Detection, characterization and survival of *Bacillus cereus* group members in spices and herbs

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by
HENDRIK FRENTZEL
from Eberswalde

2017

This dissertation was prepared under supervision of Prof. Dr. Bernd Appel within the EU research project SPICED with financial support from the 7th Framework Program of the EU.

Experimental work was performed in the laboratory for spore formers in the unit Microbial Toxins, Department Biological Safety, Federal Institute for Risk Assessment.

March 2014 to March 2017

1. Gutachter: Prof. Dr. Rupert Mutzel
Freie Universität Berlin (FU), Institut für Biologie - Mikrobiologie,
Königin-Luise-Str. 12 - 16, 14195 Berlin

2. Gutachter: Prof. Dr. Bernd Appel
Bundesinstitut für Risikobewertung (BfR), Abteilung Biologische Sicherheit,
Diedersdorfer Weg 1, 12277 Berlin

Disputation: 07.06.2017

Table of contents

List of abbreviations

AFNOR	Association Francaise de Normalisation
AGES	Austrian Agency for Health and Food Safety GmbH
AIT	Austrian Institute of Technology GmbH
ANI	average nucleotide identity
a_w	water activity
BBC	Brilliance *Bacillus cereus* agar
BHI	brain heart infusion
BIOHAZ	EFSA Panel on Biological Hazards
bpm	PCR target-sequence in *B. pseudomycoides*
CAC	Codex Alimentarius Commission
CaCo-2	human colon carcinoma cell line
CBI	Confederation of British Industry
ces	gene for nonribosomal cereulide synthetase
cfu	colony forming units
cry/*cyt*	genes for parasporal crystal or cytolytic protein toxins in *B. thuringiensis*
cspA	gene for cold shock protein A
CTAB	cetyltrimethylammonium bromide
CytK	cytotoxin K
cytK	gene for cytotoxin K
D value	time needed for a decimal reduction
DDH	DNA-DNA-hybridisation
DGHM	German Society for Hygiene and Microbiology
DNA	desoxyribonucleic acid
DSM/DSMZ	German Collection of Microorganisms and Cell Cultures
EC	European Commission
EFSA	European Food Safety Authority
EHEC	enterohaemorrhagic *Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ESA	European Spice Association
FAO	Food and Agriculture Organization
gyrB	gyrase B gene

HACCP	Hazard Analysis Critical Control Point
hbl	gene for haemolysin BL
Hbl	haemolysin BL
HPLC-MS	high-performance liquid chromatography - mass spectrometry
ICMSF	International Commission on Microbiological Specifications for Foods
K_2SO_3	potassium sulphite
KbE	Koloniebildende Einheiten
L1/L2	lytic component of Hbl
LOD	limit of detection
MLST	multilocus sequence typing
motB	gene sequence encoding a flagellar motor protein
MPN	most probable number
MYP	mannitol egg yolk polymyxin agar
NaCl	sodium chloride
nhe	gene for non-haemolytic enterotoxin
Nhe	non-haemolytic enterotoxin
PCR	polymerase chain reaction
PEMBA	polymyxin pyruvate egg yolk mannitol bromothymol blue agar
RASFF	Rapid Alert System for Food and Feed
RKI	Robert Koch Institute
S	Svedberg
s.l.	sensu lato
s.s.	sensu stricto
SBA	sheep blood agar
ST	sequence type
TAMB	total aerobic mesophilic bacteria
v	volume
w	weight
WGS	whole genome sequencing
WHO	World Health Organization

List of figures and tables

1 Introduction

1.1 Spices and herbs

1.1.1 Definition and market overview

Spices and herbs have been used since ancient times to flavour food, but also as perfumes, cosmetics or for medicinal purposes (McKee, 1995). According to the European Spice Association (ESA) culinary spices and herbs can be defined as "edible parts of plants that are traditionally added to foodstuffs for either their natural flavouring, aromatic and/or visual properties". The ESA-List of Culinary Spices and Herbs comprises more than 80 different kinds of products representing the most frequently traded dried herbs and spices in Europe. The parts of the plant mostly used for spices are seed, fruit, rhizome, flower bud, bulb, aril, parts of pistil or stem, whereas for herbs mainly leaf, flower or the whole soft part of the plant are used (ESA, 2016).

A market survey of the Confederation of British Industry gives an overview on the current status and important developments on the spice market from a European perspective (CBI, 2015). Relating to this report, the EU, with more than 500 million consumers, is one of the biggest markets for spices and herbs with a slow but steady growth. Between 2010 and 2014 the volume of imports in average grew by 3.8 % per year ending up at a volume of 533 thousand tons in 2014 with a value of 1.9 billion €. Not only the volumes increased but also the value of spice and herb commodities (10 % per year between 2010 and 2014), due to a growing global demand (CBI, 2015).

Most important suppliers to the EU are China (35 % of total imported volume from outside the EU; mainly ginger and paprika), India (17 %; mainly turmeric, pepper, paprika and cumin seeds), Vietnam (11 %; mainly pepper), Indonesia (6.9 %) and Brazil (5.0 %). The majority of imported products are "whole", meaning not crushed or ground which shows that the processing and manufacturing of spices is predominated by the importing (EU) companies. However, processing is increasingly taking place in the harvesting countries. The main exporters in the EU are the Netherlands (30 % of total exported volume), Spain (21 %) and Germany (12 %), while the main part of exports is intra-EU-trade (67 %). Within the EU 137 thousand tonnes of spices and herbs were produced in 2013. Most important primary producers are Romania (37 % of EU produced volume), Bulgaria (28 %), Hungary (19 %) and Spain (8 %). Leading consuming countries in the EU are UK, Germany, Romania and Hungary. The most important consumed spices include pepper, paprika and allspice and the leading herbs comprise oregano, thyme, parsley, dill and chives (CBI, 2009; EFSA Food Consumption Database, www.efsa.europe.eu/en/datexfoodcdb/datexfooddb.htm). The largest

part of consumed spices and herbs is used in the industrial food producing sector (55 - 60 %) followed by the retail sector (35 - 40 %) and the catering sector (10 - 15 %) (CBI, 2009).

1.1.2 Production and decontamination

Common steps for the production of spices and herbs include harvesting, sometimes pre-treatments such as curing or punching, drying, cleaning, grinding, packaging/storage and often microbial decontamination as a final processing step (Schweiggert *et al.*, 2007). Most spice plants are grown in subtropical and tropical regions. Besides local production in the temperate or Mediterranean regions this also applies to the herb cultivation. The primary production structures can vary from industrial cultivation over small scale farming to wild collection (Schaarschmidt *et al.*, 2016b).

Due to the common occurrence of microorganisms - among them also potential foodborne pathogens - in spices and herbs (see 1.1.3), microbial decontamination is a common final processing step in the spice industry. In general, three well known decontamination technologies are currently applied: fumigation with ethylene oxide, irradiation, and steam treatment (Schweiggert *et al.*, 2007; Waje *et al.*, 2008). Additionally, several alternative methods for food decontamination are described (e.g. UV-, IR-irradiation, microwave-, heat- and/or pressure treatments), however, their application on dried herbs and spices is limited as they can cause product alterations or are ineffective due to the low a_w value of these commodities (Brodowska *et al.*, 2014; Eliasson *et al.*, 2014; Erdogdu & Ekiz, 2011; Schweiggert *et al.*, 2007).

Ethylene oxide fumigation is suitable to significantly reduce the microflora in spices and herbs. However, ethylene oxide is considered as a carcinogen. Thus, its application is prohibited in the EU (not so in the USA).

In contrast, irradiation (gamma rays, electron beam and X-rays) is a permitted method within the EU for the decontamination of dried aromatic herbs, spices and vegetable seasonings with a maximum absorbed dose of 10 kGy (Directive 1999/3/EC). Irradiation would be suitable to reduce both, bacterial cells as well as spores (EFSA, 2016). However, irradiation for the decontamination of foodstuffs is scarcely used because of labelling demands and low consumer acceptance. Indeed, irradiation could cause (at least slight) sensorial and compositional alterations in spices and herbs. In addition, when applied on pre-packaged products irradiation could cause reaction products originating from the packaging material (Schweiggert *et al.*, 2007).

Due to the drawbacks of the mentioned methods, a common method of decontamination in the spice industry is steam treatment, using hot steam for a short period of exposure, e.g. 108 - 125 °C for 20 - 120 seconds (Bata-Vidács *et al.*, 2016). This method reduces the vegetative microbial flora but is not effective to inactivate spores. Additionally, steam treatment can also cause product modifications, especially with regard to moisture content, colour and aroma (Schweiggert *et al.*, 2007).

1.1.3 Contamination of spices and herbs

As stated above primary production and processing conditions for spice and herb manufacturing are very diverse causing a broad range of product quality. The most critical points influencing the burden of contamination of these commodities in the primary production are the cultivation practices including soil condition, faecal matter, irrigation-water and harvesting practices. Most critical processing points comprise cleaning steps, drying procedures, packaging, storage, personnel/equipment hygiene and antimicrobial treatments (AIT & AGES, 2013). Generally, contamination of spices and herbs can occur at any stage along the food chain and may be present as chemical, physical as well as microbiological contaminants.

Chemical compounds of concern are mycotoxins, pesticide residues, additives and illegal colorants, heavy metals, persistent organic pollutants, allergens and toxic plant compounds (Schaarschmidt, 2016).

Regarding microbial contaminations McKee *et al.* (1995) reported in a comprehensive review, besides fungal contamination, the occurrence of potentially pathogenic microorganisms such as *Salmonella* spp., *Bacillus cereus*, *Clostridium perfringens*, *Escherichia coli*, *Staphylococcus aureus* and *Shigella* spp. in a variety of spices and herbs, albeit mostly in low numbers.

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) ranked low-moisture foods (a_w below 0.85 (CAC, 2015)) from a microbiological food safety perspective (WHO/FAO, 2014). Seven food categories (making up seven possible ranks) were evaluated in this ranking: cereals and grains, confections and snacks, dried fruit and vegetables, dried protein products, nuts and nut products, seeds for consumption, and spices and dried aromatic herbs (including teas). Used criteria for the ranking of each food category were “international trade” (export value per year), “burden of disease” (based on outbreak cases caused by the food category), “vulnerabilities due to food consumption” (based on average consumption, consumption by vulnerable groups and risk of

mishandling) and "vulnerabilities due to food production" (considering decontamination steps and risk of contamination at levels to cause illness). Based on that the category "spices and dried aromatic herbs (including teas)" was placed on rank three with the criteria "vulnerabilities due to food production" and "burden of disease" as the decisive factors, as contamination during the production stages is not unlikely.

Similarly, the EFSA Panel on Biological Hazards (BIOHAZ) ranked food/pathogen combinations to evaluate the risk posed by pathogens in food of non-animal origin (EFSA, 2013). In total, 39 food categories were considered in combination with ten typical foodborne pathogens. The applied risk ranking model was based on seven criteria: "strength of associations between food and pathogen" based on the foodborne outbreak data from EU Zoonoses Monitoring (2007-2011), "incidence of illness", "burden of disease", "dose-response relationship", "consumption", "prevalence of contamination" and "pathogen growth potential during shelf life". As a result the category "spices and dry powdered herbs" connected to *Bacillus* spp. and *Salmonella* spp. can be found among the top-four ranking groups of food/pathogen combinations.

In a review carried out in frame of the "Joint FAO/WHO expert meeting on microbiological hazards in spices and dried aromatic herbs" 28 foodborne disease outbreaks associated to contaminated spices and herbs are reported for the period between 1973 and 2012. Authors of this report state that outbreaks caused by spices and herbs may be underreported. More than 2200 cases of illness were caused by these outbreaks; most of them by *Salmonella* spp. (77 %) followed by *Bacillus cereus* (20 %) and *Clostridium perfringens* (3 %). Interestingly, most outbreaks (not cases of illness) were caused by *Bacillus cereus* (Mader & Schaarschmidt, 2015).

An evaluation of the EU-RASFF (Rapid Alert System for Food and Feed) notifications between 2004 and 2014 by Banach *et al.* (2016) revealed a total of 1831 notifications within the product category "herbs and spices". The most frequent categories were mycotoxins (27 %; mainly aflatoxins and ochratoxin), pathogenic microorganisms (23 %), composition (23 %; mainly sudan dyes), pesticide residues (11 %), and food additives and flavourings (5 %). The 425 notifications for pathogenic microorganisms comprised a total of 500 hazards, most frequently *Salmonella* spp. (74 %; mainly in basil, coriander, black pepper and peppermint), followed by *E. coli* (12 %; mainly basil, mint, peppermint and coriander), and *Bacillus* spp. (8 %; mainly chili products and curry products). Most notifications resulted from products originating from Thailand, Vietnam and India.

Within a similar timeframe (2004 to 2013) EU reports summarizing zoonosis monitoring programs based on directive 2003/99/EC (Anonymous, 2003b) describe a prevalence of 1.1 % for *Salmonella* spp., 0.7 % for *Listeria monocytogenes*, 0.2 % for *E. coli* and 0.02 % for *Campylobacter* spp. in spices and herbs (*Bacillus* spp. and *Clostridium* spp. were not part of the monitoring programs) (Banach *et al.*, 2016).

In conclusion, although spices and herbs usually do not permit microbial growth due to their low a_w value, contaminated condiments can act as a vehicle to transfer pathogens to foods with optimal conditions for growth or toxin production. As condiments are added mainly as minor ingredients to various foods, including ready-to-eat products, a single contaminated batch could affect many products. Given the fact that outbreak investigations usually focus on main ingredients of dishes, condiments are unlikely to be identified as cause for foodborne diseases. Additionally, typical outbreak mitigation strategies like product forward- and backward-tracing combined with product recalls are complicated due to the long shelf-life, mixed batches from several harvesting years and/or mixed spice products (e.g. curry-products) (Schaarschmidt *et al.*, 2016b).

1.1.4 Microbiological quality standards

Although based on RASFF data the number of notifications - based on non-randomized sampling of spices and herbs - for chemical and biological hazards are comparable, large differences are prevailing with regard to existing standards for biological and chemical/physical quality of spices and herbs. For all of the top three chemical hazards ("mycotoxins", "pesticide residues and additives", and "illegal colorants") detailed limits are specified by the Codex Alimentarius Commission (CAC) and the European Commission (EC) (for an overview see Schaarschmidt *et al.*, 2016a).

In contrast, microbiological criteria specific for spices and herbs are rarely defined by public bodies. However, some public (mostly national) but also private standards are existing (for an overview see Schaarschmidt *et al.*, 2016a).

Of course, the general food safety provisions of Regulation (EC) No 178/2002 (Anonymous, 2002) on general principles of food safety, (EC) No 852/2004 (Anonymous, 2004) on the hygiene of foodstuffs and (EC) No 2073/2005 (Anonymous, 2005) on microbiological criteria for foodstuffs are also valid for spice and herb producers and products. According to these regulations, foodstuffs should not contain microorganisms or their toxins or metabolites in quantities that present an unacceptable risk for human health. Besides these general provisions, no specific mandatory microbiological criteria are applicable for spices and herbs

in the EU. However, for orientation regarding a satisfactory, acceptable and unsatisfactory bacteriological safety of condiments a European Commission recommendation is available (Anonymous, 2003a). Annex III of this recommendation concerning a coordinated program for the official control of foodstuffs for 2004 includes criteria for *Salmonella* spp., *B. cereus*, *C. perfringens* and *Enterobacteriaceae* (Table 1).

Similar general microbiological safety provisions are also part of product standards for spices and herbs like for example the Quality Minima Document of the ESA (ESA, 2016) or specifications of ISO for individual products (Schaarschmidt, 2016).

Somewhat more specific, in the CAC Code of Hygienic Practice for Spices and Dried Aromatic Plants in the version of 1995 it was provided that *Salmonella* spp. should be absent in 10 samples of 25 g in ready-to-eat spices (CAC, 1995). In the current version it is only advised that incoming commodities should not be used when *Salmonella* spp. are detected and should be free from pathogenic and toxigenic microorganisms in levels that may present a risk to health (CAC, 2015; Schaarschmidt, 2016).

On a national level additional standards are available (Schaarschmidt *et al.*, 2016a). In Germany for example, the German Society for Hygiene and Microbiology (DGHM, 2011) provides recommendations on critical microbiological contaminations in consumer-ready spices and herbs (Table 1).

Table 1: Microbiological standards for spices and herbs (adapted from Schaarschmidt *et al.*, 2016a).

Reference	**Entero-bacteriaceae**	***Escherichia coli***	***Salmonella* spp.**	**Presumptive *Bacillus cereus***	***Clostridium perfringens*/ sulphite-reducing Clostridia (s.C.)**	**Moulds**
EC 2004	m = 10 M = 10^2 (n = 5, c = 1)	-	n.d. in 25 g (n = 5, c = 0)	m = 10^3 M = 10^4 (n = 5, c = 1)	m = 10^2 M = 10^3 (n = 5, c = 1)	-
DGHM	-	m = 10^3 M = 10^4	n.d. in 25 g	m = 10^3 M = 10^4	s.C. m = 10^3 s.C. M = 10^4	m = 10^5

Values are given in cfu/g. Values are satisfactory if all the values observed (n) are <m, acceptable if maximum of c values are between m and M, and unsatisfactory if one or more values are >M or more than c values are between m and M.

n.d.: not detectable

s.C.: sulphite reducing Clostridia

Besides these public and private standards the microbiological quality of commodities is also subject of buyer-seller agreements that are commonly applied in the condiment industry and may be more stringent than legal requirements (Schaarschmidt *et al.*, 2016a).

To assure the compliance with standards and/or agreements a number of guidelines are existing which can be assigned to the following fields: 1) Good hygienic practices, 2) Good agricultural practices, 3) Good manufacturing practices and 4) HACCP (Hazard Analysis Critical Control Point) guidelines (Schaarschmidt *et al.*, 2016a).

1.1.5 Antimicrobial effects

Despite the fact that dried herbs and spices have a rich microflora, their secondary plant metabolites like essential oils are known to hold antimicrobial effects. For example the review of Tajkarimi *et al.* (2010) reports antimicrobial activity of herbs and spices containing essential oils in the range of 0.05 - 0.1% against *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*, in food systems. In line with this, Singh *et al.* (2002) describe a reducing effect of essential oils from several spices against a range of Gram positive and Gram negative organisms (*Corynebacterium diphtheriae*, *S. aureus*, *Streptococcus haemolyticus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella* species and *Proteus vulgaris*). Also sporicidal activity or inhibition of spore germination due to essential oils has been reported (Chaibi *et al.*, 1997; Lau *et al.*, 2014; Lawrence & Palombo, 2009). Antimicrobial acting substances comprise mostly phenols (e.g. eugenol, anethol, thymol), but also others such as aldehydes, ketones, ethanol or allyl isothiocyanate (Graubaum, 2003; ICMSF, 2005). Condiments reported to contain the most inhibitory essential oils are cloves, thyme, oregano, cinnamon, allspice, cumin, and caraway (ICMSF, 2005). However, the content of essential oils varies within the same spice depending on agricultural practices, geographic and climatic conditions during the growing season (ICMSF, 2005).

Antimicrobially acting substances may influence cultural microbial detection methods as these methods are based on bacterial growth. For example Graubaum (2003) reports inhibition of growth or even cell reduction for *Salmonella* spp. in enrichments for a variety of spices.

Mostly, the antimicrobial components are identical with the most important flavour compounds. Combined with the fact that relatively high concentrations of essential oils are needed for efficient inhibition, the organoleptic characteristics of food preserved with spice extracts would be strongly altered (ICMSF, 2005). In line with this Tajkarimi *et al.* (2010)

state that spice and herb compounds are currently scarcely used as food preservatives due to limited data about their effects in food, strong odour and high cost.

1.2 The *Bacillus cereus* group

As outlined in subsection 1.1.3 *B. cereus* is a frequent contaminant in spices and herbs and as such was found responsible for a number of foodborne disease outbreaks.

1.2.1 Taxonomy and species differentiation within the *Bacillus cereus* group

The genus *Bacillus* (Kingdom Bacteria, Phylum Firmicutes, Class Bacilli, Order Bacillales, Family Bacillaceae) was established by Ferdinand Cohn in 1872 and includes Gram positive, rod-shaped, aerobic and facultative anaerobic, spore-forming bacteria (at present at least 226 species) (EFSA, 2016). The *B. cereus* group (also referred to as *B. cereus* sensu lato (s.l.)) is a subdivision of the *Bacillus* genus. This group currently comprises eight genetically closely related species: *B. cereus* (sensu stricto (s.s.)), *B. thuringiensis*, *B. weihenstephanensis*, *B. toyonensis*, *B. cytotoxicus*, *B. mycoides*, *B. pseudomycoides* and *B. anthracis*. Especially the four first mentioned species are difficult to differentiate from each other. The difficulties in species differentiation are reflected in the term "presumptive *B. cereus*" commonly used in food quality standards or method titles such as ISO 7932:2005 and ISO 21871:2006 (Anonymous, 2005, 2006) (see also 1.2.3). The taxonomy of the *B. cereus* group was subject to several changes in recent years and is currently under revision. Besides the eight formally recognized species mentioned above, three further putative *B. cereus* group species are already effectively but not yet validly published: *B. gaemokensis* (Jung *et al.*, 2010), *B. manliponensis* (Jung *et al.*, 2011) and *B. bingmayongensis* (Liu *et al.*, 2014). Moreover, Liu *et al.* (2015) predict 30 *B. cereus* group species based on whole genome sequence comparisons (digital DNA-DNA-hybridisation). Within this section we summarize the current status of the validly published *B. cereus* group species (at the time of writing) and their distinction.

The species *B. cereus* (Frankland, 1887; Skerman *et al.*, 1997) was first described in 1887. *B. cereus* is a ubiquitous species which was isolated from soils (main reservoir), sediments, water, invertebrates, mammals and foods of plant and animal origin (EFSA, 2016; Vilas-Boas *et al.*, 2007). Most *B. cereus* strains are motile, use glucose as a source of carbon (but not mannitol, arabinose, or xylose), show haemolytic and lecithinase activity and are resistant to

ampicillin (Vilas-Boas *et al.*, 2007). The majority of *B. cereus* are mesophilic bacteria (3.0 - 5.0 µm in size), with a growth range of 10 to 45 °C and an optimal growth between 30 and 45 °C (EFSA, 2005; Guinebretiere *et al.*, 2008). However, also psychrotrophic strains with the ability to grow at or below 7 °C have been described (Stenfors & Granum, 2001).

Bacillus anthracis (Cohn, 1872; Skerman *et al.*, 1997) is a zoonotic pathogen causing the disease anthrax in animals and humans. Most *B. anthracis* strains can be differentiated from other *B. cereus* group members by their non-motility, capsule production, absence of haemolytic activity when growing on blood agar, and penicillin sensitivity (Vilas-Boas *et al.*, 2007). However, non-motility and missing haemolysis are not strictly refined to *B. anthracis* (Messelhäußer & Ehling-Schulz, 2014). Additionally, it is crucial to determine the virulence potential of *B. anthracis* strains as virulence determinants are plasmid coded and thus can be lost. Accordingly, PCR based methods have been developed to identify *B. anthracis* and its toxin plasmids. For example Wielinga *et al.* (2011) successfully identified *B. anthracis* based on a multiplex real-time PCR targeting the chromosomally located part of the lambda prophage type 3. Simultaneously, the two toxin plasmids pXO1 and pXO2 could be detected based on the *cya* gene and the *capB* gene, respectively.

B. thuringiensis (Berliner, 1915; Skerman *et al.*, 1997) displays the same phenotypic characteristics as mentioned above for *B. cereus* (Vilas-Boas *et al.*, 2007). The distinctive feature for *B. thuringiensis* is its ability to produce protein inclusions during sporulation, the so called parasporal crystals that can be visualized by phase-contrast microscopy. These protein crystals are mostly plasmid coded and constitute the entomopathogenic Cry and/or Cyt toxins acting against larvae of Lepidoptera, Diptera or Coleoptera (Bravo *et al.*, 2011). Due to its entomopathogenic activity several *B. thuringiensis* strains are commercially used as bio-insecticide in formulations mostly containing a mixture of living spores and parasporal crystals (EFSA, 2016). The appearance of the parasporal crystals ranges from bipyramidal, diamond-shaped to spherical and from large to very small. Especially very small crystals are hardly to distinguish from other inclusions that might be present in the cell. Thus, microscopy is not always reliable (EFSA, 2016). Consequently, molecular-biological approaches targeting the *cry*/*cyt*-genes constitute alternative methods to identify *B. thuringiensis* (e.g. Noguera & Ibarra, 2010; Thammasittirong & Attathom, 2008). However, the huge diversity of the *cry* and *cyt* genes (more than 500 *cry* sequences (Bravo *et al.*, 2011)) makes it very difficult to capture all variants. Thus, negative results in microscopy as well as *cry*/*cyt*-gene detection do not

completely rule out the presence of *B. thuringiensis*. The fact that the genes for crystal formation are located on plasmids challenges the suitability of this characteristic as a distinctive species feature (EFSA, 2016). Indeed, recent bioinformatic evaluations of the species status using data from whole genome sequences (WGS) or multi-locus sequence typing (MLST) suggest that *B. cereus* and *B. thuringiensis* should be treated as one species (Cardazzo *et al.*, 2008; Liu *et al.*, 2015).

Also *B. weihenstephanensis* (Lechner *et al.*, 1998) is very similar to *B. cereus* with regard to nutrient requirements and growth morphology. However, its growth range and optimum growth temperatures differ from most *B. cereus* strains. *B. weihenstephanensis* is a psychrotrophic member of the *B. cereus* group growing at 4 - 7 °C but not at 43 °C (Lechner *et al.*, 1998). Thus, known strains are most frequently isolated from dairy products, usually kept at refrigeration temperatures. Further characteristics of *B. weihenstephanensis* are signature sequences in their 16S rDNA and the major cold shock protein gene (*cspA*). The 16S rDNA signature sequence ^{1008}TCTAGAGATAGA and the *cspA* signature sequence ^{4}ACAGTT were believed to be distinctive for psychrotrophic strains and thus *B. weihenstephanensis*, whereas the 16S rDNA signature sequence ^{1008}CCTAGAGATAGG should represent mesophilic strains. Francis *et al.* (1998) and von Stetten *et al.* (1998) established PCR methods to detect these signature sequences. However, Pruss *et al.* (1999) as well as Stenfors & Granum (2001) found strains containing both 16S rDNA sequences - the psychrotrophic as well as the mesophilic - and not all of these strains were able to grow at 6 °C. In the study of Stenfors & Granum (2001) two of these (intermediate) strains were additionally positive for the psychrotrophic variant of the *cspA* gene and grew at 6 °C but not at 42 °C, thus matching *B. weihenstephanensis* characteristics. Still, as the strains described as *B. weihenstephanensis* by Lechner *et al.* (1998) contained exclusively the psychrotrophic 16S rDNA signature the intermediate strains were not designated *B. weihenstephanensis* by Stenfors & Granum (2001). Recent findings by Soufiane *et al.* (2013) suggest further *B. weihenstephanensis* signature sequences in housekeeping genes used for MLST that could be useful to distinguish *B. weihenstephanensis* from *B. cereus*.

Interestingly, Soufiane & Cote (2010) also found all typical *B. weihenstephanensis* markers (including the suggested MLST markers) in three strains originally assigned to the species *B. thuringiensis* based on the presence of parasporal crystals. Consequently, the authors hypothesize that this finding is the result of a transformation process in which *B. weihenstephanensis* strains acquired the *cry*-containing plasmid of *B. thuringiensis*.

Moreover, the same authors found all *B. weihenstephanensis* markers in some *B. mycoides* strains. According to the authors these strains should be reclassified as *B. weihenstephanensis* as the value of rhizoid growth as a species marker for *B. mycoides* is questionable.
Oliwa-Stasiak *et al.* (2011) suggested a real-time PCR method to distinguish *B. weihenstephanensis* from *B. cereus* based on differences in the *motB* gene sequences (encoding the flagellar motor protein). However, the suggested primer-probe-combination showed also sensitivity for some *B. mycoides* strains in the original study.
Interestingly, according to Liu *et al.* (2015) *B. weihenstephanensis* and *B. mycoides* should be treated as one species based on WGS similarity.

The species *B. mycoides* (Flügge, 1886; Skerman *et al.*, 1997) was described in 1886 as an aerobic, spore forming, rod-shaped, non-motile organism with a preference to form rhizoidal colonies (Flügge, 1886; Nakamura, 1998). However, the use of rhizoidal colony morphology as a species marker is somewhat problematic. Hendriksen & Hansen (2011) reported absence of rhizoid growth for the majority of 96 *B. mycoides* strains incubated at 37 °C on a commercial chromogenic agar used for *B. cereus* diagnostics. In contrast, when incubated at 30 °C all but one isolate showed rhizoid growth. Like *B. weihenstephanensis* also *B. mycoides* is regarded as a psychrotolerant species (Messelhäußer *et al.*, 2014). As mentioned above, genetically *B. weihenstephanensis* and *B. mycoides* do not constitute two separate species (Liu *et al.*, 2015).

Also *B. pseudomycoides* (Nakamura, 1998) is considered a psychrotolerant species with rhizoidal growth (Messelhäußer & Ehling-Schulz, 2014). In 1998 Nakamura proposed *B. pseudomycoides* as a distinct species that can be distinguished from *B. cereus* and *B mycoides* based on DNA relatedness, 16S rDNA sequences and fatty acid composition (Nakamura, 1998). The separate species status could be confirmed by other authors based on whole genome comparisons, MLST and 16S rDNA analysis (Jimenez *et al.*, 2013; Liu *et al.*, 2015). As a rapid detection and discrimination method for *B. pseudomycoides* Oliwa-Stasiak *et al.* (2011) suggested a real-time PCR targeting a sequence called *bpm* encoding a hypothetical protein.

B. cytotoxicus was proposed as a new *B. cereus* group species by Guinebretiere *et al.* (2013) in 2013. The species description is based on a strain (NVH 391-98) that was isolated during a severe diarrhoeal foodborne outbreak in France and four further strains (three of them also

linked to food poisoning and vegetable purees). Meanwhile many more *B. cytotoxicus* strains could be isolated mainly from potato products after enrichment at 50 °C (Contzen *et al.*, 2014). The distinctive features of *B. cytotoxicus* are very well defined. Of practical relevance for species identification are especially its growth temperature range of 20 - 50 °C (thermotolerance), the presence of the *cytK* gene in the *cytK-1* form, and the formation of distinct phylogenetic clusters based on MLST and also 16S rDNA sequences (Guinebretiere 2013). The detection of the *cytK-1* gene based on PCR and real-time PCR is well established and validated (e.g. Guinebretiere *et al.*, 2006; Wehrle *et al.*, 2010).

In 2013 *B. toyonensis* was proposed as a name for a new, eighth species within the *B. cereus* group (Jimenez *et al.*, 2013; Oren & Garrity, 2014). At present only one strain is formally assigned to this species. This strain (NCIMB 14858^T, previously BCT-7112^T) was isolated in 1966 in Japan and was originally referred to as *B. cereus* var toyoi. Since 1975 spores of this strain are in use as a probiotic feed additive in form of the commercial preparation TOYOCERIN®, especially in the nutrition for swine, poultry, cattle, rabbits and aquaculture. It was also authorized in the EU as feed additive for the use in swine, poultry, cattle and rabbits in 1994 (Jimenez *et al.*, 2013). The positive effect of the feed additive on the daily weight gain, final body weight and feed to gain ratio was consistently demonstrated on weaned piglets (EFSA, 2014). In contrast, the safety of the preparation for human health is not unambiguously proven; firstly, due to the risk of spreading antibiotic resistance against chloramphenicol and tetracycline based on the *catQ* and *tet(M)* genes, and secondly, due to the capability of producing the enterotoxins. Consequently, the authorization of TOYOCERIN® is currently suspended in the EU (EFSA, 2014).
The description of the species *B. toyonensis* is mainly based on pairwise genome comparisons resulting in an average nucleotide identity (ANI) value. This ANI value corresponds to DNA-DNA hybridization (DDH) values and can thus be used for species circumscriptions (Richter & Rossello-Mora, 2009). Besides the ANI value of less than 92 % compared to the closest related type strains also MLST analyses resulted in a distinct phylogenetic cluster. Apart from that, the phenotypic species characteristics such as optimal growth temperature of 35 °C, a salinity tolerance of up to 5 % NaCl or the optimal pH of 6.5 as suggested by Jimenez *et al.* (2013) are of little practical value for species differentiation.

1.2.2 Pathogenesis of *Bacillus cereus*

Besides a range of opportunistic infections *B. cereus* is an important cause of foodborne illness with two distinct syndromes: the diarrhoeal illness and the emetic illness (Logan, 2012).

The diarrhoeal symptoms - abdominal pain and watery diarrhoea - start 8 - 16 h after consumption of contaminated food and abate usually 24 - 48 h after symptom onset. The diarrhoeal disease is caused by enterotoxins produced in the small intestine by vegetative *B. cereus* cells (Ceuppens *et al.*, 2011) after ingestion of higher numbers of spores/cells (usually more than 10^5 cfu/g of food (EFSA, 2016)). In this regard, it should be noted that the enterotoxic potential is highly strain dependent (Jessberger *et al.*, 2015).

Three chromosomally encoded protein toxins are recognized as the causative enterotoxins for the diarrhoeal symptoms: non-haemolytic enterotoxin (Nhe), haemolysin BL (Hbl) and cytotoxin K (CytK). These toxins show haemolytic and cytotoxic activity and are secreted from cells via the Sec translocation pathway. Secretion relies on Sec-type signal peptides as part of the amino-acid sequence of the toxin proteins which are cleaved upon secretion (Dietrich *et al.*, 1999; Fagerlund *et al.*, 2010; Logan, 2012). As the enterotoxins are heat- (e.g. 20 min at 55 °C), acid- (e.g. pH 3.1 for 20 min) and protease-sensitive pre-formation of these toxins in food is most likely not relevant as toxins would be inactivated by cooking and/or during gastrointestinal passage (Ceuppens *et al.*, 2011).

Nhe is a tripartite pore forming protein that consists of the cytolytic component NheA and the two binding components NheB and NheC. All components are encoded in a single operon (Granum *et al.*, 1999). Maximum cytotoxicity is achieved by a ratio of 10:10:1 of the components NheA, NheB and NheC (Lindback *et al.*, 2004). The *nhe* genes are present in most *B. cereus* group strains (Ceuppens *et al.*, 2011; Guinebretiere *et al.*, 2010; Wehrle *et al.*, 2009). A specific *nhe* polymorphism is characteristic for *B. cytotoxicus* strains (Fagerlund *et al.*, 2007). Guinebretiere *et al.* (2010) assert that the *nhe* genes are a constant part of the chromosome of all *B. cereus* group members. Dissenting, Wehrle *et al.* (2009) report strains that at least lack individual *nhe* genes and Ceuppens *et al.* (2011) summarize the prevalence of *nhe* from literature data as being 84 - 100 %. Diverging observations might be a result of different methods used for the detection of *nhe* genes (e.g. southern-blotting or PCR; different PCR-primers).

Hbl is composed of three protein components (encoded in one operon): the lytic components L1 (HblD) and L2 (HblC) and the binding protein B (HblA). The three components bind independently to the cell membrane and cause osmotic cell lysis after pore formation.

Maximum cytotoxicity is reached by a ratio of 1:1:1 of the components in culture supernatants. The prevalence of *hbl* genes (29 - 92 %) in *B. cereus* is somewhat lower than for *nhe* (Ceuppens *et al.*, 2011), but can as well be found in all *B. cereus* group members except *B. cytotoxicus* (Guinebretiere *et al.*, 2010).

CytK is a single protein toxin that forms ß-barrel pores similar to *Clostridium perfringens* ß-toxins. Two variants of this toxin occur, CytK-1 and CytK-2, with a sequence homology of 89 %. The CytK-1 form shows higher cytotoxicity (at least five times more than the CytK-2 variant) and its presence is refined to *B. cytotoxicus* strains (Ceuppens *et al.*, 2011; Guinebretiere *et al.*, 2013). Compared to *cytK-1*, the *cytK-2* gene has a higher frequency (37 - 89 %) (Ceuppens *et al.*, 2011), while it appears to be absent in *B. anthracis*, *B. pseudomycoides*, *B. mycoides* and *B. weihenstephanensis* (Guinebretiere *et al.*, 2010).

The emetic illness is caused by a cyclic dodecadepsipeptide called cereulide. The basic structure of cereulide was determined to be [D-O-leucine-D-alanine-O-valine-D-valine]$_3$, however recently at least 18 structural variants (isocereulides) have been found (Marxen *et al.*, 2015). The disease is characterized by nausea and vomiting with rapid onset of symptoms 0.5 - 6 h after consumption of food containing the toxin and usually recovery within the subsequent 24 h. However, also fatal cases are reported due to liver failure partly in connection with brain oedema following consumption of contaminated pasta or rice dishes (Dierick *et al.*, 2005; Mahler *et al.*, 1997; Shiota *et al.*, 2010). Cereulide induced symptoms are ascribed to the stimulation of the vagus afferent nerves upon binding to serotonin 5-HT$_3$ receptors (nausea and vomiting) (Agata *et al.*, 1995; Logan, 2012) and toxicity to mitochondria causing inhibition of fatty acid oxidation (liver failure, brain oedema) (Ceuppens *et al.*, 2011; Mikkola *et al.*, 1999).

Cereulide is generated by the non-ribosomal cereulide synthetase encoded in the megaplasmid located *cesHPTABCD* genes cluster (Ehling-Schulz *et al.*, 2006). In contrast to the enterotoxin genes, the prevalence of the *ces* genes is much lower (< 5%, compare Ceuppens *et al.*, 2011; Wehrle *et al.*, 2010) and refined to a genetically closely related group of *B. cereus* strains (Ehling-Schulz *et al.*, 2005) and very few *B. weihenstephanensis* strains (Thorsen *et al.*, 2006).

Different to the enterotoxins, cereulide is already formed in the food under aerobic conditions and temperatures of 8 - 40 °C. The toxin is extremely heat- (e.g. 2 h at 121 °C), acid- (e.g. pH 2 - 11) and protease-stable and thus is not inactivated by cooking, most food processing steps or during gastrointestinal passage (Ceuppens *et al.*, 2011). Different ranges of cereulide concentrations are reported in food implicated in emetic outbreaks: 0.01 - 1.28 µg/g (Agata *et*

al., 2002), 0.3 - 2 µg/g (Messelhäußer *et al.*, 2014) or 3.1 - 4.2 µg/g (Delbrassinne *et al.*, 2015). Assuming a body weight of 70 kg and a consumption of 100 g of food (Ceuppens *et al.*, 2011) the intoxication dose resulting from the above cereulide concentrations ranges from 0.014 to 6.0 µg cereulide per kg body weight. Based on animal trials the minimum intoxication dose for humans is estimated to be in the range of 8 - 10 µg/g body weight (EFSA, 2016). Cell concentrations of 10^5 to 10^8 cfu/g of food are supposed to be necessary to produce illness inducing cereulide amounts (Ceuppens *et al.*, 2011). However, frequently lower *B. cereus* cell concentrations are found in implicated food (e.g. < 100 cfu/g, compare Messelhäußer *et al.*, 2014) which most likely reflects the fact that cells have been killed by food processing whereas the preformed toxin remained stable. Further explanations for the broad range of observed intoxication doses and cell concentrations may be the following: 1) toxin production potential is variable between strains (Carlin *et al.*, 2006); 2) toxin production depends on environmental factors such as food type, temperature, pH and atmospheric conditions (Ceuppens *et al.*, 2011), 3) cytotoxicity varies between the different isocereulide variants (Marxen *et al.*, 2015).

1.2.3 Detection of *Bacillus cereus* in food

Standard procedures for the detection of *B. cereus* in food rely on cultural detection methods based on ISO 7932:2005 and ISO 21871:2006 (Anonymous, 2005, 2006). Typical protocol steps include the dilution of the sample, partly enrichment (MPN method of ISO 21871:2006), plating on mannitol egg yolk polymyxin agar (MYP) or polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) followed by confirmation on sheep blood agar (SBA). The selectivity of MYP and PEMBA is based on: 1) the resistance of *B. cereus* group strains against the antibiotic polymyxin which inhibits most Gram negative organisms; 2) the inability to produce acid from mannitol resulting in the pH dependent colorization of the media and 3) the ability to hydrolyse egg yolk (lecithinase activity) leading to a precipitation zone around typical colonies. The confirmation as "presumptive *B. cereus*" on SBA relies on the haemolytic activity of most *B. cereus* strains. As mentioned above, these methods are not suitable to differentiate between individual *B. cereus* group species especially with regard to *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis* and *B. toyonensis*. Moreover, some strains are lacking the typical characteristics leading to misinterpretation and false-negatives (Fricker *et al.*, 2008; Hendriksen & Hansen, 2011). Enumeration of presumptive *B. cereus* from food with high microbial background flora is further hampered as *B. cereus* colonies might be overgrown by other species (Tallent *et al.*, 2012). Alternative selective media were recently

developed and tested. The chromogenic medium Brilliance *Bacillus cereus* agar (BBC) applies the antibiotic trimethoprim in addition to polymyxin B contributing to a better suppression of background flora (Chon *et al.*, 2014). The selectivity of BBC is further based on the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-glycopyranoside which is cleaved by the ß-glucosidase of *B. cereus* resulting in blue/green colony colour. However, apparently not all *B. cereus* strains grow on BBC and not all strains show ß-glucosidase activity (Hendriksen & Hansen, 2011). The chromogenic medium Bacara is also reported to efficiently inhibit background flora and thus being more sensitive in *B. cereus* enumeration from foods (Tallent *et al.*, 2012). This medium has also been validated by AFNOR for the enumeration of *B. cereus* group strains from food matrices (EFSA, 2016; Tallent *et al.*, 2012). However, comprehensive studies on growth characteristics of different *B. cereus* group species on Bacara are still lacking.

When analysing spices and herbs by cultural techniques high microbial background flora is indeed a problem. Moreover, plant compounds may also dissolve in the dilution medium and thus may influence the microbiological detection system by antimicrobial effects (compare 1.1.5). Accordingly, ISO 6887-4 on the preparation of test samples, initial suspension and decimal dilutions for microbiological examination (Anonymous, 2012) includes a passage on the handling of antimicrobial acting foods such as oregano or pepper. Recommendations on the handling include higher dilution factors (resulting in higher limits of detection) or the addition of K_2SO_3 to the first suspension.

In order to overcome problems associated with cultural techniques efforts have been made to develop culture independent detection methods for *B. cereus*. Hence, several PCR methods are available with different focus towards the detection of *B. cereus* group species. For example Martinez-Blanch *et al.* (2009) developed a real-time PCR assay targeting the phosphatidylcholine-specific phospholipase C gene (*pc-plc*) to detect *B. cereus* group members. This assay was further evaluated for the use of spore detection and quantification in foods (liquid egg and infant formula) (Martinez-Blanch *et al.*, 2010). Similarly, Dzieciol *et al.* (2013) proposed a real-time PCR assay for the detection of the *B. cereus* group and demonstrated its suitability for spore quantification in milk. This PCR assay is a multiplex approach that detects *B. cereus* group species based on the gyrase B gene (*gyrB*) and a 16S rDNA sequence and simultaneously differentiates emetic strains targeting the *ces* gene. With a focus towards species differentiation Oliwa-Stasiak *et al.* (2011) suggested a multiplex real-time PCR to detect the *B. cereus* group with simultaneous discrimination of

B. weihenstephanensis and *B. pseudomycoides* from *B. cereus* (s.l.) and demonstrated its quantitative character on milk samples spiked with vegetative cells.

Naturally, quantification by real-time PCR is not only depending on the efficiency of the PCR assay but much more on the DNA-extraction method. When attempting to detect *B. cereus* in food by DNA-extraction coupled to real-time PCR some potential difficulties need attention: 1) the spore-coat hampers DNA-extraction; 2) the food matrix may inhibit bacterial DNA-extraction; 3) substances from the food matrix may interfere with the subsequent PCR reaction. Taking these points into account several studies investigated the efficiency of DNA-extraction protocols for Bacilli species (e.g. Dineen *et al.*, 2010; Dzieciol *et al.*, 2013; Martinez-Blanch *et al.*, 2010; Mertens *et al.*, 2014; Rawsthorne *et al.*, 2009; Thomas *et al.*, 2013).

Regarding the detection of *B. cereus* in spices and herbs by DNA-extraction combined with real-time PCR all of the above mentioned difficulties apply: 1) in condiments *B. cereus* persists in the spore form; 2) the insoluble powdery matrices cause problems with DNA-extraction as they are difficult to homogenize and clot filters; 3) plant compounds from spices and herbs are likely to interfere with the PCR reaction mixture (compare Focke *et al.*, 2011). In this regard, Cabicarová *et al.* (2016) substantially improved the PCR based detection sensitivity for *Staphylococcus aureus* in several condiments by incorporation of a washing step in the sample preparation protocol.

1.2.4 Occurrence and survival of *Bacillus cereus* in food

B. cereus is a frequent contaminant in virtually all kinds of food e.g. vegetables, flour, bakery products, egg, milk products (also pasteurized), ready-to-eat chilled foods and also spices and herbs with concentration mostly ranging from <10 to 10^6 cfu/g (EFSA, 2005).

Soils are the primary natural reservoir where *B. cereus* proliferates under favourable conditions as vegetative cell or survives in the spore form under unfavourable conditions. Thus, *B. cereus* can easily be transferred to plant derived foods via soil contamination or colonization of plants (EFSA, 2016). In various studies, *B. cereus* was determined in cultivated soils in concentrations of 10^4 - 10^5 cfu/g (EFSA, 2016). Regarding spices and herbs the cultivation, harvesting and processing steps may cause soil/dust contaminations (see also 1.1.3). Sagoo *et al.* (2009) analysed 2090 spice and 743 herb samples from UK retail and determined a portion of 8.5 % and 7.8 % with *B. cereus* counts above 10^3 cfu/g for spices and herbs, respectively. Also production sites were sampled (109 spice and 23 herb samples) and the proportion of samples containing more than 10^3 cfu/g *B. cereus* was 15.6 % and 30.4 %,

respectively. The gap between the proportions of retail compared to production batches probably reflects a decontamination step that at least inactivates a part of present *B. cereus* spores. Table 2 shows further examples of *B. cereus* concentrations determined in various spices and herbs in the past 15 years.

Table 2: Percentage of spice and herb samples containing *B. cereus*.

	Contamination level (cfu/g)				
Spice/Herb	**$<10^2$**	**10^2 - 10^4**	**$>10^4$ - 10^6**	**No. samples**	**Reference**
Allspice	67.0	33.0	0.0	6	Banerjee & Sarkar, 2003
	100.0	0.0	0.0	60	Hampikyan *et al.*, 2009
	100.0[a]	0.0	0.0	1	Hariram & Labbé, 2015
Anise seed	20.0	60.0	20.0	5	Banerjee & Sarkar, 2003
	100.0[a]	0.0	0.0	1	Hariram & Labbé, 2015
Black pepper	29.4	70.6	0.0	17	Aksu *et al.*, 2000
	50.0	50.0	0.0	6	Banerjee & Sarkar, 2003
	90.0	8.3	1.7	60	Hampikyan *et al.*, 2009
Cardamom	60.0	20.0	20.0	5	Banerjee & Sarkar, 2003
	0.0[a]	100.0	0.0	1	Hariram & Labbé, 2015
Chili powder	66.0	17.0	0.0	6	Banerjee & Sarkar, 2003
	100.0[a]	0.0	0.0	1	Hariram & Labbé, 2015
Cinnamon	40.0	60.0	0.0	5	Banerjee & Sarkar, 2003
	100.0[a]	0.0	0.0	1	Hariram & Labbé, 2015
Coriander	66.7	33.3	0.0	6	Aksu *et al.*, 2000
	14.0	72.0	14.0	7	Banerjee & Sarkar, 2003
	90.0	8.3	1.7	60	Hampikyan *et al.*, 2009
Cumin	50.0	50.0	0.0	6	Aksu *et al.*, 2000
	14.0	86.0	0.0	7	Banerjee & Sarkar, 2003
	93.3	3.3	3.3	60	Hampikyan *et al.*, 2009
Curry powder	25.0	75.0	0.0	4	Aksu *et al.*, 2000
	100.0[a]	0.0	0.0	1	Hariram & Labbé, 2015
Fenugreek	17.0	33.0	50.0	6	Banerjee & Sarkar, 2003
	100.0[a]	0.0	0.0	1	Hariram & Labbé, 2015
Ginger	60.0	40.0	0.0	5	Aksu *et al.*, 2000
	33.0	17.0	50.0	6	Banerjee & Sarkar, 2003
	83.3	11.7	5.0	60	Hampikyan *et al.*, 2009
	100.0[a]	0.0	0.0	1	Hariram & Labbé, 2015
Oregano	100.0[a]	0.0	0.0	1	Hariram & Labbé, 2015
Parsley	40.0	60.0	0.0	5	Aksu *et al.*, 2000
	0.0[a]	100.0	0.0	1	Hariram & Labbé, 2015
Thyme	0.0[a]	100.0	0.0	1	Hariram & Labbé, 2015
Turmeric	40.0	40.0	20.0	5	Banerjee & Sarkar, 2003
	0.0[a]	100.0	0.0	1	Hariram & Labbé, 2015
White pepper	96.7	3.3	0.0	60	Hampikyan *et al.*, 2009
	0.0[a]	100.0	0.0	1	Hariram & Labbé, 2015

[a]In case of Hariram & Labbé, 2015 the category $<10^2$ includes values from 3.6 to $<10^2$ MPN/g

Banerjee & Sarkar, 2003: Indish retail spices; Hariram & Labbé, 2015: US retail spices; Hampikyan *et al.*, 2009: Turkish retail spices; Aksu *et al.*, 2000: Turkish retail spices

As can be concluded from Table 2 the majority of spices and herbs contains relatively low *B. cereus* concentrations of less than 10^4 cfu/g. Combined with the usually low amounts of consumed condiments the risk of foodborne illness by a bare consumption of spices and herbs is very low concerning *B. cereus*. Nevertheless, condiments could act as carrier for *B. cereus* spores to other foods where proliferation is possible. The opportunity for spore germination and cell proliferation is however depending on various factors: nutrients (Abee *et al.*, 2011), water availability (no growth below a_w 0.92 (EFSA, 2005)), storage temperature (no growth below 4 °C for psychrophiles and 10 °C for mesophiles), pH-value (no growth below pH 4.5 (EFSA, 2005). Temperatures reached during common cooking or pasteurization procedures kill vegetative cells but are not sufficient to inactivate all spores definitely. The heat resistance of spores is strongly dependent on the food matrix. For example oily products significantly contribute to spore heat resistance (van Asselt & Zwietering, 2006). Moreover, spore heat resistance is highly strain variable - for example the time needed for a tenfold reduction of the spore number at 100 °C (D_{100}-value) may range from < 1 min to > 10 min (Mazas *et al.*, 1999). Especially spores of emetic strains appear to show a considerably higher heat resistance (Ankolekar & Labbe, 2009; Hariram & Labbe, 2016). In order to definitely kill all spores, food must be heated to 121 °C for at least three minutes (EFSA, 2016). Mild temperature treatments (70 - 80 °C for 10 minutes) can even accelerate the germination of *B. cereus* spores (Samapundo *et al.*, 2014). However, heat treatments at higher temperatures (90 °C) combined with subsequent cooling may delay the growth (Valero *et al.*, 2002).

Due to their resistance against chemical and physical stress *B. cereus* spores may remain viable in food for very long time. For example Kim *et al.* (2014) demonstrated constant spore numbers in rice wine for at least 28 days. Likewise Jaquette & Beuchat (1998) reported unchanged spore concentrations in dry cereal for 48 weeks. In comparison, for *B. thuringiensis* half-lives of 100 - 200 days have been observed in soil (EFSA, 2016). In contrast to that, half-lives on plant surfaces in the field and in the glasshouse were strikingly shorter (one to five days) indicating that UV-light (besides rain in the field) is the decisive factor for spore inactivation (EFSA, 2016).

1.3 Aims of the study

Spores of *B. cereus* are a frequent contaminant in spices and herbs which has been linked to several foodborne disease outbreaks. The demand for spices and their use in the industrial food production is growing. Thus, condiments may serve as a carrier for *B. cereus* spores to other food that enables germination and growth. However, cultural detection of *B. cereus* in spices and herbs can be problematic due to possible antimicrobial effects, high microbial loads and difficulties to distinguish between the closely related *B. cereus* group species. Due to these obstacles it is difficult to interpret and compare results of numerous studies.

Against this background, the first aim of this thesis was to establish a culture independent method to quantitatively detect spores of *B. cereus* group species in spices and herbs. This method should additionally provide taxonomic information in order to narrow down present *B. cereus* group members.

The second aim of the thesis was to characterize the pathogenic potential of *B. cereus* species isolated from spices and herbs. For this, strains should be analysed in terms of species classification, capability to produce toxins and belonging to phylogenetic groups associated with certain cytotoxicity.

In order to further assess the risk posed by *B. cereus* group species, the third aim was to investigate their long-term survival capacities in spices and herbs. Linked to this, possible matrix-effects on cultural detection methods for *B. cereus* due to antimicrobial acting substances should be evaluated.

2 Publications

2.1 List of publications and own contribution

Publication 1: Quantification and differentiation of *Bacillus cereus* group species in spices and herbs by real-time PCR

Hendrik Frentzel, Mai Dinh Thanh, Gladys Krause, Bernd Appel, Anneluise Mader; Food Control (2016) (Article in Press)

http://dx.doi.org/10.1016/j.foodcont.2016.11.028

I took major parts in the conception of the study. I performed all experimental work to incorporate an internal amplification control and to optimise sensitivity of the multiplex real-time PCR. I accomplished most experimental work for the DNA-extraction and spore quantification studies with assistance of Silke Bechlars. I analysed and interpreted the respective results and wrote the major part of the article.

Publication 2: Phylogenetic and toxinogenic characteristics of *Bacillus cereus* group members isolated from spices and herbs

Hendrik Frentzel, Britta Kraushaar, Gladys Krause, Dorina Bodi, Heidi Wichmann-Schauer, Bernd Appel, Anneluise Mader; Food Control (2016) (Article in Press)

http://dx.doi.org/10.1016/j.foodcont.2016.12.022

I took major parts in the conception of the study. I performed all experimental work for the cultural detection and isolation of presumptive *B. cereus* in spices and herbs and carried out the major part of the genotypic and phenotypic characterization of isolates. I optimised and executed the protocol for bacterial cereulide production and extraction, while cereulide detection by HPLC-MS was taken over by D. Bodi. I conducted a part of the experimental work and data analysis (using Accelrys Gene) for MLST and contributed to the interpretation of data by choosing reference sequences and labelling of the phylogenetic tree, while the major part of the experimental and analytical work for MLST, including the calculation of the phylogenetic tree, was accomplished by B. Kraushaar. I analysed and interpreted the respective results and wrote the major part of the article.

Publication 3: Tenacity of *Bacillus cereus* and *Staphylococcus aureus* in dried spices and herbs

Mai Dinh Thanh[1], Hendrik Frentzel[1], Alexandra Fetsch, Gladys Krause, Bernd Appel, Anneluise Mader; Food Control (2016) (Article in Press)

http://dx.doi.org/10.1016/j.foodcont.2016.12.027

[1] These authors contributed equally to this work.

I performed most conceptual work for the tenacity study concerning *B. cereus* and *B. thuringiensis* spores in spices and herbs. I performed most of the experimental work on Bacilli including spore production, spiking and cultural detection (with technical and conceptual assistance to ensure spiking within a single day by M. Dinh Thanh and A. Mader). The overall microbial status of spices and herbs was determined together with M. Dinh Thanh. I performed the statistical analysis for the *B. cereus*/*B. thuringiensis* data, interpreted these data and wrote the Bacilli-part of the article.

Publication 4: Impact of spiking techniques on the survival of *Staphylococcus aureus* in artificially contaminated condiments

Mai Dinh Thanh, Hendrik Frentzel, Alexandra Fetsch, Bernd Appel, Anneluise Mader; Food Control 73 (2017) 117-126

http://dx.doi.org/10.1016/j.foodcont.2016.10.021

I was involved in the conception of the study and assisted in the experimental work, especially the spiking process. I assisted the conception and the writing of the article.

2.2 Publication 1

Quantification and differentiation of *Bacillus cereus* group species in spices and herbs by real-time PCR

Hendrik Frentzel, Mai Dinh Thanh, Gladys Krause, Bernd Appel, Anneluise Mader; Food Control (2016) (Article in Press)
http://dx.doi.org/10.1016/j.foodcont.2016.11.028

2.3 Publication 2

Phylogenetic and toxinogenic characteristics of *Bacillus cereus* group members isolated from spices and herbs

Hendrik Frentzel, Britta Kraushaar, Gladys Krause, Dorina Bodi, Heidi Wichmann-Schauer, Bernd Appel, Anneluise Mader; Food Control (2016) (Article in Press)
http://dx.doi.org/10.1016/j.foodcont.2016.12.022

2.4 Publication 3

Publication 3: Tenacity of *Bacillus cereus* and *Staphylococcus aureus* in dried spices and herbs

Mai Dinh Thanh[1], Hendrik Frentzel[1], Alexandra Fetsch, Gladys Krause, Bernd Appel, Anneluise Mader; Food Control (2016) (Article in Press)
http://dx.doi.org/10.1016/j.foodcont.2016.12.027

[1] These authors contributed equally to this work.

2.5 Publication 4

Publication 4: Impact of spiking techniques on the survival of *Staphylococcus aureus* in artificially contaminated condiments

Mai Dinh Thanh, Hendrik Frentzel, Alexandra Fetsch, Bernd Appel, Anneluise Mader; Food Control 73 (2017) 117-126

http://dx.doi.org/10.1016/j.foodcont.2016.10.021

3 General discussion

3.1 Background

The EHEC-outbreak in 2011 with more than 3800 cases of illness and 53 fatalities exemplified the dramatic consequences than can be caused by a single batch of contaminated fenugreek seeds and the sprouts made thereof (BfR, 2011; RKI, 2011). Similar to spices and herbs, fenugreek seeds and sprouts are minor food ingredients in numerous food products and therefore one batch can be distributed broadly. In general, the demand for spices and herbs is growing. The majority of condiments are used in the industrial food production. Various ready-to-eat chilled foods are seasoned while there is a trend towards milder heat treatments and lower salt concentrations to keep nutritional and organoleptic properties in these products. This, however, may facilitate survival and outgrowth of spoilage or illness inducing bacteria, especially spore formers which resist usual heat treatments (Burt, 2004; Warda *et al.*, 2015; Witkowska *et al.*, 2011; Witkowska *et al.*, 2014). Also the catering sector processes considerable amounts of spices and herbs while storage temperature abuse of heat treated meals in catering or restaurant establishments is a main cause of foodborne outbreaks by *B. cereus* (EFSA, 2005). Spices and herbs have frequently been found to contain *B. cereus* spores (Aksu *et al.*, 2000; Banerjee & Sarkar, 2003; Hariram & Labbe, 2015; Sagoo *et al.*, 2009; Tajkarimi *et al.*, 2010) what has been linked to several foodborne disease outbreaks (EFSA, 2016). This highlights the potential of condiments to serve as a carrier for pathogens, especially spore formers such as *B. cereus*.

However, microbiological analysis of spices and herbs can be challenging due to a high microbial background flora and possible antimicrobial effects of suspensions. With regard to *B. cereus,* diagnostics is further complicated by the difficulties to distinguish the closely related *B. cereus* group species. Nevertheless, taxonomic information is important for assessing the risk posed by the occurrence of presumptive *B. cereus* in spices and herbs as not all *B. cereus* group species are considered typical foodborne pathogens. Thus, (rapid) diagnostic methods are needed that can be applied to spices and herbs and hold the potential to provide taxonomic information. For a more comprehensive understanding of the risks posed by *B. cereus* (s.l.) in spices and herbs also data on the toxinogenic potential as well as survival capacities of presumptive *B. cereus* isolates from spices and herbs are needed.

This thesis was prepared within the EU research project SPICED (Securing the spices and herbs commodity chains in Europe against deliberate, accidental and natural biological and chemical contamination) with the financial support from the 7th Framework Program of the EU. In this work molecular-biological detection methods for *B. cereus* group species and their

application on condiment matrices have been investigated. Moreover, the occurrence, the toxinogenic potential, the species and the phylogenetic relationship of presumptive *B. cereus* in spices and herbs were analysed. Additionally, the long-term survival of *B. cereus* as well as *B. thuringiensis* spores in condiments was determined. The following section summarizes the main results of the four publications included in this thesis and unpublished results by providing a general discussion.

3.2 Multiplex real-time PCR to detect *Bacillus cereus* group species in spices and herbs

Aiming at a rapid method that can be applied to spices and herbs and provide taxonomic information within the *B. cereus* group an approach based on DNA extraction combined with multiplex real-time PCR was investigated.

3.2.1 DNA-extraction from spores in condiment matrices

A prerequisite for the use of PCR based methods in microbiological food diagnostics is a successful DNA extraction from the target organism present in the food matrix. Numerous kit-based and open-formula DNA extraction protocols are available for different demands. For the purpose of this study the DNA-extraction procedure should be suitable for lysis of spores, applicable to difficult food matrices including the removal of PCR inhibiting substances, and at best easy to perform. In a first step, based on a literature survey, five DNA-extraction kits were chosen for further tests: 1) the DNeasy Blood & Tissue Kit; 2) the MasterPure Gram Positive DNA Purification Kit; 3) the Invisorb Spin Plant Mini Kit; 4) the UltraClean Microbial DNA Isolation Kit and 5) the PowerSoil DNA Isolation Kit. Applied to pure spore suspensions followed by real-time PCR the DNeasy Blood & Tissue Kit performed best with an LOD of 1.4 x 10^2 cfu/ml which was at least one $\log_{10}$ cfu/ml lower compared to all other kits. However, applied to artificially contaminated spices and herbs with *B. cereus* spores the LOD was 10^5 to 10^6 cfu/g for pepper, paprika and oregano and even higher for allspice. Therefore, the open-formula CTAB (cetyltrimethylammonium bromide)-DNA-extraction method was tested, as it was previously successfully applied to extract DNA from spices and herbs (Cabicarová *et al.*, 2016; Focke *et al.*, 2011). Using this method an LOD of 10^2 to 10^3 cfu/g for spores of *B. cereus* in pepper, paprika and oregano and 10^6 cfu/g in allspice could be achieved. Moreover, the internal amplification control of the PCR method was consistently positive using CTAB-derived DNA but not Blood & Tissue Kit-derived

DNA. Overall, these results indicate, firstly, that it is necessary to incorporate a combination of proteinase and K and lysozyme-treatment or of proteinase K and CTAB-treatment for successful *Bacillus* spore lyses. This finding was also reported by other authors (Mertens *et al.*, 2014). Secondly, for PCR based detection of bacterial DNA present in spices and herbs it is essential to remove inhibitory compounds during the DNA-extraction procedure. This conclusion is substantiated by findings of Focke *et al.* (2011) and Cabicarová *et al.* (2016) in their studies on DNA extraction from spices or *S. aureus* in spices, respectively.

3.2.2 Quantitative multiplex real-time PCR

The applied multiplex real-time PCR is based on primer-probe-combinations previously described by Oliwa-Stasiak *et al.* (2011) (detection of *B. cereus* (s.l.) with differentiation of *B. weihenstephanensis/mycoides* and *B. pseudomycoides*) and Wielinga *et al.* (2011) (detection of *B. anthracis* and *cry1*-positive *B. thuringiensis*). For the analysis of presumptive *B. cereus* isolates Krause *et al.*, (data unpublished) combined both protocols to a fourplex real-time PCR to detect *B. cereus* (s.l.) with differentiation of *B. pseudomycoides*, *B. weihenstephanensis/mycoides* and *cry1*-positive *B. thuringiensis* in one reaction and by the exchange of one primer-probe-pair also *B. anthracis* in a second reaction. Within the present work an internal amplification control based on the pUC 18/19 vector was incorporated in the multiplex PCR protocol to meet the requirements for direct food testing (Maurischat *et al.*, 2015) and reaction mix components were optimised to increase the sensitivity of the PCR.

With the aim to use this method for the quantification of *B. cereus* group spores the efficiency and the LOD of the PCR was determined using pure spore suspensions and the DNeasy Blood & Tissue Kit. Similar amplification efficiencies (86.8 % to 92.4 %) and LODs (~10^2 cfu/ml) for reference strains of the species *B. cereus* (s.s.), *B. weihenstephanensis*, *B. thuringiensis* and *B. pseudomycoides* indicated that spores of these species are similar sensitive towards the applied DNA-extraction procedure and the target sequences are equally amplified (even simultaneously) in the multiplex real-time PCR.

Subsequent to CTAB-DNA-extraction from artificially contaminated condiments with spores of one *B. cereus* strain (emetic *B. cereus* (s.s.)) the PCR method showed an LOD of 10^2 to 10^3 cfu/g, a high linear response to the contamination level ($R^2 \geq 0.98$) and mean recovery rates of 17.4% for oregano, 21.6% for paprika and 3.6 % for pepper which is in a similar range like recovery rates obtained by plate-counting. This demonstrates the suitability of the method for spore quantification in spices and herbs. When applied to pepper simultaneously contaminated with spores of four different species the LOD was 10^3 to 10^4 cfu/g.

The common way to gain DNA-sequence based data from target organisms in foods is the selective cultivation of these organisms and the subsequent characterization of isolates derived from enrichments of individual colonies. Compared to this procedure CTAB-DNA-extraction coupled to real-time PCR provides much faster results (possible within one day compared to three days using the traditional method). However, especially the DNA-extraction protocol is still very laborious and time consuming. In order to provide a more rapid method, future efforts should be directed towards an optimization of the DNA-extraction protocol.

A limitation of the applied multiplex real-time PCR protocol is the inclusivity for *B. thuringiensis* since it only captures *cry1*-positive variants. Although it detects important commercial biopesticidal strains such as *B. thuringiensis* subsp. *aizawai* ABTS-1875 (Xentari®), *B. thuringiensis* subsp. *kurstaki*, ABTS-351 (Dipel®) and *B. thuringiensis* subsp. *israelensis*, AM65-52 (StechmückenFrei®) (Krause & Ureta, data unpublished) it could miss a variety of natural occurring strains (Noguera & Ibarra, 2010; Thammasittirong & Attathom, 2008). Thus, future research could be directed towards a broader detection range of *B. thuringiensis* strains. A second limitation of the PCR is the exclusivity of the applied primer-probe-combination for *B. weihenstephanensis*. Firstly, these oligonucleotides also target some *B. mycoides* strains which would necessitate a cultural prove of rhizoid growth for exact species determination (Oliwa-Stasiak *et al.*, 2011). Secondly, later findings within this work indicated that phylogenetic information obtained by MLST does not always support the species declaration as *B. weihenstephanensis* based on real-time PCR (see section 3.3) which needs further investigation. Morover, MLST analysis also indicated that the applied *bpm*-sequence might not capture all *B. pseudomycoides* strains and therefore would be of interest for further investigations.

3.3 Characterization of *Bacillus cereus* group isolates from spices and herbs

In order to assess the risk associated to the presence of *B. cereus* group species in condiments, production batches of five spices and three herbs were microbiologically analysed. Obtained presumptive *B. cereus* isolates were further characterized with regard to their species status, their toxinogenic potential and their phylogenetic relationship.

3.3.1 *Bacillus cereus* group species in spices and herbs

The spices allspice, cinnamon, nutmeg, paprika and pepper and the herbs basil, oregano and parsley were analysed on presumptive *B. cereus* based on cultural detection. For further differentiation of prevailing species a combination of cultural, microscopic, PCR-based and MLST-based methods was applied.

Presumptive *B. cereus* was found in concentrations of 10^3 cfu/g in allspice, 10^1 - 10^2 cfu/g in basil, oregano, paprika, parsley and pepper, while no presumptive *B. cereus* was detectable in cinnamon and nutmeg (LOD = 20 cfu/g). These findings were in line with previous studies mostly reporting *B. cereus* concentrations of less than 10^4 cfu/g (Aksu *et al.*, 2000; Banerjee & Sarkar, 2003; Hampikyan *et al.*, 2009; Hariram & Labbe, 2015; Sagoo *et al.*, 2009). Moreover, all samples were below the DGHM warning value of 10^4 cfu/g *B. cereus* in spices and herbs (DGHM, 2011) and also below the threshold of 10^5 cfu/g that is considered as a food safety risk by EFSA (EFSA, 2016).

For further analyses, 59 isolates were randomly picked from the different matrices. Within these 59 isolates 44 were assigned to the species *B. cereus* (s.s.), five to *B. thuringiensis*, four to *B. toyonensis*-like, two to *B. mycoides/pseudomycoides* and one to *B. weihenstephanensis*, while another three isolates were not further classified due to contradictory results. A maximum of three different species was identified within one matrix (e.g. *B. cereus*, *B. thuringiensis* and *B. mycoides/pseudomycoides* in parsley). The overall proportion of *B. thuringiensis* of 8.5 % within our 59 isolates is similar to previous studies in different food types (4.3 to 12.5 % *B. thuringiensis* in spices and herbs, rice, milk and dairy products) (Ankolekar *et al.*, 2009; Cui *et al.*, 2016; Hariram & Labbe, 2015; Zhou *et al.*, 2008). Much higher proportions (39.1 %) were found in fresh fruit and vegetable products that were believed to be treated with *B. thuringiensis* based insecticides (Frederiksen *et al.*, 2006).

Interestingly, three of all together five *B. thuringiensis* strains were isolated from paprika in the present work. Based on an identical MLST sequence type of one of these *B. thuringiensis* strains and the *B. thuringiensis* subsp. *aizawai* ABTS-1875 strain applied in the commercial bio-insecticide Xentari® it can be hypothesised that a part of the *B. thuringiensis* population in the analysed paprika originates from bio-insecticidal residues.

The assignment to individual species was based on the following assumptions (EFSA, 2016; Jimenez *et al.*, 2013; Oliwa-Stasiak *et al.*, 2011; Priest *et al.*, 2004; Wielinga *et al.*, 2011): *B. cereus* (s.s.) are all presumptive *B. cereus* isolates that do not match descriptions of other *B. cereus* group species (definition per exclusion). *B. thuringiensis* was defined by the microscopically determined presence of parasporal crystals and/or a positive PCR-signal for

the *cry1*-gene (Wielinga *et al.*, 2011). The definition "*B. toyonensis*-like species" relied on a separate MLST-cluster. Assignment to *B. mycoides/pseudomycoides* was based on rhizoid colony morphology, PCR targeting the *bpm*-sequence (Oliwa-Stasiak *et al.*, 2011) and MLST. Finally, classification as *B. weihenstephanensis* was based on a positive PCR signal targeting a sequence-polymorphism in the *motB*-gene sequence (Oliwa-Stasiak *et al.*, 2011) and on MLST. *B. cytotoxicus* was ruled out due to a missing PCR signal for *cytK-1* (Guinebretiere *et al.*, 2006, 2013) and *B. anthracis* based on haemolysis and a missing PCR signal for the PL3-target (Wielinga *et al.*, 2011).

As mentioned above MLST analysis not always supported the conclusions drawn from PCR based classification. Only one of four isolates that were initially classified as *B. weihenstephanensis* based on non-rhizoid growth and PCR using the primer-probe-combination for the *motB*-gene-polymorphism (Oliwa-Stasiak *et al.*, 2011) clustered in close proximity to other *B. weihenstephanensis* and in the expected clade III in the MLST-derived phylogenetic tree. This indicates that the applied primer-probe-combination is not exclusive for *B. weihenstephanensis* (or *B. mycoides* in case of rhizoid growth) but captures as well *B. cereus* (s.s.) strains. Hence, further strains showing the *motB* polymorphism detected by the primer-probe-combination according to Oliwa-Stasiak *et al.* (2011) should be analysed with regard to other characteristics that are specific for *B. weihenstephanensis* such as growth at ≤ 7 °C but not at 43 °C or the presence of the psychrotrophic *cspA* and 16S rDNA signature (Lechner *et al.*, 1998; Stenfors & Granum, 2001). Another disagreement between applied species markers and MLST became obvious in case of the *bpm*-target. This target sequence was meant to be specific for *B. pseudomycoides* (Oliwa-Stasiak *et al.*, 2011). However, two of our isolates with rhizoid growth were not detected by the primer-probe-combination specific for *bpm* although they clearly branched within a *B. pseudomycoides* cluster in the MLST-derived phylogenetic tree and not within the separate *B. weihenstephanensis*/*B. mycoides* cluster. This indicates that not all *B. pseudomycoides* are captured by the primer-probe-combination targeting *bpm*. This assumption needs, however, further investigations to be substantiated.

In conclusion, by using cultural, microscopic, PCR-based and MLST-based methods it was possible to classify most isolates from spices and herbs to one of the currently validated *B. cereus* group species. However, species markers used for the identification of *B. weihenstephanensis* and *B. mycoides* were not always in agreement with findings from MLST analysis. Based on recent analysis of whole genome sequencing data the taxonomy of the *B. cereus* group will most likely by revised in the future probably resulting in 30 rather

than eight species (Liu *et al.*, 2015). Hence, the possibility to differentiate *B. cereus* group species will depend on capacities to generate and process data from DNA-sequencing in the future.

3.3.2 Toxinogenic potential of *Bacillus cereus* group species isolated from spices and herbs

In order to characterize the toxinogenic potential of *B. cereus* group species from spices and herbs the presence of enterotoxin genes and the *ces* gene was investigated based on PCR (Guinebretiere *et al.*, 2006; Wehrle *et al.*, 2010). In parallel, the actual capability to produce the enterotoxins *Nhe* and *Hbl* (Duopath immuno-assay) as well as the emetic toxin cereulide (HPLC-MS (Anonymous, 2014)) was analysed.

Except the two *B. pseudomycoides/B. mycoides* strains and one emetic *B. cereus* strain all isolates carried the *nheA* and *hblD* gene indicating the capability of producing the enterotoxins Nhe and Hbl. In the two *B. pseudomycoides/B. mycoides* strains no toxin genes could be detected, whereas the emetic strain possessed the *nheA*, the *cytK-2* and the *ces* genes. Half of the isolates that carried *nheA* and *hblD* additionally harboured the *cytK-2* gene. These prevalences (96.6 % *nheA*, 94.9 % *hblD*, 50.8 % *cytK-2* and 1.7 % *ces*) are in line with previously reported toxin gene prevalences (e.g. Ceuppens *et al.*, 2011): 84 - 100 % *nhe*; 29 - 92 % *hbl*; 37 - 89 % *cytK and* 1.5 % *ces*).

Most of our strains proved positive for the capacity to actually produce the implicated toxins. The Duopath assay, detecting the NheB-component of Nhe and the L2-component of Hbl (corresponding to HblC), was positive for all strains that carried the *nheA* and/or *hblD* genes. The only exception was the *B. weihenstephanensis* strain that was negative for Hbl-L2. As could be expected from missing toxin gene detection the two *B. pseudomycoides/B. mycoides* were negative in the Duopath assay.

Maximum cytotoxicity due to Nhe requires the presence of all three genes of the three-partite *nhe*-operon (*nheA*, *B* and *C*). The same is valid for toxicity due to Hbl that requires the presence of *hblC*, *D* and *A* (corresponding to the L2-, L1- and the binding component of Hbl) (Ceuppens *et al.*, 2011). To this effect our results indicate the following:

If *nheA* is present as determined by PCR also *nheB* is present as indirectly determined by the presence of NheB using the Duopath assay. The same applies to *hblD* (PCR) and *hblC* (detection of L2-component with Duopath assay) with the only exception of the *B. weihenstephanensis* strain negative for Hbl-L2.

For *nhe* genes this is in line with results of Wehrle *et al.* (2009) who found the complete *nhe*-operon in 173 out of 176 *B. cereus* group strains. One of the three remaining strains was found to be positive for *nheA* and *nheC* and the second strain only for *nheA*. The selective occurrence of these toxin genes was substantiated by enzyme immunoassays with selective antibodies against the individual toxin components. Moreover, these strains lacked cytotoxicity in Vero-cell and HEp-2 cell assays. In the third strain the complete *nhe*-operon was missing. Accordingly, no Nhe-components were detected in the immune-assay. However, the strain showed Vero cell cytotoxicity that could be ascribed to the presence of *cytK-1* in this strain. Indeed, this strain was later described as *B. cytotoxicus* known for a pronounced polymorphism in the *nheA*, *B* and *C* gene sequences (Fagerlund *et al.*, 2007) that is not captured by the applied primers and antibodies (Wehrle *et al.*, 2009). Guinebretiere *et al.* (2010) even assert that the *nhe* genes are a constant part of the genome of all *B. cereus* group strains based on a study of 391 *B. cereus* group strains of diverse phylogenetic groups (including *B. cytotoxicus*). However, the detection of the whole *nhe*-operon in all tested strains was only possible through additionally applied southern-blotting as the used PCR primers failed to capture all *nhe* gene variants (Guinebretiere *et al.*, 2002, 2010).

Also for the *hbl* genes the results of the present work are in accordance with Wehrle *et al.* (2009): in 78 strains that carried at least one *hbl* gene, 74 harboured the complete *hbl* operon, one carried *hblC* and *A* (confirmed by a selective immunoassay) while a further tree carried only *hblA* (not detected in the selective immunoassay). Based on a combination of PCR and southern-blotting also Ehling-Schulz *et al.* (2005) and Guinebretiere *et al.* (2002) detected the complete *hbl* operon in all strains that carried at least one *hbl* gene, with only one exception - a strain that harboured only *hblC*. Based on these comprehensive studies it can be assumed that at least all of the strains that were positive for *nheA* and *B* and/or *hblD* and *C* in our study, also possess the whole *nhe* and *hbl* operon.

A second implication of the positive Duopath assay for virtually all toxin gene positive strains in our study is that present toxin genes are also expressed, at least under suitable culture conditions. Also this finding is in agreement with previous studies that confirmed the production of enterotoxins Nhe and Hbl for the majority (though not all) of strains that carried the respective genes (Ehling-Schulz *et al.*, 2005; Guinebretiere *et al.*, 2002; Hariram & Labbe, 2015; Krause *et al.*, 2010; Wehrle *et al.*, 2009).

It should be mentioned that the detection of enterotoxins with the Duopath assay provides no information about the strains capacity to produce high or low toxin amounts or the capability to produce enterotoxins in natural conditions. A way of characterising strains as high or low

toxin producers is the sandwich enzyme immunoassay described by (Moravek *et al.*, 2006). However, this method is based on in-house generated antibodies not freely available. Another approach to approximate the toxicity of strains is the cytotoxicity assay using cell cultures, because cytotoxicity is correlated to the amount of produced enterotoxin (Jessberger *et al.*, 2014). Based on a range of methods providing phylogenetic resolution combined with growth temperature studies Guinebretiere *et al.* (2008) established seven phylogenetic groups. In a later study these groups were classified concerning the cytotoxic activity of their members (Guinebretiere *et al.*, 2010). According to Tourasse *et al.* (2011) MLST analysis can be correlated to the phylogenetic groups of Guinebretiere *et al.* (2008, 2010). In this regard, most of our isolates from spices and herbs belong to the group IV including mesophilic *B. cereus* and *B. thuringiensis* strains considered as moderate to high cytotoxic (Ceuppens *et al.*, 2011; Guinebretiere *et al.*, 2010). Some of our isolates were assigned to group III comprising mesophilic *B. cereus* (including emetic strains) as well as *B. thuringiensis* strains of low and high cytotoxicity and *B. anthracis* strains of high cytotoxicity. Few isolates were part of group VI consisting of *B. weihenstephanensis*, *B. mycoides* and *B. thuringiensis* strains of low cytotoxicity. It should be noted that the cytotoxicity in the study of Guinebretiere *et al.* (2010) was determined using the human colon carcinoma cell line (CaCo-2) which seems to resemble natural conditions more realistic than the routinely used Vero cells (stemming from the kidney) (Jessberger *et al.*, 2015). Even though cytotoxicity assays provide valuable information that can be used for risk assessment, they still do not take into account the natural environment for enterotoxin production which is the gut system, especially the small intestine (Ceuppens *et al.*, 2012b). Investigations on the enterotoxin production under gastrointestinal conditions indicate that bacterial growth and subsequent enterotoxin production only occurs in close proximity to intestinal epithelial cells (Ceuppens *et al.*, 2012b). This is attributed to strongly different growth conditions close to the mucus layer compared to the intestinal lumen with respect to oxygen concentration, viscosity, nutrient availability, competing bacterial community and immunological and antimicrobial molecules (Ceuppens *et al.*, 2012a). The hypothesis of growth in vicinity to intestinal epithelial cells is supported by the fact that CaCo-2 cells induce spore germination (Wijnands *et al.*, 2007). Additionally, produced enterotoxins would be quickly degraded by digestive enzymes in the intestinal lumen whereas they could act on epithelial cells before degradation upon toxin production near the host cells (Ceuppens *et al.*, 2012a, 2012b). Yet, at present no model is available that takes all of these aspects into account; i.e. simulates the gastrointestinal tract in the presence of intestinal epithelial cells (e.g. CaCo-2 cells).

One of our strains (BfR-BA-331) carried the *ces* gene and was also able to produce the toxin as measured by HPLC-MS. Also previous findings indicate that, if the *ces* gene is present, it is also expressed resulting in measurable cereulide production (Ehling-Schulz *et al.*, 2005). Our emetic strain was characterized by the MLST sequence type 869. Hitherto, all emetic strains were found in two distinct but closely related MLST clusters formed by the STs 26 and 165 as well as STs 144 and 164 (Ehling-Schulz *et al.*, 2005; Vassileva *et al.*, 2007). Both clusters can be found in clade 1 of the phylogenetic tree according to the classification of Priest *et al.* (2004) which corresponds to the phylogenetic group III in terms of Guinebretiere *et al.* (2008). This underlines the close genetic relationship of the emetic strains. Also BfR-BA-331 branches within the same clade, but it is not part of the previously known emetic clusters.

In conclusion, our results show that 57 of 59 isolates from spices and herbs were able to produce at least one enterotoxin. Only two *B. pseudomycoides*/*B. mycoides* strains lacked toxin production. Most isolates carried the *nheA* and *hblD* genes, while half of them carried additionally the *cytK-2* gene. One strain harboured the *ces* gene. The capability to actually produce toxins (Nhe, Hbl and cereulide) reflected the genetic configuration (except one strain). MLST analysis could link phylogenetic information to groups of certain cytotoxicity.

3.4 Tenacity of *Bacillus cereus* and *Bacillus thuringiensis* spores in spices and herbs

As outlined in section 1.1.5, spices and herbs can contain antimicrobial acting substances that also affect *B. cereus* in liquid test systems (Chaibi *et al.*, 1997; Lau *et al.*, 2014; Lawrence & Palombo, 2009; Singh *et al.*, 2002; Tajkarimi *et al.*, 2010). Given that, two questions arise: 1) "Are cultural detection methods used for presumptive *B. cereus* enumeration biased by antimicrobial effects?" and 2) "Do spices and herbs also affect the survival of *B. cereus* under low-moisture conditions?".

In order to address the first question, experiments were performed to examine a possible matrix-effect on standard cultural methods applied to spices and herbs. The results of these experiments are not part of the publications within this thesis and therefore further results are presented for the discussion. For these experiments, overnight-cell-cultures or spores of the emetic *B. cereus* strain DSM 4312 or the *B. thuringiensis* strain BfR-BA-316 isolated from Xentari® were added to 1:20 w/v condiment suspensions in peptone water (0.1 % w/v; Merck, Darmstadt, Germany) and a control (peptone water) to reach a final concentration of

~10^3 cfu/ml. Subsequently, the cfu/ml were determined by plate counting after 3 min, 45 min and 24 h of incubation in the suspensions at room-temperature. The experiment was repeated three times. Results of these trials are shown in Figure 1. Reduced cfu/ml compared to the control in the suspensions of cinnamon, nutmeg, oregano, pepper and, less pronounced, also basil indicate an influence on the enumeration of vegetative cells of *B. cereus* and *B. thuringiensis*. The reduced cfu/ml in suspensions of allspice reflect difficulties to count colonies due to a high microbial background flora in allspice rather than inhibitory effects. In contrast, suspensions of paprika and parsley did not affect the cell populations but even allowed growth within 24 h, as did the control.

All condiment matrices that affected vegetative cells were also tested on spores. Within 45 min no effects could be observed in any of the matrix-spore-suspensions. After 24 h the cfu/ml remained stable in suspensions of allspice, basil and cinnamon indicating that matrix effects inhibit the spore germination and subsequent growth. Slight reductions were detected in suspensions of pepper and oregano for both strains and additionally in nutmeg suspension for the *B. thuringiensis* strain. This indicates that suspensions of pepper, oregano and nutmeg could even have a slightly reducing effect on *B. cereus* and *B. thuringiensis* spores. Yet, based on our study design it cannot be specified if this effect was due to antimicrobial acting substances or due to competitive microflora that grew in the 24 h of incubation and partly also hampered colony-counting by unspecific growth on the selective agar plates.

In the thesis of Graubaum (2003) on enrichments of *Salmonella* spp. inhibitory effects were observed in suspensions of oregano, cinnamon and basil whereas no effects were seen for pepper, paprika, allspice, parsley and nutmeg. It should, however, be noted that Gram negative organisms are slightly less susceptible to essential oils from spices and herbs compared to Gram positive organisms (Burt, 2004).

Regarding the possible bias on plate counts due to antimicrobial effects it can be concluded that vegetative cells are likely to be underestimated in some condiment solutions. However, at a_w values of usually below 0.6 in dried condiments it can be expected that *B. cereus* naturally is only present in its spore form. The spores, however, were mostly unaffected by condiment suspensions. Within 45 min, which is advised as the maximum time between first dilution and spread plating (Anonymous, 2013), the determined cfu/ml were equal to the control. Constant or slightly reduced cfu/ml determined after 24 h indicate that enrichment (for example applied in MPN methods (Anonymous, 2006)) could be hampered.

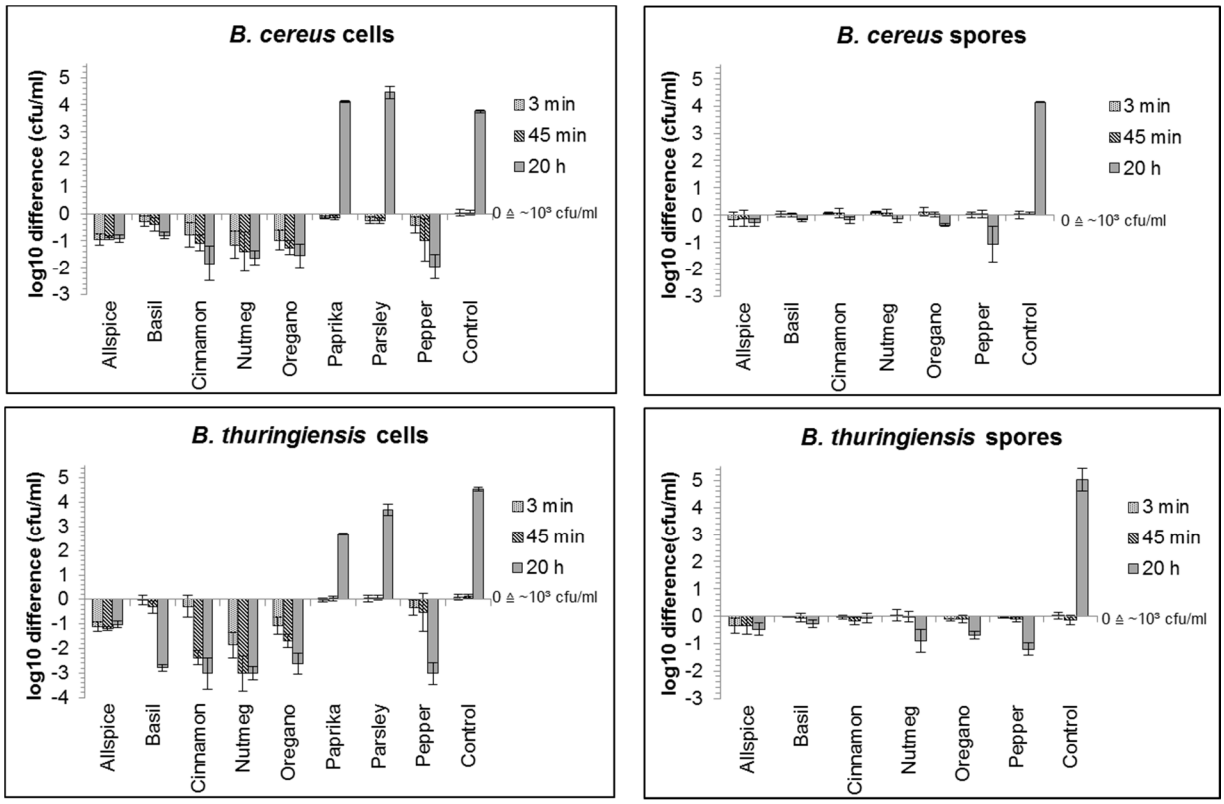

Figure 1: Influence of spices and herbs on cultural detection.

Differences in $\log_{10}$ cfu/ml between 1:20 w/v condiment suspensions and the control (peptone water) for the emetic *B. cereus* strain DSM 4312 and the *B. thuringiensis* strain BfR-BA-316 isolated from Xentari®. Cfu/ml were determined by plate counting after 3 min, 45 min and 24 h of incubation at room temperature (n = 3, LOD = 10 cfu/ml).

In order to answer the second question (influence of dry condiments), spores of the emetic *B. cereus* strain DSM 4312 or the *B. thuringiensis* strain BfR-BA-316 isolated from Xentari® were added to different spices (allspice, cinnamon, nutmeg, paprika and pepper) and herbs (basil, oregano and parsley) using a dry sand carrier to reach a final concentration of ~10^6 cfu/g. To account for possible strain variability the strains mentioned above and additionally spores of two strains previously isolated from oregano and allspice were applied to pepper, paprika and a sand control (~10^6 cfu/g). The artificially contaminated samples (with a_w values of 0.36 to 0.47) were sealed and stored in the dark at 24 ± 2 °C for 50 weeks. To demonstrate the tremendous differences between spores and vegetative cells, results of an equivalent study with *S. aureus* will be outlined below.

After 50 weeks of storage no statistical significant reduction was observed for any of the strains and any of the matrices. These results show that spores of *B. cereus* and *B. thuringiensis* remain unaffected in dry condiments. Concerning *B. cereus*, this finding is in

agreement with previous long-term studies on the natural *B. cereus* population in dried dill, basil and marjoram (Malmsten *et al.*, 1991) and artificially contaminated dry cereal (Jaquette & Beuchat, 1998). Regarding *B. thuringiensis*, significant reductions (46 - 77 %) were found within one week on greenhouse vegetables (Stephan *et al.*, 2014; Wang *et al.*, 2014) and even shorter half-lives of 16 - 38 h on vegetables in the field (EFSA, 2016; Pedersen *et al.*, 1995). In contrast, half-lives of 100 - 200 days were reported for *B. thuringiensis* spores in soil (EFSA, 2016; Pedersen *et al.*, 1995). Based on these findings and our results it can be concluded that a reduction of spores of *B. thuringiensis* strains (including commercial bio-insecticidal products) cannot be expected, if not UV-light or rain contributes to its inactivation or reduction.

In contrast to *Bacilli* spores, the effects on the survival of *S. aureus* cells spiked to dry condiments were much more pronounced. Within ten weeks of storage the cfu/g declined from the average initial contamination level of 8.1 ± 0.5 $\log_{10}$ cfu/g to 1.6 ± 1.5 $\log_{10}$ cfu/g. Moreover, significant differences in the times needed for reduction of 1 $\log_{10}$ cfu/g (*D* value) was observed between different matrices and also between different strains. In allspice, basil, nutmeg and paprika the *D* value was significantly lower compared to the sand control, whereas it was significantly higher in cinnamon.

3.5 Significance of spiking techniques on the results of tenacity studies

Data on the survival capacities of microorganisms in food are essential for assessing the risks associated to certain food-pathogen-combinations. Additionally, survival data can assist in outbreak situations when presumable causative agents must be narrowed down. Microbial survival in food can be investigated by tenacity studies which usually rely on an artificial contamination (spiking) of the concerned food matrix. As there are various options to contaminate food, the choice of the right spiking-approach is a crucial step to get evaluable results. The following aspects need consideration: 1) for most applications/questions the artificial contamination should be homogeneously distributed in the food matrix; 2) alterations of the food matrix should be kept to a minimum; 3) the spiking process should somehow resemble realistic ways of contamination. These aspects must especially be reflected in the method to transfer the microorganism to the food matrix and in the status of the cells (e.g. growing, stressed, injured or sporulated).

Given that, spiking of low-moisture foods, especially spices and herbs, is challenging. Applying a liquid inoculum directly to the food matrix (the most common way of spiking),

could alter the condiment matrix in terms of a_w-value and the release of antimicrobial acting compounds. Moreover, clump-formation may prevent homogeneous distribution of microorganisms. These drawbacks could be prevented by the use of dry inocula using freeze-dried cells which, however, strongly influences the cell conditions, or by the use of dried cells on an inert carrier which also influences cell conditions and to some extent also the food matrix.

On the example of *S. aureus* spiked to spices and herbs using all of the options mentioned above, it was shown that the spiking approach has a tremendous influence on the survival capacities (*D* values ranged from one to more than 100 days). Spiking of condiments with dried cells via a sand carrier was found to provide stable cell concentrations at the beginning of the experiment, accounted for a homogenous distribution and only slightly altered the matrix in terms of its composition.

Concerning *B. cereus*, contamination via dust or soil during harvesting and drying is a likely way of transferring spores to spices and herbs. Thus, balancing all advantages and disadvantages of different spiking-techniques, dry spiking using a sand carrier provides a practicable spiking-approach that resembles a realistic contamination path for *B. cereus* in condiments.

4 Summary

The Gram positive spore forming bacterium *B. cereus* can cause foodborne diseases with diarrhoeal and emetic symptoms. Spices and herbs have frequently been found to contain spores of *B. cereus* group species which has been linked to several foodborne disease outbreaks. The spice market is growing and a considerable part of condiments is used in the industrial food production and in the catering sector. Thus, spices and herbs can act as a vehicle to transfer *B. cereus* spores to various foodstuffs that might enable germination and growth under temperature abuse conditions.

The informative value of standard microbiological methods applied to spices and herbs is limited as they can be biased by a high microbial background flora and possible antimicrobial effects. Moreover, they neither provide information on the exact *B. cereus* group species nor on the toxinogenic potential. Hence, data on these parameters are scarce for presumptive *B. cereus* isolated from spices and herbs. To facilitate data gathering culture independent detection and characterization methods should be developed.

In this work a multiplex real-time PCR for the quantification of spores of *B. cereus* group species with simultaneous discrimination of *B. pseudomycoides*, *cry1*-positive *B. thuringiensis* as well as *B. weihenstephanensis*/*B. mycoides* was applied to artificially contaminated spices and herbs. For this, an internal amplification control was included in the PCR-method and different DNA-extraction procedures were evaluated. The best results with pure spore suspensions were achieved with the Qiagen DNeasy Blood & Tissue Kit coupled to quantitative real-time PCR (LOD ~10^2 cfu/ml). Yet, applied to spices and herbs kit based DNA-extraction yielded poor LODs of ≥10^5 cfu/g, whereas the open-formula CTAB-DNA-extraction achieved LODs of 10^2 to 10^3 cfu/g.

In order to further investigate the pathogenic potential of *B. cereus* group species naturally present in spices and herbs, we analysed presumptive *B. cereus* strains isolated from six different condiments with presumptive *B. cereus* concentrations of 10^1 to 10^3 cfu/g. In a total of 59 isolates, 44 were classified as *B. cereus*, five as *B. thuringiensis*, four as *B. toyonensis*-like, two as *B. pseudomycoides*/*B. mycoides* one as *B. weihenstephanensis*, and three as undefined *B. cereus* group species. Most isolates carried the enterotoxin genes *nheA* and *hblD* and were also able to produce the respective toxins (Nhe and Hbl). Half of the isolates additionally harboured the *cytK-2* gene. One isolate possessed the *ces* gene and was able to produce cereulide. For some isolates MLST analyses revealed disagreements between phylogenetic relationship and the classification as *B. weihenstephanensis* and *B. mycoides* based on previously described real-time PCR species markers (*motB* and *bpm* sequences).

Hence, the suitability of these markers for species differentiation should be further investigated.

For the purpose to investigate the long-term survival of *B. cereus* and *B. thuringiensis* in spices and herbs, tenacity studies were conducted using artificially contaminated condiments with spores of each species. After 50 weeks of storage in the dark no significant spore reduction could be observed for either strain. Beforehand, an appropriate spiking technique for spices and herbs was investigated and the suitability of the applied cultural detection method was evaluated. Using sand as a carrier proved to be a suitable method for dry-spiking. The examination of the cultural detection method indicated that the enumeration of vegetative cells in suspensions of spices and herbs can be biased due to substances with inhibitory effect on the bacteria. In contrast, spores remained unaffected unless stored for prolonged times in condiment suspension. This finding might be relevant for cultural enrichment.

5 Zusammenfassung

Das Gram positive sporenbildende Bakterium *B. cereus* kann lebensmittelbedingte Erkrankungen verursachen, die von Symptomen wie Durchfall, Übelkeit und Erbrechen geprägt sind. In Gewürzen und Kräutern wurden bisher häufig Spezies der *B. cereus*-Gruppe nachgewiesen, wobei deren Vorkommen bereits mit lebensmittelbedingten Krankheitsausbrüchen in Verbindung gebracht wurde. Der Gewürzmarkt wächst und ein beträchtlicher Teil der Gewürzprodukte wird in der industriellen Lebensmittelproduktion und im Gastronomiebereich eingesetzt. So können Gewürze und Kräuter als Vehikel fungieren, um *B. cereus* Sporen auf verschiedene Lebensmittel zu übertragen, in welchen sie bei Nicht-einhalten der optimalen Lagertemperaturen keimen und wachsen können.

Der Aussagewert von kulturellen mikrobiologischen Methoden, die an Gewürzen und Kräutern angewendet werden, ist begrenzt, da sie durch eine hohe mikrobielle Begleitflora und mögliche antimikrobielle Effekte beeinflusst werden. Darüber hinaus liefern sie weder Informationen über die exakte *B. cereus*-Gruppenspezies noch über die Toxinbildungs-fähigkeit. Daher fehlen Daten über diese Parameter für präsumtive *B. cereus* aus Gewürzen und Kräutern. Um solche Daten einfacher erfassen zu können, sind kulturunabhängige Detektions- und Charakterisierungsmethoden erstrebenswert.

In dieser Arbeit wurde eine Multiplex Real-Time PCR eingesetzt, um Sporen von Mitgliedern der *B. cereus*-Gruppe in künstlich kontaminierten Kräutern und Gewürzen zu quantifizieren und gleichzeitig *B. pseudomycoides*, *cry1*-positive *B. thuringiensis* sowie *B. weihenstephanensis*/*B. mycoides* von *B. cereus* zu unterscheiden. Dazu wurde eine interne Amplifikationskontrolle in die PCR-Methode integriert und verschiedene DNA-Extraktionsverfahren untersucht. Die besten Ergebnisse mit reinen Sporen-Suspensionen wurden mit dem Qiagen DNeasy Blood & Tissue Kit gekoppelt mit quantitativer Real-Time PCR erzielt (Detektionslimit ~10^2 KbE/ml). Für die mikrobielle DNA-Extraktion aus Gewürzen und Kräutern erwies sich die Kit-basierte DNA-Extraktion jedoch als ungeeignet (Nachweisgrenzen von $\geq 10^5$ KbE/g), wohingegen mit der CTAB-DNA-Extraktion Nachweisgrenzen von 10^2 bis 10^3 KbE/g erreicht wurden.

Um das pathogene Potenzial von präsumtiven *B. cereus* aus Kräutern und Gewürzen zu untersuchen, wurden Stämme aus verschiedenen Gewürzprodukten, mit präsumtiven *B. cereus*-Gehalten von 10^1 - 10^3 KbE/g, isoliert und näher charakterisiert. Von insgesamt 59 Isolaten wurden 44 als *B. cereus*, fünf als *B. thuringiensis*, vier als *B. toyonensis*-ähnlich, zwei als *B. pseudomycoides*/*B. mycoides*, eins als *B. weihenstephanensis* und drei als undefinierte

B. cereus-Gruppenspezies klassifiziert. Fast alle Isolate trugen die Enterotoxin-Gene *nheA* und *hblD* und konnten auch die entsprechenden Toxine (Nhe und Hbl) produzieren. Die Hälfte der Stämme enthielt zusätzlich das *cytK-2*-Gen. Ein Stamm besaß das *ces*-Gen und konnte Cereulid produzieren. Für einige Isolate zeigten die MLST-Analysen Widersprüche zwischen phylogenetischer Einordnung und der Klassifikation als *B. weihenstephanensis* und *B. mycoides*, basierend auf zuvor beschriebenen Real-Time PCR-Speziesmarkern (*motB* und *bpm* Sequenzen). Die Eignung dieser Marker zur Speziesabgrenzung sollte daher in weiteren Studien genauer untersucht werden.

Mit dem Ziel das Langzeitüberleben von *B. cereus* und *B. thuringiensis* in Gewürzen und Kräutern zu untersuchen, wurden Tenazitätsstudien mit Sporen dieser Spezies in verschiedenen Gewürzprodukten durchgeführt. Nach 50-wöchiger Lagerung konnte bei keiner Spezies eine signifikante Sporenreduktion beobachtet werden. Im Vorfeld wurde eine zweckmäßige Spiking-Technik für Gewürze etabliert und die Eignung der angewandten kulturellen Detektionsmethode überprüft. Die Verwendung von Sand als Träger erwies sich als eine geeignete Methode zum Trocken-Spiken. Bei der Überprüfung der kulturellen Nachweismethode stellte sich heraus, dass die Quantifizierung von vegetativen Zellen in Gewürzen und Kräutern aufgrund von antimikrobiellen Substanzen in der Erstverdünnung beeinflusst sein kann. Im Gegensatz dazu zeigte sich kein Effekt auf den Nachweis von Sporen, wenn sie nicht für längere Zeiträume der Gewürzsuspension ausgesetzt waren, wie dies beispielsweise bei Anreicherungen der Fall wäre.

6 References

Abee, T., Groot, M. N., Tempelaars, M., Zwietering, M., Moezelaar, R., & van der Voort, M. (2011). Germination and outgrowth of spores of Bacillus cereus group members: Diversity and role of germinant receptors. *Food Microbiology, 28*(2), 199-208.

Agata, N., Ohta, M., Mori, M., & Isobe, M. (1995). A novel dodecadepsipeptide, cereulide, is an emetic toxin of bacillus-cereus. *FEMS Microbiology Letters, 129*(1), 17-19.

Agata, N., Ohta, M., & Yokoyama, K. (2002). Production of Bacillus cereus emetic toxin (cereulide) in various foods. *International Journal of Food Microbiology, 73*(1), 23-27.

AIT, & AGES. (2013). Food of plant origin: production methods and microbiological hazards linked to food-borne disease. Reference: CFT/EFSA/BIOHAZ/2012/01 Lot 2 (Food of plant origin with low water content such as seeds, nuts, cereals, and spices), in Supporting Publications 2013:EN-403. 2013. p. 1-160). www.efsa.europa.eu/publications

Aksu, H., Bostan, K., & Ergün, Ö. (2000). Presence of Bacillus cereus in Packaged Some Spices and Herbs Sold in Istanbul. *Pakistan Journal of Biological Sciences, 3*(5), 710-712.

Ankolekar, C., & Labbe, R. G. (2009). Survival during Cooking and Growth from Spores of Diarrheal and Emetic Types of Bacillus cereus in Rice. *Journal of Food Protection, 72*(11), 2386-2389.

Ankolekar, C., Rahmati, T., & Labbe, R. G. (2009). Detection of toxigenic Bacillus cereus and Bacillus thuringiensis spores in US rice. *International Journal of Food Microbiology, 128*(3), 460-466.

Anonymous. (2002). Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

Anonymous. (2003a). Commission Recommendation of 19 December 2003 concerning a coordinated programme for the official control of foodstuffs for 2004.

Anonymous. (2003b). Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC.

Anonymous. (2004). Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs.

Anonymous. (2005). Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs.

Anonymous. (2006). Microbiology of food and animal feeding stuffs - Horizontal method for the determination of low numbers of presumptive Bacillus cereus - Most probable number technique and detection method (ISO 21871:2006). *Beuth Verlag GmbH, Köln, Germany*.

Anonymous. (2012). Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination (6887-4:2012-01). *Beuth Verlag GmbH, Köln, Germany*.

Anonymous. (2013). Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations (ISO 7218:2007 + Amd 1:2013). *Beuth Verlag GmbH, Köln, Germany*.

Anonymous. (2014). Microbiology of the food chain - Quantitative analysis of emetic toxin (cereulide) using LC-MS/MS (ISO 18465:2014-11 - Draft). *Beuth Verlag GmbH, Köln, Germany*.

Banach, J. L., Stratakou, I., van der Fels-Klerx, H. J., Besten, H. M. W. d., & Zwietering, M. H. (2016). European alerting and monitoring data as inputs for the risk assessment of microbiological and chemical hazards in spices and herbs. *Food Control, 69*, 237-249.

Banerjee, M., & Sarkar, P. K. (2003). Microbiological quality of some retail spices in India. *Food Research International, 36*(5), 469-474.

Berliner, E. (1915). Über die Schlaffsucht der Mehlmottenraupe (*Ephestia kühniella Zell)* und ihren Erreger *Bacillus thuringiensis n sp*. *Zeitschrift fur angewandte Entomologie, 2*, 29-56.

BfR. (2011). EHEC-Ausbruch 2011, Aufklärung des Ausbruchs entlang der Lebensmittelkette. http://www.bfr.bund.de/cm/350/ehec-ausbruch-2011-aufklaerung-des-ausbruchs-entlang-der-lebensmittelkette.pdf

Bravo, A., Likitvivatanavong, S., Gill, S. S., & Soberon, M. (2011). Bacillus thuringiensis: A story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology, 41*(7), 423-431.

Brodowska, A. J., Smigielski, K., Nowak, A., Brodowska, K., Catthoor, R., & Czyzowska, A. (2014). The impact of ozone treatment on changes in biologically active substances of cardamom seeds. *Journal of Food Science, 79*(9), C1649-C1655.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. *International Journal of Food Microbiology, 94*(3), 223-253.

Cabicarová, T., Kaclíková, E., Mader, A., Minarovičová, J., Koreňová, J., & Kuchta, T. (2016). Improvement of the detection sensitivity for staphylococcus aureus in spices and herbs. *Food Analytical Methods, 9*(7), 1980-1984.

CAC. (1995). Code of hygienic practice for spices and dried aromatic herbs. pp. 17. http://www.greenfoodec.eu/documents/codeofhygienicpracticeforspicesanddriedaromaticplants.pdf

CAC. (2015). Code of hygienic practice for low-moisture foods. pp. 10. www.fao.org

Cardazzo, B., Negrisolo, E., Carraro, L., Alberghini, L., Patarnello, T., & Giaccone, V. (2008). Multiple-locus sequence typing and analysis of toxin genes in Bacillus cereus food-borne isolates. *Applied and Environmental Microbiology, 74*(3), 850-860.

Carlin, F., Fricker, M., Pielaat, A., Heisterkamp, S., Shaheen, R., Salonen, M. S., Svensson, B., Nguyen-The, C., & Ehling-Schulz, M. (2006). Emetic toxin-producing strains of Bacillus cereus show distinct characteristics within the Bacillus cereus group. *International Journal of Food Microbiology, 109*(1-2), 132-138.

CBI. (2009). CBI market survey: the spices and herbs market in the EU. http://www.greenfoodec.eu/documents/The_spices_and_herbs_market_in_the_EU.pdf

CBI. (2015). CBI trade statistics: Spices and herbs. www.cbi.eu/sites/default/files/trade-statistics-europe-spices-herbs-2015.pdf

Ceuppens, S., Rajkovic, A., Hamelink, S., van de Wiele, T., Boon, N., & Uyttendaele, M. (2012a). Enterotoxin production by Bacillus cereus under gastrointestinal conditions and their immunological detection by commercially available kits. *Foodborne Pathogens and Disease, 9*(12), 1130-1136.

Ceuppens, S., Rajkovic, A., Heyndrickx, M., Tsilia, V., van De Wiele, T., Boon, N., & Uyttendaele, M. (2011). Regulation of toxin production by Bacillus cereus and its food safety implications. *Critical Reviews in Microbiology, 37*(3), 188-213.

Ceuppens, S., Uyttendaele, M., Drieskens, K., Heyndrickx, M., Rajkovic, A., Boon, N., & Van de Wiele, T. (2012b). Survival and germination of Bacillus cereus spores without outgrowth or enterotoxin production during in vitro simulation of gastrointestinal transit. *Applied and Environmental Microbiology, 78*(21), 7698-7705.

Chaibi, A., Ababouch, L. H., Belasri, K., Boucetta, S., & Busta, F. F. (1997). Inhibition of germination and vegetative growth of Bacillus cereus T and Clostridium botulinum 62A spores by essential oils. *Food Microbiology, 14*(2), 161-174.

Chon, J. W., Song, K. Y., Kim, H., & Seo, K. H. (2014). Comparison of 3 selective media for enumeration of bacillus cereus in several food matrixes. *Journal of Food Science, 79*(12), M2480-M2484.

Cohn, F. (1872). Untersuchungen über Bakterien *Beitrage zur Biologie der Pflanzen, 1*(1875 1 (Heft 2)), 127–224.

Contzen, M., Hailer, M., & Rau, J. (2014). Isolation of Bacillus cytotoxicus from various commercial potato products. *International Journal of Food Microbiology, 174*, 19-22.

Cui, Y. F., Liu, X. Y., Dietrich, R., Märtlbauer, E., Cao, J., Ding, S. Y., & Zhu, K. (2016). Characterization of Bacillus cereus isolates from local dairy farms in China. *FEMS Microbiology Letters, 363*(12), 6.

Delbrassinne, L., Botteldoorn, N., Andjelkovic, M., Dierick, K., & Denayer, S. (2015). An emetic Bacillus cereus outbreak in a kindergarten: Detection and quantification of critical levels of cereulide toxin. *Foodborne Pathogens and Disease, 12*(1), 84-87.

DGHM. (2011). Veröffentlichte mikrobiologische Richt- und Warnwerte zur Beurteilung von Lebensmitteln. http://www.dghm.org/m_275.

Dierick, K., Van Coillie, E., Swiecicka, I., Meyfroidt, G., Devlieger, H., Meulemans, A., Hoedemaekers, G., Fourie, L., Heyndrickx, M., & Mahillon, J. (2005). Fatal family outbreak of Bacillus cereus-associated food poisoning. *Journal of Clinical Microbiology, 43*(8), 4277-4279.

Dietrich, R., Fella, C., Strich, S., & Märtlbauer, E. (1999). Production and characterization of monoclonal antibodies against the hemolysin BL enterotoxin complex produced by Bacillus cereus. *Applied and Environmental Microbiology, 65*(10), 4470-4474.

Dineen, S. M., Aranda, R., Anders, D. L., & Robertson, J. M. (2010). An evaluation of commercial DNA extraction kits for the isolation of bacterial spore DNA from soil. *Journal of Applied Microbiology, 109*(6), 1886-1896.

Dzieciol, M., Fricker, M., Wagner, M., Hein, I., & Ehling-Schulz, M. (2013). A novel diagnostic real-time PCR assay for quantification and differentiation of emetic and non-emetic Bacillus cereus. *Food Control, 32*(1), 176-185.

EFSA. (2005). Opinion of the Scientific Panel on Biological Hazards on Bacillus cereus and other Bacillus spp in foodstuffs. *EFSA Journal, 175*, 1-48.

EFSA. (2013). Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations). *EFSA Journal, 11*(1:3025), pp. 138.

EFSA. (2014). Scientific Opinion on the safety and efficacy of Toyocerin® (Bacillus toyonensis) as a feed additive for rabbits for fattening, chickens for fattening, weaned piglets, pigs for fattening, sows for reproduction, cattle for fattening, and calves for rearing. *EFSA Journal, 12*(7), 17.

EFSA. (2016). Scientific opinion on the risks for public health related to the presence of Bacillus cereus and other Bacillus spp. including Bacillus thuringiensis in foodstuffs. . *EFSA Journal, 14*(7), 93.

Ehling-Schulz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M., & Scherer, S. (2006). Cereulide synthetase gene cluster from emetic Bacillus cereus: Structure and location on a mega virulence plasmid related to Bacillus anthracis toxin plasmid pXOI. *Bmc Microbiology, 6*.

Ehling-Schulz, M., Svensson, B., Guinebretiere, M. H., Lindback, T., Andersson, M., Schulz, A., Fricker, M., Christiansson, A., Granum, P. E., Märtlbauer, E., Nguyen-The, C., Salkinoja-Salonen, M., & Scherer, S. (2005). Emetic toxin formation of Bacillus cereus is restricted to a single evolutionary lineage of closely related strains. *Microbiology-Sgm, 151*, 183-197.

Eliasson, L., Libander, P., Lovenklev, M., Isaksson, S., & Ahrne, L. (2014). Infrared decontamination of oregano: Effects on Bacillus cereus spores, water activity, color, and volatile compounds. *Journal of Food Science, 79*(12), E2447-E2455.

Erdogdu, S. B., & Ekiz, H. I. (2011). Effect of ultraviolet and far infrared radiation on microbial decontamination and quality of cumin seeds. *Journal of Food Science, 76*(5), M284-M292.

ESA. (2016). ESA Definitions of culinary herbs and spices. In.

Fagerlund, A., Brillard, J., Fürst, R., Guinebretiere, M. H., & Granum, P. E. (2007). Toxin production in a rare and genetically remote cluster of strains of the Bacillus cereus group. *Bmc Microbiology, 7*.

Fagerlund, A., Lindback, T., & Granum, P. E. (2010). Bacillus cereus cytotoxins Hbl, Nhe and CytK are secreted via the Sec translocation pathway. *Bmc Microbiology, 10*.

Flügge, C. (1886). Die Mikroorganismen *F.C.W., Vogel, Leipzig*.

Focke, F., Haase, I., & Fischer, M. (2011). DNA-based identification of spices: DNA isolation, whole genome amplification, and polymerase chain reaction. *Journal of Agricultural and Food Chemistry, 59*(2), 513-520.

Francis, K. P., Mayr, R., von Stetten, F., Stewart, G., & Scherer, S. (1998). Discrimination of psychrotrophic and mesophilic strains of the Bacillus cereus group by PCR targeting

of major cold shock protein genes. *Applied and Environmental Microbiology, 64*(9), 3525-3529.

Frankland, G. C. F., Percy F. (1887). Studies on some new microorganisms obtained from air. *Philosophical Transactions of the Royal Society B, 178*, 257–287.

Frederiksen, K., Rosenquist, H., Jorgensen, K., & Wilcks, A. (2006). Occurrence of natural Bacillus thuringiensis contaminants and residues of Bacillus thuringiensis-based insecticides on fresh fruits and vegetables. *Applied and Environmental Microbiology, 72*(5), 3435-3440.

Fricker, M., Reissbrodt, R., & Ehling-Schulz, M. (2008). Evaluation of standard and new chromogenic selective plating media for isolation and identification of Bacillus cereus. *International Journal of Food Microbiology, 121*(1), 27-34.

Granum, P. E., O'Sullivan, K., & Lund, T. (1999). The sequence of the non-haemolytic enterotoxin operon from Bacillus cerceus. *FEMS Microbiology Letters, 177*(2), 225-229.

Graubaum, D. (2003). *Dissertation: Zur Notwendigkeit einer Voranreicherung im Verhältnis 1:100 beim Nachweis von Salmonellen in Gewürzen.* Freien Universität Berlin, Berlin.

Guinebretiere, M. H., Auger, S., Galleron, N., Contzen, M., De Sarrau, B., De Buyser, M. L., Lamberet, G., Fagerlund, A., Granum, P. E., Lereclus, D., De Vos, P., Nguyen-The, C., & Sorokin, A. (2013). Bacillus cytotoxicus sp nov is a novel thermotolerant species of the Bacillus cereus Group occasionally associated with food poisoning. *International Journal of Systematic and Evolutionary Microbiology, 63*, 31-40.

Guinebretiere, M. H., Broussolle, V., & Nguyen-The, C. (2002). Enterotoxigenic profiles of food-poisoning and food-borne Bacillus cereus strains. *Journal of Clinical Microbiology, 40*(8), 3053-3056.

Guinebretiere, M. H., Fagerlund, A., Granum, P. E., & Nguyen-The, C. (2006). Rapid discrimination of cytK-1 and cytK-2 genes in Bacillus cereus strains by a novel duplex PCR system. *FEMS Microbiology Letters, 259*(1), 74-80.

Guinebretiere, M. H., Thompson, F. L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M., & De Vos, P. (2008). Ecological diversification in the Bacillus cereus Group. *Environmental Microbiology, 10*(4), 851-865.

Guinebretiere, M. H., Velge, P., Couvert, O., Carlin, F., Debuyser, M. L., & Nguyen-The, C. (2010). Ability of Bacillus cereus group strains to cause food poisoning varies

according to phylogenetic affiliation (groups I to VII) rather than species affiliation. *Journal of Clinical Microbiology, 48*(9), 3388-3391.

Hampikyan, H., Bingol, E. B., Colak, H., & Aydin, A. (2009). The evaluation of microbiological profile of some spices used in Turkish meat industry. *Journal of Food Agriculture & Environment, 7*(3-4), 111-115.

Hariram, U., & Labbe, R. (2015). Spore prevalence and toxigenicity of Bacillus cereus and Bacillus thuringiensis isolates from US retail spices. *Journal of Food Protection, 78*(3), 590-596.

Hariram, U., & Labbe, R. G. (2016). Growth and inhibition by spices of growth from spores of enterotoxigenic Bacillus cereus in cooked rice. *Food Control, 64*, 60-64.

Hendriksen, N. B., & Hansen, B. M. (2011). Diagnostic properties of three conventional selective plating media for selection of Bacillus cereus, B. thuringiensis and B. weihenstephanensis. *Folia Microbiologica, 56*(6), 535-539.

ICMSF. (2005). Spices, dry soups, and oriental flavorings. In *Microorganisms in Foods 6* (2 ed.). New York, Boston, Dordrecht London, Moscow: Kluwer Academic / Plenum Publishers.

Jaquette, C. B., & Beuchat, L. R. (1998). Survival and growth of psychrotrophic Bacillus cereus in dry and reconstituted infant rice cereal. *Journal of Food Protection, 61*(12), 1629-1635.

Jessberger, N., Dietrich, R., Bock, S., Didier, A., & Märtlbauer, E. (2014). Bacillus cereus enterotoxins act as major virulence factors and exhibit distinct cytotoxicity to different human cell lines. *Toxicon, 77*, 49-57.

Jessberger, N., Krey, V. M., Rademacher, C., Böhm, M. E., Mohr, A. K., Ehling-Schulz, M., Scherer, S., & Märtlbauer, E. (2015). From genome to toxicity: a combinatory approach highlights the complexity of enterotoxin production in Bacillus cereus. *Frontiers in Microbiology, 6*, 15.

Jimenez, G., Urdiain, M., Cifuentes, A., Lopez-Lopez, A., Blanch, A. R., Tamames, J., Kampfer, P., Kolsto, A. B., Ramon, D., Martinez, J. F., Codoner, F. M., & Rossello-Mora, R. (2013). Description of Bacillus toyonensis sp nov., a novel species of the Bacillus cereus group, and pairwise genome comparisons of the species of the group by means of ANI calculations. *Systematic and Applied Microbiology, 36*(6), 383-391.

Jung, M. Y., Kim, J. S., Paek, W. K., Lim, J., Lee, H., Il Kim, P., Ma, J. Y., Kim, W., & Chang, Y. H. (2011). Bacillus manliponensis sp nov., a new member of the Bacillus

cereus group isolated from foreshore tidal flat sediment. *Journal of Microbiology, 49*(6), 1027-1032.

Jung, M. Y., Paek, W. K., Park, I. S., Han, J. R., Sin, Y., Paek, J., Rhee, M. S., Kim, H., Song, H. S., & Chang, Y. H. (2010). Bacillus gaemokensis sp nov., isolated from foreshore tidal flat sediment from the Yellow Sea. *Journal of Microbiology, 48*(6), 867-871.

Kim, S. A., Park, H. J., Lee, S. H., Hwang, I. G., & Rhee, M. S. (2014). Short communication: Fate of major foodborne pathogens and Bacillus cereus spores in sterilized and non-sterilized Korean turbid rice wine (Makgeolli). *Food Control, 39*, 139-145.

Krause, N., Moravek, M., Dietrich, R., Wehrle, E., Slaghuis, J., & Märtlbauer, E. (2010). Performance characteristics of the Duopath (R) Cereus Enterotoxins assay for rapid detection of enterotoxinogenic Bacillus cereus strains. *International Journal of Food Microbiology, 144*(2), 322-326.

Lau, K. Y., Zainin, N. S., Abas, F., & Rukayadi, Y. (2014). Antibacterial and sporicidal activity of Eugenia polyantha Wight against Bacillus cereus and Bacillus subtilis. *International Journal of Current Microbiology and Applied Science, 3*(12), 499 - 510.

Lawrence, H. A., & Palombo, E. A. (2009). Activity of essential oils against Bacillus subtilis spores. *Journal of Microbiology and Biotechnology, 19*(12), 1590-1595.

Lechner, S., Mayr, R., Francis, K. P., Pruss, B. M., Kaplan, T., Wiessner-Gunkel, E., Stewartz, G., & Scherer, S. (1998). Bacillus weihenstephanensis sp. nov. is a new psychrotolerant species of the Bacillus cereus group. *International Journal of Systematic Bacteriology, 48*, 1373-1382.

Lindback, T., Fagerlund, A., Rodland, M. S., & Granum, P. E. (2004). Characterization of the Bacillus cereus Nhe enterotoxin. *Microbiology-Sgm, 150*, 3959-3967.

Liu, B., Liu, G. H., Hu, G. P., Cetin, S., Lin, N. Q., Tang, J. Y., Tang, W. Q., & Lin, Y. Z. (2014). Bacillus bingmayongensis sp nov., isolated from the pit soil of Emperor Qin's Terra-cotta warriors in China. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 105*(3), 501-510.

Liu, Y., Lai, Q. L., Goker, M., Meier-Kolthoff, J. P., Wang, M., Sun, Y. M., Wang, L., & Shao, Z. Z. (2015). Genomic insights into the taxonomic status of the Bacillus cereus group. *Scientific Reports, 5*, 11.

Logan, N. A. (2012). Bacillus and relatives in foodborne illness. *Journal of Applied Microbiology, 112*(3), 417-429.

Mader, A., & Schaarschmidt, S. (2015). Bacteriological safety of spices microbiological contamination can lead to outbreaks of foodborne disease. *Fleischwirtschaft, 95*(11), 42-47.

Mahler, H., Pasi, A., Kramer, J. M., Schulte, P., Scoging, A. C., Bar, W., & Krahenbuhl, S. (1997). Fulminant liver failure in association with the emetic toxin of Bacillus cereus. *New England Journal of Medicine, 336*(16), 1142-1148.

Malmsten, T., Paakkonen, K., & Hyvonen, L. (1991). Packaging and storage effects on microbiological quality of dried herbs. *Journal of Food Science, 56*(3), 873-875.

Martinez-Blanch, J. F., Sanchez, G., Garay, E., & Aznar, R. (2009). Development of a real-time PCR assay for detection and quantification of enterotoxigenic members of Bacillus cereus group in food samples. *International Journal of Food Microbiology, 135*(1), 15-21.

Martinez-Blanch, J. F., Sanchez, G., Garay, E., & Aznar, R. (2010). Evaluation of a real-time PCR assay for the detection and quantification of Bacillus cereus group spores in food. *Journal of Food Protection, 73*(8), 1480-1485.

Marxen, S., Stark, T. D., Frenzel, E., Ruetschle, A., Luecking, G., Puerstinger, G., Pohl, E. E., Scherer, S., Ehling-Schulz, M., & Hofmann, T. (2015). Chemodiversity of cereulide, the emetic toxin of Bacillus cereus. *Analytical and Bioanalytical Chemistry, 407*(9), 2439-2453.

Maurischat, S., Baumann, B., Martin, A., & Malorny, B. (2015). Rapid detection and specific differentiation of Salmonella enterica subsp enterica Enteritidis, Typhimurium and its monophasic variant 4, 5 ,12:i: - by real-time multiplex PCR. *International Journal of Food Microbiology, 193*, 8-14.

Mazas, M., Martinez, S., Lopez, M., Alvarez, A. B., & Martin, R. (1999). Thermal inactivation of Bacillus cereus spores affected by the solutes used to control water activity of the heating medium. *International Journal of Food Microbiology, 53*(1), 61-67.

McKee, L. H. (1995). Microbial contamination of spices and herbs: a review. *Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 28*(1), 1-11.

Mertens, K., Freund, L., Schmoock, G., Hansel, C., Melzer, F., & Elschner, M. C. (2014). Comparative evaluation of eleven commercial DNA extraction kits for real-time PCR detection of Bacillus anthracis spores in spiked dairy samples. *International Journal of Food Microbiology, 170*, 29-37.

Messelhäußer, U., & Ehling-Schulz, M. (2014). *Bacillus cereus - Vorkommen, Nachweis und Präventionsstrategien* (Vol. 2). Hamburg: B. Behr´s Verlag.

Messelhäußer, U., Frenzel, E., Blochinger, C., Zucker, R., Kampf, P., & Ehling-Schulz, M. (2014). Emetic Bacillus cereus are more volatile than thought: Recent foodborne outbreaks and prevalence studies in Bavaria (2007-2013). *Biomed Research International*.

Mikkola, R., Saris, N. E. L., Grigoriev, P. A., Andersson, M. A., & Salkinoja-Salonen, M. S. (1999). Ionophoretic properties and mitochondrial effects of cereulide - The emetic toxin of B. cereus. *European Journal of Biochemistry, 263*(1), 112-117.

Moravek, M., Dietrich, R., Bürk, C., Broussolle, V., Guinebretiere, M. H., Granum, P. E., Nguyen-the, C., & Märtlbauer, E. (2006). Determination of the toxic potential of Bacillus cereus isolates by quantitative enterotoxin analyses. *FEMS Microbiology Letters, 257*(2), 293-298.

Nakamura, L. K. (1998). Bacillus pseudomycoides sp. nov. *International Journal of Systematic Bacteriology, 48*, 1031-1035.

Noguera, P. A., & Ibarra, J. E. (2010). Detection of new cry genes of Bacillus thuringiensis by use of a novel PCR primer system. *Applied and Environmental Microbiology, 76*(18), 6150-6155.

Oliwa-Stasiak, K., Kolaj-Robin, O., & Adley, C. C. (2011). Development of real-time PCR assays for detection and quantification of Bacillus cereus group species: Differentiation of B. Weihenstephanensis and rhizoid B. pseudomycoides isolates from milk. *Applied and Environmental Microbiology, 77*(1), 80-88.

Oren, A., & Garrity, G. M. (2014). List of new names and new combinations previously effectively, but not validly, published. *International Journal of Systematic and Evolutionary Microbiology, 64*(7), 2184-2187.

Pedersen, J. C., Damgaard, P. H., Eilenberg, J., & Hansen, B. M. (1995). Dispersal of Bacillus thuringiensis var kurstaki in an experimental cabbage field. *Canadian Journal of Microbiology, 41*(2), 118-125.

Priest, F. G., Barker, M., Baillie, L. W. J., Holmes, E. C., & Maiden, M. C. J. (2004). Population structure and evolution of the Bacillus cereus group. *Journal of Bacteriology, 186*(23), 7959-7970.

Pruss, B. M., Francis, K. P., von Stetten, F., & Scherer, S. (1999). Correlation of 16S ribosomal DNA signature sequences with temperature-dependent growth rates of

mesophilic and psychrotolerant strains of the Bacillus cereus group. *Journal of Bacteriology, 181*(8), 2624-2630.

Rawsthorne, H., Dock, C. N., & Jaykus, L. A. (2009). PCR-based method using propidium monoazide to distinguish viable from nonviable Bacillus subtilis spores. *Applied and Environmental Microbiology, 75*(9), 2936-2939.

Richter, M., & Rossello-Mora, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proceedings of the National Academy of Sciences of the United States of America, 106*(45), 19126-19131.

RKI. (2011). Abschließende Darstellung und Bewertung der epidemiologischen Erkenntnisse im EHEC O104:H4 Ausbruch, Deutschland 2011. Berlin 2011., http://edoc.rki.de/documents/rki_ab/reeFNxULvsdZo/PDF/262b4Pk2TGGs.pdf

Sagoo, S. K., Little, C. L., Greenwood, M., Mithani, V., Grant, K. A., McLauchlin, J., de Pinna, E., & Threlfall, E. J. (2009). Assessment of the microbiological safety of dried spices and herbs from production and retail premises in the United Kingdom. *Food Microbiology, 26*(1), 39-43.

Samapundo, S., Heyndrickx, M., Xhaferi, R., de Baenst, I., & Devlieghere, F. (2014). The combined effect of pasteurization intensity, water activity, pH and incubation temperature on the survival and outgrowth of spores of Bacillus cereus and Bacillus pumilus in artificial media and food products. *International Journal of Food Microbiology, 181*, 10-18.

Schaarschmidt, S. (2016). Public and private standards for dried culinary herbs and spices-Part I: Standards defining the physical and chemical product quality and safety. *Food Control, 70*, 339-349.

Schaarschmidt, S., Spradau, F., Mank, H., Banach, J. L., van der Fels-Klerx, H. J., Hiller, P., Appel, B., Braunig, J., Wichmann-Schauer, H., & Mader, A. (2016a). Public and private standards for dried culinary herbs and spices-Part II: Production and product standards for ensuring microbiological safety. *Food Control, 70*, 360-370.

Schaarschmidt, S., Spradau, F., Mank, H., Hiller, P., Appel, B., Bräunig, J., Wichmann-Schauer, H., & Mader, A. (2016b). Reporting of traceability and food safety data in the culinary herb and spice chains. *Food Control*.

Schweiggert, U., Carle, R., & Schieber, A. (2007). Conventional and alternative processes for spice production - a review. *Trends in Food Science & Technology, 18*(5), 260-268.

Shiota, M., Saitou, K., Mizumoto, H., Matsusaka, M., Agata, N., Nakayama, M., Kage, M., Tatsumi, S., Okamoto, A., Yamaguchi, S., Ohta, M., & Hata, D. (2010). Rapid

detoxification of cereulide in Bacillus cereus food poisoning. *Pediatrics, 125*(4), E951-E955.

Singh, G., Kapoor, I. P. S., Pandey, S. K., Singh, U. K., & Singh, R. K. (2002). Studies on essential oils: Part 10; Antibacterial activity of volatile oils of some spices. *Phytotherapy Research, 16*(7), 680-682.

Skerman, V. B. D., McGowan, V., & Sneath, P. H. A. (1997). Approved Lists of Bacterial Names and amended edition of the Approved Lists of Bacterial Names (vol 30, pg 225, 1980). *International Journal of Systematic Bacteriology, 47*(4), 1271-1272.

Soufiane, B., Baizet, M., & Cote, J. C. (2013). Multilocus sequence analysis of Bacillus thuringiensis serovars navarrensis, bolivia and vazensis and Bacillus weihenstephanensis reveals a common phylogeny. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 103*(1), 195-205.

Soufiane, B., & Cote, J. C. (2010). Bacillus thuringiensis Serovars bolivia, vazensis and navarrensis meet the description of Bacillus weihenstephanensis. *Current Microbiology, 60*(5), 343-349.

Stenfors, L. P., & Granum, P. E. (2001). Psychrotolerant species from the Bacillus cereus group are not necessarily Bacillus weihenstephanensis. *FEMS Microbiology Letters, 197*(2), 223-228.

Stephan, D., Scholz-Döbelin, H., Reintges, T., Pelz, J., Jehle, J. A., & Keßler, J. (2014). Investigations on residues of Xentari® (Bacillus thuringiensis subspec. aizawai) on greenhouse tomatoes. *Journal für Kulturpflanzen, 66*(9), 312-318.

Tajkarimi, M. M., Ibrahim, S. A., & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control, 21*(9), 1199-1218.

Tallent, S. M., Kotewicz, K. M., Strain, E. A., & Bennett, R. W. (2012). Efficient isolation and identification of Bacillus cereus group. *Journal of AOAC International, 95*(2), 446-451.

Thammasittirong, A., & Attathom, T. (2008). PCR-based method for the detection of cry genes in local isolates of Bacillus thuringiensis from Thailand. *Journal of Invertebrate Pathology, 98*(2), 121-126.

Thomas, M. C., Shields, M. J., Hahn, K. R., Janzen, T. W., Goji, N., & Amoako, K. K. (2013). Evaluation of DNA extraction methods for Bacillus anthracis spores isolated from spiked food samples. *Journal of Applied Microbiology, 115*(1), 156-162.

Thorsen, L., Hansen, B. M., Nielsen, K. F., Hendriksen, N. B., Phipps, R. K., & Budde, B. B. (2006). Characterization of emetic Bacillus weihenstephanensis, a new cereulide-producing bacterium. *Applied and Environmental Microbiology, 72*(7), 5118-5121.

Tourasse, N. J., Helgason, E., Klevan, A., Sylvestre, P., Moya, M., Haustant, M., Okstad, O. A., Fouet, A., Mock, M., & Kolsto, A. B. (2011). Extended and global phylogenetic view of the Bacillus cereus group population by combination of MLST, AFLP, and MLEE genotyping data. *Food Microbiology, 28*(2), 236-244.

Valero, M., Hernandez-Herrero, L. A., Fernandez, P. S., & Salmeron, M. C. (2002). Characterization of Bacillus cereus isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. *Food Microbiology, 19*(5), 491-499.

van Asselt, E. D., & Zwietering, M. H. (2006). A systematic approach to determine global thermal inactivation parameters for various food pathogens. *International Journal of Food Microbiology, 107*(1), 73-82.

Vassileva, M., Torii, K., Oshimoto, M., Okamoto, A., Agata, N., Yamada, K., Hasegawa, T., & Ohta, M. (2007). A new phylogenetic cluster of cereulide-producing Bacillus cereus strains. *Journal of Clinical Microbiology, 45*(4), 1274-1277.

Vilas-Boas, G. T., Peruca, A. P. S., & Arantes, O. M. N. (2007). Biology and taxonomy of Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis. *Canadian Journal of Microbiology, 53*(6), 673-687.

von Stetten, F., Francis, K. P., Lechner, S., Neuhaus, K., & Scherer, S. (1998). Rapid discrimination of psychrotolerant and mesophilic strains of the Bacillus cereus group by PCR targeting of 16S rDNA. *Journal of Microbiological Methods, 34*(2), 99-106.

Waje, C. K., Kim, H. K., Kim, K. S., Todoriki, S., & Kwon, J. H. (2008). Physicochemical and microbiological qualities of steamed and irradiated ground black pepper (Piper nigrum L.). *Journal of Agricultural and Food Chemistry, 56*(12), 4592-4596.

Wang, J., Ding, T., & Oh, D. H. (2014). Effect of temperatures on the growth, toxin production, and heat resistance of Bacillus cereus in cooked rice. *Foodborne Pathogens and Disease, 11*(2), 133-137.

Warda, A. K., den Besten, H. M., Sha, N., Abee, T., & Nierop Groot, M. N. (2015). Influence of food matrix on outgrowth heterogeneity of heat damaged Bacillus cereus spores. *International Journal of Food Microbiology, 201*, 27-34.

Wehrle, E., Didier, A., Moravek, M., Dietrich, R., & Märtlbauer, E. (2010). Detection of Bacillus cereus with enteropathogenic potential by multiplex real-time PCR based on SYBR green I. *Molecular and Cellular Probes, 24*(3), 124-130.

Wehrle, E., Moravek, M., Dietrich, R., Bürk, C., Didier, A., & Märtlbauer, E. (2009). Comparison of multiplex PCR, enzyme immunoassay and cell culture methods for the detection of enterotoxinogenic Bacillus cereus. *Journal of Microbiological Methods, 78*(3), 265-270.

WHO/FAO. (2014). Ranking of low moisture foods in support of microbiological risk management. Preliminary report of FAO/WHO expert consultation on ranking of low moisture foods. pp. 254. http://ucfoodsafety.ucdavis.edu/files/209893.pdf

Wielinga, P. R., Hamidjaja, R. A., Agren, J., Knutsson, R., Segerman, B., Fricker, M., Ehling-Schulz, M., de Groot, A., Burton, J., Brooks, T., Janse, I., & van Rotterdam, B. (2011). A multiplex real-time PCR for identifying and differentiating B. anthracis virulent types. *International Journal of Food Microbiology, 145*, S137-S144.

Wijnands, L. M., Dufrenne, J. B., van Leusden, F. M., & Abee, T. (2007). Germination of Bacillus cereus spores is induced by germinants from differentiated caco-2 cells, a human cell line mimicking the epithelial cells of the small intestine. *Applied and Environmental Microbiology, 73*(15), 5052-5054.

Witkowska, A. M., Hickey, D. K., Alonso-Gomez, M., & Wilkinson, M. G. (2011). The microbiological quality of commercial herb and spice preparations used in the formulation of a chicken supreme ready meal and microbial survival following a simulated industrial heating process. *Food Control, 22*(3-4), 616-625.

Witkowska, A. M., Hickey, D. K., & Wilkinson, M. G. (2014). Effect of variation in food components and composition on the antimicrobial activity of oregano and clove essential oils in broth and in a reformulated reduced salt vegetable soup product. *Journal of Food Research, 3*(6), 92-106.

Zhou, G. P., Liu, H. Z., He, J., Yuan, Y. M., & Yuan, Z. M. (2008). The occurrence of Bacillus cereus, B. thuringiensis and B. mycoides in Chinese pasteurized full fat milk. *International Journal of Food Microbiology, 121*(2), 195-200.

7 Danksagung

Mein herzlicher Dank gilt Prof. Dr. Bernd Appel und Prof. Dr. Rupert Mutzel für die Betreuung und die Begutachtung dieser Doktorarbeit.

Ganz besonders möchte ich mich bei Dr. Anneluise Mader, Mai Dinh-Thanh und Dr. Sara Schaarschmidt für die tolle Zusammenarbeit im SPICED-Projekt und die kontinuierliche Unterstützung bedanken.

Ich bedanke mich bei Dr. Heidi Wichmann-Schauer und der Fachgruppe 44 „Mikrobielle Toxine“ für die angenehme Arbeitsatmosphäre sowie die fachliche und praktische Unterstützung. Mein besonderer Dank gilt Dr. Britta Kraushaar, Dr. Silke Bechlars, Dr. Gladys Krause, Dr. Ylanna Kelner-Burgos, Katja Drache, Daniel Leeser, Dr. Sven Maurischat sowie Dr. Alexandra Fetsch.

Für die gute Zusammenarbeit und Hilfsbereitschaft bei der Cereulid-Detektion danke ich Dr. Dorina Bodi, Wolfgang Herkt und Dr. Angelika Preiß-Weigert aus der Fachgruppe 82 „Kontaminanten“.

Ich danke Dr. Tomáš Kuchta vom Food Research Institute, Bratislava für die theoretische und praktische Hilfestellung bei der Anwendung von DNA-Extraktionsverfahren.

Ganz lieber Dank gilt meiner Frau, meinen Kindern und meinen Eltern für ihre Geduld und die stetige Unterstützung.

8 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die hier vorgelegte Arbeit selbstständig verfasst habe und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form keiner anderen Prüfungsbehörde vorgelegt wurde.