Detection and quantification of the food-related bacterial toxins cereulide, lichenysin and botulinum neurotoxin/CD with liquid chromatography – tandem mass spectrometry

Philosophiae Doctor (PhD) Thesis

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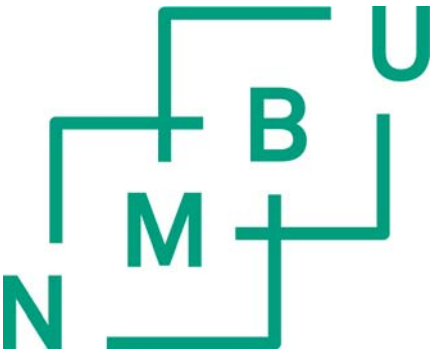
Adamstuen 2015



Thesis number 2015:103
ISSN 1894-6402
ISBN 978-82-575-1968-1

Series of dissertations submitted to the
Norwegian University of Life Sciences

Thesis number 2015:103

ISSN 1894-6402
ISBN 978-82-575-1968-1

Printed in Norway: Andvord Grafisk AS

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Acknowledgments

The work presented in this thesis has been performed at Department of Food Safety and Infection Biology at Norwegian University of Life Sciences (NMBU) from 2008-2015. The study on botulinum neurotoxin was funded by the Research Council of Norway, project no. 199375/I99. I would like to thank the Section for Food Safety for providing the resources necessary to complete this work.

Finally the day has come: my PhD thesis is done! Or at least I have to let go.

It has been an interesting and enlightening journey but also a frustrating and long one. There is one quality crucial to everyone only part-time hired to a time-consuming project like this, namely the ability to say «no». Unfortunately I do not possess this quality.

I would like to express my deepest gratitude to my supervisors Per Einar Granum and Tone Normann Asp for all your great ideas and interesting discussions. You have supported me to the bitter end and raised my spirit whenever I was certain the whole project went down the drain. Thank you both for being such optimists on my behalf!

I would also like to thank my supervisor Morten Skaugen. You have always responded promptly and thoroughly to my answers. I truly regret that you haven't been nearly as much involved as we planned to due to the change of course of the project.

Toril; my microbiology encyclopaedia. Without your patience with all my strange microbiology questions I would never have reached the finish line. Thank you!

To my co-authors Elisabeth and Elin, thank you for the collaboration providing interesting applications for the developed methods.

My dear former colleagues at the Chemistry laboratory: Kristin, Marianne, Daniela, Greta, Siri and Lojana. You all deserve a big Thank you! For all the help in the lab, the discussions, the chats, the laughter, the chocolate and the coffee breaks. I miss you all.

Tina, Olga and Marte: Thank you for all the bacterial work. I'm sorry I ruined your appetite.

John: Thank you for all the LC-MS discussions and for sharing my frustration whenever the instruments weren't cooperative.

To everyone at Section for Food Safety: Thank you for the laughter, the chatting and the hugs. Keep up the good work!

To Mum and Dad: Thank you for bringing me up making me as curious and stubborn as I am. (Otherwise I would never have finished this.) And thank you for all the babysitting, making this possible.

Last but not least: The ones who have suffered the most:

I would like to thank my wonderful husband Pål for just being you and always being there for me. And thank you for being such a great father for our sweet but mischievous girls; you have seen a lot to them lately. Finally they are getting their Mommy back too. I love you!

Helene Thorsen Rønning

Slependen, November 2015

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Determination and quantification of the emetic toxin cereulide from *Bacillus cereus* in pasta, rice and cream with liquid chromatography – tandem mass spectrometry.

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Detection of botulinum neurotoxin C/D through substrate cleavage and liquid chromatography – tandem mass spectrometry.

H. T. Rønning, T. N. Asp, T. Lindbäck, P. E. Granum

Manuscript

Summary

Food- and waterborne illness has a tremendous impact on the society, both economically and through the vast number of diseased people. Increased focus on food safety both at the consumer level and in the food producing industry is necessary to reduce the number of food poisoning outbreaks through extensive knowledge about the possible sources of food poisoning and available detection methods thereof. Bacterial toxins cause foodborne intoxications ranging from widespread disease with relatively mild symptoms to rare but possibly fatal disease.

Bacillus species and *Clostridium* species pose a distinctive challenge due to their endospore forming properties. The endospores are heat resistant and survive the food processing and preparation, enabling germination and toxin production in the prepared food. The main goals of this study have been to develop quantitative detection methods for the bacterial toxins cereulide from *Bacillus cereus* and lichenysin from *Bacillus licheniformis* and a qualitative detection method for botulinum neurotoxins from *Clostridium* spp.

A fully validated LC-MS/MS method has been developed for determination and quantification of cereulide in pasta, rice and cream. This method is now part of the analytical portfolio at Centre for Food Safety, NMBU. Two different studies have been performed to evaluate the cereulide production in food. An inoculation study of emetic *B. cereus* in rice, pasta and cream revealed cereulide production in the blank samples of rice and pasta. Repetition of the same study with eight different rice and pasta samples without inoculation showed that cereulide was produced in all eight samples. This indicates that the prevalence of emetic *B. cereus* in rice and pasta is higher than previously assumed. The second study sought to mimic the exposure of cold cuts to room temperature and evaluate the potential of toxin production and bacterial growth during lengthy holiday meals. Psychrotolerant *Bacillus weihenstephanensis* was used to evaluate the cereulide

production. Cereulide concentrations above the infective dose were first obtained after five days storage at 8 °C with daily exposure to room temperature varying from 0 -2 hours.

A fully validated LC-MS/MS method has been developed for determination and quantification of lichenysin in bacterial cell extracts. Lichenysin from 53 different *B. licheniformis* strains has been structurally examined with LC-MS/MS utilising quadrupole – time of flight. All strains produced the same lichenysin isoforms but the ratios between them vary. This finding strengthens the assumption that growth conditions have a greater influence than the genotype on which isoforms are produced. An amino acid substitution in one of the isoforms was detected; also this was present in all samples.

An extensive study has been performed on different calibrants for both cereulide and lichenysin, concluding that the use of the analyte itself as calibration standard and isotopically labelled internal standards are of utmost importance to achieve correct quantification. Other calibration standards fail to compensate for matrix effects and other possible influences on the analyte throughout the analysis.

A qualitative LC-MS/MS method for the detection of botulinum neurotoxin /CD in growth medium utilising the toxin's endopeptidase properties has been developed. Specific product peptides resulting from substrate cleavage by BoNT/CD are detected with LC-MS/MS instead of the actual toxin; hence, the toxin activity is included in the detection method.

Sammendrag

Mat- og vannbåren sykdom har en stor betydning for samfunnet, både samfunnsøkonomisk og på grunn av det store antallet mennesker som blir syke. Økt fokus på mattrygghet både på konsumentnivå og i matproduksjonsindustrien er nødvendig for å redusere omfanget av matforgiftning. Omfattende kunnskap om mulige årsaker til matforgiftning og gode deteksjonsmetoder for dem er nødvendige for å oppnå målet. Bakterietoksiner kan forårsake ulike typer matforgiftning, alt fra hyppige utbrudd med milde symptomer til sjeldne, men dødelige sykdommer.

Slektene *Bacillus* og *Clostridium* er spesielt utfordrende fordi de danner sporer. Sporene er motstandsdyktige mot varme og kan overleve både matproduksjonen og tilberedelsen av mat for deretter å germinere og danne toksiner i den ferdige maten. Hovedmålet med denne oppgaven har vært å etablere kvantitative deteksjonsmetoder for bakterietoksinene cereulide fra *Bacillus cereus* og lichenysin fra *Bacillus licheniformis* samt en kvalitativ deteksjonsmetode for botulinum neurotoxin produsert av ulike *Clostridium* arter.

En fullt validert LC-MS/MS metode har blitt etablert for deteksjon og kvantifisering av cereulide i pasta, ris og fløte. Denne metoden tilbys nå kommersielt fra Senter for Mattrygghet, NMBU. Cereulideproduksjon i mat har blitt evaluert i to ulike studier. En inokuleringsstudie med emetisk *B. cereus* i pasta, ris og fløte viste at det også ble produsert cereulide i blindprøvene av ris og pasta. Forsøket ble gjentatt uten inokulering med åtte ulike ris- og pastaprøver hvor cereulide ble funnet i alle prøvene. Dette antyder at utbredelsen av emetisk *B. cereus* i ris og pasta er større enn tidligere antatt. Den andre studien etterlignet kaldt kjøttpåleggs eksponering for romtemperatur og evaluerte toksinproduksjonen og bakterieveksten i løpet av langvarige jule- og påskefrokoster. Den kuldetolerante bakterien *Bacillus weihenstephanensis* ble brukt til å evaluere cereulideproduksjonen.

Cedeulidekonsentrasjoner høyere enn infektiv dose ble først observert etter fire døgn ved 8 °C med daglig eksponering for romtemperatur i 0-2 timer.

En fullt validert LC-MS/MS metode har blitt etablert for deteksjon og kvantifisering av lichenysin i bakterielle celleekstrakter. Molekylstrukturen til lichenysin fra 53 ulike *B. licheniformis* stammer har blitt bestemt med LC-MS. Alle stammene produserte de samme isoformene men med ulikt forhold dem imellom. Dette funnet understøtter antagelsen at vekstforholdene har større innvirkning enn genotypen på hvilke isoformer som dannes. En aminosyresubstitusjon ble oppdaget i én av isoformene, også denne ble påvist i alle prøvene.

Ved å studere kvantifisering med ulike kalibreringsstandarder for både cereulide og lichenysin er det vist at bruk av analytten som kalibreringsstandard og isotopmerket intern standard er nødvendig for å oppnå riktig kvantifisering. Bruk av lignende molekyler som kalibreringsstandarder og internstandarder klarer ikke å kompensere for matrikseffekter og annen type påvirkning på analytten i løpet av analysen.

En kvalitativ LC-MS/MS metode som utnytter toksinets endopeptidase egenskaper, har blitt etablert for deteksjon av botulinum nevrotoksin /CD i vekstmedium. BoNT/CD danner spesifikke produktpeptider ved å kløyve et substrat. Deteksjon av produktpeptidene istedenfor BoNT/CD med LC-MS/MS inkluderer toksinaktiviteten i deteksjonsmetoden.

Abbreviations

AA	Amino acid
BoNT	Botulinum neurotoxin
CFU	Colony forming units
EFSA	European food safety authorities
ELISA	Enzyme-linked immunosorbent assay
FEEDAP	EFSA panel on additives and products or substance used in animal feed
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
Mm	Molecular mass
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MWCO	Molecular weight cut-off
NRPS	Non-ribosomal peptide synthetase
PCR	Polymerase chain reaction
QqQ	Triple quadrupole
Q-ToF	Quadrupole – time of flight

1 Introduction

1.1 General introduction

Food- and waterborne illness is a common challenge worldwide, causing distress and sometimes life-threatening disease. Of the estimated 1.5 billion episodes/year of diarrhoea in children under the age of five, including three million deaths, is a substantial part of food- and waterborne origin [1]. The economic impact of food- and waterborne illness is considerable; it is estimated to US$ 15.6 billion in USA and £ 1.5 billion in UK annually [2–4]. Equivalent figures are not estimated for Norway.

Bacteria have developed several mechanisms for survival in nature. From a food safety point of view, spore formation and toxin production are probably the most important and both play a central role in food related disease. There are many different bacterial toxins with completely different characteristics. Some toxins are produced in foods prior to consumption and the bacteria might not even be present due to (heat) treatment of the foods prior to ingestion. Among the heat stable toxins are the *Staphylococcus aureus* enterotoxin (SE) and the *Bacillus cereus* emetic toxin (cereulide). Botulinum neurotoxins are also pre-formed in foods but are sensitive to heat and therefore dependent on production in non-heat treated foods or in foods after heat treatment. The majority of bacterial food poisoning toxins are proteins produced in the intestinal tract where they cause infection after ingestion of living cells, e.g. cholera toxin and many of the different toxins produced by *Escherichia coli* [1]. For some of the food poisoning diseases all symptoms are caused by the bacterial toxins, whereas for others the toxins only increase the severity of the disease.

Cereulide and lichenysin produced by *Bacillus* spp. and botulinum neurotoxins (BoNTs) produced by several *Clostridium* spp. are all bacterial toxins associated with food poisoning. BoNTs are large proteins with Mm 150 kDa. Cereulide and lichenysin are both produced by non-ribosomal peptide synthetases (NRPS) enabling their cyclic structures including non-proteinogenic D-amino acids [5]; cereulide is a cyclic dodekadepsipeptide produced by cereulide synthetase encoded by the *ces* gene

cluster [6] and lichenysin is a cyclic lactonic heptalipopeptide with a fatty acid tail containing 12-15 carbons, produced by lichenysin synthetase encoded by the *lchA* gene cluster [7].

1.2 *Bacillus*; prevalence and its endospore forming property

The genus *Bacillus* consists of saprophytic, endospore forming bacteria that is ubiquitous in nature. Traditionally considered as soil bacteria, Bacilli have lately been assessed as gut commensals following findings of large amounts of Bacilli in human faeces and ileum [8–10]. An endospore is a dormant form of a bacterial cell containing a chromosome covered by small acid soluble spore proteins, protected by several layers (cortex, coat and crust) making the spore able to withstand environmental stress like heat, desiccation, UV-radiation and acidity allowing the bacterium to survive outer challenges like nutrient depletion and habitat alterations [11]. *Bacillus cereus* endospores are frequently also covered with an exosporium that might offer additional protection against chemical and enzymatic exposure and increases the adhesive properties of the endospores by providing a hydrophobic surface [11]. Among the *Bacillus* species sporulation is mainly triggered by starvation [12]. When appropriate growth conditions are re-established the endospores germinate into vegetative cells. This poses a challenge in food safety as endospores survive the initial heat processing and might germinate, multiply and produce toxins in the prepared food.

1.2.1 Cereulide

1.2.1.1 Relevance to food poisoning

Bacillus cereus is the *Bacillus* spp. most commonly associated with food poisoning [13]. It produces four different toxins; the emetic toxin cereulide and three different diarrhoeal enterotoxins [14–16]. *Bacillus weihenstephanensis* is a psychrotolerant species in the *Bacillus cereus* group that is capable of cereulide production at 8 °C [17,18](Paper II). Cereulide is a well-known source of intoxication caused by ingestion of food containing preformed toxin [15]. The illness is usually accompanied by the relatively mild symptoms nausea and vomiting appearing 0.5 to 6 hours after ingestion of the

contaminated food [16,19,20](Paper I). The mild symptoms and the symptomatic similarity with *Staphylococcus aureus* induced food poisoning probably result in an underreporting of cereulide as causative agent [16]. Cereulide has also been confirmed as the causative agent in severe cases of liver failure and brain oedema, resulting in liver transplant [21] and fatalities [22–25]. A wide range of foods might contain cereulide due to the ubiquitous nature of *B. cereus*. Even though mostly associated with farinaceous foods, both tuna [26] and fermented black beans [27] have been recognized as the source of cereulide food poisoning and Messelhäusser et al. [28] demonstrated that a wide range of food products contained emetic *B. cereus* strains. A majority of the reported outbreaks of cereulide induced food poisoning have occurred in restaurants, cafeterias and catering establishments [13]. However, all cereulide-induced food poisoning cases with fatal outcomes have been domestic and were caused by cooked pasta or rice stored at least 24 hours at insufficiently low temperatures. The European Food Safety Authorities (EFSA) has evaluated potential risks of *B. cereus* in foodstuffs and published a Scientific Opinion including recommendations to manage the risk caused by *B. cereus* and other *Bacillus* spp. in foodstuffs [13].

1.2.1.2 Structure, physicochemical properties and mode of action

Cereulide is a cyclic dodekadepsipeptide with molecular mass 1153 Da and the structure: [D-O-Leu-D-Ala-L-O-Val-L-Val]$_3$ (Figure 1) [29,30]. This ring structure is highly stable making cereulide resistant to both acidic conditions, high temperatures (121 °C for 30 min) and proteolytic cleavage [30]. These properties enable survival during cooking and reheating of food and ensure safe passage through the gastrointestinal tract.

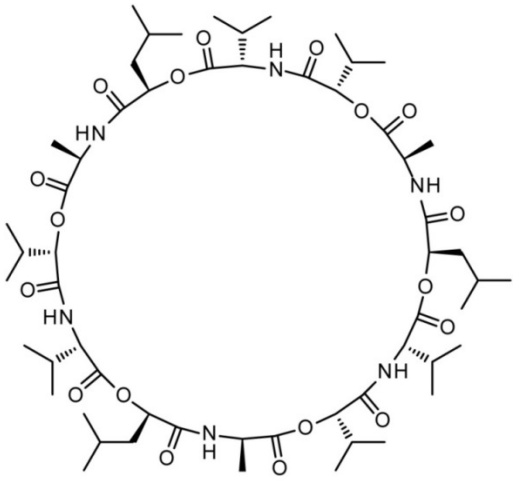

Figure 1: Molecular structure of cereulide (with permission from Chiralix B.V.)

The presumed mechanism of action that causes emesis in humans is binding of cereulide to the 5-HT_3 receptor and stimulation of the afferent vagus nerve in the duodenum [30]. Cereulide is a potassium ionophore. In the more severe food poisoning incidents transportation of K^+ into the mitochondria causes swelling and subsequently mitochondrial damage in the liver by inhibition of the fatty acid oxidation which in turn might lead to fulminant liver failure [31].

1.2.2 Lichenysin

1.2.2.1 Relevance to food poisoning and animal disease

The first involvement of *Bacillus licheniformis* in food poisoning was reported in the 1970s [32]. The virulence factor was neither detected nor described. A few more incidents of food poisoning (intoxications) involving large amounts of *B. licheniformis* are known, one of which had a fatal outcome [33]. Both *B. licheniformis* strains isolated from baby milk formula associated with the death of an infant, and other strains of *B. licheniformis* linked to food poisoning, were shown to produce lichenysin [33,34]. Lichenysin was the only toxic compound found in the cell extracts from the baby milk formula, strongly suggesting that this toxin was the causative agent of food poisoning. *B. licheniformis* has also been linked to animal abortions and bovine mastitis where lichenysin-

producing strains have been isolated from mastitic milk [35–38]. Several other *Bacillus* spp. also produce lipopeptides; *B. subtilis*, *B. mojavensis, B. amyloliquefaciens* and *B. pumilus* [39–45]. The structure and physicochemical properties of these lipopeptides are very similar. In addition to lichenysin, both pumilacidin and amylosin have been involved in food poisoning cases were the assumed mode of action is pore formation in membranes [44,46].

B. licheniformis is widely used in the industrial production of probiotics, antibiotics, proteases and amylases and some of these products are used as feed additives [47,48]. This use is regulated by the EFSA Panel on Additives and Products or Substance used in Animal Feed (FEEDAP) who in 2014 published a Scientific Opinion that recommends that the cytotoxicity of all *Bacillus* strains (non-*B. cereus*) considered as feed additives should be evaluated by an in vitro cell based method [49]. This recommendation is an alteration of the previous Scientific Opinion from 2011 where PCR-screening for NRPS-genes and testing for haemolysis were considered sufficient to reveal potential lipopeptide production in the strains of interest [50]. The revision was a direct consequence of the findings that most *B. licheniformis* strains produce lichenysin and several are non-haemolytic (Paper IV), and that several strains of *B. subtilis* and *B. pumilus* are haemolytic and produce lipopeptides [51].

1.2.2.2 Structure, physicochemical properties and mode of action

Lichenysin is a heat-stable, protease-resistant, and pH-stable cyclic lipopeptide consisting of a peptide ring with seven amino acids and a β-hydroxy fatty acid with 12 – 17 carbon atoms with possible normal, iso and anteiso branching [33,34,52–59]. Several isoforms and homologues have been observed in nature, both with different amino acid substitutions and varying length and branching of the hydrocarbon chain. Yakimov et al. [58] named the most abundant isoform lichenysin A with amino acid sequence L-Gln – L-Leu –D- Leu –L- Val – L-Asp –D- Leu – L-Ile (Figure 2) [34,56].

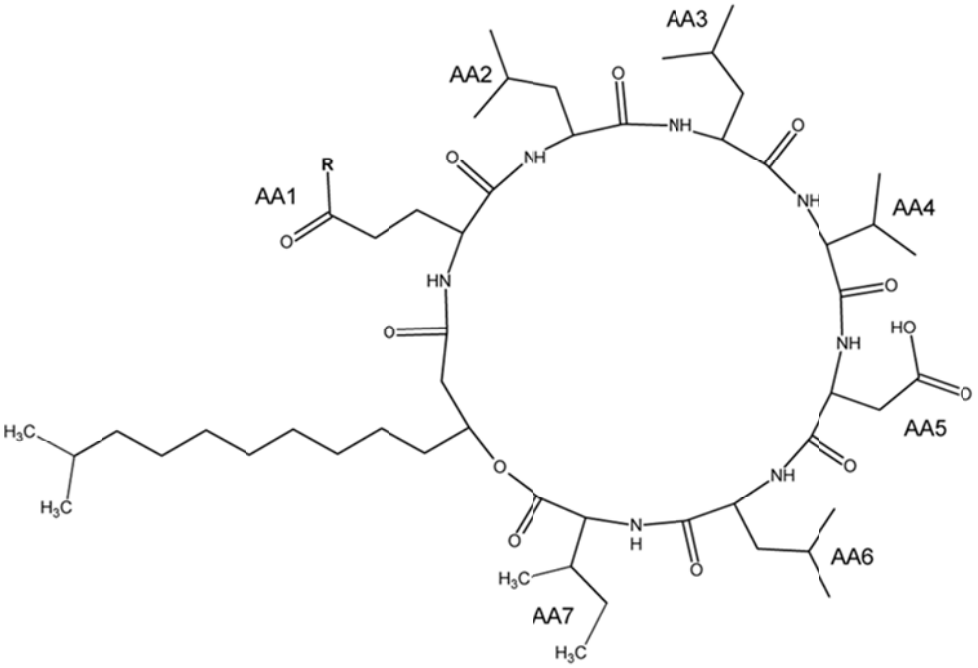

Figure 2: Molecular structure of lichenysin A (monoisotopic mass 1020, R = NH_2) and surfactin (monoisotopic mass 1021, R = OH), differing only with a glutamine (lichenysin) / glutamic acid (surfactin) substitution at amino acid position AA1.

The spatial conformation of lichenysin contains the hydrophobic amino acid residues folded towards the hydrocarbon chain while the hydrophilic amino acid residues are extending at the opposite end, making lichenysin an amphiphilic molecule [60]. The surfactant properties of lichenysin are a result of the amphiphilic nature of the molecule; lichenysin can lower the surface tension in water from 72 mN/m to 27 mN/m [59]. It is also an excellent chelating agent for Ca^{2+} and Mg^{2+} [61]. Another well-known surfactant from a *Bacillus* spp. is surfactin produced by *Bacillus subtilis* [62]. Surfactin and lichenysin are closely related; their molecular structures differing with only one amino acid where glutamine in lichenysin is substituted with glutamic acid in surfactin (Figure 2) [56,58,63]. Comparison of the physicochemical properties of surfactin and lichenysin demonstrates that this single amino acid substitution significantly improves the molecule's surfactant properties: The critical

micelle concentration (MCM) is 22 µM for lichenysin and 220 µM for surfactin. Haemolysis (100 %) is obtained with 15 µM lichenysin and 200 µM surfactin, whereas the association constant is 4 and 16 times higher for lichenysin than for surfactin with Ca^{2+} and Mg^{2+}, respectively [61]. The physicochemical properties of lichenysin enable pore formation in membranes that in turn causes the cytotoxicity by disrupting the ion flux [60].

1.3 Botulinum neurotoxins

1.3.1 Prevalence and relevance to food poisoning and animal disease

Clostridia are anaerobic sporeformers that are omnipresent in nature and often found in soil and sediments [64]. Already in 1895 *C. botulinum* was recognised as the causative agent of food poisoning after isolation of the bacterium from both ham and a human that died from botulism [65]. Botulinum neurotoxins (BoNT) are produced by six phylogenetically distinct Clostridia; *Clostridium botulinum* group I – III, *Clostridium argentinense*, *Clostridium butyricum* and *Clostridium baratii* [66]. BoNTs are the most toxic natural substances known with a lethal dose of 1 µg/kg body weight when administered orally and as low as 1.3 – 2.1 ng/kg body weight when administered intravenously [67,68]. BoNTs cause botulism in vertebrates, an illness involving flaccid paralysis that might progress to respiratory failure and death if untreated [65,69]. Eight serotypes of BoNT (BoNT/A - BoNT/H) with more than 40 subtypes are described so far [65,66]. Both reservoir and genotype varies between the serotypes and their toxicity is species-dependent: BoNT/A, -B, -F and –H are toxic to humans while BoNT/C1 and –D are mainly toxic to birds and mammals but also reported in association with infant botulism [70–72].

Avian botulism is a significant contributor to mortality in poultry and waterfowl [72,73], in the Nordic countries has BoNT/C1 mainly been associated with botulism in poultry [74,75]. Outbreaks among mink and foxes at fur farms caused by BoNT/C1 have also been reported [76,77]. Chickens grow less susceptible to BoNT as they age; hence, the majority of botulism outbreaks in poultry involve young

broilers [73]. However, a recent case report from Sweden associates a botulism outbreak among laying hens with BoNT/CD [78].

1.3.2 Structure, physicochemical properties and mode of action

BoNTs are large proteins with Mm around 150 kDa. Their structures include a heavy chain with Mm 100 kDa and a light chain with Mm 50 kDa. Activation of the toxin occurs through proteolytic cleavage whereupon the light chain and heavy chain only are connected by a disulphide bridge (Figure 3).

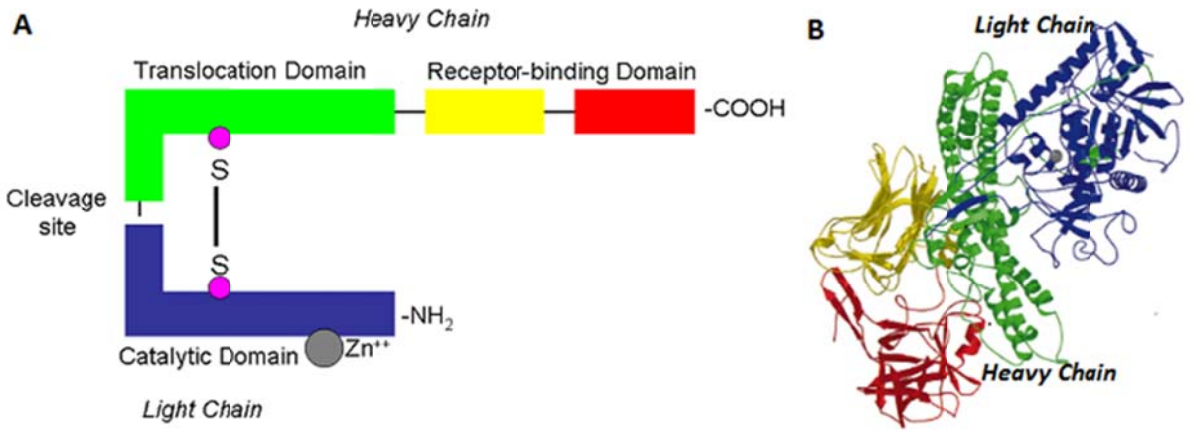

Figure 3: Structure of BoNTs represented by BoNT/A. A: Schematic structure and B: crystal structure. The light chain is coloured in blue, the heavy chain translocation domain is coloured in green, N-terminal and C-terminal receptor binding domains are coloured in yellow and red, respectively. The catalytic Zn^{2+} is represented by a grey ball. (3A: Reprinted by permission from V. Chelliah; URL: http://www.ebi.ac.uk/biomodels-main/static-pages.do?page=ModelMonth%2F2010-08. 3B: Reprinted by permission from Macmillan Publishers Ltd: [79]. Figure and caption slightly modified.)

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) is a large protein complex that facilitates vesicle fusion where the proteins syntaxin, SNAP-25 and VAMP mediate the docking of synaptic vesicles with the presynaptic membrane (Figure 4).

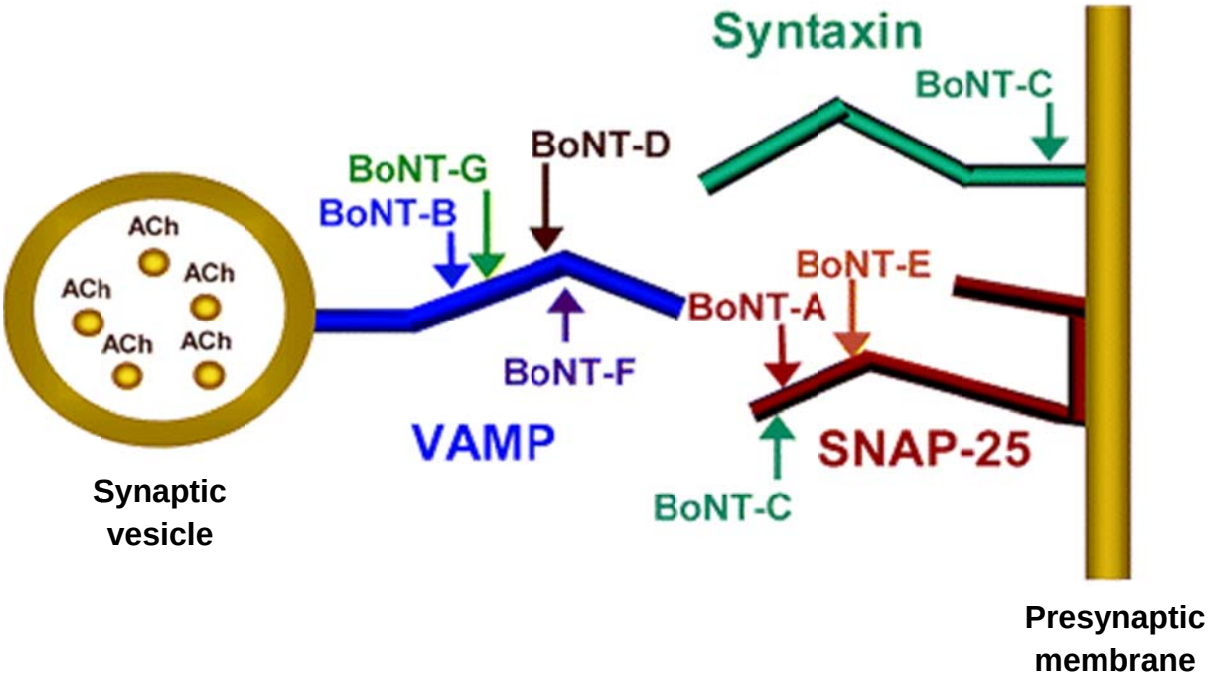

Figure 4: Inhibition of neurotransmission caused by BoNT. The synaptic vesicle carrying acetylcholine (Ach) neurotransmitter fuses with the presynaptic membrane to transmit the nerve impulse across the synaptic cleft. This fusion requires formation of the SNARE-complex, an interaction between the docking proteins VAMP on the synaptic vesicle and *syntaxin and SNAP-25 on the presynaptic membrane. Each BoNT specifically cleaves one or two of the docking proteins which prevents vesicle membrane docking and nerve impulse transmission. Reprinted with permission from [80]. Copyright 2005 American Chemical Society. (Figure and caption slightly modified.)*

BoNTs are Zn^{2+}-dependent metalloproteases that inhibit neurotransmission in the synapses by cleaving one or two of the docking proteins [66]. The light chain carries the proteolytic activity whereas the heavy chain accommodates the translocation into the synaptic vesicles. The endopeptidase reaction is serotype-dependent, both regarding target docking protein and the

specific cleavage site. Both BoNT/A and BoNT/C1 cleave SNAP-25, but at Gln197 - Arg198 and Arg198 - Ala199, respectively [81]. BoNT/C1 is the only serotype to cleave two docking proteins; both SNAP-25 and syntaxin [82]. BoNTs are sensitive to acidic pH and heat; all toxins are inactivated after heating at 85 °C for 5 minutes [69]. BoNTs survive the gastric acid barrier by complexation with non-toxic non-haemagglutinin (NTNHA) protein and three haemagglutinin (HA) proteins into big oligomers called progenitor toxin complexes (PTC) that protect BoNT through the acidic conditions and release the toxin upon entry at neutral pH in the circulation [66,77,83].

1.3.3 BoNT/CD mosaic

This study has been done on a BoNT/CD mosaic toxin from *C. Botulinum* strain BKT00287. This mosaic toxin consists of the light chain and translocation domain of the heavy chain from BoNT/C1 and the binding domain of the heavy chain from BoNT/D [66].

1.4 Analytical methods used for the detection of bacterial toxins

1.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) has been used both for the detection of emetic *B. cereus* strains [84–89], lichenysin-producing *B. licheniformis strains* [36,90–92](Paper IV) and BoNT/C-producing *Clostridium* strains [93,94]. Specific primers for the genes of interest are mixed with the sample and an amplification of present genes will occur during repeated heat cycles. This is an indirect method that does not detect the toxins, but only the genes that are encoding them. As a result, PCR for toxin detection is prone to both false positives and false negatives, both undesirable. False positives occur when the genes encoding the toxin synthetase are present, but no toxin has been produced. False negatives are the result when the toxins are present but the toxin genes do not match the used primers. In general, positive PCR results are considered reliable, whereas negative results may be caused by poor interaction with the primers, interference from the sample matrix and absence of the

genes, among others. Hence, negative results offer no actual information about the genes in the samples.

1.4.2 Toxicity assays

There are several biological assays that evaluate toxicity, from animal assays, cytotoxicity tests on different cell types to more specific tests for each toxin.

1.4.2.1 Sperm motility assay

The boar sperm motility assay has been developed for and used for detection of both cereulide and lichenysin [95–98](Paper IV). Lichenysin disrupts the spermatozoa cell membrane and causes swelling of the acrosome. If lichenysin is present in the sample, the motility of the spermatozoa is reduced but not eliminated, making the assay dependent on a skilled analyst and prone to subjective views. Cereulide causes paralysation of the sperm due to mitochondrial damage, resulting in a total motility loss making the assay less dependent on the analyst than that for lichenysin.

1.4.2.2 Cell based assays

Different cell-based assays are established for detection of cereulide and lichenysin. The same assays cannot be applied to both toxins due to their different mode of action. The Vero cell cytotoxicity assay and lysis of erythrocytes have been used for detection of lichenysin [99](Paper IV). The Vero cell assay used in our laboratory measures the inhibition of the protein synthesis by comparing the reduction of incorporated ^{14}C-leucine in toxin-added Vero cells with non-treated cells. Although *B. licheniformis* is usually considered non-haemolytic, toxigenic strains are proven to be weakly beta-haemolytic, making haemolysis a possible tool for detection of at least the highly lichenysin-producing strains. Detection of lichenysin through haemolysis does however have some challenges: Salkinja-Salonen et al. [33] found that some haemolytic strains of *B. licheniformis* were not sperm-toxic, indicating that no lichenysin were produced.

The HEp-2 cell assay has been used for detection of cereulide. The toxin causes vacuolation of the mitochondria in the HEp-2 cells which is visible under a light microscope [29,100]. Both Finlay et al. [101] and Kamata et al. [102] have later proposed improvements of the cell assay by eliminating the subjective evaluation of the vacuolation and substituting the HEp-2 cells with human hepatoma Hep-G2 cells. With the latter alteration, the limit of detection (LOD) was lowered to 0.04 ng/mL compared to 1 ng/mL for HEp-2-cells. Another cytotoxicity assay that has been applied for cereulide is the measurement of the lactate dehydreogenase (LDH) leakage in serum [103]. However, LDH-leakage might be caused by many different cytotoxic species; hence, the LDH-leakage assay is non-specific and not applicable in the search of a causative agent at a food poisoning incident.

1.4.2.3 Mouse bioassay

The mouse bioassay has been the "gold standard" for detection of BoNTs during the last three decades [104–106]. Sample extracts (1 mL) from patient samples (usually serum) or food are injected intraperitoneally into 20 g mice. Reduced mobility, laboured breathing, abdominal muscle contractions seen as wasp waist, and finally paralysis are symptoms which will often occur during the first 8-12 hours after injection but the mice must be observed for 4 days before a final reading is made. Trained personnel are vital to evaluate the symptoms in the mice, especially at toxin concentrations around the mouse LD_{50} of 1 ng/kg body weight [107]. Assessment of symptoms is increasingly subjective with diminishing toxin concentration. Reactions caused by other constituents in the injection might be confused with symptoms of botulism. The number of mice necessary for analysis is high: the measurement should be done in duplicates, dilutions are required for a quantitative assessment and antitoxins for the different serotypes must be evaluated. The use of experimental animals is ethically disputed, time consuming and costly. When alternative detection methods exist they should always be implemented to replace the use of experimental animals.

1.4.2.4 Endopeptidase assay

The most widespread alternative for BoNT/C1 detection to the mouse bioassay are the endopeptidase methods [80,108–112]. They utilise the endopeptidase property of BoNT/C1; the toxin cleaves SNAP25 and syntaxin at specific cleavage sites. The substrate peptide is incubated with the toxin samples and the amount of cleaved product is detected. Mass spectrometry (MS) is the most commonly used detection technique after the endopeptidase reaction [80,110–112] but also fluorescence [109] and an immunoassay has been used for detection [108].

1.4.3 Immunological methods

Immunological detection methods like enzyme-linked immunosorbent assays (ELISA) have been used to some extent for the detection of BoNT/C1 [94,113–116]. There are no commercial ELISA kits available but some vendors offer BoNT/C1 –antibodies. The LODs of the developed ELISA-methods ranged from 0.25 ng/mL – 3.9 ng/mL; all above the LOD of the mouse bioassay.

1.4.4 Liquid chromatography – mass spectrometry

Mass spectrometry (MS) detects molecular ions based on their molecular mass, monitored as mass to charge ratio (m/z). Coupled with liquid chromatography (LC) it is a powerful technique, also able to distinguish between most isobaric compounds due to the chromatographic separation. Tandem mass spectrometry (MS/MS) includes two mass analysers: the first mass analyser isolates the molecular ion of interest, the precursor, which is then fragmented in a collision cell followed by separation and selection of the fragments of interest, product ions, in the second mass analyser. LC-MS/MS is a valuable technique both for structural elucidation, qualitative detection and quantitative measurements when suitable calibration standards are available. By monitoring ion transitions from precursor ion to product ion instead of just molecular ions, LC-MS/MS is a highly selective technique suitable for complex matrices like food.

LC-MS has been used for the detection of cereulide since the beginning of the century. First as single MS methods [96,97,117–121] then followed by more selective tandem MS methods [122–129](Paper I and II). All these methods are quantitative, using either cereulide or valinomycin as calibration standard. MS-methods published for the detection and structural elucidation of lichenysin are mostly qualitative [45,52,54,55,58,95,130–132], but also a few quantitative LC-MS methods have been published [91,133](Paper III and IV). All of these LC-MS methods detect the relatively small molecules of cereulide and lichenysin. For large proteins like BoNT/C, the molecular mass is outside the mass range of the MS. The neurotoxin has to be enzymatically digested before analysis of the resulting peptide mixture with special emphasis on marker peptides that are specific for the analyte [112,134–136]. Another approach for LC-MS analysis of BoNT/C1 is to utilise the endopeptidase properties of the neurotoxin by detecting the specific cleaved peptides after incubation of BoNT/C1 with the substrate peptide(s), indirectly verifying the presence of BoNT/C1 [80,110–112,137]. Both techniques are qualitative with established LODs.

1.5 Validation protocol

Validation of analytical methods is important to ensure good quality data. No validation protocol has been established for the detection of bacterial toxins in food; hence, they might be regarded as contaminants and the methods validated based on Commission Decision 2002/657 regarding residues and contaminants in food and feed [138]. The following validation parameters are mandatory for quantitative, confirmatory methods: trueness/recovery, precision, selectivity/specificity, applicability/ruggedness/stability, decision limit (CCα), and detection capability (CCβ). All these parameters have been evaluated accordingly, based on 2002/657/EC and an interpretation thereof [138,139]. Both CCα and CCβ are calculated for forbidden substances or relative to the maximum residue limit (MRL) of the analyte. Bacterial toxins are neither forbidden

substances nor do they have established MRLs; hence, the limit of detection (LOD) and limit of quantification (LOQ) have been calculated instead.

2 Aim of the study

2.1 Main objective

The main objective for this PhD-project was development of chemical methods utilising liquid chromatography – tandem mass spectrometry (LC-MS/MS) for the detection and quantification of the bacterial toxins cereulide, lichenysin and BoNT/CD.

2.2 Sub-objectives

- Development of quantitative LC-MS/MS methods for cereulide and lichenysin
- Development of qualitative LC-MS/MS methods for lichenysin and BoNT/CD
- Structural elucidation of the different isoforms/homologues of lichenysin using LC-MS/MS
- Comparison of LC-MS/MS methods with biological detection methods
- Application of the developed methods on real samples
- Validation of the quantitative methods based on Commission Decision 2002/657 [138]

3 Summary of the papers

Paper I:

Determination and quantification of the emetic toxin cereulide from *Bacillus cereus* in pasta, rice and cream with liquid chromatography – tandem mass spectrometry.

H.T. Rønning, T. N. Asp, P. E. Granum

Food additives & contaminants: Part A, 2015, 32, 911-921

A fully validated quantitative method for the detection of cereulide in different food matrices was established. The introduction of commercially available cereulide as calibration standard and $^{13}C_6$-cereulide as internal standard improved the trueness and robustness of the method compared to calibration against valinomycin or in-house produced and purified cereulide. Recovery and reproducibility were enhanced through achieving a higher extraction yield and better extraction repeatability after including the starch digesting enzyme amylase in the sample preparation. The established method was used to analyse samples from a cereulide induced food poisoning outbreak in a kindergarten in Norway. In addition, eight different rice and pastas were investigated for possible cereulide production. Surprisingly, all eight matrices produced cereulide. This is in contrast to the earlier assumption that about 5 % of rice and pastas contain emetic *B. cereus*.

Paper II:

Toxin production and growth of pathogens subjected to temperature fluctuations simulating consumer handling of cold cuts.

E. Røssvoll, H. T. Rønning, P. E. Granum, T. Møretrø, M. R. Hjerpekjøn, S. Langsrud

International journal of food microbiology, 2014, 185, 82-92

A simulation of consumer exposure of cold cuts to room temperature was performed to monitor the toxin production from *Bacillus cereus*, *Bacillus weihenstephanensis* and *Staphylococcus aureus* and the bacterial growth of *Listeria monocytogenes* and *Yersinia enterocolitica*. A simple semi-quantitative method for the detection of cereulide was developed. Nutrient agar plates mimicking cold cuts were stored refrigerated and exposed to room temperature for different time intervals throughout the experimental period of ten days. The exposure scheme was founded on temperature loggings collected from 46 households during Christmas and Easter holidays in Norway. A significant increase in the growth rate of the bacteria was seen after a daily exposure of 2 hours to room temperature compared to continuous storage at 4 °C or 8 °C. Toxin production first commenced at bacterial levels above cell concentrations related to human disease; hence, the limiting factor for food safety was considered to be *L. monocytogenes* and *Y. enterocolitica.* Comparison of the bacterial growth with predicted values from ComBase Predictor revealed good correlation for some of the pathogen-temperature scenarios but resulted in underestimation for others.

Paper III:

Identification and quantification of lichenysin – a possible source of food poisoning.

H. T. Rønning, E. H. Madslien, T. N. Asp, P. E. Granum
Food additives & contaminants: Part A, 2015, 32, 2120-2130

A rapid, quantitative LC-MS/MS method for the detection of lichenysin in cell extracts was refined. (The original method was published in Paper IV.) Commercially available lichenysin was used as calibration standard for the first time, resulting in a significantly improved trueness: Together with matrix-matched calibration the trueness was increased from 30 % to 94 % for C15-lichenysin. The quantitative method was fully validated based on Commission Decision 2002/657/EC. Repeatability ranged from 10 % to 16 %. Instability of the analyte resulted in an unacceptable reproducibility, this

issue can be solved by introducing an isotopically labelled internal standard. Lichenysin produced by 53 different *B. licheniformis* strains were structurally examined. Four major isoforms with m/z 993.65, 1007.67, 1021.68 and 1035.70 were detected. The most intense peak for all isoforms had the same amino acid sequence in the peptide moiety of the molecule; hence, the 14 Da mass difference was a result of varying length of the hydrocarbon chain. The second peak of m/z 1021.68 however, was shown to have an amino acid substitution at AA7 where leucine was replaced by valine. All strains showed the same distribution pattern between the different isoforms, indicating that lichenysin production is more dependent on the growth conditions than the genotype.

Paper IV:

Lichenysin is produced by most *Bacillus licheniformis* strains.

E. H. Madslien, H. T. Rønning, T. Lindbäck, B. Hassel, M. A. Anderson, P. E. Granum

Journal of applied microbiology, 2013, 115, 1068-1080

Lichenysin production in 53 *different B. licheniformis* strains was investigated. *lchA*, a gene encoding parts of the lichenysin synthetase enzyme complex, was detected in all strains by PCR and lichenysin production was confirmed in all strains by LC-MS/MS. However, the amount of detected lichenysin varied with more than two orders of magnitude between the strains. Neither lichenysin production nor concentration showed any association with genotype. The cytotoxicity of the samples was measured both with the sperm motility assay, Vero cell assay and lysis of erythrocytes. A quantitative method utilising LC-MS/MS was developed for detection of lichenysin. Four different isoforms of lichenysin with mass differences of 14 Da were detected. The total amount of lichenysin correlated well with the measured cytotoxicity determined with all three biological methods. However, cytotoxicity was only apparent at lichenysin concentrations above 10 µg/mL; hence, cytotoxicity tests alone are inadequate for the detection of lichenysin.

Paper V:

Detection of botulinum neurotoxin /CD through substrate cleavage and liquid chromatography – tandem mass spectrometry.

H. T. Rønning, T. N. Asp, T. Lindbäck, P. E. Granum

Manuscript

Botulinum neurotoxin /CD (BoNT/CD) can cause avian botulism and is a major source to disease and mortality among poultry and wild birds. A qualitative method for the detection of BoNT/CD by combining the toxin's endopeptidase properties with LC-MS/MS has been developed. Sample clean-up is performed with 100 kDa MWCO-filtration before incubation over night with the substrate peptide SNAP-25, followed by detection of the product peptide with LC-MS. This technique ensures that only active BoNT/CD is detected. The major challenge is to maintain the activity of the heat labile toxin throughout the sample preparation and simultaneously achieve sufficient sensitivity to detect the trace amounts of BoNT/CD that might cause disease. This study has been contemplated in bacterial growth medium; the next step will be implementation of serum as matrix.

4 Results and discussion

4.1 Comparison of LC-MS and biological detection methods

Biological detection methods such as PCR, toxicity assays and immunological methods, assess the presence of the analyte by molecular interactions (e.g. antibody-analyte interactions, interaction with membranes) or by detecting genes encoding the analyte instead of detecting the molecule of interest itself. LC-MS on the other hand, confirms the presence of the actual molecule (or the activity of the molecule, for BoNT), thus it is a more reliable technique. Of the commonly used detection methods PCR is probably the least suitable to determine the presence of toxins at a food poisoning incident. At intoxications caused by heat-stable toxins like cereulide and lichenysin there are often no viable bacteria present in the food after heat-treatment. The bacterial DNA may be degraded or the food matrix may contain substances that inhibit the PCR reaction, thus the use of PCR may result in false negatives. A major disadvantage with PCR is the incompatibility with complex matrices like food. Matrix components might be bound to the genes and obstruct the transcription which results in false negatives [140]. PCR is also prone to false positives when genes encoding the toxin are present but no toxin production has occurred. Zechmeister et al [94] performed a comparison of PCR with ELISA and the mouse-bioassay for BoNT/C1 in 77 sediment samples. The study showed that PCR gave an overestimation of 13 % compared with immunostick-ELISA and 15.5 % compared with the mouse-bioassay and it was poor correlation between PCR and the mouse-bioassay ($p = 0.07$) and no correlation between immunostick-ELISA and PCR ($p = 0.46$). Good correlation has been proven between PCR and LC-MS/MS for the detection of cereulide [36,141] and lichenysin (Paper IV). However, the amount of toxin produced, described in Paper IV, ranged over three orders of magnitude, indicating the importance of quantification of the toxin production. PCR is usually a qualitative technique even though quantitative PCR has been used to show that the expression level

of *lchAA* was relative to the lichenysin production [91] and for quantification and differentiation of emetic and non-emetic *B. cereus* [89].

One shortcoming of some of the toxicity assays is the relatively low sensitivity. Comparison of the lichenysin concentration found with LC-MS/MS with concentrations determined with the sperm motility assay and the Vero cell assay revealed that the LOD of the bioassays were about 10 µg/mL lichenysin while the LOD for the LC-MS/MS method is < 1 µg/mL (Paper IV). Several lichenysin-producing *B. licheniformis* strains and at least one emetic *B. cereus* strain proven to produce cereulide were negative in the sperm motility assay [36,142] (Paper IV). Toxins present at low concentrations in food might cause discomfort for the consumers despite negative cytotoxicity tests. Different species/individuals have varying tolerance levels toward toxins. For instance cattle is more sensitive towards BoNT/C1 than mice; hence, the mouse bioassay is inadequate as detection method for BoNT/C1 [143]. Altogether this demonstrates the need for detection methods with better sensitivity.

The use of LC-MS/MS for the detection of BoNT/CD (Paper V) does in contrast to the determination of cereulide and lichenysin not detect the toxin itself. The endopeptidase property of BoNT/CD is essential for the detection of the toxin. BoNT/CD is incubated with a substrate and the resulting cleavage product is measured. This ensures that the BoNT/C1 detected is an active toxin. Despite being an indirect method LC-MS/MS analysis of BoNT/C1 shows some advantages compared to existing methods. Brooks et al. [116] demonstrated that ELISA (sELISA-2) was better than the mouse bioassay for gastrointestinal samples from cattle where the mouse bioassay showed some false-negatives. However, this ELISA-method presumes cultivation for five days which is not compatible with a rapid analysis. The mouse bioassay has also been proven able to give a false-positive, albeit for BoNT/A in human [144]. Several endopeptidase-immunoassays have been developed for BoNT/C1. The assay published by Jones et al. [108] gave good results for pure toxins but the introduction of serum as sample matrix resulted in false-negatives (Kristin O'Sullivan, personal communication).

4.2 Quantification challenges

4.2.1 The importance of suitable calibration standards

Certified standards of the analyte in question are not always available, forcing the analyst to find the best possible substitute. In Paper II and IV we were forced to use similar compounds as calibration standards as neither cereulide nor lichenysin were commercially available at the time. We have later demonstrated a significant difference in the quantified amount of both cereulide and lichenysin between values obtained with the actual analytes as calibration standards and the similar compounds valinomycin and surfactin, respectively (Paper I and III). In both cases the use of non-analyte calibration standard resulted in a significant underestimation of the toxin content: The underestimation of the cereulide content in rice and pasta samples calibrated against valinomycin was 14 % - 63 % depending on the ion transition monitored (Paper I), whereas the trueness was improved with 32 % to 48 % for the different lichenysin isoforms by changing the calibration standard from surfactin to lichenysin (Paper III). Zuberovic Muratovic et al. [128] reported an underestimation of cereulide of about 50 % calculated against valinomycin for fragment ion m/z 172 which correlates well with our finding of 60 % underestimation for this ion transition. Earlier a 10 % overestimation has been reported [120]. These different results might be due to the use of different mass analysers; triple quadrupoles and an ion trap, respectively. The ion transition in question was precursor – precursor for which we got the best quantitation with valinomycin; only 14 – 21 % underestimation. It follows that the choice of calibration is crucial to obtain correct quantification and demonstrates that the LC-MS methods developed in Paper II and IV are semi-quantitative.

In Paper II we tried to overcome the intensity-difference between valinomycin and cereulide by determining the intensity ratio between valinomycin and synthetic cereulide. (Synthetic cereulide was not commercially available at the time and synthesising was costly.) By multiplying the found amount of cereulide with the valinomycin / synthetic cereulide ratio the actual concentration of

cereulide could be determined. However, monitoring of the valinomycin / synthetic cereulide ratio over time revealed a substantial variation, proving this method inadequate.

An alternative to commercially available, certified calibration standards is in-house produced standards. Several research groups have used this approach for cereulide, either production of synthetic cereulide [120] or production and purification of cereulide from emetic *B. cereus* [117,119,123]. This requires skilled personnel and a well-equipped laboratory for purity testing of the end product. Commercial laboratories predominantly doing routine analysis seldom have this expertise, emphasising the importance of commercially available calibration standards.

4.2.2 Matrix-matched calibration

Food is a complex sample matrix with varying content of fat, proteins, carbohydrates, dyes and minerals. These matrix constituents might have considerable influence on the detection of the analyte in question compared to the analyte detection in pure solvent standards, both during the sample preparation and the LC-MS analysis. One major advantage of LC-MS is that mostly it is not necessary to preserve the biological activity of the analyte; hence, rough sample treatment can be applied to separate interfering matrix components from the analyte which is especially useful for stable molecules like cereulide and lichenysin. Even after a thorough removal of the matrix, there might be substances left that interfere with the chromatographic separation, ionisation and mass analysis. Matrix-matched calibration should be used to ensure good accuracy of the quantification: blank sample matrix is spiked with known amounts of calibration standard before the sample preparation and the calibration samples go through the same treatment as the samples. In the detection of lichenysin in cell cultures (a matrix considered relatively simple) we increased the trueness with 19-22 % through the introduction of matrix-matched calibration compared to calibration against pure solvent (Paper III).

4.2.3 Selecting a suitable internal standard

Method development is all about finding the best compromise. Even after a thorough sample preparation and matrix-matched calibration the method might still suffer from matrix effects and/or poor recovery. Internal standards are used to overcome this obstacle. A comparison of two different internal standards for cereulide was carried out in Paper I: Valinomycin, another dodeka-depsipeptide very similar to cereulide, and $^{13}C_6$-cereulide were both added to the same samples as internal standards (Figure 5).

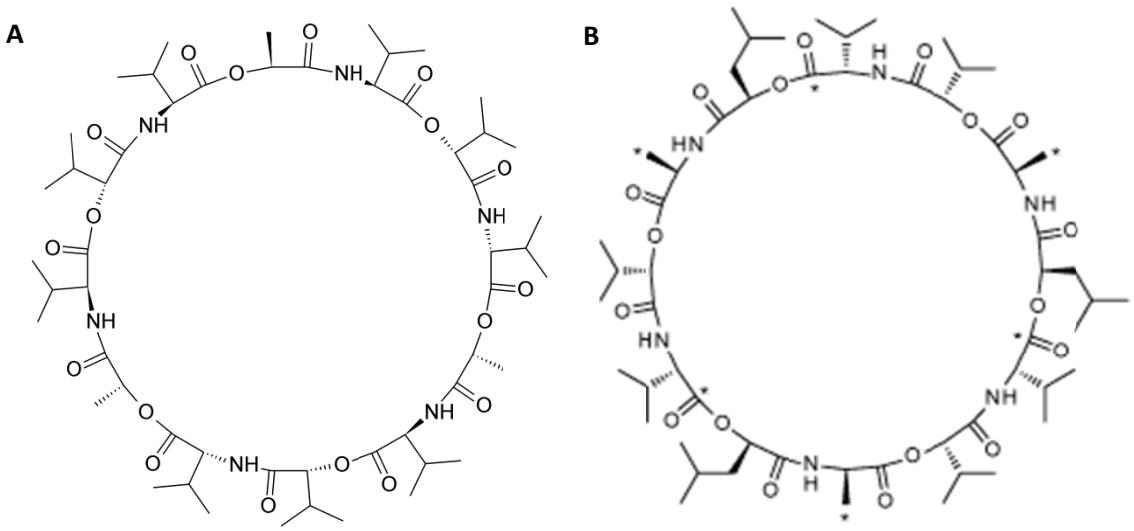

Figure 5: Molecular structures of A) valinomycin and B) $^{13}C_6$-cereulide (B with permission from Chiralix B.V.)

The introduction of an internal standard, despite which one, neutralised the variation found between the different ion transitions without an internal standard. But, the quantification of cereulide performed with valinomycin as internal standard varied from 50 to 130 % of the amounts found with $^{13}C_6$-cereulide as internal standard. This accentuates the importance of a suitable internal standard: Despite the close resemblance of cereulide and valinomycin they do not have the same physicochemical properties. Cereulide is more hydrophobic than valinomycin and elutes later in the mobile phase gradient, they might also be affected differently throughout the sample preparation. An ion suppression test was performed, but revealed no visible difference between cereulide and

valinomycin (Figure 6). Consequently, the variation between the depsipeptides is caused by different behaviour during the sample preparation.

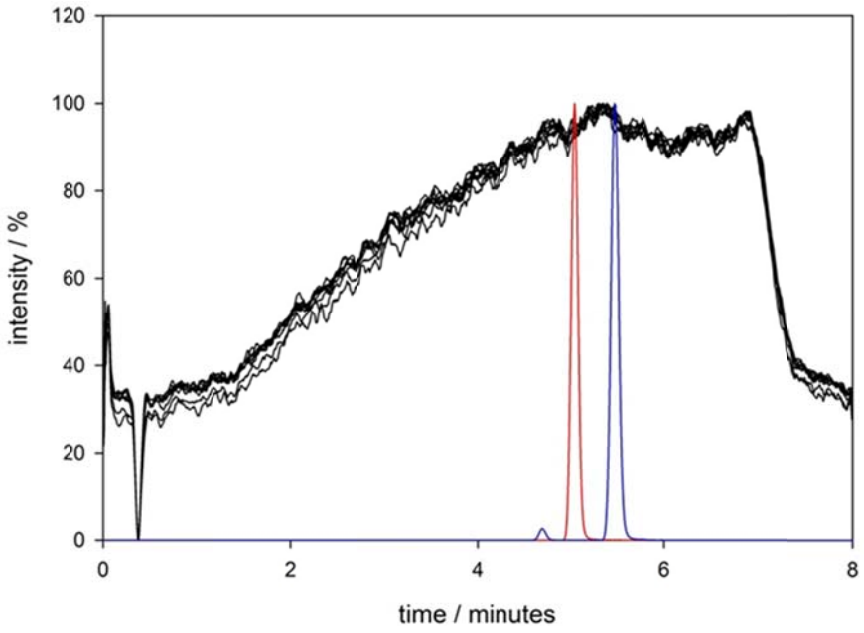

Figure 6: Ion suppression test done with continuous injection of cereulide, $^{13}C_6$-cereulide and valinomycin during an injection of blank pasta matrix. All ion transitions monitored are extracted as separate ion chromatograms (black lines). No matrix effect is observed at the retention time of the analytes, represented by the blue (cereulide) and red (valinomycin) chromatograms.

The lack of a suitable internal standard for lichenysin made the calculation of reproducibility impossible; the day-to-day variations were too high. C15-surfactin has been used as internal standard for lichenysin [133]. The mass difference between the molecules is only 1 Da making it impossible to differentiate between the first isotopic peak of lichenysin and the major peak of surfactin. This would be resolved if their retention times were different, but unfortunately we found that a small peak of a lichenysin isoform with m/z 1035 co-eluted with surfactin m/z 1036 (Paper III). Since production of the different isoforms varies with different growth conditions and maybe between strains surfactin is not suitable as internal standard for lichenysin. The use of one single surfactin-isoform as internal standard for all lichenysin isoforms is not recommended as we have shown that the matrix effect

varies with retention time and they might experience different influence throughout the sample preparation. Implementation of isotopically labelled internal standards is encouraged to ensure correct quantification.

4.2.4 Challenges regarding quantitative analysis of isoforms and homologues

Production of several isoforms and homologues of the analytes raises new quantification issues. Both lichenysin and surfactin are produced as a range of isoforms differing only with a methyl group. This 14 Da mass difference might result from either an amino acid substitution in the peptide moiety or from varying length of the hydrocarbon chain [34,52–59,63,133] (Paper III and IV). Consequently, also isobaric isoforms with different physicochemical properties and thereby varying behaviour throughout the sample preparation and LC-separation might occur. Comparison of the two major peaks from lichenysin m/z 1021.7 and surfactin m/z 1022.6 reveals opposite intensity ratios for these isoforms from the two lipopeptides (Figure 7).

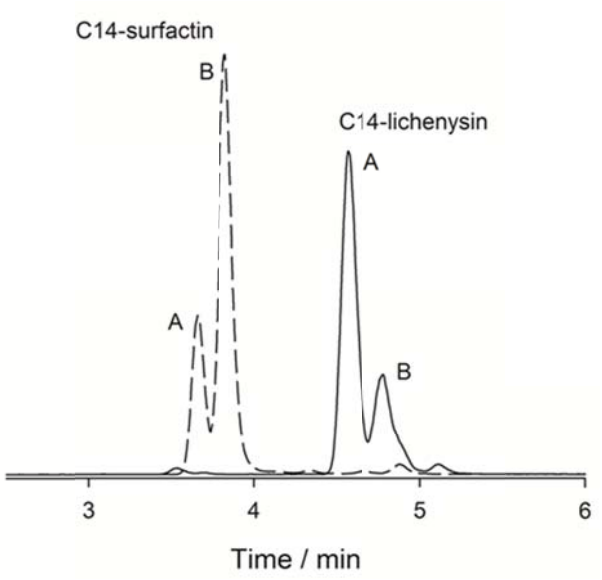

Figure 7: Peak intensity difference between surfactin m/z 1022.6 A and B and lichenysin m/z 1021.7 A and B (from B. licheniformis *ATCC 14580).*

This makes quantification of lichenysin 1021.7 A and B with surfactin 1022.6 as calibration standard impossible, illustrated by a trueness of – 0.2 % for lichenysin 1021B (ion transition m/z 1021-685) (Paper III). Lichenysin 1021.7 A was the more intense of the two isobaric isoforms produced by all 53 *B. licheniformis* strains investigated but the ratio between 1021.7 A and B varied considerably. This finding suggests that the growth conditions have a stronger influence on the lichenysin production than genotype which also supports earlier results [131]. Only one kind of commercially available surfactin was investigated (strain identification is not known). Both the commercially available surfactin and lichenysin contained a mixture of isoforms. The ratio between them might vary; hence, the quantitation might be prone to errors.

Lichenysin examined in Paper IV contained a significant proportion of m/z 993.4 whereas the strain used for the calibration standard (*B. licheniformis* ATCC 14580) produced only small amounts of this isoform under the given conditions (Paper III and IV). Consequently, quantification of this lichenysin isoform was not possible. Currently there are, to our knowledge, no data about the toxicity of the different lichenysin isoforms and homologues. Making an assumption of equal toxicity can exclude the challenge of different isoform quantitation by determining the total concentration of all isoforms present in the samples. Recent studies show that also cereulide is produced as several isoforms [129,145]. Marxen et al. [129] elucidated the molecular structure of seven isoforms of cereulide and showed that the cytotoxicity varies between them; the cytotoxicity of isocereulide A was eight times higher than for cereulide. In general, cereulide is expressed at least ten times higher than isocereulide A-G, but due to the higher cytotoxicity of isocereulide A all isoforms should be detected [129]. From et al. [44] revealed that also pumilacidin are produced as a range of isoforms with varying cytotoxicity. Cereulide, lichenysin and pumilacidin are all produced by NRPS. The production of several isoforms indicates that this production scheme is less rigid than ribosomal synthesis.

4.3 Method validation

"Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice." Huber, 1998 [146].

Through the validation study the method in question is scrutinised to reveal and eliminate shortcomings and sources of error in addition to thoroughly assess the method spesifics. The quantitative methods developed for cereulide and lichenysin are both validated based on 2002/657/EC [138](Paper I and III). Within-laboratory reproducibility was calculated for cereulide (Paper I), whereas the lack of a suitable internal standard made this impossible for lichenysin. Instead, the repeatability was used to evaluate the precision (Paper III).

Despite the thorough method validation performed at implementation of the methods, they are not infallible. Robustness must be evaluated over a long time period and it is important to maintain control charts to rule out systematic errors and reveal possible discrepancies. The need for method validation is not exclusively for chemical methods; also biological methods must be validated. However, direct comparison of method parameters between chemical and biological methods are challenging, especially due to the elevated variance associated with biological systems.

4.4 Matrix influence on the qualitative detection of BoNT/CD

The initial method development for the detection of BoNT/CD was performed with in-house produced BoNT/CD and TPGY (tryptone peptone glucose yeast extract broth) as sample matrix (Paper V). Introduction of serum as sample matrix resulted in a total loss of the product peptide signal. An ion suppression test could have revealed if the signal loss was due to severe matrix interference, unfortunately this was not possible due to a very limited amount of BoNT/CD available at the time.

Preparation of several sample parallels, each with different addition of substrate peptides and spiking with the product peptide before incubation with SNAP-25, revealed that the signal loss was a result of product peptide degradation during the incubation with substrate peptide. This might be a result of insufficient removal of proteases from the serum. It is crucial to maintain the biological activity of BoNT/CD; hence, the introduction of a denaturation step is difficult. These experiments were carried out with serum dialysed with a 100 kDa MWCO membrane. The tertiary structure of the proteases might have caused retention of the proteases on the membrane despite their molecular weights below the cut-off. MWCO-filtration should be implemented for serum.

Our primary objective is a qualitative detection of BoNT/CD in biological samples like serum, cecum and faeces from different species including poultry and horses. The secondary objective is the detection of other BoNTs, not only in biological samples but also food matrices. Food is a significantly more complex matrix than serum and plasma; here even bigger challenges regarding matrix effects than for serum awaits.

4.5 Structure elucidation of the different lichenysin isoforms

Lichenysin produced by 53 different *B. licheniformis* strains was investigated with qualitative LC-MS/MS utilising a Q-TOF mass spectrometer (Paper III). Three isoforms with molecular masses 14 Da apart were produced in adequate concentrations to perform structure elucidation: m/z 1007.67, 1021.68 A and B and 1035.70. The isoform with m/z 1021.68 elutes as a double peak, annotated A and B, with chromatographic resolution between 0.8 and 1 (Figure 7). The ratio between the isobaric peaks varies between the bacterial strains; thus, also the resolution fluctuates. The amino acid (AA) sequence elucidated from all isoforms except 1021 B was Gln - Ile/Leu - Ile/Leu - Val - Asp - Ile/Leu - Ile/Leu; the established AA-structure of lichenysin A [58]. Consequently, the 14 Da mass shifts are caused by varying length of the fatty acid chain, a finding that coincides with earlier publications

[34,58,133]. The product ion spectra from m/z 1021 B revealed an amino acid substitution where Ile/Leu in position AA7 has been substituted with Val (Figure 8).

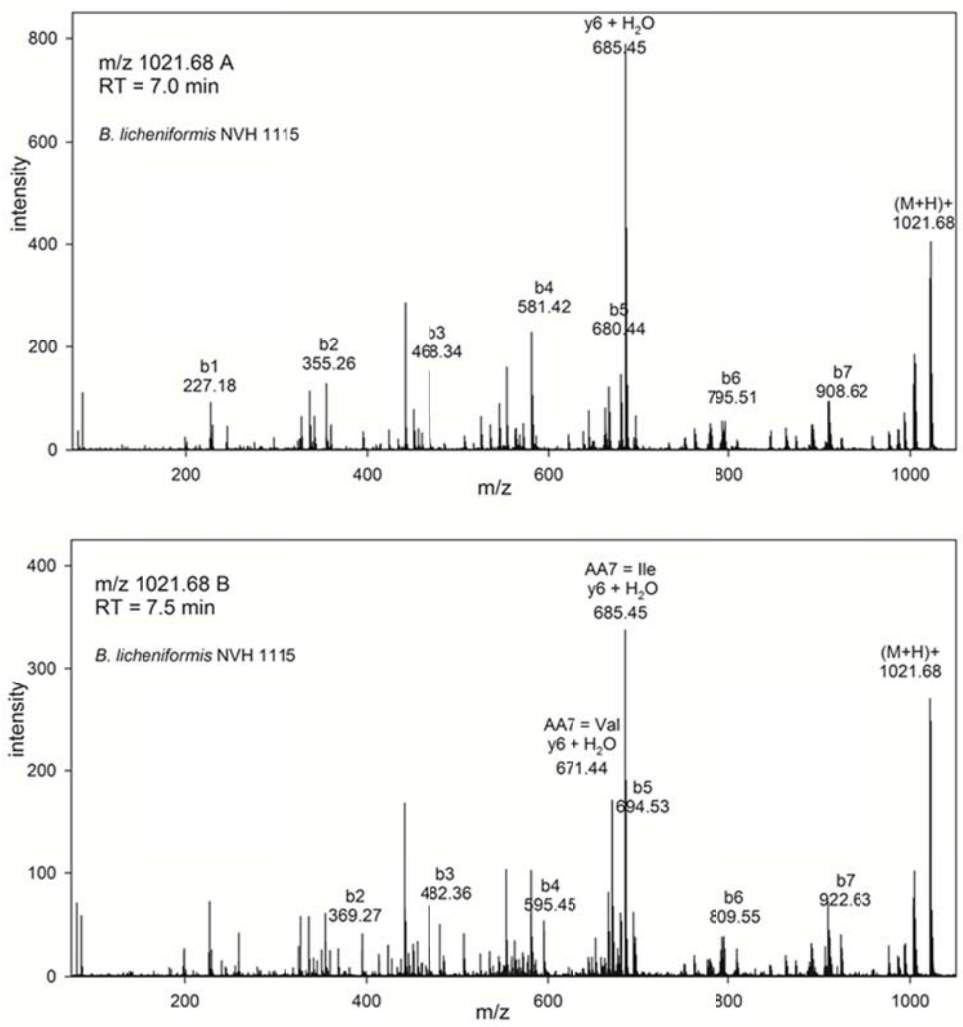

Figure 8: Product ion spectra from m/z 1021.68 A and B showing the complete b-ion series from the two different occurring isoforms: In m/z 1021 A is AA7 Ile/Leu, while in m/z 1021 B is both AA7 Ile/Leu and AA7 Val present. The most abundant fragment ion, y6 + H_2O, represented by both m/z 671 and m/z 685 from precursor m/z 1021 B indicates the presence of both AA7 Ile/Leu and AA7 Val isoforms, whereas it is only present as the AA7 Ile/Leu isoform in m/z 1021 A.

Zhang et al. [133] and Peypoux et al. [147] described the same AA-substitution for lichenysin and surfactin, respectively. However, it is interesting that both Val- and Ile/Leu-isoforms of m/z 1021

coexists in m/z 1021 B. This is shown in figure 8 through the difference in y- and b-ions identified from precursors m/z 1021 A and B, and particularly through the presence of the most intense fragment ion, y6 + H_2O, from both AA7-Val and AA7-Ile/Leu. The co-elution of the Val- and Ile/Leu-isoforms in m/z 1021 B suggests that three isoforms contribute to the m/z 1021 double peak; AA7-Ile/Leu in A, AA7-Ile/Leu in B and AA7-Val in B. The change in retention time between AA7-Ile/Leu in A and B might be caused by different branching of the fatty acid chain.

Another explanation of the presence of the AA7-Ile/Leu-isoform in m/z 1021 B is that it originates from m/z 1021 A. Collection of the mass spectrum from m/z 1021 B when m/z 1021 A was still eluting would enable this. This was ruled out by confirming that mass spectra at both front and tail of the m/z 1021 B peak revealed the same fragment ions.

4.6 Prevalence of emetic *Bacillus cereus* in rice and pasta

During the initial inoculation study with emetic *B. cereus* in Paper I we were surprised to find that cereulide was produced in the blank rice and pasta samples. To ensure that the cereulide production was not caused by contamination during the experiment, the study was repeated including eight different rice and pastas without inoculation. Cereulide was produced in all eight samples within 48 hours at room temperature, in two rice samples at concentrations 100 times higher than the level reported to cause disease [22,124]. This is a remarkable finding compared to the earlier estimation of 5 % prevalence of emetic *B. cereus* strains in rice and pasta [28]. *B. cereus* strains isolated from all eight samples were compared using exoprotein profiling on SDS-PAGE revealing distinct differences in their profiles; hence, diminishing the possibility that the cereulide production was due to contamination. An extended study including a vast number of rice and pastas should be conducted to further expand the knowledge about emetic *B. cereus* prevalence. The temperature abuse mimicked in this study was quite extreme but resembling food storage reported from fatal cases of cereulide intoxication [22–25]. More moderate levels of cereulide were found in the growth studies conducted

in Paper II where inoculated agar plates were exposed to room temperature daily at different time intervals. Cereulide concentrations above the infective dose were first obtained after five days at 8 °C with a daily exposure to room temperature for 0 – 2 hours. Cold cuts are not heat treated and normally contain competing bacteria that will inhibit growth of *B. cereus.* The agar plates were only inoculated with the psychrotolerant cereulide producing *B. weihenstephanensis*; hence, this study design might not be representative for the general cereulide production in cold cuts.

In June 2013 a small outbreak of emetic food poisoning occurred in a kindergarten in Norway [148] (Paper I). Five children and three adults out of around 100 people experienced nausea and vomiting, all of which recovered within the same day. Two samples from the lunch meal consisting of cod, reheated rice and vegetables were analysed and 1 µg/kg and 10 µg/kg cereulide were detected. These cereulide levels should have inflicted a larger outbreak and more severe and enduring symptoms. Further inquiries revealed that the meal had been consumed on a Friday and the samples collected on the following Monday, enabling additional cereulide production during the weekend.

No significant correlation between cell growth and cereulide production was observed in any of the studies (Paper I and II). Cereulide production commenced during the logarithmic growth and kept increasing throughout the stationary phase, indicating that once the *ces* gene is activated (Spo0A) and the repressor released (AbrB) [149], there is no correlation between cereulide production and cell growth. Häggblom et al. [117] reported that the cell growth reached the stationary phase before cereulide production began. That study was performed with inoculation with an overnight culture of *B. cereus* strain NC7401 in trypticase soy broth stored at room temperature, where the inoculum apparently contained about 10^6 CFU. The high initial cell count enabled the cell growth to reach the stationary phase within 24 hours and could explain why no cereulide was produced during the logarithmic growth phase.

5 Conclusions

- A quantitative LC-MS/MS method for the detection of cereulide in rice, pasta and cream has been developed and validated. This method is now part of the analytical portfolio at Centre of Food Safety, NMBU.
- An LC-MS/MS method for both qualitative and quantitative analysis of lichenysin in bacterial cell extracts has been developed and validated.
- A qualitative LC-MS/MS method for the detection of BoNT/CD in growth medium utilising the endopeptidase properties of BoNT/CD has been developed.
- The prevalence of emetic *Bacillus cereus* in rice and pasta might be higher than earlier assumed. Cereulide was produced in all eight blank samples of rice and pasta.
- All 53 *Bacillus licheniformis* strains investigated produced the same lichenysin isoforms, although in varying ratios.
- Three isoforms of lichenysin with molecular mass 1021 were detected, whereof two had Ile/Leu in the AA7 position and the third had Val in the AA7 position. The two Ile/Leu isoforms probably differ in the branching of their fatty acid chain.
- Suitable calibration standards and preferably isotopically labelled internal standards are crucial to obtain correct quantification. Even structurally very similar compounds might be affected significantly dissimilar throughout sample preparation, chromatographic separation and ionisation.

6 Future perspectives

- Implementation of new matrices for cereulide, lichenysin and BoNT/CD.
- Implementation of the isocereulides in the developed LC-MS/MS method.
- Survey of the prevalence of emetic *Bacillus cereus* and cereulide production in processed food stuffs.
- Find suitable internal standard for lichenysin, preferably isotopically labelled.
- Cytotoxicity study of the major lichenysin isoforms.
- Method development for all BoNTs using the same method.

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Enclosed papers I - V

I

Paper I

Determination and quantification of the emetic toxin cereulide from *Bacillus cereus* in pasta, rice and cream with liquid chromatography – tandem mass spectrometry

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Food additives & contaminants: Part A, 2015, 32, 911-921

Food Additives & Contaminants: Part A, 2015
Vol. 32, No. 6, 911–921, http://dx.doi.org/10.1080/19440049.2015.1022886

Determination and quantification of the emetic toxin cereulide from *Bacillus cereus* in pasta, rice and cream with liquid chromatography–tandem mass spectrometry

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(*Received 15 October 2014; accepted 22 February 2015*)

A rapid and sensitive method has been developed for determination and quantification of cereulide in cream, rice and pasta. Samples are homogenised after addition of amylase to cooked rice and pasta, and cereulide is extracted with methanol. After the removal of water with methyl-tert butyl ether/hexane and evaporation until dryness, no further purification was required before analysis with liquid chromatography–tandem mass spectrometry (LC-MS/MS). Recently, both cereulide and $^{13}C_6$-cereulide has become commercially available at high purities; hence, this method offers a more reliable quantification of positive samples than previous methods using valinomycin or in-house produced and purified cereulide as calibration standard. The introduction of amylase in the sample preparation improves both the extraction yield of cereulide from positive samples of starch-rich matrices such as pasta and rice, and the within-laboratory reproducibility of the analytical method. The LoQ of the method is 1.1 ng/g cereulide with RSDs ranging from 2.6% to 10%. The method is fully validated based on Commission Decision 2002/657/EC, suitable for routine analysis, and has been used to analyse samples from a cereulide food poisoning outbreak in a kindergarten in Norway. Cereulide production in different rice and pasta samples was investigated, showing that cereulide was unexpectedly produced by emetic *Bacillus cereus* in all eight pasta and rice samples.

Keywords: cereulide; LC-MS/MS; emetic *Bacillus cereus*

Introduction

Cereulide is an emetic toxin produced by the spore-forming bacteria *Bacillus cereus* and a well-known source of food poisoning. *B. cereus* is omnipresent in nature with the main reservoirs in the soil (Bottone 2010); therefore, it is also ubiquitous in foods such as rice and pasta. Cereulide is a cyclic dodecadepsipeptide with molecular mass 1153 Da and the structure: [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$ (Agata et al. 1995). Cereulide is produced by a nonribosomal peptide synthetase, encoded by the 24-kb cereulide synthetase (*ces*) gene cluster (Ehling-Schulz, Vukov, et al. 2005). The *ces* gene cluster is located on a megaplasmid related to the *Bacillus anthracis* pXO1 plasmid (Ehling-Schulz et al. 2006). Cereulide is highly resistant to acidic conditions, proteolysis and heat (cooking), thus it will not be destroyed by gastric acid, the proteolytic enzymes of the intestinal tract, or by reheating foods after cereulide production (Agata et al. 1995). The mechanism of action in humans has not been definitely determined, but after release from the stomach into the duodenum, cereulide binds to the 5-HT$_3$ receptor, and stimulation of the afferent vagus nerve causes vomiting (Agata et al. 1995). The toxin acts as a cation ionopore, like valinomycin, and is therefore able to inhibit mitochondrial activity by inhibition of fatty acid oxidation (Mikkola et al. 1999). This effect of cereulide was the reason for the liver failure in four lethal cases of emetic food poisoning (Mahler et al. 1997; Dierick et al. 2005; Shiota et al. 2010; Naranjo et al. 2011). In all the published cases with fatal outcome, pasta or rice left at room temperature over time has been the cause of the disease.

Cereulide production commences at the end of the logarithmic phase during vegetative growth of *B. cereus*, with the highest level of production at early stationary phase of growth. Cereulide synthesis takes place at temperatures ranging from approximately 12 to 37°C, although maximal production of emetic toxin appears to occur between 12°C and 22°C (Häggblom et al. 2002). However, two isolates belonging to the psychrotolerant species *B. weihenstephanensis* were shown to produce cereulide at 8°C (Thorsen et al. 2006; Røssvoll et al. 2014). Different foods have varying abilities to support cereulide production (Messelhäusser et al. 2014). In infant formulas, for instance, levels from 0.02 to 2 µg cereulide/ml food were reached after 24 h incubation at room temperature after inoculation with > 10^5 cfu/ml emetic *B. cereus* (Shaheen et al. 2006). Most cereulide-producing strains are unable to degrade starch (Kim et al. 2010). All the emetic strains belong to a single evolutionary lineage of closely related strains, and are closer related to *B. anthracis* than the other species of the *B. cereus* group (Ehling-Schulz et al. 2006).

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Normally, *B. cereus* emetic food poisoning symptoms are mild and thus underreporting is likely. It can also be mistaken for mild outbreaks of *Staphylococcus aureus* food poisoning, which are more commonly known to people. The infective dose is not exactly known, but is believed to be at least 10 ng/g food, depending on the food that is eaten. Recently, Messelhäusser et al. (2014) divided foods into low-, medium- and high-risk products for the production of cereulide by visualising the production using a lux-based real-time monitoring system.

Due to the widespread food poisoning caused by cereulide, it is important to have reliable detection methods for routine analysis of the toxin. There are a few biological methods for detection of cereulide available, including the sperm motility assay (Andersson et al. 1998, 2004; Jääskeläinen, Teplova et al. 2003) and HEp-2 cell assay (Finlay et al. 1999). The sperm motility assay monitors the loss of mobility of boar spermatozoa due to mitochondrial damage by cereulide, while the HEp-2 cell assay utilises the ability of cereulide to form vacuoles in this cell line. In addition, methods based on polymerase chain reaction (PCR) are developed for the detection of emetic *B. cereus* strains (Ehling-Schulz et al. 2004; Ehling-Schulz, Vukov et al. 2005; Fricker et al. 2007; Ueda et al. 2013). A severe limitation of these methods is that bioassays are based on biological actions of the toxin and PCR detects genes encoding the proteins that produce cereulide. None of them detects the actual toxin itself. Chemical methods utilising liquid chromatography–mass spectrometry (LC-MS), on the other hand, would be able to detect cereulide directly and the development and use of this methodology started in the beginning of the century (Häggblom et al. 2002; Jääskeläinen, Häggblom, et al. 2003; Jääskeläinen, Teplova, et al. 2003; Andersson et al. 2004; Hormazabal et al. 2004; Biesta-Peters et al. 2010; Delbrassinne et al. 2011). These LC-MS methods based their detection on single molecular information provided by ion-trap or single quadrupole mass spectrometers, which will not meet the selectivity criteria needed in complex sample matrices such as foods. Later, several LC-MS/MS methods were described (Rau et al. 2009; Bauer et al. 2010; Delbrassinne et al. 2012; Ueda et al. 2012; Stark et al. 2013; Yamaguchi et al. 2013; Røssvoll et al. 2014) using tandem mass spectrometry, which is more selective due to fragmentation of the molecular ions providing more structural information. Until recently, cereulide was not available commercially as reference material; hence, the quantification was often achieved using external calibration with the dodecadepsipeptide valinomycin as calibration standard (Häggblom et al. 2002; Jääskeläinen, Häggblom, et al. 2003; Jääskeläinen, Teplova, et al. 2003; Rau et al. 2009; Delbrassinne et al. 2012; Yamaguchi et al. 2013). Even though valinomycin is very similar to cereulide, it is not identical and might consequently be influenced differently during the sample preparation and LC-MS/MS analysis, leading to over- or underestimation of the cereulide concentration. In recognition of this, some of the LC-MS methods described up until now are based on cereulide quantification by in-house purified or synthesised cereulide (Hormazabal et al. 2004; Bauer et al. 2010; Biesta-Peters et al. 2010; Ueda et al. 2012; Stark et al. 2013; Røssvoll et al. 2014) and one group also used an in-house biosynthesised stable isotope of cereulide ($^{13}C_6$-cereulide) as an internal standard (Bauer et al. 2010; Stark et al. 2013). For MS quantification in complex matrices, the use of internal standard calibration will give more accurate and precise results as an appropriate internal standard would correct for variability throughout the analysis. The analyte itself and one of its stable isotopic molecules are indeed the ideal standards for quantitative work with MS; however, in-house purification or synthesis of the standards needed is a time-consuming and resource-demanding process and none of these methods are thoroughly validated. Guidelines for in-house validation of chemical methods for confirmation and quantification of contaminants in foods are given in Commission Decision 2002/657/EC (Commission decision of 12 August 2002 implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results 2002) and one LC-MS/MS method described (Delbrassinne et al. 2012) is validated according to these guidelines, but here the quantification was based on external calibration with valinomycin as calibration standard. With both cereulide and $^{13}C_6$-cereulide available commercially, the scope of this work has been to develop a simple, rapid and fully validated quantitative LC-MS/MS method based on internal standard calibration for the determination of cereulide in different food matrices for use in routine analysis.

Materials and methods

Chemicals and reagents

All chemicals were at least HPLC-grade and supplied by VWR (Poole, England) except valinomycin (98% purity) and α-amylase from porcine pancreas (Sigma, Steinheim, Germany), heptafluorobutyric acid, HFBA, (Fluka, Buchs, Switzerland) and Peptone Bacteriological (Oxoid Limited, Hampshire, UK). Cereulide and $^{13}C_6$-cereulide (> 95% purity) were purchased from Chiralix (Nijmegen, the Netherlands). The water used was grade 1 purified by a Milli-Q purification system from Millipore (Bedford, MA, USA).

Preparation of blank and inoculated food samples

Four different kinds of pasta (three brands of macaroni and one brand whole wheat fusilli), four different kinds of rice (polished rice, ecological polished rice, brown rice

and barley rice) and one kind of pasteurised whipping cream (38% fat) were bought in local grocery stores. The rice and pasta were prepared after instructions on the packages and cooled to room temperature before sampling, hereafter termed blank samples. Two hundred grams each of whipping cream, one kind of macaroni and polished rice were inoculated with 1×10^4 cfu/g *B. cereus* strain NC7401 (Nagoya City Public Health Institute, Japan). Both the inoculated and the blank samples were left at room temperature for 96 h. Every 24 h, 4 aliquots of 10 g were weighed from both the inoculated and blank samples and the cell count ($n = 2$) and cereulide concentration ($n = 4$) were measured.

Cell count

Ten grams of sample was homogenised with 90 ml sterile saline peptone water in a stomacher bag for 60 s. Decimal dilutions were made in sterile saline peptone water and appropriate dilutions were spread on blood agar plates (Colombia blood agar, Oxoid Limited, Hampshire, UK) and incubated aerobically at 30 ± 1.0°C. The resulting growth was counted and the results were noted as colony-forming units per gram (cfu/g).

Homogeneity

An inoculated rice sample collected after 72 h at room temperature was homogenised with a blender and 20 aliquots of 1 g were weighed and the cereulide content determined.

Sample preparation

After homogenisation of the sample with a blender, 1 g sample was weighed in a 50 ml centrifuge tube and 100 ng/g internal standard ($^{13}C_6$-cereulide) was added. After addition of 4 ml methanol/water (3 + 1) the sample was vortex mixed for 10 s. One hundred microlitres of 0.1 mg/ml amylase was added when rice or pasta was the matrix, and the sample was thoroughly shaken by hand. After at least 5 min resting, the sample was homogenised with Ultra-Turrax 18/10 (Janke & Kunkel KG, Ika Werk, Staufen, Germany) for 10 s followed by centrifugation at 4300 × *g* for 5 min (Rotanta 46, Andreas Hettich GmbH & Co KG, Tuttlingen, Germany). The supernatant was transferred to a 12 ml centrifuge tube before addition of 5 ml methyl-tert butyl ether/hexane (4 + 1). The sample was thoroughly shaken by hand and centrifuged at 2600 × *g* for 5 min. The organic phase was transferred to a new 12 ml centrifuge tube and evaporated to dryness at max. 60°C under a stream of air using a Pierce Reacti-Therm heating module (Pierce, Rockford, IL, USA). The dry residue was reconstituted in 1 ml methanol/water (3 + 1) by vortex mixing. The sample was filtrated through a 0.2 µm nylon centrifuge filter (VWR, Poole, England) at 9900 × *g* for 1 min and the filtrate transferred to an HPLC vial before analysis with LC-MS/MS.

Liquid chromatography

The HPLC system consisted of an Agilent 1200 SL (Agilent Technologies, Waldbronn, Germany) binary pump with degasser, thermostatted column compartment and autosampler held at 40°C and 4°C, respectively. The separation was performed on an RRHD Zorbax Eclipse Plus C18 column, 100 × 2.1 mm I.D. and 1.8 µm particles (Agilent Technologies) with a Fast Guard Eclipse Plus C18 guard column, 5 mm × 2.1 mm I.D., 1.8 µm particles. Mobile phase A consisted of 2 mM ammonium acetate and 0.2% HFBA in water, whereas mobile phase B was acetonitrile. The flow rate was 0.7 ml/min with a linear gradient from 87% to 97% B in 3.5 min. Total analysis time was 8 min. The injection volume was 1 µl. The injection needle was rinsed in methanol/water (3 + 1) for 3 s after each injection to avoid carry-over.

Mass spectrometry

The instrument used was an Agilent G6460 triple quadrupole (Agilent Technologies, Santa Clara, CA, USA) equipped with a Jet Stream electrospray ion source. The ions were monitored in positive multiple reaction monitoring mode (MRM). The major peaks used as precursor ion from both cereulide and valinomycin were their ammonium adducts $(M + NH_4)^+$, *m/z* = 1170.7 Da and 1128.7 Da, respectively. The ion transitions monitored and their individual settings are listed in Table 1. Common settings for all transitions were 50 ms dwell time, fragmentor voltage 250 eV, gas temperature 350°C, gas flow 10 l/min, nebulizer pressure 30 psi, sheath gas temperature 400°C, sheath gas flow 11 l/min, capillary voltage 5000 V and nozzle voltage 0 V.

Validation protocol

The validation was based on Commission Decision 2002/657/EC (Commission decision of 12 August 2002 implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results 2002) using the calibration curve method first proposed by Antignac et al. (2003). The calculations were performed on a total of 72 samples, whereof 23 rice samples (polished rice), 35 pasta samples (macaroni) and 14 cream samples (11 samples of whipping cream, 38% fat, and 3 samples of coffee milk, 10% fat) divided on 4 sets of samples prepared on 4 different days by 2 analysts within 2 months.

Linearity and sensitivity were evaluated from the regression coefficient (R^2) and the slope (a) of the linear

Table 1. Ion transitions monitored and their individual instrument settings. The ion transitions marked in grey were only used for the quantitative comparison between cereulide (CRL) and valinomycin (VAL).

Compound	Precursor ion	Product ion	Collision energy (V)	Cell accelerator voltage (V)	Internal standard
CRL	1170.7	172.0	120	3	A
CRL	1170.7	186.0	90	5	B
CRL	1170.7	314.2	80	5	C
CRL	1170.7	1125.4	40	5	D
CRL	1170.7	1153.3	40	5	E
CRL	1170.7	1170.7	0	5	F
VAL	1128.7	172.2	100	5	
VAL	1128.7	1083.4	40	5	
VAL	1128.7	1111.3	30	5	
VAL	1128.7	1128.7	0	5	
$^{13}C_6$-CRL (I.S.)	1176.7	172.1	120	5	A
$^{13}C_6$-CRL (I.S.)	1176.7	187.1	100	5	B
$^{13}C_6$-CRL (I.S.)	1176.7	315.1	80	5	C
$^{13}C_6$-CRL (I.S.)	1176.7	1131.4	40	5	D
$^{13}C_6$-CRL (I.S.)	1176.7	1159.3	40	5	E
$^{13}C_6$-CRL (I.S.)	1176.7	1176.7	0	5	F

calibration curve comprised of 26 samples divided on 7 concentration levels (1, 5, 10, 50, 100, 500 and 1000 ng/g). The limit of detection (LoD = 3 × SD_b, SD_b being the standard deviation of the blank signal) and the limit of quantification (LoQ = 10 × SD_b) demonstrates the sensitivity. The linearity was also evaluated using the response factor test.

The precision was assessed by the relative standard deviations (RSDs) of the area ratios and the relative retention times at all calibration levels. These RSDs cover both the repeatability and the within-laboratory reproducibility of the method; they are calculated from a total of 46 samples from 3 different matrices, divided on 7 different calibration levels ranging from 1 to 1000 ng/g, prepared on 4 different days by 2 analysts.

Injection on the LC-MS/MS of pure cereulide standard (A_S), matrix samples without fortification (A_B), prepared matrix samples fortified before injection on the LC-MS/MS (A_{MS}) and matrix samples fortified before the sample preparation (A_{SP}) made it possible to calculate four kinds of recovery: apparent recovery (R^{app})(Burns et al. 2002), total recovery without the use of internal standard ($R^T = (A_{SP} - A_B)/A_S$), recovery in the sample preparation alone ($R^{SP} = (A_{SP} - A_B)/(A_{MS} - A_B)$) and recovery of the LC-MS method ($R^{MS} = (A_{MS} - A_B)/A_S$). R^{app} is calculated against the matrix-matched calibration curve with the use of internal standard, whereas the three latter are calculated from the analyte signal alone; hence, they reflect the method without the use of internal standard.

Matrix effects

To examine the robustness and selectivity of the method as well as possible matrix effects, both during sample preparation and LC-MS/MS analysis, cereulide-containing samples were added other food products that might cause interferences. Pre-weighed 1 g pasta samples were added tomato sauce and pesto, rice samples were added olive oil and curry powder. After homogenisation, the samples were prepared as described above.

To evaluate possible matrix effects in the LC-MS/MS method, a continuous flow of cereulide, $^{13}C_6$-cereulide and valinomycin was directed to the ion source at 10 µl/min through a T-coupling while blank samples were injected.

Quantification

Quantification was performed against matrix-matched calibration curves fortified at the beginning of the sample preparation with cereulide as calibration standard and $^{13}C_6$-cereulide as internal standard. The ratio between analyte peak area and internal standard peak area was plotted against concentration. Valinomycin was added to the calibration samples to compare quantification with cereulide and valinomycin as calibration compound. Valinomycin was also added to the samples for evaluation as internal standard.

Results and discussion

Preliminary experiments

Several steps in the sample preparation were optimised during the development of the method. Different extraction solvents such as acetonitrile, methanol, acetone and tetrahydrofuran were tested together with varying extraction times. Some of the matrices were difficult to homogenise with Ultra-Turrax. This was overcome by adding the enzyme amylase to the samples prior to homogenisation. Different amounts of amylase as well as amylase

from different origins were tested, showing that only α-amylase from porcine pancreas had the desired effect. Different SPE cartridges were investigated as a sample clean-up technique, but proved to be unnecessary (data not shown). The LC-MS/MS method is the result of testing different mobile-phase solvents and additives together with different chromatographic columns to achieve the best separation of the analyte from possible interferences.

The resulting method was used in a homogeneity test done on an inoculated rice sample containing approximately 1200 ng/g cereulide, which gave an RSD = 4.1% (n = 20). This confirms that the homogenisation of the sample material with a blender and a sample size of 1 g are sufficient to achieve reproducible quantification of cereulide.

Method validation

The introductory optimisation of the LC-MS/MS method for cereulide indicated three major fragment ions suitable for further use. Throughout the validation of the method, these three ion transitions have been monitored and the most sensitive ion transition, 1170.7 → 172, was used for quantification and the second most sensitive, 1170.7 → 314, was used for confirmation together with their corresponding internal standard ion transitions. The third ion transition, 1170.7 → 186, was also validated, but is not used routinely. It still increases the robustness of the method. If an interference for one of the two major ion transitions occurs in new matrices, this third ion transition can be used instead.

Precision

The precision of the method is given as the RSD of the ratio between the analyte peak area and the internal standard peak area calculated at all calibration levels. The RSD ranges between 2.6% and 10% for the ion transition 1170.7 → 172 and 1.1% and 14% for the ion transition 1170.7 → 314. The ion ratio 314/172 was 52% with 6.7% RSD in the spiked samples (n = 47) and 53% with 3.7% RSD in the standards (n = 5). The retention time for cereulide in the samples compared to the retention time in the standards was 0.15% to 0.27% and all RSDs of the relative retention times were below 0.1%. All precision parameters are well within acceptable limits for the concentration levels in question, and the within-laboratory reproducibility values are within the requirement RSD < 16% at 1000 ng/g stated in Commission Decision 2002/657/EC (Commission decision of 12 August 2002 implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results 2002).

Recovery

The apparent recovery (R^{app}) was 90–116% throughout all matrices (Table 2). The use of an isotopically labelled internal standard compensates for the eventual loss of analyte and matrix effects occurring during the sample preparation and LC-MS analysis. In addition to R^{app}, R^{MS}, R^{SP} and R^{T} were also calculated (Table 2). The R^{MS} was 96% to 111% for all matrices; hence, the LC-MS/MS analysis is not prone to matrix effects, neither ion suppression nor signal enhancement. Ion suppression tests done on all sample matrices included in this study, illustrated with pasta in Figure 1, support this finding. The black lines are all extracted ion chromatograms monitored (Table 1) whereas the red and blue peaks are valinomycin and cereulide, respectively. No influence from the sample matrix on the analyte signal is observed for any of the matrices, i.e. the black chromatograms show neither an increase nor decrease in signal intensity at the retention time of the analytes. The R^{SP} indicates an analyte loss of approximately 45% for rice and 50% for pasta during the sample preparation, whereas preliminary experiments with coffee milk indicates an R^{SP} of 60%. Whipping cream, on the other hand, gave only 26% recovery in the sample preparation, probably due to the high fat content because there is no lipid removal step in the sample preparation. When the homogeneity of the sample and the reproducibility can be assured, and the sensitivity of the method is satisfactory, the apparent recovery is more important than the R^{T} due to the use of an isotopically labelled internal

Table 2. Recovery in the different food matrices, given as min – max values. Calculated from two sample sets for pasta (n = 10) and one sample set each for rice and whipping cream (n = 6).

Ion transition	Matrix	R^{APP}, apparent recovery (%)	R^{T}, total recovery (%)	R^{SP}, sample preparation recovery (%)
1170.7–172	Pasta	90–116	45–59	47–56
1170.7–314.2		94–113	46–59	47–55
1170.7–172	Rice	92–103	38–47	39–48
1170.7–314.2		90–100	37–46	38–47
1170.7–172	Whipping cream,	91–101	24–29	22–26
1170.7–314.2	38 % fat	90–98	24–29	22–26

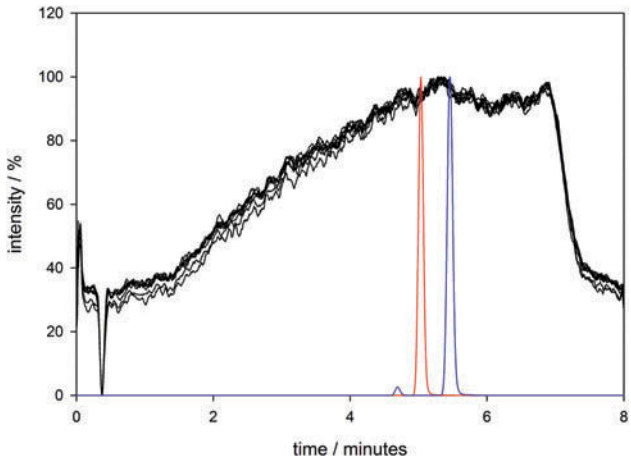

Figure 1. (colour online) Ion suppression test done with continuous injection of cereulide, $^{13}C_6$-cereulide and valinomycin during an injection of blank pasta matrix. All ion transitions (listed in Table 1) are extracted as separate ion chromatograms (black lines). No matrix effect is observed at the retention time of the analytes, represented by the blue (100 ng/g cereulide) and red (100 ng/g valinomycin) chromatograms.

standard that compensates for the analyte loss during the analysis.

The introduction of the enzyme amylase in the sample preparation has improved both the extraction yield and reproducibility of some rice and pasta matrices (data not shown). Amylase degrades the starch, thus the extraction reagent gains better access to the cereulide. In samples with easy access to the cereulide, i.e. fortified samples, this will not be recognised, but it is important in naturally positive samples. An incomplete extraction will underestimate the content of cereulide in the samples.

Selectivity, linearity and sensitivity

The selectivity was evaluated from 13 blind matrix samples: 2 cream samples, 6 pasta samples and 5 rice samples. No interferences were discovered in any of the matrices at the retention time of cereulide or valinomycin. Samples spiked with both cereulide and valinomycin show that these two similar compounds do not interfere with each other.

Linear regression resulted in a coefficient of determination, R^2, above 0.996 for both ion transitions calculated both without and with $1/x$ weighting (Table 3). The response factor test also gave satisfactory results with RSDs below 11%.

The LoD of the method was 0.7 ng/g and the LoQ was 1.1 ng/g, both calculated with $1/x$ weighting in linear regression. Calculation of the LoD and LoQ without weighting gave artificially high values due to the large concentration range in the calibration curve covering three orders of magnitude. Experimental evaluation of blank samples fortified with 1 ng/g cereulide shows that the LoD and LoQ calculated with $1/x$ weighting are indeed the actual levels. The sensitivity of this method is assessed sufficient, as the cereulide levels found in food suspected to cause illness range from 10 to 63,000 ng/g and in patient samples such as serum, faeces and intestinal content range from 4 to 800 ng/g (Mahler et al. 1997; Agata et al. 2002; Shiota et al. 2010; Naranjo et al. 2011; Yamaguchi et al. 2013). If necessary, the LoD and LoQ could be lowered by increasing the injection volume and/or reducing the reconstitution volume because there is no ion suppression in the LC-MS/MS method.

Comparison of quantification with cereulide and valinomycin

It has been common practise to use valinomycin as calibration standard when analysing cereulide (Häggblom et al. 2002; Bauer et al. 2010; Biesta-Peters et al. 2010; Delbrassinne et al. 2011, 2012; Ueda et al. 2012; Yamaguchi et al. 2013). In this study we have not only utilised commercially available cereulide as calibration standard and $^{13}C_6$-cereulide as internal standard, but also quantified the same samples with valinomycin, both as calibration standard and internal standard. In order to achieve comparable results between valinomycin and cereulide, ion transitions representing the same fragment loss

Table 3. Linearity for cereulide combined for all matrices; rice, pasta, whipping cream and coffee milk. Calculated from 26 data points distributed on 7 calibration levels.

Ion transition	R^2	Slope with standard error	Intercept with standard error	Measurement uncertainty over the calibration range (%)	Response factor test RSD (%)
1170.7–172	0.9977	0.0115 (0.000116)	−0.0458 (0.0465)	1.0	10.7
1170.7–314.2	0.9986	0.0111 (0.000086)	−0.0500 (0.0348)	0.8	10.4
1170.7–172 with $1/x$ weighting	0.9959	0.0115 (0.00015)	−0.006191 (0.00602)	1.3	n.c.[a]
1170.7–314.2 with $1/x$-weighting	0.9963	0.011016 (0.00013)	−0.006943 (0.00551)	1.2	n.c.

Note: [a]n.c., not calculated.

Table 4. Comparison of cereulide quantification with valinomycin (VAL) and cereulide (CRL) as calibration standards and valinomycin (VAL) and $^{13}C_6$-cereulide ($^{13}C_6$-CRL) as internal standards. Calculated as average of 28 inoculated or naturally positive samples of both rice and pasta with different additives.

CRL Precursor ion	CRL Product ion	VAL Precursor ion	VAL Product ion	% CRL found when calibrated against VAL compared to CRL (RSD, %)[a]	% CRL found with VAL as I.S. compared to 13C6-CRL as I.S. (RSD, %)[b]	Reference[c]
1170.7	1170.7	1128.7	1128.7	79–86 (4.0)	57–120 (21)	(Biesta-Peters et al. 2010; Yamaguchi et al. 2013)
1170.7	1153.7	1128.7	1111.7	57–63 (5.1)	50–121 (22)	(Delbrassinne et al. 2012; Yamaguchi et al. 2013)
1170.7	1125.7	1128.7	1083.7	38–40 (3.3)	56–130 (21)	(Bauer et al. 2010; Delbrassinne et al. 2012; Ueda et al. 2012)
1170.7	172	1128.7	172	37–40 (4.0)	54–116 (20)	(Bauer et al. 2010)

Notes: [a]% cereulide calculated with VAL as calibration standard of % cereulide calculated with CRL as calibration standard, without the use of internal standard.
[b]% cereulide calculated with VAL as I.S. of % cereulide calculated with $^{13}C_6$-CRL as intern standard. CRL is used as calibration standard.
[c]Publication where the ion transition in question has been used.

were chosen instead of the ion transitions validated. Altogether, four ion transitions were investigated based on use in previously published methods. The calibration results are shown in Table 4. Quantification with valinomycin as calibration standard resulted in a 14–21% underestimation of the cereulide content in the samples when the ion transitions monitored were 1170.7 → 1170.7 for cereulide and 1128.7 → 1128.7 for valinomycin (both $(M + NH_4)^+$). These ion transitions represent no fragmentation in the collision cell and are more prone to matrix effects and less selective than ion transitions where the product ion represents a specific fragment loss. This might explain why the use of a triple quadrupole mass spectrometer gives an underestimation of the cereulide content with valinomycin as calibration standard while an ion trap mass spectrometer gives a 10% overestimation of the cereulide content when the same ion transitions are used (Biesta-Peters et al. 2010). For the other three ion transitions investigated, the underestimation of the cereulide content is more severe when calibrated against valinomycin, as much as 60%. The variance in quantification between the ion transitions is probably caused by the different probability of the fragment ions to be produced in the collision cell, due to the structural differences between valinomycin and cereulide.

Valinomycin and $^{13}C_6$-cereulide were both added as internal standard to the same samples. With the addition of an internal standard, disregarding which one, the big differences in quantification between the ion transitions are eliminated. On the other hand, the quantification of cereulide with valinomycin as internal standard varies between 50% and 130% of the quantification done with $^{13}C_6$-cereulide as internal standard, independent of which ion transition was used (Table 4). This suggests that cereulide and valinomycin either do not behave the same way during the sample preparation, despite their molecular similarity, or they are differently influenced by the sample matrix in the LC-MS/MS analysis even though this could not be shown by traditional ion suppression tests. These findings emphasise the importance of an isotopically labelled internal standard to achieve reliable quantitative results.

Several methods using in-house produced synthetic cereulide and/or $^{13}C_6$-cereulide have been published (Jääskeläinen, Häggblom, et al. 2003; Biesta-Peters et al. 2010; Ueda et al. 2012); unfortunately, this requires both skilful organic chemists and a well-equipped laboratory and is not achievable in every laboratory. The use of in-house purified cereulide from emetic *B. cereus* strains as calibration standard is previously reported (Häggblom et al. 2002; Hormazabal et al. 2004; Bauer et al. 2010). Several protocols for cereulide production and purification based on the method published by Hormazabal et al. (2004) have been investigated and optimised in this study. A comparison between purified cereulide and synthetic cereulide showed that the in-house purified cereulide was without exception not nearly as pure as the synthetic cereulide (data not shown). Hence, the use of such purified cereulide as calibration standard may result in an overestimation of the cereulide content in the samples investigated.

Positive samples

Inoculated and blank rice, pasta and cream

To verify the suitability of the method for determination and quantification of cereulide in positive samples, three different inoculations of *B. cereus* strain NC 7401 were

performed in pasta, rice and whipping cream. In addition, four different pasta samples and four different rice samples were treated identically to the inoculated samples. The cereulide content and the cell count were measured every 24 h for 96 h. High amounts of cereulide were detected in the inoculated samples of pasta and rice after 24 h; 539 ng/g in the pasta and 1351 ng/g in the rice ($n = 4$) (Figures 2, 3 and 4). To our surprise, considerable amounts of cereulide were also found in all the blank pasta and rice samples after 48 h; 14–593 ng/g and 91–446 ng/g, respectively. These levels are in the concentration range earlier reported to have caused illness (Mahler et al. 1997; Agata et al. 2002; Shiota et al. 2010; Naranjo et al. 2011; Yamaguchi et al. 2013). This indicates the presence of naturally occurring emetic *B. cereus* strains in the sample materials. It has been reported that about 5% of pasta and rice contain emetic *B. cereus* (Messelhäusser et al. 2014); hence, positive findings of cereulide in all of the blank materials were unexpected. During the experiment, measures were taken to ensure there was no contamination between the samples; i.e. using disposable lab ware, reagent blanks that went through the whole sample preparation and both negative and positive controls. To ensure the origin of the bacteria

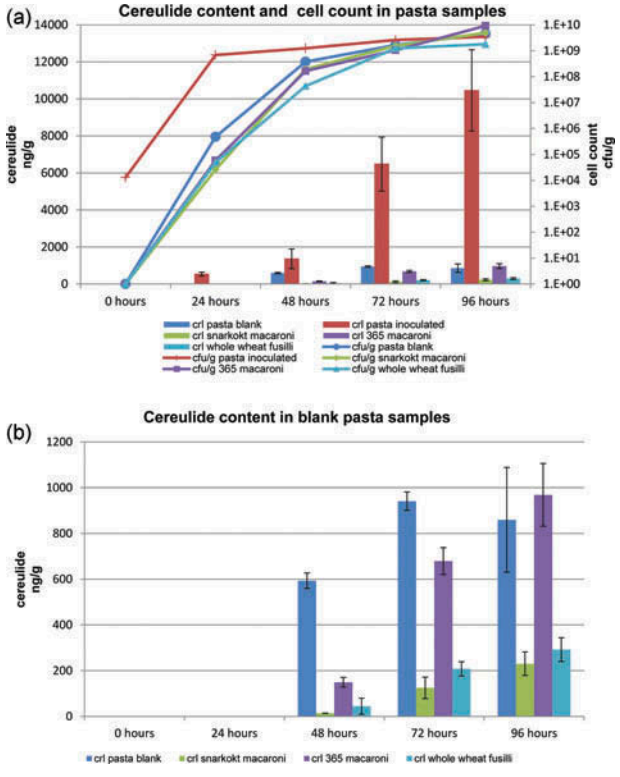

Figure 2. (A) Cereulide content and cell count in pasta samples with and without inoculation. (B) Cereulide content in the blank pasta samples (same as Figure 2A but without the inoculated sample).

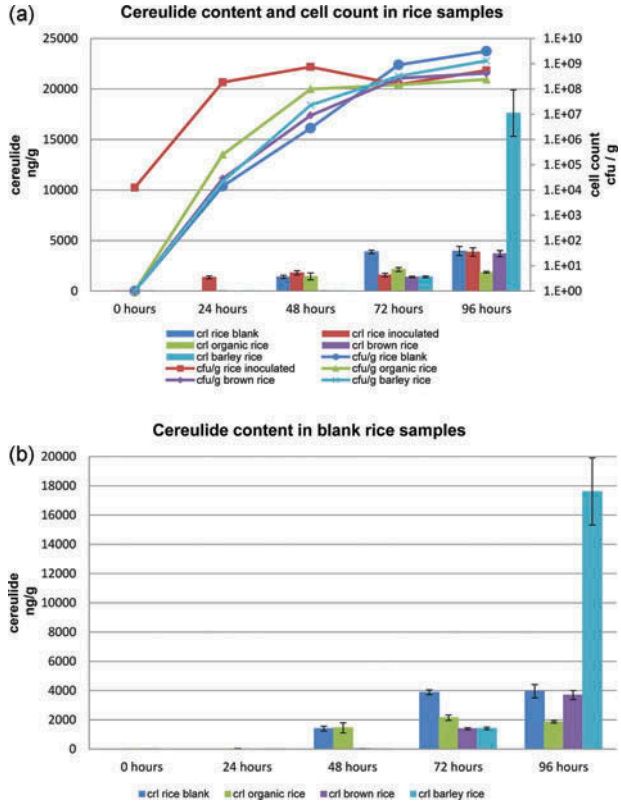

Figure 3. (A) Cereulide content and cell count in rice samples with and without inoculation. (B) Cereulide content in the blank rice samples (same as Figure 3A but without the inoculated sample).

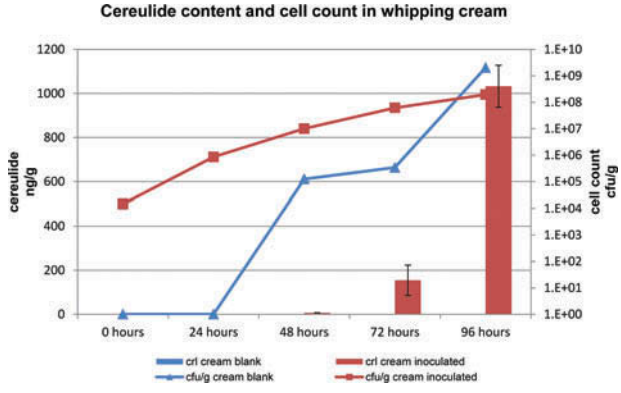

Figure 4. Cereulide content and cell count in inoculated and blank whipping cream.

was the pasta and not a contamination, blank pasta was prepared a second time and the experiment repeated, without inoculation. Also this time, similar amounts of cereulide were produced in the pasta. Finally, the *B. cereus* strains isolated from the rice and pasta samples were compared by exoprotein profiling on SDS–PAGE

(data not shown) (Ehling-Schulz, Svensson, et al. 2005), their profiles were clearly different.

Figure 2 shows that throughout the entire experiment the cereulide content in the pasta samples, both the inoculated and the blanks, increased every 24 h. Häggblom et al. (2002) showed that cereulide production commenced at the end of logarithmic growth phase. We observed cereulide production initiated during the logarithmic growth and it kept increasing during the stationary phase. This implies that once the *ces* gene is activated (Spo0A) and the repressor released (AbrB) (Lücking et al. 2009), there is no correlation between cell growth and cereulide production.

Figure 3 shows that the cereulide production is different in rice compared to pasta. This is most probably due to access to nutrients which may influence the activation of the *ces* gene. In pasta the different spores will be distributed throughout the matrix due to the production procedure, while in rice the grains will mainly be contaminated on the surface.

The same inoculation experiment was performed on pastuerised whipping cream, using a single blank sample. Both the cereulide production and the cell growth were lower and slower than in the starch rich matrices (Figure 4). After 48 h only 7 ng/g cereulide was produced in the inoculated sample. There was no cereulide production in the blank sample within the 96 h of the experiment. This supports the finding of Messelhäusser et al. (2014) categorising pasteurised cream as a medium-risk food whereas boiled rice were considered high-risk food, possibly due to the easily accessible carbohydrate nutrients in the latter.

Cereulide food poisoning in a kindergarten

In June 2013 there was a small outbreak of food poisoning related to cereulide in a kindergarten in Norway (Mellegård et al. 2014). Out of approximately 100 people, 5 children and 3 adults became sick with nausea and vomiting that for all afflicted terminated throughout the same day. The suspected culprit of the outbreak was a rice dish with cod and vegetables, where the rice was prepared two days prior to consumption followed by refrigerated storage until use. The Norwegian Food Safety Authority (NFSA) collected two samples from the rice dish from two different serving trays. The amount of cereulide present in the two samples was determined with the LC-MS method described here and quantified to approximately 1 µg/g and 10 µg/g, respectively. The cell counts in the samples were 3.0×10^6 and 8.8×10^7 cfu/g, respectively. These cereulide concentrations are in the higher range of the toxin levels earlier shown to have caused disease (Agata et al. 2002; Jääskeläinen, Teplova, et al. 2003; Naranjo et al. 2011; Yamaguchi et al. 2013). The mild symptoms and the fact that only a few people became ill suggest that the cereulide level in the food was much lower at the time of consumption than at the time of sample collection. The food was consumed on a Friday whereas the samples were collected on the following Monday. The time interval between consumption and sample collection may explain why the high concentrations of cereulide found did not cause a much more severe illness. There was no control of the temperature in the refrigerators in the kindergarten, nor is it known how long the leftovers were exposed to room temperature before refrigeration after the meal. NFSA measured 11°C in one of the kindergarten's refrigerators after the outbreak, a temperature where cereulide production is feasible (Häggblom et al. 2002; Thorsen et al. 2006; Delbrassinne et al. 2011; Røssvoll et al. 2014).

The presence of cereulide in the samples was also determined with the already established sperm motility assay (Andersson et al. 2004). The sperm motility is examined under the microscope and requires great objectivity of the analyst. The method is merely qualitative and the reported LoD is 1.5 ng/g (Andersson et al. 2004). Another limitation of the sperm motility assay is that it is developed for pure bacteria isolates. Analysis of food might offer difficulties; for instance, the presence of other bacteria or toxins that may influence the mobility of the spermatozoa. Altogether this makes the sperm motility assay suitable for screening purposes, although it is not adequate as a confirmatory analysis. The LC-MS/MS method is more rapid than the sperm motility assay; results of the analysis can be reported within 24 h after reception of the samples, whereas the sperm motility assay requires at least 48 h due to overnight growth, or longer, on agar plates prior to analysis. In severe cases of food poisoning an early establishment of the pathogen might be vital.

Conclusion

In this study we have developed a fully validated, simple, rapid and robust method for the determination and quantification of cereulide in three different matrices associated with food poisoning outbreaks. Due to the use of commercially available standards the method is easily implemented in the laboratory without the need of in-house production and purification of cereulide for calibration. Cereulide production in all of the investigated pasta and rice samples indicates that the prevalence of emetic *B. cereus* is much higher than earlier assumed.

Acknowledgements

We would like to thank Tina O'Sullivan, Toril Lindbäck, Marte Monshaugen, Shani Wilhelmina Aarek Kidd and Olga Osinska

for all help with the production of cereulide and microbiological analysis and Marianne Løken and Kristin Einarsen for the help with the chemical analysis of cereulide. We also thank Tjakko Abee for providing us with synthetic cereulide.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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II

Paper II

Toxin production and growth of pathogens subjected to temperature fluctuations simulating consumer handling of cold cuts

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International journal of food microbiology, 2014, 185, 82-92

International Journal of Food Microbiology 185 (2014) 82–92

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Toxin production and growth of pathogens subjected to temperature fluctuations simulating consumer handling of cold cuts

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ARTICLE INFO

Article history:
Received 12 August 2013
Received in revised form 18 February 2014
Accepted 25 May 2014
Available online 2 June 2014

Keywords:
Consumers
Refrigerator
Temperature exposure
RTE food
Domestic food handling

ABSTRACT

It is crucial for the quality and safety of ready-to-eat (RTE) foods to maintain the cold chain from production to consumption. The effect of temperature abuse related to daily meals and elevated refrigerator temperatures on the growth and toxin production of *Bacillus cereus*, *Bacillus weihenstephanensis* and *Staphylococcus aureus* and the growth of *Listeria monocytogenes* and *Yersinia enterocolitica* was studied. A case study with temperature loggings in the domestic environment during Easter and Christmas holidays was performed to select relevant time and temperature courses. A model for bacterial surface growth on food using nutrient agar plates exposed to variations in temperatures was used to simulate food stored at different temperatures and exposed to room temperature for short periods of time. The results were compared with predicted growth using the modeling tool ComBase Predictor.

The consumers exposed their cold cuts to room temperatures as high as 26.5 °C with an average duration of meals was 47 min daily for breakfast/brunch during the vacations. Short (≤2 h) daily intervals at 25 °C nearly halved the time the different pathogens needed to reach levels corresponding to the levels associated with human infection or intoxication, compared with the controls continuously stored at refrigerator temperature. Although the temperature fluctuations affected growth of both *B. weihenstephanensis* and *S. aureus*, toxin production was only detected at much higher cell concentrations than what has been associated with human intoxications. Therefore, growth of *L. monocytogenes* and *Y. enterocolitica* was found to be the limiting factor for safety. In combination with data on temperature abuse in the domestic environment, modeling programs such as ComBase Predictor can be efficient tools to predict growth of some pathogens but will not predict toxin production.

1. Introduction

For many foods, low temperature storage from production to consumption is necessary to maintain quality and safety. Although temperature abuse may occur in every stage in the food chain, the least controllable part is at the consumer stage. This is especially important for ready-to-eat (RTE) foods with a long shelf life based on an unbroken cold chain. If RTE food is stored periodically or constantly at a too high temperature or eaten after the "last day of consumption," the producers cannot guarantee for the safety of the product. In a study conducted in Norway in 2009 where over 2000 randomly selected consumers were surveyed, 44% reported they continued using cold cuts even after the expiry date. The majority of these consumers, 62%, considered their own sensory apparatus as an important tool to evaluate the quality of cold cuts after the last day of consumption (Jacobsen and Lavik, 2011; Røssvoll et al., 2013, 2012). This is consistent with a survey conducted in the United States, where nearly two-thirds stated that they relied on their senses whether to eat refrigerated food or not (Kosa et al., 2007). This is a hazardous practice, as growth of pathogens will normally not destroy the organoleptic quality of the food (Farber, 1991).

It is important to get a better understanding of the consequence of consumer food-handling practices for the growth and toxin production of pathogens under domestic conditions. Laws and regulations are implemented in the food chain to reduce risk and improve food safety all the way from the primary producer to retail, but as soon as the product is purchased by the consumer, there is little knowledge of how the product is treated. Garrido et al. (2010) conducted a study of *Listeria monocytogenes* growth on sliced RTE ham stored at temperatures simulating temperature variations in refrigerators. However, they did not investigate how the consumers treated the sliced RTE ham outside of the refrigerators (Garrido et al., 2010). Many studies describe the growth of pathogenic bacteria in food stored at varying temperatures. But to our knowledge nobody has collected information about rapid temperature

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http://dx.doi.org/10.1016/j.ijfoodmicro.2014.05.020
0168-1605/

variations caused by consumers' handling of food, and tested how these temperature variations influence the growth and toxin production of pathogenic bacteria.

When the product is taken out of the cooling systems in the supermarkets, it is out of the producers' control. We were therefore interested in looking at the temperatures that RTE foods are exposed to at the consumer stage over a longer period of time. Breakfast and lunch in Norway are traditionally based on bread and different cooked meat and fish products served cold. Vacations and holidays were chosen as the domestic setting in this study, as such occasions often entail longer meals as people eat more slowly and leave the food at room temperature for extended periods of time, and could therefore represent a possible "worst case" scenario. Also, food may be stored longer before consumption during Christmas and Easter holidays than in the weekends, since food stores are closed or not accessible and traditional home-made food is often prepared in advance. Temperatures from purchase to refrigerator storage by the consumers were not considered in this study. The aim of this study was to i) measure the occurrence of repeated temperature abuse connected to serving RTE food in the domestic environment, using vacations as a worst case scenario, and ii) investigate both pathogenic growth and toxin production under such temperature abuse. We focused on bacteria that are known to cause problems either by growing at low temperatures or by producing toxins after moderate temperature abuse: *L. monocytogenes* (Farber and Peterkin, 2000), *Yersinia enterocolitica* (Nesbakken, 2000) and the toxin producers *Bacillus cereus* (Granum and Baird-Parker, 2000), *Bacillus weihenstephanensis* (emetic toxin) (Stenfors et al., 2002) and *Staphylococcus aureus* (Baird-Parker, 2000). *B. weihenstephanensis* is a psychrotolerant species which belongs to the *B. cereus* group (Lechner et al., 1998). There has not been any documented foodborne outbreaks caused by *B. weihenstephanensis*, however it has been found that the organism has the ability to produce several pathogenicity factors and it was therefore included in this study (Stenfors et al., 2002).

2. Materials and methods

2.1. Refrigerator temperatures and temperature variations at the consumer stage

In order to choose relevant conditions for the laboratory experiments, a model system for measuring the temperature profile for cooled RTE products served at breakfast/brunch was established. The case study is a descriptive study involving a convenience sample of 60 households recruited from the Oslo and Akershus area in Norway. Subjects were recruited by word-of-mouth and by the snowball effect. Criteria for inclusion of subjects were that they ate cold cuts and that they were preparing most of the meals themselves. One part of the study targeted pregnant women. The subjects were provided with a temperature logger (EL-USB data logger Lascar Electronics, UK) during Easter 2009, Christmas 2009 or Easter 2010. The subjects received the following written guidance on how to treat the temperature loggers (translated here from Norwegian):

> "The temperature logger is to be treated as cold cuts during the whole period. Put the temperature logger in your refrigerator where you also keep your cold cuts. When you take the cold cuts out of the refrigerator, take the temperature logger out as well. For instance, when laying the table for breakfast and/or lunch with cold cuts, the temperature logger is to be placed on the table as well. And when the meal is over and the cold cuts are put back into the refrigerator, the temperature logger is to be placed beside the cold cuts in the refrigerator."

The temperature was logged every minute over the whole period of 11 days.

2.2. Temperature effect on growth and toxin production

2.2.1. Experimental design of growth and toxin production experiments

The effect of the temperature abuse related to daily meals and elevated refrigerator temperatures found in the case study was studied on pathogenic growth and toxin production using nutrient agar plates as a food model. The results were compared with predicted growth using the modeling tool ComBase predictor.

2.2.2. Bacterial strains and culture conditions

The pathogens *B. cereus*, *B. weihenstephanensis* and two different strains of *L. monocytogenes*, *S. aureus* and *Y. enterocolitica* were investigated (Table 1). In the first experiment, strains isolated from foodborne outbreaks in Norway were chosen, that is *L. monocytogenes* 2583/92, *S. aureus* 50089 (toxin A), *Y. enterocolitica* 1106-0129-1 O:3 and an emetic toxin producing *B. weihenstephanensis* MC67. The experiment was performed three times on different weeks with newly prepared inocula, resulting in three biological replicates. In the subsequent experiment, the other strains were chosen for comparison, see Table 1. The purpose of the latter experiment was to investigate whether the isolates chosen deviated significantly from other strains, and it was performed only once.

Stock cultures were maintained in 20% glycerol at −80 °C. For inoculum preparation, frozen suspensions were streaked on tryptic soy agar (TSA; Oxoid, Basingstoke, UK), and incubated at 30 °C for 24 h, except for *B. cereus* F3605/73 and *B. weihenstephanensis* MC67. Preliminary experiments showed that *Bacillus* spp. formed large, indistinct colonies on TSA that were difficult to count, and that they grew better when preincubated at 20 °C. Standard plate count agar (PCA; Oxoid) and a preincubation temperature of 20 °C were therefore used for *Bacillus* spp. Three to five colonies from the agar plates were transferred to 5 mL tryptic soy broth (TSB; Oxoid) and cultured for 16–18 h at 30 °C, 200 rpm (*L. monocytogenes* was cultured without shaking because of a lack of a shaking incubator at the class 3 pathogen laboratory). The strains were transferred to 4 °C and incubated for 16–18 h for cold adaptation prior to the growth and toxin experiments.

2.2.3. Surface growth

The effect of rapidly fluctuating temperatures on the growth of the selected pathogenic strains was examined on the surface of TSA (PCA for *Bacillus* spp. strains). Every plate was inoculated with 100 μL of a 10^3 CFU/mL inoculum, which gave approximately 100 bacterial cells on each plate. The agar plates were incubated at 4 ± 1 °C and 8 ± 1 °C (Innova 4230, New Brunswick Scientific Co, Edison, USA) and exposed to a temperature of 25 ± 0.5 °C (Termaks Series B8000, Bergen, Norway) every day for 30 min, 1 h or 2 h, respectively. *S. aureus* was incubated at 8 ± 1 °C and 12 ± 1 °C (Innova 4230, New Brunswick Scientific Co, Edison, USA), as it does not proliferate at temperatures below 7 °C (Halpin-Dohnalek and Marth, 1989; Rørvik and Granum, 2007). Control plates were also inoculated with cells and incubated at a constant temperature (4, 8 or 12 °C). The experiments lasted for 9–10 days.

Samples were removed daily for viable cell count. The cells were washed off the agar surface using 2 mL cold (4 °C) buffered peptone water and a sterile L-shaped spreader (VWR International, West Chester, USA). The viable cell count analysis was performed using an automatic spiral plater (WASP (Whitley Automatic Spiral Plater); Don Whitley Scientific, West Yorkshire, UK). *Bacillus* sp. made cell aggregates and was difficult to homogenize in the buffered peptone water. Preliminary experiments showed that the aggregates could be dissolved by sonication (Joyce et al., 2003) or the use of Tween 80 (Sigma-Aldrich, St. Louis, USA) (Besse and Lafarge, 2001; Oh and Cox, 2010) in the washing water. 10 min of sonication of the buffered peptone water with the cells from agar plates with *Bacillus* sp. was compared with the use of 0.2% Tween 80 instead of buffered peptone water. No significant difference in viable cell count was found between the two methods, and Tween 80 was used in further experiments.

Table 1
Strains used in this study.

Pathogen	Strain	Origin	Reference
B. cereus	F3605/73	Outbreak, boiled rice, UK	NMBU[a] Mortimer and McCann (1974)
B. weihenstephanensis	MC67	Sandy loam, Denmark	Hendriksen et al. (2006) Thorsen et al. (2006)
L. monocytogenes	2583/92	Outbreak, cold cuts of cured ham, Norway	NIPH[b] (1992)
	ILSI 36	Outbreak, frankfurters, USA	Fugett et al. (2006)
S. aureus	50089	Outbreak, goat cheese, Norway (toxin A)	NVI[c], Rode et al. (2007)
	50583	Outbreak, pizza, Norway (toxin B)	NVI, Rode et al. (2007)
Y. enterocolitica	1106-0094-1, O:9	Outbreak, processed pork, Norway	NIPH, Grahek-Ogden et al. (2007)
	1106-0129-1, O:3	Outbreak, processed pork, Norway	NIPH

[a] Norwegian University of Life Sciences.
[b] Norwegian Institute of Public Health.
[c] Norwegian Veterinary Institute.

2.2.4. Extraction and quantification of cereulide

All chemicals were of at least HPLC grade and supplied by VWR (Merck, Darmstadt, Germany) except valinomycin (Sigma, Steinheim, Germany) and heptafluorobutyric acid (Fluka, Buchs, Switzerland). The water used was grade 1 water-purified with a Milli-Q water purification system from Millipore (Bedford, MA, USA).

The *B. weihenstephanensis* MC67 can produce the intercellular emetic toxin cereulide (Thorsen et al., 2006). Samples for cereulide quantification were taken every day, starting at day 4 for one experiment and day 2 for the two replicates, to obtain at least three samples taken before the culture entered the stationary phase for all temperature courses. The method for extracting the cells with cereulide from the agar plates was modified from Thorsen et al. (2009). The cells were scraped and washed off the PCA surface using 2 mL cold (4 °C) sterile distilled water and a sterile L-shaped spreader, transferred to vials and whirly mixed for 2 min. The cells were kept at −20 °C until the day of analysis.

Cereulide was extracted from the cells as described by Dybwad et al. (2012) with the following modifications: After thawing, acetone (0.75 mL) and chloroform (1 mL) were added to each sample, and the mixture was vigorously shaken (10 s) and centrifuged at 1600 *g* for 3 min. The organic phase was transferred to another glass vial and evaporated to dryness using a Thermo Scientific Savant SpeedVac concentrator (Thermo Fisher Scientific, Waltham, USA). Methanol (1 mL) was added, and the dry residue was dissolved in the methanol by shaking the samples with 1 g sterile glass beads (2 mm). The samples were centrifuged at 8100 *g* for 1 min, and 30 μL of the supernatant was transferred to an HPLC vial. 30 μL internal control (10 ng/mL valinomycin and 2 mM ammonium acetate in acetonitrile/water: 1 + 1) was added, and the samples were whirly mixed for 25 s.

Cereulide was separated from other matrix components, identified and quantified using liquid chromatography–tandem mass spectrometry, LC–MS/MS. The instrumentation used was a 1260 series binary pump with a 1200 series thermostatted autosampler and column compartment (Agilent Technologies, Waldbronn, Germany) and a 6460 triple quadruple mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The separation was performed on an RRHD Zorbax Eclipse Plus C18 column, 100 × 2.1 mm id, with 1.8 μm particles (Agilent Technologies, Palo Alto, CA, USA) using a linear gradient from 87 to 97% mobile phase B (acetonitrile) in 3.5 min. The column temperature was 40 °C. Mobile phase A consisted of 2 mM ammonium acetate and 0.2% heptafluorobutyric acid in water, and the flow rate was 0.8 mL/min. Total analysis time was 7 min. Both cereulide and valinomycin were monitored as their ammonium adducts (NH_4^+) in positive multiple reaction monitoring mode, MRM. The transitions monitored were m/z 1170.7 → 314.2, 1170.7 → 186 and 1170.7 → 172 for cereulide and m/z 1128.7 → 343 and 1128.7 → 272.2 for valinomycin.

Though cereulide has been synthesized and is commercially available, it is too expensive for extensive use. In-house purified cereulide proved to contain impurities when compared with synthetic cereulide, hence another method for reliable quantification had to be established. Valinomycin is another cyclic dodeka depsipeptide with very similar structure and physicochemical properties as cereulide, and it is thus a suitable candidate for external calibration (Häggblom et al., 2002). Calibration curves of valinomycin and synthetic cereulide were prepared by serial dilution in 2 mM ammonium acetate in acetonitrile/water (1 + 1) from stock solutions in methanol. The calibration levels ranged from 1 to 100 ng/mL for synthetic cereulide and from 1 to 500 ng/mL for valinomycin. The ratio between valinomycin and synthetic cereulide was established, so that valinomycin could henceforth be used for the quantification of cereulide. The internal control added to the samples before analysis on LC–MS/MS provided a measure of the reproducibility of the method.

2.2.5. Extraction, detection and quantification of S. aureus enterotoxin

S. aureus 50089 is able to produce enterotoxin A. Samples for toxin detection were taken every second day to obtain at least three samples taken before the culture entered the stationary phase for all temperature courses. For 8 °C storage, toxin samples were also taken at day 10. Since enterotoxin A is secreted from the cells, it was extracted from the agar (methodology modified from Lapeyre et al. (1996) and the user manual from Transia Plate Staphylococcal Enterotoxins (BioControl Systems, Bellevue, USA)), before immunological detection. The agar was weighed and mixed with the same weight of distilled water using a stomacher, followed by incubation in a water bath at 45–50 °C for 30 min to allow the toxins to diffuse. The suspension was centrifuged at 3000 *g* for 15 min, and the supernatant was collected and kept at −20 °C until analysis. For detection and quantification of *S. aureus* toxin, the Transia Plate Staphylococcal Enterotoxins (BioControl Systems) was chosen (Hennekinne et al., 2007; Lapeyre et al., 1996). The frozen samples were thawed in a refrigerator (4 °C) over night and sterilized by filtration (0.45 μm). A 900 μL filtrated sample was added 100 μL decomplemented normal rabbit serum (Transia; BioControl Systems) and incubated for 30 min at room temperature. The pH of the solutions was checked and adjusted to 7.0–7.5. The analysis was performed with 100 μL of sample as described by the manufacturer. The optical density was measured at 450 nm by a spectrophotometer (Titertek Multiskan Plus MK, II; Titertek Instruments, Huntsville, USA). A sample was considered positive for staphylococcal enterotoxins if its optical density

was higher or equal to the threshold of 0.40, according to the manufacturer (BioControl Systems).

2.2.6. Prediction of growth using ComBase Predictor

The bacterial growth was predicted using the ComBase Predictor (ComBase, 2013). There were limitations in the permitted range of input pH for the ComBase Predictor for some of the bacterial models. *Y. enterocolitica* and *S. aureus* were modeled with pH 7.2 and 7.1, respectively, while the growth experiment was performed on TSA with pH 7.3. All strains grown on TSA were modeled with 0.5% NaCl. For *B. weihenstephanensis* no growth model was available in the ComBase Predictor. Moreover, the minimum allowed input temperature for the closely related *B. cereus* was 5 °C. As discussed by Ter Beek et al. (2011), it is difficult to use modeling tools on spore-forming bacteria as *Bacillus* spp., as it is hard to accurately predict the timing of germination and outgrowth of low numbers of spores. We therefore chose not to predict the growth of *B. weihenstephanensis* and *B. cereus* with ComBase. The starting counts used in the predictions were determined on the basis of plating to agar of the inoculum used in the experiments. The ComBase Predictor contains an input parameter for the physiological state. When using a value of 0.9, which indicates relatively adapted strains, no lag phase was predicted. This was similar to our growth experiment, where the strains were adapted to cold temperature before inoculation. For the input of the changing temperature values, we anticipated that heating to 25 °C and cooling to cold temperature took similar time. The input temperatures were included as a stepwise model with direct change in temperature between 25 °C and the specific cooling temperature (input data e.g., time 0–23 h: 4 °C; time 23.1–24 h: 25 °C; time 24.1–47 h: 4 °C, etc.).

2.3. Statistical analysis

The temperature recordings were analyzed with EasyLog USB version 4.7 (Lascar Electronics, USA). Statistical analyses were performed using Microsoft Office Excel 2003 (Microsoft, USA) and SPSS version 17 (IBM, Chicago, USA). As the growth rate of the different bacteria would vary with the different temperatures they were exposed to, an average growth rate was calculated to enable us to compare the different temperature regimes and the different bacteria. Average lag time and growth rate were calculated from a linear regression line based on minimum 3 points in the linear area of the $\log_{10}$ CFU/plate for each strain and growth condition. The lag times (X) were defined as the time point when the linear regression line ($Y = aX + b$) equals the $\log_{10}$ CFU/plate at time 0 ($X = [\log_{10}(\text{CFU / plate at time 0}) - b] / a$). The growth rate was defined as the slope (a) to the linear regression line ($a = [\log_{10}(\text{CFU / plate at time 0}) - b] / X$). Linear regression analysis was performed using Minitab 16 (Minitab, Pennsylvania, USA). The level of significance was 0.05.

3. Results

3.1. Case study

The aim of the case study was to obtain relevant background information for selection of conditions in the simulation experiment and ComBase Predictor. Information about the temperature exposure both between and during meals was determined using an automatic logging device together with instructions for use and contact information for questions. All logging devices were returned, but 14 recordings were excluded from further analysis because the subjects reported that they partly or completely forgot to handle the device according to the instructions, the logger was not working properly or the temperature fluctuations were so large and frequent that it was not possible to differentiate between variations in refrigerator temperature and meals. In total, 46 of the 60 temperature loggings were of satisfactory quality and used in further analyses. During the Easter holiday of 2009 12 temperature loggings were recorded (from 11 women and one man), 16 loggings were recorded in Christmas 2009 (from nine women and seven men), and 18 loggings were recorded in Easter 2010 (from 18 pregnant women).

The average temperature the logging devices were exposed to during the whole period of the sampled 11 days varied from 2.9 °C to 12.0 °C, with a total sample mean of 6.2 °C. The temperature loggings from the case study of the 18 pregnant women showed that they had a mean temperature of 5.8 °C. With a few exceptions, the logging devices were exposed to room temperature once daily for breakfast/brunch. The average duration of meals (that is, when the logger recorded exposure to room temperature) was 47 min. The highest average duration of meals in one household was 116 min daily during the vacation period. The maximum temperatures the cold cuts were exposed to varied from 13.5 °C to 26.5 °C, with an average room temperature of 22.0 °C for all samples. An example of the temperature logging by one of the consumers in the study is given in Fig. 1. To calculate the average temperature in the refrigerators, the recordings for a given night in the period between 00:00 and 06:00 were chosen. The average refrigerator temperatures ranged from 1.3 to 9.9 °C. The total sample mean was 5.6 °C.

On the basis of the case study and the toxin production and growth studies, 4 °C, 8 °C and 12 °C were selected as refrigerator temperatures, 30 min, 60 min and 120 min as meal durations and 25 °C as room temperature.

3.2. Growth and toxin production under different temperature regimes

As expected, the average growth rate for the different pathogens was lowest for the controls incubated at a constant temperature, and increased with the length of exposure to 25 °C. Daily exposure to 25 °C resulted in increasing cell numbers for all samples except *B. cereus* F3605/73, which did not show growth under any of the conditions in our study. No lag time was observed for the pathogens in our study, with the exception of *B. weihenstephanensis* MC67.

3.2.1. Bacillus spp.

As opposed to cultures exposed to 25 °C daily, *B. weihenstephanensis* MC67 stored at a constant temperature of 4 °C showed no growth (Table 2). Exposure to 25 °C for 2 h daily resulted in significantly higher cell counts than the samples exposed for 0.5 h. The average lag phase of the samples stored at 4 °C exposed to 25 °C for 0.5 h, 1 h and 2 h were 2.2, 1.9 and 1.0 days, respectively. At 8 °C *B. weihenstephanensis* MC67 multiplied and the cell counts increased with increasing daily exposure time at room temperature (25 °C). The samples exposed for 25 °C for 2 h daily reached the stationary phase with a cell number of 10^9 CFU per plate in an average of 5.3 days (Fig. 2B). *B. cereus* F3605/73 showed no growth at either 4 °C or 8 °C, regardless of time of exposure (0.5 h, 1 h or 2 h) to 25 °C during the 9 days of the study (results not shown).

3.2.1.1. Emetic toxin production. One single replicate of *B. weihenstephanensis* MC67 stored continuously at 4 °C for three days showed a cereulide concentration of 4 ppb. No other samples of *B. weihenstephanensis* MC67 stored at 4 °C exceeded the detection limit of 1 ppb (ng/mL), irrespective of time exposed at 25 °C. Storage at 8 °C, however, resulted in cereulide production (Fig. 2). Since *B. cereus* F3605/73 did not grow under the test conditions, the toxin production was not measured.

3.2.2. L. monocytogenes

The average growth rate of *L. monocytogenes* 2583/92 increased as the exposure time to 25 °C increased (Fig. 3). The samples stored at 4 °C exposed to 25 °C for 2 h daily showed a significantly higher cell count than the control and the samples exposed for only 0.5 h and 1 h. At 8 °C, also 1 h daily exposure to room temperature had significantly higher cell numbers than the control. The average growth rate

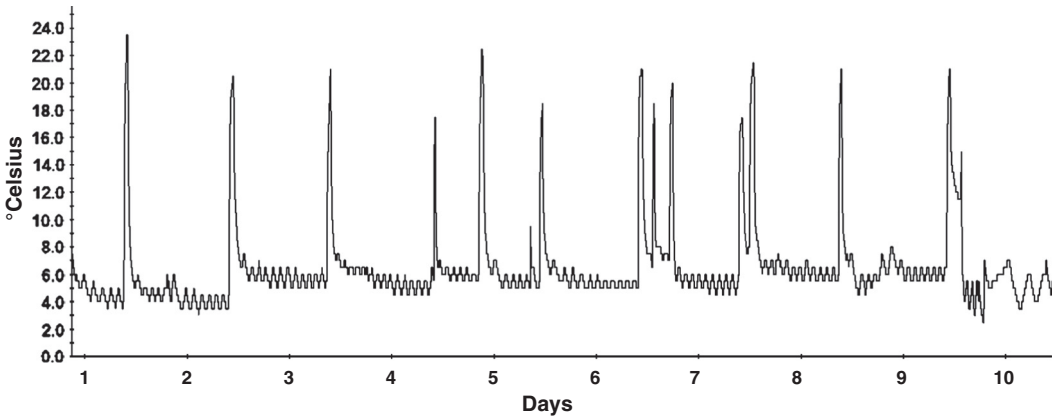

Fig. 1. An example of a temperature logging from one of the consumers in the case study.

of *L. monocytogenes* ILSI No 36 appeared slightly lower than that of *L. monocytogenes* 2583/92 (results not shown).

3.2.3. S. aureus

S. aureus 50089 incubated constantly at 8 °C showed an average growth rate of 0.33 $\log_{10}$ CFU/day. Even exposure to periods as short as 0.5 h daily resulted in significantly higher cell counts than the control at 8 °C ($P < 0.01$), with no significant differences in cell numbers between the samples exposed for 0.5, 1 and 2 h. At a temperature of 12 °C, only the samples exposed to 25 °C for 2 h daily showed significantly higher cell numbers than the controls, and the tendency was that the cell counts increased with increasing exposure to room temperature (Fig. 4). *S. aureus* 50583 showed a similar growth pattern to *S. aureus* 50089 (results not shown).

3.2.3.1. Toxin production of S. aureus. In all three replicates, *S. aureus* 50089 showed low toxin production at both 8 °C and 12 °C, with a high variability between the different replicate samples (see Fig. 4). By comparison, in the single experiment with *S. aureus* 50583 at 8 °C, no samples were positive for toxin production. At 12 °C, all plates with *S. aureus* (control, 0.5 h, 1 h and 2 h) at days 5, 7 and 9 were positive for toxin, except the 2 h plate at day 9, with an optical density at 0.40, just below the threshold limit.

3.2.4. Y. enterocolitica

Y. enterocolitica O:3 showed the fastest increase in cell numbers among the pathogens studied, at both 4 °C and 8 °C (Fig. 5). For the samples stored at 4 °C, those exposed for 1 and 2 h at 25 °C daily showed a significantly higher cell count than the control, and the 2 h samples also had significantly higher cell numbers than the 0.5 h and 1 h samples (Fig. 5). At 8 °C, the tendency was that average growth rates increased as the exposure to room temperature increased in a similar range as at 4 °C, but the effect was not statistically significant. *Y. enterocolitica* O:9 showed a similar growth pattern to *Y. enterocolitica* O:3 (results not shown).

3.2.5. Prediction of growth with ComBase Predictor

The predicted growth pattern of most bacteria showed an exponential growth to $\log_{10}$ 7.5–8, where the predicted growth ceased. The predicted growth increased with the length of the daily exposure time at 25 °C. The predicted growth of *L. monocytogenes* was similar to the observed growth for *L. monocytogenes* 2583/92 at 4 °C, but the ComBase Predictor in general underestimated the growth when stored at 8 °C. As an example, the predicted growth for the *L. monocytogenes* exposed daily for 1 h at 25 °C is compared with the observed growth in Fig. 3. ComBase Predictor underestimated the growth of *S. aureus*, especially when stored at 8 °C (Fig. 4). For both strains of *Y. enterocolitica*, there was a good correlation between the predicted data and the observed growth for all conditions tested in the present study. The predicted growth for *Y. enterocolitica* exposed daily for 1 h at 25 °C is shown in Fig. 5 and Table 2.

4. Discussion

The official standards of the Codex Alimentarius Commission declare that insufficient food temperature control is one of the most common causes of foodborne illness (Codex Alimentarius Commission, 2003). The World Health Organization (WHO) also states in its five keys to safer food that cooked food should not be left at room temperature for more than 2 h (in total), and that all cooked and perishable food should

Table 2
Average time (days) to reach given level associated with human intoxication/infection from initial inoculum of 10^2 cells.

Strain	Storage temperature	Time of daily exposure for 25 °C			ComBase
		0 h	1 h	2 h	1 h predicted
Listeria monocytogenes 2583/92[a]	4 °C	7.5	5.5	4	6.5
	8 °C	3.5	2.5	2.5	3.5
Staphylococcus aureus 50089[b]	8 °C	7.5	3.5	2.5	5
	12 °C	3	2.5	1.5	2.5
Yersinia enterocolitica O:3[c]	4 °C	7.5	4.5	3.5	4
	8 °C	3.5	2.5	2.5	2.5
Bacillus weihenstephanensis MC67[d]	4 °C	–[e]	6.5	6.5	–[f]
	8 °C	3.5	2.5	2.5	–[f]

[a] Level associated with infection: 10^6 cells.
[b] Level associated with intoxication: 10^5 cells.
[c] Level associated with infection: 10^7 cells.
[d] Level associated with intoxication: 10^5 cells.
[e] No growth.
[f] Not predicted.

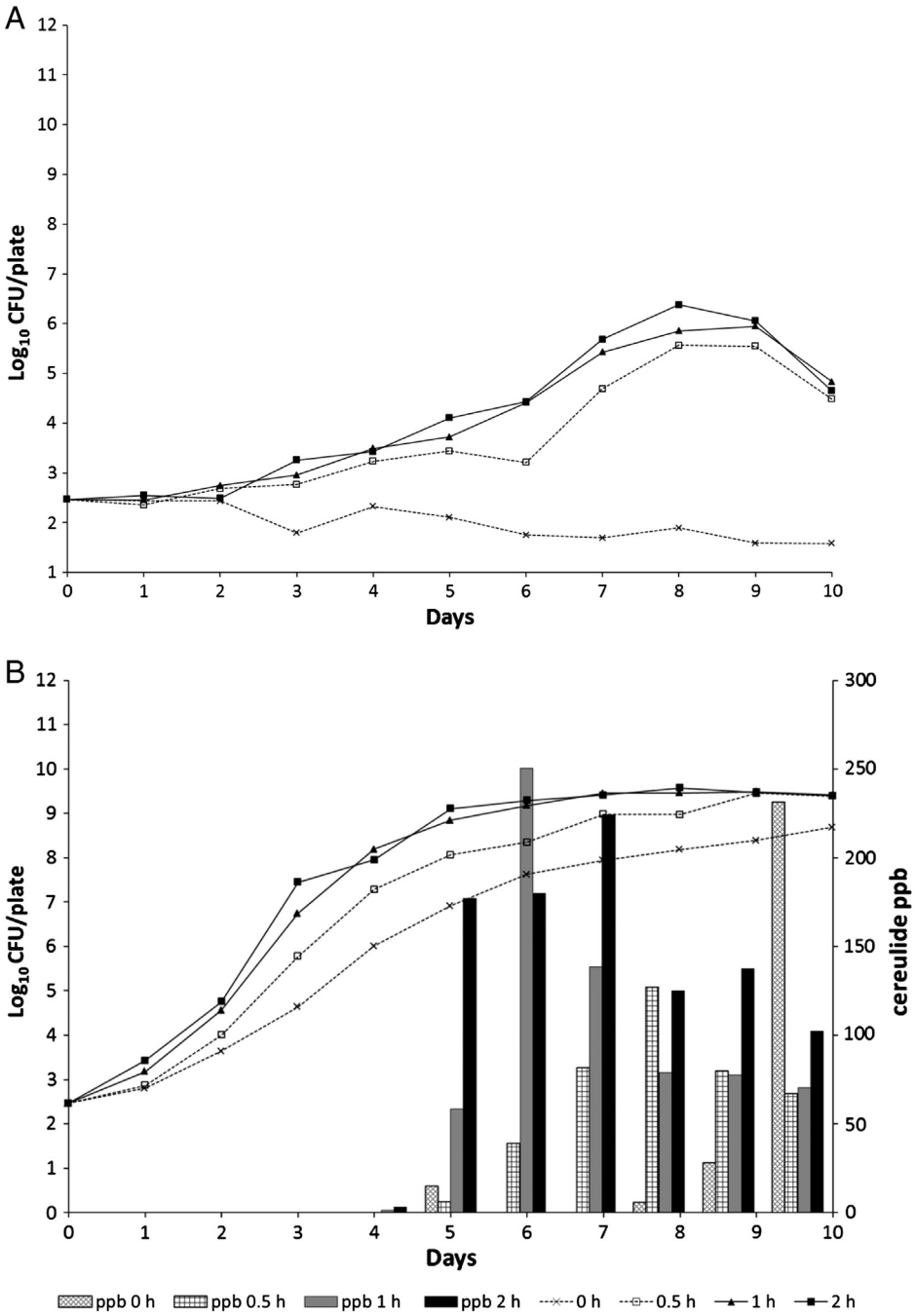

Fig. 2. Growth of *Bacillus weihenstephanensis* MC67 (log_{10} CFU/plate) and cereulide production (ppb) at a storage temperature of 4 °C (A) and 8 °C (B) with different times of daily exposure at 25 °C. No cereulide was detected from the samples stored at 4 °C. Mean standard deviations (log_{10}) of the three independent growth experiments at 4 °C were 0.84 and 0.39 at 8 °C. The measurement uncertainty (95% confidence interval) of the cereulide quantification was 9.6%.

be quickly refrigerated below 5 °C (World Health Organization, 2001). In our case study, one participant exposed the cold cuts to ambient temperatures daily in the period of 11 days during breakfast/brunch for an average of 1 h 56 min. The majority of the participants, however, exposed their cold cuts for shorter intervals. The pregnant women in the case study were found to expose their cold cuts for temperatures higher than recommended, which is of concern since pregnant women are much more susceptible to foodborne diseases (Gerba et al., 1996; Lund and O'Brien, 2011). It should be pointed out that the results of the temperature logging in our case study may underestimate the conditions in "real life," since the participants were aware of the reason for measuring the time and temperature they exposed their cold cuts to. Such studies tend to, consciously or unconsciously, change participants' behavior to provide a more positive image of themselves, as they know they are being observed or measured (Redmond and Griffith, 2003). The temperature logger measured the air temperature and not the temperature of the cold cuts. This could lead to both an over- and underestimation of the real temperature, if the cold cuts were heated up slower than the ambient air temperature, and an underestimation, if the cold cuts were cooled down slower than the air temperature.

Of the 46 temperature data-loggings collected during this study, the temperatures logged during the 11 days spanned from a minimum of −7.0 °C to a maximum of 26.5 °C. The total sample mean temperature found in our study is based on the total temperature time range the cold cuts were exposed to, that is, both inside and outside of the refrigerator. In the domestic environment, it is not just the storage temperature

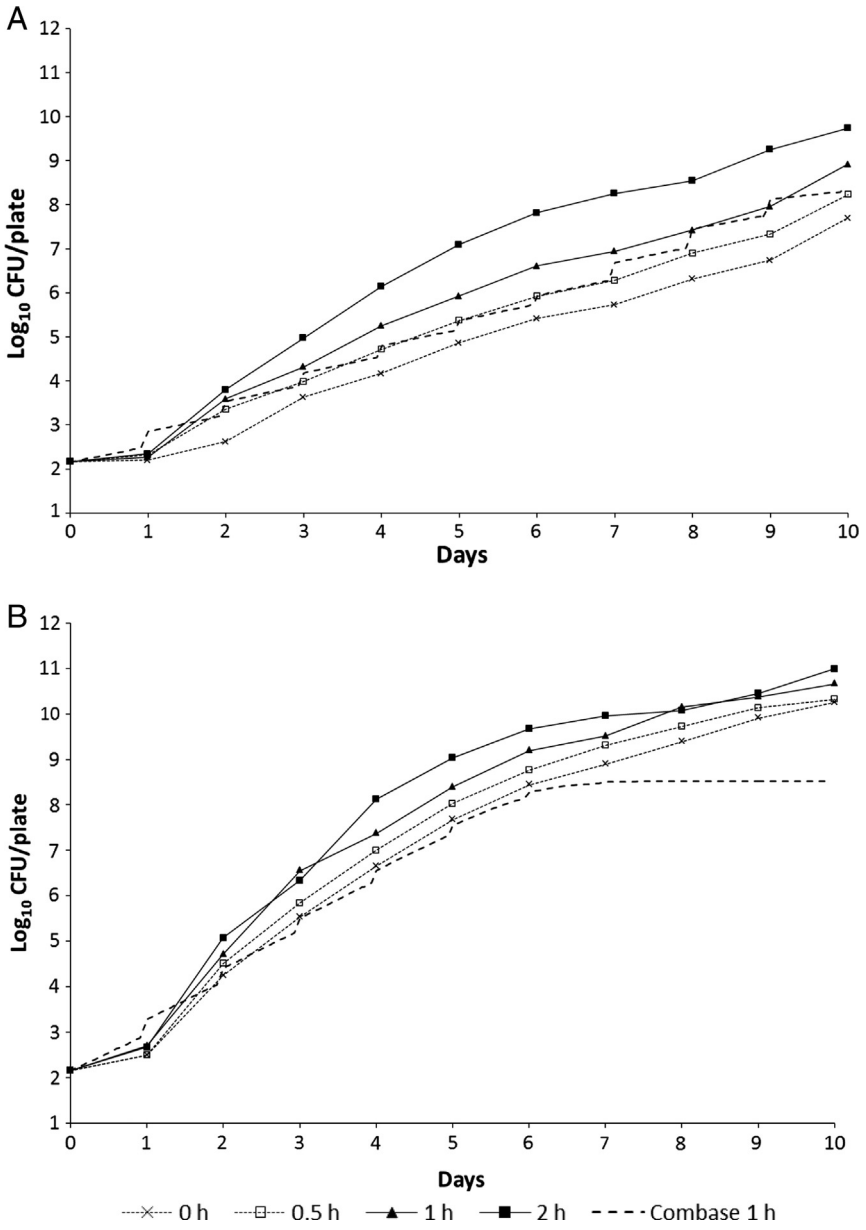

Fig. 3. Growth of *Listeria monocytogenes* 2583/92 (log_{10} CFU/plate) at a storage temperature of 4 °C (A) and 8 °C (B) with different times of daily exposure at 25 °C and the predicted growth of 1 h daily exposure at 25 °C by ComBase Predictor. Input in the model was pH 7.3 and 0.5% NaCl. Mean standard deviations (log_{10}) of the three independent growth experiments at 4 °C were 1.61 and 1.15 at 8 °C.

inside the refrigerator that is of interest, but the total temperature exposure. Additionally, a domestic refrigerator ranges from almost empty to packed full of food, which has a tremendous impact on the refrigerator's cooling capacity, and after purchase from retail stores the food is at an ambient temperature or even warm when it is loaded into domestic refrigerators (James et al., 2008).

As expected, daily exposure to room temperature had a significant effect on bacterial growth. The impact was greatest at the lowest storage temperature: 4 °C for *B. weihenstephanensis*, *L. monocytogenes* and *Y. enterocolitica*, and 8 °C for *S. aureus*. At these storage temperatures, 2 h daily exposure to an ambient temperature approximately halved the time the different pathogens used to reach the levels associated with human infection or intoxication compared with the controls. For all the pathogens, with the exception of *B. weihenstephanensis*, this reduced time to reach the level associated with human infection or intoxication was well within the recommended 3–5 days of shelf life after opening a package of cold cuts. Short (≤2 h) periods in room temperature daily did not facilitate toxin production in the investigated *S. aureus* or *B. weihenstephanensis* strains. It did however promote the growth to adequate numbers of the organism, so if contaminated food is left outside of the refrigerator for longer periods, or the refrigerator operates at a higher than recommended temperature, there is a possibility of toxin production and a risk of subsequent intoxications.

The total number of *Bacillus* spp., from the *cereus* group required to produce sufficient cereulide (emetic toxin) to cause emetic disease has not been clearly established, but data from outbreaks indicate at least 10^5 CFU/g food (Arnesen et al., 2008). Even though the number of *B. weihenstephanensis* MC67 stored at 4 °C in our experiments reached the intoxicating level after approximately 6.5 days due to the fluctuating daily periods in an ambient temperature, these rapid temperature alterations did not support cereulide production (Fig. 2). This is consistent with the results of Thorsen et al. (2009), who found that irrespective of the pre-incubation time at 5 °C, the cereulide formation of *B. weihenstephanensis* MC67 was below the quantification limit within

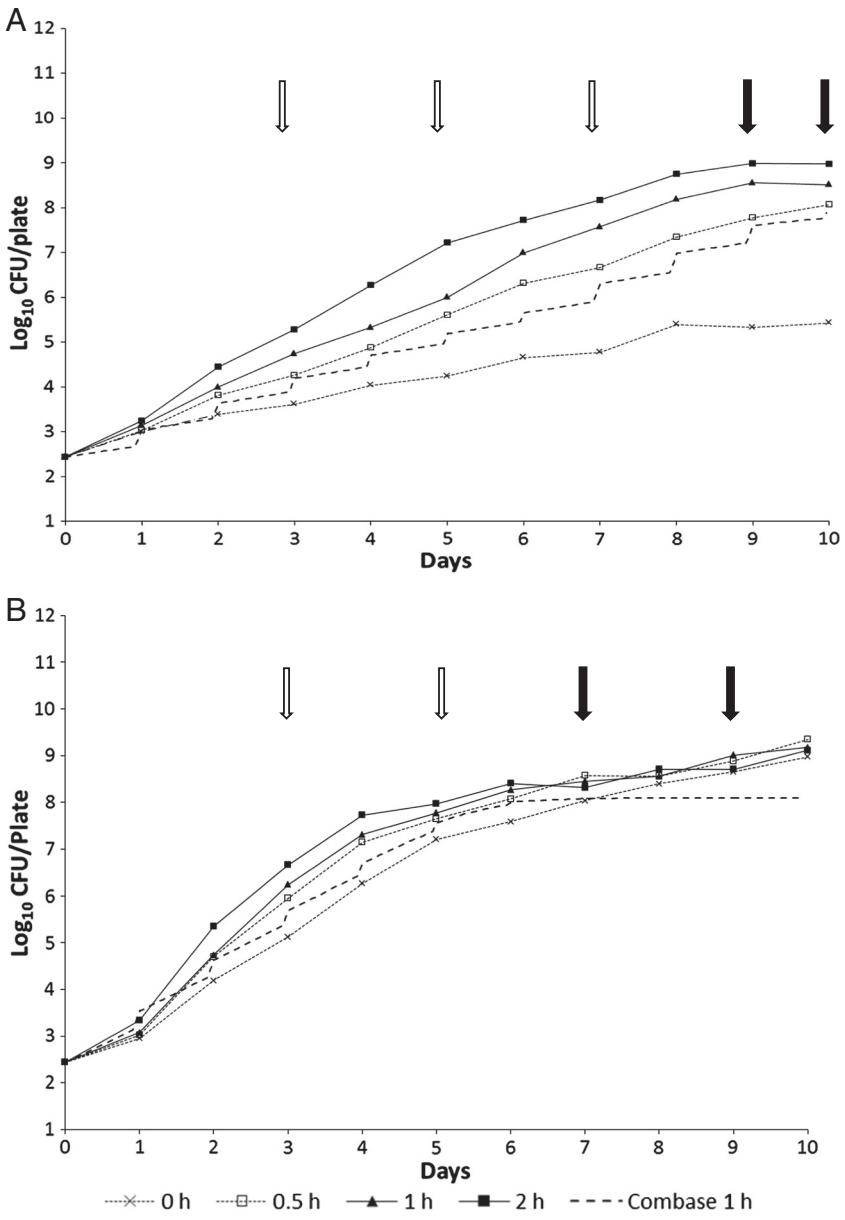

Fig. 4. Growth of *Staphylococcus aureus* 50089 (log_{10} CFU/plate) at a storage temperature of 8 °C (A) and 12 °C (B) with different times of daily exposure at 25 °C and the predicted growth of 1 h daily exposure at 25 °C by ComBase Predictor. Input in the model was pH 7.1 and 0.5% NaCl. Mean standard deviations (log_{10}) of the three independent growth experiments at 8 °C were 0.67 and 0.58 at 12 °C. Open arrow: sampling, no toxin detected. Closed arrow: toxin detected. In the first two replicates at 8 °C, toxin was detected on the plates exposed daily to 25 °C for 2 h on days 9 and 10. In the first replicate, toxin was also detected on the plate exposed daily to 25 °C for 1 h after 10 days of incubation. No toxin positive samples were detected in the third replicate experiment. At 12 °C, toxin was detected on all plates (control, 0.5 h, 1 h and 2 h) at days 7 and 9 (first replicate) and day 9 (second replicate). In the third replicate at 12 °C, only one sample, the 1 h plate at day 7, came out positive.

the first 12 h after a temperature shift to 25 °C. The emetic *B. cereus* F3605/73 did not grow at temperatures as low as 4 °C and 8 °C, irrespective of the intervals of exposure to an ambient temperature.

At constant storage of 8 °C it took approximately 3.5 days for the *B. weihenstephanensis* MC67 samples to reach the level of 10^5 cells which is associated with human intoxication (Arnesen et al., 2008). Daily exposure to an ambient temperature 2 h daily resulted in levels associated with human intoxication being reached in approximately 2.5 days. Cereulide production was first detected on the 2 h sample at day 4 (Fig. 2). In the study of Thorsen et al. (2009), cereulide formation of *B. weihenstephanensis* MC67 on Brain Heart Infusion (BHI) agar was very limited after even 3 weeks of storage at 8 °C. This is in contrast to our results and could be explained by our use of PCA (0% NaCl), as it is reported that growth of *B. cereus* on PCA yielded more cereulide than growth on BHI (0.5% NaCl) (Dommel et al., 2010). A more recent study found that salt (NaCl) has a direct influence on cereulide toxin production by down-regulation of the gene responsible for the toxin production (Dommel et al., 2011). Many studies (Apetroaie-Constantin et al., 2008; Delbrassinne et al., 2011; Dommel et al., 2011; Thorsen et al., 2011) conclude that the cereulide formation is not dependent on growth rates or cell counts alone, but is also influenced by various genetic and environmental factors. This could in part explain the high observed variability in the toxin production between the different replicate samples in our study, which also Thorsen et al. (2011) reported. We can assume that different types of food also could cause differences in cereulide production, either resulting in more or less production than what was found in the present study. However, the temperature continues to be an important factor for cereulide production (Apetroaie-Constantin et al., 2008; Thorsen et al., 2011) and is the most important factor for consumers to control in order to prevent cereulide intoxication (Røssvoll et al.,

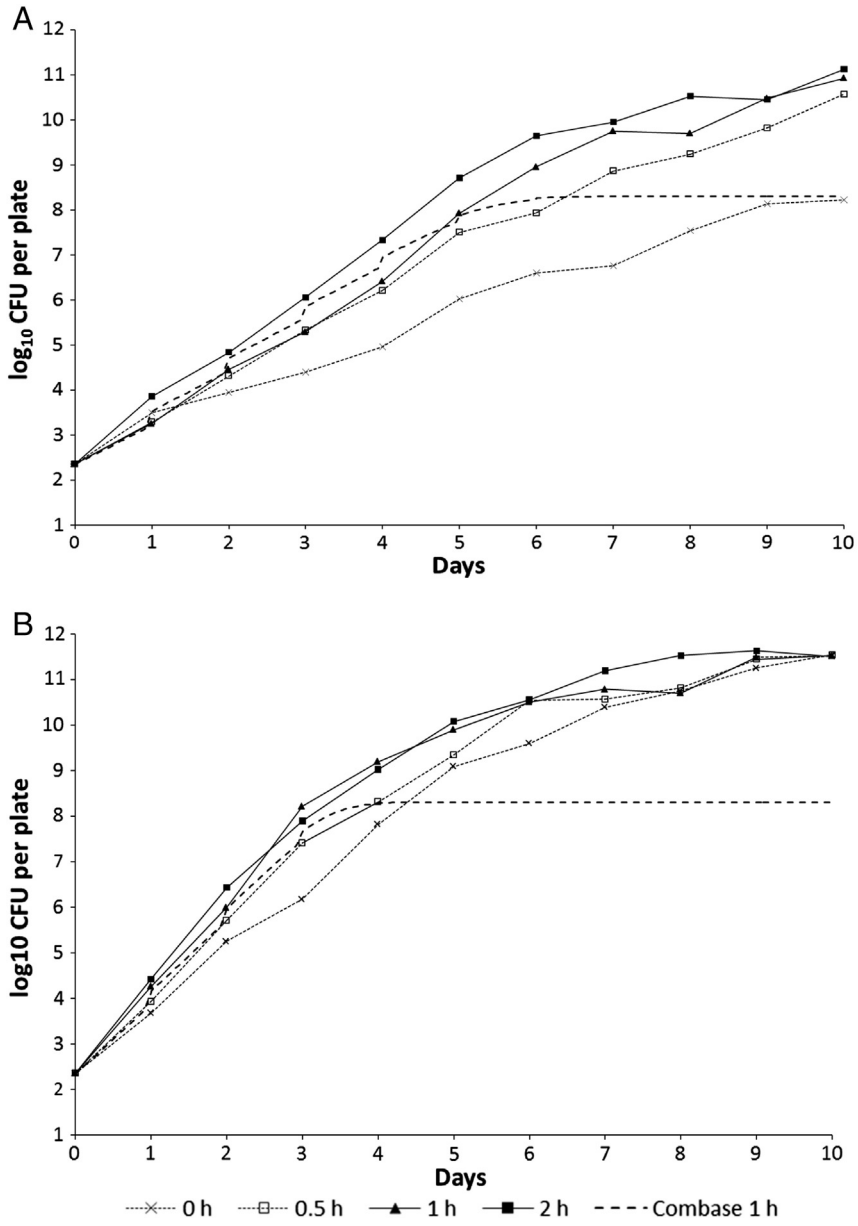

Fig. 5. Growth of *Yersinia enterocolitica* 1106-0129-1, O:3 (log_{10} CFU/plate) at a storage temperature of 4 °C (A) and 8 °C (B) with different times of daily exposure at 25 °C and the predicted growth of 1 h daily exposure at 25 °C by ComBase Predictor. Input in the model was pH 7.2 and 0.5% NaCl. Mean standard deviations (log_{10}) of the three independent growth experiments at 4 °C were 0.39 and 0.60 at 8 °C.

2012). This is clearly illustrated by the two fatal cereulide intoxications in Belgium: the death of a young man in 2008 because of a spaghetti dish stored in an ambient temperature for five days (Naranjo et al., 2011), and that of a seven-year-old girl in 2003 caused by a pasta salad stored in a domestic refrigerator that held a temperature of 14 °C (Dierick et al., 2005).

Listeriosis is a rare disease primarily affecting immunocompromised individuals, although the recently reported outbreak of febrile gastroenteritis should not be forgotten (Aureli et al., 2000). Although an active infection depends on the total intake of contaminated food and not on the CFU only, the number of *L. monocytogenes* has been as high as 10^6–10^8 CFU when associated with outbreaks (Bhunia, 2008). However, given the long incubation period of listeriosis and the time it takes between diagnosis and analysis of the food eaten, the microbes may have multiplied in the food since consumption. The real infectious dose could be much lower — as low as 10^3 cells per gram food (10^5 if eating 100 g food), depending on the immune status of the person (Schmid-Hempel and Frank, 2007; Vazquez-Boland et al., 2001). Given a level associated with human infection of 10^6 CFU, more than 7 days elapsed before the initial inoculum of approximately 100 bacteria reached this level, at a constant temperature of 4 °C. Daily exposure at 25 °C for 2 h nearly halved the time to reach the level associated with human infection compared to the control, to only 4 days. The effect of daily exposure to higher temperature was lower at 8 °C, but on the other hand a level associated with human infection was reached in approximately 3.5 days even without variation in temperature. A lag phase was not observed in this study, as also observed in a study of *L. monocytogenes* on sliced-cooked meats (Garrido et al., 2010).

It is the amount of *S. aureus* enterotoxin (SE) that is important for the development of illness of an *S. aureus* intoxication, but it is considered that at least 10^5 bacteria per gram of food is necessary to produce enough SE to cause an intoxication (Rørvik and Granum, 2007; U. S. Food and Drug Administration, 2009). More than 7 days at a constant temperature of 8 °C elapsed before *S. aureus* 50089 reached a level of 10^5 CFU from the initial inoculum of approximately 100 bacteria. SE was first detected on the plates exposed to 25 °C for 2 h daily after

9 days, for two of the replicates. None of the control plates kept constantly at 8 °C was found positive for SE. Daily exposure at 25 °C for 2 h decreased the time to reach a level associated with human intoxication dramatically, to only 2.5 days. At 12 °C the daily exposure to room temperature had less effect on the growth of *S. aureus* (Table 2). Constant incubation at 12 °C gave an average of 3 days to reach the level associated with human intoxication, while 2 h daily exposure at 25 °C reduced this time to less than 2 days. SE was demonstrated after 7 days at constant storage at 12 °C in one replicate. The toxin production of both *S. aureus* strains in our study showed a high variability between the different replicate samples. This is possibly due to the fact that 12 °C is a temperature on the border of the production of SE.

Exposure to room temperature had a considerable effect on the growth of *Y. enterocolitica* O:3 incubated at 4 °C. The infective dose of *Y. enterocolitica* is considered to be 10^7–10^9 bacterial cells (Bhunia, 2008; Todd et al., 2008). At a constant temperature of 4 °C, it took more than 7 days to reach the number of 10^7 from an initial inoculum of approximately 100 bacteria. Daily exposure at 25 °C for 2 h resulted in the level of 10^7 bacteria associated with human infection after less than 4 days. At a constant storage temperature of 8 °C, *Y. enterocolitica* O:3 reached the level associated with human infection in approximately 3.5 days. The periods of exposure in room temperature had less impact on the average growth rate when stored at such a high temperature as 8 °C (Table 2), and 2 h daily exposure reduced the time to the level associated with human infection to approximately 2.5 days.

A strength of ComBase Predictor compared with other microbial modeling programs is that growth can be predicted for a non-constant temperature time profile. While relatively good correlation of growth at varying temperatures was observed between predicted and observed growth of *Y. enterocolitica*, the predicted growth of *S. aureus* 50089 was underestimated at both temperature conditions (Fig. 4), while *L. monocytogenes* 2583/92 was underestimated when stored at 8 °C (Fig. 3B). It cannot be ruled out that companies producing food may use available tools like ComBase Predictor when deciding the shelf-life of a product. Thus, it is important that growth models are based on the fastest growing bacterial strains. Also models in Combase are in general based on growth in broth, and there may be some differences in growth between such systems and the surface growth model tested in the present study.

Bacterial growth on solid foods normally occurs at the surface. A model agar system was chosen in our study, as we wanted to investigate the effect of rapid temperature fluctuations on the growth and toxin production of foodborne pathogens. The use of agar plates with a large surface compared to volume facilitated rapid heating and cooling of the samples, which was essential for the experiment, as temperature alterations for such short periods as 30 min were investigated. This was a simple model representing a "worst case" scenario, as the agar plates contained no preservatives or competing bacterial flora and the periods of room temperature exposure found in the case study must be regarded as long.

Although *L. monocytogenes* and *Y. enterocolitica* are considered to have the highest levels associated with human infection of the pathogens investigated in this study, they showed the fastest increase in cell counts, and in that respect they determine the shelf life of products such as cold cuts. It takes quite some temperature violations before RTE foods stored in domestic refrigerators becomes hazardous, but the temperature fluctuations found in the case study did enhance pathogenic growth and could in worst cases lead to outbreaks of foodborne diseases. The ComBase Predictor may be used to model growth at varying temperatures. However, the correlation between predicted and observed growth varied for the pathogenic bacteria tested, and no growth model was available for *B. weihenstephanensis*.

Since our data are extracted from a relatively small case study and during holidays, the study cannot be regarded as representative for the whole population or for consumer handling on ordinary weekdays. During holidays one would expect a higher violation of the temperature regime with longer meals and (at least initially) full refrigerators. Christmas and Easter holidays were chosen to represent "worst case scenarios" as they include three to five days where food is purchased in advance, since most food shops in Norway are closed during the holidays. Also, the traditional Norwegian breakfast served in holidays and weekends is mainly based on bread and different cooked meat and fish products served cold, and for Christmas many of these products are home-made. Food handling and eating patterns vary between different segments of consumers, cultures, traditions and contexts, and the results from the present study cannot be extrapolated to consumers generally. However, the use of automatic logging devices to gather information about consumer handling practices could potentially be combined with food consumption questionnaires and data on growth and toxin production to improve risk analyses and food safety advice to consumers. Predictive modeling programs such as ComBase can be used to simulate the growth of some pathogens under fluctuating temperatures, but there is a lack of data on toxin production.

Acknowledgments

This project was funded by the Research Council of Norway, the Foundation for Research Levy on Agricultural Products and the Norwegian Agricultural Authority. We thank Tjakko Abee (Wageningen University and Research Centre, the Netherlands) for providing synthetic cereulide and Georg Kapperud (Norwegian Institute of Public Health) for providing the strains of *Y. enterocolitica*. We acknowledge Tone Normann Asp (Norwegian School of Veterinary Science) for valuable discussions.

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Paper III

III

Identification and quantification of lichenysin – a possible source of food poisoning

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Food additives & contaminants: Part A, 2015, 32, 2120-2130

Food Additives & Contaminants: Part A, 2015
http://dx.doi.org/10.1080/19440049.2015.1096967

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Identification and quantification of lichenysin – a possible source of food poisoning

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(*Received 3 July 2015; accepted 12 September 2015*)

Lichenysin produced by 53 different *Bacillus licheniformis* strains has been structurally examined with a qualitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) method using quadrupole-time-of-flight mass spectrometry. The same lichenysin isoforms are produced from all strains, indicating that the growth conditions have a stronger influence on the lipopeptide production than the genotype. A rapid method for the quantification of lichenysin from bacterial cell cultures with LC-MS/MS after a simple methanol extraction has been refined. For the first time commercially available lichenysin has been used as calibrant, making quantification more accurate. The trueness for C15-lichenysin has been improved to 94% using matrix-matched calibration with lichenysin compared with 30% using solvent calibration with surfactin. The quantitative method was fully validated based on Commission Decision 2002/657/EC. The LOD of the method was below 1 μg g^{-1} and the repeatability ranged from 10% to 16%.

Keywords: lichenysin; LC-MS/MS; quantification; validation; *Bacillus licheniformis*

Introduction

Bacillus licheniformis is a saprophytic soil bacterium that is widespread in nature due to its endospore-forming properties. It is widely used in the fermentation industry in the production of enzymes (proteases and amylases), antibiotics (Schallmey et al. 2004), and probiotics (Cutting 2011). *B. licheniformis* is not considered a human pathogen although it has been isolated from several human infections, in all of which the inflicted humans where immunocompromised (Park et al. 2006; Lepine et al. 2009; Idelevich et al. 2013). The first reports of the involvement of *B. licheniformis* in food poisoning came in England in the 1970s, but the virulence factor(s) were neither detected nor described (Kramer & Gilbert 1989). Large amounts of *B. licheniformis* have been associated with a few cases of food poisoning (intoxications), one of which had a fatal outcome (Salkinoja-Salonen et al. 1999). It has also been involved in animal abortions and bovine mastitis where lichenysin-producing strains were detected in mastitic milk (Johnson et al. 1994; Agerholm et al. 1995; Nieminen et al. 2007; Syrjälä et al. 2007). The exact mechanism and role of *B. licheniformis* as a causative agent of food poisoning is unknown, although lichenysin has been proposed as the virulence factor (Salkinoja-Salonen et al. 1999; Mikkola et al. 2000; From et al. 2005). Toxic lichenysin was detected in baby milk formula associated with the death of an infant, indicating that lichenysin is indeed the source of food poisoning (Mikkola et al. 2000). Further investigations are needed to clarify the role of lichenysin in food poisoning.

Lichenysin is a lipopeptide produced by most, if not all, *B. licheniformis* strains (Madslien et al. 2013). It is an excellent surfactant and a good chelating agent for Ca^{2+} and Mg^{2+} (Javaheri et al. 1985; McInerney et al. 1990; Grangemard et al. 2001). Lichenysin is also shown to have anti-inflammatory, antimicrobial, antitumor and immunosuppressive properties, but is also haemolytic (Grangemard et al. 2001). These characteristics are to a wide extent caused by the amphiphilic nature of the lipopeptide; it consists of a peptide moiety comprised by seven amino acids and a β-hydroxy fatty acid with 12–17 carbon atoms with possible normal, iso and anteiso branching (Horowitz & Griffin 1991; Jenny et al. 1991; Trischman et al. 1994; Hasumi et al. 1995; Yakimov et al. 1995, 1999; Grangemard et al. 1999; Konz et al. 1999; Mikkola et al. 2000). Several isoforms and homologues of lichenysin are found in nature, both amino acid substitutions and alterations in the length and branching of the fatty acid chain occurs. The most abundant isoform is known as lichenysin A (Figure 1) (Yakimov et al. 1999) where the amino acid sequence is Gln-Leu-D-Leu-Val-Asp-D-Leu-Ile (Konz et al. 1999; Yakimov et al. 1999; Mikkola et al. 2000). Surfactin, another lipopeptide produced by *Bacillus subtilis*, is very similar in structure to lichenysin A (Figure 1) and differs only with the substitution of glutamine with glutamic acid in the first amino acid position (AA1) (Konz et al. 1999; Peypoux et al. 1999). This small difference, however, increases the surfactant properties of lichenysin significantly; the critical micelle concentration (CMC) is 22 μM for lichenysin and 220 μM

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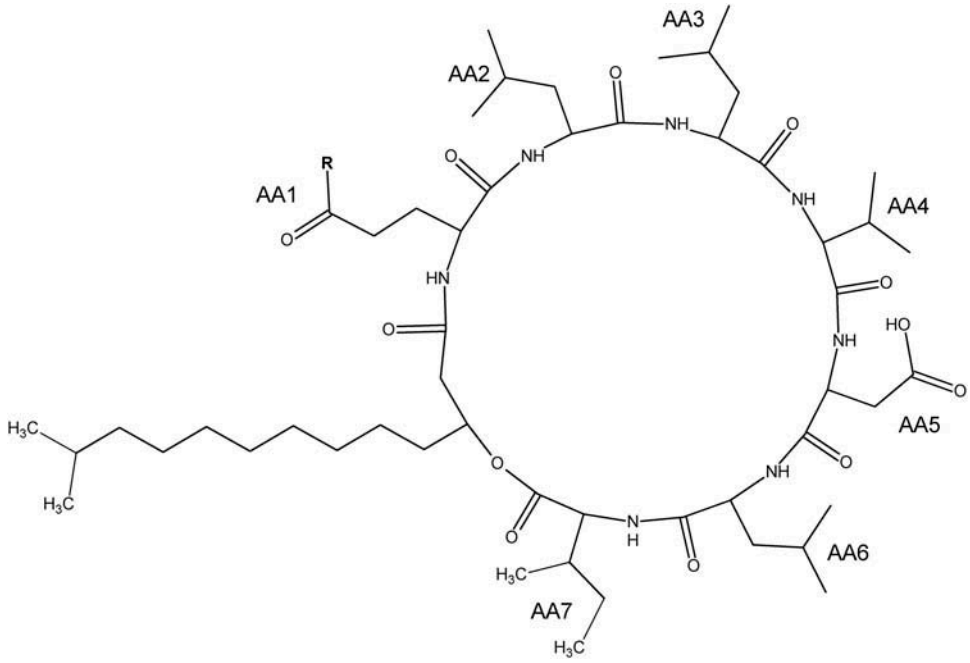

Figure 1. Molecular structure of lichenysin A (monoisotopic mass 1020, R=NH_2) and surfactin (monoisotopic mass 1021, R=OH), differing only with a glutamine (lichenysin)/glutamic acid (surfactin) substitution at amino acid position AA1.

for surfactin, 100% haemolysis is obtained with 15 μM lichenysin and 200 μM surfactin and the association constant with Ca^{2+} is four times higher for lichenysin than surfactin and 16 times higher for Mg^{2+} (Grangemard et al. 2001).

Several different detection methods for lichenysin have been developed including cytotoxicity methods like the sperm motility assay (Andersson et al. 1998; Hoornstra et al. 2003) and the Vero cell assay (Sandvig & Olsnes 1982), and PCR (Turgay & Marahiel 1994; Nieminen et al. 2007; Tapi et al. 2010; Madslien et al. 2013; Wu et al. 2015). None of these methods proves the presence of lichenysin, only a possible effect of the lipopeptide or the genes encoding lichenysin synthetase. Analytical methods utilising LC-MS detect lichenysin directly by separating the analyte of interest from interfering matrix components both based on differences in hydrophobicity and molecular mass. Several qualitative MS-based methods have been published for the detection of lichenysin (Horowitz & Griffin 1991; Jenny et al. 1991; Andersson et al. 1998; Grangemard et al. 1999; Mikkola et al. 1999; Yakimov et al. 1999; Yang et al. 2006; From et al. 2007; Li et al. 2008; Guo et al. 2014), but only a few quantitative methods, none of which uses commercially available lichenysin for calibration (Madslien et al. 2013; Zhang et al. 2014).

The use of *Bacillus* species as additives in the production of animal feed is regulated by the EFSA panel on additives and products or substances used in animal feed (FEEDAP). Until 2013 the FEEDAP scientific opinion stated that a test for haemolysis together with PCR screening for non-ribosomal peptide synthase genes were sufficient to reveal the potential of lipopeptide production in the strains of interest (EFSA FEEDAP Panel: Technical guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition 2011). After the revelation that most, if not all, *B. licheniformis* strains produces lichenysin and several strains are non-haemolytic (Madslien et al. 2013), together with indications of the same behaviour in *B. subtilis* strains (Dybwad et al. 2012), FEEDAP endorsed the need for a revision of the current scientific opinion resulting in a revised scientific opinion published in 2014 (EFSA FEEDAP Panel: Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition 2014, EFSA FEEDAP Panel: The need to revise the technical guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition 2013). It recommends that the cytotoxicity of all *Bacillus* strains (non-*B. cereus*) considered in feed production should be evaluated by an *in vitro* cell-based method like the Vero cell assay. The strain is not suitable for use as a feed additive if cytotoxicity is proven.

We have shown earlier that the cytotoxicity alone is insufficient as a marker for the lichenysin content in cell extracts (Madslien et al. 2013). The risk of illness resulting from the presence of lichenysin in food products is also yet to be evaluated. To ensure an accurate measurement of the lichenysin production from different bacterial strains, it is therefore important not only to verify the cytotoxicity but also to quantify the amount of lichenysin present in the cell extracts. Quantitative methods suitable

for routine use are necessary to enable this. To ensure an accurate quantification, the choice of calibration and a proper validation of the method is of importance.

In this study, 53 *B. licheniformis* strains have been qualitatively analysed to compare the occurrence of the different lichenysin isoforms. We have also sought further to improve a quantitative method to determine the lichenysin content in bacterial cell extracts. For the first time commercially available lichenysin has been used for calibration to ensure more accurate quantification. The method has been fully validated based on Commission Decision 657/2002/EC.

Materials and methods

This method, both the sample preparation, chromatography and quantitative MS, is a modified version of the one described by Madslien et al. (2013).

Chemicals and reagents

All chemicals were of at least HPLC grade and supplied by VWR (West Chester, PA, USA), except lichenysin A (98.2%, Lipofabrik, Villeneuve-d'Ascq, France), surfactin (≥ 98%, Sigma, Steinheim, Germany) and heptafluorobutyric acid (Fluka, Buchs, Switzerland). The water used was grade 1 purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Sample preparation

Bacterial strains were grown for 10 days at 37°C on trypticase soy agar (TSA) plates (Merck KGaA, Darmstadt, Germany). The biomass was collected and weighed before addition of 2 equivalents of methanol and homogenisation by vortex mixing for 5 min (VXR basic Vibrax, IKA Werk, Staufen, Germany). Equivalents of 50 mg biomass were weighed in centrifuge tubes. The cells were lysed by boiling in 1 ml methanol for 30 min, resulting in partially evaporation of the methanol. The residue was added 500 µl methanol and vortex mixed for 3 min before centrifugation at 14 000*g* for 3 min. The supernatant was transferred to a 12 ml centrifuge tube and evaporated to dryness at 80°C under a stream of air using a Pierce Reacti-Therm heating module (Pierce, Rockford, IL, USA). The dry residue was reconstituted in 200 µl methanol and filtered through a 0.22 µm nylon spin filter (Costar Spin-x, Costar, Corning Inc., Corning, NY, USA). Aliquots of 10 µl were injected on column for qualitative analysis and 1 µl for quantitative analysis.

Liquid chromatography

The instrumentation used for the qualitative analysis was an Agilent 1260 SL system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, thermostatted autosampler kept at 4°C and column compartment kept at 35°C. The separation was performed on an RRHD Zorbax Eclipse Plus C18 column, 100 × 2.1 mm id, with 1.8 µm particles (Agilent Technologies, Palo Alto, CA, USA). Mobile phase A consisted of 2 mM ammonium acetate and 0.2% heptafluorobutyric acid in water and mobile phase B was acetonitrile and methanol (1 + 1). The flow rate was 0.3 ml min^{-1} with a linear gradient from 90% to 93% B in 6 min. Total time of analysis was 12 min. The autosampler temperature was 4°C.

The quantitative analysis was performed with an Agilent 1290 Infinity system (Agilent Technologies, Waldbronn, Germany) with the same conditions as the quantitative analysis only with flow rate 0.4 ml min^{-1} with a linear gradient from 90% to 93% B in 4 min. Total time of analysis was 8 min.

Qualitative mass spectrometry

The structure elucidation of lichenysin was done on a G6520 quadrupole-time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) operated in 4 GHz, high resolution mode. The ionisation was performed with dual electrospray in positive mode with reference mass correction. The fragmentor voltage was 175 V, gas temperature 325°C, drying gas flow 5 L min^{-1}, nebuliser pressure 30 psi, capillary voltage 4000 V, and collision energies 25 and 35 V. The acquisition mode was targeted MS/MS in scan area 50–1200 Da with recorded 3 spectra/s in both MS and MS/MS. The targeted list is shown in Table 1. The delta retention time was 2 min for all ions.

Quantitative mass spectrometry

Lichenysin was quantified using a G6490 triple quadrupole mass spectrometer (Agilent Technologies, Singapore) equipped with a Jet Stream electrospray ion source. Data were acquired in positive MRM mode. The ion transitions monitored and their corresponding collision energies are listed in Table 2. Common instrument settings for all ion

Table 1. Targeted precursors for product ion scan for qualitative LC-MS/MS analysis on LC-Q-TOF.

	Precursor *m/z*	Retention time (min)
Lichenysin	993.65	4.8
	1007.67	5.7
	1021.68	7.0 and 7.5
	1035.70	8.6
Surfactin	994.64	3.7
	1008.65	4.3
	1022.67	5.4 and 5.7
	1036.68	6.7

Table 2. Ion transitions monitored and their corresponding collision energies for quantitative LC-MS/MS analysis on LC-QqQ.

	Precursor *m/z*	Product *m/z*	CE (eV)	Retention time (min)
Lichenysin	993.4	685.2	40	3.3
	993.4	535.4	40	3.3
	1007.7	685.4	40	3.8
	1007.7	441.1	40	3.8
	1021.7	685.4	40	4.6 and 4.8
	1021.7	581.3	40	4.6 and 4.8
	1035.7	685.3	20	5.5
	1035.7	240.1	80	5.5
Surfactin	994.6	685.2	30	2.6
	994.6	441.2	40	2.6
	1008.6	685.2	30	3.0
	1008.6	441.2	40	3.0
	1022.6	685.2	30	3.6 and 3.8
	1022.6	201.0	80	3.6 and 3.8
	1036.6	685.3	30	4.4
	1036.6	227.2	50	4.4

transitions were fragmentor voltage 380 V, dwell time 20 ms, gas flow 14 L min^{-1}, gas temperature 250°C, nebuliser pressure 20 psi, sheath gas flow 11 L min^{-1}, sheath gas temperature 400°C, capillary voltage 4000 V and nozzle voltage 0 V.

Calibration

Matrix-matched calibration was performed by adding lichenysin at five concentration levels (1, 10, 50, 100 and 500 µg ml^{-1}) to biomass from *B. cereus* strain ATCC 14579 before the extraction. Calibration curves of both lichenysin and surfactin in pure solvent (methanol/water, 3 + 1) were prepared for comparison with matrix-matched calibration. Five concentration levels (1, 10, 50, 100 and 500 µg ml^{-1}) were diluted with methanol from a stock solution of 1 mg ml^{-1} in acetonitrile (lichenysin) or ethanol (surfactin).

Validation

B. cereus strain ATCC 14579 was used as matrix for the validation samples. The biomass from seven plates was pooled, a known amount of methanol added, and homogenised to a slurry by vortexing before weighing in aliquots equivalent to 50 mg biomass. *Bacillus licheniformis* strain NVH1115 was used as positive control.

The validation of the quantitative method was performed using the calibration curve method based on 2002/657/EC (Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results 2002) and an interpretation thereof (Antignac et al. 2003). Matrix-matched calibration curves were prepared by adding lichenysin at five concentration levels (1, 10, 50, 100, 500 µg g^{-1}) to the samples before extraction. The regression coefficient (R^2) and the slope (a) of the calibration curve were used to assess the linearity of the method together with the response factor test. The repeatability was evaluated by the RSD for 12 samples from three different sample sets spiked with 10 µg g^{-1} lichenysin. The LOD = 3 × SD_b, where SD_b is the standard deviation of the blank signal; and LOQ = 10 × SD_b assesses the sensitivity. The selectivity was evaluated from six negative samples.

Determination of the recovery was done by analysing pure lichenysin standard (A_S), matrix samples without fortification (A_B), prepared matrix samples fortified before injection on the LC-MS/MS (A_{MS}) and matrix samples fortified before the sample preparation (A_{SP}). Both total recovery ($R^T = (A_{SP} - A_B)/A_S$), recovery in the sample preparation ($R^{SP} = (A_{SP} - A_B)/(A_{MS} - A_B)$) and recovery of the LC-MS/MS method ($R^{MS} = (A_{MS} - A_B)/A_S$) were established.

The lichenysin content in 21 positive samples from *B. licheniformis* strain NVH1079 was used to evaluate the homogeneity. The biomass from 18 plates were pooled and treated in the same way as the validation samples.

Results and discussion

This study was performed to validate fully and elaborate the quantitative method for lichenysin that we first published in 2013 (Madslien et al. 2013), as well as to confirm the structure of the lichenysin isoforms found by developing a qualitative LC-MS/MS method. The introduction of commercially available lichenysin as calibrant instead of surfactin has raised new methodological issues and a study of different quantification techniques has been applied. To ensure the validity of the quantification a validation study based on Commission Decision 657/2002 has been applied (Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results 2002).

During the validation study it became clear that the lichenysin isoform with precursor ion *m/z* 993 was not expressed as well by the bacterial strain used for the calibration standard (ATCC 14580) as by the strains used in the preliminary experiments (Madslien et al. 2013). As a consequence, only one ion transition was sufficiently abundant to be evaluated for *m/z* 993 and it was not possible to establish any sensible validation data due to too few calibration levels above the LOD. The ion transition *m/z* 993–685 has been included in the study but is only to be regarded as qualitative.

Structural determination of lichenysin in cell extracts from different bacterial strains

The molecular structure of lichenysin has been described in several publications during the last two decades and, as pointed out in the introduction, several isoforms and homologues of lichenysin occur in nature (Horowitz & Griffin 1991; Jenny et al. 1991; Trischman et al. 1994; Hasumi et al. 1995; Yakimov et al. 1995, 1999; Grangemard et al. 1999; Konz et al. 1999; Mikkola et al. 2000). Different bacterial strains might produce different isoforms in different ratios. Whether this is enzymatically controlled, due to growth conditions or genetics or a combination, is not certain (Konz et al. 1999). Four major isoforms of lichenysin have been separated and identified in this study (Figure 2). They are all detected as their protonated ions $(M + H)^+$, their sodium adducts $(M + Na)^+$ and the three most abundant are also detected as their 2Na adducts $(M – H + 2Na)^+$. The protonated ions are the most sensitive in the LC-MS/MS under the optimised conditions. The molecular structure of the three major peaks of lichenysin (*m/z* 1007.7, 1021.7 and 1035.7) in extracts from 53 different *B. licheniformis* strains was investigated. More information about the strains can be found in Madslien et al. (2013). The product ion mass spectra from *m/z* 1007.67, 1021.68 A and 1035.70 all show the same amino acid sequence with a good coverage of both y- and b-ions (Figure 3) after ring opening; only the b1 and y1 ions are missing from the expected ion series (Table 3). Most of the fragments are present in pairs with a mass difference of 18 Da, resulting from a possible dehydration at either end of the lipopeptide during the ring opening. From the y- and b-series of ions resulting from ring opening and dehydration at the N-terminal end, only b2, b3 and y3–6 are found. This is in accordance with the findings of Yakimov et al. (1999). y6 after dehydration at the N-terminal end, *m/z* 685.45, is by far the most abundant fragment ion from all precursors

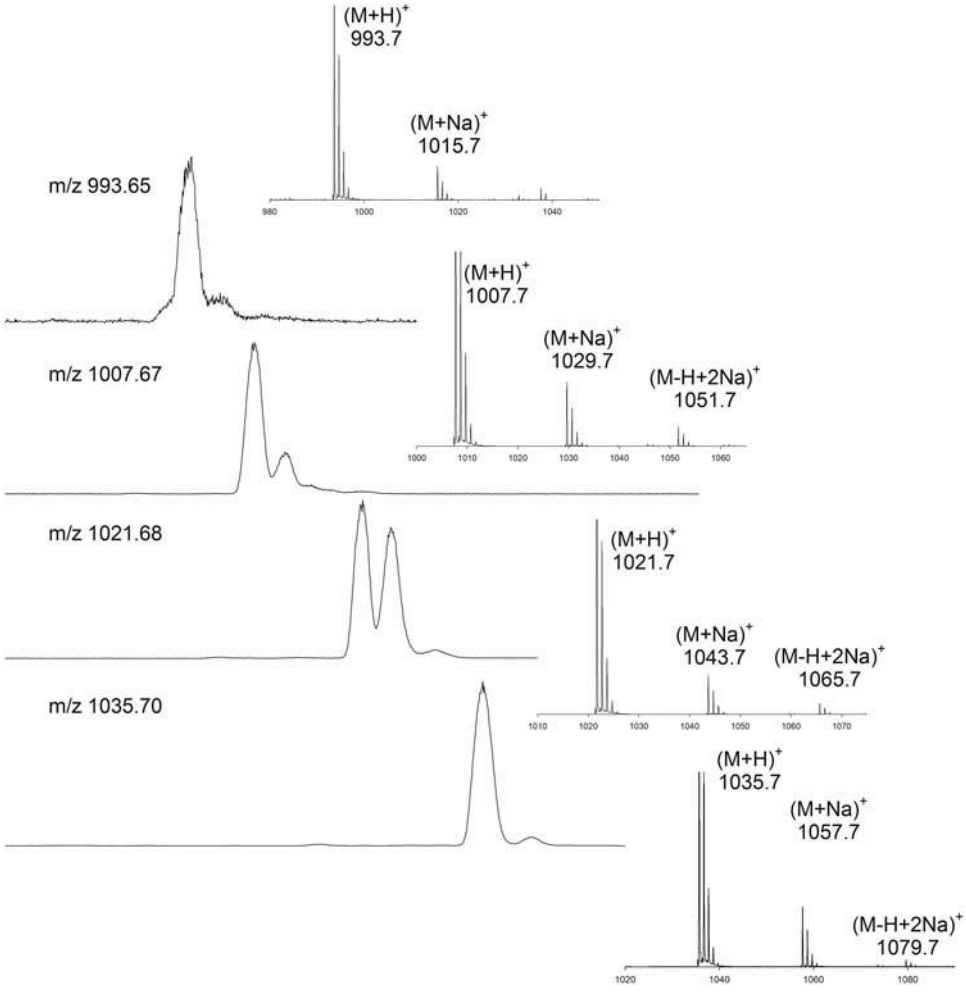

Figure 2. Extracted ion chromatograms (EICs) of the four major lichenysin isoforms found together with their mass spectra (*B. licheniformis* ATCC 14580).

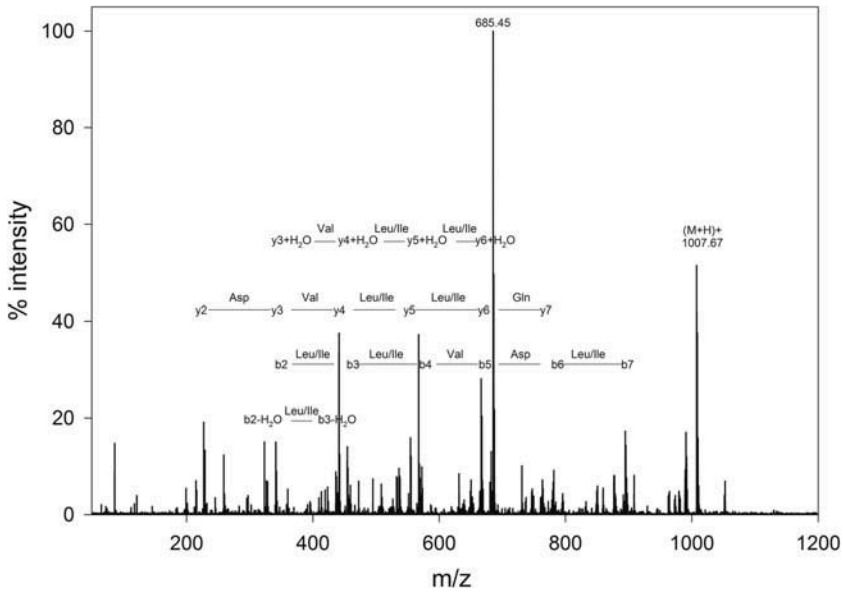

Figure 3. Q-TOF mass spectrum from lichenysin precursor *m/z* 1007.67 (*B. licheniformis* NVH 1115). Only the b1 and y1 ions are missing from the expected fragment ion series resulting from a ring opening and dehydration at the C-terminal end. From the fragment ion-series of ions resulting from ring opening and dehydration at the N-terminal end, only b2-H_2O and b3-H_2O and y3 + H_2O, y4 + H_2O, y5 + H_2O and y6 + H_2O are found. The latter is the most abundant fragment ion in the mass spectrum.

Table 3. y- and b-ions found after ring opening and fragmentation of the peptide moiety of lichenysin.

Ions found	*m/z* 1007.67	*m/z* 1021.68 A	*m/z* 1021.68 B	*m/z* 1035.70
b1	–	227	227	–
b2	341/323	355/337	355/337 (369)	369/351
b3	454/436	468/450	468/450 (482)	482/464
b4	567	581	581 (595)	595/578
b5	666	680	680 (694)	694
b6	781	795	795 (809)	809
b7	894	908	908 (922)	922
y2	227	227	227	227
y3	342/360	342/360	342/360	342/360
y4	441/459	441/459	441/459 (445)	441/459
y5	554/572	554/572	554/572	554/572
y6	667/685	667/685	667/685 (671)	667/685
y7	795	795	795	–

Note: The first fragments result from dehydration at the C-terminal end and the second fragments are products of dehydration at the N-terminal end. The fragments in parentheses are from *m/z* 1021.68 B with valine in the AA7 position.

and represents the peptide moiety after ring opening and loss of the fatty acid chain and AA1 (Yakimov et al. 1999; Hue et al. 2001). The elucidated amino acid sequence for all three major peaks was the same: Gln-Leu/Ile-Leu/Ile-Val-Asp-Leu/Ile-Leu/Ile. This is indeed the established structure for lichenysin A; henceforth, we will name the peaks C13-lichenysin (*m/z* 1007), C14-lichenysin (*m/z* 1021) and C15-lichenysin (*m/z* 1035).

As shown in Figure 2, there are two major peaks of about the same intensity from C14-lichenysin as opposed to the other three lichenysin isoforms that only show one major peak each. The product ion mass spectra from the two C14-lichenysin peaks labelled A and B (Figure 4) reveal the occurrence of an amino acid substitution where leucine in position AA7 has been substituted with valine in the second peak, 1021 B. The overall *m/z* of the isoform remains the same as for 1021 A, meanwhile the rest of the amino acid sequence remains the same; hence, the fatty acid chain has gained one methyl group. This substitution has been reported earlier (Zhang et al. 2014), also for surfactin (Peypoux et al. 1991). However, here the product ion scan from precursor 1021 B shows that the fragment *m/z* 685 is also present alongside *m/z* 671. This suggests that peak 1021 B is comprised of two different lichenysin isoforms that have the same retention properties on the C18 column, despite structural differences. This might be because the alterations only involves the addition and removal of two methyl groups, as opposed to the substitution of glutamine with glutamic acid in surfactin that leads to as much as 2 min reduction of the retention time. The product ion spectra of *m/z* 1021 A and B from all 53 *B. licheniformis* strains reveals a similar pattern: Peak A only contains the common lichenysin A amino acid sequence, while peak B contains a mixture of the AA7 leucine and AA7 valine isoforms, but in different ratios ranging from 50% to 220%. Overall, the production of the same lichenysin isoforms from all 53 bacterial strains investigated indicates that the growth conditions have a greater influence on which isoforms are produced than the genotype. This is in

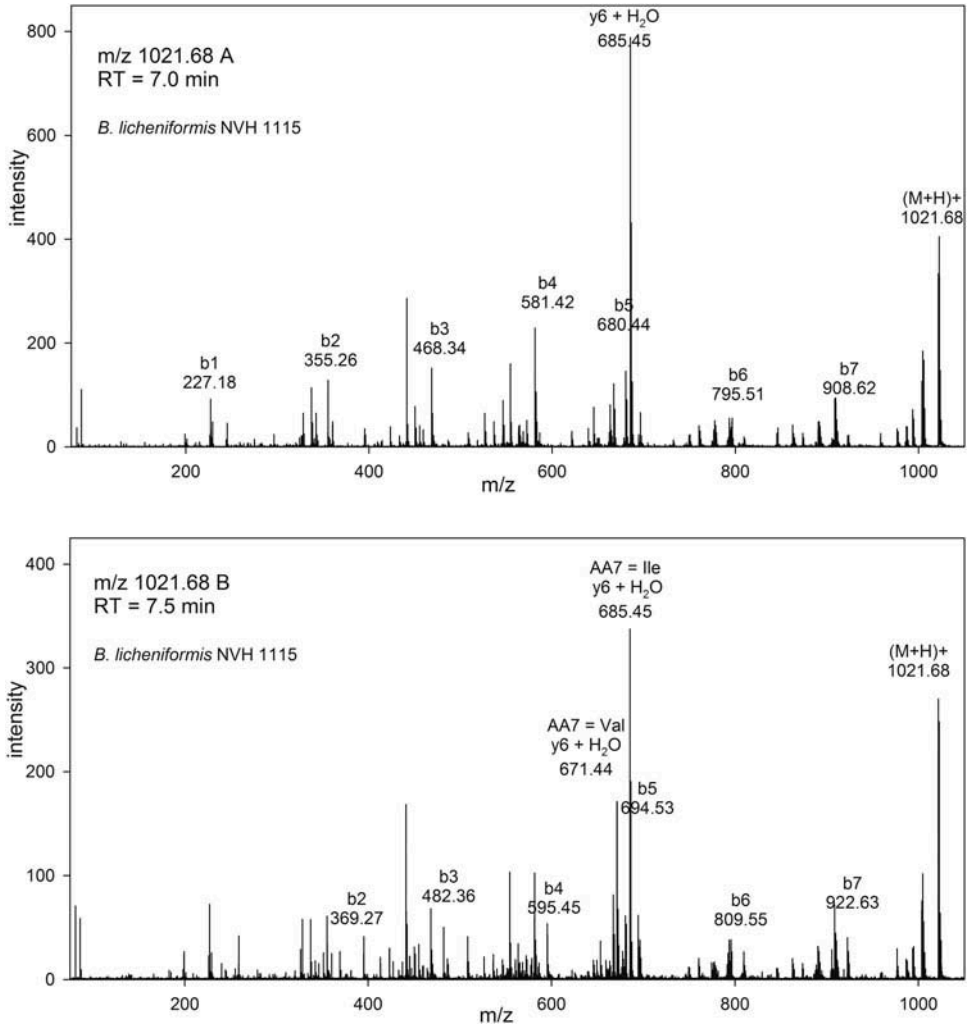

Figure 4. Product ion spectra from *m/z* 1021.68 A and B showing the complete b-ion series from the two different occurring isoforms: in *m/z* 1021 A is AA7 leucine, while in *m/z* 1021 B is both AA7 leucine and AA7 valine present. The most abundant fragment ion, y6 + H_2O, represented by both *m/z* 671 and *m/z* 685 from precursor *m/z* 1021 B indicates the presence of both AA7 Leu and AA7 Val isoforms, whereas it is only present as the AA7 Leu isoform in *m/z* 1021 A.

accordance with the findings of Li et al. (2008) who found that *B. licheniformis* strain HSN221 produced different lichenysin homologues when grown in different media. The AA7 valine isoforms of lichenysin might also be present for the *m/z* 993, 1007 and 1035 isoforms, but due to low-intensity second peaks they have not been considered for structural determination.

Quantification with surfactin and lichenysin as calibration standards

Each of the four major isoforms of lichenysin was quantified separately. Since it is not known whether all isoforms are equally potent, it is in general the total lichenysin concentration that is of interest. The certified amount of lipopeptide in commercially available lichenysin and surfactin is also given as the total amount; it is important to note that the distribution between the different isoforms present might differ.

Lichenysin has recently become commercially available and was used for calibration for the first time. Until now, surfactin (Madslien et al. 2013) and presumably in-house-purified lichenysin (Zhang et al. 2014) have been used as calibration standard for lichenysin quantification. Surfactin and lichenysin are very similar in structure (Figure 1) differing only with 1 Da in their molecular masses; hence, they appear to be good calibrants for each other. However, the substitution of glutamic acid with glutamine at AA1 alters the physicochemical properties of the molecules, as

Table 4. Average trueness (%) of lichenysin (concentration 10 µg g^{-1}, n = 12) quantified against pure solvent standards of surfactin and lichenysin, and matrix-matched calibration with lichenysin.

Ion transition	Surfactin, pure solvent	Lichenysin, pure solvent	Lichenysin, matrix-matched
1007–685	20 (9)	68 (10)	89 (10)
A 1021–685	38 (5)	70 (12)	89 (12)
B 1021–685	−0.2 (−4)	67 (14)	89 (16)
1035–685	30 (12)	74 (11)	94 (11)

Note: RSDs are shown in parentheses.

illustrated by the difference in their surface tension characteristics (Grangemard et al. 1999). Consequently, they might be affected differently during sample preparation and not be prone to the same matrix effects during the analysis on the LC-MS, the latter also due to the differences in retention times. These differences in retention times are what make surfactin a possible calibration standard for lichenysin: if they co-eluted it would be impossible to differentiate between the monoisotopic mass of surfactin and the first isotopic mass of lichenysin. To evaluate the suitability of surfactin as a calibration standard for lichenysin, we calculated the lichenysin content in 12 samples spiked with 10 µg g^{-1} lichenysin with pure solvent standards of both surfactin and lichenysin as calibrants (Table 4). The measured concentrations were 50–70% lower with surfactin as calibrant compared with lichenysin as calibrant (Table 4). This reveals a substantial underestimation of the lichenysin concentration when surfactin is used as calibration standard.

Both lichenysin *m/z* 1021 Da and surfactin *m/z* 1022 Da are double peaks. As shown through the structural determination, these peaks are representing different isoforms of lichenysin, but only one isoform of surfactin (data not shown). Consequently, the quantification of lichenysin with surfactin is a challenge for lichenysin *m/z* 1021. Not only because of the different isoforms, but also due to the difference in peak intensity: for surfactin the first peak is the less intense, while the first peak is the most intense for lichenysin (Figure 5). This pattern is seen in lichenysin produced from all 53 strains included in the study: the ratio between C14-lichenysin A and B varies but C14-lichenysin A is always the most intense. To overcome this challenge we have earlier chosen to integrate and quantify these peaks as one (Madslien et al. 2013). However, the structure elucidation performed in this study revealed that the two peaks represent different isoforms; hence, they should be quantified separately. The negative trueness found for *m/z* 1021 B with surfactin as calibration standard (Table 4) emphasises the shortcoming of surfactin as calibration standard for this lichenysin isoform.

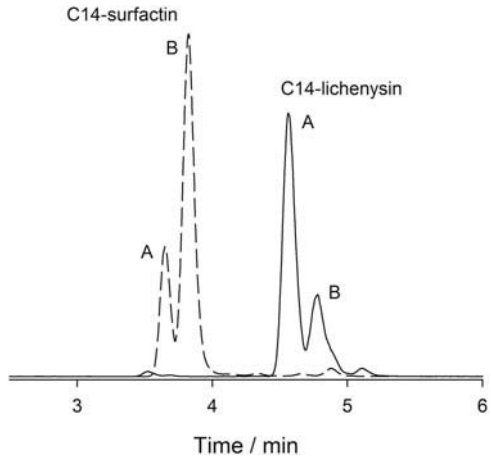

Figure 5. Peak intensity difference between C14-surfactin A and B and C14-lichenysin A and B (surfactin from *B. subtilis* and lichenysin from *B. licheniformis* ATCC 14580).

Quantification with pure solvent standards and matrix-matched calibration

Quantification against a matrix-matched calibration curve yields about 20% higher lichenysin concentrations than calculated against pure solvent standards (Table 4). This demonstrates the significance of matrix-matched calibration. In some cases, for instance at high concentration levels, it is necessary to dilute the samples to get within the concentration range of the calibration curve. A desirable side effect is the removal of possible matrix effects through dilution, and thus eliminating the difference between matrix-matched calibration and calibration against pure solvent standards. The ratio was 93–101% (n = 7) between the two calibration techniques when both samples and calibration samples were diluted 100 times before injection on the LC-MS/MS (with surfactin as standard). This implies that in cases where the samples have to be diluted 100 times due to high concentration levels of lichenysin, the most correct result will be achieved from calibration against pure solvent standard instead of undiluted matrix-matched standards.

Matrix-matched calibration with surfactin as calibrant was carried out with *B. licheniformis* NVH 1079 grown according to protocol as sample matrix; hence, the calibration samples all contained lichenysin as well. As mentioned above, a small peak with *m/z* 1035 eluted at the same time as C15-surfactin with *m/z* 1036. This is another disadvantage for surfactin as calibrant for lichenysin but poses no difficulties for the selectivity of lichenysin as the monoisotopic mass is 1 Da lower than for surfactin.

Surfactin as internal standard

Zhang et al. (2014) used C15-surfactin (*m/z* 1036) as an internal standard for the quantification of lichenysin. We

find that C15-surfactin coelutes with a low-intensity peak representing an isoform of C15-lichenysin in extracts from *B. licheniformis*. These two peaks are isobaric and not separable in the mass spectrometer; hence, C15-surfactin is not suitable for use as internal standard for lichenysin when several strains are being investigated. It would be possible to use one of the other surfactin isoforms as internal standard for all lichenysin isoforms but that would neither compensate possible matrix effects occurring at different retention times, nor variations throughout the sample preparation due to their different physicochemical properties. The validation data from this study show that variation in extraction efficiency and matrix effects that are influencing the lichenysin isoforms differently are a higher contribution to the RSD than variation between samples due to common influences on all isoforms, for instance sample loss during the sample preparation. The best solution to overcome this challenge and improve the precision and trueness of the method would be to implement isotopically labelled internal standards for each lichenysin isoform.

Validation of the quantitative LC-MS/MS method

For all lichenysin isoforms, the most abundant fragment ion was chosen for the quantitative ion transition. The qualitative ion transition was chosen not only according to abundance but also considering precision. For *m/z* 1021 B, neither the quantitative nor the qualitative fragment ion is present in the second isoform comprising the peak; subsequently, only the AA7 leucine isoform is quantified.

We have shown earlier that all of the 53 *B. licheniformis* strains tested contained the lichenysin syntethase gene (*lchAA*) and produced lichenysin, including strains thought to be non-producers such as ATCC 14580 (Madslien et al. 2013). This is probably due to our prolonged growth period; 10 days as opposed to the 24 h used by Wu et al. (2015). As a result, none of the *B. licheniformis* strains could be used as a negative control. To ensure the calibration samples did not contain any lichenysin another *Bacillus* sp., *B. cereus* ATCC 14579 was grown according to protocol and used as a negative control and blank matrix for the calibration samples.

Linearity was evaluated through the regression coefficient (R^2) and the response factor test (Table 5). The coefficient of determination (R^2) ≥ 0.99 both with and without 1/*x*-weighting for all ion transitions except *m/z* 1035–685 and 1035–240. This is the most sensitive precursor ion; hence, the highest calibration point at 500 µg g^{-1} yields counts around the saturation limit of the detector. Without this level, with 1/*x*-weighting, R^2 = 0.995 and 0.994 for the ion transitions *m/z* 1035–685 and 1035–240, respectively. The response factor test was < 15% for all ion transitions. Six matrix samples without lichenysin were used to evaluate the selectivity; no interferences were discovered at the retention times of the analytes. For all three precursors *m/z* 1007, 1021 A and 1021 B, the LOQ is higher for the qualitative ion transitions than the one used for quantification (Table 5). This is as expected as the qualitative ion transitions are the least sensitive. With this method the LODs are all below 1 µg g^{-1}. If necessary, the injection volume can be increased to achieve a higher sensitivity. We have tried this without difficulties during the method development (data not shown).

Without a suitable internal standard, it was not possible to determine the reproducibility of the method. The samples deteriorate rapidly both when stored at RT, 4°C and −21°C; hence, without an internal standard to compensate for the variation in storage time, the RSDs are above acceptable levels when validation samples from different days are compared. As a consequence, the precision was evaluated through the repeatability in three sample sets prepared separately but within the same day by the same analyst. The RSD at

Table 5. Results of the validation calculated from three different sample sets and a total of 30 samples.

Ion transition	R^2	R^2 with 1/*x*-weighting	LOD (µg g^{-1})	LOQ (µg g^{-1})	Response factor test (%)	Repeatability RSD (%), *n* = 12	ΔRT (%), *n* = 30	Ion ratio (RSD %), *n* = 21	Trueness (%), *n* = 12
993–685[a]	0.973	0.854	−4.0	17	60	67	2.1	4.2 (127)	−116–70
1007–685	0.994	0.993	0.4	1.0	12	10	0.2	0.37 (14)	72–102
1007–441	0.990	0.991	−0.1	2.5	14	16	0.3		67–117
A 1021–685	0.993	0.992	0.5	0.9	14	12	0.2	0.35 (9)	70–103
A 1021–581	0.991	0.992	0.5	1.0	13	13	0.2		83–121
B 1021–685	0.991	0.992	0.2	1.7	14	15	0.2	0.50 (15)	68–113
B 1021–581	0.995	0.994	0.8	2.1	13	13	0.3		79–121
1035–685	0.992	0.987 [0.995]	−0.1	1.2	12	10	0.2	0.79 (5)	77–109 [70–98]
1035–240	0.991	0.986 [0.996]	−0.3	0.5	13	12	0.2		79–113 [71–100]

Notes: LOD, LOQ and trueness are calculated with 1/*x*-weighting in linear regression. Numbers in brackets are calculated without 500 µg g^{-1}.
[a]Ion transition *m/z* 993–685 is only qualitative due to the low content in the calibration standard. It is included for information only.

10 μg g^{-1} was between 10% and 16% ($n = 12$). The ion ratio of *m/z* 1021 B is 0.50 with an RSD of 15%, whereas the remaining ion ratios are well within the limits stated in 2002/657/EC (Table 5). The retention times of all ion transitions in the samples are well within 2.5% of the retention time of the standard; hence, the requirement from 2002/657/EC is achieved.

According to 2002/657/EC, the trueness should be within 80–110%. Due to the lack of a certified reference material, the trueness was evaluated by spiking blank matrix samples with a known amount of lichenysin. The results are given for each ion transition in Table 5, ranging from 67% to 121% overall ($n = 12$). Both ion transitions from precursor ion *m/z* 1035 show the best trueness; 77–109% and 79–113%, respectively, but also these are slightly outside the limits. The poor trueness of the method is most likely a result of varying matrix effects and sample loss throughout the analysis. Careful studying of each measurement reveals that the different lichenysin isoforms are not influenced in the same way in each sample: the ratio in trueness ranges from 80% to 111% between two single measurements for the different lichenysin isoforms. Implementation of isotopically labelled internal standards representing each lichenysin isoform would probably improve the trueness of the method.

The total recovery of the method (R^T) was 74–83%. This is a result of the rapid and simple sample preparation; about one-third of the lichenysin was lost during the extraction as shown through the R^{SP} ranging from 57% to 68%. The R^{MS} from 111% to 142% indicates a signal enhancement that reduces the effect of the analyte loss during the sample preparation. A total recovery less than 100% emphasises the importance of matrix-matched calibration where the calibration samples undergo the same extraction procedure as the unknown samples.

Biomass from 18 plates of *B. licheniformis* strain NVH1079, a known lichenysin producer (Madslien et al. 2013), was pooled to achieve a homogenous sample material to be used as a positive control. The RSD ($n = 21$) was 30% for C12-lichenysin, 18% for C13-lichenysin, 16% for C14-lichenysin A and B and 12% for C15-lichenysin when samples prepared on three different days within 1 month were calculated together. Within each day ($n = 7$) the RSDs were 4–8%, 6–10% and 16–23%, without C12-lichenysin. This indicates that the highest contribution to the deviation is day-to-day variations and that the homogeneity of the sample material is satisfactory.

Conclusions

Lichenysin produced by 53 different *B. licheniformis* strains has been qualitatively examined. All strains produced the same lichenysin isoforms but in varying ratios. This indicates that lichenysin production is indeed more dependent on growth conditions than genotype. By introducing commercially available lichenysin as a calibration standard and performing a complete validation study based on Commission Decision 2002/657/EC we have refined a quantitative analytical method that ensures more accurate quantification of lichenysin in bacterial cell extracts.

Acknowledgements

The authors thank Kristin O'Sullivan and Toril Lindbäck for their invaluable help in performing the bacterial growth experiments.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Paper IV

Lichenysin is produced by most *Bacillus licheniformis* strains

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IV

Journal of applied microbiology, 2013, 115, 1068-1080

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Lichenysin is produced by most *Bacillus licheniformis* strains

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Keywords
Bacillus, cytotoxicity, food safety, genotyping.

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2013/0877: received 3 May 2013, revised 1 July 2013 and accepted 3 July 2013

doi:10.1111/jam.12299

Abstract

Aims: The aim of this study was to elucidate the prevalence of lichenysin production in *Bacillus licheniformis* and to see whether this feature was restricted to certain genotypes. Secondly, we wanted to see whether cytotoxicity reflected the measured levels of lichenysin.

Methods and Results: Fifty-three genotyped strains of *B. licheniformis*, representing a wide variety of sources, were included. *lchAA* gene fragments were detected in all strains by polymerase chain reaction (PCR). All 53 strains produced lichenysins with four molecular masses as confirmed by LC-MS/MS (liquid chromatography–tandem mass spectrometry) analysis. The amounts of lichenysin varied more than two orders of magnitude between strains and were irrespective of genotype. Finally, there was a strong association between lichenysin concentrations and toxicity towards boar spermatozoa, erythrocytes and Vero cells.

Conclusions: Lichenysin synthesis was universal among the 53 *B. licheniformis* strains examined. The quantities varied considerably between strains, but were not specifically associated with genotype. Cytotoxicity was evident at lichenysin concentrations above 10 μg ml^{-1}, which is in accordance with previous studies.

Significance and Impact of Study: This study might be of interest to those working on *B. licheniformis* for commercial use as well as for authorities who make risk assessments of *B. licheniformis* when used as a food and feed additive.

Introduction

Bacillus licheniformis is a facultative anaerobic, Gram-positive, rod shaped, endospore-forming bacterium, closely related to *Bacillus subtilis* (Logan and De Vos 2009). *Bacillus* spp. are found almost everywhere in the environment where they survive harsh conditions in the form of highly resistant endospores (Nicholson *et al.* 2000). Great fermentative capacity combined with low toxicity has made *B. licheniformis* a popular choice for the industrial production of enzymes, antibiotics (Schallmey *et al.* 2004) and probiotics (Cutting 2011).

Despite being generally regarded as safe, sporadic cases of *B. licheniformis*-associated systemic infections in humans have been reported (Sugar and McCloskey 1977; Kramer *et al.* 1982; Logan 1988; Santini *et al.* 1995). However, *B. licheniformis* has not been shown to be able to invade the outer barriers of the body, for example, the mucosal lining of the gastrointestinal tract, respiratory tract or the skin without previous lesions. Nevertheless, abortions in cattle and sheep have been reported (Agerholm *et al.* 1995; Syrjälä *et al.* 2007), and it has been experimentally demonstrated that *B. licheniformis* is able to infect the bovine placenta (Agerholm *et al.* 1999).

Bacillus gastrointestinal disease is most often caused by *Bacillus cereus* which, depending on the toxins present, is classified into two different types: the diarrhoeal type and the emetic type, reviewed by Stenfors Arnesen *et al.* 2008;. Foodborne disease caused by *Bacillus* species outside the *B. cereus*-group is less common (Logan 2012).

Reported cases of *B. licheniformis*-associated food poisoning have been characterized by a relatively short incubation time (2–14 h) and high infective dose (>10^5 CFU g^{-1}) followed by mild gastrointestinal symptoms lasting for 6–24 h (Kramer and Gilbert 1989; Salkinoja-Salonen *et al.* 1999).

One case of fatal illness connected to *B. licheniformis*-contaminated baby milk powder has been described (Salkinoja-Salonen *et al.* 1999). Cell extracts from this particular strain were found to be toxic towards boar spermatozoa. The only cytotoxic substance detected was lichenysin A (Yakimov *et al.* 1999; Mikkola *et al.* 2000), a small cyclic lipopeptide (CLP) structurally very similar to surfactin (Arima *et al.* 1968) and pumilacidin (Naruse *et al.* 1990). The ability of surfactin to form ion-specific channels in lipid bilayers was demonstrated in 1991 by Sheppard and colleagues (Sheppard *et al.* 1991). Also, an unspecific, 'detergent-like' membrane-disrupting effect of surfactin has been described (Hoornstra *et al.* 2003; Shaligram and Singhal 2010). Several *in vitro* studies have indicated a strong correlation between surfactin and lichenysin and their toxicity towards different mammalian cells (Mikkola *et al.* 2000; Nieminen *et al.* 2007; Apetroaie-Constantin *et al.* 2009). From and colleagues (From *et al.* 2007) showed that deletion of the *sfp* gene (encoding the enzyme that converts the surfactin synthetase complex into an active holoform) in *Bacillus mojavensis* B31 led to termination of both surfactin synthesis as well as cytotoxicity towards erythrocytes, boar spermatozoa and Vero cells. Further, Nieminen *et al.* 2007 found that purified lichenysin exerted a similar biologic effect towards boar spermatozoa as seen for surfactin, such as loss of motility, damaged plasma membrane and swelling of the acrosome. Despite the high association between surfactin and cytotoxicity *in vitro*, studies in rats and mice have shown a generally low toxicity *in vivo* (Park *et al.* 2006; Hwang *et al.* 2009). The effect of lichenysin in mammals has, to our knowledge, not been demonstrated.

The frequency of cytotoxic, lichenysin-synthetizing *B. licheniformis* strains has previously been found to be low. In a Finnish study, only 4% of 239 foodborne and natural isolates of *B. licheniformis* were toxic towards boar spermatozoa (Salkinoja-Salonen *et al.* 1999). In a similar study, the cytotoxicity of 333 *B. subtilis*-group isolates from various sources was examined, and 3% of the isolates were found to be cytotoxic towards boar spermatozoa and Vero cells (From *et al.* 2005).

Lichenysin and other *Bacillus* CLPs are synthesized nonribosomally by multimodular peptide synthetases, so called NRPSs (Stachelhaus and Marahiel 2006). Genes encoding NRPSs are organized into large (20–30 kb) gene clusters and have been detected in many *Bacillus* species (Tapi *et al.* 2009). Surfactin (SrfA) and lichenysin (LchA) synthetase are responsible for the synthesis of surfactin and lichenysin, respectively. PCR-based methods targeting NRPSs genes and the *sfp* gene have been developed in order to detect surfactin and lichenysin producers (Turgay and Marahiel 1994; Hsieh *et al.* 2004; Tapi *et al.* 2009). Although these assays have revealed a high overall distribution of NRPSs genes, little is known about the distribution within one single species.

The genetic relationship of 53 *B. licheniformis* strains was recently described using a novel multilocus sequence typing (MLST) scheme (Madslien *et al.* 2012). In order to clarify the association between lichenysin production and toxicity, we have examined the 53 *B. licheniformis* strains for a) the presence and distribution of the lichenysin synthetase gene (*lchAA*), b) the presence of lichenysin in bacterial cell extracts and c) the cytotoxicity of bacterial cell extracts towards boar spermatozoa, erythrocytes and Vero cells.

Materials and methods

Strains

Fifty-three strains of *B. licheniformis,* representing various sources, were included in this study (Table 1). Their internal phylogenetic relationship was recently characterized by multilocus sequence typing (MLST) (Madslien *et al.* 2012).

β-haemolysis

Freeze bacteria cultures were grown at 37°C on agar plates supplemented with 5% bovine blood and Columbia agar plates supplemented with 5% sheep blood (Oxoid Limited, Hampshire, UK).

The presence or absence of a zone of clearing surrounding the colonies (β-haemolysis) was recorded after 24 and 48 h.

DNA extraction, primer design and PCR against the lichenysin synthetase A (*lchAA*) gene

Strains were stored at −70°C and plated on sheep blood agar (Columbia blood agar, Oxoid Limited) and grown at 30°C for 24 h. Single-colony material was inoculated in 20 ml Luria broth (LB), grown overnight at 30°C and centrifuged at 3000 ***g*** for 10 min. The pellet was resuspended in 1 ml lysis buffer (20 mmol l^{-1} Tris–Cl, pH 8·0, 1·2% Triton X-100, 20 mg ml^{-1} lysozyme) (Sigma, Steinheim, Germany), and DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN Hamburg GmbH, Hamburg, Germany) according to the manufacturer's instruction. DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer (Thermo

Table 1 Lichenysin levels and cytotoxicity of cell extracts from 53 genotyped strains of *Bacillus licheniformis*

						Methanol extracts from 60 mg bacterial biomass				
Isolate	Source	MLST*	Group*	*lchAA* allel	β haemolysis	Lichenysin	Oil displacement	Hb release	Vero cell	Boar sperm
749	Industrial	15	A	5	Yes	1	Weak	1	Neg	Neg
S172	Unknown	1	B	2	No	1	Weak	0	ND	Neg
LMG17659	Horse blood	7	B	1	No	1	Weak	8	Neg	Neg
S170	Unknown	1	B	2	No	1	Weak	0	Neg	Neg
ATCC14580	Type strain	1	B	2	No	1	Weak	0	Neg	Neg
ATCC9945A	Industrial	1	B	2	No	1	Weak	ND	Neg	Neg
ATCC8480	Unknown	4	A	3	Yes	2	Weak	39	Neg	Neg
LMG7559	Food	4	A	3	Yes	3	Weak	0	Neg	Neg
LMG7633	Chincilla feces	4	A	3	Yes	3	Weak	0	Neg	Neg
LMG7558	Industrial	7	B	1	No	3	Weak	0	Neg	Neg
NCTC6346	Industrial	7	B	1	Yes	3	Moderate	0	Neg	Neg
NCTC962	Food	7	B	1	No	3	Weak	1	Neg	Neg
B317	Ovine abortion	5	B	2	No	3	Moderate	1	Neg	Neg
CCUG26008	Food	11	A	6	Yes	3	Weak	2	Neg	Neg
ATCC10716	Industrial	4	A	3	Yes	3	Weak	1	Neg	Neg
NVH1090	Industrial	7	B	1	No	3	Moderate	1	Neg	Neg
NVH1077	Ovine abortion	6	A	5	Yes	3	Weak	0	Neg	Neg
NVH1112	Bovine abortion	17	A	4	Yes	3	Moderate	0	Neg	Pos
LMG6934	Soil	18	A	6	Yes	3	Moderate	0	Neg	Pos
MB1	Air	21	A	5	Yes	3	Moderate	3	Neg	Neg
553/1	Food poisoning, fatal	2	B	2	No	3	Moderate	1	Neg	Neg
NVH1111	Bovine abortion	3	B	2	No	3	Strong	34	Pos	Pos
CCUG44767	Human blood	14	A	6	Yes	3	Strong	35	Pos	Pos
NVH1032	Food	8	A	3	Yes	3	Moderate	1	Pos	Pos
NVH622	Unknown	1	B	2	Yes	4	Strong	39	Pos	Pos
NVH1109	Bovine abortion	16	A	6	Yes	4	Moderate	33	Pos	Neg
NVH1078	Ovine abortion	26	B	2	Yes	4	Moderate	1	Neg	Pos
NVH1079	Ovine abortion	2	B	2	Yes	4	Strong	19	Pos	Pos
BAS50	Oil reservoir	1	B	2	Yes	4	Strong	63	Pos	Pos
NVH1113	Bovine abortion	3	B	2	Yes	4	Strong	66	Pos	Pos
F231	Food poisoning	23	B	2	Yes	4	Strong	0	Neg	Pos
NVH800	Food	27	A	6	Yes	4	Moderate	50	Pos	Pos
B316	Bovine abortion	10	B	2	Yes	4	Strong	60	Pos	Pos
B357	Water	5	B	2	Yes	4	Strong	3	Pos	Pos
LMG17661	Food	3	B	2	Yes	4	Strong	84	Pos	Pos
CCUG41412	Food poisoning	3	B	2	Yes	4	Strong	72	Pos	Pos
M3	Air	3	B	2	ND	4	Strong	86	Pos	Pos
M46	Air	19	B	2	Yes	4	Strong	92	Pos	Pos
NVH1023	Food	3	B	2	Yes	4	Strong	63	Pos	Pos
NCIB7224	Industrial	9	B	2	Yes	4	Strong	71	Pos	Pos
CCUG43512A	Food	13	B	2	Yes	4	Strong	ND	Neg	Pos
F2943	Food poisoning	22	B	2	No	4	Strong	57	Pos	Pos
F5520	Food poisoning	3	B	2	Yes	4	Strong	89	Pos	Pos
NVH1110	Bovine abortion	9	B	2	Yes	4	Strong	90	Pos	Pos
M55	Air	20	B	2	Yes	4	Moderate	71	Pos	Pos
CCUG31354	Water	2	B	2	Yes	4	Strong	97	Pos	Pos
Koskio52	Bovine mastitis	24	B	2	Yes	4	Strong	92	Pos	Pos
F287	Food poisoning	9	B	2	Yes	4	Strong	92	Pos	Pos
M23	Air	2	B	2	Yes	4	Strong	91	Pos	Pos
CCUG43486	Water	12	B	2	Yes	4	Strong	44	Pos	Pos

(*Continued*)

Table 1 (*Continued*)

						Methanol extracts from 60 mg bacterial biomass				
Isolate	Source	MLST*	Group*	*lchAA* allel	β haemolysis	Lichenysin	Oil displacement	Hb release	Vero cell	Boar sperm
Koskio51	Bovine mastitis	24	B	2	Yes	4	Strong	85	Pos	Pos
NVH1123	Bovine abortion	2	B	2	Yes	4	Strong	97	Pos	Pos
NVH1115	Bovine abortion	25	B	2	No	ND	Strong	1	Neg	Pos

MLST (group): * Genotypes represent results from Madslien *et al.* 2012.
lchAA: Allel type was determined on the bases of partial sequence of the *lchAA* gene encoding lichenysin synthetase A.
β-haemolysis: Evaluated after 48 h incubation on sheep blood agar.
Lichenysin levels: Were determined by mass spectrometry from the average of two independently prepared extracts. The values represent the total quantities of lichenysin [sum of mol.mass (Da) 993·3; 1007·3; 1021·3; 1035·4].
The quantities were categorized into three levels (μg ml^{-1}): <1: ≤3; 1: [1–9]; 2: [10, 99]; 3: [100, 999]; 4: ≥1000.
Oil displacement test: The ability of the extracts to spread oil on a watery surface was categorized into three levels: 'Weak' (<50% displacement), 'moderate' (>50% displacement) and 'strong' (complete displacement).
Hb release: Maximum release (%) of haemoglobin (Hb) was calculated relative to 100% lysis (1% Triton-X100) when incubated for 2 h with 3·3% methanol extract. ND: not determined.
Vero cell toxicity: Positive: >20% inhibition of protein synthesis after 2 h incubation with 3·3% methanol extract. ND: not determined.
Boar spermatozoa toxicity: Positive: <20% motile spermatozoa after 72 h incubation with 1% methanol extract.

Fisher Scientific, Waltham, MA, USA). PCR primers targeting the *B. licheniformis* ATCC14580 lichenysin synthetase A (*lchAA*) gene sequence NC_006270·3 (378877–389619) were designed using PRIMER 3 (Rozen and Skaletsky 1999). The primers (F: 5′-ACTGAAGCGATTCGCAAGTT-3′, R: 5′–TCGCTTCATATTGTGCGTTC-3′) complementary to the *B. licheniformis* ATCC14580 genome [NC_006270.3; (Rey *et al.* 2004)] positions 379037–379018 and 379470–379489 were synthetized by Invitrogen Life Sciences (Life Technologies Ltd., Paisley, UK).

The amplification reactions were performed in a LightCycler® 480 System using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) according to recommendations given by the manufacturer of the kit. The amplification programme was as follows: 5 min initial denaturation at 95°C followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 10 s and primer extension at 72°C for 30 s. The amplifications were terminated after a final elongation step of 7 min at 72°C. DNA from the type strain ATCC14580 was used as positive control. The PCR fragments were verified by electrophoresis using Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). PCR products were purified and sequenced by Eurofins MWG Operon (Ebersberg, Germany), using the same primer pair as for amplification.

The Staden Package (Staden 1996) was used for alignment, editing and construction of consensus sequences based on the ABI sequence chromatograms. Consensus sequences were entered into the MEGA5 (Tamura *et al.* 2011) software and aligned by CLUSTALW (Thompson *et al.* 1994). Dendograms were constructed in MEGA5 using the neighbour-joining (NJ) (Saitou and Nei 1987) algorithm with branch lengths estimated by the maximum composite likelihood method (Tamura *et al.* 2007). Branch quality was assessed by the bootstrap test using 500 replicates. Ratio of *d*N/*d*S (nonsilent *vs* silent substitutions) was calculated in S.T.A.R.T. 2 (Jolley *et al.* 2001) using the method by Nei and Gojobori (1986).

Preparation of bacterial methanol extracts

All strains were grown for 10 days at 37°C on trypticase soy agar plates (TSA) (Merck KGaA, Darmstadt, Germany). 60 mg of bacterial biomass, equivalent to 10^9–10^{10} CFU, was extracted with 1·0 ml methanol and heated for 30 min at 80°C. The dry pellet was resuspended in 0·5 ml of methanol, vortexed and centrifuged for 3 min at 13 000 ***g***. The supernatant was collected and heated at 80°C until complete evaporation (30 min). The dry residue was dissolved in 200 μl methanol and stored in dark glass vials at 4°C before use. Three independently prepared extracts were made from all strains.

Molecular mass determination and quantification of lichenysin by LC-MS/MS

Chemicals, reagents and sample preparations
All chemicals were of at least HPLC (high-performance liquid chromatography) grade and supplied by VWR (West Chester, PA, USA) except surfactin (Sigma-Aldrich, St Louis, MO, USA) and heptafluorobutyric acid (Fluka, Buchs, Switzerland). The water used was grade 1 water purified with a Milli-Q water purification system from Millipore (Bedford, MA, USA).

Bacterial methanol extracts were diluted 1 : 10 in methanol/water (1/1) before aliquots (1 μl) were injected into the LC-MS/MS apparatus.

Liquid chromatography

The instrumentation used was an Agilent 1200 SL system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, thermostatted autosampler and column compartment. The separation was performed at 35°C on an RRHD Zorbax Eclipse Plus C18 column, 100 × 2·1 mm id, with 1·8 μm particles (Agilent Technologies, Santa Clara, CA, USA). Mobile phase A consisted of 2 mmol l^{-1} ammonium acetate and 0·2% heptafluorobutyric acid in water, whereas mobile phase B was acetonitrile and methanol (1/1). The flow rate was 0·4 ml min^{-1} with a linear gradient from 90–93% B in 4 min. Total time of analysis was 8 min. The temperature of the autosampler was 4°C.

Mass spectrometry

The instrument used was G6490 triple quadrupole mass spectrometer (Agilent Technologies, Singapore) equipped with a Jet Stream electrospray ion source. The LC-MS/MS was operated in full-scan mode (from 950 to 1150 Da) to determine the molecular masses of surfactin/lichenysin present in the methanol extracts, whereas data were acquired in positive multiple reaction monitoring mode, MRM, for quantification. Total ion chromatogram and extracted ion chromatograms with mass spectra are presented in Fig. 2.

A calibration curve of surfactin was used for external calibration; hence, all concentrations are given as surfactin equivalents. Six concentration levels were diluted in methanol from a stock solution of 1 mg ml^{-1} surfactin in ethanol.

Quantification of lichenysin was performed on three independently prepared extracts.

Oil displacement

Presence of surfactant in the bacterial methanol extracts was determined semi-quantitatively by the ability to displace oil from a watery surface (Morikawa *et al.* 2000). Petri plates (diameter 90 mm) were filled with 30 ml of distilled water. 20 μl of sterile filtered (0·45 μm) corn oil (Mills, Norway) was carefully layered on top of the water phase with a micropipette. 10 μl of bacterial methanol extracts was applied in the centre of the oil surface. Immediately after application, the occurrence and size of a clearing zone appearing around the applications site were observed. The results were classified into three categories ('weak', 'moderate' and 'strong') based on the size of the clearing zone that appeared around the application site (Fig. S1). The oil displacement test was performed in duplicate on three independently prepared extracts.

Commercially available *B. subtilis* surfactin (Sigma) was dissolved in methanol at a concentration of 1 mg ml^{-1} and diluted 1:2; 1:4; 1:10 and 1:100 in methanol. The oil displacement ability of the different dilutions was tested as above, and the presence and size of the clearance zone were recorded (Fig. S1).

Haemolytic activity of methanol extracts

Haemolytic activity of the methanol extracts was measured according to Fagerlund *et al.* 2008 with minor modifications. Bovine blood cells were washed three times in PBS. 5 μl crude bacterial methanol extract was incubated with 150 μl 2% (v/v) bovine blood cells for 2 h at 37°C. After incubation, the sample was centrifuged and haemolysis was determined from the A_{540} of the supernatant. The percentage of haemolysis was calculated by comparing the A_{540} of the sample with positive [100% lysis by 1% Triton X-100 (Sigma)] and negative (5 μl methanol) controls. All experiments were performed in duplicates.

Boar spermatozoa motility test

The boar sperm motility test was performed according to the protocol of Andersson *et al.* 1998 with modifications. Briefly, 20 μl of crude methanol extract was mixed with 2 ml of extended boar semen (Norsvin LE, Norway) containing approximately 2×10^7 cells ml^{-1} and 0·25 mg ml^{-1} gentamycin to inhibit bacterial growth. The samples were incubated at 18°C for 72 h before readings. Evaluation of motility of preheated (37°C for 5 min) samples was performed in phase-contrast microscope (100 *x* objective) counting approximately 20 spermatozoa from five different fields (minimum 100 spermatozoa in total). The percentage of immotile spermatozoa was recorded. Loss of motility in >80% of the cells was considered indicative of cytotoxicity. The percentage of motile cells of control samples (20 μl methanol per 2 ml boar semen) was always >80%. The assay was performed in duplicates on two independently prepared extracts.

Commercially available surfactin from *B. subtilis* (Sigma) was dissolved in methanol (1 mg ml^{-1}) and diluted 1:2; 1:4; 1:10; 1:100 and 1:1000. The effect of surfactin on boar semen motility was tested as above, on two different batches of boar semen and compared against the toxicity of the *B. licheniformis* extracts.

Vero cell assay

Cytotoxicity was determined using a Vero cell test (Sandvig and Olsnes 1982) that monitors the inhibition of

protein synthesis by measuring the reduction of incorporated ^{14}C-leucine in the Vero cells (ECACC-European Collection of Cell Cultures, Public Health England, Salisbury, UK) upon addition of toxins. Briefly, 10 μl of bacterial methanol extract was applied to 300 μl of low-leucine medium (Gibco, Life Technologies Ltd.) covering a confluent monolayer of Vero cells. The cells were incubated for 2 h followed by washing and addition of 300 μl of low-leucine medium with ^{14}C-leucine (0·2 nCi μl^{-1}) (Hartmann Analytic GmbH, Braunschweig, Germany) and incubated further for 1 h. The radioactive medium was removed, and the cells were washed once in low-leucine medium before radioactivity (c.p.m.) in the Vero cells was counted in a scintillation counter (Pacard Liquid Scintillation Analyzer, Perkin Elmer, MA, USA) using Ultima Gold scintillation fluid (Perkin Elmer, MA, USA). The percentage of inhibition of protein synthesis was calculated using the formula [(negative control – sample)/negative control] × 100. Vero cells incubated with 10 μl methanol for 2 h prior to incubation with ^{14}C leucine were used as negative control. Samples were considered toxin positive when >20% of protein synthesis was inhibited. Assays were performed in at least two independent assays with two technical replicates in each assay.

Statistical analysis

Spearman correlation coefficients (ρ) were calculated in GraphPad Prism 6 Software package. *P*-values were determined by a two-tailed test using a 95% confidence interval.

Results

Screening for the presence of lichenysin synthetase A gene (*lchAA*)

The presence of the first structural gene (*lchAA)* of the lichenysin synthetase A operon was detected by PCR in all 53 strains. The region corresponded to amino acid positions 69 to 184 of lichenysin synthetase A (GenBank accession: YP_077640), part of the condensation domain involved in the formation of peptide bonds (Stachelhaus *et al.* 1998). All sequences have been submitted to GenBank (accession number: KC986877-KC986929). Alignment and cluster analysis of the 53-nucleotide sequences revealed six unique alleles (Fig. 1). The *lchAA*-based dendogram followed roughly the same pattern as seen for the individual MLST house-keeping gene sequences (Madslien *et al.* 2012). Base substitutions were spread over the entire sequence and the overall substitution frequency was 9·5%. The ratio of nonsynonymous (changes in amino acid sequence) *vs* synonymous (no change in amino acid sequence) substitutions (dN/dS) was 0·0780, which is slightly higher than the calculated values for the MLST loci (0·0043–0·0457) (Madslien *et al.* 2012).

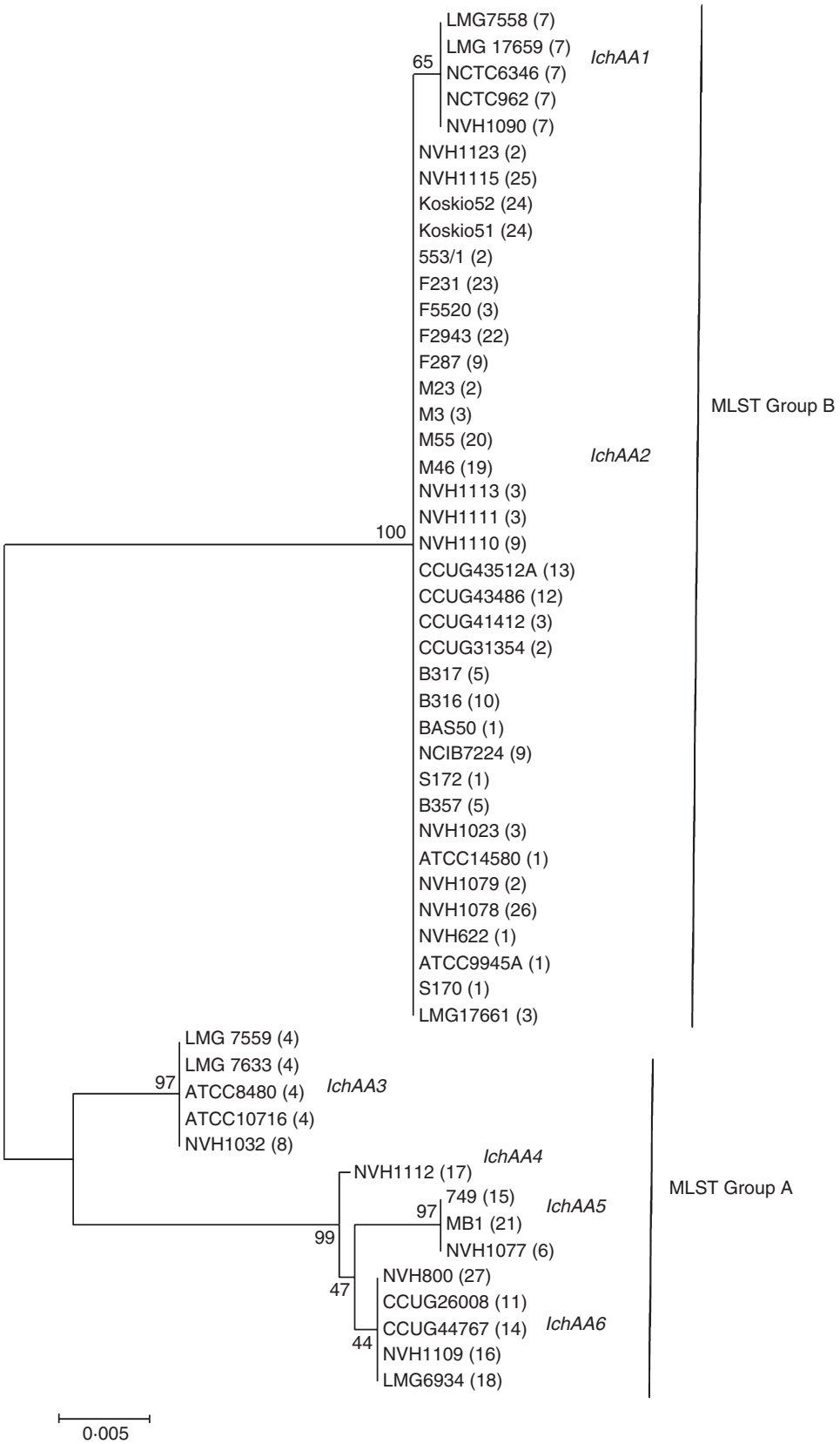
Molecular mass determination and quantification of lichenysin by LC-MS/MS

The results of the LC-MS/MS detection and quantification of lichenysin are summarized in Table 1. Four different isoforms of lichenysin with m/z [molecular mass (Da)]: 993·7 (993·3); 1007·7 (1007·3); 1021·7 (1021·3); 1035·7 (1035·4) were detected by LC-MS/MS. The isoforms were separated from each other and interfering matrix components as shown in the chromatogram (Fig. 2). The two peaks representing m/z 1021·7 had a different ratio in lichenysin compared to commercial surfactin. Hence, the double peak was integrated as one. Lichenysin was detected in methanol extracts from all of the 53 strains included, although seven of the strains produced lichenysins at concentrations below the quantification limit (LOQ, calculated as 10× standard deviation of the noise) at 1 μg ml^{-1} (Table 1). The amount of lichenysin in Table 1 is presented as the sum of the four isoforms. Log_{10}–quantities of lichenysin from three independent extractions are given in Fig. S2.

Oil displacement test

The results of the oil displacement test are listed in Table 1. We found a strong correlation ($\rho = 0{\cdot}8$; $P < 0{\cdot}0001$) between the oil displacement ability and the quantity of lichenysin (Table 2). The effect of the different extracts varied from no displacement to complete displacement. Complete displacement ('strong') was equivalent to the effect of 1 mg ml^{-1} commercial surfactin, 50% displacement ('moderate') was observed at 0·5 mg ml$^{-1,}$ and <50% displacement ('weak') was observed at concentrations

Figure 1 Cluster analysis of partial *lchAA* sequence in 53 strains of Bacillus licheniformis. The cluster analysis was constructed using the neighbour-joining method. The optimal tree with the sum of branch length = 0·06 is shown. Branch quality is estimated by using the bootstrap test (500 replicates) and is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 53 nucleotide sequences. Numbers in brackets represent MLST sequence type. There were a total of 348 positions in the final dataset. The analyses were conducted in MEGA5.


LMG7558 (7)
LMG 17659 (7)
65
NCTC6346 (7)
NCTC962 (7)
NVH1090 (7)
IchAA1
NVH1123 (2)
NVH1115 (25)
Koskio52 (24)
Koskio51 (24)
553/1 (2)
F231 (23)
F5520 (3)
F2943 (22)
F287 (9)
M23 (2)
M3 (3)
M55 (20)
M46 (19)
IchAA2
NVH1113 (3)
NVH1111 (3)
100
NVH1110 (9)
CCUG43512A (13)
CCUG43486 (12)
CCUG41412 (3)
CCUG31354 (2)
B317 (5)
B316 (10)
BAS50 (1)
NCIB7224 (9)
S172 (1)
B357 (5)
NVH1023 (3)
ATCC14580 (1)
NVH1079 (2)
NVH1078 (26)
NVH622 (1)
ATCC9945A (1)
S170 (1)
LMG17661 (3)
MLST Group B
LMG 7559 (4)
LMG 7633 (4)
97
ATCC8480 (4)
IchAA3
ATCC10716 (4)
NVH1032 (8)
IchAA4
NVH1112 (17)
749 (15)
97
IchAA5
MB1 (21)
99
NVH1077 (6)
NVH800 (27)
47
CCUG26008 (11)
CCUG44767 (14)
IchAA6
44
NVH1109 (16)
LMG6934 (18)
MLST Group A
0·005

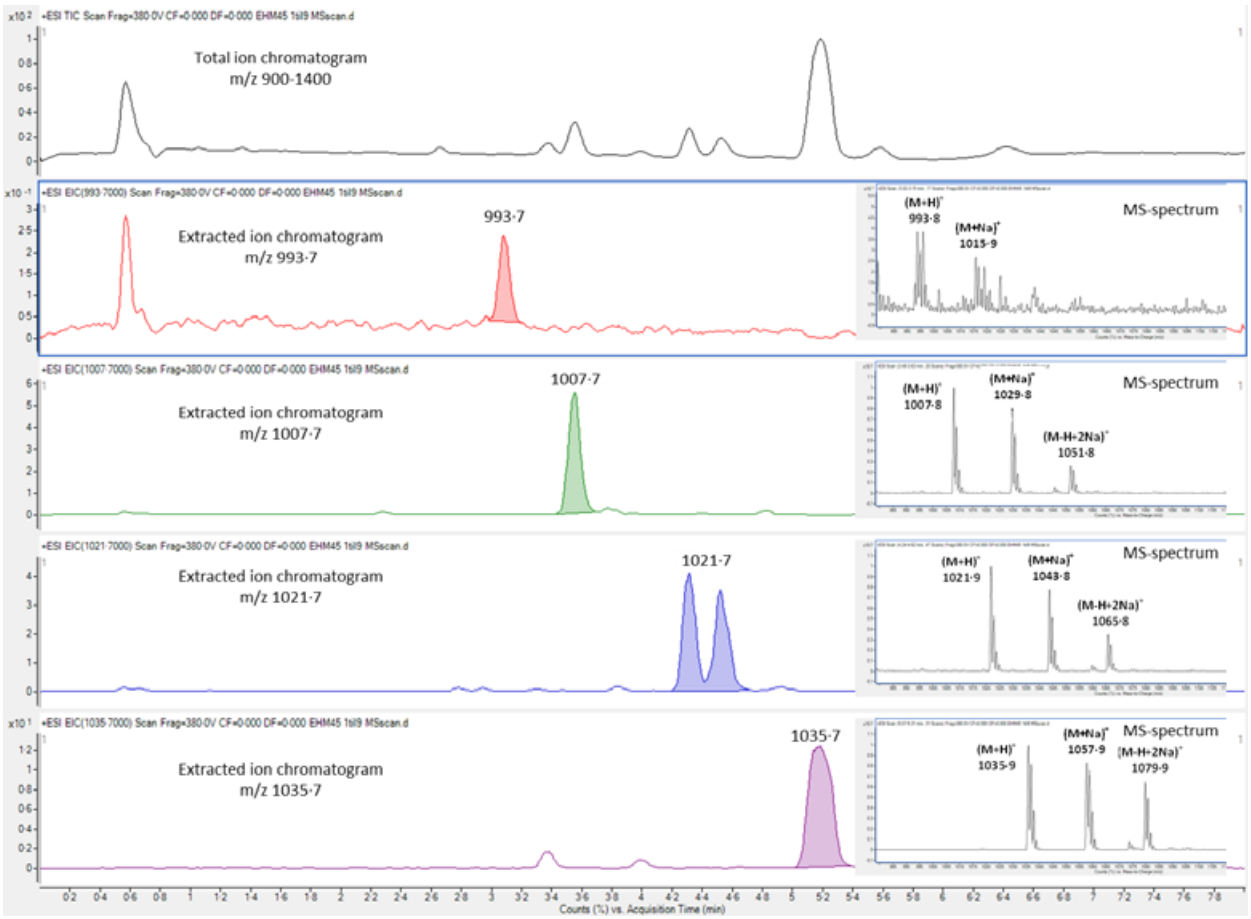


Figure 2 Chromatogram from a representative sample (strain LMG17661). Total ion chromatogram and extracted ion chromatograms with mass spectra for each of the four detected isoforms of lichenysin are displayed.

Table 2 Correlation matrix showing the association between lichenysin concentration, oil displacement and Hb release

	Lichenysin	Oil displacement
Lichenysin		
Oil displacement	0·8****	
Hb release	0·8****	0·7****

The analysis was performed in GraphPad Prism v.6.01 using Spearman rho (ρ). ****$P \leq 0{\cdot}0001$.

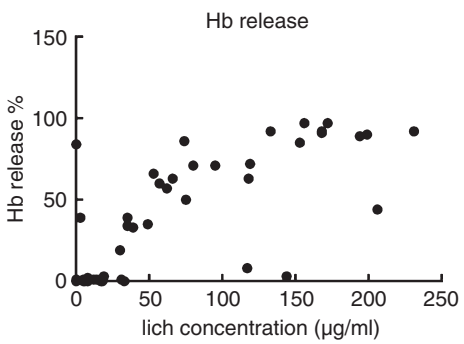


Figure 3 Hb release (%) from erythrocytes plotted against the lichenysin concentrations. The analysis was performed in the statistical software package GraphPad Prism v.6.01.

of $\leq 0{\cdot}25$ mg ml^{-1}. The effect was observed immediately after application (Fig. S1).

β-haemolysis and cytotoxicity

Thirty-nine strains of *B. licheniformis* caused β-haemolysis in sheep blood agar after 48 h of growth, whereas 13 strains did not. Because haemolytic activity on bovine blood agar plates was weak, diffuse and hard to classify compared to what observed on sheep blood agar, only the results from the sheep blood plates are included in Table 1. Generally, β-haemolysis was more evident after 48 h than 24 h. Methanol extracts from 33/53 strains

caused cytotoxicity in boar spermatozoa as could be seen from a loss of motility in more than 80% of the sperm cells (Table 1). Motility loss seemed to occur after cell membrane disruption and was accompanied by swelling of the acrosome (as observed by phase-contrast microscopy). The overlap between β-haemolysis and sperm-toxicity was 75%. Extracts from 28/52 strains were toxic to Vero cells, causing loss of ^{14}C -leucine incorporation into proteins. The overlap between sperm-toxic and Vero cell-toxic strains was 87%; only seven strains were toxic to only one of the cell types. 20/51 of the samples generated a haemoglobin (Hb) release of ≥50% (Table 1).

Cytotoxicity was generally observed at lichenysin concentrations above 10 μg ml^{-1} (20 μg lichenysin in 2 ml cell suspension) for the boar spermatozoa and 33 μg ml^{-1} (10 μg lichenysin in 300 μl cell suspension) for the Vero cells. Hb release of ≥50% was only observed in samples containing >33 μg ml^{-1} (5 μg lichenysin in 150 μl cell suspension). A sigmoid relationship between haemolysis (Hb release) and lichenysin concentrations was observed (Fig. 3). Toxicity was absent in samples containing <29 μg ml^{-1} lichenysin (Vero cell assay) or <1·8 μg ml^{-1} lichenysin (boar spermatozoa assay).

Discussion

Lichenysin synthesis is a common feature of *Bacillus licheniformis*

The type strain *B. licheniformis* ATCC14580/DSM13 and a few other *B. licheniformis* strains have previously been shown to harbour the *lchAA* gene (Nieminen *et al.* 2007). However, the presence of lichenysin synthetase genes in a large number of *B. licheniformis* strains is, to our knowledge, unknown. In this study, *lchAA* was detected in all 53 *B. licheniformis* strains examined, indicating that most, if not all, *B. licheniformis* strains are capable of producing lichenysin.

Four lichenysin isoforms with molecular masses (Da) of 993·3, 1007·3, 1021·3 and 1035·4 were detected by LC-MS/MS in all 53 *B. licheniformis* strains, showing that lichenysin synthesis is a common feature of this species. The three highest masses, 1007·3, 1021·3 and 1035·4, are identical to those classified as lichenysin A in strains BAS50 and 553/1 (Yakimov *et al.* 1995; Mikkola *et al.* 2000). Both of these strains have been included in our work. The isoform with the lowest molecular mass (993·3) was first defined as lichenysin G (Grangemard *et al.* 1999) with the C-terminal amino acid valine instead of leucine/isoleucine as in lichenysin A. On the other hand, the same isoform may also represent lichenysin A, as Yakimov *et al.* 1995 concluded that the fatty acid moiety of lichenysin might contain 12 to 17 methylene groups. To summarize, among the 53 strains included in this study, the 14 Da mass shift between the molecular masses detected might arise both from amino acid substitutions in the peptide moiety and different lengths of the fatty acid chain (Yakimov *et al.* 1995; Grangemard *et al.* 1999).

Although lichenysin was synthetized in all strains, the amounts varied more than two orders of magnitude between strains, ranging from <0·013 μg to >3·3 μg per mg biomass. The quantity produced by each strain was highly reproducible (Fig. S2) indicating that strain-dependent differences were not a random observation. The differences might arise at the a) nucleotide sequence level, b) transcriptional level, c) translational level or d) enzyme (lichenysin synthetase) activity level. In *B. subtilis,* several gene regulatory and environmental factors are known to affect the biosynthesis of surfactin, which could lead to 1) different yield from the same strain under different conditions or 2) different yield from different strains under identical conditions (Jacques 2011). Examples of important environmental factors that can affect surfactin synthesis are: pH, nitrogen/iron/manganese-ratio, carbon source and temperature. Less is known about conditions influencing lichenysin biosynthesis, although nutrient sources and temperature are thought to play essential roles (Yakimov *et al.* 1995; Joshi *et al.* 2008).

The 28 strains that produced high levels of lichenysin (above 3·3 μg mg^{-1}; level 4) were from a variety of sources (Table 1). No obvious relationship between genotype and concentrations of lichenysin was observed. However, strains belonging to MLST group A generally produced lower quantities than strains of group B. Similar lineage-dependent differences have been reported for the nonribosomally synthesized peptide antibiotic bacitracin in *B. licheniformis* (Ishihara *et al.* 2002).

The six unique *lchAA* allels were unevenly distributed among the strains. Allel type 2 (*lchAA*2) was the most abundant, carried by 34 of the strains. Most of the level-4 lichenysin-producing strains carried *lchAA*2. However, this allel was also carried by four of the strains that produced the lowest levels of lichenysin (level 1), including the type strain ATCC14580 (Table 1). Thus, the cluster analysis based on partial NRPSs sequence (*lchAA*) presented in Fig. 1 cannot be used to generally distinguish between strains producing high levels of lichenysin *vs* low producers. Interestingly, some of the most potent lichenysin-producing strains (BAS50 and NVH622) were genetically undistinguishable from the low-producing type strain ATCC14580 (Fig. 1). Presumably, the distribution of *lchAA* sequences in our study reflects the genetic relationship between the strains rather than the capacity of lichenysin synthesis.

Cytotoxicity was observed in 64% of the extracts

Previous studies have indicated an association between cytotoxicity and the ability to produce lichenysin (Mikkola *et al.* 2000). A relatively low number (0·5–4%) of *B. licheniformis* strains have previously been found to synthesize lichenysin or other heat-stable toxins at cytotoxic levels (Salkinoja-Salonen *et al.* 1999; From *et al.* 2005; Taylor *et al.* 2005). In this study, methanol extracts from 64% of the strains were found to be cytotoxic. We suggest that this large divergence was mainly due to 1) higher growth temperature prior to extraction or 2) modifications of the extraction protocol (higher ratio of biomass *vs* solvent, different solvent) leading to a higher concentration of toxins rather than to difference in sensitivity of the cell assays.

Strong association between lichenysin level and cytotoxicity

We found that toxicity of the methanol extracts was highly associated with the amount of lichenysin as determined by LC-MS/MS (Tables 1 and 2). Cytotoxicity towards boar spermatozoa, Vero cells and erythrocytes was observed at lichenysin concentrations above 10 μg ml^{-1}, which is in agreement with previous reports (4–8 μg ml^{-1}) (Mikkola *et al.* 2000; Hoornstra *et al.* 2003). Lichenysin is thought to interact with the spermatozoa plasma membrane in a surfactant-like fashion, as opposed to the emetic toxin cereulide that has been shown to act as a K-ionophore on the mitochondrial membrane (Mikkola *et al.* 1999). Our microscopic observations of membrane damage and swelling of the acrosome correspond to what has previously been described for lichenysin and other surfactants (Hoornstra *et al.* 2003). However, we did not specifically search for molecules other than lichenysin and surfactin in the extracts. Therefore, we cannot rule out that there were other methanol-soluble heat-stable toxic substances present in the extracts that could, at least partly be responsible for the observed cytotoxicity.

Although we found that more than half of the *B. licheniformis* strains produced lichenysin at cytotoxic concentrations *in vitro*, it does not necessarily explain the pathogenicity of *B. licheniformis in vivo*. This might be illustrated by the observation that extracts from strains associated with clinical disease in humans or animals were not always cytotoxic (Table 1). However, environmental factors are known to affect lichenysin production (Yakimov *et al.* 1995; Joshi *et al.* 2008). Therefore, the amounts of lichenysin detected from each strain in our study might differ from the situation *in vivo* and in foods. Interestingly, the extract from the only strain connected to the death of a human patient, 553/1 was not cytotoxic, although previous studies have found the opposite (Salkinoja-Salonen *et al.* 1999; Mikkola *et al.* 2000). Different growth temperature and modification of the extraction procedure might have generated lower toxin amount from 553/1 than previously detected. However, four other food poisoning-associated strains from the Finnish study were also included for comparison (F231, F2943, F5520 and F287). Among these strains, boar sperm cytotoxicity corresponded well to what has previously been found. We therefore speculate that the toxic ability of strain 553/1 might have been lost, possibly as a result of adaption to repeated cultivations in the laboratory (Maughan and Nicholson 2011). It is interesting that these toxins appear to be so widespread among *B. licheniformis* strains and still there are very few reports of *B. licheniformis*-associated disease. The reason for this is probably that, even for the most potent lichenysin producers, a very high number of cells are required in order to synthesize sufficient lichenysin to cause toxicity in humans.

The oil displacement test-a reliable tool for rapid detection of lichenysin at cytotoxic concentrations

Haemolysis on blood agar is a widely used method for screening strains for the presence of surfactins. However, the oil-clearance test has been proposed as a more accurate method for determination of surfactin (Youssef *et al.* 2004). We found a strong correlation between oil displacement and the amount of lichenysin (Table 2). Moderate and strong displacement always occurred in samples containing >1 mg ml^{-1} lichenysin. Interestingly, while all MLST group A strains were β-haemolytic on blood agar plates, only a few of them produced methanol-soluble, heat-stable substances at toxic levels. We could therefore speculate that, during vegetative growth, group A strains may produce haemolytic substances that are inactivated by the heat treatment. Regarding group B-strains, β-haemolysis on blood agar plates seemed to be a better indicator of lichenysin production. However, several of the nonhaemolytic strains produced considerable amounts of lichenysin. Hence, a rapid screening of lichenysin-producing strains on blood agar will most probably give false negatives. In this context, the oil displacement test seemed to be the most reliable tool for a rapid screening of strains producing lichenysin at concentrations associated with cytotoxicity.

We have shown that the ability to synthesize lichenysin is highly conserved among all 53 *B. licheniformis* strains used in this study. *In vitro* cytotoxicity of the methanol extracts was strongly associated with the presence of lichenysin. Further studies are needed to see if the *in vitro* pathogenicity of *B. licheniformis* is reflected by the *in vivo* situation in different tissues.

Acknowledgements

The work was supported by grants from the Norwegian Research Council (grant 178299/I10) and the Norwegian Defence Research Establishment (FFI). The authors wish to thank Kristin O'Sullivan (Norwegian School of Veterinary Science) for technical assistance with the toxicity assays and Avi Ring (FFI) for helpful comments to the design of this study and for kindly sharing his knowledge about bioactive compounds and their interactions with lipid membranes. We also want to thank Tone Normann Asp (Norwegian School of Veterinary Science) for valuable discussions.

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Oil displacement of surfactin. Surfactin from *Bacillus subtilis* (Sigma) was used to generate a dose-response relationship of the oil-clearance ability of surfactin.

Figure S2 Comparison of $\log_{10}$ concentrations (μg ml^{-1}) of lichenysin of three independently prepared methanol extracts. The height of the bars represents the total quantities (μg ml^{-1}) of lichenysin [sum of mol.mass (Da) 993.3, 1007.3, 1021.3 and 1035.4]. The blue, red and green bars represent each replicate. For two of the strains (F231 and CCUG26008) quantification was only determined in two of the three replicates. For six of the strains (S170, ATCC14580, S172, 749 and LMG7659) green bars are missing because the amounts of lichenysin were below the quantification limit (LOQ). Quantification of lichenysin was performed by LC-MS/MS as described in materials and methods.

Paper V

Detection of botulinum neurotoxin C/D through substrate cleavage and liquid chromatography – tandem mass spectrometry

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V

Detection of botulinum neurotoxin/CD through substrate cleavage and liquid chromatography – tandem mass spectrometry

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Abstract

Six phylogenetically distinct Clostridia can produce botulinum neurotoxins (BoNT), the most potent natural toxins known. BoNT/CD can cause avian botulism and is a major source to disease and mortality among poultry and wild birds. A qualitative method for the detection of BoNT/CD with LC-MS/MS that utilises the endopeptidase properties of BoNT/CD has been developed. Molecular weight cut-off (MWCO) filtration at 100 kDa is used as sample clean-up before incubation over night with the substrate peptide SNAP-25. The resulting cleaved product peptide is detected with LC-MS/MS. Implementation of the endopeptidase reaction of BoNT/CD ensures that only active toxin is detected. This study has been contemplated in bacterial growth medium.

1. Introduction

Botulinum neurotoxins (BoNT) are the most potent natural toxins known and can cause severe muscle paralysis in vertebrates, known as the illness botulism [1–3]. Six phylogenetically distinct Clostridia produce BoNT; *Clostridium botulinum* group I – III, *Clostridium argentinense*, *Clostridium butyricum* and *Clostridium baratii* [4]. They are anaerobic, endospore forming bacteria that is widespread in nature and found in soil and sediments [5]. Eight different serotypes of BoNT are discovered (BoNT/A to BoNT/H) with more than 40 subtypes [3,4]. The

serotypes vary in both reservoirs and genotype and affect different species: BoNT/A, -B, -F and -H are toxic to humans whereas BoNT/C1 and -D are mainly toxic to animals but are also reported in association with human disease [6–8]. In the Nordic countries, BoNT/C1 has mostly been connected to botulism in poultry [9–11] but also to mink and foxes at fur farms [12,13].

The BoNT are zinc-dependent metalloproteases that inhibit neurotransmission in the synapses by cleaving one or two of the docking proteins syntaxin, SNAP-25 and VAMP in the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) -complex resulting in paralysis [4]. They are large proteins with molecular mass (Mm) around 150 kDa, structurally divided in a light chain (LC) with Mm ~ 50 kDa and a heavy chain (HC) with Mm ~ 100 kDa connected only with a disulphide bridge after proteolytic cleavage [4]. The heavy chain is responsible for the translocation into the synaptic vesicles while the light chain contains the proteolytic properties. BoNT/C1 is the only serotype that cleaves two proteins; both syntaxin and SNAP-25 [14]. The cleavage sites are at specific positions for each BoNT, for instance both BoNT/A and BoNT/C1 cleave SNAP-25 but at different sites, Gln197-Arg198 and Arg198-Ala199, respectively [15]. In recent years, genes encoding the BoNT/CD mosaic has been isolated from several outbreaks of avian botulism [9,11,16,17]. The BoNT/CD mosaic consists of the light chain and translocation domain of the heavy chain from BoNT/C1 and the receptor-binding domain of the heavy chain from BoNT/D [4]; hence, the endopeptidase cleavage site is the same as for BoNT/C.

The “gold standard” for detection of BoNTs is the mouse bioassay that both detects BoNTs as a group and identifies the serotype by use of specific antitoxins [18,19]. Immunological methods [20–22] and polymerase chain reaction (PCR) [23] have also been used for the detection of BoNT/C1 and BoNT/C1 encoding genes, respectively, but the most prominent technique used is mass spectrometry (MS). There are several published methods utilising MS for the detection of BoNT/C, both proteomics methods that identify the neurotoxin itself after enzymatic digestion [24–27] and the so-called endopep-MS methods that detect the specific

product peptides resulting from enzymatic cleavage of substrate peptides due to the endopeptidase properties of BoNT/C1 [24,28–31]. Several of the published endopep-MS methods contain a clean-up step using an immunoaffinity method with BoNT/C-specific antibody-coated beads before the endopeptidase reaction [24,29,31]. Due to the purity of the samples in these methods, matrix-assisted laser desorption/ionisation (MALDI) is the most frequently used ionisation technique together with time of flight (TOF) MS; however, there is also one study that utilises liquid chromatography (LC) with electrospray ionisation (ESI) and triple quadrupole (QqQ) MS [28]. The major advantage of using QqQ instead of TOF for the mass analysis is improved sensitivity with a 10-fold decrease in the limit of detection (LOD) for the product peptide for three out of four BoNT [28]. This is of major importance in analysis of BoNT/C1 where it is of great interest to achieve LODs lower than achieved with the mouse bioassay. This is due to different sensitivities toward BoNT/C1 in different species; for instance cattle is much more sensitive towards BoNT/C1 than mice [32].

As the Norwegian national reference laboratory for the detection of *C. botulinum* in food and animals we aim to use state of the art analytical methods and are continuously implementing new methods and improving the existing ones. In this study we wanted to develop a chemical method utilising LC-MS for the detection of BoNT/C1 that eventually can substitute the mouse bioassay. A change of reference method for the detection of BoNT is of great importance of several reasons: Detection and serotyping of BoNT with the mouse bioassay requires a large number of mice; the use of laboratory animals should be avoided whenever possible. After injection, the mice must be monitored for 96 hours before a reliable conclusion can be drawn from the mouse bioassay, making this a time-consuming method. With the LC-MS method proposed in this study this can be reduced to 48 hours, probably even less. MS-based methods might also be able to decrease the LODs for BoNTs which is of importance for sensitive species like cattle.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of at least HPLC-grade and purchased from VWR (West Chester, PA, USA), except $ZnCl_2$ and dithiothreitol (DTT) supplied by Sigma (Steinheim, Germany). The synthetic peptides ATKMLGSG, SNAP-25 (AA137 – 206) and syntaxin-1A (AA232 – 266) were purchased from Biomatik (Cambridge, Ontario, Canada). The water used was grade 1 purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). The culture medium was tryptone peptone glucose yeast extract broth (TPGY), made of 5 % tryptone (Difco, Detroit, MI, USA), 0.5 % proteose peptone (Difco, Detroit, MI, USA), 2.5 % yeast extract (Oxoid Ltd, Basingstoke, UK), 0.5 % glucose and 0.05 % cysteine HCl in water.

2.2. Toxin production

For BoNT/CD production, *Clostridium botulinum* strain BKT002873 was incubated anaerobically in TPGY for 5 - 7 days at 37 °C. The supernatant was collected for further analysis.

2.3. Sample preparation

500 µL sample was centrifuged at 14 000 x *g* for 5 minutes in pre-washed Nanosep® 100 kDa molecular weight cut-off (MWCO) centrifuge filters (Pall Life Sciences, Ann Arbor, MI, USA). The pre-wash was accomplished by two times centrifugation of 500 µl 50 mM ammoniumformate pH 7.6 at 14 000 x *g* for 1 minute. 200 µL 50 mM ammoniumformate pH 7.6 was added to the retentate on the filter and transferred to another test tube. Aliquots of 25 µL was transferred to an eppendorf and added 2 µL 10 mM $ZnCl_2$, 3 µL freshly made 100 mM DTT in 50 mM ammoniumformate pH 7.6 and 5 µg substrate before incubation at 37 °C over night. 1 µL sample was injected on the LC-MS/MS after incubation.

2.4. Liquid chromatography

The separation was performed with an Agilent Eclipse Plus C18 RRHD column, 100 x 2.1 mm with 1.8 µm particles (Agilent Technologies, Waldbronn, Germany) using an Agilent 1290 setup consisting of a binary pump, thermostatted column compartment held at 40 °C and thermostatted autosampler held at 4 °C (Agilent Technologies, Waldbronn, Germany). Mobile phase A was 0.1 % formic acid in water and mobile phase B was acetonitrile running in a gradient from 2 – 40 % MFB in 4 minutes at a flow rate of 400 µL/min. The complete time of analysis was 9 minutes.

2.5. Mass spectrometry

The mass spectrometer used was a G6490 triple quadrupole (Agilent Technologies, Singapore) equipped with a Jet Stream electrospray ion source operated in positive multiple reaction monitoring (MRM) mode. The analyte, ATKMLGSG, was detected as its doubly charged ion with m/z 382.7, the ion transitions monitored and their collision energies are listed in Table 1. The common settings were gas temperature 250 °C, gas flow 14 L/min, nebulizer pressure 20 psi, sheath gas temperature 400 °C, sheath gas flow 11 L/min, capillary voltage 4000 V and cell acceleration voltage 5 V.

3. Results and discussion

3.1. Choice of sample clean-up and ionisation technique

The existing endopep-MS methods for detection of BoNT/C1 in real sample matrices like serum, liver, food and feed utilises antibody-coated beads for sample clean-up before analysis with MALDI-MS. [24,29,31]. This immunoaffinity purification procedure is very efficient and allows extensive cleaning when the toxin is bound to the antibodies, even in complex matrices. MALDI is a soft ionisation technique that requires high purity samples to obtain good mass spectra due to the sampling technique that ionises everything in the laser spot without any other separation besides the mass to charge ratio (m/z). MALDI produces almost solely singly-charged ions;

additional separation due to different charge states is very unlikely [33]. The endopep-MS assay is susceptible to proteolytic matrix components that may digest the substrate and/or the product peptide, thus a proper separation of BoNT/C1 from the matrix is vital [34]. By binding the BoNT/C1 to antibody-coated beads, BoNT/C1 is removed from the sample matrix prior to the endopep reaction and the probability of unspecific cleavage of the substrate peptide reduced.

In this study we wanted to use a more generic approach to sample preparation before the endopeptidase reaction; molecular weight cut-off (MWCO) - based sample preparation instead of immunoaffinity. The elimination of the immunoaffinity step enables possible detection of several BoNTs simultaneously because no analyte – antibody reaction is required. Proteins from eukaryotic cells have an average molecular weight (Mw) of 50 kDa [35] whereas the Mw of BoNT/C1 is 150 kDa. With a MWCO of 100 kDa most proteases and other matrix constituents will be removed from the sample. By using LC-separation coupled with electrospray ionisation (ESI) it is possible to use cruder sample extracts than for MALDI because LC performed with optimised conditions is able to separate the peptides of interest from other matrix components.

3.2. Sample clean-up

Two different sample clean-up methods were evaluated during the method development: dialysis and MWCO-filtration. Micro Float-A-Lyzers (100 kDa MWCO, 200 µL, SpectrumLabs, Breda, the Netherlands) were used for dialysis and Nanosep centrifuge filters of 30 kDa and 100 kDa MWCO were used for filtration. Comparison of dialysis and filtration with MWCO of 100 kDa revealed that both clean-up techniques were equally efficient with ammonium formate as dialysis buffer and to wash the retained proteins off the MWCO-filter. Purification with 30 kDa filtration resulted in a fivefold decrease of the sensitivity compared to 100 kDa filtration. These experiments were carried out using TPGY medium as sample matrix where no protease that could interfere with the endopep-reaction should be present. Thus, the decrease in sensitivity was most likely due to ion suppression in the MS caused by remaining matrix constituents with

molecular weights between 30 kDa and 100 kDa. Filtration is much more time efficient than dialysis, 15 minutes compared to minimum six hours; hence filtration was chosen for sample clean-up.

3.3. Endopeptidase reaction

The endopeptidase reaction used in this study is based on the publication by Jones et al. [21] with some alterations to make it compatible with MS. Both HEPES, Tris and ammonium formate buffer were examined as potential reaction buffers, Tris and ammonium formate were tested both with and without tween. The highest analyte yield was achieved using ammonium formate. HEPES gave the same result as ammonium formate when filtration was used, but was considered non-compatible with dialysis due to poor recovery of BoNT/C. Tris buffer resulted in a fourfold decrease of the sensitivity compared to ammonium formate. Tween is known to cause considerable contamination and ion suppression in MS; this was also the case in this study. According to this, 50 mM ammonium formate pH 7.6 gave the best results and was used as reaction buffer.

The sample clean-up includes a buffer exchange since the retentate on the MWCO-filter is collected with ammonium formate; hence the toxins are already present in the reaction buffer. To avoid unnecessary dilution of the samples both the endopep-reagents and the substrate peptide was added directly to the samples instead of preparing a solution of them before addition. This had no influence on the toxin - substrate reaction (data not shown).

A part of SNAP-25 (AA137 – 206), was synthesised and used as substrate [21]. The peptide ATKMLGSG (monoisotopic mass 763.4 Da) resulting from cleavage of SNAP-25 by BoNT/C1 was detected in TPGY medium. However, when serum was introduced as sample matrix the product peptide was not detected. Even after spiking the sample with the product peptide before the endopep-reaction, ATKMLGSG could not be recovered, indicating that the produced peptide is

rapidly degraded in the incubated samples. The degradation of the product peptide is most likely due to proteases still present in the samples after the MWCO filtration. To ensure the BoNT/C1 remains active it was not possible to include a denaturation step to inactivate unwanted proteases that might have been retained on the filter due to their tertiary structures despite having a molecular weight below 100 kDa. These possibilities need to be investigated further. If proteases are indeed present in the sample matrix, protease inhibitors could be added to quench the proteolytic activity. It is however of utmost importance to neutralise the proteases without reducing the proteolytic activity of BoNT/C. In this study we incubated the sample with the substrate at 37 °C. Increase of the incubation temperature to 42 °C, has been shown to improve the toxin-substrate reaction [24,31]. Both incubation temperature and – time should be re-evaluated.

The LOD necessary for satisfactory sensitivity is below the mouse LD_{50} (1 ng/kg body weight [36]). Wang et al. [31] increased the sensitivity of BoNT/C-detection 200fold by optimising the substrate peptide. An optimisation of the substrate might also eliminate the problem of degradation of the product peptide after substrate cleavage described above.

3.4. LC-MS

Ion transitions m/z 382.7 - 373.6 and m/z 382.7 - 592.2 both had the same sensitivity in the MS and no interferences have been observed with TPGY medium or serum as matrix. The ion transition m/z 382.7 – 129.1 however, was not suitable for use due to contamination peaks that occurred even in injections of pure ammonium formate.

4. Further work

4.1. Food /feed

The next step of this study after implementation of serum as matrix is to further expand sample matrices to include food and feed that might be the source of BoNT/C. A rapid identification of

the source is of importance during an outbreak of botulism to confine the number of inflicted animals. Liquid matrices will be tried implemented in the same sample preparation as described, whereas solid matrices will be homogenized in buffer before the sample preparation. For lipid rich matrices a lipid removal step before the described sample preparation might be necessary.

4.2. Implementation of all botulinum neurotoxin serotypes

After the method for BoNT/CD has been finalised we plan to implement the other BoNT-serotypes with their respective substrates. Optimization of every step of the analysis is crucial to obtain sufficiently low LODs for the BoNTs. The optimal temperature and pH of the substrate cleavage step varies between the BoNTs [24,37], making it impossible to ensure ideal conditions for all BoNTs in a common sample preparation. The matrices of interest also differ between the serotypes, especially between BoNT/C1 and D, and their mosaics, that are mostly involved in animal botulism and BoNT/A, B, E and F that are the cause of human illness. The sample clean-up step of the method could also be optimized for different matrices. For human serum we tried removal of the most abundant proteins with a multiple affinity removal system (MARS) Hu-2 column (Agilent Technologies, Waldbronn, Germany), but due to problems with peptide degradation during the endopeptidase reaction we have not evaluated this sample clean-up. MARS is not an alternative clean-up procedure for serum from animals, since they have different protein profiles than humans.

Ideally we would like to develop one common method for all seven BoNT serotypes in all matrices with a peptide cocktail as substrate. This may be possible using the unspecific MWCO-filtration as sample preparation in contrast to the analyte specific immunoaffinity clean-up. However, to achieve highest possible sensitivity, optimal conditions for each BoNT serotype must be implemented. The incubation temperature for the endopep reaction differs for each serotype; it is optimal at 42 °C for BoNT/C1 and at 37 °C for BoNT/A [24]. Both the endopep-reaction and the bacterial growth are optimal at temperatures near the body temperature of the

host species [24,38]. The optimal pH of the reaction buffer also varies between the BoNT serotypes. At the same time, the sample matrix also varies between the BoNT serotypes. However, in all suspected cases of botulism serum from the inflicted will be included. At outbreaks of animal botulism the main samples trying to identify the source of contamination will mostly be a narrow range of feed samples while in human botulism it will be food samples with very different matrix composition. The variety in sample matrix is probably the main challenge in this approach to BoNT/C-detection.

5. Funding

This study was funded by the Research Council of Norway, project no. 199375/I99.

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Table 1: Ion transitions monitored and their collision energies

Precursor ion m/z	Product ion m/z	collision energy /V
382.7	592.2	10
382.7	373.6	10
382.7	129.1	30