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The use of liquid chromatography coupled to mass spectrometry as a necessary tool for the assessment of cereulide in food

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ABSTRACT

Certain *B. cereus* strains are able to produce an emetic toxin named cereulide which is at the origin of numerous foodborne outbreaks with even fatal outcomes. This stressed the importance of setting up rapid and reliable quantification methods for cereulide in food products.

An optimized LC-MS² method was developed in the first part of this thesis and an in-house validation was performed according to the Commission Decision 2002/657/EC. The method was compared with other concurrent methods in terms of specificity, sensitivity and time consumption. It guaranteed low detection limits in rice (LOD of 0.5 ng/g) with a good repeatability and reproducibility. The robustness of the method was tested in other food matrices coming from industry (red beans, chili con carne, and spices) or from foodborne intoxications (*i.e.* white and fried rice, and bean soups) in which cereulide was detected at various levels (from 1.5 ng/g up to 13 µg/g). Besides, the actual concentration of cereulide was quantified in the pasta leftovers of a recent Belgian lethal case due to *B. cereus* and it helped to explicitly incriminate cereulide as the most probable cause of the death.

The study of critical conditions (temperature and time of storage, type of strain) influencing cereulide production revealed that the correlation between toxin production and bacterial counts is more complex than proportional.

The actual role of cereulide for its bacterial producer was also assessed. The emetic toxin displays a fungistatic activity against nine fungal species (*Alternaria alternata*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Magnaporthe grisea*, *Monographella nivalis*, *Mortierella isabellina*, *Sclerotinia minor*, *Stemphylium vesicarium* and *Verticillium dahliae*).

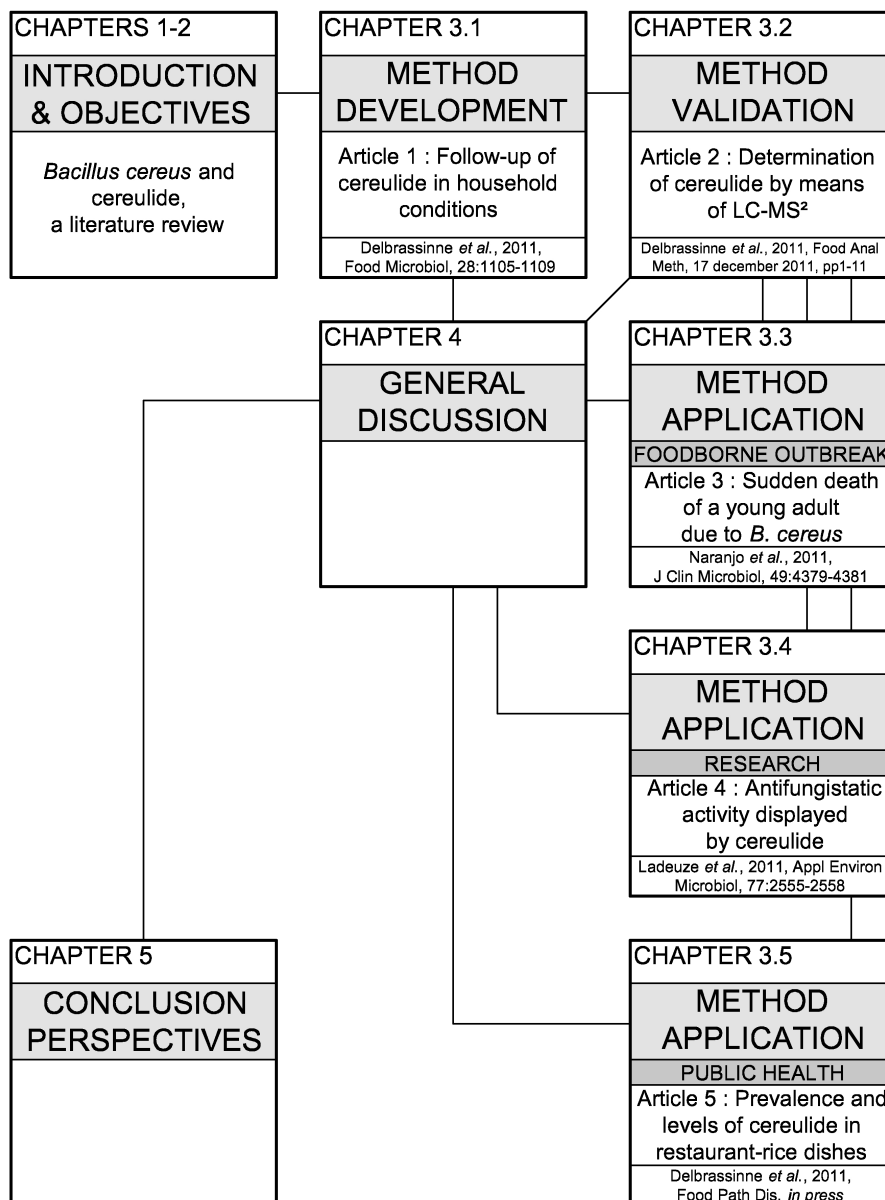
Finally, the presence and concentrations of cereulide in rice dishes were assessed in Asian restaurants of Brussels and Wallonia. The prevalence of cereulide (7.4 % of the dishes analysed on the day of consumption contained cereulide) was higher than previously studied for the cereulide-producing strains and encourages to further characterize the risk of *B. cereus* emetic food poisoning.

PREAMBLE

This work was realised in the frame of an Ylief Project at the Scientific Institute of Public Health (IPH) in Brussels, in collaboration with the Laboratory of Food and Environmental Microbiology (MIAE-MBLA) at UCL.

This thesis was dedicated to the study of cereulide, the emetic toxin produced by *Bacillus cereus*. In this context, an LC-MS² method was developed and validated for the quantification of cereulide in food. During the method development, various conditions (time and temperature of storage, type of strain) influencing cereulide production were explored. A complete validation of the LC-MS² method was performed based on several pre-experiments. As direct applications of the validated method, analyses were carried out to quantify cereulide in various food samples that were part of HACCP monitoring or intoxication cases. In parallel, experiments were designed to evaluate the prevalence of cereulide in rice dishes available in 54 restaurants in Brussels and in Wallonia. Besides, the potential biological role of cereulide was explored by growth inhibition tests of fungi. Finally, a lethal *B. cereus* food poisoning case was thoroughly analysed and, thanks to the LC-MS² quantification, cereulide was clearly incriminated as the cause of the intoxication.

A graphical outline of the thesis is displayed on the following page. The first chapter relates the state-of-the-art on cereulide, while the second chapter presents the specific objectives of this thesis. A series of publications that resulted from the experiments described above constituted the core of this thesis (Chapter 3) and are articulated as Method Development (Chapter 3.1), Method Validation (Chapter 3.2) and Method Applications (Chapters 3.3, 3.4 and 3.5). The five sections related to the results are discussed in their globality in Chapter 4. Finally, the concluding considerations on the performed study are given in Chapter 5 together with perspectives for the future research on cereulide.



Graphical outline of the thesis

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ABBREVIATIONS

aa	amino acid
AFLP	amplified fragment-length polymorphism
BHI	Brain Heart Infusion
Bw	body weight
<i>ces</i>	cereulide gene cluster
CFU	colony forming unit
Cry	crystal endotoxin
cspA	cold-shock protein A
CytK	Cytotoxin K
DNA	DesoxyriboNucleic Acid
H ₂ O	water
HBL	Hemolysin BL
ISO	International Organisation for standardization
IPH	Institute of Public Health
Kb	kilobase
kDa	kilodalton
LC-MS	Liquid chromatography mass spectrometry
LC-MS ²	Liquid chromatography tandem mass spectrometry
mM	milli molar
MAP	modified atmosphere packaging
MALDI-TOF	Matrix-assisted laser desorption/ionization- time of flight
MLST	Multi locus sequence typing
mRNA	messenger RiboNucleic Acid
MYP	Mannitol-egg yolk Polymyxin
m/z	mass-to-charge
NHE	Non-hemolytic enterotoxin
NK	natural killer
NRPS	Non-Ribosomal Peptide Synthesis
PCR	Polymerase Chain Reaction
PlcR	Phospholipase C regulator
Polyps	polyphosphates
REFED	Refrigerated Processed Foods of Extended Durability
RTE-foods	Ready-to-Eat Foods
<i>s.l.</i>	<i>sensu lato</i>
<i>s.s.</i>	<i>sensu stricto</i>
TFA	trifluoroacetic acid

1. INTRODUCTION

1.1 THE *BACILLUS CEREUS SENSU LATO* GROUP

The *Bacillus cereus sensu lato* nomenclature is used to group six closely related species corresponding to *B. anthracis*, *B. cereus sensu stricto*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis* and *B. weihenstephanensis*. It was suggested that these taxa should be considered as a single species due to their high genomic similarity (Helgason *et al.*, 2000). Their chromosomes have been shown to be similar in both gene content and gene order and, the *B. cereus* group members are globally defined by differences in plasmid encoded-features (Rasko *et al.*, 2007). However, plasmid exchange is possible between *B. cereus s.l.* members (Andrup *et al.*, 1996; Van der Auwera *et al.*, 2007; Vilas-Boas *et al.*, 2002). This possible plasmid transfer renders the phenotypic markers almost useless for the typing although the *B. cereus* members were initially considered as distinct on the basis of their phenotypic features. The speciation of the group members is therefore questionable.

In fact, these bacteria are thought to have appeared from a common ancestor thanks to the acquisition of virulence gene sets (Rasko *et al.*, 2005) and different spectra of toxicity are observed among the *B. cereus* members. *B. anthracis*, *B. cereus sensu stricto* and *B. thuringiensis* are pathogenic for humans and/or animals and their virulence genes are mainly located on large plasmids. *B. anthracis* virulence is expressed through the production of the anthrax toxins and the elaboration of a protective capsule, both encoded on two large plasmids: pXO1 (182 kb) (Okinaka *et al.*, 1999b) and pXO2 (92 kb) (Okinaka *et al.*, 1999a), respectively. *B. thuringiensis* produces the crystal insecticide, or parasporal crystals inclusions, encoded by the crystal entomotoxin genes (*cry*) which are located on large plasmids (Aronson, 2002). *B. cereus* toxicity of emetic strains is expressed via the cereulide, an emetic toxin whose genetic determinants are located on large virulence plasmids (~270 kb) similar to pXO1 and differently named according to the strain. Interestingly, some *B. weihenstephanensis* strains are able to produce cereulide (Thorsen *et al.*, 2006; Thorsen *et al.*, 2009a; Thorsen *et al.*, 2009b)

but the gene cluster responsible for this production can be present either on plasmids unrelated to pXO1, either on the chromosome (Hoton *et al.*, 2009).

1.1.1 *B. ANTHRACIS*

B. anthracis carries inglorious fame of being the most well-known *B. cereus* group member, because it has been used as a bioterrorist weapon in 2001 in the United States (Spencer, 2003). *B. anthracis* is the etiological agent of anthrax, which is primarily a disease of herbivores, but which can also affect all mammals, including humans. *B. anthracis* is persistent in the environment in a state of dormant spores which are able to survive for long periods in soils. These spores are highly resistant to adverse environmental conditions including heat, ultraviolet and ionizing radiation, pressure, and chemical agents. If ingested, the spores are able to generate vegetative forms which can multiply inside the mammalian host and express various virulence factors (Mock and Fouet, 2001), as illustrated in Fig. 1.

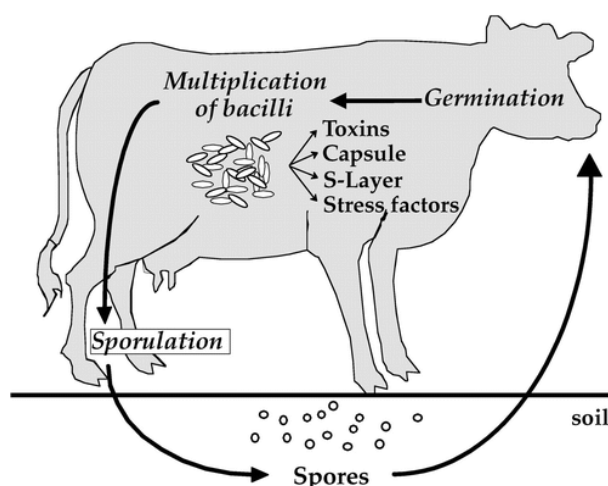


Figure 1 – *B. anthracis* life cycle (Mock and Fouet, 2001)

There are three routes of contamination with *B. anthracis* leading to different types of anthrax which may all result in a fatal outcome: cutaneous (through lesion of the skin), gastrointestinal (through ingestion of contaminated food), and respiratory anthrax (through inhalation of contaminated air). The most benign anthrax is the cutaneous form because it is treatable with appropriate antibiotics (*e.g.* penicillin and amoxicillin) if early diagnosed. Cutaneous

anthrax is implicated in about 90 % of all human cases but the mortality rate is below 1 %. It is characterized by the apparition of a small papule which undergoes ulceration and then evolves into a typical black eschar. The lesion, where the bacteria remain located, is accompanied with oedema (Spencer 2003).

The gastrointestinal anthrax follows ingestion of spores, formation of the classical eschar in the gut system, germination of the spores resulting in multiplication of vegetative cells and expression of both toxins and capsule. The pathological features reflect the actions of the different virulence factors and result in oedema, vascular leakage, and bloody diarrhea. The outcome is often fatal due to difficulty of posing a correct diagnosis.

The most severe form of anthrax is the inhalation of spores which result in more than 95 % of mortality if the disease is not treated early. The spores of 1–2 μm of diameter can deposit in the alveolar spaces and then be transported to the lymph nodes where vegetative cells can proliferate and spread through the bloodstream. Large amounts of toxins are then produced and provoke acute symptoms. The illness is therefore characterized by two phases: the first prodromal stage during which mild flu-like symptoms appear and suddenly stop after 48h to let place to the fulminant illness exemplified by fever, tachypnoea, cyanosis, tachycardia, moist rales, and evidence of pleural effusion; extremely rapidly followed by coma and death of the patient (Guichard *et al.*, 2011; Spencer, 2003b).

The anthrax toxins and the capsule formation are central to anthrax pathogenesis. The anthrax toxins are composed of three secreted proteins acting in binary combinations as shown in Fig. 2. The protective antigen (PA), the lethal factor (LF) and the oedema factor (EF). For complete toxicity, bipartite exotoxins are necessary: the lethal toxin ($\text{LTx} = \text{PA} + \text{LF}$) and the oedema toxin ($\text{ETx} = \text{PA} + \text{EF}$). PA acts as the binding domain for the receptors that allow entry of the toxin into the host cell while EF and LF elicit cell damages (Mock and Mignot, 2003).

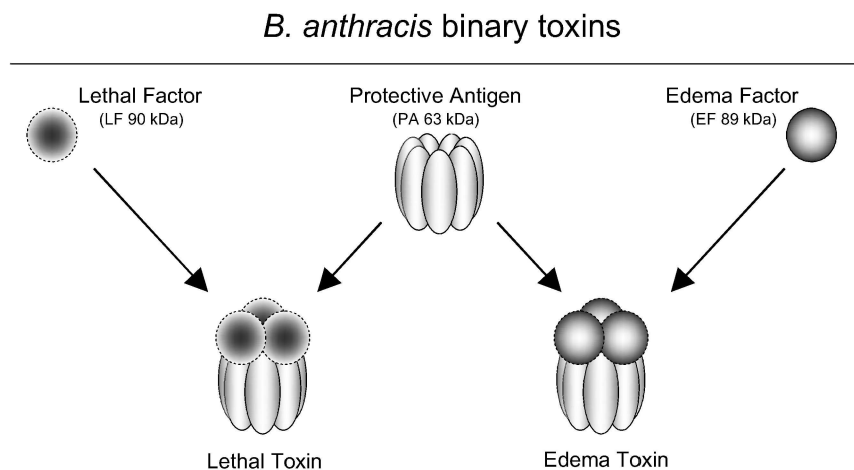


Figure 2 – *B. anthracis* bipartite toxins
Modified from Boyer *et al.*, 2011

The capsule, a polymer of γ -D-glutamic acid, contributes to pathogenicity of *B. anthracis* by enabling the bacteria to evade the host immune defences. Thanks to its negative charge, the capsule can inhibit the phagocytosis of the *B. anthracis* vegetative cells by macrophages. In addition to the lethal toxin and the oedema toxin which target macrophages and neutrophils, the capsule permits an unhampered growth of *B. anthracis* in the infected host (Ezzell and Welkos, 1999).

Two recent discoveries of *B. cereus* strains producing anthrax toxins and a capsule and giving anthrax-like diseases (Hoffmaster *et al.*, 2004; Klee *et al.*, 2010) endorse the hypothesis of a great proximity between the species by demonstrating either the proximity at the chromosome level and at the level of plasmids among the *B. cereus s.l.* members.

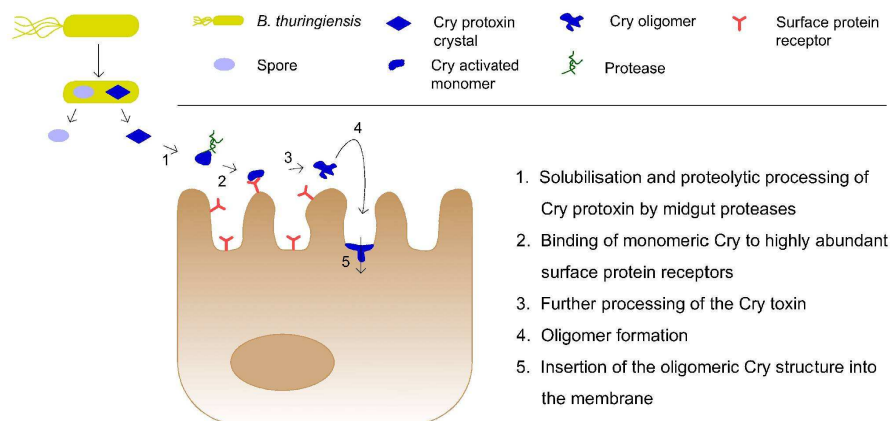
Regarding this critical speciation between the *B. cereus* group members, phenotypic tests may be performed in order to discriminate *B. anthracis* from other group members like *B. cereus* and *B. thuringiensis*. Most *B. anthracis* strains possess some typical phenotypic traits such as sensitivity to penicillin and amoxillicin, the absence of motility, the incapacity of hemolysis, and the production of the capsule (Vilas-Boas *et al.*, 2007).

1.1.2 *B. THURINGIENSIS*

B. cereus and *B. thuringiensis* are critically distinguished by phenotypic identification tests because those organisms are both positive for motility, tyrosine degradation, phosphatase production, resistance to penicillin and hemolysis, even if this latter ability is not always present (Slamti *et al.*, 2004). The only distinction between the two species is the ability of *B. thuringiensis* to generate parasporal crystals inclusions, or δ -endotoxins (Crickmore *et al.*, 1998; Schnepf *et al.*, 1998). These crystal polypeptides possess a highly specific activity against some insects, and particularly against the insect orders of *Lepidoptera*, *Diptera* and/or *Coleoptera* (Crickmore *et al.*, 1998), but also, depending on the class of the toxin molecule, against the orders of *Hymenoptera*, *Homoptera*, *Orthoptera* and *Mallophaga*. Other non insect organisms such as nematodes, mites and protozoa are also sensitive to the *B. thuringiensis* entomotoxins (Schnepf *et al.*, 1998).

The insecticidal endotoxins are packaged in intracellular inclusions called parasporal crystals, and are produced in large quantities during sporulation. These crystals are mainly composed of one or more pore-forming toxins: the crystal (Cry) and cytolytic (Cyt) proteins. Upon ingestion of the inclusions by insect larvae, the crystals are solubilised by the alkaline pH of the sensitive insect gut and they release their components as protoxins.

The Cyt toxins directly insert into membrane lipids after the formation of a pore. On the contrary, the Cry protoxins need first to be solubilized and processed by insect midgut proteases (Fig. 3). An activated structure will be consequently formed that can specifically interact with surface proteins receptors located in the microvilli of larvae epithelial cells. This specific binding leads to the formation of an oligomeric structure that can insert in the membrane through the formation of cation-selective pore. This results in destruction of the epithelial cells lining the midgut and drop of the pH leading to the death of the insect by paralysis of feeding and starvation. Besides, a parallel proliferation of *B. thuringiensis* and other bacteria may lead to septicemia (Aronson and Shai, 2001; Bravo *et al.*, 2007; Bravo *et al.*, 2011; Schnepf *et al.*, 1998)

**Figure 3 – Mode of action of Cry toxins**Modified from Bravo *et al.*, 2011

Insect specificity is largely determined by the specific interaction between Cry toxins and surface proteins receptors and therefore, the mode of action of the Cry toxins depends on the insect order.

Thanks to its pesticidal characteristic, *B. thuringiensis* has been extensively used in agriculture as a biopesticide in order to struggle insect pests during fruits and vegetables production. The use of *B. thuringiensis* insecticidal toxins in transgenic crops has been shown tremendously successful and beneficial, leading to higher yields and to significant reduction in the use of chemical insecticides which were associated with environmental pollution and chronic human health problems (Sanahuja *et al.*, 2011).

The *B. thuringiensis* entomotoxins are considered as inoffensive for humans, vertebrates and plants because they specifically bind to receptors only present in the intestines of insects (Frederiksen *et al.*, 2006). However, *B. thuringiensis* has already been implicated in several food poisonings (Hansen and Hendriksen, 2001; Jackson *et al.*, 1995; McIntyre *et al.*, 2008) and has been found in ready-to-eat foods (Rosenquist *et al.*, 2005) and in fresh fruits and vegetables (Frederiksen *et al.*, 2006). Interestingly, some strains of *B. thuringiensis* are capable of producing the same potential diarrheal enterotoxins as those produced by *B. cereus*. For instance, the

genes encoding the hemolysin BL (HBL), the non-hemolytic enterotoxin (NHE) and the cytotoxin K (CytK) were found in insecticidal commercial strains of *B. thuringiensis* (Damgaard, 1995). *B. thuringiensis* isolated from the intestines of rodents and insectivores also harbored the genes coding for these three potential diarrheal toxins with a highest frequency for *nhe* gene (Swiecicka *et al.*, 2006b). Very high occurrence of several toxin genes (100 % for *NheABC* and *entFM*; 86.80 % for *hblCDA*; and 83.90 % for *cytK*) was reported in a study on 205 *B. thuringiensis* strains (Ngamwongsatit *et al.*, 2008). Beside the gene presence, the expression of the toxin is relevant in order to evaluate the toxigenic potential of an isolate. One study evaluated the expression of the three components forming the HBL complex and some *B. thuringiensis* strains were shown to express the three HBL constituents leading to a complete toxicity of the toxin (Pruss *et al.*, 1999). Besides, the toxicity of several *B. thuringiensis* strains was tested on Vero cell lines (monkey kidney epithelial cells) and it was shown to be generally equivalent to the toxicity of *B. cereus* strains isolated from food poisonings (Gaviria Rivera *et al.*, 2000).

B. thuringiensis carries the genetic arsenal for producing potential diarrheal enterotoxins and this is of importance since this organism is widely sprayed for protecting agriculture worldwide. Although the number of food poisonings due to *B. thuringiensis* is extremely limited, the critical distinction between the *B. cereus* group members with normal culture confirmation procedures may lead to underreporting.

1.1.3 *B. MYCOIDES* AND *B. PSEUDOMYCOIDES*

B. mycoides and *B. pseudomycoides* are non motile and can be easily distinguished from close *B. cereus* species due to their colony shape, made by filaments of cells, resembling fungal hyphae and called rhizoid growth. The filaments may extend from several centimeters which is a crucial advantage to reach nutrients in soils. Depending on the strain, the rhizoid growth can curve clock- or counterclockwise and the curvature is apparently genetically determined (Di Franco *et al.*, 2002; Di Franco *et al.*, 2005). *B. mycoides* and *B. pseudomycoides* are both present in soils and more specifically they have been recovered from roots, mycorrhizae, and rhizosphere soil of conifers with which they act as plant growth-promoters (Petersen *et al.*, 1995).

B. mycoides and *B. pseudomycoides* share phenotypic traits but are genetically distinct. Although they are not distinguishable by physiological and morphological characteristics, they can be differentiated on the basis of their fatty acid composition (Nakamura, 1998).

B. mycoides presents an antagonist activity against various fungi species such as *Penicillium* spp. or *Trichoderma* spp. (Pandey *et al.*, 2001). Therefore, it has been used as a biological control agent to reduce pest and disease affecting plants. As for example, foliar applications of *B. mycoides* strains succeeded, as efficiently as standard fungicides, to reduce the Cercospora leaf spot (*Cercospora beticola*) of sugar beet (Bargabus *et al.*, 2002). *B. mycoides* also allowed to control *Glomerella cingulata* var. *orbiculare*, the causal agent of anthracnose of cucumber (Neher *et al.*, 2009). More recently, the mechanism of predation exerted by *B. mycoides* against *Microcystis aeruginosa*, a toxic cyanobacterium which is responsible for harmful algal blooms affecting global quality water by production of microcystins, has been elucidated. Promising results have been obtained that promotes the use of *B. mycoides* as a biological agent (Gumbo and Cloete, 2011).



Figure 4 – Algae bloom in Lake Erie (United States)

The green scum is the toxic algae bloom which occurred in Lake Erie on 5th October 2011

(<http://earthobservatory.nasa.gov/IOTD/view.php?id=76127>)

The implication of *B. mycoides* and *B. pseudomycoides* in food poisoning is unclear although one *B. mycoides* strain was related to an outbreak that happened in Canada (McIntyre *et al.*, 2008). The occurrence of *B. mycoides* in milk and its toxigenic potential with regard to the diarrheal toxins from *B. cereus* was evaluated in two studies. In the first study, a psychrotrophic isolate of *B. mycoides* was found in milk which harbors *nheA* and *nheC* genes (coding for the NHE toxin from *B. cereus*), but not *hbl* genes (coding for the HBL toxin) (Zhou *et al.*, 2008). In the second study, 30 % and 70 % of the *B. mycoides*/*B. pseudomycoides* isolates harbored *nheA* and *hblA* genes, respectively (Bartoszewicz *et al.*, 2008).

Besides food poisoning cases, *B. mycoides* has been implicated with other bacteria in an upper respiratory distress of grey parrots (*Psittacus erithacus*) (Burr, 1981) and it has also been isolated in channel catfish (*Ictalurus punctatus*) (Goodwin *et al.*, 1994). Regarding human pathogenesis, *B. mycoides* was implicated in an endophthalmitis infection (Ansell *et al.*, 1980).

1.1.4 *B. CEREUS SENSU STRICTO*

B. cereus s.s. can be found in a wide variety of niches from soils to intestines of healthy arthropods and healthy humans (Jensen *et al.*, 2003; Swiecicka and Mahillon, 2006a). The estimated prevalence of *B. cereus* in the intestines of healthy human individuals extends from 15 to 40 % in the population

(Apetroaie *et al.*, 2005; Wijnands *et al.*, 2002). Nevertheless, soil is probably the main source of contamination as it has been shown to contain 10^5 – 10^6 spores of *B. cereus* per g (Andersson *et al.*, 1995). Due of its spore formation and spore resistance, *B. cereus* can live in soils with a low nutrient amount (Schoeni and Wong, 2005) and it can contaminate a large variety of agricultural products. Incidentally, *B. cereus* has been isolated from a wide variety of raw and processed foods, including pasta, rice (unhusked and white rice), dairy and dried milk products, dried foodstuffs, meat, chicken, vegetables, fruits and seafood (Chen *et al.*, 2009; Fangio *et al.*, 2010; Nishikawa *et al.*, 1996; Schoeni and Wong, 2005). Two major problems for food industry are associated with *B. cereus* presence: spoilage and/or foodborne illnesses.

This bacterium is responsible for different types of disease: non-gastrointestinal infections including systemic and local infections such as septicaemia, bacteremia, endocarditis, pneumonia, pleuritis, periodontitis, osteomyelitis, meningitis, endophthalmitis and eye infections (Arnaout *et al.*, 1999; Callegan *et al.*, 1999; Callegan *et al.*, 2006; Frankard *et al.*, 2004; Hilliard *et al.*, 2003; Kotiranta *et al.*, 2000; Miller *et al.*, 1997). Although the toxins implicated in these cases are not precisely known, a large number of potential virulence factors may contribute to the pathogenesis of *B. cereus* such as hemolysins (cereolysin, hemolysin II and sphingomyelinase), phospholipases C and proteases (Granum, 1994; Michelet *et al.*, 2005; Schoeni and Wong, 2005).

B. cereus also exhibits a health risk to the consumer through the induction of the two types of gastrointestinal illnesses: emesis and diarrhea. However, the mechanism of these two syndromes is radically different leading to diverse symptoms (Table 1). The scenario is rather simple for emesis since it results from the consumption of dishes contaminated with one unique toxin, cereulide. The fact that the toxin is preformed in food implies a very short incubation time from 1 to 5 hours. On the opposite, the situation is still unclear for the diarrheal syndrome notably regarding the potential toxins responsible for the disease. It is postulated that the disease is caused by ingestion of *B. cereus* cells that grow and generate enterotoxins inside the

1. INTRODUCTION

1.1. The *Bacillus cereus sensu lato* group

intestinal tract of humans, which explains the longer incubation time of 8 to 16 hours (Granum and Lund, 1997). The infective doses of *B. cereus* are given as a range because it depends on various factors including *e.g.* the amounts of toxins produced and the susceptibility of target population. It is recognized that more severe forms of illnesses have been correlated with young adults and elderly people.

Table 1 – Characteristics of *B. cereus* diarrheal and emetic syndromes

<i>B. cereus</i> diarrheal and emetic syndromes							
Syndrome	Toxin production	Incubation	Duration	Infective dose	Symptoms	Similar food poisonings	Foods mostly implicated
Diarrhea	in small intestine of the host	8 - 16 h	12 - 24h	10 ³ to 10 ⁷ cfu ingested	Abdominal pain, watery diarrhea, moderate nausea, seldom vomiting	<i>Clostridium perfringens</i>	Milk, soup, meat products, pudding, sauces
Emesis	preformed in foods	1 - 5h	12h - 24h	10 ⁵ to 10 ⁸ cfu / g of food	Nausea, vomiting, occasional abdominal cramps and/or diarrhea	<i>Staphylococcus aureus</i>	Starchy foods (rice, pasta, pastries)

Different enterotoxins are considered as toxin-candidates for causing the diarrheal syndrome: the enterotoxin hemolysin BL (HBL), the non-hemolytic enterotoxin (NHE), the necrotic cytotoxin K (CytK), the enterotoxin FM (entFM) and the enterotoxin T (BceT) (Agata *et al.*, 1995a; Asano *et al.*, 1997; Beecher and Wong, 1994a; Beecher and Wong, 1994b; Lund *et al.*, 2000; Lund and Granum, 1996). However, the two latters have never been demonstrated to be directly implicated in diarrheal food poisoning. The CytK enterotoxin has been last discovered, following a serious outbreak of gastroenteritis causing the death of three people in France (Lund *et al.*, 2000). Two different forms of CytK, CytK-1 or CytK-2, were subsequently described and CytK-1 was considered as more toxic based on cellular tests performed on human intestinal cells (Fagerlund *et al.*, 2004).

Table 2 - *B. cereus* enterotoxins

<i>B. cereus</i> enterotoxins						
Toxin	Characteristics	Genetic determinants	Molecular mass (kDa)	Toxicity	Detection kits	References
Hemolysin BL (HBL)	tri-component protein	B encoded by <i>hblA</i> gene	38,0	-	-	Beecher and Wong, 1994
		L1 encoded by <i>hblC</i> gene	38,5	Vero-cells	-	
		L2 encoded by <i>hblD</i> gene	43,2	BCET-RPLA (Oxoid)	-	
Non-hemolytic enterotoxin (NHE)	tri-component protein	NheA encoded by <i>NheA</i> gene	41,0	BDE-VIA (Tecra)	-	Lund and Granum, 1996 ; Granum et al., 1999
		NheB encoded by <i>NheB</i> gene	39,8	Vero-cells	-	
		NheC encoded by <i>NheC</i> gene	36,5	-	-	
Enterotoxin T (boeT)	single protein	encoded by <i>boeT</i> gene	41,0	-	-	Agata et al., 1996
Enterotoxin FM (entFM)	single protein	encoded by <i>entFM</i> gene	45,0	-	-	Asano et al., 1997; Shinagawa et al., 1990;
Cytotoxin K (CytK)	single protein variant 1 (CytK-1)	encoded by <i>cytK1</i> gene	34,0	Vero-cells	-	Lund et al., 2000; Fagerlund et al., 2004
	single protein variant 2 (CytK-2)	encoded by <i>cytK2</i> gene		-	-	Guinebreière et al., 2006
Cereulide	cyclic dodecadepsipeptide	encoded by <i>ces</i> cluster gene	1,2	-	-	Agata et al., 1995

The emetic form of food poisoning was first identified in the 1970's and was associated with the consumption of fried rice (Kramer and Gilbert, 1989). It was only in 1995 that the structure of the causative toxin was identified and then named cereulide (Agata *et al.*, 1995b). A complete overview regarding the properties, the synthesis, the biological role and the effects of cereulide will be fully described in Part 2.

1.1.5 *B. WEIHENSTEPHANENSIS*

As previously mentioned, *B. cereus s.l.* possesses the ability to grow in a wide range of temperatures (from 4°C to 45°C). The strains able to grow below 7°C up to 43°C are considered as psychrotrophic strains. These strains are mostly clustered in the *B. weihenstephanensis* group (Lechner *et al.*, 1998), notably on the basis of their 16S and 23S RNAr and the presence of a particular variant of the cold shock protein A gene (*cspA*). However, all the psychrotolerant strains of the *B. cereus* group are not necessarily *B. weihenstephanensis* (Stenfors and Granum, 2001). Furthermore, some intermediate forms regarding genetic and phenotypic features between the mesophilic strains and the psychrotrophic strains may exist.

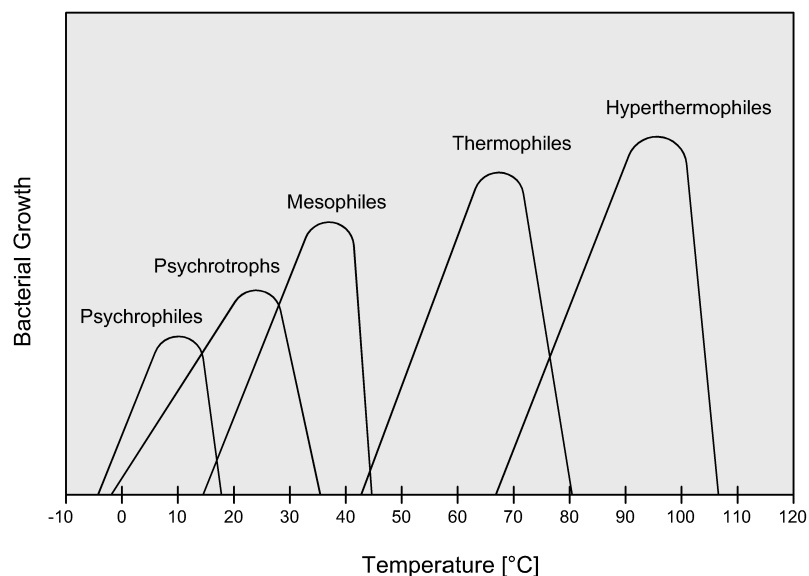


Figure 5 – Temperature scales for bacterial growth

Both enteropathogenic and emetic *B. weihenstephanensis* have been reported and this discoveries draw attention to a potential risk of food poisoning from psychrotolerant strains in cooked chilled foods. However, up to now, *B. weihenstephanensis* has not been implicated in food poisoning in spite of the presence of its virulence characters.

Some studies showed the presence of enterotoxigenic genes and/or the production of enterotoxin components by *B. weihenstephanensis* strains: some strains isolated from ice creams exhibited *nheA*, *nheB*, *nheC*, *hblA*, *hblC*, *hblD* and *cytK* genes (Zhou *et al.*, 2010) and a high fraction (60 %) of *B. weihenstephanensis* soil and milk isolates possessed *hblA* gene (Pruss *et al.*, 1999). In a third study, several strains were also tested with commercial kits for the production of the toxins. All the 23 tested strains had part of at least one of the *B. cereus* potential enterotoxins Hbl, Nhe or CytK. And some of the strains were highly cytotoxic when tested on Vero cells (Stenfors *et al.*, 2002).

Furthermore, recent evidence of cereulide production by two *B. weihenstephanensis* strains was reported (Thorsen *et al.*, 2006). Although it was recognized that cereulide is produced by mesophilic strains at temperature higher than 8°C (Häggblom *et al.*, 2002; Jäskeläinen *et al.*, 2003a), recent findings showed cereulide production by *B. weihenstephanensis* at temperature as low as 8°C (Thorsen *et al.*, 2009a; Thorsen *et al.*, 2009b). These results confirmed concern about the consumer risk of acquiring an emetic intoxication after consumption of refrigerated foods. The safety of the cold chain should therefore be carefully investigated. However, the production of cereulide by *B. weihenstephanensis* strains is considered as very rare by Guinebretière and coll., (2010).

Considering the critical speciation of the *B. cereus* group members associated with the variability in their phenotypic features and the very broad thermal range for their growth temperatures, a new structure for *B. cereus* populations based on ecological differences was proposed (Guinebretière *et al.*, 2008). The idea is based on the fact that different ecological populations may be associated in function of their temperature growth rather than their

species affiliations in the *B. cereus* group. Seven major phylogenetic groups were designed using AFLP (Amplified Fragment–Length Polymorphism), data on growth temperatures, psychrotolerance signature and thermal niches.

Temperature tolerance was shown to have an important impact of *B. cereus* ecological adaptation since this new classification was structured in two psychrotolerant groups (VI, II), three mesophilic groups (I, III, IV), an intermediate group (V) and a moderate thermotolerant group (VII). All the groups assembly more than one taxon (*e.g.* group II comprises psychrotolerant *B. thuringiensis* and *B. cereus*; group III comprises mesophilic *B. thuringiensis*, *B. cereus* and *B. anthracis* and group VI comprises psychrotolerant *B. thuringiensis*, *B. mycoides* and *B. weihenstephanensis*) with the exception of group I (*B. pseudomycoides*) and group VII (CytK–1–producing *B. cereus*). This latter cluster is constituted by highly toxic strain which are moderate thermotolerant and which can be considered as a novel bacterial species named *Bacillus cytotoxicus* (Lapidus *et al.*, 2008). More recently, additional new strains, mostly isolated from food poisoning, have been added to this *B. cytotoxicus* cluster which was confirmed to be inside the *B. cereus* group although distant (Guinebretière *et al.*, 2012).

1.1.6 *B. CYTOTOXICUS*

The natural niche of *B. cytotoxicus* is still unknown but several strains were isolated from purees associated with food poisonings. To date, only five strains are clustered in this new genomic species and three of them are originating from France, one from Germany and one from Norway. This new species shares high genetic proximity with the *B. cereus s.l.* group but displays distinct phenotypic features, notably its thermotolerant ability (minimal and maximal growth temperatures at 20 and 50°C, respectively), its inability to hydrolyse starch and its auxotrophy for tryptophan (Guinebretière *et al.*, 2012).

1.2 CEREULIDE, THE HEAT-STABLE TOXIN OF *B. CEREUS*

Cereulide is responsible for the emetic food poisoning caused by *B. cereus*. This syndrome is characterized by nausea, vomiting and abdominal cramping, resembling *Staphylococcus aureus* food poisoning (Ankolekar *et al.*, 2009), with a short incubation time from 1 to 5 h after ingestion. The disease is generally self-limiting and recovery usually occurs within 24 h which may explain the underreporting of *B. cereus* food poisoning (Granum and Lund, 1997; Nelms *et al.*, 1997).

Nevertheless, several lethal cases have occurred worldwide and most of them were related to the consumption of pasta or rice (Table 3). The victims were young (below 20 year-old) and the death occurred within hours. Starchy foods, among which rice is the most representative because it was incriminated in 95 % of intoxications, are very frequent vehicles for the emetic syndrome (Altayar and Sutherland, 2006). Many other foods can support cereulide production such as beef, poultry, vanilla sauce, pasteurized cream, milk pudding, milk product, pasteurized milk, dairy products, bread, potato, or infant formulas (Kroten *et al.*, 2010; Schoeni and Wong, 2005) or even, as described by Svensson *et al.*, (2006), cooked vegetables, grilled chicken, soy bean curd, fried fish and UHT milk. However, cereulide accumulation in these foods may vary in function of their composition as discussed in Section 1.2.6.

Non lethal outbreaks incriminating emetic *B. cereus* strains have also been globally reported with more or less severe outcome. Several of them implicated children, from 5 to 11-year-old (Ichikawa *et al.*, 2010; Posfay-Barbe *et al.*, 2008; Yotsumoto *et al.*, 2008) and several others involved a large number of people, from 37 to 116 individuals (Essen *et al.*, 2000; Kamga Wambo *et al.*, 2011; Kim *et al.*, 2010a). Although the type of involved foods involved was mainly pasta or rice products, two unexpected food products were also at the origin of foodborne outbreaks: dried figs (Hormazábal *et al.*, 2004) and tuna fish (Domenech-Sanchez *et al.*, 2011).

Table 3 – Reported lethal intoxications due to cereulide

Year	Country	Victims n	Death (age)	Time from lunch to death (h)	Incriminated food	Symptoms	Pathological features - Target Organ	Reference
1974	Japan	50	1(11)	15	Noodles	Vomiting, Diarrhea in 1h	Fatty degeneration of the liver/heart, brain edema	Takabe and Oya, 1976
1997	Switzerland	2	1(17)	48	Spaghetti pesto (prepared 4 days earlier)	Vomiting in 30 min	Liver failure, diffuse brain edema, kidney	Malher et al., 1997
2003	Belgium	5	1(7)	13	Pasta salad (prepared 3 days earlier)	Vomiting in 6 h	Liver failure, brain edema, pulmonary hemorrhage	Dierick et al., 2005
2008	Belgium	1	1(20)	10	Spaghetti bolognaise (prepared 5 days earlier)	Vomiting in 30 min	Acute cardiac insufficiency, liver necrosis, pancreas?	Naranjo et al., 2011
2010	Japan	3	1(1)	6	Fried rice	Vomiting in 30 min	Acute encephalopathy	Shiota et al., 2010

In the most recent Japanese case, one of the child victims (a 2-year-old girl) was able to recover thanks to plasma exchange and hemodialysis whereas her 1-year-old brother passed away. Interestingly, cereulide was detected in her stool (0.16 µg/g), urine (0.008 µg/ml), gastric fluid (0.004 µg/ml) but also in her blood serum (up to 0.004 µg/ml) (Shiota *et al.*, 2010).

Inappropriate holding of temperature of cooked foods is the major risk factor associated with the emetic syndrome (Ehling-Schulz *et al.*, 2004a; From *et al.*, 2005; Shinagawa, 1990). The emetic toxin possesses a remarkable heat resistance profile: it withstands heating at 121°C for 2h at pH7 and even heating at 126°C for 90 minutes, which infers that cereulide is not inactivated by normal food preparations or reheating of the cooked foods (Rajkovic *et al.*, 2008; Turnbull *et al.*, 1979). Furthermore, cereulide is not destroyed in the stomach by proteolytic enzymes and gastric acid due to its resistance towards digestive proteases and acid conditions (Agata *et al.*, 1994; Shinagawa *et al.*, 1996). It is therefore essential to prevent the bacterial outgrowth and the subsequent cereulide production in food by keeping the aliments at refrigerated temperatures and by limiting the duration of storage.

1.2.1 CEREULIDE SYNTHESIS AND MOLECULAR REGULATION

Cereulide structure consists of a 36-member ring containing three repetitions of four amino acids (two amino acids and two hydroxy-amino acids) with alternating ester and amide bonds, as showed in Fig. 6. According to the International Union of Pure and Applied Chemistry (IUPAC), a peptide with at least one peptide bond replaced by an ester bond is called a depsipeptide. Cereulide is consequently a dodecadepsipeptide (Kroten *et al.*, 2010). This type of heterocyclic structure with incorporated D-amino acids is clearly related to the non ribosomal machinery for peptide synthesis (NRPS) (Toh *et al.*, 2004). The structure of cereulide is quite similar to the one of valinomycin which is an antibiotic bacterial molecule produced by *Streptomyces griseus* and *S. fulvissimus* (Park *et al.*, 2008) and also by *Bacillus subtilis* (Kroten *et al.*, 2010).

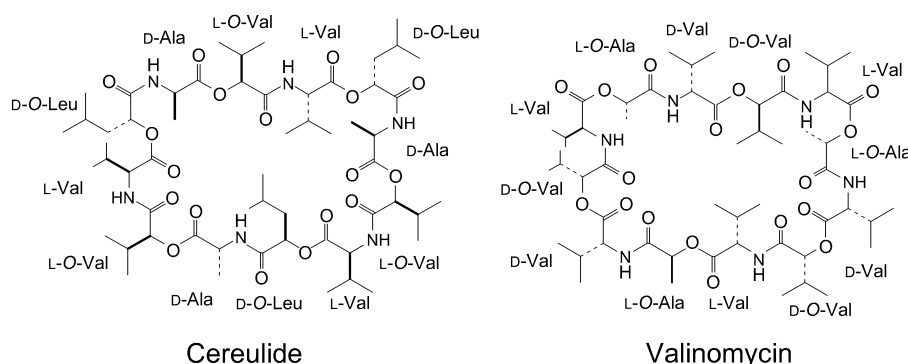


Figure 6 – Structures of cereulide and valinomycin
Reprinted from Pitchayawasin *et al.*, 2004

1.2.1.1 BIOSYNTHESIS OF CEREULIDE

Cereulide and valinomycin are both synthesized by special large enzymes called non-ribosomal peptide synthetases (Toh *et al.*, 2004). These enzymatic complexes are characterized by a multi-domain structure and typically utilize a thio-template mechanism for activation, modification, and condensation of the constituent amino acids of the peptide product (Toh *et al.*, 2004). In the case of cereulide, the non-ribosomal peptide synthase is encoded by the cereulide synthase gene cluster (*ces*) of 24kb which contains

7 genes with different activities leading to the complete construction of cereulide: *cesH*, *cesP*, *cesT*, *cesA*, *cesB*, *cesC* and *cesD* (Ehling-Schulz *et al.*, 2006a).

The structural organization of the *ces* gene locus is schematically presented in Fig. 7. Briefly, *cesA* and *cesB* are the structural genes responsible for the assembly of the peptide by incorporation of two monomers in the peptide chain. The other typical genes located on the *ces* genetic locus are: *cesP* encoding a 4-phosphopantetheinyl (4-PP) transferase for priming the NRPS and *cesT* encoding a putative thioesterase which removes misprimed monomers. Moreover, the gene cluster contains *cesH* gene encoding a putative hydrolase and *cesC* and *cesD* encoding a putative ABC transporter. The *ces-PTABCD* genes are transcribed together as an operon, while *cesH* is transcribed from its own promoter (Dommel *et al.*, 2010).

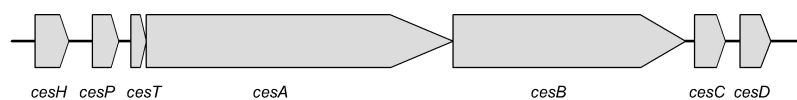


Figure 7 – Schematic representation of the *ces* gene cluster
Simplified from Ehling-Schulz *et al.*, 2006b – See text for details

The cereulide synthase (*ces*) gene cluster involved in the cereulide synthesis is located on a large virulence plasmid which is highly conserved between emetic strains and absent in non-emetic strains. This plasmid (270 kb), sharing the same backbone as the *B. anthracis* pXO1 plasmid, has been determined to carry the genetic determinants for cereulide production in various *B. cereus* strains (Ehling-Schulz *et al.*, 2006a; Horwood *et al.*, 2004; Hoton *et al.*, 2005). The megaplasmid is differently named according to the *B. cereus* strain in which it has been found : pCERE01 in the *B. cereus* emetic strain Kinrooi 5975c (Hoton *et al.*, 2005), pBC270 in *B. cereus* strain AH187 (Rasko *et al.*, 2007) and pBCE4810 plasmid in the *B. cereus* reference strain F4810/72 (Ehling-Schulz *et al.*, 2006a).

1.2.1.2 REGULATION OF CEREULIDE

As mentioned above, cereulide has been proved to be synthesized non-ribosomally by the plasmid-encoded *ces* peptide synthetase. Its production is

negatively regulated by the two sporulation regulators SpoOA and AbrB, and is independent of the well known pleiotropic transcription factor phospholipase C regulator (PlcR) which regulates a vaste panel of the *B. cereus* enterotoxins (Dommel *et al.*, 2010; Lucking *et al.*, 2009).

The SpoOA-AbrB regulon mechanism is function of the levels of expression of these respective regulators which are differently expressed during growth phases. AbrB is expressed at maximal levels at the start of the exponential growth but those levels decrease until the mid-exponential phase while SpoOA is only expressed during the stationary phase. One one hand, AbrB acts as a repressor of the cereulide production and so, overexpression of AbrB factor, as tested by Lucking *et al.*, (2009), resulted in a non-toxic phenotype of *B. cereus*. In fact, AbrB binds to the main promoter of the *ces* operon region and, in this manner, prevents the *ces* gene expression (Lucking *et al.*, 2009). One the other hand, SpoOA acts as a direct regulator of AbrB by binding it. In this way, it stops the inhibition activity of AbrB and it allows the *ces* gene transcription leading to the production of cereulide (Fig. 8).

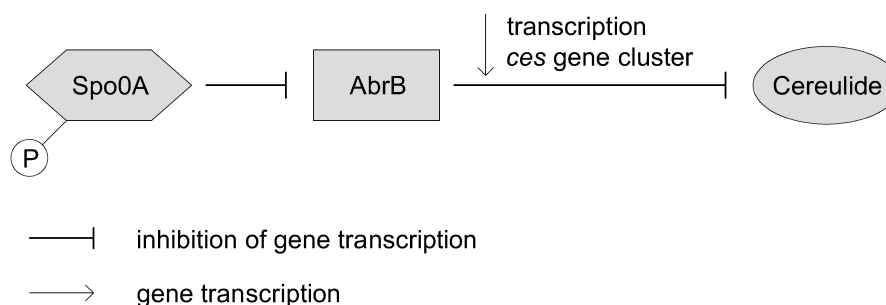


Figure 8 – Schematic overview of the SpoOA-AbrB regulation of cereulide

AbrB exerts a negative regulation of cereulide transcription by binding to the main promoter of the *ces* operon. When AbrB is inhibited by phosphorylated SpoOA, the cereulide expression is re-established.

It can be anticipated that the levels of cereulide are dependent on the transcription of the *ces* operon. Two studies showed that the transcription of the *ces* genes happens in mid exponential growth phase, is maximal at the late exponential phase and is then after strongly downregulated (Dommel *et*

al., 2011; Frenzel *et al.*, 2011). Surprisingly, the maximum levels of cereulide are accumulated in the stationary phase implying that the decrease of *ces* genes transcription in the stationary phase does not entail a complete cessation of cereulide production. Actually, other extrinsic (environmental and nutritional) factors play a role in the expression of cereulide, and this specific issue will be addressed in Section 1.2.6.

1.2.2 ANALOG NON-RIBOSOMAL PEPTIDES

The NRPS enzymes are associated with the formation of a wide variety of non-ribosomal peptides present in prokaryotes and eukaryotes, including various fungal species. The NRPS products exhibit broad structural and biological activities as illustrated in Table 4.

Some of these peptides are directly involved in the metabolism of the organism (*e.g.* the bacillibactin-related siderophores for chelating of iron). Others are involved in the pathogenicity of the micro-organism by displaying antifungal and antibacterial properties (Weber and Marahiel, 2001), as for example, β -lactam antibiotics (*e.g.* penicillin), the species-specific antibiotics tyrocidine and gramicidin S, cyclosporin, bacitracin, mycosubtilin, vancomycin or surfactants (Horwood *et al.*, 2004; Toh *et al.*, 2004). The various biological properties (antibiotic, immunomodulating (*e.g.* cyclosporin), antiviral and antitumor (*e.g.* bleomycin) activities) that NRPS products display are of utmost importance for pharmacological applications and for drug discovery. Therefore, a comprehensive database for non-ribosomal peptides has been recently built and is freely accessible at <http://bioinfo.lifl.fr/norine/> (Caboche *et al.*, 2008; Caboche *et al.*, 2010).

Table 4 – Non exhaustive list of non-ribosomal molecules
(implemented from Caboche *et al.*, 2008; 2010; Moffitt
and Neilan, 2000)

Name	Micro-organism	Primary function	Secondary function	References
Actinomycin D	<i>Streptomyces antibioticus</i>	-	Antibiotic, antitumor	Pfennig et al., 1999
Bacillibactin	<i>Bacillus subtilis</i>	Siderophore	-	May et al., 2001
Bacilysin	<i>B. subtilis</i>	-	Antibiotic	Phister et al., 2004
Bleomycin	<i>Streptomyces verticillus</i>	-	Antitumor, antibiotic	Shen et al., 2001
Cereulide	<i>B. cereus</i>	Ionophore	Antifungal, antibacterial	Agata et al., 1995
Cyclosporin	<i>Tolypocladium inflatum</i>		Antibiotic, immunomodulating	von Dohren et al., 1997
Daptomycin	<i>Streptomyces roseosporus</i>	-	Antibiotic	McHenney et al., 1998
Edein A	<i>Bacillus brevis</i>	-	Antibiotic, immunomodulating	Czajgucki et al., 2006
Enniatin	<i>Fusarium spp.</i>	Ionophore	Entomotoxin, antibiotic, anticancer	Firáková et al., 2007
Fengycin	<i>B. subtilis</i> 168	Biosurfactant	Antimicrobial, antiviral	Tosato et al., 1997
Gramicidin S	<i>Bacillus brevis</i>	-	Antibiotic	Gause and Brazhnikova, 1994
Kanosamine	<i>B. cereus</i>	-	Antibiotic	Milner et al., 1996
Lichenysin A	<i>Bacillus licheniformis</i>	Biosurfactant	Toxin	Yakimov et al., 1995
Microcystin	<i>Microcystis aeruginosa</i>	-	Toxin	Tillett et al., 2000
Monensin	<i>Streptomyces cinnamonensis</i>	-	Antimicrobial	Huczynski et al., 2008
Montanastatin	<i>Streptomyces annulatus</i>	-	Antibiotic, anticancer	Ppette et al., 1999
Mycobactin	<i>Mycobacterium tuberculosis</i>	Siderophore	Virulence factor	Quadri et al., 1998
Mycosubtilin	<i>B. subtilis</i>	-	Antifungal, antibiotic	Duitman et al., 1999
Nigericin	<i>Streptomyces hygroscopicus</i>	-	Phytotoxin	Steinrauf et al., 1968
Nodularin	<i>Nodularia spumigena</i>	-	Hepatotoxin	Moffitt and Neilan, 2004

1. INTRODUCTION

1.2. Cereulide, the heat-stable toxin of *B. cereus*

Name	Micro-organism	Primary function	Secondary function	References
Pyochelin	<i>Pseudomonas aeruginosa</i>	Siderophore	-	Serino et al., 1997
Pyoverdine C	<i>Pseudomonas putida</i>	Siderophore		Seinsche et al., 1993
Surfactin	<i>B. subtilis</i>	Biosurfactant	Antibacterial; antiviral; antifungal	Peypoux et al., 1999
Tolaasin	<i>Pseudomonas tolaasii</i>	Biosurfactant	Toxin, antibiotic	Bassarello et al., 2004
Tyrocidine	<i>Bacillus brevis</i>	-	Antibiotic	Mootz et al., 1997
Valinomycin	<i>Streptomyces spp.</i>	Ionophore	Antibiotic	Magarvey et al., 2006
Vancomycin	<i>Nocardia orientalis</i>	-	Antibiotic	Hubbard et al., 2003
Vibriobactin	<i>Vibrio cholerae</i>	Siderophore	-	Butterton et al., 2000
Yersiniabactin	<i>Yersinia pestis</i>	Siderophore	Virulence factor	Gehring et al., 1998
Zwittermicin	<i>B. cereus</i>	-	Antifungal, antiprotist, antibiotic	Emmert et al., 2004

Interestingly, enniatins produced as secondary metabolites by some *Fusarium* spp. display similar ionophore activities as those of cereulide and valinomycin. This specific function will be discussed in the next Chapter.

Globally, the non-ribosomal peptides are short (from 2 to 50 residues) and their diversity is large since they are formed by non proteogenic aminoacids (beyond the 20 classical proteogenic amino acid residues). In addition, they can be chemically modified by epimerization or methylation processes among others. Their structure can be greatly variable (linear, branched, partially or totally cyclic or even poly-cyclic) and some examples are given in Fig. 9.

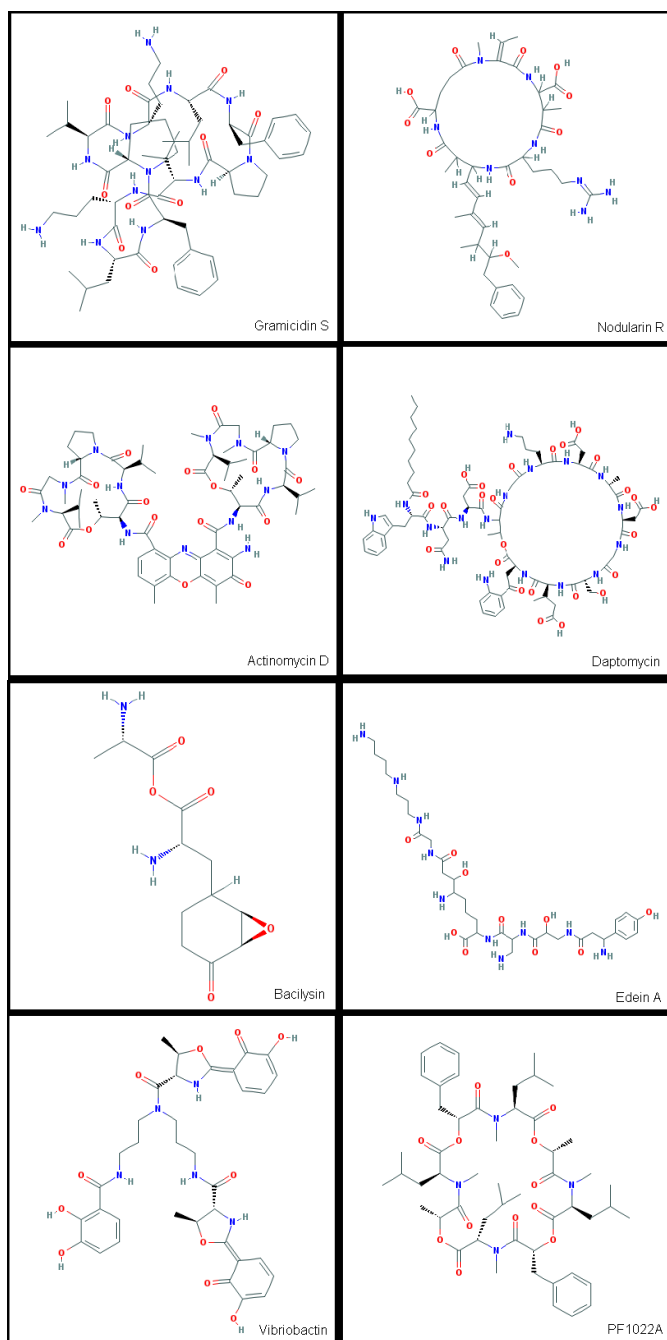


Figure 9 – Molecular structures diversity for non-ribosomal products

Panel 1 – 4: cyclic antibiotics; Panel 5 – 6: linear antibiotics;

Panel 7: branched siderophore; Panel 8: cyclic antibiotic.

The NRPS mechanism has been first described in the early 1970's through the study of two antibiotics: gramicidin S and tyrocidin (Fritz, 1981). This system is highly flexible for biosynthesis and leads to production of novel peptides. The benefit of these substances for the producer-organism is most often not known. However, a large part of these bioactive peptides possess an antibacterial or antifungal spectrum and it may be speculated that they represent a selective advantage to other rival organisms living in the same environment (Walsh, 2000).

Cereulide is a non-ribosomal peptide and questions have been raised regarding its function towards the *B. cereus* producing-strains. It has been hypothesized that cereulide may take part in competition against other organisms by displaying antibacterial or antifungal features. These properties were explored through bacterial, fungal and yeast growth inhibition experiments but remained undetermined for a time, as explained in Section 1.2.4.

1.2.3 IONOPHORETIC PROPERTIES OF CEREULIDE

An ionophore is a lipid-soluble substance that increases the permeability of biological membranes to specific ions (alkali metals and ammonium ions) and that consequently allows the entrance of these ions across the membrane. Cereulide, the emetic toxin produced by *B. cereus* and valinomycin, the antibiotic molecule produced by *Streptomyces* spp., both carry ionophoretic properties notably due to their cyclic structure and because they can form unpaired electron complexes with positively charged ions (Teplova *et al.*, 2006).

Two ways of transporting the ions across the lipid bilayer membranes are known which correspond to two types of ionophores: the shuttle/mobile carriers and the permeable channel formers (Duax *et al.*, 1996). Cereulide and valinomycin act both as channel formers with specific affinity to alkali ions such as potassium (K^+), lithium (Li^+), sodium (Na^+), cesium (Cs^+) or rubidium (Rb^+) (Mikkola *et al.*, 1999). As a consequence, these alkali ions can be transported across the inner mitochondrial membrane and can then accumulate in the mitochondria while they do usually not enter easily inside

it. This massive entry of positive ions from the cytoplasm into the mitochondria induces an efflux of protons (H^+) which results into a short-circuiting of the proton gradient across the mitochondrial membrane. This phenomenon is called the depolarization of the transmembrane electric potential leading to a blockage, or uncoupling, of the oxidative phosphorylation (Fig. 10).

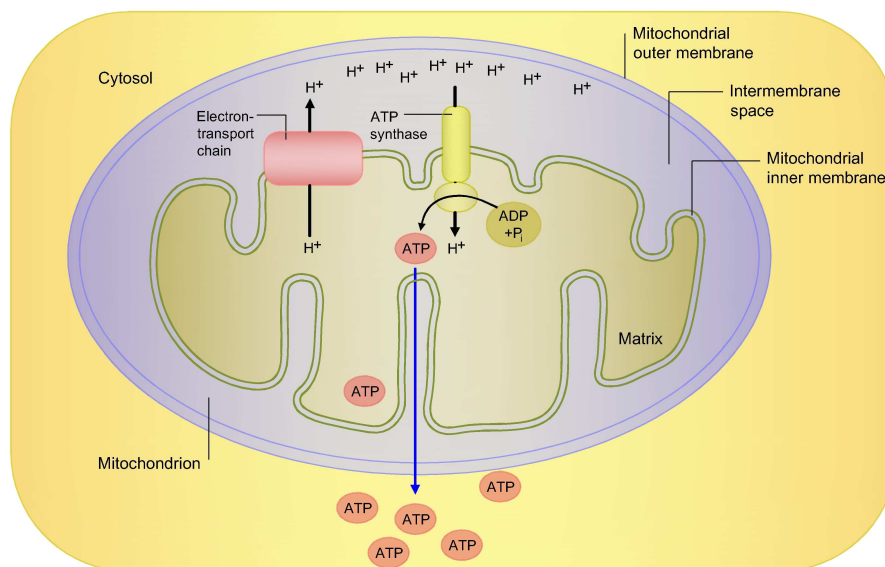


Figure 10 – Electron transport chain
(modified from Krauss et al., 2005)

The electron transport chain (in red) is localized at the inner mitochondrial membrane and results in ATP synthesis thanks to the ATP synthase indicated in yellow. If cereulide is present, it will allow the entrance of K^+ ions inside the mitochondria, while they are mainly present in the cytosol. An efflux of H^+ will take place to counterbalance the influx of K^+ inside. This proton leak will perturbate the H^+ gradient necessary for the ATP synthase and it will uncouple the process of electron transport/proton-gradient generation.

The ionophoretic properties of cereulide were demonstrated by a selective increase in K^+ permeability of phospholipid membranes. Interestingly, cereulide and valinomycin possess their own affinity profile to specific ions. For example, cereulide weakly binds lithium (Li^+), sodium (Na^+), cesium (Cs^+) and slightly more rubidium (Rb^+), while its highest affinity is expressed

towards potassium ions (K^+). The differences in ion specificity may greatly vary as, for instance, the cereulide selectivity ratio of K^+ over Na^+ is of 700:1 (Mikkola *et al.*, 1999). Furthermore, it seems that cereulide is a stronger potassium ionophore than valinomycin at low concentrations of K^+ (1 to 3 mM). This may explain why cereulide is more toxic to animals than valinomycin knowing that this range of concentration corresponds to the physiologic levels of K^+ present in blood serum (Teplova *et al.*, 2006). The same type of explanation could be attributed to enniatins, the *Fusarium* ionophoretic mycotoxins, which present an affinity ratio of K^+ over Na^+ thousand times lower than valinomycin (Pressman, 1976).

The mitochondria of various type of cells are sensitive to exposure to cereulide as for example boar sperm cells (Andersson *et al.*, 1998; Hoornstra *et al.*, 2003), human epithelial (Jääskeläinen *et al.*, 2003b) and neural cells (Teplova *et al.*, 2004). Knowing that mitochondria are key elements in the signal transduction of apoptosis and necrosis, their impairment directly affects the cell (Gottlieb, 2000a; Gottlieb, 2000b). Cereulide cytotoxicity towards sensitive cells is visible through the formation of large vacuoles in the cytoplasm actually corresponding to mitochondria swelling. So, several cell lines sensitive to cereulide have been used as in vitro tests for the detection of the toxin, as discussed in Section 1.3. Cereulide is also the only toxic agent in the studied strains of *B. cereus* which is highly toxic to Beta-cells of foetal porcine pancreatic Langerhans islets by decreasing the insuline content in the islets (Virtanen *et al.*, 2008).

Due to its ionophoretic properties, cereulide also exhibits immunomodulatory properties such as the inhibition of the activity of human natural killer (NK) cells and the induction of their apoptosis (Paananen *et al.*, 2002). Interestingly, HeLa (human epithelial cells derived from a fatal cervical carcinoma) cells and T-lymphocytes are much less sensitive to intoxication than NK cells and, at the other extreme, monocytes are not sensitive at all to cereulide. In addition, it seems that the toxin decreases the cytokine production by immune cells and thereby impairs the immunological potential of organisms (Kroten *et al.*, 2010).

The implication of cereulide in food poisoning is henceforth unquestionable and its toxic effects on mammalian cells are well recognized. In contrast, nothing was known about the biological significance of cereulide for the producing bacterial strains. This important question is explored in the next Chapter.

1.2.4 ROLE OF CEREULIDE AND TOXICOLOGY

1.2.4.1 BIOLOGICAL ROLE OF CEREULIDE

Many ionophores display antimicrobial properties and valinomycin, which is a representative molecule for cereulide, is a long recognized antibiotic (Shemyakin *et al.*, 1965). Moreover, valinomycin also exhibits antiviral, and apoptosis-inducing effects in human cells (Cheng, 2006; Klein *et al.*, 2011; Ryoo *et al.*, 2006; Wu *et al.*, 2004) as well as insecticidal and nematocidal properties (Fabre *et al.*, 1988). Because of the similarities existing between valinomycin and cereulide, it was conjectured that cereulide may also play a role in competing with other micro-organisms.

Therefore, the inhibition potential of cereulide was tested through growth inhibition studies against various organisms: a panel of bacterial, fungi and yeasts species. No antibiotic activity of cereulide could then be shown in the performed studies against the selected organisms (Altayar and Sutherland, 2006). Yet, even if antimicrobial molecules produced by bacteria may cover a broad spectrum of activity, they can, on the contrary, be very specific and only effective against certain species (Riley and Wertz, 2002).

It was only in the year 2011 and during the course of the present thesis, that one of the bioactivities of cereulide was discovered. In fact, Tempelaars and co-workers, (2011) compared the antimicrobial activity of valinomycin and cereulide on a selection of Gram-negative and Gram-positive bacteria under anaerobic and aerobic conditions over a pH range (5.5 to 9.5). They showed a clear growth inhibition of some Gram-positive bacteria, including *S. aureus*, *Listeria innocua*, *L. monocytogenes*, *B. subtilis*, and *B. cereus* ATCC 10987, in presence of both cereulide and valinomycin although at different levels and with a higher effectiveness at alkaline pH values. On the other hand, none of the tested Gram-negative bacteria were affected in their growth by the

presence of cereulide or valinomycin. In their study, they included the reference strains for diarrheal (*B. cereus* ATCC14579) and emetic (*B. cereus* F4810/72) syndromes.

Interestingly, enterotoxic *B. cereus* strain ATCC14579 and the emetic *B. cereus* strains F4810/72 showed increased resistance to both ionophores, with the latter strain reaching full resistance to cereulide. In this manner, the producing-strain would be self protected by a so-called innate immunity (Tempelaars *et al.*, 2011).

Another biological function of cereulide has been very recently advanced by Ekman and collaborators (2012). Their experiments suggested that cereulide helps to maintain K⁺ homeostasis in cereulide-producing *B. cereus* but provokes an efflux of K⁺ from non-producer *B. cereus* in absence of energy source. This effect was also observed towards *B. subtilis* but not towards the Gram-negative *E. coli*. The competitiveness of emetic strains over non-producers was more intense in K⁺-low media than in K⁺-rich media. This suggests that cereulide supports producer strains in low-potassium environments. Furthermore, it was shown in the same study that cereulide promotes biofilm formation which is a multicellular association providing extensive protection to the bacteria.

It is interesting to note that the production of an emetic toxin is not restricted to *B. cereus* s.s. As previously mentioned, *B. weihenstephanensis* strains are able to produce cereulide (Thorsen *et al.*, 2006). Although they have generally been considered of little significance in food poisoning, some *Bacillus* species outside the *B. cereus* group can also produce emetic toxins. Among those, *B. subtilis*, *B. pumilus*, *B. mojavensis* and *B. licheniformis* are often cited as being able to produce a putative emetic toxin different in size from cereulide (From *et al.*, 2005; Ouoba *et al.*, 2008). Taylor and collaborators (2005) also noticed toxin production in *B. firmus*, *B. megaterium*, *B. simplex* and *B. licheniformis*. More recently, the production of cereulide-like depsipeptides has been demonstrated in *Paenibacillus tundrae*, a bacterium which colonizes barley. Two produced molecules (paenilide and homopaenilide) were characterized as being toxic on certain

mammalian cells (boar spermatozoa, porcine kidney tubular epithelial cells and rat liver cells) at temperatures as low as 5°C (Rasimus *et al.*, 2012).

1.2.4.2 TOXICOLOGY OF CEREULIDE TOWARDS HUMANS

Due to its potassium transmembrane carrier role, cereulide is toxic to mitochondria and this means that cereulide is also extremely toxic to humans. It is reported that cereulide can inhibit the hepatic mitochondrial fatty-acid metabolism (or beta-oxidation process) and thereby suggests some liver damage. Different target organs have been reported in several lethal cases of cereulide intoxication, as reminded in Table 3. Particularly, besides liver failure, brain oedema has been seen in four of the five lethal cases caused by cereulide (Dierick *et al.*, 2005; Mahler *et al.*, 1997; Shiota *et al.*, 2010; Takabe and Oya, 1976). Moreover, acute encephalopathy has occurred in one fatal case. In fact, cereulide is thought to be a possible causative agent for Reye-like syndrome with, as one clinical feature, acute encephalopathy (Ichikawa *et al.*, 2010). Cereulide was also proven to be the cause of several clinically significant systemic infections in infants (Hilliard *et al.*, 2003).

As cereulide resists heat process, extreme acid and alkali pH and exposure to human proteases (pepsin and trypsin) (Schoeni and Wong, 2005), the toxin, which is preformed in food, is not destroyed by normal food preparation or digestion. Therefore, it will not lose its toxicity in the stomach and it can easily reach intestines. It is there, in the intestinal mucosa, that the mechanism of the pathogenicity exercised by cereulide takes place.

It was suggested that cereulide binds the 5-HT₃ receptor (or serotonin receptor) which results in the stimulation of the vagal afferent nerve (Agata *et al.*, 1995b) and provokes emesis. The 5-HT₃ receptor consists of 5 subunits arranged around a central ion conducting pore which is permeable to sodium, potassium, and calcium (Ca²⁺) ions. Binding of the neurotransmitter 5-hydroxytryptamine (serotonin) to the 5-HT₃ receptor opens the channel which in turn leads to an excitatory response in neurons (Schmitt, 2007). It is not known whether the emetic effects of cereulide are directly or indirectly dependent on its binding to the 5-HT₃ receptor. It has been hypothesized

that cereulide may exert its emetic effect through the increasing of cytosolic Ca^{2+} concentration which is necessary for translocation of the toxin to the cytosol (Schmitt, 2007).

1.2.5 CEREULIDE TOXIC DOSES AND ALLOWED LEVELS

The exact concentration of *B. cereus* cells at which cereulide starts to be produced is not known and a range of 10^5 to 10^8 CFU/g of food has been given as infective doses for the induction of emetic food poisoning (Granum and Lund, 1997). However, incriminated foods in intoxication may contain as low as 10^3 CFU/g of food (Notermans and Batt, 1998). Therefore, it was not easy to establish safety limits but, generally, any food exceeding 10^3 – 10^5 CFU/g is considered in Europe as unsafe for consumption (EFSA, 2005).

However, there is no global harmonization and allowed levels for *B. cereus* are quite different according to the type of food and to the country. Furthermore, in case of toxin-related food safety issues, it would be more reliable to set maximum levels on the basis of the toxin concentration rather than the bacterial counts.

Emesis-induced doses of cereulide were determined in Asian house shrew (*Suncus murinus*) as 8 $\mu\text{g/kg}$ body weight (bw) (Agata *et al.*, 1994; Isobe *et al.*, 1995) and in Rhesus monkey (*Macaca mulatta*) as 10 $\mu\text{g/kg}$ (Shinagawa *et al.*, 1995). These levels are higher than those of other known toxins (*e.g.* toxin concentration as low as 100 ng/kg bw may trigger *S. aureus* emetic disease) (Bergdoll, 1989; Rajkovic *et al.*, 2006a). Several food samples implicated in emetic food poisonings have been analysed for cereulide contents. According to the study, the concentrations range found in the samples was large: 0.01 – 1.6 μg of cereulide per g food (Virtanen *et al.*, 2008) and 0.01– 1.28 $\mu\text{g/g}$ food (Agata *et al.*, 2002).

1.2.6 FACTORS INFLUENCING CEREULIDE EXPRESSION

Cereulide expression is controlled by various bacterial (intrinsic) and environmental (extrinsic) factors. The production of cereulide can vastly vary from one strain to another, but also according to its growth phase, the temperature, the presence of oxygene, the type of food and the food

properties (nutrients, water activity, pH, aeration, presence of amino-acids,...) (Agata *et al.*, 1999; Apetroaie-Constantin *et al.*, 2008; Finlay *et al.*, 2000; Häggblom *et al.*, 2002; Jääskeläinen *et al.*, 2003a; Jääskeläinen *et al.*, 2004; Rajkovic *et al.*, 2006a). *B. cereus* is ubiquitous in the environment and making foods totally free of bacteria is impossible. It seems therefore crucial to determine the influence of these various parameters on cereulide production for preventing food intoxications.

1.2.6.1 INTRINSIC FACTORS

Cereulide formation relies on bacterial growth and is therefore dependent on the growth phase of a given strain. It is regularly suggested that cereulide production starts during exponential stage when *B. cereus* levels reach 10⁵ CFU/gram of food (Fretz *et al.*, 2007) or 10⁶ CFU/g (EFSA, 2005). However, the cereulide production may be initiated by lower numbers of *B. cereus*, *ca.* 10³ CFU/g (Kramer and Gilbert, 1989; Rajkovic *et al.*, 2006a).

Toxin production is not strictly dependent on the growth rate since it is also highly variable between strains and upon culture conditions. For example, significant levels of *B. cereus* will not necessarily conduct to cereulide production as illustrated by Rajkovic *et al.*, (2006a). Five groups of food products (potato puree, pasta, rice, béchamel sauce and milk) obtained from a local producer were inoculated with a cereulide-producing strain and incubated at 12 or 22°C. Although the strain was able to grow in all food products, no detectable cereulide was found in the béchamel sauce at both temperatures using the boar sperm assay.

Besides, cereulide production between individual strains may differ from 100-fold (Andersson *et al.*, 2004) up to 1000-fold (Carlin *et al.*, 2006) under identical conditions. For instance, the amount of cereulide produced among the strains tested by Svensson *et al.*, (2006) ranged from 0.01 to 2.3 µg cereulide per mg of bacterial biomass wet weight. In summary, it cannot be guaranteed that a cereulide-producing strain which grows at high levels will produce enough cereulide to be toxic.

Moreover, the toxin production may be substantially influenced by environmental factors. Several studies have investigated the respective influence of various parameters (*e.g.* culture medium, temperature or aeration) on cereulide production (Apetroaie–Constantin *et al.*, 2008; Häggblom *et al.*, 2002; Thorsen *et al.*, 2009b).

1.2.6.2 EXTRINSIC FACTORS

Temperature

Temperature is one of the most important environmental factor to which bacteria have to adapt. It is then a key parameter for bacterial growth but also for toxin production. Cereulide formation is sensitive to temperature and its synthesis occurs at temperatures ranging from 12 to 37°C for mesophilic strains (Stenfors Arnesen *et al.*, 2008). Temperatures lower than 30°C seem optimal for emetic toxin production since maximal accumulation of cereulide appears to occur between 12 and 22°C (Stenfors Arnesen *et al.*, 2008), or between 12 and 15°C (Finlay *et al.*, 2000; Finlay *et al.*, 2002a) when these respective punctual temperatures were compared to 30°C. The temperature range for cereulide production has been extended by the discovery of *B. weihenstephanensis* strains (MC67 and MC118) capable of producing cereulide at 8°C (Thorsen *et al.*, 2006). In view of the new acquired knowledge, the monitoring and respect of cold chain seem more than crucial in order to limit the emetic toxin production. Nonetheless, the temperature is not the only parameter which controls cereulide formation.

Oxygen

Oxygen is essential for cereulide formation and different packaging techniques which offer variable atmosphere compositions (variable percentage of CO₂, O₂ and N₂) have been evaluated with regards to the risk of cereulide formation during storage. The modified atmosphere packaging (MAP) assuring low oxygen atmosphere by replacing most of the head space atmosphere with nitrogen gas was shown to drastically diminish the cereulide amounts produced by mesophilic *B. cereus* strains (Jääskeläinen *et al.*, 2004). Another study showed that limiting the levels of oxygen below 1.6 % efficiently prevents cereulide production (Rajkovic *et al.*, 2006b). MAP packages which contain CO₂ atmosphere and maintain a low level of oxygen are also effective to reduce the cereulide formation by psychrotolerant

B. weihenstephanensis strains (Thorsen *et al.*, 2009a). In conclusion, anoxic packaging reduces the risk of cereulide formation and may help to enhance food safety of foods of extended durability. However, it should be noted that contradictory study on the influence of oxygen for cereulide production does exist. For example, Finlay *et al.*, (2002b) showed that cereulide production by emetic *B. cereus* strains was more induced when little free oxygen is available, which totally goes against the experiments with modified atmospheres.

Food composition

Cereulide-related foodborne poisonings have very often occurred after consumption of farinaceous foods (Agata *et al.*, 2002; Ankolekar *et al.*, 2009; Granum and Lund, 1997; Kramer and Gilbert, 1989) which suggested that the cereulide production is obviously correlated to the type of food contaminated with *B. cereus*. Several studies which assessed the influence of various parameters making part of the food composition (starch content, pH, water activity, addition of polyphosphates or salts, nutrient availability) or related to the food handling (temperature, conditions of storage, heat or acid treatment,...) are thereafter briefly described.

Agata and collaborators (2002) have tested a panel of foods regarding the potential of cereulide formation by *B. cereus*. The toxin production was very low in eggs, milk, fish and meat despite a good bacterial growth. On the other hand, high amounts of cereulide were produced in starchy foods (rice, noodles, pasta and mashed potato) confirming that starch content has a significant impact on cereulide synthesis. Furthermore, it seems that the pH of food also plays an important role since the cereulide production was inhibited in the same starchy foods cooked with vinegar, mayonnaise and ketchup (Agata *et al.*, 2002). Knowing that children are highly vulnerable to *B. cereus* emetic toxin, commercial infant food formulas were tested as matrices for cereulide formation. Cereulide was differently accumulated according to the formula's composition reaching the highest levels in infant formulas containing cereal or dairy ingredients (Shaheen *et al.*, 2006).

Besides starch content and pH of the food, water activity has also a significant impact on cereulide production in food. As demonstrated by Rajkovic *et al.*, (2006a), non-acidic foods with high water activity and high starch content are favorable conditions which lead to the highest accumulation of cereulide.

Free amino-acids provided as food supplements also affect cereulide production. For instance, only three amino-acids (leucine, valine and threonine) are necessary for *B. cereus* emetic growth and they were shown to be essential for cereulide synthesis. Interestingly, cereulide production was decreased in media supplemented with high levels of leucine, isoleucine and glutamic acid while no effect on bacterial growth was observed (Agata *et al.*, 1999; Isobe *et al.*, 1995; Jääskeläinen *et al.*, 2004).

Given that cereulide is very difficult to inactivate once it is produced, it is wise to consider several complementary techniques to prevent its formation. Several molecules are known to affect the bacterial growth but it was not known if they will directly interfere on cereulide biosynthesis. Two studies showed a direct effect on the toxin formation process without specifically affecting the bacterial growth. First, addition of salt (NaCl) at a certain concentration to the food reduced the emetic toxin levels although the *B. cereus* bacterial growth was apparently not strongly influenced (Dommel *et al.*, 2011). Second, using long-chain polyphosphates (polyps) as additives effectively delayed and reduced the cereulide formation (Frenzel *et al.*, 2011). However, the prevention of cereulide in food is not a straightforward task since it is highly dependant on intrinsic and extrinsic factors, as illustrated above.

In conclusion, multiple factors, from food composition to food handling, are actually mediating the cereulide synthesis in food matrices. All these interplaying factors attest that the toxicity of a food product cannot be predicted only from bacterial growth counts but that a good quantification method for cereulide is mandatory.

1.3 METHODS FOR DETECTION AND QUANTIFICATION OF CEREULIDE

A classical way of detecting for bacterial toxins is the use of immunology-based methods. However, a minimum size of 5 kDa is required for immunogenicity (Kramer and Gilbert, 1989; Melling and Capel, 1978). As a result of its little molecular weight of 1.2k Da, as well as its highly stable chemical structure containing D-amino acids and its high toxicity, cereulide does not induce immune response and hence, cannot be detected by immunological assays. Several methods of detection have been developed to prove the existence and the toxicity of the toxin. First, experimental animal models were used to demonstrate the activity of the emetic toxin. Then, several cytotoxicity tests or in vitro tests were developed for an easy detection of cereulide. However, those assays were only qualitative or, at best, semi-quantitative and lacked specificity. Therefore, a chemical assay using LC-MS method emerged which allowed absolute quantification of cereulide. In parallel, a molecular method was developed which targets the cereulide-producing strains of *B. cereus*. The advantages and drawbacks, together with a brief description of each method, are discussed in the following paragraphs.

1.3.1 BIOLOGICAL TESTS

Animal feeding tests are a reliable tool to verify, for example, if putative toxins display emetic properties. In the case of cereulide, feeding trials with living primates have been first used (Turnbull, 1981).

1.3.1.1 MONKEY FEEDING ASSAY AND MOUSE BIOASSAY

In the late 70's, Melling *et al.* (1978) were able to show an emetic response in monkeys while injecting rice cultures of *B. cereus* with intragastric tubes. They could also show the poor antigenicity of cereulide through these feeding experiments. Then, Kramer and Gilbert (1989) confirmed that the emetic syndrome was due to an excreted toxin because bacterial cell-free supernatants produced emetic symptoms in monkeys. Later, Shinagawa *et al.*, (1995) estimated the emesis-induced dose in rhesus monkeys (*Macaca mulatta*) as being 10 µg/g of body weight. An emesis-induced dose was also

determined in Asian house shrew (*Suncus murinus*) as being 8 µg/kg body weight (Agata *et al.*, 1994; Isobe *et al.*, 1995). Furthermore, intravenous and intraperitoneal administrations of cereulide to mice were shown to induce lethality (Shinagawa *et al.*, 1996). The great advantage of monkey feeding assays is that monkeys are very similar to humans (*e.g.* they are more sensitive to cereulide than mice) and that the activity of the toxin can be verified through the feeding trials. However, these methods are ethically problematic.

1.3.1.2 CYTOTOXICITY TESTS

Cell lines

Several in vitro pathogenicity tests have been developed based on the mitochondrial swelling due to cereulide which appears as vacuolation in the cytoplasm of sensitive cells. These assays may be combined with a cell viability indicator as the MTT test or tetrazolium bromide assay. The MTT (3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), is usually converted from yellow, water-soluble tetrazolium salt, to insoluble purple formazan by metabolizing cells. If the toxin is present, the MTT conversion might be affected and the tetrazolium salt will remain yellow (Finlay *et al.*, 1999; Taylor *et al.*, 2005). Regarding the cultured cell lines, the HEp-2 cells (cell line of human carcinoma of the larynx) have been mainly used (Agata *et al.*, 1994; Agata *et al.*, 1996; Agata *et al.*, 2002; Hughes *et al.*, 1988). But there are also some reports of cytotoxicity tests performed on CHO (Chinese Hamster Ovary) cells (Beattie and Williams, 1999). Rat liver cells (Kawamura-Sato *et al.*, 2005; Mahler *et al.*, 1997), Vero-cells (Svensson *et al.*, 2007), HeLa (human cervical carcinoma), Calu (human lung adenocarcinoma), Paju (from pleural fluid of a patient with a metastasized neuroectodermal tumor) and CaCo-2 (human epithelial colorectal adenocarcinoma) cells have also been used (Jääskeläinen *et al.*, 2003b).

The above cellular tests are rather time-consuming and not easy to perform. In addition, they are not specific due to their sensitivity to other mitochondrial toxins such as gramicidin. Furthermore, the cytotoxicity tests only offer approximate titers and are therefore merely semi-quantitative.

Boar semen bioassay

A second type of bioassay, which is much more rapid, relies on the toxicity of cereulide against mitochondria of boar spermatozoa. The activity of cereulide is visible through cessation of the boar sperm motility within 5 minutes of exposure to cereulide extracts (Andersson *et al.*, 1998; Andersson *et al.*, 2004). In fact, the uncoupling of oxidative phosphorylation prevents spermatozoa from moving. This bioassay was first not quantitative and was thereafter adapted by Rajkovic *et al.* (2006b; Rajkovic *et al.*, 2007) in order to produce quantitative results. The CASA-bioassay using a standard curve gives accurate concentrations, provided that the only reacting toxin present is cereulide.

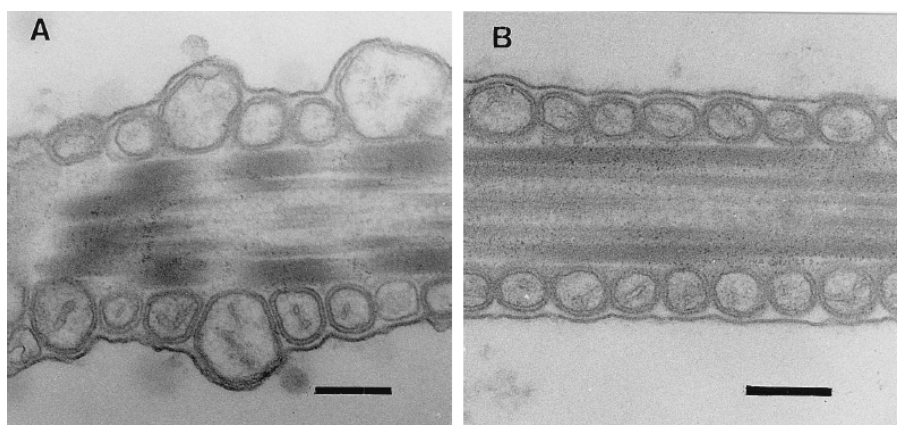


Figure 11 – Thin sections of the middle piece of a boar spermatozoon exposed to cell extracts of emetic and non emetic *B. cereus* (Andersson *et al.*, 1998)

(A) Mitochondrial damage in the middle piece of a spermatozoon exposed to cell extracts of emetic *B. cereus* strain F4810/72. (B) Middle piece of a spermatozoon exposed to cell extracts of non-emetic *B. cereus* ATCC 14579T. Mitochondria of ordinary size with intact membranes are seen. [Bars represent a length of 200 nm]

Compared with the previous cellular tests, this boar sperm bioassay is easy, not expensive but unfortunately not specific either. It detects other mitochondrial toxins such as valinomycin, the aflatoxins M1 and M3, the fusarium toxin or the gramicidin (Andersson *et al.*, 1998; Rajkovic *et al.*, 2007).

1.3.2 BIOMOLECULAR METHOD

1.3.2.1 POLYMERASE CHAIN REACTION (PCR)

The molecular method established to detect cereulide-producing strains is based on the PCR fragment specific to emetic strains which targets the NRPS cereulide synthetase gene cluster (*ces*) (Ehling-Schulz *et al.*, 2004b; Horwood *et al.*, 2004; Kim *et al.*, 2010b). Various PCR assays using different primers have been reported and each of them displays differences in specificity and accuracy (Kim *et al.*, 2010b). Another type of molecular assay based on RNA amplification has been recently designed which detects the *cesA* mRNA (Yasukawa *et al.*, 2010).

All the developed molecular assays are very specific to emetic strains but they do not reflect on the toxin expression. They do not account for the differences in the amounts of cereulide produced according to the environmental conditions or strain diversity. Yet, toxin confirmation and quantification is of utmost importance in case of toxin-related foodborne intoxications.

1.3.3 PHYSICO-CHEMICAL METHOD

Liquid chromatography coupled with mass spectrometry (LC-MS) methods are frequently used in chemical analysis for sample identification and quantification. Basically, the liquid chromatography allows the separation of the different compounds present in a mixture on the basis of their molecular properties (*e.g.* polarity, molecular size, boiling point) while the mass spectrometry provides useful information on the molecular weight of the compound of interest.

The heart of the chromatography is the column, made of a stationary phase backbone, usually silica, to which a chemical coating is fixed. The mobile phase, made of aqueous or organic solvents, percolates through the column and leads the samples molecules dissolved in it. The different compounds elute from the end of the column at different retention times (t_R) because they are more or less retained in accordance with their specific affinity for the column (Fig. 12).

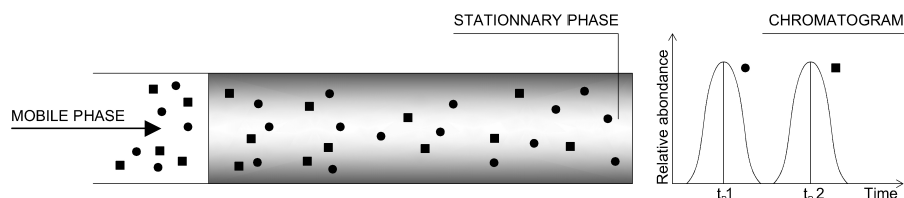


Figure 12 – Schematic illustration of the liquid chromatography leading to a chromatogram output

The mobile phase drags the mixture components through the column. In function of their polarity, the different components (square and rounds) will elute at different times. As shown on the right chromatogram, the round compound will first elute.

The mass spectrometer plays the role of detector and provides additional information on the compound of interest. After elution from the column, the sample is first ionized in gas phase thanks to the ionization source, and then the separation of the different produced ions occurs in the mass analyser according to their mass-to-charge (m/z) ratio. Finally, the abundance of each ion is measured with a detector, in a full scan mass spectrometry. A wide variety of ion sources, mass analysers and detectors is available and their choice result in different categories of mass spectrometry, as illustrated in Fig. 13.

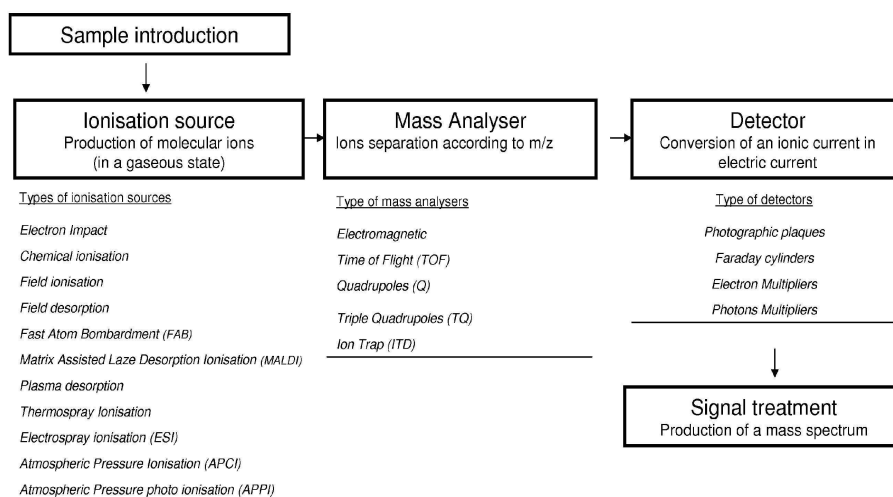


Figure 13 – Basic steps induced in the mass spectrometer and variety of equipments

In order to get more specificity and higher sensitivity of the method, a tandem mass spectrometry (LC-MS/MS or LC-MS²) may be recommended. Tandem mass spectrometry is based on the fragmentation of one selected ion (precursor ion) leading to specific fragment ions, or daughter ions. The different fragments are always generated in same proportions (*e.g.* abundance of fragment 1 > fragment 2 > fragment 3) when starting from the same ion. This guarantees the specificity of identification. The basic steps of LC-MS/MS analysis in an ion trap are illustrated in Fig. 14.

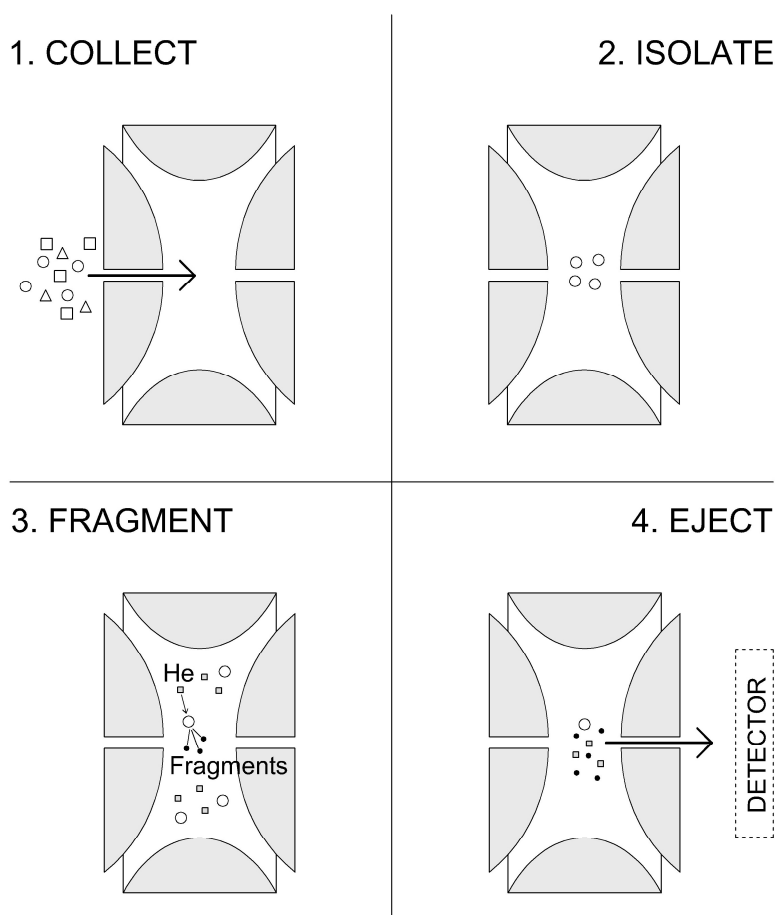


Figure 14 – Fragmentation process in an ion trap

Mass fragmentation occurs inside the ion trap in four basic steps. First, ions are collected in the trap. Then, all ions are ejected except the ion of interest by applying a specific tension. The precursor ion is then fragmented by collision with a gas (*e.g.* Helium). The fragment ions are then ejected and detected.

In 2002, Häggblom *et al.* (2002) developed a quantitative chemical assay for cereulide based on liquid chromatography ion trap mass spectrometry (LC-MS). Then, Hormazábal *et al.*, (2004) also developed a LC-MS method for the quantification of cereulide in figs. The LC-MS methods require good standards for providing accurate concentrations. Since no cereulide standard was commercially available, cereulide contents in food samples were expressed in valinomycin equivalents for both studies. Thereafter, in the progress of the present thesis, two groups improved the original methods by producing a standard of cereulide. Bauer *et al.* (2010) developed a stable isotope dilution analysis using a ¹³C₆-cereulide produced from *B. cereus* and Biesta-Peters *et al.* (2010) synthesized cereulide on the basis of the process described by Isobe and collaborators (1995). The developed methods involve different conditions and parameters for the chromatographic separation and the mass spectrometry analysis which are briefly described in Table 5.

For all the described methods, cereulide is extracted by organic solvents since this lipophilic molecule is not easily solubilised in aqueous solutions. Furthermore, the predominant ions which are monitored for cereulide determination are NH₄⁺-M and K⁺-M adducts. The peaks related to these adduct ions are more intense than the molecular ion and they are more easily fragmented than the original molecule. The four elaborated LC-MS methods allow specific detection as well as quantification of cereulide.

The amounts of cereulide produced in food products is the relevant parameter to be monitored in case of food safety issues, especially as this production has been shown to highly vary in function of bacterial features and environmental conditions. The described LC-MS methods used for cereulide quantification may help to achieve this aim. However, each of these methods presents advantages and drawbacks as regards time-consumption, cost, specificity and efficiency. The performance of an analytical method is evaluated by a validation study, as explained in the next Chapter.

Table 5 - Comparison of methods

References	Standard used	Chromatographic separation			Mass spectrometry		
		Column	Elution mode	Mobile phase	Flow rate (ml min ⁻¹)	Instrument	Monitored ions for cereulide
Hägglom <i>et al.</i> , 2002	valinomycin	Supelco Discovery C8 (100mm; 2.1mm; 5µm)	Isocratic	95% acetonitrile, 4.9% water (H ₂ O) and 0.1% trifluoroacetic acid (TFA)	0.15	Buker ESI electrospray	1171.1 (NH ₄ ⁺ -M); 1191.8 (K ⁺ -M); 1153.8 (H ⁺ -M)
Hormazabal <i>et al.</i> , 2004	valinomycin	Zorbax SB-C18 (50mm; 4.6 mm, 1.8µm)	Isocratic	92% methanol, 8% H ₂ O with 100µl/l TFA	0.8	API 100 LC/MS system (Applied Biosystems)	1170.9 (NH ₄ ⁺ -M)
Biesta-Peters <i>et al.</i> , 2011	synthesized cereulide, valinomycin	Supelco Discovery C18 (100mm; 2.1 mm; 5µm); Acquity UPLC C18 (100 mm; 2.1mm, 5µm)	Gradient	Acetonitrile; H ₂ O with ammonium formate 10mM and 2% TFA	0.2	LCQ Advantage (ThermoFinnigan); LTQ XL Setup (Thermo Fisher)	1170.6 (NH ₄ ⁺ -M); 1191.67 (K ⁺ -M)
Bauer <i>et al.</i> , 2011	¹³ C-cereulide, valinomycin	Synergi Fusion-RP (150 mm; 2mm, 4µm)	Gradient	Methanol; H ₂ O	0.25	API 4000QTrap LC-MS/MS (Applied Biosystems)	1170.9 (NH ₄ ⁺ -M) -> 172.3; 1176.9 (NH ₄ ⁺ -M) -> 173.3

1.4 VALIDATION OF AN ANALYTICAL METHOD

A developed method must be validated in order to demonstrate that it provides reliable and accurate results and that it is fit for the intended purpose. In this scope, several performance parameters have to be compared to acceptance criteria. The validation of analytical methods can be performed within the lab (in-house validation) or it can be a subject of a bigger scale study (interlaboratory study). The performance characteristics that should be settled for an in-house validation (single laboratory study) are briefly described in the following paragraphs. The indicated characteristics are prescribed by the European Commission Decision 2002/657/EC for assessment of the method performances in the analyses of veterinary residues.

1.4.1 LINEARITY OF THE METHOD

Evaluation of the linear range of the calibration curve occurs by visual or statistical tests, as for instance, the Mandel's fitting test (Mandel, 1964). The obtained Mandel's test value is compared to the F-critical value and, in case of inferior obtained value, a linear correlation is accepted. The linearity is described by the equation $y = ax + b$ where y is the signal surface and x the standard concentration. The equation is provided with a correlation coefficient R^2 , which is usually accepted at the level higher than 0.98.

1.4.2 MATRIX EFFECT

The matrix effect reflects the influence of the matrix on the signal for a sample to which a standard has been added. The typical matrix effects in mass spectrometry are enhancement or suppression of ions. This may lead to errors when using calibration with standards in solution. The matrix effect is evaluated by comparing the calibration curve with standard solutions and the calibration curve with standards in sample extracts. A statistical t-test is performed to compare the slopes of those calibration curves. If a matrix effect is observed, it is recommended to use calibration curve with standards in sample extracts (matrix matched standards) for further calibration and quantifications.

1.4.3 LIMIT OF DETECTION (LOD)

Limit of detection is the smallest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty (EC/2007/333). Several definitions may be used as, for instance, the LOD may be determined by analyzing samples with decreasing concentrations. The analyte yields a certain signal on the calculated noise and this signal to noise (S/N) ratio diminishes with the decreasing concentrations. The limit of detection can be estimated at a S/N of three (LOD: $S/N = 3$).

1.4.4 LIMIT OF QUANTIFICATION (LOQ)

Limit of quantification is the lowest content of the analyte which can be measured with reasonable statistical certainty (EC/2007/333). The limit of quantification can be estimated at a signal to noise (S/N) of six (LOQ: $S/N = 6$).

1.4.5 PRECISION

1.4.5.1 REPEATABILITY

Repeatability refers to the degree of correspondence between single test results obtained under optimal conditions (i.e. same test sample, same operator, same equipment, same laboratory and short interval of time) (2002/657/EC). Standard deviation (SD_R) and relative standard deviation (RSD_R) are calculated from the results generated under repeatability conditions according to ISO 5725-2.

1.4.5.2 REPRODUCIBILITY

Reproducibility refers to data obtained under reproducibility conditions (i.e. same test material in different laboratories using the standardized test method) (2002/657/EC). In the case of an in-house validation, within-laboratory reproducibility is performed. The intra-laboratory reproducibility data are obtained under longer period of times conditions, and/or different calibration, operator, equipment. Standard deviation (SD_w) and relative standard deviation (RSD_w) are calculated from the results generated under reproducibility conditions according to ISO 5725-2.

The acceptance criteria with respect to precision are settled by the Horwitz equation. The calculated coefficients ratio for RSD_R and RSD_w should not exceed 14.7 % and 22.0 %, respectively (Horwitz and Albert 2006).

1.4.6 RECOVERY

The recovery is the percentage of the effective concentration of the analyte which is recovered from the analytical procedure (2002/657/EC). This may be specified as the extraction yield (yield of extraction for an analyte divided by the added amount of analyte in the original sample) or by the apparent recovery (observed value derived from an analytical procedure by means of a calibration graph divided by reference value).

1.4.7 SPECIFICITY

The specificity stands for the ability of the method to determine accurately the analyte of interest in the presence of other components in a sample matrix under the stated conditions of the test (2002/657/EC).

1.4.8 ROBUSTNESS/RUGGEDNESS

The robustness refers to the susceptibility of an analytical method to deliberate changes in environmental and/or operating conditions (temperature, reagents, extraction time, composition in the sample,...) as described in the 2002/657/EC document.

For official controls, other limits criteria are established by the European Commission Decision 2002/657/EC, as for instance, the decision limit $CC\alpha$, the detection capability $CC\beta$, the measurement of uncertainty in order to account for all the biases that could appear during calculations/measurements.

2. OBJECTIVES

The current legislations regarding food chain safety towards *B. cereus* vary between countries but are all establishing the norms on bacterial counts, not on the cereulide toxin concentration itself. A first question emerges: does this represent adequate information on the degree of dangerousness of a product for the consumer ? Besides, the effect of prolonged storage on cereulide production needs to be evaluated, as illustrated by recent outbreaks that befell in households. Is the toxin still produced when bacterial cells are dying off during long storage ? Finally, the number of reported cereulide-outbreaks raises a third question: are the emetic strains of *B. cereus* indeed rare in the environment or is the consumer more frequently exposed than previously thought ?

These issues may all be tackled by using a reliable quantification method for cereulide. The first objective of this work is therefore to develop a LC-MS analytical approach for cereulide determination in food in order to overcome drawbacks of existing methods. The method will be used in various applications of research and routine fields in order to shed light on the aforementioned food safety issues associated with cereulide:

- Evaluation of toxin production under various storage conditions (Chapter 3.1).
- Validation of the LC-MS method according to EU directives and screening of real samples in the scope of industrial monitoring plan or following foodborne intoxications (Chapter 3.2).
- Characterization of a recent lethal Belgian case and cereulide determination in the remaining leftovers (Chapter 3.3).
- Prevalence study to determine the exposure of the Belgian population to cereulide through consumption of rice dishes available in restaurants (Chapter 3.5).
- Evaluation of the potential antifungal activity of cereulide in a quest for its biological (ecological) role (Chapter 3.4).

3. RESULTS

3.1 METHOD DEVELOPMENT – FOLLOW-UP OF CEREULIDE PRODUCTION UNDER HOUSEHOLD CONDITIONS

In the scope of developing the LC-MS chemical method for the direct quantification of cereulide in food, it made sense to design experiments in which emetic *B. cereus* strains were inoculated in a food matrix and to follow the variability of their respective cereulide production. In parallel, two lethal intoxication cases which occurred in Belgium showed the necessity of estimating the toxin production during long periods of storage and in function of temperature. In particular, in 2003, a 7-year-old girl died after consuming pasta salad which had been kept in a defective fridge (14°C) for 3 days while in 2008, a 20-year old man died following consumption of a spaghetti meal which had been prepared 5 days before and left at room temperature in the kitchen. Therefore, it was decided to use pasta as food matrix and to inoculate it with the two strains isolated from those lethal cases in order to characterize their toxin production during long periods (7 days). Knowing that *B. cereus* growth is usually inhibited at cold temperatures, the effect of chill storage (at 4 and 8°C) on cereulide production was also evaluated in order to get relevant indications for the safety of foods chilled during extended periods.

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¹ Delbrassinne L., M. Andjelkovic, A. Rajkovic, N. Botteldoorn, J. Mahillon and J. Van Locho. 2011. Follow-up of the *Bacillus cereus* emetic toxin production in penne pasta under household conditions using liquid chromatography coupled with mass spectrometry. Food Microbiology, 28:1105–1109.

Summary

Two outbreak-related *Bacillus cereus* emetic strains were investigated for their growth and cereulide production potential in penne pasta at 4, 8 and 25°C during 7 day storage. Cereulide production was detected and quantified by LC-MS method (LOD of 1 ng/ml, LOQ of 5 ng/ml) and growth was determined by culture-based enumeration. Inoculated *B. cereus* strains (10^5 CFU/g) were able to reach counts of more than 10^8 CFU/g and cereulide production of about 500 ng/g already after 3 days of storage at 25°C. Interestingly, a constant increase of the toxin was noticed during incubation at ambient temperature storage: the cereulide was continuously produced during the bacterial stationary growth phase reaching maximal amounts at the end of the experiment (7 days, concentration of about 1000 ng/g). Strictly respected cold chain temperature as 4°C did not allow any detectable cereulide production for any of the two tested strains. At the limited temperature abuse of 8°C, a detectable amount of cereulide was observed after two days for one of the strain (TIAC303) (< LOQ). These results confirm that cereulide production is controlled by multiple factors (from type of strain to temperature) and that prolonged storage time plays a crucial role for consumer safety.

Keywords

cereulide, LC-MS, *B. cereus* emetic strains, food matrices, household storage

3.1.1 INTRODUCTION

B. cereus is a common foodborne pathogen causing two types of foodborne diseases: emesis, in which the main symptoms are nausea and vomiting, and diarrhea. The emetic syndrome is caused by cereulide, a heat resistant cyclic dodecadeptide toxin (Agata *et al.*, 1995), while the diarrheal syndrome is associated with several heat labile enterotoxins. The emetic toxin is not inactivated by food preparations because of its heat resistance (Rajkovic *et al.*, 2008). Moreover, it is not destroyed in the stomach by proteolytic enzymes and gastric acid because of its resistance towards proteases and acid conditions (Agata *et al.*, 1994; Shinagawa *et al.*, 1996). Cereulide is one of the most toxic substances among the known heat-stable toxins of microbial origin (Andersson *et al.*, 2007).

The food composition has major influence on cereulide production. Non-acidic foods with high water activity and high starch content will generally allow the highest accumulation of cereulide (Rajkovic *et al.*, 2006a). It is rice and pasta that carry inglorious fame of most incriminated foods in cereulide poisoning. Two fatal cases reported in Belgium were directly linked to pasta (Dierick *et al.*, 2005; Naranjo *et al.*, in preparation), as well as one of the first described cases (Mahler *et al.*, 1997). For this reason the focus of the current research was placed in penne pasta, a popular pasta type for consumers.

Emetic strains of *B. cereus* were shown to share several common traits like negative starch hydrolysis and similar ribotype (Pirttijärvi *et al.*, 2000; Pirhonen *et al.*, 2005) and were previously considered as a homogenous group of mesophilic strains (Ehling-Schulz *et al.*, 2005). These views have been questioned in recent report by Kim *et al.*, (2010a) who found that there are few emetic strains that could produce HBL and NHE and can hydrolyze starch and salicine. Moreover, psychrotolerant strains of *Bacillus weihenstephanensis* were recently reported as cereulide producers (Thorsen *et al.*, 2006; Hoton *et al.*, 2009), adding up a new dimension to food safety issues related to *B. cereus* (*sensu lato* in this case). While mesophilic strains may produce higher amounts of cereulide at room temperature,

psychrotolerant strains may pose a new threat to REfrigerated Processed Foods of Extended Durability (REFPED) kind of products.

In the past decades, cereulide has been mostly detected and measured by bioassays such as cytotoxicity cell assay including HEp-2 (Hughes *et al.*, 1988; Agata *et al.*, 2002), CHO (Chinese Hamster Ovary) (Beattie and Williams, 1999), rat liver (Mahler *et al.*, 1997; Kawamura-Sato *et al.*, 2005) and boar spermatozoa cells (Andersson *et al.*, 1998; 2004; Rajkovic *et al.*, 2006b). These bioassays are only qualitative or semi-quantitative. Several PCR assays were developed for the rapid detection of emetic toxin producing *B. cereus* strains (Ehling-Schulz *et al.*, 2004; Kim *et al.*, 2010b) but these molecular tools do not allow quantification of the toxin produced. In 2002, Häggblom and coll. developed a quantitative chemical assay for cereulide based on liquid chromatography mass spectrometry (LC-MS). Such method proved to be useful for studying the effects of various parameters on cereulide production such as growth temperature and media, strain diversity or food matrices (Jääskeläinen *et al.*, 2003; Pirhonen *et al.*, 2005; Jääskeläinen *et al.*, 2003b; Shaheen *et al.*, 2006; Rajkovic *et al.*, 2006a; Thorsen *et al.*, 2009; Bauer *et al.*, 2010).

The primary goal of this study was to evaluate the rate of cereulide production of different recognized emetic strains in one popular pasta type (penne) in various simulated household storage conditions. Cereulide concentration was followed by LC-MS analysis in penne pasta during 7 days.

3.1.2 MATERIALS AND METHODS

3.1.2.1 EMETIC STRAINS AND GROWTH CONDITIONS

Two emetic strains kinrooi 5975c and TIAC303 were used as inocula for penne pasta in parallel experiments. The strains were isolated from two Belgian lethal food poisoning cases, in 2003 (Dierick *et al.*, 2005) and in 2008 (Naranjo *et al.*, in preparation), respectively. Two cultures were grown overnight in brain heart infusion broth (BHI, Difco Laboratories; BD Diagnostic Systems, Le point de Claix, France) at 30 °C. Appropriate dilution

in peptone buffer water (PBW) was performed for each overnight culture to provide inoculum for pasta at a level of *ca.* 10⁵ CFU/g.

3.1.2.2 BACTERIAL INOCULATION OF COOKED PASTA AND INCUBATION CONDITIONS

Penne pasta, purchased at a supermarket in Brussels, were cooked in boiling water for 15 min and then divided in three parts. Two parts were inoculated with *ca.* 10⁵ CFU/g with appropriate dilution of kinrooi 5975c or TIAC303. The third part of pasta was used as negative control. All samples were homogenized using a Stomacher (Bag Mixer, Interscience, France) and incubated during 7 days at three temperatures: 4°C, 8°C and 25°C. The experiment was repeated twice under the same conditions.

3.1.2.3 ENUMERATION OF *B. CEREUS* FROM FOOD SAMPLES

Enumeration of *B. cereus* was performed for all samples (including negative samples) at time zero (D0), and every 24 h for 7 days. Daily, 10 grams of each sample were aseptically added to 90 ml of PBW in sterile stomacher bags (International Medical, Brussels, Belgium) and homogenized for 1 min. From appropriate tenfold serial dilutions, 100 µl were spread onto *B. cereus* selective Mannitol–Egg Yolk–Polymixin (MYP, Bio–Rad, Marnes–la–Coquette, France) agar plates and incubated for 24 h at 30°C. Bacterial counts were determined after enumeration of colony forming unit (CFU) displaying characteristic pink color surrounded by white area on MYP medium.

3.1.2.4 CEREULIDE EXTRACTION

At each time point, the emetic toxin was extracted in duplicate: 6 ml of methanol was added to 3 g of sample and then mixed in stomacher bags. The homogenized liquid was collected in glass tubes and placed in boiling water for 15 min, followed by evaporation under N₂ atmosphere until dryness. The residue was resuspended and diluted in 3 ml methanol, vortexed and centrifuged. The supernatant was collected and stored at –20°C prior to analyses.

3.1.2.5 LC–MS ANALYSIS

Cereulide content of each extract was analyzed by LC–MS method as described by Häggblom *et al.* (2002) with the following modifications. The LC–MS analysis was performed on a LCQ Deca–XP Plus ion trap mass analyser

(ThermoFinnigan, USA). Chromatographic separation was done on the Symmetry C8 column, 2,1 mm x 150 mm, 5 μ m (Waters, USA). The mobile phase of the isocratic method was a mixture of 95 % acetonitrile, 4.9 % water, 0.1 % trifluoroacetic acid at a flow rate of 0.2 ml min⁻¹ with a sample injection volume of 5 μ l. Because cereulide standard was not commercially available, valinomycin (Sigma, Fluka, Germany), dissolved in pure methanol, was used as an external standard to plot a calibration curve in the range 1 to 1,000 ng/ml. A full mass spectrum was recorded from 500 to 1300 m/z in positive electrospray mode (ESI⁺). The total ion chromatogram was smoothed with a Gaussian function. For detection of cereulide, the m/z values for adduct ions 1170.5 (NH₄⁺ adduct) and 1191.5 (K⁺ adduct) were monitored.

3.1.3 RESULTS

The LC-MS method characterized with a limit of detection (LOD) of 1 ng/ml and a limit of quantification (LOQ) of 5 ng/ml was used for the quantitative follow-up of cereulide production in penne pasta under conditions mimicking household conservation. Two distinct emetic strains, three temperatures and long period of storage were tested.

3.1.3.1 INFLUENCE OF STORAGE TIME AND TYPE OF STRAIN ON CEREULIDE PRODUCTION IN PASTA

Cereulide production by two pasta-outbreak related emetic strains (Dierick *et al.*, 2005; Naranjo *et al.*, in preparation), kinrooi 5975c and TIAC303 at 25° C, was followed daily in pasta for 7 days, mimicking extended storage in a household. The same conditions were used for both strains.

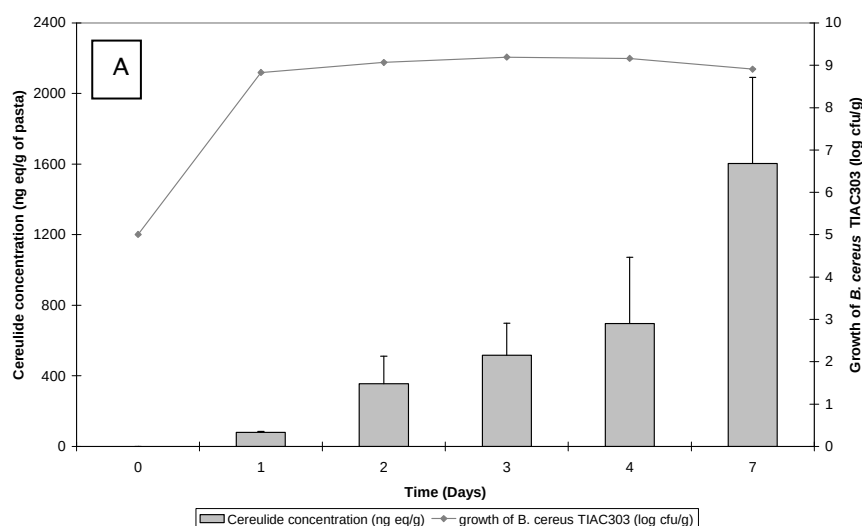
The enumeration of the growth of TIAC303 and kinrooi 5975c strains and their respective cereulide production at 25°C are presented in Fig. 15. The growth curves show that *B. cereus* TIAC303 and kinrooi 5975c were able to reach counts of more than 10⁸ CFU/g by the first day of storage at 25°C. In all cases, no cereulide was detected at time of inoculation (D0) indicating that cereulide production may require sufficient amount of bacterial cells and that no detectable cereulide was transferred into food with inoculum. The quantifiable production of cereulide could be observed in the first 24 h of

3. RESULTS

3.1. Follow-Up of cereulide production under household conditions

storage at 25°C for both strains, corresponding to 3 log CFU/g increase in *B. cereus* counts. Already at day 1, intoxicative amounts were found (Agata *et al.*, 2002 reported 10 ng/g as intoxication level). After only one day, cereulide produced by kinrooi 5975c strain reached concentrations of 145 ng/g cereulide while TIAC303 formed 80 ng/g. As shown in Fig. 15A, the amount of cereulide produced by the emetic strain TIAC303 steadily increased during the storage period reaching the highest concentration of 1,603 ng of cereulide per g of pasta at the end of the experiment (day 7).

Cereulide production by the emetic strain kinrooi 5975c (Fig. 15B) also increased steadily reaching a maximum of 1,346 ng/g of cereulide on the last day. These results highlight the ability of both strains to gradually increase their cereulide production during growth and especially during the very late stationary phase of bacterial growth. These results also show that contamination with emetic strains can already appeared after 24h at room temperature and produce intoxicative concentrations of cereulide.



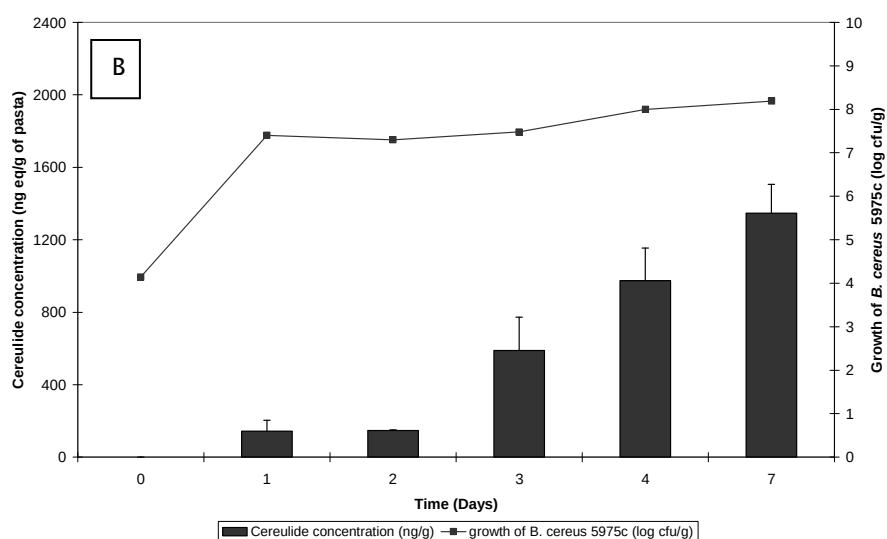


Figure 15 – Production of cereulide in pasta incubated at 25°C

Penne pasta was inoculated with two *B. cereus* emetic strains: TIAC303 (A) and kinrooi 5975c (B) at 10^5 CFU/g. Bacterial growth curves and cereulide concentrations (assayed by LC-MS with detection and quantification limits of 1 ng/ml and 5 ng/ml respectively) are presented. LC-MS quantitative results are the average of two individually analyzed samples. The error bars indicate the deviation of cereulide contents in the two replicates. Enumeration was done on MYP plates with detection limit of 10 CFU/ml.

3.1.3.2 INFLUENCE OF TEMPERATURE ON CEREULIDE PRODUCTION IN PASTA

As temperature is a key extrinsic factor in controlling microbial behavior, cereulide production in pasta was also evaluated under incubation at different temperatures (4°C, 8°C and 25°C). Table 6 shows results of cereulide detection in pasta inoculated with strain TIAC303 or kinrooi 5975c and incubated at the three different temperatures during 7 days.

At 4°C, cereulide was not noticed in both inoculated pasta during whole incubation time. However, at 8°C cereulide production was not detected for kinrooi 5975c strain while cereulide produced by TIAC303 could be detected after 2 days of incubation. Regarding the bacterial growth, both mesophilic strains died off during the long storage at 4°C and 8°C (data not shown) though the TIAC303 strain showed a slower reduction of bacterial counts at

both temperatures. At 25°C, the two emetic strains produced higher concentrations of cereulide.

Table 6 – Production of cereulide in pasta at 4, 8 and 25°C

Time (day)	TIAC303			kinrooi 5975c		
	Cereulide concentration			Cereulide concentration		
	(ng/g)			(ng/g)		
	4°C	8°C	25°C	4°C	8°C	25°C
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	<LOD	<LOD	80	<LOD	<LOD	145
2	<LOD	<LOQ	355	<LOD	<LOD	148
3	<LOD	<LOQ	516	<LOD	<LOD	589
4	<LOD	<LOQ	696	<LOD	<LOD	974
7	<LOD	<LOQ	1603	<LOD	<LOD	1346

Production of cereulide [ng Valinomycin equivalent/g] at 4°C, 8°C, and 25°C in pasta inoculated with *B. cereus* TIAC303 and *B. cereus* kinrooi 5975c. Cereulide was detected by LC–MS (detection limit LOD of 1 ng/ml; quantification limit LOQ of 5 ng/ml). <LOD (below detection limit; not detected); <LOQ (below quantification limit; detected). LC–MS quantitative results are the average of two individually analyzed samples

3.1.4 DISCUSSION AND CONCLUSION

In this work, the cereulide production related to time and temperature was analyzed in artificially inoculated pasta using two different emetic strains. Both emetic strains tested in the study were isolated from lethal food poisoning cases that occurred in Belgium and were caused by improper storage (time and temperature abuse) of pasta meal. It was demonstrated with LC–MS that both strains could rapidly accumulate cereulide to 80 ng/g and 145 ng/g, amounts that outreach the minimum acute toxic level of cereulide (10ng/g) quantified by Agata *et al.*, (2002) in samples implicated in different outbreaks. After less than 24 h of incubation at 25°C, both emetic strains produced cereulide in the range of emesis-inducing dose described above. After a long period of storage (7 days), concentrations of at least 1 µg of cereulide per g of pasta were produced by the strains. This corresponds to the results of Jääskeläinen *et al.*, (2003b) who found cereulide production in

the range of 0.02 µg/g to 5.5 µg/g, depending on the food matrix (in bread and meat pastry after 8 and 4 days, respectively).

Although it is understood that cereulide gets produced at temperature higher than 8°C (Häggbloom *et al.*, 2002; Jääskeläinen *et al.*, 2003b), recent findings showing cereulide production by *Bacillus weihenstephanensis* at lower temperature (Thorsen *et al.*, 2009) required to investigate the actual safety of cold chain regarding prevention of cereulide production. In this work however, two cereulide-producing strains displayed slight differences. At 8°C, although no cereulide could be detected for the strain kinrooi 5975c, small amounts of cereulide were produced already after 2 days by the TIAC303 strain. These results suggest that, in contaminated food samples stored at temperatures of 8°C, cereulide could be present in small concentrations for which it is not known if they can lead to intoxications. Furthermore, Finlay *et al.* (2000) and Häggbloom *et al.* (2002) proved that there was already significant cereulide formation at 12°C in laboratory media and Rajkovic *et al.* (2006a) measured cereulide with the boar semen bioassay in penne incubated at 12°C. Based on these observations, it would be interesting to assess the toxin production of both mesophilic strains at 12°C and to determine if their toxin level would exceed the quantification limits. An additional comparison with the toxins levels produced by psychrotrophic strains at the same low temperature would precise the risk incurred by the consumer. Not only temperature but also type of strain and food conditions will be determinant for cereulide production and will influence actual risk for the consumer.

Cereulide emesis-induced dose was calculated in *Suncus murinus* as 8 µg/kg body weight (Agata *et al.*, 1994; Isobe *et al.*, 1995) and in Rhesus monkeys as 10 µg/kg body weight (Shinagawa *et al.*, 1995). Assuming that humans would be similarly sensitive (Jääskeläinen, 2003a), only a portion of about 300 to 500 g of contaminated pasta left at room temperature for several days would already result in acute emetic syndrome. However, foods implicated in intoxications were reported to contain lower amounts of cereulide (In't Veld *et al.*, 2010). Therefore, these doses should be revisited for human applications and safety factors, as used in risk assessment for

transposing the doses from one species to another, should be defined and applied.

The time of storage also played a key role in cereulide production. The amounts of cereulide steadily increased during the 7 days of incubation at ambient temperature, independently of the strain. This increase could have perhaps happened also at 8°C for strain TIAC303, up to LOQ level. Similar findings have been observed during shorter periods of time as reported elsewhere (Agata *et al.*, 2002; Bauer *et al.*, 2010). Agata *et al.* (2002) used the HEp-2 cell vacuolation assay to measure cereulide production in boiled rice during 24h. They showed that the production steadily increased to reach a maximum after 24 h. More recently, Bauer *et al.* (2010) developed a stable isotope dilution analysis and quantified cereulide in cooked rice during 96 h at 24°C. The toxin started to be produced only after 24h and constantly increased up to 6.12 µg/g after 96 h. In our study in inoculated pasta, we showed that cereulide production continued to increase even during the late stationary phase. Similar observation of very late toxin production was recently reported for the staphylococcal enterotoxin A (SEA) by Wallin-Carlquist and coll. (2010) with a constant increase of toxins amounts during 7 days of storage at room temperature.

Although previous studies focused mainly on the detection of bacteria and occasionally on toxin production either after expiration date of the food or during maximum 4 days (Jääskeläinen *et al.*, 2003b; Agata *et al.*, 2002; Bauer *et al.*, 2010), recent intoxications cases show the need of evaluating the production during longer periods (Naranjo *et al.*, in preparation). In addition, cooked rice has been more often studied because of its ethnological implication in cereulide food poisoning. However, Rajkovic *et al.* (2006a) showed pasta penne to be a significantly better substrate for *B. cereus* growth and cereulide production compared to boiled rice, using a *B. cereus* strain isolated from a fatal outbreak.

In this study, the importance of a limited shelf life for cooked meals was highlighted since cereulide production increased constantly with storage time. Appropriate storage should be followed in households in order to avoid

acute intoxications due to high cereulide concentrations. Cereulide formation in food samples is dependent on different factors where temperature is a key parameter but cereulide production is also tightly linked to type of strain, growth phase, type of food and food composition, pH, water activity, consistency, aeration, atmospheric conditions, presence of amino-acids and cations ratios (Finlay *et al.*, 2000; Rajkovic *et al.*, 2006a; Häggblom *et al.*, 2002; Jääskeläinen *et al.*, 2003b, Agata *et al.*, 1999; Jääskeläinen *et al.*, 2004; Apetroaie-Constantin *et al.*, 2008). By investigating those different parameters, challenge tests could be performed to adapt shelf life. Moreover, and this is an important issue, there is no evidence for a simple correlation between the *B. cereus* counts and the actual cereulide concentration in foodstuffs since cereulide is continuously produced while counts remain stable in stationary phase. This crucial observation means that in the scope of risk assessment, the cereulide concentration should be measured instead of bacterial counts. For this purpose also further optimization, mainly regarding increased sensitivity of quantitative LC-MS based methods, is needed.

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3.2 METHOD VALIDATION – DETERMINATION OF THE *BACILLUS CEREUS* EMETIC TOXIN BY MEANS OF LC-MS² METHOD

During the past decades, cereulide was mainly detected with cytotoxicity assays or with the boar semen bioassay. In 2002, a first LC-MS method, which used valinomycin as a standard for the quantification of cereulide was described. This method was a promising start but let some place to optimisation in terms of specificity, sensitivity and standard utilisation. In the midst of the present study, two methods were developed which used synthesized and ¹³C-cereulide standards, respectively. Each technique possesses its own advantages and drawbacks regarding *e.g.* time of extraction, solvents, and specificity of the method. Therefore, we comparatively assessed those methods in pre-validation tests prior to the validation characteristics of our own method. The core of the following section describes the validation of the method but also the pre-validation tests that have been performed in order to compare the method with the two parallel published methods.

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² Delbrassinne L., M. Andjelkovic, A. Rajkovic, P. Dubois, E. Nguessan, J. Mahillon and J. Van Lo. 2011. Determination of *Bacillus cereus* emetic toxin in food products by means of LC-MS². Food Analytical Methods, 17 december 2011, pp1–11.

Summary

Cereulide is the heat-stable toxin produced by certain strains of *Bacillus cereus*. It is the main virulence factor of emetic *B. cereus* strains, which causes the emetic food poisoning syndrome, including rare fatal cases of food intoxications. Due to presumably low intoxication doses, a sensitive, specific and robust technique is needed for its detection. In 2002, a LC-MS method was developed which allowed absolute quantification of cereulide using valinomycin as standard. This study describes the validation, according to the Commission Decision 2002/657/EC, of the LC-MS² method, a tandem mass spectrometry technique, which guarantees lower detection limit and higher specificity. The LC-MS² method, calibrated with valinomycin, was validated in rice and tested on various matrices (*i.e.* red beans, spices and chili con carne) containing cereulide. The process combines a simple extraction step from the food matrix followed by LC-MS² analysis and detection by ion-trap mass spectrometer. The detection limit for cereulide in rice was 0.5 ng eq/g, which is 20 to 2,500 times lower than currently understood intoxicative doses between 10 and 1.280 ng/g previously reported for cereulide. The validated method was specific, sensitive, repeatable and reproducible with recoveries ranging from 77 to 100 %.

Keywords

cereulide, LC-MS², food matrices, method validation

3.2.1 INTRODUCTION

Bacillus cereus is a Gram-positive spore forming bacterium that belongs to Hazard group 2 organisms as defined by the European legislation (European Commission 1993). It can cause systemic and local infections and two types of food-borne illness, *i.e.*, the emetic (intoxication) and diarrheal (toxico-infection) syndrome. Different toxins are implicated in two food-borne diseases (Ceuppens *et al.* 2011). The toxin responsible for emetic syndrome is a cyclic dodecadeptide identified as cereulide by Agata *et al.* (1995) and characterized by a notable resistant profile including heat stability to temperatures up to 150°C (Rajkovic *et al.* 2008; Turnbull 1981), proteolytic resistance towards pepsine and trypsin, as well as resistance to extreme pH conditions (Rajkovic *et al.* 2008; Schoeni and Wong, 2005; Shinagawa *et al.* 1995). This toxin is preformed in food and, and because of its extreme stability, it does not lose its biological toxic activity in human stomach upon ingestion of contaminated food.

B. cereus can be found in a wide variety of foods, resulting in a versatility of food vehicles involved in emetic disease of *B. cereus* food poisonings. However, so far the vast majority of emetic intoxications have been related to starchy foods. Among these, contaminated rice dishes represent the main culprit in reported cases (about 95 % of cases) (Altayar and Sutherland 2006). The underreporting of *B. cereus* related diseases has been acknowledged as an actual issue (Granum and Baird-Parker, 2000; Granum and Lund, 1997; Nelms *et al.*, 1997), caused by number of factors such as generally mild symptoms of *B. cereus* foodborne diseases and by the fact that *B. cereus* food poisoning is a non-reportable disease in Europe (Nissen *et al.*, 2002). Sporadic cases are not reportable in US, hence, in US the only cases reported are those related to outbreaks (Mead *et al.*, 1999). Moreover, food poisoning due to *B. cereus* may be misdiagnosed due to the similarity of symptoms with other types of food poisoning. In the case of emetic syndrome, the key element to better characterize *B. cereus* related food poisonings is to have reliable method for toxin detection. Moreover, a reliable detection method should also be able to determine the intoxication dose for cereulide and be

able to distinguish foods containing amounts of cereulide higher than the intoxicative dose.

The emesis-inducing dose in humans is currently not known, but on the basis of animal studies and in vitro experiments (Agata *et al.* 1994; Isobe *et al.* 1995, Paananen *et al.* 2002; Shinagawa *et al.* 1995) it can be estimated to be around 8 µg of cereulide / kg body weight (Andersson *et al.* 2007; Jääskeläinen *et al.* 2003a). However, outbreak data suggest that lower concentration of 5.2 ng of cereulide/g of food could induce food intoxication (in't Veld *et al.* 2010). This dose may even be lower in cases of food heterogeneity and/or imperfect recovery (extraction) rates.

Previously described detection methods for cereulide include cytotoxicity-based assays using cell cultures of HEp-2 (Agata *et al.* 2002; Hughes *et al.* 1988), CHO (Chinese Hamster Ovary) (Beattie and Williams 1999), or rat liver cells (Kawamura-Sato *et al.* 2005; Mahler *et al.* 1997). The presence of cereulide can also be detected using the boar spermatozoa motility assay (Andersson *et al.* 1998; Andersson *et al.* 2004; Rajkovic *et al.* 2006). However, no immuno-assays exist at the moment, mainly due to the cereulide small molecular weight of 1,153 g/mol (a minimum size of 5 kDa is required for immunogenicity, Melling and Capel 1978), its chemical structure, the ability to be processed by immune cells capturing antigens and its high toxicity. Bioassays are not always easy to perform with the exception of the boar semen test and they are not specific because they can cross-react with other mitochondrial toxins. Moreover, they do not provide accurate concentrations but only approximate toxicity titers, with the exception of the CASA-bioassay, using a standard curve with the condition that the only reacting toxin present is cereulide (Rajkovic *et al.* 2007). Therefore, the method of choice for quantitative determination should be based on chemical methods. Several quantitative assays based on liquid chromatography ion trap mass spectrometry (LC-MS) have been developed (Hägglom *et al.* 2002; Bauer *et al.* 2010; Biesta-Pieters *et al.* 2010). This technique gives quantitative results and was used in several studies to analyze cereulide in various food commodities (Jääskeläinen *et al.* 2003b, Jääskeläinen *et al.* 2004; Pirhonen *et al.* 2005; Shaheen *et al.* 2006). Although a synthetic

cereulide standard has been recently developed (Biesta-Peters *et al.* 2010), no cereulide standard is currently commercially available, and the contents of cereulide in food samples were therefore expressed in valinomycin equivalents, a highly similar dodecadepsipeptide produced by *Streptomyces* spp.

In case of samples containing unknown components or in case of complex matrices, the single molecular weight information provided by LC-MS is not always sufficient. Tandem mass spectrometry (LC-MS/MS or LC-MS²) is then usually preferred because it utilizes multiple stages of mass analysis and yields structural information of the compound through fragmentation process. Fragmentation spectra obtained can indeed provide compound specific information needed for identity, structural elucidation and specificity for quantification (Willoughby *et al.* 1998). In the first stage of mass analysis (MS¹), selected ions specific to the compound of interest are isolated and monitored. These parent ions are then excited by interaction with a collision gas leading to fragmentation processes. Fragment ions are subsequently formed and monitored in a second stage of mass analysis (MS²). It can be anticipated that LC-MS ion trap instrument would provide LC-MS² analyses for cereulide with better sensitivity (with lower detection limits), with higher specificity and the robustness of this method should decrease the influence of possible matrix interferences. Although foods implicated in emetic food poisoning are principally rice and pasta, cereulide has also been found in other food products such as beans, dairy products or infant formulas (Schoeni and Wong 2005).

The method presented and validated in this study consists of cereulide determination in rice by LC-MS², using low amount of samples and with inexpensive and readily available valinomycin as a reliable surrogate standard. The method was also tested on different complex food matrices coming from monitoring plans and foodborne outbreaks (chili con carne, red beans and spices) containing naturally produced cereulide.

3.2.2 MATERIALS AND METHODS

3.2.2.1 SUPPLIES AND REAGENTS

Valinomycin was obtained from Sigma-Aldrich (Bornem, Belgium). For chromatographic separation, dilution and extraction, HPLC grade solvents (methanol, ethanol and acetonitrile), produced by BioSolve (Valkenswaard, the Netherlands) were used. Deionised water (Milli-Q, Millipore Corp.) of 18.0 MΩ/cm resistivity and Trifluoroacetic acid (TFA) of certified purity of at least 99 % (spectrophotometric grade) from Sigma-Aldrich (Belgium) were used in the mobile phase. Basmati rice was purchased from a local supermarket and other samples (chili con carne, red beans, and spices) were supplied frozen in the scope of an industrial food safety control as a response to outbreak or as a part of the HACCP monitoring.

3.2.2.2 METHOD VALIDATION

For the validation experiments *B. cereus* emetic strain kinrooi 5958b isolated from a lethal food poisoning case in Belgium (Dierick *et al.* 2005) was used. The 30°C overnight culture grown in brain heart infusion (BHI) broth (Difco Laboratories) was used to inoculate cooked (100°C, 15 minutes) rice at *ca.* 10⁵ CFU/g. The rice was then incubated at 25°C during 24 h, mimicking food contamination conditions that could lead to cereulide production. A negative control (not inoculated rice) was included in the experimental setup.

3.2.2.3 CEREULIDE/VALINOMYCIN EXTRACTION FROM FOOD SAMPLES

Since there is no commercially available standard of cereulide, valinomycin was used as a surrogate standard for this study. Validation experiments were done on the basis of spiked valinomycin. Freshly cooked rice was divided into independent samples: three samples for precision measurements (R1, R2 and R3), one sample to evaluate the food matrix effect and one to be used as a negative control. The cooked rice inoculated with the emetic strain kinrooi 5958b was used as a positive control for cereulide. For each sample, 3 g of rice was weighed in 50 ml plastic tubes (Falcon® from VWR, Belgium). Three different concentrations of valinomycin were spiked (10, 20 and 40 ng/g) in triplicate to R1, R2 and R3 samples, while negative control and matrix samples were not spiked. For extraction, 15 ml of pure methanol was added to the samples, including the positive control, in which cereulide, produced

by the inoculated strain, was present. All samples were then vigorously vortexed for 1 min. The homogenized liquids were centrifuged at 3,200 g for 15 min and supernatants were collected in glass balloons. The pellet remaining after centrifugation was re-dissolved in 10 ml of methanol, vortexed and centrifuged again at 3,200 g for 25 min. The supernatant was added to the previous one and methanol was evaporated in a rotary evaporator (Rotavap® Büchi, from Q-Lab, Belgium). Decontamination (by methanol rinsing) was performed between each sample. All residues were diluted in 3 ml methanol, vortexed and filtered on 0.22 µm filters. Supernatants of all samples, except for the matrix sample, were collected and stored at 4°C prior to analyses. The matrix sample was divided in 5 aliquots and a second evaporation was performed under N₂ flow until dryness. The same concentrations of valinomycin used for standard calibration curve in solution were then used to reconstitute each matrix sample and formed the addition curve (from 1 to 40 ng/g). After vortex step, all samples were stored at 4°C until the moment of analysis.

Some optimization tests were carried out prior to the validation in which the sample size (3, 5 or 10 g), the solvent of extraction (ethanol or methanol) and the ratios of sample size to solvent volume (1:2, 1:5 and 1:10) were compared. When the sample size was assessed, a fixed ratio of sample size to solvent volume (ratio of 1:2) was applied in order to extract 3, 5 or 10 g of matrix. The methanol extraction procedure described above was applied to the samples. A sample size of 3 g was consequently selected for the next experiments. For solvent comparison, 3 g of rice spiked with valinomycin were extracted with 15 ml of methanol or ethanol and then treated as explained above. The influence of the ratios of sample size to solvent volume were also tested on 3 g of sample by adding either 6 ml (ratio 1:2), 15 ml (ratio 1:5) or 30 ml (ratio 1:10) of solvent, before proceeding to the rest of the procedure.

3.2.2.4 METHOD VALIDATION CRITERIA

The following parameters were considered when performing an in-house validation: linearity, matrix effect, recovery, precision (repeatability and reproducibility), limit of detection (LOD) and limit of quantification (LOQ).

These parameters were evaluated on the basis of valinomycin spiking, in accordance with the performance criteria suggested in the 2002/657/EC document.

Linearity of the method was investigated by determining the calibration curve in methanol (standard calibration) in the range of 1 to 40 ng/ml. The range was voluntarily limited to avoid the saturation of the detector which occurred after 40 ng/ml. Injections were performed in duplicate. The matrix effect was evaluated by comparison of standard calibration curve with calibration curve in rice. Valinomycin spiked concentrations ranged from 1 to 40 ng/g. Injections were performed in duplicate. Recovery and precision of the method were determined with samples spiked with valinomycin at 10, 20 and 40 ng/g. The procedure was performed in triplicate and repeated on 3 different days.

Instrumental LOD and LOQ were determined by analysing standard solutions with decreasing concentrations and were additionally compared to LOD and LOQ of the method, determined in valinomycin equivalents from the concentration of cereulide present in the positive rice sample. The signal to noise ratio (S/N) method was used to calculate those limits according to the Commission Decision 333/2007/EC. The principle is based on the measurement of the peak-to-peak noise around the analyte retention time and reporting the concentration of the analyte yielding a certain signal on the calculated noise. While a S/N of 3 is accepted for LOD estimation, a S/N of 6 is required for LOQ estimation. The minimal concentrations tested that satisfy the identification and the S/N ratio criteria are the LOD and LOQ.

For quality control purposes each batch of analysis was accompanied with a negative control (not inoculated, not spiked rice sample) and a positive control (inoculated rice with emetic *B. cereus* kinrooi 5958b at *ca.* 10⁵ CFU/g leading to cereulide production). Positive and negative samples were processed with each batch on three different days as described in Section 3.2.2.3.

3.2.2.5 METHOD APPLICATION

The method was used to determine cereulide production in inoculated rice at two different inoculum levels 10^3 and 10^6 CFU/g, under two different storage temperatures (23 and 30°C during 24h) and with presence/absence of other *B. cereus*. After cooking, one set of rice (described as “cooked rice” in the study) was kept at low temperature ($< 4^\circ\text{C}$) to avoid germination of the *B. cereus* spores eventually present in rice and/or contamination by other bacteria. The other set of rice (described as “contaminated rice” in the study) was left at room temperature for 48h for natural contamination to occur. The background *B. cereus* flora of the latter was enumerated on MYP (Mannitol Egg Yolk Polymyxin) media before inoculation. *B. cereus* k5975cR, a spontaneous rifampicin resistant mutant of strain k5975c was used as inoculum to allow an easier recovery and discrimination from the resident *B. cereus* microflora. Both types of rice were inoculated with mutant k5975cR, either at 10^3 and 10^6 CFU/g, to follow the *B. cereus* growth behavior and to compare the total cereulide production in cooked rice and in naturally contaminated rice. The total *B. cereus* flora and the inoculated k5975cR mutant were both enumerated after incubation of the two types of rice on MYP medium and on LB (Luria Bertani, Oxoid) supplemented with rifampicin agar plates, respectively. This experiment was repeated on three independent occasions.

Moreover, the method was applied on various matrix samples (chili con carne, red beans and barbecue spices) that were part of probable cereulide outbreak or part of HACCP monitoring. For this purpose, the methanol extraction protocol, followed by two centrifugation steps as described in Section 3.2.2.3, was applied. A negative control (consisting in a not contaminated, not spiked matrix) and an analytical control sample (not contaminated matrix spiked with valinomycin) were added to each batch. The recoveries were calculated on the basis of the valinomycin spiking (20 ng/g). Since the method was validated in rice, the calculated concentrations of cereulide in the different matrices are only indicative.

3.2.2.6 LC-MS² ANALYSIS

Samples were injected into the LCQ Deca-XP Plus ion trap mass analyser (ThermoFinnigan, USA). Analysis protocol used for cereulide and valinomycin determination was inspired from the procedure described by Häggblom *et al.* (2002). Chromatographic separation was done on the Symmetry C8 column, 2.1 mm x 150 mm, 5 μ m (Waters, USA). The mobile phase of the isocratic method was a mixture of 95 % acetonitrile, 4.9 % water, 0.1 % trifluoroacetic acid at a flow rate of 0.2 ml/min with a sample injection volume of 5 μ l.

Valinomycin was dissolved in pure methanol and used as an external standard. The retention times were 9.8 min for valinomycin and 9.6 min for cereulide. Fundamental difference resided in the chosen mode of mass analysis. Since LC-MS² allows more sensitive quantification, the MS² analysis was performed by monitoring ion fragments of cereulide or valinomycin generated by the fragmentation of the chosen parent ion through collision with helium gas. The MS² parameters were tuned for each compound (cereulide and valinomycin) by optimising the fragmentation of the M-NH₄⁺ adduct ion leading to specific product ions. During analysis, a full mass spectrum from 500 to 1300 m/z values and a MS² fragmentation mass spectrum were recorded in positive electrospray mode (ESI+). For detection of valinomycin and cereulide, the m/z values monitored in the full scan mode were for valinomycin adduct ions 1128.5 (M- NH₄⁺ adduct) and 1149.5 (M- K⁺ adduct) and for cereulide 1170.5 (M- NH₄⁺ adduct) and 1191.5 (M- K⁺ adduct). During MS² mass analysis, fragmentation of valinomycin parent ion 1,128.5 or cereulide parent ion 1,170.5 was induced and the m/z values recorded were respectively 1,083.3 and 1,111.2 (valinomycin fragment ions corresponding to the loss of a CO and to the loss of the NH₄⁺ adduct, respectively) or 1,125.3 and 1,153.3 (cereulide fragments ions corresponding to the loss of a CO and to the loss of the NH₄⁺ adduct, respectively). For both cereulide and valinomycin, the peaks corresponding to the loss of a CO (m/z values 1,125.3 and 1,083.3, respectively) were always the highest peaks generated through the fragmentation. The chromatograms were smoothed with a Gaussian function.

Presence of carry over, and variability of the peak area in the chromatograms, were verified throughout the sequence of injections by analyzing methanol sample (for which no peak should be detected) and valinomycin standard controls in solution (for which the variation in the area should be below 25 %) at the beginning, in the middle and at the end of the sequence.

3.2.3 RESULTS AND DISCUSSION

3.2.3.1 VALIDATION OF THE CEREULIDE ANALYTICAL METHOD

The following parameters were determined on the basis of valinomycin spiking: specificity, linearity, matrix effect, precision, recovery, limit of detection (LOD) and limit of quantification (LOQ).

Specificity

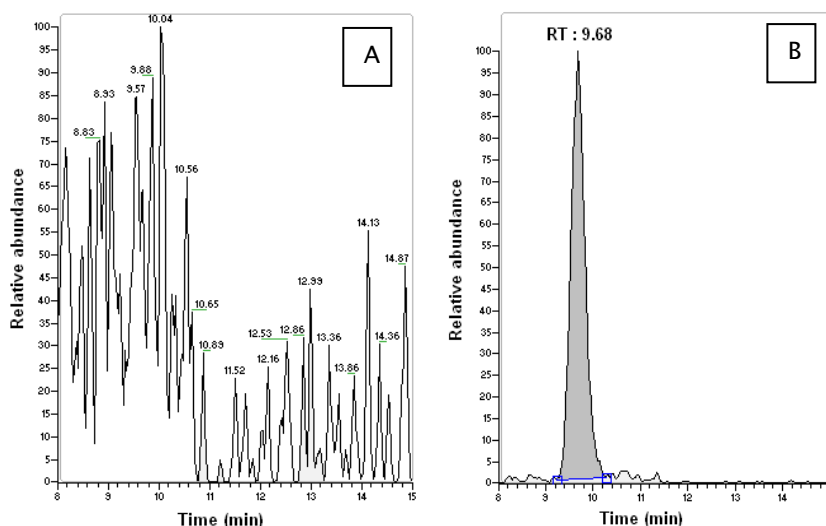


Figure 16 – MS² chromatogram for cereulide in the negative and positive rice samples

MS² Chromatograms for A: Negative (not inoculated, not spiked) rice sample; B: positive (containing cereulide) rice sample. The relative abundance (in y-axis) is normalized to the highest peak, and is presented in function of time.

Specificity of the method was determined by comparing spiked rice sample with negative rice samples, in which valinomycin, dissolved in methanol, or only methanol were added. Since no interfering peak at the retention time of

valinomycin was detected in MS² chromatogram in the negative control (data not shown), the method was considered as specific for valinomycin. The MS² chromatograms of the rice sample containing cereulide (positive sample) and the corresponding toxin-free rice sample (negative sample) were also compared. No interfering peak was present in the MS² chromatogram at the retention time of cereulide (Fig. 16A compared to 16B). Based on these results, the method was considered specific to cereulide as well.

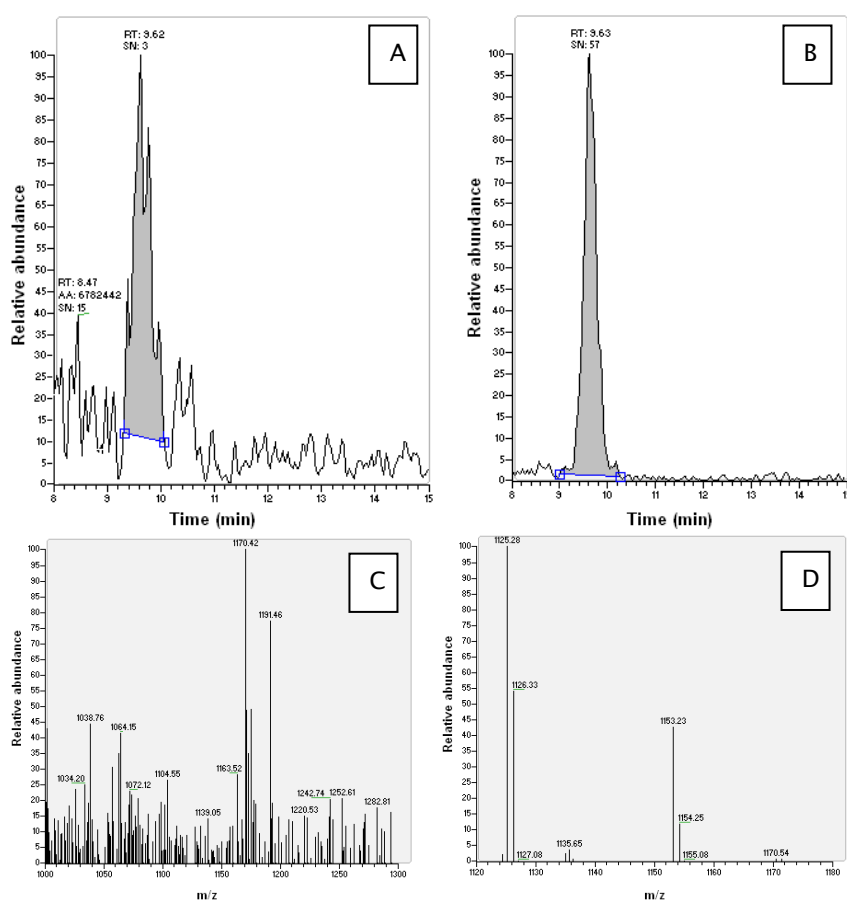


Figure 17 – Comparison of MS and MS² chromatograms and mass spectra for cereulide in the same positive sample

Chromatograms and mass spectra for the positive rice sample by LC-MS analysis (A, C) and by LC-MS² analysis (B, D), respectively. The characteristics m/z values monitored for cereulide are 1170.5 (M- NH₄⁺ adduct ion) and 1191.5 (M-K⁺ adduct ion) in the LC-MS analysis, and 1125.3 and 1153.3 (cereulide fragments ions) in the MS² analysis.

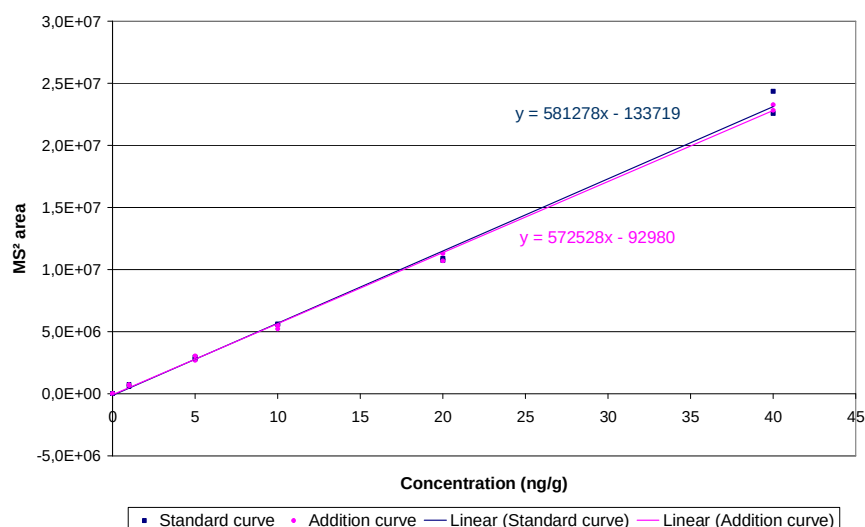
Furthermore, as shown in Fig. 17, the advantages of LC-MS² analysis compared to LC-MS were visible through striking reduction of interferences either in the chromatograms or in the mass spectra of the samples containing cereulide. The peak corresponding to cereulide was clearly better shaped, and the noise reduced, in the MS² chromatogram (Fig. 17B) compared to the MS chromatogram (Fig. 17A), leading to an increase of sensitivity illustrated by the signal to noise ratio (from S/N 3 to S/N 57). In the MS spectra, the two mass ions representing cereulide (1,170.5 and 1,191.5) were slightly above the noise (Fig. 17C). In contrast, the characteristic fragment masses of cereulide (1,153.3 and 1,125.3) were clearly visible in the MS² spectra of the same sample (Fig. 17D).

Linearity

A calibration curve consisting of seven standard solutions with nominal concentrations ranging from 1 to 40 ng/ml was used to determine instrument linearity. Above these limits, saturation of the detector was observed. The Mandel's fitting test (1964), used to assess the linearity of the calibration curve, gave a value of 0.2597. Since this value is lower than $F(99.9\%) = 9.0738$, the hypothesis of a linear correlation was accepted. Linearity was described by the equation $y = 76.4296 x + 298.572$ with $R^2 = 0.994$ (data not shown).

Matrix effect

The presence of the food matrix may induce some systematic errors regarding concentration, *i.e.* ion enhancement or ion suppression. Matrix effect was evaluated by comparing the slope of the standard calibration with the slope of the matrix curve by means of a statistical t-test. The calculated t-value is compared with the tabulated t-value at 95 % confidence level. The standard calibration was performed in methanol while the matrix curve was performed in rice. The t-test value obtained for rice 0.66 was below the T crit, 95 % = 2.09, which indicates that there is no matrix effect and which allows to quantify cereulide in samples on the basis of a calibration curve made in solution (Fig. 18).

**Figure 18 – Matrix effect**

The standard calibration (blue line) was compared with the calibration curve in rice (purple line). The injections were performed in duplicate.

Precision and recovery

For the determination of precision parameters and recovery within the validation, several replicate analyses are required. The results were used to establish repeatability (r) and intra-laboratory reproducibility (w) standard deviations. In this work, each replicate used for repeatability stemmed from a separate extraction performed on the same day. For the intra-laboratory reproducibility, the replicates were extracted separately on three different days. Repeatability standard deviation (sr) and intra-laboratory reproducibility standard deviation (sw) were calculated according to ISO 5725-2.

The recovery of the method was determined on the basis of valinomycin spiking in rice samples and the recovery values evaluated at three spiking levels (10, 20 and 40 ng/g) were in the range of 77 to 101 %, as calculated from Table 7. These values are within the limits of acceptance according to the European Commission decision 2002/657/EC.

As indicated in Table 7, the coefficients of variation for repeatability (CVr) ranged from 1.56 to 4.80 % and the coefficient of variation for intra-

3. RESULTS

3.2. Determination of the *Bacillus cereus* emetic toxin by means of LC-MS² method

laboratory reproducibility (CVw) ranged from 3.35 to 8.24 %. These values were compared with the Horwitz ratio, a normalized performance parameter indicating the acceptability of analysis methods with respect to precision (Horwitz and Albert 2006). The calculated coefficients were about 3 times lower than the 14.7 and 22.0 % determined by the Horwitz ratio for CVr and CVw, respectively.

Table 7 – Precision of valinomycin determination in cooked rice by LC-MS² method at three concentration levels

	10 ng/g (n=3x3)	20 ng/g (n=3x3)	40 ng/g (n=3x3)
mean (ng/g)	7.71	16.01	40.39
sr (ng/g)	0.37	0.26	0.63
CVr (%)	4.80	1.60	1.56
sw (ng/g)	0.64	0.55	1.36
CVw (%)	8.24	3.44	3.35

Sr: intraday standard deviation (repeatability), sw: interday standard deviation (reproducibility), CVr and CVw: coefficients of variation for repeatability and reproducibility, respectively

Table 7 presents average, standard deviation and coefficient of variation for repeatability and reproducibility and shows that the method is repeatable and reproducible in rice on the basis of valinomycin spiking. Furthermore, precision parameters were also verified for the inoculated rice sample containing cereulide. This positive sample was extracted after 24 h of incubation at 25°C in triplicate and on three different days (after placing the samples at 4°C to suspend cereulide production). The calculated concentration of cereulide in the rice was an average of 707.87 ng/g and the coefficient of variation for repeatability (CVr) and reproducibility (CVw) were 6.13 % and 11.24 %, respectively (data not shown). These coefficients of variation still did not exceed the Horwitz ratio for CVr and CVw.

LOD and LOQ

The signal to noise ratio (S/N) method was used to determine LOD and LOQ according to the Commission Decision 333/2007/EC. The 0.4 ng/ml valinomycin solution with an S/N of 4 (in accordance with $S/N \geq 3$

requirement for LOD) and the 1 ng/ml valinomycin solution displaying an S/N of 6 (in accordance with $S/N \geq 6$ requirement for LOQ) were determined as instrumental LOD and LOQ, respectively. LOD and LOQ of the method were determined from the positive rice sample containing cereulide. The positive control sample (cereulide concentration of about 7 ng valinomycin equivalent/g of food) displayed a S/N of 50, leading to extrapolation of LOD and LOQ for cereulide as being ≤ 0.5 ng/g and ≤ 1 ng/g, respectively.

3.2.3.2 OPTIMIZATION AND APPLICATION OF THE METHOD

Extraction solvents (on rice samples)

Recently, Bauer *et al.* (2010) developed a stable isotope dilution analysis using a ¹³C₆-cereulide produced from *B. cereus* as internal standard for quantification and Biesta-Peters *et al.* (2010) synthesized cereulide to use as standard in LC-MS quantification. The methods by Bauer *et al.* (2010) and Biesta-Peters *et al.* (2010) used ethanol and acetonitrile as extraction solvents in a 15 h and 1 h-extraction step, respectively. Possible drawbacks are, for the fast extraction step method, the use of acetonitrile as solvent which can represent an important financial cost, and in the case of the ethanol-based method, the extraction step that is rather time-consuming (15 h).

The protocol described above (see 3.2.2.3) and consisting in a run of about 2.5 h was tested using methanol and ethanol in parallel. We excluded the use of acetonitrile because of its higher toxicity and cost. The recovery efficiency was evaluated on the basis of valinomycin spiking. The average recoveries of the extracted triplicates were 98.72 and 100.03 %, respectively (data not shown). There was no significant difference between extraction with ethanol or methanol (p -value > 0.5).

Analysis of various food samples

The method was applied to 70 different food samples suspected to contain cereulide, which are listed in Table 8. On the 70 analyzed samples, 24 were detected as containing cereulide. The concentrations found in red beans were very low and close to the quantification limits (LOQ): on 14 positive red beans, 8 samples were shown to contain cereulide but the level could not be

quantified (< LOQ). However, only one sample (on 5 positive samples) was close to the LOQ in chili con carne and the 5 detected cereulide amounts in spices were all above the LOQ. The highest amounts of cereulide quantified in the different matrices were: 21.3 ng eq/g of food (ng valinomycin equivalent per g of food) in spices, 14.7 ng eq/g in chili con carne and 3.2 ng eq/g in red beans. The respective recoveries for chili con carne, red beans and spices were calculated on the basis of valinomycin spiking (20 ng/g) to be about 55, 45 and 65 %, respectively. The presence of cereulide in the spices is an interesting result because it implies either the growth of *B. cereus* to levels at which cereulide could be produced, either that cereulide was carried over by contact with other contaminated food.

Table 8 – Detection of cereulide in different food samples analyzed by LC-MS²

Matrices	Analyzed samples	Samples with detected cereulide	Concentrations in the positive samples (ng eq / g)	Samples < LOQ	Samples < LOD
Chili con carne	N = 40	5	2.4 – 14.7	1	35
Red Beans	N = 24	14	1.5 – 3.2	8	10
BBQ spices	N = 6	5	4.8 – 21.3	0	1

Cereulide concentrations ranges are expressed in ng of valinomycin equivalent/g of food, < LOQ: below limit of quantification, < LOD: below limit of detection

Minimum representative sample quantity

The samples sizes used by Bauer *et al.* (2010) and Biesta-Peters (2010) were 10 and 5 g, respectively. However, in case of foodborne intoxications, the amount of contaminated sample generally available may be very limited. For this reason, we investigated the influence of sample sizes on the recovery efficiency (calculated on the basis of valinomycin spiking). Samples of 3, 5 and 10 g were extracted with a fixed ratio of sample size: solvent volume of 1:2. Similar recoveries (p-value > 0.05) were obtained independently of the sample quantity (data not shown). A representative size of 3 g was consequently chosen for the next experiments. In order to assess the influence of the ratio sample size to solvent volume, 3 g of rice was extracted

with either 6 ml (ratio 1:2), 15 ml (ratio 1:5) or 30 ml (ratio 1:10) of methanol. As illustrated in Fig. 19, the recoveries calculated on the basis of valinomycin spiking were significantly higher (p-value < 0,05) for the ratio 1:5 than for the ratio 1:2. The ratio 1:10 was not significantly different than the ratio 1:5, probably due to the higher standard deviation observed. The method was optimised and the parameters were chosen as 3g of sample (matrix) to be extracted with 15 ml of methanol.

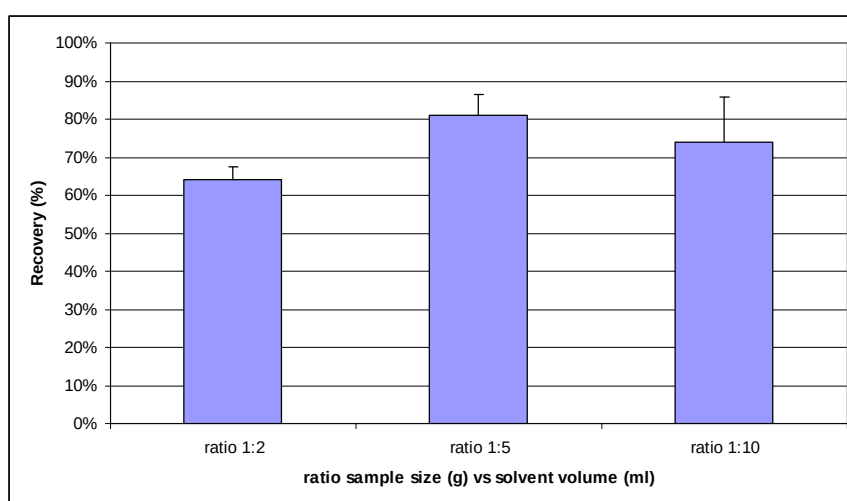


Figure 19 –Influence of solvent volume (ml) ratio on cereulide recovery (expressed in %) from rice samples

Cereulide recovery (expressed in %) from rice in function of the applied ratio. Presented recoveries are the average of three replicates and the error bars indicate the standard deviation for the triplicates

Influence of growth parameters on cereulide production in rice

Production of cereulide in inoculated rice samples together with the bacterial counts of the inoculated *B. cereus* emetic rifampicin-resistant strain is shown in Fig. 20. The amount of cereulide produced was a function of inoculum level, temperature and presence of naturally resident *B. cereus*. The background flora in naturally contaminated rice before inoculation was enumerated on MYP medium as being $2.5 \cdot 10^7$ CFU/g; $1.6 \cdot 10^8$ CFU/g and $8.7 \cdot 10^6$ CFU/g in the three independent experiments.

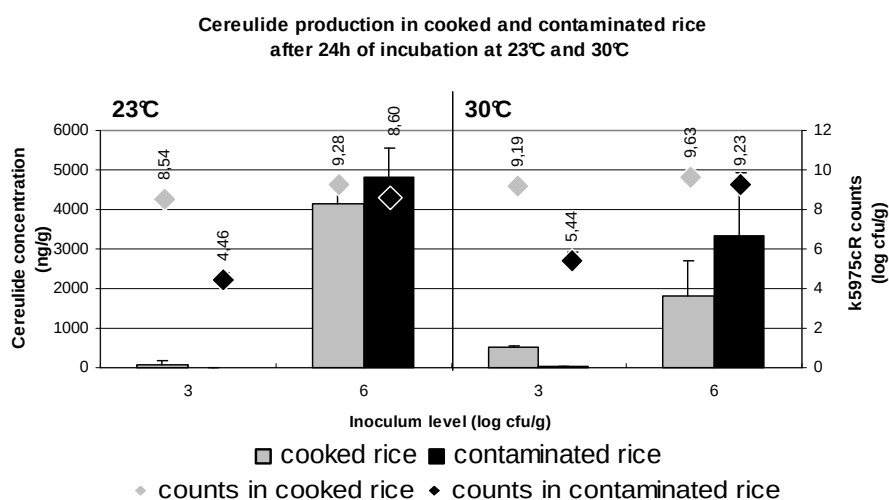


Figure 20 – Cereulide production in cooked and contaminated rice

Production of cereulide (expressed in ng of valinomycin equivalent/g of rice) at 23°C and 30°C in cooked and naturally contaminated rice, inoculated with either *ca.* 3 log or 6 log CFU/g of the rifampicin-resistant cereulide-producing strain k5975cR. Cereulide concentrations and bacterial counts of the k5975cR strain are the average of three individually repetitions and the error bars indicate the standard deviation in the cereulide contents for the three experiments.

For samples inoculated with 10^6 CFU/g, a higher amount of cereulide was found, in both pre-contaminated and not pre-contaminated samples, during storage at 23 °C (about 4,000 ng equivalent of valinomycin/g of food) than at 30°C. For the inoculum of 10^3 CFU/g, the amount of cereulide per gram after 24 h was greater at 30 °C than at 23 °C (less than 100 ng) and always higher in the not pre-contaminated samples. Pre-contamination of the rice (containing more than 7 log of resident *B. cereus*) did not significantly ($p > 0.05$) influence the final cereulide concentration in the case of an inoculum of 10^6 CFU/g of the k5975cR emetic strain at both 23°C and 30°C. With an inoculum level of 10^3 CFU/g, cereulide was quantifiable in not pre-contaminated samples but was hardly detected in the pre-contaminated samples (Fig. 20).

These observations indicated that the inoculated emetic strain produced emetic toxin within 24 h, both in cooked and naturally contaminated rice,

except when a low inoculum level of *ca.* 10³ CFU/g was used in contaminated rice. The growth of the inoculated strain was then impaired by the resident *B. cereus* and restricted at 4.46 log CFU/g, and the cereulide production was undetectable. This is in accordance with previous study, which showed that cereulide production occurs only from 10⁵ CFU/g (Fretz *et al.* 2007; Granum and Lund 1997). The amounts of cereulide produced reached toxin levels previously shown to induce emesis (0.01–1.6 µg cereulide/g food) in humans (Agata *et al.* 2002). Interestingly, there is no strict correlation between the amount of cereulide produced and the number of *B. cereus* CFU, compared with temperature. For inoculum of 10⁶ CFU/g, higher cereulide amounts were found in both types of rice at 23°C than at 30°C while the opposite situation was observed for the inoculated *B. cereus* 5975cR mutant counts. This indicates that caution should be taken in assessing the toxicity of a food product by enumerating its *B. cereus* content and that rather the toxin contents should be quantified, as previously mentioned by Delbrassinne *et al.* (2011).

3.2.4 CONCLUSION

A very specific and sensitive LC-MS² method for the determination of cereulide in food product was presented in this study. This approach was proved to be robust and applicable to various food samples. Cereulide concentrations could be expressed in valinomycin equivalents with low detection and quantification limits. Although cereulide standard is the ideal standard, valinomycin is a good commercially available standard and the LC-MS² method described in this study is easily performed. Regarding the parameters such as cost, time and matrix availability, the method allowed to use lower samples quantities (3g), cheaper solvent of extraction (methanol) and is less time-consuming than previously published methods. Furthermore, a higher sensitivity and specificity were reached by using the tandem mass spectrometry in the LC-MS² method. Determination of cereulide/valinomycin in rice was validated in-house according to the 2002/657/EC and the method is ready to be used in routine analysis in the scope of protecting public health.

Since cereulide is a highly lipophilic molecule and can rapidly be absorbed into the bloodstream (Paananen *et al.* 2002), there may be a possible

accumulation in human body if cereulide is regularly ingested in low amounts from contaminated foods. LC-MS² would permit to follow such sub-clinical concentrations possibly present in consumed food. Accurate detection could also assist in the determination of cereulide concentration in blood and help possible detoxification (Shiota *et al.* 2010).

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3.3 METHOD APPLICATIONS – FOODBORNE OUTBREAKS – REPORTED OUTBREAK

Although *B. cereus* infections are not commonly reported due to its usually mild symptoms, five known fatal cases due to cereulide occurred in Europe and Asia in the last two decades. All these intoxications have been associated with some organ damages such as liver failure or brain oedema. However, fatality is quite a seldom outcome albeit it demonstrated the possible severity of the emetic syndrome. Just before the start of the present study, a fatal case occurred in Belgium where a 20-year-old man died after ingestion of contaminated pasta. It was easily demonstrated that *B. cereus* was at the origin of the death since significant counts were found in the leftovers and the isolated strains were all emetic *B. cereus*. Yet, the presence of the toxin and its concentration in the leftovers could not be revealed with the usual methods available for routine. One of the first applications of the validated LC-MS² method was therefore to verify and quantify the amounts of cereulide in the remaining spaghetti dish collected from the case. This performed analysis could point to cereulide as the most likely cause for the lethal outcome of the healthy young man.

This manuscript has been published in Journal of Clinical Microbiology³.

My contribution to this paper consisted in the managing and organising the analytical determination of cereulide in the pasta leftovers. I also actively participated in the writing process in collaboration with the first author.

³ Naranjo M, S. Denayer, N. Botteldoorn, L. Delbrassinne, J. Veys, J. Waegenaere, N. Sirtaine, R.B. Driesen, K.R. Sipido, J. Mahillon, and K. Dierick. 2011. Sudden death of a young adult associated with *Bacillus cereus* food poisoning. Journal of Clinical Microbiology, 49:4379–4381.

Summary

A lethal intoxication case, which occurred in Brussels (Belgium) is described. A 20-year-old man died following the ingestion of pasta contaminated with *Bacillus cereus*. Emetic strains of *B. cereus* were isolated and high levels of cereulide (14.8 µg/g) were found in the spaghetti meal.

Keywords

B. cereus, enterotoxins, cereulide

3.3.1 CASE REPORT

On October 1st, 2008, a twenty-year-old man became sick after eating a meal of leftovers of spaghetti with tomato sauce, which had been prepared 5 days before and left in the kitchen at room temperature. After school, he warmed up the spaghetti in the microwave. Immediately after eating, he left home for his sports but had to return home 30 minutes later because of headache, abdominal pain and nausea. At his arrival, he vomited profusely during some hours and at midnight had two episodes of watery diarrhea. He did not receive any medication, and only drank water. After midnight, he fell asleep. The next morning at 11:00 AM his parents were worried because he did not get up. When they went to his room, they found him dead.

Legal examination determined the time of death presumably at 4:00 AM, approximately 10 hours after ingestion of the suspected meal. An autopsy could only be performed 5 days later. Macroscopically, brownish and moderately softened liver and ascites (550 cc of citrine liquid) were found. The heart was macroscopically normal. A total lysis of the pancreas was also found, but it could not be excluded that this finding was due to the autopsy delay.

Microscopic findings were: moderate centro-lobular liver necrosis without inflammatory signs and discrete biliary stasis, significant vascular congestion of the lungs probably due to acute cardiac insufficiency, significant necrosis from all layers of colon mucosa and submucosa alternating with better preserved zones, mixed intestinal flora but no evidence of invasive bacterial lesions. Significant lysis of the adrenal glands was reported too. The exact cause of the death could not be determined by the autopsy because the interpretation of findings was very difficult due to the autopsy delay.

Five faecal swabs and two faeces samples were taken post-mortem and samples were tested for the presence of *Bacillus cereus* by growth on MYP (Mannitol Egg Yolk Polymyxin) agar. *B. cereus* was only found in two of the five faecal swabs and the strains isolated were named ISP321 and ISP322. No *B. cereus* was cultured from the faeces samples.

Pasta and tomato sauce samples, the leftovers of the dinner, were also sent for analysis to the National Reference Laboratory for Food-borne outbreaks (NRLFBO). For enumeration of *B. cereus* in food samples, the ISO 7932 method (17) was used. Significant *B. cereus* counts (9.5×10^7 CFU/g) were found in the pasta while *B. cereus* was absent in the tomato sauce. The strain isolated from the pasta meal was named ISP303.

PCR assays that detect the presence of toxin genes were applied to DNA from the pasta isolate (ISP303) and the two human isolates (ISP321 and ISP322) and the results are presented in Table 9. The presence of genes encoding non-haemolytic enterotoxin NHE (*nheA*), phospholipase C (*plcA*), cytotoxin K (*cytK*), hemolysin II (*hem2*) and hemolysin BL (*hblA*) was investigated (12, 13, 15). All three strains tested positive for the presence of the *nheA* and *plcA* genes. The strains were all negative for the other enterotoxin genes (*cytK*, *hem2*, *hblA*). A specific cereulide-associated PCR test (7) was also used to target the *ces* genetic determinants which are necessary for cereulide synthesis. The PCR test to detect *ces* gave positive results for all three isolates.

Table 9 – Characterization of the *B. cereus* isolates

Isolate	Source	<i>nheA</i>	<i>plcA</i>	<i>cytK</i>	<i>hem2</i>	<i>hblA</i>	<i>ces</i>
ISP303	spaghetti	+	+	–	–	–	+
ISP321	faecal swab	+	+	–	–	–	+
ISP322	faecal swab	+	+	–	–	–	+

Toxin genes presence was assessed by PCR analysis. The + and – symbols refer to the presence and absence of the gene, respectively

The actual production of cereulide was also confirmed by the boar sperm assay (4) (data not shown). These results suggest that all three *B. cereus* isolates ISP303, ISP321 and ISP322 are emetic strains. The concentration of the cereulide toxin in the spaghetti was determined by LC-MS² (5, Delbrassinne *et al.*, unpublished results) as being 14.8 µg/g of pasta. In comparison with the cereulide concentration of 1.6 µg/g previously found in a contaminated pasta dish (18), the amount of cereulide determined in the

spaghetti by the present study was almost 10 times higher and seems consequently to be within the upper range of cereulide amounts reported in foods implicated in outbreaks.

Further characterization of the three isolates was performed using the repetitive sequence-based PCR (rep-PCR) and pulsed-field gel electrophoresis (PFGE) (10, 11, 25) of genomic DNA. All three strains displayed the same rep-PCR and PFGE profiles (data not shown).

B. cereus is a well-known food poisoning organism. It can cause two types of food poisoning described as the emetic and the diarrheal syndromes (12). The emetic type is caused by a heat-stable cereulide toxin that is preformed in food (2). The symptoms are usually mild but can be severe in some cases. Four fatal cases attributed to cereulide have been reported (6, 22, 24, 26). The present results provided evidence for *B. cereus* food poisoning in a young healthy man. Clinical data and the rapid onset of symptoms, together with the microbiological and molecular study, point to *B. cereus* as the most likely cause for this fatal outcome.

Emetic strains of *B. cereus* are known to cause mitochondrial damage. Because the macroscopical methods used in autopsy could not reveal any specific cardiac symptoms, we performed further specific microscopical staining. Immunostaining for activated caspase 3, a marker for apoptosis (1, 27), was negative indicating the absence of mitochondrial damage. The structure of cardiac myocytes was also intact, which suggested that the sudden cardiac arrest was caused indirectly, *e.g.* due to acidosis.

Fulminate hepatic failure is one of the most feared complications caused by emetic *B. cereus* strains. In this case, the liver damage was mild and could not be the only explanation for the sudden death of a healthy young man. Therefore, and because of the presence of diarrhea, together with the severe colonic necrosis, PCR assays that detect the presence of enterotoxin genes were applied to the samples.

As indicated above, PCR products could be found for the *nheA* and *plcA* genes, but not for *cytK*, *hem2* and *hblA*. These observations are rather difficult to interpret since the actual implication of putative enterotoxins in diarrhea remains a matter of debate. The absence of HBL enterotoxin in *B. cereus* strains which produce cereulide is common whereas the emetic strains usually produce NHE toxin (8). The pattern of toxin production for the strains isolated in the present case is presumably classical for strains implicated in emetic poisoning. Nevertheless, some emetic strains producing NHE toxin were found very cytotoxic on Caco-2 cells as previously reported (14). Furthermore, the simultaneous presence of the genetic determinants for cereulide and the genes encoding for potential enterotoxins has been recently demonstrated in a *B. cereus* strain (19, 20), which appeared to be particularly virulent. Could this fact have played a role in the necrosis of the colon, even though the *cytK* PCR was negative, knowing that this toxin is the one that has been implicated in necrotic enteritis (21) ? That is an important question for which no answer can be given at the moment.

Although we cannot incriminate *B. cereus* as the direct and unique cause of death, the present case illustrates the severity of the emetic and diarrheal syndromes and the importance of adequate refrigeration of prepared food. Because the emetic toxin is preformed in food and is not inactivated by heat treatment (2, 23), it is important to prevent *B. cereus* growth and its cereulide production during storage. This toxin production is closely linked to temperature (9) and is not strictly correlated with bacterial counts as recently demonstrated by Delbrassinne *et al.*, (5, unpublished results). The cereulide amounts produced by a *B. cereus* emetic strain inoculated at 10⁶ CFU/g in cooked rice were higher at 23°C than at 30°C whereas the opposite situation was observed for the cereulide-producer counts. These results suggest that cereulide may be more actively produced at ambient temperatures. In this case, the spaghettis had been kept at room temperature for several days: this allowed *B. cereus* to grow to the previously mentioned very high concentrations and produced the high toxin concentration (14.8 µg/g) found in the pasta and likely responsible for the fatal outcome. According to previously published reports, toxin concentrations that induce emesis in humans, as determined by the

cytotoxicity test on HEp-2 cells on the basis of several contaminated foods, extended from 0.01 to 1.28 µg cereulide/g of food (3), and from 0.005 to 32 µg cereulide/g of food (16), determined by LC-MS. Although those concentrations did not implicate lethal cases, we do not know what were the amounts actually ingested. In the fatal case described by Mahler *et al.*, (22), strains isolated from the pan residue produced enterotoxin NHE (detected by the BCE-VIA assay) and the emetic toxin when grown on cooked rice. This highlighted the virulence of strains capable of this simultaneous production, as reported in this study.

In conclusion, although the autopsy results were not conclusive, probably due to the delay of analysis, the large number of *B. cereus* cells and the significant cereulide concentration found in the leftovers are the most likely cause of death of the young healthy man. Further investigation of these isolates and this case may provide insights into the virulence mechanisms of emetic *B. cereus* isolates.

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3.4 METHOD APPLICATIONS – RESEARCH – ANTIFUNGAL ACTIVITY OF CEREULIDE

The biological role of cereulide for the producing strains has long remained unclear. It was suggested that cereulide could provide a competitive advantage in virulence or in environmental adaptation of *B. cereus*. But, during several years, no antibiotic role could be attributed to the toxin despite the numerous growth inhibition experiments that have been performed against various species of fungi, yeast and bacteria. Contrary to this, we were successful to highlight a fungistatic function exerted by cereulide against specific fungi. The inhibition effects on the growth of several fungi species (*Alternaria alternata*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Magnaporthe grisea*, *Monographella nivalis*, *Mortierella isabellina*, *Sclerotinia minor*, *Stemphylium vesicarium* and *Verticillium dahliae*) was evaluated for cereulide extracts and valinomycin solutions. A similar approach, performed in parallel to our own experiments, demonstrated an antibacterial activity of cereulide against Gram-positive bacteria. The combination of these two discoveries allowed to lift the veil on the cereulide “raison d’être”.

This manuscript has been published in Applied and Environmental Microbiology⁴.

My contribution to this paper consisted in managing, planning and performing the analysis of cereulide extracts to confirm the unambiguous presence of the toxin and to quantify the levels of cereulide with valinomycin standard. This helped to perform the growth inhibition experiments.

⁴ Ladeuze S., N. Lentz, L. Delbrassinne, X. Hu and J. Mahillon 2011. Antifungal activity displayed by cereulide, the emetic toxin produced by *Bacillus cereus*. Applied and Environmental Microbiology 77:2555–2558.

Summary

In this study, the fungistatic activity of *Bacillus cereus* cereulide-producing strains was demonstrated against nine fungal species. The role of cereulide was confirmed using plasmid-cured derivatives and *ces*-knockout mutants. The fungistatic spectra of cereulide and valinomycin, a chemically-related cyclododecapeptide, were also compared and found to be similar but distinct.

Keywords

antifungal activity, *Bacillus cereus*, cereulide, valinomycin

Food poisoning by emetic *Bacillus cereus* strains has been shown to cause fatal liver failure (6, 15, 25). Cereulide, the emetic toxin of *B. cereus*, is a cyclic dodecadepsipeptide composed of three repetitions of two amino acids and two hydroxyacids (D-O-Leu-D-Ala-L-O-Val-L-Val)₃. Its chemical structure is closely related to that of valinomycin, which is produced by various *Streptomyces* spp. (16). Cereulide is a heat-stable toxin (4, 21, 24, 27) and is synthesized enzymatically by a non-ribosomal peptide synthesis (NRPS) whose genetic determinants are located on the pCERE01 plasmid of the *B. cereus* emetic strain Kinrooi 5975c (9), the pBC270 element from *B. cereus* strain AH187 (23) and the pBCE4810 plasmid from *B. cereus* reference strain F4810/72 (8). These elements contain a 23-kb gene cluster (*ces*) involved in the cereulide synthesis. The two largest genes, named *cesA* (~10 kb) and *cesB* (~8 kb), lead to the incorporation and modification of D-O-Leu, D-Ala (*cesA*), L-O-Val, and L-Val (*cesB*), which compose the basic tetradepsipeptide motif of cereulide (7, 14).

Cereulide can cause stomach pain and vomiting, respiratory distress, occasional loss of consciousness, possibly leading to coma and ultimately death of the individual (6). This toxin is also an ionophoric molecule with a high degree of affinity for K⁺ ions (26). It is lipophilic and specifically targets mitochondrial membranes (4) where it leads to cellular dysfunctions, such as swelling mitochondria and blocking oxidative phosphorylation (2, 17).

It has been shown that valinomycin and cereulide display similar activities since they block the motility of boar spermatozoa through the dissipation of mitochondrial intern membrane potential, a property often used for the detection of cereulide (3, 4, 13, 22). Given these similarities, it was interesting to consider the known activities of valinomycin to hypothesize on the actual “raison d’être” of cereulide. Valinomycin is toxic for some insects, nematodes and Gram-positive bacteria at very low concentrations (18, 19, 20). Although the antifungal activity of valinomycin has been reported (18), Altayar and Sutherland (1) showed that cereulide does not have any antifungal activity on rich medium. Since fungi are ubiquitous organisms living in the same environment as *B. cereus* (11, 12), a possible competition between these organisms could however be expected. The purpose of this

study was to assess the potential antifungal activity of three emetic *B. cereus* strains and to highlight the specific contribution of cereulide using isogenic cereulide-producing and cereulide-minus (cured or mutated) strains. Comparison with the antifungal activity spectrum of valinomycin was also performed.

Six strains of *B. cereus* were used in this study. The emetic strain Kinrooi 5975c came from a fatal food intoxication and is considered as a high producer of cereulide (6). Strain KC1 (Kinrooi Cured 1) was obtained by curing Kinrooi 5975c from its pCERE01 plasmid that carries the cereulide genetic determinants (9). The reference emetic strain, F4810/72 was isolated from a victim of food poisoning caused by cereulide (23) and strain IS075 was isolated from a vole (10). The *cesA* knockout derivatives (*cesA*KO) of strains F4810/72 and IS075 were also constructed, the *cesA* synthetase genes were inactivated via homologous recombination by an inactivated copy bearing an internal deletion and the insertion of a spectinomycin-resistance gene marker. The final construction was checked by PCR that could discriminate between the wt and tagged versions.

27 fungi comprising different families were tested for their sensitivity to cereulide: 19 Ascomycota (10 different orders), 2 Zygomycota, 1 Basidiomycota and 5 Oomycota. Non-diluted, diluted 5 (PDA-0.2) and 10 times (PDA-0.1) Potatoes Dextrose Agar (PDA, Oxoid) were used for fungal culture. Oomycota growth was obtained in "V8 medium" that contains (per liter) 100 ml of V8 juice.

BHI (Brain Heart Infusion) medium, solidified with 2 % of agar, was used for the biomass production of the six *B. cereus* strains. Cereulide was extracted from the bacterial cultures using a protocol adapted from Andersson *et al.* (3) and extract concentrations were determined by LC-MS, using commercial valinomycin as external standard (L. Delbrassinne, M. Andjelkovic, A. Rajkovic, N. Botteldoorn, J. Mahillon, and J. Van Loco, submitted for publication). 7,281 ng/ml and 3,987 ng/ml were obtained for the Kinrooi 5975c and F4810/72 emetic strains, respectively, in contrast to the 52 ng/ml of isolate IS075. For KC1, a Kinrooi 5975c derivative cured of its "cereulide"

plasmid (9) and for both *ces4*KO mutants, results of LC–MS were inferior to the LOD (1 ng/ml). Cereulide activity of each extract was also assessed by boar semen bio-assay (3).

Antifungal activity was tested as follow. Fungi were inoculated at the centre of a petri dish and incubated at 25°C to reach a diameter of *ca.* 3 cm. The contour of the strain was marked and 20 µl of extracts were spotted as a 90° arc along the edge of the colony. For each colony, two facing 90° arcs (including a negative control) were deposited at the edge. After a second incubation period, their growth was recorded. In case of inhibition, the fungus stopped growing where the toxin was placed, but displayed a continuous growth on the negative control. All tests were performed in triplicate and no difference was observed among them.

The potential antifungal activities of the Kinrooi 5975c extract and its cured derivative KC1 were first evaluated in three growth conditions of the fungi: PDA, PDA–0.2 and PDA–0.1. For each fungus, 20 µl of bacterial extract were spotted along the edge of the colonies and their potential effect was recorded as a growth inhibition of the fungus. As shown in Table 10, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Magnaporthe grisea*, *Monographella nivalis*, *Mortierella isabellina* and *Sclerotinia minor* displayed inhibition by the methanol/water extract from the Kinrooi 5975c strain on all media. *Alternaria alternata* and *Verticillium dahliae* were sensitive to the Kinrooi 5975c extract on both PDA–0.2 and PDA–0.1, while *Stemphylium vesicarium* was inhibited by the extract only on the most diluted medium PDA–0.1. Interestingly, none of the fungal strains had their growth inhibited by the extract of strain KC1, cured of the pCERE01 plasmid.

Table 10 – Sensitivity of fungi to cereulide and valinomycin.

	PDA			PDA-0.2			PDA-0.1		
	Kinrooi	KC1	Val.	Kinrooi	KC1	Val.	Kinrooi	KC1	Val.
<i>Alternaria alternata</i>	-	-	+	+	-	+	+	-	+
<i>Aspergillus niger</i>	-	-	+	-	-	+	-	-	+
<i>Botrytis aclada</i>	-	-	-	-	-	-	-	-	-
<i>Botrytis allii</i>	-	-	-	-	-	-	-	-	-
<i>Botrytis cinerea</i>	+	-	+	+	-	+	+	-	+
<i>Cladosporium cucumerinum</i>	+	-	+	+	-	+	+	-	+
<i>Colletotrichum gloeosporioides</i>	-	-	+	/	/	/	/	/	/
<i>Didymella lycopersici</i>	-	-	-	-	-	-	-	-	-
<i>Fusarium graminearum</i>	-	-	+	-	-	+	-	-	+
<i>Macrophomina phaseolina</i>	-	-	-	-	-	+	-	-	+
<i>Magnaporthe grisea</i>	+	-	+	+	-	+	+	-	+
<i>Monographella nivalis</i>	+	-	+	+	-	+	+	-	+
<i>Mortierella isabellina</i>	+	-	+	+	-	+	+	-	+
<i>Nectria radiculicola</i>	-	-	-	-	-	-	-	-	-
<i>Penicillium chrysogenum</i>	-	-	-	-	-	-	-	-	-
<i>Rhizoctonia solani</i>	-	-	-	-	-	-	-	-	-
<i>Sclerotinia cinerea</i>	-	-	-	-	-	-	-	-	-
<i>Sclerotinia minor</i>	+	-	+	+	-	+	+	-	+
<i>Stemphylium vesicarium</i>	-	-	-	-	-	+	+	-	+
<i>Trichoderma viride</i>	-	-	-	-	-	-	-	-	-
<i>Verticillium dahliae</i>	-	-	-	+	-	-	+	-	-
<i>Zygorhynchus moelleri</i>	-	-	-	-	-	-	-	-	-

3. RESULTS

3.4. Antifungal activity of cereulide

Sensitivity of 22 fungi to cereulide extract from *B. cereus* Kinrooi 5975c and its cured derivative KC1 and to valinomycin (Val.) on rich (PDA) and diluted (PDA-0.2 and PDA-0.1) media. For Kinrooi 5975c extract and valinomycin, 20 µl corresponding to 146 ng of toxin were dropped on the edge of every tested fungus. The + or – symbols refer to the presence or absence of growth inhibition, respectively. A slash indicates the absence of fungal growth.

In order to further demonstrate that the toxic activity displayed in Table 10 was due to cereulide and not to another toxic component carried by the “cereulide” plasmid, all the 22 fungal species were tested against methanol/water extracts from the *B. cereus* emetic strains F4810/72 (*ca.* 80 ng) and IS075 (*ca.* 1 ng) and their *ces4KO* derivatives. Only those species that were sensitive to Kinrooi 5975c are reported, all the others were not sensitive to *B. cereus* F4810/72 or IS075 (data not shown).

As shown in Table 11, six species were inhibited by the extract of F4810/72, which contained approximately 50 % less cereulide than Kinrooi 5975c extract. Two species were also sensitive to the much less concentrated cereulide extract from IS075 strain. The data also clearly indicated that no inhibition was observed with both *ces4KO* mutants, confirming that the observed fungal growth inhibition was indeed due to the presence of the cereulide.

Table 11 : Fungal sensitivity to cereulide extracts from two emetic strains and their *ces4KO* derivatives.

	F4810/72	F4810/72 <i>ces4KO</i>	IS075	IS075 <i>ces4KO</i>
<i>Alternaria alternata</i>	+	–	–	–
<i>Botrytis cinerea</i>	+	–	–	–
<i>Cladosporium cucumerinum</i>	+	–	–	–
<i>Magnaporthe grisea</i>	+	–	+	–
<i>Monographella nivalis</i>	+	–	–	–
<i>Mortierella isabellina</i>	+	–	+	–
<i>Sclerotinia minor</i>	–	–	–	–
<i>Verticillium dahliae</i>	–	–	–	–

Sensitivity of the 8 fungi (that were previously shown to be sensitive to the cereulide extract from *B. cereus* Kinrooi 5975c, see Table 10), to extracts from the F4810/72 and IS075 emetic strains (containing 80 and *ca.* 1 ng, respectively in the 20 µl used) and their corresponding *ces*AKO mutants. The growth medium used was PDA-0.2

It was also interesting to compare these inhibition activities observed with the cereulide with those reported for valinomycin. As shown in Table 10, using the same amount of both toxins (146 ng), the effects were similar, but slightly different. In all growth conditions, the six species sensitive to Kinrooi 5975c on all media were also inhibited by valinomycin. The *A. alternata* and *S. vesicarium* species, sensitive to Kinrooi only under PDA-0.2 and/or PDA-0.1 growth conditions, were also sensitive to valinomycin, but under different growth conditions (Table 10). Interestingly, the cereulide and valinomycin toxins clearly differentiated for five species: while *V. dahliae* was only inhibited by cereulide (on PDA-0.2 and PDA-0.1), *Aspergillus niger*, *Fusarium graminearum* and *Colletotrichum gloeosporioides* (in all tested growth conditions) and *Macrophomina phaseolina* (on diluted media) turned out to be sensitive to valinomycin only.

Five Oomycota (*Pythium ultimum* var *sporangiiferum*, *P. ultimum* var *splendens*, *Phytophthora cactorum*, *Phytophthora drechsleri* and *Phytophthora citrophthora*) were also tested against valinomycin and cereulide extracts. However, in the conditions used, no growth inhibition could be detected (data not shown).

Using the cured, cereulide-minus derivative of an emetic strain first, and subsequently the *ces*AKO mutants of two cereulide-producing *B. cereus* as negative controls, it was possible to demonstrate that the growth inhibition of several fungal species was specifically due to the presence of cereulide. The 22 fungal species used in this work have been placed in the phylogeny tree for kingdom fungi from Blackwell *et al.* (5). The parasitic strategy (biotrophic, hemibiotrophic, necrotrophic or saprophytic) of each species has also been examined, but no correlation could be drawn between these two classifications and the cereulide sensitivity of fungi.

Another interesting observation of the present work is the differential sensitivity of the fungal species to both cereulide and valinomycin. For instance, *M. isabellina* and *M. grisea* were highly sensitive and were inhibited by 1 ng of cereulide (extract from IS075 strain); *A. alternata*, *B. cinerea*, *C. cucumerinum* and *M. nivalis* were inhibited by 80 ng of cereulide (from strain F4810/72), while *S. minor* and *V. dahliae*, the less sensitive to cereulide were only blocked by the Kinrooi 5975c extract (ca. 146 ng of cereulide). Similar observations can be made for valinomycin.

Using commercial valinomycin as reference molecule is convenient, but results should be interpreted with care. Although their chemical structure and major properties are related, their activity spectrum on fungi differed. Teplova *et al.* (26) showed that cereulide affinity for K⁺ ion is higher than the one of valinomycin. At the same concentration, cereulide should thus show a higher toxicity than valinomycin; however, *A. niger*, *C. gloeosporioides*, *F. graminearum* and *M. phaseolina* were inhibited by valinomycin (Table 10) but not by cereulide at the same concentration. On the contrary, *V. dahliae* seemed to be insensitive to the action of valinomycin whereas it was sensitive to the Kinrooi 5975c extract on diluted media.

The fungistatic activity of cereulide is an important piece of information that can help us to suggest a hypothesis about its biological “*raisons d’être*”. In the environment, or in starchy food products, *B. cereus* shares its niches with various types of fungi. It is reasonable to assume that cereulide could help the emetic strains to settle more efficiently in these environments thanks to their fungistatic effect. This issue however first requires further experimental investigations.

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3.5 METHOD APPLICATIONS – PUBLIC HEALTH – PREVALENCE OF CEREULIDE IN RESTAURANT-RICE DISHES

The emetic syndrome provoked by *B. cereus* is mainly associated with rice dishes although the prevalence of the emetic strains in food and in the environment has been shown to be very low. This prevalence was mostly determined by PCR assays, which target genes encoding for cereulide, and sometimes the cytotoxic potential of the isolated strains was evaluated on growth media with biological tests. However, none study assessed the presence of the toxin in the dishes. In order to evaluate the potential hazard posed by emetic *B. cereus* in ordinary food, 54 Asian restaurants were visited in Brussels and in Wallonia and rice dishes were ordered. The cereulide content and the bacterial count were determined on the day of sampling and after five days of incubation at 25°C in order to evaluate the effect of long abused storage on cereulide formation. In parallel, a family foodborne outbreak which occurred in Belgium after the consumption of Asian ordered dishes was fully analysed. The levels of cereulide found in the rice dishes of the outbreak were compared with those found in the restaurant dishes collected for the prevalence study.

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⁵ Delbrassinne, L., M. Andjelkovic, S. Denayer, K. Dierick, J. Mahillon and J. Van Loco (2012). Prevalence and levels of *Bacillus cereus* emetic toxin in rice dishes collected randomly from restaurants and comparison with the levels measured in a recent foodborne outbreak, in press.

Summary

Whereas the prevalence of *Bacillus cereus* emetic strains in the environment has been shown to be very low, there is a lack of information on the prevalence of its toxin, cereulide, in food. Yet, the rice leftovers of a family outbreak which occurred after the consumption of dishes taken away from an Asian restaurant revealed significant amounts of cereulide, reaching up to 13,200 ng/g of food. The occurrence of cereulide in rice dishes collected from various restaurants was therefore evaluated using LC-MS² method that allows for the direct quantification of the toxin in food. The cereulide prevalence was found to be 7.4 % when samples were analysed at the day of sampling, but reached 12.9 % when exposed to temperature abuse conditions. The cereulide concentrations observed in cooked rice dishes were low (*ca.* 4 ng/g of food). However, since little is known yet about the potential chronic toxicity of cereulide, one needs to be very careful and vigilant.

Keywords

Prevalence, foodborne outbreak, foodborne pathogen, rapid detection

3.5.1 INTRODUCTION

Bacillus cereus is a spore-forming pathogen which possesses a facultative anaerobic fitness and the ability to grow in a large range of temperature (from 5 to 45°C). This explains its ubiquitous presence in environments and in food products. *B. cereus* can cause two types of foodborne illnesses: emesis, caused by cereulide, is mainly associated with starchy foods (Schoeni and Wong, 2005), and diarrheal syndromes are induced by a variety of enterotoxins and are associated with protein rich foods. Emetic strains of *B. cereus* have been shown to be very rare in the environment (Altayar and Sutherland, 2006) and several studies have determined this low frequency to be around 1–2 % (Hoton *et al.*, 2009; Nguyen-The, 2009). Yet, the incidence of *B. cereus* toxin-related food poisonings is steadily increasing in Europe (EFSA, 2009) and lethal cases of emetic intoxications, associated with the consumption of rice or pasta dishes, have been described worldwide (Dierick *et al.*, 2005; Mahler *et al.*, 1997; Naranjo *et al.*, 2011; Shiota *et al.*, 2010).

Detection of the pathogenic organism in food is a standard measure in industry and is the first control to be made after intoxication cases are signalled. Besides the detection of cereulide by biological or molecular assays, its quantification has so far not been frequently performed due to lack of accurate and sensitive methods. Recently though, a chemical method based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS or LC-MS²) has been developed for the appropriate and accurate quantification of cereulide in food matrices (Delbrassinne *et al.*, 2011a; Bauer *et al.*, 2010).

Cooked rice and its improper storage represent one of the major risk factors in emetic food poisoning. Indeed, rice is implicated in 95 % of the emetic food poisoning cases (Altayar and Sutherland, 2006; Ehling-Schulz *et al.*, 2004; Schoeni and Wong, 2005; Shinagawa, 1990). Furthermore, two very recent prevalence studies (Chang *et al.*, 2011; Perera and Ranasinghe, 2011) performed in Chinese-style restaurants revealed that *B. cereus* was frequently present (37.5 % and 56 %, respectively) in the served fried rice dishes. Unfortunately, the toxigenic potential of the isolated strains was not

assessed. Since rice is regularly served as main or side dish in many restaurants (among which Asiatic restaurants are quite representative), it is relevant to evaluate the incidence of cereulide in served rice dishes.

The aim of the present study was to assess the prevalence and concentration of cereulide in rice dishes collected in restaurants in several cities of Wallonia and Brussels. This quantification was performed thanks to an analytical LC-MS² method specifically targeting cereulide in food. Food samples recovered from a recent family outbreak due to emetic *B. cereus* were similarly analysed to allow for comparison.

3.5.2 MATERIALS AND METHODS

3.5.2.1 FOOD SAMPLES FROM FAMILY OUTBREAK AND FROM RANDOMLY PICKED RESTAURANTS

Two types of food samples were analysed following a family outbreak: four samples from leftovers (white rice #1, fried rice, noodle sauce and Wong Tong soup) that had been partially eaten, and three samples from inspection (white rice #2, noodles, and chickpeas) that were collected by food inspectors in the incriminated restaurant 24 h after the official complaint. The intoxication affected several members of the same family (from 14 to 80 year-old) after ingestion of meals collected in an Asian take-away restaurant in Belgium. Eight of the eleven exposed people became ill within 2 h, with vomiting episodes, while some of them experienced additional diarrhea. One elderly person (80 year-old) needed hospitalisation.

In the scope of a small-scale survey study, a total of 54 randomly chosen restaurants were visited in Brussels and Wallonia during Spring and Summer 2011 (Supplementary Table S1). Prepared rice dishes (13 white rice and 41 fried rice dishes) were collected and submitted to direct analysis (at the day of sampling or within 24h of purchase). In order to evaluate the incidence of cereulide in rice in case of prolonged storage at temperature abuse, the food remnants of the analysed restaurant samples were subsequently placed for 5 days in an incubator at 25°C.

3.5.2.2 ENUMERATION OF *B. CEREUS* FROM FOOD SAMPLES

The ISO7932 method was used for the *B. cereus* enumeration. After 24h of incubation on Mannitol egg Yolk Polymyxin (MYP) agar media at 30°C, the typical pink colonies surrounded by a zone of lecithin hydrolysis were enumerated.

3.5.2.3 EXTRACTION OF CEREULIDE FROM FOOD SAMPLES AND ANALYSIS BY LC–MS²

The protocol recently described by Delbrassinne *et al.* (2011a) was applied followed by injection in the LC–MS LCQ Deca–XP Plus ion trap mass analyser (ThermoFinnigan, USA) for cereulide determination. Valinomycin (Sigma–Aldrich, Belgium), diluted in methanol, was used as a surrogate standard for quantification. For each batch, the recovery efficiency was verified on the basis of valinomycin spiking (cooked rice spiked with valinomycin at 20 ng/g of rice) and a negative control (not contaminated cooked rice) was also included for evaluating the matrix interferences. The presence of cereulide was evaluated by monitoring the following m/z values: 1125.3, 1153.3, and 1170.5 in the MS² mode of analysis. The chromatograms were smoothed thanks to a Gaussian function.

3.5.3 RESULTS

3.5.3.1 ANALYSIS OF SAMPLES IMPLICATED IN A BELGIAN FAMILY OUTBREAK

The food samples related to a recent family outbreak in Belgium were analysed for their bacterial content and cereulide concentrations. *B. cereus* was present among six (fried rice, Wong Tong soup, noodle sauce, chickpeas and two white rice dishes) of the seven collected samples and was absent in the noodles, as indicated in Table 12. The highest counts (2.4×10^7 ; 1.5×10^7 ; 1.3×10^7 and 2.8×10^5 CFU/g) were found in the leftovers eaten by the family (white rice dish #1, fried rice dish, soup and noodle sauce, respectively). In the other food products that were collected in the same restaurant 24 h after the complaint (inspection samples), the counts were at least 10^3 to 10^4 times lower than in the leftovers.

The six samples containing *B. cereus* also contained cereulide with the exception of the chickpea sample. Cereulide was found in concentrations ranging from 9 ng/g to 13,184 ng/g (Table 12). As a general trend, the leftovers contained more cereulide than the inspection samples. The fried and white rice #1 displayed the highest cereulide concentration (13,184 and 8,693 ng/g, respectively). The obvious discrepancy between these concentrations measured in the rice leftovers and the concentrations found in the white rice #2 and noodle sauce (9 and 152 ng/g, respectively) are in accordance with the lower *B. cereus* counts. Interestingly, the soybean soup (one of the leftovers of the outbreak) and the chickpea sample (inspection sample) displayed *B. cereus* counts but contained only low or no amount of cereulide.

Table 12 – *B. cereus* enumeration and cereulide determination in samples related to the recent Belgian food poisoning.

	Samples	<i>B. cereus</i> counts (CFU/g)	Cereulide concentration (ng eq/g)
Leftovers ^a	White rice #1	2.4E+07	8,693
	Fried rice	1.5E+07	13,184
	Soybean soup	1.3E+07	16
	Noodle sauce	2.8E+05	152
Inspection samples ^b	White rice #2	3.6E+04	9
	Chickpea	3.0E+03	< LOD
	Noodles	< 1.0E+02	ND

B. cereus counts (enumerated on MYP medium) and cereulide production (assayed by LC-MS² and expressed in ng valinomycin equivalent/g of food) are presented. ^aLeftovers were directly consumed by the family after delivery and then stored in the households fridge. ^bInspection samples were collected by food inspectors from restaurant and stored in the lab fridge (4°C) until analysis. The detection limit (LOD) for LC-MS² method was 0.5 ng/g. ND stands for Not Determined.

3.5.3.2 INCIDENCE OF *B. CEREUS*-LIKE ORGANISMS IN RESTAURANT RICE SAMPLES

A small scale study was performed in 54 Belgian restaurants in order to evaluate *B. cereus* presence and the potential cereulide production in rice dishes. Presumptive *B. cereus* strains were found in 10 of the 54 samples

(18.5 %) when analysed within 24h after sampling. The concentrations of *B. cereus* were low and ranged from 100 to 3,500 CFU/g. After 5 days of incubation at 25°C, 41/54 rice samples (75.9 %) contained *B. cereus* at concentrations ranging from 2×10^5 to 2×10^9 CFU/g.

3.5.3.3 INCIDENCE OF CEREULIDE IN RESTAURANT RICE SAMPLES

The rice dishes collected in the various restaurants were also analysed by LC-MS² for the presence of cereulide, before and after 5 days of storage at 25°C. For all the analyses, the toxin recovery was always above 75 %. As shown in Table 13, cereulide was detected in 4 samples (7.4 %) prior to incubation and in three additional samples after incubation. The concentrations of cereulide were very low in all the samples: below 2 ng/g for the samples which were not incubated (samples 1, 2, 3, and 4) and below 4 ng/g for the samples stored at 25°C (samples 5, 6, and 7). As indicated in Table 13, four samples that contained cereulide before incubation turned out to be negative after incubation at 25°C.

Table 13 – Cereulide concentrations and *B. cereus* counts in the seven cereulide-positive rice dishes before and after incubation at 25°C.

Samples	1	2	3	4	5	6	7
Type of rice	Fried rice						White rice
Cereulide concentration (ng eq/g) ^a	<LOQ	<LOQ	1.57	1.96	<LOD	<LOD	<LOD
Presumptive <i>B. cereus</i> (CFU/g) ^a	<100	300	<100	3500	<100	<100	<100
Cereulide concentration (ng eq/g) ^b	<LOD	<LOD	<LOD	<LOD	<LOQ	3.75	3.94
Presumptive <i>B. cereus</i> (CFU/g) ^b	5.0 E+07	6.4 E+08	1.0 E+07	2.7 E+08	< 100	4.2 E+08	4.8 E+08

Cereulide was detected in seven dishes originating from seven various restaurants. Cereulide concentrations (assayed by LC-MS² and expressed in ng valinomycin equivalent/g of food) and *B. cereus* counts (by enumeration on MYP medium and expressed in CFU/g) are given ^abefore and ^bafter 25°C incubation. The limits of quantification (LOQ) and detection (LOD) for LC-MS² were 1 ng/g and 0.5 ng/g, respectively.

3.5.4 DISCUSSION

The leftovers from the outbreak contained significant *B. cereus* counts but variable cereulide concentrations. Except for the rice dishes that displayed the highest toxin amount, low levels of cereulide were found in the soybean soup and it could not be detected in the chickpea sample. That may be explained by a less efficient cereulide extraction, as suggested by additional spiking experiments performed with these food matrices (unpublished results). Another possible explanation is a lower cereulide production in these matrices. Food composition has indeed been pointed out to have an important influence on toxin production (Rajkovic *et al.*, 2006).

Temperature abuse storage of the dishes in the restaurant may be at the origin of the outbreak since it was reported by the food inspectors that the prepared dishes were conserved in a badly refrigerated counter. The controlled temperature was around 9°C whilst it has been reported that cereulide can be produced at temperatures as low as 8°C (Thorsen *et al.*, 2009; Delbrassinne *et al.*, 2011b). Furthermore, a *B. cereus* cross contamination may have occurred between dishes because of the general bad hygiene: foods were uncovered in the refrigerated counter, dustbins were fully open, the fridge, microwave and many other surfaces were dirty, as reported by the food inspectors.

The cereulide amounts present in two rice dishes were comparable to those reported in one of the lethal cases (Naranjo *et al.*, 2011) in which a spaghetti meal containing cereulide at a concentration of 14,800 ng/g caused the death of a 20 year-old man. The people involved in this family outbreak reported vomiting and some diarrhea episodes, with hospitalisation of the oldest person (80 year-old). Cereulide is known to be highly toxic to certain groups such as children and elderly people. In this case, the children were not strongly affected because they had not completely eaten their meals. This underlines the importance of knowing the total amount of ingested toxin and the necessity to establish the toxic dose of cereulide for human beings (in µg/kg of body weight). Furthermore, the significant levels of cereulide measured in rice dishes from the outbreak justify the need for an accurate assessment of its prevalence in rice-based meals served in restaurants.

The low incidence of *B. cereus* (18.5 %) found in the restaurant dishes at the time of consumption is not surprising and is in line with the findings of Kim *et al.*, (2009) who could not detect any *B. cereus* contamination in rice without performing enrichment. After incubation, the same samples displayed a higher incidence of *B. cereus* (75.9 %). However, samples that contained cereulide did not always display *B. cereus* counts. This might be explained by the elimination of vegetative cells in a reheating step of rice, which is a quite common practice in Asian restaurants. Part of the discrepancies between cereulide–positive samples before storage that were negative after storage (Table 13) could be due to the heterogeneity of *B. cereus* repartition and consequently of its cereulide production in the samples. Three samples were only detected positive after incubation which may be explained by the growth of emetic strains that were present in the samples. Cereulide production was probably promoted by suitable conditions. However, for sample 5 which contained small amounts of cereulide, no *B. cereus* could be detected because there was an overgrowth of other background bacteria which displayed yellow colonies (data not shown).

According to previous studies, summarised in Table 14, it is generally recognised that emetic strains of *B. cereus* are rather rare in the environment. However, these *B. cereus* isolates were obtained on specific media selecting presumptive strains. Emetic strains may be overlooked by those standard methods for *B. cereus* detection which are based on positive lecithinase and positive haemolysis. It is indeed now recognised that haemolysis is often weak or absent in the cereulide–producing *B. cereus* (Apetroaie *et al.*, 2005; Kim *et al.*, 2010; Pirhonen *et al.*, 2005) hence some lecithinase–negative emetic strains have also been found (Apetroaie *et al.*, 2005). This implies that cultural methods, upon which the PCR and cytotoxic assays rely, are likely to lead to an underestimation of the emetic strains prevalence.

Although some studies failed to isolate any emetic strains of *B. cereus* (Ankolekar *et al.*, 2009; Kim *et al.*, 2009; De Jonghe *et al.*, 2010; Samapundo

et al., 2011), the frequency of emetic strains was generally evaluated to be between 1 to 2.5 %, using PCR, bioassay or cytotoxicity assays on both foodborne and environmental *B. cereus* strains (Rosenquist *et al.*, 2005; Altayar and Sutherland, 2006; Svensson *et al.*, 2006; Rahmati and Labbe, 2008; Hoton *et al.*, 2009; N'guessan, 2010). However, as discussed above, the random selection of isolates or the choice of the hemolysis and/or lecithinase activities as a basis for the selection may also have led to an underestimation of the actual number of emetic strains. In addition, drawbacks can be associated with the use of PCR since a sample free of bacteria but containing the toxin (*e.g.* after heat treatment) might happen to yield a negative result. Similarly, the toxin genetic determinants present in the strains may be overlooked by specific primers because of variability in targeted DNA sequences, as observed by Hoton *et al.* (2009).

Interestingly, a few reports have indicated a slightly higher prevalence of cereulide-producing strains in specific food products (Table 14): *ca.* 4 % of cereulide-producing strains were detected in dried infant formula (Shaheen *et al.*, 2006) and German ice creams (Messelhausser *et al.*, 2010), while the highest occurrence of emetic strains (8.2 %) was found in Dutch retail food samples (Wijnands *et al.*, 2006), using HEp-2 cell assay. This frequency is five times higher than the generally reported 1.5 % (Nguyen-The, 2009; Hoton *et al.*, 2009). Unfortunately, cereulide concentrations in these samples were not determined and no comparison could therefore be established with results of the present study. The levels of cereulide found in the randomly selected rice dishes were measured to be in the order of ng/g. However, the reported family outbreak should lead the authorities to emphasize the risk of cereulide food intoxication in cases where strict hygiene rules and cold storage temperatures were not appropriately followed.

Table 14 – Review of previous studies on *B. cereus* emetic strains prevalence in the environment and in food.

References	Method ^a	Source	<i>B. cereus</i> Isolates (n)	Emetic isolates (n)	Frequency of emetic <i>B. cereus</i> (%)
Ankolekar <i>et al.</i> (2009)	PCR	Raw rice from retail food store	83	0	0
Kim <i>et al.</i> (2009)	PCR	Cooked rice and sunsik (powdered raw grains)	163	0	0
De Jonghe <i>et al.</i> (2010)	PCR, cytotoxicity test on Vero cell line	Raw milk from 10 farms	7	0	0
Samapundo <i>et al.</i> (2011)	PCR	Lasagna, béchamel and bolognaise sauces, pasta, rice, minced beef and carrots	80	0	0
Hoton <i>et al.</i> (2009)	PCR, bioassay	Random food samples	1,863	28	1.5
N'guessan (2010)	PCR	Pasta, rice, meat, potato, milk, spices, and honey	409	6	1,5
Rahmati and Labbe (2008)	PCR	Seafood samples	62	1	1.6
Altayar and Sutherland (2006)	MTT assay on HEP-2 cells	Soil, animal faeces, raw and processed vegetables	177	3	1,7

Svensson <i>et al.</i> (2006)	RAPD-PCR, bioassay, LC-MS on strains*	Farms and dairy plants	1,757 3911	34 44	1.9 1.1
Rosenquist <i>et al.</i> (2005)	PCR	48901 RTE-foods	40	1	2,5
Shaheen <i>et al.</i> (2006)	Bioassay LC-MS on strains*	Dried infant formula	100	4	4
Messelhauser <i>et al.</i> (2010)	Real-time PCR	Ice creams in Bavaria	508 (samples)	24 (samples)	4,7
Wijnands <i>et al.</i> (2006)	HEP-2 cell test	Pastry, vegetables, RTE-foods, milk, meat and fish.	796	65	8,2

^a MTT: metabolic staining assay using the 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as cell viability indicator, HEP-2: vacuolization assay using cells originating from human carcinoma of the larynx, bioassay: sperm motility assay using boar semen cells, RTE-foods: Ready-to-eat foods. * LC-MS analysis was performed on a selected number of strains from plates as a confirmation of the bioassay

3.5.5 CONCLUSION

To our knowledge, this is the first study on prevalence of cereulide which directly targets the toxin in restaurant food, while previous studies had mainly targeted cereulide-producing strains. Although it has been shown that emetic *B. cereus* strains are rare, cereulide was found in several rice dishes randomly collected from restaurants. These results suggest that the prevalence of cereulide is probably higher than previously thought. Although the cereulide concentrations found in these randomly picked dishes were low, cereulide may reach hazardous levels in rice, as indicated by the reported outbreak. Therefore, additional studies on factors that affect the growth of emetic *B. cereus* and the production of cereulide are needed. On account of the cereulide prevalence reported in the present study, it seems essential to follow strict temperature conditions for the storage of cooked rice in order to ensure food safety. This message should be more efficiently passed on to restaurants, catering and domestic consumers.

3. RESULTS

3.5. Prevalence of ceulide in restaurant–rice dishes

Supplementary Table – List of visited restaurants and details of sampling.

Restaurant	District	Period of sampling	Type of dish	<i>B. cereus</i> counts at day 0 (CFU/g)
1	Brussels	May 2011	white rice	400
2	Brussels		rice jasmin	<100
3	Brussels		white rice	<100
4	Brussels		mixed rice	<100
5	Brussels		white rice	<100
6	Brussels		white rice	<100
7	Brussels		white rice	<100
8	Namur		cantonese rice	<100
9	Brussels		fried rice with vegetables	<100
10	Brussels		white rice	<100
11	Namur		cantonese rice	<100
12	Brussels		white rice	1200
13	Brussels		white rice	<100
14	Brussels		white rice	<100
15	Namur		fried rice	<100*
16	Namur		fried rice	100
17	Namur		cantonese rice	2800
18	Namur		cantonese rice	<100
19	Namur		cantonese rice	<100
20	Brussels		white rice	<100
21	Namur		cantonese rice	<100
22	Namur		cantonese rice	<100*
23	Namur	June 2011	cantonese rice	<100*
24	Namur		cantonese rice	<100*
25	Namur		cantonese rice	<100
26	Dinant		fried rice with chicken	<100
27	Namur		fried rice with chicken	<100*
28	Namur		cantonese fried rice	<100
29	Namur		fried rice with chicken	<100*
30	Namur		fried rice with chicken	200*
31	Brussels	July 2011	white rice	<100
32	Namur		cantonese rice	3500
33	Brussels		fried rice with chicken	<100

34	Brussels		fried rice with chicken	<100
35	Namur		fried rice with chicken	<100
36	Brussels		fried rice with chicken	<100
37	Brussels		fried rice with chicken	<100
38	Namur	August 2011	fried rice with chicken	<100*
39	Namur		fried rice with chicken	<100
40	Namur		cantonese fried rice	<100*
41	Namur		fried rice with chicken	300
42	Namur		fried rice with chicken	200
43	Namur		fried rice with chicken	660000
44	Namur		fried rice with chicken	<100
45	Namur		fried rice with chicken	<100
46	Brussels		fried rice with chicken	<100*
47	Brussels		fried rice with chicken	<100*
48	Brussels		tonkonese rice	<100*
49	Brussels		fried rice with chicken	<100
50	Brussels		cantonese fried rice	200
51	Brussels		cantonese fried rice	<100
52	Brussels		fried rice with chicken	<100*
53	Brussels		fried rice with chicken	<100*
54	Brussels		cantonese fried rice	<100

When samples were collected at lunchtime, they were directly analysed (within one hour) and when they were collected in the evening, they were put at 4°C and analysed the day after (within 24h). For each restaurant, an entire dish was ordered. Bacterial counts (CFU/g) are given after 24h of incubation at 30°C on MYP medium. * indicates the presence of other bacteria (yellow colonies)

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4. GENERAL DISCUSSION

4.1 SCIENTIFIC CONTEXT

In recent years, there has been a surge in the number of publications related to cereulide. Several avenues were explored in the field of cereulide synthesis, regulation and expression but also in the search for its biological role.

In this study, we tackled several issues that emerged from the cereulide-producing *B. cereus* context. As illustrated in Fig. 21, *B. cereus* is commonly present in soils and certain strains, named emetic *B. cereus*, are able to naturally produce the toxin cereulide. The exact role of this toxin production (why ?) with regards to the producing-bacterium was part of our interests. Since *B. cereus* is ubiquitous in soil, it may contaminate a large variety of foods but, although emetic strains are thought to be very rare, the occurrence of cereulide in food is not known (how frequent ?). Production of the emetic toxin can be induced by the *B. cereus* cells present in the contaminated food and may vary according to the type of food and the environmental conditions (how much ?). This may pose food safety issues for the consumer leading to a diversified severity according to food handling (food storage and/or food processing such as heat treatment) or in case of direct consumption. Three situations are displayed in Fig. 21: if the contaminated food is directly consumed, both *B. cereus* cells and emetic toxin would be ingested by the human host leading to diarrheal and emetic syndromes, respectively. If a contaminated food entering the food chain is treated with appropriate methods such as pasteurization, elimination of the vegetative cells of *B. cereus* and other resident bacteria will occur, but the toxin will remain intact. The absence of competing germs would aid the outgrowth of sporulating bacteria, such as *B. cereus*. The consumer would therefore be exposed to increased risk of emetic food poisoning. If contaminated foods are stored without preliminary treatment (e.g. cooking in households), the resident bacteria may multiply and, in case of *B. cereus*, continue to produce the toxin throughout the storage. This may in turn lead to toxin accumulation (to what extent ?).

This toxin production is variable under various conditions (type of food, type of strain or temperature of storage) and cereulide may reach hazardous levels as described in the recent lethal cases. Therefore, it seemed important to determine the key parameters that influence the toxin production, as addressed in this study. In case of breach in the food control measures, risk of food intoxication is high. Determining the exact and unambiguous origin of an outbreak is a tricky task that we also addressed in this work (origin ?).

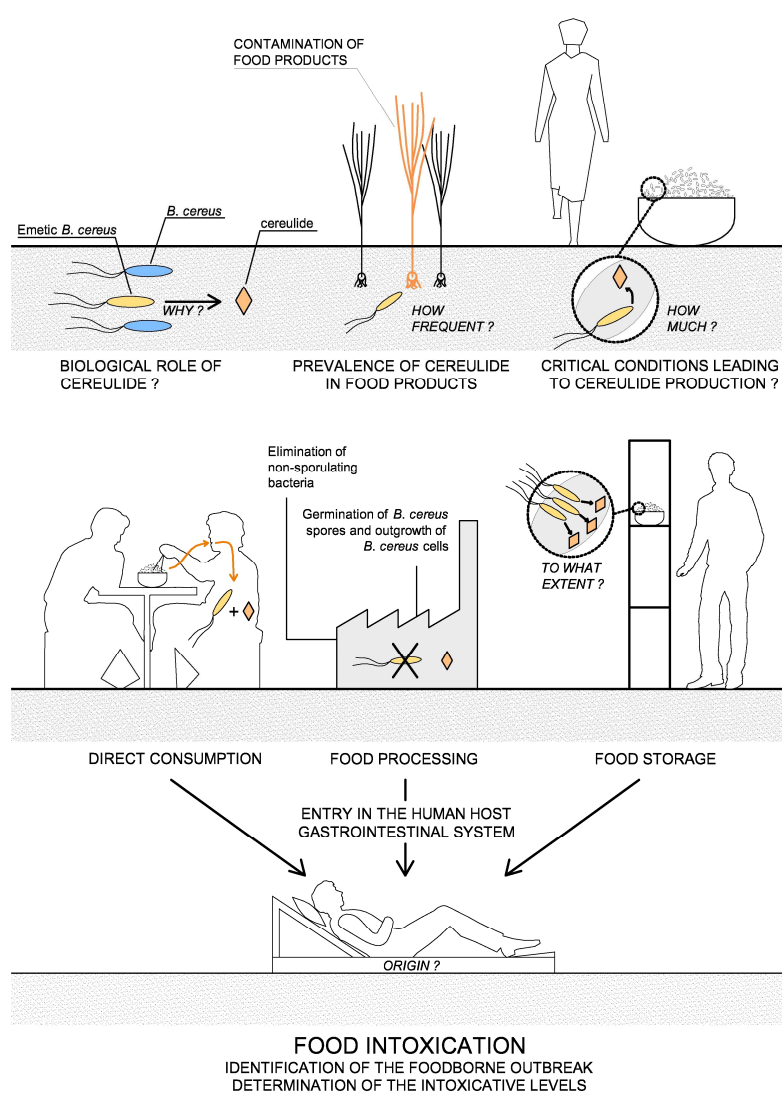


Figure 21 – Food safety issues due to cereulide-producing *B. cereus*

4.2 BETTER INSIGHTS INTO CEREULIDE MEDIATED SYNDROME: QUANTIFICATION, EXPRESSION, PREVALENCE AND BIOLOGICAL TOXICITY OF CEREULIDE

A good quantification method is mandatory to tackle these issues, and the development of a quantitative LC-MS method for the determination of cereulide by Häggblom *et al.*, (2002) has paved the way with regards to the study of emetic toxin expression.

In view of the aforementioned problems, we mainly focused on developing a robust method for the quantification of cereulide which offers good specificity and low detection levels.

4.2.1 METHODS OF QUANTIFICATION FOR CEREULIDE

When starting the present study, two methods had been described for cereulide determination: one had been used on the bacteria and the other on fig fruits (Häggblom *et al.*, 2002; Hormazábal *et al.*, 2004). As explained in the Introduction, two additional methods have been developed and validated in parallel to our validated protocole. Table 15 compares the different validation criteria that were reached by each method.

Table 15 – Validation of the quantification methods for cereulide

References	Standard used	Ions followed for cereulide identification and quantification	Type of food	LOD (ng/g of food)	LOQ (ng/g of food)	Linearity range	Spiking level (ng/g)	Recovery (% ± SD)	Repeatability RSD (%)	Reproducibility RSD (%)
Hägglom et al., 2002	valinomycin	1171.0; 1191.8; 1153.8	bacterial strains	ND	ND	1 to 1000 ng/ml	ND	ND	ND	ND
Hormazabal et al., 2004	valinomycin	1170.9	Figs	1	2	2 to 20 ng/g	5 10 20	93 ± 2 89 ± 1 93 ± 3	ND	ND
Biesta-Pieters et al., 2010	synthesized cereulide, valinomycin	1170.70; 1191.67	cooked rice	ND	4	1.5 to 7.5 ng/ml	6 - 18 31 - 92	97 98	2 3	3 5
Bauer et al., 2010	¹³ C-cereulide, valinomycin	1170.9 -> 172.3 (for ¹³ C-cereulide); 1176.9 -> 173.3 (for ¹³ C-cereulide)	cooked rice	3	30	1 to 100 ng/ml	1000 2000 5000	81 ± 4 111 ± 12 82 ± 10	<8 ND <8	ND <8 ND
Delbrassinne et al., 2011	valinomycin	1170.5 -> 1153.3 and 1125.3	cooked rice	0.5	1	1 to 40 ng/ml	10 20 40	77 ± 0.4 80 ± 0.3 101 ± 0.6	5 2 2	8 3 3

LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation, ND: Not Determined, Repeatability: intra-day variability, Reproducibility inter-day variability

The five methods were comparable in terms of recoveries, repeatability and reproducibility. Yet, our LC-MS² method, using an ion trap instrument, provides the lowest detection limits and guarantees good specificity by following fragment products. However, it should be noted that the loss of the NH₄⁺-adduct (fragment 1, m/z=1153.3) is less specific than a fragmentation inside the molecule (fragment 2, m/z= 1125.3). The other methods were not validated for low amounts of food matrix which is still relevant in case of foodborne intoxications because the amounts of leftovers might be very limited. Attempts have been made in this study to extract cereulide from various foods but yields were variable according to the extraction method that was carried out. For instance, recovery of 45 and 55 % were obtained for beans and chili con carne in extractions performed with methanol, respectively. These yields were improved up to 90 % by using acetonitrile instead of methanol.

Although the isotopic cereulide (¹³C-cereulide) is the ideal standard for LC-MS analysis, the commercial availability of such standard has been so far limited due to very high costs. Therefore, no standard method for the quantification of cereulide in food has been established and the comparison between the different LC-MS techniques is then difficult.

The European mandate on Food Microbiology will precisely focus on standardisation and validation of microbial methods. For that reason, an inter-laboratory study (CEN working group) to which we are currently participating has been recently created by The Dutch Food and Consumer Product Safety Authority (nVWA) and will be ended in 2013. The goal is to standardize the analytical method for cereulide using LC-MS and/or LC-MS-MS analysis. In that scope, we have received a certified cereulide standard from Dr E. Biesta-Peters (nVWA) and we are currently transferring the method to a UPLC-MS/MS (Ultra Performance Liquid Chromatography tandem Mass Spectrometry) triple quadrupole instrument which provides rapid runs (the retention time of cereulide is about 3 minutes) with higher sensitivity and specificity. A very first test has been performed to examine which LOD and LOQ can be reached with the various equipments. The results shown in Table

16 have been obtained by injecting a calibration curve of cereulide and valinomycin standards in both ion trap and recent triple quadrupole mass spectrometers. The LOD reached with the UPLC-MS/MS is 0.04 ng/g which is a ten-fold improvement of our previous LOD (0.5 ng/g).

Table 16 – Comparison of XEVO-TQ-S instrument with the LCQ Deca XP Plus ion trap

Standard cereulide	Concentration ng/ml	LC-MS ² (LCQ Deca XP ion trap)	UPLC/MS-MS (XEVO-TQ-S triple quad)
		MS-MS area	MS-MS area*
std1	0.04	not found	1.12E+06
std2	0.08	not found	2.41E+06
std3	0.41	Detected	1.49E+07
std4	0.83	9.23E+06	3.11E+07
std5	1.65	1.01E+07	6.20E+07
std6	4.13	8.78E+07	1.58E+08
std7	8.25	2.02E+08	3.19E+08

Cereulide and valinomycin standards dissolved in acetonitrile were injected in the ion trap (LCQ Deca XP Plus ion trap) and in the triple quad mass spectrometer (XEVO-TQ-S). * Standards were injected twice in the XEVO-TQ-S to test the first utilisation of the instrument with the help of Dr T. Reyns. When injecting the cereulide standard in the ion trap, only the last five points of the curve (0.41; 0.83; 1.65; 4.13; 8.25 ng/ml) were detected. MS-MS area: smoothed peak area.

The UPLC-MS/MS allows for the monitoring of specific transitions (Multiple Reaction Monitoring or MRM) instead of detecting all fragment ions. This targeted MS analysis usually enhances detection limits.

4.2.2 CORRELATION OF LC-MS² METHOD WITH OTHER METHODS

During its development, the LC-MS² method was compared on several occasions to other techniques for detection of cereulide: the PCR assay targeting the *ces* gene cluster which governs the cereulide synthesis (Ehling-Schulz *et al.*, 2004b) and the boar semen bioassay (Andersson *et al.*, 2004; Rajkovic *et al.*, 2006b; Rajkovic *et al.*, 2007).

PCR assays

All the strains isolated from the first lethal Belgian incident, which occurred in Kinrooi in 2003, were tested by PCR analysis and by LC-MS². A full

correlation was observed between PCR results and LC-MS² analysis which confirmed that the presence of the *ces* gene cluster is indispensable for cereulide formation. Similarly, 15 strains implicated in a large foodborne outbreak in September 2011 were strictly consistent in both kinds of analysis: 14 strains that were positive in LC-MS² harbored the *ces* gene cluster in PCR analysis, while one PCR-negative strain did not show cereulide in the LC-MS² chromatogram.

Boar semen bioassay

Many samples (red beans, spices, chili con carne) that were analysed by LC-MS² in the scope of HACCP control or after intoxications were also analysed in Ghent University by Dr Andreja Rajkovic using the CASA-boar semen bioassay (Rajkovic *et al.*, 2007). A good correspondence was observed for the majority of the samples, except for the samples which contained cereulide in very low amounts. Those were unnoticed by the CASA-bioassay due to its higher detection limit (LOD 20 ng/ml) compared to LC-MS² (LOD 0.5 ng/g). These experiments demonstrated the usefulness of LC-MS² analysis for assessing very low doses of cereulide in foods which may have been overlooked when using other detection techniques.

Yet, each method has advantages and drawbacks. LC-MS equipment requires a substantial financial investment while PCR assays and cytotoxicity tests are more affordable. This investment may be profitable if an important number of samples are analysed. Nonetheless, LC-MS instrument requires enough space in the lab, annual maintenance and adequate trainings of technicians. On the opposite, PCR assays are easily performed and a large number of samples may also be screened at one time. However some disadvantages can be identified: i) they rely on a random selection of isolates since it is practically not possible to test all the colonies from a plate, ii) they specifically detect emetic strains and their genetic toxic potential but nothing is known about the expression of the toxin by these isolates. LC-MS and toxicity tests could confirm the actual toxin production but the quantification is accurate only up to the titers with toxicity assays (excepted for the CASA-bioassay) while LC-MS offers a reliable quantification of the toxin. Still, contrary to LC-MS, the bioassays demonstrate that the toxicity of the toxin

remains active; provided that cereulide is the only toxin present in the sample. Bioassays lack indeed specificity (*e.g.* they are potentially sensitive to other toxins).

It appears that using the different techniques in combination offers a global overview of the potential toxicity of a food product. However, when a choice or priority is needed, a balance between the aforementioned aspects should be made. For instance, if a rapid detection is required (*e.g.* hospitalisation of a patient), LC-MS method should be preferred to conventional microbiological assays (Fig. 22).

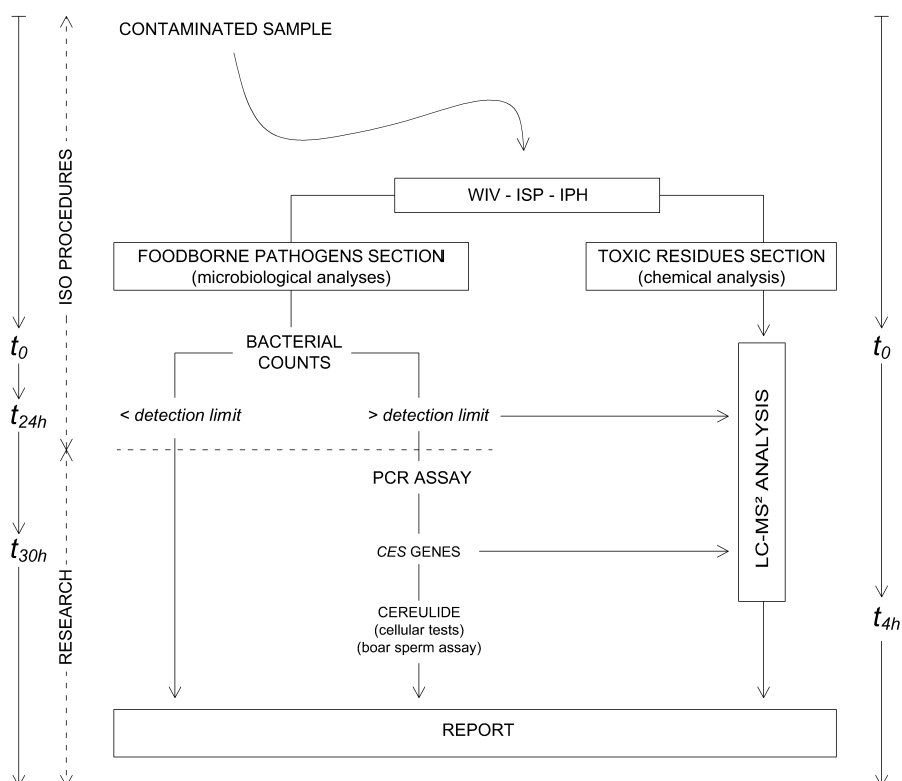


Figure 22 – Microbiological and chemical analyses of food samples at IPH

Possible routes of analysis for a contaminated food sample are described.

Conventional route: A first bacterial assessment is performed on selective media (ISO7932 method). If *B. cereus* counts exceed detection limit, several

colonies are picked up for PCR assay (targeting *ces* genes) in a research perspective. A further confirmation of cereulide production by isolates may be performed using cellular tests or boar semen assay.

Alternative route: The sample may be sent to Toxic Residues section for LC-MS² analysis at different time points: i) directly at its arrival (*e.g.* in case of emergency), ii) if *B. cereus* are detected by classical cultures (after 24h of incubation), iii) if emetic strains are detected by PCR analysis (after 30h from t_0).

LC-MS analysis appears as a powerful tool at various levels. First, it allows the accurate quantification of cereulide in food contrary to conventional microbiological methods, PCR assays and cell tests. This is an important issue, for instance, for tracing and/or preventing intoxications (*e.g.* if vegetative cells of *B. cereus* have been eliminated from the food by a reheating step, which is quite a common practice in Asian restaurants). Secondly, it provides a clear time-saving compared with other methods that rely on bacterial cultures (4h versus at least 24h). Thirdly, the LC-MS does not require specific preparations, such as cell cultures, and is ready-to-use at any moment. Finally, the cereulide concentration appears as the most relevant parameter to be followed for emetic food poisoning since it has been demonstrated in this work that the correlation between bacterial counts and emetic toxin production is not proportional. Only LC-MS analysis is able to provide specific and quantitative proof that cereulide is present in a food product.

4.2.3 CEREULIDE EXPRESSION

As described in the Introduction, the process of emetic toxin production by *B. cereus* is highly sensitive to environmental and bacterial factors. In the scope of method development, liquid and solid media were tested with regards to cereulide formation (Delbrassinne *et al.*, Proceedings of the 14th Conference on Food Microbiology, June 2009, Belgium). In liquid medium, it appeared that the production of the toxin was absent in the supernatant and was mainly associated with the pellet. Four solid media (Nutrient Agar, Mannitol Egg Yolk Polymyxin, Brain Heart Infusion-agar, and Tryptic Soy Agar) were compared in terms of cereulide production and purity of the obtained mass spectra. Tryptic Soy Agar medium came out as the best

medium for cereulide formation and purity of the mass spectrum (data not shown).

Similar observations have been made in several studies that assessed the cereulide formation in laboratory media. For instance, the cereulide production tested by others was high on TSA, BHI and bovine blood agar contrarily to MYP medium (Andersson *et al.*, 2004; Jääskeläinen *et al.*, 2004).

The production of cereulide by *B. cereus* cells grown on laboratory media (V8, PDA, Glucose and Saccharose media) which are especially favorable for fungi growth was also tested at 25 and 30°C in order to determine the best conditions for investigating the antifungal effect of cereulide (Table 17).

Table 17 – Comparison of cereulide production in various solid media at 25 and 30°C

Laboratory media	Temperature (°C)	Cereulide concentration (µg/g biomass)
v8	25	4.50
	30	1.71
PDA	25	13.49
	30	13.31
Glucose	25	9.15
	30	4.29
Saccharose	25	4.34
	30	1.12

V8: juice product of Campbell Soup Company; PDA: potato dextrose agar (Oxoid); Glucose and Saccharose media contained 10 g/l of yeast extract, 10 g/l of tryptone and 20 g/l of glucose or saccharose, respectively.

PDA turned out to be the best medium for cereulide production independently of the temperature, whilst 25°C seemed optimal for all the other tested media. This simple experiment confirmed that temperature is also a key parameter to be tested in further experiments.

Cereulide formation takes place in the temperature range between 8°C and 40°C but only minimal production is observed at 8 to 10°C or above 35°C.

Different optimal temperatures for cereulide formation have been reported, probably due to the influence of other factors like the type of medium or food, the incubation time and the growth phase. Finlay *et al.*, (2000) showed that cereulide production was higher at 12 to 15°C compared with 30°C while 21°C seemed better when compared to 11, 40, and 42°C (Hägglom *et al.*, 2002). Psychrotrophic strains able to produce cereulide at 8°C have also been described (Thorsen *et al.*, 2006). Quite interestingly, we found traces of cereulide produced in pasta by a mesophilic strain (TIAC303) that was incubated at 8°C. This fact had never been reported before in the studied mesophilic strains, with the exception of cells pregrown at high temperatures (Hägglom *et al.*, 2002). Although the measured levels of cereulide were very low (< LOQ), this may raise concern regarding refrigerated food products of extended durability. However, additional cereulide production is unlikely at this temperature, and the traces of cereulide found in the pasta never crossed the LOQ threshold, even after 7 days of storage.

The expression of cereulide has been intensively studied in various laboratory media. Nevertheless, transposing the expression of cereulide in these artificial media to food is a delicate task. Several foods have been tested in the literature as matrices for cereulide production and a non-exhaustive summary is given in Table 18. Comparing these data is not straightforward since different methods and different inoculated strains have been used. However, it is evident that the susceptibility of foods to accumulate the toxin is highly variable according to their composition: foods with high starch content such as pasta, rice, potato or pastries are favorable for strong cereulide production while milk seems less prone to accumulate cereulide (Delbrassinne *et al.*, 2011; Jääskeläinen *et al.*, 2003a; Rajkovic *et al.*, 2006a).

The storage conditions (shaking, duration and temperature of storage) influence considerably the concentration of toxin reached in the food as, for example, no cereulide production was seen in milk kept under stationary conditions while it significantly increases under shaking (Agata *et al.*, 2002). However, conflicting results have been obtained showing lower or no

cereulide production under shaking conditions in infant food formulas (Shaheen *et al.*, 2006) and in milk (Rajkovic *et al.*, 2006a), respectively.

When foods are acidified (*e.g.* spaghetti with ketchup, rice with vinegar), cereulide seems to be less accumulated (Agata *et al.*, 2002). This was also observed for foods with low pH (< 5.6) and low water activity (< 0.953) as for instance rye bread (Jääskeläinen *et al.*, 2003a). Concern should be also raised as regards infant food formulas, and more precisely, cereal based infant food formulas, which support significant cereulide levels (Shaheen *et al.*, 2006).

Table 18 - Cereulide expression in various foods assessed with different methods

Reference	Type of Food	Inoculated strain	Inoculation level (log cfu/g)	T° of storage (°C)	Time of storage (h)	Type of storage	Maximal concentration of cereulide (ng/ml; ng/g)	Method
Agata et al., 2002	Boiled rice	NC7401	3	30	24	Stationary	320	Hep-2 assay (toxin titers)
	Bread & cake						20	
	Spaghetti						160	
	Spaghetti with ketchup						ND	
	Rice with vinegar						20	
	Milk						ND	
Jaaskelainen et al., 2003a	Milk	F4810/72	8	21-23	Shelflife + 1day	Stationary	640	Bioassay and LC-MS
	Bakery products (meat and rice pastries, types of bread)						6700 (meat pastries)	
							5500 (meat pastries)	
							170 (rye bread)	
Shaheen et al., 2006	Cereal and dairy based infant food formulis	F4810/72	1 to 7	21-23	24	Stationary	3000	Bioassay and LC-MS
	Penne pasta					Shaking	2000	
Rajkovic et al., 2006a	Penne pasta	5964a	6	28	48	Stationary	3300	HPLC-MS
	Potato						4080	
	Rice						2010	
	Milk						1140	
	Penne pasta					Shaking	210	
	Potato						210	
	Rice						710	
	Milk						ND	
Rajkovic et al., 2007	Moussaka	5964a	4	30	24	Stationary	11	CASA-bioassay
	Polenta						5	
	Infant food formula						12	
	Soy & vegetable crockot						11	
	Béarnaise sauce						8000	
	Cooked rice						1800	
Dommel et al., 2010	Camembert cheese	F4810/72		24	24	Stationary	600	Real-time PCR and Hep-2 assay
	Crème fraîche						ND	
	Penne pasta						1346	
	Rice						1600	
This study		5975c	5	25	7 days	Stationary	4160	LC-MS
		TIAC303					1802	
		5975cR					1802	

ND: Not Detected

CASA-bioassay : Computer-Aided boar Semen Analysis

As explained earlier, the toxic potential of strains is highly variable with up to a 1000-fold difference from one strain to another (Carlin et al., 2006). All the strains used as inocula that are reported in Table 18 have been isolated from food poisonings. However, emetic strains may be isolated from routine food samples as well. In the scope of screening emetic strains from farms and egg product industry (in collaboration with Dr S. Jan from INRA), seventy *B. cereus* isolates, including psychrotrophic and mesophilic strains, have been analysed for cereulide production with both methods (LC-MS² and UPLC-MS/MS). The strains have been incubated during 18h at 30°C and during 5 days at 10°C but none of the seventy strains was able to produce cereulide at those temperatures.

The correlation between bacterial counts and cereulide levels is not constantly proportional as highlighted by two concurrent experiments performed in this work. First, the follow-up of cereulide production in pasta during 7 days of storage revealed a constant increase of the toxin amounts contrary to the stable bacterial counts in the stationary phase. A second experiment in inoculated rice (ca . 10⁶ CFU/g) showed that the cereulide levels were higher at 25°C than at 30°C while the opposite situation was observed for bacterial counts. Furthermore, similar discrepancy between bacterial counts and cereulide production was observed in the recent Belgian outbreak. An entire family who had ordered dishes in an Asian Take-away restaurant experienced vomiting episodes after their lunch. The leftovers were analysed in this study and revealed that, despite equivalent bacterial counts, the rice dishes contained far higher levels of cereulide compared to the soybean soup (Table 12).

These independent experiments reminded that the cereulide production is not strictly dependent on bacterial growth and that the toxin concentration should be considered as the most relevant parameter in case of toxin-related food intoxications. However, establishing norms is not a straightforward task as several parameters (*e.g.* food composition, type of strain, duration and temperature of storage) should be taken into consideration in order to minimize the risk of emetic food poisoning.

4.2.4 CEREULIDE PREVALENCE

Acute toxicity of cereulide has been illustrated in several outbreaks with some very serious and even fatal outcomes, although the prevalence of emetic *B. cereus* strains was thought to be very low in the environment. By directly detecting the toxin in rice dishes served in restaurants, a higher prevalence of cereulide was found than previously studied by targeting cereulide-producing strains.

The LC-MS results that we obtained for cereulide prevalence in rice were not totally in line with the previous PCR results reporting low prevalence of cereulide-producing strains in food and in the environment. As explained earlier, PCR assays rely first on bacterial cultures with selective medium and subsequent random selection of colonies for analysis. Provided that emetic and non emetic *B. cereus* are both present in the sample, and that emetic strains are clearly the minority (*e.g.* ratio of 1:100), these latter may be overlooked by the random picking up. It may also be hypothesized that LC-MS analysis overestimates the occurrence of cereulide because of food matrix interferences leading to possible false-positive results. This weakness may be avoided by verification of food matrix blanks and by using tandem mass spectrometry (LC-MS² or LC-MS/MS) which monitors very specific fragmentation patterns.

Similar discrepancy showing negative PCR results but positive antibody tests that detect components of the *B. cereus* enterotoxins, has already been observed. For instance, toxin expression was detected in a certain percentage of strains (up to 36 %) that had been showed gene-negative by PCR assay (Guinebretière *et al.*, 2002; Hansen and Hendriksen, 2001; Wehrle *et al.*, 2009). Possible reasons at the origin of these discrepancies might be: toxin gene polymorphism which would bias the PCR results or cross-reactivity of the antibody tests. Such false positive and false negative results may be avoided by using LC-MS² method.

Besides, polymorphism of the genetic determinants for cereulide has already been observed in a great collection of strains (Hoton *et al.*, 2009). Interestingly, a very recent study showed a remarkable high prevalence of

emetic strains in sunsik samples (powdered food prepared from grains, fruits and vegetables) although they found only a low *B. cereus* contamination level of 10 to 200 CFU/g (Lee *et al.*, 2012). They were able to detect 36 emetic strains out of 39 *B. cereus* strains isolated from the samples by using general primers of *ces* gene cluster different than those used previously (Lee *et al.*, 2008). This suggests that previous PCR analysis might have overlooked some toxic isolates due to technical limitations. These new findings tend to imply the relevance of our hypothesis that emetic *B. cereus* may be more prevalent, and possibly diverse, than previously thought.

4.2.5 CEREULIDE BIOLOGICAL ROLE

Although the emetic effects of cereulide towards human are well recognized, the function of cereulide for the bacterial producers was still unknown at the start of this thesis. However, the hypothesis that producing a toxin should increase the fitness of the producer in its natural reservoirs seemed worth testing.

Research has first focused on competitiveness of cereulide-producing strains against bacterial, yeast or fungi species. Through concurrent experiments of growth inhibition on bacteria (Tempelaars *et al.*, 2011) and fungi (this study), antibacterial and fungistatic properties of cereulide have been demonstrated. This suggests that cereulide-producing strains may benefit from toxin production to outcompete other species present in their natural niches. Additional tests should be performed to better understand the actual ecological role of cereulide since several other fungal and bacterial species that had been tested were not affected by the presence of cereulide (Altayar and Sutherland, 2006; Ekman *et al.*, 2012; Ladeuze *et al.*, 2011; Tempelaars *et al.*, 2011).

Besides antibacterial and antifungal properties, the competitiveness of emetic strains over non-producers has also been explained by potassium fluxes induced by the presence of cereulide. It seems that cereulide helps the maintaining of K⁺ homeostasis in cereulide-producers and provokes an efflux of K⁺ from non-producer *B. cereus* in absence of energy source.

4.2.6 DETERMINATION OF OUTBREAKS

The causative agent of foodborne intoxications is rather difficult to determine for several reasons. One of these reasons is that the bacteria used for tracing the intoxication might have been eliminated from the product by cooking steps while the produced toxins may still be present in the food. The LC-MS method may facilitate the identification of outbreaks, as illustrated in Chapter 3.3 of this thesis describing the recent lethal Belgian case. This has been the first time that cereulide was quantified in the leftovers of a reported lethal outbreak and, in this manner, that emetic *B. cereus* could be clearly pointed out as the most probable cause of death.

Table 19 summarizes the different levels of cereulide that were quantified in leftovers of foodborne outbreaks using different methods (HEp-2 assay, LC-MS and bioassay). The concentrations range is wide, probably because the victims ate different portions sizes and/or because some victims were more subject to intoxications (*e.g.* children, elderly people). Unfortunately, this information is not always reported. The Belgian lethal case implied the death of a young adult who does not fit to the sensitive target group. This underlines that the total amount of toxin ingested ($\mu\text{g}/\text{kg}$ bw) is an essential value to take into account in order to estimate the risk of intoxication.

Table 19 – Cereulide levels in foodborne outbreaks

Reference	Type of Food	Country	Victims age (years)	Concentration of cereulide (ng/g)	Method
Agata et al., 2002	Fried rice	Japan	Unknown	160 - 1280	Hep-2 assay
	Boiled rice			10 - 640	
	Curry and rice			80	
	Spaghetti			40 - 80	
	Noodle			20	
	chow mein			640	
Jaaskelainen et al., 2003	Pasta dish	Finland	Adult	1000 - 2000	Bioassay and LC-MS
In't Veld et al., 2010	Chinese rice dish	Netherlands	Unknown	32000	LC-MS
	Kebab rice dish	Finland		2000 - 5000	
This study	Spaghetti	Belgium	20	14800*	LC-MS ²
	White rice		14 - 80	8693	
	Fried rice			13184	
	Soybean soup			16	
	Noodle sauce			152	
	chili con carne		Unknown	15	
	spices		Unknown	21	

* lethal case

Non-lethal outbreaks due to emetic *B. cereus* have been reported worldwide. Severe outcome was generally observed for children (Posfay-Barbe *et al.*, 2008; Ichikawa *et al.*, 2010) while adults, who had consumed the same meal, recovered. General outbreaks did also occur and affected up to 116 people (Essen *et al.*, 2000). The common thread among these intoxications was the type of incriminated foods: mainly pasta or rice products including, for instance, rice puddings. However, atypical food products were also involved in emetic food poisonings: dried figs (Hormazábal *et al.*, 2004) and tuna fish (Domenech-Sanchez *et al.*, 2011). Taking these recent findings into account together with detection of cereulide in red beans, chili con carne or spices highlighted by this work, the panel of foods susceptible to support cereulide production and to potentially induce an emetic food poisoning, can definitely be enlarged.

The ubiquity of *B. cereus* ensures that the number of foodborne outbreaks due to this organism is not negligible. Sporadic cases are however more common than general outbreaks (Logan, 2012). Due to resistance of *B. cereus* spores to heat, radiation, disinfectants, dessication and their strong ability to adhere to surfaces (Andersson *et al.*, 1995), *B. cereus* is a common contaminant of food production equipment.

A total elimination of *B. cereus* contamination from food and food production is impossible. Given that normal cooking procedures will make spores germinate and that a subsequent multiplication of vegetative cells and toxin production may occur under improper storage conditions, concern should be raised about practices of boiled rice storage in restaurants. The reported outbreak from Take-Away Asian restaurant, where very high levels of cereulide (up to 13 µg/g) were measured, should invite the authorities to address a better communication with restaurants, catering and end users.

5. CONCLUSIONS AND PERSPECTIVES

The total number of outbreaks caused by *Bacillus* spp. toxins within the EU has dramatically increased by 41.9 % since 2006 (EFSA, 2009). Although the diarrheal and the emetic syndromes caused by *B. cereus* are generally mild, some serious outbreaks leading to fulminant death of healthy people have amplified attention on cereulide.

Food safety is based on random food testing in order to confirm that the food meets certain control criteria. These criteria should be scientifically determined, for example by a risk analysis approach which includes several steps from hazard identification to risk characterization. For microbiological risk assessment, the *hazard* is the causative agent present in the food and the *risk* is the probability of adverse health conditions, as stated by the Commission of the *Codex Alimentarius* and the World Organisation for Animal Health. In the case of *B. cereus*, the exposure assessment would often be based on the levels of food contamination expressed in bacterial counts, as practically performed by Notermans and coll. (1997).

As illustrated in this work, the number of *B. cereus* present in the food does not strictly reflect the risk of emetic food poisoning. It seems therefore relevant to measure toxin production by suitable tests as LC-MS analysis in complement to the detection of the bacterium itself. Those tests may be performed in food samples as well as in human samples. The LC-MS² method built in this study delivers a sensitive alternative for the determination of low levels of cereulide in various foods. Yet, cereulide has been recently detected in human clinical samples (stool, urine, gastric fluid and blood serum) of outbreak patients (Shiota *et al.*, 2010). Detection of cereulide in human samples may allow prompt reaction for better consumer protection. For instance, in the Japanese case, one of the child victims was saved thanks to plasma exchange and hemodialysis.

The presence of cereulide in human clinical samples and more particularly in blood is a good indicator that the toxin may reach diverse organs in the human body. Since no human toxin is known to detoxify cereulide, its activity

will remain intact. However, ingestion of cereulide will not necessarily lead to an acute intoxication since it depends on the total amount of toxin ingested and on the target group victims. It has been seen in several outbreaks that children were hospitalised while adults recover rapidly. Detrimental effect of cereulide on the function of liver and kidney has been reported in severe outbreaks, probably because cereulide hampers the mitochondrial fatty-acid metabolism. Cereulide mitochondrial toxicity is probably not confined to the liver. In fact, two additional organs were affected in the case of the recent Belgian lethal case of 2008: the healthy young man died of a sudden cardiac arrest and a total lysis of the pancreas was observed in the microscopic analysis. Since cereulide is a mitochondrial toxin, it was hypothesized that it may also affect cardiac mitochondria. However, histology analysis of the heart revealed absence of mitochondrial damage and the death of the young man was probably caused indirectly. Yet, heart failure due to myocardial fatty degeneration, together with the less unusual fatty degeneration of the liver and the kidneys, was observed after autopsy of an 11-year old boy (Takabe and Oya, 1976). These two lethal examples encourage further research about cereulide effect on cardiac mitochondria. Interpretation of the pancreas lysis observed in the Belgian case was difficult due to autopsy delay. But, since cereulide has been shown to decrease the insuline content in the pancreatic Langerhans islets (Virtanen *et al.*, 2008), it may be suggested that cereulide may damage pancreatic cells and/or play a role in pancreatic chronic diseases such as diabetes. Moreover, it can also be assumed that cereulide may have been overlooked as a possible cause of other liver and/or pancreatic failures of unknown aetiology.

In the described lethal cases, the consumer was exposed to acute doses of cereulide. As demonstrated by the small-scale survey study conducted in Asian restaurants, the consumer may also be exposed to subclinical doses of cereulide on a more regular basis. Nothing is known about long term effects due to a chronic exposure to cereulide. It would be very interesting to redesign the study on a larger scale including the monitoring of parameters such as the mode of cooking, food storage conditions, kitchen hygiene and food packing. A well-established questionnaire would be necessary to determine the consumption habits of the restaurant clients. This, in turn, will

show how important is the issue of chronic exposure to (low doses of) cereulide. Then, a feeding trial on animals would help to determine which organs are targeted and how they are affected following a daily exposition to doses of cereulide. Blood and urine should be sampled since cereulide is rapidly absorbed in the bloodstream and excreted in urine. Liver, kidney, pancreas and heart tissues should also be collected since those organs were affected in severe outbreaks. A very sensitive LC-MS method, such as the UPLC-MS/MS method described in the discussion section, appears as the best method for tackling these issues.

The developed LC-MS² method has been successful for detecting low doses (LOD of 0.5 ng/g) of cereulide in white and fried restaurant-rice dishes which are the most incriminated foods in emetic food poisoning. However, the method had to be slightly adapted for more complex food matrices such as chili con carne. In these foods, the recovery was less satisfactory and the matrix effect more intense. Hopefully, the use of ion tandem mass spectrometry (LC-MS²) increased the specificity by detecting specific fragmentation pattern. In the case of biological samples such as liver, new extraction methods should probably be designed. In order to ensure high sensitivity and high specificity of detection, the UPLC-MS/MS should be preferred because it allows for the monitoring of specific transitions (Multiple Reaction Monitoring or MRM) instead of detecting all fragment ions. This targeted MS analysis usually enhances detection limits.

Besides, mention is made in the literature of the existence of cereulide homologs. The molecular weight of cereulide is 1152 Da but homologs have been observed at 1138 Da and 1166 Da, displaying a 14 mass variability (Pitchayawasin *et al.*, 2004). Nonetheless, very little attention has been addressed to these compounds. Two strains (*B. cereus* NC7401 and a marine *B. cereus* NS-58 strain) that are able of producing them have been reported (Pitchayawasin *et al.*, 2004; Wang *et al.*, 1995) but it is not known whether the production of homologous compounds of cereulide is a frequent phenomenon inside the group of emetic strains. Although the homocereulide produced by *B. cereus* NS-58 has not been associated with food poisoning, it is a very cytotoxic molecule (Wang *et al.*, 1995). A study performed on a large collection of emetic strains would help to get better insights into the

prevalence of these compounds and their toxicity towards mammalian cells. MALDI-TOF (Matrix-assisted laser desorption/ionization- time of flight) method may be a very simple way to distinguish between cereulide homologs.

Beside toxin production, another major issue posed by *B. cereus* for food safety is its capacity to sporulate. Contrary to vegetative cells, spores are able to resist to high temperatures, dessiccation, radiation and physical stresses (Schoeni and Wong, 2005). Therefore, they can survive food processing and germinate when returned to favorable conditions. New vegetative cells can be then generated and toxin production may occur in processed foods. Moreover, it has been reported that spores of emetic strains displayed a higher resistance to heat than non-producing strains (Altayar and Sutherland, 2006; Andersson *et al.*, 2004; Carlin *et al.*, 2006; Shinagawa, 1993). Do spores from emetic strains pose a higher risk for the consumer ? This interesting question should also be addressed in further experiments.

The relation between spore formation and production of cereulide is still unclear. Several contradictory results have been obtained: some claimed that there is an association between sporulation and toxin formation (Christiansson, 1992; McKillip, 2000) while others were not able to find any correlation (Finlay *et al.*, 2000; Häggblom *et al.*, 2002; Lucking *et al.*, 2009). What is clear is that the toxin formation can occur before sporulation (Turnbull *et al.*, 1979) although Finlay *et al.* (2002b) showed that emetic toxin production concurred with spore production. Yet, they were unable to show a correlation between spores counts and toxin levels but it is now recognized that toxin production is not systematically proportional to bacterial counts. Interestingly, Dommel and coll. (2011) showed that sporulated pre-cultures induced higher levels of cereulide. All these considerations suggest that the sporulation is not crucial for toxin production but that a complex interplay exists between those two events.

Although they have not yet been implicated in food poisoning, several *Bacillus* spp are able to form emetic ring peptides similar to cereulide. Inside the *B. cereus* group, psychrotolerant *B. weihenstephanensis* is actually

producing cereulide whereas it was long thought that cereulide production was restricted to emetic *B. cereus* s.s. The recent discovery of paenilides and homopaenilides, cytotoxic molecules produced by *Paenibacillus tundrae* (Rasimus et al., 2012) sharing structural features with cereulide and homologs, has extended this production outside the *Bacillus* genus. *B. weihenstephanensis* and *P. tundrae* are both able to grow at refrigeration temperatures and have therefore drawn attention to emetic food poisoning risk related to foods conserved under chilled conditions. Considering the increasing consumer demand for convenient foods with extended durability under refrigeration and the recent success of RTE-foods (Ready-to-Eat Foods), this topic deserves as well consideration.

Concern about *B. cereus* foodborne outbreak is still largely underestimated despite several fatal cases in recent times. The present work has helped to raise awareness on several levels. On one hand, the implication of cereulide in the fatal case involving the death of a twenty-year-old man has proven that emetic food poisoning could be very brutal and that it does affect other target groups than only children or elderly people. On the other hand, thanks to the sensitive LC-MS² analysis, light was shed on a not negligible exposure to low doses of cereulide through consumption of restaurant-rice dishes, leading to, although not yet identified, potential chronic consequences.

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