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The *Bacillus cereus* toxin cereulide: Quantification and its biological actions

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“CE SONT LES MICROBES, QUI AURONT LE DERNIERE MOT”

Louis Pasteur (1822-1895)

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PUBLICATIONS

Parts of this thesis have been or will be published in peer-reviewed journals

I. **Bauer, T.**, Stark, T., Hofmann, T., Ehling-Schulz, M. (2010) Development of a stable isotope dilution analysis (SIDA) for the quantification of the *Bacillus cereus toxin* cereulide in foods. *J Agric Food Chem* **58:** 1420-1428

II. **Bauer, T**. Silbermayr, K., Joachim, A. Ehling Schulz M. (2013) Broad toxicological spectrum of cereulide, the emetic toxin of *Bacillus cereus*, *in preparation*

III. **Bauer, T**., Sipos, W., Stark, T., Käser, T., Knecht, C., Saalmüller, A. Hofmann, T., Ehling-Schulz M. (2013) Swine as a model for studying cereulide intoxications.*,in preparation*

III Publications

SUMMARY

The pathogenicity of the emetic (vomiting provoking) *Bacillus cereus* arises from the small dodecadepsipeptide cereulide. This cyclic toxin is resistant to heat, proteolysis as well as to acid and basic conditions. Due to these properties, cereulide, once preformed in food, will not be destroyed by reheating of food products or in the gastrointestinal tract after consumption. During the last years, severe food borne intoxications caused by cereulide are increasingly reported; occasionally even involving hospitalization or death a few hours after ingestion. Intoxications with cereulide are presumably underreported because of misdiagnosis and/or the lack of rapid detection and toxin quantification methods. Therefore, in this work a stable isotope dilution analysis (SIDA) was developed, which enables a precise quantification of cereulide in complex matrices such as foods or clinical specimens, using an isotope labelled $^{13}C_6$-cereulide as internal standard. Cereulide and $^{13}C_6$-cereulide were biosynthetically produced by *B. cereus* F4810/72 and a protocol was established for a rapid and efficient purification of both substances in high quality and quantity. The produced isotope labelled standard $^{13}C_6$-cereulide gave good recovery rates, thus demonstrating the robustness and accuracy of the developed SIDA for food analyses.

Hitherto, the benefit of cereulide synthesis for the producer strain in its typical habitat soil is unknown. Therefore, the impacts of cereulide on bacteria, nematodes and insects were analysed and compared to the biological action of the structure analogue valinomycin. Both depsipeptides demonstrated strain specific antibacterial activity towards a set of Gram-positive bacteria indicated by an elongation of lag phase duration in response to increasing cereulide or valinomycin concentrations. In contrast, all tested Gram-negative bacteria were resistant towards cereulide or valinomycin treatment and it could be demonstrated that the outer membrane of Gram-negative bacteria was responsible for the observed toxin resistance. Exposure of cultures of the nematode *Oesophagostomum dentatum* to cereulide or valinomycin resulted in an inhibition of development from third to fourth instar larvae. Additionally, treatment with cereulide caused a distinct larval phenotype characterised by a malformation of the anterior part of third instar larvae. After injections of cereulide and valinomycin into insect larvae of *Galleria mellonella* paralysis of the insects was observed a few hours after treatment. While the paralysis caused by valinomycin was fully reversible, insect larvae treated with cereulide died. For almost all analysed actions of cereulide, the observed effects seemed not to be related solely to the ionophoretic activity of cereulide. Furthermore, this work showed that potassium plays an important role in the toxicity of cereulide.

To analyse the action of cereulide on a mammal host, a swine intoxication model was established. Here, cereulide seemed to possess a specific neuromodulating activity. A few hours after oral administration of cereulide (10 – 150 µg kg bw^{-1}), piglets showed

symptoms like seizures and trembling, which point to disturbances of the central nervous system (CNS). However, cereulide did not influence biochemical markers or the cell composition in blood samples. Although most of the cereulide was excreted via faeces, low amounts could also be detected in several organs indicating the possibility of cereulide entering the body. Altogether, the swine intoxication experiments showed that screening of faeces samples may represent an easy non-invasive method for detection of cereulide intoxications not only in swines but also in humans.

ZUSAMMENFASSUNG

Die Pathogenität von emetischen (Erbrechen auslösenden) *Bacillus cereus* ist auf das Dodekadepsipeptid Cereulid zurückzuführen. Dieses zyklische Toxin zeichnet sich durch seine hohe Resistenz gegenüber Hitze, Proteolyse sowie sauren und basischen Bedingungen aus. Aufgrund dieser Eigenschaften kann Cereulid, einmal im Lebensmittel gebildet, auch nicht durch Erhitzen des Lebensmittels oder im Gastrointestinaltrakt nach der Nahrungsaufnahme zerstört werden. In den letzten Jahren nahm die Anzahl an Fällen von Cereulid assoziierten Intoxikationen mit einem schweren Krankheitsverlauf, die teilweise im Krankenhaus oder sogar im Tod wenige Stunden nach Verzehr der Mahlzeit endeten, stark zu. Die Intoxikationen sind meist unterrepräsentiert wegen einer Fehldiagnose und/oder dem Fehlen von geeigneten Detektions- und Toxin-quantifizierungsmethoden. Aufgrund dieser Tatsache wurde eine Stabilisotopen-verdünnungsanalyse (SIVA) für eine präzise Quantifizierung von Cereulide in komplexen Matrizes wie Lebensmittel oder Proben mit klinischem Hintergrund mittels eines Isotopen-markierten Cereulids als internen Standard entwickelt. Sowohl Cereulid als auch $^{13}C_6$-markiertes Cereulid wurden biosynthetisch durch Kultivierung von *B. cereus* F4810/72 produziert und ein Protokoll für eine schnelle und effiziente Reinigung der beiden Substanzen in hoher Qualität und Quantität wurde etabliert. Der produzierte Isotopen-markierte Standard $^{13}C_6$-Cereulid ergab gute Wiederfindungsraten, was die Stabilität und Präzision der entwickelten SIVA für die Lebensmittelanalyse wieder-spiegeln.

Bis heute sind die Vorteile der Cereulidsynthese für den Produzenten *B. cereus* in seinem typischen Habitat nicht bekannt. Daher wurde die Wirkung von Cereulid auf Bakterien, Nematoden und Insekten analysiert und mit der Wirkung des Strukturanalogs Valinomycin verglichen. Beide Depsipeptide wiesen eine stammspezifische antibakterielle Wirkung gegenüber Gram-positiven Bakterien auf, die sich durch eine Verlängerung der lag-Phase bei ansteigenden Cereulid- und Valinomycin-konzentrationen zeigte. Alle untersuchten Gram-negativen Bakterien waren resistent gegenüber Valinomycin und Cereulid und es konnte gezeigt werden, dass die äußere Membran von Gram-negativen Bakterien für die beobachtete Toxinresistenz verantwortlich ist. Eine Zugabe von Cereulid und Valinomycin zu Kulturen des Nematoden *Oesophagostomum dentatum* resultierte in einer Hemmung der Entwicklung vom dritten zum vierten Larvenstadium. Weiterhin konnte bei einer Cereulidzugabe ein bestimmter Larvenphänotyp beobachtet werden, der durch eine Fehlbildung im anterioren Teil des dritten Larvenstadiums in Erscheinung trat. Eine Injektion von Cereulid und Valinomycin löste bei Larven des Insekts *Galleria mellonella* eine Lähmung ein paar Stunden nach der Behandlung aus. Diese Lähmungserscheinungen waren reversibel bei Injektion von Valinomycin, jedoch irreversibel bei Cereulid und führten

zum Tod der Larven. Nahezu alle beobachteten Effekte von Cereulid scheinen nicht nur mit der ionophoretischen Aktivität erklärbar zu sein und lassen auf weitere Wirkungsweisen von Cereulid schließen. Zusätzlich konnte gezeigt werden, dass Kalium eine wichtige Rolle bei der Toxizität von Cereulide spielt.

Um die Wirkung von Cereulid auf Säugetiere zu untersuchen, wurde ein Schweineintoxikationsmodell etabliert. Es zeigte sich, dass Cereulid eine neuromodulatorische Aktivität besitzt, da Symptome wie Streckkrämpfe und Zittern bei einer Gabe von 10-150 µg kg^{-1} Körpergewicht beobachtet wurden, die auf eine Störung des Zentralnervensystems hinweisen. Cereulid zeigte keinen Einfluss auf biochemische Marker oder auf die Zellzusammensetzung im Blut. Bei der Quantifizierung von klinischen Proben konnte ein großer Teil des Cereulids im ausgeschiedenen Kot, aber auch in einzelnen Organen nachgewiesen werden, was auf eine Aufnahme in den Körper hindeutet. Anhand des Schweinemodells konnte demonstriert werden, dass die Analyse von Stuhlproben eine einfache nicht-invasive Methode zur Detektion von Cereulidintoxikationen nicht nur im Schwein sondern auch im Mensch darstellt.

1. INTRODUCTION AND RESEARCH OBJECTIVES

1.1 *BACILLUS CEREUS* AND ITS PATHOGENICITY

Food-borne diseases are a public health problem, which comprise a broad group of illnesses. Among them, gastrointestinal diseases are the most frequent clinical syndromes, which can be attributed to a wide range of microorganisms, including bacteria, viruses and parasites. One important agent of food borne diseases worldwide is the toxin producing, endospore-forming, Gram-positive bacterium *Bacillus cereus.* Its name derived from the cell shape (bacillus, gr.: rod) and colony appearance (cereus, gr.: wax). The facultative anaerobic *B. cereus* has been isolated from almost all categories of foodstuff, as it is able to grow in very diverse habitats like soil and sediments (Kotiranta *et al.*, 2000; Stenfors Arnesen *et al.*, 2008; von Stetten *et al.*, 1999). *B. cereus* spores can reach concentrations of up to 10^3-10^5 cells per gram soil (von Stetten *et al.*, 1999). Moreover, *B. cereus* has been isolated from the insect gut of several arthropod species in high frequency and a commensal lifestyle has been proposed for this bacterium (Luxananil *et al.*, 2003; Margulis *et al.*, 1998; Swiecicka & Mahillon, 2006).

The toxicological profile of *B. cereus* strains ranges from non-pathogenic strains used as probiotics in animal feed (Baum *et al.*, 2002; Lodemann *et al.*, 2008) to highly pathogenic isolates, which are responsible for major outbreaks such as 173 cases of intoxication after a banquette (Ghelardi *et al.*, 2002) or severe individual cases leading to hospitalization and even death (see 1.3.3). *B. cereus* is mainly known to evoke two types of gastrointestinal food borne poisonings. The emetic type indicated by nausea and forceful vomiting shortly after ingestion is caused by the small dodecadepsipeptide cereulide (Agata *et al.*, 1994), which is produced by a subgroup of *B. cereus* (Ehling-Schulz *et al.*, 2005a). The diarrheal syndrome is caused by several heat labile enterotoxins produced during growth of *B. cereus* in the intestine. The hemolysin BL (HbL) and the pore forming non-hemolytic toxin (Nhe) belong to the class of three-component enterotoxins, whereas cytotoxin K (CytK) represents a β-barrel channel forming one-component enterotoxin (Beecher & Wong, 1997; Lund & Granum, 1997; Lund & Granum, 1999; Lund *et al.*, 2000). In general, 6 to 12 hours after ingestion of about 10^5 to 10^7 cells, abdominal cramps and diarrhoea occur, but the course of the disease is normally relatively mild and symptoms disappear within 24 hours (Stenfors Arnesen *et al.*, 2008). The extent and duration of the disease depend on the infection dose and the amount of produced enterotoxins, which seem to differ strongly among different *B. cereus* strains (Ehling-Schulz *et al.*, 2011a; Granum, 2001).

Besides the known toxins, *B. cereus* also produces several enzymes like sphingomyelinase, phosphatidylinositol- and phosphatidylcholine-specific phospholipases and several proteases that are so far not directly associated with gastrointestinal diseases, but may play an important role in non-gastrointestinal infections such as wound and eye infections,

bacteremia, pneumonia, meningitis, periodontitis, and endocarditis (Drobniewski, 1993; Gilmore *et al.*, 1989; Haase *et al.*, 2005; Helgason *et al.*, 2000; Miller *et al.*, 1997; Pillai *et al.*, 2006).

In recent years, the number of food borne outbreaks caused by *B. cereus* has increased in industrial countries (Kotiranta *et al.*, 2000; Stenfors Arnesen *et al.*, 2008). The ability to generate protecting biofilms and to form endospores, which are metabolic inactive and resistant to harsh conditions such as heat (>100°C), many chemicals, radiation as well as desiccation, allows *B. cereus* to survive e.g. treatments of the food industry like conservation, chemical disinfection and preservation (Abee *et al.*, 2011; Carlin *et al.*, 2006; Ehling-Schulz *et al.*, 2011b; Novak *et al.*, 2005; Stenfors Arnesen *et al.*, 2008; Wijman *et al.*, 2007). Most notably, the high hydrophobic character of the spores seems to increase their adherence to the surface of food processing machines and equipment, pipelines as well as tanks leading to contamination of food products by direct contact with these different sources (Faille *et al.*, 2007). Consequently, once spores have entered the food, pasteurization or normal sanitation processes will not contribute to their elimination (Ehling-Schulz *et al.*, 2011b; Novak *et al.*, 2005).

B. cereus is one of six members of the *Bacillus cereus* group within the genus *Bacillus*. The other members of this genetically closely related group are *Bacillus anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides* and *B. pseudomycoides* (Didelot *et al.*, 2009; Gordon *et al.*, 1973; Lechner *et al.*, 1998; Nakamura, 1998; Priest *et al.*, 2004). Although a clear separation of these species by phenotyping or classical DNA hybridization studies failed, these bacilli differ significantly in their ecological features such as the synthesis of virulence factors, specialized morphology and cold adaption. (Ash *et al.*, 1991; Kaneko *et al.*, 1978; Priest *et al.*, 1988). These special features are mainly encoded by genes located on megaplasmids like e.g. pXO1 and pXO2 of *B. anthracis*. The causative agent of the fatal mammalian disease anthrax*, B. anthracis*, arrested attention in 2001 for its use as bioterrorism agent and biological weapon developed by several countries (Hoffmaster *et al.*, 2002). The insect pathogen *B. thuringiensis* produces toxic crystals (δ-endotoxins), which are encoded on a plasmid and lyse midgut epithelial cells (Berry *et al.*, 2002). *B. thuringiensis* is routinely used as agent to control agricultural insect pests (Bravo *et al.*, 2011).

1.2 DEPSIPEPTIDES

The *B. cereus* toxin cereulide belongs to the class of depsipeptides found in several organisms including fungi, bacteria and marine sponges (Davidson, 1994; Fusetani & Matsunaga, 1993). Prominent and well-characterised depsipeptides are for example vancomycin (Nicolaou *et al.*, 1999), valinomycin (Duax *et al.*, 1996), enniatins (Firakova *et al.*, 2007) fusaricidins (Kajimura & Kaneda, 1996) and destruxins (Pedras *et al.*, 2002). Depsipeptides comprise hydroxy and aminoacids, which are connected by amide or

esterbonds (Ballard *et al.*, 2002; Sarabia *et al.*, 2004). Especially cyclic depsipeptides possess unique structural features and it has been demonstrated that the cyclic structure is important for their biological activities such as e.g antiplasmodial, insecticidal or cytotoxic activities (Ballard *et al.*, 2002; Hamann *et al.*, 1996; Sarabia *et al.*, 2004). In addition, some depsipeptides exhibit antiviral actions and it has even been shown that several depsipeptides are able to inhibit HIV (Oku *et al.*, 2004; Rashid *et al.*, 2001). Furthermore, cyclodepsipeptides have been discovered as potential drugs for anticancer therapy in combination with other substances and some have entered clinical trials (Fotie & Morgan, 2008). However, due to their extreme toxicity many cyclic depsipeptides fail in preclinical studies. Interestingly, almost all cyclodepsipeptides exhibit ionophoretic properties and are able to transport mono- or divalent cations through the membrane, but the pharmacologically pertinent activity seems not to be due to this feature (Lemmens-Gruber *et al.*, 2009).

1.3 CEREULIDE AND ITS TOXICITY

1.3.1 Biosynthesis

Like other cyclic depsipeptides, cereulide is produced enzymatically by large multifunctional protein complexes, so called nonribosomal peptide synthetases (NRPS) (Marahiel *et al.*, 1997). The NRPS complexes are composed of repetitive modules, which in turn mainly consist of three functional distinct domains: 1. Adenylation domain for activation of the monomer, 2. Thiolation or peptidyl carrier (PPC) domain for attachment of the 4'-phosphopantetine cofactor and covalent binding and 3. Condensation domain for peptide bond formation (Koglin & Walsh, 2009). Each module recognizes and incorporates one amino acid, α- hydroxyl acid or carboxylic acid into the NRPS product. The last module called the termination module consists of a adenylation-, PPC- and thioesterase domain, which releases the newly produced non ribosomal peptide from the NRPS complex (Strieker *et al.*, 2010).

The cereulide NRPS genes were found within a 24 kb multigene cluster on the megaplasmid pCER270 (Ehling-Schulz *et al.*, 2005b; Ehling-Schulz *et al.*, 2006a). The *ces* gene cluster is composed of the seven following genes: *cesH* encodes a putative hydrolase, *cesP* a 4'-phosphopantetheinyl transferase, *cesT* a type II thioesterase, *cesA* and *cesB* encode for the functional NRPS modules and *cesC* and *cesD* for a putative ABC transporter. The *ces*PTABCD genes are transcribed as a single operon from the main $cesP_1$ promotor (Dommel *et al.*, 2010)

1.3.1 Structure and Characteristics

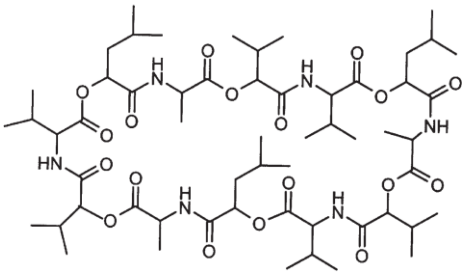


Figure 1: Structure of cereulide (Ehling-Schulz *et al.*, 2004)

The chemical structure of cereulide (Figure 1) is composed of a cyclic trimer consisting of the repeating tetrapeptide units [$_{L}$-*O*-Val-$_{L}$-Val-$_{D}$-*O*-Leu-$_{D}$-Ala-] (Isobe *et al.*, 1995; Suwan Sathorn 1995). Thus, cereulide resembles the macrolide antibiotic valinomycin produced by *Streptomyces spec.* which consists of [$_{L}$-Lac-$_{L}$-Val-$_{D}$-*O*-Hiv-$_{D}$-Val-]$_3$ (Pinkerton *et al.*, 1969). In accordance with the ionophoretic properties of valinomycin, cereulide was found to be able to form complexes with monovalent cations, preferentially with potassium, in a highly ordered intramolecularly hydrogen-bonded structure (Saris *et al.*, 2009). Due to the alternating ester and amide bonds, its cyclic structure and various intramolecular hydrogen bondings, cereulide is highly resistant towards many environmental influences: it exhibits a high heat stability (150°C; 60 min; pH < 9.5 (Rajkovic *et al.*, 2008), resistance to acid and basic conditions (pH 2-11)(Melling *et al.*, 1976; Mikkola *et al.*, 1999; Rajkovic *et al.*, 2008) as well as proteases such as trypsin (Melling & Capel, 1978; Mikami *et al.*, 1994), pepsin (Shinagawa *et al.*, 1995), pronase (Sakurai *et al.*, 1994) and proteinase K (Andersson, 1998). In consequence, when cereulide is present in contaminated food products, it will neither be destroyed by thermal processing nor through the passage in the intestinal tract after food consumption. Additionally, cereulide cannot be removed by filtration from food products because of its low molecular mass of 1152 Da. Moreover, cereulide is highly hydrophobic with an octanol/water coefficient of log K_{ow} ~6 (Teplova *et al.*, 2006) and can attach to many hydrophobic surfaces of e.g. food processing equipment and pipelines leading to serious problems in the food industry.

1.3.2 Known Biological Actions

Cereulide is also known as the *B. cereus* emetic toxin, because it evokes nausea and emesis half to six hours after ingestion of contaminated food but symptoms generally disappear after 24 hours. The disease is usually mild and self-limiting, but there also have been some major outbreaks with involvement of up to several hundred people, mainly linked to caterers or canteens (Fricker *et al.*, 2007; Kamga Wambo *et al.*, 2011; Okahisa *et al.*, 2008).

Feeding experiments with rhesus monkeys (*Macaca mulatta*) and Asian house shrew (*Suncus murinus)* indicate an emesis inducing dose of about 10 µg kg^{-1} bw after ingestion (Agata *et al.*, 1995; Shinagawa *et al.*, 1995). Ingestions of higher concentrations can cause severe diseases; some with a fatal outcome (cf. 1.3.3). In feeding experiments it was further demonstrated that a prevention of emesis caused by cereulide can be obtained by blocking of 5-HT_3 receptors, but the underlying mechanism of receptor activation by cereulide is still unclear (Agata *et al.*, 1995).

Cereulide is able to complex monovalent cations, preferably potassium, and its highly hydrophobic character allows cereulide to diffuse through cell membranes (Mikkola *et al.*, 1999). These two features make it an optimal ionophore, which can destroy the electrochemical gradient of membranes. For instance, it has been shown that cereulide disrupts the mitochondrial membrane potential by uncoupling the ATP synthesis, which results in the inhibition of the fatty acid metabolism in mammalian cells (Andersson *et al.*, 2007; Mahler *et al.*, 1997; Teplova *et al.*, 2006). Besides its influence on the mitochondrial membrane potential, cereulide has been reported to inhibit human natural killer cells (Paananen *et al.*, 2002) and cause cellular damage (Shinagawa *et al.*, 1996). Most of the biological cereulide detection methods, such as the cytotoxicity assays employing human larynx carcinoma cells (HEp-2) or rat liver cells (Finlay *et al.*, 1999; Kawamura-Sato *et al.*, 2005), as well as the boar sperm motility assay (Hoornstra, 2003), are based on ionophoretic properties of cereulide, especially its mitochondrial toxicity. Recently, there have been some hints about a potential antifungal and antibacterial activity of cereulide (Ladeuze *et al.*, 2011; Tempelaars *et al.*, 2011).

So far, the biological function of cereulide synthesis and its advantage for emetic *B. cereus* strains has not been determined. Interestingly, another soil dwelling Gram-positive bacteria *Streptomyces spec.* produce the structurally highly similar cyclic peptide valinomycin (see 1.3.1). Several biological actions of valinomycin on other organisms have been demonstrated: antimicrobial activity, anti-viral effects on the human coronavirus (Wu *et al.*, 2004), anti-insecticidal activity (Pansa *et al.*, 1973), anti-nematodal activity, effect on mites (Patterson & Wright, 1970) and an apoptosis inducing effect on human and hamster cells (Abdalah *et al.*, 2006; Ryoo *et al.*, 2006). Due to the similar structure of valinomycin and the related habitats of the producers, it might be possible that cereulide has analogous effects on organisms.

1.3.3 Severe Case Reports

Emesis after an extremely short incubation period of 15-40 min after consumption of contaminated food is mainly due to food poisonings caused by *Staphylococcus aureus* or *B. cereus*. Besides the generally mild course of the disease, some severe cases have been described even leading to death (Table 1). Notably, although *B. cereus* is not able to hydrolyze starch, all severe cases are associated with starch rich food such as rice and noodles.

Table 1: Overview of severe cereulide intoxications

Year and *Country*	Food details	Person(s) affected (age)	Symptoms and (→) therapy	Toxin and bacteria	Reference
1997 *Switzerland*	Reheated noodles with pesto	Father & boy (17) Boy (17)	Acute gastroenteritis 30 min after ingestion Died on fulminant liver failure	Cereulide was found in food, bile, plasma ,intestinal content	(Mahler *et al.*, 1997)
2003 *Belgium*	Noodle salad stored 24 h	Five children (7, 9, 9, 10, 14) Girl (7)	Vomiting after 6 hours Died on severe metabolic acidosis and liver failure	Detection of emetic *B. cereus* in the gut in spleen of the girl	(Dierick *et al.*, 2005)
2008 publ. *Switzerland*	Reheated noodles & sauce stored 48 h	Man (23) & girl (9) Girl (9 years old)	Severe abdominal pain, emesis after 2 hours Nonbloody diarrhoea followed by unconscious in bed with tonic-clonic seizures after 6 hours Diagnosis: fulminant hepatitis, renal and pancreatic insufficiency	Emetic *B. cereus* was detected by PCR	(Posfay-Barbe *et al.*, 2008)
2008 *Japan*	Reheated fried rice stored 24h	Boy (1) Girl (2) Mother (26)	Emesis, lethargy followed by unconsciousness after 6 h Transfer to the hospital Diagnosis: severe hyperkalemia, hypoglycemia, mixed acidosis → Intensive resuscitation Died after 6.5 h Emesis, lethargy and unconsciousness after 6 h Severe hypoglycemia with metabolic and lactic acidosis → Plasma exchange followed by hemodialysis Full consciousness on the 2nd day Frequently vomiting	Detection of cereulide in rice and serum, vomitus, intestinal content serum, urine, stool, gastric fluid	(Shiota *et al.*, 2010)
2008 *Belgium*	Reheated noodles & tomato sauce stored 5 d at RT	Boy (20)	Abdominal pain, nausea after 0.5 h and frequently vomiting Dead after 10h during sleep	Detection of cereulide (14.8 µg/g) and emetic *B. cereus* in noodles	(Naranjo *et al.*, 2011)
2009 *Japan*	Reheated fried rice stored 24 h in the fridge	Boy (11)	Abdominal cramps and vomiting after 30 min Drowsiness and convulsion followed by gastroenteritis. Diagnosis: Acute encephalopathy, liver failure, systematic organ damage diagnosed on first day →Methylprednisolone therapy, infusions and cyclosporine A Severe brain edema on 5th day →Hypothermia therapy over 5 days After 41 days "recovery" just with mild intelligence impairment	Emetic *B. cereus* could be isolated from gastric fluid, a stool specimen and fried rice	(Ichikawa *et al.*, 2010)

These case reports demonstrate a wide range of disease types caused by cereulide reaching from liver failure to encephalopathy. Today it is not known how cereulide causes these different types of diseases. Its lipophilic character could help cereulide to pass through the membranes, to enter the bloodstream and therefore reach different organs in the whole body. However, up to now, it is not known, if ingested cereulide is excreted or accumulated in the body. Due to its high stability, cereulide probably cannot be degraded in the body and an accumulation in organs or tissue, where it is able to affect metabolic functions, may be possible.

Interestingly, all severe cases occurred in only three countries (Belgium, Switzerland and Japan). This local limitation is certainly not due to the absence of emetic *B. cereus* strains and/or emetic outbreaks in other countries, but rather to a lack of detection methods or to a misdiagnosis of the symptoms. Since there exists no obligation of report in any country, it is evident that the number of food poisonings triggered by cereulide is seriously underestimated and underreported. Thus, in many cases the symptoms may be misdiagnosed, also because of a lack of healthcare facilities that can specifically detect cereulide or emetic strains. Although there exists a toxin gene profiling system for rapid detection of emetic toxin producing *B. cereus* strains (Ehling-Schulz *et al.*, 2006b; Fricker *et al.*, 2007), it is not routinely used in hospitals. Furthermore, a standard detection method for cereulide in complex matrices has not yet been developed.

1.4 Principle of Stable Isotope Dilution Analysis (SIDA)

Stable isotope dilution analysis is becoming more and more popular for a precise detection of toxins in complex matrices such as food, human or animal samples because of its excellent specificity and sensitivity. In general, the separation of most substances in a complex mixture of compounds is not a huge problem anymore using liquid or gas chromatography (Wolthers & Kraan, 1999). By knowing the mass of the searched substance the detection is easy with mass spectrometric analysis. The liquid chromatography-tandem mass spectrometer (LC-MS/MS) provides a high-degree of accuracy and precision when making quantitative measurements and the detection of even very low levels of a specific compound is possible. An accurate quantification of a compound in a complex mixture proves to be more difficult and an internal standard can be useful to improve the precision. The addition of a known amount of an internal standard to a sample allows quantification by comparison of the signal of the analyte with the signal from the internal standard after mass spectrometric analysis. The purpose of an internal standard is to act chemically and physically as similar as possible to the target analyte, but to provide a signal that can be distinguished from that of the analyte. Therefore, the requirements for an optimal internal standard are very high and for precise quantification the standard has to be nearly identical to the analyte (Ciccimaro & Blair, 2010).

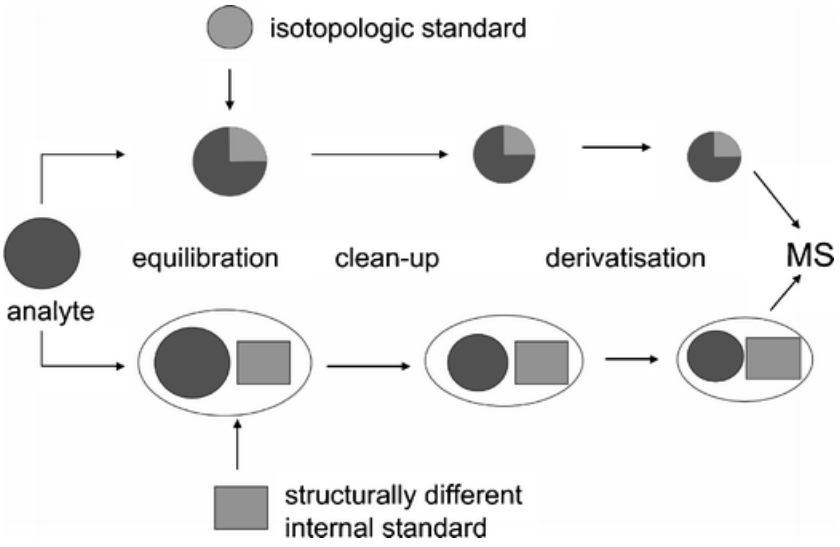


Figure 2: Principle of the stable isotope dilution analysis. For description see text. Taken from (Rychlik & Asam, 2008).

For the LC-MS/MS analysis of samples consisting of complex matrices such as food, urine or blood, there exist a huge number of competing effects in the ionization source that can influence the ionization of the target analyte. Such matrix effects can induce a suppression or enhancement of the signal of the target and are likely to distort the quantitative measurement results (Halliday & Rennie, 1982; Wang *et al.*, 2007). The use of a stable isotope internal standard represents the best method to overcome these problems, because structural analogues will have different retention times and ionization properties compared to the target analyte (Rychlik & Asam, 2008). In contrast, stable isotope labelled internal standards (SIL standards) are identical to the target analyte, except for the mass (Stokvis *et al.*, 2005). Generally, SIL standards are labelled by ^{13}C or deuterium for SIDA. Then, the SIL standard is spiked into a sample at a known concentration. After equilibration with the target analyte, the mass spectrometric analysis allows a differentiation between the standard and the analyte. Finally, the signal ratio between both can be interpolated onto a standard curve to calculate the absolute amount of the target analyte in the sample. Due to the nearly identical physico-chemical properties the ratio between both components remains stable during all sample preparation steps as well as in the final mass spectroscopic analysis. In contrast, the use of a structural analogue as IS would presumably result in different losses of IS and analyte during sample preparation resulting in inaccurateness (for review see (Rychlik & Asam, 2008) .

1.5 RESEARCH OBJECTIVES

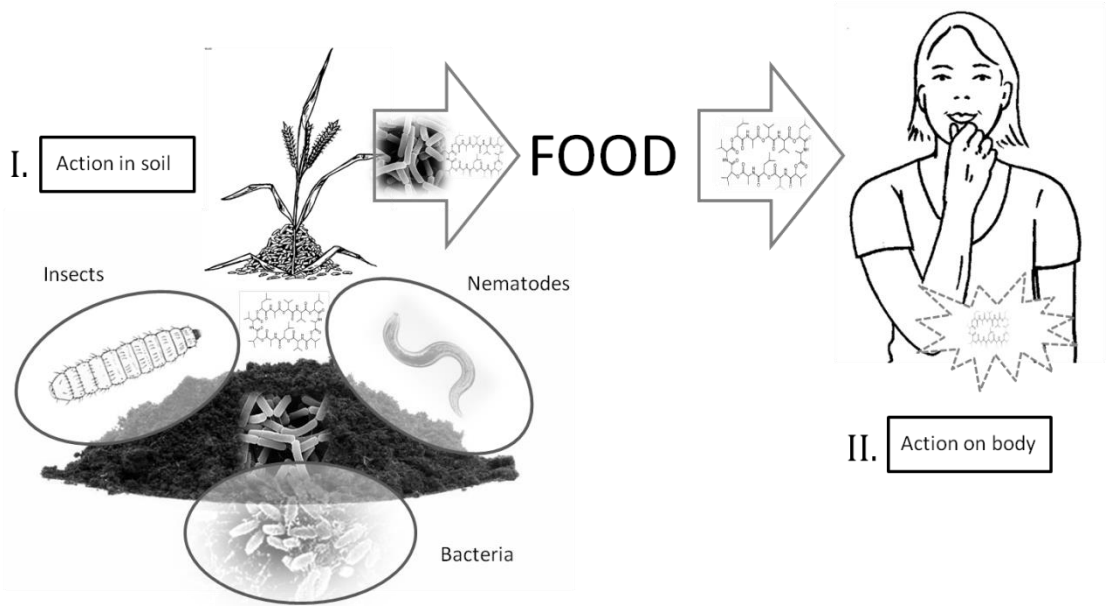


Figure 3: Research objectives of the thesis[1] I. Impact of cereulide on habitat of *B. cereus* II. Actions on the mammal host. Emetic *B. cereus* strains or the toxin can enter the food chain and ingestion of cereulide contaminated food may cause severe intoxications even involving in hospitalization or death.

The pathogenicity of emetic *Bacillus cereus* arises from the small dodecadepsipeptide cereulide. This cyclic toxin is resistant to heat, proteolysis as well as to acid and basic conditions. Because of these properties cereulide is stable against many environmental factors and once preformed in food, it will not be destroyed in the intestinal tract or by reheating of food.

The first aim of the thesis was to establish an effective bio fermentation system for cereulide production in high quality and quantity, and to develop a precise quantification system for cereulide in complex matrices such as food and clinical samples.

After establishment of the fundamental methods, the action of the toxin on other organisms was characterised. Since the ecological role of cereulide as well as the mode of its action on other organisms was hitherto unknown, the influence of cereulide and cereulide producing *B. cereus* strains on different soil living organisms such as insects, nematodes and bacteria was investigated. In addition, purified cereulide toxin was used to study the toxin action of cereulide on the body. For the latter task a pig feeding model was established and, in addition, the in vitro effect of cereulide on gastric muscle activity was analysed.

[1] Figure was created of illustrations taken from following sources: http://www.themicrobiologist.tumblr/ post/ 12210008690; http://www.iter.kbs.msu.edu/who-we-are/research.highlights/soil-microbes; http://www.sign.lang.uni-hamburg.de/alex/lemmata/e_lemma/essen.html; http://www.4to40.com/coloring_ book/Flora.asp?counter=60; http://www.ipm.ncsu.edu/AG369/keys.html (Status 17.02.2012)

1 Introduction

2. MATERIALS AND METHODS

2.1 ORGANISMS AND GROWTH CONDITIONS

2.1.1 *B. cereus* Strains

The emetic reference strain *B. cereus* F4810/72, originally isolated from vomit (Public Health Laboratory Service; London, UK (Turnbull *et al.*, 1979)), was used for cereulide and $^{13}C_6$-cereulide production. Other *B. cereus* strains used in this study are listed in table 2. Strains tested for antimicrobial analysis of cereulide are listed in Table 8 with culture conditions and references.

Table 2: *B. cereus* strains

Strain	Description	Reference
F4810/72	Emetic reference strain	(Turnbull *et al.*, 1979)
F4810/72 pCER270$^-$	F4810/72 without pCER270 plasmid	(Lücking, 2009)
F4810/72 pMDX [cspA/*lux*]	F4810/72 containing the *cspA* promotor upstream of lux operon (*luxABCDE*) on pMDX plasmid Cm^r	(Dommel, 2008)
F4810/72 pMDX [P_1/*lux*]	F4810/72 containing the cereulide synthetase central P_1 promotor upstream of lux operon (*luxABCDE*) on pMDX plasmid Cm^r	(Dommel *et al.*, 2010)
F4810/72 Δ*plcR*	F4810/72 plcR deletion mutant: Spc^r	(Lücking *et al.*, 2009)
F4810/72 Δ*plcR* pMDX [cspA/*lux*]	F4810/72 Δ*plcR* containing the *cspA* promotor upstream of lux operon (*luxABCDE*) on pMDX plasmid Cm^r ; Spc^r	This study
F4810/72 Δ*plcR* pMDX [P_1/*lux*]	F4810/72 Δ*plcR* containing the cereulide synthetase central P_1 promotor upstream of lux operon (*luxABCDE*) on pMDX plasmid Cm^r ; Spc^r	This study

2.1.2 Bacterial Growth Conditions for *B. cereus*

B. cereus F4810/72 strain was routinely grown in lysogeny broth (LB) (150 rpm), on LB agar or plate count (PC) agar at 30 °C. To analyse cereulide production LB broth (100 mL) was inoculated with nearly 10^3 cfu mL^{-1} from on overnight culture (3 mL LB broth, 150 rpm, 24 °C or 30°C) and cultures were incubated in baffled flasks (Schott, 500 mL) with rotary shaking (150 rpm) at 24 or 30°C for up to 24 hours. Antibiotics were used when appropriate (see Table 2): spectinomycin (100 µg ml^{-1}) and chloramphenicol (5 µg mL^{-1}).
For long time storage of bacteria an overnight bacterial culture was supplemented with nearly 15 % glycerol (v/v; from sterilized 87 % glycerol solution), frozen in fluid nitrogen and stored at -80°C.
Liquid media (Table 3; except MOD) were prepared with dH_20 and sterilized by auto-claving (121°C, 17 min). For solid medium, 15 g agar per litre of liquid media was used. Modified MOD medium (Glatz & Goepfert, 1976; Rosenfeld *et al.*, 2005) was adjusted to pH 7.2 and autoclaved at 110°C for 10 min. Trace elements for MOD stock solutions were solved in ddH_2O, sterilized by filtration (0.22 µm pore size) and added to the autoclaved and cooled MOD medium. Chemicals for media were purchased from Oxoid, Sigma-Aldrich and Merck.

2.1.3 Chemicals & Materials

Chemicals were purchased from AppliChem, Baker, Carl Roth GmbH, Merck, Serva, and Sigma-Aldrich. Plastic and related articles: plastic tubes (Greiner), PTFE-filters and SPE-cartridges (Phenomenex,), MF- filters (Millipore). Cell culture equipment was ordered by Biochrom. The cereulide structure analogue valinomycin and Polymyxin-B-nonapeptide (PMBN) were obtained from Sigma-Aldrich. Valinomycin was solved in ethanol (≥99.9 %; Baker) at 20 mg ml^{-1} and stored at 8°C. The stable isotope labelled $^{13}C_1$-valine was from Euroiso-top (Saint Aubin Cedex, France). Dimethyl sulfoxide (DMSO; ≥ 99 %; Sigma-Aldrich) was sterilized by filtration (PTFE; Sartorius) and stored in the dark at RT. Buffers and solutions were sterilized by autoclavation (17 min, 121°C) or filtered (0.22 µm pore size). Purified cereulide and $^{13}C_6$-cereulide (see 2.3.2) powder was stored at -20°C. Stock solutions of cereulide (3 mg mL^{-1}) and $^{13}C_6$-cereulide (0.1 mg mL^{-1}) were prepared in DMSO (stored at RT) or in ethanol (≥99.9 %; Baker) and stored at 8°C.

Table 3: Used Media

LB:	5 g L^{-1}	Yeast extract
	10 g L^{-1}	Peptone (from casein, enzymatic digest)
	10 g L^{-1}	NaCl
K^+ LB:	5 g L^{-1}	Yeast extract
	10 g L^{-1}	Peptone (from casein, enzymatic digest)
	12.7 g L^{-1}	KCl
PC:	5 g L^{-1}	Peptone (from casein, enzymatic digest)
	2.5 g L^{-1}	Yeast extract
	1 g L^{-1}	D-Glucose
TSB:	15 g L^{-1}	Tryptone
	10 g L^{-1}	Soy peptone
	10 g L^{-1}	NaCl
BHI:	37 g L^{-1}	Brain heart broth (Merck, Germany)
MRS:	52 g L^{-1}	Lactobacilli broth acc. to De Mas, Rogasa and Sharp (Oxoid)
MOD:	0.39 g L^{-1}	L-glycine
	2 g L^{-1}	L-glutamic acid
	0.91 g L^{-1}	L-valine
	0.91 g L^{-1}	L-threonine
	0.4 g L^{-1}	L-methionine
	0.36 g L^{-1}	L-histidine
	0.46 g L^{-1}	L-arginine
	0.91 g L^{-1}	L-aspartic acid
	0.04 g L^{-1}	L-cysteine
	0.46 g L^{-1}	L-arginine
	0.7 g L^{-1}	L-isoleucine
	1.37 g L^{-1}	L-leucine
	0.28 g L^{-1}	L-phenylalanine
	1.18 g L^{-1}	L-lysine
	0.66 g L^{-1}	L-serine
	0.042 g L^{-1}	L-tyrosine
MOD trace elements (1000x)	0.675 g L^{-1}	$FeCl_3$ x 6 H_20
	0.05 g L^{-1}	$MnCl_2$ x 4 H_20
	0.03 g L^{-1}	Na_2MoO_4 x 2 H_20
	0.275 g L^{-1}	$CaCl_2$
	0.085 g L^{-1}	$ZnCl_2$
	0.03 g L^{-1}	$CoCl_2$ x 6 H_2O
	0.04 g L^{-1}	$CuSo_4$
	0.024 g L^{-1}	$NaSeO_4$

2.2 DNA ISOLATION AND TRANSFORMATION

2.2.1 Plasmid Isolation

Cells of pre-cultures of *B. cereus* F4810/72 pMDX [cspA/*lux*] *or* F4810/72 pMDX [P_1/*lux*] were disrupted using zirconium silica beads (diameter 0.1 mm) in a RiboLyser homogenizer (Hybaid) (time: 45 s; speed 6.5 m/s). Plasmid DNA was isolated from disrupted cells using the GeneElute™ Plasmid Miniprep kit (Sigma-Aldrich) following the manual instructions.

2.2.2 Transformation of DNA

B. cereus Δ*plcR* was transformed by electroporation. Therefore, cells were grown until OD_{600} of 0.5 in LB supplemented with 2 % glycine, washed twice with ice cold glycerol solution (2.5 %, 5 % and 10 %) and resuspended in 10 % glycerol solution and frozen in liquid nitrogen and stored at -80 °C. Electroporation was performed with 1 µg plasmid DNA and 100 µl electro-competent *B. cereus* cells using a 0.4 cm cuvette and a BioRad Gene Pulser (2.0 kV, 25 µF, 200 Ω, 4.6-4.8 ms). After pulsing cells were resuspended in warm LB broth and incubated 2 hours at 30°C. Cells were spread on LB agar plates supplemented with chloramphenicol and spectinomycin and incubated for 20 hours at 30°C. Transformed cells were identified by luminescence signal using the Xenogen IVIS Lumina system.

2.3 IDENTIFICATION OF CEREULIDE VIA HPLC

100 mL broth was inoculated with 10^3 cfu mL^{-1} of a *B. cereus* F4810/72 overnight culture and incubated at 30°C and 150 rpm. After 24 h the cultures were autoclaved (120°C; 19 min) to destroy heat-labile proteins and other substances and centrifuged (10 min; 8600g; 20°C). Cell pellets were frozen in liquid N_2 and stored at -18 °C until used. Thawed pellets were solved in 10 mL ethanol (>99.8%, Baker), extracted overnight on a rocking table, centrifuged (10 min; 8600g; 20°C) and filtered (Phenex; 0.2 µm) to remove cell debris. The ethanol extract was concentrated to a final volume of 1 mL.

To identify the cereulide containing fraction the *B. cereus* extract was analysed by FPLC (Fast protein liquid chromatography) using the ÄKTA purifier 10 (GE Healthcare). Controlling of the FPLC system and evaluation of the runs were performed via the Unicorn 5.11 software (GE Healthcare). Separation of metabolites by reversed phase took place on a silica based C12 column (Jupiter Proteo 250 x 4.6 mm, particle Size 4µm, pore Size 90Å; Phenomenex) with a C12 guard column (SecurityGuard cartridge; 4 x 3 mm, Phenomenex). The *B. cereus* extract was 1:10 (v/v) diluted in MilliQ-water and 5 mL was injected into the FPLC system. The mobile phases ethanol and MilliQ-water were degassed in an ultrasonic

bath. Before use the FPLC system was flushed with mobile phases to make sure that there are no air bubbles in the pumps and lines. First, the column was washed with ethanol, then with a mixture of ethanol and water (1/10, v/v) with a flow rate of 0.5 mL min^{-1}. For cereulide purification following protocol was established: After equilibration with 10 mL of a mixture of ethanol and MilliQ-water (1/10, v/v) 5 mL of the extract was injected. The column was washed with 15 mL of equilibration solution to remove all hydrophilic substances followed by an isocratic gradient from 10-100 % ethanol solution in 60 min to elute hydrophobic and strong hydrophobic substances. Then the column was washed with ethanol to remove nearly all remaining substances from the column. Running conditions: flow rate of 0.5 mL min^{-1}, 24°C. The absorption of the eluents was measured at 210 and 280 nm. Fractions displaying peaks over 1200 mAU (210 nm) were collected automatically using the fraction collector Frac-950 (GE Healthcare) and stored at 8 °C.

2.4 Development of a Quantification System for Cereulide[2]

2.4.1 Biosynthetic Production of Cereulide and $^{13}C_6$-Cereulide

For production of cereulide, LB broth (100 mL) supplemented with 0.2 % glucose (11 mmol) was inoculated with nearly 10^3 cfu mL^{-1} from an overnight pre-culture and cultures were incubated in baffled flasks (500 mL) whilst rotary shaking (150rpm) at 24°C for 24 h. For production of the $^{13}C_6$-labelled cereulide, the MOD broth (Glatz & Goepfert, 1976) (550 mL) without added valine and additional C-source was supplemented with $^{13}C_1$-L-valine (0.5 mg) and was inoculated with 10^3 cfu mL^{-1} from an overnight pre-culture. The cultures were incubated with shaking (150 rpm) at 24 °C for 48 h. The cultures were autoclaved (20 min, 120 °C) to denature heat labile substances and centrifuged (8600g, 20°C, 10 min). The pelletized cells were frozen in liquid nitrogen and stored at -18°C until used. An aliquot (1 g) of the *B. cereus* biomass was extracted with ethanol (10 mL) by shaking at 20°C for 15 h, the extract was centrifuged twice at 8.600 g for 15 min, the supernatant obtained was centrifuged at 14.000 g for 5 min, then membrane filtered (0.2 µm; PTFE membrane, Phenomenex, Aschaffenburg, Germany) to remove remaining spores and cell debris. The solvent was evaporated under reduced pressure, the residue was dissolved in a methanol/water mixture (100 mL; 10/90, v/v), and aliquots (10 mL) were applied onto the top of a C18-SPE cartridge (60 mL, 10g, Strata C18-E, Phenomenex, Aschaffenburg, Germany) conditioned with methanol, followed by water. The cartridge was rinsed with

[2] Part 2.4 of this thesis was taken from "Bauer, T., Stark, T., Hofmann, T., Ehling-Schulz, M. (2010) Development of a stable isotope dilution analysis (SIDA) for the quantification of the *Bacillus cereus* toxin cereulide in foods. J Agric Food Chem 58: 1420-1428". The experiments were done, and the text was written, in collaboration with T. Stark.

water (40 mL), the target compound was eluted with ethanol (100 mL), and after the effluent was concentrated under reduced pressure to 10 mL, it was isolated by means of RP-HPLC (Jasco, Groß-Umstadt, Germany).

2.4.2 Preparative Purification via HPLC System

The HPLC system consisted of a HPLC-pump system PU 2087, a high-pressure gradient unit and a PU-2075 UV-detector using a preparative RP-18 column, Microsorb 100-5 C18, 21.2 × 250 mm, 5 µm (Varian, Darmstadt, Germany) as the stationary phase. Monitoring the effluent at 210 nm, chromatography was performed with a mixture (90/10, v/v) of methanol and water for 5 min, increasing the methanol content to 100 % over 5 min, and, thereafter, eluting with methanol for 10 min at a flow rate of 20.0 mL/min. After removing the solvent in vacuum, the target compound was suspended in water (10 mL), and freeze-dried twice to afford cereulide and $^{13}C_6$-cereulide (Figure 9), respectively, as white, amorphous powder in high purities of more than 98 %.

Cereulide, **1** (Figure 9). UV/Vis (MeOH): λ_{max}=204 nm; LC-TOF-MS: *m/z* 1175.6669 ([M+Na]$^+$, measured), m/z 1175.6673 ([$C_{57}H_{96}N_6O_{18}Na$]$^+$, calculated); MS (ESI$^+$): *m/z* 1170.9 (100, [M+NH_4]$^+$), 596.6 (70, [M+H+K]$^{2+}$), 1175.9 (40, [M+Na]$^+$), 1191.7 (25, [M+K]$^+$), 1153.8 (10, [M+H]$^+$), 588.6 (10, [M+Na+H]$^{2+}$); MS (ESI$^-$): *m/z* 1198.3 (100, [M+HCOOH-H$^+$]$^-$), 1188.3 (71, [M+Cl-H$^+$]$^-$), 1152.2 (12, [M-H$^+$]$^-$; MS/MS of *m/z* 1153.9 (ESI$^+$, CE=+52 V; intensities >20 %): *m/z* (%) 1153.8 (100), 172.2 (59), 357.2 (47), 314.2 (36), 158.2 (28), 154.2 (27), 186.2 (26), 499.4 (26), 200.2 (25), 385.2 (24), 72.0 (21); MS/MS of *m/z* 1151.8 (ESI$^-$, CE=-54 V; intensities >15 %): *m/z* (%) 216.2 (100), 202.2 (90), 1151.8 (57), 970.6 (41), 785.6 (26), 984.6 (25), 131.0 (17), 116.0 (16), 401.2 (15); ^{1}H NMR (500 MHz, DMSO-d_6, COSY, 27°C): δ0.80 [d, 9H, *J*=7.15 Hz, H-C(8b)], 0.82 [d, 9H, *J*=6.20 Hz, H-C(2c)], 0.84 [d, 9H, *J*=6.20 Hz, H-C(2d)], 0.86 [d, 9H, *J*=6.65 Hz, H-C(8c)], 0.88 [d, 9H, *J*=6.70 Hz, H-C(11b)], 0.90 [d, 9H, *J*=6.70 Hz, H-C(11c)], 1.35 [d, 9H, *J*=7.30 Hz, H-C(5a)], 1.54 [m, 3H, *J*=12.50 Hz, H-C(2a_1)], 1.61 [m, 3H, *J*=6.30 Hz, H-C(2b)], 1.65 [m, 3H, *J*=12.15 Hz, H-C(2a_2)], 2.11 [m, 3H, *J*=4.80, 6.65 Hz, H-C(8a)], 2.18 [m, 3H, *J*=6.70 Hz, H-C(11a)], 4.29 [dd, 3H, *J*=7.35, 7.45 Hz, H-C(11)], 4.36 [qd, 3H, *J*=7.10, 7.30 Hz, H-C(5)], 4.83 [d, 3H, *J*=4.47 Hz, H-C(8)], 5.00 [dd, 3H, *J*=3.73, 9.58 Hz, H-C(2)], 7.99 [d, 3H, *J*=7.66 Hz, H-N(10)], 8.31 [d, 3H, *J*=6.70 Hz, H-N(4)]. ^{13}C NMR (125 MHz, DMSO-d_6, HSQC, HMBC, 27°C): δ 16.59 [C(8b)], 16.69 [C(5a)], 18.08 [C(11c)], 18.49 [C(8c)], 18.85 [C(11b)], 20.97 [C(2c)], 22.93 [C(2d)], 23.81 [C(2b)], 29.46 [C(11a)], 30.07 [C(8a)], 40.18 [C(2a)], 48.00 [C(5)], 57.60 [C(11)], 71.65 [C(2)], 77.09 [C(8)], 168.83 [C(9)], 169.33 [C(3)], 170.49 [C(12)], 171.32 [C(6)].

$^{13}C_6$-Cereulide, **$^{13}C_6$-1** (Figure 9). UV/Vis (MeOH): λ_{max}=204 nm; LC-TOF-MS: *m/z* 1181.6987 ([M+Na]$^+$, measured); MS (ESI$^+$): *m/z* 1177.0 (100 %, [M+NH_4]$^+$), 1182.0 (44 %, [M+Na]$^+$), 1197.9 (22 %, [M+K]$^+$), 1159.8 (12 %, [M+H]$^+$), 591.7 (10 %, [M+Na+H]$^{2+}$); MS/MS of *m/z* 1160.0 (ESI$^+$, CE=+52 V; intensities >10 %): *m/z* (%) 1160.0 (100), 173.2 (36), 358.2 (28), 316.4 (36), 1131.0 (23), 387.2 (16), 186.2 (15), 155.2 (14), 202.2 (14),

298.4 (12), 501.4 (12); ^{1}H NMR (500 MHz, DMSO-d_6, COSY, 27°C): δ 0.84 [d, 9H, *J*=6.80 Hz, H-C(8b)], 0.86 [d, 9H, *J*=6.07 Hz, H-C(2c)], 0.88 [d, 9H, *J*=6.33 Hz, H-C(2d)], 0.90 [d, 9H, *J*=6.87 Hz, H-C(8c)], 0.92 [d, 9H, *J*=6.80 Hz, H-C(11b)], 0.94 [d, 9H, *J*=6.63 Hz, H-C(11c)], 1.39 [d, 9H, *J*=7.31 Hz, H-C(5a)], 1.58 [m, 3H, *J*=12.77 Hz, H-C(2a$_1$)], 1.65 [m, 3H, *J*=6.20 Hz, H-C(2b)], 1.69 [m, 3H, *J*=12.77 Hz, H-C(2a$_2$)], 2.14 [m, 3H, *J*=1.80, 4.95, 6.69 Hz, H-C(8a)], 2.22 [m, 3H, *J*=2.22, 6.70 Hz, H-C(11a)], 4.34 [ddd, 3H, *J*=2.20, 2.40, 6.32, 7.60 Hz, H-C(11)], 4.40 [qd, 3H, *J*=7.27, 7.39 Hz, H-C(5)], 4.85 [dd, 3H, *J*=3.80, 4.00 Hz, H-C(8)], 5.03 [m, 3H, *J*=3.13, 9.72 Hz, H-C(2)], 8.06 [dd, 3H, *J*=3.50, 7.34 Hz, H-N(10)], 8.37 [d, 3H, *J*=6.72 Hz, H-N(4)]. ^{13}C NMR (125 MHz, DMSO-d_6, HSQC, HMBC, 27°C): δ 16.49 [C(8b)], 16.63 [C(5a)], 17.98 [C(11c)], 18.39 [C(8c)], 18.75 [C(11b)], 20.87 [C(2c)], 22.84 [C(2d)], 23.68 [C(2b)], 29.35 [C(11a)], 30.96 [C(8a)], 40.07 [C(2a)], 47.97 [C(5)], 57.55 [d, *J*=61.91 Hz, C(11)], 71.54 [C(2)], 76.96 [d, *J*=55.67 Hz, C(8)], 168.78 [C(9)], 169.27 [C(3)], 170.38 [C(12)], 171.21 [C(6)].

2.4.3 Sample Clean-Up and HPLC-MS/MS Analysis of Cereulide in Rice

A sample of rice (10 g), prepared by boiling parboiled rice (Le Gusto, Van Sillevoldt Rijst B.V., Netherlands) for 18 min, was spiked with different amounts of purified cereulide (0, 10, 20 and 50 µg), homogenized, then spiked either with $^{13}C_6$-cereulide (20 µg), or valinomycin (20 µg) as the internal standard. To simulate the matrix of an oil-rich food, a sample of rice (10 g) was supplemented with sunflower oil (1.1 g; Thomy, Nestle) and intimately mixed prior to spiking with cereulide and the internal standard. After equilibration on a rocking table for 2.5 h at 20°C, each sample was extracted by shaking with ethanol (10 mL) on a rocking table for 15 h at 20°C, and after centrifugation and membrane filtration as described above, the supernatant was 1:10 diluted with water. An aliquot (1.0 mL) of the diluted extract was applied onto the top of a C18-SPE cartridge (6mL; 1000 mg; Strata C18-E, Phenomenex, Aschaffenburg, Germany) and, after rinsing with water (4 mL) and methanol/water (2 mL; 70/30, v/v), the target compounds were eluted with ethanol (1 mL) and aliquots (5 μL) were analysed by means of LC-MS/MS on a Synergi Fusion-RP, 2 x 150 mm, 4 µm, column (Phenomenex, Germany) operating at a flow rate of 0.25 mL/min. Chromatography was performed using a solvent gradient starting with methanol/water (92/8, v/v) for 3 min, thereafter, the methanol content was increased to 100 % within 7 min, and, finally, held at 100 % for 10 min.

2.4.4 LC-MS /MS

LC-MS/MS analysis was performed using an Agilent 1200 HPLC-system connected to the API 4000QTrap LC-MS/MS (Applied Biosystems, Darmstadt, Germany) running in the positive electrospray ionization (ESI$^+$) mode. Zero grade air served as nebulizer gas (45 psi) and turbo gas (425°C) for solvent drying (55 psi), nitrogen was used as curtain (20

psi) and collision gas (8.7x10^{-7} psi), respectively. Both quadrupoles were set at unit resolution. By means of the multiple reaction monitoring (MRM) mode, cereulide (**1**, *m/z* 1170.9→172.3), $^{13}C_6$-cereulide (**$^{13}C_6$-1**; *m/z* 1176.9→173.3), as well as valinomycin (**2**, *m/z* 1128.8→172.2) were analysed using the mass transitions (given in brackets) monitored for a duration of 55 ms. In addition, three further mass transitions of the singly charged pseudomolecular ions ($[M+H]^+$) and to the corresponding daughter fragment ions and of the corresponding ammonium ions ($[M+NH_4]^+$) to the same daughter fragment ions were recorded (Table S1). ESI$^+$ mass and product ion spectra were acquired with direct flow infusion. For ESI$^+$, the ion spray voltage was set at +5500 V in the positive mode. The MS/MS parameters were tuned for each individual compound, detecting the fragmentation of the $[M+H]^+$ and $[M+NH_4]^+$ molecular ions into specific product ions after collision with nitrogen (8.7x10^{-7} psi). The declustering potential (DP), the collision energy (CE), and the cell exit potential (CXP) were set as given in Table S1.

2.4.5 Quantitative Analysis of Cereulide in Food Samples

To investigate the cereulide production by *B. cereus* F4810/72 in food system, boiled rice samples (10 g) were inoculated with 150 cfu g^{-1} rice from an overnight pre-culture diluted in saline buffer (0.9 % NaCl) and incubated at 24 °C. After 0, 6, 12, 24, 36, 48, 60, 72, and 96 h, the samples were spiked with the internal standard $^{13}C_6$-cereulide (10 µg), equilibrated for two hours while shaking at 4 °C, and then extracted by ethanol (10mL) on a rocking table for 24 h. The samples were centrifuged, the supernatants were membrane filtered and concentrated to a volume of about 3 mL, and after C18-SPE cartridge cleanup as detailed above, aliquots (5 µL) were analysed by means of LC-MS/MS as described above. In addition, a sample of milk pudding and liver sausage were artificially contaminated and analysed as follows: 15 g of milk pudding or liver sausage was inoculated with 150 cfu/g food of a *B. cereus* F4810/72 overnight culture. After incubation at 24 °C for 24 h, the food samples were spiked with $^{13}C_6$-cereulide (10 µg), equilibrated while shaking for two hours at 4 °C, and extracted with ethanol (15 mL). Sample cleanup and analysis were performed as shown above for rice samples.

2.4.6 Calibration

For quantitation, the analyte cereulide (**1**) and either $^{13}C_6$-cereulide (**$^{13}C_6$-1**), or valinomycin (**2**) as an internal standard was mixed in five molar ratios from 0.1 to 10 (1-100 ng/mL in EtOH) and analysed by means of HPLC-MS/MS in the MRM mode in triplicates. Calibration solutions were stored in the dark at 4 °C, and the stability was checked via HPLC and LC-MS before use. Calibration curves were prepared by plotting peak area ratios of analyte to internal standard against concentration ratios of each

analyte to the internal standard using linear regression. The equations obtained were y = 0.485 x (**1**/^{13}**C$_6$-1**,R= 0.997) and y = 0.137 x (**1**/**2**, R = 0.998), respectively.

2.4.7 Method Validation

Linearity was evaluated using mixtures of internal standards and analyte in five molar ratios from 0.1 to 10. To study the repeatability (intraday precision) of the method, a sample of boiled rice (10 g) and a sample of boiled rice supplemented with 10 % sunflower oil were spiked with two different concentrations of cereulide (1 and 5 µg/g) using three replicates for each concentration in 1 day. Reproducibility (interday precision) was studied with a sample of rice (10 g) and a sample of rice supplemented with 10 % sunflower oil, respectively, both spiked with cereulide (2 µg/g) and analysed at four different days. The recovery determination was performed using a rice sample (10 g) and a sample of rice supplemented with 10 % sunflower oil, respectively, both spiked with cereulide (1, 2, and 5 µg/g) each in three replicates. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the concentrations for which signal-to-noise ratios were 3 and 10, respectively.

2.4.8 Investigations of Matrix Effects

To study possible matrix effects during the LC-MS/MS analysis of the ethanolic rice extracts, a sample of boiled rice (10 g) inoculated with 1x10^7 cfu of *B. cereus* F4810/72 was analysed using the same LC-MS/MS parameters as listed above. A constant flow of 10 µL/min of a solution of valinomycin (**2**, 0.6 µmol/L) was introduced to the solvent flow via a three-way valve by means of a PHD 4400 Hpsi type syringe pump (Harvard Apparatus).

2.4.9 LC/Time-of-Flight Mass Spectrometry (LC/ESI-TOF-MS)

Mass spectra of the target compounds were measured on a Bruker Micro-TOF-Q (Bruker Daltronics, Bremen,Germany) mass spectrometer with flow injection referenced on sodium formate (5 mmol). The compounds were dissolved in MeOH, and 10 µL of a saturated solution of Naformiat in MeOH was added to measure the exact mass of the sodium adducts. Data processing was performed by using Daltonics Data Analysis software (version 3.4; Bruker).

2.4.10 NMR Spectroscopy

^{1}H, gs-COSY, gs-HSQC, gs-HMBC, ^{13}C, and DEPT-135 NMR measurements were performed on an Avance 3 DRX 500MHz spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts were referenced to tetramethylsilane or the solvent signal. Data processing was

performed by using Topspin Version 1.3 (Bruker, Rheinstetten) and MestReNova version 5.2.3 software (Mestrelab Research, Santiago de Compostela, Spain).

2.5 Effect of Cereulide on Other Organisms

2.5.1 Antimicrobial Activity

The antimicrobial activity of cereulide and valinomycin was determined by growth experiments using the Bioscreen C analyser (Oy Growth Curves AB Ltd, Helsinki, Finland). Broth and growth temperature are provided in Table 8. To determine the minimum inhibitory concentration (MIC) the bacteria were generally grown in K^+LB media containing KCl instead of NaCl (Table 3). From cereulide and valinomycin stock solutions (3 mg ml^{-1} in DMSO) serial dilutions were made in microtitre plates (100 well) resulting in final concentrations of 0.1 to 50 mg ml^{-1}. After addition of diluted overnight cultures of bacteria to an optical density (OD_{600}) of 0.1-0.12, the plates were incubated at the desired temperature for 24 or 30 hours with the analyser recording the optical density (OD_{600}) of each well every half hour. Pure medium and DMSO in a final concentration of 3 % served as controls. To investigate the influence of different chlorides on the antimicrobial activity of cereulide, *Brevibacterium casei* (food isolate; *WS 3023*) was incubated with cereulide in different LB broth variants containing LiCl, NaCl, KCl, $MgCl_2$, NH4Cl or CsCl in a final concentration of 171 mM. Growth curves were generated by mean of two experiments with duplicates. Minimum inhibitory concentrations were determined on the basis of the growth curves after 24 hours of incubation. To analyse the barrier function of the outer membrane of Gram negative bacteria in response to cereulide and valinomycin, *E. coli* MG1655 (Blattner *et al.*, 1997) was incubated with different concentrations of cereulide and valinomycin as described above, but with and without addition of 10 µg mL^{-1} polymixin B nonapeptide (PMBN). Duration of lag phase (λ) and maximal growth rate (μ_{max}) were determined after growth fitting via the modified Gompertz function according to (Kleer & Hildebrandt, 2002).

2.5.2 Cytotoxicity Assay

The toxicity of cereulide and valinomycin towards HEp-2 cells in response to different KCl concentrations (5, 25, 50 mM) was analysed using the HEp-2 cytotoxicity assay (Lücking *et al.*, 2009). Various dilutions of cereulide (0.1-50 ng ml^{-1} in 5 and 25mM KCl; 0.01-5 ng ml^{-1} in 50 M KCl) and valinomycin (1-500 ng ml^{-1} in 5 and 25 mM KCl; 0.1-50 ng ml^{-1} in 50 mM KCl) were made in 96 well microtitre plates using Eagle's minimum essential medium with Earl salts and glutamine (Biochrom) supplemented with 1 % fetal calf serum (Thermo), 1 % sodium pyruvate (Biochrom), 0.4 % penicillin-streptomycin (Biochrom) and 2 % ethanol to ensure solubility of both toxins. After addition of HEp-2 cells to a final

concentration of 2.8 x10^5 cells ml^{-1}, plates were incubated for 48 h at 37°C and 5 % CO_2. Cytotoxicity was determined by adding the cell proliferation reagent WST-1 (Roche). The produced formazan was measured in a microplate reader at A_{450} after 20 min incubation at 37°C. The 50 % inhibitory concentration was calculated from three independent experiments on the basis of a dose response curve (Dietrich *et al.*, 1999).

2.5.3 Antiparasitic Activity

Antiparasitic activity of cereulide and valinomycin was tested on the Institute of Parasitology of the Vetmeduni Vienna by Katja Silbermayr. A monospecific laboratory strain of *O. dentatum* (OD-Hann) is propagated continuously at the Institute of Parasitology of the Vetmeduni Vienna for the production of worm eggs (by permission of the Austrian Ministry of Science and Research, GZ BMWF-68.205/242/3b/2010). All animal experimentation was conducted in accordance with national Animal Welfare Regulations and approved by the Animal Ethics Committee.

The ensheathed third-stage larvae (L3) were harvested and purified from coprocultures (Talvik *et al.*, 1997) of experimentally infected pigs and cleaned worms were stored in distilled water at 11 °C for a maximum of six month. For culture experiments the L3 (100 L3/ml medium) were purified using a small-scale agar-gel migration and exsheathed with sodium hypochlorite (10-14 % free chlorine) to 1 ml of a larval suspension (Joachim et al., 1997). A defined larval population of exsheathed L3 was thus obtained and further maintained in 24-well culture plates in 1 ml medium containing 75 % LB-medium (Roth, Lactan, Graz, Austria), 25 % inactivated pig serum and 1 % antibiotics and antimycotics (10 0000 U penicillin, 10 mg streptomycin and 25 µl amphotericin B per 50 ml of medium; PAA, The Cell Culture Company, Pasching, Austria). Plates were incubated at 38.5 °C and 10 % CO_2 for 14 days with medium change on days 4 and 11.

To determine the antiparasitic effect toxins were added in decreasing concentrations. Cereulide was applied to the culture medium in concentrations of 20 µg ml^{-1} – 2 µg ml^{-1} and valinomycin from 2 µg ml^{-1} – 0.5 µg ml^{-1}. To avoid precipitation of the cereulide the sodium chloride (NaCl) in the LB culture medium was substituted with potassium chloride (KCl). The rate of development of L3 to fourth-stage larvae (L4) development was determined on days 5, 7, 11 and 13 of culture. The effects on the nematode development, the parasite vitality and the survival rate were observed and assessed visually using an inverted light microscope (Diaphot 300, Nikon Corporation) and the percentage of dead L3 and developed L4 was calculated. Each treatment group consisted of three replicate wells and appropriate solvent controls were used for each assay.

2.5.4 *Galleria mellonella* as Model Host

Last instar larvae of the greater wax-moth *Galleria mellonella* (Kerf-Terraristik, Hamm, Germany) were sorted by weight in groups (± 50 mg) and stored with wood shavings in the dark at 15°C. All larvae were used within 3 weeks of receipt.

2.5.4.1 Toxin Injection Experiments

Larvae (0.3-0.35 g) in groups of ten were injected with each 5 µl of cereulide or valinomycin solved in 10 % or 30 % (for 10 µg g^{-1} bw) ethanol through the last left pro-leg into the haemocoel using a 25 µl syringe with a needle diameter of 0.31 mm (Hamilton,) as described previously (Cotter *et al.*, 2000; Ratcliffe, 1983). Three controls were used in all assays: untouched larvae, larvae with 5 µl 10 % ethanol and with 30 % ethanol. After injection, the larvae were incubated in petri dishes with wood shavings at 24 °C and observed over a period of 2 days. Larvae were considered paralysed if they were white and failed to respond to tweezers stimulation. Dead larvae could be differentiated from the paralysed phenotype by brownish discoloration. Each condition was tested twice with triplicates.

2.5.4.2 Analysis of *ces* Promotor Activity in *G. mellonella*

For investigation of cereulide synthetase promotor activity expression experiments larvae (0.4-0.45 g) were injected with 5 µl bacterial cultures F4810/72 or *B cereus F48ΔplcR* (Lücking *et al.*, 2009) transformed with pMDX[P_1/*luxABCDE*] (Dommel *et al.*, 2010) or pMDX[*cspA*/*luxABCDE*] incubated for 16 h at 24°C and adjusted to an optical density at 600 nm of 0.5. The treated larvae were incubated at 24°C and luciferase activity of injected strains was visualized in live or dead *G. mellonella* larvae with a photon-counting intensified-charge-coupled-device (ICCD) camera (Xenogen IVIS Lumina 100, Caliper Life Science, USA). Images were acquired for 10 s with a binning factor of 3 (without filter; relative aperture, 1), and the bioluminescence intensity was superimposed as false-color rendering. Editing of pictures was performed by the Living Image software (Caliper Life Science, USA).

2.6 Intoxication Studies with Pigs

2.6.1 Experimental Animals and Design of Study

Five Piglets (Austrian Large White x Pietrain; Schweinehof Klein Bonnleiten Betriebs GmbH, Stössing, Austria) weighing 10-13 kg were used in each experiment. They were placed in metabolic single cages three days before the start of the experiment for acclimatization. Water and feed (Ferkelkorn, Garant Tiernahrung, Pöchlarn, Austria) were available before and during the whole experiment. One hour before administration blood samples (~4 mL EDTA stabilized blood; ~5 mL heparin-stabilized blood and ~3 mL heparin blood for serum; Primavette V, KABE Labortechnik GmbH, Germany) were taken and the piglets were weighed for calculation of the cereulide concentration (t=0h). In the intoxication experiment with 150 µg kg^{-1} blood samples were taken 68 h before the experiment to reduce the distress for the animals on the day of the experiment start.
On weight calculated cereulide concentrations were mixed with milk (UHT milk ; 3.5 % fat), which was checked for *B. cereus* contamination prior to the experiment by plating on LB as well as MYP agar plates, and offered for feeding to four piglets. One piglet served as control and was fed with milk supplemented with similar ethanol concentration as the experimental piglets. The experiment started after the uptake of the whole milk cereulide mixture.

Table 4: Overview of the pig feedings experiments

Experiment	Cereulide conc. per bodyweight	Administration	Duration	Blood samples
1	10 µg kg^{-1}	one-time	48 h	0, 24, 48 h
2	30 µg kg^{-1}	one-time	48 h	0, 24, 48 h
3	150 µg kg^{-1}	one-time	48 h	0, 8, 24, 48 h
4	10 µg kg^{-1}	each day	7 days	0, 3, 7 d

2.6.2 Clinical Parameters and Sampling

After oral administration of cereulide piglets were monitored for unusual behaviour an several clinical parameters including internal body temperature, pulse and respiration every two to three hours. Blood samples were taken from *Vena cava cranialis*. Urine and faeces were collected during the whole experiment and stored at -80°C. After the experiment the pigs were anaesthetised by intramuscular injection of a mixture of ketamine (10 mg per kg bw) and azaperone (1.3 mg per kg bw). When sedation was deep enough cerebrospinal fluid (liquor) was taken by lumbar puncture. Finally, the pigs were euthanized by intracardial injection of T61®. Dead piglets were dissected and parts of the following organs and tissue were observed and removed for SIDA analysis and histology:

liver, lung, heart, kidney, brain, spleen, small and large intestine, lymph nodes, stomach as well as intestine content, muscle tissue, and abdominal and subcutaneous fat.

2.6.3 Histological Analyses

During the pathological analysis organs were checked for abnormalities and sections of liver, lung, heart, kidney, brain, spleen, small and large intestine were collected for histological analyses performed by the Institute of Pathology, University of Veterinary Medicine Vienna.

2.6.4 Blood Analysis

1 mL EDTA stabilised blood and 3 mL heparin stabilised blood were screened for the content of erythrocytes, haemoglobin, haematocrit, mean cellular volume (MCV), mean cellular heamoglobine (MCH), mean cellular heamoglobine concentration (MCHC), leukocytes, mean peroxidase index (MPXI), monocytes, lymphocytes, eosinophils, basophils, lymphoblasts, juvenils, neutrophils and segmented neutrophils, glucose, urea, creatinine, total protein, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma lactate dehydrogenase (GLDH), gamma glutamyl transferase (GGT), bile acid, lipase, creatine kinase, sodium, potassium, chloride, calcium and phosphor at the central diagnostic unit of University of Veterinary Medicine Vienna.

2.6.5 Immunological Analysis

2.6.5.1 Isolation of Porcine PBMCs from Blood Samples

Dulbecco PBS (PAA Laboratories, Austria) was added to Heparin stabilized blood samples (5-8 mL) adjusted to a final volume of 30 mL. Porcine PBMCs were isolated by gradient centrifugation (900 g, 30 min, 20°C, without break) using lymphocyte separation medium "LSM 1077" (PAA Laboratories, Austria) (cf. (Saalmuller *et al.*, 1987)). Cells were solved in cold PBS solution, pelleted, washed with PBS (350 g, 10 min , 4°C) and resuspended in cell culture medium (RPMI 1640 (PAA Laboratories, Austria) supplemented with 10 % FCS (PAA), 100 IU/mL Penicillin, and 0.1 mg/mL Streptomycin Penicillin/Streptomycin solution (Sigma). Cells were stained with 10 % trypan blue solution and living cell concentration was determined using Neubauer counting chamber. After pelleting isolated PBMCs were resuspended in concentrations of approximately $3x10^7$ cells mL^{-1} in freezing medium (50 % RPMI 1640, 40 % FCS, 10 % DMSO). Then, cells were placed in -80°C freezer and stored at -150°C after 24 to 48 hours.

2.6.5.2 Defrosting of Isolated PBMCs

Frozen cells were placed in a water bath (37°C) until the cell suspension was nearly melted. 1 mL warm RPMI 1640 medium was slowly added and the whole suspension was transferred in 10 mL pre-heated RPMI 1640 medium. After pelletizing (1300 rpm, 10 min, RT) cells were resuspended in porcine plasma buffer.

2.6.5.3 Flow Cytometric (FCM) Staining

Approximately $4x10^5$ cells in (200 µL) porcine plasma buffer were placed in each well of a microtitre plate. Primary antibodies (see Table 5 for first and secondary antibody description) and controls (porcine plasma buffer) were added. After an incubation of 20 minutes on ice cells were washed twice with 200 µL porcine plasma buffer, centrifuged (1300rpm, 4 min, 4°C) and the supernatant was discarded. After addition of secondary antibodies (Table 5) cells were incubated on ice for 20 min and washed twice with 200 µl porcine plasma buffer. Cells without additional treatments were solved in 200 µL porcine plasma buffer for flow cytometric analysis.

For characterization of B cells, cells had to fixed and permeabilized for staining of intracellular antigens cells (cf. (Gerner *et al.*, 2008; Käser *et al.*, 2008)). Therefore, cells were resuspended in 200 µL FACS-Fix + 0.1% Saponin solution (4.5 mL sterile Aqua dest.; 600 µL 10x PBS without Ca/Mg (PAA Laboratories, Austria) ; 150 µL Formaldehyde solution (37 %; Merck) and 5.25 mg Saponin (Sigma-Aldrich)) and incubated for 30 minutes on ice. Cells were washed two times with 200 µL Saponin wash buffer (PBS without Ca/Mg; 2 % FCS and 0.1 % Saponin), antibody for intracellular antigen staining CD79-PE was added and incubated for 30 minutes on ice. Then cells were washed two times with Saponin wash buffer and resuspended in 200 µL Saponin wash buffer for FCM measurements.

For characterization of regulatory T cells (Tregs) cells had to be stained for the Treg differentiation marker Forkhead Box P3 (Foxp3). Therefore, cells were resuspended in 200 µL ebioscience Fix&Perm solution (1 part Fix/Perm concentrate and 3 parts Fix/Perm diluent). After 30 minutes incubation on ice cells were washed with 200 µl of ebioscience Perm/Wash buffer (1 part Perm/wash concentrate and 9 part water) (1300 rpm; 4min, 4°C). After a second wash step the Foxp3 antibody (FJK-16s-Al647) was added and cells were incubated for 30 minutes on ice. Cells were washed two times with Fix/Perm wash buffer and then resuspended in 200 µL wash buffer for analysis on flow cytometer.

2.6.5.4 FCM Analysis

Samples were analysed immediately after the final washing steps. Flow cytometric analyses were performed on a FACSCantoII (BD Bioscience). 50,000 events were collected

for each sample. Data were analysed using FACSDiva software 6.1.3 (BD Bioscience) and FlowJo software 7.6 by Tobias Käser (Institute for Clinical Immunology, University of Veterinary Medicine Vienna.).

Table 5: Three colour staining: Antibody combinations

Staining of Cell populations	First antibodies	Source	Second antibodies	Source	Fix /Perm	Intracell. Antigen staining	Source
Negative control	-		-		-	-	
Isotype control	Isotyp control IgG2b Isotyp control IgG Isotyp control IgG2a	**1** **1** **1**	Anti-IgG2b-Alexa488 Anti-IgG1-RPE Anti-IgG2a-Alexa647	**2** **3** **2**	-	-	
T helper cells NK cells	Anti-CD4 (74-12-4; IgG2b) Anti-CD3 (PPT3; IgG1) Anti-CD8α (11/295/33; IgG2a)	**4** **5** **6**	Anti-IgG2b-Alexa488 Anti-IgG1-RPE Anti-IgG2a-Alexa647	**2** **3** **2**	-	-	
T-helper Cytotoxic T lymphocytes	Anti-CD4 (74-12-4; IgG2b) Anti-CD45RC (3a56; IgG1) Anti-CD8ß (PG164A; IgG2a)	**4** **7** **8**	Anti-IgG2b-Alexa488 Anti-IgG1-RPE Anti-IgG2a-Alexa647	**2** **3** **2**	-	-	
γδ T cells	Anti-TcR-gd (PPT16; IgG2b) Anti-CD3 (PPT3; IgG1) Anti-CD8α (11/295/33; IgG2a)	**8** **5** **6**	Anti-IgG2b-Alexa488 Anti-IgG1-RPE Anti-IgG2a-Alexa647	**2** **3** **2**	-	-	
Dendritic cells monocytes	Anti-CD4 (74-12-4; IgG2b) Anti-SWC3/CD172a (74-22-15; IgG1) Anti-CD14 (TYK4; IgG2a)	**4** **8** **9**	Anti-IgG2b-Alexa488 Anti-IgG1-RPE Anti-IgG2a-Alexa647	**2** **3** **2**	-	-	
Regulatory T cells	Anti-CD4 (74-12-4; IgG2b) Anti-CD25(3B2; IgG1)	**4** **10**	Anti-IgG2b-Alexa488 Anti-IgG1-RPE	**2** **3**	Yes Ebioscience Fix Perm	Anti-Foxp3 (FJK-16s; IgG2a)-AIexa647	**12**
B-cells	Anti-CD21-APC (B-ly4; IgG1)	**11**	-		Yes saponin	Anti-CD79 (HM57; IgG1)-PE	**13**

1 Dianova, Hamburg, DE **2** Invitrogen, Carlsbad **3** Southern Biotech, Birmingham, UK **4** (Pescovitz *et al.*, 1984) **5** (Yang *et al.*, 1996) **6** (Jonjic & Koszinowski, 1984) **7** (Zuckermann *et al.*, 1994a; Zuckermann *et al.*, 1994b) **8** Institute for Clinical Immunology, University of Veterinary Medicine Vienna **9** Serotec Raleigh, NC, **10** (Bailey *et al.*, 1992) **11** BD Bioscience, San Jose, CA **12** eBioscience. San Diego, CA **13** Dako Glostrup, DK

2.6.6 Cereulide Quantification by SIDA

Collected blood samples, urine, feces, tissue and organs were stored at -80 °C until analysis. Therefore, samples were freeze dried and stored at -80°C. Before extraction liquid nitrogen was added to frozen samples and they were crushed to a powder using a mortar. 10 µg $^{13}C_6$-cereulide was added and samples were incubated on a rocking table for two hours. Then samples were solved in ethanol (volume depending on weight 10-40 mL) and incubated 14 hours. The suspension was centrifuged (RT, 8500 g, 10 min) and the supernatant was transferred to a new tube.

For pre-purification the ethanolic extract was 1:5 diluted with water and applied onto the top of a methanol equilibrated C18-SPE cartridge (6 mL; 1g; Strata C18-E; Phenomenex, Aschaffenburg, Germany). The cartridge was washed with 6 mL water followed by methanol/water (4 mL; 70/30, v/v) and target compounds were eluted with ethanol (2.5 mL). Finally, samples were membrane filtrated (0.2 µm; PTFE membrane; Phenomenex, Aschaffenburg, Germany) and analysed by means of LC-MS/MS (see 2.4.3).

2.7 Action on Enteric Nervous System

2.7.1 Preparation of Stomach

To analyse the influence of cereulide on the stomach muscle activity *in vitro,* muscle motility recordings were performed in organ baths. The stomach was isolated from guinea pigs (obtained from Charles River). Prior to the experiment the animals were kept in isolation cages at 20-24°C with a night/day cycle of 14/10 hours, in which dried fodder and water were available ad libitum. Animals were killed by cervical dislocation and subsequent cutting of throat. Stomachs were removed and stored in cold Krebs solution (117 mM NaCl, 4.7mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 $CaCl_2$, 11 mM Glucose) at 4°C before starting the experiment. Stomachs were opened, washed and then placed in a Sylgard coated petri dish filled with Krebs solution. During dissection the Krebs solution was perfused by CO_2/O_2 (5 %/95 %) gas mixture. One half of the stomach was pinned out as flat sheet preparation with the mucosa on top. At one half of the stomach the mucosa was removed at the corpus part. Then two sheets (each 1 x 1.5 cm) of corpus parts with mucosa and two without mucosa were cut. Then the borders were rolled to the middle and fixed at the ends with a polyamide thread, resulting in a roll with circular muscles lengthwise.

2.7.2 Measurement of Muscle Activity

Before mounting the muscle preparations, the force transducers were calibrated with a 2 g weight. The experimental setup consisted of an organ bath with two stimulation electrodes, which was filled with 37°C-temperated and carbogen-gassed Krebs solution. One end of the stomach preparation was fixed to the holder and the second end was connected with the force transducer. To simulate physiological conditions a preload of 15 mN was applied. Subsequently, after 60 minutes equilibration, the first electrical stimulation (10Hz for 10 s with pulse duration of 0.5 ms) was performed to test the muscle activity. One hour later, 13 µM cereulide or 0.5 % DMSO (corresponding to the DMSO supplemented concentration of cereulide) was added and half an hour later the muscles were electrically stimulated. Three additional stimulations were executed each

half an hour. The motility of gastric muscle stripes was recorded during the whole experiment using the LabChart software. Each experiment was performed four times. Analyses of muscle motility were performed by LabChart (v.6.1.3).

2.7.3 Evaluation of Muscle Activity

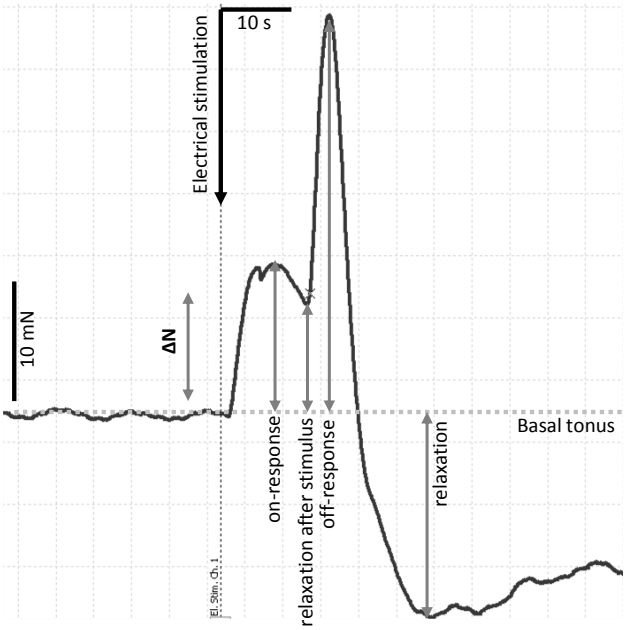


Figure 4: Activity of muscle stripes of proximal gastric corpus after electrical stimulation (10Hz for 10 s with pulse duration of 5 ms) in an organ bath with carbogen gassed Krebs solution and addition of 13 µM cereulide. Description see text.

Electrical stimulations of gastric muscle stripes induced a response consisting of several responses: On response (early contraction during stimulation), relaxation after electrical stimulus (post stimulation), off response (second contraction after electrical stimulus) and a final relaxation. For analysis of the muscle activity after electrical stimulation the maximal deviation of each response from the basal tonus (ΔN) was determined (Figure 4). The (mean) basal tonus was calculated for 120 s immediately before and after electrical stimulation using LabChart (v.6.1.3). Significance differences between control stimulation and stimulation after DMSO or cereulide addition was calculated with the paired Student's t-test.

3. RESULTS

3.1 CEREULIDE ISOLATION AND PRODUCTION

3.1.1 Isolation of Cereulide

For isolation of cereulide, *B. cereus* F4810/72 (Bc WT) was grown in LB broth for 24 h and extracted with ethanol after pelleting of cells by centrifugation. Due to the highly hydrophobic character of cereulide the concentrated ethanol extract was separated by reversed phase chromatography using a C12-column and a fast protein liquid chromatography (FPLC-)system. Hydrophobic substances from the *B. cereus* ethanol extract were separated using an isocratic gradient from 10-100 % ethanol in 60 min followed by 15 min at 100 % ethanol. To identify cereulide in the *B. cereus* spectrum, a pCer270 cured *B. cereus* strain F4810/72 pCER270$^-$ (Bc pCER270$^-$), which is not able to produce cereulide, was analysed. Comparison of absorption spectra at 210 nm of Bc WT and Bc pCer270$^-$ revealed one high and two small peaks found by WT (Figure 5). These three peaks eluting between 54 to 57 ml were analysed for cytotoxic activity using a cell culture assay with human larynx carcinoma cells (HEp-2; cf. 3.3.4). Prior to ethanol extraction the cell pellets were autoclaved to destroy heat-labile proteins and other substances. Fraction eluted after 54.6 mL exhibited a high cytotoxicity in contrast to the other analysed fractions (data not shown) and suggested the presence of the heat stable cereulide in this fraction.

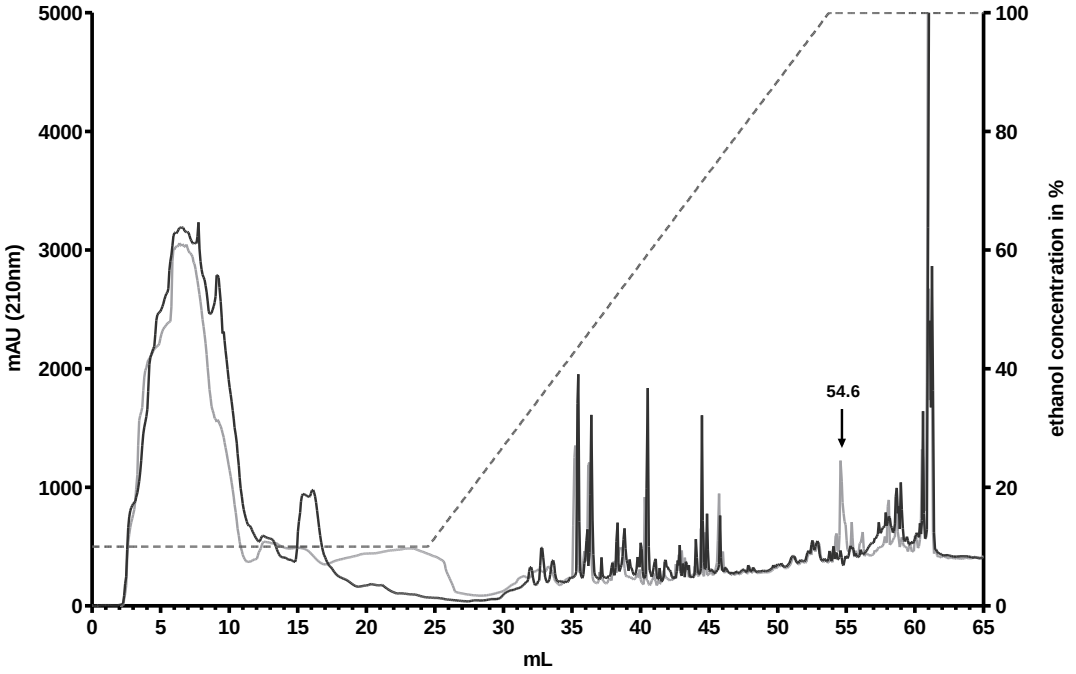


Figure 5: Comparison of FPLC-chromatogram of ethanol cell extracts from wildtype *B. cereus* 4810/72 (grey) and plasmid cured strain *B. cereus* F4810 pCer270$^-$ (black). Strains were incubated in LB broth at 30 °C for 24 h, After incubation cultures were autoclaved, pelletized and extracted with ethanol. Extracts were concentrated 10-fold, diluted 1:10 in water (v/v) and 5 mL of the extracts were analysed by FPLC.

3.1.2 Optimising of Culture Conditions for Cereulide Production

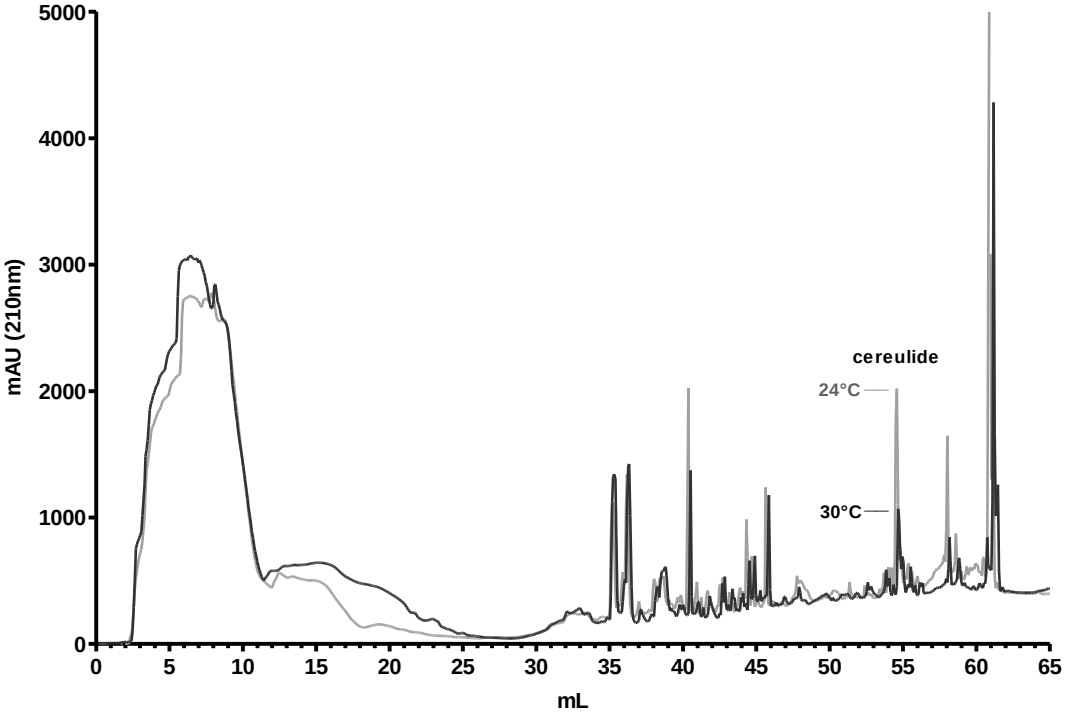


Figure 6: Influence of temperature on cereulide production. Comparison of ethanol extracts of *B. cereus* F4810/72 cultivated in LB broth for 24 h at 24°C (grey) and 30°C (black).

To gain cereulide in high quantity, production conditions were optimized in LB broth by varying the growth temperature and glucose concentration. The reduction of growth temperature to 24°C led to a two to three-fold enhancement of cereulide production in LB medium (Figure 6). An additional increase of cereulide synthesis was achieved by supplementation of 0.2 % glucose to the LB medium (Figure 7). Similar amounts of cereulide were produced by addition of 0.4 and 1 % glucose whereas 2 % glucose resulted in a lower cereulide production after 24 h (Figure 7). Therefore, cereulide was produced for almost all analysis in LB broth added with 0.2 % glucose at 24 °C for 24 h.

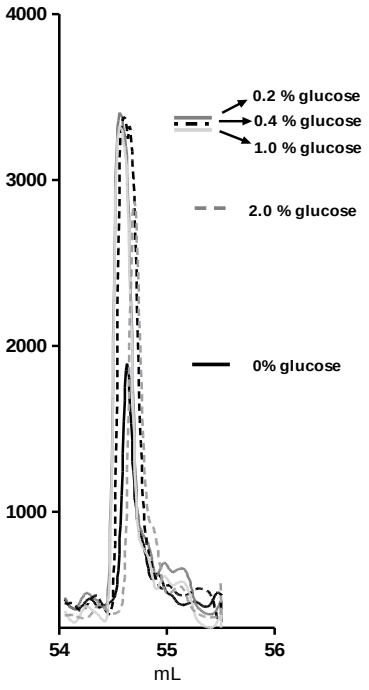


Figure 7: Influence of glucose addition on cereulide production of *B. cereus* F4810/72 in LB broth after 24 h at 24 °C.

3.1.3 Purification of Cereulide

Cereulide was prepared biosynthetically by means of a *B. cereus* F4810/72 culture in LB broth with 0.2 % glucose. After incubation for 24 h, the culture was autoclaved, centrifuged, and the cereulide produced was extracted with ethanol and purified by means of RP18 solid-phase extraction and RP-HPLC with a preparative column (C-12, Jupiter Proteo, Phenomenex), thus affording the toxin as a white, amorphous powder in a yield of 4 mg L^{-1} culture and a purity of more than 98 %, determined by NMR-spectroscopy (Figure 8). To confirm the identity of cereulide, UV-vis, LC-MS/MS, and 1D/2D NMR experiments were performed. LC-MS (ESI^+) revealed an intense $[M+ NH_4]^+$ ion with *m/z* 1170.9 as well as sodium, potassium, and proton adducts with *m/z* 1175.9, 1191.7, and 1153.8, respectively. LC-TOF-MS analysis of the sodium adduct *m/z* 1175.6669 confirmed the target compound to have the molecular formula $C_{57}H_{96}N_6O_{18}$. 1D/2D NMR data were in accordance with literature reported earlier (Agata *et al.*, 1994; Suwan Sathorn *et al.*, 1995). Taking all of these spectroscopic data into consideration, the structure of cereulide (Figure 9A) could be unequivocally identified as the 36-membered cyclic depsipeptide with the sequence cyclo-[$_L$-*O*-Val--$_L$-Val--$_D$-*O*-Leu--$_D$-Ala-]$_3$, thus being well in line with previously published data (Agata *et al.*, 1994; Andersson *et al.*, 1998; Jääskeläinen *et al.*, 2003b; Pitchayawasin *et al.*, 2004; Suwan Sathorn *et al.*, 1995).

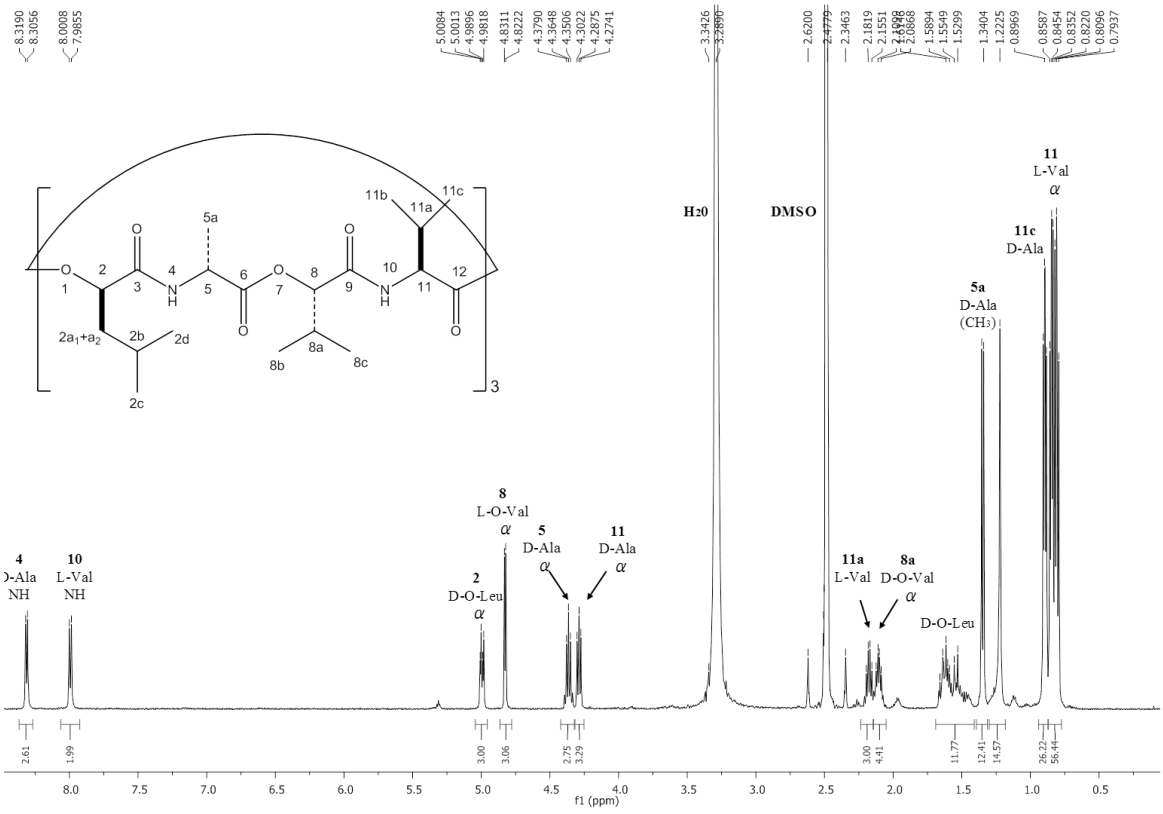


Figure 8: Determination of purity of the isolated toxin cereulide using ^{1}H-NMR-spectroscopy (500 MHz, d_6-DMSO.). Shown are ^{1}H-resonances of amino and oxyamino acids of cereulide.

This cultivation conditions were therefore used for production of cereulide throughout the complete project. The high quality of the *in vivo* produced cereulide was the basis for all performed experiments in this study and ensured that no other components, which are present in rude purified cereulide or *B. cereus* extracts, might influence the results of the experiments.

3.2 Development of a Quantification System for Cereulide[3]

3.2.1 Production of ^{13}C-labelled Cereulide

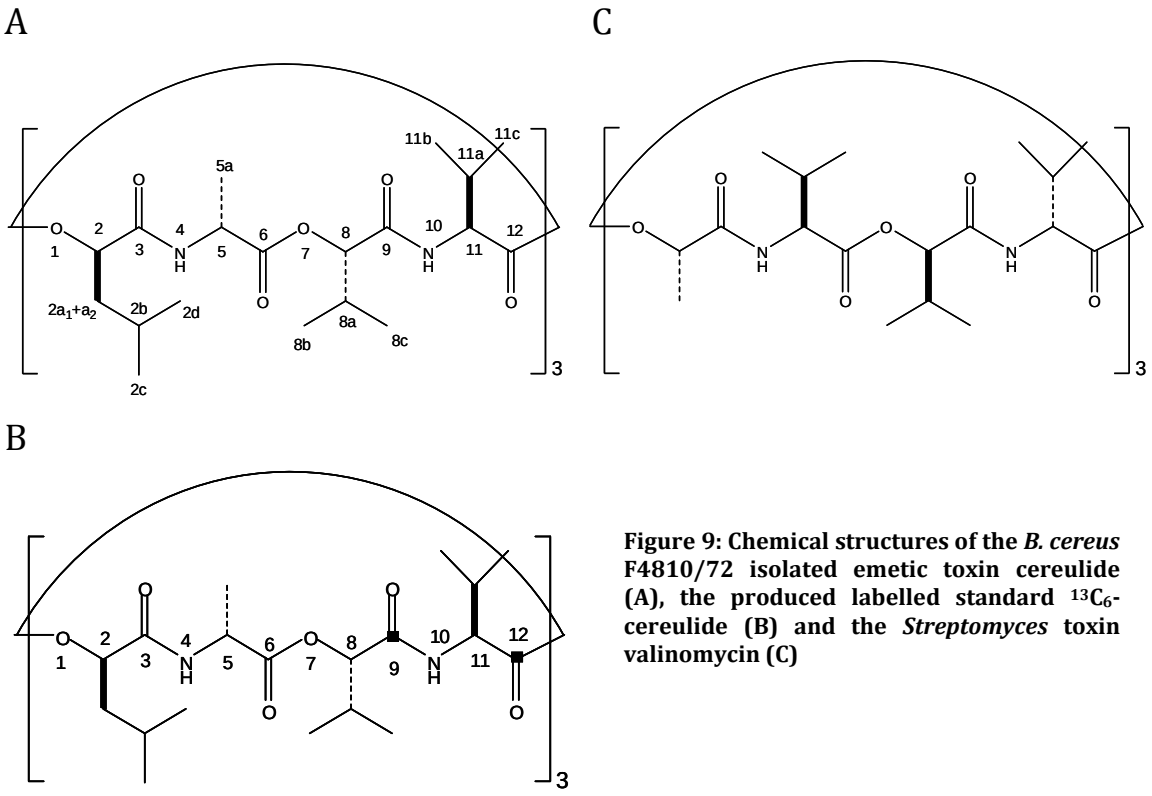


Figure 9: Chemical structures of the *B. cereus* F4810/72 isolated emetic toxin cereulide (A), the produced labelled standard $^{13}C_6$-cereulide (B) and the *Streptomyces* toxin valinomycin (C)

To develop a SIDA for cereulide quantitation in foods, a stable isotope-labelled internal standard was produced by inoculating a valine-depleted, $^{13}C_1$-L-valine enriched MOD broth without an additional carbon source with an overnight pre-culture of *B. cereus* for 48 h at 24 °C while shaking. Thereafter, the culture was centrifuged, and the target compound was extracted with ethanol and purified by means of RP18 solid-phase extraction and RP-HPLC, thus affording the ^{13}C-labelled toxin as a white, amorphous powder in a purity of more than 98 %. To confirm the chemical structure of the ^{13}C-labelled cereulide and to identify the position of the ^{13}C atoms in the molecule, LC-MS/MS as well as NMR spectroscopic experiments were performed. When compared to cereulide, the MS spectrum of ^{13}C-cereulide ($^{13}C_6$-1) measured in the ESI$^+$ mode showed an increase of the pseudo molecular ions *m/z* 1176.9 ([M + NH_4]$^+$), 1181.9 ([M + Na]$^+$), and 1197.9 ([M

[3] Part 3.2 of this thesis was taken from "Bauer, T., Stark, T., Hofmann, T., Ehling-Schulz, M. (2010) Development of a stable isotope dilution analysis (SIDA) for the quantification of the *Bacillus cereus* toxin cereulide in foods. J Agric Food Chem 58: 1420-1428". The experiments were done, and the text was written, in collaboration with T. Stark.

+ K]+) by six units, thus demonstrating $^{13}C_6$-cereulide as the predominant isotopologue. LC-MS analysis using an enhanced resolution scan confirmed the 6-fold ^{13}C-labelled cereulide with its pseudo molecular ion *m/z* 1159.6 ([M + H]+) as the major isotopologue, whereas the less frequent ion *m/z* 1158.6 indicated an incorporation of five ^{13}C carbon atoms into the molecule. Considering the natural ^{13}C abundance in these pseudomolecular ions, the sample was found to consist of 94.2 % 6-fold and 5.8 % 5-fold ^{13}C-labelled cereulide.

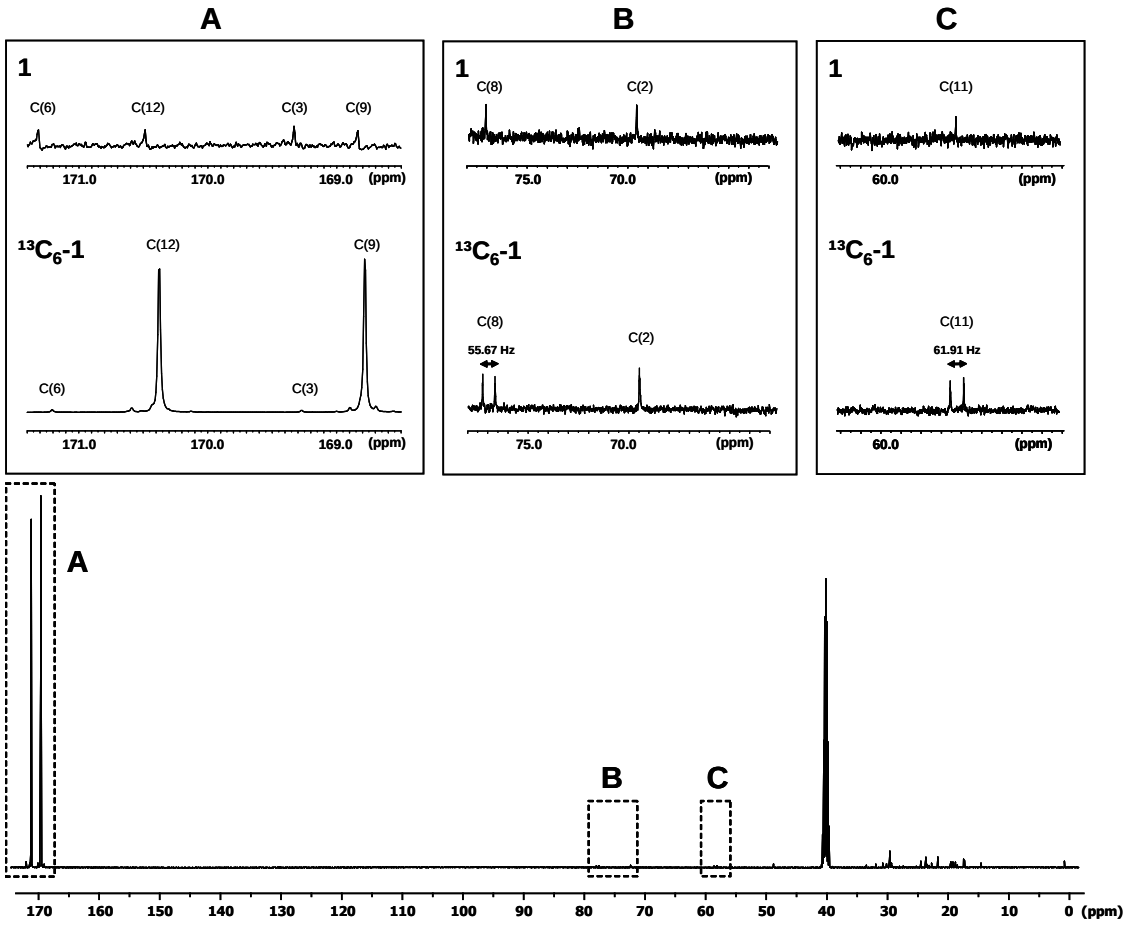


Figure 10: ^{13}C NMR spectrum (500 MHz, DMSO-d6) of $^{13}C_6$-cereulide ($^{13}C_6$-1) and enlarged spectrum excerpts A, B, and C representing selected carbon resonances of $^{13}C_6$-cereulide ($^{13}C_6$-1) and cereulide (1), respectively

To identify the positions of the incorporated ^{13}C atoms in the target molecule, the cereulide as well as a sample of ^{13}C-labelled cereulide were analysed by means of ^{1}H broadband-decoupled ^{13}C NMR spectroscopy (Figure 10). Comparison of the ^{13}C NMR spectrum (Figure 10A) obtained from cereulide (1), exhibiting four carbonyl carbon signals resonating as singlets with similar relative intensities, with that of the isotopologue $^{13}C_6$-1 revealed two highly intense ^{13}C signals for the carbonyl atoms C(12) and C(9)of L-Val and L-O-Val, respectively. In comparison, the signals of the natural ^{13}C-abundant

carbonyl atoms C(3) and C(6) were hardly detectable in the NMR spectrum of $^{13}C_6$-1, thus demonstrating the incorporation of the carboxygroup of $^{13}C_1$-labelled L-valine into the molecule. Furthermore, the spectra excerpts Band C (Figure 10) between 57.0 and 77.5 ppm in the ^{13}C NMR spectrum of cereulide (1) showed three carbon signals resonating as singlets with comparable signal intensities, whereas the same spectrum segments obtained from $^{13}C_6$-1 exhibited a signal duplet for carbon atoms C(11) and C(8), respectively, thus indicating a $^1J_{c,c}$-coupling to the ^{13}C-labelled carbonyl atoms C(12) and C(9).

LC-MS/MS analysis of cereulide revealed that compound fragmentation starts by ring opening of the cyclodepsipeptide by cleavage of an ester bond, followed by the formation of straight chain fragments preferably from the C terminus as reported earlier (Pitchayawasin *et al.*, 2004)(Figure 11 and Figure 10A). The most predominant fragments were found to be *m/z* 172.2, 314.2, 357.2, 499.4, and 1125.8. In comparison to cereulide, fragmentation of the isotopologue $^{13}C_6$-1 gives the corresponding fragment ions *m/z* 173.2, 316.4, 358.2, 501.4, and 1132.0 (Figure 10B). By comparison, the fragments of natural ^{13}C-abundant with $^{13}C_6$-labelled cereulide, it was possible to reconstitute the straight chain and assign the fragments originating from one or the other possible straight chain. For example, the fragment ion *m/z* 1131.0 formed from $^{13}C_6$-cereulide was found to contain five ^{13}C labelled carbonyls and, therefore, derived from the upper chain in Figure 11B. In contrast, the fragment ion *m/z* 1132.0 indicated the presence of six ^{13}C carbons and, therefore, was deduced to originate from the lower chain. The relative frequencies of the fragment ions *m/z* 1131.0 and 1132.0 were 24 and 12 %, respectively. In addition, the isotopic shift of the fragment ion *m/z* 357.2 derived from 1 to *m/z* 358.2 or *m/z* 359.4 generated from $^{13}C_6$-ceeulide demonstrated the presence of one or two ^{13}C carbonyl atoms, respectively. Interestingly, the fragment ion *m/z* 358.2 originates from the upper chain, whereas the fragment ion *m/z* 359.4 is derived from the lower chain (Figure 11B). By comparing the intensities of both fragments, again, the fragment of the upper chain (*m/z* 358.2) is favored with a frequency of 28 % when compared to *m/z* 359.4 with 8 %. These observations suggest that upon fragmentation the ring opening of cereulide starts by cleavage of the ester bond between D-O-Leu and L-Val, which is 2-fold favored in contrast to the ester bond between L-O-Val and D-Ala. In conclusion, LC-MS/MS as well as NMR experiments confirmed the position of the ^{13}C-labelled carbons in the structure of $^{13}C_6$-cereulide and demonstrated that *B. cereus* incorporated the carbon skeleton of six $^{13}C_1$-L-valine molecules into the toxin. These findings do confirm previous studies on the incorporation of the ^{13}C-labelled L-amino acids in cereulide upon cultivation of *B. cereus* in synthetic media (Kuse *et al.*, 2000).

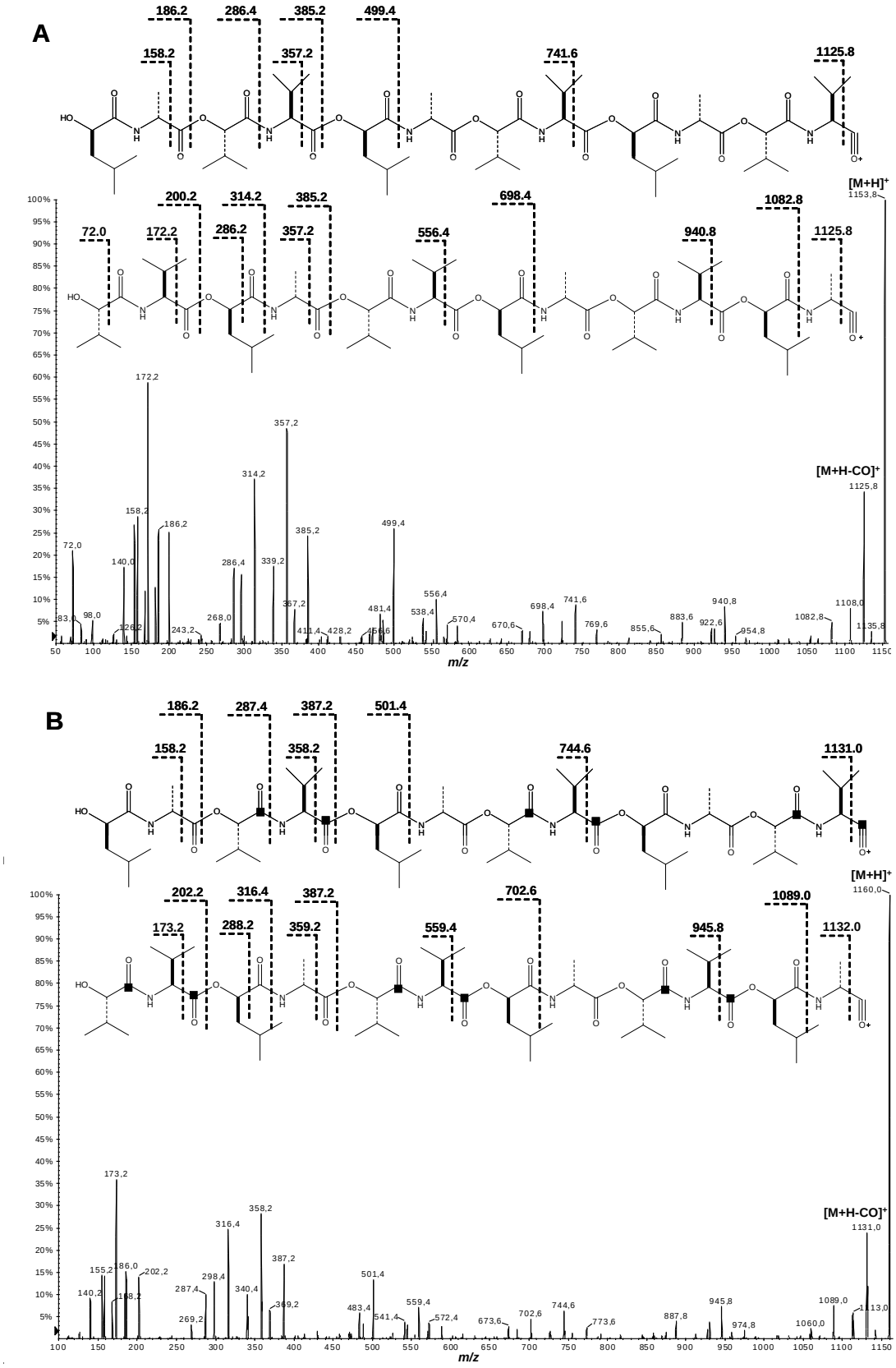


Figure 11: LC-MS/MS spectrum (ESI+) of (A) cereulide and (B) $13C_6$-cereulide (■ indicates the presence of a ^{13}C-atom)

3.2.2 Development of a Stable Isotope Dilution Analysis (SIDA)

To compare the suitability of $^{13}C_6$-cereulide as an internal standard with that of valinomycin, both compounds as well as the analyte cereulide (1) were individually infused into the mass spectrometer using a syringe pump. Optimum intensities of pseudomolecular ions and respective fragments were obtained by software-assisted ramping of the DP, the cell entrance potential, the CE, and the CXP, respectively (see Table S1). As the ion $[M + NH_4]^+$ of cereulide and valinomycin was the most predominant pseudomolecular ion, these were selected for the optimization. Tuning of the ammonium adducts and the pseudomolecular ions $[M + H]^+$ revealed similar fragment patterns and fragment intensities. To convert the measured ion intensities into the mass ratios of the internal standards $^{13}C_6$-cereulide and valinomycin (2), respectively, and the analyte cereulide (1), a graph was calculated from calibration mixtures of known mass ratios and the corresponding peak area ratios in HPLC-MS/MS. To achieve this, fixed amounts of the internal standards were mixed with the analytes in ratios of 0.1-10 on a molar basis, and the quotients of area internal standard/area analyte obtained by HPLC-MS/MS were plotted against the quotient concentration analyte/concentration internal standard.

To develop and validate the LC-MS/MS method for quantitative analysis of cereulide, samples of cooked rice and cooked rice supplemented with 10 % sunflower oil, respectively, were spiked with increasing amounts of cereulide and then homogenized. For the quantitative analysis of cereulide, the internal standards $^{13}C_6$-cereulide and valinomycin, respectively, were added, and each sample was equilibrated and then extracted with ethanol at 20 °C. After 15 h, nonsolubles were separated by centrifugation, the supernatant was membrane filtered, and after cleanup using a C18-SPE cartridge with ethanol as the eluent, the target compounds were analysed by means of HPLC-MS/MS on a RP-18 phase. The mass chromatograms showing the mass transitions for the analyte cereulide (A) as well as both of the internal standards $^{13}C_6$-cereulide (B) and valinomycin (2) looked rather clean as no major peaks appeared within the respective traces of the compounds under investigation (Figure 12). As the analyte cereulide and the isotope-labelled standard $^{13}C_6$-cereulide coelute, a similar influence of matrix compounds on the ionization of both molecules is to be expected. However, as valinomycin elutes somewhat later in the chromatogram, the question arises as to whether coeluting matrix components do affect the ionization of the internal standard valinomycin (2). To visualize such matrix effects (Bonfiglio *et al.*, 1999; Hsieh *et al.*, 2001), a constant flow of a solution of valinomycin (2) was introduced into the LC-MS/MS system via a syringe pump during the analysis of rice extract spiked with sun flower oil. As shown in Figure 12D, severe influence of eluting matrix compounds on the ionization of valinomycin could be observed depending on the retention time, thus indicating that the use of valinomycin as an internal standard might be not suitable to overcome matrix effects during LC-MS/MS analysis.

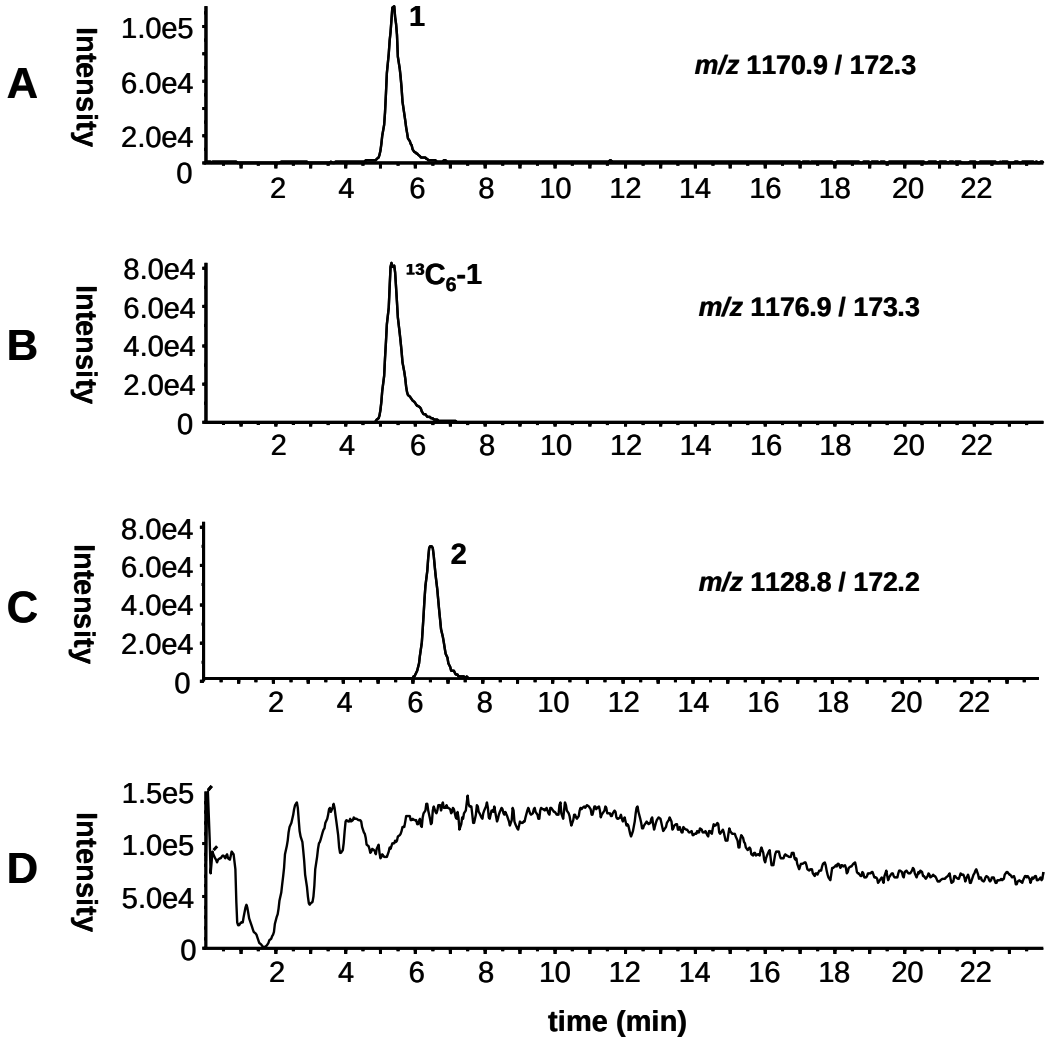


Figure 12: HPLC-MS/MS (ESI$^+$) analysis of a *B. cereus* infected boiled rice sample and MRM traces of (A) cereulide (1) and the internal standards (B) $^{13}C_6$-cereulide ($^{13}C_6$-1) and (C) valinomycin (2). (D) HPLC-MS/MS (MRM) chromatogram recorded for a rice sample whilst a continuous flow of valinomycin (2) was introduced into the LC-MS/MS system by means of a syringe pump.

3.2.3 Performance of the SIDA

To check the performance of the developed LC-MS/MS method linearity, trueness, intraday and interday precision, sensitivity, and selectivity were investigated for both internal standards $^{13}C_6$-cereulide and valinomycin, respectively. Calibration curves, obtained by linear regression analysis of the peak area versus concentration, showed a linear response with correlation coefficients of >0.99 for both internal standards. To check the trueness of the analytical method, recovery experiments were performed as follows. Purified reference material of cereulide was added to samples of cooked rice (blank) and, in addition, to rice containing 10 % sunflower oil to mimic an oil-rich food product at three concentrations (1, 2, and 5 µg g^{-1}), each prior to quantitative analysis, and the amounts determined after workup were compared to those found in the blank rice samples. The

recovery rates, calculated on the basis of the content of cereulide added to the rice samples prior to workup, were found to be 104.4 (7.1 % for the internal standard $^{13}C_6$-cereulide, whereas a lower value of 90.7 (8.5 % was found when valinomycin was used as an internal standard (Table 6). Interestingly, even lower recovery rates of only 79.9 (6.3 % were found when the rice sample supplemented with 10 % sunflower oil was analysed by using valinomycin as the internal standard, whereas the use of the ^{13}C-labelled cereulide still gave an excellent recovery rate of 110.7 (5.3 % (Table 6). To study the repeatability (intraday precision) of the analytical method, samples of cooked rice as well as samples of cooked rice supplemented with 10 % sunflower oil were spiked with two different concentrations of cereulide (1 and 5 µg g^{-1}) and then analysed using the SIDA developed above using three replicates for each concentration in 1 day. Reproducibility (interday precision) was studied with a sample of cooked rice and a sample of cooked rice supplemented with 10 % sunflower oil, both spiked with cereulide (2 µg g^{-1}), and they were analysed by the developed SIDA method at four consecutive days. For repeatability as well as reproducibility, relative standard deviations of less than 8 % were found for cereulide quantitatively determined in both food matrices by using $^{13}C_6$-cereulide as the internal standard. In comparison, analysis of cereulide by means of valinomycin as an internal standard revealed a value of less than 10 % for intraday as well as interday precision (Table 6).

In addition, limit of quantification (LOQ) and limit of detection (LOD) were determined in cooked rice as well as rice supplemented with 10 % sunflower oil as the concentration at which the peak height of the internal standard was at least 3 and 10 times higher than the underground noise (Table 6). To achieve this, a reference material of cereulide was added to both food matrices in different concentrations prior to LC-MS/MS analysis. The LOD and LOQ were found to be 3 and 30 ng g^{-1} for cooked rice and 5 and 50 ng g^{-1} for the oil-containing rice sample, respectively.

3.2.4 Quantitative analysis of cereulide in food samples

To investigate the cereulide production of the emetic *B. cereus* strain in rice, cooked rice samples were inoculated with an overnight culture of *B. cereus* and incubated at 24 °C for up to 96 h. At different time points, samples were taken, spiked with the internal standard $^{13}C_6$-cereulide, and equilibrated as described in the Materials and Methods. The amount of cereulide was determined using the SIDA developed. The production of cereulide in rice was found to be induced after 24 h (Figure 13). With increasing incubation time, the amount of the emetic toxin increased continuously up to 6.12 µg g^{-1} at 96 h.

Table 6: Validation criteria for the quantitative analysis of cereulide using ^{13}C-labelled cereulide or valinomycin as internal standard.

Internal standard		food matrix used for experiments			
		boiled rice		boiled rice suppl. with 10% oil	
		Recovery rate[a]			
	amount added (µg/g)	amount recovered(µg/g)	recovery rate (%)	amount recovered (µg/g)	recovery rate (%)
$^{13}C_6$-cereulide	0	n.d.	n.d.	n.d.	n.d.
	1	1.00 (± 0.09)	100.4 ±8.7	1.05 (± 0.03)	104.9±2.8
	2	1.97 (± 0.09)	98.7 ±4.5	2.23 (± 0.11)	111.5±5.0
	5	5.71 (± 0.47)	114.2 ±8.3	5.78 (± 0.47)	115.6±8.2
			mean 104.4 ±7.1		mean 110.7±5.3
valinomycin	0	n.d.	n.d.	n.d.	n.d.
	1	0.81 (± 0.32)	80.5 ±4.0	0.92 (± 0.07)	91.6±8.2
	2	2.21 (± 0.26)	110.5 ±11.9	1.66 (± 0.06)	83.1±3.8
	5	4.08 (± 0.39)	81.5 ±9.6	3.26 (± 0.23)	65.1±7.0
			mean 90.7 ±8.5		mean 79.9±6.3
		Repeatability (intraday precision)[b]			
$^{13}C_6$-cereulide		<8%		<8%	
valinomycin		<8%		<10%	
		Reproducibility (interday precision)[c]			
$^{13}C_6$-cereulide		<8%		<8%	
valinomycin		<8%		<10%	
		Limit of detection (LOD)[d]			
$^{13}C_6$-cereulide		3 ng/g		5 ng/g	
		Limit of Quantification (LOQ)[e]			
$^{13}C_6$-cereulide	0	30 ng/g		50 ng/g	

[a] concentrations are given as the mean of three independent clean-ups with triple injection, [b] Repeatability (intraday precision as relative standard deviation in %) was determined spiking blank rice and rice containing 10% sunflower oil at 1 and 5 µg/g, using three replicates for each concentration in one day, [c] Reproducibility (interday precision as relative standard deviation in %) was evaluated at 2 µg/g for both matrices, and spiked samples were analysed at four consecutive days, [d], [e] Limit of detection (LOD) and quantification (LOQ) were calculated analyzing blank samples and were determined as the lowest concentrations of analyte for which signal-to-noise ratios were 3 and 10.

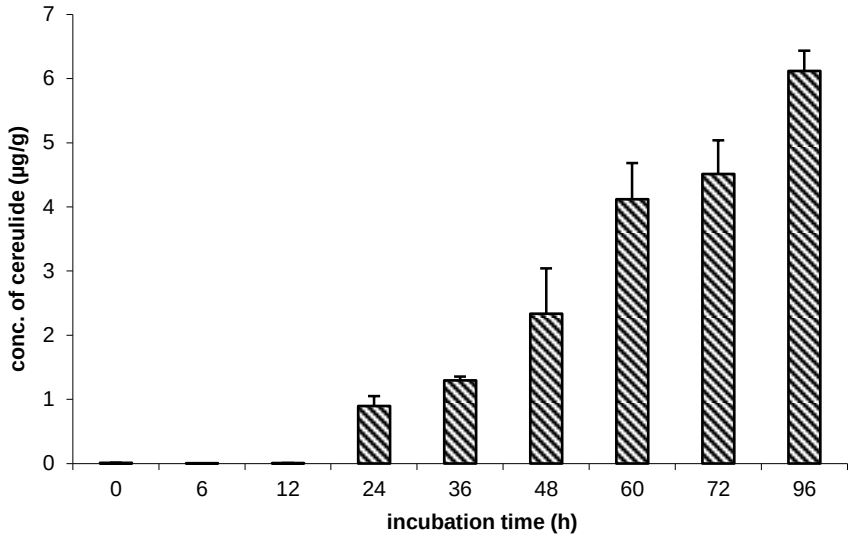


Figure 13: Influence of incubation time on the production of cereulide in boiled rice inoculated with *B. cereus* strain F4810/72. Concentrations were calculated as the mean of three independent experiments with double injection.

In addition to rice, two additional food products obtained from a German retailer, milk pudding and liver sausage, were artificially contaminated with *B. cereus* and incubated for 24 h at 24 °C (cf. inoculation in rice). Prior to inoculation with emetic *B. cereus*, foods were checked for cereulide contamination. Cereulide was not detectable in any of the food samples analysed (Table 7). After an incubation of 24 h, cooked rice exhibited the highest amount of cereulide (896 ng cereulide g^{-1} food). A somewhat lower amount of 219 ng g^{-1} was detectable in the inoculated milk pudding, whereas a comparatively low amount of 3 ng cereulide g^{-1} was found in liver sausage.

Table 7: Influence of *B. cereus* inoculation on cereulide concentration in selected food samples

	Concentration of cereulide in ng g^{-1}*	
Food samples	**Prior to incubation**	**after 24h**
Cooked rice	not detectable	896±150
Milk pudding	not detectable	215± 20
Liver sausage	not detectable	3± 0.9

*concentrations are given as the mean of two independent clean-ups with double injection

3.3 Effect of Cereulide on Different Organisms

3.3.1 Antibacterial Activity

A test panel of 31 bacterial strains mainly derived from similar environmental habitats as *B. cereus* or *Streptomyces* was compiled to study the species specific action of the *B. cereus* emetic toxin cereulide on other bacteria. For reason of comparison, the well-known ionophore valinomycin, produced by certain *Streptomyces* sp. was included in this study.

3.3.1.1 Influence of Different Ions on Toxin Activity

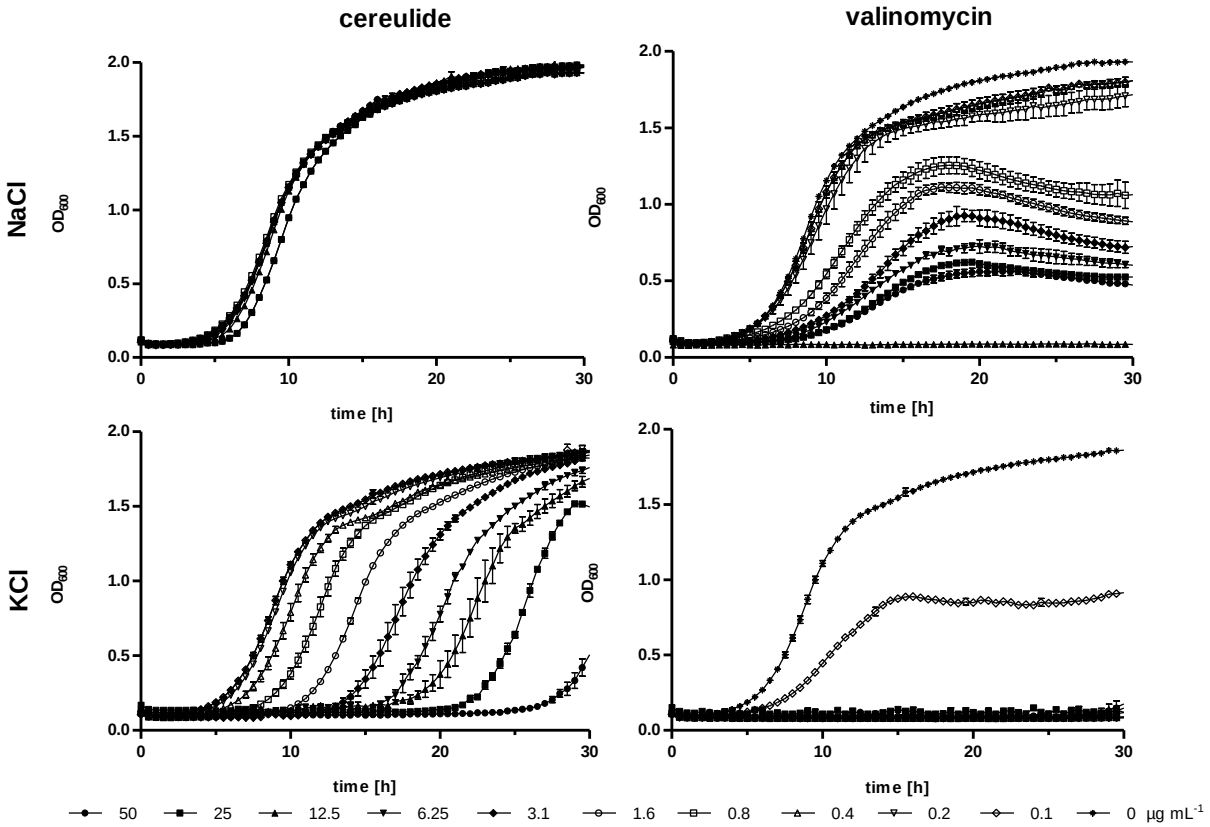


Figure 14: Impact of NaCl and KCl on the bacterial toxicity caused by cereulide and valinomycin: OD_{600} values of *Brevibacterium casei* cultures grown in LB (171 mM NaC) or or K^+-LB (171 mM KCL) supplemented with increasing concentrations (0.1-50 µg ml^{-1}) of cereulide and valinomycin, respectively. Standard deviations are derived from two parallel experiments.

First, the effect of different chlorides on the toxicity caused by cereulide and valinomycin was tested. Therefore, growth behaviour of bacteria was analysed in broth supplemented with different chlorides to visualize the influence of the complexed cations. LB broth was chosen as basis medium and NaCl (171 mM) was replaced by other chlorides, including KCl, MgCl, LiCl, NH_4Cl or CsCl. *Brevibacterium casei* turned out to be a suitable candidate for this investigation, because its growth was affected, but not completely inhibited by the presence of cereulide or valinomycin (Figure 14). In conventional LB broth growth of *B. casei* was not significantly influenced by cereulide (Figure 14). However, the exchange of

NaCl to KCl in LB broth (K^+-LB) enhanced cereulide toxicity clearly, indicated by the prolonged lag phases in a concentration dependent manner, but nearly stable growth rates. In contrast, valinomycin already exhibited toxicity in conventional LB, but the inhibitory impact on growth was also enhanced in K^+–LB (Figure 14). The other analysed chlorides MgCl, NH_4Cl and CsCl did not impair growth of *B. casei* in response to cereulide or valinomycin, whereas addition of LiCl completely inhibited growth of *B. casei* (data not shown).

3.3.1.2 Influence of Potassium Concentration on Bacterial Toxicity

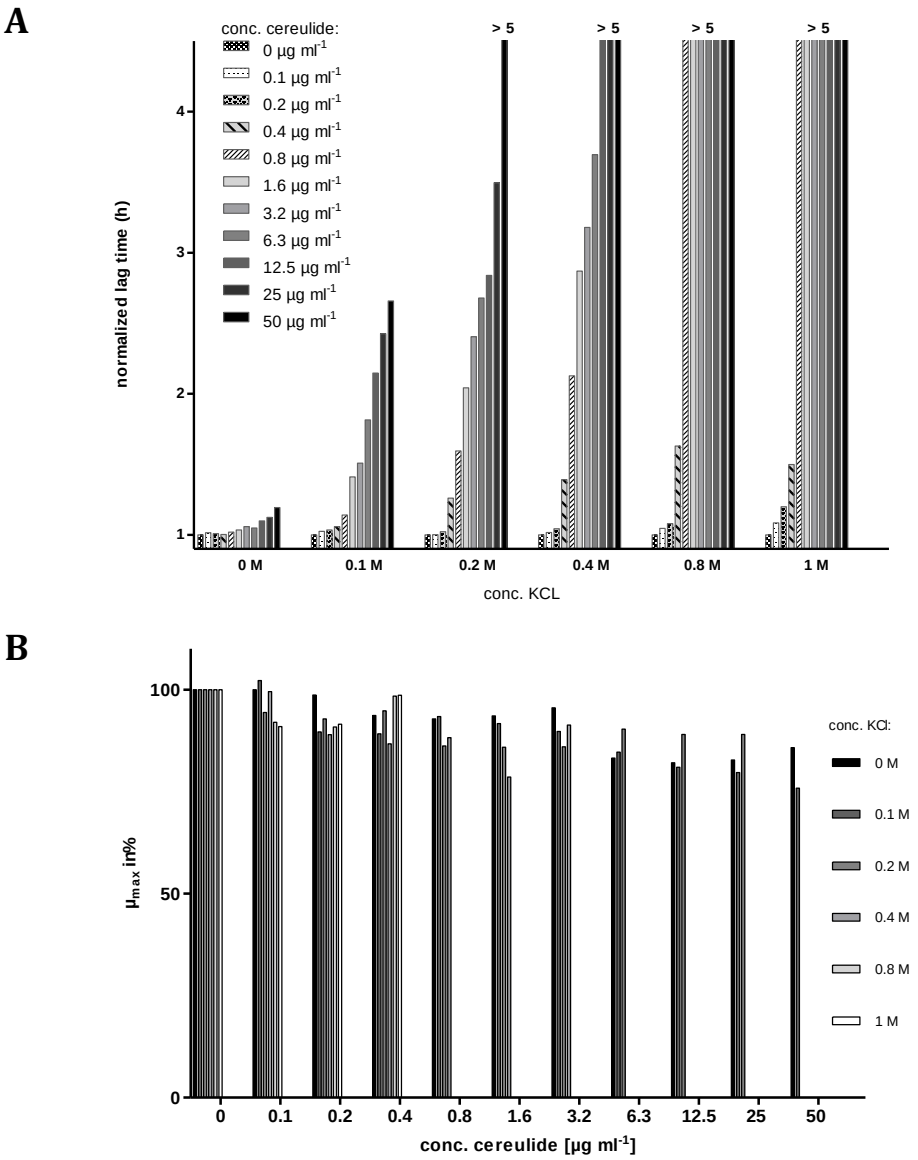


Figure 15: Influence of K^+ concentration (0-1M) on (A) lag phase duration and (B) maximal growth rate of *Brevibacterium casei* incubated with different cereulide amounts (0-50µg ml^{-1}). Lag phase durations were calculated by curve fitting via Gompertz function and data normalized by deviation over control results

To analyse the effect of potassium on the antibacterial activity of cereulide, *B. casei* was incubated in LB broth with increasing KCl and cereulide concentrations. For all experimental conditions lag time duration λ and maximal growth rate μmax were calculated by the modified Gompertz function. Cereulide caused in a KCl concentration dependent manner an increase of lag time duration, as for instance the raise of KCl from 0.1 to 0.4 M resulted in a doubled λ (Figure 15 A). At 0.8 M KCl growth of *B. casei* could only be observed up to 0.4 µg ml^{-1} cereulide within the analysed 30 h. In contrast, the maximal growth rate showed only a slight influence on higher KCl concentration (Figure 15 B).

3.3.1.3 Growth Inhibiting Effect on Gram-positive Bacteria

Based on the *B. casei* data indicating an increase of cereulide and valinomycin action at higher KCl concentrations, additional 30 bacterial strains were examined in K^+-LB broth. For determination of the minimum inhibitory concentration (MIC) of both toxins, the effect caused by different toxin concentrations (reaching from 0.1 to 50 µg ml^{-1}) was analysed in growth experiments after 24 hours. The growth of all analysed Gram-negative bacteria analysed was found not to be influenced by the addition of valinomycin nor cereulide (Table 8). In contrast, the growth of Gram-positive bacteria was differently affected by the toxins ranging from no influence to nearly complete growth inhibition at low concentration of both substances. Under culture conditions described above the analysed Gram-positive bacteria could be grouped into three classes: (I.) cereulide insensitive bacteria including the producer strain and near relatives e.g. *B. thuringiensis* and *B. weihenstephanensis,* but also pathogenic organisms such as *Staphylococcus aureus* and *Listeria monocytogenes*, (II.) cereulide sensitive bacteria, e.g. *Brevibacterium casei* or *Arthrobacter ureafaciens,* which revealed a concentration dependent lag time duration, and (III.) cereulide highly sensitive strains, which were able to grow only at very low cereulide concentrations (Figure 16 and Table 8).

Although organisms of class I. are sensitive towards cereulide, nevertheless some strains, such as *B. weihenstephanensis* and *B. thuringiensis*, appeared to be sensitive towards high concentrations of valinomycin, indicated by an elongation of the lag phase. Additionally, pathogenic strains like *S. aureus* and *L. monocytogenes* were resistant towards cereulide, but their growth was impaired in the presence of valinomycin. Strains of class II showed a high taxonomic diversity and exhibited a concentration dependent elongation of the lag phase in response to increasing amounts of either valinomycin or cereulide (Table 8). Generally, valinomycin revealed a significant higher toxicity towards Gram-positive bacteria than cereulide. Valinomycin was about 30- to 60-fold more toxic for about 55% of the analysed strains of analysed class II, while 25% of strains belonging to this group were about 2- to 8-fold more sensitive towards valinomycin compared to cereulide. The MIC of valinomycin was over the limit for the remaining strains and it was therefore not possible

to determine the valinomycin/cereulide ratios. Additionally, two very sensitive strains, namely *Arthrobacter globiformes* and *Rhodococcus fascians*, were found with a MIC below 0.4 µg ml^{-1} for both substances and were grouped into class III (Table 8).

Table 8: Impact of cereulide and valinomycin on growth of Gram-positive and Gram-negative bacteria

Indicator organisms	Broth	Incubation temperature (°C)	Minimal inhibitory concentration (µg mL^{-1}) cereulide	valinomycin
Gram-negative bacteria				
***Escherichia coli MG1655 (*Blattner *et al.*, 1997)**	**K+LB**	**37**	**res[3]**	**res**
***Proteus mirabilis* (WS 3316)**	**K+LB**	**37**	**res**	**res**
***Pseudomonas stutzeri* (WS 1844)**	**K+LB**	**37**	**res**	**res**
***Pseudomonas fluorescens* (WS 2201)**	**K+LB**	**30**	**res**	**res**
***Salmonella enterica subsp. enterica serovar Typhimurium* (ATCC 14028)**	**K+LB**	**37**	**res**	**res**
Yersinia enterocolitica (Cornelis & Colson, 1975)	**K+LB**	**30**	**res**	**res**
Gram-positive bacteria				
I a. Insensitive strains				
Bacillus cereus F4810/72	**K+LB**	**30**	**res**	**res**
***Micrococcus luteus* (WS 1937)**	**K+LB**	**30**	**res**	**res**
I b. Cereulide insensitive strains				
Listeria monocytogens EGDe (Schlech et al., 1983)	**K+BHI***	**37**	**res**	**3.1**
***Bacillus pumilus* (WSBC 25001)**	**K+LB**	**30**	**res**	**>50[1]**
***Bacillus thuringiensis* (WSBC 28001)**	**K+LB**	**30**	**res**	**50**
***Bacillus weihenstephanensis* (WSBC 10204)**	**K+LB**	**30**	**res**	**>50**
Staphylococcus aureus (Novick, 1967)	**K+LB**	**37**	**res**	**6.3**
II. Sensitive strains				
***Arthrobacter nicotianae* (ATCC 31021)**	**K+LB**	**30**	**50**	**1.6**
***Arthrobacter oxydans* (WS 1106)**	**K+LB**	**30**	**1.6**	**0.8**
***Arthrobacter ureafaciens* (ATCC 7562)**	**K+LB**	**30**	**25**	**0.4**
***Bacillus licheniformes* (WSBC 23001)**	**K+LB**	**30**	**>50**	**6.3**
Bacillus sp ATCC 15294 (Arthrobacter viscosus)	**K+LB**	**30**	**25**	**0.8**
***Bacillus subtilis* (WS 26001)**	**K+LB**	**30**	**50**	**1.6**
***Brevibacterium casei* (WS 3023)**	**K+LB**	**30**	**25**	**0.4**
***Brevibacterium helvolum* (ATCC 19390)**	**K+LB**	**30**	**25**	**0.8**
Brevibacterium linens (Rattray et al., 1997)	**K+LB**	**30**	**0.8**	**0.2**
***Brevibacterium oxydans (food isolate)* (WS 1998)**	**K+LB**	**30**	**25**	**0.4**
***Corynebacterium casei* (WS 3539)**	**K+LB**	**30**	**6.3**	**0.8**
***Corynebacterium glutamicum* (WS 1084)**	**K+LB**	**30**	**>50**	**3.1**
***Microbacterium lacticum* (WS 1914)**	**K+LB**	**30**	**50**	**25**
***Micrococcus aurantiacus* (ATCC 11731)**	**K+LB**	**30**	**12.5**	**0.4**
***Propionibacterium freudenreichii* (WS 1723)[2]**	**K+LB**	**30**	**25**	**0.4**
***Staphylococcus epidermidis* (WS 2892)**	**K+LB**	**37**	**>50**	**6.3**
III. Highly sensitive strains				
***Arthrobacter globiformes* (WS 1457)**	**K+LB**	**30**	**0.2**	**< 0.1**
***Rhodococcus fascians* (ATCC 12975)**	**K+LB**	**30**	**0.2**	**< 0.1**

[1]meaning a concentration dependent growth behavior was observed but no growth inhibiting concentration could be determined.

[2]On ATCC filed under *Micrococcus freudenreichii*

[3] resistant: bacterial growth is unaffected

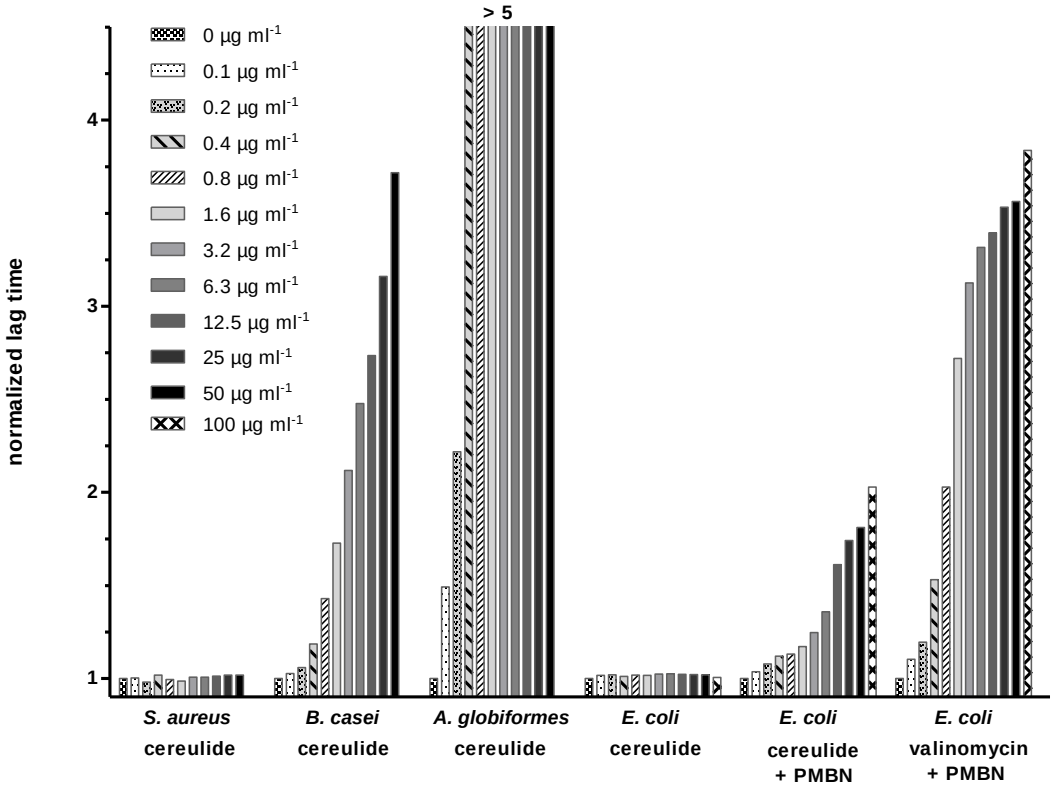


Figure 16: Normalized lag phase duration (λ) of *S. aureus* (class I: Insensitive strain), *B. casei* (class II: Sensitive strain), *A. globiformes* (class III: Highly sensitive strain) and *E. coli* incubated in K⁺LB supplemented with different cereulide concentrations. The outer membrane permeability of *E. coli* was enhanced by addition of 10 µg mL^{-1} PMBN to the growth medium, supplemented with different cereulide and valinomycin concentrations. Lag phase durations were calculated by curve fitting via Gompertz function and data normalized by deviation over control results.

3.3.1.4 Gram-negative Bacterial Outer Membrane Prevents Accessibility of Cereulide and Valinomycin

To investigate the resistance of Gram-negative bacteria in response to cereulide or valinomycin in more detail, the barrier function of *E. coli's* cell envelope was analysed. *E. coli* was cultivated in K⁺-LB broth supplemented with different concentrations of cereulide or valinomycin and polymyxin B nonapeptide (PMBN: 10 µg mL^{-1}) in order to enhance the outer membrane permeability. In general, PMBN disrupts the outer membrane of Gram-negative bacteria leading to a weak leakage of periplasmic proteins without significantly affecting cell growth (Sahalan & Dixon, 2008; Vaara, 1992). Addition of PMBN to cereulide and valinomycin treated *E. coli* caused a toxin concentration dependent elongation of the lag phase (Figure 16). The effect on the growth of *E. coli* was stronger with valinomycin than with cereulide. Thus, the elongation of lag phases in response to cereulide resembled the growth behaviour of Gram-positive bacteria of class II when incubated with increasing amounts of cereulide.

3.3.2 Insect Model *Galleria mellonella*

3.3.2.1 Toxin Injection Experiments

The larvae *Galleria mellonella* is routinely used as model organisms for *in vivo* studies of pathogenic bacteria and fungi as well as toxic substances produced by these organisms (Joyce & Gahan, 2010; Mollier *et al.*, 1994; Ratcliffe, 1983). The infection process of *G. mellonella* can be easily followed by a stepwise melanisation of the larvae. However, the injection of cereulide and valinomycin led to a paralysis of the larvae after a short time and resulted in death at a later point in time. The paralysis caused by concentrations of 3.3 to 10 µg g^{-1} cereulide and 10 µg g^{-1} valinomycin was linked to a distinct phenotype showing a swollen and bent body in lateral position (Figure 17). At concentrations of 10 µg g^{-1} of either valinomycin or cereulide, paralysis occurred one hour after injection. Treatments with 1.6 and 3.3 µg g^{-1} bw of cereulide led to paralysis symptoms two to three hours post injection (Figure 18). Cereulide treated larvae remained in this paralysis state followed by a direct transition into death, which could be observed by melanisation (cf. Figure 17 C). In contrast, paralysis caused by valinomycin disappeared after 15 hours and larvae returned to an almost normal behaviour with a slight impairment regarding motility after 15-40 h (Figure 18).

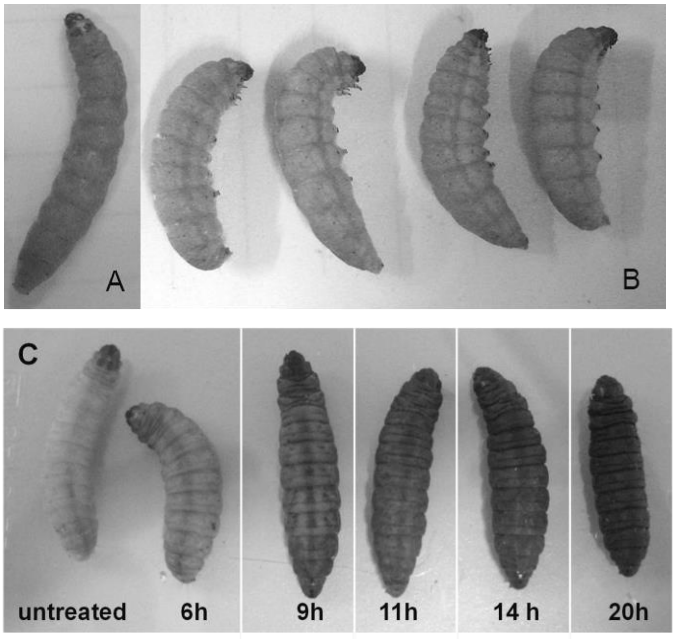
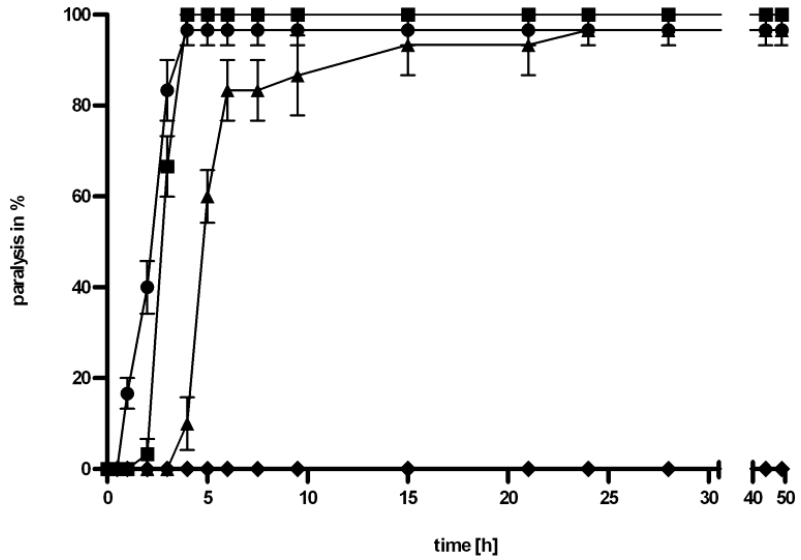


Figure 17: Phenotypes of fifth instar larvae of *Galleria mellonella* after cereulide treatment (B) or *B. cereus* infection (C). Larvae of *Galleria mellonella* before **(A)** and two hours after inoculation of 10 µg g^{-1} bw cereulide **(B)**. A few hours after injection of cereulide into the larval haemocoel, larvae exhibited a curvature and swelling of body.
Melanisation status of *G. mellonella* larvae infected with *Bc 4810/72* pMDX [P_1/*lux*] after 6, 9, 11, 14 and 20 hours after infection and incubation at 24°C **(C)**. Generally, the larvae died 10 hours after injection with cells of *Bc 4810/72* pMDX [P_1/*lux*]

A



B

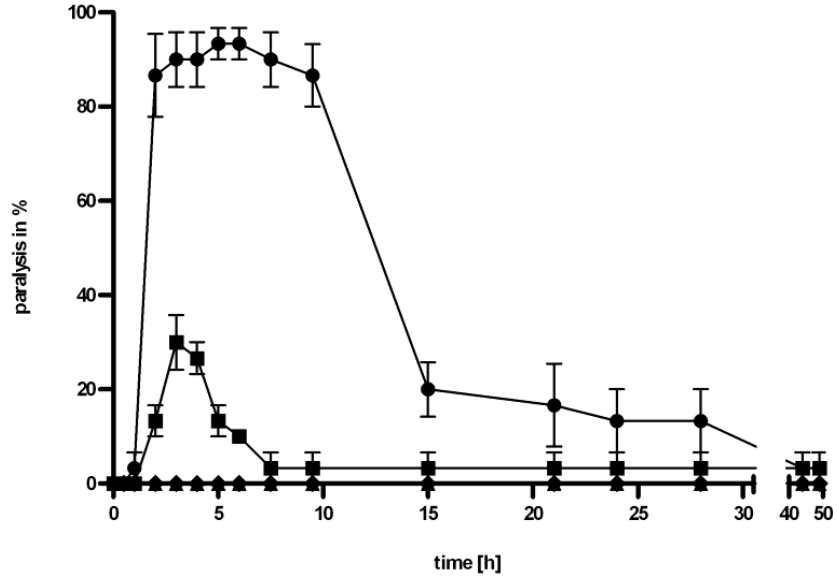


Figure 18: Paralysis of *G. mellonella* larvae in response to cereulide (A) and valinomycin (B). Larvae were injected with 10 µg g^{-1} bw (circles), 3.3 µg g^{-1} bw (squares), 1.6 µg g^{-1} bw (triangles), 0.5 µg g^{-1} bw (crosses), 0.25 µg g^{-1} bw (diamonds) and incubated at room temperature up to 50 h. Standard deviations are derived from three parallel experiments.

3.3.2.2 *In vivo* Cereulide Production in Insects

To monitor the *in vivo* cereulide synthesis in insects, *G. mellonella* larvae were infected with different luciferase reporter strains of *B. cereus*. Therefore, larvae were infected with Bc 4810/72 (Bc WT) transformed with the pMDX [P_1/*lux*] vector, which contains the main promotor region of the *ces* gene cluster fused to a luciferase cassette, to visualize *ces* gene expression (Dommel *et al.*, 2010). The bacterial growth of *B. cereus* in larvae was monitored by infection with Bc 4810/72 containing the *cspA* promotor region fused to the

luciferase cassette on the plasmid pMDX [*cspA*/lux]. The gene *cspA* encodes a cold shock protein, which is expressed at temperatures under 30°C. Infected larvae with Bc WT died 10 hours post infection (Figure 17 C) (80 % of larvae were dead) and growth of *B. cereus* was recognizable by *cspA* promoter activity after 12 hours. The *cspA* promotor activity increased continuously up to 18 hours and then remained nearly stable indicating a persistence of bacterial cells in the larvae (Figure 19 A). At 14 hours *ces* gene promotor became active, visible by the appearance of luminescence (Figure 19 B) and the bioluminescent signal increased strongly up to 36 hours demonstrating an enhanced *ces* expression. Highest cereulide syntethase promotor activity was found in the head and abdomen of the larvae (Figure 19 B). The role of cereulide synthesis in killing of the insects is unclear. Therefore, larvae were infected with a *ces* deficient knockout mutant Bc Δ*cesP*/polar containing pMDX [P_1/*lux*] meaning that this strain is unable to produce cereulide but the cereulide syntethase promotor activity can be monitored by luciferase gene expression. This strain killed larvae after 10 hours and revealed a nearly identical *ces* gene promotor activity course as the Bc WT pMDX [P_1/*lux*], indicating that cereulide plays a minor role in the infection of the larvae (data not shown; cf. Figure 19 B).

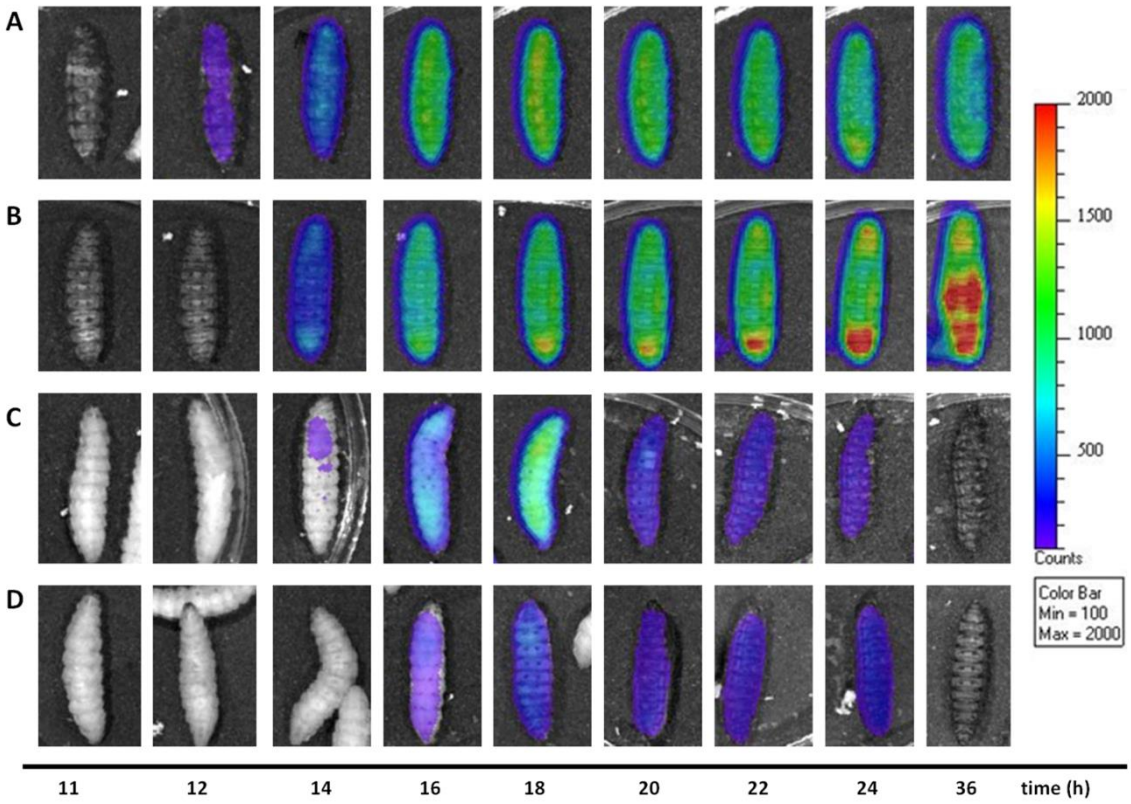


Figure 19: Real time monitoring of cereulide synthetase and cold shock protein (CspA) promoter activity in *G. mellonella* using a photon-counting ICCD camera. Larvae were infected with F4810/72 pMDX [cspA/*lux*] to visualize the bacterial growth **(A)**, with F4810/72 pMDX [P_1/*lux*] to analyse the *ces* promoter activity in Bc WT **(B)**, with F4810/72 Δ*plcR* to investigate the influence of cereulide production without other virulence factors with plasmids pMDX [cspA/*lux*] **(C)** and pMDX[P_1/*lux*] **(D).** Two independent test series were performed at 24°C and one representative larva is shown for each reporter strain from 11 to 36 hours.

To confirm the observations of cereulide production in insects, cereulide concentrations were determined using the developed SIDA (3.2) by analysing larvae from four different time points: at experimental start phase (0 h), dying phase (11 h), high *ces* gene promotor activity phase (20 h), and at the end of the experiment after 36 hours (cf. Figure 19). In living insects no cereulide production was found, but cereulide could be detected in concentration of 6 µg g^{-1} larva after 20 hours and 8 µg g^{-1} larva after 36 hours (Figure 20).

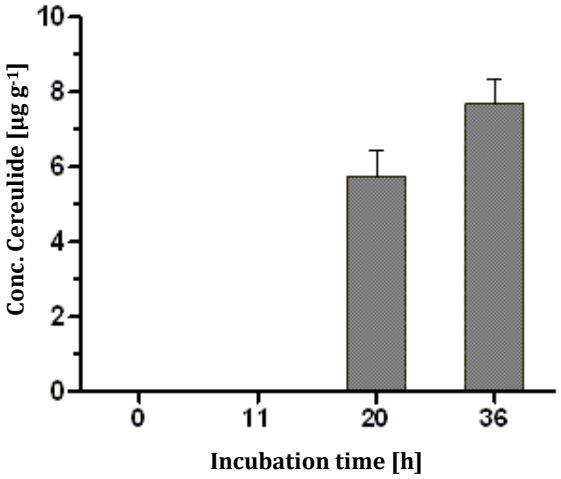


Figure 20: Cereulide production in larvae of *G. mellonella* after injection of *B.cereus F4810/72* [P_1/*lux*] determined by SIDA. Means and standard deviations are derived from measurements of three injected larvae with LC-MS/MS double injection.

In *B. cereus* many virulence factors such as the enterotoxins, lipases, proteinases and haemolysins are under transcriptional control of the pleiotropic regulator (PlcR), but whilst *ces* gene transcription is not controlled by PlcR (Gohar *et al.*, 2008; Lücking *et al.*, 2009). To investigate the influence of cereulide synthesis in insects and larval mortality in the absence of PlcR regulated virulence factors, larvae were infected with the *plcR* deletion mutant F4810/72 Δ*plcR* (Bc Δ*plcR*) harbouring the plasmids pMDX [cspA/*lux*] or pMDX[P_1/*lux*] (Figure 19 C, D). Generally, death of larvae was not detected within the first 18 hours after Bc Δ*plcR* mutant infection (Figure 19 C,D). In contrast to Bc WT infected larvae, luminescence from *cspA* promotor activity could already be observed in living larvae after 14 hours. The bioluminescent signal increased until the death of the larvae after 18 hours and thereafter decreased and disappeared after 35 hours (Figure 19 C). The cereulide synthetase promotor activity resembled the course of *cspA* promotor activity but with a time delay of 2 hours. Hence, *ces* gene expression was detectable in living larvae after 16 hours, was then slightly enhanced after 18 hours, remained nearly stable up to 24 hours and disappeared after 36 hours (Figure 19 D). In comparison to the Bc WT, bacterial growth indicated by *cspA* expression and *ces* gene expression of Bc Δ*plcR* was significantly reduced in insects.

3.3.2 Antiparasitic action

The nodular worm *Oesophagostomum dentatum* is a frequently used *in-vitro* model for larval development research. Especially the larval development of this swine parasite from third stage (L3) to the fourth stage (L4) is suitable for investigations of parameters influencing the growth and development of nematodes.

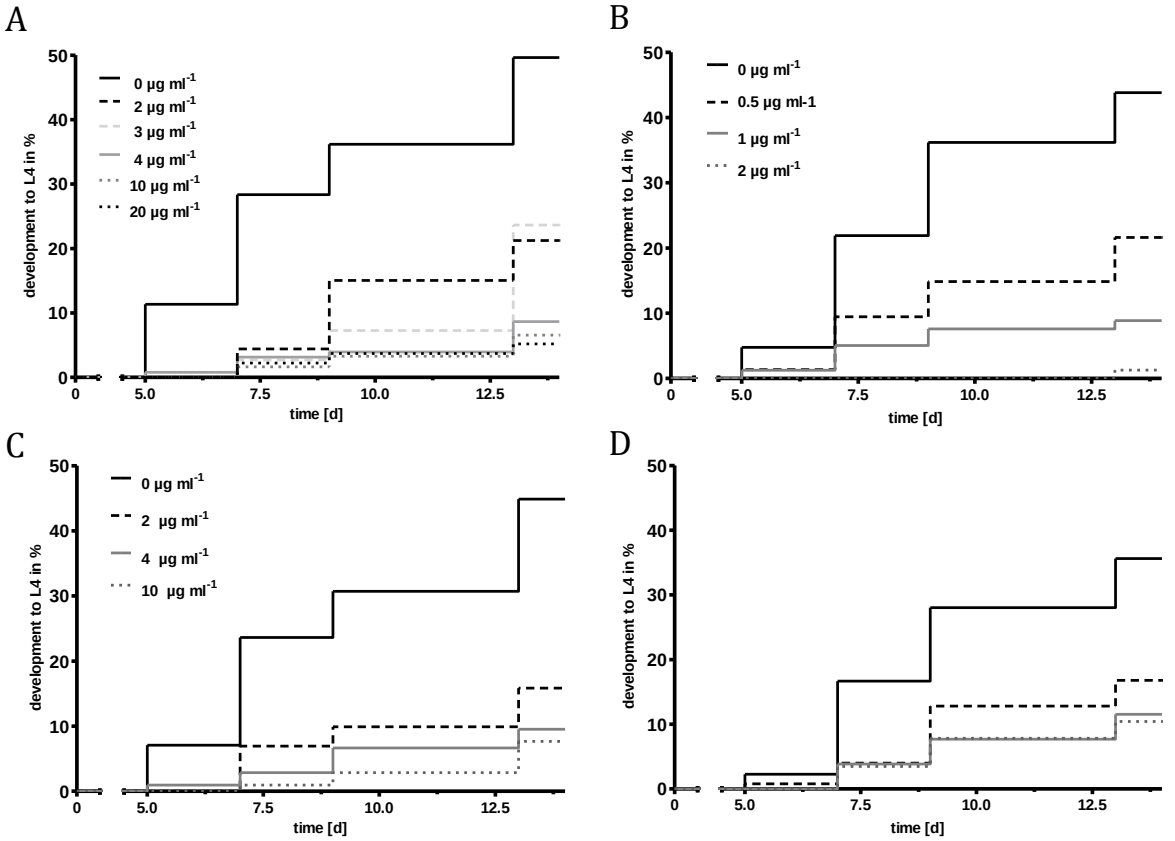


Figure 21: Development to L4 larvae of *O. dentatum* in response to addition of cereulide and valinomycin. Comparison of development of larvae after addition of different concentrations of cereulide **(A)** or valinomycin **(B)** cultivated in LB supplemented with 60 mM KCL. Influence of external potassium concentration on larval development in response to different concentration of cereulide in LB with 5 mM KCL **(C)** and with 100 mM KCl **(D)**; concentrations cf. legend C). Kaplan-Meier-analysis displayed the results of three parallel experiments.

First, the impact of cereulide and valinomycin on the development from L3 (cf. Figure 22 A) to L4 larvae (cf. Figure 22 B) was analysed. Addition of cereulide led to a delay in larval development of 2 days compared to untreated larvae. Moreover, a complete inhibition of larval development was observed for 57 % of the larvae at 2 µg mL^{-1} of cereulide and for 90 % at 20 µg mL^{-1} compared to the L4 development of untreated larvae in LB (60 mM KCl) after 13 days incubation (Figure 21 A). In addition, L3 larvae reacted very sensitive towards valinomycin and similar effects as with cereulide were already observed by addition of 0.5 µg mL^{-1} to 2 µg mL^{-1} (Figure 21 B). These concentrations inhibited the development of 50 % (0.5 µg mL^{-1}) and 97 % (2 µg mL^{-1}) of the larvae after 13 days of

incubation, indicating a 4-12-fold higher impact of valinomycin on the larval development in comparison to cereulide. An increase of the external potassium concentration to 100 mM KCl led to a 25 % inhibition of the L4 development compared to 60 mM KCl whereas only a little influence on the development was observed by reducing the KCl concentration to 5 mM over a period of 13 days. At 5 mM KCl addition of cereulide resulted in an inhibition of larval development of 64 % of the larvae at 2 µg mL^{-1} cereulide and 82 % at 10 µg mL^{-1}. However, an increase of the external potassium concentration to 100 mM KCl reduced the impact of cereulide on the larval development, leading to an inhibition of 52 % of the larvae at 2 µg mL^{-1} of cereulide and 70,8 % at 10 µg mL^{-1} in comparison to the untreated control group after 13 days of incubation (Figure 21 C & D).

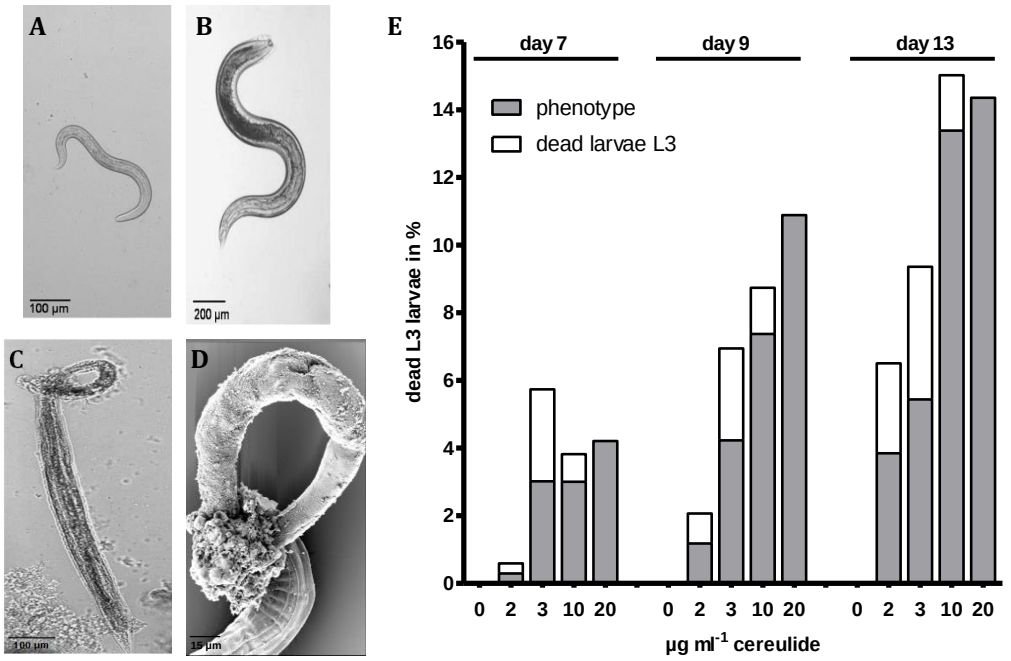


Figure 22: Influence of cereulide on L3 larvae of *O. dentatum*. Inverted light microscope images of *O. dentatum* L3 larva **(A)**, L4 larva **(B)** and the observed phenotype after cereulide treatment **(C)** in LB (60mM KCl). Electron microscope image of the phenotype of a cereulide treated L3 larva with malformation of the cuticle **(D)**. Percentage of total dead L3 larvae (white) and dead L3 larvae which exhibited a malformation of the cuticle (grey) in response to different concentrations of cereulide **(E)**. Data of one representative cultivation are shown. Microscopic images were made by Katja Silbermayr (vetmeduni Vienna)

Besides the inhibition of larval development, cereulide exhibited a nematocidal effect on L3 larvae of *O. dentatum* after 7 days of incubation ranging from 0.6 % dead L3 larvae at 2 µg mL^{-1} of cereulide to 5.7 % at 3 µg mL^{-1} (Figure 22 E). The amount of L3 larvae killed by cereulide increased strongly with higher toxin concentrations (from 6.3 % dead larvae at 2 µg mL^{-1} to 15 % at 10 µg mL^{-1} cereulide), whereas untreated larvae displayed no loss of vitality after an incubation time of 13 days in LB broth (60 mM KCl) (Figure 22 E). Most of the dead L3 larvae treated with cereulide exhibited a distinct phenotype characterised by an anterior malformation of the cuticle in the larvae (Figure 22 C&D). The percentage of dead L3 larvae revealing this phenotype rose accordingly to the increased cereulide

concentration in the external medium (Figure 22). At the highest analysed cereulide concentration of 20 µg mL^{-1} all dead L3 larvae exhibited the characteristic phenotype depicted in Figure 22 D. Interestingly, this phenotype was not observed in experiments with valinomycin treated L3 larvae (data not shown).

In addition, the influence of low and high external potassium concentrations on the toxicity of cereulide towards L3 larvae was investigated. The nematoxicity of cereulide on L3 larvae after incubation in LB with 5 mM KCl was similar to that of LB with 60 mM KCl. In contrast, supplementation of 100 mM KCl to LB medium caused an increase of cereulide's toxicity of 23-41 % on the L3 larvae. Moreover, cereulide treated L3 larvae died two days earlier in LB supplemented with 100 mM KCL compared to larvae incubated in lower potassium concentrations (Figure 22 and 23). The amount of dead L3 larvae displaying malformations caused by cereulide was not influenced by changes in KCl concentration (5-100 mM) of the external medium (Figure 22 and 23). It should also be noted that several dead untreated L3 larvae and a decrease in L4 development were observed at 100 mM KCl compared to lower KCl concentrations indicating suboptimal cultivation conditions for *O. dentatum.* Experiments with even higher external potassium concentrations caused a strong increase of dead L3 larvae and a drastic inhibition of larval development, which allowed no distinction between cereulide and potassium triggered toxicity on nematodes (data not shown).

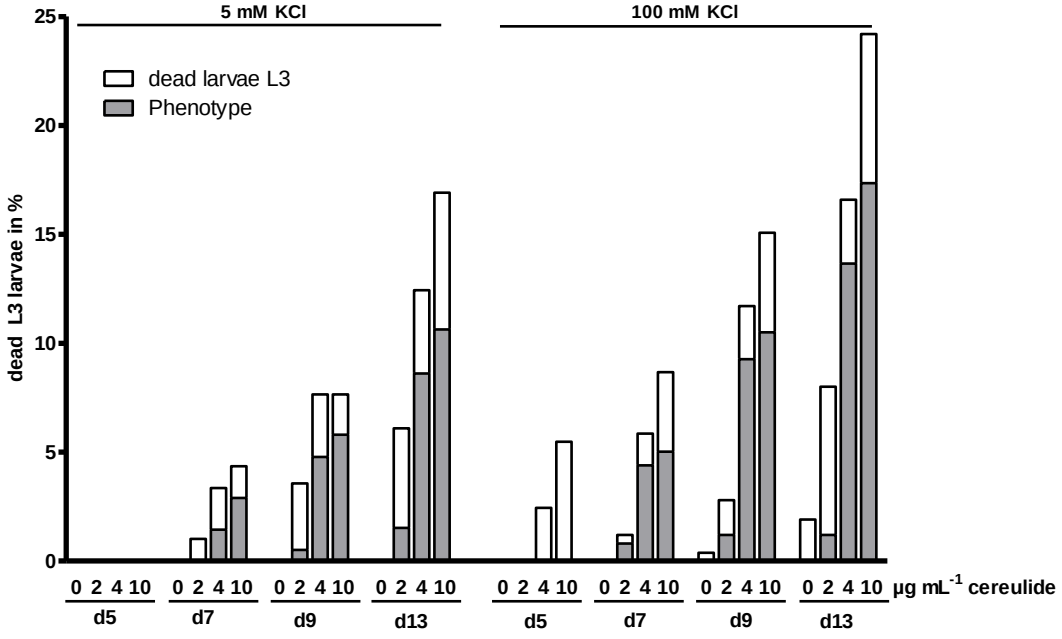


Figure 23: Nematocidal effect of cereulide on L3 larvae in LB with 5 mM and 100 mM KCl. Amount of total dead L3 larvae (white) and of L3 dead larvae, which exhibited a malformation (grey). Data derived from one representative cultivation.

3.3.4 Human Cells

The influence of various external potassium concentrations on the cytotoxicity of cereulide and valinomycin towards human cells was investigated using the human larynx carcinoma cell line HEp-2. Toxin concentrations, which inhibited 50% of the HEp-2 cells, were calculated for each tested potassium concentration. In regular cell culture medium (MEM; 5mM KCl) cereulide exhibited an almost 10-fold higher toxicity than valinomycin (Figure 24). When the external potassium concentration was raised to 25 mM, cereulide's toxicity increased nearly three-fold and valinomycin's toxicity 1.5-fold. Furthermore, a 10-fold potassium concentration (50 mM) in the cell culture medium led to a nearly 10-fold increase in cereulide toxicity. A similar effect was observed for valinomycin showing an 8-fold increase in toxicity.

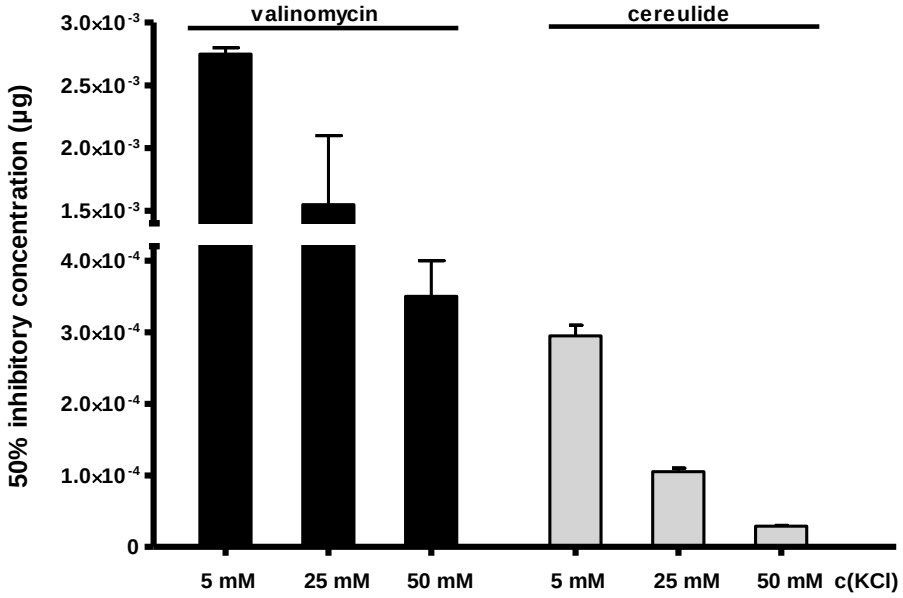


Figure 24: Impact of potassium on toxicity of cereulide and valinomycin to HEp-2 cells. Cells were treated with one of two toxin and different potassium concentrations. After incubation for 48 h at 37 °C the cell viability was measured using a calorimetric assay (WST-1). Mean of two independent experiments with double analysis. 50 % inhibitory concentrations were normalized by dividing through potassium control results.

3.4 Intoxication Studies with Pigs

A pig model was established for intoxication studies to gain insights into cereulide's action on mammal host. To get a nearly complete picture of cereulide's action, clinical findings, immunological-, blood-, and histological analyses as well as cereulide quantification measurements in organs, tissue, urine and faeces were carried out. Altogether, four experiments, each including four treated animals and one control pig, were performed. To analyse the acute toxicity, pigs weighing between 10-15 kg were fed with a single dose of either 10, 30 or 150 µg kg^{-1} bw cereulide or for analysis of chronic toxicity with the until now suspected emesis inducing dose of 10 µg kg^{-1} bw cereulide each day over a period of 7 days.

The experimental procedures were carried out in accordance with the international guidelines for the care & use of laboratory animals and approved by internal and Austrian governmental ethic committees. All piglets in this study were obtained legally from an official breeder. During the whole experiment animals were under constant supervision of a veterinarian. If pigs would have suffered constantly from severe pain, the experiment would have been terminated at any time.

3.4.1 Clinical Findings

Acute Toxicity

All piglets treated with 10 µg kg^{-1} cereulide per bw exhibited a transient depressiveness for 4.5 to 6 hours or 9 hours (one piglet) after oral administration. Additionally, seizures involving all four extremities for about 2-3 seconds were observed in half of the treated animals for about 20 minutes. Time periods of these neurological symptoms alternated with phases of typical vivid behaviour characterised by uptake of feed or playing behaviour. One animal vomited after 11 hours.

An increase of the applied cereulide dose to 30 µg kg^{-1} bw led to an earlier appearance of symptoms such as shivering after one hour (2 of 4 piglets) followed by seizures an additional hour later. The remaining two piglets displayed seizures 4-6 hours after cereulide administration. All symptoms disappeared after 7 – 9 hours. Between periods with seizures the piglets showed almost normal behaviour. One piglet, which shiver after 1 hour and suffered from seizures after six hours, additionally exhibited grinding of teeth and an increased salivation up to 24 hours after the oral administration of cereulide.

Application of 150 µg kg^{-1} bw led to a lethargic behaviour after 1.5 - 2 hours observed for all treated piglets. First symptoms were twitching of the snouts followed by convulsions of the whole body combined with seizures. Two piglets remained lying up to 2 hours after appearance of the first symptoms, meanwhile exhibiting profound lethargy, refusal of feed and up to 4.5 hours seizure attacks. One piglet displayed attacks over 4 hours and up to 12 hours transient depressiveness. After 6-7 hours, 3 of the 4 piglets gradually recovered.

Symptoms of the fourth piglet vanished more rapidly after 3.5 hours, but after 24 hours gasping respiration, foam at the mouth and strong grinding of teeth was observed. The latter symptoms disappeared slowly within 8-10 hours.

Chronic Toxicity

To analyse the chronic toxicity, cereulide was administered daily in a concentration of 10 µg kg^{-1} bw for a period of 7 days. Similar to the acute toxicity experiment with 10 µg kg^{-1} bw all piglets developed a transient depressiveness after 2-6 hours. Seizures could be observed for all four pigs to varying extents after 2-4 hours and a change in appearance was noticed from day to day. Two piglets exhibited seizures on one day, one piglet on two days and one piglet showed besides other symptoms, such as grinding of teeth, increased salivation, shivering of the body and increased respiration, the described seizures on four days. The latter piglet displayed the lowest weight of the group and a reduced appetite and depressiveness were observed on the first day of the experiment. For the remaining three pigs the occurring symptoms disappeared 6 hours after toxin administration every day.

All animals displayed normal pulse, respiration and internal temperature during the experiment, except of the time periods in which the neurobehavioral abnormalities such as seizures and convulsions appeared.

Histology

Histological examinations of the liver, lung heart, kidney, brain, spleen and gastrointestinal tract did not reveal any pathological changes. Two piglets treated with 150 µg kg^{-1} bw showed slight abnormalities of the lungs.

3.4.2 Blood Analysis

Haematology and Clinical Chemistry

Blood samples taken before and during the experiment were analysed with regard to possible changes of the following parameters: erythrocytes, haemoglobin, haematocrit, mean cellular volume (MCV), mean cellular heamoglobine (MCH), mean cellular heamoglobine concentration (MCHC), leukocytes, concentration of sodium, potassium, chloride, calcium and phosphor. Additionally, following biochemical markers were investigated: mean peroxidase index (MPXI), monocytes, lymphocytes, eosinophils, basophils, lymphoblasts, juvenils, neutrophils and segmented neutrophils, glucose, urea, creatinine, total protein, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma lactate dehydrogenase (GLDH), gamma glutamyl transferase (GGT), bile acid, lipase and creatine kinase.

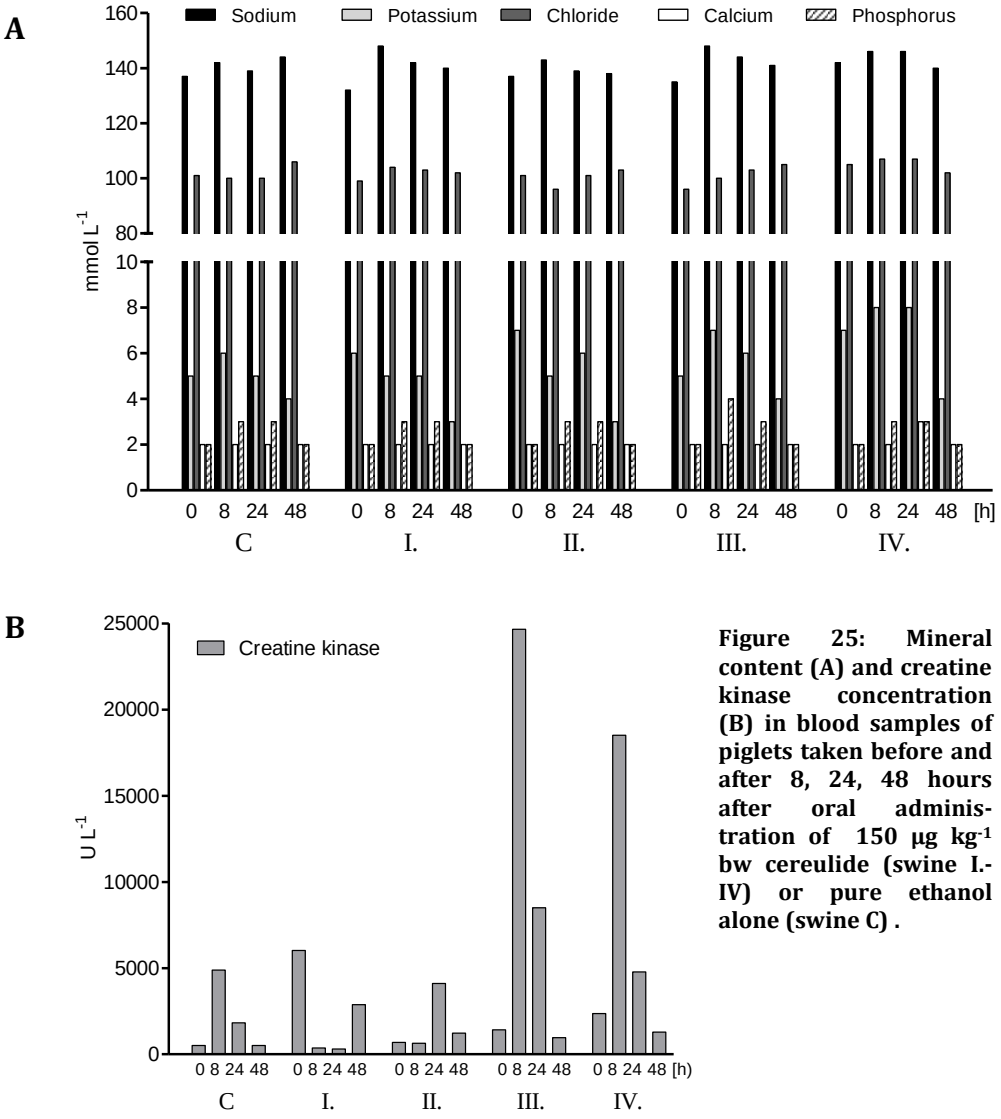


Figure 25: Mineral content (A) and creatine kinase concentration (B) in blood samples of piglets taken before and after 8, 24, 48 hours after oral administration of 150 µg kg^{-1} bw cereulide (swine I.-IV) or pure ethanol alone (swine C) .

No significant influence of cereulide on the blood cell composition and biochemical markers in blood samples taken before and after 24 and 48 hours could be observed by piglets treated with 10 and 30 µg kg^{-1} bw cereulide. For analysis of blood samples immediately after the relief of the symptoms, additional blood samples were taken 8 hours after treatment of 150 µg kg^{-1} bw cereulide. However, also the administration of 150 µg kg^{-1} bw cereulide caused no significant difference regarding the analysed parameters between treated and control pigs (example for minerals Figure 25 A). The only differing parameter was the creatine kinase, which was upregulated 8 hours post administration of the toxin in half of the pigs, but decreased continuously up to 48 hours (Figure 25 B). Blood parameter of samples taken before, after 3 and 7 days were also not affected by daily administration of 10 µg cereulide kg^{-1} bw in the chronic toxicity experiment.

Immunological Analysis

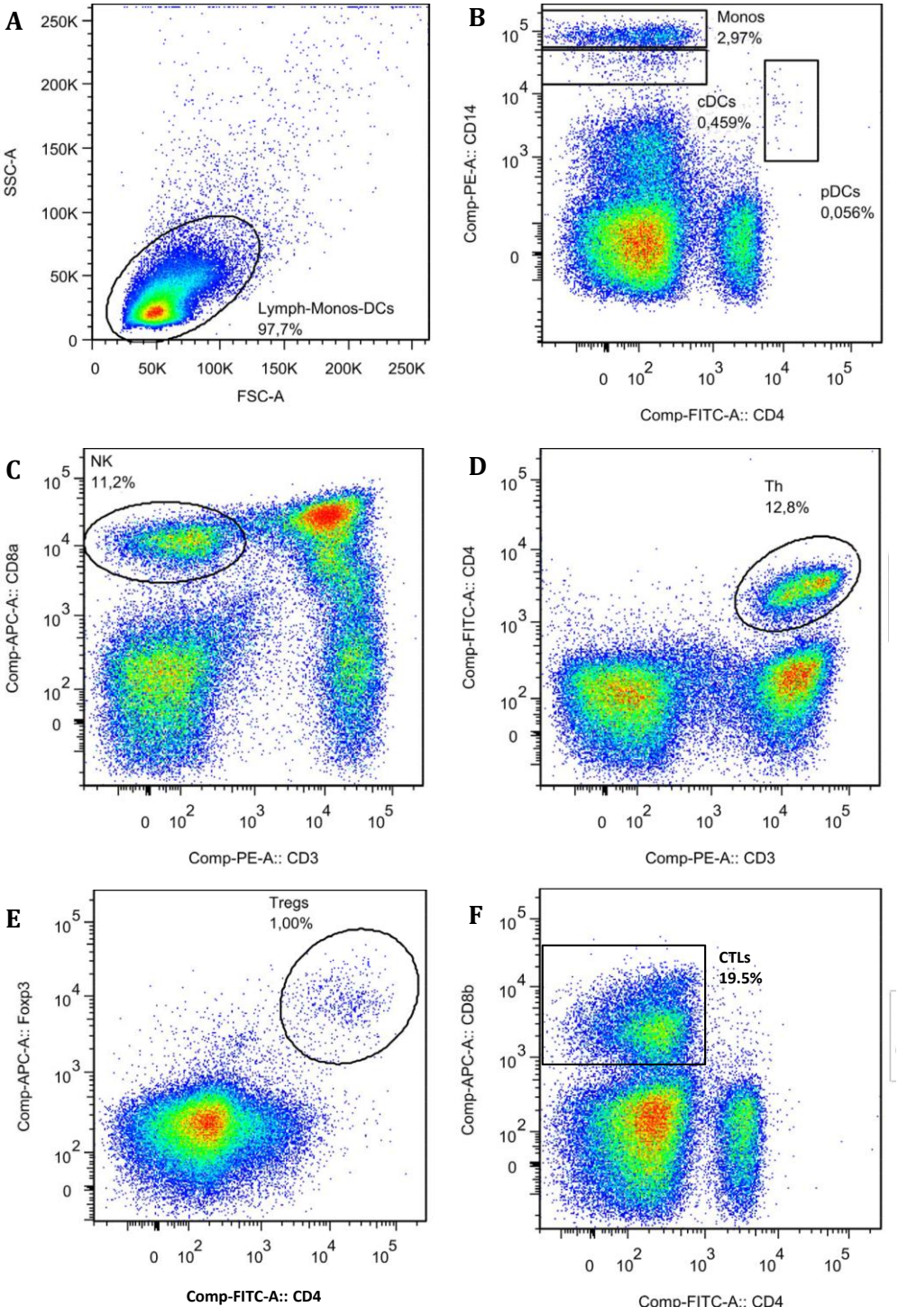


Figure 26: Leukocyte populations and lymphocyte subpopulation of cereulide treated pigs. Main PBMC population **(A)**, monocytes and dendritic cells (DC) **(B)**, natural killer cells **(C)**, T helper cells **(D)**, regulatoric T cells (T regs) **(E)** and cytotoxic T lymphocytes (CTLs) **(F)**. PMBCs were isolated from blood sample of piglet fed with 150 µg cereulide kg^{-1} bw taken 8 hours after cereulide administration.

The cell composition of peripheral blood mononuclear cells (PMBCs) isolated from blood samples of untreated and cereulide treated piglets was determined using flow cytometry analysis before intoxication and after 8 (only at 150 µg kg^{-1} bw), 24 and 48 hours after cereulide administration (examples are shown in Figure 26). The percentages of analysed leukocyte populations and lymphocyte subpopulations were not significantly influenced by oral administration of cereulide in concentrations of 10, 30 and 150 µg kg^{-1} bw (shown for 150 µg kg^{-1} bw in Table 9). Proportions of cell populations of blood samples were nearly stable in the same piglet, but differed more strongly between the piglets resulting in high standard deviations for some cell populations (for instance monocytes or CTLs in Table 9). Generally, leukocyte populations and subpopulations are subject to strong fluctuations as shown in untreated piglet (Table 9). Similar to the data of the acute toxicity experiments, the proportions of cell populations remained also stable after daily oral administration of 10 µg cereulide kg^{-1} bw over a period of 7 days for analysis the chronic toxicity of cereulide (data not shown).

Table 9: Leukocyte populations and lymphocyte subpopulations in % of total leukocytes of untreated and intoxicated piglets

Cell populations	untreated piglet				piglets fed with 150 µg cereulide kg^{-1} bw (Mean and SD)*			
	0 h	8 h	24 h	48 h	0 h	8 h	24 h	48 h
B cells	26.20	25.40	27.00	18.10	28.33 ± 5.46	23.13 ± 5.52	26.05 ± 3.95	25.03 ± 5.13
B cells $CD21^+$	14.25	15.67	13.4	9.15	15.54 ± 0.15	15.8 ± 0.31	17.7 ± 0.25	16.21 ± 0.31
Plasmacytoid DCs	0.90	1.17	0.81	1.10	0.66 ± 0.11	0.46 ± 0.09	2.19 ± 1.44	1.48 ± 0.52
Conventional DCs	0.11	0.18	0.19	0.21	0.16 ± 0.10	0.11 ± 0.07	0.11 ± 0.04	0.22 ± 0.08
Monocytes	14.10	12.90	10.80	12.00	11.53 ± 1.11	7.58 ± 4.51	10.89 ± 3.14	14.45 ± 2.51
T helper cells	16.10	15.30	11.40	17.20	14.83 ± 1.14	16.43 ± 3.31	16.70 ± 3.75	15.05 ± 3.18
CTLs	11.00	16.00	19.10	21.80	9.17 ± 3.92	17.73 ± 8.48	15.16 ± 7.09	13.65 ± 3.44
TCR γδ T cells	10.30	10.30	13.30	9.79	12.62 ± 5.91	14.05 ± 3.25	12.58 ± 3.60	10.68 ± 4.12
TCR αβ T cells	26.20	28.40	26.50	37.90	22.20 ± 4.34	31.48 ± 5.59	30.08 ± 5.32	27.60 ± 2.58
T regs	0.58	0.53	0.54	0.60	0.71 ± 0.09	0.87 ± 0.08	0.68 ± 0.19	0.70 ± 0.26
T regs $CD4^+$	0.48	0.44	0.44	0.51	0.60 ± 0.002	0.74 ± 0.001	0.56 ± 0.007	0.57 ± 0.011
NK cells	9.44	n d	8.22	10.50	7.23 ± 3.45	9.12 ± 5.45	8.11 ± 5.00	5.83 ± 4.32

* Mean and SD are derived from blood samples of four pigs

3.4.3 Quantification of Cereulide in Organs, Blood, Faeces and Urine

Due to the high stability and hydrophobic character of cereulide it is not known, if absorption of the toxin from the intestinal tract into the bloodstream occurs, resulting in distribution of cereulide in the whole body and potentially organ damage. The other possibility would be the excretion of cereulide. Therefore, organs, muscles, fat, blood, faeces and urine were analysed to quantify cereulide using the developed SIDA (3.2).

Highest cereulide concentrations were found in faeces in pigs fed with 10 and 30 µg kg^{-1} bw. After 12 hours 20.35 µg and after 24 hours 26.35 µg cereulide were excreted in comparison to the intake of 390 µg cereulide suggesting an uptake of cereulide into the body. Lower amounts of cereulide were detected in the intestinal content and the intestine. In a few cases low cereulide concentrations were also found in other organ samples indicating an uptake into the intestinal tract, entry into the bloodstream and distribution in the body. For example, in one pig fed with 30 µg kg^{-1} bw, cereulide was detected in a concentration of 26 ng g^{-1} in the heart, 7.8 ng g^{-1} in the small intestine and 3.6 ng g^{-1} in brain. Another pig showed toxin amounts of 25.9 ng g^{-1} in the muscle and 8.7 ng g^{-1} in the abdominal fat. These results clearly indicate an uptake of cereulide. Quantification of organs, faeces and urine of pigs treated with 150 µg kg^{-1} bw cereulide was not completed by submission of this work.

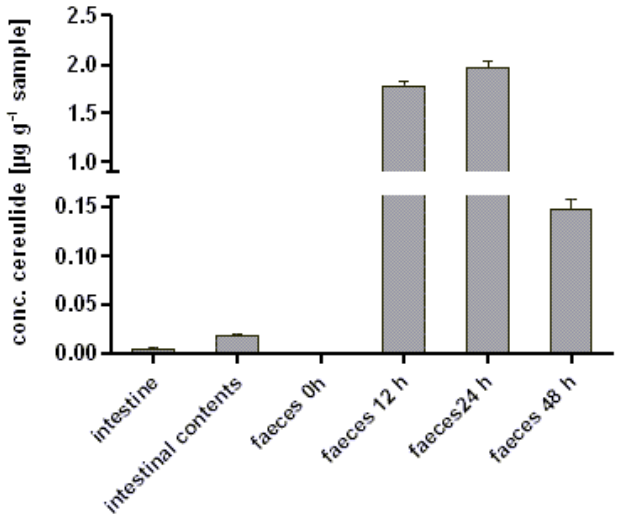


Figure 27: Cereulide quantification in intestine and faeces of pigs. Cereulide was mainly found in the intestine and the intestinal contents (48 h). High amounts of cereulide were excreted via faeces up to 48 hours after oral administration. Figure 27 shows a representative result of one pig after administration of 30 µg kg^{-1} bw. Samples were freeze-dried, crushed, extracted with ethanol and analysed using the developed SIDA (cf. 3.2).

3.5 Action on Enteric Nervous System

Cereulide is known to evoke emesis in humans. In feeding experiments it was demonstrated that cereulide induced emesis can be prevented by blocking of 5-HT_3 receptor. However, the underlying mechanism that leads to receptor activation by cereulide is still unclear. The enteric nervous system (ENS) represents an independent nervous system, which controls gut motility, secretion and blood flow and interacts with gastric muscles, epithelium and the immune system. To obtain insights into the influence of cereulide on the enteric nervous system, gastric muscle motility recordings in response to cereulide were performed. Because of its own nerve supply, gastric motility can be determined under *in vitro* conditions using an organ bath.

Addition of cereulide dissolved in DMSO to circular muscle stripes, which were prepared from guinea pig stomach, exhibited no significant effect on the muscle activity indicated by no influence of the basal tonus. Similarly, addition of DMSO (0.5 % final concentration) alone had no influence on the basal tonus (data not shown). In a next step the muscle activity was analysed after electrical stimulation before and after cereulide addition. The stimulation resulted in a response comprising an early contraction (on response) during the electrical stimulation followed by a decline of tonus after stimulus (post stimulation), a second contraction (off response) and finally, by a large relaxation (off relax) (cf. Figure 4). This described muscle response was compared before and after addition of cereulide or DMSO to the gastric muscle stripes. Although cereulide led to significantly enhanced responses to the electrical stimulation (Figure 28A), there was no difference to muscle preparations which were treated with DMSO alone.

To investigate if factors produced and/or released by the mucosa are necessary for cereulide action, experiments were repeated with circular muscle stripes without removing of the mucosa. Similar effects could be observed after supplementation of cereulide to gastric muscle stripes with mucosa, except with an enhanced effect at off relaxation (Figure 28 B). The comparison of the muscle response towards cereulide and towards DMSO revealed barely any differences regarding the impact on the muscle motility ($p>0.6$), which was almost the same in experiments with and without mucosa. Therefore, the observed effect cannot be due to cereulide alone.

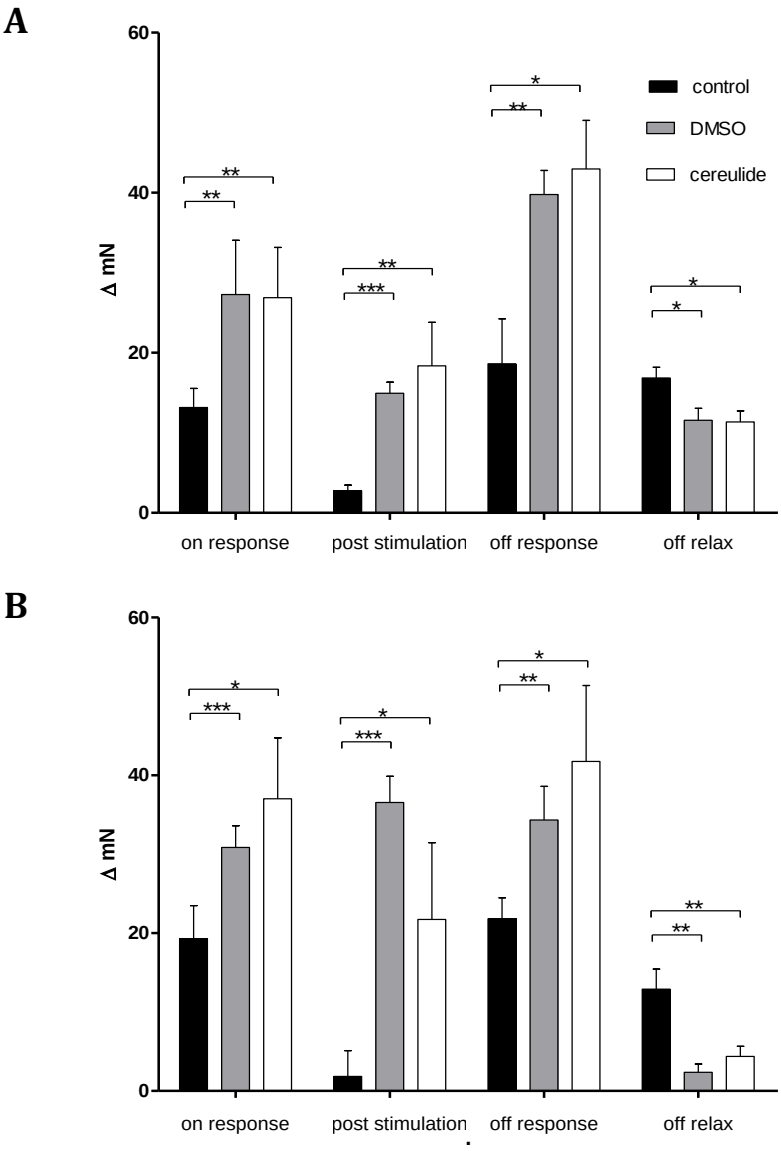


Figure 28: Circular muscle activity of guinea pig gastric corpus without mucosa (A) and with mucosa (B) after treatment with 0.5 % DMSO (grey bars) and 13 µM cereulide (white bars). Mean with SEM of four independent experiments. Significance differences (∗: p< 0.05; ∗∗: p<0.01; ∗∗∗: p<0.005) between control and DMSO or cereulide was calculated with the paired Student's t-test.

4. DISCUSSION

4.1 BIOSYNTHETIC PRODUCTION OF CEREULIDE REFERENCE MATERIAL AND STABLE ISOTOPE DILUTION ANALYSIS (SIDA) BASED ASSAY FOR DETECTION AND QUANTIFICATION OF CEREULIDE TOXIN

As cereulide poses a health risk to humans, the development of an appropriate method for the analysis of this toxin is mandatory. To establish a precise quantification system (cf. 3.2) and to analyse the action of cereulide (cf. 3.3-3.5), reference material of the toxin needed to be prepared in high quantity and purity. As previously reported procedures for cereulide preparation are rather laborious, time consuming and costly (Agata *et al.*, 1994; Andersson *et al.*, 1998; Häggblom *et al.*, 2002; Hormazabal *et al.*, 2004), this toxin should be prepared using a biotechnological approach, followed by rapid downstream purification. Therefore, the reference material of cereulide as well as its $^{13}C_6$-isotopologue was prepared by means of a biosynthetic approach using a *B. cereus* culture, followed by a rapid but efficient downstream purification. Using $^{13}C_6$-cereulide as internal standard, a stable isotope dilution analysis (SIDA) was developed for the precise quantification of cereulide in foods.

So far, for a quantitative analysis of cereulide in complex matrices such as food, urine or tissue, several biological cytotoxicity assays employing human larynx carcinoma cells or rat liver cells (Finlay *et al.*, 1999; Kawamura-Sato *et al.*, 2005), as well as a boar sperm motility assay (Hoornstra *et al.*, 2003), have been used. However, as the *in vitro* assays are based only on the toxic action of cereulide to mitochondria of eukaryotic cells and do not allow a direct measurement of the toxin, cytotoxic effects of other substances might influence the result of these assays when applied to more complex matrices, as e.g. food samples. Furthermore, bioassays are time-consuming and critically depend on the viability of cells or the motility of boar sperm, which derogates the reproducibility. To overcome these problems and analyse the target toxin directly, high-performance liquid chromatography-mass spectrometry (HPLC-MS) procedures using an ion trap mass spectrometer (Häggblom *et al.*, 2002; Jääskelainen *et al.*, 2003a; Shaheen *et al.*, 2006) or a single quadrupole mass spectrometer (Hormazabal *et al.*, 2004) have been developed. Since reference materials for cereulide have not been available, the antibiotic valinomycin was used instead as a surrogate standard compound for quantification, although its suitability has not been proven yet.

Therefore, the $^{13}C_6$-labelled standard developed in this work was compared to valinomycin for quantification of cereulide in complex matrices using LC-MS/MS analysis. However, valinomycin cannot to be considered as the ideal internal standard as this compound did not coelute with the cereulide (Figure 12) and is not suitable to overcome matrix effects

during LC-tandem mass spectrometry (MS/MS) analysis of complex food samples. However, such matrix effects could be counterbalanced by the use of a $^{13}C_6$-cereulide as internal standard instead of valinomycin. In detail, trueness, repeatability, and reproducibility expressed as relative standard deviation showed values <10 or <8 % for valinomycin and <8 % for $^{13}C_6$-cereulide, respectively (Table 6). Furthermore, the MS response of the valinomycin was found to be significantly influenced by the food matrix, thus leading to rather low recovery rates of 91 % from boiled rice and 80 % from boiled rice supplemented with 10 % sunflower oil (Table 6). In contrast, the use of $^{13}C_6$-cereulide as an internal standard gave good recovery rates of 104 and 111 % from both matrices, thus demonstrating the robustness and accuracy of the developed SIDA. These data clearly demonstrate the advantage of using $^{13}C_6$-cereulide rather than valinomycin as the internal standard for quantitative analysis of cereulide and revealed the developed SIDA as a reliable tool enabling a rapid and accurate quantitative determination of cereulide in low fat as well as high-fat food products.

Since the SIDA requires the availability of a stable isotope labelled internal standard, there might be the possibility to chemically synthesize ^{13}C-labelled cereulide using a complex sequence of reactions starting from suitable hydroxyl and amino acid derivatives (Biesta-Peters *et al.*, 2010). However, this total synthesis is rather time-consuming and expensive, and the biosynthetic production of ^{13}C-labelled cereulide using *B. cereus* strains and ^{13}C-labelled precursor amino acids might be a fast and cost-saving alternative.

To test the developed SIDA cereulide production in rice inoculated with *B. cereus* was monitored up to 96 hours. *B. cereus* synthesized cereulide in high quantities up to 6.12 µg/g rice at 96 hours at room temperature (Figure 13). This underpins that even weak infestations of emetic *B. cereus* in food products, stored at ambient temperature over a period of 1 day, are able to trigger fatal food poisonings. Hitherto, the poisonous dose of cereulide in humans is unknown, but feeding experiments with *Suncus murinus* and rhesus monkeys (Agata *et al.*, 1994; Isobe *et al.*, 1995; Shinagawa *et al.*, 1995) indicate an emesis-inducing dose of about 8-10 µg per kg body weight. This demonstrates that the consumption of even small amounts of rice stored at ambient temperature can induce emesis or other severe food borne intoxications in humans. Other analysed food products exhibited a very low (liver sausage) and middle production of cereulide in food after inoculation with low amounts of *B. cereus* (Table 7). These data clearly demonstrate the importance of the food matrix for cereulide production in processed foods.

In summary, a rapid biosynthetic procedure for the production and downstream purification of cereulide as well as $^{13}C_6$-labelled cereulide was developed and used for the quantification of cereulide in food matrices by means of a SIDA with HPLC-MS/MS detection. Method validation data confirmed the advantage of $^{13}C_6$-cereulide as the internal standard over valinomycin used for calibration in previous investigations (Häggblom *et al.*, 2002).

4.2 Effect of Cereulide on Different Organisms

The benefit of cereulide synthesis for the producer strain in its typical environment is still unclear. However, recently published work points toward an ecological role of cereulide, since it has been reported to prevent growth of some fungi and some bacteria (Ladeuze *et al.*, 2011; Tempelaars *et al.*, 2011). In the work presented here, cereulide activity on different organisms was analysed in more detail.

The small peptide cereulide exhibited a broad action on bacteria reaching from resistance to high sensitivity. In this study all analysed Gram-negative bacteria exhibited a resistance both towards cereulide and towards valinomycin. In contrast to the envelope of Gram-positive bacteria, the outer membrane of Gram-negative bacteria represents a permeability barrier, which impedes the entry of possibly harmful substances such as detergents, antibiotics or dyes into the cell. Both, the hydrophilic outer surface composed of densely packed LPS molecules and the inner surface consisting of glycerophospholipids prevent hydrophobic substances from entering the cell (Nikaido, 1996). By enhancing the permeability of the outer membrane via exposure to PMBN, Gram-negative bacteria became sensitive towards cereulide and valinomycin (Figure 16), indicating that the outer membrane of Gram-negative bacteria prevents the depsipeptide toxins cereulide and valinomycin from reaching their targets in the cell.

In contrast to Gram-negative bacteria, a strain specific antimicrobial impact of cereulide and valinomycin on Gram-positive bacteria was demonstrated reaching from nearly resistant (*S. aureus* and *L. monocytogens*) to very sensitive (*A. globiformes* and *R. fascians;* Figure 16 and Table 8). The cell envelope of Gram-positive bacteria consists of a thick, relatively porous structure, the peptidoglycan layer, which is accessible for small peptides (Lambert, 2002) and could not be responsible for the resistance of some Gram-positive bacteria towards the two depsipeptides. In nature a protection mechanism of the producer strain and relatives is a common observed trait (Abee, 1995; Marshall *et al.*, 1998; Riley, 1998). Likewise, the producer strain and related strains such as *B. thuringiensis* and *B. weihenstephanensis* were found to be resistant against cereulide treatment. Some protection mechanisms are known from other antimicrobial peptides such as membrane transporter e.g. antiporter (RosA/RosB, *Yersinia*) or ABC transporter (*Streptomyces*), intracellular degradation, modification of the specific intracellular target (gyrase B subunit, *Escherichia*) or modified cell membrane (*Staphylococcus*) (Mendez & Salas, 2001, for review see Yeaman & Yount, 2003). To explain this resistance mechanism the specific target of cereulide in the cell has to be found and compared to the intracellular presence of the potential target in other cereulide resistant Gram-positive bacteria, such as *S. aureus* and *L. monocytogenes*.

The majority of the strains (class II and III; Table 8) analysed showed a concentration dependent elongation of the lag phase duration λ in response to cereulide. Recently, it was shown that, due to their ionophoretic properties, cereulide and valinomycin are able to

decrease the membrane potential of Gram-positive bacteria (Tempelaars *et al.*, 2011). In the current study, antibacterial assays were performed using an extracellular potassium concentration of 170 mM KCL, a level similar to the potassium concentration in the cytoplasm of the cells, indicating that in our experiments the ionophoretic properties of cereulide and valinomycin did not play a major role in terms of potassium transport through membranes. Additionally, this fact cannot explain the high variability regarding the impact of the toxins on different bacteria. Generally, valinomycin showed a higher toxicity towards bacteria than to cereulide. The toxicity ratio (valinomycin: cereulide) was highly variable, depending on the analysed strain: 30-60-fold for 55 % of the tested strains and 2-8-fold for 25 % of the analysed strains (Table 8). Although the chemical structures of both toxins reveal a high similarity, in its biological activity cereulide differs from that of valinomycin. This demonstrates that valinomycin is not a suitable surrogate to study the activity spectrum and exact mode of action of cereulide. All organisms of class II and III had an elongation of the lag phase duration in response to increasing cereulide concentrations in common, but their maximal growth rate remained nearly constant. A shift in the growth start indicates a gradual adjustment process mostly by activation of a resistance mechanism that allows normal growth of the cells after adaption.

In contrast to bacterial cells, cereulide and valinomycin may act at two sites in intact eukaryotic cells, the mitochondrial membrane and the plasma membrane. In intact eukaryotic cells both toxins influence the electrical properties of cell membranes leading for example to an uncoupling of energy in mitochondria (Mikkola et al., 1999; Teplova et al., 2006). Additionally, the difference in mitochondrial toxicity of cereulide to valinomycin corresponds nearly to a factor of 2-4 (Teplova *et al.*, 2006), whereas in our cytotoxicity assay cereulide revealed a 10-fold higher toxicity compared to cereulide, indicating an additional action of cereulide besides the mitochondrial toxicity on cells described previously. Similar cytotoxicity ratios in HEp-2 cells were also observed with synthetic cereulide in comparison to valinomycin (Biesta-Peters *et al.*, 2010).

The nematocidal activity of cereulide and valinomycin was tested using the nodular worm *O. dentatum*. Developmental inhibition to fourth stage larvae and dead of third stage larvae were observed by addition of cereulide and valinomycin to the cultivation medium (Figure 21 and 22). Valinomycin exhibited higher toxicity compared to cereulide and act in very low concentrations reaching from 0.375 to 2 µg ml^{-1} (Figure 21). Similar concentrations of valinomycin in an earlier study led to a complete acetylcholine esterase inhibition and nematocidal activity towards *Nippostrongylus brasiliensis* and *Trichinella spiralis* (Geßner *et al.*, 1996). In contrast to valinomycin action, cereulide treated larvae exhibited a distinct phenotype in third stage: a malformation of the larval anterior part (Figure 22 C, D, E). For cyclic depsipeptides it was shown previously, that the ionophoretic and anthelmintic activity are not related (Geßner *et al.*, 1996). For instance, for the cyclic octadepsipeptide PF1022A binding on the gamma-aminobutyric acid receptors, which induces paralysis in *Ascaris suum,* was demonstrated (Chen *et al.*, 1996) and emodepsides have been reported

to be able to influence the function of latrophilin-like transmembrane receptors in nematodes (Harder *et al.*, 2005). Due to the specific phenotype of cereulide treated L3 larvae a receptor mediated action seems possible. For instance, the latrophilin-like receptor effects the pharyngeal pumping that is necessary for the feeding process of nematodes (Harder *et al.*, 2003; Harder *et al.*, 2005; Martin, 1997) and a similar mechanism of action might be expected at the specific cereulide phenotype malformation of the L3 larvae. However, a precise mechanism of action for this cereulide triggered phenotype needs further analysis.

Cereulide's impact turned out to be specific to L3 larvae of *O. dentatum* by inhibition of development and the characteristic phenotype resulting in dead of larvae. For instance the addition of cereulide in concentrations of 33-50 µg g^{-1} faeces from infected pigs did not significantly influence the development of *O. dentatum* from egg to the third instar larvae (L3) (K. Silbermayr, unpublished data). For example another microbial toxin of *Bacillus thuringiensis* completely inhibits the development from eggs to L3 larvae of *Haemonchus contortus* (O'Grady *et al.*, 2007). In the cultivation medium the availability of cereulide is increased and *O. dentatum* as detritus feeder actively takes up its nutrients from the medium and thus also an active uptake of cereulide in L3 larvae is expected. The nematocidal effect of cereulide is specific to L3 larvae and allows a control of larval development in nutrient rich environments such as faeces. Recently, it was demonstrated that 3.4 % of *B. cereus* isolated from stool belonged to the emetic type (Chon *et al.*, 2012).

The larvae of *Galleria mellonella* are routinely used as *in vivo* model organisms for investigations of pathogenic bacteria and fungi as well as toxic substances produced by these organisms (Joyce & Gahan, 2010; Mollier *et al.*, 1994; Ratcliffe, 1983). In the current study it was shown that larvae of *G. mellonella* were sensitive to cereulide and valinomycin. Both toxins caused a paralysis of larvae (at concentrations of 10 µg g^{-1} per larva valinomycin and 1.6 to 10 µg g^{-1} per larva for cereulide) within 3 hours (Figure 18). It is noteworthy that, in contrast to its action on bacteria, cereulide exhibited an approximately 8-fold higher toxicity towards *G. mellonella* larvae than valinomycin. Furthermore, cereulide displayed a different type of action after paralysis: cereulide injected larvae died, whereas valinomycin treated larvae almost fully recovered after 15 hours (Figure 18). These observations propose different actions of the toxins on larvae, suggesting that cereulide may have different or additional targets in insects. In accordance to the results of this study, the paralysis and recovering effect caused by valinomycin was also shown by feeding of *Bombyx mori* larvae with 1.5-3 µg g^{-1} toxin per larva (Angus, 1968). Similar to our described larval phenotype in response to high concentrations of cereulide and valinomycin, a study by Pansa *et al.* reported a motor paresis and curved body after injection of valinomycin into cockroaches (Pansa *et al.*, 1973). Other depsipeptides have also been linked to paralysis as for example PF1022A in *A. suum* and emodepside in *C. elegans*. The action of the latter two depsipeptides was independent from their ionophoretic properties (Geßner *et al.*, 1996; Harder *et al.*, 2005). For emodepsides a

receptor mediated mechanism is postulated. Latrophilin receptor activation triggers a signal chain that causes transmitter release resulting in relaxation of somatic body wall muscle and finally flaccid paralysis (Wilson *et al.*, 2004). A related receptor mediated mechanism might be responsible for a cereulide caused paralysis in insects.

B. cereus has been isolated from a huge number of insects such as mosquito larvae and sow bug and hence is able to colonize the gut of insects (Angus, 1956; Margulis *et al.*, 1998; Rae *et al.*, 2010; Swiecicka & Mahillon, 2006). Since in the referred studies mentioned strains were not analysed for the presence of *ces* gene cluster, it is so far unknown whether emetic strains are present in insects and if cereulide production plays a role in the infection process. Therefore, *B. cereus* infection assays were performed and *ces* gene expression as well as cereulide amounts measured in insects. Cereulide synthetase promotor activity and cereulide production of Bc WT pMDX [$cesP_1$/lux] was only be observed in dead insects (Figure 19 and Figure 20). After the death of larvae, *B. cereus* exhibited a high metabolic activity in larvae reflected in strong *cspA* promotor activity and *ces* gene transcription was strongly enhanced between 20 to 36 hours after infection. Infection assays and promotor activity analyses using a *ces* deficient knockout mutant Bc 4810/72 *ΔcesP* pMDX [$cesP_1$/lux] showed that cereulide production was not involved in the infection process resulting in killing of larvae, indicating that other virulence factors play an important role in insect killing. Dead larvae seemed to be an optimal nutrient source, as bacterial growth as well as cereulide production were observed, suggesting a saprophytic lifestyle of emetic *B. cereus* in insects. 36 hours after infection of pMDX [$cesP_1$/lux] the amount of cereulide in larva (Figure 20) was almost identical to the cereulide production in rice after 96 hours (Figure 13) indicating that dead larvae represent a good source for cereulide synthesis. Consequently, the question arises: why is cereulide production strongly enhanced in dead larvae? An answer might be the antibacterial and antifungal action of cereulide (cf. 3.3.1 (Ladeuze *et al.*, 2011)). Dead insects represent a good nutrient source in the soil. By cereulide production *B. cereus* may retard or even prevent the growth of other microorganisms and together with its high growth rate it can ensure the predominance in this nutrient rich environment. Moreover, dead insects can also contribute to a dispersion of bacteria and spores in the environment by being eaten and excretion by insectivore animals.

Furthermore, it could be demonstrated that PlcR controlled virulence factors are important for the infection process in the larvae and bacterial growth. It was shown previously that PlcR is not directly involved in the regulation of cereulide synthesis (Lücking *et al.*, 2009), but in *G. mellonella* larvae bacterial growth seems to be enhanced by PlcR controlled factors and as a consequence also cereulide production. PlcR controls the transcription of a huge number of secreted proteases, lipases, hemolysins and enterotoxins (Gohar *et al.*, 2002; Gohar *et al.*, 2008; Okstad *et al.*, 1999). In contrast to PlcR knockout studies of *B. thuringiensis* (Salamitou et al., 2000*)* it could be demonstrated that these produced factors obviously contribute to an early larval death after injection of

bacteria and to a decomposition of larval components resulting in a better acquisition of nutrients in larvae. These effects seem to promote drastically bacterial growth and finally cereulide production.
It has been reported recently, that another structure analogue of cereulide was isolated from *Paenibacillus tundrae* called paenilide, which is not a product of the pCer270 plasmid (Rasimus *et al.*, 2012). These bacterial cyclododecadepsipeptides seems to be common in nature and need further investigations regarding the exact ecological role of these secondary metabolites.

Influence of Potassium on Biological Activity of Cereulide

In the present study the impact of cereulide on different organism was analysed. It was demonstrated that the observed activity of cereulide strongly depended on potassium concentration in the environment. At low potassium concentrations (5 mM) cereulide was almost non-toxic towards analysed bacteria, however by enhancing of external potassium concentration the impact on bacteria indicating by a prolongation of lag phases. In contrast, valinomycin already exhibited antimicrobial activity at low potassium concentrations, but addition of potassium also enhanced its toxicity to a certain extend (Figure 14). However, cereulide has a higher affinity for potassium than valinomycin and is more toxic to eukaryotic cells at low potassium concentration (1-3 mM) (Mikkola *et al.*, 1999; Teplova *et al.*, 2006), at these concentrations cereulide did not exhibit antibacterial activity (Figure 15). Potassium occurs in the environment in rather low concentrations, typically ranging from 0.1 – 5 mM in soil (Blume *et al.*, 2010; Liebeke *et al.*, 2009; Maathuis, 2009). At these low KCl concentrations cereulide would probably exhibit a slight antimicrobial activity, however, higher potassium concentrations can be found in potash rich soils or in plants as well as their fruits e.g. seeds and beans. Equally, high concentrations up to 0.2 M potassium were found in humus, animal dung and insect gut (Harvey & Nedergaard, 1964; Leigh & Jones, 1984; Walker *et al.*, 1996; Wang & Wu, 2010), where *B. cereus* cells have been frequently isolated (Angus, 1956; Margulis *et al.*, 1998; Rae *et al.*, 2010; Swiecicka & Mahillon, 2006). Furthermore, an emetic *B. cereus* strain could be isolated from a potato, which is known for its high potassium content, especially in the peel (Altayar & Sutherland, 2006). These environments represent an optimal nutrient source for organisms such as bacteria and fungi. Only recently, an antifungal action of cereulide could be demonstrated (Ladeuze *et al.*, 2011). Exactly in these described potassium and nutrient rich environments, a rivalry among different organisms is likely and cereulide producing *B. cereus* strains may be able to dominate by inhibiting the growth of other organisms. Additionally, in the current study it could be demonstrated that emetic *B. cereus* are able to kill and growth in insects (Figure 19). In dead larva the cereulide production was induced and high amounts of cereulide in larvae could be detected. Recently, it was postulated that cereulide supports the fitness of emetic *B. cereus* strains by scavenge potassium in environments with low potassium (Ekman *et al.*, 2012).

Previous studies indicated a higher cereulide production in nutrient-poor environments (Shaheen *et al.*, 2006), but in emetic *B. cereus* cereulide synthesis is not upregulated under low potassium concentrations (0.1-5 mM) in minimal broth (T. Bauer, A. Rütschle, unpublished data). Cereulide is also produced under nutrient rich conditions for instance a higher cereulide production was demonstrated by addition of glucose (3.1.2) or addition of amino acids (Jääskelainen *et al.*, 2004). In these nutrient rich environments cereulide is able to inhibit the growth of typical soil organisms such as sensitive bacteria and fungi (Ladeuze *et al.*, 2011). Therefore, a delay in the growth of these organisms is sufficient for the fast growing bacterium *B. cereus* to prevail in this environment.

The impact of cereulide on eukaryotic cells was also increased by enhancing the external potassium concentration. For instance, an enhancement of external potassium of 5-10-fold increased the cytotoxicity in the same ratio (Figure 24). Cereulide is known to dissipate the mitochondrial membrane potential by its ionophoretic activity (Mikkola *et al.*, 1999; Saris *et al.*, 2009). Higher external potassium concentrations may contribute to a lower decrease in mitochondrial membrane potential in response to the ionophoretic activity of cereulide and should not enhance the toxicity. Therefore, an additional target of cereulide action in the cell is to be expected. A potential target could be the potassium amount in cytosol itself. There is some evidence that changes in intracellular potassium concentrations may play a role in induction and implementation of apoptosis (Bortner *et al.*, 1997; Bortner & Cidlowski, 1999; Vu *et al.*, 2001). It has also be shown, that staurosporine induced apoptosis, is characterised by a decrease in cytosolic potassium concentrations (Arrebola *et al.*, 2005; Bortner *et al.*, 1997). Moreover, it has been reported that a decrease in cytosolic potassium concentration enhances caspase-3 activation, resulting in apoptosis (Bortner & Cidlowski, 1998; Remillard & Yuan, 2004).

In insects paresis and paralysis was observed after treatment with cereulide. This effect could also be due to potassium efflux from cytosol to extracellular space where it can influence many chemical and electrical processes in the organisms such as muscle activity. In humans these effects are described for acute hyperkalemia (Choudhury & Visveswaran, 2009). In further studies it should be clarified if the cereulide induced paralysis and killing of insect larvae is due to the ionophoretic activity or a receptor mediated mechanism known from other depsipeptides (Harder *et al.*, 2005; Wilson *et al.*, 2004). A combination of both mechanisms might also be possible and could explain the different impact of valinomycin and cereulide on larvae (cf. 3.3.2.1). In experiments with nematodes an enhancement of external concentrations from 5 mM to 60 mM KCl did not influence the activity of cereulide. Interestingly, an additional increase of external potassium to 100 mM decrease the inhibition of larval development but increase the nematocidal effect on L3 larvae and the malformation of the cuticle, indicating a different target or other activities of cereulide are involved in both processes.

4.3 Pig Model for Intoxication Studies

Little is known about the impact of cereulide on the human body. Therefore, intoxication experiments with different doses of cereulide were performed using pig model and a broad spectrum of parameters reaching from blood analysis to toxin quantification in a set of organs was investigated. The model organism swine is very similar to humans in terms of size, alimentation, biochemistry, physiology and anatomy. Especially, their cardio-vascular system and digestive tract represent optimal models for studying human diseases (Swindle & Smith, 1998). Due to the omnivorous diet, metabolic functions, intestinal transport times and characteristics of nutrient absorption of swine resemble accordant processes in humans. Also, the response of pigs to drugs and chemicals is similar to the symptoms of humans, which makes the pig the model of choice for toxicology studies, whereas rodents often differ in disease responses or in its manifestations (Milano *et al.*, 1995; Walters *et al.*, 2011).

In this study piglets were fed with the previously described emesis inducing cereulide dose of 10 µg kg bw^{-1} (Agata *et al.*, 1995; Shinagawa *et al.*, 1995) and subsequently with higher concentrations of 30 µg kg^{-1} bw and 150 µg kg^{-1} bw, respectively. In haematological, immunological and histopathological analyses no difference to the control group could be observed at the concentrations analysed except for creatine kinase activity, which was strongly increased in 2 out of 4 pigs 8 hours after treatment with 150 µg kg^{-1} bw cereulide. Enhanced creatine kinase activity can often be observed in animals that lie down for long periods as is known for recumbency (personal communication Prof. Sipos), hence, the high activity could be due to the fact that the pigs were lying down for 2-4 hours in response to the ingested cereulide.

The developed SIDA (see 3.2) was used for the detection and quantification of cereulide in organs, faeces, urine and tissues of pigs fed with 10 and 30 µg kg^{-1} bw cereulide. The main part of cereulide was excreted via faeces, suggesting a low absorption from the intestine. However, low amounts of cereulide could also be detected in the heart, brain, muscles and fat indicating the ability of cereulide to enter the bloodstream after absorption and reach different organs and tissues, but a distinct organ as target could not be determined. This may be due to the low cereulide concentrations offered and/or the large size of organs, which did not allow the analysis of the whole organ, but only in small parts of diverse organs analysed. Experiments with higher concentration could underline the detection in some organs.

Cereulide was not detected in blood samples and cereulide did not significantly influence the blood composition such as cell types, protein concentration and trace elements (cf. 3.4.2 and Figure 21 and Table 9). Blood and urine samples are routinely analysed in clinical settings and diagnostic labs. However, the pig feeding experiments demonstrated that analysis of blood composition or urine does not provide information about cereulide intoxications. In contrast, due to its hydrophobic character the main part of the ingested

cereulide probably adheres to digested food and is consequently excreted via faeces. This study showed for the first time that screening of faeces samples represents a non-invasive method for detection of cereulide intoxications.

Remarkably, administration of the concentrations analysed in this work did not provoke emesis in piglets. In previously performed feeding experiments, cereulide induced emesis after oral administration in *Suncus murinus* (house musk shrew) at a concentration of 12.9 µg kg^{-1} bw (ED_{50})(Agata *et al.*, 1995) and in *Macaca mulatta* (rhesus monkey) with 5-7 µg kg^{-1} bw (calculated according to information of (Agata *et al.*, 1994; Agata *et al.*, 1995; Shinagawa *et al.*, 1995). Piglets seem to be more resistant towards cereulide than monkeys and shrews or the vomiting provoking mechanism in pigs differs to that of monkeys and shrews. The exact mechanism that evokes vomiting in humans in response to cereulide is unclear, but in feeding experiments with *Suncus murinus* it was shown that the 5-HT_3 receptor might play an important role (Agata *et al.*, 1995). Blocking of this receptor led to disappearance of emesis after treatment with cereulide, indicating an influence of cereulide on the receptor activation process. This could be a direct interaction of the toxin with the receptor or indirectly by influencing the activation process (see 4.4). It is known that pigs also possess the 5-HT_3 receptor and the emesis provoking mechanism is thought to be similar to the one of humans, because pigs are common model organisms to test antiemetic drugs used for chemotherapy (Girod *et al.*, 2000; Girod *et al.*, 2002; Grelot *et al.*, 1996; Szelenyi *et al.*, 1994). In this study, cereulide was added to milk to ensure its solubility and then offered to pigs. So far it is not known if and how complex matrices such as milk are influencing the activity of cereulide. For example, it might be possible that cereulide enters the lipid droplets in milk and this effect may influence the target and decrease the symptoms. A further explanation might be that pigs are tolerating higher toxin levels until vomiting is induced in comparison to monkeys and shrews. This effect has already been reported from different animal studies using the enterotoxin A of *Staphylococcus aureus* (SEA), a toxin that shows high similarity to cereulide with respect to the described symptoms in humans and the expected emesis provoking mechanism (Popoff & Poulain, 2010). In case of SEA, feeding experiments with piglets, monkeys and shrews demonstrated species-specific emetic doses. In detail, the 50 % emetic dose (ED_{50}) for piglets in response to SEA was nearly 4 to 10-fold higher, depending on the weight of the piglets, than the ED_{50} for *Suncus murinus,* and 10-fold higher than the ED_{50} for monkeys (Hu *et al.*, 2007; Suglyama & Hayama, 1965; Taylor *et al.*, 1982). This indicates that pigs probably tolerate higher toxin concentration until vomiting is induced. Therefore, it needs to be clarified which model organism corresponds best to human toxicity levels. In case reports, the emesis inducing dose or the cereulide concentration linked to other symptoms is generally not given (cf. Table 1). In very few cases the contaminated food was checked for cereulide, but in just one case the cereulide concentration was quantified in food (Naranjo *et al.*, 2011). However, in the latter case it is not known how much of the contaminated food was ingested by the person.

The pigs in this study exhibited neurobehavioral abnormalities such as alternating periods of lethargy and drowsiness, sometimes refusal of feed and the characterised seizures with increased salivation 2-3 hours after ingestion of cereulide. Manifestation and duration of these symptoms increased with rising cereulide concentrations. Some of these observed symptoms such as periods of lethargy, drowsiness and refusal of feed were also noted by pigs treated with SEA underpinning the hypothesis about a very similar target and mode of action in the body (Taylor *et al.*, 1982). Additionally, a disappearance of neuro-behavioral symptoms was found by an intraduodenal administration of SEA demonstrating an uptake of this toxin into the body within the passage from mouth to stomach (Taylor *et al.*, 1982). In the current pig feeding study, cereulide administration resulted primarily in trembling and seizures indicated by stretching out all four extremities. These symptoms point towards disturbances of the central nervous system (CNS). In low concentrations cereulide was detected in the brain and heart, indicating an uptake of the toxin in the bloodstream, thereby reaching the brain, where it may influence potassium concentrations by its ionophoretic properties. Animal epilepsy models revealed that one cause for seizures is aberrations in the potassium regulation in the brain (Frohlich *et al.*, 2008). Due to its lipophilic character cereulide might be able to cross the blood-brain-barrier (BBB) by transmembrane diffusion (Oldendorf, 1974), possibly leading to a disturbance of the potassium content of the cerebrospinal fluid. Potassium is normally prevented by the BBB from reaching the cerebrospinal fluid (Durand *et al.*, 2010). The ionophoretic properties of cereulide could affect that barrier function and potassium may be transported from blood (5 mM K^+) to the cerebrospinal fluid (2.9 mM K^+). Interestingly, it was shown previously that high potassium concentrations are involved in the generation of synaptic seizures (Durand *et al.*, 2010), which might explain the observed seizures in response to cereulide. Some of the observed symptoms such as lethargy, seizures and convulsions after oral administration of cereulide to pigs (3.4.1) resemble the described symptoms from human case reports (cf. Table 1). Lethargy could be observed in almost all patients a few hours after ingestion and in two of six cases seizures /convulsions were noticed up to six hours after ingestion. In all cases the amount of ingested cereulide is unknown and most of the cases are associated with a *B. cereus* infection. Therefore, in these cases it is not possible to draw clear conclusions about which symptoms are attributed directly to cereulide. The results of the pig feeding experiments and the observations of the case reports suggest additional targets of cereulide besides the $5HT_3$ receptor in the intestine. Pigs show neurobehavioral abnormalities indicating the CNS as possible target, whereas in some case reports the liver seems to play an important role, but these cases are mainly associated with a *B. cereus* infection (cf. Table 1). In one dramatically case report in 2009, the first symptoms resemble the results of the pig feeding experiments indicating the CNS as the main target: The boy showed drowsiness, abdominal cramps and convulsions after a few minutes after ingestion contaminated food and an acute encephalopathy was diagnosed (Ichikawa *et al.*, 2010).

Case reports linked to very severe intoxications resulted mainly in death suggest the ingestion of higher doses of cereulide than the ones used in the pig study described in this work. The observed symptoms in piglets occurred within 1.5-3 hours and were self-limiting after 7-9 hours after ingestion. Interestingly, the severe symptoms in case reports were appeared in the same time period, which may indicate either a higher uptake of cereulide or a higher sensitivity towards cereulide in humans.

4.4 Action of Cereulide on Enteric Nervous System

The emetic reflex represents a protective system of the body in order to recognise and finally eliminate ingested toxins (Andrews & Hawthorn, 1988). To gain the first insights into cereulide action on gastric tissue muscle recording experiments were performed with proximal gastric corpus. Addition of DMSO-dissolved Cereulide led to an increased response of contractions after electrical stimulation. However, since a similar reaction could be observed with DMSO alone, the observed effect may be caused by DMSO. This effect may overlay a possible action of cereulide at the concentration used in this experiment or cereulide may not influence muscle activity after electrical stimulation or basal tone. Therefore, it was not possible to draw any firm conclusion on influence of cereulide on the gastric motility using this method. Due to the limited solubility of cereulide in DMSO, it was not possible to use higher cereulide concentrations or to reduce the concentration of the solvent DMSO in cereulide solution.

The exact vomiting provoking mechanism of cereulide is unknown. In previous studies it was demonstrated that emesis caused by cereulide can be prevented by offering a blocker of the 5-HT_3 receptor (ondansetron) (Agata *et al.*, 1995). It was previously shown that by activation of the 5-HT_3 receptors in the stomach of guinea pigs the "on"- response (cf. 2.7.3) was enhanced in comparison to the control "on"-response (Michel *et al.*, 1997). As the current study showed no comparable effect of cereulide on gastric muscle stripes, this indicates that cereulide binds not directly to the 5-HT_3 receptor in the stomach or the increased response caused by the solvent DMSO overlaid the possible 5-HT_3 binding response of cereulide. A recent study showed that the vomiting provoking toxin SE type A of *S. aureus* (SEA) increased the release of serotonin (5-HT) in the intestine of *S. murinus*. A direct interaction of SEA to enterochromaffin (EC) cells or neurons to trigger the 5–HT release was postulated (Hu *et al.*, 2007). The symptoms of cereulide resemble that of SEA intoxications: both food poisonings are characterised by severe vomiting up to six hours after ingestion of contaminated food. Therefore, a possible influence of cereulide on the 5-HT metabolism or release of these signal molecules cannot be excluded. For other emetics such as tromadol or morphine it is known that they inhibit the re-uptake of transporters for norepinephrine and 5-HT (Barann *et al.*, 2006a; Barann *et al.*, 2006b). The mechanism of cereulide which provokes emesis has still to be elucidated.

5. CONCLUSION AND PERSPECTIVES

In this thesis a method for cereulide production in high quality and quantity was established and a precise cereulide quantification system was developed. In previous studies cereulide was detected indirectly using bioassays or by analytical techniques such as HPLC-MS, which allow detection, but not a precise quantification in complex matrices (Finlay *et al.*, 1999; Häggblom *et al.*, 2002; Kawamura-Sato *et al.*, 2005). Via the biosynthetic production of $^{13}C_6$ labelled cereulide, a stable isotope dilution analysis (SIDA) with a precise HPLC-MS/MS detection was established and validation of this method confirmed the advantage of the developed $^{13}C_6$-cereulide as internal standard over valinomycin used as standard in previous investigations. In this work it was shown that the developed SIDA is very suitable for the precise quantification of cereulide in food (3.2.4) and environmental samples (insects; 3.3.2.2). Moreover, in the pig intoxication studies it was shown that the SIDA allowed accurate toxin quantification in samples with clinical background such as organs, urine and faeces and thus may represent a helpful tool for analysis of clinical specimens of severe human cereulide intoxications. This demonstrates the possibility of a successful implementation of this method for rapid and accurate cereulide diagnostics, which would be very useful e.g. for the food industry or in clinical settings.

At the beginning of this thesis the benefit of cereulide production for emetic *B. cereus* was unclear and little was known about the biological actions of cereulide. Thus, the ecological role of cereulide was characterised by analysing its impact on different soil organisms. It was shown that cereulide exhibited a broad range of biological actions ranging from antibacterial, antihelmintical to insecticidal activity. Therefore, production of cereulide could represent a mechanism, which ensures and promotes the growth and distribution of the producer strain in its habitat or nutrient rich environments. High cereulide production and growth of *B. cereus* were also detected in dead insects in this work, suggesting a saprophytic lifestyle of emetic *B. cereus*.
For bacteria, a strain specific impact of cereulide on Gram positive bacteria was observed in growth experiments. Furthermore, it was demonstrated that the outer membrane of Gram-negative bacteria protects the cell against the impact of cereulide, whereas the resistance mechanisms of some Gram positive bacteria remain to be determined. Therefore, comparison of known resistance strategies would be informative to gain insights into resistance mechanisms of these bacteria. Insects and nematodes exhibited a specific phenotype after cereulide treatment that differed from the valinomycin induced phenotype indicating a cereulide specific, possibly ionophore independent mechanism. One possible impact of cereulide may be a receptor mediated activity, which was

previously shown for other cyclic depsipeptides (Harder *et al.*, 2005). To identify these receptors or mechanisms that activate receptors would be very helpful to unravel the molecular actions of cereulide in more detail. Furthermore, analyses of the impact of cereulide to additional organisms e.g. yeast or viruses may be helpful for a more detailed characterisation of the ecological role of cereulide in the habitat of *B. cereus*.
Interestingly, the toxicity of cereulide was enhanced by increasing the potassium concentration in the external medium in almost all performed biological assays. It remains to be examined whether this increase of toxicity is due to its ionophoretic action alone or potassium is involved in a receptor mediated mechanism. The detailed role of potassium in these processes requires further investigations.

In this work a swine model was established to analyse the impacts of cereulide on the mammal body. It was demonstrated that typical diagnostic human specimens such as blood samples and urine give no specific indication about cereulide intoxications. Since most of the ingested cereulide was excreted via faeces in the swine intoxications experiments, screening of faeces samples may represent a simple and non-invasive method for detection of cereulide intoxications in clinical settings. Additionally, low amounts of cereulide were detected in organs and an influence on the CNS was observed, indicating a possible absorption of the toxin from the intestinal tract and the ability of cereulide to enter the bloodstream in order to reach organs and tissues after ingestions. The ongoing quantitative analyses of organs and tissues of pigs fed with 150 µg cereulide kg^{-1} bw may provide further informations regarding the distribution in the mammal body and this information may give clues about the site of action. Also the impact of cereulide on the CNS, has to be analysed in more detail; for instance, cereulide's accessibility to the CNS through the blood-brain-barrier, its impact on neurons or the influence on the potassium concentration in the CNS are open questions that need to be clarified.
Analysis of cereulide's impact on the enteric nervous system using guinea pig stomach demonstrated no influence on gastric muscle activity that was specifically due to cereulide. It was proposed previously that the $5\text{-}HT_3$ receptor plays a role in cereulide provoked emesis, but data of this study suggest an indirect mechanism. To elucidate the distinct emesis inducing mechanism of cereulide in the gastrointestinal tract, further experiments must be carried out, such as the determination of 5-HT release in the intestine after addition of cereulide or the search of other gastrointestinal receptors that may be involved in the emesis provoking process.

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7. APPENDIX

Table S1: Optimized mass spectrometric parameters for the LC-MS/MS analysis of cereulide, $^{13}C_6$-cereulide, and valinomycin.

Compound	Mass transition (m/z)[a]	DP[b]	EP[c]	CE[d]	CXP[e]
Cereulide	$[M+H]^+$: 1153.7 → 1125.8	201	10	43	12
	$[M+H]^+$: 1153.7 → 172.3	201	10	123	6
	$[M+H]^+$: 1153.7 → 357.3	201	10	79	8
	$[M+H]^+$: 1153.7 → 314.2	201	10	87	8
	$[M+NH_4]^+$: 1170.9 → 1125.8	201	10	43	12
	$[M+NH_4]^+$: 1170.9 → 172.3	201	10	123	6
	$[M+NH_4]^+$: 1170.9 → 357.3	201	10	79	8
	$[M+NH_4]^+$: 1170.9 → 314.2	201	10	87	2
$^{13}C_6$-cereulide	$[M+H]^+$: 1159.9 → 1130.8	226	10	41	12
	$[M+H]^+$: 1159.9 → 173.3	226	10	113	10
	$[M+H]^+$: 1159.9 → 358.3	226	10	83	14
	$[M+H]^+$: 1159.9 → 316.2	226	10	83	2
	$[M+NH_4]^+$: 1176.9 → 173.3	176	10	119	10
	$[M+NH_4]^+$: 1176.9 → 358.3	176	10	85	12
	$[M+NH_4]^+$: 1176.9 → 316.2	176	10	85	10
	$[M+NH_4]^+$: 1176.9 → 501.4	176	10	83	14
Valinomycin	$[M+H]^+$: 1111.7 → 1083.8	166	10	39	24
	$[M+H]^+$: 1111.7 → 172.2	166	10	99	10
	$[M+H]^+$: 1111.7 → 343.3	166	10	77	14
	$[M+H]^+$: 1111.7 → 144.1	166	10	129	24
	$[M+H]^+$: 1111.7 → 371.2	166	10	67	12
	$[M+NH_4]^+$: 1128.8 → 1083.8	86	10	51	32
	$[M+NH_4]^+$: 1128.8 → 172.2	86	10	109	12
	$[M+NH_4]^+$: 1128.8 → 343.3	86	10	81	8
	$[M+NH_4]^+$: 1128.8 → 144.1	86	10	123	10
	$[M+NH_4]^+$: 1128.8 → 371.2	86	10	65	6

[a] Mass transition from pseudomolecular ion selected in Q1 and fragment ion detected in Q3
[b] Declustering potential [in V].
[d] Entrance potential [in V].
[c] Collision energy [in V].
[f] Cell exit potential [in V].

7 Appendix

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EDUCATION

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RESEARCH AND WORKING EXPERIENCES

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