1999) Semiautomated metabolic staining assay for *Bacillus cereus* emetic toxin. *Applied and Environmental Microbiology* **65,** 1811-1812.

90. Finlay, W.J.J., Logan, N.A. and Sutherland, A.D. (2000) *Bacillus cereus* produces most emetic toxin at lower temperatures. *Letters in Applied Microbiology* **31,** 385-389.

91. Frankland, G.C. and Frankland, P.F. (1887) Studies on some new micro-organisms from air. *Philosophical Transactions of the Royal Society of London* **173**, 257-287.

92. Fricker, M., Messelhäusser, U., Busch, U., Schere, S. and Ehling-Schulz, M. (2007) Diagnostic real-time PCR assay for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. *Applied and Environmental Microbiology* **73**, 1892-1898.

93. Garcia Arribas, M.L. and Kramer, J.M. (1990) The effect of glucose, starch, and pH on growth, enterotoxin and haemolysin production by strains of *Bacillus cereus* associated with food poisoning and non-gastro-intestinal infection. *International Journal of Food Microbiology* **11**, 21-33.

94. Gaulin, C., Bonnier-Viger, Y. and Fillion, L. (2002) An outbreak of *Bacillus cereus* implicating a part-time banquet caterer. *Canadian Journal of Public Health* **93**, 353-355.

95. Gezondheidsraad (2000) Voedselinfecties. Den Haag: Gezondheidsraad, publicatie nr 2000/09. (In Dutch with English abstract):

96. Ghelardi, E., Celandroni, F., Salvetti, S., Barsotti, C., Baggiani, A. and Senesi, S. (2002) Identification and characterization of toxigenic *Bacillus cereus* isolates responsible for two food-poisoning outbreaks. *FEMS Microbiology Letters* **208,** 129-134.

97. Gilbert, R.J. and Kramer, J.M. (1984) *Bacillus cereus* enterotoxins: present status. *Biochemical Society Transactions* **12**, 198-200.

98. Glatz, B.A., Spira, W.M. and Goepfert, J.M. (1974) Alteration of vascular permeability in rabbits by culture filtrates of *Bacillus cereus* and related species. *Infection and Immunity* **10**, 299-303.

99. Goepfert, J.M. (1974) Monkey-feeding trials in the investigation of the nature of *Bacillus cereus* food poisoning. *Proceedings of the IV International Congress of Food Science and Technology* **38**, 449-452.

100. Granum, P.E. (2007) *Bacillus cereus*. In: Doyle, M.P., Beuchat, L.R. and Montville, T.J., (Eds.) *Food Microbiology: fundamentals and frontiers*, 3rd edn. pp. 445-455. Washington DC: ASM Press.

101. Granum, P.E., Andersson, A., Gayther, C., Giffel, M.te., Larsen, H., Lund, T. and O'Sullivan, K. (1996) Evidence for a further enterotoxin complex produced by *Bacillus cereus. FEMS Microbiology Letters* **141,** 145-149.

102. Granum, P.E., Brynestad, S., O' Sullivan, K. and Nissen, H. (1993) Enterotoxin from *Bacillus cereus*: production and biochemical characterization. *Netherlands Milk and Dairy Journal* **47**, 63-70.

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

104. Granum, P.E., Naestvold, A. and Nordi Gundersby, K. (1995) An outbreak of *Bacillus cereus* food poisoning during the Norwegian ski championships for juniors. *Norsk Veterinaer Tidskrift* **107**, 945-948.

105. Granum, P.E., O' Sullivan, K. and Lund, T. (1999) The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. *FEMS Microbiology Letters* **177,** 225-229.

106. Granum, P.E., Tomas, J.M. and Alouf, J.E. (1995) A survey of bacterial toxins involved in food poisoning: a suggestion for bacterial food poisoning toxin nomenclature. *International Journal of Food Microbiology* **28**, 129-144.

107. Grein, T. (2001) Outbreak of *Bacillus cereus* food poisoning. Department of Public Health, Ireland.

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

109. Guinebretiere, M.-H., Fagerlund, A., Granum, P.E. and 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.

110. Häggblom, M.M., Apetroaie, C., Andersson, M.A. and Salkinoja-Salonen, M.S. (2002) Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus*, produced under various conditions. *Applied and Environmental Microbiology* **68**, 2479-2483.

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

112. Hao, W.-L. and Lee, Y.-K. (2004) Microflora of the gastro-intestinal tract: a review. In: Spencer, J.F.T. and Ragout de Spencer, A.L., (Eds.) *Public Health Microbiology: Methods and Protocols*, Totowa, NJ: Humana Press Inc.

113. Hardy, S.P., Lund, T. and Granum, P.E. (2001) CytK toxin of *Bacillus cereus* forms pores in planar lipid bilayers and is cytotoxic to intestinal epithelia. *FEMS Microbiology Letters* **197**, 47-51.

114. Hasler, W.L. (2003) Physiology of gastric motility and gastric emptying. In: Yamada, T., (Ed.) *Textbook of Gastroenterology*, Fourth edition edn. pp. 195-219. Philadelphia: Lippincott Williams and Wilkins.

115. Hauge, S. (1955) Food poisoning caused by aerobic spore-forming Bacilli. *Journal of Applied Bacteriology* **18**, 591-595.

116. Heinrichs, J.H., Beecher, D.J., MacMillan, J.D. and Zilinskas, B.A. (1993) Molecular cloning and characterization of the hblA gene encoding the B component of hemolysin BL from *Bacillus cereus*. *Journal of Bacteriology* **175,** 6760-6766.

117. Helgason, E., Caugant, D.A., Olsen, I. and Kolstø, A.B. (2000a) Genetic structure of population of *Bacillus cereus* and *B. thuringiensis* isolates associated with periodontitis and other human infections. *Journal of Clinical Microbiology* **38,** 1615-1622.

118. Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I. and Kolstø, A.B. (2000b) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* – one species on the basis of genetic evidence. *Applied and Environmental Microbiology* **66**, 2627-2630.

119. Holbrook, R. and Anderson, J.M. (1980) An improved selective and diagnostic medium for the isolation and enumeration of *Bacillus cereus* in foods. *Canadian Journal of Microbiology* **26**, 753-759.

120. Hornstra, L.M., de Vries, Y.P., de Vos, W. and Abee, T. (2006) Characterization of the germinant receptors in *Bacillus cereus* ATCC 14579. *Applied and Environmental Microbiology* **72**, 44-53.

121. Hornstra, L.M., de Vries, Y.P., de Vos, W.M., Abee, T. and Wells-Bennik, M.H.J. (2005) *GerR*, a novel *ger* operon involved in L-alanine- and inosine-initiated germination of *Bacillus cereus* ATCC 14579. *Applied and Environmental Microbiology* **71**, 774-781.

122. Horwood, P.F., Burgess, G.W. and Oakey, H.J. (2004) Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. *FEMS Microbiology Letters* **236,** 319-324.

123. Ingels, F., Deferme, S., Destexhe, E., Oth, M., van den Mooter, G. and Augustijns, P. (2002) Simulated intestinal fluid as transport medium in the Caco-2 cell culture model. *International Journal of Pharmaceutics* **232**, 183-192.

124. Isobe, M., Ishikawa, T., Suwan, S., Agata, N. and Ohta, M. (1995) Synthesis and activity of cereulide, a cyclic dodecadepsipeptide ionophore as emetic toxin from *Bacillus cereus*. *Bioorganic and Medicinal Chemistry Letters* **5,** 2855-2858.

125. Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatrai, V., Bhattarcharyya, A., Reznik, G., Mikhallova, N., Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N., D'Souza, M., Walunas, T., Grechkin, Y., Pusch, G., Haselkorn, R., Fonstein, M., Dusko-Ehrlich, S., Overbeek, R. and Kyrpides, N. (2003) Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature* **423**, 87-91.

126. Jansen, H.A.P.M. and in 't Veld, P.H. (2002) Microbiologisch onderzoek van kant-en-klaar maaltijden in Nederland, 2001 (in Dutch). Report nr. SAZD/01/50/21/K&K, 's-Hertogenbosch, The Netherlands: Keuringsdienst van Waren Zuid.

127. Jaradat, Z.W. and Bhunia, A.K. (2003) Adhesion, invasion, and translocation characteristics of *Listeria monocytogenes* serotypes in Caco-2 cell and mouse models. *Applied and Environmental Microbiology* **69**, 3640-3645.

128. Jobin, M.-P., Clavel, T., Carlin, F. and Schmitt, P. (2002) Acid tolerance response is low-pH and late-stationary growth phase inducible in *Bacillus cereus* TZ415. *International Journal of Food Microbiology* **79**, 65-73.

129. Johnson, A.E., Donkin, A.J., Morgan, K., Lilley, J.M.N.R.J., Page, R.M. and Silburn, R. (1998) Food safety knowledge and practice among elderly people living at home. *Journal of Epidemiology and Community Health* **52**, 745-748.

130. Johnson, K.M. (1984) *Bacillus cereus* foodborne illness - An update. *Journal of Food Protection* **47**, 145-153.

131. Jääskeläinen, E.L., Häggblom, M.M., Andersson, M.A. and Salkinoja Salonen, M.S. (2004) Atmospheric oxygen and other conditions affecting the production of cereulide by *Bacillus cereus* in food. *International Journal of Food Microbiology* **96**, 75-83.

132. Jääskeläinen, E.L., Häggblom, M.H., Andersson, M.A., Vanne, L. and Salkinoja-Salonen, M.S. (2003a) Potential of *Bacillus cereus* for producing an emetic toxin, cereulide, in bakery products: quantitative analysis by chemical and biological methods. *Journal of Food Protection* **66**, 1047-1054.

133. Jääskeläinen, E.L., Teplova, V., Andersson, M.A., Andersson, L.C., Tammela, P., Andersson, M.C., Pirhonen, T.I., Saris, N.-E.L., Vuorela, P. and Salkinoja-Salonen, M.S. (2003b) In vitro assay for human toxicity of cereulide, the emetic mitochondrial toxin produced by food poisoning *Bacillus cereus*. *Toxicology In Vitro* **17**, 737-744.

134. Kalser, M.H., Arteaga, I., Yawn, E., Hoffert, W., Frazier, D., Cohen, R. and Mayoral, L. (1965) Microflora of the small intestine. *American Journal of Digestive Disease* **10**, 839-843.

135. Kotiranta, A.K., Ito, H., Haapasalo, M.P.P. and Lounatmaa, K. (1999) Radiation sensitivity of *Bacillus cereus* with and without a crystalline surface protein layer. *FEMS Microbiology Letters* **179,** 275-280.

136. Kramer, J.M. and Gilbert, R.J. (1989) *Bacillus cereus* and other *Bacillus* species. In: Doyle, M.P. (Ed.) *Foodborne bacterial pathogens*, pp. 21-70. New York: Marcel Dekker Inc.

137. Langeveld, L.P.M., Spronsen, W.A.v., Beresteijn, E.C.H.v. and Notermans, S.H.W. (1996) Consumption by healthy adults of pasteurized milk with high concentration of *Bacillus cereus:* a double-blind study. *Journal of Food Protection* **59,** 723-726.

138. Larsen, H.D. and Jorgensen, K. (1997) The occurrence of *Bacillus cereus* in Danish pasteurized milk. *International Journal of Food Microbiology* **34,** 179-186.

139. Larsen, H.D. and Jorgensen, K. (1999) Growth of *Bacillus cereus* in pasteurized milk products. *International Journal of Food Microbiology* **46,** 173-176.

140. Lechner, S., Mayr, R., Francis, K.P., Pruss, B.M., Kaplan, T., Wiessner Gunkel, E., Stewart, G.S. and 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.

141. Lee, I.S., Slonczewski, J.L. and Foster, J.W. (1994) A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella typhimurium*. *Journal of Bacteriology* **176**, 1422-1426.

142. Lereclus, D., Agaisse, H., Grandvalet, C., Salamitou, S. and Gominet, M. (2000) Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis*. *International Journal of Medical Microbiology* **290,** 295-299.

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

144. Lin, H.C., Prather, C., Fisher, R.S., Meyer, J.H., Summers, R.W., Pimentel, M., McCallum, R.W., Akkermans, L.M.A. and Loening-Baucke, V. (2005) Measurement of gastro-intestinal transit. *Digestive Diseases and Sciences* **50**, 989-1004.

145. Lubenau, C. (1906) *Bacillus peptonificans* als Erreger einer Gastroenteritis-Epidemie. *Zentralblatt für Bakteriologie, I Originale* **40**, 433-437.

146. Lund, T., DeBuyser, M.-L. and Granum, P.E. (2000) A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Molecular Microbiology* **38**, 254-261.

147. Lund, T. and Granum, P.E. (1996) Characterization of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiology Letters* **141,** 151-156.

148. Lund, T. and Granum, P.E. (1997) Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. *Microbiology* **143,** 3329-3336.

149. Lund, T. and Granum, P.E. (1999) The 105-kda protein component of *Bacillus cereus* non-haemolytic enterotoxin (nhe) is a metalloprotease with gelatinolytic and collagenolytic activity. *FEMS Microbiology Letters* **178.** 355-361.

150. Mahler, H., Pasi, A., Kramer, J.M., Schulte, P., Scoging, A.C., Bar, W. and Krahenbuhl, S. (1997) Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. *New England Journal of Medicine* **336,** 1142-1148.

151. Manzano, M., Cocolin, L., Cantoni, C. and Comi, G. (2003) *Bacillus cereus, Bacillus thuringiensis* and *Bacillus mycoides* differentiation using a PCR-RE technique. *International Journal of Food Microbiology* **81**, 349-354.

152. McClane, B.A. (1997) *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R. and Montville, T.J., (Eds.) *Food Microbiology: fundamentals and frontiers.* 1st edn. pp. 305-326. Washington DC: ASM Press.

153. McMinn, L.H., Hodges, G.M. and Carr, K.E. (1996) Gastro-intestinal uptake and translocation of microparticles in the streptozotocin-diabetic rat. *Journal of Anatomy* **189**, 553-559.

154. Meer, R.R., Baker, J., Bodyfelt, F.W. and Griffiths, M.W. (1991) Psychrotrophic *Bacillus* spp. in fluid milk products: a review. *Journal of Food Protection* **54**, 969-979.

155. Melling, J., Capel, B.J., Turnbull, P.C.B. and Gilbert, R.J. (1976) Identification of a novel enterotoxigenic activity associated with *Bacillus cereus. Journal of Clinical Pathology* **29**, 938-940.

156. Mikkola, R., Saris, N.E.L., Grigoriev, P.A., Andersson, M.A. and Salkinoja Salonen, M.S. (1999) Ionophoretic properties and mitochondrial effects of cereulide - the emetic toxin of *B. cereus*. *European Journal of Biochemistry* **263,** 112-117.

157. Minnaard, J., Humen, M. and Perez, P.F. (2001) Effect of *Bacillus cereus* exocellular factors on human intestinal epithelial cells. *Journal of Food Protection* **64**, 1535-1541.

158. Minnaard, J., Lievin-Le Moal, V., Coconnier, M.-H., Servin, A.L. and Pérez, P.F. (2004) Disassembly of F-actin cytoskeleton after interaction of *Bacillus cereus* with fully differentiated human intestinal Caco-2 cells. *Infection and Immunity* **72**, 3106-3112.

159. Montville, T.J. (1997) Principles which influence microbial growth, survival, and death in foods. In: Doyle, M.P., Beuchat, L.R. and Montville, T.J., (Eds.) *Food Microbiology: fundamentals and frontiers*, 1st edn. pp. 13-29. Washington DC: ASM Press.

160. Moravek, M. , Dietrich, R., Buerk, C., Brousolle, V., Guinebretiere, M.-H., Granum, P.E., Nguyen-the, C. and Märtlbauer, E. (2006) Determination of the toxic potential of *Bacillus cereus* isolates by quantitative analyses. *FEMS Microbiology Letters* **257**, 293-298.

161. Mossel, D.A.A., Koopman, M.J. and Jongerius, E. (1967) Enumeration of *Bacillus cereus* in foods. *Applied Microbiology* **15**, 650-653.

162. Nakamura, L.K. (1998) *Bacillus pseudomycoides* sp. nov. *International Journal of Systematic Bacteriology* **48**, 1031-1035.

163. Notermans, S.H.W., Dufrenne, J., Teunis, P., Beumer, R., Giffel, M.t. and Peeters Weem, P. (1997) A risk assessment study of *Bacillus cereus* present in pasteurized milk. *Food Microbiology* **14,** 143-151.

164. O'Driscoll, B., Gahan, C.G.M. and Hill, C. (1996) Adaptive acid tolerance response in Listeria monocytogenes: isolation of an acid-tolerant mutant which demonstrates increased virulence. *Applied and Environmental Microbiology* **62**, 1693-1698.

165. Okstad, O.A., Gominet, M., Purnelle, B., Rose, M., Lereclus, D. and Kolsto, A.B. (1999) Sequence analysis of three *Bacillus cereus* loci carrying plcr-regulated genes encoding degradative enzymes and enterotoxin. *Microbiology* **145,** 3129-3138.

166. Oomen, A.G. , van Twillert, K., Hofhuis, M.F.A., Rompelberg, C.J.M. and Versantvoort, C.H.M. (2003) Development and suitability of in vitro digestion models in assessing bioaccessibility of lead from toy matrices. RIVM report 320102 001, Bilthoven, the Netherlands: National Institute for Public Health and the Environment (RIVM).

167. Ouib, O., Clavel, T. and Schmitt, P. (2006) The production of *Bacillus cereus* enterotoxins is influenced by carbohydrate and growth rate. *Current Microbiology* **53**, 222-226.

168. Oxford, A. (2005) Thesis. Studies on the survival of *Bacillus cereus* enterotoxin HBL in artificial stomach and intestinal juices. Caledonian University, Glasgow, UK:

169. Pandiripally, V.K., Westbrook, D.G., Sunki, G.R. and Bhunia, A.K. (1999) Surface protein p104 is involved in adhesion of *Listeria monocytogenes* to human intestinal cell line, Caco-2. *Journal of Medical Microbiology* **48**, 117-124.

170. Pielaat, A., Fricker, M., Nauta, M.J. and van Leusden, F.M. (2005) Biodiversity in *Bacillus cereus*. RIVM report 250912 004, Bilthoven, The Netherlands: National Institute for Public Health and the Environment (RIVM).

171. Pielaat, A., Wijnands, L.M., Takumi, K., Nauta, M.J. and Van Leusden, F.M. (2005) The fate of *Bacillus cereus* in the gastro-intestinal tract . RIVM Report 250912 005, Bilthoven, The Netherlands: National Institute fro Public Health and the Environment (RIVM).

172. Pinto, M., Robine-Leon, S., Appay, M.-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen K., Fogh, J. and Zweibaum, A. (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biology of the Cell* **47**, 323-330.

173. Powell, D.W. (1987) Enterogenic diarrhea: mechanisms and prospects for therapy. In: Dorner, F. and Drews, J., (Eds.) *Pharmacology of bacterial toxins*, pp. 173-183. Oxford: Pergamon.

174. Preston, R.A. and Douthit, H.A. (1988) Functional relationships between L- and D-alanine, inosine and NH_4Cl during germination of spores of *Bacillus cereus* T. *Journal of General Microbiology* **134**, 3001-3010.

175. Prüss, B.M., Francis, K.P., Von Stetten, F. and Scherer, S. (1999) Correlation of 16S ribosomal DNA signature sequences with temperature-dependent growth rates of mesophilic and psychrotolerant strains of the *Bacillus cereus* group. *Journal of Bacteriology* **181,** 2624-2630.

176. Rajkovic, A., Uyttendaele, M. and Debevere, J. (2007) Computer aided boar semen motility analysis for cereulide detection in different food matrices. *International Journal of Food Microbiology* **114**, 92-99.

177. Rajkovic, A., Uyttendaele, M., Deley, W., Van Soom, A., Rijsselaere, T. and Debevere, J. (2006) Dynamics of boar semen motility inhibition as a semi-quantitative measurement of *Bacillus cereus* emetic toxin (Cereulide). *Journal of Microbiological Methods* **65**, 525-534.

178. Ripabelli, G., McLauchlin, J., Mithani, V. and Threlfall, E.J. (2000) Epidemiological typing of *Bacillus cereus* by amplified fragment length polymorphism. *Letters in Applied Microbiology* **30,** 358-363.

179. Rössler, D., Ludwig, W., Schleifer, K.H., Lin, C., McGill, T.J., Wisotzkey, J.D., Jurtshuk Jr, P. and Fox, G.E. (1991) Phylogenetic diversity in the genus *Bacillus* as seen by 16S rRNA sequencing studies. *Systematic and Applied Microbiology* **14**, 266-269.

180. Rotard, W., Christmann, W., Knoth, W. and Mailahn, W. (1995) Bestimmung der resorptionsverfügbaren PCDD/PCDF aus Kieselrot. *Zeitschrift für Umweltchemie und Ökotoxikologie* **7**, 3-9.

181. Rousset, E. and Dubreuil, J.D. (2000) Bacterial enterotoxin receptors. *Veterinary Research* **31,** 413-415.

182. Rowan, N.J., Deans, K., Anderson, J.G., Gemmell, C.G., Hunter, I.S. and Chaithong, T. (2001) Putative virulence factor expression by clinical and food isolates of *Bacillus* spp. after growth in reconstituted infant milk formulae. *Applied and Environmental Microbiology* **67**, 3873-3881.

183. Russell, T.L., Berardi, R.R., Barnett, J.L., Dermentzoglou, L.C., Jarvenpaa, K.M., Schmaltz, S.P. and Dressman, J.B. (1993) Upper gastro-intestinal ph in seventy-nine healthy, elderly, north American men and women. *Pharmaceutical Research* **10,** 187-196.

184. Rusul, G. and Yaacob, N.H. (1995) Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. *International Journal of Food Microbiology* **25,** 131-139.

185. Ryan, P.A., Macmillan, J.D. and Zilinskas, B.A. (1997) Molecular cloning and characterization of the genes encoding the L(1) and L(2) components of hemolysin BL from *Bacillus cereus*. *Journal of Bacteriology* **179,** 2551-2556.

186. Salamitou, S., Ramisse, F., Brehélin, M., Bourguet, D., Gilois, N., Gominet, M., Hernandez, E. and Lereclus, D. (2000) The plcR regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. *Microbiology* **146**, 2825-2832.

187. Schlegel, H.G. (1997) *General Microbiology*, 7th edn. Cambridge, 2nd ed.: Cambridge University Press.

188. Seitz, M. (1913) Pathogener *Bacillus subtilis*. *Zentralblatt für Bakteriologie, I Originale* **70**, 113-114.

189. Setlow, P. (2003) Spore germination. *Current Opinions in Microbiology* **6**, 550-556.

190. Shinagawa, K. (1990a) Analytical methods for *Bacillus cereus* and other *Bacillus* species. *International Journal of Food Microbiology* **10,** 125-141.

191. Shinagawa, K. (1990b) Purification and characterization of *Bacillus cereus* enterotoxin and its application to diagnosis. In: Pohland, A.E.e.al., (Ed.) *Microbial Toxins in Foods and Feeds*, pp. 181-193. New York: Plenum Press.

192. Shinagawa, K., Sato, K., Konuma, H., Matsusaka, N. and Sugii, S. (1991a) Fluid accumulation in mouse ligated intestine inoculated with the vascular permeability factor produced by *Bacillus cereus*. *Journal of Veterinary Medical Science* **53,** 167-171.

193. Shinagawa, K., Ueno, S., Konuma, H., Matsusaka, N. and Sugii, S. (1991b) Purification and characterization of the vascular permeability factor produced by *Bacillus cereus*. *Journal of Veterinary Medical Science* **53,** 281-286.

194. Shinagawa, K., Ueno, S., Matsusaka, N. and Sugii, S. (1991c) In vitro stability in biological activity and antigenicity of the vascular permeability factor produced by *Bacillus cereus*. *Journal of Veterinary Medical Science* **53,** 317-319.

195. Simone, E., Goossen, M., Notermans, S.H.W. and Borgdorff, M.W. (1997) Investigations of foodborne diseases by food inspection services in The Netherlands. *Journal of Food Protection* **60**, 442-446.

196. Slamti, L. and Lereclus, D. (2002) A cell-cell signalling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. *EMBO Journal* **21**, 4550-4559.

197. Slamti, L. and Lereclus, D. (2005) Specificity and polymorphism of the PlcR-PapR quorum-sensing system in the *Bacillus cereus* group. *Journal of Bacteriology* **187,** 1182-1187.

198. Slamti, L., Perchat, S., Gominet, M., Vilas Boas, G., Fouet, A., Mock, M., Sanchis, V., Chaufaux, J., Gohar, M. and Lereclus, D. (2004) Distinct mutations in PlcR explain why some strains of the *Bacillus cereus* group are nonhemolytic. *Journal of Bacteriology* **186,** 3531-3538.

199. Smith, D.P., Berrang, M.E., Feldner, P.W., Phillips, R.W. and Meinersmann, R.J. (2004) Detection of *Bacillus cereus* on selected retail chicken products. *Journal of Food Protection* **67**, 1770-1773.

200. Spira, W.M. and Goepfert, J.M. (1972) *Bacillus cereus*-induced fluid accumulation in a rabbit ileal loop. *Applied Microbiology* **24**, 341-348.

201. Steen, M.K. , Bruno-Murtha, L.A., Chaux, G., Lazar, H., Bernard, S. and Sulis, C. (1992) *Bacillus cereus* endocarditis: report of a case and review. *Clinical Infectious Diseases* **14**, 945-946.

202. Sutherland, A.D. and Limond, A.M. (1993) Influence of pH and sugars on the growth and production of diarrhoeagenic toxin by *Bacillus cereus*. *Journal of Dairy Research* **60,** 575-580.

203. Takumi, K., Jonge, R.d. and Havelaar, A. (2000) Modelling inactivation of Escherichia coli by low pH: application to passage through the stomach of young and elderly people. *Journal of Applied Microbiology* **89**, 936-943.

204. Te Giffel, M.C., Beumer, R.R., Bonestroo, M.H. and Rombouts, F.M. (1996) Incidence and characterization of *Bacillus cereus* in two dairy processing plants. *Netherlands Milk and Dairy Journal* **50,** 479-492.

205. Te Giffel, M.C., Beumer, R.R., Granum, P.E. and Rombouts, F.M. (1997a) Isolation and characterization of *Bacillus cereus* from pasteurised milk in household refrigerators in The Netherlands. *International Journal of Food Microbiology* **34,** 307-318.

206. Te Giffel, M.C., Beumer, R.R., Klijn, N., Wagendorp, A. and Rombouts, F.M. (1997b) Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on variable regions of 16S rRNA. *FEMS Microbiology Letters* **146,** 47-51.

207. Te Giffel, M.C., Beumer, R.R., Slaghuis, B.A. and Rombouts, F.M. (1996) Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands. In: Anonymous *Symposium on Bacteriological Quality of Raw Milk, Proceedings*, pp. 40-45. International Dairy Federation.

208. Teplova, V.V., Mikkola, R., Tonshin, A.A., Saris, N.E. and Salkinoja-Salonen, M.S. (2006) The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at physiological plasma concentrations. *Toxicology and Applied Pharmacology* **210**, 39-46.

209. Teunis, P.F.M., van der Heijden, O.G., van der Giessen, J.W.B. and Havelaar, A.H. (1996) The dose response relation in human volunteers for gastro-intestinal pathogens. RIVM report 284550 002, Bilthoven, The Netherlands: National Institute for Public Health and the Environment (RIVM).

210. Thadepalli, H., Lou, M.A., Bach, V.T., Matsui, T. and Mandal, A.K. (1979) Microflora of the human small intestine. *American Journal of Surgery* **138**, 845-850.

211. Toh, M., Moffitt, M.C., Henrichsen, L., Raftery, M., Barrow, K., Cox, J.M., Marquis, C.P. and Neilan, B.A. (2004) Cereulide, the emetic toxin of *Bacillus cereus*, is putatively a product of nonribosomal peptide synthesis. *Journal of Applied Microbiology* **97**, 992-1000.

212. Tuomola, E.M. and Ouwehand, A.C.S.S.J. (1999) The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunology and Medical Microbiology* **26**, 137-142.

213. Turnbull, P.C., Kramer, J.M., Jorgensen, K., Gilbert, R.J. and Melling, J. (1979) Properties and production characteristics of vomiting, diarrheal, and necrotizing toxins of *Bacillus cereus*. *American Journal of Clinical Nutrition* **32,** 219-228.

214. Ueda, S. and Kuwabara, Y. (1993) An ecological study of *Bacillus cereus* in rice crop processing. *Journal of Antibacterial and Antifungal Agents* **21**, 499-502.

215. Van Kreijl, C.F., Knaap, A.G.A.C. and Van Raaij, J.M.A. (2004) Our food, our health - Healthy diet and safe food in the Netherlands. RIVM report 270555009, Bilthoven, the Netherlands: National Institute for Public Health and the Environment (RIVM).

216. Van Netten, P.v.M.A.v.d., Hoensel, P.v., Mossel, D.A. and Perales, I. (1990) Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *Journal of Applied Bacteriology* **69,** 73-79.

217. von Stetten, F., Francis, K.P., Lechner, S., Neuhaus, K. and Scherer, S. (1998) Rapid discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group by PCR targeting of 16S rDNA. *Journal of Microbiological Methods* **34,** 99-106.

218. Waterman, S.R. and Small, P.L.C. (1998) Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. *Applied and Environmental Microbiology* **64**, 3882-3886.

219. Wegscheider, M. (2004) PhD-Thesis. Untersuchungen zu *Bacillus cereus* Enterotoxin-Komplexen auf zellulärer Ebene. Ludwig Maximilians Universität, Munich, Germany.

220. Weisbrodt, N.W. (2001a) Gastric emptying. In: Johnson, L.R. and Gerwin, T.A., (Eds.) *Gastro-intestinal physiology.,* 6th edn. pp. 37-46. St. Louis: Mosby, Inc.

221. Weisbrodt, N.W. (2001b) Motility of the small intestine. In: Johnson, L.R. and Weisbrodt, N.W., (Eds.) *Gastro-intestinal Physiology*, 6th edn. St. Louis: Mosby, Inc.

222. Wilcks, A., Hanse, B.M., Hendriksen, N.B. and Licht, T.R. (2005) Fate and effect of ingested *Bacillus cereus* spores and vegetative cells in the intestinal tract of human-flora-associated rats. *FEMS Immunology and Medical Microbiology* **46,** 70-77.

223. Zaid, A., Hughes, H.G., Porceddu, E. and Nicholas, F. (1999) Glossary of biotechnology and genetic engineering. FAO Research and Technology Paper 7, Rome: FAO.

224. Zwart, L.L.d., Rompelberg, C.J.M., Sips, A.J.A.M., Welink, J. and Engelen, J.G.M.v. (1999) RIVM report 623860 010, Bilthoven, the Netherlands: National Institute of Public Health and the Environment (RIVM).

Samenvatting

Bacillus cereus is een sporenvormende bacterie die voorkomt in het milieu en als gevolg daarvan veelvuldig wordt aangetroffen in en op voedsel. Daar kan de bacterie zorgen voor bederf of leiden tot ziekte bij de mens als gevolg van de consumptie van voedsel besmet met deze bacterie. Voedselgerelateerde ziekte door *B. cereus* kan bestaan uit twee verschillende aandoeningen, namelijk een diarreesyndroom geassocieerd met algehele malaise en diarree, of een braaksyndroom geassocieerd met misselijkheid en braken. Beide aandoeningen worden veroorzaakt door toxinen. Het diarreesyndroom wordt veroorzaakt door toxinen die gevormd worden tijdens groei van *B. cereus* in de dunne darm, zogenaamde enterotoxinen. Een toxine, cereulide, dat tijdens groei van *B. cereus* in voedsel wordt geproduceerd vóór consumptie veroorzaakt het braaksyndroom.

Dit proefschrift behandelt, binnen de kaders van kwantitatieve risicoschatting, een schatting van de blootstelling aan *B. cereus* en een gevarenkarakterisering voor het diarreesyndroom van *B. cereus*. Schatting van de blootstelling is gebaseerd op een inventarisatie van het vóórkomen van potentieel pathogene *B. cereus* stammen in voedsel dat in Nederland gekocht kan worden. De karakterisering van het gevaar op het diarreesyndroom is onderzocht door processen die zich in het maagdarmkanaal afspelen te simuleren.

Voor een inventarisatie van het vóórkomen van pathogene *B. cereus* stammen is in samenwerking met de Voedsel en Waren Autoriteit een groot aantal stammen verzameld en onderzocht op aanwezigheid van genen die coderen voor enterotoxinen, op de mogelijkheid tot productie van cereulide, en op het groei-temperatuur-profiel van de stammen. De resultaten staan beschreven in hoofdstuk 2. Alle stammen blijken wel één of meer virulentie-eigenschappen te bezitten: de mogelijkheid om één of meer toxinen te produceren. Daarnaast blijkt het merendeel van de stammen (89%) een mesofiele signatuur te bezitten, i.e. stammen die niet in staat zijn te groeien bij temperaturen beneden 10ºC, maar die uitstekend groeien bij 37ºC (darmcondities) en hoger. Bovendien blijkt in voedsel dat gekoeld bewaard moet worden, zoals zuivel, vaker psychrotrofe stammen voor te komen, i.e. stammen die kunnen groeien bij temperaturen beneden 10ºC, maar die matig groeien bij 37ºC.

In hoofdstuk 3 is beschreven dat niet alle stammen van *B. cereus* even goed kunnen groeien in omstandigheden die de condities in de dunne

darm simuleren. Het is gebleken dat psychrotrofe stammen minder goed in staat zijn te groeien onder omstandigheden die voorkomen in de dunne darm. Temperatuur blijkt niet de enige factor te zijn die voor slechtere groei bij 37ºC zorgt, ook stoffen die uitgescheiden worden voor de vertering van voedsel, gal en pancreassap, verhinderen een goede groei. Mesofiele stammen lijken veel minder gevoelig te zijn voor omstandigheden die voorkomen in de dunne darm.

Over de processen die leiden tot het diarreesyndroom was tot voor kort niet veel bekend. Algemeen werd aangenomen dat het werd eroorzaakt door de consumptie van voedsel besmet met sporen, een overlevingsvorm van het micro-organisme. Van vegetatieve cellen van het micro-organisme werd algemeen aangenomen dat die de maagpassage niet zouden overleven als gevolg van de daar aanwezige lage pH, en daarom geen rol zouden spelen bij het ziekmakende proces. In hoofdstuk 4, echter, wordt aangetoond dat vegetatieve cellen wel degelijk een rol van betekenis kunnen spelen. In rusttoestand is de pH van de maag weliswaar dusdanig laag dat vegetatieve cellen dat niet zouden overleven, maar tijdens de consumptie van voedsel loopt de pH zodanig op dat vegetatieve cellen niet worden aangetast en naar de dunne darm getransporteerd kunnen worden.

Sporen moeten, aangekomen in de dunne darm, ontkiemen om uit te kunnen groeien en enterotoxinen te kunnen produceren. Om tot ontkieming te komen, moeten receptoren worden geactiveerd door stoffen als L-alanine, inosine en andere. Het is echter gebleken dat gedifferentieerde Caco-2 cellen, humane carcinoma cellen uit de dikke darm die de eigenschappen van epitheelcellen van de dunne darm bezitten, eveneens in staat zijn (een) stof(fen) te produceren die *B. cereus* sporen kan(kunnen) aanzetten tot ontkieming. Tussen de verschillende *B. cereus* stammen bestaan wel verschillen: waar sommige zeer snel tot ontkieming worden aangezet, worden andere niet geactiveerd om te ontkiemen. Dit is beschreven in hoofdstuk 5.

Diarree wordt veroorzaakt door enterotoxinen die geproduceerd worden in de dunne darm. Twee van deze enterotoxinen blijken zeer gevoelig voor proteolytische activiteit zoals die door de pancreas wordt uitgescheiden in het darmlumen. Om toch het epitheel van de dunne darm te kunnen aantasten zouden deze enterotoxinen dus zo dicht mogelijk bij het darmepitheel gevormd moeten worden. Dit kan gerealiseerd worden door hechting van *B. cereus* cellen aan het epitheel. *B. cereus* cellen, sporen en vegetatieve cellen, blijken tot hechting in staat, met een efficiëntie van ongeveer één procent. En daarmee is een belangrijke

voorwaarde voor het optreden van epitheelschade vervuld. Daarnaast lijken ook alleen de cellen die gehecht zijn aan het epitheel van belang voor het optreden van ziektesymptomen, aangezien in een modelsysteem een duidelijke correlatie werd gevonden tussen het aantal *B. cereus* cellen, dat gehecht was aan gedifferentieerde Caco-2 cellen, en het optreden van cytotoxische effecten.

De resultaten zoals beschreven in dit proefschrift dragen bij aan kennis over de factoren en processen in de dunne darm die een rol spelen bij het optreden van het diarreesyndroom als gevolg van het eten van voedsel besmet met *B. cereus.* Tevens wordt op basis van de gevonden resultaten over vóórkomen en gedrag in gesimuleerde maag-darm omstandigheden een schatting gegeven van het aantal ziektegevallen voor het diarreesyndroom, namelijk 17.000 tot 310.000 per jaar. Bij deze berekening is rekening gehouden met het in Nederland geldende tolerantieniveau.

Op basis van gegevens over vóórkomen van *B. cereus* stammen, zoals gepresenteerd in hoofdstuk 2, en gegevens uit de literatuur is het aantal gevallen van het braaksyndroom geschat op 630.000 tot 3 miljoen per jaar. Ook bij deze berekening is rekening gehouden met het Nederlandse tolerantie niveau. Daarnaast worden processen die kunnen bijdragen tot het vóórkomen en factoren die kunnen bijdragen tot het voorkómen van het braaksyndroom bediscussieerd. Hierbij wordt aangegeven welke kennishiaten nog ingevuld moeten worden om tot een betere risicoschatting te komen.

List of Publications

Wijnands, L.M., Zwietering, M.H., Van Leusden, F.M., Abee, T.
Caco-2 cell surface-associated growth of enterotoxic *Bacillus cereus* is a prerequisite for initiation of cytotoxic effects in simulated intestinal fluid.
To be published.

Wijnands, L.M., Pielaat, A., Dufrenne, J.B., Zwietering, M.H., Van Leusden, F.M.
Modelling the number of viable vegetative cells of *Bacillus cereus* passing through the stomach.
Submitted for publication.

Wijnands, L.M., Dufrenne, J.B., Van Leusden, F.M., Abee, T. (2007).
Germination of *Bacillus cereus* spores is induced by germinants from differentiated Caco-2 cells, a human cell line mimicking the epithelial cells of the small intestine.
Applied and Environmental Microbiology, **73** (15), 5052-5054

Wijnands, L.M., Dufrenne, J.B., Zwietering, M.H., Van Leusden, F.M. (2006).
Spores from mesophilic *Bacillus cereus* strains germinate better and grow faster in simulated gastro-intestinal conditions than spores from psychrotrophic strains.
International Journal of Food Microbiology, **112** (2), 120-128.

Wijnands, L.M., Dufrenne, J.B., Rombouts, F.M., In 't Veld, P.H., Van Leusden, F.M. (2006).
Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in the Netherlands.
Journal of Food Protection, **69** (11), 2587-2594.

Apetroaie, C., Andersson, M.A., Spröer, C., Tsitko, I., Shaheen, R., Jääskeläinen, E.L., Wijnands, L.M., Heikkilä, R., Salkinoja-Salonen, M.S. (2005).
Cereulide-producing strains of *Bacillus cereus* show diversity.
Archives of Microbiology, **184** (3), 141-151.

Andersson, M.A., Jääskeläinen, E.L., Shaheen, R., Pirhonen, T., Wijnands, L.M., Salkinoja-Salonen, M.S. (2004).
Sperm bioassay for rapid detection of cereulide-producing *Bacillus cereus* in food and related environments.
International Journal of Food Microbiology, **94** (2), 175-183.

Wijnands, L.M., Deisz, W.D.C., Van Leusden, F.M. (2000).
Marker antigens to assess exposure to molds and their allergens. II. *Alternaria alternate*.
Allergy: European Journal of Allergy and Clinical Immunology, **55** (9), 856-864.

Wijnands, L.M., Deisz, W.D.C., Van Leusden, F.M. (2000).
Marker antigens to assess exposure to molds and their allergens. I. *Aspergillus fumigatus*.
Allergy: European Journal of Allergy and Clinical Immunology, **55** (9), 850-855.

Douwes, J., Van Der Sluis, B., Doekes, G., Van Leusden, F., Wijnands, L., Van Strien, R., Verhoeff, A., Brunekreef, B. (1999).
Fungal extracellular polysaccharides in house dust as a marker for exposure to fungi: Relations with culturable fungi, reported home dampness, and respiratory symptoms.
Journal of Allergy and Clinical Immunology, **103** (3 II), 494-500.

Wijnands, L.M., Deisz, W.D.C., Van Leusden, F.M. (1999).
Immunoblotting technique for the detection of allergens of *Aspergillus fumigatus*: Influence of Nonidet P-40 on the sensitivity.
Journal of Investigational Allergology and Clinical Immunology, **9** (3), pp. 195-196.

Haynes, K.A., Tuinstra, P., Hughes, T.A., Wijnands, L.M., Rogers, T.R., Allen, A.K. (1996).
Purification and characterization of a 93 kDa *Aspergillus fumigatus* antigen with diagnostic potential.
Journal of Medical and Veterinary Mycology, **34** (6), 421-426.

Wijnands, L.M., Van Leusden, F.M., Puyk, R.J.T., Hofstee, M.P.M., Engel, H.W.B. (1994).
Pitfalls in immunoblot detection of *Aspergillus* antigens associated with invasive infection.
Journal of Clinical Microbiology, **32** (9), 2339-2340.

Notermans, S.H.W., Dufrenne, J.B., Wijnands, L.M., Engel, H.W.B. (1988).
Human serum antibodies to extracellular polysaccharides (EPS) of moulds.
Journal of Medical and Veterinary Mycology, **26** (1), 41-48.

Nawoord

Een promotie-onderzoek plus bijbehorend proefschrift doe je niet alleen. Veel mensen zijn daarbij van belang geweest. Allereerst drie Fransen. De eerste is Frans van Leusden: vanaf het begin van mijn RIV(M) tijd heb ik met je samengewerkt aan uiteenlopende onderzoeken. Je hebt heel wat inspanningen verricht om dit onderzoek mogelijk te maken. De tweede is Frans Rombouts, jij kon je wel vinden in het onderzoeksplan voor *Bacillus cereus* en was bereid als mijn promotor op te treden. Dat je uiteindelijk het stokje hebt doorgegeven aan Marcel Zwietering heeft niets met verminderde interesse te maken, integendeel. Je ging gewoon van je pensioen genieten. De derde is Frans van Knapen, voormalig hoofd van het laboratorium voor Parasitologie en Mycologie. Door jouw aanmoediging nog een jaartje bij te studeren heeft uiteindelijk dit proefschrift het levenslicht gezien. Alle drie heel erg bedankt.
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although you speak Dutch as well, I'll address you in English. Laymen are indispensable for finalizing a thesis. Your help is much appreciated. Cheers.
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Curriculum vitae

Lucas Maria Wijnands werd op 15 september 1956 geboren te Nijmegen. Hij behaalde in 1975 zijn gymnasium-β diploma aan het Sint Canisius College te Nijmegen. Hij startte in 1977 zijn HBO opleiding en behaalde in 1981 het HBO-B diploma, richting biochemie aan de HMLS te Oss. Zijn eerste en tot nog toe enige werkgever werd het toenmalige Rijks Instituut voor Volksgezondheid, RIV, hetgeen nu door de wereld gaat als het Rijksinstituut voor Volksgezondheid en Milieu, het RIVM. In eerste instantie deed hij onderzoek naar het opzetten van een chemische multimethode voor de detectie van antibiotica in vlees, met de nadruk op sulfonamiden. Bij een reorganisatie, waarbij het Laboratorium voor Parasitologie en Mycologie (LPM) het daglicht zag, werd het werkveld verlegd naar onderzoek aan opportunistische systemische schimmel-infecties, voornamelijk aspergillose, cryptococcose en candidose. Na een aantal jaren werd het werkveld verlegd naar de opsporing van schimmel-allergenen in het binnenhuismilieu. Rond die tijd werd ook het HBO-B diploma opgewaardeerd tot een HLO diploma, eveneens richting biochemie. Helaas werd het allergenen onderzoek abrupt beëindigd, ondanks veelbelovende resultaten. De aandacht werd verlegd naar de mogelijke gezondheidseffecten van mycotoxinen in voedsel. Ondertussen werd met een nieuwe reorganisatie een deel van LPM samengevoegd met LWL (Laboratorium voor Water en Levensmiddelenmicrobiologie) tot het Microbiologisch Laboratorium voor Gezondheidsbescherming (MGB), nu bekend als Laboratorium voor Zoonosen en Omgevingsmicrobiologie (LZO). Vanwege prioritering van de onderzoeks-capaciteit binnen het nieuw opgerichte laboratorium werd een einde gemaakt aan het onderzoek naar gezondheidseffecten van mycotoxinen. Lucas werd gevraagd zich te richten op onderzoek naar kwantitatieve aspecten van blootstelling en gevarenkarakterisering van *Bacillus cereus* in relatie tot voedsel gerelateerd aandoeningen. Hij stelde direct de tegenvraag of dit onderzoek dan zou kunnen dienen voor promotie onderzoek. Met medewerking van Frans Rombouts werd dit een feit. Waartoe dit alles heeft geleid staat beschreven in dit proefschrift. Tijdens dit onderzoek werd ook deelgenomen aan een Europees project gericht op de mogelijke gevaren van besmetting van voedsel met *B. cereus*.

The research described in this thesis was conducted at the Microbiological Laboratory for Health Protection of the National Institute for Public Health and the Environment within the framework of project V/250912, "Quantitative research of *Bacillus cereus* within the scope of hazard characterization and exposure assessment" by order and on the account of the Food and Consumer Product Safety Authority.

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