



Report for the Superior Health Council Belgium

## RISK PROFILE OF THE *BACILLUS CEREUS* GROUP IMPLICATED IN FOOD POISONING

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"CSS-HGR 8316 *Public health implications of Bacillus cereus in food*"

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# 1. Introduction

*Bacillus cereus* belongs to a group of closely related aerobic sporeforming species, which is being referred to as the *B. cereus* group. Although *B. cereus* is a well-known cause of food-borne illness it is not commonly reported because of its usually mild symptoms. It can cause two types of food poisoning, known as the emetic and the diarrhoeal types. For the emetic type, a heat-stable emetic toxin named cereulide, preformed in the food, is responsible for the symptoms similar to those of *Staphylococcus aureus* intoxication, and is characterized by a short incubation period (Granum and Lund, 1997). This type is probably the most dangerous since it has also been associated with life-threatening acute conditions of fulminating liver failure and rhabdomyolysis (Mahler *et al.* 1997). Heat-unstable enterotoxins, produced in the gut by vegetative cells cause the diarrhoeal syndrome, with symptoms resembling those of the *Clostridium perfringens* food poisoning, with a 6 to 24h incubation period. The emetic type is frequently associated with the consumption of food rich in carbohydrates such as rice and pasta (Mahler *et al.* 1997; Raevuori *et al.* 1976), whereas the diarrhoeal type is often associated with cooked meat and meat products (Debuono *et al.* 1988; Gianella and Brasile, 1979; Luby *et al.* 1993). It is generally admitted that contamination levels above  $10^5$  CFU g<sup>-1</sup> are required to provoke illness.

*B. cereus* is a widespread Gram-positive spore-forming bacterium, commonly present in soil and the environment and found in low numbers in raw, dried or prepared food (Priest, 1993). Cooking of food eliminates the competitive flora in a foodstuff and thus favours the germination and growth of this bacterium from its spores.

*B. cereus* is often present as an intrinsic contaminating microorganism in Refrigerated Processed Foods of Extended Durability (REPFED), pasteurized milk, rice dishes and pastas. During shelf life it may become a major part of the microflora. Because of its resistant spores, significant numbers of *B. cereus* have also been found in herbs and spices, vegetables and dehydrated foods. The presence of both vegetative cells and spores in food commodities has been reported and their role in food safety and food spoilage elaborated.

There is controversy in the fact that public health authorities and legislation do a clear distinction between *B. cereus sensu stricto*, being categorized as a potential food poisoning agent of risk group 2, and the other species of the *B. cereus* group, while the currently available internationally recognized methods for presumptive *B. cereus* (ISO 7932, ISO 21871) acknowledge the limitation of detection and enumeration to the *B. cereus* group as a whole. As a matter of fact, routine investigations do not distinguish between the different members of the *B. cereus* group.

The present document aims, by collecting information available in recent literature reports, at the determination of the risks associated with presence of strains belonging to the *B. cereus* group in foods. In addition, it aims at revealing from analysis of reported outbreaks and the characteristics of the *B. cereus* strains, critical points in the food chain that need enhanced attention in order to decrease the risk.

## 2. *B. cereus* toxins

Most *B. cereus* strains produce a whole series of extracellular enzymes such as proteases, amylases, phospholipases, haemolysins and several toxins or virulence factors.

As indicated above, *B. cereus* is a causative agent of two distinct types of food poisonings, both being related to *B. cereus* toxigenesis. Emetic and diarrhoeal syndromes are caused by two different types of respective peptide and protein toxins, produced by pathogenic strains of *B. cereus*. While the emetic syndrome belongs to the intoxication type of food poisoning, the diarrhoeal syndrome belongs to the toxin-mediated infections or toxicoinfections. The most important features of the two syndromes are outlined in Table 1. Characteristics of the toxins involved in the syndromes are summarized in Table 2, enabling deduction of the relationship between properties of each of the toxin and the corresponding type of foodborne disease. Recently, a comprehensive review of the *B. cereus* toxins, dealing with a.o. structure, biological activity and mechanism, detection, genetic organization and regulation, has been published (Stenfors Arnesen et al., 2008).

### 2.1 Diarrhoeal toxin-mediated infection

The diarrhoeal type of *B. cereus* food poisoning has been related to single proteins, as well as, to protein complexes as causative agents. Haemolysin BL (HBL) and Non-Haemolytic Enterotoxin (NHE) are two different enterotoxin complexes, each consisting of three exoproteins. The proteins of NHE and HBL show certain degree of homology, with amino acid identities ranging from 18 to 44% (Arnesen et al. 2008). Both HBL and NHE belong to general class of A-B toxins, which consist of two parts (A and B) each playing different roles in toxin action. The B component binds to molecules on the surface of target cells. The toxins are then taken up into the target cells, at which point the A component enters the cytoplasm and carries out characteristic toxic enzymatic reaction inside the cell. For both of these tripartite proteins all three components are needed for maximum biological activity. Cytotoxin K (cytK) and enterotoxin T (bceT) are single proteins (Kramer and Gilbert 1989; Beecher et al. 1995; Fermanian and Wong 2000; McKillip 2000; Lund et al. 2000; Lindbäck et al. 2004; Fagerlund et al. 2004; Schoeni and Wong 2005; Michelet et al. 2006). CytK belongs to the family of  $\beta$ -barrel pore-forming toxins. In addition, several other protein cytotoxins, haemolysins and enzymes have been described and their possible role in increased pathogenicity of *B. cereus* has been suggested (Arnesen et al. 2008 and references therein).

HBL contains three protein components: a binding component B, and two lytic components L1 and L2. Each component is encoded by its own gene, with *hbIA* encoding B component (Heinrichs et al. 1993), *hbIC* and *hbID* encoding L1 and L2 component, respectively, all being cotranscribed and located on the same operon. A fourth gene, *hbIB* (encoding the B' protein), which shows high degree of homology with *hbIA*, was reported to be part of the same operon (Ryan et al. 1997), but its function still needs to be clarified. Ryan et al. (1997) have also suggested that the component B of HBL is produced as prototoxin. Protein of approx. 100 kDa, which reacts with antibodies against component B is produced in the early exponential growth

phase. In later growth stages the formation of component B takes place on the account of degradation of the 100 kDa protein, indicating the later as a precursor in the formation of component B. The relationship between this precursor, its possible toxicity, growth phase and numbers of *B. cereus* required for measurable and dose-effect relevant toxin production requires further research.

Regarding detection, PCR detection of genes encoding for HBL components was reported (Prüß *et al.* 1999; in't Veld *et al.* 2001; Corona *et al.* 2003). Also, specific antisera for each of the peptides were reported (Beecher *et al.* 1995; Prüß *et al.* 1999). Commercially available BCET RPLA test (Oxoid) is shown to detect only the L2 peptide (Beecher and Wong 1994) providing therefore only limited information, both in research and routine analysis.

NHE also consists of three different proteins, NheA (41.0 kDa), NheB (39.8 kDa), and NheC (36.5 kDa). Initial findings reported by Lund and Granum (1996; 1997; 1999) suggested NheC to be a 105kDa collagenase; they also suggested somewhat different molecular masses of other NHE components, but later reports brought about different results (Lindbäck *et al.* 2004). Genes of all three components (*nheA*, *nheB* and *nheC*) were sequenced (Granum *et al.* 1999; Dietrich *et al.* 2005) and PCR detection has been described (Hansen and Hendriksen 2001). The genes encoding the NHE components have been cloned and characterized, and, similarly to HBL, they are transcribed as one operon (Granum *et al.* 1999; Lindbäck *et al.* 2004). Antibodies against each of the three subunits of NHE were produced and characterized (Dietrich *et al.* 2005). Commercially available BDE ELISA test (Tecra) reacts mostly with the NheA peptide (Hansen and Hendriksen 2001), but certain cross-reactivity with another component was reported. Two reactive components were designated as the 40kDa and 41kDa constituents by Beecher and Wong (1994). Due to inconsistency in identification of the enterotoxins and their components it is not obvious what the second component is, and whether the reactivity of antibodies is significantly different between these components. Therefore, care should be taken when referring to and analyzing different published information to avoid confusion and incorrect conclusions. Regarding the detection of the *nhe* genes, PCR detection has been reported by several groups (Prüß *et al.* 1999; in't Veld *et al.* 2001; Corona *et al.* 2003).

The genes encoding the HBL and NHE complexes are clustered. While it has been demonstrated that the maximum biological activity of HBL was reached with a 1:1:1 ratio for its three components (Beecher *et al.* 1995), the optimal ratio for the NheA, NheB and NheC proteins was shown to be 10:10:1, respectively (Lindbäck *et al.* 2004).

Being sensitive to the low pH of the gastric juice, and to the proteolytic enzymes, it is quite likely that in the vast majority of cases preformed diarrhoeal toxins will not pass through the stomach and retain their biological activity. Moreover, average incubation period of 12 h is rather long for intoxication *sensu stricto* (Granum and Lund 1997; Granum 1997). However, under certain conditions, the pH in the stomach may raise just enough to allow the toxin to pass undamaged to the small intestines (Wijnands *et al.* 2002). These particular conditions can occur with elderly consumers and with an intake of large volumes of foods, which in addition can shelter the toxin molecule from the proteolytic gastric enzymes (Alouf 2000). Nevertheless, etiology of food poisoning caused by HBL and NHE, in general, follows the scenario of toxin-mediated infection referring to the consumption of food harboring sufficient counts of HBL/NHE producing *B.*

*cereus*.

Apart from HBL and NHE, also Enterotoxin T and Cytotoxin K belongs to the group of diarrhoeal enterotoxins. Although, Enterotoxin T (bceT) is questioned for its role as enterotoxin (no actual outbreaks have been reported), its toxicity in laboratory tests has been confirmed (Choma and Granum 2002; Lindbäck *et al.* 2004). Contrary to bceT, Cytotoxin K (CytK) was implicated in a food poisoning outbreak and has been involved in cases of potentially fatal bloody diarrhoea (Lund *et al.* 2000; Hardy *et al.* 2001). There are two forms of CytK: CytK1 is highly toxic to human epithelial cells and CytK2 is more common but far less toxic to human epithelial cells (Fagerlund *et al.* 2004; Guinebretière *et al.* 2006).

Less is known about verotoxic and haemolytic Enterotoxin FM (entFM) reported by Asano *et al.* (1997). Several other putative (entero-)toxins have been described (haemolysin II, haemolysin III, cereolysin AB, cereolysin O), but their actual implication in *B. cereus* food toxico-infections remains to be confirmed (Michelet *et al.* 2006).

## 2.2 Emetic intoxication

The causative agent of emesis (*B. cereus* foodborne poisoning characterized with vomiting) is a ring-structured dodecadepsipeptide (a lacton) of ca. 1.2 kDa named cereulide (Isobe *et al.* 1995). This small peptide consists only of three repeats of 4 amino/oxy amino acids (D-O-Leu-D-Ala-L-O-Val-L-Val) (Isobe *et al.* 1995; Agata *et al.* 1995; Granum and Lund 1997). In its chemical structure, composition and mitochondrial toxicity, cereulide is similar to valinomycin, which is produced by *Streptomyces fulvissimus*. Both cereulide and valinomycin are potassium selective ionophores (Isobe *et al.* 1995; Granum and Lund 1997; Andersson *et al.* 1998b; Mikkola *et al.* 1999; Hoornstra *et al.* 2004). It is well established that cereulide, similarly to valinomycin, mediates the uptake of K<sup>+</sup> in respiring mitochondria with ejection of H<sup>+</sup> and stimulation of respiration (Teplova *et al.* 2006 and references therein). The cavity within the cyclic dodecadepsipeptide molecule accommodates K<sup>+</sup> ions, forming positively charged complexes whose uptake was found to be driven by the membrane potential of the inner membrane, negative on the matrix side. Lowering of the mitochondrial membrane potential and induction of apoptosis, which has been shown in several cell lines (Inai *et al.* 1997 and Paananen *et al.* 2000), is part of the toxic scenarios of cereulide. The inhibition of respiration and lowering of the mitochondrial membrane potential by K<sup>+</sup> influx into the mitochondrial matrix, causes mitochondrial swelling. At physiological concentrations of K<sup>+</sup>, the swelling amplitude induced by cereulide was about three times that of valinomycin (Teplova *et al.* 2006).

Cereulide is highly hydrophobic and lipophilic. A direct relationship between lipophilicity and permeability through the membrane was reported (Spacie *et al.* 1995). Only hydrophobic substances can pass through the hydrophobic interior of the mitochondrial membrane by simple diffusion down a concentration gradient. Hydrophilic molecules, which repel the hydrophobic interior, must be moved by facilitated diffusion or active transport through the proteins lodged in the membrane (Timbrell 2002).

Cereulide is resistant to heat, pH, and proteolytic activity of pepsin and trypsin (Kramer and

Gilbert 1989; Rajkovic *et al.* 2008) and thanks to these properties, the toxin preformed in food will reach the intestines without losing its biological (toxic) activity in the stomach. Once in the intestine, cereulide will presumably bind to 5-HT3 receptors inducing an emetic response in the host (Agata *et al.* 1995). In high concentrations cereulide has been reported to cause fatal liver failure and respiratory distress leading to the patient's death (Mahler *et al.* 1997; Dierick *et al.* 2005).

Cereulide, due to its structure and small size, has no antigenic properties (Granum and Lund 1997; Andersson *et al.* 1998c; Mikkola *et al.* 1999). Hence, immunological detection of the toxin has been so far impossible. Currently, the most reported detection and quantification methods are those based on biological boar semen motility assay (Andersson *et al.* 1998b; Andersson *et al.* 2004; Rajkovic *et al.* 2006b) or chemical assay based on HPLC-MS (Hagglom *et al.* 2002). Cereulide is produced by Non-Ribosomal Peptide Synthesis (NRPS), whose genetic determinants are located on a large plasmid (Hoton *et al.* 2005). Recently, the plasmid-borne peptide-synthetase gene (*ces*) responsible for the non-ribosomal production of cereulide have been identified and characterized (Ehling-Schulz *et al.* 2005a and 2006). Also, the DNA sequence of the 204 kb plasmid (named pCERE01 or pBC270) has been determined and has revealed that it belongs to the family of pXO1, the largest virulent plasmid of *Bacillus anthracis* (Rasko *et al.* 2007). Primers sequences derived from these DNA sequences have also been used for the development of conventional and real-time PCR assays to provide a rapid and sensitive method for the specific identification and detection of emetic *B. cereus* in food (Ehling-Schulz *et al.* 2004 and 2006; Fricker *et al.* 2007).

Food poisoning caused by cereulide belongs to the group of intoxications. That implies that cereulide is formed and released in food, as a consequence of proliferation of present cereulide producing strains of *B. cereus* under favourable conditions. Whether consumption of cereulide producing *B. cereus* cells can also lead to the disease (toxin-mediated infection) has not been proved or disapproved yet.

Tables 1 and 2 show some of the characteristics of emetic poisoning and properties of cereulide.

### **2.3 Distribution of toxin encoding genes in *B. cereus* strains**

Reported prevalence of diarrhoeal strains of *B. cereus* in foods and participation in outbreaks seem to have changed trend in the last decade. While the general perception used to be that HBL strains were more prevalent and also more involved in outbreaks, it seems that latest reports indicate NHE producing strains as more frequent among foodborne isolates. In fact, genes encoding NHE are now thought to be present in most *B. cereus* strains. Yet, such a statement requires to the opinion of these authors additional time for confirmation, as in the past reports of *nhe* negative strains were published. *Hbl* and *cytK* seem to be present in less than 50% of randomly sampled strains. Difference in the prevalence patterns can be observed when food and clinical isolates are compared (Arnesen *et al.* 2008 and references therein). An interesting conclusion can be drawn from the results of Guinebretière *et al.* (2002) who analyzed enterotoxigenic profiles of 37 food poisoning and 25 foodborne *B. cereus* strains. A great overlap of virulence factors was found, indicating that one strain can carry a multiple

pathogenic armoury. Of 37 food poisoning strains 36 (97.3 %) had genes for all three NHE components, 27 for all three HBL components and *cytK* (73 %), and 21 (56.8%) for *bceT*. All strains that had genes for HBL components had them also for NHE components. Of 27 strains positive for *cytK* gene, 26 were also positive for *nhe* genes and 23 for *hbl* genes. Of 37 food poisoning *B. cereus* strains, 21 harboured *bceT* gene, of which 20 were positive for *nhe* genes, 19 for *hbl* genes and 18 for *cytK* gene. In none of food poisoning isolates was *bceT* found alone. From 25 foodborne strains, 23 possessed all three *nhe* genes, 21 all three *hbl* genes, 2 *cytK* gene and 12 *bceT* gene. Of 21 *hbl* positive strains, 9 also harboured *hblB* gene, but in no strain was *hblB* found alone (same as with food poisoning *B. cereus*). The presence of all three genes coding for three components of HBL and NHE was noted in all HBL and NHE positive isolates. Another important finding is that presence/absence of *hblB* gene did not influence presence of *hblA* gene.

In a recent study by Moravek *et al.* (2006) on 100 *B. cereus* strains, including clinical isolates and isolates from food remnants connected to food-borne outbreaks, as well as isolates from diverse foodstuffs and the environment, it was found that 42% of the tested strains harboured genes encoding for HBL and 99% for NHE. The production of all NHE and HBL components were analyzed with component specific antibodies and, in culture supernatants, detectable levels of HBL and NHE were found for 100% of the *hbl*-positive and 96% of the *nhe*-positive strains.

Wijnands *et al.* (2006a) reported that of all tested 796 isolates, originating from 182 different food samples ca. 95% contained genes for NHE (either as a single virulence factor or in combination with other toxins), approx. 66% and 50% were found positive for genes of HBL and *cytK*, respectively.

The high prevalence of *nhe* genes was found also by Smith *et al.* (2004) who reported that at least one of the *nhe* genes was found in all 27 isolates from different chicken products at the point of sale. Sixteen out of 27 isolates (59%) contained *hbl* and/or *bceT* genes. The presence of all three genes coding for all three components of NHE and/or HBL toxins was found in approximately 67% and 60% of isolates, respectively.

It is interesting to note that similar observations were made for *Bacillus thuringiensis*, a bacterium closely related to *B. cereus* (see next chapter). Indeed, among 74 *B. thuringiensis* strains isolated from small mammals, most carried the *nhe* genes and about 75% harboured the *hbl* and/or *cytK* toxin genes (Swiecicka *et al.* 2006b). A recent study on 411 *B. cereus* strains (from food and soil) and 205 *B. thuringiensis* strains (from the same sources) using newly designed primers indicated that in 65% of these strains all eight toxin genes (*hbl* and *nhe* operon, *cytK*, *entFM*) were present, that the *hbl* operon was significantly more present in *B. thuringiensis* (86%) than in *B. cereus* (66%), but that there were no significant differences for the other toxin genes, and that the *nhe* operon and *entFM* were present in all investigated strains, while *cytK* was present in 83-89% of the strains (Ngamwongsatit *et al.*, 2008).

In comparison to diarrhoeal strains, lower prevalence was noted for emetic strains. About 8% of isolates from different food commodities in the Netherlands tested positively on cereulide production (HEp-2 test) (Wijnands *et al.* 2006a). None of the isolates from potato puree reported by Rajkovic *et al.* (2006a) showed ability to produce cereulide, while in total, 30.4% of isolates expressed enterotoxic components (HBL and/or NHE).

Several reports emphasize strain-based differences in cereulide production (Andersson *et al.* 2004; Carlin *et al.* 2006; Rajkovic *et al.* 2006a). Previous phenotypic and molecular studies have shown that the “emetic” strains form a distinct cluster within the *B. cereus* group (Agata *et al.* 1996; Ehling-Schulz *et al.* 2005b). However, more recent findings have suggested that the emetic strains are phenotypically and genetically more diverse than previously thought (Apetroiae *et al.* 2005; Vassileva *et al.* 2007; Hoton *et al.* *in preparation*).

### Conclusions:

- A variety of diarrhoeal toxins are produced, but it is not clear which ones are responsible for diarrhoeal symptoms, and it is not clear which combinations of genes are needed to be present to cause diarrhoeal symptoms.
- It is not clear, when the potential toxin genes are present, whether they are expressed and the corresponding toxin produced. Therefore, the mere presence of genes is not enough for a *B. cereus* strain to be pathogenic: the expression of the genes depends on the strain (and its full genetic potential to express genes) and this may be influenced by environmental circumstances. The risk of illness is therefore a function of actual toxin production.

### 3.Taxonomy, growth and spore characteristics of *B. cereus*

#### 3.1. Taxonomy

*B. cereus* is a Gram-positive, motile, spore-forming (central, ellipsoid) rod, with a granular internal structure. According to the classical classification, bacilli were defined into groups based on spore and sporangium morphology. Group I bacilli (which includes *B. cereus*) are defined as having a sporangium that is not swollen by the spore. Within this group, subdivision of the species can be obtained on the basis of cell diameter. The large-celled species (including *B. cereus*) have cell diameters > 0.9 µm. The other large-celled Group I bacilli are *B. anthracis* (non-motile), *Bacillus mycoides* (rhizoid growth) and *B. thuringiensis* (parasporal crystal).

*B. cereus* belongs to the species cluster commonly known as the “*B. cereus* group”. This group encompasses six species (see below), which are, according to the current bacterial taxonomy, regarded as valid species (Euzéby, 1997). However, the taxonomy of this group is controversial since several authors have suggested that at least some of these species (*B. cereus*, *B. thuringiensis* and *B. anthracis*) should be regarded as a single taxon based on genetic evidence (e.g. Helgason *et al.* 2000). Furthermore, discrimination between these species is not easy and is rather laborious relying on phenotypic or chemotaxonomic characteristics, which may be lost or exchanged among the different species of this group (Van der Auwera *et al.* 2007). It is well-known among numerical taxonomists that differentiating phenotypic characteristics are valid for most but not all of the strains of a species. Table 3 summarizes the differential characteristics to be used for identification of the member species of the *B. cereus* group. Also, the ISO-selective isolation method used only enables to isolate and recognize the *B. cereus* group as a whole, without possibilities to discriminate between its members (see also further). Nevertheless, further discrimination on species level is important in epidemiological studies and would be useful in food testing and monitoring of food safety. This would indeed be helpful to define more precisely the critical control points, to apply preventive measures and to define microbiological criteria for certain food stuffs.

The six species of the *B. cereus* group, also called *B. cereus* *sensu lato* (*s.l.*), are: *B. cereus* *sensu stricto* (*s.s.*), *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *Bacillus weihenstephanensis*. The former species “*B. medusa*”, described by Delaporte (1969), has not been taken up in the list of valid species. The first four species are the most known and constitute the classical *B. cereus* group. The main reasons why these four different species are still maintained despite obvious genetic evidence of overall genomic similarity (Helgason *et al.* 2000) are their clear clinical and economic differences. *B. cereus* *s.s.* contains pathogenic strains (referred to as “pathotypes”) which may cause foodborne gastrointestinal disease, but also soft tissue infections such eye infections (Drobniewski, 1993) and periodontal disease (Helgason *et al.* 2000) and it has been associated with other local infections and systemic diseases such as

bacteraemia, septicaemia, endocarditis, central nervous system infection and respiratory infection (Drobniewski, 1993). *B. thuringiensis* is commonly known as a commercial biopesticide or as a producer of crystal toxin preparations. As the crystalline toxin genes are plasmid encoded, these plasmid(s) can be lost spontaneously during culture (Gonzalez *et al.* 1981). It has been found that *B. thuringiensis* strains in commercial bioinsecticide products harbour complete *hbl* and *nhe* operons and *cytK* (Frederiksen *et al.* 2006), and also produce enterotoxins *in vitro* (Damgaard, 1995). *B. anthracis* is the well-known causal agent of anthrax in mammals and feared as potential bioterrorism agent. The rhizoid colony forming and non-motile *B. mycoides*, and the very closely related *B. pseudomycoides*, have not been associated with any disease or harmful or beneficial effect. Although these differences seem clear-cut according to the species, food poisoning or pathogenic *B. thuringiensis* strains have been described (Jackson *et al.* 1995), while *B. cereus* s.s. also contains psychrotolerant strains which can provoke food spoilage, but which have also been associated with food poisoning (Guinebretière *et al.* 2007). Recently, it has been deduced that the genome of *B. thuringiensis* 97-27, isolated from a severe tissue necrosis case (Hernandez *et al.* 1998), contains the full *hbl* operon (Han *et al.* 2006). Although this strain was identified as *B. thuringiensis* based on the initial discovery of toxin crystals, a subsequent isolate from the same patient lacking crystalline toxin and recent comparative sequence analysis indicates that this strain is more likely a pathogenic *B. cereus* strain than an insecticidal *B. thuringiensis* strain (Han *et al.* 2006). *B. anthracis* can loose its toxicity plasmids pXO1 and pXO2 (Marston *et al.* 2005). There are also *B. anthracis* strains lacking the toxin and capsule virulence plasmids pXO1 and pXO2 (Makino *et al.* 1993) as there are *B. cereus* and *B. thuringiensis* strains which are genetically closely related to *B. anthracis* and which can display anthrax-like virulence traits (Daffonchio *et al.* 2006; Han *et al.* 2006). As will be discussed later in more detail, it seems that the species boundaries between *B. cereus* and *B. thuringiensis* are rather diffuse. Moreover, differences in virulence between *B. anthracis*, *B. cereus* and *B. thuringiensis* are also due to alteration in gene expression, largely regulated by the transcriptional activator PlcR, rather than simply the gain or loss of gene functions. While *B. cereus* and *B. thuringiensis* contain an intact PlcR encoding gene, this gene is inactivated by a frameshift mutation in *B. anthracis* (Mignot *et al.* 2001; Han *et al.* 2006). The situation has also become more complicated with the description of *B. weihenstephanensis* that accounts for a large part of the psychrotolerant strains, previously identified as *B. cereus*.

Since their description in 1998, *B. pseudomycoides* and *B. weihenstephanensis* were added to the *B. cereus* group. *B. pseudomycoides* (Nakamura, 1998) was described as a new species on the basis of DNA relatedness studies within *B. mycoides*, which showed the existence of two genetically distinct groups. *B. pseudomycoides* and *B. mycoides* cannot be differentiated on physiological and morphological characteristics, nor from their 16S rDNA sequences, but they can be separated on the basis of fatty acid composition of the cells, especially in the content of 12:0 iso and 13:0 anteiso fatty acids. Also from *B. cereus* s.s., this species can be differentiated on the basis of 12:0 iso, 12:0, 15:0 iso and 16:0 fatty acid composition. *B. pseudomycoides* has an optimal growth temperature at 28°C, a maximum at 40°C and a minimum at 15°C and usually produces rhizoidal colonies. Generally speaking, this species contains the mesophilic rhizoidal colony forming strains of the *B. cereus* group. It has been isolated from soil and raw and pasteurized milk and belongs to hazard group 1.

*B. weihenstephanensis* (Lechner *et al.* 1998) was described as a new psychrotolerant species of the *B. cereus* group. Like the mesophiles, psychrotolerant strains have an optimal growth temperature at ca. 25-35°C, but they can be distinguished from the mesophilic strains by their ability to grow at temperatures of 7°C or below. *B. weihenstephanensis* strains grow at 4-7°C, but not at 43°C. Although DNA-DNA hybridization experiments showed large heterogeneity within the *B. cereus* group and that both mesophilic and psychrotolerant *B. cereus* strains as well as *B. mycoides* shared high DNA homology levels above the species threshold of 70%, the new species *B. weihenstephanensis* was proposed on the basis of colony morphology, growth temperature and molecular analyses. The authors justified this new species to consider the unusual importance of *B. cereus* in food poisoning and food spoilage. *B. weihenstephanensis* can be differentiated from *B. cereus* by its ability to grow aerobically at 7°C in agitated liquid culture, absence of growth at 43°C, by the presence of a psychrotolerant signature in the 16S rDNA sequence (<sup>1003</sup>TCTAGAGATAGA) and in the major cold shock protein gene cspA (<sup>4</sup>ACAGTT). Two PCR tests have been designed on the basis of these signature sequences (von Stetten *et al.* 1998; Francis *et al.* 1998). It can be differentiated from *B. mycoides* solely by its non-rhizoidal colony morphology, as both species are phylogenetically very high related and identical in all other characteristics. Unlike *B. mycoides*, which is grouped in hazard group 1, *B. weihenstephanensis* is grouped in hazard group 2, due to its food poisoning potential.

Since its original description, it has been shown that not all psychrotolerant strains belonging to the *B. cereus* group are systematically *B. weihenstephanensis* (Stenfors and Granum, 2001). According to these authors, psychrotolerant strains of the *B. cereus* group can be identified as *B. weihenstephanensis* on the basis of two PCR tests (cspA and 16S rDNA based) designed to discriminate between mesophilic and psychrotolerant strains, but some psychrotolerant strains remain in the *B. cereus* s.s. species, and there are even intermediate strains between both species based on the differentiating criteria. For the moment, these intermediate strains have to be classified as *B. cereus* according to the authors. Likewise, Sorokin *et al.* (2006) found on the basis of a phylogenetic study with multiple-locus sequence (MLST) typing that strains previously identified as *B. weihenstephanensis* in the original description of the species, were to be assigned to *B. cereus* s.s. On the other hand, these authors found some non-psychrotolerant strains in the MLST-cluster corresponding to *B. weihenstephanensis*, indicating that strains cannot be unambiguously assigned to this species based solely on the results of pschrotropy testing.

One of the four identified *B. weihenstephanensis* strains was highly cytotoxic and was positive in the L2 Oxoid toxin detection kit, while another one was low cytotoxic (Stenfors and Granum, 2001). From a recent whole genome analysis, it has been deduced that the chromosome of the *B. weihenstephanensis* strain KBAB4, isolated from a forest soil near Paris, contains 2 toxin operons, encoding HBL and NHE, as well as a second operon encoding NHE on a 400 kb plasmid, which is the first report of this toxin being detected on a plasmid (Lapidus *et al.* 2008). It has been speculated on the basis of population structure analysis that a more active genetic recombination exchange occurs in nature between psychrotolerant strains belonging to *B. weihenstephanensis* compared to mesophilic strains of *B. cereus* and *B. thuringiensis* (Sorokin *et al.* 2006).

To complete the description of the *B. cereus* s.l. group, it is quite worthy to note that both *B. cereus* and *B. thuringiensis* have been suggested to be natural inhabitants of the digestive tract of arthropods, including isopods (Swiecicka and Mahillon, 2006a) and insects. In the latter case, a filamentous symbiont known since 1849 as "Arthromitus" was shown to be a genuine *B. cereus* (Margulis *et al.* 1998).

### **3.2. Population structure of the *B. cereus* group based on genetic studies**

In recent years, several molecular genetic techniques have been used to unravel the genetic population structure of the *B. cereus* group and many studies have tackled this problem. Most studies have used either whole genome fingerprinting techniques such as AFLP (Hill *et al.* 2004; Ticknor *et al.* 2001) and rep-PCR (Cherif *et al.* 2003a; Kim *et al.* 2001; Reyes-Ramirez and Ibarra, 2005), or MultiLocus Sequence Typing of housekeeping genes (MLST) (Priest *et al.* 2004; Bavykin *et al.* 2004; Sorokin *et al.* 2006; Helgason *et al.* 2000 and 2004). All studies showed that the *B. cereus* group could be divided in three major phylogenetic clades or groups or lines of descent (Fig. 3):

- Clade 1 (= *B. cereus* clade of Priest *et al.* 2004) comprising *B. anthracis*, numerous strains of *B. cereus* and a few strains of *B. thuringiensis*. *B. anthracis* strains are more closely related to each other than to any other isolate of the *B. cereus* group (monomorphic nature of *B. anthracis*) and seem to represent an independent lineage within this clade 1 which predominantly contains *B. cereus* isolates. This clade also contains a few psychrotolerant *B. cereus* strains (Sorokin *et al.* 2006) and contains all emetic toxin producing strains of which some are closely related to *B. anthracis* (Vassileva *et al.* 2007).
- Clade 2 (= *B. thuringiensis* clade of Priest *et al.* 2004) comprising the majority of the *B. thuringiensis* strains together with some *B. cereus* strains.
- Clade 3 comprising *B. mycoides* and *B. weihenstephanensis* and a few *B. cereus* and *B. thuringiensis* strains. Almost all psychrotolerant isolates of the *B. cereus* group belong to this clade, but this clade does not contain exclusively psychrotolerant strains (Sorokin *et al.* 2006).
- A fourth clade is formed by *B. pseudomycoides*, which at the time of description was the most distantly related to the other species of the *B. cereus* group (Bavykin *et al.* 2004).
- Very recently, a fifth clade has been proposed, which is composed of at present only a few thermotolerant *B. cereus* strains, among which a pathogenic strain isolated from a severe food poisoning outbreak (Guinebretière *et al.* 2007). There is a proposal to consider this clade as a new species, for which the name "*Bacillus cytotoxis*" or "*Bacillus cytotoxicus*" is proposed, being the seventh member of the *B. cereus* group (Lapidus *et al.* 2008). The pathogenic strain, responsible for the foodborne outbreak with three fatal

cases, was shown to synthesise only one, and at that time a novel toxin, cytotoxin K (Lund *et al.* 2000).

According to a MLST-based analysis of foodborne *B. cereus* group strains, nearly all foodborne isolates belonging to 50 different sequence types (ST) clustered in clades 1 and 2 described above, with more than half of them in clade 1, while only foodborne isolates belonging to two ST's belonged to clade 3 (Cardazzo *et al.* 2008).

A remarkable finding is that some toxigenic strains of *B. cereus* isolated from food and from periodontal disease as well as a few unusual isolates of *B. thuringiensis* (isolated from a human wound or belonging to particular serotypes), are closely related to *B. anthracis* (Hill *et al.* 2004; Ticknor *et al.* 2001; Priest *et al.* 2004; Kim *et al.* 2001). The interspersing of *B. cereus* and *B. thuringiensis* isolates within the phylogenetic tree suggests that phenotypic traits used to distinguish between these two species do not reflect the genomic content of the isolates and that horizontal gene transfer in the *B. cereus* group plays an important role in establishing the phenotype (Hill *et al.* 2004) and in the evolution and distribution of toxin genes (Cardazzo *et al.* 2008; Rosso *et al.* 2000). Conversely, other authors found a weakly clonal population structure within the *B. cereus* group, with evidence of only limited genetic exchanges through recombination (Priest *et al.* 2004; Helgason *et al.* 2004). While some authors argue on the basis of the above findings that at least *B. anthracis*, *B. cereus* and *B. thuringiensis* should be united on the species level (Helgason *et al.* 2000), others disagree with unification of all three species (Bavykin *et al.* 2004; Ticknor *et al.* 2001). It is however fair to say that the actual contribution of horizontal gene transfers (conjugation, transduction or transformation) to the genome flexibility and recombination among members of the *B. cereus* group remains largely unknown and will certainly require further in-depth investigation.

Recently, it was shown that the above described population structure of the *B. cereus* group corresponds well with the thermal niches or habitats of the defined genetic groups, or in other words, that these genetic groups may represent ecotypes with distinct pathogenic potential or risk. This is illustrated in Fig. 4 where the association between seven major genetic groups, as delineated by AFLP, with the distribution and characteristics of the strains in each group is shown (Guinebretière *et al.* 2007). Psychrotolerant strains (i.e. strains able to grow at  $\leq 7^{\circ}\text{C}$ ) are restricted to two major genetic groups: one group composed of *B. cereus* and *B. thuringiensis* strains, and another group, composed of *B. weihenstephanensis* and *B. mycoides* strains. However, only the first genetic group contains strains, which have been isolated from food poisoning. These two genetic groups can be differentiated from each other by means of the psychrotolerant *cspA* signature, being present in 100% of the second group strains only. A small genetic group contains thermotolerant strains, able to grow from 20 to 50°C, of which one isolated from a fatal outbreak as described above. The other four major genetic groups contain mesophilic strains which can grow starting from temperatures in the range 8–15°C depending on the group. One group represents *B. pseudomycoides* (rhizoidal colonies) and no food poisoning strains, while the other three major groups contain *B. cereus*, *B. thuringiensis* and (for one group) *B. anthracis* strains of which several have been isolated from food poisoning. These data can be regarded as a good starting point for tentatively identification of risk groups and

discrimination within the *B. cereus* group by reference to the seven major genetic groups defined in this study.

### Conclusions:

Microorganisms of the *B. cereus* group are still largely identified on the basis of phenotypic features, but these characteristics are not reliable. Hence, the phenotypic identification may differ from the genotypic clustering based on molecular methods. This has important consequences for the assessment of potential risk. What can be concluded from these relationship dendrograms is:

- The *B. cereus* emetic type clusters with a group of atypical strains.
- *B. cereus* is not significantly different from *B. thuringiensis*, the latter is used as bio-insecticide and may be isolated as such from treated vegetables (e.g. *Brassicaceae*) and will be identified as presumptive *B. cereus* according to ISO-methods (thus vegetables may be contaminated with high numbers of presumptive *B. cereus*).
- There are lateral gene transfers among members of the *B. cereus* group, including toxin genes, but their extent needs to be further investigated.
- The majority of psychrotolerant *B. cereus* are defined as *B. weihenstephanensis*, but some psychrotolerant *B. cereus* also group with other *B. cereus* sensu stricto strains.
- There is a need of genetic markers to distinguish pathogenic *B. cereus*, especially those which might be psychrotolerant.

### **3.3. *Growth, sporulation, germination, spore characteristics and biofilm formation***

#### **Growth**

The optimum temperature for growth is between 28°C and 37°C, with minima between 4°C and 7°C and maxima between 47°C and 53°C, the highest temperature being restricted to a remote thermotolerant pathogenic strain of the *B. cereus* group, with the proposed name "*B. cytotoxicus*" (Auger *et al.* 2008). Typical *B. cereus* strains, originated from the environment, normally do not grow at temperature below 10°C. However, lately, psychrotolerant strains were isolated from milk products and chilled pasteurised meals, were shown to grow at refrigerating temperatures. Experimentally, growth was detected for *B. weihenstephanensis* between 8°C and 38°C, while a Ratkowsky growth model extended this range from 1-4°C to 40-46°C (Auger *et al.* 2008). For mesophilic *B. cereus* strains, experimentally growth was detected between 12°C and 47°C, while the Ratkowsky model indicated growth between 2-7°C and 49-53°C. For the thermotolerant "*B. cytotoxicus*" strain, experimentally growth was detected from 18 to 53°C, while the Ratkowsky model indicated growth between 8-15°C to 58°C.

Carlin *et al.* (2006) have showed significant differences between emetic and diarrhoeal enterotoxin producing strains with regard to the temperature growth limits. While none of 17 tested emetic strains were able to grow at 7°C and below, 50 (28 diarrhoeal and 22 food-environment strains) out of 83 non-emetic toxin-producing were able to grow at 4°C and/or 7°C. It is however important to stress that the number of strains tested remains modest, and that more psychrotolerant isolates should be sought and characterized (see also section 5.2).

*B. cereus* grows between pH = 4.9 and pH = 9.3. The minimum  $a_w$  for growth is 0.90, which corresponds to 15 % NaCl.

Being facultative anaerobes, members of the *B. cereus* group are capable of growth under anaerobic and aerobic atmospheres. A study by Jääskeläinen *et al.* (2004) showed that growth did not differ significantly under various atmospheres ranging from 99.5% nitrogen to ambient air. In contradiction to this study Rosenfeld *et al.* (2005) reported that due to the greater acidification during anaerobic glucose fermentation, the apparent growth of *B. cereus* was lower than that which took place under aerobic conditions. Beattie and Williams (2002) observed that *B. cereus* growth did not take place under anaerobic conditions in a chemically defined medium whereas it took place under aerobic conditions. The same isolate was able to grow in more complex media indicating that the effect of oxygen level in the atmosphere on growth of *B. cereus* may only become apparent when other stress factors are present such as nutrient limitation in this case. The level of carbon dioxide in the atmosphere is very important and is typically manifested as an increase in the duration of the lag phase and generation time (Bennik *et al.* 1995). Enfors and Molin (1980) even suggested that some low levels of CO<sub>2</sub> may actually have a growth stimulating effect on the growth of *B. cereus*. Atmospheres with more than 50%

$\text{CO}_2$  have been found to completely inhibit the growth of *B. cereus*. Finally, it should also be reminded that *B. cereus* sustains anaerobic respiration using nitrate as electron acceptor. Recent studies have therefore investigated the effect of anaerobiosis (fermentation *versus* anaerobic respiration) on *B. cereus* growth and toxinogenesis and found the FnR redox regulator to be required for enterotoxin synthesis in a foodborne *B. cereus* strain (Rosenfeld *et al.*, 2005; Zigha *et al.*, 2007).

### **Sporulation**

Several types of differentiated cell types are formed by bacteria to survive harsh conditions such as starvation, high temperatures, ionizing radiation, chemical solvents, detergents, desiccation, pH extremes and antibiotics (Setlow, 2000; Errington, 2003). Of these, bacterial endospores represent the pinnacle of evolution as they are the toughest and certainly survive the longest (Scheldeman, 2004). A typical spore is shown in Fig. 1A and B. As can be seen in this figure, the bacterial spore structure consists of an inner core surrounded by the inner membrane, a cortex surrounded by the outer membrane, and an exterior coat. The coat of members of the *B. cereus* group is surrounded by a loosely attached exosporium (Kutima and Foegeding, 1987). *B. cereus* endospores are of great concern to the food industry since they are omnipresent in the environment (de Vries, 2006) and possess remarkable resistance, which enables them to survive most food processing and conservation methods.

### **Factors influencing sporulation**

Although starvation is considered to be the main stimulus or trigger for sporulation, no single nutrient effect acts a trigger (Setlow, 2003; Scheldeman, 2004). Experimental evidence from a study by de Vries *et al.* (2005) showed that the sporulation of *B. cereus* in a chemically defined medium can take place in the absence of starvation. Rather, an extremely complex and sophisticated decision making machinery incorporating an enormous array of internal and external organs triggers sporulation. The complex nature determining the initiation of sporulation is thought to be a result of the bacterial cell being able to utilize several survival strategies i.e. motility or the synthesis of antibiotics, with sporulation appearing to be the 'last resort'. Some chromosomal proteins have been identified such as inosine monophosphate dehydrogenase which seems to control the initiation of sporulation by *B. cereus*. A high population density is also required for sporulation to take place (Scheldeman, 2004). This has been demonstrated by Røssland *et al.* (2003) who found that sporulation of *B. cereus* only took place when the total count was above  $10^7 \text{ CFU ml}^{-1}$  in co-cultures with *Lactobacillus acidophilus*. Similarly Finlay *et al.* (2002) found that sporulation started when the counts of *B. cereus* reached values greater than  $10^6 \text{ CFU ml}^{-1}$ .

Several other factors peculiar to the medium or environment in which growth of *B. cereus* takes place also have an impact on sporulation. Mazas *et al.* (1997) showed that although sporulation and the amount of spores obtained were high in the pH range 6.5-8.0, the highest rates of sporulation and the maximum spore yield on an artificial medium were obtained at near neutral pH. They suggested that higher or lower pH values resulted in smaller sporulation rates and amounts of spores as they could arrest synthesis or inactivate the enzymes responsible for the

use of organic acids during sporogenesis. Røssland *et al.* (2005) also showed that a rapid decrease of pH to a value of at least 5.0, in co-cultures with lactic acid bacteria, was sufficient to inhibit sporulation. The addition of glucose to a complex medium has also been reported to repress the inhibition of sporulation of *B. cereus* (Bursik and Nemec, 1999). The temperature at which sporulation takes place also influences the rates and yields of sporulation of *B. cereus*. Lower rates and yields being obtained the lower the temperature (Borge *et al.* 2001). The same authors observed that although growth of *B. cereus* took place at 10°C in brain heart infusion (BHI) broth, sporulation did not occur within 40 days. This lead them to state that from a food safety point of view, as vegetative cells are unlikely to develop endospores in refrigerated media, the spores in a food product are most likely contaminants from the environment rather than those produced by vegetative *B. cereus* cells.

### **Heat resistance of *B. cereus* spores**

Spores are thermoresistant and are consequently of great importance to the food industry; in particular the canning sector. The heat resistance of spores has been attributed to its unique architecture and composition. In particular, the relative dehydration of the spore core due to its high content and dipicolinic acid (DPA) (~10% of spore dry weight) has been identified as the most important contributor to heat resistance (Moir *et al.* 2002; Margosch *et al.* 2004).

Bacterial spore heat resistance is affected by some inherent traits and by various environmental factors, which can exert their influence before, during and after the heat treatment itself (Scheldeman, 2004). Before the heat treatment, the heat resistance can be affected by the sporulation conditions and the general physiological state. Valero *et al.* (2002) characterized *B. cereus* isolated from fresh vegetables and refrigerated minimally processed foods and observed that strains without the ability to hydrolyze starch were the most heat-resistant ( $D_{90}$ -values > 10.8 minutes). This relationship has also been reported by Choma *et al.* (2000). In addition, González *et al.* (1999) found that large differences in heat resistance can also occur between members of the same species. Differences have also been reported between emetic toxin-producing strains of *B. cereus* on the one hand and diarrhoeal and food-environment strains on the other hand (Carlin *et al.* 2006). The emetic toxin-producing strains were found to have higher heat resistances at 90°C than the diarrhoeal and food-environment strains. The extent of the variation that can occur is clearly illustrated in a study by de Vries (2006) on the heat resistance of spores from naturally occurring *B. cereus* strains. The  $D_{95}$ -values varied from around 5 to as high as 80 minutes. An extensive compilation of the D-values of *B. cereus* spores under various conditions and food types is provided by ICSMF (1996).

Any pre-heating conditions, particularly the temperature at which sporulation takes place strongly influence the heat resistance of the resulting spores. In general, it has been stated that for a given species the spores will be more heat resistant when it is grown at its maximum growth temperature compared to when it is grown at the optimum or minimum growth temperatures (Scheldeman, 2004). Alternatively it can simply be stated that the higher the sporulation temperature the greater the heat resistance (Beaman and Gerhardt, 1986; Raso *et al.* 1998; González *et al.* 1999). The composition of the sporulation medium is also a factor that plays an important role on the heat resistance of spores. Mazas *et al.* (1997) observed that the

$D_{100}$ -values of *B. cereus* spores from three strains decreased with the pH of a buffered medium; decreasing by 65% per pH unit. Although the mechanisms by which the sporulation pH could affect heat resistance are still not known, it has been suggested that it could be a result of changes in the spore mineralization levels due to differences in the availability of some transition metals such as iron and copper capable of sensitizing spore to heat (Kihm *et al.* 1990). In support of this hypothesis, Cazemier *et al.* (2001) stated that the metal content (cations) strongly affected the heat resistance of various *Bacillus* spores. They determined that the heat resistance of the spores was generally greater when growth was done on nutrient agar supplemented with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{K}^+$  compared to those obtained when  $\text{Mn}^{2+}$  was used. The increase in heat resistance observed correlated with a decrease in the water content of the spore protoplasts. The levels of glutamate in the growth medium have also been found to have an influence on the heat resistance of spores of *B. cereus* (de Vries *et al.* 2005), with high glutamate levels of 20 mM resulting in spores with a higher heat resistance and germinating more rapidly than those from a medium with low glutamate levels of 2.5 mM.

During heating the additional use of high pressures (600 – 800 MPa) has been observed to result in high level of inactivation of several species of *Bacillus* including *B. cereus* (Margosch *et al.* 2004; Scurrah *et al.* 2006) and high pressure has been suggested as a potential alternative to traditional heat-only processes. It has been reported by Margosch *et al.* (2004) that at a pressure between 600 and 800 MPa and temperatures greater than 60°C, DPA is released by a predominantly physicochemical rather than physiological process. Thereafter the DPA-free spores are inactivated by moderate heat independent of the pressure level. The nature of the medium or food in which the heating is performed as well as the recovery conditions after the heating will all have an impact on the observed heat resistance (Scheldeman, 2004). An example being that  $D_{100}^{\circ\text{C}}$ -values of 40, 5.5 and 4.2-6.3 minutes were obtained when *B. cereus* spores were heated in pumpkin pie, distilled water and rice broth, respectively (FSAI, 2007). After heating, the medium in which the heat injured spores recover will influence the apparent D-value (and therefore heat resistance) observed. González *et al.* (1997) investigated the role of several additives in the recovery medium on the heat resistance of *B. cereus*. They observed that although sodium citrate, monosodium and disodium phosphate effectively inhibited heat injured *B. cereus* spores, only the latter two caused a significant reduction in the D-values. Sodium chloride was even more effective with concentrations as low as 0.5% resulting in significantly reduced spore recoveries. Starch was found to have the opposite effect, as little as 0.1% significantly increased the counts.

### **Germination of *B. cereus***

The dormancy of a spore is normally broken by irreversible, degradative biochemical changes (collectively referred to as germination), which occur when favourable conditions for the growth of the vegetative cells occur or when the spores are exposed to germinants (Coote *et al.* 1995). The spores are able to monitor their environment by means of an alert sensory mechanism, which can detect when favourable germination and growing conditions occur (Scheldeman 2004; Setlow, 2003; Hornstra *et al.* 2005). The exact mechanism of spore activation is not yet well understood (Setlow, 2003). Although spore germination has been found to occur in response to low concentrations of chemical germinants i.e. nutrients and non-nutrients (Moir *et*

*al.* 2002), it is most likely that spores in nature will germinate in response to the former (Setlow, 2003).

Nutrient germinants that have been identified to date include single amino acids, sugars, nucleosides and small molecules (Setlow, 2003). Up to 20 different amino acids including L-alanine, L-cysteine, L-threonine and L-glutamate have been found to be able to induce germination (Hornstra *et al.* 2006). Of simple nucleosides, inosine is generally the most powerful germinant and has been shown to show strong synergy when it is used as a co-germinant with L-alanine (Abel-Santos and Dodatko, 2007). Non-nutrient germinants include lysozyme, salts, high hydrostatic pressures, calcium dipicolinate ( $\text{Ca}^{2+}$ -DPA) and cationic surfactants such as dodecylamine. It has been proposed that it is possible for  $\text{Ca}^{2+}$ -DPA released from one spore to induce the germination of spores within its vicinity (Setlow, 2003). Prior to the addition of a germinant, spores often need to be activated to condition or potentiate them to germinate (Blocher and Busta, 1983; Coote *et al.* 1995). Spore activation can also be performed by exposure to aqueous alcohols, reducing agents and heat. Heat treatments, usually in the order of pasteurizations (Laurent *et al.* 1999), are the simplest and most widely used activation methods. Heat activation of spores is thought to be a result of the loss of protein from the spore coat and a change in their ultrastructure (Hashimoto *et al.* 1971). Activation has been observed to result in an increase of the rate of germination and the proportion of germinated endospores (Cook and Pierson, 1983). The heating regime applied during heat activation is also important as isothermal heat activation of *B. cereus* spores has been found to result in lower level of germination than non-isothermal activation (Fernández *et al.* 2001). Once activated, germination of the spores can then be triggered by the germinants described above or also by other methods such as abrasion and hydrostatic pressure (Coote *et al.* 1995). The life cycle is then completed by outgrowth and the formation of vegetative cells.

The rates of germination vary considerably from strain to strain (Dufrenne *et al.* 1995). A study in milk and brain heart infusion broth by Dufrenne *et al.* (1995), showed that the most heat resistant strains of *B. cereus* tend to have the slowest germination rates. Differences have also been found between the abilities of spores of mesophilic and psychrotolerant *B. cereus* strains to germinate in conditions simulating the gastro-intestinal tract (Wijnands *et al.* 2006b). In this study the spores of mesophilic strains were found to germinate better and grow faster than those of psychrotolerant strains. Carlin *et al.* (2006) reported that emetic toxin-producing *B. cereus* strains had a lower germination rate than diarrhoeal or food-environment strains at 7°C. Some inhibitors of germination of the *B. cereus* group strains have been identified and include potassium sorbate (Smoot and Pierson, 1981), 6-thioguanosine (an inosine analogue) (Akoachere *et al.* 2007), essential oils i.e. extracts from rosemary, eucalyptus, chamomile, oranges and carrots (Chaibi *et al.* 1997), and fatty acids i.e. stearic, oleic, linoleic, lauric, and linolenic acid (Ababouch *et al.* 1994).

### **Adhesion of *B. cereus* spores**

The ability of *B. cereus* spores to adhere and act as an initiation stage for biofilm formation on a wide variety of materials commonly encountered in food processing plants is well known (Faille *et al.* 2001; Peng *et al.* 2001; Faille *et al.* 2002), and is the reason why there have been frequent

reports of persistent spore or vegetative cell contamination of food processing lines (Tauveron *et al.* 2006). The formation of such biofilms and the frequency of their formation suggest that food processing line contamination by *B. cereus* biofilms could be a serious threat due to its pathogenic potential, especially in foods that undergo mild processing such as minimally heat-treated foods (Tauveron *et al.* 2006). The strong adhesion properties of spores of the members of *B. cereus* have been attributed to the hydrophobic character of the exosporium (Faille *et al.* 2001; Peng *et al.* 2001; Hüsmark and Rönner, 1992), which varies from strain to strain (Anderson and Rönner, 1998; Tauveron *et al.* 2006). The presence of appendages on the surface of the spores is also thought to play a very important role in biofilm formation as they are thought to initiate contact with surfaces by overcoming the potential barrier (van Loosdrecht *et al.* 1989; Hüsmark and Rönner, 1992). The ability to adhere is generally greater when the spores where surrounded by long appendages (Tauveron *et al.* 2006).

Ryu and Beuchat (2005) investigated biofilm formation of *B. cereus* on stainless steel coupons and its sporulation in these biofilms as affected by nutrient availability, temperature and relative humidity. The relative humidity played an important role in sporulation in the biofilms as it was observed that at after 4 days at 22°C, biofilms exposed to air at 100 or 97% relative humidity had 10 and 2.5% spores, respectively. Biofilms exposed to 93 and 85% relative humidity at the same temperature did not sporulate. Exposure to air also was important as cultures immersed in tryptic soy broth (TSB) and incubated at 22°C only contained 0.02% spores. The study also determined that spores embedded in biofilms were protected against disinfectants such as chlorine, chlorine dioxide and a peroxyacetic acid based sanitizer. In line with this, the efficacy of cleaning in place procedures with regards to *B. cereus* spore viability were demonstrated by Hornstra *et al.* (2007) to be greatly improved when a germinant mixture of L-alanine and inosine were used prior to the cleaning. Anderson and Rönner (1998) have also determined that dormant spores are 33-48% more adhesive than germinated spores, an observation that would also support the intentional germination of the spores prior to the cleaning to increase the level of cleaning attained.

### Conclusions:

- As a survival strategy, sporulation will occur in response to harsh conditions, but under favourable conditions sporulation will occur when the number of vegetative cells become very high.
- A multitude of factors play very important often interacting roles in the heat resistance of *B. cereus* spores, and these can exert their influence before, during and after a heat treatment.
- Spore heat resistance will vary from species to species and from strain to strain. The conditions during sporulation (water activity, pH or temperature), heating medium and recovery medium all contribute significantly to the heat resistance observed.
- Germination can be triggered by the occurrence of favourable conditions for growth or the presence of nutrient or non-nutrient germinants.

- Biofilm formation by spores is proven for *B. cereus* and should present important challenges where foods are mildly or inadequately heat processed. The use of germinants can significantly increase the efficiency of cleaning in place processes with regards to *Bacillus* biofilms.

## 4. Detection method and pitfalls

Direct plate counts of *B. cereus* can be made using a selective agar such as mannitol egg-yolk polymyxin (MYP). The polymyxin is added to suppress the growth of other microorganisms, while *B. cereus* is highly resistant to this antibiotic. Mannitol is not used by most *B. cereus*, and therefore colonies are pink, as opposed to yellow for mannitol fermenting bacteria. Egg-yolk is a substrate for lecithinase, an enzyme found in *B. cereus*, leading to formation of a precipitate around the colony. Confirmation of *B. cereus* requires completion of a number of biochemical tests generally performed in identification galleries.

In the ISO 7932 International Standard (Fig. 2), confirmation of presumptive *B. cereus* is performed by the haemolysis test on sheep blood agar. Some pitfalls are described in the standard itself:

- The standard acknowledges the fact that the confirmatory stage does not allow the distinction of *B. cereus* from the other species of the *B. cereus* group and prescribes a motility test to help differentiate *B. cereus* from *B. anthracis* if the latter is suspected to be present.
- If the plates contain numerous mannitol-fermenting microorganisms leading to the production of acid, the characteristic pink colour of *B. cereus* colonies may be reduced or even disappear leading to potential false negatives.
- Some colonies of *B. cereus* produce only little or no lecithinase and are thus not surrounded by a precipitation zone. The standard describes that also such colonies should be confirmed. It has been reported that emetic strains often display these atypical lecithinase phenotypes (Ehling-Shulz *et al.* 2004).

Emetic strains have also been found to show atypical colony morphology on other plating media such as PEMBA with deficiency in typical precipitate and characteristic blue colony colour (Ehling-Shulz *et al.* 2004).

Another pitfall comes from observations that emetic *B. cereus* strains show only weak or no haemolysis on Columbia agar containing 5% sheep blood (Ehling-Shulz *et al.* 2004). It has been shown that ca. 1% of *B. cereus* or *B. thuringiensis* strains as well as all *B. anthracis* isolates contain mutations in the pleiotropic regulator gene *plcR* that controls the expression of several extracellular proteins such as haemolysins and phospholipases (Slamti *et al.* 2004). These *PlcR* deficient strains are haemolysis- and lecithinase-negative, but their relation with emetic toxin production has not been studied. Furthermore, another 1% of *B. cereus* or *B. thuringiensis*

strains are also haemolytic- and lecithinase-negative, although they do not contain mutations in the *plcR* gene (Slamti *et al.* 2004). It has been shown that cereulide producing strains show two different phenotypes of which one phenotype completely lacked haemolysis and was unable to hydrolyse lecithin (Apetroaie *et al.* 2005). Using diagnostic media based on lecithin hydrolysis and haemolytic activity is therefore rather inappropriate, especially in the case of emetic *B. cereus* strains.

Specific tests are needed to distinguish among the various members of the *B. cereus* group (Table 3). Although several authors have reported PCR-based methods, a.o. on the basis of *gyrB*, for the discrimination between *B. cereus* s.s., *B. thuringiensis* and *B. anthracis* (Cherif *et al.* 2003a, 2003b, 2007; Daffonchio *et al.* 2006; Jensen *et al.* 2006; La Duc *et al.* 2004), extreme caution should be taken in the use of these approaches as they are not fully validated on all members of the *B. cereus* group or only on a limited number of strains. Similarly, molecular typing methods such as PFGE, RAPD, ribotyping or MLST (Helgason *et al.* 2004) have also shown various efficiency in discriminating among the *B. cereus* group members indicating a dynamic population structure (Tourasse *et al.* 2006). Finally as already mentioned, the mere presence/absence of putative enterotoxin genes is not a guarantee for an accurate identification of a pathogenic strain.

#### Conclusions:

- In the case of foodborne outbreaks, there is a need to pick all presumptive *B. cereus*, i.e. haemolytic, weakly haemolytic or not haemolytic at all, some atypical strains may even miss lecithinase activity.
- In principle, all the strains should be fully characterized with regard to the presence of virulence genes and the potential expression/ production of the corresponding toxins, which is understandably not possible to implement in routine analyses.

## 5. Conditions for toxin production by *B. cereus*

### 5.1. *Diarrhoeal toxin*

Surely for the enterotoxins, mere presence of the toxin genes is not sufficient to predict the pathogenic character of a strain: this is largely dependent on the genetic background of the strain and the environmental conditions. Large differences in production of toxin components HBL-L2 and NheB were observed with strains from food poisonings showing significantly more NheB production than food and environmental strains (Moravek *et al.*, 2006). It thus seems that the level of NHE production is to a large extent responsible for the cytotoxic activity of *B. cereus* and is an indication of a high diarrhoeal causing potential.

According to recent investigations, the regulation of enterotoxin production seems to be very complex with the involvement of different transcriptional regulators. The expression of the enterotoxin genes encoding NHE, HBL and CytK is strongly upregulated by the PlcR-PapR quorum sensing system (Gohar *et al.*, 2008), but for NHE and HBL also by other environment dependent systems such as the redox regulators ResDE and Fnr (Duport *et al.*, 2006; Zigha *et al.*, 2007), which also regulate the fermentative growth of *B. cereus* and which are at least partially independent from PlcR. Additionally, *hbl* and *nhe* operon expression seems to be repressed by the catabolic regulator CcpA, which also controls the glucose metabolism (van der Voort *et al.*, 2008). These findings correlate with the observation that the growth rate as well as the sugar source influence the enterotoxin production of *B. cereus* - a.o. sucrose enhances NHE production (Ouhib *et al.*, 2006) - and that a low redox potential stimulates the enterotoxin production (especially HBL) (Zigha *et al.*, 2006).

The diarrhoeal syndrome is in general a toxicoinfection (or toxin-mediated infection) which results from the ingestion of vegetative cells or spores of *B. cereus*. Depending on the type of the food, as well as on handling of a food product, either *B. cereus* vegetative cells or spores or a combination of both will be present in food. This can have an implication on the dynamics of possible diarrhoeal toxin production in the small intestine, in relation to the survival, germination, growth and intestinal adhesion of *B. cereus* spores and vegetative cells (Andersson *et al.* 1998a; Clavel *et al.* 2004, Arnesen *et al.* 2008). In seldom cases the syndrome can occur as intoxication, implying passage of biologically active toxin through the stomach. Later scenario is related to individuals with lowered stomach acidity, e.g. elderly people and persons suffering from achlorhydria.

Infective dose required for diarrhoeal syndrome is estimated to be  $10^5$ - $10^8$  cells. A recent report of Pielaat *et al.* (2006) presented the model of *B. cereus* behaviour and production of diarrhoeal enterotoxins in the host gastrointestinal tract. Outcome of the model showed that diarrhoeal toxin production is dependent on the number and type of ingested cells (vegetative

cells versus spores and mesophilic vs. psychrotolerant strains). According to this model an exposure to a total of  $10^5$  psychrotolerant cells (100 g of food contaminated with  $10^3$  CFU g $^{-1}$ ) would not lead to the toxin-mediated infection, while the same number of mesophilic cells presents a higher risk. Consumption of 100 g of foods contaminated with  $10^5$  CFU g $^{-1}$  (Dutch standard, (Anonymous 1994)) is suggested to pose a health hazard in any case. Several reports from the past link lower counts of *B. cereus*,  $10^3$ - $10^4$  CFU/g, with food poisoning outbreaks (Gilbert and Kramer, 1984; Arnesen *et al.* 2008 and references therein). However, doubts exist on the relevance of these findings, as no information is available on the amount of food consumed and possible analytical errors were suggested.

## **5.2. Emetic toxin (*cereulide*)**

The emetic syndrome is a typical example of intoxication where toxin is formed in foods prior to the consumption. Prerequisites are the presence of sufficient numbers of cereulide producing *B. cereus* and appropriate environment formed by favorable extrinsic, intrinsic and implicit factors that will allow outgrowth and toxin production. The numbers of *B. cereus* required for the production of dose-response relevant amounts of cereulide has not yet been determined. However, literature reports wide variation with numbers ranging between  $10^3$  and  $10^{10}$  CFU/g food, with  $10^5$  CFU/g being the most often reported value (Arnesen *et al.* 2008). Moreover, no simple and straightforward relationship exists between the growth phase and/or critical numbers of *B. cereus* and cereulide production. The growth conditions (temperature, atmosphere, composition of food and related growth factors-pH and  $a_w$ ) play an important role on overall amounts of cereulide produced (Rajkovic 2006; Rajkovic *et al.* 2006c). The amounts of cereulide suggested as intoxicative are around 8 µg/kg body weight (Agata *et al.* 1994, 1995; Shinagawa *et al.* 1985; Jääskeläinen *et al.* 2003a).

Some authors reported that exclusion or limitation of oxygen to the levels below 1.6% might prevent cereulide production. Static incubation at 28°C for 48 hours allowed cereulide production of ca. 5 µg g $^{-1}$  in potato puree, ca. 3 µg g $^{-1}$  in penne and 2 µg g $^{-1}$  in rice. This difference occurred despite the fact that *B. cereus* counts of more than 8 log CFU g $^{-1}$  were found in all three products. In the samples incubated under 150 rpm orbital shaking lower cereulide production or complete lack of the production was observed, while growth was in general as high as in statically incubated samples. The opposite was found by some other authors, who found that cereulide production could be enhanced by shaking (Finlay *et al.* 2002; Hagglom *et al.* 2002). *B. cereus* counts at which cereulide was detected were different in different foods and at different temperatures. At 12°C counts had to be in all foods higher than 7 log CFU g $^{-1}$ . At 22°C required counts were food dependent, but were ca. 6 log CFU g $^{-1}$  (Rajkovic *et al.* 2006c).

The screening for the potential of different foods to support growth of cereulide producing *B. cereus* and cereulide production clearly contributed to the understanding that *B. cereus* growth to ca. 8 log CFU g $^{-1}$  will not necessarily result in cereulide production (Rajkovic *et al.* 2007). Data obtained for cereulide production indicated that stationary phase commenced at counts higher than 8 log CFU g $^{-1}$  and that cereulide production can set in already in the earlier stages of exponential phase. Thus, preceding growth to high counts seems to be a necessary condition for

cereulide production, but is not the only factor to influence it. The amounts of toxin found in food differ from each other. Certain rice containing bakery products were found to hold cereulide in concentration of 5-8 µg g<sup>-1</sup> (Jääskeläinen *et al.* 2003a). Even much lower concentrations of cereulide, ranging from 0.01 µg g<sup>-1</sup> up to 1.280 µg g<sup>-1</sup>, were reported in foods implicated in emetic type of food poisoning (Agata *et al.* 2002).

The recognized problems for food safety increased as the research attention focused on other members of *B. cereus* group. An example of this was recently offered by Thorsen *et al.* (2006) who reported two *B. weihenstephanensis* strains able to produce cereulide. Both strains produced cereulide at temperatures of as low as 8°C. These findings bring new information regarding cereulide production questioning previously established knowledge that cereulide is not formed at temperatures below 12°C. However, since low amounts of cereulide were produced and long time was needed for the production, temperatures should be taken into account when interpreting these findings. Moreover, Thorsen *et al.* (2006) found that both *B. weihenstephanensis* as cereulide producers differed genetically from the stereotype strain by harbouring the Hbl enterotoxin complex genes *hblA* and *hblD*. Until now it was generally admitted that cereulide producing strains did not possess the genes encoding haemolysin BL (Hbl), were unable to degrade starch and to ferment salicin (Ehling-Schulz *et al.* 2005b).

## 6. Prevalence of *B. cereus* in foods

*B. cereus* is found in a wide range of habitats with air and soil being probably the primary source of food contamination. Especially soil is heavily contaminated with *B. cereus* spores as demonstrated by a study on Swedish dairy farms: the median content of *B. cereus* spores in soil was 1450 spores per gram (72 samples analysed, mean spore content 9980 spores per gram), with occasionally contents up to 380.000 spores per gram, and between 61 and 87% of the isolates was psychrotolerant (Christiansson *et al.* 1999). Due to its ubiquitous nature, *B. cereus* can be found in a wide variety of foods. Transmission routes for *B. cereus* in the food chain are illustrated in Fig. 5. Humans and animals are not significant reservoirs of *B. cereus* but may occasionally carry them in low levels. Being a soil resident, *B. cereus* is part of the microflora of plant raw materials such as fresh vegetables, sprouts (Becker and Holzapfel 1997), spices and herbs, raw potatoes (King *et al.* 2007). In a recent investigation it has been shown that *B. thuringiensis* strains indistinguishable from the commercial strains in the microbial bioinsecticides were present on fresh vegetables in Danish retail shops to a level, which may exceed  $10^4$  CFU g<sup>-1</sup> (Frederiksen *et al.* 2006). Since these strains harbour genes encoding enterotoxins, vegetable producers and food safety authorities should consider the residual amount of *B. thuringiensis* insecticide on vegetable products after harvest. Overall, *B. cereus* is not the dominating microorganism in the microflora of plant raw materials and fresh produce is not associated with *B. cereus* foodborne outbreaks or reported cases.

During the manufacture of dried vegetable products, the spores of *B. cereus* (and other *Bacillus* spp. or sporeforming organisms) that are resistant to heat and desiccation may survive the process and contaminate the final product. *B. cereus* has been commonly isolated from dried potato products: levels between below the limit of detection (10 CFU g<sup>-1</sup>) to  $4 \times 10^3$  CFU g<sup>-1</sup> have been reported (King *et al.* 2007). The same phenomenon can explain the presence of *B. cereus* in dried spices, dehydrated soups, starch, rice, cereals in which increased levels may be found also due to the concentration effect of *B. cereus* spores during dehydration. If dried spices or herbs (as well as fresh spices or herbs) are used as seasonings of previously heat treated foods, the *B. cereus* spores introduced as such may find, because of the absence of competitive vegetative microflora due to heat treatment, an ecological niche for germination and growth in these food stuffs and pose a risk.

Although the dairy production chain such as silo tanks (Svensson *et al.* 2006) and post-pasteurization at the dairy factory is also a contributing factor (reviewed by Heyndrickx and Scheldeman, 2002), it has been established by different studies that the farm environment is the main source of contamination of raw milk and other dairy products with *Bacillus* species, mostly those of the *B. cereus* group (Arnessen *et al.* 2007). Especially soil was found to be the major contamination source of raw milk in a Swedish study (Christiansson *et al.* 1999). Some critical points at the farm and the production, storage and handling of pasteurized milk are indicated in Table 4. Note that subsequent contamination of milk powder and derived products is difficult to be avoided (Hammer *et al.* 2001).

The widespread prevalence of *B. cereus* in a variety of food products was also demonstrated in a survey in the Netherlands including a total of 229 samples of milk, yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices. Of these 109 (48%) contained *B. cereus*. The contamination level ranged from  $10^2$  to  $10^6$  bacteria per g or ml. Valero *et al.* (2007) found one out of 12 refrigerated deli salad (containing vegetables and mustard) positive for *B. cereus* at less than  $5 \times 10^3$  CFU g<sup>-1</sup>.

Due to its sporeforming character *B. cereus* is a usual contaminant of many Ready-To-Eat or Ready-To-(Re)Heat foods. These food products are produced with mild heat treatments such as thermisation (15 seconds at 57-68°C) applied for dairy desserts or pasteurization for 2 min at 70°C for short shelf-life foods (<10 days) such as pasteurized milk or for 10 min at 90°C for prolonged shelf life (> 10 days) foods such as Refrigerated Processed Foods of Extended Durability (REPFEDs), also known as cooked chilled foods. These thermally processed food products of high organoleptic quality rely on refrigeration for their safety and quality. The presence of *B. cereus* has been demonstrated in potato puree made from whole potatoes (King *et al.* 2007). Carlin *et al.* (2000) isolated *B. cereus* from 80% to 100% of samples of cooked pasteurized and chilled vegetable purees of leek, zucchini, broccoli, split pea, carrot and potato purees stored at 10°C. Del Torre *et al.* (2001) reported isolation of *B. cereus* from 33% of tested gnocchi in Italy. Large number of REPFEDs involved in *B. cereus* transmission implies that cereulide producing *B. cereus*, which are mostly related to "take a way" rice dishes, should not be overlooked in REPFED

Data for *B. cereus* from the Belgian Federal Agency for the safety of the Food Chain (FASFC) in the transformation and distribution sector in a variety of food products are shown in Table 5. The prevalence of *B. cereus* in sampled food items in Belgium is given in relation to respective limits for these food items in recent years. According to these criteria, from 0.5 % to 4.6 % of the samples did not conform in certain years for pasteurized milk, ready-to-eat meals, dehydrated mushrooms, prepacked and cut fruit and vegetables including germinated seeds, or spices and herbs.

It is important to mention that sporeforming bacteria, primarily of the genus *Bacillus* and related genera, are also used and commercialized as human probiotics (see review by Sanders *et al.* 2003 and by Hong *et al.* 2004). The use of *Bacillus* spores as probiotic implies the direct consumption of high concentrations of viable cells. One of these commercial products produced in Europe and marketed in at least three EU countries (a.o. Belgium), namely Bactisubtil, seems to contain *B. cereus* spores, although the product label originally mentioned *B. subtilis*. The same strain as in Bactisubtil has also been used in the animal feed product Paciflor, which has been withdrawn in 2002 from production due to the ability of the strain to produce diarrhoea enterotoxins HBL and NHE. Two other human probiotics produced outside Europe (Biosubtyl and Subtyl in Vietnam and Biovicerin in Brazil) also contain *B. cereus* spores. Besides human probiotic products, there are several *Bacillus* containing probiotic products for animal use (see review of Hong *et al.* 2004 and Cartman *et al.* 2007). Unlike human probiotic products, probiotic products for animal use are under strict regulations<sup>1</sup> with a scientific assessment of the

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<sup>1</sup> Regulation (EC) No. 1831/2003 establishes the rules governing the Community authorization of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lies down that any person seeking an authorization for a feed additive or

European Food Safety Authority (EFSA) (reviewed by Cartman *et al.* 2007). Toyocerin, a Japanese product containing *B. cereus* var. *toyoi*, is licensed in the EU for use in calves, poultry, rabbits and swine (and possibly also for aquaculture). The *toyoi* strain has been deemed safe for animal use because of its failure to produce enterotoxins and its inability to transfer antibiotic resistance. It is unclear to what extent the legitimate use of this product in animal production may lead to contamination of the food product through fecal excretion.

Whatever the safety issues of these strains and probiotic products might be, it is important that great care should be taken during the fermentation process of these preparations (Anadón *et al.* 2006). Indeed, contamination by undesired strains of *B. cereus* could potentially be undetected and therefore represent a serious problem for the animal and/or human consumers. This aspect has probably been largely underestimated so far.

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for a new use of a feed additive shall submit an application in accordance with Article 7.

## 7. *B. cereus* foodborne outbreaks

*B. cereus* may cause two types of food poisoning in humans: a diarrhoeal and an emetic form. The mechanisms of action for both syndromes are illustrated in Fig. 6. A literature review of published reports on emetic (Table 6a), diarrhoeal (Table 6b) and mixed outbreaks (Table 6c) is given.

Impressive prevalence of *B. cereus* in different food commodities endorses connotations made by many authors on underreporting, which represents an actual problem in the assessment of danger posed to public health. Contribution to this is given by generally mild symptoms of *B. cereus* foodborne diseases and by the fact that *B. cereus* food poisoning is a non-reportable disease in Europe. Sporadic cases are not reportable in US, hence, in US the only cases reported are those related to outbreaks.

*B. cereus* (or *Bacillus* spp.) implication has been shown in 33% of all bacterial food poisonings in Norway (1988-1993), 47% in Iceland (1987-1992), 22% in Finland (1992), 8% in the Netherlands (1991). In USA, Japan, Canada, England and Wales, and Denmark this figure is in the range of 0.7-5%.

Among foods of animal origin, the dairy products are most often involved in *B. cereus* contamination. Foodborne outbreaks related to the presence of *B. cereus* in different REPFED (Refrigerated processed Foods of Extended Durability) have been reported. Large number of REPFED (RTE) foods involved in *B. cereus* transmission implies that cereulide producing *B. cereus*, which are mostly related to "take a way" rice dishes, should not be overlooked in this kind of products.

Overall *B. cereus* is a well known cause of foodborne illness, although infections with this organism are not commonly reported because of its usual mild symptoms. Overall *B. cereus* is responsible for 0.9%-6.9% of the reported foodborne outbreaks in Belgium. *Salmonella* stays one of the most important agents reported in foodborne outbreaks, but a large decrease in *Salmonella* outbreaks is noticed the last years and other agents become more and more important (Collard *et al.* 2008). At European level the same decrease in the number of *Salmonella* outbreaks was seen from 74% in 2004 to 54% in 2006 (Table 7). In Belgium 22 foodborne outbreaks caused by *B. cereus* have been reported during the last six years, (from January 2002 till December 2007) resulting in 386 ill people, 21 hospitalizations and 3 deaths. Except one in December, all outbreaks occurred between the beginning of June and the end of October. The attack rate (AR) varied between 8% in the large general outbreaks and 100% in the smaller family outbreaks. At European level *B. cereus* is also underreported in number of foodborne outbreaks (Table 7). In the first EFSA report of 2004 only 6 outbreaks (0.08%) caused by *B. cereus* were noticed on a total of 6860 outbreaks reported. For 2005 and 2006 this number increased respectively to 74 and 77 outbreaks (1.4% and 1.3%). Compared to other agents causing foodborne intoxications (*Staphylococcus* spp. and *Clostridium* spp.) *B. cereus* is responsible for on average 18% of the outbreaks. The hospitalization rate is very low for *B. cereus* (1.8%) compared to the hospitalization rate after a *Staphylococcus* intoxication (12%) but

is in the same order as after a *Clostridium perfringens* toxicoinfection (2.5%). The *B. cereus* strains causing the diarrhoea type of symptoms are more reported than the emetic type and are rather less severe in pathology.

For Belgium, in seven outbreaks, the emetic type was involved and the food vehicle was pasta in 3 outbreaks, rice in two other outbreaks and cream pastry and mashed potatoes in the last two (Table 8). The *B. cereus* counts in the suspected food varied between  $3.5 \cdot 10^4 \text{ g}^{-1}$  in a witness dish and  $4.2 \cdot 10^8 \text{ g}^{-1}$ . Temperature abuse was demonstrated clearly in 3 of those outbreaks. The hospitalization rate was 4% and one child died (Dierick *et al.* 2005). The occurrence of a fatal case due to liver failure after the consumption of pasta salad by a 7 year-old girl demonstrates the potential severity of the emetic syndrome. Investigation of the circumstances surrounding the case suggested that considerable increases in *B. cereus* counts and levels of emetic toxin took place in the pasta salad during transport at room temperature and refrigerated storage at inadequate temperature ( $14^\circ\text{C}$ ).

In 13 other outbreaks (Table 8), the diarrhoeal type was at the origin of the symptoms and the implicated foodstuffs were meat (5), composite meals (4), milk (1), salad (1), fish (1) and pasta (1). The number of *B. cereus* detected in the sampled food was between  $10^2 \text{ cfu g}^{-1}$  (salad sample<sup>2</sup> from salad dryer some days later) and  $3.9 \cdot 10^5 \text{ cfu/g}$  in the milk. In the outbreak with the fish papillote  $8.8 \times 10^7$  colonies were counted but these were the leftovers found in the dust-bin. The hospitalization rate was 7% and two elderly people died from acute dehydratation in one outbreak caused by cooked minced meat. Bad hygienic conditions and temperature abuse are the most important contributing factors. In almost all the *B. cereus* outbreaks the agent is only confirmed in the food, but most of the time no human feces samples were taken, which complicates the determination of a dose-response relationship for the diarrhoeal food poisoning type.

In 2004, only 6 *B. cereus* outbreaks were reported to EFSA (20 member states (MS) and Norway) resulting in 96 human cases but without hospitalizations. Belgium reported the largest outbreak with 50 ill persons after eating contaminated pasta and also Norway reported one *B. cereus* outbreak. In 2005, reporting of food-borne outbreaks to EFSA was mandatory for the first time and 74 *B. cereus* outbreaks were reported (23 MS and Norway) with 1180 human cases and 28 hospitalizations. Finland reported 8 outbreaks caused by bacterial toxins, with the largest outbreak caused by ham and involved 20 cases. Norway reported five *B. cereus* outbreaks. The largest one involved 22 people at a private party; the stew was identified as the source of the intoxication. Denmark reported 3 outbreaks, 21 persons became ill after having a buffet meal in a restaurant. In 2006, in total 78 outbreaks were reported caused by *B. cereus*. In 33 outbreaks the source of the infection was unspecified and in 18% the source was reported as unknown. Meat, meat products, cereals products and poultry were identified as source of the infection. The most common exposure place were restaurants (57%).

Overall it can be concluded that many of the described foodborne outbreaks were due to the disruption of the cold or the hot chain by consumers or collective catering, keeping food for

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<sup>2</sup> This food item was probably not the primary cause of the outbreak but cross contaminated by the implicated food item responsible for the food poisoning , resulting in a lower *B. cereus* count.

hours at temperatures above 12°C or under 50°C. This temperature abuse enabling rapid multiplication of pathogenic *B. cereus* to high numbers was the determining factor in many outbreaks. Consumer education on proper handling and storage of food seems to be important in managing the risk for *B. cereus* intoxication.

### Conclusions:

There are still many questions concerning the number of *B. cereus* cells necessary to cause foodborne illness:

- Is a contamination level between  $10^3$  and  $10^5$  cfu g<sup>-1</sup> necessary as in the EFSA opinion (2005)? Is there sound evidence for these numbers? Must a distinction be made between mesophilic and psychrotolerant strains?
- Dose-response data/studies are available, but these differ among population group. What is the importance of reports on <math><10^5</math> cfu g<sup>-1</sup> contaminated food products involved in *B. cereus* foodborne outbreaks? This may be pertinent only for a more susceptible population with underlying illness. Maybe there are low numbers in the analysed control meals or food items but not in the actually consumed product leading to the food poisoning, which is however in many cases no longer available for analysis at the time of sampling.
- Concerning the emetic toxin cases, there are sometimes high numbers without toxin production: the influence of food composition and environmental conditions are therefore very important!

## 8. Risk potential of *B. cereus* in the food chain

FAO/WHO (1995) defines **risk** as a function of the probability of an adverse effect and the magnitude of that effect. In other words the risk is a statistical or probabilistic concept, which is directly linked to a hazard, being essentially a mathematical function of two components: probability (P) of occurrence of an undesired event (adverse effect) and the effect (E), also called severity, the latter considered as the magnitude of the consequence of its occurrence. Incorporation of this simple quantitative risk categorization (rather than determination) into HACCP for the determination of control measures (prerequisite program, control point or critical control point) has been adopted already for some time.

### 8.1. Probability (P)

The **probability** can be related to the prevalence of *B. cereus* in food. Since *B. cereus* is a ubiquitous microorganism in the environment, the pathogen is often encountered in foods, although often in low numbers < 10-100 CFU g<sup>-1</sup>. As in raw foods such as raw milk, raw vegetables and raw potatoes, *B. cereus* is only a (minor) part of the microflora and the competition for nutrients with consequences for cell density seems to be important. In these type of foods, where overall the indigenous Gram negative microflora or lactic acid bacteria dominate, *B. cereus* does not have the ability to multiply to elevated levels and produce toxins. Pasteurized foods, where the competitive vegetative flora has been eliminated by heat treatment and where the surviving spores may germinate and dominate the microflora, are at risk products for *B. cereus*. These include e.g. pasteurized milk, cream, dairy desserts, REPFED's such as vegetable purees, prepared dishes with seafood, meat, meat substitutes, fried rice/Chinese noodles/pasta (salads). Also dehydrated foods (herbs/spices, sprouted seeds, soups, sauces, vegetables) where vegetative microflora is eliminated or hindered by reduction of water activity and (*B. cereus*) spores may survive for prolonged periods can be categorized as at risk food stuffs for *B. cereus* because upon rehydration they may support the germination and growth of *B. cereus*. Both the pasteurized and the to be reconstituted dry foods, to be categorized as at risk food stuffs are regularly found to be contaminated with *B. cereus* (spores) either introduced by the raw material used for production or by contamination from the processing environment (either from the air flow or due to (post)contamination from biofilms on food contact surfaces).

In order to restrict the presence and numbers of *B. cereus* in these at risk food stuffs, attention is needed to Good Manufacturing Practices (GMP) and HACCP in the production process encompassing e.g. hygienic design of equipment, adequate cleaning and disinfection, development of high care zones for aseptic filling, rapid cool down after heat treatment and control of the cold chain during storage and transport. These control measures are not only essential in the food processing plant to control the toxigenic *B. cereus*, but for all *B. cereus* and *Bacillus* spp. since the genus holds a number of important spoilage microorganisms limiting the

shelf-life of pasteurized food. If the food business operator does not control these basic requirements that are necessary for the production and processing of safe foods, a continuous post contamination with *B. cereus* may occur. For example, in a potato puree production line a filling step at the dosage pump at which hot potato puree was portioned into the packages was found to be a source of *B. cereus* contamination of the final product (Rajkovic *et al.* 2006a) showing that if the control at dosage pump fails the final product of the given batch will be systematically affected by *B. cereus*.

In order to verify the effectiveness of the above mentioned control measures in the frame of its food safety management system it is recommended that the food business operator establishes a microbial assessment scheme to monitor *B. cereus* in raw materials, in processing line or processing environment and in intermediate or end products on a regular basis. Overall *B. cereus* can be considered as a kind of hygiene indicator organism, representative for aerobic spore forming microorganisms which monitors for the (1) general hygiene of the environment (contamination through dust in the air, ...), (2) the presence of biofilm formation, and (3) the correct cooling down of heated products (time/temperature period). However, since certain strains of *B. cereus* are psychrotolerant (cold tolerant) and thus multiply during shelf life (even under respect of the cold chain), *B. cereus* can only be considered as an indicator of good manufacturing practices during and at the end of production.

As the intrinsic characteristic (pH and  $a_w$ ) of mild heat-treated milk and dairy desserts enable the multiplication of *B. cereus*, these dairy products mainly rely on proper refrigeration temperatures and restricted shelf life for food safety. The storage under conditions of significant temperature abuse or ambient temperatures fosters growth of *B. cereus* thus making this group of foods vulnerable for contamination at high numbers at the post-processing level.

In fermented milk and cheese products, vegetative *B. cereus* cells are rapidly eliminated during the production process (Rossland *et al.* 2005; Schoder *et al.* 2007).

*B. cereus* is also isolated from milk powder and infant formula foods, but usually at lower numbers. Upon rehydratation, again storage of reconstituted product for too long periods at ambient temperature should be avoided. The same is true of other dehydrated products (such as dehydrated soups or herbs), which can hold initial elevated levels of *B. cereus*. Upon reconstitution or its usage as seasoning to prepare or garnish ready-to-eat meals storage time at ambient temperature should be restricted according to good hygienic practices in the preparation of foods in the home or with collective restaurateurs.

In non-dairy products, growth inhibition of *B. cereus* can be accomplished by hurdle technology. The presence of antimicrobial compounds (plant essential oils and some of their isolated constituents) in combination with refrigeration temperatures and acidic pH can prevent multiplication in vegetable based REPFEDs (Valero and Frances 2006; Valero and Salmeron 2003; Varelo *et al.* 2003). Valero *et al.* (2006) also confirmed the effectiveness of the preservative potassium sorbate to prevent growth under acidic conditions in an industrially manufactured deli-salad.

Maintaining the cold chain is an important management operation to be taken by each food business operators involved in the food chain being processing plant, transport, retail, caterer and consumer in order to reduce the risk potential of *B. cereus*. In addition food processors may

exploit the hurdle technology to limit the growth of *B. cereus* during shelf life.

## **8.2. Effect (E)**

The EFFECT (also called severity) of *B. cereus* can be related to the consequences of its occurrence in the food chain. Both food poisoning and food spoilage is linked to the presence of *B. cereus* in the food chain.

On the aspects of spoilage, *B. cereus* is best known as a spoilage organism in the dairy chain. It affects the shelf-life of pasteurized milk and cream. At counts above  $2 \times 10^5$  cfu ml<sup>-1</sup> *B. cereus* causes off flavors such as unclean, fruity, bitter, putrid, rancid and yeasty. During further growth of *B. cereus*, a coagulation deviation, so called sweet curdling, in homogenized low-pasteurized milk will occur. It has been estimated that more than 25% of the shelf-life problems encountered with pasteurized milk are due to the proliferation of *Bacillus* species. Sweet curdling is certainly not only caused by *B. cereus*, as reported by Hanson *et al.* (2005) who found an important role of *B. mycoides* in this phenomenon. Apart from coagulation of milk ("sweet curdling") also formation of flakes in cream ("bitty cream") in low-temperature pasteurized milk is attributed to *B. cereus*. However, it is important to note that in non-dairy products even at high counts of spoilage or pathogenic *B. cereus* sensorial properties of food may not be alarming. In these circumstances consumer cannot realize the unfitness of the food product for consumption and the food may present a potential risk for the consumer.

Overall there are several factors which determine the ecological opportunity for *B. cereus* to grow and dominate in foods. Whether *B. cereus* reaches high levels in milk and cream and other cooked chilled products depends upon the condition of storage (respect of the cold chain ( $\leq 7^\circ\text{C}$ ) limits growth of mesophilic *B. cereus* strains), the presence of psychrotolerant strains (with ability to multiply at  $7^\circ\text{C}$  at neutral pH) and the presence of other microbial flora, including other *Bacillus* spp. The extent to which the cold chain is respected has a significant impact on the growth of psychrotolerant *Bacillus* spoilage or pathogenic species. Storage at a temperature of  $7^\circ\text{C}$  revealed a dominating population of *Bacillus circulans*, which was able to partially inhibit the growth of one of the two tested *B. cereus* strains. The temperature abuse of  $10^\circ\text{C}$  favors the fast outgrowth of mesophilic *B. cereus*, reaching counts of more than  $10^5$  CFU g<sup>-1</sup> within approximately 70 h of storage (Rajkovic *et al.* 2005). The overall effect of interaction between spoilage and pathogenic *Bacillus* spp. will depend upon the psychrotolerant character of both cultures influencing finally the time needed by *B. cereus* to grow to critical population density becoming a dominant bacterium.

*B. cereus* is linked to food poisoning (either characterized by emesis or by diarrhoeal symptoms) due to the consumption of contaminated food. In case of emesis the response is linked to the ingestion of pre-formed toxin in the food (= food intoxication). With regard to dose-response from evidence from foodborne outbreaks the concentration of (highly heat resistant) cereulide triggering emesis is overall considered to be in the order of ca.  $10 \mu\text{g kg}^{-1}$  body weight or  $0.01 \mu\text{g g}^{-1}$  of food.

In case of diarrhoeal symptoms it is generally assumed that the response is linked to the

ingestion of viable toxigenic *B. cereus* cells present in elevated numbers in contaminated food as both the HBL and NHE toxin are protein complexes known to be heat-labile and easily degraded by proteolytic enzymes.

For both the production of cereulide at a concentration to cause emesis as well as for the triggering of diarrhoeal symptoms it is generally acknowledged that elevated numbers are needed.

As such, low numbers (100 - 1000 cfu/g) can be tolerated. However, still it is unclear how to define the exact number of elevated levels of *B. cereus* that may cause food poisoning. This is because the numbers of *B. cereus* are only part of the predisposing factor linked to *B. cereus* food poisoning, whether symptoms (response) will occur due to ingested of contaminated food is not only linked to the numbers of *B. cereus* present but is in fact linked to three variables: the pathogen (*B. cereus*), the food and the host ingesting the contaminated food.

It is well acknowledged that dose-response of biological hazards is variable depending upon the immuno-response/the susceptibility of the host. However also the food (and the presence or absence of certain food constituents) may influence the virulence and toxin production of the hazard. Last but not least there are many variables that determine the pathogenicity of a biological hazard which are in the case of *B. cereus* largely unknown and need to be clarified.

The ability for and the extent of toxin production by a *B. cereus* strain is influenced by its genetic background (which provides the arsenal of toxin genes and its regulatory systems) and its immediate environment (food or gastro-intestinal tract) which does not only provides the substrates and conditions for growth but as well the environment conditions (in terms of temperature, redox potential, substrates, etc) will dictate whether and which of these genetic determinants will be switched on.

One of the problems which leads to confusion with regard to levels of *B. cereus* that are linked to food poisoning, is the fact that *B. cereus* is a micro-organism with an enormous genetic complexity and large biodiversity.

The enormous genetic complexity has been referred to in above sections and is related to two aspects of *B. cereus*.

The first aspect relates to the taxonomic position of the isolates and relates to the definition of its genotype and the use of molecular typing methods or PCR tools in order to differentiate, classify and identify isolates of the *B. cereus* group to (sub)species level.

The second aspect relates to the pathotype of *B. cereus* i.e. the description of toxigenic potential of the isolates by detection of the arsenal of toxin genes and its regulatory systems in the isolate. This also refers to gene expression studies and the fact that food constituents and conditions (for emetic toxin production) or GI environment (for diarrhoeal toxin production after ingestion) may to a great extent define whether along with growth to elevated levels toxin production occurs and to which extent.

The biodiversity of *B. cereus* strains rather refers to the variety observed in the phenotype of the *B. cereus* group isolates. This is in the frame of food microbiology most relevant in two aspects.

Firstly, there is the classical detection and identification of *B. cereus* isolates using morphological-phenotypic aspects which is insufficiently reliable to identify isolates of the *B. cereus* group to the species level. Thus as mentioned above the ISO method indeed is limited to detection and enumeration of presumptive *B. cereus*. Reliable identification to species level needs the introduction of appropriate PCR methods.

Secondly, there is the aspect on the ability or not to grow at refrigeration temperatures ( $\leq 7^{\circ}\text{C}$ ) also referred to as the psychrotolerant character of the strains. Another phenotypic characterization relates to the demonstration of functional toxic activity by the isolate either using a cytotoxicity test (for diarrhoeal strains) or a boar sperm assay (for emetic strains).

This means that depending upon isolation of a presumptive *B. cereus* from a food, first the identity to species level need to be established by appropriate PCR methods. Next, further characterization of the isolate to unravel the genotype, the pathotype and the phenotype (psychrotolerant character or not, toxin production or not) will be informative to determine the effect in terms of possibility to provoke food poisoning due the presence of the isolate at the determined numbers in the food.

With regard to emetic strains the risk estimation is overall more clear than with regard to the diarrhoeal strains. Results of a BACILLUS CEREUS EU project (Preventing *Bacillus cereus* foodborne poisoning in Europe: Detecting hazardous strains, tracing contamination routes and proposing criteria for foods, QLK1-2001-00854) that investigated the biodiversity of emetic *B. cereus*, show that emetic strains often form a distinct genogroup. This was also confirmed from the literature data in the above mentioned section on taxonomic position of emetic strains and genotype as mentioned in Fig. 3. Further the EU biodiversity study of the emetic strains showed these strains do not grow at temperatures  $< 8^{\circ}\text{C}$ . This assumption must however be critically reviewed since a few emetic producing psychrotolerant strains belonging to *B. weihenstephanensis* have been described (Thorsen *et al.*, 2006). Overall, spores of emetic strains are more heat resistant. Emetic strains will, therefore, predominantly form a risk when heated products would be kept outside the fridge or serious temperature abuse ( $>12^{\circ}\text{C}$ ) is encountered during storage. Portioned chilling to rapidly lower the temperatures of precooked food is recommended to prevent growth of emetic strains and, with that, emetic toxin production.

An overview of the information at present available relating biodiversity (especially psychrotolerant character) of diarrhoeal strains with genetic complexity and toxin production is summarized in the following section.

Characteristics concerning psychrotolerant nature and diarrhoeal potential were investigated in *B. cereus* dairy strains. While the majority of the strains were able to grow at refrigeration temperatures, none of the thirty-nine strains isolated from whipping cream were highly cytotoxic at  $37^{\circ}\text{C}$  as determined by Vero cell cytotoxicity assay (Arnesen *et al.* 2007). Compared to the high counts ( $> 10^6 \text{ cfu ml}^{-1}$ ) of *B. cereus* regularly observed in milk, cases of food poisoning are relatively rare. One might speculate that the apparently low production of enterotoxins at  $37^{\circ}\text{C}$  from psychrotolerant strains could partly explain this observation.

Psychrotolerant strains can grow well at refrigeration temperatures but grow at  $37^{\circ}\text{C}$  with difficulty. The germination potential of psychrotolerant and mesophilic spores in simulated

intestinal fluid does not differ much. Under conditions simulating the gastro-intestinal passage, 5 out of 6 mesophilic strains showed growth and only 2/6 psychrotolerant strains (Wijnands *et al.* 2006b) did. From these experiments it could be concluded that mesophilic strains, when ingested in equal amounts as psychrotolerant strains, may be more important for the onset of diarrhoeal symptoms caused by *B. cereus*.

A double blind experiment with 34 healthy volunteers using *B. cereus* naturally present in pasteurized milk following storage for 3 to 14 days at 7.5°C was conducted to obtain information about the allowable concentration in pasteurized milk (Langeveld *et al.* 1996). It was concluded that for healthy adults the probability of becoming diseased from cold-stored pasteurized milk is small. From the results no evidence is obtained that *B. cereus* concentrations less than  $10^5$  cfu ml<sup>-1</sup> will cause intoxication. Pure cultures of *B. cereus* strains isolated from samples with high *B. cereus* counts also showed a low production of enterotoxin.

One of the conclusions of the EU project on *B. cereus* within the scope of hazard characterization and exposure assessment was that growth of diarrhoeal strains can be prevented when standard safety measures are being considered being 1) well regulated fridge temperatures ( $\leq 7^\circ\text{C}$ ) during storage, and 2) producing food products with a non-neutral pH (Pielaat *et al.* 2005). Also Guinebretière *et al.* (2007) stressed that the compliance with low storage temperatures (4°C) should prevent spoilage and growth of potential human pathogens such as *B. cereus*.

## 9. Conclusions

From the risk profile developed in this report, the following general conclusions can be made:

- Clearly, the fact that ***B. cereus* as a sporeforming organism is a ubiquitous microorganism** in the (production) environment, the **heterogeneity of the food categories** involved in food poisoning, together with the **versatility of *B. cereus*** itself represents a **real challenge for control of the micro-organism in the food chain**.
- Overall, **at risk foods** are either foods which have been subjected to a mild heat treatment (pasteurized dairy foods or cooked chill foods) with an extended shelf life under refrigeration or dehydrated foods such as milk powder or powder for dairy desserts or dehydrated soups or spices and herbs. These foods are at risk because the competitive vegetative microflora is inactivated either by heat treatment or by reduced water activity but enables the survival of the spores of *B. cereus*. These spores are **able to support germination, growth and toxin production of *B. cereus*** during storage or after reconstitution (if the food forwards appropriate conditions). Particular potential at risk products, which have been recognized in foodborne outbreaks are pasteurized milk and dairy products, REPFED's, potato puree, dishes containing rice or pasta or salads and milk powder.
- From the literature review no clear evidence is obtained that *B. cereus* concentration less than  $10^5$  CFU ml<sup>-1</sup> will cause food poisoning. Compared to the high counts ( $> 10^6$  CFU ml<sup>-1</sup>) of *B. cereus* regularly observed in milk, cases of food poisoning are relatively rare. High numbers of *B. cereus* may be obtained, even if storage conditions encompassed respect of the cold chain ( $\leq 7^\circ\text{C}$ ) due to the multiplication of psychrotolerant strains.
- Considering pasteurized milk or dairy products, REPFED's or dehydrated **food containing more than  $10^4$  CFU ml<sup>-1</sup> *B. cereus* as hazardous needs further substantiation**. However, there is **insufficient information available to define the exact number of elevated levels of *B. cereus* that may cause food poisoning**. This is because the numbers of *B. cereus* are only part of the predisposing factor linked to *B. cereus* food poisoning: whether **symptoms (response to exposure to defined levels)** will occur due to ingestion of contaminated food is not only **linked to the numbers of *B. cereus* present**, but is in fact linked to three variables: the pathogen (**the *B. cereus* strain**), the food and the host ingesting the contaminated food.
- With regard to the **emetic strains** it is well acknowledged that there is variety in the amount of cereulide produced (**high producer versus low producer**) by various established emetic toxin producing ***B. cereus* strains**. However also the exact **food composition** have been shown to **influence the toxin production** for the same *B. cereus* strain.
- Overall there are many variables that determine the pathogenicity of a biological hazard which are in the case of *B. cereus* largely unknown and need to be clarified. The **ability for and the**

**extent of toxin production by a *B. cereus* strain is influenced by its genetic background** (which provides the arsenal of toxin genes and its regulatory systems) **and its immediate environment** (food or gastro-intestinal tract) which does not only provide the substrates and conditions for growth, but also the environmental conditions (in terms of temperature, redox potential or substrates) which **will dictate whether and which of these genetic determinants will be switched on**. One of the **bottlenecks** that leads to confusing information with regard to levels of *B. cereus* that are linked to foodborne poisoning is the fact that ***B. cereus* is a micro-organism with an enormous genetic complexity and large biodiversity**.

- *B. cereus* comprises both mesophilic and psychrotolerant strains. Although **psychrotolerant strains are most important as food contaminants, mesophilic strains are most important for the onset of disease**. Whereas both types can produce diarrhoeal toxins, emetic toxin seemed to be restricted to mesophilic strains of *B. cereus*. However, one recent report indicated possible emetic toxin production by psychrotolerant bacilli at low incubation temperatures. Public health significance of this new finding needs further investigation. Capacity of psychrotolerant *B. cereus* to produce diarrhoeal toxins in human intestines is suggested to be weaker than that of mesophilic strains. This hypothesis also requires verification under experimental condition mimicking condition of the human GIT.
- **Other factors than the mesophilic or psychrotolerant character of the strains are of importance with respect to the onset of disease:** although the level of contamination will definitely play a role, the ability of *B. cereus* strains to produce enterotoxins and the conditions that trigger enterotoxin production are as important. Toxin production is linked to environmental conditions, type of food matrix, conditions in the GI tract, cell numbers and properties of the strains. **At present however, the full extent of the regulatory mechanisms for enterotoxin production is not completely known in *B. cereus***, but very recently this topic is under investigation in the literature.
- **In recent years, considerable knowledge has been gained regarding *B. cereus* emetic toxin, mainly as a result of optimized detection methods** (computerized boar semen bio-assay and HPLC-MS). **Lack of quantitative, robust and reproducible detection methods for diarrhoeal toxins (HBL, Nhe, EntT or CytK) hindered real progress in the research of these toxins**. It is therefore an imperative of future research to provide effective detection tools that will help answering some of the crucial questions, such as i) what are the amounts of diarrhoeal toxins produced in intestines, ii) what is intoxicative dose of different diarrhoeal toxins, and iii) how can we model the relationship between *B. cereus* counts and toxin production.
- Detection and isolation of presumptive *B. cereus*** seems to be straightforward using the ISO method, but there are **taxonomic problems** or issues in the whole *B. cereus* group to be taken into account. There is a **need to a more precise characterization of the pathogenic target organism(s) within the *B. cereus* group and hence for reliable identification and detection methods at the species level and for definition of the genotypes, pathotypes and phenotypes**.

- Whenever **outbreaks occur**, it is important to pick multiple and if feasible all isolates (also consider atypical non or weak haemolytic isolates) and **apply all the appropriate phenotypic and molecular methodologies**: i.e. classical isolation and biochemical confirmation, PCR detection for confirmation of identity to species level, detection of toxin genes (pathotype); verification of toxin production capacity of the isolates (phenotype) and the psychrotolerant character of the isolated strains. More specific isolation media should be developed which allow a more direct evaluation of important phenotypes.
- ***B. cereus* can produce multiple toxins and one strain can produce more than one toxin.** It is currently believed that HBL and emetic toxin production are mutually exclusive. It is not clear whether this statement can be understood as an absolute rule. Further characterization of strains is necessary to elucidate what is the relation between genotype or origin of a strain and its ability to produce toxins or the compatibility of various toxins especially for the diarrhoeal strains. Furthermore there is only a limited knowledge on the **prevalence of toxin genes among other strains and species of the *B. cereus* group**. This needs to be further investigated, including the potential impact, in term of public health, of their presence (or not) in the food chain. For this, quicker and specific methodologies are needed to evaluate the pathogenic potential of a strain.
- The *B. cereus* group is widespread in the environment and is a very heterogeneous species group. Yet, it is apparently the combination of the prevalence of a particular type and its toxin producing capacity with the type of food and environmental factors (temperature abuse), which determine the risk profile. **At present, and unlike other foodborne pathogens such as *Salmonella* spp., there is no indication that a particular type is or becomes dominating in foodborne outbreaks.**
- In order to restrict the presence and numbers of *B. cereus* in these at risk food stuffs, **attention is needed to Good Manufacturing Practices (GMP) and HACCP in the production process** encompassing e.g. hygienic design of equipment, adequate cleaning and disinfection, development of high care zones for aseptic filling, rapid cool down after heat treatment and control of the cold chain during storage and transport. If the food business operator (FBO) does not control these basic requirements that are necessary for the production and processing of safe foods a continuous post contamination with *B. cereus* may occur.
- Overall ***B. cereus* as a hazard and the management of the risk should be part of the concerned FBO's obligatory food safety management system.** In order to **verify the effectiveness of** the above mentioned **control measures in the frame of its food safety management system**, it is recommended that the food business operator active in the production of at risk foods such as pasteurized dairy, cooked chilled or dehydrated foods establishes a **microbial assessment scheme to monitor *B. cereus*** in raw materials, in processing line or processing environment and in intermediate or end products on a regular basis.

- As mentioned by the BACILLUS CEREUS EU project on *B. cereus* within the scope of hazard characterization and exposure assessment and also supported by the analyses of foodborne outbreaks, **at present the most efficient management option for control of *B. cereus* in the food chain seems to be well regulated fridge temperatures ( $\leq 7^{\circ}\text{C}$ ) throughout the food chain** (during processing, storage and transport) **being a responsibility of the food processor, retailer, caterer and consumer.**
- **Especially for emetic strains, it is of importance that heated products are not kept outside the fridge or that no serious temperature abuse ( $> 10^{\circ}\text{C}$ ) is encountered during storage for a short period. Portioned chilling to rapidly lower the temperatures of precooked food is recommended to prevent growth of emetic strains.**
- ***B. cereus* growth may also be inhibited by producing food products with a non-neutral pH or develop product formulation according to hurdle technology to restrict growth of *B. cereus* throughout the shelf life.** Appropriate product formulation and shelf life conditions are a management option, which is available to the food business operator to control the potential risk of *B. cereus* in the food chain.
- **Infant foods should be considered as a high risk food class** due to growth capabilities of *B. cereus* in these products and to the particularities of this group of consumers. Recently legislation was issued with regard to a **process hygiene criterium at the end of the production process for presumptive *B. cereus* in dried infant formulae and dried dietary foods for medical purposes intended for infants below six months of age** (Commission Regulation (EC) No 1441/2007 amending Regulation (EC) No 2073/2005 on microbial criteria for foodstuffs) as follows:  **$m = 50 \text{ cfu/g}$  to  $M = 500 \text{ cfu/g}$  ( $n=5, c=1$ ).**
- In contrast to the use of **probiotics**, amongst which sporeforming organisms such as *B. cereus*, in animal feed which are evaluated scientifically by EFSA and comprehensively regulated by the European Commission, **up to now no strict regulations for their use in human food are applied.** Initial steps of **guidelines for a probiotic product to be used for humans**, which are taken on worldwide level by the Food and Agricultural Organisation of the United States (FAO) and the World Health Organisation (WHO) (available through the WHO website, [ftp://ftp.fao.org/es/esn/food/probio\\_report\\_en.pdf](http://ftp.fao.org/es/esn/food/probio_report_en.pdf)) and on the European level by EFSA (Qualified Presumption of Safety or QPS approach<sup>3</sup>, available on the EFSA website, [http://www.efsa.eu.int/EFSA/efsa\\_locale-1178620753812\\_1178667590178.htm](http://www.efsa.eu.int/EFSA/efsa_locale-1178620753812_1178667590178.htm)) should be further elaborated and implemented.

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<sup>3</sup> The Scientific Committee is of the opinion that the use of strains from the *B. cereus* group should be avoided whenever there is a possibility of human exposure whether intended or incidental. The *B. cereus* group is therefore excluded from consideration for QPS status.

## 10. Needs for further research

### 10.1. *Factors and regulatory mechanisms influencing the (extent of) toxin production by *Bacillus cereus* strains*

There is insufficient information available to define the exact number of elevated levels of *B. cereus* that may cause food poisoning. This is because the numbers of *B. cereus* are only part of the predisposing factor linked to *B. cereus* food poisoning: whether symptoms (= response to exposure to defined levels) will occur due to ingestion of contaminated food is not only linked to the numbers of *B. cereus* present but is in fact linked to three variables, the pathogen (the *B. cereus* strain), the food and the host ingesting the contaminated food.

Overall there are many variables that determine the pathogenicity of a biological hazard which are in the case of *B. cereus* largely unknown and need to be clarified. One of the bottlenecks which leads to confusing information with regard to levels of *B. cereus* that are linked to foodborne poisoning is the fact that *B. cereus* is a micro-organism with an enormous genetic complexity and large biodiversity.

Moreover, at present the full extent of regulatory mechanisms for enterotoxin production is not yet fully known in *B. cereus* and this aspect is only now gaining more attention in the scientific literature. Especially the factors influencing infection mediated diarrhoeal toxin production requires further study under experimental conditions mimicking the conditions of the human gastrointestinal tract. Also the relation between the type of food and the food constituents that trigger emetic toxin production needs further study.

Whereas both mesophilic and psychrotolerant types can produce diarrhoeal toxins, emetic toxin production seems to be restricted to mesophilic strains of *B. cereus*. However, one recent report indicated possible emetic toxin production by psychrotolerant bacilli at low incubation temperatures. Public health significance of this new finding needs further investigation.

The capacity of psychrotolerant *B. cereus* to produce diarrhoeal toxins in human intestines is suggested to be weaker than that of mesophilic strains. This hypothesis also requires verification under experimental conditions mimicking the condition of the human GIT.

## **10.2. Methodology for identification and characterization of *Bacillus cereus* isolates and toxin detection**

- In recent the years, considerable knowledge has been gained regarding *B. cereus* emetic toxin, mainly as a result of optimized detection methods (computerized boar semen bio-assay and HPLC-MS). On the other hand, lack of quantitative, robust and reproducible detection methods for diarrhoeal toxins (HBL, NHE; entT and cytK) hindered real progress in the research of these toxins. It is an imperative of future research to provide effective detection tools of expression of toxin genes and toxin detection itself that will help answering some of the crucial questions, such as 1) what are the amounts of diarrhoeal toxins produced in intestines, 2) what is intoxicative dose of different diarrhoeal toxins, and 3) how can we model the relationship between *B. cereus* counts and toxin production. Detection of toxin genes is an important screening approach, but actual toxin detection and quantification is needed to provide data for food safety policy makers.

-Detection and isolation of presumptive *B. cereus* seems to be straightforward using the ISO method, but there are taxonomic problems or issues in the whole *B. cereus* group to be taken into account. There is a need to a more exact characterization of the pathogenic target organism(s) within the *B. cereus* group and hence a reliable detection method at species level and/or at the level of genotype, pathotype or phenotype (psychrotolerant character and functional toxic activity by biological assays).

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Table 3: Principal distinguishing characteristics between members of the *B. cereus* group (adapted from Drobniowski, 1993, Lechner *et al.* 1998 and Nakamura, 1998).

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Fig. 1: A. Electron microscopic image of a spore of a *B. cereus* mastitis milk isolate (146 000 x). B. Typical structure and components of a bacterial spore

Fig. 2: Horizontal method for the enumeration of presumptive *B. cereus*-colony count technique at 30°C (schematic representation of method described in ISO 7932:2004)

Fig. 3: Phylogenetic tree based on MLST of gene sequences of *glpF*, *gmk*, *ivID*, *pta*, *pur*, *pycA* and *tpi* (taken from Priest *et al.* 2004).

Fig. 4: Dendrogram showing relationship between seven genetic groups (as defined in part A of the figure by UPGMA clustering of Dice similarity coefficients of AFLP fingerprints and represented as light grey triangles at 60% similarity level) in the *B. cereus* group and key characteristics (part B of the figure) of the corresponding strains (taken from Guinebretière *et al.* 2007). Dark grey triangles represent genetic subgroups. Legend: BpT, *B. pseudomycoides* DSM 12442T; BwT, *B. weihenstephanensis* WSB 10204T; BcT, *B. cereus* ATCC 14579T; BtT, *B. thuringiensis* CIP 53.137T; Ba, *B. anthracis* CEB 94-0040; BmT, *B. mycoides* CIP 103472T.

<sup>a</sup> psychrotolerant *rrs* (= 16S rDNA) sequence signature; <sup>b</sup> psychrotolerant *cspA* (cold shock protein) sequence signature; <sup>c</sup> Bc, *B. cereus*; Bt, *B. thuringiensis*; Ba, *B. anthracis*; Bw, *B. weihenstephanensis*; Bm, *B. mycoides*; Bp, *B. pseudomycoides* (p): psychrotolerant, (m):

mesophilic;<sup>d</sup> according to a rapid screening test (growth at 7°C on J-agar plates after 21 days).

Fig. 5: Transmission routes of *B. cereus* in the food chain.

Fig. 6. Mechanisms of *B. cereus* foodborne outbreaks.

**Table 1: Comparison of syndromes caused by toxins of *B. cereus***

Characteristics	Syndrome / Toxin	
	Diarrhoeal syndrome / (HBL, NHE, bceT, cytK)	Emetic syndrome/ (cereulide)
Symptoms	Abdominal pain, cramps, watery diarrhoea (secretory type) and occasionally nausea	Nausea, vomiting, malaise and ultimately a fatal liver failure
Incubation time (h)	8-24 (or longer depending on the dose and host susceptibility)	0.5-5
Resolution time (h)	12-24 ( up to several days in severe cases)	6-24
Intoxication/Infection dose	Ingestion of more than $10^5$ CFU of diarrhoeal toxin producing <i>B. cereus</i> strains	ca. $10 \mu\text{g kg}^{-1}$ bw, $0.01 \mu\text{g g}^{-1}$ of food <i>B. cereus</i> concentration more than $10^5$ CFU g <sup>-1</sup> food, depending on the strain, food and conditions
Toxin produced	In the small intestine of the host	Preformed in the food
Food involved	milk and meat containing products, soups, vegetables, puddings	Rice, pasta, potato puree, noodles, sauces, paella, meat, polenta
Reference	(Kramer and Gilbert 1989; Granum 1990; Granum 1994; Granum and Lund 1997)	(Mahler <i>et al.</i> 1997; Paaninen <i>et al.</i> 2002; Agata <i>et al.</i> 2002; Jääskeläinen <i>et al.</i> 2003a; Dierick <i>et al.</i> 2005; Rajkovic <i>et al.</i> 2006c)

1 **Table 2: Overview of foodborne protein/peptide enterotoxins of *B. cereus***

Toxins and components	Gene localization <sup>a</sup>	AA	Mass (kDa)	Possible mode of action	Chemistry	Overall function <sup>b</sup>	Toxin detection methods reported	Resistance	Reference	
HBL <sup>c</sup>	B	C <sup>e</sup>	344	37,5	Pore-forming, membrane damaging or catabolic activity of L component(s) within the target	Multifactor toxin	E, H, VP, DN, CYT	Immunological assay – RPLA, ELISA, immuno-blot	5 minutes at 56°C pH 4-8	(Heinrichs et al. 1993; Beecher and Wong 1997; Ryan et al. 1997; Økstad et al. 1999; Schoeni and Wong 1999; Moravek et al. 2004)
	L <sub>1</sub>		384	38,2				Proteinases: Animal assays	Not resistant	
	L <sub>2</sub>		447	43,5						
NHE <sup>d</sup>	NHEA	C <sup>e</sup>	360	41,0	Inhibition of protein synthesis	Multifactor toxin	E, CYT	Immunological assays -ELISA		(Granum et al. 1999; Rowan et al. 2001; Rowan et al. 2003; Lindbäck et al. 2004)
	NHEB		372	39,8						
	NHEC		329	36,5						
Cereulide (emetic toxin)	P <sup>f</sup>	12	1,2	Membrane damaging ionophore	K <sup>+</sup>	Single cyclopeptid e	E, MT, VF, limited VP <sup>g</sup>	Chemical assays (HPLC-MS); Bio-assays (boar semen motility)	80 min at 121°C and 60 min at 150°C at pH 9.5 pH 2-11	(Isobe et al. 1995; Agata et al. 1995; Hoton et al. 2005)
									Proteinases:	Rajkovic et al. (2008)

Resistant

EnterotoxinT (bceT)	NA <sup>h</sup>	366	41,	NA <sup>f</sup>	Single chain	NA <sup>h</sup>	NA, detection of encoding genes	Heat: 5 minutes at 56°C  pH: 4-8	(Ombui <i>et al.</i> 1997b; Hansen and Hendriksen 2001; Choma and Granum 2002)
Cytotoxin K (cytK)	cytK1 NA <sup>h</sup> cytK2	336	36,9	Pore-forming and necrosis of epithelial cells	Single chain	CYT, H	Cell cultures (CACO2, Vero); CytK2 is shown to be less toxic than CytK1	Proteinases: Not resistant	(Ombui <i>et al.</i> 1997a; Lund <i>et al.</i> 2000; Hardy <i>et al.</i> 2001; Fagerlund <i>et al.</i> 2004)

<sup>a</sup> C-chromosomal; P-plasmid; B-bacteriophage. All sequences and data are as stored in UniProt database on 10.03.2006 and do not include signal sequence when possible; <sup>b</sup> E-enterotoxic; VP-Vascular permeability factor; DN-dermonecrotic; CYT-cytotoxic; H-haemolytic; VF-Vacuole formation, T-Mitochondrial toxin, <sup>c</sup> B- Binding component, L1 and L2-lytic components; <sup>d</sup> B-Binding component, A and C-lytic components. The optimal ratio reported is 10:10:1 (NheA:NheB:NheC); <sup>e</sup> Each component is encoded by own gene. Three genes are grouped in one operon; <sup>f</sup> Indicated gene localization is that of NPRS gene; <sup>g</sup> VP activity was noted as strain dependent, but was limited in comparison to diarrhoeal strains. Higher VP activity of some emetic strains could be due to production of diarrhoeal enterotoxins, too; <sup>h</sup> Not Available

**Table 3: Principal distinguishing characteristics between members of the *B. cereus* group (adapted from Drobiewski, 1993, Lechner *et al.* 1998 and Nakamura, 1998).**

Symbols used: +, ≥ 85% of the strains positive; (+), 50-84% of the strains positive; v, variable (15-49% of the strains positive); -, <15% of the strains positive; w, usually weakly positive; NA, no data available.

Species	Colony appearance on blood agar	motility	Hemo-lysis	Crystalline parasporal inclusion	Citrate utilization	Growth in 7% NaCl	Tyrosine decomposition
<i>B. cereus</i>	Slight green tinge	+ <sup>e</sup>	+	-	+	+	+
<i>B. anthracis</i> <sup>a</sup>	Gray-white to white, generally smaller than <i>B. cereus</i>	-	-	-	v	+	-
<i>B. thuringiensis</i>	as <i>B. cereus</i>	+	+	+	+	+	+
<i>B. mycoides</i>	Spreading rhizoid with marked tailing	-	w	-	(+)	(+)	(+)
<i>B. pseudomycoides</i> <sup>b</sup>	White to cream, usually rhizoid	-	NA	NA	+/v <sup>e</sup>	+	+
<i>B. weihenstephanensis</i> <sup>c</sup>	as <i>B. cereus</i>	+	NA	-	+	+	+

<sup>a</sup> *B. anthracis* is the only species from the *B. cereus* group which is positive for lysis by gamma phage, penicillin susceptibility, elaboration of anthrax and capsule and possession of plasmids pXO1 and pXO2, virulence in mice and M'Fadyean reaction in killed mice.

<sup>b</sup> *B. pseudomycoides* can be distinguished from *B. mycoides* by differences in C<sub>12:0</sub> iso and C<sub>13:0</sub> anteiso cellular fatty acid levels and from *B. cereus* by differences in C<sub>12:0</sub> iso, C<sub>12:0</sub>, C<sub>15:0</sub> iso and C<sub>16:0</sub> cellular fatty acid levels (Nakamura, 1998).

<sup>c</sup> *B. weihenstephanensis* can be differentiated from *B. cereus* by its ability to grow aerobically at 7°C in agitated liquid culture, absence of growth at 43°C, the presence of a 16S rDNA signature sequence (<sup>1003</sup>TCTAGAGATAGA) and the signature sequence <sup>4</sup>ACAGTT of the major cold shock protein gene *cspA* (Lechner *et al.* 1998).

<sup>d</sup> on sheep or horse blood agar after 24h.

<sup>e</sup> listed in the same publication as positive and as variable (Nakamura, 1998).

**Table 4: General critical points in Dairy Supply Chain regarding *B. cereus* contamination**

Dairy Supply Chain
<b>Feed origin</b>
When feed supplier is changed or when these facts are interpreted over different geographical and climatic locations dairy farm should know that possible increase in the load of <i>B. cereus</i> spores can occur.
<i>Note:</i>
<ul style="list-style-type: none"> <li>• <i>Soil is the primary source of food contamination, with one gram of soil being able to host <math>5 \times 10^1</math>-<math>4 \times 10^5</math> <i>B. cereus</i> spores (Christiansson et al. 1999)</i></li> <li>• <i>Feed being in contact with soil will almost in all cases carry contamination with <i>B. cereus</i> spores.</i></li> <li>• <i>Feed of different geographic origins (different soils and different ambient temperatures) will carry different levels of contamination.</i></li> </ul>
<b>Feeds (includes rendering, processing, packaging, etc.)/Pasture/Fodder</b>
<b>Animal and Environment (includes breeding, husbandry, hygiene, housing, water supply etc.)</b>
<ul style="list-style-type: none"> <li>• Hygiene of stables</li> </ul>
<b>Milking (by machine or by hand)</b>
<ul style="list-style-type: none"> <li>• Hygiene of stables</li> <li>• Hygiene of the udder (see Note 1)</li> <li>• Hygiene of hands and/or equipment and recipients (see Note 2)</li> </ul>
<i>Note 1:</i>
<ul style="list-style-type: none"> <li>• <i>Transfer of contamination from the udder that was previously in contact with grass and soil (Granum 1997; Christiansson et al. 1999)</i></li> <li>• <i>Faecal contamination of the udder (Altayar and Sutherland 2006).</i></li> </ul>
<i>Note 2:</i>
<ul style="list-style-type: none"> <li>• <i>In the milking machines and/or pipes in more automated milking systems the hydrophobicity of <i>B. cereus</i> spores may allow them to attach to the surfaces (Faille et al. 2002) creating conditions for bio-films formation that will become a resource for a repeatable in-house contamination</i></li> </ul>
<b>Milk storage (on-farm)</b>
<ul style="list-style-type: none"> <li>• Storage at temperature of 4°C and lower to prevent germination and/or growth of mesophilic <i>B. cereus</i> and sufficient retardation of growth of psychrotrophic strains</li> </ul>
<i>Note: It is recommended that pasteurized RTE foods are cooled down within 2 h to 10°C or below and within 12 h to 3°C and below</i>
<b>Transport of milk</b>
<ul style="list-style-type: none"> <li>• Storage at temperature of 4°C and lower to prevent germination and/or growth of mesophilic <i>B. cereus</i> and sufficient retardation of growth of psychrotrophic strains</li> </ul>
<b>Consumer product preparation: milk/cheese/fermented milks</b>
Milk pasteurization: 80°C/10minutes to assure removal of vegetative cells
Immediate cooling to 4°C to prevent germination of spores and outgrowth of germinated cells

No contact between pasteurized and raw milk
Cleaning in Place or alternative cleaning and disinfection of the collecting (silo tanks), handling and processing system to assure removal of bio-films
In fermented products correct pH and/or water activity should be obtained to inhibit germination and growth of <i>B. cereus</i>
<b>Storage, retail (includes packaging)</b>
Maintenance of cold chain; Intact packaging; Properly indicated shelf-life and storage conditions on the label
<b>Distribution to consumer/catering outlets</b>
Maintenance of cold chain; Intact packaging
<b>Domestic, trade and retail catering</b>
Maintenance of cold chain; Intact packaging
<b>Consumers</b>
Maintenance of cold chain; Intact packaging; Prevention of cross-contamination upon opening of the package; Respect of the "use by date"

**Table 5: Overview of *B. cereus* contamination in some selected food products (data Belgian FASFC 2001-2006 monitoring programme)**

N° <sup>1</sup>	Food type	Limit <sup>2</sup>		Result <sup>3</sup>		
		m	M	S	A	U
186	Infant food Formula	≤100		100%		
308	RTE <sup>4</sup> infant milk	≤100		100%		
155	RTE meal for infants (6m -18m)	100	1000	92.6%	2.8%	4.6%
419	Pasteurized milk	100	1000	96.3%	1%	2.7%
135	Chinese take away dishes	1000	10.000	100%		
484	RTE dishes	1000	10.000	97.5%		2.5%
266	Cooked chilled meals	1000	10.000	100%		
402	Pasta salads	1000	10.000	98.8%	1.2%	
164	Quorn, tofu based preparations	1000	10.000	100%		
252	Precut packed fruits & vegetables including sprouted seeds	1000	10.000	96.6%	0.8%	2.6%
140	Fresh herbs	1000	10.000	100%		
323	Herbs and spices	1000	10.000	99.5%		0.5%
58	Tea (Vrac & prepacked)	1000	10.000	100%		
114	Dehydrated mushrooms	1000	10.000	92.6%	3.7%	3.7%
50	Dehydrated fruits	1000	10.000	100%		

<sup>1</sup> Number of samples analysed.

<sup>2</sup> Unit is cfu/g

<sup>3</sup> S: satisfactory; A: acceptable; U: unacceptable

<sup>4</sup> Ready-to-Eat

**Table 6a: Literature reports on emetic outbreaks**

1

Type of Food / Main Component of the dish	Range reported of <i>B. cereus</i> count	Outbreak/syndrome and number of people involved	Country, year <sup>a</sup>	Reference
Take a way meal	$10^5$ in the faeces of the consumers	Vomiting / 2 people	UK, 1997-1999	(Ripabelli <i>et al.</i> 2000)
Meal with meat substitute	$10^2$	Vomiting / 7 people		
Meal with rice	$10^6$	Vomiting / 4 people		
Take a way beef dish	$>10^4$	Vomiting / 2 people		
Fried rice	$>10^6$ in fried rice	Vomiting/4 people	NA	(EFSA 2005) and references therein
Pasta salad	$10^7$ - $10^8$	Vomiting, respiratory distress, liver failure. Outbreak involving 5 young children, hospitalization and one fatality	Belgium, 2003	(Dierick <i>et al.</i> 2005)
Home made minced meat pasta dish	$7 \times 10^6$ (1500-3000 ng cereulide present in the pasta determined with bioassay and HPLC-MS)	Vomiting involving 2 people	Finland	(Jääskeläinen <i>et al.</i> 2003b)
Fried rice	ca. $10^6$ ( $10^4$ in vomits), 60-1280 ng cereulide	Vomiting involving 2625 people with 1 death, in 686 cases	Japan, 1971-1982	(Shinagawa <i>et al.</i> 1985; Agata <i>et al.</i> 2002);
Boiled rice	ca. $10^{6-7}$ , 10-640 ng cereulide			
Chow main	640 ng of cereulide			
Curry and rice	$5.7 \times 10^7$ , 80 ng of cereulide			
Spaghetti	40-80 ng of cereulide			
Noodle	20 ng of cereulide			
Catered box lunch	$1.2 \times 10^6$ (in vomits)			
Rice ball (with sushi)	$1.6 \times 10^7$ - $8 \times 10^8$			
Roast chicken	$1.3 \times 10^6$			
Omelette and rice	$1.1 \times 10^7$			

NA: not available

**Table 6b: Literature reports on diarrhoeal outbreaks**

Type of Food / Main Component of the dish	Range reported <i>B. cereus</i> count	Outbreak/syndrome and number of people involved	Country, year <sup>a</sup>	Reference
Chicken & rice meal	$10^2$ in chicken and $10^6$ in rice <sup>b</sup>	Diarrhoea / 300 people	UK, 1997-1999	(Ripabelli <i>et al.</i> 2000)
Cooked chicken meal	Not known	Diarrhoea / 30 people		
Banquet cakes served at 2 banquettes in the same area (the same catering service used)	> $10^2$ (same DNA profile of food, kitchen and faeces isolates)/100% (food samples tested)	2 simultaneous diarrhoeal outbreaks involving 95 and 78 people. All isolates produced HBL (tested with BCET-RPLA and HBL agar plates) and phospholipase C. No emetic toxin, but genes for NHE were found.	Italy, 2001	(Ghelardi <i>et al.</i> 2002)
Mayonnaise used for potato salad	$10^3$	Diarrhoea and gastroenteritis involving 25 (70%) of attending people	Quebec, Canada	(Gaulin <i>et al.</i> 2002)
Meal served during Norwegian Ski Championship	$10^4$ - $10^5$ per serving of incriminated food <sup>d</sup>	Diarrhoea involving 152 people. Isolate involved produced NHE detected with TECRA-VIA and Vero cells assay	Norway	(Lund and Granum 1996)
Vegetable puree	$3 \times 10^5$	Diarrhoea where 44 people were ill. 6 had bloody diarrhoea, and 3 died. Isolate produced cytK enterotoxin and contained no genes for HBL and NHE.	France, 1998	(Lund <i>et al.</i> 2000)
Cornish game served as a catering service	$10^5$ (data confirmed) not	Gastroenteritis involving 140 people	CA, 1989 USA,	(Slater <i>et al.</i> 1992)
Cod fish	$4 \times 10^5$	Diarrhoea involving 4 people	NA	(EFSA 2005) and references therein
Pudding	$2 \times 10^6$	Diarrhoea involving more than 1860 people	Japan, 1971-1975	(Shinagawa <i>et al.</i> 1985)
Catered lunch box	$5.7 \times 10^6$ - $3 \times 10^7$	with 3 deaths, in total of 425 cases		

<sup>a</sup> Chicken and rice isolates were not sharing the same DNA profile, indicating different source of contamination

<sup>b</sup> Detected with mouse ligated ileal loop assay

NA: Not Available

**Table 6c: Literature reports on mixed outbreaks**

Type of Food / Main Component of the dish	Range reported <i>B. cereus</i> count	Outbreak/syndrome and number of people involved	Country, year	Reference
Spaghetti with pesto	NA	Gastroenteritis, diarrhoea and vomiting involving 2 people (1 died due to the fulminant liver failure)	Switzerland, 1996	(Mahler <i>et al.</i> 1997)
Chinese noodles	6x10 <sup>7</sup> in cooked noodles	Vomiting and diarrhoea /50 people	NA	(EFSA 2005) and references therein
Chicken and fried rice	>10 <sup>6</sup> from chicken fried rice	Vomiting, cramps, diarrhoea involving 14 (21%) of people eating lunch showed symptoms. The median incubation period was 2 hours.	Virginia, USA, 1993	(Khodr <i>et al.</i> 1994)
Wedding meal: suspect quiche (eaten by 56% of ill people) and beef (cross-contamination)	<i>B. cereus</i> was isolated from implicated foods, but exact count was not determined	79 persons (58%) of which 91% with nausea, 84% with diarrhoea and cramps, 73% with vomiting, 71% with headache, 63% with chills and 49% with fever.	Mississippi, USA, 1993	(Penman <i>et al.</i> 1996)

NA: Not Available

**Table 7: Reported foodborne outbreaks in the EU (collated from Anonymous 2006; 2007a and b<sup>4</sup>)**

Year	2004			2005			2006		
agent	Nr of outbreaks	ill <sup>b</sup>	hos <sup>c</sup>	Nr of outbreaks	ill	hos	Nr of outbreaks	ill	hos
<i>Salmonella</i>	5067	30638	3304	3406	25760	3554	3131	22705	3185
<i>Campylobacter</i>	1243	3749	157	494	2478	150	400	1304	65
<i>Fviruses<sup>a</sup></i>	86	3010	146	312	6812	255	587	13345	553
<i>S. aureus</i>	35	778	14	164	1692	365	236	2057	277
<i>B. cereus</i>	6	96	0	74	1180	28	77	964	34
<i>Clostridium</i> <i>spp.</i>	18	650	17	79	1633	38	81	1651	44
<i>E. coli</i> pathogenic	87	799	54	60	796	125	48	750	103
Other	101	2076	205	193	1733	267	294	2816	572
Unknown	217	2681	472	573	5699	557	952	9437	947
Total	6860	44477	4369	5355	47783	5339	5807	55029	5780

<sup>a</sup> Foodborne viruses (Hepatitis A, Rotavirus and Calicivirus, including Norovirus)

<sup>b</sup> number of persons ill

<sup>c</sup> number of persons hospitalised

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<sup>4</sup> Available through the EFSA website [http://www.efsa.eu.int/EFSA/efsa\\_locale-1178620753812\\_home.htm](http://www.efsa.eu.int/EFSA/efsa_locale-1178620753812_home.htm)

**Table 8: Overview of foodborne outbreaks caused by *B. cereus* in Belgium (2002-2006) (data collated from the Belgian National Reference Laboratory on foodborne outbreaks, Institute for Public Health, Brussels)**

Food vehicle	Food category	Type	Number of <i>B. cereus</i> CFU g <sup>-1</sup>	Cereulide gene	Persons exposed	ill	died	Hospit-alized	AR	Setting	Contributing factors
Pasta salad	cereals	E	2.1x10 <sup>8</sup>	P	5	5	1	5	100	household	Pasta left over from picnic 2 days before, fridge at 12°C
Pasta salad	cereals	E	NR	NT	240	50	0	1	21	NR	
Rice	cereals	E	1.5x10 <sup>6</sup>	P	6	6	0	0	100	Take-away Chinese restaurant	Refrigerated storage at 15°C
Spaghetti	cereals	E	3.5x10 <sup>4</sup>	P	280	60	0	0	21	hospital	Cross contamination
Cream pastry	pastry	E	4.2x10 <sup>7</sup>	P	20	9	0	0	45	school	No refrigeration
Nasi, bami panga, chop soy	mixed	E	1.6x10 <sup>5</sup>	P	5	5	0	0	100	Chinese restaurant	
Mashed potatoes	potatoes	E	7.7x10 <sup>4</sup>	A	NR	2	0	0	NR	At work	
Minced meat	meat	D	1.5x10 <sup>5</sup>	A	78	17	2	11	22	Home for elderly people	Insufficient water in bain-marie
Cold dishes salmon, Pasta salad	mixed	D	NR	NT	22	6	0	4	27	Banquet, outdoor party	
Durum pita	meat	D	NR	NT	3	3	0	0	100		
Beef stew	meat	D	NR	A	104	38	0	0	37	Home for elderly people	
Milk	milk	D	3.9x10 <sup>5</sup>	A	70	70	0	0	100	Scouting camp	
Salad	vegetables	D	100	A	6	4	0	0	67	Café-snack	Bad hygienic conditions
Mexican grill	mixed	D	200	A	40	27	0	0	68	Restaurant	
Merguez	meat	D	7.6x10 <sup>3*</sup>	A	12	11	0	0	92	Household	
Chinese meal	mixed	D	5.2x10 <sup>4 **</sup>	A	2	2	0	0	100	Household	
Merguez	meat	D	1.0x10 <sup>3*</sup>	A	5	2	0	0	40	Household	
Papillote of fish	fish	D	8.8x10 <sup>7</sup>	A	130	10	0	0	8	Scouting camp	Prolonged storage at room temperature
Pasta	cereals	D	2.1x10 <sup>5 **</sup>	A	NR	2	0	0	NR	Restaurant	No refrigeration of the fresh pasta
Vietnamese dish	mixed	D	1.8x10 <sup>4 **</sup>	A	44	30	0	0	68	At work	No refrigeration

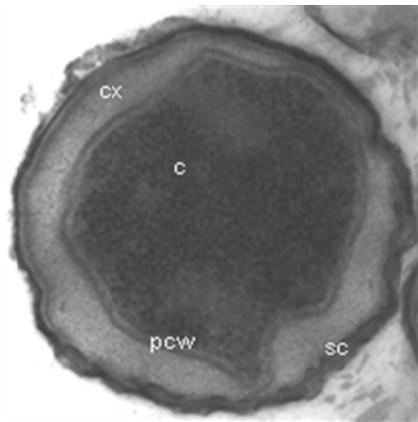
E:emetic type; D:diarrhoea type; NR: Not reported; NT: not tested; P: Present; A: Absent; AR: attack rate;

\*, these low *B. cereus* counts are probably due to cross contamination of the sampled control meal or food item by the food item implicated in the foodborne outbreak.

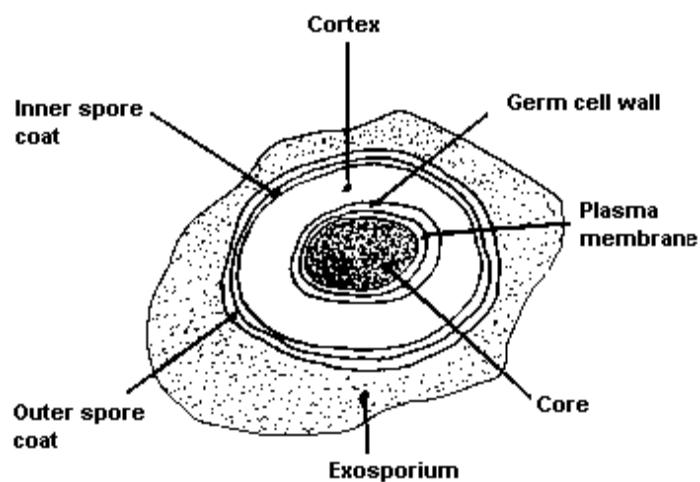
\*\*, toxin detected in the food by BCET-RPLA

**Fig. 1A:** Electron microscopic image of a spore of a *B. cereus* mastitis milk isolate  
(146 000 x)

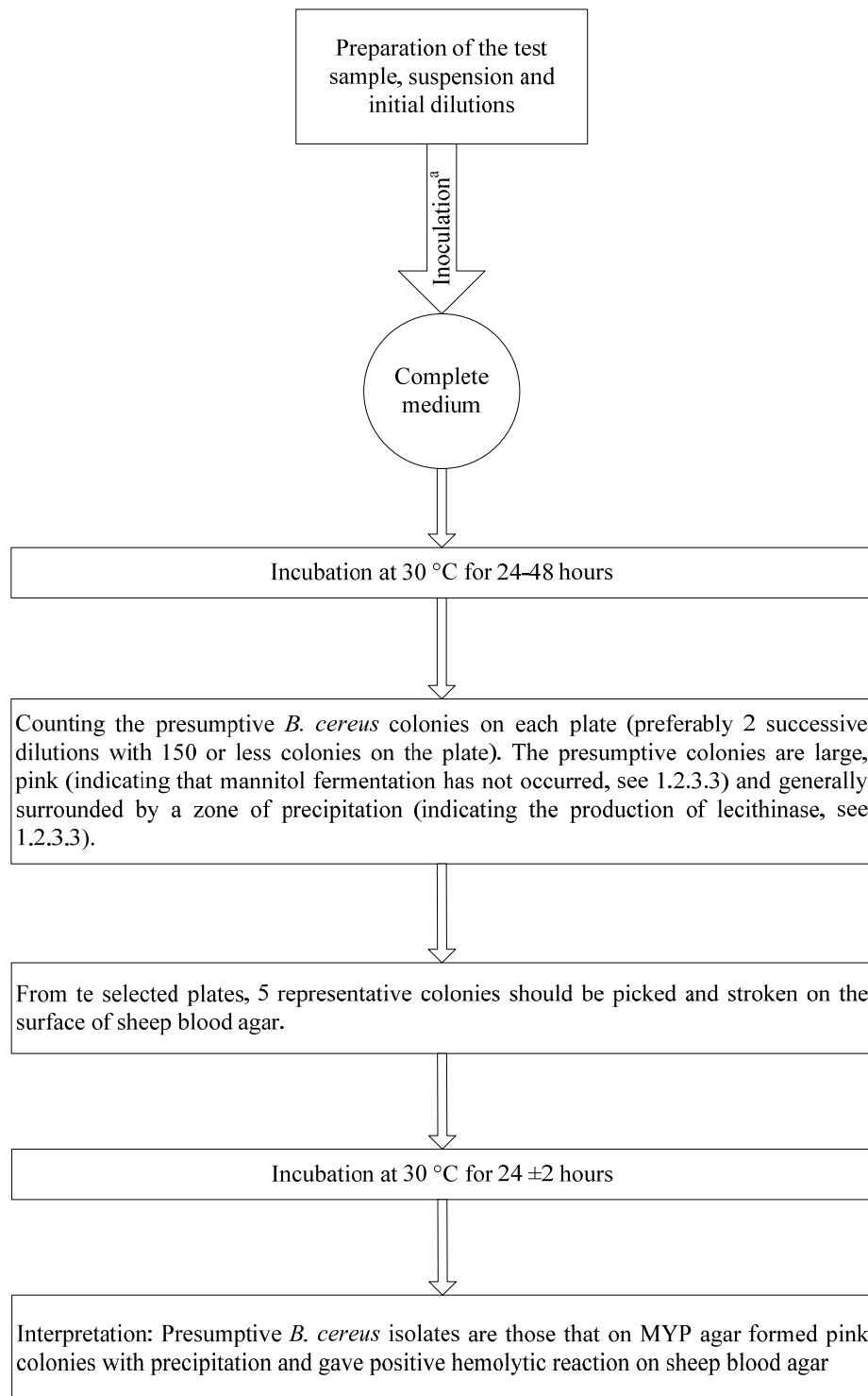
C, spore core; cx, spore cortex; pcw, primordial cell wall; sc, spore coat. (taken from Scheldeman *et al.* 2006).



**Fig. 1B.:** Typical structure and components of a bacterial spore.

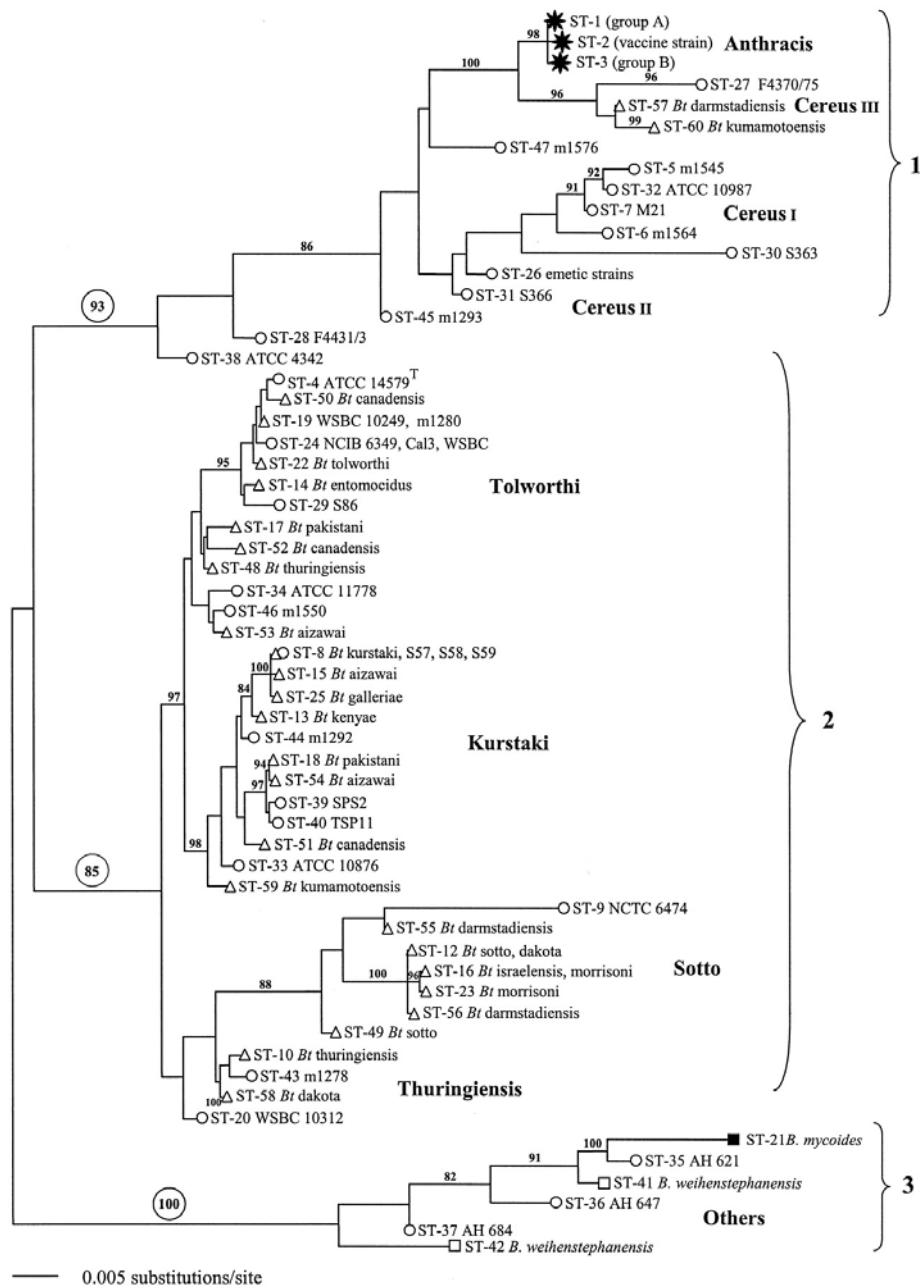


**Figure 2: Horizontal method for the enumeration of presumptive *B. cereus*-colony count technique at 30°C (schematic representation of method described in ISO 7932:2004)**

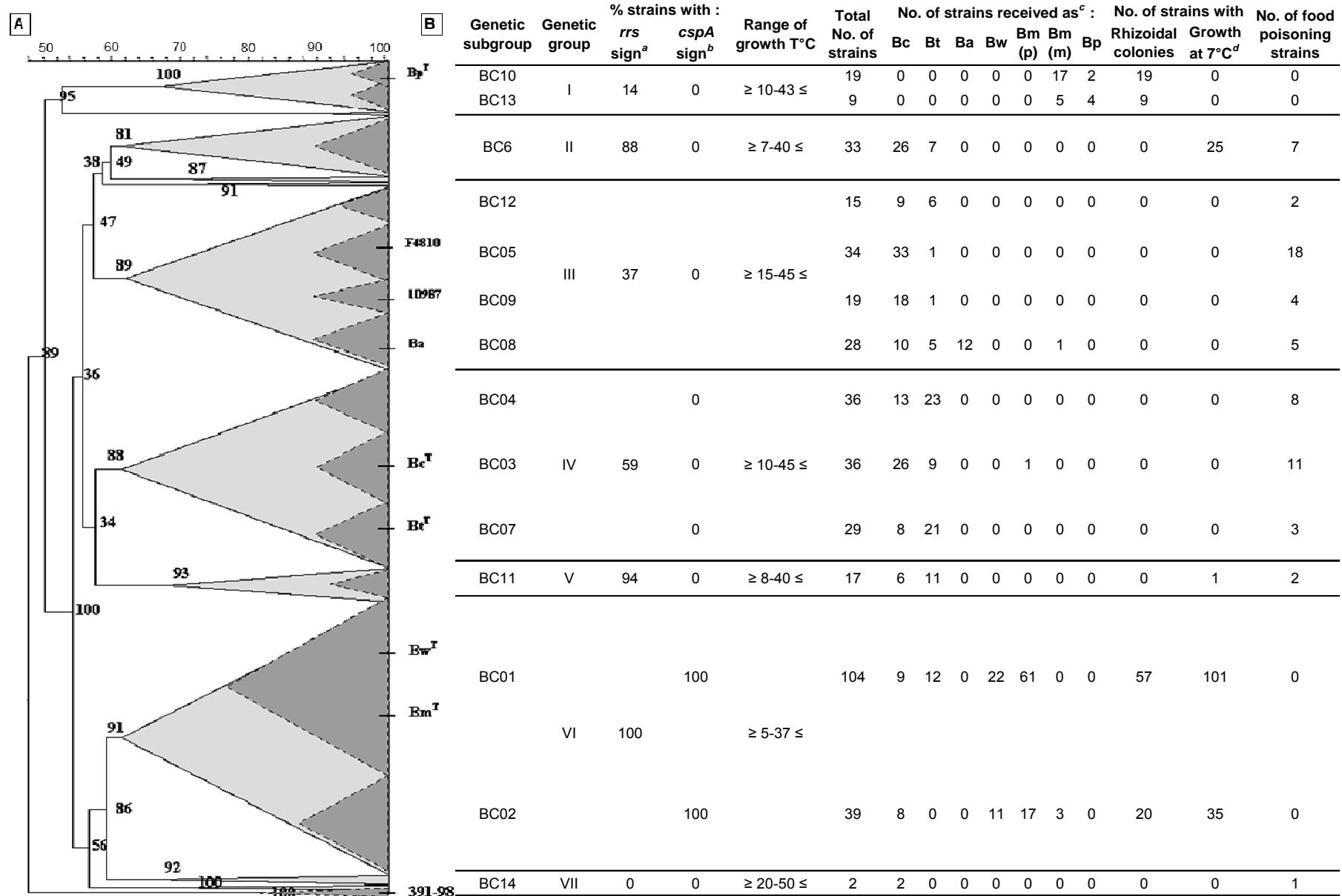


<sup>a</sup> 0.1 ml on 1 plate of 90 mm with sterile pipette (or 1 ml on 1 140mm-plate or on 3 90mm-plates)

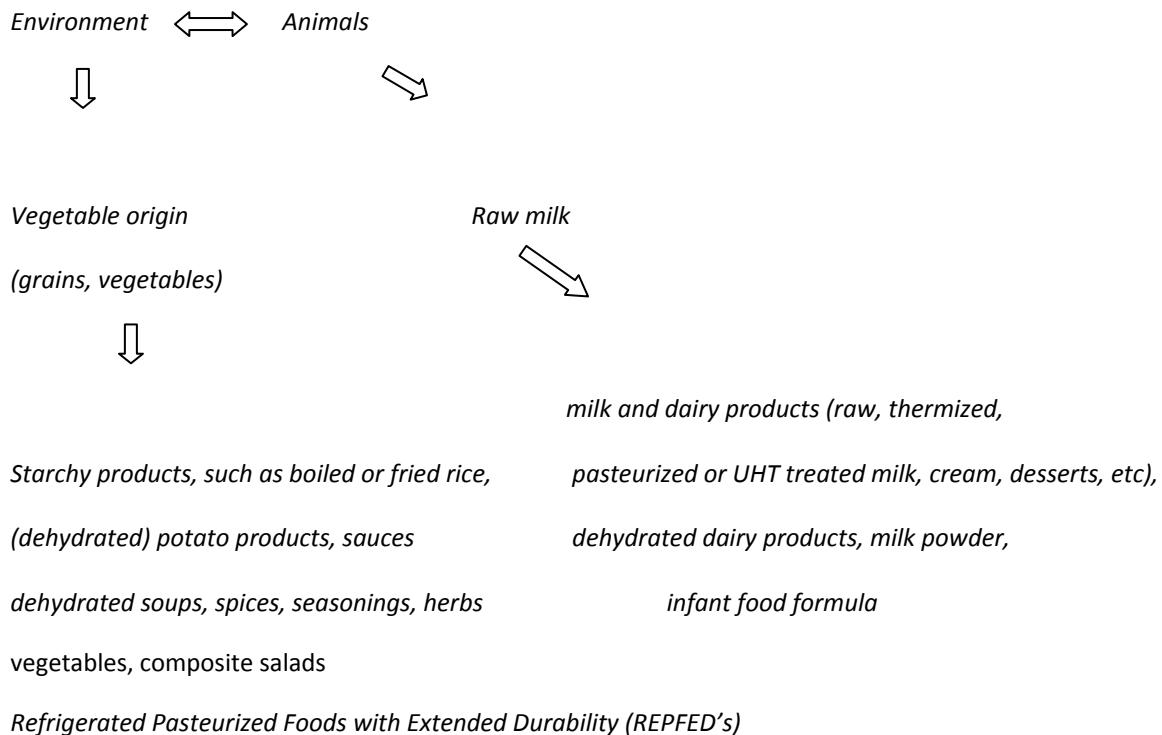
**Figure 3: Phylogenetic tree based on MLST of gene sequences of *glpF*, *gmk*, *ivID*, *pta*, *pur*, *pycA* and *tpi* (taken from Priest *et al.* 2004).**



**Fig. 4: Relationship between genetic groups in the *B. cereus* group and key characteristics of the strains (taken from Guinebretière *et al.* 2007).**



**Figure 5: Transmission routes of *Bacillus cereus* in the food chain**



## **Figure 6. Mechanisms of *B. cereus* foodborne outbreaks**

### *B. cereus* Diarrhoea toxin – Toxicoinfection

- Food contaminated with spores
  - preparation (insufficient heat treatment)
- Survival of thermoresistant spores
  - slow cooling down (too long period at 10-45°C)
- Germination, growth (and diarrhoeal toxin production)
- Food + high numbers vegetative cells (+ toxin)
  - insufficient heat treatment
- Food + high numbers vegetative cells (+ toxin)
  - Consumption  $\Rightarrow$  (proteolysis of toxin)
  - Multiplication/Sporulation + Toxin production (*Clostridium perfringens*)
  - Multiplication + Toxin production in GI tract
- Symptoms

### *B. cereus* emetic toxin - Intoxication

- Food contaminated with spores
  - $\Rightarrow$  preparation (insufficient heat treatment)
- Survival of thermoresistant spores
  - $\Rightarrow$  slow cooling down (too long period at 10-45°C)
- Germination, growth and toxin production: Food + high numbers of *B. cereus* vegetative cells + emetic toxin
  - $\Rightarrow$  heat treatment
- Food + emetic toxin
  - $\Rightarrow$  Consumption
- Symptoms

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