Opinion of the Scientific Panel on Biological Hazards on *Bacillus cereus* and other *Bacillus* spp in foodstuffs. [1]

(Question N° EFSA-Q-2004-010)

Adopted on 26-27 January 2005

SUMMARY

Bacillus cereus is the cause of two kinds of foodborne diseases, an emetic (vomiting) intoxication due to the ingestion of a toxin (cereulide) pre-formed in the food and a diarrhoeal infection due to the ingestion of bacterial cells/spores which produce enterotoxins in the small intestine. Other *Bacillus* spp., *B. subtilis, B. licheniformis, B. pumilus* have more rarely beeen identified as agents of foodborne diseases characterized by diarrhoea and/or vomiting. Emetic intoxication is caused by a very homogeneous group of strains of *B. cereus* identified by their ability to produce cereulide. In contrast, *B. cereus* strains able to cause diarrhoea are not easy to identify because the mechanisms leading to infection are complex and diverse. Very little is known on the virulence mechanisms of other *Bacillus* spp and therefore it is not possible to identify the strains able to cause foodborne poisoning.

In most instances, foodborne diseases caused by *B. cereus* were associated with 5 log to 8 log cells/spores per g of the food vehicle. However, in some outbreaks, lower numbers in the food (3 – 4 log per g) were reported. Foodborne poisoning caused by other *Bacillus* spp. has always been linked to high numbers of cells/spores in the food vehicle (equal or more to 6 log per g).

Bacillus cereus is ubiquitous and low numbers of its spores, too low to cause foodborne poisoning, can be found in a wide range of foodstuffs. Spores can germinate and multiply in humid, low acid foods, from 4-5°C to 55°C. However, strains able to multiply below 7°C, and strains able to multiply above 45°C, are not the most common. Emetic *B. cereus* are presumably unable to grow and produce their toxin cereulide below 10°C, or in the absence of oxygen. Other *Bacillus* spp. involved in

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foodborne poisoning cases are also frequent causes of food spoilage. Almost all kind of foods have been implicated in *B. cereus* foodborne poisoning. However, a majority of reported outbreaks were linked to the consumption of heat treated foods and frequently occurred in restaurant and catering establishments. Failure in refrigeration was frequently suspected. Cooked dishes containing pasta or rice were the main, but not the only, foods implicated in emetic intoxications.

The major control measures are to control temperature and to establish HACCP system. Only heat treatments used for canning of low acid foods will ensure a complete destruction of spores of *B. cereus*. The number of spores in other processed foods must be kept as low as possible by proper cleaning and disinfection of equipments. Rapid cooling is necessary to prevent germination and growth of *B. cereus* spores. Low pH (below 4.5), reduction in a_w (below 0.92) would inhibit *B. cereus*. In other cases, refrigeration below 4°C is necessary to prevent growth of all types of *B. cereus*, including psychrotrophic strains. However, below 10°C, lag time and generation times are very significantly increased, particularly whenever other factors (i.e. pH, a_w, nutrient content of the food) are not optimum for *B. cereus*. This should be verified by microbiological testing. Control measures for *B.cereus* would contribute to control other *Bacillus spp.*

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BACKGROUND

Bacillus cereus is an important cause of food-borne illnesses in humans. It is a ubiquitous bacterium in the environment, and it can thus be present in a wide range of different foodstuffs. Food-borne outbreaks caused by *B. cereus* have been associated with many types of foodstuffs including both food of animal and plant origin. Also some other *Bacillus* species, such as *B. licheniformis* and *B. subtilis*, have occasionally been reported as a cause of food-borne illnesses in humans.

Several Member States have in their national legislation or guidelines, criteria for *B. cereus* in various foodstuffs. However, current Community legislation does not include any specific provisions on *B. cereus* or other *Bacillus* sp. in foodstuffs.

Community legislation on food hygiene is currently under revision. In this framework a revision of the microbiological criteria in Community legislation is taking place. The revised hygiene legislation provides also, among other things, a legal basis to set specific temperature control requirements for foodstuffs, when appropriate.

Question

The European Food Safety Authority is asked to:

- identify the categories of foodstuffs and the food manufacturing and preparation processes where *Bacillus cereus* or other *Bacillus* spp. may pose a risk for human health;
- establish, for the different categories of foodstuffs identified, the relation between the number of *Bacillus* spp. bacteria in the foodstuffs and the ability of these foodstuffs to cause food-borne illness;
- list and evaluate specific control measures, including microbiological testing and temperature requirements, to manage the risk caused by *Bacillus cereus*, other *Bacillus* spp. and their toxins in foodstuffs. In doing so attention should be paid to different species of *Bacillus* spp.

ASSESSMENT

1. HAZARD IDENTIFICATION

Among *Bacillus* species identified as a cause of foodborne poisoning, most of the knowledge available concerns *Bacillus cereus*. The relative incidence of *B. cereus* foodborne poisoning in developed countries are with a few exceptions a minority of the total reported foodborne disease outbreaks. Some examples are given below and in Table 7 in annex.

Norway: in the period 1988 to 1993 around 33% of reported bacterial foodborne poisoning cases were linked to *B. cereus* (Granum and Baird-Parker, 2000). However no outbreak due to *B. cereus* was reported in 1999-2000 (WHO 2000a).

Netherlands: in the period 1993-1998 2% of reported outbreaks were caused by *B. cereus* (WHO 2000b) and in 2003 no outbreak among 86 reported was attributed to *B. cereus* (Brandsema *et al.* 2004).

England and Wales: in the period 1993-1999, over 1093 foodborne outbreaks with known causative agent, 2% were caused by *B. cereus* and 1% by *B. subtilis* (WHO 2000b)

In France, from 1998 to 2000, *B. cereus* represented 4 to 5 % of foodborne poisoning outbreaks of known origin (Haeghbaert *et al*., 2001, 2002a and 2002b).

In Northen America, *B. cereus* represented 1 to 2% of outbreaks of identified origin (Granum and Baird-Parker, 2000).

The incidence of *B. cereus* foodborne poisoning per capita is presumably not so different among European countries. However, comparison of data from different countries is difficult, because foodborne poisoning caused by *B. cereus* is usually mild, and reporting of sporadic cases is not mandatory. Therefore it is presumably under reported in most countries.

Foodborne cases caused by other *Bacillus* species (*B. subtilis*, *B. licheniformis* and *B.pumilus*) have also been reported (Kramer and Gilbert, 1989; Salkinoja-salonen *et al.*, 1999).

As a pathogen, *B. cereus* is not only involved in foodborne poisoning but also in various clinical human infections such as septicemia, meningitis, gingival and ocular infections (Teyssou *et al*., 1998).

B. cereus is also considered as a beneficial bacterium, some strains being used as probiotics (Hoa *et al*. 2000, Kniehl *et al*., 2003) or as plant growth promoter (Halverson and Handelsman, 1991).

Bacillus foodborne poisoning hazard is therefore difficult to identify because it concerns several *Bacillus* species, not solely *B. cereus*, and it presumably does not involve all *B. cereus* strains.

Bacillus anthracis will not be considered in this report.

1.1. Foodborne poisoning caused by *Bacillus cereus*

Two kinds of foodborne poisoning have been attributed to *B. cereus.*

- Vomiting (emetic) poisoning caused by a ring-formed peptide, heat stable toxin, named "cereulide";

- Diarrhoeic poisoning most presumably caused by several heat labile proteins with enterotoxic activities.

Emetic poisoning is characterised by vomiting, followed with diarrhoea after 8 to 16 h in approximately a third of the cases, and occurs within 1 to 5 hours after ingestion of contaminated foods (Kramer and Gilbert, 1989). Emetic syndrome is usually mild but rare fatal cases have been reported (Malher *et al.* 1997). The emetic toxin "cereulide" is produced in the food and poisoning occur after ingestion of the toxin.

Diarrhoeal poisoning is characterized by watery diarrhoea associated with abdominal pain and occurs within 8 to 16 hours after ingestion of the contaminated food (Granum and Lund, 1997). It is generally mild, but bloody diarrhoea leading to some fatal cases has been described (Lund *et al.*, 2000). It is generally recognized that diarrhoeal poisoning occurred through production of enterotoxins in the intestine by ingested bacterial cells (Granum 2001), see § 7.1 for more details.

Therefore, *B. cereus* represents two distinct hazards:

- emetic poisoning, a foodborne intoxication for which amount of emetic toxin (cereulide) ingested is important,

- diarrhoeal poisoning, a foodborne infection, for which number of bacterial cells/spores ingested is important.

1.2. Taxonomy of the *Bacillus cereus* group

B. cereus and the 5 species *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides* form a very homogeneous group with 99% similarity in their 16s rRNA sequences (Ash *et al.*, 1991; Ash and Collins, 1992). These 6 species can be distinguished by phenotypic and genotypic features (Claus and Berkeley, 1986; Lechner *et al*., 1998; Nakamura, 1998)

The distinction among these species within *B. cereus sensu lato* has important implications for food and human safety: *B. cereus* is currently considered as a foodborne disease agent, *B. thuringiensis* produces a parasporal crystal toxic against insects and is used as a bio-pesticide, *B. anthracis* is the agent of anthrax, and *B. weihenstephanensis* is able to grow at 4-5 ° C. However, the distinction of the species *B. thuringiensis*, *B. cereus* and *B. anthracis* is not supported by recent results in taxonomy (Guinebretière and Sanchis, 2003, Helgason *et al.* 2004). In particular, the ability to produce the parasporal crystal, the distinctive feature between *B. thuringiensis* and *B. cereus* can be carried by a plasmid. In such case, loss of the plasmid would turn a *B. thuringiensis* into a *B. cereus* and vice versa.

In contrast, *B. weihenstephanensis*, which regroup the strains form *B. cereus* group able to grow at 4-5°C, is presumably disctinct from *B. cereus* sensu stricto (Lechner *et al*. 1998, Guinebretière *et al*. in preparation[2]).

Standard methods (ISO7932 and 21871) for detection and enumeration of *B. cereus* do not distinguish *B. cereus* from *B. thuringiensis* and *B. weihenstephanensis*. Therefore, most reports of incidence of *B. cereus* in foods or of *B. cereus* foodborne poisoning could concern any of these three species.

1.3. Virulence factors of *Bacillus cereus* linked to foodborne poisoning

1.3.1. Emetic foodborne intoxication

The emetic toxin cereulide is a small cyclic peptide (Agata *et al*. 1994 and 1995). Because cereulide is very stable, it may persist in heat treated foods after death of the *B. cereus* cells.

All *B. cereus* involved in emetic foodborne infections produce cereulide and form a very homogenous group with distinct phenotypic properties (Pirttijarvi *et al*., 1999; Raevuori *et al*., 1977). They presumably represent a clonal lineage (Pirttijarvi *et al*,. 1999, Ehling-Schulz *et al*., 2004a and b) hereafter called "emetic strains" or "emetic *B. cereus*".

1.3.2. Diarrhoeal foodborne infection

Several proteins produced by *B. cereus* have been identified as virulence factors involved in diarrhoeal poisoning (Table 1). These proteins exhibit a cytotoxic activity on Caco2 human epithelial cell lines. Enterotoxic activity was demonstrated for some of them on ileal loop test. An extensive description of the various potential enterotoxins of *B. cereus*, can be found in Granum and Baird-Parker (2000).

[2] Also in the final report of the European project QLK1-2001-00854, that will be available at www.avignon.inra.fr/BACILLUS_CEREUS/Page1/Accueil.htm.

Table 1 – *Bacillus cereus* toxins involved in diarrhoeal poisoning

Toxin names				
Hemolysin BL (HBL)	*Genes* / Proteins	*hblC* / L2	*hblD* / L1	*hblA* / B
	Toxin formed by 3 proteins, coded by 3 genes organised in 1 operon			
Non haemolytic enterotoxin (NHE)	*Genes* / Proteins	*nheA* / A	*nheB* / B	*nheC* / C
	Toxin formed by 3 proteins, coded by 3 genes organised in 1 operon			
Cytotoxin K (CytK)	*Gene* / Protein	*CytK* / CytK		
	One single protein.			

A large proportion of strains from the species *B. cereus, B. thuringiensis* and *B. weihenstephanensis* have the genes for at least one of these diarrhoeal toxins (Guinebretière *et al*. 2002; Gaviria Rivera *et al*., 2000; Stenfors *et al*., 2002). The ability to cause diarrhoeal foodborne poisoning is presumably distributed over *B. cereus* and some related species, in particular *B. thuringiensis*. Several strains of *B. thuringiensis* have diarrhoeal virulence features similar to those of *B. cereus* strains that have been implicated in diarrhoeal foodborne infections (production of diarrhoeal toxins and cytotoxic activity, Gaviria Rivera *et al*. 2000) and a strain of *B. thuringiensis* was implicated in a case of foodborne diarrhoea (Jackson *et al.* 1995).

However, all *B. cereus* strains certainly do not have the same ability to cause diarrhoea. Amounts of enterotoxins produced and cytotoxic activity on Caco2 cells vary considerably among strains (Guinebretière *et al.* 2002, Choma *et al*. 2000).

The determinism of diarrhoeal foodborne infection is more complex than that of emetic foodborne intoxication. It is currently not possible to easily identify a "diarrhoeal strain".

1.4. Other *Bacillus* spp

Food poisoning caused by other *Bacillus* spp (*B. subtilis, B. licheniformis and B. pumilus*) has been poorly investigated. Most of our knowledge comes from investigations of 82 incidents, mostly in the UK and to a lesser extent in Norway and Finland (Kramer and Gilbert, 1989; Salkinoja-Salonen *et al*., 1999). Symptoms of food poisoning were vomiting and/or diarrhoea. These

cases were attributed to *Bacillus* spp on the basis of their presence in large numbers in the faeces and/or vomit of patients, the absence of other pathogens in the food and the high numbers of *Bacillus* cells in the food.

As in the case of *B. cereus*, poisoning was mild, except one fatal case linked to infant food formulae. Apart from a heat stable, non proteinaceous toxin produced by some of the strains implicated in foodborne poisoning, nothing is known on the virulence factors of these *Bacillus* spp.

2. HAZARD CHARACTERISATION INCLUDING DOSE-RESPONSE RELATIONSHIP

2.1. Aetiology of *B. cereus* intoxications and toxico-infections

Nature of B. cereus *cells present in foods at consumpion*

Diarrhoeal poisoning is most probably due to *B. cereus* cells ingested with the food (see §1). Most foods will be contaminated with spores of *B. cereus* (see § 3.1.2) and whenever conditions are not favourable to spore germination and growth (see §4), *B. cereus* would be ingested as spores. In contrast, whenever conditions in the food permit germination and growth, *B. cereus* would be ingested as vegetative cells. In particular, refrigeration, considering a mean temperature of refrigerators of 4-7°C (Notermans *et al.* 1997) would select the psychtrotrophic strains of *B. cereus*.

Conclusively, the number of viable *B. cereus* cells and the nature of the organisms (vegetative or spores, respectively psychrotrophy or mesophily) that may be ingested is determined by the combination of initial contamination and the way the food is handled prior to ingestion. In the case of emetic poisoning, production of emetic toxin in the food would depend of the presence of strains carrying the genes responsible for emetic toxin production and of food conditions (see § 3.1.4 and 3.1.5).

Fate of B. cereus cells in the digestive tract

After consumption food reaches the stomach where the ingested *B. cereus* cells and toxins are exposed to low pH and the action of pepsin. In case the food was handled properly only spores will be present and irrespective of the pH of the stomach spores will reach the small intestine. If, on the other hand, the food has been handled improperly spores, vegetative cells and toxins will be present. In this case the emetic toxin, depending on the concentration, will bind to the 5-HT_3 receptors in the stomach and causes the emetic intoxication. It depends on the pH of the stomach whether only spores or spores and vegetative cells pass the stomach to reach the small intestine. A sufficiently low pH will kill the vegetative cells, but "higher" pH's give the opportunity to vegetative cells to reach the small intestine (Clavel *et al.* 2004). Due to enzymatic activity in the stomach the diarrhoeal toxins will be destroyed.

pH of the stomach during digestion of the contaminated food (depending on the consumer and on the composition of the meal) and nature of *B. cereus* cells in the food (vegetative cells or spores) would certainly influence the number of *B. cereus* cells needed to cause diarrhoea. This certainly complicates the determination of the relation between number of *B. cereus* and disease.

The process in the small intestine leading to a diarrhoeal infection is not completely understood. But, germination of spores, growth and toxin production have to occur within a certain period of time based on the following information on the kinetics of the diarrhoeal syndrome:

- The onset of symptoms is 6 – 24 hours after consumption (Kramer and Gilbert 1989).

- The mean transit time through stomach and small intestine has been calculated to be 6 hours (Takumi 2000).

- Production of enterotoxins takes place during the exponential growth phase (Granum, 1994).

2.2. Influence of subgroups of *B. cereus* on the diarrhoeal type of disease

It is not possible to easily identify *B. cereus* strains able to cause diarrhoeal poisoning and therefore it is not possible to determine their incidence in foods. However, all strains present in foods may not be able to cause diarrhoea. The majority of strains isolated from foods produced only low amounts of enterotoxins at 35°C, whereas strains that have been isolated from food poisoning cases were found to produce high amounts (Guinebretière *et al*. 2002). Similarly, Table 2 illustrates the diversity of potential virulence factors among strains present in foodstuffs.

An interesting observation is that among strains isolated from foods, all strains able to grow at 5°C had very low or undetectable toxic activity on Caco2 cells, presumably indicating a low ability to cause diarrhoea (Choma *et al*. 2000).

In addition, considering the information we have on the kinetics of diarrhoeal syndrome, psychrotrophic strains (able to grow at 4-5°C) can be considered less pathogenic in the diarrhoeal type of disease, due to their lower growth rate at 37°C (maximum growth temperature) and the fact that they do not produce (or only low amount) of enterotoxins at 37°C. The mean passage time in the small intestine is *ca* 4 h. In this period spores have to germinate and grow to the end logaritmic phase to produce enough diarrhoeal toxins to cause disease. This is nearly impossible for psychrotropic strains (Wijnands, *et al.* 2004). For vegetative cells of psychrotrophic strains this is also the case, due to a long lag time in combination with the above mentioned lower growth rate.

In conclusion, psychrotrophic strains (able to grow at 4-5°C) might be less able to cause diarrhoeal syndrome that mesophilic strains. Concerning, emetic syndrome, psychrotrophic strains do not seem able to produce emetic toxin.

Table 2 Characteristics of strains from rice products at retail in the Netherlands contaminated with *B. cereus.* Data from RIVM report 250912008 Prevalence- and virulence factors of *Bacillus cereus* in food commodities in retail in the Netherlands (will be published in 2005).

		Number of strains containing:						
Contamination level [1]	**Sample number**	**Total number of strains[2]**	**Complete set of HBL genes [3]**	**Complete set of NHE genes [4]**	**Genes for Cytotoxin K**	**Capability to produce emetic toxin [5]**	**Genes for mesophily**	**Genes for psychotrophy**
1-2	1	5	5	0	5	0	5	0
2-3	1	5	5	0	3	0	5	0
	2	5	5	5	3	1	5	2
4-5	1	3	2	3	3	0	3	0
	2	5	2	5	5	0	5	0
5-6	1	5	3	0	1	0	4	1
	2	5	0	0	0	0	5	0
	3	5	4	0	4	0	5	0
	4	5	0	0	0	0	5	0
	5	5	0	0	4	0	5	0
	6	5	5	5	5	0	5	0
	7	5	3	3	3	1	5	0
	8	5	5	0	5	0	5	0
	9	5	0	0	0	0	1	4
	10	5	0	0	0	0	5	0
	11	5	0	5	5	0	5	0
	12	5	0	5	5	0	5	0
	13	5	3	4	4	0	5	0
6-7	1	5	0	0	0	0	5	0

[1)] colony forming units of *B. cereus* g^{-1} food; [2)] number of colonies from the enumerated plates, tested for different characteristics; [3)] the 3 genes encoding the 3 parts of the HBL toxin were detected by PCR; [4)] the 3 genes encoding for the 3 parts of the NHE toxin were detected by PCR; [5)] emetic toxin was detected in an extract of colony material by Hep cell test.

2.3. Dose response relations

2.3.1. Emetic toxin dose response assessment

In the summer of 2000 an outbreak of *B. cereus* intoxication occurred in the Netherlands. Within 2 hours after consumption of a vegetarian rice dish around 100 students out of a group of about 1200 suffered from vomiting and abdominal pain. In the food samples emetic toxin was detected in concentrations of 0.03–13.3 μg valinomycin equivalents g^{-1} food. From *in vitro* experiments it was calculated that 10^5–10^8 cells of emetic toxin producing *B. cereus* strain(s) g^{-1} food were necessary. This is consistent with observation that in laboratory, emetic toxin was detected only at the end of the growth phase of *B. cereus* (see §3.1.4).

Due to the great stability of emetic toxin, *B. cereus* might have been killed, by heating of the food before consumption for instance, and emetic toxin would still be present. This might explain the emetic poisoning cases in Table 9 (annex) with low numbers (10^2 per g) found in the food. Therefore, number of *B. cereus* in food does not necessarily reflect the risk of emetic poisoning. However, important growth of *B. cereus* during the food history is necessary. In table 9 in annex, a great number of food-borne outbreaks *B. cereus* toxico-infections and intoxications with a short incubation period contains 10^5.10^8 *B. cereus* per g.

2.3.2. Diarrhoeal toxin dose response assessment

<u>*Based on epidemiological data*</u>

Pathogenic organisms or their toxic products entering the human body via ingestion meet a system of barriers of the host. The organism or its toxic product has to reach the parts of the gastro-intestinal tract suitable to attachment, growth, toxin production and/or absorption, before it is capable to cause adverse health effects. As described in section 2.1 the mechanism leading to disease is very complex and not completely understood. Also, epidemiological data as presented in the literature are not always useful, mostly due to the incompleteness of the clinical signs, ingested dose of micro-organisms as well as toxins, and incubation periods. Based on reliable epidemiological data and laboratory experiments dose response assessments for both types of *B. cereus* food poisoning are performed.

After the first recognized diarrhoeal outbreak of *B. cereus* foodborne illness in Oslo (caused by contaminated vanilla sauce), Hauge isolated the bacterium, grew it to 4 x 10^6 cells per ml, and drank 200 ml of the bacterial culture. After about 13 h he developed abdominal pain and watery diarrhoea that lasted for about 8 h. The infective dose was about 8 x 10^8 cells. Later studies of outbreak related incriminated foods revealed *B. cereus* counts ranging from 200 to 10^9 per g (or ml), with calculated infective doses ranging from 5 x 10^4 to 10^{11} cells (Table 9). The infective dose may vary from 10^5 to 10^8 viable cells or spores in

part because of the large differences in the amount of enterotoxin produced by different strains. Hence, food containing more than 10^4 cells/spores per g may sometimes pose a risk (Granum and Lund, 1997).

Little is known about susceptible populations, but more severe symptoms have been associated with young athletes submitted to intensive training (<19 years old) and the elderly (>60 years old) (Granum and Lund, 1997).

Based on modelling from experimental data

It may be concluded from the RIVM report 250912007 that for 50% epithelial cells death in the first meter of the small intestine, leading to diarrhoea, 1 x 10^{10} *B. cereus* are needed. With the model described in RIVM report 250912 005, it is calculated that this number of cells can be reached by mesophilic *B.* cereus within 8 h after consumption of 10^9 spores. This means, by consumption of a meal of 500 g, a contamination level of 2 x 10^6 spores g^{-1} food. This is partly confirmed by the number of cfu g^{-1} food in the outbreaks in 9b (Annex) with an higher incubation time and diarrhoea as clinical sign.

3. EXPOSURE ASSESSMENT.

3.1. Ecology of Bacillus cereus and other foodborne poisoning Bacillus spp

3.1.1. Primary Reservoir

Soil can contain between 10^3 and 10^5 spores of *B. cereus* per gram (Guinebretière and Nguyen-the, 2003; Te Giffel *et al*. 1995; Christiansson *et al*. 1999). Development of some strains of *B. cereus* and of some strains of the very close species *B. thuringiensis* was observed in the rhizosphere of plants and in the gut of earthworms (Hendriksen and Hansen, 2002; Halverson *et al.*, 1993). The climate presumably influences the *B. cereus* population in soil, psychrotrophic strains being more frequent in soil from cold regions (von Stetten *et al*., 1999).

3.1.2. Incidence in the food production chain

Soil is the primary source of contamination of foods with spores of *B. cereus*. For instance, the same genotypes were found in the soil of dairy farms and in the milk (Christiansson *et al*., 1999), and in the soil on which vegetables were grown and in cooked chilled foods containing the vegetables (Guinebretière and Nguyen-the, 2003).

Additional contamination during processing may occur because spores of *B. cereus* have strong adhesion properties, might form biofilms and may persist on the surface of processing equipment (Andersson *et al.* 1995). For instance, raw milk can be contaminated by *B. cereus* strains that persist in milk silo tanks (Svensson *et al.,* 2004). Contamination of pasteurised and powdered milk by *B. cereus* strains persisting in pasteurising and drying equipments was also suspected (Eneroth *et al*., 2001; Svensson *et al*., 1999; Te Giffel *et al*., 1996a).

In complex foods, some ingredients have been identified as important source of contamination with *B. cereus* spores, such as texturing agents, (Guinebretière and Nguyen-the, 2003), liquid eggs, herbs and spices (ICMSF, 2005)

Spores of *B. cereus* were also found in paper mill industries and in packaging materials (Pirttijarvi *et al.*, 2000) which could represent an additional route of contamination for foods.

In conclusion, *B. cereus* is ubiquitous and its presence in most raw foods appears as inevitable. Additional contamination during some processing steps indicates that good hygienic practices and hygienic design of equipments are essential to reduce contamination of some processed foods.

3.1.3. Incidence in various foods, in relation to processing conditions

***Bacillus cereus* in general**. *B. cereus* has been isolated in almost all the categories of foodstuffs (Table 3). Raw fruits and vegetables, raw herbs, dry foods, raw milk and processed foods before storage usually contain < 100 spores/g. However, in some herbs and spices, presence > 1000 spores g^{-1} was reported. (ICMSF, 2005). *B. cereus* is frequently present as spores either because vegetative cells have been killed by preparation of ingredients (for instance mild heat treatment, drying…) or because conditions do not permit spore germination and growth (dry product such as spices and herbs for instance, see § 4 for more details on conditions permitting growth of *B. cereus*). The presence of spores in such raw materials is usually not a concern as such. However, *B. cereus* spores would survive cooking (see section 4 on "Control measures" for more details on heat resistance) and high numbers of spores in spices and herbs can be a problem whenever they are used in processed foods allowing growth of *B. cereus*.

Storage of the processed product, or use of raw materials in complex foods with conditions suitable for *B. cereus* (for instance spices in recipe dishes, or liquid eggs in refrigerated custards or cream caramel) permit spore germination and growth of *B. cereus* to numbers that might represent a hazard for consumers. *B. cereus* is not particularly tolerant to acid and low water activity (see section 4 for more details). Most foods with high humidity and not acidified would support its growth. It is worth noting that innovation in food processing might create favorable conditions in foods previously considered as unfit for growth of *B. cereus*. For instance, in the case of bread produced by the "partial baking process" which consist in two baking steps, the temperature in the middle of the dough never goes over 100°C, allowing spores to survive, and the finished bread has a water activity of 0.96, favorable for *B. cereus* growth (Leuschner *et al.* 1997).

Growth to numbers representing a hazard is limited by refrigeration. Below 10°C, only a minority of the strains present in a food product will be able to grow (Table4). Refrigeration will therefore reduce the diversity of *B. cereus* populations present in foods at consumption. For instance, in pasteurized dairy

cream, *B. cereus* was the dominant species of the microflora for samples stored at 12°C but only a minor component for samples stored at 7°C (Dommett, 1992). However, some strains of *B. cereus* and related species are able to grow between 10 and 4°C. *B. cereus* might reach high numbers (10^5 CFU / g) if the storage period is long enough, and if psychrotrophic strains are present, as shown from examples in Table 3, but it would reduce the probability to find such high levels of *B. cereus*. Effect of refrigeration to limit growth of *B. cereus* is described more in detail in the section on control measures.

Some strains of *B. cereus* are able to grow at temperatures as high as 55°C (Table 3) and might multiply rapidly during cooling of heat treated foods.

Food poisoning *B. cereus*. Emetic *B. cereus* can be distinguished from other *B. cereus* by their ability to produce cereulide. Most investigations did not specifically enumerate emetic *B. cereus*. However, as all emetic *B. cereus* are unable to hydrolyse starch, incidence of starch negative *B. cereus* could provide an estimate of emetic *B. cereus*. Starch negative *B. cereus* represented at most 2% to 11% of strains isolated from dairy products and from dairy farms (Te Giffel *et al*., 1995). Starch negative strains represented only 4% of *B. cereus* from various origins (Logan and Berkeley, 1984). We can assume that incidence of emetic *B. cereus* is below these figures as all starch negative *B. cereus* are not necessarily emetic. In contrast, no easy method can distinguish *B. cereus* able to cause diarrhoea. How frequent they can be in foods in therefore not known.

Table 3 – Examples of incidence of *B. cereus* in various foods

Food categories	% of positive samples	Numbers of *B. cereus* in positive samples (cfu g^{-1}	References

	(limit of detection cfu g^{-1})	or ml^{-1})	
Spices	42 % (10^2)	most samples < 10^4 - few samples >10^4	Van Netten *et al.* 1990
Herbs and spices	100% (10^2)	10^2 to 10^6	Te Giffel *et al.* 1996b
Ready to eat chilled foods in self service restaurants	6 to 21% (all categories positive: vegetables, rice, pasta, meat and fish)	10^3 to 10^5	Amodio-Cocchieri *et al.* 1998
Fresh vegetables	0 to 100% (10)	10^2 to 8 x 10^3	Valero *et al.* 2002
Vegetable salads	2 % (10^2)	< 10^3	Van Netten *et al.* 1990
Cooked chilled foods (unstored)	0% (10)	< 10	Choma *et al.* 2000
(stored at 4°C until use by date)	0% (10)	< 10	
(stored at 10°C until use by date)	0 to 100%	10^4 to 10^6	
Flour	55% (10^2)	10^3	Te Giffel *et al.* 1996b
Liquid egg yolk pasteurised	24% (10^2)	< 10^3	Van Netten *et al.* 1990
Bakery product	90% (10^2)	10^3 to 10^4	Te Giffel *et al.* 1996b
Pasteurised milk after storage at 7°C until "best before" date	8% (10^2)	From < 10^3 to > 10^5	Van Netten *et al.* 1990
Pasteurised milk after 8 days at 7°C	56% (10)	From 10^3 to 3 x 10^5	Larsen and Jorgensen 1997
Milk powder	27% (10)	4 spores g^{-1} (mean), up to 40 spores g^{-1}	Van Netten *et al.* 1990
Powdered infant formulae	75% (0.04)	0.04 to 1 MPN g^{-1}	Harmon and Kautter, 1991

Table 4 – Percentage of strains of *Bacillus cereus* able to grow at various temperatures

Origin (number)	Temperatures tested (°C)	References

of strains tested

of strains tested								
Various foods and diarrhoeal poisoning (17)	-	-	-	47	100	99	47	*[1]*
Various foods and foodborne poisoning (27)	0	7	52	-	100	-	-	*[2]*
Pasteurised milk (106)	-	53	100	100	100	38	-	*[3]*
Various foods (596)	-	15	-	-	100	85	-	*[4]*
Various foods and foodborne poisoning (31)	13	16	45	100	-	-	-	*[5]*
Various foods and food poisoning (11)	2	72	-	-	100	90-81	9	*[6]*
Cooked chilled foods (83)	10	-	62	-	100	81	-	*[7]*

-: not tested. [1]: Rajkowski and Milolajcik (1987), [2]: Foegeding and Berry (1997), [3]: Te Giffel *et al*. (1997), [4]: Van Netten *et al*. (1990), [5]: Dufrenne *et al*. (1994), [6]: Andersen Borge *et al*. 2001, [7]: Choma *et al*. (2000).

No emetic *B. cereus* able to grow below 10°C has been described so far[3] (Carlin *et al*., in preparation). Therefore we could assume that refrigeration should prevent development of emetic *B. cereus*.

Among *B. cereus* strains previously implicated in diarrhoeal foodborne poisoning, no strains able to grow at 4°C have been described so far[3] (Nguyen-the *et al*., in preparation). However, several of these strains were able to multiply at 7°C.

Other *Bacillus* spp. Other Bacillus *spp* that have been the cause of foodborne poisoning (*B. subtilis, B. licheniformis, B. pumilus*) were identified as cause of spoilage of foods (Guinebretière *et al*. 2001, Rodriguez *et al*. 1992). In particular, some strains are able to multiply in acid foods such as canned tomato juice (Rodrigez *et al*., 1992). These species are also involved in traditional fermented beans dishes in Africa and India (Ogbadu and Okagbue *et al*. 1988, Sarkar *et al*. 2002). However, it is not known if the strains implicated

[3] Also in the final report of the European project QLK1-2001-00854, that will be available at www.avignon.inra.fr/BACILLUS_CEREUS/Page1/Accueil.htm.

in spoilage or fermations were able to cause poisoning. No strains from these species able to grow below 10°C have been described so far.

3.1.4. Toxin production in foods

Diarrhoeal infection. Several evidences support the assumption that diarrhoeal infection occurs through production of enterotoxins in the small intestine and not by toxins produced in the food (Granum and Lund 1997). Production of enterotoxins in foods by *B. cereus* is possible (van Netten *et al*. 1990), but is presumably of little importance to assess the risk of diarrheal infection.

Emetic intoxication. Emetic intoxication occurs through ingestion of emetic toxin (cereulide) preformed in the food. Therefore determining conditions in the foods that would lead to production of cereulide by emetic *B. cereus* is important for risk assessment of emetic intoxication. Cereulide is not easily destroyed by heat treatments. For instance, it can resist 90 min at 126°C (Turnbull *et al*., 1979; ICMSF, 1996). It is also resistant to acid conditions. Cereulide will therefore not be eliminated from foods in which it had been produced.

Conditions permitting emetic toxin production in foods by emetic strains of *B. cereus* are still not elucidated. The few conclusions that can be drawn from published work are the following:

- Cereulide became detectable at the end of the growth of *B. cereus* (Häggblom *et al*, 2002).
- The range of conditions permiting cereulide production is narrower than conditions permitting growth of *B. cereus*. Anaerobic conditions and temperatures above 37°C did not permit cereulide production (Finlay *et al*. 2000, Finlay *et al*. 2002a and b, Jääskeläinen *et al*. 2004)
- Not all foods can permit cereulide production even if growth of *B. cereus* is possible (Agata *et al*., 2002). Milk, cooked rice and pasta supported important cereulide production at 30°C (Finlay *et al*. 2002a and b).
- Production of cereulide below 10°C does not seem possible.

This clearly shows that presence and even growth of emetic *B. cereus* does not always mean cereulide accumulation in foods.

Foodborne poisoning caused by other *Bacillus* spp. Nothing is known on the ability of other *Bacillus* spp to produce toxins in foods.

3.2. Food categories that have caused foodborne poisoning

Some examples of foods linked to *B. cereus* food borne poisoning are presented in Tables 9a and b (Annex). However, uncertainties on the identification of the food implicated are still possible. Because *B. cereus* is ubiquitous and might be

present in several foods consumed by patients, it is particularly difficult to trace the food involved in foodborne poisoning.

Considering both emetic and diarrhoeal *B. cereus* foodborne poisoning, food categories implicated are frequently heat-treated foods (recipe dishes, stews, purées...). However, other food categories (salad sprouts, orange juice, mayonnaise dressing) have also been implicated. Foodborne poisoning frequently occurred in restaurant or in catering services. Failure in refrigeration and/or too long delay before preparation and consumption are presumably factors that led to poisoning in several cases.

Emetic *Bacillus cereus* foodborne intoxications were frequently linked to rice and pasta dishes. In particular, cooking rice and keeping it unrefrigerated several hours before frying or re-heating led to several emetic intoxication outbreaks, because the emetic toxin cereulide was produced during storage of the cooked rice but was not destroyed by the frying or re-heating step (Kramer and Gilbert, 1989).

A wide range of foods have been implicated in poisoning from other *Bacillus* spp (Kramer and Gilbert, 1989; Salkinoja Salonen *et al.*, 1999), including various recipe dishes, meat products, pastries, dairy products, infant food formulae, sandwich, pizzas, canned tomato juice. In all cases, suspected foods contained between 10^6 and 10^9 cfu/g

4. SPECIFIC CONTROL MEASURES

4.1. Growth limitation of *Bacillus* in the food chain.

4.1.1. Effect of temperature on growth.

Mesophilic strains of *B. cereus* can grow between 10 ºC and 42 ºC, some strains being able to grow at 50 - 55ºC (Kramer and Gilbert 1989, Nguyen-the and Carlin, 2003), with an optimal growth temperature between 30 and 37 ºC. Phychrotrophic strains would grow below 10 ºC, at temperatures as low as 4 ºC (Kramer and Gilbert 1989, Nguyen-the and Carlin, 2003; Andersson *et al* 1995, van Netten *et al*, 1990; Te Giffel *et al*., 1996, 1995a, 1997; Dufrenne *et al*., 1994; Francis *et al*., 1998; Andersen Borge *et al*., 2001). Psychrotrophic strains have optimal growth temperatures between 30 and 37 ºC. Maximum growth temperature ranged from between 37 and 42°C.

Refrigeration reduces growth of *B. cereus* by increasing the generation time. For instance, doubling times of a cocktail of 5 strains of *B. cereus*, including psychrotrophic and mesophilic strains in laboratory media were: 1.6 h at 19.5°C, 2.9 h at 14.2°C, 4 h at 9.6°C and 6.7 h at 6.5°C (Choma *et al*. 2000). Refrigeration also considerably increase lag time. Valero *et al* (2000) reported lag times of respectively 148.77 h and 1.96 h at 5 and 30 ºC.

Growth of some strains of *B. cereus* was observed at 55°C, but growth kinetics have not been characterised. At 50°C and below, important variations were observed among

strains. Generation times for *B. cereus* in laboratory media were: 0.3 to 3.6 h at 50°C (for strains able to grow), 0.3 to 0.7 h at 40°C, 0.4 to 1.3 h at 35°C (Rajkowski and Mikolajcik, 1987; Johnson *et al*. 1983). These generation times are similar or slightly lower than those reported for *Clostridium perfringens*: 0.5 h at 51°C and 0.2 h at 40°C in cooked meat (Willardsen *et al.*, 1978), 0.3 to 0.4 h at 35°C in laboratory media (Beuchat *et al*. 1980).

4.1.2. Effect of pH on growth

B. cereus is not a particularly acid tolerant bacterium. pH limit for growth in carrot substrate acidified with citric acid was between 4.5 and 4.75 (Valero *et al*. 2000). In milk acidified with HCl, slight growth was observed at pH 4.1 at 37°C (Clavel *et al*. 2004). At 25°C, growth rate of a strains of *B. cereus* was approximately constant between pH 7 and pH 5.5 but dropped dramatically below this level (Lindsay *et al*. 2000)

4.1.3. Effect of water activity on growth

Bacteria are more sensitive to low water activity than yeast and moulds. In the particular case of *Bacillus cereus*, the water activity must be higher than 0.92 for growth (Kramer and Gilbert 1989). For other *Bacillus* spp, at 20°C and pH 6.4 in Tryptose-Caseine-Soja broth, a mixture of 9 strains isolated from bakery products had lag times of 10 days and 15-18 days respectively for aw of 0.92 and 0.91 (Quintavalla and Paroli, 1993).

4.1.4. Effect of sodium chlorine

Regarding the effect of NaCl concentration on the growth of *Bacillus cereus* there are inconsistent results. Raevuori and Genigeorgis (1975) and Claus and Berkeley (1986) reported that 11-89% of strains of *B. cereus* grew in 7% of NaCl. Mossel *et al* (1967) suggested that 5% NaCl included in isolation agars was a useful selective agent for *Bacillus cereus* but that 10% was too inhibitory. Peters *et al* (1991) identified the temperature range over which *Bacillus cereus* could grow at different NaCl concentrations. These ranged from growth at all temperatures examined (14-41 ºC) at NaCl concentration of 0.5% (w/v) with pH 4.7 the lowest permitting growth. At 5% (w/v) NaCl, the temperature range supporting growth was observed only between 21-39 ºC and the minimum pH allowing growth was 5.5, while at 7% (w/v) NaCl no growth was recorded at any temperature.

4.1.5. Effect of modified atmosphere packaging

Bennink *et al*. (1995) studied the effect of CO_2 and O_2 concentrations on the growth of foodborne pathogens. For *B. cereus*, a strong reduction in maximum population density was observed only under 50% CO_2. Effect of CO_2: O_2 ratio on growth of foodborne pathogens including *Bacillus cereus* was studied by Ogihara *et al.* (1993). Growth of *B. cereus* was not completely inhibited by the CO_2, O_2 concentrations tested, however growth rate was reduced as the proportion of CO_2 in the gas mixture increased.

4.2. Inactivation of *Bacillus* in the food chain

4.2.1. Effect of heating

Heat is the more common method used to kill bacterial spores in foods. However, spores of *B. cereus* have a broad range of heat resistance, being a problem for producers to develop consistent cooking or pasteurization processes (Table 5). For instance, among a set of strains from various origins, time to reduce 10 fold the number of cultivable spores at 90°C, pH 7 (D value) varied from a few min to >100 min (Dufrenne *et al*. 1994). Strains from foodborne outbreaks had D values at 100°C ranging from 6 to 27 minutes (Rajkowski and Mikolajcik, 1987). Strains isolated from spoiled canned vegetables had D values at 130°C around 0.3 min (Bradshaw *et al*. 1975). Spores isolated from vegetables showed a D_{105^oC} value of 0.63 min in reference substrate (pH 7) (Fernandez *et al*, 1999). Heat resistance of *Bacillus cereus* spores can be modified by the pH. Survival of *Bacillus cereus* spores at 95 ºC decreased by three fold when the pH of the heating substrate was decreased from 6.2 to 4.7 (Fernandez *et al* 2002). Mazas *et al* (1998) found that acidification from pH 7 to 4 produced a five fold decrease in D_{103^oC} values.. According to those results, pasteurisation or cooking would not eliminate *Bacillus cereus* spores. In low acid foods, the process used to eliminate *Clostridium botulinum*, 121°C for *ca* 3 min (F_0 = 3) would also eliminate spores of *B. cereus*. Foods subjected to less severe heat treatments will occasionally carry spores of *B. cereus*.

4.2.2. Effect of other processes

The effect of non thermal technologies on bacterial spores seems to be variable. Very high pressures are needed to inactivate spores (Knorr 1995). The inactivation of bacterial spores by High Pressure combined with moderate heat occurs in two steps. First pressure causes spore germination and/or dipicolinic acid release, which permit spore inactivation (Gould and Sale 1970, Raso *et al* 1998, Margosch *et al*. 2004). However, always remains an ungerminated fraction "super-dormant fraction" that under temperature abuse could germinate and grow. Electric Pulsed Fields does not kill bacterial spores and their effect on germinated forms is very limited (Ruiz *et al* 2003).

Spores of *B. cereus* have a resistance to irradiation similar to that of *C. perfringens*. The dose needed to cause a 10-fold reduction was around 1.6 kGy with a shoulder (minimum dose to apply before starting to kill the spores) of 2 kGy (WHO, 1999).

4.2.3. Effect of food additives

Food additives as nisin produce the inactivation of *Bacillus cereus* vegetative cells while others as carvacrol have a very little effect (Ray 1992, Pol and Smith 1999, Periago and Moezelaar 2001).

4.3. Preventing build up of spores by Good Hygienic Practices (GHP) and Good Manufacturing Practices (GMP)

Cleaning is an essential step in preventing machines and equipments used to move food inside the processing plant to support growth of *Bacillus cereus*. Whenever

possible the use of hypochlorite (pH<8) is recommendable at least in pipelines. This will eliminate or dramatically reduce the number of spores. The use of weak acids at 30-40 ºC for 20-30 min can be an alternative to chemicals that can harm the pasteurizer or other equipments (Andersson *et al* 1995). Spores of *Bacillus cereus* have a pronounced ability to adhere to the surface of stainless steel material commonly used to build processing equipment for the food industry, which may become a reservoir of spores. Therefore the attachment of *Bacillus cereus* to online processing equipment may present a major problem for the food industry (Peng *et al*, 2001). It is a major cause for its presence and the difficulty of control. To provide consumers with wholesome and safe products, it is essential to control microorganisms present not only in food products but in the processing equipment as well. This might imply increasing the cleaning frequency and intensity (Pontefract 1991, Lelieveld 1985).

4.4. Discussion on specific control measures

Sterilization is the most effective way to control *Bacillus cereus* spores. Considering heat resistance data (Fernandez *et al* 1999), 3 min at the constant temperature of 105 ºC can produce 5 log reductions in the population of a high resistant *Bacillus cereus* strain. Temperatures higher than 105 ºC should protect food from this microorganism in most instances. However, only canning can ensure complete destruction of *B. cereus* spores. Other heating processes such as normal cooking, mild heat application on refrigerated processed food of extended durability (REPFED's) or pasteurization are not enough to kill all *Bacillus cereus* spores. These treatments will activate spores, thus readily triggering germination and enhancing further vegetative cell multiplication. Therefore, a rapid cooling process is required, followed by storage at temperatures of refrigeration, to avoid the multiplication of vegetative cells to a level that could endanger the safety of the product.

Establishing standard cooling procedures for heat treated foods is advisable (Collado *et al.* 2003). As the growth rate of *B. cereus* is similar and not higher that that of *Clostridium perfringens* in the range of temperatures critical during cooling (see § 4.1.1), the procedures developed for *C. perfringens* would likely also prevent *B. cereus* foodborne poisoning.

Table 5 – Examples of heat resistance of *B. cereus* spores, expressed as time for one decimal reduction of the initial number of spores (D).

Origin of strains	Number of strains tested	Heating temperature (°C)	D (min)		
			Mean	Minimum	Maximum
Milk [1]	6	95	2,0	1,8	2,8
		100	0,8	0,7	1,5
Various dairy	25	100	3,5	2,0	5,4

products [2]					
Rice [3]	6	92 100	22 4,8	16 4,2	36 6,3
Rice [4]	13	95	2,8	1,5	6,0
Various foods [5]	12	90	Not calculated	2,2	> 100
Cooked vegetables [6]	52	90	Not calculated	0,7	5,9
Spoiled canned vegetables [7]	2	129,4	Not calculated	0,19	0,28
Foodborne diarrhoeal cases [8]	6	100	6,7	0,6	27
Vegetables [9]	2	90	4,0	21,5	39,0

1 – Janstova et Lukasova (2001) ; 2 – Wong *et al.* (1988) ; 3 – Chung et Sun (1986) ; 4 – Parry et Gilbert (1980) ; 5 – Dufrenne *et al.* (1994) ; 6 – Choma *et al.* (2000) ; 7 – Bradshaw *et al.* (1975) ; 8 – Rajkowski et Mikolajcik (1987), 9- Fernandez et al (1999).

Whenever refrigeration is the major factor to control growth of *B. cereus* in foods, maintaining a low temperature, is essential. Only storage below 4°C would ensure that no *B. cereus* growth is possible. Above 4 ºC both the lag phase and the growth rate vary with the temperature. According to Andersson *et al*, (1995) the increase in the concentration of *Bacillus cereus* is tremendous when the storage temperature is raised just 2ºC, from 6ºC to 8ºC. However, slight reduction in pH or water activity might be sufficient to prevent growth of *B. cereus* at temperatures of refrigeration. For instance at 8°C, using 3 *B. cereus* strains, not growth was observed at pH 6.5 whereas growth occurred after 4 days lag time at pH 7 (Benedict *et al*. 1993). The nature of the food also influences the effect of refrigeration. Strains of *B. cereus* able to grow at 7°C in laboratory media did not grow in vegetable broth at the same pH (Choma *et al*. 2000). Therefore, refrigeration might be much more efficient than expected to delay or prevent *B. cereus* growth in some foods.

Nevertheless, even if food composition and storage conditions prevent growth of *B. cereus*, maintaining a low initial number of cells remains important. Consequently, proper cleaning and disinfection (for instance hypochlorite at pH<8) is essential to prevent high levels of *Bacillus cereus* in food products. Well designed pipelines to improve the cleaning process can help to fight against the formation of biofilms as reservoirs of bacteria.

4.5. Discussion on the need and possibility of microbiological testing / criteria.

Referring to the Codex recommendations for setting criteria (FAO, 1997) in which Microbiological criterion have the purpose of "defining the acceptability of a product or a food lot, based on the absence or presence, or number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot." Such microbiological criteria can be applied i) by regulatory authorities where no other more effective tools are available and where they are

expected to improve the degree of protection offered to the consumer; ii) by food business operator to validate the efficacy of HACCP plan (FAO, 1997).

- There is epidemiological evidence that a high proportion of strains of *B. cereus* and a few strains of other *Bacillus* spp can cause foodborne poisoning. However, many strains among these species are presumably not foodborne pathogens.

- All categories of consumers are at risk. Symptoms are generally mild and of short duration but more severe symptoms have been described among elderly persons including deaths.

- Un-processed foods will almost always be contaminated with low numbers of spores of *B. cereus* and/or other potentially pathogenic *Bacillus* spp., but these levels are usually too low to represent a risk for consumers.

- Heat processes, apart from canning, will not eliminate spores of *B. cereus* and other *Bacillus* spp. In addition, accumulation of *B. cereus* on processing equipment surfaces can occur.

- Multiplication of *B. cereus* and other potentially pathogenic *Bacillus* spp. during storage and handling of foods is possible and can lead to hazardous levels of bacteria or toxins at consumption.

- Methods to enumerate and detect *B. cereus* are available (ISO 7932, ISO 21871) but give no indication of the ability of the strains to produce toxins, and fail to differentiate *B. cereus* from closely related species that have not been associated with illness. Rapid methods to selectively detect emetic *B. cereus* have been developed (Ehling-Schulz *et al.* 2004a). In contrast, methods to identify *B. cereus* strains potentially diarrhoeic are not yet suitable for routine laboratories. No method can easily detect and enumerate other species of *Bacillus* that have been involved in foodborne poisoning (i.e. *B. subtilis, B. licheniformis, B. pumilus*). In addition, methods to distinguish pathogenic strains among these species are not available.

- Protecting consumers from exposure to hazardous levels of *B. cereus* and of potentially dangerous *Bacillus* spp can be achieved by means other than testing foods against legal criteria: (i) preventing accumulation of spores in the food processing chain by adequate design and cleaning of equipments and (ii) limiting growth of *Bacillus* by refrigeration, rapid cooling of heat treated foods or maintaining the cooked food above *ca*. 60°C until consumption.

Testing by food business operators might be necessary to determine the adequate shelf life whenever foods are likely to support growth of *B. cereus* or of other potentially pathogenic *Bacillus* spp. Thus food business operators should ensure that numbers of *B. cereus* between 10^3 and 10^5 per g are not reached at the stage of consumption under anticipated conditions of storage and handling.

Food business operators may also decide to determine maximum limits for the contamination of raw materials or ingredients they use. As an example, spices, herbs and

vegetable seasonings may carry substantial numbers of *B. cereus* spores. As these products have so far not been implicated in outbreaks of human illnesses due to *B. cereus*, routine microbiological testing of spices in international trade would not improve consumer safety. However, for some processed foods in which *B.cereus* can multiply, spices have been identified as a source of contamination. In such cases food processors might decide to set their own limits based on experience.

Dehydrated foods (soup, purées, infant formulae) in which presence of spores of pathogenic *Bacillus* is frequent, might permit growth of *B. cereus* once re-hydrated in warm water. Food processors have been aware of this risk and for instance in the case of dehydrated soups, the AIIBP (Association International de l'Industrie des Bouillons et Potages) have set up limits for *B. cereus* with $m=10^3$, $M=10^5$, n=5 and c=3 (AIIBP, 1992). Some dehydrated foods are consumed by potentially fragile consumers, such as neonates and elderly. For instance, three fatal cases occurred in France in a home for the elderly and were due to vegetable purées (Lund *et al*. 2000). Therefore, in addition to good practices designed to reduce delay between preparation and consumption, it might be advisable to recommend that numbers of *B. cereus* spores in such dehydrated foods should be as low as possible.

5. CONCLUSIONS BY ANSWERING THE TERMS OF REFERENCE

ToR 1: Identify the categories of foodstuffs and the food manufacturing and preparation processes where Bacillus cereus or other Bacillus spp. may pose a risk for human health;

- *B. cereus* is ubiquitous and its spores will not be eliminated from food materials by heat treatment, apart from canning. Spores are present in almost all categories of foods before storage, generally in numbers too low to cause foodborne poisoning. Risk for human health could arise from unusually high initial contamination of foods but, more usually, from multiplication of *B. cereus* after temperature abuse.

- *B. cereus* will grow in most foods under favourable pH and water activities, from 4 to 48°C (55°C for a few strains). The number of *B. cereus* to which consumers will be exposed is a function of storage duration and storage temperature. In addition some strains multiply during cooling of heat treated foods.

- The *B. cereus* group is very diverse. Storage of foods below 10°C prevents growth of strains that produce emetic toxin (responsible for the vomiting poisoning). Hence, reducing the storage temperature reduces the diversity of *B. cereus* population able to multiply.

- Published reports indicate that foods linked to *B. cereus* diarrhoeal or emetic foodborne poisoning were frequently, *but not exclusively*, heat treated, and/or not adequately refrigerated after preparation and before consumption. Rice and pasta dishes have frequently caused emetic poisoning.

- Dry foods such as dry soups, dried dairy products, infant formulae, spices, herbs and seasonings are frequently contaminated with *B. cereus* and other *Bacillus* species although at different levels.

- Some strains of other species of *Bacillus* (*B. subtilis, B. licheniformis* and *B. pumilus*) have caused foodborne poisoning with symptoms similar to *B. cereus*. Their spores are as ubiquitous as *B. cereus* but the foods at risk might be different. None of these species are known to grow below 10°C.

- There are uncertainties in reporting. Many cases of *B. cereus* foodborne poisoning are not reported because it is not mandatory and poisoning is usually mild (although rarely fatal cases have occurred). The vehicle for poisoning is rarely confirmed.

ToR 2: Establish, for the different categories of foodstuffs identified, the relation between the number of Bacillus *spp. bacteria in the foodstuffs and the ability of these foodstuffs to cause food-borne illness.*

- *B. cereus* strains cause a diarrhoeal poisoning linked to the number of cells ingested and/or an emetic (vomiting) poisoning linked to the amount of an emetic toxin (cereulide) produced in the food. Because cereulide is very stable, it might cause vomiting poisoning even if all *B. cereus* cells have disappeared.

- Published reports of *B. cereus* foodborne poisoning cases show that 10^5-10^6 cells or spores/g of food clearly can cause foodborne poisoning. In rare cases, 10^3 spores/g of food caused illness.

- No food processing treatments will inactivate cereulide. Emetic strains of *B. cereus* have to reach at least 10^5 cells/g to produce sufficient cereulide to cause food poisoning.

- Not all *B. cereus* strains are able to cause foodborne illness.

- Concentration of other *Bacillus* species required to cause foodborne illness exceeded 10^6 cells/g

ToR 3: List and evaluate specific control measures, including microbiological testing and temperature requirements, to manage the risk caused by Bacillus cereus*, other* Bacillus *spp. and their toxins in foodstuffs. In doing so, attention should be paid to different species of* Bacillus *spp.*

- One of the major control measures is to control temperature and to establish HACCP system. The major control measure is an appropriate combination of storage temperature and storage duration, to prevent growth of *B. cereus* to hazardous level, or to prevent emetic toxin production, at the stage of consumption. This should be verified by microbiological testing.

- Inactivation of spores of *B. cereus* can only be guaranteed by a heat process equivalent to that used for low acid canned foods (F_0=3). However, this control measure cannot be used in most foods without dramatically altering their quality.

- Inhibition of growth of *B. cereus* can be achieved by reducing water activity, pH and/or temperature. Temperature below 10°C greatly slows multiplication and temperatures below 4°C prevent it. Rapid cooling of heat treated foods through the temperature range supporting growth will also minimise multiplication before storage. After cooking, cool through the temperature range 55°C to 10°C as quickly as possible; store below 10°C (ideally below 4°C). Rapid cooling can only be achieved if the portion size is relatively small.

- *B. cereus*, mainly spores, can persist on the surface of some processing equipment, which may increase the contamination of processed food. Good hygienic practices, HACCP, GMP, and using equipment designed to allow efficient cleaning, could reduce the initial number of *B. cereus* in processed foods.

- Other pathogenic *Bacillus* spp. have some features similar to *B. cereus*: they produce heat resistant spores and they must be present in high numbers in foods to cause diseases. Control measures presented above for *B. cereus* certainly contribute to control other pathogenic *Bacillus* spp. However, there is little information in the literature on other pathogenic *Bacillus* spp. No routine methods easily detect and enumerate other species of *Bacillus* that could be involved in foodborne poisoning and no methods distinguish pathogenic strains among these species. The temperature and pH ranges allowing growth of pathogenic *Bacillus* other than *B. cereus* are not known. Therefore control measure specific to other pathogenic *Bacillus* spp are not available.

6. RECOMMENDATIONS

- To avoid high numbers of *B. cereus* at the stage of consumption, cooked foods should be eaten soon after cooking, or kept hot (above 63°C) or cooled rapidly and kept below 7-8°C (ideally below 4°C), preferably for a short time, e.g. a few days, depending on products.

- For the development of new food product, or food product that support growth of *B. cereus*, either by their nature or their conditions of storage (e.g. extended shelf life), processors should ensure that numbers of *B. cereus* between 10^3 and 10^5 per g are not reached at the stage of consumption under anticipated conditions of storage and handling. This should also apply for dehydrated foods reconstituted by hot water before consumption.

- The maximum limit at consumption described in the above bullet point should be used as a target for food business operators to verify their HACCP system and could be considered as microbiological criteria to test the acceptability of a process.

- Code of Good Manufacturing Practices (GMP) should include recommendations on *B. cereus* and other *Bacillus* species.

- Additional research is needed to provide data to extend knowledge on other food poisoning *Bacillus* species up to the level of knowledge of *Bacillus cereus*.

- Refrigerated foods have seldom been incriminated in *Bacillus* foodborne outbreaks. There are uncertainties on the pathogenicity of psychrotrophic *Bacillus* spp. Generation of data on growth range of human pathogenic *Bacillus* is needed.

- Efforts by some Member States to improve monitoring of *Bacillus* species foodborne outbreaks should be considered.

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ANNEX

Table 6 *B. cereus* counts in products in the Netherlands

Product	**Year**	**Number of samples**	**Number of samples with CFU $^{-1}$ g**			
			$< 10^5$	10^5 - 10^6	10^6 – 10^7	$> 10^7$
Products (to be) served in restaurants, snackbars etc.	2000	11,039	10,996	31	12	
	2001	19,313	19,244	70	19	

	2002	17,111	17,058	46	7	
	2003	7,634	7,625	9		
Milk and milk products	2000	1,372	1,366	4	2	
	2001	4,085	3,996	36	49	4
	2002	3,460	3,448	9	3	
	2003	1,044	1,044			
Bakery products	2000	733	731	1	1	
	2001	1,978	1,974	2	2	
	2002	2,828	2,828			
	2003	1,072	1,071	1		

Extracted from KRIS database, Microbiological Laboratory for Health Protection, National Institute for Public Health and the Environment,PO Box 1, 3720 BA Bilthoven, The Netherlands.

Reported foodborne outbreaks in Europe with identified agents

Table 7. Causative agents identified in investigated outbreaks period 1993–1998. (Source WHO surveillance program for control of foodborne infections and intoxications in Europe. Seventh report 1993-1998)

Causative agent	Outbreaks		Cases in outbreaks	
	Number	%	Number	%
Salmonella total	18,159	77,1	126.303	54.6
Shigella	633	2,7	17.786	7.7
Staphylococcus total	970	4.1	11,001	4.8
Clostridium perfringens	522	2.2	9.019	4,0
Bacillus cereus	**278**	**1,2**	**2,918**	**1.3**
Escherichia coli total	193	0,9	2,109	0.9

Campylobacter	204	0,9	1.703	0.8
Clostridium botulinum	**238**	**1.0**	**700**	**0.3**
Vibrio parahaemollyticus	23	0,1	350	0.2
Streptococcus	10	0	234	0.1
Yersinia	13	0.1	327	0.1
Bacillus subtilis	**8**	**0**	**0**	**0**
Brucella	47	0,2	0	0
Listeria	3	0	5	0
Proteus	6	0	18	0
Other bacteria	340	1.1	8,581	3.7
Bacteria total	**21,565**		**181.603**	**78.3**
Viruses total	230	1.0	6,790	2.9
Parasites total	749	3.2	2,025	0.8
Mushroom poisoning	485	2.1	1.609	0.7
Waterborne	127	0.5	34.126	14.8
Others total	377	1,7	4.894	2.1
Agent known total	**23.538**	**100,0**	**231,330**	**100**

Table 8. Places were outbreaks by *B. cereus* occurred in the period 1993 – 1998. (WHO surveillance program for control of foodborne infections and intoxications in Europe. Seventh report 1993-1998).

Places	**Percentage**
School, kindergarten, home for children	22
Restaurants, hotels, bars, cafes	22
Canteens	16
Private home	11
Medical care facilities	4
Various places	25
Total	**100**

Table 9a– Examples of foods linked to *Bacillus cereus* poisoning-

Food categories (number of persons affected)	Type of poisoning	cfu *B. cereus* in the food (Comments)	References
Cod fish (4)	Diarrhoea	4×10^5 g^{-1}	Van Netten *et al*. 1990
Chicken and rice meal (300)	Diarrhoea	10^2 g^{-1} chicken, 10^6 g^{-1} rice	Ripabelli *et al.* 2000
Quiche (79)	Diarrhoea and vomiting	> 100 g^{-1} (refrigerator not working, food not heated sufficiently, poor restaurant hygiene)	Penman *et al*. 1996
Cakes at 2 banquets (95 and 78)	Diarrhoea	> 10^2 g^{-1} foods (same strain in the foods, in the confectioner's kitchen and in patients' faeces)	Ghelardi *et al*. 2002
Vegetable purée (44)	Bloody diarrhoea (3 persons died)	3×10^5 g^{-1}	Lund *et al*. 2000
Potato salad with mayonnaise at a banquet (25)	Diarrhoea	10^3 g^{-1} mayonnaise	Gaulin *et al*. 2002
Stew (17)	Diarrhoea (3 persons hospitalised for up to 3 weeks)	10^4 to 10^5 per serving of incriminated food	Granum *et al.* 1995
Salad sprouts (4)	Vomiting and diarrhoea	10^5 to 10^7 g^{-1}	Portnoy *et al*. 1976
Orange juice from concentrate (43)	Vomiting and diarrhoea	100 ml^{-1}	Talarmin *et al.* 1993
Onion powder (18)	Diarrhoea and vomiting (mixed Norwalk virus, *B. cereus* and *B. thuringiensis* outbreak)	ND	Jackson *et al*. 1995
Various foods from a meal (77)	Diarrhoea and/or vomiting	3 to 6 x 10^6 g^{-1} of foods (foods not properly refrigerated)	Pena Gonzalez *et al*. 1998
Pasteurised milk (280)	Nausea,vomiting (complication in one patient with gastric ulcer)	4×10^5 g^{-1}	Van Netten *et al*. 1990
* Spaghetti with pesto (2)	Diarrhoea and vomiting (1 died from fulminant liver failure)	ND (*B. cereus* producing emetic toxin isolated from food residues. Food left unrefrigerated)	Mahler *et al*. 1997
* Chinese noodles (50)	Vomiting and diarrhoea (1 died from heart failure)	6 x 10^7 g^{-1} cooked noodles	Takabe and Oya 1976
* Fried rice (4)	Vomiting	> 10^6 g^{-1} fried rice	Grein 2001
* Cooked and fried rice (14)	Vomiting and Diarrhoea	> 10^6 g^{-1} chicken fried rice (food not refrigerated)	Khodr *et al*. 1994

* Vegetarian dish (7)	Vomiting	10^2 g^{-1} meat substitute	Ripabelli *et al.* 2000
* Restaurant meal with cooked rice (5)	Vomiting	$6x10^3$ g^{-1} cooked rice	Ripabelli *et al.* 2000
* Rice dishes, spaghetti and noodles (14 outbreaks from 1974 to 1999 in Japan)	Vomiting	(1280 to 10 ng emetic toxin g^{-1} implicated foods)	Agata *et al*. 2002
* Home made pasta dish (2)	Vomiting	7 x 10^6 g^{-1} (1500 to 3000 ng emetic toxin g^{-1} implicated foods	Jääskeläinen *et al*. 2003

ND – Not determined (*) Indication of evidences for emetic poisoning (short incubation period, symptoms)

3 **Table** 9b: Description of outbreaks of *Bacillus cereus*

(A)Outbreak ; (B)Exposed ; (C)Illness ; (D)Death ; (E) Mortality % ;;
Dose Log 10 (F) Faeces ,(G)Vomitus ; (H) Food ;
(I) Incubation time hrs,
CLINICAL SIGNS (J) Duration time hrs; (K): Gastrointestinal, abdominal cramp; (L): Gastrointestinal, Diarrhoea, (M): Gastrointestinal, nausea; (N): Gastrointestinal, vomiting; (O): Neurological, muscle weakness

Incriminated food (ingredient) Place	Year Country	A	B	C	D	E	Dose Log 10			I	CLINICAL SIGNS						Reference
							F	G	H		J	K	L	M	N	O	
spaghetti, pesto home	96 Switzerland	1	2	2	1	50			2.6 (p)	0.5		f+	f+		s+		(1)
chicken, roasted	78 Japan/Osaka		1	1					6.1	0.5-1					+		(2)
rice, fried	77 Japan/Osaka	1	2	2	1	50			6.1	0.5-1					+		(2)
rice and omelette	81 Japan/Osaka	1	4	4					+	0.5-1					+		(2)
lunch box, catered	77 Japan/Osaka	1	13	9					7.1	0.5-2					+		(2)
rice and curry	77 Japan/Fukuoka	1	2	2					7.8	0.5-2					+		(2)
rice, fried	79 Japan/Osaka	1	4	4			5.3			0.5-2					+		(2)
rice ball	77 Japan/Osaka	1	6	6					7.2	0.5-3					+		(2)
rice, boiled Canteen	75 Finland	1	36	18			neg		8.2 (r) / 6.0 (m)	0.5-4	<24	+	+		+		(3)
rice fried with curried shrimps	71 UK			1						1.5			+		+		(4)

Chinese restaurant														
rice, fried	78 Japan/Aichi	1	2	2		+	1.5-2					+		(2)
rice, fried	78 Japan/Osaka	1	2	2	4.3		1.5-2					+		(2)
Meatloaf fraternity house	69 USA/California	1	31	15		7.8	10 m	<24	+	+	+	(+)		(5)
rice, fried	82 Japan/Miyagi	1	2	2		+	1-1.5					+		(2)
rice, fried	81 Japan/Kyoto	1	53	35		6.1	1-2					+		(2)
rice, fried	79 Japan/Osaka	1	6	6		7.9	1-2					+		(2)
yakisoba (dumpling with cabbage and beef)	74 Japan/Aichi	1	52	51		8.2	1-2					+		(2)
rice and chicken	81 Japan/Aichi	1	61	46	6		1-2.5					+		(2)
chicken enchilada with gravy cafetaria, hospital	85 USA/Tennessee	1	249	160			12.5 m	24.3m	90%	96.3%	50.6%	13.8%	24.7%	(6)
lunch box, catered	75 Japan/Osaka	1	48	24	6.1		1-3					+		(2)
rice, fried	78 Japan/Osaka	1	206	94	4.4		1-4					+		(2)
soy bean curd	81 Japan/Chiba	1	338	172		9.7	1-5					+		(2)
rice fried with beef. bean shoots chinese restaurant	71 UK			5			1-6			+ (3x)		+		(4)
rice fried with beef, beanshoots or curried shrimps chinese restaurant	71 UK			3			2	24		+		+		(4)
rice fried with chickenegg foo yung	71 UK			1			2			+		+		(4)

chinese restaurant														
lunch box, catered	74 Japan/Osaka	1	33	12			7.5	2-12			+			(2)
milk, pasteurized	86-89 Netherlands	1	4200	280			5.6	2-14	<36			+	+	(7)
lunch box, catered	75 Japan/Yamagata	1	422	130	3	2.3	+	2-17			+			(2)
rice ball and shushi	77 Japan/Osaka	1	1809	211			8.9	2-2.5					+	(2)
tempura (fried vegetables with shrimps and/or fish)	74 Japan/Osaka	1	3	3			5.7	2-3					+	(2)
rice, fried	73 Japan/Osaka	1	5	5			6.8	2-4					+	(2)
rice and omelette	82 Japan/Saitama	1	6	5			7.0	2-4					+	(2)
Pudding	71 Japan/Osaka	1		89			6.1	3-7			+			(2)
vegetable pie, home made home	86-89 Netherlands	1	3	3			5.3	4	24	+	+			(7)
rice fried with curried shrimps, beanshoots chinese restaurant	71 UK			2				4			+ (1x)		+	(4)
lunch box, catered	74 Japan/Mie	1	1407	194			6.8	4-10			+			(2)
Roastbeef elderly home	67 Netherlands	1	150	18			3.5	8-20	<24	+	++			(8)

(1)Mahler *et al.* 1997; (2) Shinagawa et al. 1985; (3) Raevuori *et al.* 1976 ; (4) Mortimer and McCann 1974; (5) Midura *et al.* 1970; (6) Baddour *et al.* 1986 (7) Netten *et al.* 1990 ; (8) Zijl and Wolff 1968

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