To my wife - my scientist, my inspiration

To my beloved parents and sister

To my grandmother and to my grandfather whom I miss a lot

To my family and friends
Promoters: **Prof. dr. ir. Mieke Uyttendaele** and  
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Rector: **Prof. dr. Paul Van Cauwenberge**
Msc. ing. Andreja RAJKOVIĆ

Biological and immuno-molecular techniques for monitoring of *Bacillus cereus* emetic toxin and *Staphylococcus aureus* enterotoxin

Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Applied Biological Sciences
Titel van het doctoraat in het Nederlands:

**Biologische en immuno-moleculaire methoden voor monitoring van het emetisch toxine van *Bacillus cereus* en het enterotoxine van *Staphylococcus aureus***

Illustration: Graphical composition with molecules of cereulide and SEB prepared by Mirjana and Andreja Rajković on the basis of published images by Paananen, A. (2004) and Papageorgiou *et al.* (1998) for cereulide and SEB, respectively.

To refer to this thesis:

Rajkovic, A. 2006. Biological and immuno-molecular techniques for monitoring of *Bacillus cereus* emetic toxin and *Staphylococcus aureus* enterotoxin. Thesis submitted in fulfillment of the requirements for the degree of doctor (Ph.D.) in Applied Biological Sciences. Faculty of Bioscience Engineering, University of Ghent


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Andreja Rajkovic was supported by the fellowship from Special Research Funds of Ghent University (Bijzonder Onderzoeksfonds, BOF).

Analysis of cereulide were supported by Hypor Belgium (Olsene, Belgium) and Leja Products B.V. (Nieuw-Vennep, The Netherlands).

The construction of the index of terms used in this thesis was performed with TExtract provided by Texyz Indexing Software (Utrecht, The Netherlands).

The research was performed at the:

Laboratory of Food Microbiology and Food Preservation, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Centre de Recherche sur les Protéines Prions (CRPP) - Service d'Histologie Humaine, Université de Liège-CHU, Liège, Belgium

Department of Animal Product Quality and Transformation Technology, ILVO, Ghent, Belgium

Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium
PREFACE AND ACKNOWLEDGEMENTS

In last four thousand years there were about nine thousand Sun eclipses. It seems as such a long period of time, but it is just a short span of history of mankind. And the history has a tendency to keep only relevant information. Today, the analytical power of human-computer teamwork resulted in all of us being snowed under information volume. And still each day seems to bring another breakthrough to be considered. So much we know, and we still strive for more - because we know how few we know.

Was it ever that so many brains and hands got engaged in the science? Can’t be! It is in latest decades that scientific community faced such evolutionary expansion. See only the number of different scientific journals: general ones, more and less specific ones. See how much is published therein. Just like the science community got fueled by super-octane power source. A renewable and sustainable source, hopefully it is. “Never before in the history of science have so many scientists been as active as today” - a book said. If we were not so many, we just would not keep up with the pace with which we change the nature and ourselves.

Now, the information is there. What to do with it?! What ever the course of action is taken in broadening, deepening and questioning the existing scientific information it will be very unlikely that one will achieve much by being all alone. The scientist must sustain and follow the vision, but the effort in the realization of ideas and experimental setups requires a team.

How lucky it was for me to be a part of a good team. In fact, of more than one: a team on the scene and the team behind the scene. Each single member being essentially worth. I thank them all: for being supportive when they were supportive and for not being that, when they thought that was more appropriate; for letting me do things the way I did them - by letting me do it my way and not letting me, too; for their words and for their silence; for often being right and for rarely being wrong. We all learned! I’ve been having my trust given to you. I’ve never regretted it! You gave your trust to me. I hope regrets you had none, too.

This research we have performed yielded some nice results. Publications we published did not remain unnoticed. Prof. Uyttendaele, Mieke, we did it together. It does not seem all that long ago when you said how four PhD years will go fast away. I said I knew and I took a strong grip, clutched the gear in, and started the run. I was sure that I understood. And, I did, but just did not see at that moment how fast it actually was. Thank you for helping me not to slide of the track on this research runway. But, that is not all. It was more than once that you stood up to give a hand, even when science was not the one in need.

In the LFMFP we are with many heads and all similar, but different among each other. It is the pure scientific power, we have here. But the power without the control would not mean much, would it? Dear Professor Debevere you managed to keep our numerous and diverse group under one roof, providing us with what many I know, can only desire for. And although so powerful, you turned not to be distant. In fact you traveled 4000 kilometers to break the distance and be with us on a day - the day my wife and me, and so many of our staff will keep in our memories.

From a shadow of the work pile fast and lucid hints were coming. While under his own work load Prof. Devlieghere maintained his omnipresence in all the research
areas of LFMFP putting also my work into new perspectives. Frank, thank you for this added value.

Dear professors not only I thank you for what we have achieved in the past, I also thank you for your trust in what we are about to achieve together in days to come.

In this solemn work there was another ace in my sleeve that pulled me through when it was needed. Colleagues, friends and my thesis students and trainees! I wish to thank to all my unreasonable mates at LFMFP. Be even more unreasonable, I should say. The reasonable man adapts himself to the world: the unreasonable one persists in trying to adapt the world to himself. Therefore all progress depends on the unreasonable man. So, in this small community of ours, we may not have explicitly realized that, but we often found that what once was strange became a familiar fact today, and what was familiar may seem today odd. No surprise here. We have been exercising science.

For all the help, guidance and advices around iqPCR a thank goes to the whole team of Prof. E. Heinen, leaded by dr. W. Zorzi and dr. B. El Moualij, at the University of Liege. The same way, I thank to Prof. A. Van Soom and dr. T. Rijsselaere from the UGent, for their help with experiments with boar semen motility. For their genereous support in the research my gratefulness goes to Wouter Deley in Hypor Belgium (Belgium) and to Leja Products B.V. (The Netehrlands). For providing me with TExtract software that yielded Index of terms in this dissertation, a special thanks goes to Harry Bego.

And coming closer and closer to an end of this already long preface I desire to state my believe that Kam nije dugme and I gratefully acknowledge my devotion to Olja and Ivan (and Jovan and Milena), Ljuba and Ivana, Minja and Vesna (and Marko), Tom, Natasa and Aleksa (and Dimitrije)! To my dear hard time mate Milivoj. To Marko and Stasa. To Nikola. To beloved D’Haeyer family for all they did for me, us and many others; for being who they are! To Prof. Lale Radovanovic who was a childhood friend of my father, then became my professor and mentor, and today he is my friend. To Prof. D. Obradovic for opening the doors of food related microbiology to me. To Prof. M. Todorovic for so many things she did and let me be part in some! To ir. M. Remaut and her team for everything they did for us international students at Food Safety and Food Quality Department. To all my great IAAS friends.

At last, but not least I have some very personal notices to make. But, that simply cannot be put on a paper with all the niceties. It just would not be appropriate. But, I want to thank my parents and my sister for accepting and supporting my international ways. They so courageously accepted my absence from home. And they supported it in any way they could. The great people they have been! With love and dignity they went through the harsh times of more than a decade lasting dusk on Balkan. The strong people they have been! The love and care they never lacked. The loving people they have been! The human values they endorsed. The great family we are!

A special word of thanking is for family Andjelkovic. How stronger we are together!

For virtually everything that I could think of, to the personification of what one lives and works for, to my wife, my Mira! She helped with her love and kindness and with her sharp intelligence that critically questioned all I did in my research.

Andreja, June 23, 2006
TABLE OF CONTENTS
# TABLE OF CONTENTS

PREFACE AND ACKNOWLEDGEMENTS....................................................................................... ix

TABLE OF CONTENTS ........................................................................................................ xiii

INTRODUCTION AND OBJECTIVES ..................................................................................... xix

LIST OF ABBREVIATIONS........................................................................................................ xxv

1 BACTERIAL TOXINS & ENTEROTOXIGENIC BACILLUS CEREUS: A REVIEW....3

  1.1 TOXINS ........................................................................................................................3

    1.1.1 Introduction to microbial (bacterial) pathogenesis .................................................3

    1.1.2 Types of toxins........................................................................................................6

    1.1.3 Structural characteristics of bacterial protein toxins.............................................7

    1.1.4 Genetic determinants of protein toxins...................................................................9

    1.1.5 Mechanisms of action of protein toxins on target (eukaryotic) cells....................13

    1.1.6 Role of bacterial protein toxin in foodborne diseases...........................................18

    1.1.7 Analytical methods for bacterial protein toxins causing intoxications and
          toxin-mediated infections...........................................................................................20

    1.1.8 Biological toxins other than bacterial ...................................................................28

  1.2 BACILLUS CEREUS, A TOXIN PRODUCING FOODBORNE PATHOGEN ........31

    1.2.1 Taxonomy, morphology and biochemical characteristics of B. cereus group ......33

    1.2.2 B. cereus properties...............................................................................................35

    1.2.3 B. cereus and foodborne poisoning.......................................................................36

    1.2.4 Enumeration of B. cereus and toxin detection ......................................................43

    1.2.5 Breach in the food safety and food contamination with B. cereus.......................47

    1.2.6 Control of B. cereus in RTE foods........................................................................57

2 CHAPTER 2: PREVALENCE AND CHARACTERIZATION OF BACILLUS
          CEREUS IN VACUUM PACKED POTATO PUREE.....................................................65

  2.1 INTRODUCTION.......................................................................................................66

  2.2 MATERIALS AND METHODS ................................................................................67

    2.2.1 Potato puree ..........................................................................................................67

    2.2.2 Characterization of isolates...................................................................................69

    2.2.3 Statistical analysis.................................................................................................71

  2.3 RESULTS ...................................................................................................................71

    2.3.1 Physical parameters of potato puree .....................................................................71

    2.3.2 Microbiological analyses ......................................................................................71

    2.3.3 Fingerprinting of B. cereus isolates ......................................................................74

    2.3.4 Psychrotrophic and toxigenic character of B. cereus isolates...............................74

  2.4 DISCUSSION .............................................................................................................76

  2.5 CONCLUSIONS.........................................................................................................78

3 CHAPTER 3: ANTIMICROBIAL EFFECT OF Nisin AND Carvacrol AND
          COMPETITION BETWEEN BACILLUS CEREUS AND BACILLUS CIRCULANS
          IN VACUUM PACKED POTATO PUREE.....................................................................83

  3.1 INTRODUCTION.....................................................................................................84

  3.2 MATERIALS AND METHODS .............................................................................86

    3.2.1 Bacterial strains and enumeration.......................................................................86
Biological and immuno-molecular methods for detection of \textit{B. cereus} and \textit{S. aureus} enterotoxins

3.2.2 Determination of minimal inhibitory concentration (MIC) value of carvacrol and nisin ................................................................................................................87
3.2.3 Growth characteristics of \textit{B. cereus} and \textit{B. circulans} and co-cultures thereof at 7 and 10 °C.................................................................88
3.2.4 Antimicrobial effect of nisin and carvacrol in the potato puree and growth characteristics of \textit{B. cereus} and \textit{B. circulans} .........................................................................................89
3.3 RESULTS ...................................................................................................................90
3.3.1 Determination of MIC value and combination effect of antimicrobial substances .............................................................................................................90
3.3.2 Growth characteristics of \textit{B. cereus}, \textit{B. circulans} and co-cultures thereof at 7 and 10 °C in BHI...................................................................................................92
3.3.3 Antimicrobial effect of nisin and carvacrol and growth characteristics of \textit{B. cereus} and \textit{B. circulans} in potato puree ................................................................95
3.4 DISCUSSION .............................................................................................................97
3.5 CONCLUSIONS.........................................................................................................99

4 CHAPTER 4: DYNAMICS OF BOAR SEMEN MOTILITY INHIBITION AS A SEMI-QUANTITATIVE MEASUREMENT OF \textit{BACILLUS CEREUS} EMETIC TOXIN (CEREULIDE)...................................................................................................103
4.1 INTRODUCTION.....................................................................................................104
4.2 MATERIALS AND METHODS ..............................................................................105
4.2.1 Optimization of the bioassay and of the semi-quantitative approach ..........105
4.2.2 Effect of extraction and cereulide recovery from \textit{B. cereus} cultures ..........107
4.2.3 Parameters of Hamilton-Thorne Ceros (HTR) 12.1............................................108
4.2.4 Standard curve with valinomycin .......................................................................108
4.2.5 \textit{B. cereus} growth and cereulide production.........................................................109
4.3 RESULTS .................................................................................................................110
4.3.1 Optimization of parameters.................................................................................110
4.3.2 Shaken vs. static incubation of BHI cultures at different temperatures and different pH values..............................................................................................114
4.3.3 Quantification of cereulide production on different solid media ........................114
4.4 DISCUSSION ...........................................................................................................117
4.5 CONCLUSIONS.......................................................................................................119

5 CHAPTER 5: INFLUENCE OF TYPE OF FOOD ON THE KINETICS AND OVERALL PRODUCTION OF \textit{BACILLUS CEREUS} EMETIC TOXIN .....................123
5.1 INTRODUCTION.....................................................................................................124
5.2 MATERIALS AND METHODS ..............................................................................125
5.2.1 \textit{B. cereus} strains and preparation of inoculation .................................................125
5.2.2 Food samples and incubation conditions ............................................................126
5.2.3 Monitoring of \textit{B. cereus} growth in foods............................................................126
5.2.4 Boar semen motility assay and HPLC-MS analysis of cereulide production .....127
5.3 RESULTS .................................................................................................................129
5.3.1 Bacterial enumeration and physical parameters of food..................................129
5.3.2 Detection of cereulide in food samples...............................................................130
5.4 DISCUSSION ...........................................................................................................134
5.5 CONCLUSIONS.......................................................................................................136

6 CHAPTER 6: COMPUTER AIDED BOAR SEMEN MOTILITY ANALYSIS FOR CEREULIDE DETECTION IN DIFFERENT FOOD MATRICES...............................139
6.1 INTRODUCTION ..................................................................................................... 140
6.2 MATERIALS AND METHODS ................................................................................ 141
  6.2.1 Bacterial strains and enumeration .................................................................... 141
  6.2.2 Computer aided semen boar motility analysis .................................................. 142
  6.2.3 Specificity of computer aided boar semen motility analysis .............................. 142
  6.2.4 Cereulide production and robustness of CASA and boar semen bioassay in various foods ....................................................................................................... 146
  6.2.5 Computer aided boar semen motility analysis of suspected foods ..................... 148
  6.2.6 Cereulide extraction and CASA analysis ............................................................ 149
  6.2.7 Statistical interpretation ...................................................................................... 150
6.3 RESULTS ................................................................................................................. 150
  6.3.1 Specificity of boar semen and CASA for cereulide detection ............................ 150
  6.3.2 Robustness of computer aided boar semen motility analysis .............................. 151
6.4 DISCUSSION ........................................................................................................... 153
6.5 CONCLUSIONS ....................................................................................................... 154

7 CHAPTER 7: IMMUNO-QUANTITATIVE REAL-TIME PCR FOR DETECTION AND QUANTIFICATION OF STAPHYLOCOCCUS AUREUS ENTEROTOXIN B IN FOODS ....................................................................................................................... 159

7.1 INTRODUCTION....................................................................................................... 160
7.2 MATERIALS AND METHODS ................................................................................ 161
  7.2.1 Bacterial strains and culture conditions .............................................................. 161
  7.2.2 Antibodies, antigens and in-house Enzyme Linked Immuno Sorbent Assay (ELISA) ............................................................................................................. 162
  7.2.3 Immuno quantitative real-time PCR (iqPCR), primers and DNA ...................... 164
  7.2.4 Commercial SE detection systems ...................................................................... 166
  7.2.5 Determination of SEB production in food and laboratory media ....................... 166
  7.2.6 SEB and microbiological analysis of naturally contaminated foods .................. 167
  7.2.7 Statistical analyses .............................................................................................. 168
7.3 RESULTS ................................................................................................................. 168
  7.3.1 Development of an in-house ELISA and commercial assays ............................. 168
  7.3.2 Development of immuno quantitative PCR ......................................................... 168
  7.3.3 Determination of SEB production in food and laboratory media ....................... 170
  7.3.4 SEB and microbiological analysis of naturally contaminated foods .................. 175
7.4 DISCUSSION ........................................................................................................... 175
7.5 CONCLUSIONS ....................................................................................................... 178

8 GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES ......................... 181
REFERENCES ...................................................................................................................... 193
INDEX ................................................................................................................................... 227
SUMMARY ............................................................................................................................... I
SAMENVATTING ................................................................................................................. VI
CURRICULUM VITAE........................................................................................................... XV
INTRODUCTION AND OBJECTIVES
INTRODUCTION AND OBJECTIVES

Food production crucially relates to three equally important aspects. These aspects, being safety, quality and security of food supply, represent a general right of every human. At the same time, their fulfillment remains a responsibility of everyone involved in the food production chain.

Foodborne (and waterborne) diseases are major cause of illness and death around the World. In fact, they are the leading causes of illness and death in Third World Countries, killing approximately 1.8 million people annually (WHO, 2005), most of whom are children. However, they are not limited to one geographic, political or social part of the world, but in different forms and extents remain overall present. Estimated 9000 deaths in USA, due to foodborne and waterborne diseases are certainly an indicator that the food safety is an issue in developed World, as well (Cary et al., 2000a). Thus, it is not surprising that in industrialized countries, the percentage of people suffering from foodborne diseases each year has been reported to be up to 30% (WHO, 2002). Worldwide, foodborne diseases, and more especially diarrheal/emetic diseases, are an important cause of morbidity and mortality. Recent trends in global food production, processing, distribution and preparation are creating an increasing demand for food safety research in order to ensure a safer global food supply.

This Ph.D. research was designed with a purpose to contribute to the food safety assurance, providing new tools for detection of harmful toxins of bacterial foodborne pathogens. Namely, emetic toxins of \textit{Bacillus cereus} and \textit{Staphylococcus aureus}, cereulide and \textit{Staphylococcus} enterotoxin B (SEB) respectively, were the central focus of this research. The targeted toxins are causative agents of foodborne intoxications, where toxin preformed in the food enters the human gastrointestinal tract, causing a disease. By consequence, the need for a sensitive, specific and robust detection technique and method arises. The technique is to provide fast and accurate, qualitative and quantitative information on the possible toxin presence in the investigated food and to provide essential insight on mechanisms and factors underlying toxin production.
The first objective defined was to come up with a detailed and comprehensive review of the current knowledge on bacterial toxins, especially those that may be of relevance to the safety of food and/or water consumption. The first part of the literature review had to allow food microbiologist to grasp essentials of chemistry, genetics and activities of the most prominent bacterial protein exotoxins (lipopolysaccharide endotoxins were here not taken into account). The second part of it had to introduce *B. cereus* as a foodborne pathogen and its toxins as virulence factors. This part was designed in much of its structure and content as a comparative study of data available on *B. cereus* enterotoxins (diarrheal and emetic), *S. aureus* enterotoxins (SE) and *Clostridium perfringens* enterotoxin (CPE), as all these had certain properties in common. Diarrheal enterotoxins of *B. cereus* and CPE are both causative agents of toxin-mediated infection, requiring precedent growth of *B. cereus* and *C. perfringens*, respectively, to high counts. In addition they also share the same illness symptoms and both are linked to protein rich foods. Similar observation is valid for *B. cereus* emetic toxin (cereulide) and *S. aureus* enterotoxins, which share intoxicative nature, illness symptoms and high potency to cause severe damage in the host.

The second objective set for this Ph.D. research was determination and characterization of prevalence of *B. cereus* in the frame of hazard analysis for Ready-To-Eat (RTE) potato puree, as a possible vector of cereulide intoxication. The information on the psychrotrophic properties of *B. cereus* isolates and toxigenesis were to provide an essential insight in the possible safety problems with this type of food. Possibility to control the growth of present *B. cereus* and spoilage causing *B. circulans* was investigated with application of natural antimicrobial compounds, nisin and carvacrol, in combination with different pH and temperature values. Growth characteristics of *B. cereus* and *B. circulans* strains and the competitive effect that might occur between the two *Bacillus* species at refrigerated temperatures were also studied. Furthermore, microbial challenge testing (MCT) was performed in vacuum packed potato puree supplemented with (or without) antimicrobial substances, to determine the ability of the food product to support the growth of spoilage organism, *B. circulans*, and the pathogen, *B. cereus*. These objectives were reached in the research described in Chapters 2 and 3.
However, when the control fails and when outgrowth of present *B. cereus* reaches levels that can lead to the toxin production, it is of utmost importance to have a reliable toxin detection method and technique. Therefore, **the following objective**, described in Chapter 4, was to develop a quantitative detection method using boar semen motility bioassay, originally described by Andersson *et al.* (1998b), with computer aided semen analysis (CASA) and valinomycin as an internal standard. A CASA-system in correlation with the effect of valinomycin on boar semen had to establish detection of cereulide concentrations in the range that would allow detection of dose-response relevant amounts (ca 10 µg per kg body weight). The next goal was to use optimized computer aided analysis of boar semen motility to investigate production of cereulide with different strains of *B. cereus* (food isolates and isolates from foodborne outbreaks, (Dierick *et al.*, 2005)), different food products, incubation temperature, shaken or static incubation, and level of *B. cereus* growth. In Chapter 5 attention was given to starch rich products, in order to understand potential threats vectored by Ready-To-Eat meals containing sauces, pasta, rice and potato, all being rich in starch content. Milk which is frequently associated with *B. cereus* (Notermans *et al.*, 1997) was also included in the presented study.

It is not beyond a reasonable doubt that cereulide production may occur in foods other than starch based. In fact, extensive literature review in Chapter 1 indicated that many other foods could harbor cereulide producers and allow eventually cereulide production. With a very complex modern food chain and extensive mixture of different ingredients, increased consumption of different forms of Ready-To-Eat foods, cereulide production may further emerge in unexpected food items. But, before CASA system could be used to examine possible cereulide production in “untypical” food matrices, different factors had to be examined for interference with boar semen motility and data procured with CASA. Therefore, the research presented in Chapter 6 had an **objective** to examine the extent of possible interference of factors like preservatives, dioxin, acrylamide, *S. aureus* enterotoxins (causing symptoms resembling those of cereulide intoxication), *B. cereus* diarrheal enterotoxins (HBL and NHE), and foodborne mycotoxins with the CASA bioassay for detection of cereulide. Chapter 6 addressed then the potential of various food matrices, susceptible to *B. cereus* contamination, to support production of hazardous concentrations of cereulide.
Due to the fact that cereulide has no immunogenic properties (Ehling-Schulz et al., 2005b) no immunological test was possible to develop within the framework of this Ph.D. However, inspired by a large scale outbreak of *S. aureus* enterotoxins (SE) in Japan, where only 4 ng of enterotoxin per gram of milk powder caused intoxication in consumers using different products into which incriminated milk powder was incorporated (Anonymous, 2001; Asao *et al.*, 2003), the new **objective** was set, aiming to **develop** a highly sensitive immuno-quantitative polymerase chain reaction (iqPCR) against one of SE. The rationale included also the fact that SEs are, similarly to cereulide, causative agents of foodborne intoxications (exposure to toxin in foods causes disease). This **objective** was pursued in the research conducted as described in Chapter 7.
LIST OF
ABBREVIATIONS
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AFLP</td>
<td>AMPLIFIED FRAGMENT LENGTH POLYMORPHISM</td>
</tr>
<tr>
<td>ALOP</td>
<td>APPROPRIATE LEVEL OF PROTECTION</td>
</tr>
<tr>
<td>ASP</td>
<td>AMNESIC SHELLFISH POISONING</td>
</tr>
<tr>
<td>BCET</td>
<td>BACILLUS CEREUS ENTEROTOXINS</td>
</tr>
<tr>
<td>BHI</td>
<td>BRAIN HART INFUSION</td>
</tr>
<tr>
<td>BoTN</td>
<td>BOTULIN NEUROTOXIN FROM CLOSTRIDIUM BOTULINUM</td>
</tr>
<tr>
<td>BSA</td>
<td>BOVINE SERUM ALBUMIN</td>
</tr>
<tr>
<td>bw</td>
<td>BODY WEIGHT</td>
</tr>
<tr>
<td>CASA</td>
<td>COMPUTER AIDED SEMEN ANALYSIS</td>
</tr>
<tr>
<td>CFP</td>
<td>CIGUATERA FISH POISONING</td>
</tr>
<tr>
<td>CFU</td>
<td>COLONY FORMING UNIT</td>
</tr>
<tr>
<td>CLDT</td>
<td>HEAT LABILE CYTOLETHAL DISTENDING TOXIN PRODUCED BY <em>E. COLI</em></td>
</tr>
<tr>
<td>CNF</td>
<td>CYTOTOXIC NECROTIZING FACTORS</td>
</tr>
<tr>
<td>CPE</td>
<td>CLOSTRIDIUM PERFRINGENS ENTEROTOXIN</td>
</tr>
<tr>
<td>Ct</td>
<td>CYCLE THRESHOLD</td>
</tr>
<tr>
<td>CytK</td>
<td>CYTOTOXIN K</td>
</tr>
<tr>
<td>Da</td>
<td>DALTON</td>
</tr>
<tr>
<td>DMSO</td>
<td>DIMETHYL SULFOXIDE</td>
</tr>
<tr>
<td>DNA</td>
<td>DEOXYRIBONUCLEIC ACID</td>
</tr>
<tr>
<td>DSP</td>
<td>DIARRHEIC SHELLFISH POISONING</td>
</tr>
<tr>
<td>EAST</td>
<td>ENTEROAGGREGATIVE <em>E. COLI</em> HEAT STABLE ENTEROTOXIN</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>EFFECTIVE DOSE (THE DOSE CAUSING A 50% RESPONSE IN A BIOLOGICAL SYSTEM)</td>
</tr>
<tr>
<td>EFSA</td>
<td>EUROPEAN FOOD SAFETY AUTHORITY</td>
</tr>
<tr>
<td>ELISA</td>
<td>ENZYME LINKED IMMUNOSORBENT ASSAY</td>
</tr>
<tr>
<td>bceT</td>
<td>ENTEROTOXIN T (<em>B. cereus</em>)</td>
</tr>
<tr>
<td>ET</td>
<td>EXPOSURE TIME</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>EU</td>
<td>EUROPEAN UNION</td>
</tr>
<tr>
<td>FSO</td>
<td>FOOD SAFETY OBJECTIVE</td>
</tr>
<tr>
<td>GC</td>
<td>GAS CHROMATOGRAPHY</td>
</tr>
<tr>
<td>GMP</td>
<td>GOOD MANUFACTURING PRACTICE</td>
</tr>
<tr>
<td>HACCP</td>
<td>HAZARD ANALYSIS CRITICAL CONTROL POINT</td>
</tr>
<tr>
<td>HBL</td>
<td>HEMOLYSIN BL</td>
</tr>
<tr>
<td>HC</td>
<td>HEMORRHAGIC COLITIS</td>
</tr>
<tr>
<td>HPLC</td>
<td>HIGH PERFORMANCE LIQUID CHROMATOGRAPHY</td>
</tr>
<tr>
<td>HTR</td>
<td>HAMILTON THORNE RESEARCH</td>
</tr>
<tr>
<td>HUS</td>
<td>HEMOLYTIC UREMIC SYNDROME</td>
</tr>
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<td>LC</td>
<td>LIQUID CHROMATOGRAPHY</td>
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<tr>
<td>LD₅₀</td>
<td>LETHAL DOSE (THE DOSE CAUSING THE DEATH OF 50% OF TEST ANIMALS)</td>
</tr>
<tr>
<td>LPS</td>
<td>LIPOPOLYSACCHARIDE COMPLEX (ENDOTOXIN OF GRAM NEGATIVE BACTERIA’S CELL WALL)</td>
</tr>
<tr>
<td>LT</td>
<td><em>ESCHERICHIA COLI</em> HEAT LABILE ENTEROTOXIN</td>
</tr>
<tr>
<td>MA</td>
<td>MILK AGAR</td>
</tr>
<tr>
<td>MAPK</td>
<td>MITOGEN ACTIVATED PROTEIN KINASE</td>
</tr>
<tr>
<td>MCT</td>
<td>MICROBIAL CHALLENGE TESTING</td>
</tr>
<tr>
<td>MHC</td>
<td>MAJOR HISTOCOMPATIBILITY COMPLEX</td>
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<tr>
<td>MIC</td>
<td>MINIMAL INHIBITORY CONCENTRATION</td>
</tr>
<tr>
<td>mNB</td>
<td>MODIFIED NUTRIENT BROTH</td>
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<tr>
<td>MPN</td>
<td>MOST PROBABLE NUMBER TECHNIQUE</td>
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<tr>
<td>mRNA</td>
<td>MESSENGER RIBONUCLEIC ACID</td>
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<td>MRS</td>
<td>DEMAN, ROGOSA SHARPE AGAR</td>
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<td>MASS SPECTROSCOPY</td>
</tr>
<tr>
<td>NA</td>
<td>NUTRIENT AGAR</td>
</tr>
<tr>
<td>NB</td>
<td>NUTRIENT BROTH</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NHE</td>
<td>NON-HEMOLYTIC ENTEROTOXIN</td>
</tr>
<tr>
<td>NSP</td>
<td>NEUROTOXIC SHELLFISH POISONING</td>
</tr>
<tr>
<td>NTC</td>
<td>NO TEMPLATE CONTROL</td>
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<td>PCB</td>
<td>POLYCHLORINATED BIPHENYLS</td>
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<td>PC PLC</td>
<td>PHOSPHATIDYLCHOLINE PREFERENCE PHOSPHOLIPASE C</td>
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<td>PCR</td>
<td>POLYMERASE CHAIN REACTION</td>
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<tr>
<td>PDA</td>
<td>POTATO DEXTROSE AGAR</td>
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<tr>
<td>pET</td>
<td>PRE EXPOSURE TIME</td>
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<tr>
<td>PI PLC</td>
<td>PHOSPHATIDYLINOSITOL SPECIFIC PHOSPHOLIPASE C</td>
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<tr>
<td>PMOT%</td>
<td>PROGRESSIVE MOTILITY PERCENTAGE</td>
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<td>PO</td>
<td>PROCESS OBJECTIVE</td>
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<td>PPS</td>
<td>PEPTONE PHYSIOLOGICAL SOLUTION</td>
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<td>PSP</td>
<td>PARALYTIC SHELLFISH POISONING</td>
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<tr>
<td>RAPD</td>
<td>RANDOM AMPLIFICATION OF POLYMORPHIC DNA</td>
</tr>
<tr>
<td>REPFEDE</td>
<td>REFRIGERATED PROCESSED FOOD OF EXTENDED DURABILITY</td>
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<tr>
<td>RNA</td>
<td>RIBONUCLEIC ACID</td>
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<tr>
<td>RPLA</td>
<td>REVERSE PASSIVE LATEX AGGLUTINATION</td>
</tr>
<tr>
<td>RTE</td>
<td>READY TO EAT</td>
</tr>
<tr>
<td>SE (SET)</td>
<td>STAPHYLOCOCCUS AUREUS ENTEROTOXINS</td>
</tr>
<tr>
<td>SF</td>
<td>SCARLET FEVER</td>
</tr>
<tr>
<td>SNAP 25</td>
<td>SYNAPTOSOMAL ASSOCIATED PROTEIN</td>
</tr>
<tr>
<td>SSS</td>
<td>SCALDED SKIN SYNDROME</td>
</tr>
<tr>
<td>ST</td>
<td>ESCHERICHIA COLI HEAT STABILE ENTEROTOXIN</td>
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<tr>
<td>STR</td>
<td>STRAIGHTNESS</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8 TETRACHLORODIBENZO P DIOXIN</td>
</tr>
</tbody>
</table>
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR</td>
<td>T CELL RECEPTOR</td>
</tr>
<tr>
<td>TLC</td>
<td>THIN LAYER CHROMATOGRAPHY</td>
</tr>
<tr>
<td>TSA</td>
<td>TRYPTONE SOYA AGAR</td>
</tr>
<tr>
<td>TSB</td>
<td>TRYPTONE SOYA BROTH</td>
</tr>
<tr>
<td>TSS</td>
<td>TOXIC SHOCK SYNDROME</td>
</tr>
<tr>
<td>TSST 1</td>
<td>TOXIC SHOCK SYNDROME TOXIN 1</td>
</tr>
<tr>
<td>UTI</td>
<td>URINARY TRACT INFECTION</td>
</tr>
<tr>
<td>VAMP</td>
<td>VESICLE ASSOCIATED MEMBRANE PROTEIN</td>
</tr>
<tr>
<td>VAP</td>
<td>VELOCITY AVERAGE PATHWAY</td>
</tr>
<tr>
<td>YGC</td>
<td>YEAST GLUCOSE CHLORAMPHENICOL AGAR</td>
</tr>
<tr>
<td>YOPI</td>
<td>YOUNG OLD PREGNANT IMMUNODEFICIENT PART OF POPULATION</td>
</tr>
</tbody>
</table>
BACTERIAL TOXINS &
ENTEROTOXIGENIC *Bacillus cereus*:
A REVIEW
1 BACTERIAL TOXINS & ENTEROTOXIGENIC

BACILLUS CEREUS: A REVIEW

Foodborne bacteria can be divided into foodborne pathogens and spoilage causing microorganisms. While typical pathogens like *Listeria monocytogenes*, *Campylobacter jejuni* and *Staphylococcus aureus* are explicitly seen as disease causing microorganisms, some bacteria like *Bacillus* spp. can be categorized as both spoilage causing and pathogenic microorganisms. The virulence factors that are behind bacterial pathogenicity determine modes of action and different mechanism via which pathogenic effect is imposed onto the host. Some bacteria partially or completely rely on the harmful toxic compounds, as virulence factors. These toxins can induce variety of health and life threatening effects. They can induce vomiting and cause diarrhea, they can affect nerve cells and paralyze the host, or cause a muscular cramp, or cause severe pain, or fever, etc. Different bacteria produce different toxins. In some cases toxin production occurs during the bacterial growth in food, causing illness even without ingestion of living bacteria. In other type of food poisoning, bacteria invade host cells.

1.1 TOXINS

1.1.1 Introduction to microbial (bacterial) pathogenesis

The pathogenic nature of microorganisms stipulates the involvement of structural and biochemical mechanisms, as a result of which microorganisms can cause disease. Pathogenicity within the bacteria is related to unique structural components (e.g. capsules, fimbriae, lipopolysaccharide-LPS or other components of cell wall) or active secretion of substances that either damage host tissues or protect the bacteria against host defenses (Gray, 1997).

When a foodborne bacterium (or eventually toxin) enters a human host through the consumption of contaminated food, it must survive a variety of physiological stress factors before reaching the target tissue (or cells) and initiating the syndrome (disease). The first significant non-specific barrier is intact integument, as well as mucous membrane system, motility systems, low pH (pH ca. 2 in healthy adults) of
hydrochloric gastric juice in the human stomach, bile acids, lysozyme, mechanical clearance and similar (Opal and Yap, 2003). In these terms, one must consider that this barrier may be impaired in elderly persons due to the decreased production of stomach acid, as well as that certain foods may offer protection to pathogens or their toxin by buffering stomach acid. The second barrier and first immunological line of defense is the innate immune system that acts unspecific on pathogenic intruders. However, even if pathogens evade or overcome the relatively nonspecific constitutive defenses, they may yet face the third line of the more specific inducible defenses, once they have developed. The inducible defenses are so-called for the reason that they are induced upon primary exposure to a pathogen or one of its products. They must be triggered in a host and initially take time to develop (acquired immunity), as suggested by Opal and Yap (2003). If the phagocytes are successful in destroying the invader, no further damage is done to the host. But, if pathogen overcomes the host defense, the microorganism can damage the host cell. Two broad qualities of pathogenic bacteria exist, underling the means by which they cause disease, namely invasiveness and toxigenesis (Toddar, 2002).

1.1.1.1 Invasiveness

Under the term invasiveness the ability of bacteria to invade tissues is implied. This encompasses mechanisms for colonization (adherence and initial multiplication), ability to bypass or overcome host defense mechanisms, and the production of extracellular substances ("invasins") which facilitate the actual invasive process (Oelschlaeger and Hacker, 2000b). Once a pathogen is attached to host cells it can pass through them to invade other tissues. During this invasion, the pathogens metabolize and multiply to kill host cells. Some bacteria such as *Escherichia coli*, *Shigella* and *Salmonella* can induce host epithelial cells to engulf them by a process that resembles phagocytosis. These pathogens can also be extruded from the host cells by reverse phagocytosis in order to enter to other host cells. Some bacteria can also penetrate host cells by excreting enzymes and by their own motility; such penetration can itself damage the host cell (Oelschlaeger and Hacker, 2000b). Classically seen, there are two aspects of invasiveness, namely colonization and invasion.
**Colonization**

The first stage of microbial infection is colonization: the establishment of the pathogen at the appropriate portal of entry. Pathogens usually colonize host tissues that are in contact with the external environment. Sites of entry in human hosts include the urogenital tract, the digestive tract, the respiratory tract and the conjunctiva. Organisms that infect these regions have usually developed tissue adherence mechanisms and some ability to overcome or withstand the constant pressure of the host defenses on the surface.

**Invasion**

The invasion of a host by a pathogen may be aided by the production of bacterial extracellular substances, so called invasins, which act against the host by breaking down primary or secondary defenses of the body. Invasins are proteins (enzymes) that act locally to damage host cells and/or have the immediate effect of facilitating the growth and spread of the pathogen. The damage to the host as a result of this invasive activity may become part of the pathology of an infectious disease. These types of invasins are utilized by pathogens such as *Listeria, Yersinia* and *Shigella* (Oelschlaeger and Hacker, 2000b).

The extracellular proteins produced by bacteria which promote their invasion are not clearly distinguished from some extracellular protein toxins ("exotoxins") which also damage the host. Invasins usually act at a short range (in the immediate vicinity of bacterial growth) and may not actually kill host cells in their range of activity; exotoxins are often cytotoxic and may act at remote sites (removed from the site of bacterial growth). Also, exotoxins typically are more specific and more potent in their activity than invasins. Even so, some classic exotoxins (e.g. diphtheria toxin, anthrax toxin) may play some role in invasion in the early stages of an infection, and some invasins (e.g. staphylococcal leukocidin) have a relatively specific cytopathic effect (Toddar, 2002).

Most damage by bacteria, however, is done by actual toxins (Cary *et al.*, 2000b).
Bacterial toxins are differentiated in two major classes, on the basis of their chemical nature, regardless of their cellular location and staining features of bacteria that produce them (see further 1.1.2).

### 1.1.1.2 Toxigenesis

Under the term toxigenesis the ability of bacteria to produce toxins is implied. Toxic substances, produced by certain microorganisms, both soluble and cell-associated, may be transported by blood and lymph and cause cytotoxic effects at tissue sites remote from the original point of invasion or growth (Granum et al., 1995; Cary et al., 2000b; Alouf, 2000). Bacterial toxins are often the primary virulence factors of these microorganisms. In microbiology the term bacterial toxin designates special class of bacterial macromolecules that when produced during natural or experimental infection of the host, or introduced parenterally, orally (bacterial food poisoning) or by any other route in the organism, results in the impairment of physiological functions or overt damage to tissues. These effects can lead to the disease or even the death of the host.

### 1.1.2 Types of toxins

Essentially, toxins can be grouped into two classes, based on their chemical nature. They can either belong to the class of lipopolysaccharide complexes or to the class of proteins. Older terminology introduces exotoxins (proteins in their nature) as toxin components excreted from the living cells shortly after their production (exo=outside gr.). Protein class comprises currently more than 300 different entities (323 according to Alouf (2000)), which were for a long time considered only in relation to Gram positive bacteria, though produced by both Gram positive and Gram negative bacteria (Table 1-1).

Although, mostly released from bacterial cells during their growth (exotoxins, or secreted effectors), 25% of protein toxins remain attached to the cell and are secreted into the environment after the cell population enters into the stationary phase or complete cell lyses. Exotoxins are the main object of the research conducted here.

Endotoxins (endo=inside gr., cell-bound toxin) are on the contrary not proteins, but LPS, which is invariably a part of the outer membrane of Gram negative bacteria. It
Biological and immuno-molecular methods for detection of \textit{B. cereus} and \textit{S. aureus} enterotoxins

consists of three parts: A lipid portion (lipid A) from the outer membrane, the core and the O-antigen which extends outwards from the bacterial surface. As lipid A is the toxic component and an integral part of the outer membrane, no toxic effect is seen until bacterial lysis occurs. When lipid A is released it will exert its effect which is to activate complement and stimulate the release of bioactive host-proteins such as cytokines; resulting in a fever.

| Table 1-1: Repertoire of protein/peptide bacterial toxins (323 known proteinaceous toxins (Alouf, 2000)). |
|-----------------|-----------------------------------------------|
| Quality         | Number (percentage)                          |
| Gram positive bacteria | 148 (46%)                                    |
| Gram negative bacteria | 175 (54%)                                    |
| Extracellular toxins | (75%)                                        |
| Intracellular toxins | (25%)                                        |

\[1.1.3 \textbf{Structural characteristics of bacterial protein toxins}\]

Within more than 300 known protein/peptide toxins, literature reports a high versatility in their structural characteristics and multiple approaches for rendering groups of related toxins (Tortora \textit{et al.}, 1997; Cary \textit{et al.}, 2000b; Alouf, 2000; Oelschlaeger and Hacker, 2000a; Toddar, 2002). Essentially, toxin molecules can exhibit single chain, oligomeric (polymeric) structural attributes (Alouf, 2000), with one bacteria being able to produce multiple forms. Variations in each of the forms exist, rendering general classification difficult.

\[1.1.3.1 \textbf{Single chain molecules}\]

A number of toxins, such as \textit{E. coli} heat-stable enterotoxin, \textit{Clostridium difficile} toxin A and B, \textit{Staphylococcus aureus} \(\delta\) toxin, tetanus and botulinum neurotoxins produced by \textit{C. tetani} and \textit{C. botulinum}, respectively, belong to this group. Although all single chain molecules, these toxins can largely vary in the composition of amino acids constituting the chain, as well as in their molecular weight (from ca. 2 kDa up to ca. 300 kDa).
1.1.3.2 Oligomeric molecules

Bacterial protein exotoxins can also occur in a specific oligomeric form consisting of two or more subunits (protomers) joined in a holotoxin. Different variations within oligomeric toxins exist, including variations in which different subunits are synthesized and joined to each other. Additional variations occur in the number of the subunits (Leece and Hirst, 1992; Alouf, 2000; Toddar, 2002). Shiga toxin, verotoxins, \textit{E. coli} thermolabile enterotoxins I and II are some of the oligomeric toxins.

1.1.3.3 Multifactorial toxins

Multifactorial toxins (binary toxins) are practically seen two single chain molecules that are synthesized and excreted separately from each other by the microorganism and in culture solution exist as non bound independent entities. In this way they are different from classical oligomeric toxins, protomers of which are joined in a defined structure of a holotoxin. Two single chain proteins are both required in a concerted action to provide toxicity, while each protein separately provides little or no toxicity (Alouf, 2000; Barth \textit{et al.}, 2004; Blanke, 2006). Many Gram positive bacteria produce such binary toxins: \textit{C. perfringens} iota toxins, \textit{C. botulinum} C2 toxin, \textit{S. aureus} \( \gamma \) toxin, \textit{S. aureus} leucocidin, \textit{Enterococcus faecalis} hemolysin etc. The molar ratio between the components is reported as an important factor for toxic activity (Lindbäck \textit{et al.}, 2004).

1.1.3.4 Progenitor toxins

Progenitor toxins are macromolecular complexes consisting of both toxic and nontoxic moieties. \textit{C. botulinum} strains produce immunologically distinct neurotoxins (types A to G). Neurotoxins associate with nontoxic components in the culture fluids and form large complexes which are designated progenitor toxins (Inoue \textit{et al.}, 1996; Oguma \textit{et al.}, 1997; Hines \textit{et al.}, 2005).

1.1.3.5 Protoxin forms

Several toxins are excreted into the culture medium in their inactive form. These toxins can be activated by action of endogenous microbial proteases present in the medium (or other proteolytic enzymes in the environment). Such toxins are
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Chapter 1: Bacterial toxins & enterotoxigenic *Bacillus cereus*: a review

*C. perfringens* ε- and iota-toxins, C2-II component of C2 toxin of *C. botulinum*, and aerolysin of *Aeromonas hydrophila*.

Structurally and functionally seen, many protein toxins, notably those that act intracellularly (with regard to host cells), consist of two components: one component (subunit A) is responsible for the enzymatic activity of the toxin; the other component (subunit B) is responsible for binding to a specific receptor on the host cell membrane and transferring the active component across the membrane (Toddar, 2002; Barth *et al.*, 2004). The active component is not active until it is released from the native (A+B) toxin. Isolated A subunits are active but lack binding and cell entry capability. Isolated B subunits may bind to target cells, but they are nontoxic. The active component is often characterized with enzymatic activity: ADP-ribosyltransferase, adenylcyclases, metalloproteases, ribonucleases, glucosyl transferases, deamidases etc. (Alouf, 2000).

Different ways exist through which toxin subunits can be synthesized and arranged: “A + B” indicates that the toxin is synthesized and secreted as two separate protein subunits that interact at the target cell surface; “A-B” or “A-5B” indicates that the A and B subunits are synthesized separately, but associated by non-covalent bonds during secretion and binding to their target; 5B indicates that the binding domain of the protein is composed of 5 identical subunits. “A/B” denotes a toxin synthesized as a single polypeptide, divided into A and B domains, which may be separated by proteolytic cleavage (Toddar, 2002). It is obvious that with this designation A/B toxins belong to the above defined group of single protein chain toxins, while “A+B”, “A-B” and “A-5B” toxins can be grouped either under oligomeric or multifactorial toxins (Table 1-2).

1.1.4 **Genetic determinants of protein toxins**

The investigation of genetic determinants of toxin production faced a major breakthrough in last two decades. More than 150 different genes encoding for toxin proteins have been cloned, sequenced and described (Alouf, 2000). It seems that most of the toxin encoding genes are located on mobile genetic elements such as plasmids, phages, and pathogenicity islands (Novick, 2003; Barth *et al.*, 2004; Herold *et al.*, 2004).
Table 1-2: Example of bacterial protein toxins exhibiting A-B or A/B form (Barth et al., 2004).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Toxin</th>
<th>Chemical structure of the protein</th>
<th>Molecular mass (kDa) / Molar ratio of A and B subunit</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Botulinum neurotoxins A–G</td>
<td>Single</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium difficile</em></td>
<td>Toxins A and B</td>
<td>Single</td>
<td>ca. 270–308</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium novyi</em></td>
<td>Alpha toxin</td>
<td>Single</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium sordellii</em></td>
<td>Hemorrhagic and lethal toxins</td>
<td>Single</td>
<td>ca. 260–300</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium tetani</em></td>
<td>Tetanus neurotoxin</td>
<td>Single</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td><em>Corynebacterium diphtheriae</em></td>
<td>Diphtheria toxin</td>
<td>Single</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Exotoxin A</td>
<td>Single</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>Pertussis toxin</td>
<td>Multifactorial</td>
<td>105 /1:4</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Heat-labile enterotoxin</td>
<td>Multifactorial</td>
<td>85 /1:5</td>
<td></td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em></td>
<td>Shiga and Shiga-like toxins</td>
<td>Multifactorial</td>
<td>ca. 70 /1:5</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Cholera toxin</td>
<td>Multifactorial</td>
<td>85 /1:5</td>
<td></td>
</tr>
</tbody>
</table>

a X-the exact ratio is not known

An example of bacterial toxins encoded on mobile genetic elements is given in Table 1-3. It is common for pathogenic strains to express multiple toxins (Gilligan et al., 2000; Gill et al., 2005) and these toxins can be encoded on different DNA structures. For example, the ETEC *E. coli* produces two antigenic heat-labile enterotoxins, being heat-labile enterotoxin-I and heat-labile enterotoxin-II, which are plasmid and chromosomally encoded, respectively (Chart, 1998). *E. coli* heat-stable enterotoxins are reported to be encoded on transposons (Chart, 1998; Alouf, 2000).

The presence of toxin genes on such mobile DNA carriers requires additional considerations, such as an emergence of new pathogens that have recently acquired some of the virulence genes. It is possible that the determinants of these toxins have been imported from other species and therefore are not components of the basic genome of the existing producing organisms. This raises the question of the biological roles of these toxins and whether the toxin-carrying units can spread among different (though probably related) species. The possible transfer of *cpe* plasmid (encoding for *C. perfringens* enterotoxin) from few infecting *cpe*-positive isolates to predominantly *cpe*-negative *C. perfringens* isolates present in the normal intestinal flora, may convert these commensal (part of the normal flora) isolates to enteropathogens (Smedley et al., 2005).
A well known example of gene acquisition is seen with *Vibrio cholerae*. This bacterium is widely distributed in the environment in non-pathogenic form. Conversion to pathogenicity appears to result from infection with a specific bacteriophage, which inserts into one of the two *V. cholerae* chromosomes. The gene coding for cholera toxin (CT) is carried on this bacteriophage, and acquisition of the gene lead to horizontal transfer of this genetic element (Peterson, 2000).

### Table 1-3: Examples of bacterial toxin encoded on mobile genetic elements.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Toxin (gene product)</th>
<th>Location of genetic determinant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>Cytotoxin</td>
<td>Plasmid</td>
<td>(Haque et al., 1996)</td>
</tr>
<tr>
<td><em>Aeromonas caviae</em></td>
<td>Cytotoxin</td>
<td>Plasmid</td>
<td>(Novick, 2003)</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>Antrax toxin</td>
<td>Plasmid</td>
<td>(Peterson, 2000)</td>
</tr>
<tr>
<td><em>C. botulinum</em></td>
<td>Botulin toxin</td>
<td>Prophage</td>
<td>(Novick, 2003)</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>CPE</td>
<td>Transposon</td>
<td>(Peterson, 2000)</td>
</tr>
<tr>
<td><em>Clostridium tetani</em></td>
<td>Enterotoxin</td>
<td>Pathogenic island</td>
<td>(Novick, 2003)</td>
</tr>
<tr>
<td><em>Corynebacterium diphtheriae</em></td>
<td>Diptheria toxin</td>
<td>Prophage</td>
<td>(Novick, 2003)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Heat-stable enterotoxin (ST)</td>
<td>Transposon/plasmid</td>
<td>(Chart, 1998; Novick, 2003; Braga et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Shiga-like toxin I</td>
<td>Prophage</td>
<td>(Unkmeir and Schmidt, 2000; Wagner et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Shiga-like toxin II</td>
<td>Prophage</td>
<td>(Noel and Boedeker, 1997; Novick, 2003)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>SEA</td>
<td>Prophage/plasmid</td>
<td>(Gilligan et al., 2000; Alouf, 2000)</td>
</tr>
<tr>
<td></td>
<td>SEB, SECC</td>
<td>Pathogenic island/chromosome</td>
<td>(Gilligan et al., 2000; Novick, 2003)</td>
</tr>
<tr>
<td></td>
<td>SED</td>
<td>Prophage/plasmid</td>
<td>(Gilligan et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>SEI</td>
<td>Plasmid</td>
<td>(Zhang et al., 1998)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Cholera toxin</td>
<td>Prophage</td>
<td>(Goldberg and Mekalanos, 1986; Novick, 2003)</td>
</tr>
</tbody>
</table>
1.1.4.1 Genetic regulation of toxin expression

The specific levels of particular enzymes and structural proteins of any organism are determined by many factors, such as the nutritional environment of the cell or its stage of development. The same is valid for bacterial toxins. Thus, in the interests of cellular economy, enzymes and other proteins are, generally, only synthesized when they are needed. Bacterial pathogens have numerous regulatory mechanisms for controlling the expression of virulence genes (the ability of a gene to produce a biologically active toxin). These mechanisms enable them to respond rapidly to the environmental changes, such as entry of the host (Dirita and Mekalanos, 1989; Chuang et al., 1993; Casadevall and Pirofski, 1999; Liang et al., 2005). This permits the organism not only to resist hostile factors of the host defense system, but to switch on the production of specific virulence factors (pathogenic features of the organism are due, either directly, or indirectly, to the products of these virulence genes). The gene expression moves through many stages, any of which offers an opportunity for regulation (Snyder and Champness, 1997). By far the most common type of regulation occurs at transcription stage. Many regulatory mechanisms have been identified and found to be widespread among bacterial species, allowing either positive (activator genes) or negative (repressor genes) regulation or combination. The transcription of bacterial operons (region on the DNA that includes genes cotranscribed into the same mRNA) is regulated by products of regulatory genes. They can be grouped into superfamilies of bacterial transcriptional regulators, such as the two-component regulatory systems, e.g. the LysR group of regulatory elements and AraC group (Higgins et al., 1992; Cotter and Dirita, 2000). The two-component transcriptional regulators usually consist of a sensor kinase protein that detects particular environmental signals and phosphorylates a response regulator DNA-binding protein that coordinately regulates the transcription of a number of genes. All of the genes under the control of a common regulator are defined as a regulon (Cotter and Dirita, 2000; Sanchez et al., 2004). Literature reported recently that ArlR functions as a positive regulator and also as a negative repressor to directly and/or indirectly mediate the expression of at least 114 genes involved in different functions, including autolysis, cell division, growth, and pathogenesis of S. aureus (Liang et al., 2005). Induction of virulence gene expression can involve the “sensing” of host molecules or environmental cues (pH, osmolarity, temperature etc). The production of
virulence factors including cholera toxin and the toxin-coregulated pilus in the human pathogen *V. cholerae* is strongly influenced by environmental conditions. The *V. cholerae* protein ToxR is an integral membrane protein that acts as a transcription activator in response to environmental signals; it controls expression of a variety of genes related to pathogenicity and is responsible for sensing and integrating the environmental information and controlling the virulence regulon (Zhu *et al.*, 2002; Bina *et al.*, 2003). Also different virulence genes of *B. cereus* are regulated with a pleiotropic PlcR regulator. Among the genes regulated by *plcR* are: *plcA*, encoding phosphatidylinositol-specific phospholipase C (PI-PLC); *plc*, encoding phosphatidylcholine-preferring phospholipase C (PC-PLC); *nhe*, encoding the Non-Haemolytic-Enterotoxin (NHE); *hbl*, encoding Hemolytic enterotoxin BL (HBL); and genes specifying a putative S-layer like surface protein and a putative extracellular RNase (Okstad *et al.*, 1999). Role of PlcR regulon in the transcription of at least other 15 genes potentially involved in bacterial bacterial virulence was suggested for *B. thuringiensis* (Agaisse *et al.*, 1999). The PlcR is reported to be activated by a cell-cell signaling peptide, PapR (Slamti and Lereclus, 2002; Slamti and Lereclus, 2005).

### 1.1.5 Mechanisms of action of protein toxins on target (eukaryotic) cells

Toxin effects on target eukaryotic cells can be grouped in two major categories:

- type I effects induced by toxins strictly working on the cell surface (at and from the cell cytoplasmic membrane) without entering the inner cell perimeter (cytosol)

- type II effects induced by toxins ultimately affecting specific molecular targets within the cytosol, after binding to the specific sites on the cell surface and translocation through the membrane

#### 1.1.5.1 Toxins strictly working on the surface of the host cell

Toxins belonging to this group can exhibit toxic effect via two different molecular mechanisms: either by damage of the cytoplasmic membrane or by biological effects through the signal transduction process.
Toxins causing disruption of the cell membrane (cytolysins)

These toxins, produced by both Gram positive and negative bacteria, constitute ca. 35% of the protein toxin repertoire. Their nomenclature (although proper nomenclature requires more attention (Granum et al., 1995)) can be derived from the type of the target cells (hemolysins targeting red blood cells, leucotoxins acting on leucocytes etc). Three major factors (i.e. presence of specific toxin-binding sites, capacity of cells to withstand the attack and accessibility of cells) determine the types of cells that will become targets for toxin attack (Bhakdi et al., 1998). The membrane damage induced by protein toxins can be caused by enzymatic action of toxins on phospholipids in the membrane or by pore (channel) formation via insertion of toxins into the phospholipid-cholesterol bilayer (Songer, 1997; Bhakdi et al., 1998; Goldfine et al., 1998; Titball, 1999; Alouf, 2000; Tweten et al., 2001; Tweten, 2005).

Membrane damaging toxins with enzymatic activity

These toxins belong to the group of phospholipases A, C and D. Their activity hydrolyzes phospholipids in the membrane bilayer, leading to the membrane destabilization (Sears and Kaper, 1996; Balfanz et al., 1996; Songer, 1997). An example of these toxins and their respective producing microorganisms is given in Table 1-4.

Pore-forming toxins

These toxins are widely distributed proteins which form lesions in biological membranes and can be of two types, namely RTX (repeats-in-structural-toxins) toxins (produced by Gram negative bacteria and either secreted into the culture fluid or attached to the cell surface) and cholesterol binding toxins (produced by Gram positive bacteria).

RTX toxins are a family of related exotoxins with hemolytic, leucotoxic and leukocyte-stimulating activities that are produced by a diverse array of Gram negative bacteria. Most RTX toxins are proteins with a molecular mass of 100-200 kDa (Frey and Kuhnert, 2002).
The cholesterol-dependent cytolysins (CDCs) constitute a large family of pore-forming toxins produced by Gram positive pathogens, which function exclusively on cholesterol-containing membranes. A member of this family, listeriolysin O (LLO), is produced by *Listeria monocytogenes* (Dramsi and Cossart, 2002). *B. cereus* group is also known to produce these cytolysins, namely cereolysin O and anthrolysin O, produced by *B. cereus* and *B. anthracis*, respectively (Beecher *et al.*, 2000; Shannon *et al.*, 2003). In the case of lack of cholesterol these toxins fail in their biological activity. Reported data (Giddings and Tweten, 2003) revealed that significant depletion of cholesterol from the erythrocyte membrane stalls these toxins in the prepore complex. Therefore, the depletion of membrane cholesterol prevents the transmembrane pore formation (Giddings *et al.*, 2003). Table 1-5 shows a number of pore-forming toxins.

**Table 1-4: Toxins causing enzymatic damage of host’s cell membrane (Lucchesi and Domenech, 1994; Ingham and Pemberton, 1995; Alouf, 2000).**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Toxin (name from the literature)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>α</td>
</tr>
<tr>
<td><em>Clostridium sordellii</em></td>
<td>β</td>
</tr>
<tr>
<td><em>Clostridium novyi</em></td>
<td>β &amp; γ</td>
</tr>
<tr>
<td><em>Clostridium haemolyticus</em></td>
<td>Cytolytic phospholipase C</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Phospholipase C</td>
</tr>
<tr>
<td><em>Pseudomonas aureofaciens</em></td>
<td>Phospholipase C</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>Non-haemolytic phospholipase C</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Hemolysins</td>
</tr>
<tr>
<td><em>Vibrio damsela</em></td>
<td>Phospholipase C</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>β toxin</td>
</tr>
</tbody>
</table>

**Receptor targeting toxins**

These toxins bind to specific receptors on the cell surface and trigger intercellular processes via transmembrane signaling and without first being internalized (Alouf, 2000). Into this groups *E. coli* heat-stable enterotoxin I and superantigens
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

(enterotoxins and toxic-shock 1 toxin of *S. aureus, S. pyogenes* pyrogenic exotoxins A and C) as indicated in Table 1-6.

**Table 1-5: Examples of bacterial membrane damaging protein toxins (pore-formation).**

<table>
<thead>
<tr>
<th>Organism/toxin</th>
<th>Target</th>
<th>Disease</th>
<th>Toxin implicated in disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas hydrophila/aerolysin</em></td>
<td>Glycophorin</td>
<td>Diarrhea</td>
<td>(yes)</td>
</tr>
<tr>
<td><em>Clostridium perfringens/perfringolysin O</em></td>
<td>Cholesterol</td>
<td>Gas gangrene</td>
<td>sugested yes</td>
</tr>
<tr>
<td><em>Bacillus anthracis/anthrolysin O</em></td>
<td>Cholesterol</td>
<td>Anthrax</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus/cereolysin O</em></td>
<td>Cholesterol</td>
<td>Gastrointestinal and non-gastrointestinal diseases</td>
<td>?</td>
</tr>
<tr>
<td><em>Escherichia coli/hemolysin</em></td>
<td>Plasma membrane</td>
<td>UTIs</td>
<td>(yes)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes/listeriolysin O</em></td>
<td>Cholesterol</td>
<td>Foodborne systemic illness Meningitis</td>
<td>(yes)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus/α-toxin</em></td>
<td>Plasma membrane</td>
<td>Abscesses</td>
<td>(yes)</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae/pneumolysin</em></td>
<td>Cholesterol</td>
<td>Pneumonia</td>
<td>(yes)</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes/streptolysin O</em></td>
<td>Cholesterol</td>
<td>Strep throat SF</td>
<td>?</td>
</tr>
</tbody>
</table>

* a UTI-Urinary tract infection, SF-Scarlet fever; b (yes), role in pathogenesis has been shown in animal model or appropriate cell culture; ?, unknown.

**Table 1-6: Examples of bacterial superantigenic toxins.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Toxin</th>
<th>Mode of action</th>
<th>Target</th>
<th>Disease</th>
<th>Toxin implicated in disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Enterotoxins</td>
<td>Superantigen</td>
<td>TCR</td>
<td>Food poisoning</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Exfoliative toxins</td>
<td>Superantigen</td>
<td>SSS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toxic-shock toxin</td>
<td>Superantigen</td>
<td>MHC II</td>
<td>TSS</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Superantgens</td>
<td>SF/TSS</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>Pyrogenic exotoxins</td>
<td>Superantgen</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a TCR-T cell receptor, MHC-II-Major Histocompatibility Complex class II ; b Yes, strong causal relationship between toxin and disease

Processes referred to as signal transduction often involves a sequence of biochemical reactions inside the cell, which are carried out by enzymes and linked through second messengers. Some bacterial toxins can influence host cell differentiation process being also controlled by signaling pathways. Similarly, signal transduction that is normally initiated by extracellular regulators that bind to cell surface receptors is intimately involved in the choice between apoptosis and cell growth and division, so it is not surprising that some of the toxins that interfere with signaling can affect that...
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

process as well. As a result, some toxins can induce or inhibit apoptosis. Within signal transduction toxin converts one kind of signal or stimulus into another. Examples of toxins acting as second messenger activators are shown in Table 1-7.

Toxins that stimulate growth or inhibit apoptosis or differentiation in many ways behave like tumor promoters or inhibitors of tumor suppression. This is of particular concern for chronic infections. The possible role of bacteria in carcinogenesis has a long and controversial history that began shortly after the linkage between bacteria and disease was established (Lex, 2005).

Table 1-7: Examples of bacterial protein toxins acting as activators of second messenger pathways. Modified from Schmitt et al. (1999).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Toxin a</th>
<th>Mode of action</th>
<th>Target</th>
<th>Disease a</th>
<th>Toxin implicated in disease b</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>CNF</td>
<td>Deamidase</td>
<td>Rho G-proteins</td>
<td>UTIs ?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LT</td>
<td>ADP-ribosyltransferase</td>
<td>G-proteins</td>
<td>Diarrhea</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>ST</td>
<td>Stimulates guanylate cyclase</td>
<td>guanylate cyclase receptor</td>
<td>Diarrhea</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>CLDT</td>
<td>G2 block</td>
<td>Unknown</td>
<td>Diarrhea</td>
<td>(yes)</td>
</tr>
<tr>
<td></td>
<td>EAST</td>
<td>ST-like²</td>
<td>Unknown</td>
<td>Diarrhea</td>
<td>?</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>edema factor</td>
<td>Adenylate cyclase</td>
<td>ATP</td>
<td>Anthrax</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>dermonecrotic toxin</td>
<td>Deamidase</td>
<td>Rho G-proteins</td>
<td>Rhinitis (yes)</td>
<td></td>
</tr>
<tr>
<td><em>Cordesia pertussis toxin</em></td>
<td>pertussis toxin</td>
<td>ADP-ribosyltransferase</td>
<td>G-protein(s)</td>
<td>Pertussis</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>C2 toxin</td>
<td>ADP-ribosyltransferase</td>
<td>Monomeric G-actin</td>
<td>Botulism</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>C3 toxin</td>
<td>ADP-ribosyltransferase</td>
<td>Rho G-protein</td>
<td>Botulism</td>
<td>?</td>
</tr>
<tr>
<td><em>Clostridium difficile</em></td>
<td>toxin A</td>
<td>Glucosyltransferase</td>
<td>Rho G-protein(s)</td>
<td>Diarrhea/PC (yes)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>toxin B</td>
<td>Glucosyltransferase</td>
<td>Rho G-protein(s)</td>
<td>Diarrhea/PC</td>
<td>?</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>cholera toxin</td>
<td>ADP-ribosyltransferase</td>
<td>G-protein(s)</td>
<td>Cholera</td>
<td>Yes</td>
</tr>
</tbody>
</table>


b Yes, strong causal relationship between toxin and disease; (yes), role in pathogenesis has been shown in animal model or appropriate cell culture; ?, unknown.
1.1.5.2 Toxins acting ultimately in the cytosol

A great number of bacterial protein toxins enters the cell cytosol inducing intracellular damages, often by enzymatic activity imposed onto cell mechanisms causing failure of essential cell functions (Alouf, 2000). “AB” toxins with all variations mentioned belong to this group.

Regardless of the mechanism, the substrate for the toxic activity (in the host) may be a component of tissue cells, organs, or body fluid. Usually the site of damage caused by the toxin indicates the location of the substrate for that toxin. Terms such as enterotoxin, neurotoxin, leukocidin or hemolysin are sometimes used to indicate the target site of some well-defined protein toxins.

Some protein toxins exhibit very specific cytotoxic activity by attacking only specific types of host cells. For example, tetanus or botulinum toxins attack only neurons. Some other bacterial toxins (as produced by staphylococci, streptococci, clostridia, etc.) have fairly broad cytotoxic activity and cause nonspecific death of various cells and tissues, eventually resulting in necrosis. A few bacterial toxins that are obviously causing animal death are simply referred to as lethal toxins, and even though the tissues affected and the target sites may be known, the precise mechanism by which death occurs is not understood (e.g. anthrax toxin lethal factor-LF).

1.1.6 Role of bacterial protein toxin in foodborne diseases

Toxin associated diseases account for a big part of the public health problems related to the foodborne diseases. As already briefly mentioned in the beginning of this chapter toxigenic bacteria can be characterized according to the different variants of clinical effects that are recognized in regards to foodborne bacterial diseases:

1. Bacteria causing infection
2. Bacteria colonizing host before producing toxins
3. Bacteria producing toxins in intestines without interaction with the host
4. Bacteria producing toxins in foods, which as such upon ingestion cause a disease
The necessity of toxin presence is obvious for the last three types of disease, while necessity of ingestion of live bacterial cells (or possibly spores) is a requirement in the first three types. The second and the third type, which require live bacterial cells to be present in the host prior to the toxin production that will actually cause a disease, are seemingly designated as foodborne toxin mediated (or associated) infection. When presence of live bacteria cells is not required (type 4 in the above given list), but instead preformed toxin is a causative agent of a disease, one deals with a foodborne intoxication (Mossel et al., 1995; Casadevall and Pirofski, 1999). Number of toxigenic microorganisms, such as B. cereus, can exhibit the potential to induce both toxin-mediated infections and intoxications (Hammer et al., 2001).

One of the clinically important differences in characteristics of intoxications and toxin mediated infection is a shorter incubation time with rapid onset characteristic symptoms of the former one. It is believed that the toxin production in the host is a result of a microbial attempt to exploit the host as an ecological niche rather than inter-microbe competition (Williams and Clarke, 1998).

Certain toxin producing bacteria are selectively pathogenic. This could be either due to the disability of the toxin to provoke disease in one host (e.g. lack of receptors on sites for toxin action for example) or the toxin is selectively expressed in different host. A classical example can be found with Shiga-like toxin of E. coli 0157, which has emerged as a severe human pathogen, but not effecting cattle which is a natural vector of this toxin. A possible explanation may be found in the food preparation methods, usage of antibiotics and human hygiene which result in a significantly different environment for microbes in human host as opposed to those in animals. This can result in the selective pressure for toxin production (Williams and Clarke, 1998).

Foodborne diseases may induce different symptoms, most of them being related either to emesis and/or diarrhea with different severity and other accompanying symptoms (see part 1.2 of this literature study). Although the largest number of foodborne toxins are enterotoxins (toxins acting on mucosal cells of intestinal tract), some very potent toxins which are transmitted by foods, do not belong to this group, as is the case with neurotoxic botulinum toxin (Oguma et al., 1997; Alouf, 2000; Barth et al., 2004).
1.1.7 Analytical methods for bacterial protein toxins causing intoxications and toxin-mediated infections

Food poisoning due to bacterial toxins can be caused by the ingestion of toxins (e.g. S. aureus enterotoxin, B. cereus and C. botulinum food poisoning) which are preformed in the food, or by the ingestion of food containing large numbers of bacterial cells which then release toxins in the gastrointestinal tract (e.g. C. perfringens food poisoning). Therefore, a need to detect toxins in food and clinical samples requires permanent improvements in existing techniques and methods, as well as the development of new ones.

In general, detection methods for bacterial toxins can be grouped on the basis of underlying principle into bioassays, immunological assays, chemical assays, and instrumental analysis, variations of which are numerous. As a special category, molecular PCR methods are developed for detection of genes encoding for toxin production (Berrada et al., 2006). All these techniques can be also used for detection of other natural toxins, such as mycotoxins, phytotoxins etc. Often a combination of techniques and principles is brought together to provide better (faster, more sensitive, more reliable, easier-to-use, more specific, more robust) detection of a given toxin. An example is immuno PCR (Gofflot et al., 2004; Gofflot et al., 2005) that brings together the sensitivity of classic or real-time PCR amplification with specificity of antigen-antibody recognition.

All existing methods result in an array of sensitivities, specificities and robustness. None of the methods meets completely all the requirements for every application and often a combination of two is needed for a final judgment on toxin presence in the sample (Alouf, 2000). In the scope of this Ph.D. research the attention has been given to the bioassays and immunological assays for detection of B. cereus emetic toxin and S. aureus enterotoxins and therefore some more detailed information is provided thereon.

1.1.7.1 Bioassay and related methods

Qualitative (presence or absence) and quantitative (extent of presence, e.g. concentration) information on presence of bacterial toxin (or other virulence factors
for that matter) would ideally include an additional qualitative parameter, defined as a presence or absence of biological (toxic) activity. While other assays mentioned above provide information on whether the sought toxin is present in the inspected food sample, they do not give essential information on residual biological activity, which could have been lost during the processing of food. For this purpose, experimental animals are employed were feasible. When it is not feasible to use whole animals or animal systems, a variety of tissue and cell cultures can be deployed to provide information on the biological activity of present toxins (Jay, 2000a). Application of bioassays has however two major drawbacks, both being worth of consideration. Firstly, an inherent limitation of bioassay is its indirect detection of toxins, as well as, detection of the overall sample toxicity. While this kind of qualitative characterization provides valuable information on potential threat to the public health, posed by the analyzed sample, it hardly provides assurance of unique presence of the sought toxin. In other words, other toxic compounds may result in the same effect in the testing animal, tissue or cell, although the mechanism of action can be different. For this reason a bioassay would ultimately seek a confirmation by other detection techniques. Secondly, the use of animals in research is a significant ethical and political matter. Much of the debate on this issue revolves around the relative value, often referred to as 'moral value', of humans and animals. The main question arising is who takes the precedence when the rights of human beings come into conflict with the rights of laboratory animals. Today there exists a wide spectrum of views on this subject, ranging from those concerned with animal 'rights' to those who view animals only as a resource to be exploited. The raise in the global awareness of these ethical issues will have eventually an implication on the consumer perception of the safety in the food chain. Seeing the historical approach reflected in quotation from Matthew “No one is defined by what goes into his mouth; only by what comes out of it...” advances in the foods safety perception and thereby related research become apparent (Grigorakis, 2005). An overview of some bioassays, with whole animals (Table 1-8), and assays using tissue-cell cultures (Table 1-9), is provided.
### Table 1-8: Whole-animal bioassay methods for detection of bacterial toxins.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Toxin a</th>
<th>Method</th>
<th>Sensitivity b</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Diarrheal enterotoxins</td>
<td>Monkey feeding</td>
<td>NA</td>
<td>(Kramer and Gilbert, 1989; McKillip, 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rabbit ileal loop</td>
<td>NA</td>
<td>(Beecher et al., 1993b; McKillip, 2000; Rowan et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vascular permeability</td>
<td>NA</td>
<td>(Kramer and Gilbert, 1989; Granum et al., 1993; Beecher and Wong, 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guinea-pig skin emesis</td>
<td>NA</td>
<td>(Glatz and Goepfert, 1973)</td>
</tr>
<tr>
<td></td>
<td>Emetic toxin (cereulide)</td>
<td>Suncus murinus ED&lt;sub&gt;50&lt;/sub&gt; 12.9 µg kg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>(Agata et al., 1995; Jay, 2000a)</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>A, B, E, F, G</td>
<td>Mouse lethality</td>
<td>5-10 pg of BoTN/A toxin</td>
<td>(Gilligan et al., 1983; Brin, 1997; Wictome and Shone, 1998; Kelch et al., 2000; Hall et al., 2004)</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>CPE</td>
<td>Mouse ileal loop</td>
<td>1.0 µg (90 minutes test)</td>
<td>(Yamamoto et al., 1979)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rabbit ileal loop</td>
<td>6.25 µg</td>
<td>(Duncan and Strong, 1968; Miyakawa et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guinea-pig skin</td>
<td>60-125 µg</td>
<td>(Labbe, 1989)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>LT</td>
<td>Rabbit ileal loop</td>
<td>NA</td>
<td>(Cabalar et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>ST</td>
<td>Suckling mouse</td>
<td>NA</td>
<td>(Lovett and Peeler, 1984; Al Majali et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rabbit ileal loop</td>
<td>NA</td>
<td>(Whipp et al., 1975)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>SEB</td>
<td>Guinea-pig skin</td>
<td>0.1-1.0 pg</td>
<td>(Jay, 2000a)</td>
</tr>
<tr>
<td></td>
<td>All enterotoxins</td>
<td>Rhesus monkey emesis</td>
<td>5 pg per 2-3 kg body weight</td>
<td>(Minor and Marth, 1976; Jay, 2000a)</td>
</tr>
<tr>
<td></td>
<td>SEA, SEB</td>
<td>Suckling kitten emesis</td>
<td>0.1-0.5 µg per kg body weight</td>
<td>(Kuse et al., 2000; Jay, 2000a)</td>
</tr>
<tr>
<td></td>
<td>SEA, SEB, SEC</td>
<td>Mouse assay with 150 µg LPS/mouse</td>
<td>10-15 µg/mouse</td>
<td>(Stiles et al., 1993)</td>
</tr>
</tbody>
</table>

a CPE-C. perfringens enterotoxin, LT-Heat labile enterotoxin, ST-Heat stable enterotoxin, SE(A, B, C)-S. aureus enterotoxin (A, B, C); b BoTN/A-Botulinum neurotoxin A, NA-Not Available
### Table 1-9: Cell based bioassay methods for detection of bacterial toxins.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Toxin</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Diarrheal enterotoxins</td>
<td>Human fetal intestinal cells</td>
<td>(Jay, 2000a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vero cells</td>
<td>(Lindbäck et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caco-2 cells</td>
<td>(Choma et al., 2000; Guinebretiere et al., 2002; EFSA, 2005b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emetic toxin (cereulide)</td>
<td>(Andersson et al., 1998b; Andersson et al., 2004; Rajkovic et al., 2006b; Rajkovic et al., 2006c).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Boar semen cells</td>
<td>(Andersson et al., 1998b; Andersson et al., 2004; Rajkovic et al., 2006b; Rajkovic et al., 2006c).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HEP-2 cells</td>
<td>(Mikami et al., 1994; Finlay et al., 1999; Rowan et al., 2003)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Cholera toxin</td>
<td>CHO cells</td>
<td>(Jay, 2000a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y-1 adrenal cells</td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Enterotoxin</td>
<td>CHO cells</td>
<td>(Johnson and Lior, 1986; Jay, 2000a)</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>Enterotoxin</td>
<td>Vero cells</td>
<td>(Haque et al., 1996; Jay, 2000a)</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Enteritidis Enteroxin</td>
<td>Vero cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CHO cells</td>
<td>(Rumeu et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y-1 adrenal cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vero cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HeLa cells</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>CPE</td>
<td>Rat hepatocytes</td>
<td>(Jay, 2000a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rabbit intestine epithelial cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vero cells</td>
<td>(McClane and Mcdonel, 1981; Singh et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CaCo2 cells</td>
<td>(Singh et al., 2001)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> b</td>
<td>ETEC LT</td>
<td>CHO cells</td>
<td>(Chart, 1998; Nardi et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vero cells</td>
<td>(Cameron et al., 2001; Zanetti et al., 2003; Jo et al., 2004; Fabio et al., 2004; Normanno et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y-1 adrenal cells</td>
<td>(Chart, 1998; Tauschek et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VTEC (STEC)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vero cytotoxin 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HeLa cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vero cytotoxin 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HeLa cells (not all sub-variants)</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>SEA, SEB, SEE</td>
<td>Human peripheral lymphocytes</td>
<td>(Warren, 1977)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Murine spleen cells</td>
<td>(Langford et al., 1978)</td>
</tr>
</tbody>
</table>

a CPE-Clostridium perfringens enterotoxins; LT- Escherichia coli heat-labile enterotoxin
b ETEC-Enterotoxigenic E. coli; VTEC-Verotoxigenic E. coli; STEC-Shiga like E. coli

Chapter 1: Bacterial toxins & enterotoxigenic *Bacillus cereus*: a review

23
1.1.7.2 Immunological assays

Bacterial antigens may be divided into three main types according to their location:

- Cell surface antigens, which are readily accessible to antibody reactions such as the somatic (O), flagellar (H), capsule (K), fimbriae antigens and outer membrane proteins (OMP);
- Non-exposed cellular antigens, present inside the cell wall or in the cytoplasm and inaccessible in an intact organism to antibody reactions;
- Extracellular antigens such as exotoxins, bacteriocins and enzymes (e.g. streptokinase, haemolysins, collagenase, DNase, etc.)

Exotoxins of bacteria are generally highly immunogenic (there are exceptions); the antibody is the antitoxin. The cell wall antigens (O antigens) of Gram negative bacteria are components of LPS (toxicity is associated with the lipid component and immunogenicity is associated with the polysaccharide components). Extracellular polysaccharides (EPS) are produced by moulds during growth in food and culture media can be easily detected immunologically, thus providing a suitable assay for detecting *Aspergillus* and *Penicillium* spp. (Maraz et al., 2005).

An antigen must be of a certain minimum molecular weight (approximately 5000 Da) to induce an immune response. Although the actual area of the antigen that is recognized by the antibody is relatively small, antibodies can distinguish between proteins that differ by only a single amino acid. Many small organic molecules are not antigenic by themselves but can become antigenic and a specific antibody is produced if they are covalently bound to a larger carrier molecule such as a protein. Such small molecules capable of eliciting antibody production are termed hapten. An antibody specific to the hapten moiety of the antigen will bind not only to the conjugated antigen but also to the free hapten (Shen and Louie, 1999; Maraz et al., 2005).

In this case specificity is the ability of an antibody preparation to recognize a single antigen. A desirable level of specificity implies that the antibody is specific for a single antigen, and will not cross-react with any other antigen, and therefore will not provide false positive results. Sensitivity defines the lowest amount of antigen that can
be detected. The most desirable level of sensitivity is that the antibody in the test is capable to identify a single antigen molecule. High sensitivity prevents false negative reactions. Sensitivity and specificity of immuno-assays are mainly determined by the antiserum used. In this respect the use of well selected monoclonal antibodies can be of advantage. Two major disadvantages of immunoassays are being prone to the non-specific reactions, recognition of false positive results is necessary and that they determine antigenicity, which may differ from the actual toxicity (Notermans and Wernars, 1991).

Immunoassays come in many formats and serve for numerous applications (Pohland et al., 1990; Bonwick and Smith, 2004), of which some common are: Enzyme Linked Immuno Sorbent Assay (ELISA), Reverse Passive Latex Agglutination (RPLA), Immuno blotting, Radio Immuno Assay (RIA) etc. The performance of all these assays will be mainly determined by antigen and antibody characteristics. The comparison of different immunological techniques is difficult at least due to three factors of which each one contributes with its advantages and disadvantages to the overall sensitivity of an assay:

- Material used to make a support matrix (wells produced of different plastic materials with different hydrophobic and affinity characteristics, gels etc)
- Biochemical preparation of the antigen and epitope exposure
- Revelation methods (colorimetric, luminescence, fluorescence, radioactivity)

Bacterial protein toxins are one of the most powerful human poisons known and the importance of their accurate detection is obvious beyond any reasonable doubt. However, detection limit, i.e. sensitivity of a given assay for target toxin moiety must be seen in the perspective to the actual needs and goals set as objective of detection. An exposure assessment will give an estimation of the level of bacterial toxin consumed in food, while dose-response relationship will indicate actual intoxicative amounts. Sensitivity of different immunoassays in detection of bacterial and mycotoxins is shown in Table 1-10 and intoxicative doses for some bacterial pathogens and their toxins are outlined in Table 1-11.
Table 1-10: Immunological assays for detection of bacterial toxins and mycotoxins.

<table>
<thead>
<tr>
<th>Method</th>
<th>Organism</th>
<th>Toxin (matrix)</th>
<th>Sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>C. perfringens</td>
<td>Type A toxin</td>
<td>1 ng ml⁻¹</td>
<td>(Jay, 2000b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Type alpha toxin</td>
<td>16 -25 ng ml⁻¹</td>
<td>(Liu and Blaschek, 1996; Naylor et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Type C toxin</td>
<td>100 ng ml⁻¹</td>
<td>(Vaikosen and Ikhhatua, 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Type D toxin</td>
<td>100 ng ml⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE</td>
<td>0.64 ng ml⁻¹</td>
<td>(Piyankarage et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 ng ml⁻¹</td>
<td>(Jay, 2000b)</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
<td>SEA, SEB, SEC, SED, SEE</td>
<td>0.1-1 ng ml⁻¹</td>
<td>(de Oliveira et al., 1994; Goyache et al., 1994; Giletto and Fyffe, 1998; Jay, 2000b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SEH (mashed potato with raw milk)</td>
<td>NA</td>
<td>(Jorgensen et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>B. cereus</td>
<td>NHE</td>
<td>1 ng ml⁻¹</td>
<td>(Rajkovic et al., 2006a)</td>
</tr>
<tr>
<td></td>
<td>C. botulinum</td>
<td>BoNT/A</td>
<td>9 mouse LD₃₀ ml⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BoNT/E</td>
<td>100 mouse LD₃₀</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>LT</td>
<td>290 pg ml⁻¹</td>
<td>(Chart, 1998; Jay, 2000a; Klipstein et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST</td>
<td>140 pg ml⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vero cytotoxin</td>
<td>NA</td>
<td>(Yamada et al., 1994; Chart, 1998)</td>
</tr>
<tr>
<td>Aspergillus spp.</td>
<td></td>
<td>Vero cytotoxin VT₂ VP₁</td>
<td>200 pg ml⁻¹</td>
<td>(Cao et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Fusarium spp.</td>
<td>Aflatoxin B1 (peanut butter)</td>
<td>2.5 ng g⁻¹</td>
<td>(Jay, 2000b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aflatoxin M1 (milk)</td>
<td>0.25 ml⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ochratoxin (barley)</td>
<td>1 ng ml⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fumonisins (feed)</td>
<td>250 ng g⁻¹</td>
<td></td>
</tr>
<tr>
<td>RPLA</td>
<td>C. perfringens</td>
<td>CPE</td>
<td>NA</td>
<td>(Ridell et al., 1998; Sanz et al., 2002; Stagnitta et al., 2002)</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>LT</td>
<td>NA</td>
<td>(Thampuran et al., 2005)</td>
</tr>
<tr>
<td>S. aureus</td>
<td></td>
<td>SEA, SEB, SEC, SED</td>
<td>0.35-0.65 ng ml⁻¹</td>
<td>(Sanjeev and Surendran, 1992; Jay, 2000b)</td>
</tr>
<tr>
<td>B. cereus</td>
<td></td>
<td>HBL</td>
<td>2-4 ng ml (g)⁻¹</td>
<td>(Rajkovic et al., 2005; EFSA, 2005b)</td>
</tr>
</tbody>
</table>

a ELISA-Enzyme Linked Immunosorbent Assay; RPLA-Reverse Passive Latex Agglutination Assay

b NA-Not Available
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

**Table 1-11: Overview of reported toxin doses required for intoxication and/or lethal effect in different hosts.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Toxin a</th>
<th>Minimal dose to induce toxic effect or (ED50 per kg body weight) / host b</th>
<th>LD50 per kg bw or (MLD) / host b</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. hydrophila</em></td>
<td>Aerolysin</td>
<td>0.0275 µg/ rat</td>
<td>7 µg / mice</td>
<td>(Gill, 1982; Ferguson et al., 1997)</td>
</tr>
<tr>
<td><em>B. anthracis</em></td>
<td>Lethal factor</td>
<td>NA</td>
<td>&lt;114 µg/rat</td>
<td>(Gill, 1982)</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>Cereulide (9.8 mg-12.9 mg) / <em>S. murinus</em></td>
<td>15 mg</td>
<td></td>
<td>(Gill, 1982; Agata et al., 1995)</td>
</tr>
<tr>
<td><em>Bordetella</em></td>
<td>pertussis Heat-labile toxin</td>
<td>NA</td>
<td>1-2 µg dose / 11 days chicken embryo</td>
<td>(Calver et al., 1993)</td>
</tr>
<tr>
<td><em>C. botulinum</em></td>
<td>BoNT/A</td>
<td>NA</td>
<td>ca. 1 ng / humans</td>
<td>(Gill, 1982)</td>
</tr>
<tr>
<td></td>
<td>BoNT/B, C, D, E</td>
<td>NA</td>
<td>0.5-1.2 ng / mice</td>
<td>(Gill, 1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.6-1.1 ng / guinea pigs</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.08-1.1 ng rabbits</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5-40 ng / monkeys</td>
<td></td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td>CPE</td>
<td>NA</td>
<td>81-140 µg / mice</td>
<td>(Gill, 1982)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>SEA</td>
<td>(25 µg) / Rhesus monkey</td>
<td>NA</td>
<td>(Harris et al., 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10 µg) / Rhesus monkey</td>
<td></td>
<td>(Stewart, 2005) and references therein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ca. 10 mg) / Rhesus monkey</td>
<td></td>
<td>(Humber et al., 1975)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(144 ± 50 ng) / Humans</td>
<td>0.60 ng ml— of chocolate milk</td>
<td>(Holeckova et al., 2002), therein (Evenson et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>SEB</td>
<td>(0.4 µg) / Humans</td>
<td>NA</td>
<td>(Stewart et al., 2003) and references therein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.9 µg) / Rhesus monkey</td>
<td></td>
<td>(Stewart, 2005) and references therein</td>
</tr>
<tr>
<td></td>
<td>SEC1</td>
<td>(1.0 µg) / Pigtail monkey</td>
<td>&lt; 50 µg</td>
<td>(Gill, 1982; Schlievert et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>SEG</td>
<td>ca. 80 µg / Rhesus monkey</td>
<td>NA</td>
<td>(Munson et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>SEI</td>
<td>150 µg / Rhesus monkey</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SET</td>
<td>0.05-1.0 ng ml— of milk / Humans</td>
<td>NA</td>
<td>(Anonymous, 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1-1.0 µg / Humans</td>
<td></td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>Cholera tx</td>
<td>NA</td>
<td>250 µg / mice</td>
<td>(Gill, 1982)</td>
</tr>
</tbody>
</table>

a Names of toxins are indicated in the abbreviation list;  
b MLD-Minimal lethal dose, NA-Not Available;  
c Rhesus monkeys of 2-3 kg were administrated 100 µg of SEA per animal (2 out of 3 animals exhibited vomiting reaction)
1.1.8 Biological toxins other than bacterial

Except bacterial toxin, also mycotoxins and algae toxins (phycotoxin or marine toxins) are part of the natural toxins repertoire. All these toxins share the potential of entering the food chain and endangering the health of the food consumer. Due to their importance on the basis of prevalence and high consumption of incriminated foods, marine toxins and mycotoxins will be given certain consideration in following headings.

1.1.8.1 Marine biotoxins

Although the most fish or shellfish associated incidents are of viral or bacterial origin (including bacterial toxins), the proportion of intoxications caused by marine biotoxins is increasing (Scoging, 1998). Marine biotoxins are a group of natural toxins that sometimes accumulate in fish and shellfish. Many biotoxins are produced by microscopic marine algae (phytoplankton, including diatoms and dinoflagellates) and can accumulate in fish or shellfish if they ingest these algae. Some of the features of the five most relevant marine biotoxin syndromes are presented in Table 1-12. Common feature of all of them is a lack of sensorial evidence of spoilage (Scoging, 1998; FDA, 2001).

1.1.8.2 Mycotoxins

Mycotoxins are secondary metabolites of certain fungi. They are nonvolatile, relatively low-molecular weight products that may affect exposed persons in a variety of ways. The functions of mycotoxins have not been clearly established, but they are believed to play a role in eliminating other microorganisms competing in the same environment. They are also believed to help parasitic fungi to invade host tissues. The amount of toxins needed to produce adverse health effects varies widely among toxins, as well as among each person’s immune system. Although none of the mycotoxins expresses acute toxicities of the extent of bacterial toxins, they may be wide spread in foods, or in the raw materials for food production (Moss, 1998; Moss, 2002; CAST, 2003).

Some of the most important mycotoxins in food/feed production are outlined in Table 1-13.
## Table 1-12: Overview of the most important syndromes of marine biotoxin poisonings (Scoging, 1998).

<table>
<thead>
<tr>
<th>Disease⁴</th>
<th>Clinical symptoms</th>
<th>Toxins</th>
<th>Associated micro-organisms</th>
<th>Shellfish/ fish</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSP</td>
<td>Numbness in mouth and extremities, ataxia, dizziness, floating sensation, headache, respiratory distress, paralysis, death (gastrointestinal symptoms)</td>
<td>Saxitoxins Neo-saxitoxin Gonyautoxins and carbacoyl derivatives</td>
<td><em>Alexandrium spp.</em> <em>Gymnodinium spp.</em> <em>Pyrodinium spp.</em></td>
<td>Mussels, cockles, clams, scallops, oysters, crab, lobster</td>
<td>Global</td>
</tr>
<tr>
<td>DSP</td>
<td>Diarrhoea, nausea, vomiting, abdominal pain</td>
<td>Okadiac acid Dinophysitis toxins Yessotoxin Pectenotoxins</td>
<td><em>Dinophysis spp.</em> <em>Prorocentrum spp.</em></td>
<td>Mussels, cockles, oysters</td>
<td>Europe, Japan, USA, South America, Australia, New Zealand, Indonesia</td>
</tr>
<tr>
<td>NSP</td>
<td>Numbness in mouth and extremities, ataxia, vomiting, diarrhoea, nausea, abdominal pain, hot/cold temperature reversal, paralysis (death)</td>
<td>Brevetoxins Breve-like toxins</td>
<td><em>Prychodiscus brevis</em> <em>Pseudonitzschia australis</em></td>
<td>Oysters, clams, mussels</td>
<td>USA, Caribbean, Spain, New Zealand</td>
</tr>
<tr>
<td>ASP</td>
<td>Diarrhoea, vomiting, abdominal cramps, headache, disorientation, short-term memory loss, paralysis (death)</td>
<td>Domoic acid</td>
<td><em>Pseudonitzschia spp.</em></td>
<td>Oysters, clams</td>
<td>Canada, USA</td>
</tr>
<tr>
<td>CFP</td>
<td>Tingling/numbness in mouth and extremities, hot/cold temperature reversal, cramps, malaise, ataxia, temporary blindness, death</td>
<td>Ciguatoxins Maiotoxins Scaritoxins Palytoxins</td>
<td><em>Gambierdiscus toxicus</em> <em>Amphidinium spp.</em> <em>Ostreopsis spp.</em> <em>Prorocentrum spp.</em></td>
<td>Groupers, sea bass, snappers, jacks, eels, barracudas, mullet, parrot fish, surgeon fish</td>
<td>Indigenous in tropical regions</td>
</tr>
</tbody>
</table>

⁴ PSP- Paralytic shellfish poisoning, DSP- Diarrhetic shellfish poisoning, NSP- Neurotoxic shellfish poisoning, ASP- Amnesic shellfish poisoning, CFP- Ciguatera fish poisoning.
### Table 1-13: Mycotoxins in food and feed (Mossel et al., 1995; Moss, 1998; Weidenbörner, 2001; Moss, 2002).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mycotoxin/LD$_{50}$ in one day old 50 g duckling (p.o.)</th>
<th>Disease and Effect</th>
<th>Host affected</th>
<th>Lethal doses kg$^{-1}$ food / number of deaths$^a$</th>
<th>Related commodities</th>
<th>Detection techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Aflatoxin B1 / 12 µg</td>
<td>Aflatoxicosis</td>
<td>Humans, dairy cattle, swine</td>
<td>200 µg / 3 (Rice)</td>
<td>Rice, cassava, maize,</td>
<td>ELISA, HPLC, RIA, TLC</td>
</tr>
<tr>
<td><em>A. parasiticus</em></td>
<td>M1 / 16.6 µg</td>
<td>o Primary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. nomius</em></td>
<td>B2 / 84.8 µg</td>
<td>o Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2 / 62 µg</td>
<td>o Chronic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B3 / 12 µg</td>
<td>o Secondary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G1 / 40 µg</td>
<td>Genotoxic, Carcinogenic, Teratogenic, Hepatogenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G2 / 172.5 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium spp.</em></td>
<td>Fumonisin FA$_{1,3}$ 10 mg kg$^{-1}$ bw</td>
<td>Leukoencephalomalacia in horses</td>
<td>Horses, swine, humans</td>
<td>6250-15600 µg / 106 (Maize)</td>
<td>Maize, spices, cereals, beer, bread, noodles</td>
<td>ELISA, GC-MS, HPLC, LC, TLC</td>
</tr>
<tr>
<td></td>
<td>FC$_{1,4}$</td>
<td>Pulmonary endema syndrome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FP$_{1,3}$</td>
<td>Liver cancer in rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oesophageal cancer in humans</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibitors of ceramide synthesis.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Zearalenone</em></td>
<td>4000-10000 mg/rat (No acute toxicity)</td>
<td>Premature thelarche</td>
<td>Swine</td>
<td>NA</td>
<td>Bananas, barley, beans, beer</td>
<td>ELISA, GC-MS, LC, TLC</td>
</tr>
<tr>
<td><em>A. ochraceus</em></td>
<td>Ochratoxin A 20-22 mg kg$^{-1}$ bw/rat</td>
<td>Ochratoxicosis, emetic, nephrotoxic, hepatotoxic, immunoospressive</td>
<td>Pigs, Poultry, Humans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Penicillium verrucosum</em></td>
<td>Ochratoxin A 5.53 mg kg$^{-1}$ bw/trout</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ NA Not Available; $^b$ and some other *Aspergillus* spp.
1.2 **BACILLUS CEREUS, A TOxin PRODUCING FOODBORNE PATHOGEN**

*Bacillus cereus* is a spore-forming, facultative anaerobic microorganism with rod-shaped vegetative cells. Its name is derived from the cell shape (“Bacillus”, gr. “rod”) and colony appearance on the agar surface (“cereus”, gr. “wax”). It became recognized as a food poisoning organism, following publications that described four large outbreaks in Norway (Kramer and Gilbert (1989) and references therein). Since its first reported isolation in 1887, and proofs of food poisoning potential recognized after diarrheal outbreaks in 1950s (ICMSF, 1996a) its role in the food poisoning outbreaks became a growing concern. Today, *B. cereus* is recognized as a versatile pathogen with a long history of food poisoning incrimination (Agata *et al.*, 2002; EFSA, 2005b). Newspapers headlines of deadly intoxications (such as those shown in Figure 1-1: “Too warm refrigerator caused food poisoning, eng., and Figure 1-2: “*Bacillus cereus* was guilty in Kinrooi”, eng.) brought it to the general public attention, increasing the pressure on authorities to set up strict criteria for its presence in the food chain. *B. cereus* food poisonings occur with no constrains year-round and are without any particular geographic distribution.

Although *B. cereus* is indeed known mainly as an agent of food poisoning, other infections caused by this organism have been documented in immuno-compromised patients, including sepsis, meningitis, pneumonia, and wound infections (Musa *et al.*, 1999; Ripabelli *et al.*, 2000; Tuladhar *et al.*, 2000; Gaur *et al.*, 2001; Srivaths *et al.*, 2004). Beecher *et al.*(1995a) suggested that *B. cereus* exotoxins, including hemolysin BL, can cause ocular damage during fulminant endophthalmitis. In these studies, however, Hemolysin BL accounted for only half of the retinal toxicity of *B. cereus* supernatants, suggesting that full virulence is likely a multifactorial process.

Chapter 1: Bacterial toxins & enterotoxigenic *Bacillus cereus*: a review

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31
Biological and immuno-molecular methods for detection of \textit{B. cereus} and \textit{S. aureus} enterotoxins

Chapter 1: Bacterial toxins & enterotoxigenic \textit{Bacillus cereus}: a review

Figure 1-1: Extract from Belgian daily newspaper “De Standaard” on 21st of August, 2003 reporting fatal foodborne outbreak caused by \textit{Bacillus cereus} in cold pasta salad where five children were hospitalized and one did not survive (De Standaard, 2003a).

Figure 1-2: Extract from Belgian daily newspaper “De Standaard” on 22nd of August, 2003 reporting fatal foodborne outbreak caused by \textit{Bacillus cereus} in cold pasta salad where five children were hospitalized and one did not survive (De Standaard, 2003b).
1.2.1 Taxonomy, morphology and biochemical characteristics of *B. cereus* group

*Bacillus cereus* group of Gram positive, aerobic and sporeforming bacteria was increased in number in 1998 to total of 6 species. Namely, classically accepted *B. anthracis*, *B. cereus*, *B. mycoides* and *B. thuringiensis* were joined by *B. pseudomycoides* (Nakamura, 1998) and *B. weihenstephanensis* (Lechner et al., 1998). The expansion of the *B. cereus* group was proposed on the basis of DNA-DNA hybridization and 16S and 23S rRNA sequence similarity of tested isolates (Prüß et al., 1999; Heyndrickx and Scheldeman, 2002; Rasko et al., 2005). Differentiation between the members of *B. cereus* group can be based on the biochemical, morphological and microscopic analysis (Drobniowski, 1993). Methods to determine genetic diversity among environmental isolates of *B. anthracis*, *B. cereus* and *B. thuringiensis* have been demonstrated (Carlson et al., 1994; Helgason et al., 2000; Radnedge et al., 2003). A method was developed to differentiate between *B. cereus*, *B. mycoides* and *B. thuringiensis* using the polymerase chain reaction combined with a restriction endonuclease technique (Manzano et al., 2003). Cloning and nucleotide sequence analysis of *gyrB* of *B. cereus*, *B. thuringinesis*, *B. mycoides*, and *B. anthracis* were reported as able to distinguish between these species (Yamada et al., 1994). Lechner et al. (1998) reported specific sequence differences between the 16S rDNA, the 23S rDNA, the 16S-23S rDNA spacer region and the genes of the major cold-shock protein homologue CspA in a variety of psychrotrophic and mesophilic *B. cereus* and *B. mycoides* strains. Randomly amplified polymorphic DNA analysis separated psychrotrophic strains of both species from the rest of the *B. cereus* group, which were designated *B. weihenstephanensis*, as reported by Lechner et al. (1998). However, not every psychrotrophic species of *B. cereus* group is *B. weihenstephanensis*. Seventeen of the 26 strains tested by Stenfors and Granum (2001) were able to grow 6 °C, but only four were found to be *B. weihenstephanensis*.

Table 1-14 shows classic tests for differentiation among *B. cereus* group members.

Recent reports revealed that cereulide (emetic toxin) producing *B. cereus* strains are restricted to a single evolutionary lineage of closely related strains, which possibly could indicate their recent origin through acquisition of specific virulence factors such as the cereulide synthetase gene. In contrast to these strains, diarrheal toxin producers
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins seem to be very heterogenic and group into different clusters during molecular typing (Ehling-Schulz et al., 2005a).

Table 1-14: Differentiation of members of traditional *B. cereus* group (modified from Drobniewski (1993) and references therein)

<table>
<thead>
<tr>
<th>Properties</th>
<th><em>B. cereus</em></th>
<th><em>B. anthracis</em></th>
<th><em>B. thuringiensis</em></th>
<th><em>B. mycoides</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Lysis by Gamma phage</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penicillin susceptibility</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Crystalline paraspore inclusion</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Growth in 7% NaCl</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine decomposition</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Anthrax toxin and possession of plasmids</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Virulence in mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M’Fadyean reaction in killed mice</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phosphatidylinositol specific</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>phospholipase C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannitol fermentation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Food safety implication</td>
<td>Foodborne</td>
<td>Anthrax</td>
<td>Crystal toxin</td>
<td>Unknown, but isolated from</td>
</tr>
<tr>
<td></td>
<td>disease</td>
<td>insecticide</td>
<td>insecticide</td>
<td>spices</td>
</tr>
</tbody>
</table>

* Plasmids encoding for anthrax toxin, as well as for δ toxin for *B. thuringiensis* can be transferred between species making species to look alike (Prüß et al., 1999) and references, therein);

* +, > 85% of strains positive; +/-, 50% or more of strains positive; +/-, 50% or less of strains positive; (+), usually weakly positive, - more than 90% of strains negative;

* B. mycoides colonies have typical rhizoidal growth on agar surface;

* Although considered no-hemolytic biologically relevant jemolysin mounts were reported (Shannon et al., 2003). The production is thought to be growth medium dependent.

* (EFSA, 2005b; Iurlina et al., 2006)
1.2.2 *B. cereus* properties

1.2.2.1 Microbial description

Seen under the microscope, cells of *B. cereus* are typically 1.0-1.2 µm in diameter and 3.0-5.0 µm in length. Single endospores produced by this microorganism are formed either in central or pericentral position without distention of sporangium (Kramer and Gilbert, 1989) and they are much more resistant than their vegetative counterparts (Brown, 2000). The most important properties of *B. cereus* vegetative cells, as well as, of spores are presented in Table 1-15 and Table 1-16.

<table>
<thead>
<tr>
<th>Property</th>
<th>Parameters</th>
<th>Generally accepted values (ICMSF, 1996a)</th>
<th>Values reported</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative cell growth pH</td>
<td>Lower range</td>
<td>5.0</td>
<td>4.35-4.90</td>
<td>(Kramer and Gilbert, 1989; Fermanian et al., 1994; ICMSF, 1996a; Granum, 1997; Notermans and Batt, 1998)</td>
</tr>
<tr>
<td></td>
<td>Upper range</td>
<td>8.8</td>
<td>9.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Optimum</td>
<td>6.0-7.0</td>
<td>6.0-7.0</td>
<td></td>
</tr>
<tr>
<td>aw</td>
<td>Lower range</td>
<td>0.93</td>
<td>0.912-0.950</td>
<td>ICMSF, 1996a; Granum, 1997; Notermans and Batt, 1998</td>
</tr>
<tr>
<td>T (°C)</td>
<td>Lower range</td>
<td>4</td>
<td>4-15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Upper range</td>
<td>55</td>
<td>35-55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Optimum</td>
<td>30-40</td>
<td>28-40</td>
<td></td>
</tr>
<tr>
<td>Spore T (°C)</td>
<td>Heat resistance</td>
<td>$D_{100}$ 0.3-8 min</td>
<td>$D_{100}$ 0.3-40 min</td>
<td>(Bradshaw et al., 1975; ICMSF, 1996a; Brown, 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium dependent</td>
<td>$D_{100}$ 144 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>large variations</td>
<td>$D_{121.1}$ 2.35-30 min</td>
<td></td>
</tr>
</tbody>
</table>

The characteristics of cereulide and diarrheal toxin producing strains show that these strains differ from each other in psychrotrophic character, heat resistance of their spores, spore germination properties etc (Carlin *et al.*, 2006). These important differences will be in more details shown in section 1.2.6.
Table 1-16: Differences in the resistance between endospores and vegetative cells of *B. cereus* (Toddar, 2005)

<table>
<thead>
<tr>
<th>Property</th>
<th>Vegetative cells</th>
<th>Endospores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic activity</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Macromolecular synthesis</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Heat resistance</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Resistance to chemicals and acids</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Radiation resistance</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Sensitivity to lysozyme</td>
<td>Some sensitive; some resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>Sensitivity to dyes and staining</td>
<td>Sensitive</td>
<td>Resistant a</td>
</tr>
</tbody>
</table>

*a* Spores have a tough outer covering made of keratin and are highly resistant to heat and chemicals. The keratin also resists staining, so specialized procedures are necessary to stain endospores

### 1.2.2.2 Habitat

*B. cereus* is found in a wide range of habitats. Soil is probably the primary source of food contamination, with one gram of soil being able to host $5 \times 10^1$-$4 \times 10^5$ *B. cereus* spores (Christiansson et al., 1999). Consequence of its omnipresence in the nature is a frequent assault of the food chain, influencing both safety and the quality of food production (Drobniewski, 1993; McKillip, 2000). The literature review by Jensen et al. (2003) reported intestinal carriage of *B. cereus* in up to 43% of feces of tested children, as a consequence of *B. cereus* in many food products.

### 1.2.3 *B. cereus* and foodborne poisoning

*B. cereus* (or *Bacillus* spp.) implication has been shown in 33% of all bacterial food poisonings in Norway (1988-1993), 47% in Iceland (1987-1992), 22% in Finland (1992), 8% in the Netherlands (1991). In USA, Japan, Canada, England and Wells, and Denmark this figure is in the range of 0.7-5% (Adams and Moss, 2000).

*B. cereus* is a causative agent of two distinct types of food poisonings, both being related to *B. cereus* toxigenesis. Emetic and diarrheal syndromes are caused by two different types of respective peptide and protein toxins, produced by pathogenic strains of *B. cereus*. While emetic syndrome belongs to the intoxication type of food poisoning, diarrheal syndrome belongs to the toxin-mediated infections. The major difference between two forms of food poisoning is whether the toxin itself (intoxication) or toxin producing cells (toxin-mediated infection) have to be ingested.
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

with the food, to cause the illness (Granum *et al.*, 1995; Alouf, 2000). In this way emetic syndrome of *B. cereus* resembles intoxication with *S. aureus* enterotoxins (SE) and diarrheal syndrome resembles *C. perfringens* enterotoxin (CET) mediated infection. The Table 1-17 shows the most important features of two syndromes, and (Table 1-18) shows that of related virulence factors in comparison to enterotoxins of *S. aureus* and *C. perfringens*, allowing deduction of the connection between properties of each of the toxin and resulting type of foodborne disease. The data reported in Table 1-18 for number of amino acids in the protein chain and its molecular weight may vary between reports of different authors. Differences may partially exist due to the heterogeneity that was already observed in the components of HBL (Schoeni and Wong, 1999).

1.2.3.1 Diarrheal foodborne infection

Haemolysin BL (HBL), Non-Haemolytic Enterotoxin (NHE), cytotoxin K (cytK) and enterotoxin T (bceT) have been grouped into diarrheal enterotoxins (Kramer and Gilbert, 1989; Beecher *et al.*, 1995b; Fermanian and Wong, 2000; McKillip, 2000; Lund *et al.*, 2000; Lindbäck *et al.*, 2004; Fagerlund *et al.*, 2004; Schoeni and Wong, 2005). HBL contains three protein components: a binding component B, and two lytic components L1 and L2. Each component is encoded by its own gene, with *hblA* encoding B component (Heinrichs *et al.*, 1993), *hblC* and *hblD* encoding L1 and L2 component, respectively (Ryan *et al.*, 1997). NHE also consists of three different proteins, A, B, and C and all genes (*nheA*, *nheB* and *nheC*) were sequenced (Granum *et al.*, 1999). Genes encoding for HBL and NHE are grouped on closely related operons (Hansen and Hendriksen, 2001).

In recent study involving 100 *B. cereus* strains, including clinical isolates and isolates from food remnants connected to food-borne outbreaks, as well as isolates from diverse foodstuffs and the environment, it was found that 42% of the tested strains harbored genes encoding for HBL and 99% for NHE. The production of all NHE and HBL components were analyzed and, in culture supernatants, detectable levels of HBL and NHE were found for 100% of *hbl*-positive and 96% of *nhe*-positive strains (Moravek *et al.*, 2006).
### Table 1-17: Comparison of syndromes caused by toxins of B. cereus, S. aureus and C. perfringens

<table>
<thead>
<tr>
<th>Bacteria / Syndrome / Toxin</th>
<th>B. cereus</th>
<th>S. aureus</th>
<th>C. perfringens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms</td>
<td>Abdominal pain, cramps, watery diarrhea and occasionally nausea</td>
<td>Nausea, vomiting, malaise and ultimately a fatal liver failure</td>
<td>Intense abdominal cramps, diarrhea and flatulence</td>
</tr>
<tr>
<td>Incubation time (h)</td>
<td>8-24 (or longer depending on the dose and host susceptibility)</td>
<td>0.5-5</td>
<td>1-5</td>
</tr>
<tr>
<td>Resolution time (h)</td>
<td>8-24 (up to several days in severe cases)</td>
<td>6-24</td>
<td>Within 24</td>
</tr>
</tbody>
</table>
| Intoxication/Infection dose | Ingestion of more than 10^6 CFU of diarrheal toxin producing B. cereus strains | 100 ng of ingested toxin, 0.05 ng ml^{-1} of food (produced when S. aureus counts reach ca. 10^5 CFU ml^{-1} (g^{-1}) | 10^6-10^7 CFU g^{-1} of food (ingested vegetative cells produce CPE during intestinal sporulation possibly under impact of bile and stomach acids)
| Food involved               | Milk and meat containing products, soups, vegetables, puddings | Meat and milk containing products, creams, chickens salads, canned mushrooms | Meats, meat products, gravy, cooked beans, stews, soups |
| Toxin stability             | 5 minutes at 56 °C | 90 min at 121 °C | 5 minutes at 60 °C |
| pH                          | 4.8-8      | 2.4-11     | 5-10           |
| Proteinases                 | Non-resistant | Resistant | Increased activity |

Reference

- Kramer and Gilbert, 1989; Granum, 1990; Granum, 1994; Granum and Lund, 1997
- Mahler et al., 1997; Paananen et al., 2002; Agata et al., 2002; Jakschelainen et al., 2003a; Dierick et al., 2005; Rajkovic et al., 2006c
- Bergdoll, 1989; de Almeida and Nader, 2000; Anonymous, 2001; Capita et al., 2002; Cenci-Goga et al., 2003; Le Loir et al., 2003; Bennett, 2005

---

**Notes:**

- a 8-10 mg with condition that gastric juice is neutralized with sodium bicarbonate;
- b These characteristics are dependent on the SE type, concentration, purity, solvent etc.
- Resistance to proteolytic enzymes vary with pH, too (Baird and Lee, 1995; Kijek et al., 2000).
### Table 1-18: Overview of foodborne protein/peptide enterotoxins of S. aureus, B. cereus and C. perfringens

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Toxins and components</th>
<th>Gene localization&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AA (kDa)</th>
<th>Mass (kDa)</th>
<th>Possible mode of action</th>
<th>Chemistry</th>
<th>Overall function&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td>SEA</td>
<td>B</td>
<td>233</td>
<td>27.1</td>
<td>Stimulates vagus nerve inducing emetic response.</td>
<td>Single chain</td>
<td>E, SA</td>
<td>(Alouf, 2000)</td>
</tr>
<tr>
<td></td>
<td>SEB</td>
<td>B</td>
<td>239</td>
<td>28.3</td>
<td>Superantigenic over-stimulation of T-cells.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEC</td>
<td>C</td>
<td>239</td>
<td>27.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEG</td>
<td>C</td>
<td>239</td>
<td>27.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>C</td>
<td>239</td>
<td>27.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>C</td>
<td>239</td>
<td>27.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SED</td>
<td>P</td>
<td>228</td>
<td>26.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEE</td>
<td>B</td>
<td>230</td>
<td>26.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>NA</td>
<td>233</td>
<td>27.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEH</td>
<td>NA</td>
<td>218</td>
<td>27.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEI</td>
<td>NA</td>
<td>218</td>
<td>24.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. cereus</strong></td>
<td>HBL&lt;sup&gt;e&lt;/sup&gt;</td>
<td>B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>344</td>
<td>37.5</td>
<td>Pore-forming, membrane damaging or catabolic activity of L component(s) within the target</td>
<td>Multifactorial toxin</td>
<td>E, H, VP, DN, CYT</td>
<td>(Heinrichs et al., 1993; Beecher and Wong, 1997; Ryan et al., 1997; Okstad et al., 1999; Schoeni and Wong, 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L&lt;sub&gt;1&lt;/sub&gt;</td>
<td>384</td>
<td>38.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L&lt;sub&gt;2&lt;/sub&gt;</td>
<td>447</td>
<td>43.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHE&lt;sup&gt;e&lt;/sup&gt;</td>
<td>NA</td>
<td>360</td>
<td>41.0</td>
<td>Inhibition of protein synthesis</td>
<td>Multifactorial toxin</td>
<td>E, CYT</td>
<td>(Granum et al., 1999; Rowan et al., 2001; Rowan et al., 2003; Lindbäck et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>C</td>
<td>360</td>
<td>41.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>NA</td>
<td>372</td>
<td>39.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHEB</td>
<td>NA</td>
<td>329</td>
<td>36.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
<td>329</td>
<td>36.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHEC</td>
<td>NA</td>
<td>329</td>
<td>36.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
<td>329</td>
<td>36.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cereulide</td>
<td>P&lt;sup&gt;e&lt;/sup&gt;</td>
<td>12</td>
<td>1.2</td>
<td>Membrane damaging K&lt;sup&gt;+&lt;/sup&gt; ionophore</td>
<td>Single cyclopeptide</td>
<td>E, MT, VF</td>
<td>(Isobe et al., 1995; Agata et al., 1995; Hoton et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
<td>12</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EnterotoxinT</td>
<td>NA</td>
<td>366</td>
<td>41.0</td>
<td>Pore-forming and necrosis of epithelial cells</td>
<td>Single chain</td>
<td>NA&lt;sup&gt;&lt;sup&gt;1&lt;/sup&gt;&lt;/sup&gt;</td>
<td>(Choma and Granum, 2002)</td>
</tr>
<tr>
<td></td>
<td>Cytotoxin K</td>
<td>NA</td>
<td>356</td>
<td>36.9</td>
<td></td>
<td>Single chain</td>
<td>NA&lt;sup&gt;&lt;sup&gt;1&lt;/sup&gt;&lt;/sup&gt;</td>
<td>(Lund et al., 2000; Hardy et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
<td>356</td>
<td>36.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C. perfringens</strong></td>
<td>CPE</td>
<td>P</td>
<td>319</td>
<td>35.3</td>
<td>Pore-formation, Ca&lt;sup&gt;2+&lt;/sup&gt; influx, cell oncosis and apoptosis, DNA cleavage, mitochondrial membrane depolariz.</td>
<td>Single chain</td>
<td>E, CYT, HP, E. electrolyte secretion</td>
<td>(Melville et al., 1994; Smedley et al., 2005; Miyakawa et al., 2005)</td>
</tr>
</tbody>
</table>

<sup>a</sup> C-chromosomal; P-plasmid; B-bacteriophage. All sequences and data are as stored in UniProt database on 10.03.2006 and do not include signal sequence when possible; <sup>b</sup> E-enterotoxigenic; VP-Vascular permeability factor; DN-dermonecrotic; CYT-cytotoxigenic; H-hemolytic; SA-superantigen; VF-Vacuole formation, T-Mitochondrial toxin, HPE-Histopathological effects; <sup>c</sup> B-Binding component, L1 and L2-lytic components; <sup>d</sup> B-Binding component, A and C-lytic components. The optimal ratio reported is 10:10:1 (NheA:NheB:NheC); <sup>e</sup> Each component is encoded by own gene. Three genes are grouped in one operon; <sup>1</sup> Not Available; <sup>2</sup> Indicated gene localization is that of NPRS gene.
Depending on the type of the food, as well as on the handling manner of a food product, consumer exposure can be either to *B. cereus* vegetative cells, spores or combination of the two. This might have an implication on the dynamics of possible diarrheal toxin production in the small intestine, in relation to the survival, germination, growth and intestinal adhesion of *B. cereus* spores and vegetative cells (Andersson et al., 1998a; Clavel et al., 2004). Recent report of Pielaat et al. (2006) presented the hypothetical model of *B. cereus* behavior, fate and production of diarrheal enterotoxins in the host gastrointestinal tract. Outcome of the model showed diarrheal toxin production being dependent on the number of ingested vegetative cells and (or) spores (or combination), and above all on whether ingested *B. cereus* isolates are mesophilic or psychrotrophic. Exposure to the total of $10^5$ (100 g of food contaminated with $10^3$ CFU g$^{-1}$) psychrotrophic cells would according to this model not lead to the toxin-mediated infection, while the same number of mesophilic strains would present a higher risk with bigger probability of causing the disease. Ingestion of psychrotrophic *B. cereus* cells at above mentioned contamination level would more likely cause a problem in the form of $10^5$ ingested vegetative cells, or with $10^5$ vegetative cells plus $10^5$ spores, than with $10^5$ spores only. Consumption of 100 g of foods contaminated with $10^5$ CFU g$^{-1}$ (Dutch standard, (Anonymous, 1994)) is suggested to pose a food hazard in any case. According to the same model, low pH of human stomach would not have a major influence on the final number of vegetative cells reaching the small intestine (even when vegetative cell inactivation is taken into account). The probability that viable *B. cereus* cells will enter the small intestine causing diarrhea is by Clavel et al. (2004) reported to be largely dependent on whether spores or vegetative cells are ingested, on the type of the ingested food and the level of stomach acidity. Using the model gastric medium, Clavel et al. (2004) found that *B. cereus* spores were highly resistant to acidity simulated to be as in human stomach after ingestion. The survival was enhanced when using milk gastric medium and chicken meat gastric medium in comparison to split-pea puree and J-broth gastric medium. In contrast to the spores, vegetative cells were highly susceptible, but less in milk gastric medium. Milk apparently had a protective effect against inactivation by low pH.

No clear evidence, but rather suspicion has been offered for a possible action of preformed diarrheal enterotoxins. While no doubt exists on *B. cereus* ability to
produce these toxins in foods (Rowan et al., 2001), what remains unclear is the impact of the gastrointestinal passage on the biological activity of toxins. Being sensitive to pH of the gastric juice, or to the proteolytic enzymes, it is quite likely that in the vast majority of cases preformed toxin will not pass through the stomach and retain its biological activity. In addition, literature reports that number of B. cereus needed to produce intoxicative amounts of diarrheal enterotoxins is that high that the sensorial properties of implicated food would not be acceptable to the consumer. Moreover, average incubation period of 12 h is too long for intoxication (Granum and Lund, 1997; Granum, 1997). However, under certain conditions the pH in the stomach may raise just enough to allow the toxin to pass to the small intestines undamaged (Wijnands et al., 2002). These particular conditions can occur with elderly consumers and with an intake of large volumes of foods, which in addition can shelter the toxin molecule from the proteolytic gastric enzymes (Alouf, 2000).

1.2.3.2 Emetic foodborne intoxication

The causative agent of emesis causing (inducing vomiting) foodborne poisoning is B. cereus ring structured dodecadepsipeptide (lacton) of ca. 1.2 kDa named cereulide (Isobe et al., 1995). This small peptide (Figure 1-3) consists only of three repeats of 4 amino/oxy amino acids (D-O-Leu-D-Ala-L-O-Val-L-Val) with relatively high presence of L amino acids is noticeable (Isobe et al., 1995; Agata et al., 1995; Granum and Lund, 1997). Vacuolation activity and mitochondrial toxicity are some of the characteristics (Table 1-18) of cereulide that have helped elucidation of its role in food poisoning. In its chemical structure, weight and mitochondrial toxicity is cereulide similar to valinomycin, which is produced by Streptomyces fulvissimus. Both cereulide and valinomycin are potassium selective ionophores (Isobe et al., 1995; Granum and Lund, 1997; Andersson et al., 1998b; Mikkola et al., 1999; Hoornstra et al., 2004). It is exactly this property that is an underlying mechanism of mitochondrial toxicity, used for cereulide detection as shown later in Chapters 2-5. The toxin is highly lipophilic and has no antigenic properties (Granum and Lund, 1997; Andersson et al., 1998c; Mikkola et al., 1999). Cereulide is resistant to heat, pH, and proteolytic activity of pepsin and trypsin (Kramer and Gilbert, 1989) and thanks to these properties, the toxin preformed in food will reach the intestines without losing its biological (toxic) activity in the stomach. Once in the intestine, cereulide will bind
with 5-HT3 receptors inducing an emetic response in the host (Agata et al., 1995). The effect of toxin on natural killer (NK) cells was recently reported, inhibiting the cytotoxicity and cytokine production of NK cells. The mechanism behind was swelling of NK cell mitochondria and ultimate induction of NK cell apoptosis (Paananen et al., 2002). In high concentrations cereulide was reported to cause fatal liver failure and respiratory distress leading to a patient’s death (Mahler et al., 1997; Dierick et al., 2005).

Figure 1-3: A: Cereulide structure in interaction with potassium ion (Paananen, A., 2004), B: Amino acids in cereulide molecule (Isobe et al., 1995)

For a long time it was believed that cereulide is not under the genetic determination, but that it was rather a product of the enzymatic transformations of components occurring in food or culture medium environment (Granum and Lund, 1997). The most recent findings based on the chemical structure of cereulide, which is typical for non-ribosomal synthesized peptides (Marahiel et al., 1997), suggested that the gene of
non-ribosomal peptide synthetase (NRPS) is responsible for cereulide production in emetic strains of *B. cereus* (Horwood *et al.*, 2004; Toh *et al.*, 2004; Ehling-Schulz *et al.*, 2005b). One module of NRPS contains all catalytic activities which are necessary for incorporation of one amino acid residue into the peptide product. Valine activation module in cereulide synthetase (*ces*) gene was reported to be specific for cereulide producing *B. cereus* (one copy of this module being present in the genome of implicated strains). The partially sequenced *ces* was reported to be highly conserved between ten tested emetic strains and as shown with Southern blot was invariably absent in tested non-emetic strains. Although the gene involved in cereulide synthesis was found, its location remained unknown until the work of Hoton *et al.* (2005). This research investigated a potential extrachromosomal location of cereulide genetic determinants using *B. cereus* strains involved in foodborne outbreaks of cereulide intoxication (such as, *B. cereus* 5975c involved in a lethal intoxication case (Dierick *et al.*, 2005)). Performing hybridization with a probe specific for *B. cereus* cereulide positive strains (Ehling-Schulz *et al.*, 2004a), Hoton *et al.* (2005) reported that plasmid, thereby named pCERE01, was found in all five tested strains of cereulide producing *B. cereus*. Additional curing experiment showed that previously cereulide positive strains, once cured from pCERE01 did not show anymore positive results in cereulide specific PCR. They remained negative in boar semen bioassay, as well. These results confirmed direct positive involvement of pCERE01 in cereulide production (Hoton *et al.*, 2005).

### 1.2.4 Enumeration of *B. cereus* and toxin detection

The official method for enumeration of *B. cereus* is currently the one described by ISO 7932:2004 (ISO, 2004). A schematic overview of this protocol is presented in Figure 1-4. The method is based on the usage of Mannitol Egg Yolk Polymyxin (MYP) agar, which apart from the base agar medium contains one selective and one elective supplement, polymixin B sulfate (10^6 IU) and lecithin (egg yolk), respectively. Egg yolk emulsion can be obtained pre-made or can be self made following protocol in ISO 7932. Agar base contains D-mannitol and phenol red as pH indicator. Since *B. cereus* cannot ferment mannitol, no acid will be formed and phenol red will not turn yellow. *B. cereus* colonies will appear as white-pinkish colonies with pink precipitate around them.
However, few notes should be taken into account. If the plates contain numerous mannitol-fermenting microorganisms leading to the production of acid, then the characteristic pink color of *B. cereus* colonies may be reduced or disappear entirely. Some strains of *B. cereus* produce only little or no lecithinase. Colonies of these strains will not be surrounded by a precipitation zone. These colonies should also be subjected to confirmation tests. Confirmation of presumptive colonies is done on sheep blood agar plate on the basis of positive hemolytic reaction, which is characteristic for *B. cereus*. Nevertheless, attention should be paid that not only the size of hemolytic zone may vary between different *B. cereus* isolates, but also that recent findings suggest that strain producing emetic toxin, might have less pronounced (or be completely deprived from) hemolytic properties (Ehling-Schulz *et al.*, 2005a).

As, for the toxin detection, different detection methods for bacterial toxins, including those of *B. cereus*, are extensively elaborated in the first part of this literature study, as well as throughout other chapters of this dissertation (Chapters 2-5). It is, however, interesting to point out the advantages and pitfalls of existing commercial assays that are widely used in research and routine analyses. Careless interpretation of these results may lead to both underestimation and overestimation of problems related to the presence of *B. cereus* toxins.
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Chapter 1: Bacterial toxins & enterotoxigenic *Bacillus cereus*: a review

Figure 1-4: Horizontal method for the enumeration of presumptive *B. cereus*-colony count technique at 30 °C

---

Preparation of the test sample, suspension and initial dilutions

Incubation at 30 °C for 24-48 hours

Counting the presumptive *B. cereus* colonies on each plate (preferably 2 successive dilutions with 150 or less colonies on the plate). The presumptive colonies are large, pink (indicating that mannitol fermentation has not occurred, see 1.2.3.3) and generally surrounded by a zone of precipitation (indicating the production of lecithinase, see 1.2.3.3).

From the selected plates, 5 representative colonies should be picked and stroked on the surface of sheep blood agar.

Incubation at 30 °C for 24 ±2 hours

Interpretation: Presumptive *B. cereus* isolates are those that on MYP agar formed pink colonies with precipitation and gave positive hemolytic reaction on sheep blood agar

*0.1 ml on 1 plate of 90 mm with sterile pipette (or 1 ml on 1 140 mm-plate or on 3 90 mm-plates)*
All of the currently available kits for the detection of *B. cereus*, *C. perfringens* and staphylococcal enterotoxins are portable immunoassays (Brett, 1998). The sandwich ELISA with a solid phase of a microtitre plate is a common format while coated tubes or polystyrene balls are used in some kits. The result may be read by eye (positive or negative) or by computerized measurements of optical density. The other common system is the reversed passive latex agglutination assay (RPLA), where latex beads coated with specific antibody or with normal serum are added to doubling dilutions of the test sample in a microtitre plate. If antigen is present, the latex beads form a diffuse layer due to antigen–antibody reactions creating a lattice, while if antigen is absent the latex beads form a tight button. The difference in the reaction with control and sensitized latex is read by eye. Two rapid tests, based on sandwich ELISA and RPLA, for detection of *B. cereus* enterotoxins are commercially available. Both of the kits are made to detect diarrheal enterotoxins, but the fact that they detect different antigens was not initially evident. Work of Day *et al.* (1994) with fourteen strains of *B. cereus* examining their ability to produce diarrheal enterotoxin by two commercial immunoassay kits and the microslide immunodiffusion assay confirmed that the two commercial assays (BCET-RPLA marketed by Oxoid, Basingstoke, UK and produced by Denka, Japan; and TECRA-VIA, Bioenterprises Pty Ltd, Roseville, Australia) did not detect the same antigen. These results were in accordance with results of Buchanan and Schultz (1992; 1994) who found that the positive controls of the BCET-RPLA and the TECRA-VIA kits are not detected by the other kit. Notermans and Tatini (1993) also showed no correlation between the two kits showing that isolates from a particular outbreak were giving only positive reaction with TECRA-VIA kit. It is now well established that HBL is detected with RPLA and NHE is detected with ELISA based visual assay and that both HBL and NHE can be involved in foodborne outbreaks (Beecher and Wong, 1994; Schoeni and Wong, 2005; EFSA, 2005b). The antiserum used in the BCET-RPLA reacts against L2 component (Table 1-18) of the enterotoxin (Granum *et al.*, 1993). Beecher and Wong (1994) reported that the TECRA-VIA kit antiserum reacted with two proteins of 40 and 41 kDa, which were nontoxic in a vascular permeability assay, and did not react with enterotoxin components. From these results it appears that the BCET-RPLA detects one component of the enterotoxin complex and the TECRA-VIA detects two apparently nontoxic components. It is possible that the TECRA-reactive components participate in human diarrhea, despite being non-reactive in the vascular permeability assay, but
it is also possible that there will be false-negative and false-positive results. Although in general use, the usefulness of two commercial immunoassays for the detection of diarrheal enterotoxin of *B. cereus* remains somewhat unclear and the information provided by these tests is more indicative than final.

Due to the lack of immunogenic properties of cereulide, no kit is currently available for its detection (Kramer and Gilbert, 1989; Rajkovic *et al.*, 2006b).

**1.2.5 Breach in the food safety and food contamination with *B. cereus***

Food contamination originates either directly from the soil or via contaminated equipment that harbors *B. cereus* (Guinebretiere and Nguyen-The, 2003; Moussa-Boudjemaa *et al.*, 2004). Guinebretiere *et al.* (2003) found that soil in which zucchinis were grown held very high counts of *B. cereus* spores (almost $3.9 \times 10^4$ CFU g$^{-1}$). Much less of contamination is coming from the air ($<100$ CFU/m$^3$) (Christiansson *et al.*, 1999). Therefore, foods, such as vegetables and spices that are in direct contact with the soil seem to be the most important carriers of *B. cereus* cells (vegetative and spores) into the food chain. However, occurrence in dairy and meat products is not to be neglected (Kramer and Gilbert, 1989; Notermans *et al.*, 1997; Notermans and Batt, 1998; Christiansson *et al.*, 1999), where it mostly happens via cross-contamination. The problems in milk and milk products are often caused by transfer of contamination from the udder that was previously in contact with grass and soil (Granum, 1997) or possibly due to the fecal contamination of the udder (Altayar and Sutherland, 2006). The numbers of *B. cereus* in feces, $(1.5-5) \times 10^2$ CFU g$^{-1}$ (59-70% of samples found positive), and in soil samples $3-3.9) \times 10^3$ CFU g$^{-1}$ (75-80% of samples found positive) were comparable. The same contamination route can be noted for meats at slaughter. The origin of the contamination (soil, feces etc.) will have an impact on the characteristics of *B. cereus* contaminants. Isolates from feces are more likely to be mesophilic, while those from the soil can be influenced by the period of the year, geographical location, culture grown on the land etc. Different findings in the toxigenic potential of *B. cereus* isolates from different soils are to be observed when results from different parts of the world are compared, and when cultures grown on the specific locations are compared (Altayar and Sutherland, 2006).
Realizing *B. cereus* omnipresence in the environment, its sporeforming character and potential of many strains to elaborate one (or more) of its toxins, problems that food industry has with it becomes an evident issue. The hydrophobicity of its spores allows them to attach to the surfaces of processing equipment (Faille *et al.*, 2002) and ability to form bio-films provide further resource for a repeatable in-house contamination (Andersson *et al.*, 1995; Oosthuizen *et al.*, 2002; Ryu and Beuchat, 2005; Auger *et al.*, 2006). This problem reflects both food safety (caused by toxins produced) and food quality (caused by the proteinase, lipase and phospholipase enzymes produced).

Overview of many foods reported to hold *B. cereus* is given in Table 1-19, Table 1-20 and Table 1-21.

**Table 1-19: Some of the raw materials and half-fabricates used in further processing reported to hold *B. cereus* cells**

<table>
<thead>
<tr>
<th>Group representative</th>
<th>Range of reported <em>B. cereus</em> count (CFU g⁻¹ or ml⁻¹)/ percentage of samples found positive</th>
<th>Involved in outbreak/syndrome[b]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spices</td>
<td>Most samples below 10⁴ and some samples above 10⁴</td>
<td>NR</td>
<td>(Van Netten <em>et al.</em>, 1990) in (EFSA, 2005b)</td>
</tr>
<tr>
<td>Herbs and spices</td>
<td>10⁴ - 10⁷/100%</td>
<td>NR</td>
<td>(te Giffel <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td>Onion powder</td>
<td>ND</td>
<td>YES/diarrhea and vomiting[c]</td>
<td>(Jackson <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Fresh vegetables</td>
<td>10⁴ - ca. 10⁵</td>
<td>NR/ Potential to produce HBL was found positive in ca. 80% of <em>B. cereus</em> isolates (RPLA test)</td>
<td>(Valero <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td>Flour</td>
<td>10⁴/55%</td>
<td>NR</td>
<td>(te Giffel <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td>Cocoa</td>
<td>10⁴ - 10⁵</td>
<td>NR</td>
<td>(Van Netten <em>et al.</em>, 1990) in (EFSA, 2005b)</td>
</tr>
<tr>
<td>Yeast</td>
<td>ND</td>
<td>NR</td>
<td>(Van Netten <em>et al.</em>, 1990) in (EFSA, 2005b)</td>
</tr>
<tr>
<td>Liquid egg yolk</td>
<td>Less than 10⁴/24%</td>
<td>NR</td>
<td>(Van Netten <em>et al.</em>, 1990) in (EFSA, 2005b)</td>
</tr>
</tbody>
</table>

[a]ND-sample was found positive, but exact *B. cereus* count was not determined (or NA-not available to us); [b] NR-Not Reported; [c] mixed Norwalk virus, *B. cereus* and *B. thuringiensis* outbreak; All *B. cereus* isolates belonged to the same phage type and expressed cytotoxic activity characteristic for *B. cereus* enterotoxins; [d] In total, 229 samples (milk, yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices) were analyzed and of these 109 (48%) contained *B. cereus*.
Table 1-20: Examples of RTE meals and prepared foods reported to hold *B. cereus* (except dairy products)

<table>
<thead>
<tr>
<th>Type of Food / Main Component of the dish</th>
<th>Range of reported <em>B. cereus</em> count (CFU g⁻¹ or ml⁻¹) / percentage of samples found positive</th>
<th>Involved in outbreak/syndrome</th>
<th>Occasion</th>
<th>Country, year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>REPFED from restaurants, cafeterias and buffets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPFED puree based on vegetables at the moment of visual spoilage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veggies</td>
<td>10²-10⁷/7.7% of 26 samples</td>
<td>NR</td>
<td>Control: Determination of prevalence and characterization of <em>B. cereus</em> in REPFED (taken on day 0 from the processing site)</td>
<td>Italy, 1997-1998</td>
<td>(Amodio-Cocchieri et al., 1998)</td>
</tr>
<tr>
<td>Rice</td>
<td>10¹-10¹²/12% of 25 samples</td>
<td>NR</td>
<td>Control: Evaluation the level of contamination by <em>B. cereus</em> in RTE foods on the point of sale</td>
<td>France, 1998-1999</td>
<td>(Choma et al., 2000)</td>
</tr>
<tr>
<td>Pasta</td>
<td>10⁶/8.9% of 17 samples</td>
<td>NR</td>
<td>Control: Evaluation the level of contamination by <em>B. cereus</em> in RTE foods on the point of sale</td>
<td>Italy, 1997-1998</td>
<td>(Amodio-Cocchieri et al., 1998)</td>
</tr>
<tr>
<td>Fish</td>
<td>10⁶-10²/21.4% of 14 samples</td>
<td>NR</td>
<td>Control: Evaluation the level of contamination by <em>B. cereus</em> in RTE foods on the point of sale</td>
<td>Italy, 1997-1998</td>
<td>(Amodio-Cocchieri et al., 1998)</td>
</tr>
<tr>
<td>Meat</td>
<td>10⁶/16.7% of 6 samples</td>
<td>NR</td>
<td>Control: Determination of prevalence and characterization of <em>B. cereus</em> in REPFED (taken on day 0 from the processing site)</td>
<td>Italy, 1997-1998</td>
<td>(Amodio-Cocchieri et al., 1998)</td>
</tr>
<tr>
<td>Broccoli</td>
<td>2.5x10⁶/40% of 5 samples after 5 days-22 (25)°C</td>
<td>NR</td>
<td>s of 83 isolates ca. 81% and 87% were positive with BCET-RPLA and TECRA-VIA, respectively; 79% were cytotoxic to Caco cells</td>
<td>France, 1998-1999</td>
<td>(Choma et al., 2000)</td>
</tr>
<tr>
<td>Carrot</td>
<td>1.6x10⁶/60% of 5 samples after 5 days-22 (25)°C 1.6x10⁵/20% of 5 samples after 32 days-10 °C</td>
<td>NR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Courgette</td>
<td>3.2x10⁶/20% of 5 samples after 5 days-22 (25)°C &lt;10⁷/0% of 5 samples after 32 days-10 °C</td>
<td>NR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato</td>
<td>3.2x10⁶/60% of 5 samples after 5 days-22 (25)°C 7.9x10⁵/40% of 5 samples after 32 days-10 °C</td>
<td>NR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Split peas</td>
<td>6.3x10⁶/20% of 5 samples after 5 days-22 (25)°C 1.3x10⁶/20% of 5 samples after 32 days-10 °C</td>
<td>NR</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 1-20: Examples of RTE meals and prepared foods reported to hold *B. cereus* (except dairy products) CONTINUED

<table>
<thead>
<tr>
<th>Type of Food³ / Main Component of the dish</th>
<th>Range of reported <em>B. cereus</em> count (CFU g⁻¹ or ml⁻¹) / percentage of samples found positive</th>
<th>Involved in outbreak/syndrome⁵</th>
<th>Occasion</th>
<th>Country, year²</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMPF gazpacho</td>
<td>MPN ca. 3/22.2%</td>
<td>NR</td>
<td>Control: Prevalence of <em>B. cereus</em> in RMPF gazpacho at the point of sale</td>
<td>Spain, 2000-2001</td>
<td>(Valero et al., 2002)</td>
</tr>
<tr>
<td>RMPF salmorejo</td>
<td>MPN ca. 8/44.4%</td>
<td>NR/4 isolates positive with BCET-RPLA</td>
<td>Control: Prevalence of <em>B. cereus</em> in RMPF salmorejo at the point of sale</td>
<td>Spain, 2000-2001</td>
<td>(Valero et al., 2002)</td>
</tr>
<tr>
<td>RMPF ajoblanco</td>
<td>MPN ca. 3.5/25%</td>
<td>NR</td>
<td>Control: prevalence and behavior of <em>B. cereus</em> in RMPF ajoblanco</td>
<td>Italy, 1998-1999</td>
<td>(Del Torre et al., 2001)</td>
</tr>
<tr>
<td>REPfed gnocchi</td>
<td>Initial contamination and storage at 8°C: &lt;10⁶/33%; at temperature abuse growth in some samples to: &gt;10⁶</td>
<td>NR</td>
<td>Control: prevalence and behavior of <em>B. cereus</em> in REPfed gnocchi</td>
<td>Italy, 1998-1999</td>
<td>(Del Torre et al., 2001)</td>
</tr>
<tr>
<td>REPfed vegetable purees</td>
<td>Bacillus spp. was dominant flora in ca 10⁶ of AMB⁺, 53 isolated <em>B. cereus</em>. 13-24 of <em>B. cereus</em> isolates in products stored at room temperature, 2-9 at 10 °C and 2 at 4 °C</td>
<td>NR</td>
<td>Control: Prevalence of Sporeformers in REPfed</td>
<td>France, 1997-1999 (2 experiments in 2 years)</td>
<td>(Carlin et al., 2000b)</td>
</tr>
<tr>
<td>Cooked foods</td>
<td>(1-5) x 10² of which 70% confirmed as <em>B. cereus</em></td>
<td>82.1% of <em>B. cereus</em> isolates positive with TECRA-VIA and 57.1% with BCET-RPLA</td>
<td>Control: prevalence of enterotoxigenic <em>B. cereus</em> in cooked foods at University canteen</td>
<td>Malaysia, 1992-1994</td>
<td>(Rusul and Yaacob, 1995)</td>
</tr>
<tr>
<td>Chicken and rice meal</td>
<td>10⁵ in chicken and 10⁶ in rice³</td>
<td>Diarrhea involving 300 people</td>
<td>Development of AFLP typing</td>
<td>UK, 1997-1999 (incidences of food poisonings occurred in a wider and different range of time)</td>
<td>(Ripabelli et al., 2000)</td>
</tr>
<tr>
<td>Take a way meal</td>
<td>10⁵ in the faeces of the meal consumers⁶</td>
<td>Vomiting involving 2 people</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meal with meat substitute</td>
<td>10⁵ g</td>
<td>Vomiting involving 7 people</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meal containing rice</td>
<td>10⁵ g</td>
<td>Vomiting involving 4 people</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Take a way beef dish</td>
<td>&gt;10⁶</td>
<td>Vomiting involving 2 people</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooked chicken meal</td>
<td>Not known</td>
<td>Nausea, diarrhoea and abdominal pain involving 30 people</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 1-20: Examples of RTE meals and prepared foods reported to hold *B. cereus* (except dairy products)  CONTINUED

<table>
<thead>
<tr>
<th>Type of Food&lt;sup&gt;a&lt;/sup&gt; / Main Component of the dish</th>
<th>Range of reported <em>B. cereus</em> count (CFU g&lt;sup&gt;-1&lt;/sup&gt; or ml&lt;sup&gt;-1&lt;/sup&gt;) / percentage of samples found positive</th>
<th>Involved in outbreak/syndrome&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Occasion</th>
<th>Country, year&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Street food” (unknown type of on the street sold foods)</td>
<td>mostly below 10&lt;sup&gt;4&lt;/sup&gt;, but in some samples 10&lt;sup&gt;5&lt;/sup&gt;-65% samples positive</td>
<td>NR/ca. 60% isolates positive with BCET-RPLA</td>
<td>Control: Evaluation of the microbiological safety and quality of street foods</td>
<td>Botswana, 2001-2002</td>
<td>(Murindamombe et al., 2005)</td>
</tr>
<tr>
<td>RTE moist foods</td>
<td>(0.1-5.8) x 10&lt;sup&gt;7&lt;/sup&gt;/83%</td>
<td>NR</td>
<td>Control: Simulation of temperature abuse in RTE moist foods</td>
<td>USA, 1988-1990</td>
<td>(Harmon and Kautter, 1991)</td>
</tr>
<tr>
<td>Banquet cakes served at 2 banquets in the same area (the same catering service used)</td>
<td>&gt;10&lt;sup&gt;7&lt;/sup&gt; (same DNA profile of food, kitchen and feces isolates)/100% (food samples tested)</td>
<td>2 simultaneous diarrheal outbreaks involving 95 and 78 people. All isolates produced HBL (tested with BCET-RPLA and HBL agar plates) and phospholipase C. No emetic toxin, but genes for NHE were found.</td>
<td>“Outbreak” investigation</td>
<td>Italy, 2001</td>
<td>(Ghelardi et al., 2002)</td>
</tr>
<tr>
<td>RTE street foods</td>
<td>&lt;10&lt;sup&gt;4&lt;/sup&gt;/22% of 51 tested foods (16% hold <em>C. perfringens</em>, 2% hold <em>Salmonella</em> spp. and <em>E. coli</em>)</td>
<td>NR</td>
<td>Control: Evaluation of quality and safety of RTE street foods (at the point of sale)</td>
<td>South Africa, 1997-1999</td>
<td>(Mosupye and von Holy, 1999)</td>
</tr>
<tr>
<td>Wedding meal: suspect quiche (eaten by 56% of ill people) and beef (cross-contamination)</td>
<td><em>B. cereus</em> was isolated from implicated foods, but exact count was not determined</td>
<td>79 persons (58%) of which 91% with nausea, 84% with diarrhea and cramps, 73% with vomiting, 71% with headache, 63% with chills and 49% with fever.</td>
<td>Outbreak investigation from a wedding party</td>
<td>Mississippi, USA, 1993</td>
<td>(Penman et al., 1996)</td>
</tr>
<tr>
<td>Mayonnaise used for potato salad</td>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Diarrhea and gastroenteritis involving 25 (70%) of attending people</td>
<td>Outbreak investigation at banquet party</td>
<td>Quebec, Canada</td>
<td>(Gaulin et al., 2002)</td>
</tr>
<tr>
<td>Type of Food⁴ / Main Component of the dish</td>
<td>Range of reported <em>B. cereus</em> count (CFU g⁻¹ or ml⁻¹) / percentage of samples found positive⁵</td>
<td>Involved in outbreak/syndrome⁶</td>
<td>Occasion</td>
<td>Country, year⁷</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------------------</td>
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</tr>
<tr>
<td>Meal served during Norwegian Ski Championship</td>
<td>10⁴-10⁵ per serving of incriminated food¹</td>
<td>Diarrhea involving 152 people. Isolate involved produced NHE detected with TECRA-VIA and vero cells assay</td>
<td>Outbreak investigation</td>
<td>Norway (Lund and Granum, 1996)</td>
<td></td>
</tr>
<tr>
<td>Vegetable puree</td>
<td>3x10⁵</td>
<td>Diarrhea where 44 people were ill. 6 had bloody diarrhea, and 3 died. Isolate produced cytK enterotoxin and contained no genes for HBL and NHE</td>
<td>Outbreak investigation in elderly home</td>
<td>France, 1998 (Lund et al., 2000)</td>
<td></td>
</tr>
<tr>
<td>Orange juice from a concentrate</td>
<td>100 ml⁻¹</td>
<td>Vomiting and diarrhea</td>
<td>NA</td>
<td>NA</td>
<td>(EFSA, 2005b) and references therein</td>
</tr>
<tr>
<td>Cornish game served as a catering service</td>
<td>10⁴ (data not confirmed)</td>
<td>Gastroenteritis involving 140 people</td>
<td>Outbreak investigation at the wedding</td>
<td>CA, USA, 1989 (Slaten et al., 1992)</td>
<td></td>
</tr>
<tr>
<td>Chicken and fried rice</td>
<td>&gt;10⁶ from chicken fried rice</td>
<td>Vomiting, cramps, diarrhea involving 14 (21%) of people eating lunch showed symptoms. The median incubation period was 2 hours</td>
<td>Outbreak investigation at 2 child day-care centers</td>
<td>Virginia, USA, 1993 (Khodr et al., 1994)</td>
<td></td>
</tr>
<tr>
<td>Fried rice</td>
<td>&gt;10⁷ in fried rice</td>
<td>Vomiting/4 people</td>
<td>NA</td>
<td>NA</td>
<td>(EFSA, 2005b) and references therein</td>
</tr>
<tr>
<td>Chinese noodles</td>
<td>6x10⁶ in cooked noodles</td>
<td>Vomiting and diarrhea/50 people</td>
<td>NA</td>
<td>NA</td>
<td>(EFSA, 2005b) and references therein</td>
</tr>
<tr>
<td>Cod fish</td>
<td>4x10⁶</td>
<td>Diarrhea involving 4 people</td>
<td>NA</td>
<td>NA</td>
<td>(Mahler et al., 1997)</td>
</tr>
<tr>
<td>Spaghetti with pesto</td>
<td>NA</td>
<td>Gastroenteritis, diarrhea and vomiting involving 2 people (1 died due to the fulminant liver failure)</td>
<td>Outbreak investigation at home</td>
<td>Switzerland, 1996</td>
<td>(Mahler et al., 1997)</td>
</tr>
</tbody>
</table>
## Table 1-20: Examples of RTE meals and prepared foods reported to hold *B. cereus* (except dairy products)  CONTINUED

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<th>Type of Food¹ / Main Component of the dish</th>
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<th>Occasion</th>
<th>Country, yearᶜ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasta salad</td>
<td>10⁻¹⁰⁸</td>
<td>Vomiting, respiratory distress, liver failure, Outbreak involving 5 young children, hospitalization and one fatality</td>
<td>Outbreak investigation at family home</td>
<td>Belgium, 2003</td>
<td>(Dierick et al., 2005)</td>
</tr>
<tr>
<td>Home made minced meat pasta dish</td>
<td>7x10⁶ (1500-3000 ng cereulide present in the pasta determined with bioassay and HPLC-MS)</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Fried rice</td>
<td>ca. 10⁻⁹ (10⁴ in vomitus), 60-1280 ng cereulide</td>
<td>Vomiting involving 2625 people with 1 death, in 686 cases</td>
<td>Study of biological activities of <em>B. cereus</em></td>
<td>Japan, 1971-1982</td>
<td>(Shinagawa et al., 1985; Agata et al., 2002);</td>
</tr>
<tr>
<td>Boiled rice</td>
<td>ca. 10⁻⁹, 10-640 ng cereulide</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Chow main</td>
<td>640 ng of cereulide</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Curry and rice</td>
<td>5.7x10⁻⁶, 80 ng of cereulide</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Spaghetti</td>
<td>40-80 ng of cereulide</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Noodle</td>
<td>20 ng of cereulide</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Catered box lunch</td>
<td>1.2x10⁶ (in vomitus)</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Rice ball (with sushi)</td>
<td>1.6x10⁻⁶-8x10⁶</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Roast chicken</td>
<td>1.3x10⁶</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Omelet and rice</td>
<td>1.1x10⁶</td>
<td>Vomiting involving 2 people</td>
<td>Bioassay and HPLC-MS evaluation.</td>
<td>Finland</td>
<td>(Jaaskelainen et al., 2003b)</td>
</tr>
<tr>
<td>Pudding</td>
<td>2x10⁶</td>
<td>Diarrhea involving more than 1860 people with 3 deaths, in total of 425 cases</td>
<td>Study of biological activities of <em>B. cereus</em></td>
<td>Japan, 1971-1975</td>
<td>(Shinagawa et al., 1985)</td>
</tr>
<tr>
<td>Catered box lunch</td>
<td>5.7x10⁻³-3x10³</td>
<td>Diarrhea involving more than 1860 people with 3 deaths, in total of 425 cases</td>
<td>Study of biological activities of <em>B. cereus</em></td>
<td>Japan, 1971-1975</td>
<td>(Shinagawa et al., 1985)</td>
</tr>
<tr>
<td>Honey</td>
<td>25% /70 samples held <em>B. cereus</em> and 39% held <em>Bacillus</em> spp.</td>
<td>NR</td>
<td>Control: prevalence of <em>Bacillus</em> spp. in manufactured foods</td>
<td>Argentina</td>
<td>(Iurlina et al., 2006)</td>
</tr>
<tr>
<td>Post Salut Argentino cheese meal</td>
<td>50% of 30 samples</td>
<td>NR</td>
<td>Control: prevalence of <em>Bacillus</em> spp. in manufactured foods</td>
<td>Argentina</td>
<td>(Iurlina et al., 2006)</td>
</tr>
</tbody>
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<th>Occasion</th>
<th>Country, year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken and potato</td>
<td><em>B. cereus</em> isolated, but specific counts not reported</td>
<td>Unspecified syndrome/3 people</td>
<td>Outbreak</td>
<td>AL, USA, 2002</td>
<td>(CDC-Foodborne Outbreak Response and Surveillance Unit, 2002)</td>
</tr>
<tr>
<td>Fried rice</td>
<td></td>
<td>Unspecified syndrome/8 people</td>
<td>Outbreak</td>
<td>FL, USA, 2002</td>
<td></td>
</tr>
<tr>
<td>Pizza meat</td>
<td></td>
<td>Unspecified syndrome/2 ppl people</td>
<td>Outbreak</td>
<td>HI, USA, 2002</td>
<td></td>
</tr>
<tr>
<td>Chicken and rice</td>
<td></td>
<td>Unspecified syndrome/6 ppl people</td>
<td>Outbreak</td>
<td>MN, USA, 2002</td>
<td></td>
</tr>
<tr>
<td>Chicken fried</td>
<td><em>B. cereus</em> isolated, but specific counts not reported</td>
<td>Unspecified syndrome/4 people</td>
<td>Outbreak</td>
<td>PA, USA, 2002</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td></td>
<td>Unspecified syndrome/11 people</td>
<td>Outbreak</td>
<td>PA, USA, 2002</td>
<td></td>
</tr>
<tr>
<td>Fried potato</td>
<td><em>B. cereus</em> isolated, but specific counts not reported</td>
<td>Unspecified syndrome/42 people</td>
<td>Outbreak</td>
<td>FL, USA, 2003</td>
<td>(CDC-Foodborne Outbreak Response and Surveillance Unit, 2003)</td>
</tr>
<tr>
<td>Chicken and rice</td>
<td></td>
<td>Unspecified syndrome/8 people</td>
<td>Outbreak</td>
<td>OH, USA, 2003</td>
<td></td>
</tr>
<tr>
<td>Chicken, roasted</td>
<td><em>B. cereus</em> isolated, but specific counts not reported</td>
<td>Unspecified syndrome/11 people</td>
<td>Outbreak</td>
<td>CT, USA, 2004</td>
<td>(CDC-Foodborne Outbreak Response and Surveillance Unit, 2004)</td>
</tr>
<tr>
<td>Deli meat, sliced turkey ham; beans, baked</td>
<td></td>
<td>Unspecified syndrome/56 people</td>
<td>Outbreak</td>
<td>FL, USA, 2004</td>
<td></td>
</tr>
<tr>
<td>Pizza, cheese; Pizza, meat and vegetable</td>
<td></td>
<td>Unspecified syndrome/4 people</td>
<td>Outbreak</td>
<td>PA, USA, 2004</td>
<td></td>
</tr>
<tr>
<td>Chicken and pasta</td>
<td><em>B. cereus</em> isolated, but specific counts not reported</td>
<td>Unspecified syndrome/2 people</td>
<td>Outbreak</td>
<td>PA, USA, 2004</td>
<td>(CDC-Foodborne Outbreak Response and Surveillance Unit, 2004)</td>
</tr>
<tr>
<td>Fried rice</td>
<td></td>
<td>Unspecified syndrome/26 people</td>
<td>Outbreak</td>
<td>TN, USA, 2004</td>
<td></td>
</tr>
</tbody>
</table>
### Table 1-20: Examples of RTE meals and prepared foods reported to hold *B. cereus* (except dairy products) CONTINUED

<table>
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<tr>
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<th>Range of reported <em>B. cereus</em> count (CFU g⁻¹ or ml⁻¹) / percentage of samples found positive</th>
<th>Involved in outbreak/syndrome⁵</th>
<th>Occasion</th>
<th>Country, year⁶</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTE vegetarian foods</td>
<td>Soybean</td>
<td>5.1% of 102 samples</td>
<td>10⁻⁰⁵⁷  In total 3.4% of samples held <em>B. cereus</em></td>
<td>NR, but 1 isolate produced diarrheal enterotoxin¹ and 4 isolates induced mouse death</td>
<td>Control: safety and quality of vegetarian foods</td>
</tr>
<tr>
<td></td>
<td>Cereals</td>
<td>9.4% of 45 samples</td>
<td>20.9% held <em>Bacillus</em> spores (10⁻¹⁰³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Konjac</td>
<td>2.5% of 66 samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>so-called &quot;18 °C RTE food products&quot;</td>
<td>Hand rolled sushi</td>
<td>1.2x10⁻⁰⁻6x10⁴/41.7% of 161</td>
<td>NR</td>
<td>Control: products bought at retail level (produced by 16 different factories)</td>
<td>Taiwan, 1999-2000 (Fang et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Sandwich</td>
<td>1.2x10⁻⁰⁻8.9x10⁴/53.3% of 161</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold noodles</td>
<td>1.2x10⁻⁰⁻8.21x10⁴/66.7% of 161</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rice balls rolled in seaweed</td>
<td>1.2x10⁻³⁻3.2x10⁵/56% of 161</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sushi</td>
<td>1.2x10⁻³⁻3.2x10⁴/18.2% of 161</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁴ REPFED Refrigerated Processed Foods of Extended Durability; RMPF Refrigerated Minimally Processed Foods (*between these two terms no real difference exist, prim. auth.*); ⁵ NR-Not Reported; ⁶ Year of if not clearly indicated in the reference was estimated from the year of publication and one-two year before; ⁷ 10% of strains was able to grow at 5 °C and 63% at 10 °C; ⁸ AMB Aerobic Mesophilic Bacteria; ⁹ Isolates from chicken and rice were not sharing the same DNA profile, indicating different contamination origin; ¹⁰ Same DNA profile of isolates from food and feces of consumers (determined with amplified fragment length polymorphism); ¹¹ The counts of *B. cereus* taken over from EFSA(2005b); ¹² detected with mouse ligated ileal loop assay.
Table 1-21: Some dairy products reported to hold *B. cereus*

<table>
<thead>
<tr>
<th>Type of dairy product</th>
<th>Percentage of positive samples/contamination level with <em>B. cereus</em> vegetative cells and/or spores (CFU ml$^{-1}$ or g$^{-1}$)</th>
<th>Virulence genes/toxins reported (or illness) $^b$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>7-35%$^a$$&lt;10^{-2}$</td>
<td>Enterotoxin production</td>
<td>(Christiansson <em>et al.</em>, 1989; Notermans and Batt, 1998; Christiansson <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>2-35%$^a$$&lt;10^{-1}$ (53% of 106 isolates were able to grow at 7 °C) 4x10$^2$</td>
<td>HBL production, cytotoxicity towards Vero cells, bceT gene presence Nausea, vomiting/280 ppl</td>
<td>(te Giffel <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td>Skimmed milk</td>
<td>NR</td>
<td>nheA, B, C, hbl, bceT</td>
<td>(Andersen Borge <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>Semiskimmed milk</td>
<td>NR</td>
<td>nheA, B, C, hbl, bceT</td>
<td>(Andersen Borge <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>Cream</td>
<td>5-11%$^a$$&lt;10^{-4}$</td>
<td>hblA</td>
<td>(Notermans and Batt, 1998) and references therein; (Torkar and Mozina, 2000) reported hblA gene in ice cream isolates</td>
</tr>
<tr>
<td>Ice cream</td>
<td>20-25%$^a$$&lt;10^{-4}$</td>
<td>hblA</td>
<td>(Notermans and Batt, 1998) and references therein; (Torkar and Mozina, 2000) reported hblA gene in ice cream isolates</td>
</tr>
<tr>
<td>Soft ice cream from a vending machine</td>
<td>5% of 1246 samples (Bacillus spp. or <em>B. cereus</em>)$&lt;10^4$</td>
<td>NR</td>
<td>(Little and de Louvois, 1999)</td>
</tr>
<tr>
<td>Mix for ice cream</td>
<td>6% of liquid mixes and 15% of powder mixes$&lt;10^4$</td>
<td>NR</td>
<td>(Andersen Borge <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>Whipped cream</td>
<td>NR</td>
<td>nheA, B, C, hbl, bceT</td>
<td>(Andersen Borge <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>Heat treated cream (90°C/15min) stored at 8°C after treatment</td>
<td>&gt;2x10$^4$ after 14 days storage 10$^6$ after 21 days storage</td>
<td>All 6 <em>B. cereus</em> isolates gave positive reactions with BCET-RPLA and TECRA-VIA. When inoculated into cream positive reaction was seen at 10 °C.</td>
<td>(Nissen <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>Hot chocolate from a vending machine</td>
<td>1.7x10$^4$ / 7 of 39 vending machines were positive</td>
<td>Illness: nausea and vomiting.</td>
<td>(Nelms <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Up to 40 spores</td>
<td>NR</td>
<td>(Van Netten <em>et al.</em>, 1990 in (EFSA, 2005))</td>
</tr>
<tr>
<td>Milk powder</td>
<td>NA/ca. 44% of samples hold <em>B. cereus</em> $^a$</td>
<td>NR</td>
<td>(Bedi <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td>Milk powder</td>
<td>NA/ca. 11% samples hold <em>B. cereus</em> (1365 milk powder samples)</td>
<td>NR/TECRA-VIA assay showed almost 98% of strains positive on NHE and cell culture showed ca. 85% of strains positive</td>
<td>(Hammer <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>Milk powder</td>
<td>10-10$^7$/1 sample</td>
<td>NR</td>
<td>(Helmy <em>et al.</em>, 1984)</td>
</tr>
</tbody>
</table>

$^a$ RAPD typing showed that isolates were originating from a daily input of fresh milk

$^b$ NR-Not Reported; italic font designates genes encoding for respective toxins

Chapter 1: Bacterial toxins & enterotoxigenic *Bacillus cereus*: a review 56
As seen in Table 1-19, Table 1-20 and Table 1-21, \textit{B. cereus} can be found in a wide variety of foods, resulting in a versatility of food vehicles involved in diarrheal and emetic diseases of \textit{B. cereus} food poisonings. It seems therefore obvious that connotations made by many authors (Granum and Lund, 1997; Nelms \textit{et al.}, 1997; Granum and Baird-Parker, 2000) on underreporting of \textit{B. cereus} related diseases are indeed an actual problem. Contribution to this is given by generally mild symptoms of \textit{B. cereus} foodborne diseases and by the fact that \textit{B. cereus} food poisoning is a non-reportable disease in Europe (Nissen \textit{et al.}, 2002). Sporadic cases are not reportable in US, hence, in US the only cases reported are those related to outbreaks (Mead \textit{et al.}, 1999).

Apart from its ubiquitous nature, two other problems related to \textit{B. cereus} are its ability to form spores and existence of psychrotrophic strains that can endanger products kept at low temperature during extended shelf-life. However, psychrotrophic strains are usually less heat resistant and have less potential to grow and produce toxins in human body (37 °C), as reported in Pielaat \textit{et al.} (2006).

For the foods that are properly reheated prior to the consumption this will eliminate the threat of diarrheal toxin mediated infection, unless foods were heavily contaminated with heat resistant spores. \textit{B. cereus} emetic toxin is reported not to be produced at temperature lower than 12 °C (Finlay \textit{et al.}, 2000; Finlay \textit{et al.}, 2002a), while possible enterotoxin production at 8 °C was suggested by Christiansson \textit{et al.} (1989).

\textbf{1.2.6 Control of \textit{B. cereus} in RTE foods}

 Appropriately much attention has been given to the \textit{B. cereus} related problems in dairy products, by many researchers. Table 1-21 shows just some of dairy products from which \textit{B. cereus} was isolated and no doubt exists that the control of these products is a necessity. Some reports (Torkar and Mozina, 2001) indicate that almost all isolated \textit{B. cereus} from milk and dairy products had a genetic potential and actually had expressed HBL and NHE, as detected with BCET-RPLA and TECRA-VIA (for limitations of these kits see 1.2.4). This is not only because of the established contamination route, but also because of the fact that wide population consumes milk and dairy products.
However, in the last decade RTE foods (and alike) have been becoming more and more popular. Both the industry (due to the added value) and the consumer, who has less time and interest in the traditional home preparation of foods, are choosing for RTE foods. There is also a world-wide trend for increased consumption of food outside the home, and restaurant are increasingly using *sous vide* (heat treatment of vacuum packed foods) or other kind of minimal processing in their preparation of the food (Nissen et al., 2002). Due to only mild heat treatment used in their production these types of products essentially rely on the cold chain maintenance and strict temperature control throughout the whole chain (including manufacture and distribution as well as household treatment). The identification of hazards that may endanger safety of food products is to be done through the risk assessment process. The risk assessment principles for *B. cereus* and its toxins are well elaborated (Notermans and Batt, 1998; Notermans et al., 1998; Carlin et al., 2000a). The control of identified hazards is achieved through Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Points (HACCP) safety system by incorporating quantitative elements obtained in stepwise analysis of the health risks associated with a specific type of food product, into HACCP (Notermans and Mead, 1996). Although there is insufficient data for detailed risk assessment for *B. cereus* in RTE foods, such analysis were performed for milk (Notermans et al., 1997), for which also predictive models were build (Zwietering et al., 1996). HACCP can very effectively minimize the probability of occurrence of sporadic problems with *B. cereus* and contribute to establishing a higher level of control over hazards and hazardous conditions, making the food production process safer. To achieve this HACCP needs to be supported by managerial decisions, referring to the nature of hazards, the definition of an acceptable level of the hazard (governing the design of products, processes and control measures) and the value of an improved safety level versus the cost of achieving it. To be meaningful and effective, HACCP needs to be directed by the choice of an outcome target or Food-Safety-Objective (FSO). FSO is defined as the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection (ALOP) (Jouve, 1999; FAO, 2003). Process objective (PO) is defined as a level of foodborne hazard that can not be exceeded at the given point in the food chain (ICMSF, 2005). However, ALOP are not defined yet for bacterial pathogens, as it is difficult political decision to publically proclaim the level of risk a society is willing to accept. For *B.
cereus, currently no FSO is defined either, but recent EFSA report (EFSA, 2005b) shows that there is a trend and initiative within scientific, governmental, consumer and industrial milieu to establish these criteria. The safety limit at the moment of consumption for foods which can support growth of B. cereus and that will not undergo any substantial heat treatment prior to the consumption seems to subsist between $10^3$ and $10^5$ CFU g$^{-1}$. However, the type of the food and its intrinsic factors, as well as characteristics of B. cereus strains present in the product, can differ so much from each other that setting general and omni-applicable criteria may be difficult. In recent report of Carlin et al. (2006) emetic toxin-producing strains were shown to be rather thermophilic in contrast to diarrheal strains. None of the 17 cereulide producing strains tested by Carlin et al. (2006) was able to grow at temperatures lower than 10 °C, all being able to grow at 48 °C. 70% of tested diarrheal strains were able to grow at 7 °C. These data are in accordance with the findings of Rajkovic et al. (2006a). Of 44 B. cereus isolates (not producing cereulide) from food and environment, tested by Carlin et al. (2006) ca. 20% was able to grow at 4 °C. The spores of cereulide producing strains were also more heat resistant. An average D$_{90}$ value and its 95% confidence interval, calculated from the data of 17 cereulide producing strains, reported by Carlin et al. (2006) was 424 ± 104 minutes, with the reported range of 68 minutes to 917 minutes. In contrast, D$_{90}$ of spores of diarrheal B. cereus strains were in average 123 ± 42 minutes, ranging from 29 to 817 minutes. In addition, spores of cereulide producing strains showed lower ability to germinate than diarrheal strains and other non-emetic food and environment isolates.

An additional factor to consider, when analyzing risk of B. cereus related illness, is the typical amount and form of the particular food consumed and relevant consumer group.

The magnitude in number, diversity, and complexity of RTE products that exist in the market place make general implementation of HACCP impossible and each product and production line must be treated individually (this is in fact not only typical for RTE foods). However, the production and distribution principle for these foods has much in common (mild heat treatment among others) and according to the International Commission on Microbiological Specifications for Foods (ICMSF), “Every well-documented report of B. cereus intoxication has described
time/temperature abuse that has enabled relatively low (innocuous) levels of *B. cereus* in foods greatly to increase” (ICMSF, 1996a). Therefore, the main conclusion is, since the complete absence of *B. cereus* can not be warranted (only heat treatments used for canning of low acid foods will ensure a complete destruction of spores of *B. cereus*), rapid cooling is necessary to prevent germination and growth of *B. cereus* spores and consequent strict maintenance of the cold chain is required to provide safety of RTE foods (EFSA, 2005b). In its essence the temperature must be such that it prevents spore germination and vegetative outgrowth. Juneja et al. (1997) reported that no germination and growth of *B. cereus* and *C. botulinum* spores (neither of vegetative cells of *L. monocytogenes*, *S. aureus* and *Salmonella* spp.) occurred if ground beef samples were chilled from 54.4 °C to 7.2 °C within 21 hours. However, this report must be analyzed more in depth and the actual drop to the temperature of 10 °C should be indicated.

The suggested minimum temperature to control the growth of present *B. cereus* is 4 °C, but below 10 °C lag time and generation times are significantly increased, particularly whenever other factors (i.e. pH, *a*<sub>w</sub>, nutrient content of the food) are not optimal for *B. cereus* growth. It is recommended that pasteurized RTE foods are cooled down within 2 h to 10 °C or below and within 12 h to 3 °C and below (Anonymous, 1994). Multiple hurdles are in fact often set in action to prevent *B. cereus* outgrowth and toxin production. Together with temperature, this also includes control by pH and *a*<sub>w</sub> (pH below 4.5, reduction in *a*<sub>w</sub> below 0.92 would inhibit *B. cereus*) different preservatives, packaging techniques, protective cultures etc. Control measures for *B. cereus* would contribute to control other *Bacillus* spp., which is of significant public health importance (also from the food quality aspect), as other *Bacillus* spp., isolated from foods, water, and food plants, have been reported to produce toxins under similar conditions as *B. cereus* (Beattie and Williams, 1999; Phelps and McKillip, 2002; From et al., 2005).

In conclusion, the choice of measure to control *B. cereus* does not belong to the will but to the reason of eminent threat that this bacterium poses to food safety, and to the food quality. However, *B. cereus* omnipresence, sporeforming character and versatility make it difficult to be controlled. Depending on the type of the food product, emetic or diarrheal toxin producing strain can be expected with higher or
lower likelihood of occurrence. Nevertheless, large number of outbreaks involving different foods, both starch and protein rich, has been implicated in both emetic and diarrheal outbreaks. The complexity of the food chain, mixture of different ingredients and consumption of RTE complete meals comprising two or three different sub-meals lead to transfer of strains from one type of food to another. Therefore, it seems safer not to add this constraint and to rightfully “expect” *B. cereus* emetic and diarrheal toxin producers equally in all foods that can support growth and toxin production. And although the emetic toxin producing strains of *B. cereus* are rather thermophilic in contrast to diarrheal and seemingly less expected problem in RTE foods with respected cold chain they can not be overseen due to higher thermal resistance of their spores and frequent temperature abuse that can lead to germination and outgrowth of present *B. cereus*. These considerations can be extended to all heat-processed foods that are kept warm or can be potentially exposed to temperature abuse.

Although the control of *B. cereus* vegetative cells and spores may seem difficult and this is with a reason, the following are summarized “7C-ES” essential control principles that should be respected:

- Control of ingredients for presence of *Bacillus* spp. and *B. cereus*
- Correct cleaning and disinfection to remove in-house contamination sources
- Control of process (cross-contamination)
- Control of time/temperature parameters during the heat treatment
- Control of rapid cooling after the heating process (bulk vs. packaged products)
- Cold chain maintenance
- Correct labeling indicating storage temperature
- Exactly determined shelf-life
- Strict refrigeration at the consumers’
PREVALENCE AND CHARACTERIZATION OF
*BACILLUS CEREUS* IN VACUUM PACKED
POTATO PUREE

Redrafted from:

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(2006)

*International Journal of Food Science and Technology*, in press
CHAPTER 2: PREVALENCE AND CHARACTERIZATION OF BACILLUS CEREUS IN VACUUM PACKED POTATO PUREE

Summary

Refrigerated Processed Foods of Extended Durability (REPFED) potato puree was analyzed for Bacillus cereus contamination along the production line and during the product shelf-life. Isolated B. cereus strains were tested for their psychrotrophic character and the ability to produce enterotoxins. B. cereus contamination during four subsequent productions was in the range of 2.3-4.0 log CFU g\(^{-1}\). Productions five and six were significantly less contaminated with B. cereus (\(\leq 1\) log CFU g\(^{-1}\)). All B. cereus isolates from the first four productions were able to grow at 7 and 10 °C, whereas the majority of the isolates from productions five and six did not. No B. cereus isolates grew at 4 °C. RAPD fingerprinting showed that the most of B. cereus contamination originated from one source. In total 30.4% of isolates expressed enterotoxigenic character producing HBL and/or NHE diarrheal enterotoxins.

The present study points out the necessity to prevent an “in house” colonization and contamination during food processing in order to accomplish the safety of REPFED throughout the shelf-life. It also indicates the most critical steps in the production line of Ready-To-Eat potato puree and impact of hygienic failures regarding the food safety.

The data provided can be used for risk assessment studies regarding B. cereus in REPFED.

Keywords: Bacillus cereus, enterotoxin, psychrotrophic character, RAPD, REPFED.
2.1 INTRODUCTION

Processing of Refrigerated Processed Foods of Extended Durability, REPFEDs, generally combines mild heat treatment and subsequent storage at temperatures of 4 to 7 °C, providing shelf-life ranging from a few days to three months. Pasteurization in their final packaging eliminates hazards from vegetative pathogenic bacteria, but it is insufficient for elimination of Bacillus cereus spores. Packaging after the heating step imposes a danger of post-contamination. The problem is further enlarged by the psychrotrophic character of some B. cereus strains (Van Netten et al., 1990; Dufrenne et al., 1994) that can compromise the safety of REPFEDs during the prolonged storage at refrigeration temperatures.

Products based on vegetables account for an important part of REPFEDs (Choma et al., 2000). Carlin et al. (2000b) have isolated B. cereus from 80% to 100% of samples of cooked pasteurized and chilled vegetable purees of leek, zucchini, broccoli, split pea, carrot and potato purees stored at 10 °C. Del Torre et al. (2001) reported isolation of B. cereus from 33% of gnocchi in Italy. Foodborne outbreaks related to the presence of B. cereus in REPFED have been reported (Van Netten et al., 1990; Griffiths, 1990; Harmon and Kautter, 1991; Ripabelli et al., 2000), and extensive review thereof is summarized in section 1.2.5 and in tables therein. Large number of REPFED (RTE) foods involved in B. cereus transmission implies that cereulide producing B. cereus, which are mostly related to “take away” rice dishes, should not be overlooked in REPFEDs. Apart from a self limiting emesis they could cause life threatening intoxications (Mahler et al., 1997; Dierick et al., 2005).

For tracing the contamination routes and investigations of outbreaks it is important to differentiate B. cereus strains. Several fingerprinting methods were developed, such as RAPD-PCR, ribotyping (Stephan, 1996; Pirttijarvi et al., 2000; Senesi et al., 2001) and amplified fragment length polymorphism (Ripabelli et al., 2000), and successfully applied.

The present work was performed in the frame of hazard analysis in order to follow up the prevalence and the persistence of B. cereus in cooked vacuum packed potato puree from one particular production unit and to evaluate the ability of the B. cereus isolates for survival and growth in this food item. As it will be shown in Chapters 3, 5 and 6,
potato puree, as well as some other RTE foods, represents an excellent substrate for growth and cereulide production.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Potato puree

In order to determine the trend of the microbiological quality in time and persistence of *B. cereus* contamination six different production lots of pasteurized, vacuum packed potato puree were investigated in the period September-November 2002. Samples (one 300 g sample per control point) were taken at six critical points or critical control points in the production line (Figure 2-1). Due to well established and long-time existing follow up of the known production process no duplicate samples were taken. Between sampling and microbial analyses all samples were stored for two days at 2 °C. In order to evaluate the evolution of the microbiological quality during the shelf life, three additional samples of the end-product for each production lot were taken and subjected to storage from day 0 (day of production) until day 16 (factory’s internal shelf-life) at 2 °C and subsequently stored at 7 °C for another 11 days until the ultimate date of consumption. One sample was analyzed at day 18 (2/3 of the predicted shelf life), while remaining two samples were analyzed at day 27 (the end of predicted shelf-life).

#### 2.2.1.1 Physical parameters

The pH and a$_w$ value of potato puree were determined simultaneously with microbiological sampling (days 2, 18 and 27), using an Ingold stab-electrode (Ingold, 104063123, Urdorf, Switzerland) and fully automated cryometer (AWK-20, NAGY Messysteme GmbH, Gäufelden, Germany), respectively. Argentometric method for determination of chlorides with 0.1 N AgNO$_3$ (Merck KgaA, Darmstadt, Germany) and 2 ml of 5% K$_2$CrO$_4$ (Vel, Leuven, Belgium) was employed to determine NaCl concentration (day 2). Sample preparation consisted of resuspending 4 g of potato puree in 100 ml of boiling water and stirring prior to titration until temperature dropped to 50 °C. The end point of titration was occurrence of the bright lemon-yellow color starting to turn orange.
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Chapter 2: *Bacillus cereus* in Ready-To-Eat potato puree

Figure 2-1: Flow chart of potato puree production
2.2.1.2 Microbial analyses

Potato puree samples (30 g) were homogenized in 9 volumes (270 ml) of peptone saline solution, PSS, (8.5 g l\(^{-1}\) NaCl, Vel, Leuven, Belgium and 1 g l\(^{-1}\) peptone, Oxoid, Hampshire, UK) using a Colworth Stomacher 400 (Steward Laboratory, London, UK). Enumeration of the total aerobic mesophilic count and aerobic spore count on Plate Count Agar pour plates (PCA, Oxoid, Basingstoke, England, UK), was done from non-heated and heated (80 °C/10 minutes) homogenates, respectively. Presumed \textit{B. cereus} was enumerated from the non-heated homogenates on BCA plates (\textit{Bacillus cereus} selective agar, Mannitol Egg Yolk Polymixin Agar, LAB M, Bury, UK). By spreading 1 ml from the first tenfold dilution step over 3 BCA plates, detection limit of enumerations was established at 10 CFU g\(^{-1}\). All incubations were done at 30 °C with appropriate time intervals. Typical \textit{B. cereus} colonies were subjected to purification, motility and hemolytic reaction test and selected isolates were identified using API 50 CHB system (Biomérieux, Marcy- L'Etoile, France). The ability of \textit{B. cereus} isolates to hydrolyze starch was tested by inoculating \textit{B. cereus} isolates on 0.2% (w/v) starch supplemented NA.

2.2.2 Characterization of isolates

2.2.2.1 Psychrotrophic character of \textit{B. cereus}

Isolates confirmed as \textit{B. cereus} were tested for their ability to grow at 4 °C, 7 °C and 10 °C in Nutrient broth (Oxoid). Two of the potato puree isolates, \textit{B. cereus} 1.91 (isolated in production 1) and \textit{B. cereus} 4.9 (isolated in production 4) were also tested in modified NB, mNB, where pH and \(a_w\) values were adjusted to respectively 6.0 and 0.994, corresponding to those of potato puree. mNB adjustments were made with 1 N HCl and NaCl, respectively (both obtained from VWR International, Leuven, Belgium) for pH and \(a_w\), respectively.

For the two mentioned isolates, 200 µl of NB and mNB containing 10-100 CFU ml\(^{-1}\), were pipetted in duplicate into each of three microtitre plates. The same culture was plated on NA and incubated at 30 °C to determine the exact inoculum level.

Daily determined optical density (OD), using VERSAmax microplate reader (Molecular Devices, Sunnyvale, California, USA), served for growth monitoring
Biological and immuno-molecular methods for detection of \textit{B. cereus} and \textit{S. aureus} enterotoxins

during six, three and two weeks of incubation at 4 °C, 7 °C and 10 °C, respectively. The time to attain an OD of 0.1 (equivalent to ca. 10^6 CFU ml^{-1} of \textit{B. cereus}) at 660nm was noted.

\subsection*{2.2.2.2 Toxin production of \textit{B. cereus} strains}

Two commercially available toxin detection kits, Visual Immunological Assay, VIA, (Tecra, Bioenterprises Pty. Ltd., Roseville, New South Wales, Australia) and \textit{Bacillus cereus} Enterotoxin Reverse Passive Agglutination assay, BCET-RPLA, (Oxoid), were used to determine the presence of Non-Hemolytic-Enterotoxin (NHE) and Hemolysin BL (HBL) toxins respectively.

The cereulide production has been tested using the bioassay, employing the known inhibitory effect of the toxin to the boar spermatozoa motility (Andersson \textit{et al.}, 1998b). Only strains that were found negative on HBL production and that gave negative starch hydrolyzes reaction were tested for cereulide production (Agata \textit{et al.}, 1996; Pirttijarvi \textit{et al.}, 2000).

\subsection*{2.2.2.3 Randomly Amplified Polymorphic DNA (RAPD) typing of \textit{B. cereus} isolates}

Modified method of Stephan (1996) and Senesi \textit{et al.}(2001) with three different 10 bp primers: RPO2 (5’-GCGATCCCCA-3’), HLWL 74 (5’-ACGTATCTGC-3’) and HLWL 85 (5’-ACAACTGCTC-3’), all obtained from Invitrogen Life Technologies (Merelbeke, Belgium), were run in duplicate to determine whether the \textit{B. cereus} contamination in the potato puree could be catalogued as an “in house strain”. Total genomic DNA from purified \textit{B. cereus} strains was isolated by using the method of Pitcher \textit{et al.} (1989). PCR reaction mixtures (25 µl containing: 1 µM of selected primer, 200 mM of each deoxynucleoside triphosphate and 2.5 mM MgCl₂ reaction buffer (Applied Biosystems, Foster City, CA, US), 1.25 U of GoldStar™ DNA-polymerase (Eurogentec, Liège, Belgium) and 0.05 µg genomic DNA were amplified in PTC 0200 DNA Engine (MJ Research, Inc. Waltham, Massachusetts, US) through the following program: 1 cycle at 94 °C for 4 min, 30 cycles consisting of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min, followed by final hold at 72 °C for 10 min. Visualized, ethidium bromide stained, electrophoretic patterns of PCR products (1.5% agarose gels, Life Sciences International, Zelik, Belgium run in TBE buffer, pH 8.0)
were used for computation of the similarity levels by Gel Compare II software (Applied Maths BVBA, Kortrijk, Belgium) using the Dice coefficient and clustering data by unweighted pair group method with arithmetic averages clustering algorithm.

2.2.3 Statistical analysis

Statistical interpretation of the growth of isolates at 7 °C and 10 °C (where required) was based on the mean value of the growth enumerated from different samples and thereof calculated 95% confidence interval. For the calculations Microsoft Excel (version 2003) was used.

2.3 RESULTS

2.3.1 Physical parameters of potato puree

The overall average pH, a_w value and NaCl concentration of six productions of the potato puree was 6.00 ± 0.03, 0.9940 ± 0.0003 and 6.25 ± 0.42 mg g⁻¹, respectively.

2.3.2 Microbiological analyses

Results of the microbiological analyses at different sampling sites in the production line and throughout the shelf-life at various time intervals for six productions of potato puree are shown in Table 2-1. The major ingredient, potato granules, constituting 62% of potato puree mix was affected by non-\textit{B. cereus} spore contamination in three out of six productions (production 2, 3 and 5). Even though no \textit{B. cereus} was found in the raw potato granules of production 1, the enumeration after the pasteurization revealed 3.56 log CFU g⁻¹. Pasteurization of raw potato granules reduced the spore count in productions 2 and 3, indicating that spores present were not very heat resistant. It has also generally decreased numbers of vegetative cells present, but productions 1 and 6 still seized in the half fabricate relatively high numbers of bacteria (Table 2-1). In production 6, high aerobic mesophilic counts in the half fabricate were not attributed to \textit{B. cereus}. A noticeable introduction of \textit{B. cereus} (most probably spores) in the potato puree in productions 2, 3 and 4 occurred during portioning of the pasteurized potato puree (process step 3 in Figure 2-1).

The average numbers of \textit{B. cereus} in samples from the first four productions ranged from $> 10^2$ CFU g⁻¹ to approximately $10^4$ CFU g⁻¹, after being stored for two days at
2 °C (Table 2-1). Within the first 18 days of a shelf life (16 days stored at 2 °C and additional 2 days at 7 °C), a steady level and even a small decrease (max 1.3 log CFU g⁻¹) in *B. cereus* numbers was observed indicating the inability of *B. cereus* spores to germinate and grow at 2 °C. These levels were, however, sufficient to initiate growth of psychrotrophic *B. cereus* strains at 7 °C, resulting in an increase of colony count (0.55-2.36 log CFU g⁻¹) during the further storage at 7 °C to counts varying from to 2.25 log CFU g⁻¹ to 5.44 log CFU g⁻¹ at the end of the shelf life. Comparison of *B. cereus* counts with aerobic mesophilic counts at the ultimate date of consumption reveals a similar contamination level indicating that the major part of the contamination can be attributed to *B. cereus* for all productions, except for production 2 were a much higher aerobic mesophilic count (4.90 log CFU g⁻¹) was found at the end of a shelf life. Variation in the increase and in the level of final *B. cereus* contamination among different productions may be attributed to the difference in psychrotrophic character of *B. cereus* strains present. In production 5 and 6 where no significant contamination with *B. cereus* was established, low aerobic mesophilic counts were also noticed.

On one occasion (production 1) other ingredients (spices and potato flakes) were also subjected to the microbiological analysis. For spices added to the mix (white pepper, muscat-nut aroma and curcuma extract) both mesophilic and spore counts were below the detection limit (10 CFU g⁻¹). Although *B. cereus* was not isolated, it was shown that potato flakes (2% of the potato puree mix) may also introduce sporeforming bacteria into the potato puree mix. Potato flakes contamination with mesophilic aerobic spores was on the level of 3.36 log spores g⁻¹, while aerobic mesophilic count found in the flakes was 0.14 log CFU g⁻¹ higher.
Biological and immuno-molecular methods for detection of B. cereus and S. aureus enterotoxins

**Table 2-1: Microbiological quality of potato puree (log CFU g⁻¹) followed through the production line until the ultimate date of consumption**

<table>
<thead>
<tr>
<th>Sampling number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sampling site/time interval</strong></td>
<td>Raw potato granules</td>
<td>Half fabricate after the pasteurization</td>
<td>Half fabricate after the dosage pump</td>
<td>Closed packages before microwave oven</td>
<td>Product after the microwave oven</td>
<td>2 days after production a (storage at 2 °C)</td>
<td>18 days after the production a (storage at 2 °C)</td>
<td>27 days after the production a (storage at 7 °C)</td>
</tr>
<tr>
<td><strong>Aerobic mesophilic counts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production 1</td>
<td>5.64</td>
<td>3.65</td>
<td>3.32</td>
<td>3.52</td>
<td>3.76</td>
<td>3.83</td>
<td>3.36</td>
<td>4.97</td>
</tr>
<tr>
<td>Production 2</td>
<td>3.92</td>
<td>1.30</td>
<td>3.00</td>
<td>3.38</td>
<td>3.20</td>
<td>3.10</td>
<td>3.59</td>
<td>4.90</td>
</tr>
<tr>
<td>Production 3</td>
<td>3.75</td>
<td>1.70</td>
<td>3.41</td>
<td>3.38</td>
<td>3.62</td>
<td>3.91</td>
<td>2.90</td>
<td>5.73</td>
</tr>
<tr>
<td>Production 4</td>
<td>4.23</td>
<td>1.78</td>
<td>2.46</td>
<td>2.38</td>
<td>1.78</td>
<td>2.49</td>
<td>2.46</td>
<td>3.67</td>
</tr>
<tr>
<td>Production 5</td>
<td>3.53</td>
<td>1.60</td>
<td>1.48</td>
<td>1.48</td>
<td>1.48</td>
<td>1.00</td>
<td>1.00</td>
<td>ND</td>
</tr>
<tr>
<td>Production 6</td>
<td>4.23</td>
<td>3.30</td>
<td>2.67</td>
<td>2.53</td>
<td>2.57</td>
<td>1.78</td>
<td>2.07</td>
<td>2.39</td>
</tr>
<tr>
<td><strong>Aerobic spore counts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production 2</td>
<td>2.74</td>
<td>&lt; 1.00</td>
<td>2.30</td>
<td>2.53</td>
<td>2.47</td>
<td>2.72</td>
<td>4.04</td>
<td>2.40</td>
</tr>
<tr>
<td>Production 3</td>
<td>3.11</td>
<td>1.00</td>
<td>2.70</td>
<td>2.78</td>
<td>3.10</td>
<td>3.77</td>
<td>2.60</td>
<td>2.92</td>
</tr>
<tr>
<td>Production 4</td>
<td>&lt; 1.00</td>
<td>1.00</td>
<td>2.07</td>
<td>2.08</td>
<td>1.30</td>
<td>2.34</td>
<td>2.49</td>
<td>3.84</td>
</tr>
<tr>
<td>Production 5</td>
<td>1.00</td>
<td>1.00</td>
<td>1.48</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt; 1.00</td>
<td>1.30</td>
<td>3.15</td>
</tr>
<tr>
<td>Production 6</td>
<td>&lt; 1.00</td>
<td>1.00</td>
<td>1.48</td>
<td>2.00</td>
<td>1.60</td>
<td>1.30</td>
<td>1.30</td>
<td>1.48</td>
</tr>
<tr>
<td><strong>Presumptive B. cereus counts on BCA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production 1</td>
<td>&lt; 1.00</td>
<td>3.56</td>
<td>3.32</td>
<td>3.36</td>
<td>3.51</td>
<td>3.36</td>
<td>3.20</td>
<td>4.51</td>
</tr>
<tr>
<td>Production 2</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
<td>2.95</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>1.70</td>
<td>2.25</td>
</tr>
<tr>
<td>Production 3</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
<td>3.53</td>
<td>3.64</td>
<td>3.86</td>
<td>3.95</td>
<td>3.08</td>
<td>5.44</td>
</tr>
<tr>
<td>Production 4</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
<td>2.04</td>
<td>2.81</td>
<td>2.14</td>
<td>2.26</td>
<td>2.38</td>
<td>3.80</td>
</tr>
<tr>
<td>Production 5</td>
<td>&lt; 1.00</td>
<td>1.30</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt; 1.00</td>
<td>1.00</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
</tr>
<tr>
<td>Production 6</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
<td>1.30</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
<td>&lt; 1.00</td>
</tr>
</tbody>
</table>

a Samples were kept for the first 16 days (factory’s internal shelf life) at 2 °C and than until the ultimate date of consumption at 7 °C (day 27); ND: not determined
2.3.3 Fingerprinting of *B. cereus* isolates

Randomly amplified polymorphic DNA (RAPD) analysis of the genetic similarity between 23 *B. cereus* isolates, with primers RPO2 and HLWL 85 (Figure 2-2) revealed two major DNA profiles comprising in average four to five DNA bands. The computer aided comparison of DNA patterns revealed two major types A (18 isolates) and B (5 isolates) and perhaps one sub-type (isolate 5.21) being very similar to the type B. All tested isolates coming from the first four productions belonged to the type A. These isolates shared also common psychrotrophic character and the same biochemical profile, pointing to one strain being implicated in the contamination of the first 4 productions. Most of the isolates from productions 5 and 6 were also clustered in the type A with three exceptions that were clustered in the type B.

2.3.4 Psychrotrophic and toxigenic character of *B. cereus* isolates

Of 38 isolates from the first four productions, 33 showed ability to grow at 7 °C (Table 2-2). Starting from an inoculum concentration of 1.0-2.0 log CFU ml⁻¹ isolates needed ca. 320 h at 7 °C and 120 h at 10 °C to reach OD of 0.1. Only four isolates (out of nine) from production 5 and 6 were able to grow at 7 °C and five out of nine at 10 °C. *B. cereus* strains sharing the same biochemical and RAPD profile (type A) were able to grow at 7 and 10 °C. Most of the strains belonging to the RAPD type B were characterized by inability to grow at 7 °C and 10 °C. None of the *B. cereus* isolates were able to grow at 4 °C.

Adjustment of pH and a_w values induced 40-45 h increase in time needed for *B. cereus* isolates to reach OD 0.1. Results confirmed also inability of *B. cereus* strains to grow at 4 °C (Table 2-3).

Out of 23 tested isolates, four (one of type B and three of type A) were found to produce HBL, while another four strains of type A produced NHE. All isolates expressing neither enterotoxin activity nor starch hydrolyses were tested on cereulide production and none of them was found to be positive.
Figure 2-2: RAPD-PCR patterns of 23 *B. cereus* potato puree isolates with primers RPO2 and HLWL85. The first number in the ID of the isolate indicates the number of the production from which the isolate was isolated; 2 indicates all isolates that were isolated from potato granules; 3 indicates all isolates that have shown negative reaction in the starch hydrolyzes test. Type of toxin produced: nt-not tested, HBL-Hemolysin BL, NHE-Non-Hemolytic-Enterotoxin.
Table 2-2: Growth of *B. cereus* potato puree isolates in NB at 7 and 10 °C

<table>
<thead>
<tr>
<th>Production</th>
<th>Total number of isolates</th>
<th>Growth at 7 °C</th>
<th>Growth at 10 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N° of isolates able to grow</td>
<td>time (h)</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>12</td>
<td>320 ± 31</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>4</td>
<td>320 ± 37</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>9</td>
<td>324 ± 37</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>8</td>
<td>352 ± 30</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>3</td>
<td>302 ± 95</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>1</td>
<td>340 ± NA</td>
</tr>
</tbody>
</table>

a An average time (h) to OD=0.1 ± 95% confidence interval; NA-Not Available

Table 2-3: Growth of *B. cereus* in NB and mNB

<table>
<thead>
<tr>
<th><em>B. cereus</em> strain</th>
<th>Start conc. log (CFU ml⁻¹)</th>
<th>Growth in NB</th>
<th>Growth in mNB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time to reach OD=0.1 (h)</td>
<td>Time to reach OD=0.1 (h)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 °C</td>
<td>7 °C</td>
</tr>
<tr>
<td>1.91</td>
<td>1.65</td>
<td>--</td>
<td>210</td>
</tr>
<tr>
<td>4.9</td>
<td>1.70</td>
<td>--</td>
<td>200</td>
</tr>
</tbody>
</table>

a NB with pH 6.0 and aw 0.994

2.4 DISCUSSION

The main ingredient in the potato puree mix, potato granules, showed a rather high mesophilic count. Downstream pasteurization of 10 minutes at 90 °C has been designed to accomplish a reduction of 6 log units in vegetative cells, therefore the source of the half-fabricate contamination laid somewhere else. Post-contamination is evident in production 1, as no *B. cereus* were detected in raw materials, but ca. 3.50 log CFU g⁻¹ of *B. cereus* were enumerated in the half fabricate. During the transfer of potato puree from the heating kettles into eurocar recipients and after the portioning into individual packages by the dosage pump post-contamination with *B. cereus* was also introduced into productions 2, 3 and 4. This can result from the existence of persistent biofilms established on the processing equipment (Logan and de Vos, 1998). These biofilms may harbor highly resistant *B. cereus* endospores enabling survival in the harsh environment and providing a continuous source for *B. cereus* transmission to the food product. Samples that were taken in the consequent production steps (process steps 4 and 5) showed relatively constant counts indicating that the time interval between portioning and final microwave pasteurization before transfer to the cooling unit was short enough to prevent significant multiplication of the present...
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

microbial flora. It may be also concluded that the microwave pasteurization is insufficient to accomplish a decrease in spores and vegetative cells still present in the closed packaged potato puree portions. Thus, *B. cereus* post-contamination could not have been eliminated during the downstream process operations. Molecular typing of *B. cereus* isolates revealed only one major type being present in the first 4 productions. There is an indication that the cleaning and disinfection process in the dosage pump had been successfully performed between productions 4 and 5, as there was a noticeable decrease in *B. cereus* count. Also a change in the DNA fingerprint was observed in *B. cereus* isolates from productions 5 and 6. It seems that a predominantly psychrotrophic *B. cereus* population with enterotoxin production potential was partially replaced by non-psychrotrophic *B. cereus* of which only one strain has been identified as an HBL producer. In the present study in total 30.4% of strains expressed enterotoxic character (13% were positive with BCET-RPLA and 17.4% with TECRA-VIA test), which is significantly less than results reported elsewhere. Valero *et al.* (2002) confirmed enterotoxin production for 71.9% of isolated *B. cereus* strains, using BCET-RPLA test, while Choma *et al.* (2000) reported 81% and 87% of *B. cereus* being positive to BCET-RPLA and TECRA-VIA tests, respectively.

Although the growth was slightly reduced when compared to the optimal conditions, the intrinsic factors of potato puree mimicked in NB supported the growth of *B. cereus*. This is in agreement with the fact that total *B. cereus* counts in the vacuum packed potato puree at the end of the shelf life exceeded $10^4$ CFU g$^{-1}$ in two out of the first four productions. In the other two productions counts remained below $10^4$ CFU g$^{-1}$, although no major difference in psychrotrophic character between respective isolates was noted. In the production 2 high counts of the psychrotrophic competitive flora, e.g. *B. circulans* may have contributed to the inhibition of the *B. cereus* growth (see Chapter 3). *Bacillus cereus* counts in the present study were higher than those reported by Del Torre *et al.* (2001) who found *B. cereus* in 33% of gnocchi puree present, but with the contamination level at the last storage day lower than $10^2$ CFU g$^{-1}$ (stored 50 days at 8 °C) and up to more than $10^5$ CFU g$^{-1}$ with storage at 12 °C. None of the isolates, isolated by Del Torre *et al.* (2001) was able to grow at 5 °C and only 27.3% showed detectable growth at 10 °C in 21 days. The work of van Netten *et al.* (1990) and Dufrenne *et al.* (1994) showed ability of some *B. cereus* strains to grow at 5 and 7 °C. Although generally regarded as dangerous (Forsythe,
2002) elevated levels of *B. cereus* (10⁴-10⁵ CFU g⁻¹) in our experiment did not make spoilage of potato puree visible, failing to alarm unfitness of the product for human consumption. This is in agreement with results published by Choma *et al.* (2000) who reported *B. cereus* numbers reaching 10⁴-10⁶ CFU g⁻¹ at 10 °C before spoilage of cooked-chilled and pasteurized vegetable products appeared.

### 2.5 CONCLUSIONS

Because it is ubiquitous in the environment, *B. cereus* will be occasionally found, in low numbers, in Ready-To-Eat foods and processing environments, mostly in the form of endospores. Therefore the main control mechanism will lay in prevention of spore germination and subsequent multiplication in Ready-To-Eat meals. Limited shelf life and storage at temperature below 7 °C (preferably at 4 °C) should provide inhibition of *B. cereus* growth in REPfed. Application of antimicrobial substances such as nisin (Jaquette and Beuchat, 1998) in combination with pH and a₅ may also be used to prevent outgrowth of *B. cereus* during the storage (see Chapter 3). However, methods to prevent the contamination and post-contamination during processing especially planned and thoroughly performed cleaning and disinfection procedures of the processing equipment, should be applied in order to eliminate the “in house” flora. However, the ability of bacterial spores and vegetative cells to adhere to inert surfaces and their resistance to cleaning or rinsing procedures is an important factor to be taken into account when designing cleaning and disinfecting procedures. The resistance of both *B. cereus* and *B. subtilis* spores to a cleaning procedure was significantly affected by the material, as reported by Faille *et al.* (2002). Hydrophobic materials were harder to clean. The same authors reported the number of adhering *B. cereus* hydrophobic spores and their resistance to a cleaning procedure being 10 times greater than those of the *B. subtilis* hydrophilic spores.

As shown in this chapter, the contamination in production of potato puree (or other REPfed (RTE) products) can originate from the ingredients. If the bulk product (not in the final package) is subjected to the heating step, which for this type of food products is of a mild character, the post-contamination may occur down the production line. In addition, mild heat treatment may activate already present *B. cereus* spores and if the cooling rate is not high enough, the spores may germinate and multiply. An additional heating in microwave may not be sufficient to eliminate this
hazard. As already mentioned, cleaning and disinfection are crucial to eliminate post-contamination. However, a correct product formulation that would include antimicrobial compounds is another and often used way to enhance shelf life and safety of the food. The following Chapter 3, will show the effect of natural antimicrobial compounds, nisin and carvacrol, on *B. cereus* and *B. circulans*, pathogen and spoilage, bacterium, respectively, as well as competition between these two organisms, all under different environmental conditions.
ANTIMICROBIAL EFFECT OF NISIN AND CARVACROL AND COMPETITION BETWEEN BACILLUS CEREUS AND BACILLUS CIRCULANS IN VACUUM PACKED POTATO PURE

Redrafted from:

A., Rajkovic, M. Uyttendaele, T. Courtens, & J. Debevere

(2005)

*Food Microbiology*, 22, vol. 2-3, 189-197
CHAPTER 3: ANTIMICROBIAL EFFECT OF NISIN AND CARVACROL AND COMPETITION BETWEEN BACILLUS CEREUS AND BACILLUS CIRCULANS IN VACUUM PACKED POTATO PUREE

Summary

A study was undertaken to investigate the growth and competition between Bacillus cereus and Bacillus circulans strains in both BHI and Ready-To-Eat potato puree. In addition, antimicrobial potential of supplemented nisin and carvacrol was evaluated against inoculated B. cereus and B. circulans strains. The accomplished growth inhibition was observed for both B. cereus and B. circulans, where B. circulans strains were more sensitive. MIC values were decreased by lowering the incubation temperature in separate applications of nisin and carvacrol, while the effect of combined application of nisin and carvacrol appeared to be more obvious at higher temperatures. The overall effect of interactions between spoilage and pathogenic Bacillus spp. was dependent on the psychrotrophic character of both cultures. The complete inhibition of B. circulans in the co-culture experiments corresponded to B. cereus population density of approximately 6 log units. Microbial challenge testing in potato puree showed that, at both 7 and 10 °C, B. cereus TZ415 and B. circulans 4.1 could grow to high counts when no antimicrobial substances (nisin and carvacrol) were applied, while no visible spoilage occurred. A more pronounced antimicrobial activity of nisin and nisin-carvacrol combination was observed in potato puree compared to the BHI medium.

Keywords: Bacillus cereus, Bacillus circulans, psychrotrophic character, nisin, carvacrol, microbial challenge testing
3.1 INTRODUCTION

*Bacillus cereus* is a spore forming food-borne pathogen associated with vegetable purees, soups, rice dishes, dairy products and complex meals containing different ingredients (see Table 1-19, Table 1-20 and Table 1-21). Some *B. cereus* strains produce either a heat labile diarrhoeal enterotoxin or a heat stable emetic toxin and their involvement in foodborne outbreaks was documented (Van Netten *et al.*, 1990; Griffiths, 1990; Harmon and Kautter, 1991; Granum *et al.*, 1993). Between 1980 and 1997, 2715 cases of *B. cereus* food poisonings in England and Wales were reported to the Public Health Laboratory Service (Ripabelli *et al.*, 2000) and the annual totals varied between 27 and 418 cases per year. Due to the difficulties in diagnosis of bacterial foodborne intoxications, it is likely that published figures represent an underestimation of the actual situation (Tompkins *et al.*, 1999). Growth of vegetative cells usually occurs within the temperature range of 10 to 50°C. However, psychrotrophic variants of *B. cereus* have been identified (Dufrenne *et al.*, 1994; Rusul and Yaacob, 1995), implying that the maintenance of the cold chain is of the utmost importance (Carlin *et al.*, 2006). Mild preservation technologies are increasingly used in food industries, endorsing the safety problem related to the spore-forming microorganisms such as *B. cereus*. Refrigerated Processed Food of Extended Durability (REPFEDs) produced using mild heat technologies (see Chapter 2) are recognized as a potential vehicle for *B. cereus* intoxications (Beuchat *et al.*, 1997).

Attempts have been made to identify processes and preservatives, either alone or in combination, that eliminate or control the growth of *B. cereus* in raw and pasteurized foods. Thomas *et al.* (1993) reported sorbate as the most effective preservative tested against *B. cereus*, whose inhibitory activity increased with an increase in acidity and NaCl concentration. Ababouch *et al.* (1992) tested 11 different fatty acids and their salts on their inhibitory activity against bacterial spores, reporting linolenic and lauric acid to be the most effective. Also, a garlic extract was reported to perform an antimicrobial activity against Gram-positive bacteria, such as *B. cereus* (Yoshida *et al.*, 1999). An alternative approach to reduce the proliferation of microorganisms is the use of essential oils. The antifungal and antibacterial effects of these components on different microorganisms have been described in several studies (Conner, 1993; Juven *et al.*, 1994; Kim *et al.*, 1995; Sivropoulou *et al.*, 1996; Ultee *et al.*, 1998).
Among the diverse group of chemical components in essential oils, carvacrol exerts a distinct antimicrobial action. Carvacrol is the major component of the essential oil fraction of oregano (60 to 74% carvacrol) and thyme (45% carvacrol) (Lagouri et al., 1993; Arrebola et al., 1994). With its hydrophobic characteristics it appears to have an influence on the biological membranes in bacterial cells (Ultee et al., 1999). Recently, Ultee et al. (1998) showed the effect of carvacrol on growth and diarrheal toxin production of *B. cereus* in Brain Hart Infusion (BHI) and soup. To accomplish the effect in soup, 50 times higher concentration of carvacrol was needed. Ultee et al. (2000b) found that concentrations of 0.15 mg g⁻¹ and higher of carvacrol in rice inhibited the growth of *B. cereus*. The extent of inhibition depended on the initial inoculum size. A synergistic antimicrobial effect was observed when 0.30 mg g⁻¹ carvacrol was combined with 0.27 mg g⁻¹ cymene. The same authors have demonstrated that a common taste enhancer soya sauce also increased the antimicrobial action of carvacrol toward *B. cereus*. The antimicrobial activity of carvacrol with cymene or soya sauce was additionally influenced by the addition of NaCl (Ultee et al., 2000b).

Several bacteriocins were investigated for their activity against *B. cereus* and nisin was reported as the most widely effective by Beuchat et al. (1997) and Wong et al. (1999). Nisin was effective in controlling both the growth of *B. cereus* in crumpets (a high-moisture, baked, flour-based product) (Jensen et al., 1994) and liquid egg (Delves-Broughton et al., 1996). Addition of nisin to the batter at levels of 3.75 µg g⁻¹ and above effectively prevented the growth of *B. cereus* to the levels capable of causing food poisoning (Jensen et al., 1994).

REPFEDs not only need control of *Bacillus* species for food safety reasons. From the food quality aspect, the main limitation and failure to meet the extended shelf life is the growth of psychrotrophic *Bacillus* spp., such as *B. circulans*. Carlin et al. (2000b) reported *B. circulans*, *B. macerans* and *B. polymyxa* to be dominating species in commercial vegetable based purees stored at 4 and 10 °C.

It was the aim of the present study to address both problems of food safety related to the outgrowth of *B. cereus* and economic losses as a consequence of food spoilage caused by development of psychrotrophic *Bacillus* spp. in REPFEDs. The potential of natural antimicrobial substances, namely carvacrol and nisin to inhibit the growth of *B.*
and B. circulans was investigated. Growth characteristics of B. cereus and B. circulans strains and the competitive effect that might occur between the two Bacillus species at refrigerated temperatures were also studied. Furthermore, microbial challenge testing (MCT) was performed in vacuum packed potato puree supplemented with (or without) antimicrobial substances, to determine the ability of the food product to support the growth of spoilage organism, B. circulans, and the pathogen, B. cereus. Challenge testing was to provide valuable information for risk assessment related to the RTE potato puree and B. cereus as the pathogen of concern (Notermans et al., 1993; Uyttendaele et al., 2004).

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Bacterial strains and enumeration

Four B. cereus strains, namely two potato puree isolates 1.94 and 4.9 (Laboratory for Food Microbiology and Food Preservation-LFMFP, Culture Collection, Ghent University, Belgium) and strains TZ415 and Z4222 isolated from minimally processed courgette puree, provided by dr. F. Carlin (Unité de Technologie des Produits Végétaux, INRA, France), were used throughout the study. In addition, B. circulans strains 2.4, 4.1 and A1 (potato puree isolates) and B. circulans strain W1 (carrot puree isolates) from LFMFP culture collection were used. Reference stock cultures, kept on Nutrient agar slants (NA, Oxoid, Basingstoke, England, UK) at 7 °C were activated in Nutrient broth (NB, Oxoid) by incubation at 30 °C for 24 h, making working stock culture. Enumeration was done by plating appropriate ten-fold dilutions (in Peptone Physiological Solution, PPS) of the sample on NA, if working with the pure culture, or Bacillus cereus agar (BCA, Mannitol Egg Yolk Polymyxin agar, Oxoid) in the case of mixed cultures. B. cereus colonies on BCA gave typical pink color surrounded by a white zone as a consequence of the B. cereus lecithinase activity, while B. circulans remained yellow colored and without white surrounding zone. On NA plates B. cereus colonies were bigger and mat, comparing to smaller and slimy appearing B. circulans strains. The counts on NA and BCA were, for both B. cereus and B. circulans comparable, indicating no inhibition of strains on the selective BCA medium.
3.2.2 Determination of minimal inhibitory concentration (MIC) value of carvacrol and nisin

Nisin, in the form of Nisaplin was obtained from Aplin and Barret, Ltd., Trowbridge, UK and was dissolved in 50% ethanol (VWR, Leuven, Belgium), centrifuged and filter sterilized through 0.2 µm pore size filter (Nalge Nunc International, New York, US). The nisin stock solution of 1 mg ml⁻¹ (40 000 IU nisin ml⁻¹) was stored at -20 °C. Carvacrol was obtained from Sigma-Aldrich chemicals GmbH, Steinheim, Germany, dissolved in 100% ethanol and stock solutions of 1.00 M concentration were stored at 4 °C. The antimicrobial activity was determined using a modified critical-dilution assay in microtiter plates (Gänzle et al., 1996). In short, twofold serial dilutions of the analytes, nisin and carvacrol, were prepared in NB starting from the concentration of 100 µg ml⁻¹ and 3 mM (0.451 g l⁻¹, molecular weight of carvacrol being 150.22 g mol⁻¹) for two analytes respectively. From the prepared dilution serial, 100 µl of each concentration was added to the wells of a microtiter plate. Subsequently 100 µl of an appropriate dilution of B. cereus (TZ415, Z4222, 1.91 and 4.9) or B. circulans (2.4, 4.1, A1 and W1), in NB, was inoculated to each well in order to obtain an OD (at 660 nm) of 0.02 (corresponding to ca. 5x10⁶ CFU ml⁻¹). For each strain and each concentration of analytes four replicates were included. Growth of the strains was further monitored by measuring the optical density at 660 nm. Antimicrobial activity was evaluated at 7, 10 and 22 °C during an incubation period of four times the time needed by the control sample (no addition of antimicrobial analytes) to show an increase in microbial population density of 1 log unit (experimentally set as attaining an OD of 0.210 for B. cereus and 0.065 for B. circulans). The lowest concentration of antimicrobial component at which the OD did not reach the critical value was set as the MIC. As the MIC value for each of the individual components was revealed, subsequently the combination effect of antimicrobial substances was investigated. The same principle as for MIC determination of individual components and the same test conditions were applied to test different combinations of nisin-carvacrol (Table 3-1) in order to elucidate inhibitory effect on B. cereus and B. circulans. The antimicrobial effects of nisin-carvacrol combinations were compared to the effects imposed by each of the antimicrobial substances individually, looking to optimize concentrations that would meet requirements of successful “combination technology”.

Table 3-1: Concentrations of nisin (µg ml⁻¹/IU) and carvacrol (mM) applied together to investigate possible combination effect in their antimicrobial effect against *B. cereus* and *B. circulans*

<table>
<thead>
<tr>
<th>Applied concentration of nisin (µg ml⁻¹/IU)</th>
<th>0.00/0.00</th>
<th>2.00/80</th>
<th>5.00/200</th>
<th>10.00/400</th>
<th>25.00/1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied concentration of carvacrol (mM)</td>
<td>0.00</td>
<td>a</td>
<td>a/b</td>
<td>a/b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>a/b</td>
<td>a</td>
<td>a/b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>a/b</td>
<td>a</td>
<td>a/b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

a: the combination applied only on *B. circulans*; b: the combination applied only on *B. cereus*

a/b: the combination applied on both *B. cereus* and *B. circulans*; nt: not tested

3.2.3 Growth characteristics of *B. cereus* and *B. circulans* and co-cultures thereof at 7 and 10 °C

The inoculation ratios of *B. cereus: B. circulans* 1:0, 10:1, 1:1, 1:10 and 0:1 were used in the study. The ratio 1:0 and 0:1 were obtained by transfer of 0.1 ml of an appropriate dilution of a respectively *B. cereus* and *B. circulans* 24 h culture to 12 ml fresh BHI to obtain 10² CFU ml⁻¹ of *B. cereus* and *B. circulans*, respectively. For the ratio 10:1, 1:1 and 1:10, 0.1ml volumes of the appropriate dilution of the corresponding culture were mixed in 12 ml BHI in order to obtain ca 10² CFU ml⁻¹ in absolute numbers as a unit of the ratio. In order to know the exact inoculum appropriate dilutions were enumerated on NA. Mixed cultures at different ratios were incubated at test temperatures of 7 and 10 °C and subsequently enumerated by plating on BCA at regular time intervals. The experimental setup was performed in triplicate. Mixed cultures included different ratios of *B. cereus* TZ415/*B. circulans* 4.1 and *B. cereus* 1.91/*B. circulans* 4.1. Enumeration at different time intervals were plotted into growth curves which were graphically inspected, without usage of models for growth rate determination.
3.2.4 Antimicrobial effect of nisin and carvacrol in the potato puree and growth characteristics of *B. cereus* and *B. circulans*

To verify the reliability of the data obtained through the tests performed in the culture media, the last sequence of the experiments was performed as a challenge test in vacuum packed, Ready-To-Eat (RTE) potato puree. Potato puree contained potato granules as a major ingredient, potato flakes and spices such as white pepper, muscat nut aroma and curcuma extract.

The growth of *B. cereus* TZ415 and *B. circulans* 4.1, both as single strains and co-cultures (1:1), was monitored in vacuum packed RTE potato puree stored at 7 and 10 °C, both in the presence and absence of nisin (10 µg g⁻¹) and carvacrol (75 µg g⁻¹), with antimicrobial components added in combination or apart. Potato puree was obtained from the local company at the date of production, in packages of 500 g and stored at 2 °C until the results of natural contamination with *Bacillus* spp., as determined by plating on BCA, were available. The pH and aw were determined using an Ingold stab-electrode (Ingold, 104063123, Urdorf, Switzerland) and a fully automated cryometer (WK-20, NAGY Messysteme GmbH, Gäufelden, Germany), respectively. Inoculation of potato puree was done in duplicates in the following order: original 500 g packages were repacked into packages of 200 g, followed by addition of nisin (1 mg nisin per 100 g potato puree) and/or carvacrol (0.5 ml of 0.1 M carvacrol solution, corresponding to 7.51 mg carvacrol per 100 g potato puree), if applicable. The uniform distribution of the antimicrobial substances was assured by mechanical treatment of one minute in a stomacher (Colworth Stomacher 400, Steward Laboratory, London, UK). The aseptic inoculation with ca. 2.5 log CFU g⁻¹ of *B. cereus* and/or *B. circulans* under the air flow of a biohazard (Biosafe 1, 2 Bio Hazard Holton, Allerød, DK) was performed by distribution of 1 ml of an appropriate dilution over 100 g potato puree. Following the inoculation, packages were mixed for one minute in the stomacher, vacuum sealed (Multivac A300/42, Vacuum compensation chamber, Hagenmüller KG, Germany) and forwarded to an air-ventilated, temperature-controlled refrigerator for storage at either 7 or 10 °C. Samples were taken for enumeration of *B. cereus* and *B. circulans* at regular time intervals by plating on BCA. At the start of the experiment the detection limit of the
Biological and immuno-molecular methods for detection of \textit{B. cereus} and \textit{S. aureus} enterotoxins

Enumeration method was lowered to ca. 10 CFU g\(^{-1}\) by spreading 1 ml of the primary dilution (food homogenate in PPS) over three BCA plates.

3.3 RESULTS

3.3.1 Determination of MIC value and combination effect of antimicrobial substances

Although both \textit{B. cereus} and \textit{B. circulans} were in the inhibitory spectrum of nisin and carvacrol, \textit{B. cereus} exhibited stronger resistance towards nisin (Table 3-2). Small variations in the MIC value of carvacrol were observed both among species and strains and between incubation temperatures. As expected, the incubation at reduced, non-optimal temperatures of 7 and 10 °C may decrease the MIC value, although not consistently for all of the strains.

Subsequently, possible combined antimicrobial effect was determined by testing combinations of nisin and carvacrol and comparing the results with those of the repeated test for the individual antimicrobial substances. Combination effects in Table 3-2 were defined and reported as the lowest and decreased nisin and carvacrol concentration that may be applied in together to accomplish the growth inhibition of \textit{Bacillus} spp. Combination effect was observed for all \textit{B. cereus} strains at 22 °C. If used together, 25.0 µg ml\(^{-1}\) nisin and 0.50 mM (75.11 µg ml\(^{-1}\)) or 0.75 mM (112.66 µg ml\(^{-1}\)) carvacrol respectively were sufficient to inhibit growth of TZ415 and Z4222 strains, whereas for strains 1.91 and 4.9, already 10 µg ml\(^{-1}\) nisin and 0.50 mM carvacrol produced an inhibitory effect.

Except for strain 2.4, a combination of 2.0 µg ml\(^{-1}\) nisin and 0.25 mM (37.56 µg ml\(^{-1}\)) carvacrol was sufficient to establish growth inhibition of \textit{B. circulans}. When either nisin or carvacrol were applied individually, \textit{B. cereus} and \textit{B. circulans} strains required higher concentrations for an inhibitory effect, respectively 5.0 to 10.0 µg ml\(^{-1}\) nisin and up to 1.0 mM (150.22 µg ml\(^{-1}\)) carvacrol for \textit{B. circulans} and more than 50 µg ml\(^{-1}\) nisin and 1.0 mM carvacrol for \textit{B. cereus}. At 10 °C no combination effect was noted for \textit{B. cereus} strain TZ415, while at 7 °C also for strain Z4222 no combination effect was observed. For all four \textit{B. circulans} strains no combination effect was noted at both 7 and 10 °C.
Table 3-2: MIC values of nisin and carvacrol in nutrient broth with different nisin-carvacrol concentrations resulting in possible combination effect (CE), monitored at 7, 10 and 22 °C

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>Incubation temperature</th>
<th>Nisin and carvacrol as single components</th>
<th>Concentrations of nisin-carvacrol combinations resulting in CE a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MIC nisin (µg ml⁻¹)</td>
<td>MIC carvacrol (mM)</td>
</tr>
<tr>
<td>B. cereus</td>
<td>TZ415</td>
<td>7 °C</td>
<td>25,0</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 °C</td>
<td>25,0</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 °C</td>
<td>&gt; 50,0</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td>Z4222</td>
<td>7 °C</td>
<td>25,0</td>
<td>0,75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 °C</td>
<td>&gt; 50,0</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 °C</td>
<td>&gt; 50,0</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td>1.91</td>
<td>7 °C</td>
<td>25,0</td>
<td>0,50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 °C</td>
<td>25,0</td>
<td>0,50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 °C</td>
<td>25,0</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td>4.9</td>
<td>7 °C</td>
<td>25,0</td>
<td>0,50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 °C</td>
<td>&gt; 50</td>
<td>0,75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 °C</td>
<td>&gt; 50</td>
<td>1,00</td>
</tr>
<tr>
<td>B. circulans</td>
<td>2.4</td>
<td>7 °C</td>
<td>2,0</td>
<td>0,50</td>
</tr>
<tr>
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<tr>
<td></td>
<td></td>
<td>22 °C</td>
<td>5,0</td>
<td>0,75</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>7 °C</td>
<td>2,0</td>
<td>0,50</td>
</tr>
<tr>
<td></td>
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<td>10 °C</td>
<td>5,0</td>
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</tr>
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<td></td>
<td></td>
<td>22 °C</td>
<td>5,0</td>
<td>0,75</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>7 °C</td>
<td>10,0</td>
<td>0,75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 °C</td>
<td>10,0</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 °C</td>
<td>10,0</td>
<td>1,25</td>
</tr>
<tr>
<td></td>
<td>W1</td>
<td>7 °C</td>
<td>10,0</td>
<td>0,75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 °C</td>
<td>10,0</td>
<td>0,75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 °C</td>
<td>10,0</td>
<td>1,00</td>
</tr>
</tbody>
</table>

a Three out of four repetitions showed no growth

b CE: combination effect; No CE observed: The effect of all applied nisin-carvacrol combinations resulted in the same antimicrobial effect as with individual application;
3.3.2 Growth characteristics of *B. cereus*, *B. circulans* and co-cultures thereof at 7 and 10 °C in BHI

Growth curves of *B. cereus* at 10 °C elucidated from Figure 3-1(A and C), both as a pure culture and in co-culture with *B. circulans*, revealed that *B. cereus* TZ415 had a higher growth rate at 10 °C compared to *B. cereus* 1.91, obtaining counts of ca. $10^7$ CFU ml$^{-1}$ within 72 h, whereas *B. cereus* 1.91 nearly reaches counts of ca. $10^7$ CFU ml$^{-1}$ after 128 h incubation in BHI at 10 °C, both starting from an initial inoculation level of $10^2$ CFU ml$^{-1}$. The presence of *B. circulans* 4.1 in the co-culture did not alter the growth curve of the *B. cereus* strains at 10 °C.

On the contrary, *B. cereus* TZ415 suppressed growth of *B. circulans* if grown in co-culture and growth inhibition and subsequent decrease in *B. circulans* numbers was observed at an earlier stage in the growth curve of *B. circulans* as the ratio of *B. cereus*: *B. circulans* increased (Figure 3-1B). Growth inhibition of *B. circulans* in the co-culture was noticed at the time when *B. cereus* TZ415 numbers reached $10^5$-$10^6$ CFU ml$^{-1}$. *B. cereus* 1.91 did not have a similar inhibitory effect on *B. circulans* growth if grown in co-culture (Figure 3-1D).

Both *B. cereus* 1.91 and *B. circulans* 4.1 showed a similar growth curve at 10 °C, *B. circulans* even obtaining higher numbers than *B. cereus* by the end of the experiment. As expected, at the temperature of 7 °C, near to the temperature limit for *B. cereus* growth, data provided in Figure 3-2 (A and C) indicate that the growth rate of *B. cereus* TZ415 and 1.91 decreased when compared to the growth rate at 10 °C, as realized from Figure 3-1 (A and C). At 7 °C, a rapid growth of *B. circulans* 4.1 was noticed and the inhibitory effect of *B. cereus* TZ415 was evident only when inoculation ratio of *B. cereus*: *B. circulans* was 10:1 (Figure 3-2B). *B. cereus* 1.91 did not induce an inhibitory effect on *B. circulans* 4.1 in the co-culture at 7 °C (Figure 3-2D). On the contrary the opposite effect was noted: *B. circulans* 4.1 forced *B. cereus* 1.91 to enter the stationary phase at an earlier stage, inducing further a steady decrease in *B. cereus* 1.91 counts under *B. circulans* 4.1 domination in the co-culture. Co-culture experiments, performed both at 7 and 10 °C, indicated that *B. cereus* TZ415 seems to be a better competitive organism in comparison to *B. cereus* 1.91.
Biological and immuno-molecular methods for detection of B. cereus and S. aureus enterotoxin

Chapter 3: Control of Bacillus cereus

Figure 3-1: A: Growth of B. cereus TZ415 in competition with B. circulans 4.1 at 10 °C in BHI with different B. cereus to B. circulans ratios: 1:0 (♦), 10:1 (×), 1:1 (■) and 1:10 (▲). B: Growth of B. circulans 4.1 in competition with B. cereus TZ415 at 10 °C in BHI with different B. cereus to B. circulans ratios: 0:1 (♦), 10:1 (×), 1:1 (■) and 1:10 (▲). C: Growth of B. cereus 1.91 in competition with B. circulans 4.1 at 10 °C in BHI with different B. cereus to B. circulans ratios: 1:0 (♦), 10:1 (×), 1:1 (■) and 1:10 (▲). D: Growth of B. circulans 4.1 in competition with B. cereus 1.91 at 10 °C in BHI with different B. cereus to B. circulans ratios: 0:1 (♦), 10:1 (×), 1:1 (■) and 1:10 (▲). Detection limit of enumeration methodology was 1 log CFU ml⁻¹ and 0 log CFU ml⁻¹ represents results beneath the detection limit.
Biological and immuno-molecular methods for detection of \textit{B. cereus} and \textit{S. aureus} enterotoxin

Chapter 3: Control of \textit{Bacillus cereus}

Figure 3-2: A: Growth of \textit{B. cereus} TZ415 in competition with \textit{B. circulans} 4.1 at 7 °C in BHI with different \textit{B. cereus} to \textit{B. circulans} ratios 1:0 (♦), 10:1 (x), 1:1 (■) and 1:10 (▲). B: Growth of \textit{B. circulans} 4.1 in competition with \textit{B. cereus} TZ415 at 7 °C in BHI with different \textit{B. cereus} to \textit{B. circulans} ratios 0:1 (♦), 10:1 (x), 1:1 (■) and 1:10 (▲). C: growth of \textit{B. cereus} 1.91 in competition with \textit{B. circulans} 4.1 at 7 °C in BHI with different \textit{B. cereus} to \textit{B. circulans} ratios 1:0 (♦), 10:1 (x), 1:1 (■) and 1:10 (▲). D: Growth of \textit{B. circulans} 4.1 in competition with \textit{B. cereus} 1.91 at 7 °C in BHI with different \textit{B. cereus} to \textit{B. circulans} ratios 0:1 (♦), 10:1 (x), 1:1 (■) and 1:10 (▲). Detection limit of enumeration methodology was 1 log CFU ml\(^{-1}\) and 0 log CFU ml\(^{-1}\) represents results beneath the detection limit.
3.3.3 Antimicrobial effect of nisin and carvacrol and growth characteristics of *B. cereus* and *B. circulans* in potato puree

The potato puree used in the study had a pH of 5.91 ± 0.02 and a water activity of 0.9940 ± 0.0007. Both at 10 and 7 °C no colonies were observed on BCA for the control samples (non-inoculated with *B. cereus* TZ415 and/or *B. circulans* 4.1) when subjected to microbial analyses, throughout the shelf life study. Figure 3-3 and Figure 3-4 indicate that combination of 10 µg g⁻¹ of nisin and 75 µg g⁻¹ (0.5 µmol g⁻¹) carvacrol inhibited the growth of both *B. cereus* TZ415 and *B. circulans* 4.1 at 10 and 7 °C. Moreover, it imposed a bactericidal effect decreasing the inoculum (log 2.5 CFU g⁻¹) to non-detectable numbers (<10 CFU g⁻¹) within 25 h.

Upon repetition of the challenge test in potato puree at 7 °C, with either separate or no addition of nisin and carvacrol, it became obvious that addition of carvacrol (75µg g⁻¹) did not accomplish any bactericidal or bacteriostatic effect towards *B. cereus* TZ415 or *B. circulans* 4.1. The observed bactericidal effect could be fully attributed to the antimicrobial effect of nisin alone at a concentration of 10 µg g⁻¹ (Figure 3-5).

![Figure 3-3](image1.png)

![Figure 3-3](image2.png)

**Figure 3-3:** A: Growth of *B. cereus* TZ415 at 10 °C in potato puree: in absence of nisin and carvacrol without *B. circulans* 4.1 (♦); with *B. circulans* 4.1 (♠); in presence of 10 µg/g nisin and 75 µg/g (0.5 mM) carvacrol: without *B. circulans* 4.1 (▲), with *B. circulans* 4.1. (×). B: Growth of *B. circulans* 4.1 at 10 °C in potato puree: in absence of nisin and carvacrol without *B. cereus* TZ415 (♦), with *B. cereus* TZ415 (♠); in presence of 10 µg/g nisin and 75 µg/g (0.5 mM) carvacrol without *B. cereus* TZ415 (▲), with *B. cereus* TZ415 (×). Detection limit of enumeration methodology was 1 log CFU g⁻¹ and 0 log CFU g⁻¹ represents results beneath the detection limit.
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Chapter 3: Control of *Bacillus cereus*

Figure 3-4: A: Growth of *B. cereus* TZ415 at 7 °C in potato puree: in absence of nisin and carvacrol without *B. circulans* 4.1 (♦), with *B. circulans* 4.1 (■); in presence of 10 µg/g nisin and 75 µg/g (0.5 mM) carvacrol without *B. circulans* 4.1 (▲), with *B. circulans* 4.1 (×). B: Growth of *B. circulans* 4.1 at 7 °C in potato puree: in absence of nisin and carvacrol without *B. cereus* TZ415 (♦), with *B. cereus* TZ415 (■); in presence of 10 µg/g nisin and 75 µg/g (0.5 mM) carvacrol without *B. cereus* TZ415 (▲), with *B. cereus* TZ415 (×). Detection limit of enumeration methodology was 1 log CFU g⁻¹ and 0 log CFU g⁻¹ represents results beneath the detection limit.

Figure 3-5: A: Growth of *B. cereus* TZ415 at 7 °C in potato puree in presence of *B. circulans* 4.1: in absence of nisin and carvacrol (♦), with 75 µg/g (0.5 mM) carvacrol (■), with 10 µg/g nisin (▲), with a combination of 75 µg/g (0.5 mM) carvacrol-10 µg/g nisin (×). B: Growth of *B. circulans* 4.1 at 7 °C in potato puree in presence of *B. cereus* TZ415: in absence of nisin and carvacrol (♦), with 75 µg/g (0.5 mM) carvacrol (■), with 10 µg/g nisin (▲), with a combination of 75 µg/g (0.5 mM) carvacrol-10 µg/g nisin (×). Detection limit of enumeration methodology was 1 log CFU g⁻¹ and 0 log CFU g⁻¹ represents results beneath the detection limit.
3.4 DISCUSSION

Carvacrol and nisin accomplished growth inhibition of *B. cereus* and *B. circulans*, although *B. circulans* strains were shown to be more sensitive. The antimicrobial effect was strain dependent. Also Periago and Moezelaar (2001) had found strain dependency of the antimicrobial effect of nisin on *B. cereus*. In the present study an addition of 10 µg g⁻¹ nisin in potato puree inhibited growth of both the spoilage organism *B. circulans* and the pathogenic *B. cereus*.

The MIC values for nisin towards *B. cereus* were not directly comparable with those determined in BHI by Pol and Smid (1999), finding nisin MIC-values of 10 µg ml⁻¹ and 5 µg ml⁻¹, at 20 °C and 8 °C respectively. Although nisin is a natural preservative with restricted applications due to its lack of activity towards Gram negative bacteria and the fact that it is immobilized by fats and other food components it was found that 0.05% nisin was sufficient to prevent outgrowth of 10³ spores of *B. cereus* per gram of infant formula for 5 days (Wong et al., 1999). The three-strain cocktail was slightly more resistant to nisin, and although the spores were not completely inactivated, a level of 0.1% nisin could reduce outgrowth to less than 10² CFU ml⁻¹ after 5 days at 25°C. The MIC values of carvacrol in the present study (0.5-1.0 mM) were comparable with reported values of 1.25 mM and 0.63 mM carvacrol at respective temperatures (Pol and Smid, 1999). Also Ultee et al. (1998) found MIC values for *B. cereus* at 8 °C to be 0.75 mM of carvacrol. The effects of various concentrations of borneol, carvacrol, cinnamaldehyde, eugenol, menthol, thymol, and vanillin were examined by Valero and Giner (2006), reporting 5 µl of cinnamaldehyde, 15 µl of carvacrol, or 30 mg of thymol per 100 ml of inoculated carrot broth completely inhibited *B. cereus* growth for more than 60 days at 16 °C. Lower concentrations of the three antimicrobials prolonged the lag phase and reduced both the exponential growth rate and the final population densities of cultures. Same authors reported absence of adverse effects of antimicrobial compounds on the sensorial properties of carrot broth. Valero and Frances (2006) reported that when carvacrol alone was applied in carrot broth concentration of 5 µl of carvacrol per 100 ml of inoculated carrot broth were unable to inhibit bacterial *B. cereus* at 8 °C. They also found that no development of *B. cereus* in carrot broth was observed at 12 °C in the presence of 2 µl in 100 ml cinnamaldehyde only.
The influence of the temperature is of high significance on the action of nisin and carvacrol. Whilst in separate usage lower temperature decreased the MIC values, a combination effect noted in this study, was more obvious at higher temperatures. Although the action mode of this combination effect is still to be explained, literature suggests dissipation of proton motive force as a possible clarification (Pol et al., 2002). Both nisin and carvacrol share properties that can cause disturbance of membrane integrity, further influencing alternation in ATP synthesis and extra ATP depletion. A more pronounced antimicrobial activity of nisin and nisin-carvacrol combination in potato puree compared to BHI medium corresponds to the expected beneficial influence of lower pH on the nisin stability and activity (pH 5.91 ± 0.02 in potato puree versus 7.0 ± 0.02 in BHI medium). This is in accordance to findings of Jaquette and Beuchat (1998). However, the observed bactericidal effect was also noted when 10 µg g⁻¹ of nisin was applied only. If only carvacrol (75 µg g⁻¹) was applied strains were still able to grow to high counts, similar to the control sample (no antimicrobial substances applied), although this concentration of carvacrol was sufficient to inhibit all *B. cereus* and *B. circulans* strains when tested in NB at 7 °C. The difference in produced effect could be perhaps explained by lipophilic nature of carvacrol (Pol et al., 2001) that possibly became associated with fat particles in potato puree, thus becoming unavailable for *Bacillus* spp. and hampered to induce alteration in membrane fluidity, as suggested mechanism of carvacrol activity against *B. cereus* (Ultee et al., 2000a).

The complete inhibition of *B. circulans* in the co-culture experiments corresponded to *B. cereus* population density of approximately 6 log units, which appeared to be a critical concentration, possibly required for mechanism of quorum sensing (Schauer and Bassler, 2001; Miller and Bassler, 2001). The difference between effects imposed by two different *B. cereus* strains on *B. circulans* at 10 °C could be attributed to the significant difference in growth characteristics at low temperatures. Slow growth of *B. cereus* 1.91 enabled *B. circulans* to grow to the higher counts (more than 10⁶ CFU g⁻¹) before *B. cereus* reached the critical concentration of 10⁶ CFU g⁻¹. The other strain of *B. cereus* was shown to be a better competitive organism at 10 °C, although it should be noted that for a pathogen like *B. cereus* to be a competitive flora is not a merit. The influence of the test temperature was also evident in the co-culture of *B. cereus* and *B. circulans*, where *B. circulans* strain was able to reduce the
growth of one of the two *B. cereus* strains. This indicates that the extent to which refrigeration temperatures are maintained and the cold chain is respected has a significant impact on the growth of psychrotrophic *Bacillus* spoilers, such as *B. circulans* and the multiplication of pathogenic *B. cereus* strains. Storage at a temperature of 7 °C revealed a dominating population of *B. circulans*, which was able to partially inhibit the growth of one of the two tested *B. cereus* strains. Also Guinebretiere *et al.* (2001), stressed that the compliance with low storage temperatures (4 °C) should prevent spoilage and growth of potential human pathogens such as *B. cereus*. The temperature abuse of 10 °C favors the fast outgrowth of pathogenic *B. cereus*, reaching counts of more than $10^5$ CFU g$^{-1}$ within approximately 70 h of storage. The overall effect of interaction between spoilage and pathogenic *Bacillus* spp. will depend upon the psychrotrophic character of both cultures influencing finally the time needed by *B. cereus* to grow to critical population density becoming a dominant bacterium.

Microbial challenge testing in potato puree showed that both at 7 and 10 °C *B. cereus* and *B. circulans* could grow to high counts when no antimicrobial substances (nisin-carvacrol) were applied. At 7 °C, *B. cereus* reached within 150 h counts higher than $10^5$ CFU g$^{-1}$, which is the critical value suggested by Code for production, distribution and retailing of REPFED (Anonymous, 1994). These high counts did not create visible signs of the spoilage, while Guinebretiere *et al.* (2001) reported spoilage, shown by pack swelling, of zucchini pure after storage at 10 °C.

### 3.5 CONCLUSIONS

In conclusion, REPFEDs may suffer from contamination by *Bacillus* spp. imposing both problems of food safety and food spoilage. In the present study it was shown that both aspects of microbial quality are closely related and that a small variation of one of the parameters in the “combination technology”, on which the safety and preservation of these minimally processed foods relies, might cause a serious shift in the microbial population. Temperature abuse clearly favors the growth of *B. cereus* raising a food safety issue, whereas the respect of the cold chain causes a dominance of the psychrotrophic *B. circulans*, hampering the product to meet the economically
important extended shelf life. However, the growth of these *Bacillus* species can be inhibited by application of natural antimicrobial substances, such as carvacrol and nisin, with the effect being species and strain dependent. Nevertheless, microbial challenge testing of the food sample under consideration is essential to validate the antimicrobial effect observed in defined culture media. The food matrix might complicate the application of these antimicrobial substances, as was the case with carvacrol. Nisin on the other hand showed potential to be used as a preservative in this type of food products.

When the control measures fail to impose sufficient inhibitory effect on *B. cereus* and growth to high counts occurs, cereulide production may result if cereulide producing strains were present. In such a case reliable detection method to detect cereulide in food is needed. The chapter 4 introduces computer aided optimized boar semen motility analyzes as a computerized bioassay to quantitatively determine cereulide presence and production in laboratory media, under different food relevant conditions (different packaging atmospheres, aeration, different solid and liquid growth media etc). In the chapters thereafter, the same method will be applied in foodstuffs.
DYNAMICS OF BOAR SEMEN MOTILITY INHIBITION AS A SEMI-QUANTITATIVE MEASUREMENT OF *Bacillus Cereus* EMETIC TOXIN (CEREULIDE)

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(2006)

*Journal of Microbiological Methods*, vol 65, 3, 525-534
CHAPTER 4: DYNAMICS OF BOAR SEMEN MOTILITY INHIBITION AS A SEMI-QUANTITATIVE MEASUREMENT OF *BACILLUS CEREUS* EMETIC TOXIN (CEREULIDE)

Summary

Qualitative and quantitative application of a computer assisted sperm analyzer (CASA) for detection and quantification of cereulide was described. The plot of the decrease of the percentage of boar semen progressive motility (PMOT%) in function of time and the visual inspection of curves provided a qualitative comparison between different samples (curve slope corresponds to the amount of cereulide in the sample). If the change of PMOT% over a time required for achieving PMOT% drop to 10% ($\Delta$PMOT%/Δτ) is plotted against the standard curve (obtained with known concentrations of valinomycin), a semi-quantitative estimation of the amount of cereulide in the sample is obtained. An optimized CASA method was applied to determine the production of cereulide under different conditions. No cereulide was found in aerated samples and in samples incubated at 12 °C. The amount of cereulide produced depended on the agar medium used, type of *B. cereus* strain and the amount of oxygen present in the atmosphere.

Keywords: *Bacillus cereus*, cereulide, emetic toxin, bioassay, boar semen motility
4.1 INTRODUCTION

The lack of suitable, easy to handle cereulide detection method makes routine detection of this exotoxin in laboratory media and food samples difficult. A simple, yet powerful assay based on the cereulide toxicity towards mitochondria was published (Andersson et al., 1998b; Haggblom et al., 2002; Andersson et al., 2004). The assay is monitoring cease of boar sperm motility when exposed to cereulide. The toxin acts as an ionophor, transporting $K^+$ ions via the ion-carrier system into the mitochondria downstream of the electrical and concentration gradients. In this way it resembles the mode of action of valinomycin (Hoornstra et al., 2003). The damaged mitochondria fail in oxidoreductive functioning, causing changes in macroscopic behavior of semen cells, expressed as a decrease in cell motility (Andersson et al., 1998b; Andersson et al., 2004). A possible observation of the semen motility can be done visually, as well as by means of the computer assisted sperm analysis (CASA) systems. The main problems related to classical microscopic methods (i.e. subjectivity and variability) may result in non reproducible results, especially when done on inter-laboratory bases as it is dependent on the evaluator’s skills. This in return results in high variations in the estimation of the motility parameters of same ejaculates assessed by different observers (Rijsselaere et al., 2003). Computerized measuring devices yield more objective results and allow for monitoring of multiple parameters simultaneously. One of the computer-aided semen analyzers, the Hamilton-Thorne, was previously validated for the evaluation of boar semen (Abaigar et al., 1999) and semen of some other mammalian species (Farrell et al., 1996). The main problems related to CASA application are the extreme needs for standardization of procedures, optimization of timing and validation of the system before any practical use is possible (Rijsselaere et al., 2003).

In the present study a CASA-system (Hamilton Thorne Ceros 12.1) was used to determine the dynamics of boar semen motility changes under the toxic impact of cereulide on the semen mitochondria. In correlation with the effect of valinomycin on boar semen a semi quantitative system to detect cereulide concentrations in the range of 20–400 ng ml$^{-1}$ was established. The detection system was further evaluated to determine the cereulide production by B. cereus under various, food relevant, growth conditions.
4.2 MATERIALS AND METHODS

4.2.1 Optimization of the bioassay and of the semi-quantitative approach

4.2.1.1 Test protocol, material and apparatus

The boar semen motility test was applied with minor modifications to the originally described protocol (Andersson et al., 1998b; Andersson et al., 1998c; Andersson et al., 2004). In short 195 µl of sperm was mixed with 5 µl of solution (blank or containing cereulide or valinomycin) in wells of a 37 °C pre-warmed microtiter plate (96 wells format) and immediately transferred into a Leja-slide (standard count 2-chamber 20 micron slide, Leja, Nieuw-Vennep, The Netherlands). Slides were pre-warmed on a portable stage warmer (automatic calibration to 37 °C). The HTR Ceros 12.1 (Hamilton Thorne Research, Beverly, US) consists of a phase-contrast microscope (Olympus, 10X magnification objective), a camera, a MiniTherm stage warmer, an image digitizer and a computer to save and analyze obtained data (Figure 4-1). If only a qualitative toxicity evaluation was required, undiluted samples were subjected to the test and results were, depending on concentration of cereulide present, obtained within 10 to 300 seconds. When a quantitative estimation was required the sample was serially diluted in two-fold dilution series requiring two consecutive dilution steps to give results within a semi linear range of a valinomycin standard curve.

4.2.1.2 Boar Semen

Boar semen (produced and provided by Hypor KI, Olsene, Belgium), either of the Belgian Piétrain extra muscled or the Beau-Pi boar, was on the production site diluted with extender containing sugar (to keep the spermatozoa alive), Na-EDTA as pH buffer, ions for the maintenance of osmotic balance and antibiotic (gentamycine) to control bacterial contamination. The sperm was standardized to a concentration of approximately 30 millions cells per milliliter with overall motility of more than 80% and packed in 100 ml plastic capped flasks. As recommended by Hypor KI the boar sperm was kept at temperatures between 15 °C and 20 °C (optimum 17 °C) and the fresh suspension was used for every day of analyses.
4.2.1.3 Effect of organic solvents and exposure time

The following organic solvents were used to dissolve and dilute cereulide: methanol (Fisher Scientific, Leicestershire, UK), ethanol (Vel, Leuven, Belgium) and dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany). All three solvents were tested in a ratio 40 : 1 (semen : solvent), as described above, in order to determine the one with least influence on the boar spermatozoa motility. The contact time of solvent and semen is divided into two sequential periods. The period of the first 5 seconds, during which mixing of semen with solvent occurs and transfer of 7 µl of the mixture into the chamber of Leja slides, forms the pre-exposure time (pET). Leja slides are preset onto a heating-stage providing a uniform temperature of 37 °C on the slide. Since sperm motility and velocity are highly dependent on the temperature, assessment of the motility should be performed as close to 37 °C as possible (Igerouada and Verstegen, 2001). At the moment of injection of the mixture into one of the Leja chambers, exposure time (ET) and observation of the semen behavior start.
Motility changes are monitored on the computer screen by capturing the image and motility parameters every 5-10 seconds. The impact of the solvent on the percentage of semen progressive motility (PMOT%) is evaluated by calculating the average PMOT% and its standard deviation. The solvent inducing the smallest decrease in the PMOT% was chosen for further experiments.

4.2.2 Effect of extraction and cereulide recovery from *B. cereus* cultures

4.2.2.1 *Bacillus cereus* strains

*B. cereus* NS117, NS115, F4810, NC7401 (cereulide producers) and *B. cereus* F528 (non producer) were obtained courtesy of Prof. Mirja Salkinoja (Department of Applied Chemistry and Microbiology, University of Helsinki). *B. cereus* 5964a (cereulide producer), 5958c, 5969a and 5972a (non producers) are food (pasta salad) isolates from a foodborne outbreak (Kinrooi, Belgium in 2003) (Dierick *et al.*, 2005) and were kindly provided to us by Dr. Katelijne Dierick from the Belgian Institute of Public Health. Strains *B. cereus* LMG 12334 (cereulide producer) and LMG 12335 (non producer) were obtained from LMG culture collection (Ghent University, Ghent, Belgium).

4.2.2.2 Liquid media-Brain Hart Infusion (BHI)

All *B. cereus* strains used were grown for 24 h at 30 °C in Brain Heart Infusion (BHI, Oxoid, Basingstoke, England).

Cereulide production was evaluated from the pellet, the supernatant and the filter-sterilized supernatant. A bacterial pellet was obtained by centrifugation at 8000 g for 10 minutes, in duplicate. Cereulide was extracted from the pellet by addition of 1 ml of methanol and further following the procedure described above. The supernatant was transferred into 4 ml glass vials (2 ml in each vial) and one of the duplicates was additionally filter-sterilized (0.2 µm pore size) to remove the total amount of bacterial cells, spores and the toxin adhered thereon. To half of the volumes of supernatants methanol was added in a ratio of 1:1 and the mixture was brought to boiling for 15 minutes in order to extract cereulide. The other half of the supernatants volume was not subjected to methanol extraction. The total volume of supernatant (filtrate) and
methanol (or only supernatant for non extracted samples) was evaporated under N₂ and dry residues were resuspended in 200 µl of DMSO prior to cereulide analyses by boar semen bio assay, as described above. Each sample was done in duplicate.

4.2.2.3 Solid media

When grown on the solid (agar) media the entire *B. cereus* biomass was collected from the agar surface by flooding the surface with 2 ml of methanol and collecting suspended colonies into 4 ml screw capped glass vials. Cereulide was extracted from the suspension during 20 minutes of boiling at 100 °C. The residual methanol was evaporated under N₂ and the dry residue of biomass was suspended in 200 µl DMSO prior to cereulide analyses. Unless otherwise indicated biomass was grown at 30 °C during 24 h. The samples were analyzed in duplicate and confirmation was sought by HPLC-MS (performed by the research group of Prof. dr. Mirja Salkinoja of University of Helsinki, as described in Haggblom *et al.* (2002)).

4.2.3 Parameters of Hamilton-Thorne Ceros (HTR) 12.1

Of fourteen parameters measured and calculated by HTR 12.1 (Rijsselaere *et al.*, 2003) four (PMOT%, RAPID, MEDIUM and SLOW) directly describing the motility were chosen to evaluate the effect of cereulide on the boar semen motility. Two different combinations of settings for the velocity average pathway (VAP) and the straightness (STR), namely setting 1 with VAP>45 µm/s and STR>45%; and setting 2: cells with both VAP>50 µm/s and STR>70% were tested in order to elucidate the impact of their values on the test outcome. The resulting value for PMOT% was taken as a major factor for measuring the cereulide concentration. RAPID, MEDIUM and SLOW motility were used only as the control parameters.

4.2.4 Standard curve with valinomycin

Commercially obtained valinomycin (Sigma Chemical, St. Louis, MO, US) was dissolved in DMSO and diluted along the two fold dilution series. In order to obtain intermediate concentrations, additional dilution combinations were created from several steps in the dilution serial (by taking 2/3 or 1/3 of a previous dilution step and mixing with 1/3 or 2/3 of DMSO). All dilutions were tested by bioassay and their impact on the boar semen motility was recorded, as described above.
4.2.5 *B. cereus* growth and cereulide production

4.2.5.1 Cereulide production in liquid culture media

BHI (100 ml broth in 250 ml Scott bottles) with pH values of 6.0, 6.8 and 7.4 (adjustments with 1 M HCl for pH 6.0 and 6.8) was inoculated with *B. cereus* strains (5964a or F528, ca. 3.5 log CFU ml⁻¹). Inoculated cultures were incubated on a shaken (150rpm) and static manner at 12, 22 and 30 °C. While cultures at 22 and 30 °C were sampled every two hours, cultures at 12 °C were sampled once a day. Samples were used for both *B. cereus* enumeration (spread plate technique on Nutrient agar (NA-Oxoid), from a tenfold dilution serial with incubation at 30 °C) and for cereulide determination following the above described procedure.

4.2.5.2 Cereulide production on different solid media

Plates of NA, Milk Agar (MA, Oxoid), Tryptone Soy Agar (TSA, Oxoid), Potato Dextrose Agar (PDA, Oxoid) were inoculated (flooded) with 1 ml of a 24 h old culture of *B. cereus* 5964a or 1 ml of *B. cereus* NS117. Additionally a medium made of 10% PDA and 90% MA was subjected to the test in order to elucidate whether combination of a starch and protein rich medium has an effect on the cereulide production. NA plates were in addition inoculated with *B. cereus* strains LMG 12334, LMG 12335, 5958c, 5969a, 5972a, NC7401, F528, F4810, ATCC 14579 and aerobically incubated.

4.2.5.3 Cereulide production on TSA under different atmospheric conditions

Plates of TSA inoculated with *B. cereus* 5964a and NS117 (as described above) were packaged under four different modified atmospheric conditions containing 10.6% O₂, 4.5% O₂, 1.6% O₂ and 0.7% O₂ (the rest of the atmosphere being N₂) (Airproducts, Villvode, Belgium) and under aerobic conditions. The aseptic packaging of petri dishes was done using a Multivac A300/42 (Hagenmüller, Wolfertschwenden, Germany) gas packaging machine combined with a gas mixer (WITT MG18-3MSO, Goretexnik, Germany) in a high barrier film (NX90, Euralpak, Wommelgem, Belgium) of 90 µm thickness. The gas composition was controlled with CO₂/O₂ gas analyzer (Servomex Food Package Analyzer Series 1450 CISMA, Zoetermeer, The...
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Netherlands). After incubation for 24 h at 30 °C the complete biomass was collected from the surface of the plates with methanol, extracted and subjected to cereulide analysis by bioassay as described above.

### 4.3 RESULTS

#### 4.3.1 Optimization of parameters

##### 4.3.1.1 Effect of different organic solvents on boar semen motility

Methanol, ethanol and DMSO were applied as organic solvents for dilution of cereulide upon its extraction and were used as blank controls after being added to the semen in a ratio of 1:40. Results show that the DMSO imposed the smallest effect on the PMOT%. The decrease of motility with methanol and ethanol was approximately 20% higher than with DMSO. Methanol induced stress in boar semen was recently described (Sutkeviciene *et al.*, 2005).

##### 4.3.1.2 Effect of extraction and cereulide recovery

Figure 4-2 (A, B and C) shows that most of the toxin for *B. cereus* strain NS 117 was retained in the pellet, requiring 60 seconds of exposure time to induce total loss in spermatozoa progressive motility. Already after pre-exposure time, the pellet had induced PMOT% drop to 35% (Figure 4-2A). On figures this is shown as a difference between fixed, ideal value of 100% and measured PMOT%. To induce the same effect, the supernatant required about 250 seconds, while no measurable effect on the PMOT% in the first 100 seconds of exposure time was observed. Filter sterilized supernatant showed no effect on the progressive motility (PMOT% remained on the level of ± 50%). In all three cases, the methanol extraction did not cause any significant difference on the exposure time required to impose total motility loss when compared to the non-extracted samples. However, a difference of 20% in immediate PMOT% loss (after pre-exposure time) was observed when extracted and non-extracted samples of the supernatant were compared. The observed difference played no role in the dynamics of the motility loss between the extracted and the non-extracted supernatant, as seen from the visual inspection of the slopes of the inhibition curves in Figure 4-2. Similar findings were found for *B. cereus* strains NS115 and F4810 (data not shown). Therefore, pellet was chosen for further investigation.
4.3.1.3 Hamilton-Thorne Ceros 12.1 parameters

Two different sets of criteria for the monitored parameters of motile spermatozoa and progressive spermatozoa were set in the software of the Hamilton-Thorne CEROS 12.1 analyzer in order to compare output information. According to our expectations the less constrained setting 1 for a threshold determination of progressive spermatozoa (cells with both VAP>45 µm/s and STR>45%) gave better results than the setting 2 (cells with both VAP>50 µm/s and STR>70%) resulting in a smaller deviation of measurements. The average PMOT% of semen alone when measured during 5 minutes in the area of the central microscopic field (positioned 3-4 fields away from the edges of Leja counting chamber) with settings 1 had a higher value and a lower standard deviation when compared to the settings 2 (80 ± 6 versus 74 ± 15). This was valid for both boar breeds (boar sperm of Belgian Piétrain extra muscled and sperm of Beau-Pi boars). If the PMOT% measurements were done in the microscopic fields closer to the chamber edges a variation in the results was getting much higher, especially with more stringent combination of settings 2.

4.3.1.4 Valinomycin standard curve

A standard curve (Figure 4-3) was plotted with known concentrations of valinomycin given on the X-axis against the ratio of progressive motility change over the time change (ΔPMOT%/Δτ) on the Y-axis. The standard curve application was limited to the concentration range of 20-400 ng ml⁻¹. Above 400 ng ml⁻¹ the curve remained parallel with the concentration axis indicating that a further increase in the valinomycin concentration would not result in measurable changes of the progressive motility, thus approaching an asymptote at about ΔPMOT%/Δτ=-6.00.
Figure 4-2: Loss of PMOT% after exposure of spermatozoa to the non-diluted DMSO suspension of extracted (♦) or non-extracted (■) pellet (A), supernatant (B), filter-sterilized supernatant (C) of the *B. cereus* NS117 culture grown in BHI at 30 °C/24 h. Ratio semen:sample = 40:1. Loss of PMOT% = 100% - measured PMOT%.
Figure 4-3: Standard curve plotted with known concentrations of valinomycin against the average ratio of PMOT% change (ΔPMOT%) over change in time (Δt) required to realize a PMOT% drop from the value of a blank sample to a PMOT% of 10%.
4.3.2 Shaken vs. static incubation of BHI cultures at different temperatures and different pH values

A more stringent temperature of 12 °C and less favorable pH 6.0 imposed a profound effect on the growth of *B. cereus*, both in the static and shaken cultures, allowing it to reach only ca. 6.0 - 6.5 log CFU ml⁻¹. In all the other tested conditions *B. cereus* grew to ca. 8-9 log CFU ml⁻¹. Results showed no toxin production in shaken cultures, regardless of the incubation temperature. In static cultures, toxin production was observed at 22 and 30 °C, but not at 12 °C. Data given in Figure 4-4 indicate the impact of different pH values in statically incubated cultures at 22 and 30 °C on toxin production. The final cell counts were for pH 7.4 and pH 6.8 almost the same at all three incubation temperatures. Toxin production at 22 °C was detectable as of 24 h incubation time for pH 7.4 and as of 26 h for pH 6.8 and 6.0 with *B. cereus* 5964a. At the same temperature counts of ca 8.0 log CFU ml⁻¹ for all three pH values were reached in the period 20-26 h (stationary phase of the culture). At 30 °C motility inhibition was observed after exposing semen to the pellet incubated for 20 h at pH 7.4 and for 24 h at pH 6.8. After 24 h incubation no toxin production was observed for pH 6.0. Enumeration, however, revealed almost identical counts at all three pH values (ca. 8 log CFU ml⁻¹) in the period of 20-24 h. In the experimental setup the negative control (*B. cereus* strain F528) grown under identical conditions showed no toxin production.

4.3.3 Quantification of cereulide production on different solid media

Table 4-1 shows the total exposure time (the sum of pET and ET) required for achieving a decrease of the progressive motility to 10%. This was transformed to a cereulide concentration using the standard curve obtained with known concentrations of valinomycin. The final toxin concentration was calculated from standard curve readings multiplied by the dilution factor of the extract and divided by the weight of the biomass used in extraction. Results indicate an influence of different nutrient agars on the cereulide production by *B. cereus* 5964a and NS117 strains at an incubation temperature of 30 °C. On all agars tested, strain 5964a produced more cereulide per
mg of biomass than NS117 (except on NA), with the maximum produced on TSA plates for both strains.

Figure 4-4: Loss of PMOT% after exposure of spermatozoa to the non-diluted DMSO suspension of extracted *B. cereus* 5964a biomass statically grown in BHI with pH 7.4 (♦), 6.8 (■) and 6.0 (▲) and at A: 12 °C after 6 days, B: 22 °C after 24 h C: 22 °C after 26 h, D: 30°C after 20 h, E: 30°C after 22 h and F: 30 °C after 24 h. A negative control (x) is represented by the extract of biomass of a cereulide non-producing *B. cereus* F528 grown in BHI with pH 7.4. Loss of PMOT% = 100%-measured PMOT%.
Table 4-1: Cereulide production by biomass of *B. cereus* 5964a and NS117 harvested from 5 different solid (agar) media.

<table>
<thead>
<tr>
<th>Agara</th>
<th>B. cereus strain</th>
<th>Exposure time (s)</th>
<th>Cereulide concentration (ng mg⁻¹) corrected with dilution and per mg biomassb</th>
</tr>
</thead>
<tbody>
<tr>
<td>90%PDA+10%MA</td>
<td>5964</td>
<td>110</td>
<td>216 ± 78</td>
</tr>
<tr>
<td></td>
<td>NS117</td>
<td>113</td>
<td>151 ± 21</td>
</tr>
<tr>
<td>MA</td>
<td>5964</td>
<td>130</td>
<td>213 ± 7</td>
</tr>
<tr>
<td></td>
<td>NS117</td>
<td>142</td>
<td>142 ± 32</td>
</tr>
<tr>
<td>PDA</td>
<td>5964a</td>
<td>63</td>
<td>576 ± 94</td>
</tr>
<tr>
<td></td>
<td>NS117</td>
<td>49</td>
<td>102 ± 23</td>
</tr>
<tr>
<td>NA</td>
<td>5964</td>
<td>147</td>
<td>87 ± ndc</td>
</tr>
<tr>
<td></td>
<td>NS117</td>
<td>84</td>
<td>134 ± 9.5</td>
</tr>
<tr>
<td>TSA</td>
<td>5964</td>
<td>127</td>
<td>1007 ± 45</td>
</tr>
<tr>
<td></td>
<td>NS117</td>
<td>108</td>
<td>396 ± 36</td>
</tr>
</tbody>
</table>

a PDA-Potato Dextrose Agar, MA-Milk Agar, NA-Nutrient Agar and TSA-Tryptone Soy Agar

b Mean concentration and its standard deviation calculated from two successive dilutions on the basis of the valinomycin standard curve given in Figure 2

c nd-Not determined

On NA plates the most of cereulide was produced by *B. cereus* NS117, followed closely by 5964a, LMG 12334 and F4810, requiring less than 60 seconds to induce the loss of PMOT% to the value of 10%. Strain NC7401 showed least of the motility inhibition (100 seconds to PMOT%=10%). Negative strains LMG 12335, ATCC14579, F528, 5958c, 5969a and 5972a did not influence the motility during the observation time.

When TSA plates were incubated in the environment containing different O₂ concentrations, subsequent growth and toxin production showed a major dependence on oxygen presence. Growth under aerobic conditions proved again that strain 5964a produces more cereulide than NS117 (in this experiment ca. 960 ng mg⁻¹ ± 57 versus ca. 570 ng mg⁻¹ ± 77). No significant difference was observed with atmosphere containing 4.5% O₂ where strain NS117 produced ca. 1225 ng mg⁻¹ ± 247, while strain 5964a produced ca. 1150 ng mg⁻¹ ± 42. At 10.6% O₂ strain 5964a produced almost equal amounts of cereulide as did strain NS117 (±1120 ng mg⁻¹), which was more than in aerobic conditions for the later strain. No toxin was found in atmospheres containing 1.6 and 0.7% of oxygen for none of two strains tested. HPLC-
MS data indicated that at 28 °C after 24 h strain 5964a produced cereulide in amounts of 350 ng mg⁻¹ biomass on TSA in aerobic conditions.

4.4 DISCUSSION

The use of ejaculated mammalian spermatozoa for studying the cytotoxic effect of biological toxins may provide several advantages compared to other widely used cell types and in vitro systems. This is especially true for their swimming activity, which is dependent upon intact cellular structures and functions, and which consequently offers an endpoint for semi-quantitative evaluation of the cytotoxic potential of various substances. The present approach represents a modification of the boar semen motility assay first developed and described by Andersson et al. (1998b). The modification offers an advantage by applying CASA in the evaluation of cereulide impact on the boar semen motility, providing numerical interpretation of the observed motility changes. Approach comprises also joined pre-incubation of semen and cereulide containing samples, measuring changes of semen motility within 5 seconds from the exposure onset. Software generated data describe the dynamics with which the progressive motility drops from the starting value to the level of 10%. The calculation of the change of PMOT% in function of time provides a useful numerical parameter for evaluation of the sample toxicity. A standard curve in the useful range of 20-400 ng ml⁻¹ is obtained for the determination of cereulide concentration by plotting these values for known concentrations of valinomycin and assuming that cereulide and valinomycin impose an inhibitory effect of the same extent on the boar semen motility (Haggblom et al., 2002; Hoornstra et al., 2003; Hoornstra et al., 2004). Valinomycin was also elsewhere suggested as a reference compound in cereulide detection using the described bioassay (Jaaskelainen et al., 2003a), as well as a standard in the routine HPLC-MS analyzes (Haggblom et al., 2002). Higher concentrations were not possible to differentiate due to the total motility loss, already within the pre-exposure time. The range measurable in the presented CASA system largely covers the range of reported dose-response data (Paananen et al., 2002; Agata et al., 2002; Jaaskelainen et al., 2003b).

The decline in the percentage of progressive motile sperm was dependent on the chemical solvent used. Of the three solvents tested the lowest decline was observed with DMSO. The application of less stringent settings in the CASA software yielded
results of better repeatability. If too stringent criteria were set for PMOT% (higher VAP and STR values) extrinsic and intrinsic factors influencing PMOT% became too limiting. This was resulting in the false positive results. Furthermore, the position of the microscopic field under observation proved to be very important as all measurements made in the peripheral areas of the Leja slide had much lower total and progressive motility than areas closer to the centre of a slide.

Due to very poor water solubility, cereulide remained in the pellet after centrifugation of the BHI culture and it stuck to the biomass and filter. This resulted in the faster onset of motility inhibition when exposing semen to the pellet. Results also showed an immediate release of cereulide into DMSO and no need for the extraction step when cereulide is tested directly from \textit{B. cereus} biomass. In addition the biomass collected from agar plates gave no detectable differences in cereulide content between extracted and non-extracted samples. This significantly shortened the test procedure, which was further challenged to investigate the influence of incubation temperature, growth media, type of \textit{B. cereus} strain, and oxygen presence on cereulide production.

Cereulide was not detected after 6 days of incubation at 12 °C in any of the samples tested, regardless the growth counts of more than $10^6$ CFU ml$^{-1}$. Also Finlay \textit{et al.} (2000) found that incubation at temperatures lower than 15°C failed to induce production of emetic toxin in skimmed milk medium. At 22 and 30 °C, pH values of the BHI medium imposed a crucial influence on the cereulide production, allowing much faster production at pH 7.4 than at less favorable pH 6.8 and 6.0. No significant impact on \textit{B. cereus} counts in the stationary phase was observed. However it was shown that when high counts of cells are present the cereulide production may come up from undetectable to detectable levels in a two hours span. Therefore, for a particular \textit{B. cereus} strain cereulide production is not solely influenced by the growth phase and the maximal level of cells obtained, but also by factors such as the nutritious composition of the culture media. Somewhat lower amounts of cereulide found by HPLC-MS in comparison to bioassay can be explained by slightly lower incubation temperature used (28°C versus 30°C). Also present amount of oxygen influenced cereulide production. Results presented indicate that exclusion or limitation of oxygen to the levels below 1.6% may prevent cereulide production.
Whether or not a degree of aeration of a liquid culture medium has a positive impact on the cereulide production is unclear at the moment. Different authors (Finlay et al., 2002b; Haggblom et al., 2002; Jaaskelainen et al., 2004) reported well elaborated results, but conclusively different from the findings in the present study. If quorum sensing is the underlying mechanism of *B. cereus* virulence (Gominet et al., 2001; Dunn and Handelsman, 2002; Bernardo et al., 2004; Slamti and Lereclus, 2005) than perhaps the local concentration of bacterial cells needs to be above a certain level in order to signal for an onset of cereulide production. Disturbance and continual dispersion of cells during horizontal shaking may possibly impair the local communication process and hamper the cereulide production. Due to the precipitation and the local accumulation of cells, the local cell concentration is higher in the static cultures than in a vortexed culture, allowing cereulide production in the static cultures. Therefore this effect may actually not be related to the aeration but to physical cell dispersion.

### 4.5 CONCLUSIONS

The present research confirms the convenience of the usage of boar semen motility for the detection of cereulide and introduces an optimized CASA approach for cereulide quantification. The quantification is based on software utilization for data generation allowing real time observation of semen motility behavior. Under experimental conditions none of the negative controls showed a positive reaction and the usage of blank samples for every test allows clear differentiation between samples containing and not-containing cereulide. Results indicate complex influence of different parameters on cereulide production.

In Chapter 5, CASA is used to detect and quantify cereulide production in starch rich Ready-To-Eat foods, namely potato puree, pasta (penne and farfalle), rice and béchamel sauce. Starch rich foods are often considered as main vectors in cereulide intoxications. Additionally, milk that is often related to *B. cereus* spores contamination was included in the study.
INFLUENCE OF TYPE OF FOOD ON THE KINETICS AND OVERALL PRODUCTION OF *BACILLUS CEREUS* EMETIC TOXIN

Redrafted from:


(2006)

*Journal of Food Protection*, vol. 69, 4, 847–852
CHAPTER 5: INFLUENCE OF TYPE OF FOOD ON THE KINETICS AND OVERALL PRODUCTION OF \textit{BACILLUS CEREUS} EMETIC TOXIN

Summary

Potato puree and penne were inoculated with cereulide producing \textit{Bacillus cereus} 5964a and \textit{B. cereus} NS117. Static incubation at 28 °C proved these two foods to be a better substrate for higher cereulide (emetic toxin) production (4080 ng g$^{-1}$ in puree and 3200 ng g$^{-1}$ in penne were produced by \textit{B. cereus} 5964a during 48 h of incubation) in comparison to boiled rice (2000 ng g$^{-1}$). This difference occurred despite the fact that \textit{B. cereus} counts of more than $10^8$ CFU g$^{-1}$ were found in all three products. Aeration of cultures had a negative impact on cereulide production causing even more than tenfold lower concentrations than in some statically incubated samples. Cereulide production remained undetectable in shaken milk, while reaching 1140 ng ml$^{-1}$ in statically incubated milk. At 12 and 22 °C, presence of background flora was also a determinative factor. A total \textit{B. cereus} count of more than $10^6$ CFU ml$^{-1}$ not necessarily led to uniform cereulide production and was also dependent on the \textit{B. cereus} strain involved. The present study confirms that number of factors play a crucial role in determination of the extent to which, if at all, cereulide will be produced. Among those factors, type of the food, temperature, pH, additional aeration (via incubation on an orbital shaker) and cereulide producing strain were shown to have a key effect on the final cereulide production.

Keywords: \textit{Bacillus cereus}, emetic toxin, cereulide, bioassay, food
5.1 INTRODUCTION

*Bacillus cereus* is a versatile foodborne pathogen that can exhibit a variety of pathogenic mechanisms, most of which are related to extracellular peptide or protein like substances. Small (1.2kDa) dodecadepsipeptide named cereulide is an emesis causing exotoxin produced by some strains of *B. cereus* and represents potentially the most dangerous of its virulence factors. It has been recently confirmed that it is produced by non-ribosomal peptide synthesis (Horwood *et al.*, 2004; Toh *et al.*, 2004) and that non-chromosomal, plasmid borne *ces* gene is involved in cereulide production (Hoton *et al.*, 2005).

The reported heat stability of cereulide (126 °C during 90 minutes), its resistance to extreme pH (pH 2-11) and stated resistance to proteolytic enzymes (Granum, 1997) render cereulide difficult to eradicate or inactivate in the foods. Therefore, the major threat is posed by a cereulide pre-formed in foods. The clinical dose, suggested to be about 10 µg kg⁻¹ body weight (Paananen *et al.*, 2002), is higher than those of some other known toxins (for example, *S. aureus* intoxication that resembles symptoms of cereulide caused intoxication, induces mild effects already at concentrations of 100 ng kg⁻¹ body weight, (Bergdoll, 1989). Certain rice containing bakery products were found to hold cereulide in concentration of 5-8 µg g⁻¹ (Jaaskelainen *et al.*, 2003a). Even much lower concentrations of cereulide, ranging from 0.01 µg g⁻¹ up to 1.280 µg g⁻¹, were reported in foods implicated in emetic type of food poisoning (Agata *et al.*, 2002).

For all of the foods reported by Agata *et al.* (2002) high starch content was a common feature, no matter the fact that cereulide strains are known to be unable to hydrolyze starch (Agata *et al.*, 1996; Pirttijarvi *et al.*, 1999). Epidemiology has established well known connections between cereulide related foodborne intoxications and farinaceous foods (Kramer and Gilbert, 1989; Granum, 1994; Agata *et al.*, 2002; Haque and Russell, 2005). However, pure starch may just not be enough to provide nutrients needed for cereulide production and three amino acids, valine, leucine and threonine, were found essential for growth and toxin production by *B. cereus* (Agata *et al.*, 1999).

Above the counts of 10⁵ CFU g⁻¹ emetic producing strain may generate substantial toxin amounts that can cause illness 0.5-6 h after the ingestion of a pre-formed toxin.
Literature however suggests that actual cereulide production can be already initiated by lower numbers of *B. cereus*, $10^3$ CFU g$^{-1}$ (Kramer and Gilbert, 1989). An explanation for these low counts may likely originate in dye-off of *B. cereus* cells due to antimicrobial treatments.

Biological activity of cereulide has been so far the most employed feature for its detection (Table 1-8 and Table 1-9). Ceasing of boar sperm motility in the presence of cereulide has been shown to be useful for cereulide detection, both in laboratory media and foods (Andersson et al., 1998b; Andersson et al., 2004). In order to characterize cereulide production and elucidate amounts of cereulide present with regards to the type of food product, incubation temperature, shaken or static incubation, strain involved and level of *B. cereus* growth, Computer Aided Semen Analyzes (CASA) of boar semen and High Performance Liquid Chromatography with Mass Spectroscopy (HPLC-MS) were used to study cereulide production under different incubation conditions and with different cereulide producing *B. cereus* strains involved. Attention was given to starch rich products, in order to understand potential threats vectored by Ready-To-Eat meals containing sauces, pasta, rice and potato, all being rich in starch content. Milk which is frequently associated with *B. cereus* (Notermans et al., 1997) was also included in the study.

**5.2 MATERIALS AND METHODS**

**5.2.1 *B. cereus* strains and preparation of inoculation**

Two cereulide producing strains were employed in the experimental setup. *B. cereus* 5964a, isolated from a cold meal implicated in fatal foodborne outbreak (De Schrijver et al., 2004; Dierick et al., 2005) (provided by dr. K. Dierick, Belgian Institute of Public Health) and NS117 (culture collection of research group of M. Salkinoja, University of Helsinki). As a non cereulide producing strain *B. cereus* F528 (culture collection of research group of M. Salkinoja, University of Helsinki) was used as a negative control. The strains were grown in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, England) for 24 h at 37 °C. From the appropriate dilution in Peptone Saline Solution, PPS, (8.5% NaCl, (Vel, Leuven, Belgium) and 1% Peptone (Oxoid)), 0.1 ml of culture was taken and used as inoculum for foods, providing an inoculation.
level of ca. 3.5 log CFU per gram (milliliter) of food in the first two experiments and ca. 6 log CFU in the third experiment.

### 5.2.2 Food samples and incubation conditions

Five different groups of commercial RTE food products, namely potato puree, pasta (penne and farfalle), rice, béchamel sauce and milk were obtained from a local producer at the day of production. Experimental setup comprised a total of 224 samples tested in three separate experiments. While in the first experiment impact of background flora was included in the test, in the second and the third experiment a heating step (80 °C during 10 minutes) was introduced prior to inoculation in order to eliminate the vegetative background flora. In the first and second experiment 25g portions of food (penne and farfalle in the first experiment; penne, potato puree and béchamel sauce in the second experiment) were inoculated in duplicate with *B. cereus* 5964a or F528 strain, packaged in Stomacher bags (180 by 300mm, Novolab, Geraardsbergen, Belgium) and further incubated at either 12 °C (representing severe temperature abuse) or 22 °C (representing the room temperature). For every analysis, also a blanc (not inoculated) sample of each food was foreseen. In the third experiment 50 g (ml) of foods (penne, potato puree, rice and milk) were mixed with Peptone Saline Solution (PPS) in 500 ml SCOTT bottles in ratio 1:5 in order to enhance the effect of aeration induced on a horizontal orbital shaker. Following heat treatment of 10 minutes at 80 °C, food was inoculated with *B. cereus* 5964a and F528 and SCOTT bottles with inoculated foods were incubated statically or on a 150 rpm orbital shaker at 28 °C. In addition potato puree and milk were inoculated with *B. cereus* NS117 strain impact on cereulide production when all other conditions are kept identical. For all foods, pH and a_w were determined with pH-meter (type 763, Knick, Berlin, Germany) using electrode (Ingold 104063123, Urdorf, Switzerland) and cryometer (AW-Kryometer, type AWK-20, NAGY Messsysteme GmbH, Gäufelden, Germany), respectively.

### 5.2.3 Monitoring of *B. cereus* growth in foods

Food samples from the first two experiments were tenfold diluted and homogenized in PPS using Stomacher (Lab-Blender 400, Led Techno, Eksel, Belgium). A tenfold dilution serial was made and from the appropriate dilution, 100 µl was spread plated
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

on a *Bacillus cereus* agar medium (BCA, Lab M, Bury, Lancashire, UK) (establishing a detection limit of 100 CFU g⁻¹). Following the incubation for 24 h at 30 °C typical *B. cereus* colonies were enumerated. For every food product and every strain involved duplicates were made and blank samples were included. Samples incubated at 12 °C were analyzed once a day during six days while samples incubated at 22 °C were analyzed after 18, 24, 42 and 48 h of incubation. Mesophilic aerobic counts on Plate Count Agar (PCA, Oxoid; 24-72 h/30 °C), yeasts and molds count on Yeast Glucose Chloramphenicol agar (YGC, Bio-Rad, Marnes-la Coquette, France; 3-5 days/30 °C) and lactic acid bacteria count on DeMan, Rogosa Sharpe agar (MRS, Oxoid; 72 h/30 °C) also were determined at every analysis for foods containing background flora (experiment 1).

Food samples that were in SCOTT bottles incubated at 28 °C (static and shaken) were sampled every 6 h for *B. cereus* enumeration either directly from the food suspension (detection limit of 10 CFU g⁻¹) or from an appropriate dilution step following the procedure described above. This protocol is a standard procedure in the lab and therefore no replicated analysis were made.

5.2.4 Boar semen motility assay and HPLC-MS analysis of cereulide production

Boar semen motility assay was performed with minor modifications (see chapter 4) to the original protocol (Andersson *et al.*, 1998b; Andersson *et al.*, 2004). CASA using Hamilton-Thorne computer-aided semen analyzer, version 12.1 Ceros (HTR Ceros 12.1, Hamilton Thorne Research, Beverly, CA, US), was employed for relative-quantitative and semi-quantitative determination of cereulide presence in the sample by measuring change in the percentage of progressive motility (PMOT%) of semen in function of time (see 4.2.1). In short 195 µl of sperm (Belgian Piétrain extra muscled standardized to a concentration of approximately 30 millions cells per milliliter) was mixed with 5 µl of solution (blank or containing cereulide or valinomycin) in wells of a microtiter plate (96 wells format) and immediately transferred into a Leja slide (standard count 2-chamber 20 micron slide, Leja, Nieuw-Vennep, The Netherlands). If only relative-quantitative toxicity evaluation was required, undiluted sample was subjected to the test and results were depending on amount of cereulide present obtained within 10 to 300 seconds. For quantitative determination sample was serially
diluted with dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) in a two-fold dilution serial (see Chapter 4). Two consecutive dilution steps giving PMOT% change in a semi linear range of a valinomycin standard curve were used to calculate cereulide concentration. Valinomycin standard curve was plotted with known concentrations of valinomycin given on the X-axis against the ratio of PMOT% change over the time period (ΔPMOT%/Δτ) on the Y-axis. The standard curve application was limited to the concentration range of 20-400 ng ml⁻¹. Above 400 ng ml⁻¹ the curve approached an asymptote at about ΔPMOT%/Δτ=−6.00.

HPLC-MS analyzes were performed as described by Jaaskelainen et al (2003a), with additional analyses in Thermofinnigan LCQ Advantage with PDA detector (Surveyor). Column used was Discovery C-18, 100 mm x 2.1 mm and 5µm particle size. The mobile phase consisted of solvent A, 2%TFA (Sigma Aldrich, Bornem, Belgium) in water and solvent B, acetonitrile (Labscan, Dublin, Ireland). At a flow rate of 0.2 ml min⁻¹, with a sample injection volume of 20 µl and column temperature of 30 °C the mobile phase was changed as following: 0-15min, A: 13% and B: 87%; 15-20min, A:13-0% and B:87-100%; 20-40min, both solvents constant, 40-45min, A: 0-13% and B: 100-87%; and till 60min both solvent were kept constant. After LC separation sample was introduced to electrospray ion trap mass analyzer (positive ionization), with following conditions: MS run time 15 min., delay 5 min., scanning area 1000-1200 m/z, capillary temperature 350 °C, and capillary voltage 48V. The ions were detected at m/z ([M+NH₄⁺]) 1128.5 for valinomycin and m/z ([M+NH₄⁺]) 1170.7 for cereulide.

Samples for cereulide determination were taken in parallel to those intended for B. cereus enumeration. Sampling consisted of taking 3 g (3 ml) of a food product into a 10 ml conical flask, mixing with double volume of methanol and extraction at 100°C during 20 minutes (or until liquid phase was evaporated). When all liquid was evaporated additional 3 ml of methanol were added, well mixed during ca. 2 minutes, the whole volume of methanol was transferred into a 4 ml screw neck glass vial and evaporated under N₂. The dry residue was suspended in 200-500 µl of DMSO and kept at -20 °C until analyzes.
5.3 RESULTS

5.3.1 Bacterial enumeration and physical parameters of food

Counts of *B. cereus* 5964a in potato puree, penne and sauce at 12 and 22 °C (Figure 5-1) showed unhindered growth when no background flora was present to compete with *B. cereus*. At both incubation temperatures and in all three food products cereulide producing *B. cereus* 5964a was able to reach counts of more than $10^7$ CFU $g^{-1}$. While at 22 °C 13 h were sufficient to cross the safety limit of $10^5$ CFU $g^{-1}$, it required ca. 24 h in penne and sauce and ca. 36 h in potato puree to cross this limit at 12 °C.

In thermally non-treated samples *B. cereus* counts at 12 °C remained below detection limit in penne reaching ca. 6 log CFU $g^{-1}$ in farfalle after 96 h of incubation. Counts of total mesophilic flora and lactic acid bacteria at the end of the incubation at 12 °C were respectively 9.7 log CFU $g^{-1}$ and 6.9 log CFU $g^{-1}$ for penne and 9.05 log CFU $g^{-1}$ and 5.4 log CFU $g^{-1}$ for farfalle. At 22 °C *B. cereus* counts reached ca. 7.4 log CFU $g^{-1}$ in penne and ca. 8.1 log CFU $g^{-1}$ in farfalle after 48 h of incubation. At the same time total mesophilic count and lactic acid bacteria counted 8.9 log CFU $g^{-1}$ and 7 log CFU $g^{-1}$, respectively, for penne and 8 log CFU $g^{-1}$ and less than 5 log CFU $g^{-1}$, respectively for farfalle.

Figure 5-4 shows growth of *B. cereus* 5964a in semi liquid potato puree, penne, rice and liquid milk at 28 °C. *B. cereus* NS117 (data not shown) grew to lower counts (maximum difference being 1 log unit after 48 h of incubation at 28 °C) in tested potato puree. In milk the growth of NS 117 was at every sampling point ca. 0.5 log CFU ml$^{-1}$ higher than the growth of 5964a.

While pH value of thermally non treated penne dropped during incubation from ca. 6.5 to ca. 5.7 both at 12 and 22 °C pH of farfalle remained stable at ca. 6.5. Water activity of both penne and farfalle remained at ca. 0.996.

In thermally treated foods incubated at 12 and 22 °C initial pH value of sauce was the lowest (5.8). Sauce also had the lowest $a_w$ of ca. 0.994. Potato puree and penne had $a_w$ of ca. 0.997 and pH of ca. 5.9 and 7.4, respectively. Potato puree incubated at 22 °C had initial pH of 6.4. These values remained stable during incubation.
5.3.2 Detection of cereulide in food samples

No cereulide was detected in thermally non-treated samples incubated at 12 °C. At 22 °C only farfalle induced loss of the motility in semen. Sample of penne did not show presence of cereulide.

High *B. cereus* 5964a counts in penne and potato puree resulted in cereulide production at both 12 °C (after 4 and 5 days respectively) and 22 °C (after 24 and 42 h respectively). Results of cereulide detection by cease in boar semen motility when exposed to the food extracts of potato puree, penne and béchamel sauce inoculated with *B. cereus* 5964a and incubated at 12 °C and 22 °C are shown in Figure 5-2 and Figure 5-3 for respective incubation temperatures.

No detectable cereulide concentrations were found in sauce at any of the incubation temperatures. Boar semen motility inhibition was neither observed with negative controls (samples inoculated with non emetic strain, *B. cereus* F528).
Inoculation of thermally treated semi liquid (1:5 diluted), potato puree, penne and rice, as well as of commercially obtained UHT sterilized milk, with $10^6$ CFU ml$^{-1}$ of cereulide producing *B. cereus* 5964a resulted in cereulide production that was largely dependent on the food product and incubation manner (at 150 rpm vs. static, both at 28 °C).

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**Figure 5-2**: Toxin production by *B. cereus* 5964a: evolution of boar semen motility when exposed to the extract of potato puree (A), penne (B) and sauce (C) after incubation at 12 °C of 6 (♦), 5 (■), 4 (▲) and 3 (×) days. Y axes error bars indicate 95% confidence interval (n=6-10)
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Chapter 5: Production of *B. cereus* emetic toxin in different foods and under different conditions

Figure 5-3: Toxin production by *B. cereus* 5964a: evolution of boar semen motility when exposed to the extract of potato puree (A), penne (B) and sauce (C) after incubation at 22 °C of 66 (∗), 48 (■), 42 (▲), 24 (x) and 18 hours (□).
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Chapter 5: Production of *B. cereus* emetic toxin in different foods and under different conditions

Figure 5-4: Production of cereulide by *B. cereus* 5964a at 28 °C in A: statically, B: shaken incubated: potato puree ( ), penne ( ), rice ( ) and milk ( ) with therein enumerated *B. cereus* 5964a growth: potato puree ( ), penne ( ), rice ( ) and milk ( ). Unless otherwise indicated cereulide was detected with HPLC-MS (detection limit 10 ng ml⁻¹) and enumeration was done in duplicate with a classical spread plate technique with detection limit of 10 CFU ml⁻¹.
The highest cereulide production as determined with HPLC-MS (Figure 5-4) was for every food product observed in the statically incubated samples, ranging from below the detection limit (10 ng g⁻¹) up to 4080 ng g⁻¹ for potato puree, 1000-3300 ng g⁻¹ for penne, 120-2010 ng g⁻¹ in rice, and 55-1140 ng ml⁻¹ in milk. For strain 5964a potato puree and penne were better substrate for growth and cereulide production, although rice is the most incriminated food. Amount of cereulide found in rice after 48 h of static incubation was in the level of 2000 ng g⁻¹ (concentration detected with boar semen bioassay). Also, in statically incubated milk large amount of cereulide was detected (1140 ng ml⁻¹).

Additionally inoculated strain of *B. cereus* NS117 showed somewhat lower extent of cereulide production in tested potato puree. In milk *B. cereus* NS117 after 48 h of incubation produced cereulide in amount of 260 ng ml⁻¹.

### 5.4 DISCUSSION

It was shown in this study study that growth of *B. cereus* to 5 log CFU g⁻¹ will not necessarily result in cereulide production. However, when the extrinsic, intrinsic and implicit factors comprise a favorable environment resulting cereulide production may lead to accumulation of intoxicative amounts of cereulide in foods. These results remain in agreement with findings of others (Agata *et al.*, 2002; Jaaskelainen *et al.*, 2003a). Of all tested foods by Agata *et al.* (2002) rice was reported as the food containing most of the cereulide when artificially inoculated with the cereulide producing strain NC7401. The cereulide amounts, in the present study, found in potato puree and penne stress that rice is not necessary the food supporting best cereulide production. *B. cereus* 5964a grew to the highest counts and produced high amounts of cereulide at highest pace in penne. This was to be expected as the strain was originally isolated from a cold pasta salad, implicated in a fatal foodborne outbreak in Kinrooi, Belgium (De Schrijver *et al.*, 2004; Dierick *et al.*, 2005). These results remained consistent at both 12 and 22 °C. Also at 28°C strain 5964a produced very high amounts in penne, which were after first 12 h of incubation higher than in potato puree and rice. However, the final amount after 48 h of incubation at 28 °C was found to be higher in potato puree. Here not only the temperature played a role, but also different pH values of a potato puree used at 28°C, being 6.40 instead of 5.89 as...
in the experiments at 12 and 22 °C. Lack of detectable cereulide amounts in sauce can be partially explained by lowest pH and a_w value of all tested foods. Although not too low, this pH may contribute to the slower rate of cereulide production (Agata et al., 2002). These findings contribute to the current understanding of type of foods implicated in cereulide intoxications.

In 13 out of 14 food samples implicated in cereulide induced foodborne intoxications in Japan amounts of cereulide found ranged from 0.01 to 1.28 µg g^-1 (Agata et al., 2002). This is in agreement with findings of this study, in which amounts of 1 to 3 µg g^-1 were found in tested foods. Results of this work also agree with the low levels of cereulide found in milk with that difference that Agata et al. (2002) found more cereulide in shaken samples and no cereulide in curdled milk.

The same authors found that at tested temperatures (30 and 35 °C) B. cereus in boiled rice rapidly increased to 10^7 to 10^8 CFU g^-1 and produced emetic toxin. The data of Jaaskelainen et al. (2003a) reported rice-containing pastries as a reservoir of high cereulide concentrations (0.3 to 5.5 µg g^-1 [wet weight]) when stored at room temperatures. Finley et al. (2002b) reported detectable amounts of cereulide after 48 h incubation at 15 °C in comparison to 24 h incubation at 20 and 30 °C. Current data indicated penne and potato puree as foods that were able to support cereulide production even at 12 °C when no background flora was present.

Although literature reports that production of cereulide can be enhanced by incubation with shaking in contrast with static conditions (Finlay et al., 2002b; Haggblom et al., 2002), results presented here showed, as seen in all food products and both tested strains, that cereulide concentrations found in statically incubated samples were 14 to 15 times higher than in shaken samples after 48 h of incubation. The data reported in Chapter 4 supports current findings. While for solid foods effect of shaking is of no relevance, for liquid or semi-liquid foods, taking into account that although limited, some shaking still may occur during the production process (reconstitution of a milk powder in standardization of a milk batch or similar), here reported findings bring another insight in possible impact of shaking on cereulide production. Although we report here that shaking at 150 rpm had a negative impact on cereulide production it remains unclear where the difference with the findings of some other authors come from.
B. cereus counts at which cereulide was detected were different in different foods and at different temperatures. At 12 °C counts had to be in all foods higher than 7 log CFU g⁻¹. At 22 °C required counts were food dependent, but were ca. 6 log CFU g⁻¹. Since stationary phase only commenced at counts higher than 8 log CFU g⁻¹ it is apparent that cereulide production may begin already in earlier stages of exponential phase. Although, detected concentrations of cereulide were always related to B. cereus counts of 6 log units and above, no exclusive connection between higher counts and higher cereulide concentrations was noticed. This was especially the case in shaken foods, where aeration and physical dispersion of cells induced lower cereulide production. Therefore, no clear evidence can be given that even when cereulide producing strain attains levels of 6-7 log CFU g⁻¹ there will be enough of cereulide produced to cause intoxication.

5.5 CONCLUSIONS

It is clear that the regulatory mechanism of cereulide production is not straightforward and that the role played by type of culture medium, type of food, environmental conditions, as well as presence of background flora is of utmost importance for cereulide production.

Application of the boar semen bioassay to detect cereulide directly in foods requires investigation of potential interference of food components, preservatives and other microbial and chemical food contaminants with the bioassay. In the following chapter 6, Staphylococcus aureus enterotoxins A, B, C and D, Bacillus cereus Hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE) and six mycotoxins (Sterigmatocystin, Fumonisins B1, Aflatoxins M1 and M3, Zearalenone and Patulin) were examined for their impact on the boar semen progressive motility. In addition, ten commonly used preservatives, as well as, acrylamide and 2,3,7,8-tetrachlorodibenz-p-dioxin (TCDD) were tested for interference with the assay.
COMPUTER AIDED BOAR SEMEN MOTILITY ANALYSIS FOR CEREULIDE DETECTION IN DIFFERENT FOOD MATRICES

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(2006)

International Journal of Food Microbiology, submitted for publication
CHAPTER 6: COMPUTER AIDED BOAR SEMEN MOTILITY ANALYSIS FOR CEREULIDE DETECTION IN DIFFERENT FOOD MATRICES

Summary

Application of Computer Aided Semen Analysis (CASA) to detect cereulide directly in foods requires investigation of potential interference of food components, preservatives and other microbial and chemical food contaminants with semen motility. None of here included Staphylococcus aureus enterotoxins A, B, C and D, nor B. cereus Hemolysin BL (HBL) and Non-Hemolytic-Enterotoxin (NHE) and three mycotoxins (Sterigmatocystin, Fumonisin B1 and Patulin) exhibited an impact on semen progressive motility. Aflatoxin M1, M3 and Zearalenone impaired semen motility only at concentrations (0.004 mg ml⁻¹, 0.1 mg ml⁻¹ and 10 mg ml⁻¹, respectively) much higher than those found in foods and those permitted by legislation, in comparison to cereulide which induces motility cease at concentrations lower than 20 ng ml⁻¹. Ten commonly used preservatives, namely potassium sorbate, sodium benzoate, (DL) malic acid, citric acid, (L+) tartaric acid, acetic acid, (DL) lactic acid, (L+) ascorbic acid, sodium chloride and sucrose induced no cease in spermatozoa motility even at preservative concentrations higher than permitted by legislation. Dioxins, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and acrylamide had no acute effect on spermatozoa motility at concentrations of 500 and 10000 mg ml⁻¹, respectively. Robustness of computer aided boar semen motility analysis, tested with 14 different foods inoculated with cereulide producing B. cereus, showed distinct cereulide production in seven samples (although B. cereus growth to counts higher than 8 log CFU g⁻¹ was noted in 11 samples), in amounts close to those reported in foodborne outbreaks. Test evaluation in 33 food samples suspected to hold cereulide showed actual cereulide presence in ten samples and no interference of food matrix with the assay.

Keywords: B. cereus, cereulide, food, CASA, preservatives, acrylamide, dioxin
6.1 INTRODUCTION

Cereulide, emetic toxin of *Bacillus cereus*, is recognized as the most perilous virulence factor from a wide spectrum of *B. cereus* pathogenesis. It is a causative agent of *B. cereus* related intoxications and is most commonly associated with farinaceous and starch rich products, where spores have survived cooking, germinated and were allowed sufficient time at moderate temperature for growth and cereulide production to occur (Gibbs, 2003; Schoeni and Wong, 2005). The clinical dose, suggested to be about 10 µg kg⁻¹ body weight (bw) (Paananen *et al.*, 2002), is higher than those of some other known toxins (*S. aureus* intoxication that resembles symptoms of cereulide intoxication, induces mild effects already at concentrations of 100 ng kg⁻¹ bw (Bergdoll, 1989)). The cereulide doses found in foods (see 5.1), although rarely, were in few occasions reported as lethal (Mahler *et al.*, 1997; Dierick *et al.*, 2005).

Inhibition of boar semen motility due to the mitochondrial dysfunction and morphological and functional alterations following acute cereulide intoxication, has been described and applied in boar semen motility bioassay (Andersson *et al.*, 1998b; Hoornstra *et al.*, 2003; Andersson *et al.*, 2004).

The Computer Aided Semen Analysis (CASA) approach acquires a real-time image, tracking the motility behavior. The image processing software detects and tabulates the tracks and immotile spermatozoa. Observation of boar semen progressive motility percentage with application of CASA allows transformation of motility data for detection and determination of cereulide in food (see Chapters 4 and 5).

Being essentially a biological test, boar semen bioassay provides information on overall sample toxicity, unless exclusively specific for cereulide. To investigate the specificity and robustness of the CASA-bioassay, the present study included a number of possible interfering factors, including different food matrices. The choice of bacterial toxins and mycotoxins included in the study was either based on reported effects of certain mycotoxins on mitochondria (Kawai *et al.*, 1984; Sajan *et al.*, 1995; Macri *et al.*, 1996; Abid-Essefi *et al.*, 2004) or as in the case of *S. aureus* enterotoxins on the intoxication symptoms similarity (both being emetic toxins). Two other major toxins produced by *B. cereus*, Hemolysin BL (HBL) and Non-Hemolytic Enterotoxin...
(NHE) were also included in the study. Moreover, the specificity study included food preservatives commonly used in food preservation, as well as dioxins and acrylamide, due to their potential occurrence in the foods where *B. cereus* growth also occurs. Dioxins are mostly related to animal products (Baars *et al.*, 2004; Maitani, 2004; Chovancova *et al.*, 2005), among which milk and milk products; the former also being associated with *B. cereus* contamination (Notermans *et al.*, 1997; Beutling and Bottcher, 1998; McElroy *et al.*, 1999; Bedi *et al.*, 2005). Acrylamide is mostly related to fried or baked starch rich products in which also cereulide production is predominantly reported (Agata *et al.*, 2002; Toh *et al.*, 2004). Polychlorinated biphenyls (PCBs) are reported to affect the reproductive system in animals and in exposed humans (Hsu *et al.*, 2003). While chronic effect was reported, the acute effect of PCBs and non-dioxin like CBs was not evident. Also with acrylamide (Tyl *et al.*, 2000; Tyl and Friedman, 2003) the impact on semen beat/cross frequency (motility parameter) was found significant when laboratory rats were administrated 60 mg kg$^{-1}$ day$^{-1}$ of acrylamide (monomer), whereas overall motility did not seem to be influenced.

The objective of the present study was to examine the extent of possible interference of above mentioned factors with the CASA bioassay for detection of cereulide. In addition, the potential of various food matrices, susceptible to *B. cereus* contamination, to support production of hazardous concentrations of cereulide was determined.

### 6.2 MATERIALS AND METHODS

#### 6.2.1 Bacterial strains and enumeration

Fourteen *B. cereus* and eleven *S. aureus* strains, producing different exotoxins (Table 6-1) were stored at −80°C and resuscitated in Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK) by incubation for 48 hours, at 30 °C for *B. cereus*, and at 37 °C for *S. aureus*.

Strains were checked on purity and the stock cultures were maintained on Tryptone Soya Agar (TSA, Oxoid) slants, stored at 4 °C. The stock culture was reactivated and
cultivated by inoculating a loopful of the culture into fresh TSB and 24 hour incubation at respective temperatures for *B. cereus* and *S. aureus*.

Enumeration of pure cultures was performed with spread plate count method on TSA (Oxoid).

### 6.2.2 Computer aided semen boar motility analysis

CASA using Hamilton-Thorne computer-aided semen analyzer (HTR Ceros 12.1, Hamilton Thorne Research, Beverly, CA, USA) was employed for relative-quantitative and semi-quantitative determination of cereulide, as described previously (see Chapter 4). In short, 195 µl of sperm (30 millions cells per milliliter, with initial progressive motility of 60-70%) were mixed with 5 µl of test solution (blank or testing sample) and injected into the chambers of Leja slide (standard count 2-chamber 20 micron slide, Leja, Nieuw-Vennep, The Netherlands). If only relative-quantitative toxicity evaluation was required, undiluted sample was subjected to the test and exposure time required for PMOT% to drop to 10% was noted. The sample was considered negative if the PMOT% remained higher than 10% within 10 minutes of exposure time. For quantitative estimation the sample was serially diluted with dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) in a two-fold dilution serial. Two consecutive dilution steps giving PMOT% change in a linear range of a valinomycin standard curve were used to calculate cereulide concentration (see Chapter 4). The effect of valinomycin on boar semen motility was reported identical to that of cereulide (Hoornstra *et al.*, 2003; Andersson *et al.*, 2004). Only test samples that induced PMOT% decrease within five minutes were quantified.

### 6.2.3 Specificity of computer aided boar semen motility analysis

#### 6.2.3.1 Influence of *B. cereus* diarrheal toxins and *S. aureus* enterotoxins on boar semen motility

All strains (Table 6-1) were cultured on TSA plates by flooding the surface of a plate with 1 ml of 24 hours old TSB culture containing ca. $10^9$ CFU ml$^{-1}$. Plates inoculated with *B. cereus* were incubated for 24 hours at 30 °C and those of *S. aureus* at 37 °C.
### Table 6-1: Overview of tested *B. cereus* and *S. aureus* strains and their impact on PMOT%

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Toxin produced</th>
<th>Effect on PMOT%&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>IPH 5964&lt;sup&gt;abx&lt;/sup&gt;</td>
<td>cereulide</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>IH 14385&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>NS117&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>NS115&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>LMG12334&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>NC 7401&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LMG12335&lt;sup&gt;e&lt;/sup&gt;</td>
<td>HBL</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LFMFP 2.8&lt;sup&gt;dy&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LFMFP 1.8&lt;sup&gt;dy&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LFMFP 3.3&lt;sup&gt;dy&lt;/sup&gt;</td>
<td>NHE</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IPH 5972&lt;sup&gt;x&lt;/sup&gt;</td>
<td>Non-toxigenic</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IPH 5958&lt;sup&gt;x&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>F528&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>F 837&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>LFMFP 085&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SEA</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LFMFP 6645&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ATCC13565&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ATCC14458&lt;sup&gt;g&lt;/sup&gt;</td>
<td>SEB</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SK64&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SK71A&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C&lt;sup&gt;g&lt;/sup&gt;</td>
<td>SEC</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ATCC 19099&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ATCC 23235&lt;sup&gt;f&lt;/sup&gt;</td>
<td>SED</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A&amp;D&lt;sup&gt;f&lt;/sup&gt;</td>
<td>SED and SEA</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ATCC27664&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Non-toxigenic</td>
<td>-</td>
</tr>
</tbody>
</table>

---

<sup>a</sup>+++ strong positive reaction; ++ medium positive reaction; + positive reaction; - negative reaction;

<sup>b</sup>Gift by dr. K. Dierick, Belgian Institute of Public Health;

<sup>c</sup>Gift from the culture collection of Prof. M. Salkinoja, University of Helsinki, Finland;

<sup>d</sup>Culture collection of Laboratory of Food Microbiology and Food Preservation, Ghent University;

<sup>e</sup>BCMM, Laboratory for Microbiology, Ghent University;

<sup>f</sup>American Type Culture Collection, gift from Dr. K. Cudjoe, Norwegian National Veterinary Institute, Department of Feed and Food Hygiene, Section of Feed and Food Microbiology, Oslo, Norway;

<sup>g</sup>Food isolate, gift from Prof. L. De Zutter, Department of Veterinary Public Health and Food Safety, Ghent University;

<sup>x</sup>Isolated from a foodborne outbreak (Dierick <i>et al.</i>, 2005);

<sup>y</sup>Isolates from a potato puree production line (see Chapter 2 and 3)
Grown biomass was collected as described earlier (see Chapter 4) using methanol (>99.8%, Fluka Chemie GmBH, Buchs, Switzerland) for *B. cereus* strains producing hydrophobic cereulide and using Phosphate Buffer Saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.45) for *B. cereus* and *S. aureus* strains producing hydrophilic enterotoxins. Briefly, cereulide extraction was performed by boiling the biomass of cereulide producing strain in 2 ml methanol during ca. 20 minutes at 100 °C (see Chapter 4), while extraction of hydrophilic enterotoxins in PBS was performed by incubating biomass of respective *B. cereus* and *S. aureus* strains in 2 ml PBS for 1 hour at the room temperature on a rotary shaker (150 rpm). Extracts were stored at 2 °C and analyzed in duplicate within 24 hours. Production of *B. cereus* and *S. aureus* enterotoxins was confirmed with BCET-RPLA and SET-RPLA kits (Oxoid). In addition, positive controls of *B. cereus* HBL (BCET-RPLA, Oxoid) and NHE (Tecra visual immunoassay, BCET-VIA; Bioenterprises Pty. Ltd., Roseville, New South Wales, Australia) test kits, as well as *S. aureus* enterotoxins SEA, SEB, SEC and SED (test kit SET-RPLA, Oxoid) and pure SEB (Toxin Technology, Inc., Sarasota, United States) were tested in CASA bioassay, as described above.

### 6.2.3.2 Impact of mycotoxins on boar semen motility

To investigate the cereulide specific response of boar semen and CASA, samples of six commercially (Sigma-Aldrich, Steinheim, Germany), obtained mycotoxins, dissolved in their respective solvents, were subjected to the analyses by boar semen bioassay, as described above. Solutions of mycotoxins were prepared in different concentration ranges, analyzed in duplicate and the lowest concentration influencing semen motility was recorded (Table 6-2).

### 6.2.3.3 Impact of food preservatives on boar semen motility

Ten different food preservatives were tested for their impact on boar semen motility using CASA application. Concentrations of preservatives to be used (Table 6-3) were higher or in the range of those allowed by the legislation (EU, 1995). All solutions were made by mixing adequate mass or volume of a solute in sterile demineralized water.
Table 6-2: Interference of mycotoxin solutions with boar semen motility, measured in CASA application

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Solvent</th>
<th>Tested concentration range (mg ml⁻¹)ᵃ</th>
<th>Minimal effective concentration (mg ml⁻¹)ᵇ</th>
<th>Time (seconds) to PMOT% = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin M1</td>
<td>DMSO</td>
<td>0.1-0.0004</td>
<td>0.004</td>
<td>120</td>
</tr>
<tr>
<td>Aflatoxin M3</td>
<td>DMSO</td>
<td>0.1-0.004</td>
<td>0.1</td>
<td>60</td>
</tr>
<tr>
<td>Sterigmatocystin</td>
<td>Methanol</td>
<td>12.5</td>
<td>No effect</td>
<td>/</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>Methanol</td>
<td>100-1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Fumonisin B1</td>
<td>Acetonitril water (1:1)</td>
<td>5</td>
<td>No effect</td>
<td>/</td>
</tr>
<tr>
<td>Patulin</td>
<td>Ethanol</td>
<td>0.01</td>
<td>No effect</td>
<td>/</td>
</tr>
</tbody>
</table>

ᵃ If no effect was observed with the highest concentration no further dilutions were tested
ᵇ The lowest concentration of mycotoxins that caused cease of boar semen progressive motility within 10 minutes of observation

Table 6-3: The most common preservatives tested for their impact on boar semen motility and CASA

<table>
<thead>
<tr>
<th>Preservative</th>
<th>Concentration range tested</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium sorbate</td>
<td>0.2%</td>
<td>Fluka, Buchs, Switzerland</td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td></td>
<td>Pharmachemic, Antwerpen, Belgium</td>
</tr>
<tr>
<td>(DL) Malic acid</td>
<td>0.5%</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(L+) Tartaric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL Lactic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(L+) Ascorbic acid</td>
<td>0.3%</td>
<td>VWR International, Fontenay sous Bois, France</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>19%</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>65%</td>
<td>RPL, Leuven, Belgium</td>
</tr>
</tbody>
</table>

6.2.3.4 Impact of dioxins and acrylamide on boar semen motility

The most notorious 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Accu Standard, New Haven, CT, USA) was tested with CASA, under the conditions described above, in the concentration range 0.075 pg ml⁻¹ to 500 pg ml⁻¹ of DMSO.

In addition, aqueous acrylamide solutions (Sigma-Aldrich, Bornem, Belgium) in the range of 50-10000 µg L⁻¹ were made and subjected to the test, as described above.
All samples were analyzed in duplicate. Positive and negative controls used were mixture of semen with cereulide extract from \textit{B. cereus} 5964a biomass and solvent used to dissolve tested sample, respectively.

6.2.4 Cereulide production and robustness of CASA and boar semen bioassay in various foods

6.2.4.1 Preparation of food samples

Fourteen food products (Table 6-4), purchased at a local supermarket (“point of sale”) were tested in duplicate for their support of \textit{B. cereus} growth and cereulide production. Simultaneously, an interference of food matrix with boar semen bioassay was investigated by subjecting non-inoculated samples to the CASA. Refrigerated foods were stored at the required temperature (indicated on the label) and used for inoculation and analysis within indicated shelf life. Prior to portioning and inoculation, frozen foods (paella, moussaka, braded soy and vegetable crockets) were thawed in their original packages, at room temperature for ca. two hours. Braded chicken fingers were fried in oil according to instructions on the package simulating conditions of at home preparation. Polenta was prepared according to the manufacturers’ instructions (250 g of polenta was added to 1 liter of boiling demineralized water containing 2.5 g of sodium chloride, and cooked for 10 minutes). Infant formula with cereals (40 g) was reconstituted with 200 ml of sterile demineralized water and left to soak for 30 minutes with occasional shaking. All other food items were used in their native state and sampled from the original packages.

For every food product, pH and \textit{a}_w values were measured from non-inoculated samples after incubation (30 °C / 24 h) using a pH-meter (type 763, Knick, Berlin, Germany) with electrode (Ingold 104063123, Urdorf, Switzerland) and cryometer (AW-Kryometer, type AWK-20, NAGY Messsysteme GmbH, Gäufelden, Germany), respectively.
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Table 6-4: Microbial and cereulide analysis in different foods inoculated with ca. 4 log CFU g⁻¹ (ml⁻¹) of cereulide producing *B. cereus* 5964a after 24 hours of incubation at 30 °C (growth data given as mean ± standard deviation).

<table>
<thead>
<tr>
<th>Food</th>
<th><em>B. cereus</em> (log CFU g⁻¹)</th>
<th>Total aerobic mesophilic count (log CFU g⁻¹)</th>
<th>Cereulide (ng g⁻¹)</th>
<th>pH</th>
<th>a_w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit yoghurt</td>
<td>No growth</td>
<td>4.8 ± 0.1</td>
<td>-</td>
<td>4.09</td>
<td>0.999</td>
</tr>
<tr>
<td>Yoghurt with fibres</td>
<td>No growth</td>
<td>5.3 ± 0.4</td>
<td>-</td>
<td>4.26</td>
<td>0.984</td>
</tr>
<tr>
<td>Cooked wheat grains</td>
<td>No growth</td>
<td>No growth</td>
<td>-</td>
<td>4.56</td>
<td>0.979</td>
</tr>
<tr>
<td>Canned mushrooms</td>
<td>8.1 ± 0.2</td>
<td>8.1 ± 0.1</td>
<td>-</td>
<td>5.24</td>
<td>0.999</td>
</tr>
<tr>
<td>Vanilla pudding</td>
<td>8.5 ± 0.1</td>
<td>8.7 ± 0.3</td>
<td>BQL⁴</td>
<td>6.55</td>
<td>0.991</td>
</tr>
<tr>
<td>Moussaka</td>
<td>8.5 ± 0.2</td>
<td>8.6 ± 0.1</td>
<td>11.34</td>
<td>5.4</td>
<td>0.995</td>
</tr>
<tr>
<td>Pet food</td>
<td>8.7 ± 0.2</td>
<td>8.7 ± 0.2</td>
<td>-</td>
<td>6.02</td>
<td>0.999</td>
</tr>
<tr>
<td>Rice pudding</td>
<td>8.6 ± 0.1</td>
<td>8.5 ± 0.1</td>
<td>BQL⁴</td>
<td>6.68</td>
<td>0.992</td>
</tr>
<tr>
<td>Polenta</td>
<td>8.7 ± 0.0</td>
<td>8.7 ± 0.2</td>
<td>4.72</td>
<td>6.55</td>
<td>NAb</td>
</tr>
<tr>
<td>Infant formula with cereals</td>
<td>8.7 ± 0.3</td>
<td>8.5 ± 0.1</td>
<td>12.00</td>
<td>6.92</td>
<td>NAb</td>
</tr>
<tr>
<td>Soy and vegetable crocket</td>
<td>9.2 ± 0.0</td>
<td>9.2 ± 0.0</td>
<td>10.8</td>
<td>6.41</td>
<td>0.978</td>
</tr>
<tr>
<td>Chicken fingers</td>
<td>9.2 ± 0.1</td>
<td>9.2 ± 0.4</td>
<td>BQL⁴</td>
<td>5.91</td>
<td>0.983</td>
</tr>
<tr>
<td>Canned corn</td>
<td>9.1 ± 0.5</td>
<td>9.1 ± 0.6</td>
<td>-</td>
<td>6.38</td>
<td>0.995</td>
</tr>
<tr>
<td>Paella</td>
<td>9.6 ± 0.1</td>
<td>8.8 ± 0.1</td>
<td>-</td>
<td>5.88</td>
<td>0.995</td>
</tr>
</tbody>
</table>

⁴ Cereulide was detectable, however the concentration was below quantification limit (BQL) of bioassay given by a standard curve (see Chapter 4).

⁵ NA-Not Available

6.2.4.2 Inoculation and microbial analysis of food

Each food product was divided into four portions of 40 g distributed in Stomacher bags or in 200 ml sterile plastic containers (BD Bioscience, Erembodegem, Belgium), depending on the food composition. The native microbial flora was determined from one food sample on the day of inoculation. Two samples were inoculated with *B. cereus* strains (cereulide producing 5964a and non producing F528, respectively) and one sample served as a negative control. Sample inoculation was done with 1 ml of an appropriate dilution (tenfold dilution serial in PPS) of a 24 hour *B. cereus* culture in...
TSB, in order to obtain inoculum level of ca. 4 log CFU per g or ml of food. Inoculated and control samples were incubated under the same conditions (30 °C for 24 hours).

Microbial enumerations of samples prior to inoculation and after incubation were done from a 30-g sample of food, homogenized for 2 min with a Stomacher (Lab-Blender 400, Led Techno, Eksel, Belgium) in 270 ml of PPS. Total mesophilic aerobic count, \textit{B. cereus} count and yeasts and molds count were determined using standard spread plate count method on Plate Count Agar (Oxoid), \textit{B. cereus} agar medium (BCA, Lab M, Bury, Lancashire, UK) and Yeast Glucose Chloramphenicol agar (YGC, Bio-Rad, Marnes-la Coquette, France), respectively. For fruit yoghurt, yoghurt with fibres, vanilla pudding and rice pudding also lactic acid bacteria were enumerated on DeMan, Rogosa Sharpe agar (MRS, Oxoid) with pour plate count method. All plates were incubated at 30 °C, for 24 hours for \textit{B. cereus} and total mesophilic count, and for 2-5 days for yeasts and molds count, and lactic acid bacteria enumeration.

6.2.5 Computer aided boar semen motility analysis of suspected foods

Besides artificially inoculated foods, naturally contaminated food samples suspected to hold cereulide or \textit{B. cereus} were included in the study to evaluate the CASA bioassay in routine analyses. 33 different foods, obtained directly from the producers or restaurants (Table 6-5) were analyzed in duplicate for cereulide and presence of \textit{B. cereus}. Three randomly taken positive samples were confirmed with HPLC-MS as described in Chapter 5 (see 5.2.4), on the basis of elsewhere reported protocols (Haggblom \textit{et al.}, 2002; Jaaskelainen \textit{et al.}, 2003a). All samples that were found positive with bioassay were additionally analyzed on the presence of \textit{S. aureus} cells (as described above) and enterotoxins (VIDAS-SET2™, bioMérieux, Marcy-l’Etoile, France, following manufacturer’s instructions).
Table 6-5: Cereulide determination with CASA analysis of food samples originating from production site or restaurants suspected to hold cereulide

<table>
<thead>
<tr>
<th>Food</th>
<th>pH</th>
<th>$a_w$</th>
<th>Cereulide presence</th>
<th>Time (seconds) to PMOT%≤10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoked salmon</td>
<td>6.2</td>
<td>0.965</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>Smoked crab</td>
<td>6.8</td>
<td>0.966</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Asparagus cream soup</td>
<td>6.2</td>
<td>0.981</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Fruit yoghurt</td>
<td>4.5</td>
<td>0.980</td>
<td>+</td>
<td>60</td>
</tr>
<tr>
<td>Pasta salad with chicken breast</td>
<td>6.1</td>
<td>0.994</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Black olives on Greek manner *</td>
<td>6.8</td>
<td>NA$^d$</td>
<td>+</td>
<td>60</td>
</tr>
<tr>
<td>Chateaubriand</td>
<td>6.2</td>
<td>0.992</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Boiled chicken in vacuum package</td>
<td>6.5</td>
<td>0.990</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mackerel filet in breadcrumbs</td>
<td>6.3</td>
<td>0.995</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Boiled chicken white</td>
<td>6.6</td>
<td>0.997</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lettuce salad</td>
<td>6.1</td>
<td>0.989</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Crab</td>
<td>6.9</td>
<td>0.998</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Moussaka</td>
<td>5.8</td>
<td>0.993</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pre-roasted turkey file *</td>
<td>6.5</td>
<td>NA$^d$</td>
<td>+</td>
<td>40</td>
</tr>
<tr>
<td>Moussly with fruits and nuts</td>
<td>5.3</td>
<td>0.615</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sweet canned corn</td>
<td>6.0</td>
<td>0.993</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Raw veal meat</td>
<td>5.9</td>
<td>0.994</td>
<td>+</td>
<td>60</td>
</tr>
<tr>
<td>Ice cream + cornette (mokka)</td>
<td>5.7</td>
<td>NA$^d$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Goulash</td>
<td>6.8</td>
<td>0.996</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Croquette</td>
<td>6.0</td>
<td>0.982</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Roulae ardenaise</td>
<td>NA$^d$</td>
<td>NA$^d$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Bread with provencal herbs</td>
<td>5.9</td>
<td>0.945</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Milk powder</td>
<td>6.2</td>
<td>0.246</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Oligo fructose</td>
<td>NA$^d$</td>
<td>NA$^d$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cassis sorbet</td>
<td>3.2</td>
<td>0.964</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Bolognaisae sauce</td>
<td>5.1</td>
<td>0.986</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Camembert cheese *</td>
<td>7.6</td>
<td>0.961</td>
<td>+</td>
<td>50</td>
</tr>
<tr>
<td>Infant formula (pollack + vegetables)</td>
<td>5.2</td>
<td>0.999</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Butter milk</td>
<td>4.5</td>
<td>NA$^d$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pasta salad (chicken + vegetables)</td>
<td>4.9</td>
<td>0.981</td>
<td>+</td>
<td>20$^b$</td>
</tr>
<tr>
<td>Canned mushroom soup</td>
<td>6.2</td>
<td>NA$^d$</td>
<td>+</td>
<td>20$^b$</td>
</tr>
<tr>
<td>Bacon$^c$</td>
<td>5.8</td>
<td>0.952</td>
<td>+</td>
<td>30$^b$</td>
</tr>
<tr>
<td>Emmental cheese</td>
<td>5.3</td>
<td>0.971</td>
<td>+</td>
<td>20$^b$</td>
</tr>
</tbody>
</table>

$^a$ Randomly taken samples and confirmed with HPLC-MS  
$^b$ Visually determined total stop of total motility  
$^c$ Positive on S. aureus enterotoxins (determined with VIDAS-SET2™, bioMérieux)  
$^d$ NA-Not Available

### 6.2.6 Cereulide extraction and CASA analysis

Samples for cereulide determination were taken from the same package as those intended for microbial enumeration. Sampling consisted of taking 10 g (10 ml) of a
food product into an open 100 ml conical flask, mixing with double volume of methanol (see Chapter 5) and extraction at 100°C during ca. 20 minutes (or until liquid phase was evaporated). When all liquid was evaporated additional 3 ml of methanol were added, intensively mixed during ca. 2 minutes and the whole volume of methanol transferred into a 4 ml screw neck glass vial. Open vials were immediately subjected to the evaporation under N2. The dry residue was suspended in 200-500 µl of DMSO and kept at -20 °C until analyses.

Additional 10 g portion of native food was prior to the extraction spiked with 1 ml methanol extracts of *B. cereus* 5964a biomass (cereulide concentration of 100 µg ml⁻¹) to serve as a positive control in bioassay. Recovery percentage (equation 1) was calculated for two representative foods, namely goulash and camembert cheese, as detected with CASA bioassay.

Equation 1:

\[
\text{Recovery (\%)} = \frac{\text{Cereulide concentration in spiked sample} - \text{Cereulide concentration in non-spiked sample}}{\text{Spiked cereulide concentration}} \times 100\%
\]

### 6.2.7 Statistical interpretation

All analyses were performed in duplicate. Mean and standard deviation values were obtained using Microsoft Excel version 2003.

### 6.3 RESULTS

#### 6.3.1 Specificity of boar semen and CASA for cereulide detection

To determine the toxic effect of food relevant toxic compounds, other than cereulide, on boar semen motility, *S. aureus* and *B. cereus* enterotoxins, as well as six mycotoxins were tested with CASA. PMOT% of blank and negative controls remained on its initial level of ca. 60% for all tested samples during ten minutes of exposure time. Results indicated that none of *S. aureus* enterotoxins imposed
inhibitory effect on boar semen motility (Table 6-1). PMOT% remained in all *S. aureus* biomass extracts containing SEA, SEB, SEC and/or SED on the same level of ca. 60% during ten minutes. Also, no effect was noticed when semen was exposed to the purified 1 µg ml⁻¹ SEB (Toxin technology) or positive controls of SEA, SEB, SEC and SED (SET-RPLA, Oxoid). No change in PMOT% was observed when semen was exposed to *B. cereus* HBL and NHE enterotoxins, produced by respective *B. cereus* strains, neither when exposing it to the positive controls of HBL and NHE from RPLA detection (Oxoid) and BCET-VIA (Tecra) kits, respectively.

Effect of six mycotoxins diluted to different concentration in different organic solvents (Table 6-2) was found to be measurable only with Aflatoxin M1 and M3, and with Zearalenone, the first one being the most reactive. No effects were observed with Sterigmatocystin, Fumonisin B1 and Patulin. Exposure time for all samples was 10 minutes, unless PMOT% dropped below 10% in a shorter time, as in the case of positive controls.

Beside toxins, ten food preservatives were included in the study. No inhibition of boar semen motility during ten minutes of exposure was observed for any of the tested preservatives (Table 6-3). Negative control (semen mixed with water) remained negative and positive control (semen mixed with cereulide extract) remained positive.

Moreover, chemical contaminants, dioxins and acrylamide, included in the study, did not interfere with boar semen motility even in the concentrations of 500 pg ml⁻¹ and 10000 ng ml⁻¹, respectively. PMOT% did not change in comparison to the negative control (semen mixed with DMSO for dioxin control or semen mixed with water for acrylamide control), remaining on the level of ca. 60%, during 10 minutes of exposure time.

### 6.3.2 Robustness of computer aided boar semen motility analysis

Using CASA in boar semen bioassay and valinomycin standard curve, cereulide production was investigated in 14 different foods. Results indicated the ability of inoculated *B. cereus* to grow in all tested food products, except in two yoghurt samples, where high counts of LAB and low pH were observed, and in cooked wheat grains where also low pH was noted (Table 6-4). Observed growth resulted in detectable cereulide production in seven foods, of which four samples held amounts
higher than the quantification limit of CASA (20 ng cereulide ml\(^{-1}\) of solvent). In two samples, namely, rice and vanilla pudding, cereulide amounts were too low to be quantified with established standard curve, resulting in complete PMOT% drop only between 5\(^{th}\) and 10\(^{th}\) minute of exposure time. Positive controls and food spiked with the cereulide extract, remained positive (average recovery percentage of ca 88% ± 10% was measured for goulash and camembert cheese). Food inoculated with cereulide non-producing *B. cereus* F528 and non-inoculated foods, remained negative. Extracts of native foods (negative control) incubated at 30 °C for 24 h, also did not impose inhibition on boar semen motility.

In none of the inoculated foods yeasts and mould growth was observed with respective counts remaining below detection limit of 2 log CFU g\(^{-1}\). High number of lactic acid bacteria was observed in all tested yoghurt (ca. 5.5 log CFU ml\(^{-1}\)) and pudding (ca. 8.5 log CFU g\(^{-1}\)) samples after 24 h incubation at 30 °C.

Results of CASA evaluation showed that of 33 tested food samples suspected to hold cereulide, presence was confirmed in 10 samples (Table 6-5). Three randomly taken positive samples were analyzed by HPLC-MS confirming cereulide presence. Control samples (foods of the same type purchased at the local supermarket) and foods spiked with methanol extract of non-cereulide producing *B. cereus* F528 biomass that were indigenously negative remained negative. Positive controls (tested foods spiked with methanol extract of cereulide producing *B. cereus* 5964a biomass) were at all times positive indicating no cereulide loss or modification during extraction or inhibition with food components. Detection of cereulide in the suspected foods was not accompanied with viable *B. cereus* cells (detection limit 100 CFU g\(^{-1}\)). Most of the foods were thermally treated or conserved in another way, except in the case of raw beef meat.

One sample (bacon) found to be positive on cereulide, was also positive on *S. aureus* enterotoxins as determined with VIDAS-SET2\textsuperscript{TM} (bioMérieux, Marcy-l’Etoile, France). The same sample harbored more than 3 log CFU g\(^{-1}\) of *S. aureus*. 


6.4 DISCUSSION

Of all tested toxins, other than cereulide, only Aspergillus aflatoxins M1 and M3 and Fusarium toxin zearalenone, expressed toxic effect on boar semen, inducing cease in the semen motility. The concentrations in which toxic effect occurred were fairly high. Provisional maximum tolerable daily intake for zearalenone of 0.5 µg per kg of bw (JECFA, 2000) appears much lower than amounts of zearalenone detectable with CASA (10 mg ml⁻¹). Also maximal doses of zearalenone reported in animal feed were 2 mg kg⁻¹. Even when lactating cows were fed with 6000 mg of zearalenone (ca. 12 mg kg⁻¹ bw) only 6.1 µg l⁻¹ zearalenone, 4 µg l⁻¹ alpha-zearalenol, and 6.6 µg l⁻¹ beta-zearalenol were found in milk (JECFA, 2000). Reported data and results of the current study allow concluding that the relevancy of zearalenone interference with cereulide detection may not be of any importance, as the concentrations needed to affect the bioassay were found too high to be expected in foods. Aflatoxin M series are major metabolites of aflatoxin B series, found in milk of animals that have consumed feed contaminated with aflatoxins. The maximum limit of 5 µg kg⁻¹ for aflatoxin B1 and of 10 µg kg⁻¹ aflatoxin total for some species of spices is determined (EU, 1998). Literature further reports that concentration of aflatoxins in raw milk are usually 100 ng l⁻¹ in Europe (Moss, 2002). Seeing that boar semen motility was influenced only at aflatoxin M1 concentration higher than 4 µg ml⁻¹ and M3 concentration of 100 µg ml⁻¹, also interference of aflatoxins with cereulide detection can be regarded as of no significant importance for cereulide detection in the food chain.

Although all the mycotoxins presently analyzed have been reported in literature (Kawai et al., 1984; Sajan et al., 1995; Macri et al., 1996; Abid-Essefi et al., 2004) to affect mitochondria, none of them posses ionophoretic properties. This could be the reason for smaller extent or complete disability of tested mycotoxins to induce the motility impairment.

With regard to the tested bacterial toxins, although inducing the same symptoms as cereulide food poisoning, S. aureus enterotoxins had no impact on measured PMOT%. The same was observed for B. cereus enterotoxins HBL and NHE. Apart from a different mode of action and no ionophoretic properties, an important and related factor for a lack of impact of these exotoxins on mitochondria is their hydrophilic nature, due to which these molecules may not be able to form pores in phospholipids.
of mitochondrial outer membrane. There is a direct relation between lipophilicity and permeability through the membrane (Spacie et al., 1995). Only hydrophobic substances can pass through the hydrophobic interior of the mitochondrial membrane by simple diffusion down a concentration gradient. Hydrophilic molecules, which repel the hydrophobic interior, must be moved by facilitated diffusion or active transport through the proteins lodged in the membrane (Timbrell, 2002).

Although reports exist on impact of dioxins on semen motility, no such acute effect was noted when exposing boar semen to TCDD for 10 minutes in the concentration of 500 pg ml\(^{-1}\). Recent study in The Netherlands showed the estimated median life-long-averaged intake of the sum of dioxins and dioxin-like PCBs in Dutch population was 1.2 pg WHO-TEQ (toxic equivalents) per kg bw per day, while the estimated median life-long-averaged intake of indicator-PCBs is 5.6 ng per kg bw per day (Baars et al., 2004). Results of the current study indicate that no interference of dioxins with cereulide detection with CASA in boar semen bioassay is expected, regarding reported data. Same conclusion can be drawn for acrylamide, which had no impact on the semen motility even in the concentration of 10000 ng ml\(^{-1}\), whereas concentrations that are often found in food and are relevant for dietary intake are much smaller (Matthaus et al., 2004; Hilbig et al., 2004), being maximally 4000 ng g\(^{-1}\) in potato chips.

Neither one of the tested foods nor tested food preservatives influenced PMOT%, indicating that the described assay can be used to test cereulide presence in large variety of foods. Inoculation of a cereulide producing strain into fourteen different foods, showed ability of non-typical food matrices to support \textit{B. cereus} growth and cereulide production. The amounts of cereulide produced were lower or around lowest amounts found in foods implicated in cereulide outbreaks (10 ng g\(^{-1}\) (Agata et al., 2002)), indicating the possibility that these foods may lead both to an exposure to the sub-intoxicative doses of cereulide, as well as to the acute intoxications.

\section*{6.5 CONCLUSIONS}

Current research provides further evidence of a wide applicability of boar semen bioassay and CASA in detection of cereulide in foods. As a rapid test it provides valuable screening possibilities and due to the biological detection of toxic activity it
provides information relevant to the public health hazard. Being a biological assay it detects cereulide indirectly, requiring an absolute negative control and HPLC-MS verification of cereulide presence in positive samples. Its application has revealed potential of variety of foods to support cereulide production. Furthermore, cereulide was found in number of naturally contaminated foods.

Development of immunological assays for cereulide detection is at the moment not feasible due to the lack of antibodies against cereulide. Therefore, another toxin, causing foodborne intoxication and having food poisoning symptoms reassembling those of cereulide, was targeted. Namely, *S. aureus* enterotoxin B was used to develop immuno-quantitative PCR described in Chapter 7. The newly developed method had to be very sensitive, as intoxication dose of SEB can possibly be as low as 0.05 ng per gram of food (Anonymous, 2001). Developed iqPCR served to characterize SEB production and would have its potential with adequate antibodies also in cereulide investigation.
IMMUNO-QUANTITATIVE REAL-TIME PCR
FOR DETECTION AND QUANTIFICATION
OF STAPHYLOCOCCUS AUREUS
ENTEROTOXIN B IN FOODS

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(2006)

Applied and Environmental Microbiology, accepted for publication
CHAPTER 7: IMMUNO-QUANTITATIVE REAL-TIME PCR FOR DETECTION AND QUANTIFICATION OF STAPHYLOCOCCUS AUREUS ENTEROTOXIN B IN FOODS

Summary

Staphylococcus aureus enterotoxins are the cause of one of the most frequent acute foodborne intoxications. A real-time immuno quantitative PCR (iqPCR) method for detection of S. aureus enterotoxin B (SEB) was developed and evaluated both in laboratory cultures and foods. The assay consists of immunocapture of SEB and real-time PCR amplification of the DNA probe linked to the detection antibody. iqPCR was compared to an in-house ELISA using the same couple of capture-detection antibodies and to commercial kits for detection of S. aureus enterotoxins (SE). iqPCR measurements were ca. 1000 times more sensitive (<10 pg ml⁻¹) than the in-house ELISA and had a dynamic range of 10 pg ml⁻¹ to 30000 pg ml⁻¹. iqPCR was able to detect SEB production in all different foods tested and anti-SEB antibodies showed no cross reactivity with food compounds and other S. aureus enterotoxins. Application of iqPCR for detection of SEB in cultures of S. aureus revealed onset of SEB production already after 4 hours of incubation at 22, 37 and 42 °C being the first half of the exponential growth phase. Total amounts of SEB produced were for both strains higher at 42 °C than at 37 °C. Final amounts of SEB produced were strain dependent.

Keywords: Staphylococcus aureus, enterotoxin, ELISA, immuno quantitative, real-time PCR
Chapter 7: Real-time iqPCR for detection of *S. aureus* enterotoxin B

### 7.1 INTRODUCTION

*Staphylococcus aureus* intoxication remains one of the most common causes of foodborne diseases (Bennett, 2005). *S. aureus* food poisoning is caused by the consumption of food containing heat stable enterotoxin(s). Although many foods harbor small numbers of *S. aureus* and can be eaten safely, time and temperature abuse of a food product can result in sufficient growth of present *S. aureus* causing formation of enterotoxins. Quantities between 100 and 200 ng of consumed enterotoxin can cause symptoms of staphylococcal intoxication. These toxin levels are reached when *S. aureus* population exceeds $10^5$-$10^6$ CFU per gram (Bennett, 2005). The presence of relatively large numbers of enterotoxigenic *S. aureus* is a good circumstantial evidence that the food contains toxins, but the absence of *S. aureus* cells is not an evidence of toxin absence, as the bacterial load might be eliminated/lowered by antimicrobial actions, which do not affect the toxin concentration and its biological activity.

The classical antigenic types of *S. aureus* enterotoxins (SE) incriminated in staphylococcal food poisoning are SEA, SEB, SEC, SED and SEE. New serological types of SEs (SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER and SEU) were identified in recent years (Blaiotta *et al.*, 2004; Lovseth *et al.*, 2004; Omoe *et al.*, 2004).

Detection techniques for staphylococcal enterotoxins are commercially available and require from one and a half to twenty-four hours to be completed. Reported detection limits range from 0.5 to 2 ng enterotoxin per gram of food (Wieneke, 1991; Mathieu *et al.*, 1992; Park *et al.*, 1994; Vernozy-Rozand *et al.*, 2004; Di Pinto *et al.*, 2004). The sensitivity of detection methods and their ability to deliver quantitative information on the amounts of toxin present may require an improvement, as the possibility exists that established intoxication dose is underestimated by currently available detection limits. Immuno polymerase chain reaction (iPCR) is a detection method that combines the specificity of antibody-antigen recognition and the sensitivity of PCR. iPCR methods using end-point detection through classical PCR and electrophoresis of amplicons for detection of *Clostridium botulinum* neurotoxin type A were reported (Wu *et al.*, 2001; Chao *et al.*, 2004a; Chao *et al.*, 2004b). The quantitative immuno-PCR technology (iqPCR) previously described in the patent WO0131056 (Zorzi *et al.*, 2001), couples an antibody detection step, similar to an ELISA, with a nucleic acid amplification of a DNA probe linked to the detection antibody by real-time PCR procedure. Application of real-time PCR platforms for
quantitative amplification (Gofflot et al., 2004; Gofflot et al., 2005) unlike end-point analysis provides data required for quantification of the target DNA. The results can be expressed in absolute terms, by reference to an external quantified standard, or in relative terms compared to another target sequence present in the sample (Saunders, 2004).

In the present work an iqPCR method for detection of \textit{S. aureus} enterotoxin B was developed and evaluated both in laboratory cultures and in foods. Sensitivity of iqPCR was compared with two commercially available kits (SET-RPLA, Oxoid, Basingstoke, UK and VIDAS-SET2\textsuperscript{TM}, bioMérieux, Marcy-l’Etoile, France), as well as to an own in-house ELISA that served as an internal reference method to iqPCR.

\section*{7.2 MATERIALS AND METHODS}

\subsection*{7.2.1 Bacterial strains and culture conditions}

Two SEB producing \textit{S. aureus} strains: ATCC 14458 (obtained from American Type Culture Collection) and SK64f (gift from Dr. K. Cudjoe, Norwegian National Veterinary Institute, Department of Feed and Food Hygiene, Section of Feed and Food Microbiology, Oslo, Norway) were used throughout the study. For the determination of the iqPCR specificity additional strains were used, namely \textit{S. aureus} ATCC 13565, ATCC 19095, ATCC 23235 and ATCC 27664 that are classified by ATCC Bacteria and Bacteriophage Reference Guide (ATCC, 1996) as SEA, SEC, SED and SEE producing strains, respectively. However, extensive work of Becker et al. (2003) revealed that the most of these strains produce and harbor genes of other SE, too. These authors found strain ATCC 13565 positive also for SED production and presence of \textit{sej} gene; strain ATCC 19095 harbored \textit{seg}, \textit{seh} and \textit{sei} gene; and strain ATCC 23235 harbored \textit{seg}, \textit{sei} and \textit{sej} gene. Assuming expression of all these genes sensitivity of iqPCR was to be screened for all SEA-SEH enterotoxins. All mentioned strains were gifts from Dr. K. Cudjoe (Norwegian National Veterinary Institute, Department of Feed and Food Hygiene, Section of Feed and Food Microbiology, Oslo, Norway). Non-toxigenic \textit{S. aureus} strain 171 (food isolate from the culture collection of Laboratory of Food Microbiology and Food Preservation, Ghent University, giving negative reaction in both SET-RPLA and VIDAS-SET2\textsuperscript{TM} assays) was used as a negative control strain for classical SEA-SEE enterotoxins. All strains were stored at –80°C and resuscitated in Tryptone Soya Broth (TSB, Oxoid) by incubation for 48 hours
at 37 °C. Strains were checked on the purity and the reference stock culture maintained on TSA slants.

Enumeration of pure cultures and determination of S. aureus count in sterilized food samples (removed background flora) used for monitoring of S. aureus growth and SE production were done by spread plate method on TSA (Oxoid) incubated at 37 °C for 24 h. Presumptive S. aureus and total aerobic mesophilic counts from naturally contaminated foods were determined on Baird Parker agar with egg-yolk tellurite (Merck, Darmstadt, Germany) and on plate count agar (Oxoid, Basingstoke, UK) using classical plate count method, respectively.

### 7.2.2 Antibodies, antigens and in-house Enzyme Linked Immuno Sorbent Assay (ELISA)

In order to develop sensitive and reproducible basis for SEB detection with iqPCR, antigen-antibody reactions were first formulated in different ELISA formats. Besides five different sandwich formats using capture and detection antibodies, also three direct ELISA formats using only a detection antibody were tested (Table 7-1). Different formats were performed in duplicate and compared using SEB in the standard solution (Tox in Technology, Inc., Sarasota, United States) as an antigen.

Wells (96-well flat bottom microtiter plate, Microlon 600, extra high binding capacity, or strip plates, Microlon 600, high binding capacity, Greiner bio-one B.V., Wemmel, Belgium) in sandwich ELISA (Table 7-1) were coated either with mouse monoclonal antibodies (2-5 µg ml⁻¹) or with sheep polyclonal antibodies (10 µg ml⁻¹) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 4 °C for 16 hours, in a total volume of 50 µl. Coated wells were blocked with 300 µl phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.45) containing 3% (w/v) Bovine Serum Albumin (BSA) well⁻¹ for 1 hour at 37 °C and then washed three times with 300 µl per well of PBS containing 1% (w/v) BSA. Antigens, either standard SEB solution or broth or food extract containing SEB, were added into the wells and two-fold serially diluted in PBS-BSA 1%. Four known positive (standard SEB solution of known concentration) and one negative reference (PBS-BSA 1%) samples were added. The plates were incubated for 1 hour at room temperature, washed three times, as described above and detection antibodies were added and incubated for another hour at room temperature. Following washing with PBS + 0.1% Tween-20, a
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

streptavidin-peroxidase (POD) conjugate was added and incubated for 30 minutes at room temperature (1/7500 diluted in PBS-BSA 1%) to reveal biotinylated detection antibodies. The wells were five times washed with PBS (300 μl/well) and TMB was added to each well. Reaction was stopped after an incubation period of 30 minutes at room temperature by adding 2N H₂SO₄ (25 μl well⁻¹).

In direct ELISA, wells were coated with standard SEB solution diluted to 50 μg ml⁻¹. A two fold dilution serial was further performed with coating buffer as a diluent. The rest of the protocol was performed as for sandwich ELISA.

### Table 7-1: Overview of tested ELISA formats, antibodies and resulting sensitivities

<table>
<thead>
<tr>
<th>Format</th>
<th>Coating material: Capture Antibody or Antigen</th>
<th>Detection Antibody</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mouse monoclonal antibodies, clone S222 (2S4) at 5 μg ml⁻¹</td>
<td>Mouse monoclonal antibodies –biotinylated, clone S643 (2S4) at 0.2-1 μg ml⁻¹</td>
<td>No reaction observed</td>
</tr>
<tr>
<td>2</td>
<td>Sheep Polyclonal antibodies -biotinylated (SBBC202) at 0.5 μg ml⁻¹</td>
<td>Sheep Polyclonal antibodies -biotinylated (SBBC202) at 0.5 μg ml⁻¹</td>
<td>ca. 1.5 ng ml⁻¹</td>
</tr>
<tr>
<td>3</td>
<td>Sheep Polyclonal antibodies at 10 μg ml⁻¹ (SLBI202)</td>
<td>Mouse monoclonal antibodies –biotinylated, clone S643 (2S4) at 0.2-1 μg ml⁻¹</td>
<td>No reaction observed</td>
</tr>
<tr>
<td>4</td>
<td>Sheep Polyclonal antibodies –POD conjugate (SLBC202) at 3 μg ml⁻¹</td>
<td>Sheep Polyclonal antibodies –POD conjugate (SLBC202) at 3 μg ml⁻¹</td>
<td>ca. 7.5 ng ml⁻¹</td>
</tr>
<tr>
<td>5</td>
<td>Sheep Polyclonal antibodies –POD conjugate (SLBC202) at 3 μg ml⁻¹</td>
<td>Sheep Polyclonal antibodies –POD conjugate (SLBC202) at 3 μg ml⁻¹</td>
<td>ca. 7.5 ng ml⁻¹</td>
</tr>
<tr>
<td>6</td>
<td>Direct coating of SEB (BT202) (first well 50 μg ml⁻¹)</td>
<td>Mouse monoclonal antibodies –biotinylated, clone S643 (2S4) at 0.2-1 μg ml⁻¹</td>
<td>No reaction observed</td>
</tr>
<tr>
<td>7</td>
<td>Sheep Polyclonal antibodies -biotinylated (SBBC202) at 0.5 μg ml⁻¹</td>
<td>Sheep Polyclonal antibodies -biotinylated (SBBC202) at 0.5 μg ml⁻¹</td>
<td>ca. 12 ng ml⁻¹</td>
</tr>
<tr>
<td>8</td>
<td>Sheep Polyclonal antibodies –POD conjugate (SLBC202) at 3 μg ml⁻¹</td>
<td>Sheep Polyclonal antibodies –POD conjugate (SLBC202) at 3 μg ml⁻¹</td>
<td>ca. 97 ng ml⁻¹</td>
</tr>
</tbody>
</table>

*a* Internal code of the producer: HyTest, Ltd., Turku, Finland; *b* Internal code of the producer: Toxin technology, Inc. Sarasota, FL, US
The optical density (OD) of the ELISA products was measured at 450 nm using VERSAmax™ tunable microplate reader (Molecular Devices, Sunnyvale, California, USA). Plates were considered valid if negative reference wells had an average OD lower than 0.100 and the four positive reference wells had an average OD higher than a value of OD of wells containing no SEB plus three standard deviations. Quantification of SEB in samples was based on a standard of known concentration run in the same reaction.

7.2.3 Immuno quantitative real-time PCR (iqPCR), primers and DNA

To increase the sensitivity obtained with immunological detection in ELISA, a real-time iqPCR was developed using the same capture-detection couple of antibodies (format 4 in Table 7-1). The ability to monitor the real-time progress of the PCR allowed PCR-based quantification of 5’ biotinylated reporter double stranded DNA (bound to biotinylated detection antibody via streptavidin), as shown in Figure 1 (Gofflot et al., 2005), with SYBR Green sequence non specific chemistry. The 246 base pairs (bp) chimeric reporter double-stranded biotinylated DNA (EMBL accession number of the sequence: AX133313) used was original, built by association of 2 DNA fragments from eukaryotic and prokaryotic origin (Zorzi et al., 2001; Gofflot et al., 2004; Gofflot et al., 2005) decreasing the risk of aspecific amplification. The primers were designed with Primer Express Software, version 1.0 (Applied Biosystems, Foster City, USA). The sequence of the forward primer was: 5’-AAGCCTTGCAGGACATCTTCA-3’ and that of the reverse primer was 5’-GCCGCCAGTGTGATGGATAT-3’. The size of the amplicons to be obtained was 67 bp.

IqPCR was carried out in Robostrips® (Roboscreen, Leipzig, Germany) coated with IgG-SLBI202 and saturated with optimized buffer. As in ELISA, the reaction volume was 50 µl and the wells were prepared identically up to the point of addition of the biotinylated detection antibody (IgG-SBBC202). After 1 hour incubation with detection antibody at room temperature, the strips were washed 3 times with PBS containing 1 ml l⁻¹ Tween 20 and 3 times with PBS containing 15 g l⁻¹ bovine serum albumin. Briefly, 1/500000 diluted recombinant streptavidin (Roche, Vilvoorde, Belgium) was incubated for 45 min on ice with biotinylated reporter DNA (10 ng ml⁻¹) in a 1:2 molar ratio for streptavidin/reporter DNA (Gofflot et al., 2005). The resulting streptavidin–DNA complex was added to the wells and incubated for 30 min at room temperature. The strips were washed 5 times with PBS and 10 times with distilled water, and were then subjected to PCR (ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Foster City, USA) under the following
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Chapter 7: Real-time iqPCR for detection of *S. aureus* enterotoxin B

conditions: 25 µL of SYBR Green PCR Master Mix 2X (Applied Biosystems, Lennik, Belgium), 0.3 µM each of forward and reverse primer, and 19 µL of water in a total volume of 50 µL. The temperature program was as follows: 10 min at 95 °C, followed by 40 PCR cycles of denaturation at 95 °C for 15 s and 60 °C for 1 min for the annealing and extension phases. The external standard (DNA of known concentrations) was used in iqPCR at a dilution range from 2 pg l⁻¹ to 2 fg l⁻¹. One no-template control (NTC) contained the real-time PCR mastermix, without reporter-DNA complex. Other four negative controls were samples without capture antibody, without antigen, without detection antibody, or without streptavidin–DNA complex. Amplification curves were analyzed with Sequence Detection System Software (version 1.9.1, Applied Biosystems, Foster City, USA), and the baseline was determined to circumvent background signals. The parameter Ct (threshold cycle), defined as the fractional cycle number at which the fluorescence passes the fixed threshold, was used to produce quantitative determination of initial SEB concentration. Standard curve was produced by plotting average Ct values of three repetitions against known SEB concentrations and its linear range was used for SEB quantification in tested samples. Standard curve was confirmed using the same conditions in a different laboratory and with a different cycler (ABI Prism SDS 7000, Applied Biosystems, Foster City, USA).

7.2.4 Commercial SE detection systems

Staphylococcal enterotoxin test kit for detection of staphylococcal enterotoxins A, B, C, and D by reversed passive latex agglutination (SET-RPLA, Oxoid) and Enzyme Linked Fluorescent Assay VIDAS™-SET2 with Mini VIDAS Compact Automated Immunoassay Analyzer (bioMérieux) for SEA, SEB, SEC, SED and SEE were used to compare and confirm results obtained with iqPCR. Assays were performed according to the manufacturers’ protocols.

7.2.5 Determination of SEB production in food and laboratory media

Foodborne intoxications with *S. aureus* enterotoxins require presence of SE in foods. While cells of *S. aureus* are readily destroyed by heat treatments applied in the food preparation, its enterotoxins are heat stable and may remain biologically active within the food matrix. It is therefore essential to be able to detect even low amounts of SE present in the food samples. SEB production and detection were investigated in five different foods. Cooked ham, tuna and paella were obtained from a local producer on the day of production. Additionally milk reconstituted from a milk powder and mix of caramel-coffee creamer (mass ratio of 1:4) were both prepared in the laboratory. pH and 

\[ a_w \]

values of foods were measured using pH-meter (type 763, Knick, Berlin, Germany) with electrode (Ingold 104063123, Urdorf, Switzerland) and cryometer (AW-Kryometer, type AWK-20, NAGY Messysteme GmbH, Gäufelden, Germany), respectively. Portions of 50 g of each food product were sterilized (121 °C for 15 minutes) to assure removal of the background flora and inoculated with 1 ml of appropriate dilution of overnight culture of *S. aureus* strain (incubated at 37 °C) providing an inoculum level of ca. 6 log CFU g⁻¹ (ml⁻¹). Inoculated foods were incubated under optimal growth temperature (37 °C) for 24 hours. Two additional samples of milk were inoculated and incubated at 22 and 42 °C for 24 hours.

Extraction of SEB from inoculated foods was done by mixing 10 g of food with VIDAS-SET2™ extraction buffer (1:1, mass to volume ratio). The mixture was incubated for 20 minutes at room temperature and centrifuged at 16000 g (22 minutes at 22 °C). Supernatant was separated by injection through the syringe with pre-wetted cotton in its cylinder. Collected eluate was immediately analyzed for SEB presence (or stored max 24 hours at -20 °C). All samples were analyzed with iqPCR, in-house ELISA, VIDAS-SET2™ and SET-RPLA. Besides food samples, a standard solution of SEB spiked in TSB or milk was
simultaneously run in qPCR allowing quantification of SEB under the same experimental conditions. qPCR measurements were performed in duplicate or triplicate. For VIDAS-SET2™ extraction was done from another 10 g of each sample, following manufacturer’s instructions.

Moreover, a sensitive detection method for SE enables the investigation of the mechanisms and factors influencing the onset of SE production. To understand the impact of different incubation temperatures on SEB production, two SEB producing strains, S. aureus ATCC 14458 and SK64f TSB, were inoculated in TSB with inoculum level of ca. 2 log CFU ml⁻¹. A non enterotoxigenic S. aureus 171 served as a negative control. Cultures were incubated at different temperatures (10, 22, 37 and 42 °C) and analyzed in duplicate after 4, 8, 12 and 24 hours for SEB production with qPCR and with in-house ELISA.

Preliminary investigation of qPCR specificity comprised a limited experimental set-up with filter sterilized cultures of S. aureus strains producing SEA, SEC, SED, SEE and possibly SEI, SEJ, SEG and SEH (strains harbored these genes, but actual toxin expression was not confirmed due to the limitations of SET-RPLA and VIDASSET2™, which are not armoured with corresponding antibodies), as well as with non enterotoxigenic strain (producing none of SEA, SEB, SEC, SED and SEE), inoculated in TSB (ca. 6 log CFU ml⁻¹) and incubated 24 hours at 37 °C.

7.2.6 SEB and microbiological analysis of naturally contaminated foods

To test naturally contaminated food with qPCR, additional six different foods were obtained at the point of sale or directly from the producer at day 0 (strawberry-vanilla ice-cream, peach pie cream, chocolate-desert, RTE salmon based salad, RTE tuna based salad, RTE surimi salad, and cooked ham). Foods were distributed in two portions of 20 and 30 g each. 20 g portions was additionally divided into 2 portions of 8 and 10 g and 8 g portion was spiked with pure 2 ml SEB (1 ng g⁻¹) to obtain final concentration in food of 0.2 ng g⁻¹. Both portions of 10 g were extracted as described above and analyzed by qPCR and VIDAS-SET2™. Portion of 30 g served to determine microbiological load of foods, namely aerobic mesophilic count and presumptive S. aureus count, as described in 7.2.1
7.2.7 Statistical analyses

Statistical calculations of mean values, standard deviations and 95% confidence intervals were performed with Microsoft Excel 2003.

7.3 RESULTS

7.3.1 Development of an in-house ELISA and commercial assays

Double antibody sandwich format (format 4) using sheep polyclonal IgG as a capture antibody at concentration of 10 µg ml\(^{-1}\) and sheep polyclonal IgG-biotinylated as a detection antibody at concentration of 0.5 µg ml\(^{-1}\) was preferred over other formats and was used throughout the study. Although format 2 and 4 showed the same sensitivity, the choice to use format 4 was based on the polyclonal recognition of multiple epitopes, making it probably more tolerant to possible small changes in the nature of the toxin molecule that could be induced during food preparation and offering a more robust reactivity profile compared with monoclonal format 2.

Figure 7-2 shows average ELISA results as OD measurements at wavelength of 450 nm in function of SEB concentration obtained with pure SEB spiked in TSB. Although detection limit was about 0.6 ng ml\(^{-1}\), the actually established quantification limit was ca. 1.5 ng ml\(^{-1}\). The same sensitivity was obtained with milk spiked with SEB. SET-RPLA and VIDAS\(^{TM}\) SET2 detected SEB in standard solution, spiked TSB and spiked reconstituted milk powder (same sample as used for ELISA) until ca. 0.6 and 0.15 ng ml\(^{-1}\), respectively.

Using described antibodies in the ELISA format 4, no signal higher than the noise (background + 3 standard deviations) with strains producing SE other than SEB (filtrate of cultures incubated 24 hours at 37 °C) was observed.

7.3.2 Development of immuno quantitative PCR

In the real-time PCR, cycle threshold (Ct) correlates to the sensitivity of the PCR experiment. The higher the initial amount of enterotoxin, the more binding of the detection antibody and thereon linked DNA target, the sooner a significant increase in the fluorescence is observed. Detection of SEB with iqPCR started at about Ct 20 (Figure 7-3) allowing quantitative determination in the linear range of ca. Ct 23-30. Average measurements obtained in iqPCR with standard SEB spiked in TSB showed ca. 1000 times
Biological and immuno-molecular methods for detection of \textit{B. cereus} and \textit{S. aureus} enterotoxins

higher sensitivity (less than 10 \( \text{pg ml}^{-1} \)) in comparison with ELISA (Figure 7-2). No difference was observed when SEB was in the first step spiked into TSB or into PBS with 1\% BSA (dilution buffer). Known concentrations of SEB spiked into reconstituted milk powder (starting concentration of 750 \( \text{ng ml}^{-1} \)) were detectable with iqPCR in \( 10^5 \) times diluted sample (ca. 7.5 \( \text{pg ml}^{-1} \)).

In iqPCR, all tested samples containing SE other than SEB had a \( \text{Ct} \) value above 30, being comparable to the negative control sample. Neither were positive results observed in the SET-RPLA test with anti-SEB antibodies. This limited study indicated no cross reactivity of used SEB antibodies with other SE or formed metabolites.

![Figure 7-2: Standard curves of ELISA format 4 (■), presented as OD values at 450 nm, and iqPCR (▲), presented as \( \text{Ct} \) values, in detection of SEB spiked in TSB, showing detection range and sensitivity of two detection techniques. Arrow dashed lines indicate start of the dynamic (quantitative) range. Sample with no SEB added (Δ) was used as a control in iqPCR.](image-url)
7.3.3 Determination of SEB production in food and laboratory media

SEB production was further assessed in five different foods with pH and $a_w$ values higher than 5.8 and 0.98, respectively. Inoculated (ca. 6 log CFU $S. aureus$ g$^{-1}$) 50 g portions of sterilized paella, cooked ham, tuna fish, caramel-coffee creamer mix (1:4) and reconstituted milk powder (incubated at 37 °C for 24 hours) were analyzed for SEB production by iqPCR, VIDAS-SET2™ and SET-RPLA, and enumerated to determine $S. aureus$ growth.

In paella and cooked ham inoculated $S. aureus$ ATCC 14458 grew to ca. 9 log CFU g$^{-1}$, facilitating SEB production, as detected with iqPCR (Figure 7-4). Quantitative interpretation of data presented in Figure 7-4 (by comparison of Ct values for different dilutions of food samples with Ct values of known SEB concentrations in TSB-run at the same time) showed that the amount of SEB produced in paella was 15 ng g$^{-1}$ and in cooked ham 6 ng g$^{-1}$. Although in samples of caramel-coffee creamer mix and tuna fish no growth of $S. aureus$ occurred, presumably a very small SEB amounts were detected. These amounts were below quantification limits to quantify them precisely (Ct value for non-diluted sample of ca. 30 and about 1 Ct value lower than the control without SEB) and their interpretation could indicate possible transfer of SEB with inoculum. When tuna fish and caramel-coffee creamer mix were spiked with pure SEB (750 ng ml$^{-1}$) iqPCR showed positive reaction until dilution of 48 pg ml$^{-1}$, indicating that PCR reactions were not
Biological and immuno-molecular methods for detection of B. cereus and S. aureus enterotoxins

Inhibited by matrix and components of these two foods. Also VIDAS™ SET2 in inoculated tuna fish and caramel-coffee creamer mix where no S. aureus growth was observed, showed weak but positive reaction. The same samples remained negative when tested with an in-house ELISA. Negative controls (food inoculated with S. aureus 171) and blank controls (not inoculated foods) remained negative at all times. Production of SEB at 22 °C and 42 °C in comparison to the optimal growth temperature of 37 °C was further tested in milk (inoculated with S. aureus ATCC 14458, ca. 6 log CFU ml⁻¹). At all three temperatures S. aureus counts of ca. 7 log CFU ml⁻¹ were reached, resulting in the SEB production of ca. 75 ng ml⁻¹ (Figure 7-5). With maximal difference between Ct values for the same dilution being 0.6 and growth difference being less than 0.5 log CFU ml⁻¹ at 37 °C in comparison to 22 and 42 °C no significant statistical difference (95% confidence interval) in SEB production and growth at three temperatures tested was noted.

Two S. aureus SEB producing strains (ATCC 14458 and SK64f) were inoculated into TSB (ca. 2 log CFU ml⁻¹) and incubated at 10, 22, 37 and 42 °C. Figure 7-6 shows results of enumerations and analyses of SEB production. Samples that showed negative results in ELISA were retested with more sensitive iqPCR. Results showed that none of the two tested strains produced SEB at 10 °C (also the number of cells for both strains remained on the level of initial inoculum, being ca. 2 log CFU ml⁻¹). At 22, 37 and 42 °C SEB production differed during incubation time and among two tested strains. Growth was at each incubation temperature similar for both strains. SEB production after 4 hours (the first half of exponential phase) with ELISA was detected only for strain ATCC 14458 at 42 °C. Application of iqPCR revealed SEB production of less than 1.5 ng ml⁻¹ (around detection limit of ELISA) in other samples of 4 hours. For strain ATCC 14458 no SEB was found with ELISA in the samples incubated at 22 °C throughout the whole incubation period and only in the SK64f inoculated sample of 24 hours was SEB detected with ELISA. All samples of 22 °C were found positive with iqPCR. Results showed that strain ATCC 14458 produced final SEB amounts already after 8 hours which did not further increase during the incubation at 22 °C. Samples taken after 8, 12 and 24 hours at 37 and 42 °C were found positive with ELISA and amounts detected were in general higher for S. aureus ATCC 14458 than for S. aureus SK64f. Amounts detected in TSB were higher at 42 °C in comparison to 37 °C for both strains.
Figure 7-4: A: iqPCR results showing SEB production in paella (♦), cooked ham (x), tuna fish (△) and caramel-coffee creamer mix (○) presented as Ct values in function of sample dilution. Not inoculated food samples served as a control without SEB. B: Standard curve of pure SEB spiked in TSB (●) run in the same experiment served for SEB quantification and PBS-BSA 1% without SEB (○) served as a control.
Figure 7-5: A -iqPCR results showing SEB production in milk at 22 (♦), 37 (■) and 42 °C (▲) presented as Ct values in function of sample dilution. Not inoculated milk sample (x) served as control. B: Standard curve obtained with SEB spiked in the milk (●) served for sample quantification and the control milk without spiked SEB served as a control (○).
Figure 7-6: Influence of incubation time and temperature on growth (symbols)-SEB production (bars) of *S. aureus* ATCC 14458 (▲ - gray bars) and *S. aureus* SK64f (x - white bars). Error bars denote standard deviation (n=2) of average measurements of *S. aureus* counts and SEB concentration. SEB production was determined with ELISA or with iqPCR for samples where ELISA gave negative results.
7.3.4 SEB and microbiological analysis of naturally contaminated foods

Results of iqPCR analysis of naturally contaminated foods showed no SEB presence in any of the tested foods. Non-spiked foods had a Ct value of ca. 30, while all spiked samples (0.2 ng g⁻¹) had a Ct values between 27 and 28, as shown in Figure 7-7 for peach pie cream and RTE salmon salad. Ct value of 28 corresponds to SEB concentration of ca. 0.12 ng g⁻¹ (see above Figure 7-2), indicating possibly minor loss of spiked SEB during extraction. Extracts of foods not spiked with SEB showed negative reaction also in VIDAS-SET2™ assay.

Microbial analysis showed no *S. aureus* presence in any of the tested foods with overall less than 10³ CFU g⁻¹ of total aerobic mesophilic count.

7.4 DISCUSSION

Specificity and sensitivity of immunological tests is mainly based on the properties of antibodies. A sensitivity of monoclonal antibodies is in certain degree hampered by their high specificity, due to which only few molecules of antibody bind to the target antigen (Zola and Vanhoutte, 1987). The described iqPCR utilizes epitopes on the SEB molecule that are in solution exposed to both capture and detection antibody. While most monoclonal antibodies are seen as an improvement in comparison to polyclonal antibodies (Bonwick and Smith, 2004), in this research satisfactory sensitivity and specificity were obtained by use of polyclonal antibodies, both for SEB capture and detection. Sensitivity obtained with in-house ELISA (ca. 1.5 ng ml⁻¹) although able to detect total amounts needed for intoxication of ca. 200 ng (Evenson *et al.*, 1988) was insufficiently sensitive to detect the lowest concentration of SEB reported (Bergdoll, 1989; Vernozy-Rozand *et al.*, 2004) as intoxicative (0.5 ng g⁻¹ or ml⁻¹). These concentrations are nevertheless detectable with VIDAS-SET2™ having a detection limit below 0.5 ng ml⁻¹ (Vernozy-Rozand *et al.*, 2004) or immunomagnetic flow cytometric detection of SEB having a detection limit of 0.25 ng ml⁻¹ (Miyamoto *et al.*, 2003). However sensitivity of all methods described is flanking with minimum intoxication dose. This could perhaps indicate that the actual intoxication dose is lower, but that so far was not detectable.
Figure 7-7: IqPCR amplification plots of A: naturally contaminated peach pie cream not spiked with SEB; B: naturally contaminated peach pie cream spiked with SEB; C: naturally contaminated RTE salmon salad not spiked with SEB; D: naturally contaminated RTE salmon salad spiked with SEB
A large scale outbreak in Japan involving more than 13000 notified cases indicated that in different final products involved 0.05-1.6 ng ml\(^{-1}\) of enterotoxin (SEA) was present, corresponding to ca. 4 ng g\(^{-1}\) of milk powder used to produce the final products (Anonymous, 2001; Asao et al., 2003). *S. aureus* cells were however not isolated from any of the samples in the mentioned outbreak. This suggests firstly a need for a reconsideration of conventional analyses which mainly focus on detection of *S. aureus*, and secondly the need for establishment of a more sensitive method for enterotoxin detection.

The real-time immuno quantitative PCR measurements conducted in the present study were highly sensitive (less than 10 pg ml\(^{-1}\)) and comprised a wide dynamic range providing a quantification range from ca. 10 pg ml\(^{-1}\) to 30000 pg ml\(^{-1}\), determined by the Ct. The dynamic range stretched from Ct values of ca 23 to 30, not being able to commence at lower Ct value as the ROX signal of the real-time cyclers was calibrated to start from Ct 7 to 15 (values near 13 in the current setup). A Ct value of more than 30 for the control sample containing no SEB was expected due to the minor cross reactivity of the antibodies, allowing fixation of some DNA in the well and causing a consequent background signal.

The indicated high sensitivity and wide quantification range enabled study of SEB production in correlation with the growth status, extrinsic and intrinsic factors such as type of food, storage temperature and *S. aureus* strain involved. Application of iqPCR for detection of SEB in early growth phases at different temperatures revealed the onset of SEB production already after 4 hours of incubation at 22, 37 and 42 °C, being the first half of the exponential phase. Some other findings suggest that SEB production is related to the late exponential phase and transition into the stationary phase (Zhang and Stewart, 2000; Klotz et al., 2003; Bennett, 2005), which can be the case for some other members of SE family. The significant increase in SEB production was noticed when *S. aureus* counts reached level of 5 log CFU ml\(^{-1}\). These counts were at 37 and 42 °C crossed already after 4 hours of incubation, while at 22 °C time required was longer than 8 hours. Temperature abuse of 24 hours at 10 °C did not result in measurable SEB production, while at room temperature (22 °C) already 4 hours was enough to produce amounts reported in a recent outbreak (Anonymous, 2001; Asao et al., 2003), corresponding to *S. aureus* counts of
ca. 3 log CFU ml\(^{-1}\). This is specially the case when the background flora is absent or hampered to compete with \textit{S. aureus}, as shown in the present study. A temperature of 42 °C favored SEB production in comparison to 22 and 37 °C.

Analysis of naturally contaminated foods with iqPCR showed that none of the foods tested inhibited PCR reaction or impaired antigen-antibody recognition. This limited robustness test indicates that iqPCR was well performing in different food matrices and with background flora present.

### 7.5 CONCLUSIONS

IqPCR showed the potential for detection and quantification of low amounts of SEB in both laboratory cultures and food samples. To our knowledge this is currently the most sensitive technique for detection of \textit{S. aureus} enterotoxin B reported. Further research will have to prove the relevancy of this low detection limit with regard to the SEB amounts found in the food or other samples and their relation to the exact intoxication dose and dynamics in which SEB is produced under the impact of different factors. Similar applications for other members of SE family are foreseen.
GENERAL DISCUSSION,
CONCLUSIONS AND PERSPECTIVES
8 GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

Bacterial foodborne diseases (infections, toxin-mediated infections and intoxications) remain an ongoing, but underestimated public health problem. Despite the fact that the most toxin-involving foodborne diseases have generally mild acute symptoms of gastro-enteritis (emesis and diarrhea), in certain cases the more severe consequences, including fatalities can occur. A case of *B. cereus* cereulide intoxication with deadly outcome was recently reported in Belgium (Dierick *et al.*, 2005). Moreover the growing demand for convenience foods and foods which are as close as possible to the fresh state, drives the orientation of the food industry towards Ready-To-Eat, i.e. RTE, foods (in particular minimally processed foods with extended durability under refrigeration conditions) creating a niche for *B. cereus* (aerobic sporeformer) intoxications, when the process and the storage conditions are insufficiently controlled. The diversity of RTE foods and combination of different ingredients and technologies make traditional safety concepts not always applicable. The main pillars in the food safety control of these foods are appropriate refrigerated storage and limited shelf life. The application and respect of this control measures is on the producer, the distribution/retail, as well as on the final consumer. Additionally, all these actors in the food chain must assure that all hygienic principles are set in motion.

*B. cereus* prevalence and its characteristics in RTE food

Chapter 2 of this Ph.D. demonstrated the occurrence and prevalence of *B. cereus* in RTE, REPFED, pasteurized and vacuum packed potato puree. It was seen that the contamination can originate from different ingredients. However, in the production line of this particular production unit, the major problem for end-product contamination was posed by the continuous cross(post)-contamination from the dosage pump, situated after the pasteurization step. This was shown by RAPD fingerprinting, indicating that GMP set to control proper cleaning and disinfection failed to assure control at this process point. The cleaning and disinfection had to be thoroughly performed in order to eliminate the contamination source. *B. cereus* contamination of the final product before cleaning and disinfection was in the range of ca. 2.3-4.0 log CFU g⁻¹, with spore count as high as ca. 4 log CFU g⁻¹ in some of the
productions. The development of *B. cereus* counts during the predicted shelf-life showed that even at the refrigerated storage vegetative cells of *B. cereus* at the moment of consumption can be higher than 5 log CFU g⁻¹, which is considered a safety limit for RTE foods at the moment of consumption (Anonymous, 1994). Yet this safety limit may underestimate the potential for foodborne illness related to *B. cereus* (Pielaat et al., 2006). The presence of psychrotrophic isolates has been confirmed through further investigation, showing that 33 out of 38 isolates from productions before cleaning and disinfection, were able to grow at 7 °C and all of them were able to grow at 10 °C. After the cleaning and disinfection were thoroughly performed, not only a decrease in the level of post-contamination occurred, but also the change in the character of *B. cereus* contamination. Less than 50% of the isolates from later productions was able to grow at 7 °C. None of the tested isolates, from any of the productions, was able to grow at 4 °C. However, latest results of Carlin et al. (2006) showed growth of food and environment *B. cereus* isolates at 4 °C. These results should be put into perspective with that fact that *B. cereus* is known to be a poor competitor and especially psychrotrophic strains would be in a non-optimal environment under the conditions of the human digestive tract, taking into account body temperature, anaerobic conditions, low pH in duodenum part of the small intestine and well established microflora in the intestines. Spores and vegetative cells from psychrotrophic strains were shown to be less prone to develop in the duodenal conditions than those from mesophilic strains. This was confirmed by modeling the effect of gastro-intestinal tract conditions on *B. cereus* (Pielaat et al., 2006). In addition, it gives the impression that psychrotrophic strains even when harboring genes encoding for diarrheal enterotoxins, do not have the ability to express these toxins in dose-response relevant amounts, as otherwise more outbreaks would occur. As for *B. cereus* cereulide producing strains it seems well established that they do not grow and do not produce toxin at temperatures below 10 °C. Therefore, it appears that under refrigerated conditions no problem of *B. cereus* intoxication or toxin-mediated infection would occur, indicating a necessity to evaluate the duration of the refrigerated shelf-life with regards to *B. cereus* (and *Bacillus* spp.) more for the sake of food quality than safety. The actual temperature required to control growth of *B. cereus* is 4 °C or lower, with a limited shelf life, to prevent outgrowth of psychrotrophic strains.
The presence of vegetative cells and spores of enterotoxigenic *B. cereus* at levels reported above, can, according to the mathematical and hypothetical model of Pielaat *et al.* (2006) (see Chapter 1), result in diarrheal toxin-mediated infection. This model however was not validated in gastrointestinal conditions and must be therefore seen only as an indication.

Although, none of the tested potato puree isolates produced cereulide, 30.4% of isolates produced HBL and/or NHE. This was despite the fact that puree as starch rich product would be rather associated with cereulide producing strains. The high potential of potato puree to support growth of cereulide producing strains and cereulide production at temperature abuse was shown in Chapters 4 and 5. The temperature abuse (10 °C and higher) will favor mesophilic strains which pose more obvious danger to the food safety.

**Effect of antimicrobial compounds and of competition**

REPFED can apart from *B. cereus* harbor other psychrotrophic *Bacillus* spp., such as spoilage microorganism *B. circulans*. In Chapter 3 was shown that both aspects of microbial quality (safety and spoilage) are closely related and that a small variation in one of the parameters in the “combination technology”, on which the safety and preservation of these minimally processed foods relies, might cause a serious shift in the microbial population. Temperature abuse favored the growth of *B. cereus* raising a food safety issue, whereas the respect of the cold chain caused dominance of the psychrotrophic *B. circulans*. In the first case public health was endangered and in the second the product was hampered to meet the economically important extended shelf life. The overall effect of interaction between spoilage and pathogenic *Bacillus* spp. will depend upon the psychrotrophic character of both cultures.

An additional hurdle to the storage temperature is to be imposed in order to control the *Bacillus* spp. contamination level. In Chapter 3 efficiency of natural antimicrobial substances, carvacrol and nisin, was tested and found to be species and strain dependent. In comparison to *B. cereus*, *B. circulans* strains were shown to be more sensitive. During challenge testing in vacuum packed potato puree, combination of 10 µg g⁻¹ of nisin and 75 µg g⁻¹ carvacrol inhibited the growth of both *B. cereus* TZ415 and *B. circulans* 4.1 at 10 and 7 °C. Application of carvacrol alone at concentration of
75 µg g\(^{-1}\) had no antimicrobial effect. In laboratory media the same concentration of carvacrol had strain dependent antimicrobial effect. The application of carvacrol and other lipid soluble antimicrobial compounds may be hindered in food matrixes. Nisin on the other hand showed potential to be used as a preservative in RTE potato puree. However, the activity of nisin and carvacrol was influenced by the storage temperature. Whilst in separate usage lower temperature decreased the MIC values, a combination effect was more obvious at higher temperatures.

When the control measures fail to impose sufficient inhibitory effect on cereulide producing strains of *B. cereus* growth to high counts may result in cereulide production. The issue of toxin detection and toxin production gets more and more attention in the framework of food safety. Especially, in the case of cereulide, detection directly in the food samples is crucial.

**Cereulide detection**

In regard to the detection method, the computer aided boar semen analysis for cereulide detection was described in Chapter 4, and tested and applied through the Chapters 4, 5 and 6. This assay ultimately shows the toxicity of the sample towards boar sperm mitochondria and has potential to be used to detect cereulide in food. Using the rate of the decrease of semen progressive motility, measured by optimized software, a semi-quantitative estimation of the amount of cereulide in the sample was obtained. These data, as shown in Chapter 4, can be related to the standard curve of valinomycin, providing further quantitative values, with limit of quantification of ca. 20 ng ml\(^{-1}\). The actual limit of detection can be lowered to below 1 ng ml\(^{-1}\) by extending the exposure time, but taking into account that suggested intoxication dose is ca. 10000 ng kg\(^{-1}\) body weight (roughly 3000 ng g\(^{-1}\) food), lowering detection limit would only have its purpose for investigation of cereulide production and less for routine analyses. Using the described method, the production of cereulide in different laboratory media and various foods, and under various conditions, has been confirmed. Furthermore, no interference with food relevant chemical and bacterial contaminants was observed, as shown in Chapters 4, 5 and 6.
**B. cereus growth, toxin production and food matrix**

The growth conditions (temperature, atmosphere, composition of food and related growth factors-pH and $a_w$) play an important role and overall amounts of cereulide produced were shown to be also highly dependent on the strain involved. Results presented indicate that exclusion or limitation of oxygen to the levels below 1.6% may prevent cereulide production. Whether or not a degree of aeration of a liquid culture medium and liquid food has a positive impact on the cereulide production is unclear at the moment. Results obtained throughout this Ph.D. research indicate negative correlation between shaking during incubation and cereulide production. However, results reported elsewhere in the literature (as discussed in Chapter 4 and 5), suggest opposite. Again, this may be an indication of multifactorial regulation of cereulide production. Chapter 5 showed that static incubation at 28 °C for 48 hours allowed cereulide production of ca. 5 µg g$^{-1}$ in potato puree. Same experiments showed cereulide production of ca. 3 µg g$^{-1}$ in penne and 2 µg g$^{-1}$ in rice. This difference occurred despite the fact that *B. cereus* counts of more than 8 log CFU g$^{-1}$ were found in all three products. In the samples incubated under shaken conditions lower cereulide production or complete lack of the production was observed, while growth was in general as high as in statically incubated samples. In conclusion, *B. cereus* counts at which cereulide was detected were different in different foods and at different temperatures. At 12 °C counts had to be in all foods higher than 7 log CFU g$^{-1}$. At 22 °C required counts were food dependent, but were ca. 6 log CFU g$^{-1}$. The screening of potential of different foods to support growth of cereulide producing *B. cereus* and cereulide production in Chapter 6 clearly contributed to the understanding that *B. cereus* growth to ca. 8 log CFU g$^{-1}$ will not necessarily result in cereulide production.

Comparison of growth data obtained in Chapters 4, 5 and 6 with data obtained for cereulide production indicates that stationary phase commenced at counts higher than 8 log CFU g$^{-1}$ and that cereulide production can onset already in the earlier stages of exponential phase. The production of *S. aureus* enterotoxin B (SEB) also commenced earlier than the end of exponential phase, as shown in Chapter 7. Results from Chapter 7 revealed the onset of SEB production already after 4 hours of incubation at 22, 37 and 42 °C, being the first half of the exponential phase. These data indicate that
intoxicative strains of *B. cereus* and *S. aureus* can produce their toxins sooner than elsewhere reported data suggest. A method as sensitive as iqPCR should be employed to investigate the actual onset of cereulide production in order to identify the factors that can control growth and toxigenesis. Risk of *B. cereus* foodborne intoxication (= presence of toxin in the foods) is generally connected to the possibility of excessive growth of present *B. cereus* to high numbers (determined as a result of epidemiologic investigations and also seen here in Chapters 4-6). Similar is valid for *S. aureus*.

From the presented results it became obvious that *B. cereus* counts of 5 log CFU per gram of food (safety limit at the moment of consumption for RTE foods, (Anonymous, 1994)) will not always result in cereulide production, but whenever detectable cereulide amounts were produced, the level of *B. cereus* in this study was always higher than 5 log CFU per gram of food. Thus, preceding growth to high counts seems to be a necessary condition for cereulide production, but not the solely factor to influence it.

**Future**

As seen in Chapters 4-6, there was no simple and straightforward relationship between the growth phases and/or critical numbers of *B. cereus* present with cereulide production. Chemostat culture of *B. cereus* in steering fermenters would allow maintenance of constant number or growth phase of *B. cereus* with standard process control utilities (pH, oxygen, redox potential, temperature control and composition of the growth medium); these factors would be linked with cereulide production. Intentional and controlled variation of each individual factor alone or in combination should bring clarity on their interactive impact on the toxin production.

Insufficient knowledge is available on the regulation of cereulide production at cellular level (biochemical and molecular) and to what extent this is regulated by the environmental factors, the competitive flora and for which part strain variation accounts. The next step in the continuation of the research in this field should fill in the gaps in understanding of the factors which regulate cereulide production on biochemical and molecular level.

Although major improvements for cereulide detection were suggested here, restrictions of the available detection methods, remain a hampering factor in
quantitative and absolutely specific detection of cereulide. Bioassays indicate an active toxic biological activity (and intrinsically provide highly valuable information on a potential danger for the public health), but form “only” an indirect manner to trace bacterial toxin and are prone to the interferences with other components with similar toxic impact. An alternative is detection of cereulide with analytical technique such as HPLC-MS, as done in Chapter 6. The bioassays and HPLC-MS are in fact methods that can be used in combination, where screening can be done with bioassays and confirmation can be performed with HPLC-MS. Lack of a standard solution of cereulide hampers the absolute quantification of toxin with any of the methods.

An immunological method (ELISA) has been so far not available, such as the systems that are commercially available for \textit{B. cereus} enterotoxins, because of the low antigenic potential of cereulide itself (Melling and Capel, 1978; Ehling-Schulz \textit{et al.}, 2005b). No antibodies against cereulide have been so far reported. To respond to the above mentioned obstacles further research should be formulated to target development of additional bioassays and ultimately immunological detection method for cereulide. Development and validation of ELISA and immuno-quantitative PCR for cereulide detection, on the basis of protocols and results demonstrated for detection of \textit{S. aureus} enterotoxin B (Chapter 7), would be currently seen as a great improvement. IqPCR for SEB detection was highly sensitive (detection limit of less than 10 pg SEB ml$^{-1}$) and comprised a wide dynamic range providing a quantification range from ca. 10 pg ml$^{-1}$ to 30000 pg ml$^{-1}$. IqPCR showed the potential for detection and quantification of low amounts of SEB in both laboratory cultures and food samples. Therefore, its possible application in the investigation of cereulide production is assumed to be of great potential value. The possibility to use the synthesis protocol (Isobe \textit{et al.}, 1995) to synthesize cereulide in several forms (open and closed ring structure, multimolecular cereulide form, coupled or not with a carrier), which might induce an antigenic activity, would need to be investigated. Being a lacton type molecule, the possibility exists that under the conditions of high pH and high temperature cereulide ring would hydrolyze and open, allowing formation of potential antigenic sites or at least functional groups that can be used to bind carrier molecule. The structure of formed cereulide is to be verified by means of NMR. Different forms of cereulide should be tested for possibility to induce development of antibodies that could be used to create an immunological method to
detect cereulide, being specific, sensitive and robust. This is necessary in order to generate precise epidemiological data within the framework of the implementation of a quantitative risk analysis for cereulide and B. cereus. Similarly, for S. aureus enterotoxins other than SEB iqPCR could contribute to a better understanding of production mechanisms and dynamics, especially for “newly” described serotypes. Their role in food intoxications, types of food in which they occur, prevalence and frequency of occurrence requires further investigation.

A possibility to develop a bioassay on the basis of inhibition of luminescence of genetically modified marine bacteria Vibrio fisheri and V. harveii could provide a new assay for toxicity of cereulide containing sample. Such assay, if shown to be sufficiently sensitive, specific and robust could complement toxicological information of cereulide and allow its detection and quantification (using computer based luminometer). Other food associated biological toxins (marine toxins, fytotoxins, etc.) and possible toxic (chemical) components with foods (e.g. associated dioxins, acrylamide etc.) will have to be examined (as done in Chapter 6 for CASA) to estimate the occurrence of false positive results. However, “false positive” results may in fact indicate an overall sample toxicity, caused by another toxic compound (chemicals, heavy metals, other biological toxins…). It is therefore worthwhile considering a need not only for specific assays, but also for those that could serve the purpose of integrated tools for determination of the toxic potential in a given sample. Such qualitative information would be the one most relevant for public health protection.

In addition, quorum sensing is one of the regulation scenarios which could be interesting to follow in the future, the importance of which has been recently demonstrated for expression of virulence genes of B. cereus (Slamti and Lereclus, 2005), C. perfringens (Ohtani et al., 2002) and S. aureus (Balaban et al., 2005) and other Gram positive bacteria (Sturme et al., 2002). The activation of the expression of extracellular virulence factors in B. cereus and resulting cereulide production should be investigated in relation to different intrinsic, extrinsic and implicit factors. This could clarify perhaps conditional relationship between counts of B. cereus and cereulide production.
With regard to the genetic background of cereulide production, it was recently shown that plasmid (pCERE01) related sequence is invariably present in cereulide producing strains. Loss of the plasmid resulted also in the loss of cereulide production. The raising question is whether a possibility of a lateral plasmid transfer exists, which could potentially result in unexpected cereulide production in other *Bacillus* spp. It was already suggested that species outside *B. cereus* group, e.g. *B. subtilis*, *B. mojavensis* and *B. pumilus*, can produce emetic toxin (From et al., 2005). Although screening for genes involved in cereulide production will provide very valuable information, such as the potential of in-house flora to produce cereulide that can be brought to motion when specific conditions are met, it is essentially important to be able to detect cereulide in food. Due to an important inter-strain variation in cereulide production, detection of genes would not be sufficient for quantitative risk assessment.

One of the issues that should be further addressed is the reported negative correlation between HBL and cereulide production (Ehling-Schulz et al., 2005a; Carlin et al., 2006), whereas no such a relation is reported between NHE and cereulide production. Distribution of genetic cereulide determinants in diarrheal enterotoxin isolates should be investigated. In order to understand the relationship between taxonomy and toxin production, pCERE01 plasmid reported by Hoton et al. (2005) could be perhaps inserted into HBL and NHE producing strains in attempt to induce cereulide production. Plasmid transfer can give an answer to the possible growth and cereulide production at lower temperatures (whereas HBL producers can be psychrotrophic, for cereulide producing strains was this up to this day not obvious). Then, mechanisms of the expression of the toxin genes would be investigated to explain the difference between high and low toxin producing strains taking into account possible synergies between toxins and their polymorphism within *B. cereus*.

The results of future research must lead to a better understanding of factors which are determinative for toxin production and will permit a better hazard characterization. Monitoring of their quantitative effect on cereulide production should eventually result in the evaluation of cereulide related risks to the safety of various types of food and food formulations. The risk assessment is to be brought into the connection with food processing parameters and may perhaps result in definition of the appropriate microbiological criteria for *B. cereus*. Currently no such criteria are given in “H1”
(EU, 2004) and therewith related regulations (EU, 2005). While for most of the experts concern about *B. cereus* in food is relevant to the actual state of art, the wide public having probably fairly accurate idea of the risks associated with the well-known hazards (e.g. *Salmonella* and *E. coli*) may still considerably underestimate the risk associated with *B. cereus*.
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Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of B. cereus and S. aureus enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


References


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


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References 214
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


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Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


Biological and immuno-molecular methods for detection of \textit{B. cereus} and \textit{S. aureus} enterotoxins


Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins


INDEX OF TERMS

OCCURING IN CHAPTERS 1 - 7
INDEX

A
Acrylamide 136, 139, 141, 145, 151,
Activity, enzymatic 9, 14, 18
Aeration 100, 119, 123, 126, 136
Aerobic mesophilic 72, 167
Aflatoxins 30, 153
Animals 19, 21, 27, 141, 153
Anthracis 15, 33, 34
Antibodies 24, 25, 46, 155, 162, 164,
Antigen 24, 25, 46, 162, 165
Antimicrobial
activity 84, 85, 87
effect 87, 88, 91, 97, 100
substances 78, 83, 86, 87, 89, 90,
98-100
Assays 21, 24, 25, 104, 136, 139, 159,
immunological 20, 24, 26, 155
Aureus 8, 12, 16, 26, 27, 37, 60, 141,
142, 150, 152, 159, 160, 162, 166,
167, 170, 171
enterotoxins SEA 144
growth 162, 170, 171
strains 141, 143, 144, 161, 166, 167
a_w 35, 60, 69, 76, 78, 89, 126, 129

B
Bacillus
Cereus 22, 31, 32, 65-72, 74-79, 83,
84, 103, 123, 124, 140, 143
group 33
spores 66
strains 107
Circulans strains 83
spp 3, 34, 36, 50, 53, 56, 60, 61, 89,
90, 98, 99
Background flora 123, 126, 127, 129,
135, 136, 166
Bacterial toxins 3-16, 18-22, 24-28,
31-37, 40-48, 56-61, 140
BCA 86, 88, 89, 95, 127, 148
Bcet-Rpla 46, 49-51, 57, 70, 77, 144
Bioassay 20, 21, 53, 70, 103, 105,
108, 110, 118, 123, 136, 147, 148,
150, 153
Biological activity 15, 21, 41, 53, 125,
160
Biomass 108, 114-116, 118, 144, 146,
150, 152
Biotoxins, marine 28
Boar semen 103-105, 110, 125, 136,
140, 144, 150, 153
bioassay 134, 136, 140, 144, 146,
151, 154
motility 103, 108, 110, 117, 119,
130-132, 139-155
analysis, aided 139, 142, 151
assay 117, 127
inhibition 101, 130, 140, 151
dynamics 101

C
Carvacrol 79, 81, 83, 85, 87-91, 95-
98, 100
CASA 103, 104, 119, 125, 127, 139,
140, 144-146, 150-154
Cereulide
analyses 108, 110, 147
concentration 108, 113, 114, 116,
117, 128, 135, 136, 142, 150
detection 41, 103-119, 125, 130,
139-155
producers 107
production 43, 67, 70, 74, 100, 109,
114, 118, 119, 123-127, 130, 131,
134-136, 139-141, 146, 151, 154,
155
Cereus
cells 35, 40, 47, 48, 125, 152
colonies 43, 44, 69, 86, 127
contamination 65, 67, 70, 72, 141
emetic toxin 20, 57, 123-136
group 15, 33, 34
growth 60, 77, 78, 92, 97, 109, 125,
126, 139, 141, 146, 154
potato puree 76
spores 36, 40, 47, 60, 72, 78
strains 59, 65, 66, 70, 72, 74, 76, 77,
83, 84, 89, 90, 92, 95, 96, 98, 99,
107, 109, 110, 123, 125, 126
CFU 38, 40, 47, 48, 50-56, 69-74, 76-
78, 87-90, 92-99, 114, 125-127,
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

Circulans 77, 79, 83, 85-90, 92, 95-99
growth 92
strains 83, 86, 90, 97, 98
Co-cultures 88, 89, 92, 98
Colonization 4, 5
Competition 79, 81, 83, 93, 94
Components 3, 9, 10, 18, 24, 37, 39, 42, 46, 49-51, 53-55, 84, 85, 87, 171
active 9
Computer 74, 100, 103-105, 139, 142, 151
aided analyses of boar semen
motility 103-117, 119, 139-155
Concentrations 20, 58, 85, 87, 88, 90, 91, 95, 97, 98, 105, 116, 117, 139, 140, 144, 151, 153, 154, 164, 165, 168, 169
of carvacrol 85, 98
lowest 87, 144, 145
of valinomycin 111, 113, 114, 117, 128
Consumers 41, 55, 58, 59, 61
Consumption 3, 40, 57-59, 61, 67, 72, 160
ultimate date of 67, 72, 73
Contamination 47, 49, 65, 72, 74, 78, 99
Control 12, 46, 57, 58, 60, 61, 68, 83-92, 95, 97-100, 105, 170
negative 114, 115, 125, 147, 151, 152, 161, 167
positive 150, 151
Cooked ham 166, 167, 170
CPE 22, 26, 38, 39
Ct 11, 145, 168-173
Cultures 47, 69, 83, 86, 88, 92, 97, 99, 109, 112, 114, 123, 125, 142, 167, 168
mixed 86, 88
Curve, standard 105, 108, 111, 113, 114, 116, 117, 128, 142, 147, 151, 152, 165, 173

D
Dairy products 49-53, 55-57, 84
Detect 12, 46, 119, 166
Detection antibody 159, 160, 162, 164, 165, 168
biotinylated 163, 164
Diarrhea 3, 19, 38, 40, 50-53
Diarrheal enterotoxins 22, 37, 40, 41, 46, 47
strains 59
syndromes 36, 37
Dilutions 46, 87-89, 108, 110, 116, 125, 126, 145, 147, 166, 170, 171
Dioxins 139, 141, 145, 151, 154
Disease 3, 4, 6, 16-19, 30, 34, 40, 57
foodborne 18, 19, 37, 160
DMSO 106, 108, 110, 117, 118, 128, 142, 145, 150, 151

E
ELISA 25, 26, 30, 159, 160, 162, 164, 168, 169, 171
in-house 159, 161, 166-168, 171
Emetic syndrome 36-38
toxin 22, 33, 51, 61, 103, 118, 123, 140
Endospores 36, 78
Enterotoxin productions 56, 57, 77
Enterotoxins 16, 18, 19, 22, 37, 38, 46, 65, 148, 157, 159, 160, 166, 168
Enumeration 43, 45, 69, 71, 86, 88, 89, 114, 133, 141, 142, 162, 171
Exotoxins 5, 6, 14, 24, 104, 124, 141, 153
Experiments 50, 78, 89, 92, 107, 126, 127, 135, 172
third 126
Exposure time 106, 110, 116, 142, 150-152
Extract 32, 114, 115
Extraction 107, 110, 114, 128, 144, 150, 152, 166

F
Food poisoning 3, 20, 31, 36, 41, 50, 85
safety 34, 35, 47, 48, 60, 65, 85, 99, 143
samples 51, 100, 104, 126, 127, 130, 135, 139, 146, 147, 149, 166, 170
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Foods</strong></td>
<td>3, 155, 162, 167</td>
</tr>
<tr>
<td>Contaminated</td>
<td>3, 155, 162, 167</td>
</tr>
<tr>
<td>Tested</td>
<td>51, 134, 135, 152, 154</td>
</tr>
</tbody>
</table>

**G**
- Growth: 5, 6, 12, 17, 24, 34, 60, 66, 67, 71, 72, 76, 77, 83-87, 95, 96, 99, 100, 129, 130, 133, 134, 170, 171
- Bacterial characteristics: 3, 5
- Curves: 88, 92
- Inhibition: 90, 92
- Rate: 92
- Support: 59, 61

**H**
- HACCP: 58, 59
- Hbl: 13, 37, 46, 48, 56, 70, 74, 136, 139, 140, 143, 151
- HPLC-MS: 53, 108, 118, 125, 128, 133, 134, 148, 149

**I**
- Immunoassays: 25
- Incubation: 69, 70, 86, 90, 92, 109, 110, 114, 118, 123, 127, 129, 131, 132, 134, 135, 141, 146, 148
- Conditions: 125, 126
- Static: 114, 123, 125, 134
- Temperatures: 83, 90, 114, 118, 125, 129, 130, 167, 171
- Infection, toxin-mediated: 19, 20, 36, 40
- Ingestion: 3, 19, 20, 38, 40, 124
- Ingredients: 61, 68, 71, 72, 76, 78, 84, 89
- Inhibition: 39, 60, 77, 78, 85, 86, 97-99, 152
- Inhibitory effect: 70, 90, 92, 100, 117, 151
- Inoculation: 89, 125, 126, 131, 146-148, 154
- Interference: 136, 141, 145, 153, 154
- Intestines: 18, 38, 40, 41
- Intoxications: 19, 20, 27, 28, 31, 36, 37, 41, 66, 136, 140
- IQPCR: 155, 159-162, 164-171

**K**
- Kits: 46, 47, 57, 151, 161
### Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

**Character** 35, 65, 66, 69, 72, 77, 83, 99  
**strains** 57

### R
- **Ready-To-Eat potato puree** 65-72, 74-79, 83
- **Real-time iqPCR** 159-173
- **REPFEDs** 49, 50, 65, 66, 78, 84, 85, 99
- **Resistance** 35, 36, 38, 78, 90, 124
- **Resistant** 35, 36, 38, 40, 41, 76, 97
- **RPLA** 25, 26, 46
- **RTE foods** 57-61, 67

### S
- **Sauce** 38, 119, 125, 126, 129-132, 135
- **SE** 22, 37, 159-161, 166, 167, 169
- **SEA** 22, 26, 27, 38, 39, 143, 151, 160-163, 166, 167
- **SEB** 11, 22, 26, 27, 143, 144, 151, 155, 159-162, 164, 166-171  
  - **concentrations** 165, 168-170, 172-174  
  - **production** 155, 159, 166, 167, 170-174
- **SEC** 22, 26, 143, 144, 151, 160, 161, 166, 167
- **SED** 26, 143, 144, 151, 160, 161, 166, 167
- **Semen** 104, 106, 107, 110, 111, 117, 127, 130, 139, 146, 151
- **SET-RPLA** 151, 161, 166-168, 170
- **Shelf life** 65-67, 71, 72, 77, 79, 95
- **Specificity** 20, 24, 25, 140-142, 150, 160
- **Spermatozoa** 105, 110, 112, 115
- **Spices** 30, 34, 47, 48, 72, 89, 153
- **Spoilage** 3, 28, 78, 79, 83, 99
- **Spores** 19, 35, 36, 40, 47, 48, 56, 59-61, 69, 71, 72, 77, 78, 84, 97, 107, 140
- **Staphylococcus aureus** 3, 7, 11, 15, 16, 22, 143, 159  
  - **Enterotoxin** 136, 139, 159
- **Thuringiensis** 13, 33, 34
- **Toxicity** 8, 24, 25  
  - **mitochondrial** 41
  - **Toxin production** 9, 19, 20, 60, 61, 70, 114, 124
- **Vegetables** 38, 47, 49, 149
- **Vegetative cells** 36, 38, 40, 60, 71, 76-78, 84  
  - **ingested** 40
- **Vidas-Set2tm** 148, 149, 152, 161, 166, 167, 170
- **Virulence factors** 3, 12, 13, 20, 33, 37, 124  
  - **genes** 10, 12, 13
- **Vomiting** 3, 29, 38, 48, 50-53, 56

### Z
- **Zearalenone** 30, 136, 139, 151, 153

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SUMMARY – SAMENVATTING
SUMMARY

The literature study presented in Chapter 1 covers an introduction to the bacterial toxins and to the foodborne pathogen *B. cereus*. The first part of this chapter gives a relatively short overview of the current state of the art relevant to bacterial protein toxins. An attempt was made to provide insight into the nature and differences of several bacterial toxins and bring this into correlation with food safety. General considerations of microbial pathogenicity and virulence factors are given. Pathogenicity based on toxigenesis and toxins as virulence factors were studied more in detail, including chemical properties of different toxins, genetic determinants and genetic regulation of toxin expression and different modes of toxin action. Overview and examples of some of the detection methods is given together with some of disease/death causing toxin concentrations. At the end of part one, marine toxins and mycotoxins are briefly presented. The second part of this chapter introduces *Bacillus cereus* as a foodborne pathogen and gives insight into characteristics of this bacterium and its toxins, trying to explain its threat to public health. Extensive overview of outbreaks and foods related to *B. cereus* growth and toxin production is given. Short description of current protocols for detection and enumeration of *B. cereus*, as well as description of commercially available detection methods for diarrheal enterotoxins is given. Chapter 1 ends with control aspects with regards to *B. cereus* in Ready-To-Eat (RTE) food products.

Chapter 2 presents research performed in the frame of hazard analysis conducted to follow up the prevalence and the persistence of *B. cereus* in REPFED (Refrigerated Processed Foods of Extended Durability) cooked vacuum packed potato puree from one particular production unit. At the same time, the ability of *B. cereus* isolates to survive and grow in this food product was evaluated. Potato puree was analyzed for *B. cereus* contamination along the production line and during the product shelf-life. Isolated *B. cereus* strains were tested for their psychrotrophic character and the ability to produce enterotoxins. *B. cereus* contamination during four subsequent productions was in the range of 2.3-4.0 log CFU g⁻¹. Productions five and six were significantly less contaminated with *B. cereus* (≤1 log CFU g⁻¹). All *B. cereus* isolates from the first four productions were able to grow at 7 and 10 °C, whereas the majority of the isolates from productions five and six did not. No *B. cereus* isolates grew at 4 °C.
RAPD fingerprinting showed that the most of *B. cereus* contamination originated from one source. In total 30.4% of isolates expressed enterotoxic character.

Results described in Chapter 2 point out the necessity to prevent an “in house” colonization and contamination during food processing in order to accomplish the safety of REPFED throughout the shelf-life. It also indicates the most critical steps in the production line of Ready-To-Eat potato puree and impact of failures regarding the food safety. However, *Bacillus* spp. are not only a food safety issue, but also represent a food quality problem, by causing spoilage. Both problems are endorsed by the ability of some species (and strains) to grow at refrigeration temperatures. The interaction between present *Bacillus* spp. under different storage conditions may have an important effect on the fate of present flora, determining the organism that will prevail.

Chapter 3 outlines the investigations of growth and competition between foodborne pathogen *B. cereus* and food spoilage bacterium *B. circulans* in both BHI and Ready-To-Eat potato puree. In addition, antimicrobial potential of supplemented nisin and carvacrol was evaluated against inoculated *B. cereus* and *B. circulans* strains. The accomplished growth inhibition was observed for both *B. cereus* and *B. circulans*, where *B. circulans* strains were more sensitive. Minimal Inhibitory Concentration (MIC) values were decreased by lowering the incubation temperature in separate applications of nisin and carvacrol, while the effect of combined application of nisin and carvacrol appeared to be more obvious at higher temperatures. The overall effect of interactions between spoilage and pathogenic *Bacillus* spp. was dependent on the psychrotrophic character of both cultures. The complete inhibition of *B. circulans* in the co-culture experiments corresponded to *B. cereus* population density of approximately 6 log CFU ml\(^{-1}\). Microbial challenge testing in potato puree showed that, at both 7 and 10 °C, *B. cereus* TZ415 and *B. circulans* 4.1 could grow to high counts when no antimicrobial substances (nisin and carvacrol) were applied, while no visible spoilage occurred. A more pronounced antimicrobial activity of nisin and nisin-carvacrol combination was observed in potato puree compared to the BHI medium.

Due to ubiquitous nature of *B. cereus* and its sporeforming character its occurrence in the food chain is virtually unavoidable. When designed control measures (such as
specific application of antimicrobial compounds described in Chapter 3 and in general application of GMP and HACCP principles) fail to prevent its multiplication, it is possible that present B. cereus may elaborate one or more of its toxins. Of toxins of B. cereus, only cereulide is known to act as a sensu stricto intoxicative compound. Therefore, its detection directly in food is of utmost importance. In Chapter 4, qualitative and quantitative application of a computer assisted sperm analyzer (CASA) for detection and quantification of cereulide was described. The plot of the decrease in the percentage of boar semen progressive motility (PMOT%) in function of time and the visual inspection of curves provided a qualitative comparison between different samples (curve slope corresponds to the amount of cereulide in the sample). If the change of PMOT% over a time required for achieving PMOT% drop to 10% (ΔPMOT%/Δτ is plotted against the standard curve (obtained with known concentrations of valinomycin)), a semi-quantitative estimation of the amount of cereulide in the sample is obtained. An optimized CASA method was applied to determine the production of cereulide under different conditions in laboratory media. No cereulide was found in aerated samples and in samples incubated at 12 °C. The amount of cereulide produced depended on the agar medium used, type of B. cereus strain and the amount of oxygen present in the atmosphere.

Using method presented in Chapter 4, further investigation of cereulide production in foods was pursued. In Chapter 5, potato puree and pasta (penne type) were inoculated with cereulide producing B. cereus 5964a and B. cereus NS117. Static incubation at 28 °C proved these two foods to be better substrates for higher cereulide production (4080 ng g⁻¹ in puree and 3200 ng g⁻¹ in penne were produced by B. cereus 5964a during 48 h of incubation) in comparison to boiled rice (2000 ng g⁻¹). This difference occurred despite the fact that B. cereus counts of more than 10⁸ CFU g⁻¹ were found in all three products. Aeration of cultures had a negative impact on cereulide production causing even more than tenfold lower concentrations than in some statically incubated samples. Cereulide production remained undetectable in shaken milk, while reaching 1140 ng ml⁻¹ in statically incubated milk. At 12 and 22 °C presence of background flora was also a determinative factor. A total B. cereus count of more than 10⁶ CFU ml⁻¹ not necessarily resulted in uniform cereulide production, yield of cereulide being also dependent on the B. cereus strain involved. The present study confirms that number of factors play a crucial role in determination
of extent to which, if at all, cereulide will be produced. Among those, type of the food, temperature, pH and additional aeration (via incubation on an orbital shaker) played an important role. Important impact was also induced by cereulide producing strain involved.

In previous chapters it has been demonstrated that Computer Aided Semen Analysis (CASA) study of the boar semen motility is an appropriate assay for detection of cereulide (B. cereus emetic toxin). Application of the boar semen bioassay to detect cereulide directly in foods required at this point additional investigation of potential interference of food components, preservatives and other microbial and chemical food contaminants with the bioassay. Chapter 6 provides an evidence that none of included Staphylococcus aureus enterotoxins A, B, C and D nor B. cereus Hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE) and three mycotoxins (Sterigmatocystin, Fumonisin B1 and Patulin) exhibited an impact on semen progressive motility. Aflatoxin M1, M3 and Zearalenone impaired semen motility only at concentrations (0.004 mg ml\(^{-1}\), 0.1 mg ml\(^{-1}\) and 10 mg ml\(^{-1}\), respectively) much higher than those found in foods and those permitted by legislation, in comparison to cereulide which induces motility cease at concentrations lower than 20 ng ml\(^{-1}\). Ten commonly used preservatives, namely potassium sorbate, sodium benzoate, (DL) malic acid, citric acid, (L+) tartaric acid, acetic acid, (DL) lactic acid, (L+) ascorbic acid, sodium chloride and sucrose induced no cease in spermatozoa motility even at preservative concentrations higher than permitted by legislation. Dioxins, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and acrylamide had no acute effect on spermatozoa motility at concentrations of 500 and 10000 mg ml\(^{-1}\), respectively.

Experiments investigating robustness of computer aided boar semen motility analysis, tested with 14 different foods inoculated with cereulide producing B. cereus, showed distinct cereulide production in seven samples (although B. cereus growth to counts higher than 8 log CFU g\(^{-1}\) was noted in 11 samples), in amounts close to those reported in foodborne outbreaks. Test evaluation in 33 samples suspected to hold cereulide showed actual cereulide presence in ten samples and no interference of food matrix with the assay.
frequent acute foodborne intoxications and the intoxicative dose is very low (could be as low as 0.05 ng per g of food). Therefore, in Chapter 7 a real-time immuno quantitative PCR (iqPCR) method for detection of \textit{S. aureus} enterotoxin B (SEB) was developed and evaluated both in laboratory cultures and foods. The assay consists of immunocapture of SEB and real-time PCR amplification of the DNA probe linked to the detection antibody. IqPCR was compared to an in-house ELISA using the same couple of capture-detection antibodies and to commercial kits for detection of \textit{S. aureus} enterotoxins (SE). IqPCR measurements were ca. 1000 times more sensitive (10 pg ml\(^{-1}\)) than the in-house ELISA and had a dynamic range of 10 pg ml\(^{-1}\) to 30000 pg ml\(^{-1}\). IqPCR was able to detect SEB production in all different foods tested and anti-SEB antibodies showed no cross reactivity with food compounds and other \textit{S. aureus} enterotoxins. Application of iqPCR for detection of SEB in cultures of \textit{S. aureus} revealed onset of SEB production already after 4 hours of incubation at 22, 37 and 42 °C corresponding to the first half of the exponential growth phase. Total amounts of SEB produced were for both strains higher at 42 °C than at 37 °C. Final amounts of SEB produced were strain dependent.

At the end final considerations and conclusions of the preformed research together with new perspectives for the follow-up are discussed. Special attention is given to the detection techniques, such are new possible bioassays and hopeful progress in the immunological detection of cereulide, on the basis of results obtained with SEB (see Chapter 7). Also, future efforts are suggested in direction of clarification of molecular and biochemical mechanisms underlying cereulide production. The influence of cell status, in the sense of the growth phase and of the total cell count, as well as intercellular communication are to be coupled with the effect of intrinsic, extrinsic and explicit factors in order to understand differences in behavior of \textit{B. cereus} in different food matrices and under different environmental conditions. This knowledge is necessary to provide quantitative and qualitative data that should serve for \textit{B. cereus} risk analysis and possible establishment of the food safety criteria.
SAMENVATTING

De literatuurstudie weergegeven in hoofdstuk 1 omvat een introductie tot bacteriële toxines en tot de voedselgerelateerde pathogeen B. cereus. Het eerste deel van dit hoofdstuk geeft een relatief kort overzicht van de huidige stand van zaken met betrekking tot eiwittoxines van bacteriële oorsprong. De aard van en de verschillen tussen bacteriële toxines en hun relatie met voedselveiligheid wordt beschreven. Pathogeniciteit gebaseerd op toxigenese en de werking van toxines als virulentiefactoren worden in detail weergegeven. Ook de chemische eigenschappen van verschillende toxines, de genetische determinanten en de regulatie van toxine-expressie worden samengevat. Tevens worden kort de detectiemethoden van diverse toxines op een rijtje gezet en gelinkt aan de minimale concentraties voor symptomen. Op het einde van deel één worden marine toxines en mycotoxines kort aangeklaard.

Het tweede deel van dit hoofdstuk beschrijft B. cereus als een voedselgerelateerde pathogeen en geeft inzicht in de eigenschappen van deze bacterie en zijn toxines. Hierbij wordt er beoogd het risico van de pathogeen naar volksgezondheid toe te verklaren. Een uitgebreid overzicht van voedselvergiftigingen en levensmiddelen, gerelateerd met de groei en toxineproductie van B. cereus wordt weergegeven. Een korte beschrijving van de huidige protocols voor detectie en telling van B. cereus, alsook een beschrijving van commercieel beschikbare detectiemethoden voor diarree veroorzakende enterotoxines, wordt gegeven. Hoofdstuk 1 eindigt met een visie op de beheersmaatregelen voor B. cereus in kant-en-klare maaltijden.

Hoofdstuk 2 beschrijft het onderzoek dat uitgevoerd werd in het kader van de gevarenanalyse om de aanwezigheid en de persistentie van B. cereus in gekoelde, verwerkte levensmiddelen met verlengde houdbaarheid te beoordelen. Als voorbeeld werd hittebehandelde, vacuüm verpakte aardappelpuree van één bepaalde productie-eenheid gekozen. Tegelijkertijd werd nagegaan of B. cereus isolaten kunnen overleven en groeien in deze producten. De B. cereus contaminatie in de aardappelpuree werd nagegaan op verschillende plaatsen in de productielijn en tijdens de volledige houdbaarheidstermijn. Het psychrotroof karakter en het vermogen voor enterotoxineproductie van de geïsoleerde B. cereus stammen werden onderzocht. De B. cereus contaminatie tijdens de eerste vier opeenvolgende producties bedroeg 2.3-4.0 log KVE g⁻¹. Productiebatch vijf en zes waren significant minder gecontamineerd.
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

met *B. cereus* (≤1 log KVE g⁻¹). Alle *B. cereus* stammen van de eerste vier producties konden groeien bij 7 en 10 °C, terwijl dit niet zo was voor de meerderheid van de isolaten uit de productiebatchen vijf en zes. Geen enkel *B. cereus* isolaat kon groeien bij 4 °C. RAPD typering toonde aan dat het merendeel van de *B. cereus* contaminatie afkomstig was van één bron. In totaal vertoonde 30.4% van de isolaten enterotoxische eigenschappen.

De resultaten van **Hoofdstuk 2** tonen de noodzaak aan van het vermijden van ‘in huis’ kolonisatie en nabesmetting tijdens de voedselverwerking om op deze manier de veiligheid van gekoelde, verwerkte levensmiddelen met verlengde houdbaarheid te kunnen garanderen tijdens de volledige houdbaarheidstermijn. Het toont eveneens de meest kritische stappen aan in de productielijn voor kant-en-klare aardappelpuree en de impact van gebrek aan hygiëne op de voedselveiligheid. *Bacillus* spp. is echter niet alleen van belang in het kader van de voedselveiligheid, maar kan ook voor kwaliteitsproblemen zorgen door bederf te veroorzaken. Beide problemen worden veroorzaakt door enkele species (en stammen) die in staat zijn te groeien bij koeltemperaturen. De interactie tussen de aanwezige *Bacillus* spp. bij de verschillende bewaaromstandigheden kan een belangrijk effect hebben op de uiteindelijke dominantie van één micro-organsime uit de aanwezige flora. Dit werd in **hoofdstuk 3** onderzocht.

**Hoofdstuk 3** geeft het onderzoek weer van groei en competitie tussen de voedselpathogeen *B. cereus* en de bederver *B. circulans* in zowel BHI medium als in kant-en-klare aardappelpuree. Antimicrobiële effecten van nisine en carvacrol op geïnoculeerde *B. cereus* en *B. circulans* stammen werden eveneens bestudeerd. Hierbij werd zowel bij *B. cereus* als bij *B. circulans* groei-inhibitie waargenomen; *B. circulans* stammen bleken echter gevoeliger te zijn. Minimale Inhiberende Concentratie (MIC) waarden daalden bij aparte toediening van nisine en carvacrol bij dalende incubatietemperaturen. Simultane toediening van nisine en carvacrol had, in tegenstelling tot incubatie bij lage temperaturen, een meer uitgesproken effect naarmate de temperatuur hoger was. Het uiteindelijke effect van interacties tussen de bederver en de pathogene *Bacillus* spp. was afhankelijk van het psychrotroof karakter van beide organismen. Een volledige inhibitie van *B. circulans* in de co-cultuur experimenten trad op bij een *B. cereus* populatiedensiteit van 6 log CFU ml⁻¹.
Biological and immuno-molecular methods for detection of B. cereus and S. aureus enterotoxins


Ten gevolge van de wijdverspreide aanwezigheid van B. cereus en zijn mogelijkheid om sporen te vormen, is het vermijden van B. cereus in de voedselketen niet steeds haalbaar. Wanneer de voorziene beheersmaatregelen (zoals het doelmatig gebruik van antimicrobiële agentia, beschreven in hoofdstuk 3, en meer algemeen het naleven van GMP en HACCP) er niet in slagen om de groei van B. cereus te verhinderen, bestaat de mogelijkheid dat de aanwezige B. cereus één of meerdere van zijn toxines zal produceren. Cereulide is het enige door B. cereus geproduceerd toxine dat bekend staat als een intoxicant sensu stricto. Bijgevolg is de detectie van dit toxine in het levensmiddel zelf uitermate belangrijk. Het gebruik van een computergestuurd sperma-analysator of CASA als een kwalitatieve en kwantitatieve detectiemethode voor cereulide wordt beschreven in hoofdstuk 4. De visuele interpretatie van de curves bekomen door het verloop van de afname van het aandeel zich progressief voortbewegend varkenssperma of PMOT% uit te zetten in functie van de tijd maakt het kwalitatief vergelijken tussen verschillende monsters mogelijk. Wanneer de verandering van PMOT% over de tijd die nodig is om de PMOT% te doen afnemen tot 10% (ΔPMOT%/Δτ) wordt uitgezet ten opzichte van de standaardcurve (opgesteld aan de hand van gekende valinomycineconcentraties), wordt een semi-kwantitatieve inschatting van de hoeveelheid cereulide aanwezig in het monster bekomen.

Een geoptimaliseerde versie van de CASA-techniek werd gehanteerd om de productie van cereulide te bepalen onder verschillende omstandigheden in nutriëntmedium. Cereulide werd niet teruggevonden in beluchte monsters en in monsters bewaard bij 12 °C. Verder is de mate waarin cereulide gevormd wordt ook afhankelijk van het type voedingsmedium, de stam en de hoeveelheid zuurstof aanwezig in de omgeving.

De methode voorgesteld in hoofdstuk 4, werd gebruikt om cereulideproductie in levensmiddelen verder te onderzoeken. In hoofdstuk 5 werden aardappelpuree, pasta (penne type) en rijst geïnoculeerd met cereulideproducerende B. cereus 5964a en B.
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

*cereus* NS117. Statische incubatie bij 28°C toonde aan dat in aardappelpuree en pasta hogere concentraties cereulide gevormd werden (4080 ng g^{-1} in puree en 3200 ng g^{-1} in penne door *B. cereus* 5964a gedurende 48 h incubatie) in vergelijking met gekookte rijst (2000 ng g^{-1}). Dit verschil trad op ondanks het feit dat *B. cereus* was uitgegroeid tot meer dan 10^8 KVE g^{-1} in de drie levensmiddelen. Aëratie van de culturen had een negatief effect op de cereulidevorming en veroorzaakte meer dan 10 keer lagere concentraties dan in statisch geïncubeerde monsters. Cereulideproductie was niet detecteerbaar in geschudde melk, terwijl 1140 ng ml^{-1} gevormd werd in statisch geïncubeerde melk. Bij 12 en 22 °C speelde de aanwezigheid van achtergrondflora eveneens een rol bij de cereulidevorming. Een concentratie van *B. cereus* hoger dan 10^6 KVE ml^{-1} leidde niet noodzakelijk tot uniforme cereulide productie en bovendien was ook de hoeveelheid van geproduceerde cereulide afhankelijk van de *B. cereus* stam. Deze studie bevestigt dan ook dat een aantal factoren een cruciale rol spelen in het al dan niet vormen van cereulide en de mate waarin dit toxine gevormd wordt. Cruciale factoren zijn het type levensmiddel, temperatuur, pH en additionele aëratie (via schudcultuur). Ook de aard van de cereulide producerende stam heeft een belangrijke impact.

In voorgaande hoofdstukken werd aangetoond dat de microscopische analyse van de beweeglijkheid van varkens sperma met een aangepast softwareprogramma (CASA – Computer Aided Semen Analysis) een geschikte techniek is voor de detectie van cereulide (*Bacillus cereus* emetisch toxine). Toepassing van de bioassay met varkens sperma voor directe detectie van cereulide in levensmiddelen vereist bijkomend onderzoek naar de potentiële interferentie van levensmiddelcomponenten, bewaarmiddelen en andere microbiologische of chemische contaminanten. **Hoofdstuk 6** toont aan dat geen van de geteste *Staphylococcus aureus* enterotoxines A, B, C and D noch *Bacillus cereus* Hemolysine BL (HBL) en niet-hemolytisch enterotoxine (NHE) noch twee mycotoxines (sterigmatocystine en Fumonisine B₁) een impact hadden op de progressieve beweeglijkheid van varkens sperma. Aflatoxine M₁, M₃ en zearalenon waren in staat om de beweeglijkheid van varkens sperma te verzwakken, maar dit slechts bij concentraties (respectievelijk 0.004 mg ml^{-1}, 0.1 mg ml^{-1} en 10 mg ml^{-1}) die hoger zijn dan deze die in levensmiddelen voorkomen en toegelaten zijn door de wetgeving. Ter vergelijking was cereulide reeds in staat om de beweeglijkheid te stoppen bij concentraties lager dan 20 ng ml^{-1}. Tien veelvuldig
Biological and immuno-molecular methods for detection of *B. cereus* and *S. aureus* enterotoxins

gebruikte bewaarmiddelen namelijk kaliumsorbaat, kaliumbenzoaat, (DL) malonzuur, citroenzuur, (L+) tartarzuur, azijnzuur, (DL) melkzuur, (L+) ascorbinezuur, natriumchloride en sucrose, waren niet in staat om de beweeglijkheid van spermatozoa te stoppen, zelfs niet bij concentraties die hoger zijn dan deze toegestaan door de wetgeving. Dioxines, 2,3,7,8-tetrachloordibenzo-p-dioxine (TCDD) en acrylamide hadden geen effect op de beweeglijkheid van spermatozoa bij concentraties van respectievelijk 500 en 10 000 mg ml⁻¹.

Onderzoek van de robuustheid van computer geassisteerde analysen van de mobiliteit van varkens sperma, getest op 14 verschillende levensmiddelen geïnoculeerd met cereulide producerende *B. cereus*, toonde duidelijke cereulide productie in zeven stalen (alhoewel *B. cereus* groei tot tellingen hoger dan 8 log KVE g⁻¹ genoteerd werd in 11 stalen). De cereulide concentratie vastgesteld, benaderde deze gerapporteerd in voedselgerelateerde uitbraken. De evaluatie van de varkens sperma gebaseerde detectietechniek in 33 stalen die cereulide kunnen bevatten, toonde aan dat er effectief cereulide aanwezig was in 10 stalen en dat er geen invloed was van de voedingsmatrix op de analyse.

Naast cereulide veroorzaken enterotoxines, geproduceerd door *Staphylococcus aureus*, gelijkaardige symptomen van voedselvergiftiging. *S. aureus* enterotoxines veroorzaken één van de meest frequent voorkomende voedselgerelateerde intoxicaties. De intoxicatiedosis is zeer laag (tot 0.05 ng per g levensmiddel). Om deze redenen werd in hoofdstuk 7 de real-time immuno kwantitatieve PCR (iqPCR) methode ontwikkeld voor de detectie van *S. aureus* enterotoxine B (SEB). Deze methode werd zowel in laboratoriumcultures als in levensmiddelen geëvalueerd. De analyse bestaat uit immunocapture van SEB en real-time PCR amplificatie van de DNA-probe gelinkt met het detectie antilichaam. IqPCR werd vergeleken met een ELISA methode, die gebruik maakt van hetzelfde koppel antilichamen, en met commerciële kits voor de detectie van *S. aureus* enterotoxinen (SE). IqPCR metingen waren ongeveer 1000 keer gevoeliger (10 pg ml⁻¹) dan de ELISA en hadden een dynamische bereikbaarheid van 10 pg ml⁻¹ tot 30000 pg ml⁻¹. Het was mogelijk om met iqPCR SEB te detecteren in verschillende types levensmiddelen en anti-SEB antilichamen toonden geen kruis reactiviteit met voedingscomponenten en andere *S. aureus* enterotoxines. Gebruik van iqPCR voor de detectie van SEB in culturen van *S. aureus* toonde aan dat SEB
productie reeds start na een incubatie van 4 uur bij 22, 37 en 42 °C. Dit komt overeen met de eerste helft van de exponentiële groeifase van *S. aureus*. Bij 42 °C was de totale geproduceerde hoeveelheid van SEB hoger dan deze bij 37 °C. Finale hoeveelheden SEB waren stamafhankelijk.

Ten slot werden bij de beëindiging van dit doctoraatsproefschrift een aantal overwegingen en conclusies van het uitgevoerde onderzoek en perspectieven voor de voortgang van het onderzoek in de toekomst besproken. Speciale aandacht werd hierbij gegeven aan bepaalde opsporingstechnieken zoals nieuwe bioassays en de noodzaak tot vooruitgang in immunologische opsporing van cereulide. Ook werden toekomstige onderzoeksthema’s voorgesteld in de richting van verduidelijking van de moleculaire en biochemische mechanismen die aan de basis liggen van cereulideproductie. Invloed van de celstatus, met de betrekking tot de groeifase en de totale celtingeling, evenals de intercellulaire communicatie dienen gekoppeld te worden met intrinsieke, extrinsieke en expliciete factoren om verschillen in het gedrag van *B. cereus* in verschillende voedselmatrices en in verschillende omstandigheden te begrijpen. Deze kennis is noodzakelijk om kwantitatieve en kwalitatieve gegevens te verstrekken die zouden moeten dienen voor *B. cereus* risicoanalyse en de eventuele vastlegging van de microbiologisch criteria.
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**Higher Education**

- Basic studies (4 years): Faculty of Agriculture, Belgrade University, Department for Food Technology, Group of Animal Origin Foods.
  **Diploma: Graduated Agricultural Engineer in Food Technology of Animal Origin Foods.** Average mark of all exams 9.65 (out of 10.00) and grade 10.00 for the Final Diploma Exam. Award for one of the best students at the University of Belgrade. Thesis title: *HACCP in meat industry, case study: primary processing of beet meat;* promotor: Prof. dr. ing. R. Radovanovic.

- Master studies: Ghent University, Department of Food Quality and Food Safety: **MSc in Food Science and Technology** (title of thesis: *Challenge testing of Listeria monocytogenes in meat products;* promotor: Prof. dr. ir. M. Uyttendaele). Graduated with **Great distinction.**

- **PhD studies:** Ghent University, Department of Food Quality and Food Safety: Project title: Biological and immuno-molecular methods for monitoring of *Bacillus cereus* emetic toxin and *Staphylococcus aureus* enterotoxin, Promoters: Prof. dr. ir. M. Uyttendaele and Prof. dr. ir. J Debevere
  - PhD training with additional activities also followed.
  - Guidance of 4 thesis students (2 as a co-promoter) and 2 trainees

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**Language skills**

- Serbian-native language
- English-fluent
- Dutch-good (7th grade in PCVO Language School, Ghent)
- Russian-good
- German-medium level
- French-beginners’ level (2nd grade of PCVO Language School, Ghent)
<table>
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<tr>
<th align="left"><strong>Public presentations of student works</strong></th>
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<tr>
<td align="left">HACCP in meat industry- December 2001, European Conference of IAAS in Subotica, Yugoslavia</td>
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<tr>
<th align="left"><strong>Student Congresses and Seminars</strong></th>
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<tbody>
<tr>
<td align="left">Summer school on renewable energy sources and sustainable agriculture, food chain and food for all Belgrade 1997, 1999. (UNESCO World Solar programme). Person in charge: Prof. Dr Marija Todorovic, Faculty of Agriculture, Belgrade University</td>
</tr>
<tr>
<td align="left">Agriculture and European Union legislation conference-Sarvas, Hungary, December 1997</td>
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<tr>
<td align="left">Biotechnology: pro and contra symposium-Vienna, Austria-November 1998</td>
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<td align="left">Biotechnology in agriculture-Loreto, Italy-December 1998-January 1999</td>
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<td align="left">III Food technology symposium-Belgrade, Yugoslavia, 4-6 February 1998.</td>
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<td align="left">Food and health congress -Alnarp, Sweden-July 1999</td>
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<td align="left">Conference and working meeting for co-operation between IAAS and ICA in the field of higher education in agriculture-Copenhagen, Denmark-October 1999</td>
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<tr>
<td align="left">Conference and working meeting for international co-operation between International Students’ Organizations-IMISO-Leuven, Belgium-November 1999.</td>
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<tr>
<td align="left">“World nutrition” congress- Fiesch, Switzerland-December 1999-January 2000-08-31</td>
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<tr>
<td align="left">Meeting for improvement of international student exchange program-Goettingen, Germany-February 2000.</td>
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<tr>
<td align="left">Leadership training program: May, 2000- Leuven, Belgium</td>
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<td align="left">Congress: Water, the challenge for the next century-Mexico, July 2000</td>
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<tr>
<td align="left">Congress: Tropical agriculture-South-East Mexico, August 2000</td>
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<tr>
<td align="left">The Bologna Process: A European Higher Education Area-Perspectives and developments for agricultural and related sciences and the competencies of the graduates-March 2002, Brussels, Belgium, by CEDIA &amp; ICA*</td>
</tr>
</tbody>
</table>
• 1996-2000 - Active in IAAS from local up to the international level; from July 1999 till July 2000 on the post of the vice president of IAAS Headquarters in Leuven, Belgium; from July 2000 till July 2001 on the post of the control committee; from July 2001 till July 2002 member of the Advisory Board. In year 2000, 2001 and 2002 main editor (together with M. Andjelkovic) of international magazine *IAAS World* (ISSN 0789-8355).

• 15 International meetings within IAAS and its partners (FAO, UNESCO, ICA and other student organizations) in Europe, Asia and Latin America.

• IMISO Secretary in 2000.

**Proceedings, posters, extended abstracts**


• Rajkovic, A., Uyttendaele, M. and Debevere, J. Impact of non typical food matrices and cell density on *Bacillus cereus* emetic toxin production. Communications in Agricultural and Applied Biological Sciences, 70 (2), pp. 10-13 (oral presentation, 06.10.2005.)
**AI peer reviewed publications**


**Chapters in books**


### Lectures, oral presentations and courses given

- Impact of non typical food matrices and cell density on *Bacillus cereus* emetic toxin production. Symposium on Applied Biological Sciences (oral presentation), October 2005, Leuven.
- Number of lectures for the Tempus projects: Master in Food Quality and Food Safety (Master en qualité et sécurité alimentaire); Contracting institution: AGRENA, France, Coordinating institution: Belgrade University, Serbia; Partner: Ghent University, Belgium. November 2005.

### PC related skills

- HTML, PHP, ASP and MySQL programming; web design with database infrastructure
  - [http://www.foodmicrobiology.UGent.be](http://www.foodmicrobiology.UGent.be)
  - [http://www.rajkovic.net](http://www.rajkovic.net)
- PC hardware maintenance and mounting

### Extra activities

- IT and web design responsible at the Laboratory of Food Microbiology and Food Preservation
- Member of the Faculty of Bioengineering Sciences (UGent) Commission of Internationalization in academic years: 2004-2005; 2005-2006

### Perspectives

- “Pathogen combat” EU project

*IAAS stands for International Association of Students in Agricultural and Related Sciences; IMISO stands for Intersectorial Meeting of Internationals Student Organizations; ICA stands for Interuniversity Conference of Agricultural and Related Sciences in Europe; CEDIA stands for The European Confederation of Agronomist Associations.*