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Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs

EFSA Panel on Biological Hazards (BIOHAZ)

Abstract

The *Bacillus cereus* group, also known as *B. cereus sensu lato*, is a subdivision of the *Bacillus* genus that consists of eight formally recognised species: *B. cereus sensu stricto, B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus* and *B. toyonensis.* The current taxonomy of the *B. cereus* group and the status of separate species mainly rely on phenotypic characteristics. *Bacillus thuringiensis* strains display a similar repertoire of the potential virulence genes on the chromosome as *B. cereus sensu stricto* strains and it has been shown that these genes can also be actively expressed in *B. thuringiensis* strains. *Bacillus cereus* and *B. thuringiensis* strains are usually not discriminated in clinical diagnostics or food microbiology. Thus, the actual contribution of the two species to gastrointestinal and non-gastrointestinal diseases is currently unknown. Most cases of food-borne outbreaks caused by the *B. cereus* group have been associated with concentrations above 10^5 CFU/g. However, cases of both emetic and diarrhoeal illness have been reported involving lower levels of *B. cereus.* The levels of *B. cereus* that can be considered as a risk for consumers are also valid for *B. thuringiensis*. There is no evidence that *B. thuringiensis* has the genetic determinants for the emetic toxin cereulide. The Panel has recommended the application of whole genome sequencing to provide unambiguous identification of strains used as biopesticides and the detailed characterisation of outbreak strains allowing discrimination of *B. thuringiensis* from *B. cereus.* Data gaps include: dose–response and behavioural characteristics of *B. cereus* group strains and specifically of *B. thuringiensis.* Field studies after application of *B. thuringiensis* biopesticides are needed to enable the establishment of pre-harvest intervals.

Keywords: *Bacillus cereus*, *Bacillus thuringiensis*, food-borne outbreaks, biopesticide

Requestor: European Commission

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Summary

The European Commission asked the Panel on Biological Hazards (BIOHAZ) to deliver a scientific opinion on the risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs, providing an update of the opinion of the Scientific Panel on biological hazards (BIOHAZ) on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs, published in 2005. In particular, the European Commission requested the European Food Safety Authority (EFSA) to: (i) provide an update of information available on pathogenicity, and contributing virulence factors, in the genus *Bacillus* (with the exclusion of *B. anthracis*) and specifically to evaluate the risk to public health arising from the presence of *B. thuringiensis* in food; (ii) review the microbiological methods available to distinguish between the members of the *B. cereus* group, to identify different *B. thuringiensis* strains, and the methods to identify the presence of toxins produced by these microorganisms; (iii) review existing data on natural background prevalence and levels of *B. thuringiensis* in the environment, and rates of transfer to foodstuffs, including conditions under which this transfer may take place; (iv) indicate, if possible, the maximum levels of *Bacillus*, and specifically of *B. thuringiensis*, in food that could be regarded as safe for human consumption; (v) evaluate what would be the *B. thuringiensis* levels in food, at all stages of the food chain, if this microorganism was applied as PPP (plant protection product), and (vi) provide an update on specific control options, to manage the risk caused by *B. cereus*, *B. thuringiensis*, and other *Bacillus* spp. and their toxins.

The European Commission also asked EFSA to consider and evaluate in the scientific opinion the confidential information shared with the WG via CIRCABC (Communication and Information Resource Centre for Administrations, Businesses and Citizens) Pesticides concerning an alleged food-borne outbreak in a family which occurred in a Member State (MS), for which a salad containing *B. thuringiensis* was suspected to be the source of the outbreak.

Several sources of information were used for the assessment: (i) *Bacillus* spp. food-borne outbreak data (2007–2014), reported to EFSA's Zoonoses database; (ii) data retrieved through a questionnaire distributed in autumn 2015 to 31 countries (28 EU MSs, Iceland, Norway and Switzerland) via the Pesticide Steering Network and the Pesticide Monitoring Network; (iii) data obtained from 25 MS via the European Commission on microbiological criteria or guideline microbiological limits for *B. cereus* or Bacillus spp.; (iv) data obtained via a technical consultation with representatives of the International Biocontrol Manufacturers Association (IBMA); and (v) publications concerning the presence and levels of *B. thuringiensis* in food, retrieved through an extensive literature review.

The Panel reviewed the classification and nomenclature of the *Bacillus cereus* group, also known as *B. cereus sensu lato* (or *B. cereus* group), which is a subdivision of the *Bacillus* genus that consists of eight formally recognised species: *B. cereus sensu stricto* (or *B. cereus* as it is usually called), *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus* and the recently validated new species *B. toyonensis.* However, the current taxonomy of the *B. cereus* group and the status of separate species for the different bacteria that constitute the *B. cereus* group, for historic reasons, mainly rely on phenotypic characteristics established before the era of genome sequencing and without the knowledge of important traits being plasmid-borne. In addition, the economic and medical importance of the individual species and in particular the separate species status of *B. thuringiensis* is clearly relevant to the use of *B. thuringiensis* as a biopesticide. *Bacillus thuringiensis* strains display a similar repertoire of the potential virulence genes on the chromosome as *B. cereus sensu stricto* strains and it has been shown that these genes can also be actively expressed in *B. thuringiensis* strains. These features clearly question the relevance of the taxonomic segregation of the *B. cereus* group into separate species. The phylogenetic relationship between the bacteria is based on genes located on the chromosome, while important genes for virulence (such as the cereulide toxin synthesis genes) or for useful products like biopesticides (*cry* genes) are located on plasmids.

The species *B. thuringiensis* is characterised by the production of crystal inclusions in parallel with spore formation. The insecticidal proteins in the crystal bodies have been shown to mainly contain two types of insecticidal proteins. The taxonomy and classification of *B. thuringiensis* genes refer to the most common type as *cry* (for crystal) genes, along with the *cyt* (for cytolytic) genes present in *B. thuringiensis* subsp. *israelensis*. The commercial *B. thuringiensis* products are powders containing a mixture of dried spores and toxin crystals. Presently there are over 400 *B. thuringiensis*-based formulations that have been registered in the market and most of them contain insecticidal proteins and viable spores.

The panoply of potential toxins and virulence factors found in the *B. cereus* group is broadly distributed among the different members of the group and it is not possible to draw any firm conclusion about the pathogenic potential of a certain strain based on the sole presence of potential virulence factors. Some species contain additional toxins defining the species, namely the anthrax toxins restricted to *B. anthracis* and a few *B. cereus* strains, and the insecticidal crystal-forming toxins (Cry and the Cyt toxins) characteristic of *B. thuringiensis*.

Nearly all *B. cereus* strains harbour the non-haemolytic enterotoxin complex (*nhe*) genes, while haemolysin BL complex (*hbl*) and cytotoxin K (*cytK*) are detected in about 30–70% of isolates. In contrast to the emetic toxin cereulide, these potential enterotoxins are also frequently found in other *B. cereus* group members, including *B. thuringiensis.* So far the cereulide toxin synthesis (*ces*) gene has not been found in *B. thuringiensis*.

As *B. cereus* and *B. thuringiensis* strains are genetically intermingled, these species are usually not discriminated in routine clinical diagnostics or food microbiology. Thus, the actual contribution of the two species to gastrointestinal (GI) and non-GI diseases is currently unknown.

In the alleged food poisoning outbreak described in the Background section of this Opinion, it appears that the only bacteria that were found above the generally accepted level were *B. cereus* group bacteria, identified as *B. thuringiensis* in the salad samples. The *B. thuringiensis* isolated from the salad were characterised by FTIR spectroscopy and could not be discriminated from *B. thuringiensis* subsp. *aizawai* (XenTari) which had been sprayed on the salad on the field. It is not clear if people eating salad also ate cheese noodles, and therefore a synergistic effect between *B. thuringiensis* and another *B. cereus* group strain cannot be excluded. It also cannot be excluded that another *B. cereus* group strain was present at low levels in the salad, although no such strain was detected in any of the samples.

Bacillus cereus group strains are widespread in the environment and can be isolated from soil and vegetation. From the soil, they can be transferred to various associated items, including plants and a variety of raw materials used for food processing. Their spores can survive the intense processing of dehydrated foods and subsequently contaminate diverse foodstuffs via dehydrated ingredients as well as cleanrooms used by many industries. The fate of *B. thuringiensis* on a specific crop after an application is hard to predict, as it is dependent on many factors including the crop, the climatic conditions and the cultivation practice. It is not possible to estimate the number of *B. thuringiensis* present on a specific crop at a specific locality after a certain period of time.

The levels of *B. cereus* group posing a health risk to consumers are highly strain-dependent due to the highly diverse pathogenic potential. The possibility of multiplication in foods after storage and/or handling must be taken into account when defining safe levels for human consumption, as well as the composition of the food, which can affect toxin production. All these factors can be responsible for the large variation in the estimated infectious doses, which makes a valid dose–response relationship difficult to establish.

Taking into account the available information, the Panel confirmed, as stated in the 2005 Opinion, that most cases of food-borne outbreaks caused by the *B. cereus* group have been associated with bacterial concentrations above 10^5 CFU/g foodstuff. However, it is important to highlight that cases of both emetic and diarrhoeal illness have been reported, involving between 10^3 and 10^5 CFU/g of *B. cereus.*

Following these considerations, the Panel concluded that, taking the enterotoxigenic potential into account as well as that *B. thuringiensis* cannot be distinguished from *B. cereus* at the chromosomal level, the levels of *B. cereus* that can be considered as a risk for consumers are also likely to be valid for *B. thuringiensis*. There is, however, no evidence that *B. thuringiensis* has the genetic determinants for the emetic toxin cereulide.

The Panel reviewed the control options for managing the risk caused by *Bacillus* spp. and their toxins, such as, in the case of primary production, the correct application, according to label directions, of commercial formulations of *B. thuringiensis* used as biopesticide, using the doses and the time intervals between commercial application and harvest recommended by the manufacturer.

At postharvest, the main management option for controlling *B. cereus group* strains in the food chain is to maintain the foods and leftovers refrigerated at ≤ 7°C (and preferably at ≤ 4°C). Other efficient control measures include heat treatment, high hydrostatic pressure, pulsed light, irradiation and chemical sanitisers. Most of these treatments are relatively efficient against vegetative cells but some of them fail to inactivate spores and so far no commonly used control option used in the food industry can inactivate cereulide toxins. Combinations of high pressure and high temperature are needed to inactivate the most resistant bacterial spores.

The Panel has recommended the application of whole genome sequencing in order to provide unambiguous identification of strains used as biopesticides and their further safety assessment. These strains and respective sequences should be available for laboratories specialising in the *B. cereus* group. Moreover, markers should be identified for commercial *B. thuringiensis* strains to allow regular monitoring and easy differentiation in suspect outbreak situations.

In cases of food-borne outbreaks associated with the *B. cereus* group, the in-depth molecular characterisation of strains to allow discrimination of *B. thuringiensis* from *B. cereus,* as well as the identification of strains related to commercial *B. thuringiensis* used as biopesticides*,* would allow precise identification of the organism involved. *Bacillus cereus* group food-borne outbreak strains should be kept in accessible culture collections preferentially managed by reference laboratories.

The Panel suggested the development of research on dose–response and behavioural characteristics of *B. cereus* group strains, and specifically of *B. thuringiensis*, to facilitate risk characterisation and to monitor and characterise the factors that lead to/favour the transfer of *Bacillus* species from the environment to foodstuffs and identify the routes and critical steps of contamination in the food industry.

The development of field studies after application of *B. thuringiensis* biopesticides would inform the possible establishment of pre-harvest intervals.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Bacillus cereus is a ubiquitous bacterium in the environment that can be present in a wide range of foodstuffs. *Bacillus cereus* originates an important number of food-borne illnesses in humans. In January 2005, the BIOHAZ Panel of EFSA issued an opinion on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs. The opinion concluded that the *Bacillus cereus* group was very diverse and that there was little information in the literature on other pathogenic *Bacillus* spp. The opinion also concluded that no routine methods easily detect and enumerate other species of *Bacillus* that could be involved in food-borne poisoning and no methods distinguish pathogenic strains among these species.

Bacillus thuringiensis species are a naturally occurring species of microorganisms belonging to the same group of microorganisms as *B. cereus.* Standard methods for detection and enumeration of *B. cereus* do not distinguish *B. cereus* from *B. thuringiensis*.

Bacillus thuringiensis (Bt) is a soil-dwelling bacterium which occurs naturally in the gut of caterpillars of various types of moths and butterflies, as well as on leaf surfaces, aquatic environments, animal faeces, insect-rich environments, flour mills and grain-storage facilities.

Several *B. thuringiensis* strains show insecticidal activity via the production of δ-endotoxins. *Bacillus thuringiensis* subsp*. aizawai* strains ABTS-1857 and GC-91*, Bacillus thuringiensis* subsp. *israelensis* (serotype H-14) strain AM65-52, *Bacillus thuringiensis* subsp. *kurstaki* strains ABTS 351, PB 54, SA 11, SA12 and EG 2348 and *Bacillus thuringiensis* subsp. *tenebrionis* strain NB 176 (TM 14 1) are active substances included in the fourth stage of the review programme covered by Commission Regulation (EC) No 2229/2004[1], as amended by Commission Regulation (EC) No 1095/2007[2].

Bacillus thuringiensis subsp*. aizawai* strains ABTS-1857 and GC-91*, Bacillus thuringiensis* subsp. *israelensis* (serotype H-14) strain AM65-52, *Bacillus thuringiensis* subsp. *kurstaki* strains ABTS 351, PB 54, SA 11, SA12 and EG 2348 and *Bacillus thuringiensis* subsp. *tenebrionis* strain NB 176 (TM 14 1) were included in Annex I to Directive 91/414/EEC on 8 December 2008 pursuant to Article 24b of Regulation (EC) No 2229/2004 and have subsequently been deemed to be approved under Regulation (EC) No 1107/2009[3], in accordance with Commission Implementing Regulation (EU) No 540/2011[4], as amended by Commission Implementing Regulation (EU) No 541/2011[5]. Formulations containing these active substances may have been approved at Member State (MS) level before that date.

As no specific MRL was fixed for these active substances under Reg. (EC) No 396/2005, according to Art. 18(1)(b) of that Regulation, the default MRL of 0.01 mg/kg is applicable to all food products included in Annex I to that Regulation. As originally the default level was intended for chemical substances, and not for microorganisms, neither the level nor the unit (mg/kg) are appropriate for microorganisms. Discussions are ongoing in the pesticides residues section of the Standing Committee for Plants, Animals, Food and Feed on how to handle microorganisms in general and *B. thuringiensis* in particular.

In the context of these discussions, a MS informed the Committee of an alleged food poisoning which was followed up by the control authorities with a number of actions. As discussions on the appropriate MRL setting for *B. thuringiensis* are ongoing, including the possibility to include *B. thuringiensis* into Annex IV[6] to Regulation (EC) No 396/2005, the case raises questions on the risk to public health related to the presence of *B. thuringiensis* in food which need clarifying before a risk management decision can be taken. Details of the specific case and the follow-up given to it by the control authorities are summarised here below.

[1] Commission Regulation (EC) No 2229/2004 of 3 December 2004 laying down further detailed rules for the implementation of the fourth stage of the programme of work referred to in Article 8(2) of Council Directive 91/414/EEC. OJ L 379, 24.12.2004, p. 13–63.

[2] Commission Regulation (EC) No 1095/2007 of 20 September 2007 amending Regulation (EC) No 1490/2002 laying down further detailed rules for the implementation of the third stage of the programme of work referred to in Article 8(2) of Council Directive 91/414/EEC and Regulation (EC) No 2229/2004 laying down further detailed rules for the implementation of the fourth stage of the programme of work referred to in Article 8(2) of Council Directive 91/414/EEC. OJ L 246, 21.9.2007, p. 19–28.

[3] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1–50.

[4] Commission Implementing Regulation (EU) No 540/2011 of 25 May 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the list of approved active substances. OJ L 153, 11.6.2011, p. 1–186.

[5] Commission Implementing Regulation (EU) No 541/2011 of 1 June 2011 amending Implementing Regulation (EU) No 540/2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the list of approved active substances. OJ L 153, 11.6.2011, p. 187–188.

[6] Annex which lists substances for which no MRL is necessary.

1.1.1. Details of the specific case

Three family members of a family of five, who ate cheese noodles and salad during the evening, got ill with nausea and diarrhoea at 1 a.m. Only the persons who had eaten from the salad got ill. This salad was bought and eaten on 28 July 2012. The respective official laboratory received the sample on 30 July 2012 and primarily analysed it for presumptive *B. cereus* which were then identified as *B. thuringiensis* (3×10^4 CFU/g). Other pathogenic germs were not detected. The producer of this salad was traced back by the competent authority. The application of the *B. thuringiensis* containing plant protection product (PPP) XenTari (*Bacillus thuringiensis* subsp. *aizawai*) was confirmed.

Also, the cheese noodles were analysed by the control laboratory. *Bacillus cereus* was found at a concentration of 6.0×10^3 CFU/g.

On the same day, 30 July 2012, two additional samples of lettuce were taken by the official food inspection services from the same supermarket and were sent to the official control laboratory for analysis. These two samples were in their sealed original packaging and originated from the same producer and the same batch as the salad which had caused the food poisoning incidence. The official control laboratory identified *B. thuringiensis* in concentrations of 4×10^4 and 1.5×10^5 CFU/g, respectively.

On 7 August 2012, three further salad samples were taken from the respective supermarket which originated from the same producer but from a different batch. In these samples the official control laboratory determined *B. thuringiensis* in concentrations of 2.5×10^4, 4×10^4 and 1.4×10^5 CFU/g.

Altogether, six salad samples were taken in connection with the case of food poisoning and *B. thuringiensis* was identified in all samples. In two samples concentrations of $1.4–1.5 \times 10^5$ CFU/g were found, whereas concentrations of $2.5–4 \times 10^4$ CFU/g were detected in the other four samples.

As a follow-up, the control authorities performed trials to examine the concentration of spores and also the decomposition of *B. thuringiensis* on greenhouse tomatoes and salad after application of XenTari. Spore concentrations up to 8.65×10^2 CFU and 2×10^5 CFU were found before the first treatment for tomato or in the untreated salad heads, respectively. Spore concentrations above 1×10^5 CFU were reached in the treated plots for lettuce but not for tomatoes.

Furthermore, the control authorities submitted a study on the presence of enterotoxin genes and respective enterotoxins in *Bacillus thuringiensis* subsp. *aizawai* ABTS-1857. All isolates produced a positive result for the *cytK2* and *nhe/hbl* genes factors known to trigger diarrhoea. It should be noted that presence of the *ces* gene, which causes production of emetic toxins, could not be proved while the family in the alleged food poisoning also experienced nausea.

1.1.2. Terms of Reference

The diagnosis of *Bacillus* food-borne poisoning is difficult because it may be related to several *Bacillus* species, mainly but not solely to *B. cereus*. *Bacillus thuringiensis* (Bt) is a species belonging to the *B. cereus* group, and analytical distinction between the two is difficult. An update of the opinion of the Scientific Panel on biological hazards (BIOHAZ) on *B. cereus* and other *Bacillus* spp. in foodstuffs (EFSA, 2005) is requested. In particular, EFSA is asked to:

1) Provide an update of information available on pathogenicity, and contributing virulence factors, in the genus *Bacillus* (with the exclusion of *B. anthracis*). Specifically to evaluate the risk to public health arising from the presence of *B. thuringiensis* in food (including in the evaluation information from the specific case of illness described above).
2) Review the microbiological methods available to distinguish between the members of the *B. cereus* group, to identify different *B. thuringiensis* strains, and the methods to identify the presence of toxins produced by these microorganisms.
3) Review existing data on natural background prevalence and levels of *B. thuringiensis* in the environment (e.g. soil), and rates of transfer to foodstuffs, including conditions under which this transfer may take place.
4) Indicate, if possible, the maximum levels (number) of *Bacillus*, and specifically of Bt, in food that could be regarded as safe for human consumption.
5) Evaluate what would be the *B. thuringiensis* levels in food, at all stages of the food chain, if this microorganism was applied as PPP.
6) Provide an update on specific control options, to manage the risk caused by *B. cereus*, *B. thuringiensis*, and other *Bacillus* spp. and their toxins.

1.2. Interpretation of the Terms of Reference

1.2.1. Term of Reference 1

When evaluating the risk to public health arising from the presence of *B. thuringiensis* in food only the food-borne route of exposure has been considered. The non-food-borne route of exposure for operators, workers and others has been excluded.

1.2.2. Term of Reference 2

The limitations of the methods currently available have also been discussed in the opinion.

1.2.3. Term of Reference 3

It was agreed with DG SANTE that foodstuffs would be understood here as foods: (i) during harvesting, (ii) before minimal processing and (iii) during minimal processing. Minimal processing is defined as in the previous Opinions on the risk posed by pathogens in food of non-animal origin (EFSA BIOHAZ Panel, 2014a–e), namely as 'any action applied to the initial product (e.g. cleaning, coring, peeling, chopping, cutting, slicing or dicing, freezing and washing) and which is not included in the definition of processing according to Regulation (EC) No 852/2004 (e.g. heating, smoking, curing, maturing, drying, marinating, extraction, extrusion or a combination of those processes). Minimal processing may occur at harvest as well as on farm postharvest and at processing'.

1.2.4. Term of Reference 4

DG SANTE understands that this Term of Reference (ToR) will be answered by a qualitative approach.

1.2.5. Term of Reference 5

It was agreed with DG SANTE that in the scope of this ToR this draft opinion covers all foods (including processed food).

1.3. Documents listed in the Mandate letter shared with the WG via CIRCABC (Communication and Information Resource Centre for Administrations, Businesses and Citizens) Pesticides

Documents already available to EFSA:

- Data and comments uploaded on CIRCABC for the SCOFCAH pesticides residues of 24–25 February 2014, point A.15.
- Data and comments uploaded on CIRCABC for the SCOFCAH pesticides residues of 12–13 June 2014, point B.08.00.
- Data and comments uploaded on CIRCABC for the PAFF (Standing Committee on Plants, Animals, Food and Feed) pesticides residues of 22–23 September 2014, point A.08.03.
- Data and comments uploaded on CIRCABC for the PAFF pesticide residues of 24–25 November 2014, point A.15.03.
- Data and comments uploaded on CIRCABC for the PAFF pesticide residues of 12–13 February 2015, point A.13.03.
- Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. EFSA Journal 2014;12(5):3665, 10 pp. doi:10.2903/j.efsa.2014.3665

2. Data and methodologies

2.1. Data

2.1.1. *Bacillus* spp. food-borne outbreaks (2007–2014) (reported to EFSA's Zoonoses database)

Information on strong-evidence[7] food-borne outbreaks caused by *Bacillus* spp. reported in accordance with Directive 2003/99/EC[8] has been summarised in Appendix A. Overall, 413 strong-evidence food-borne outbreaks where *B. cereus* was implicated as causative agent were reported in the European Union (EU) MSs and European non-MSs from 2007 to 2014. In total, 6,657 human cases, 352 hospitalisations and no deaths were reported. 'Mixed food[9] or buffet meals[10] were the most commonly implicated food vehicle categories (27.6% of outbreaks), followed by 'cereal products' (10.9% of outbreaks) and 'red meat and products thereof' (8.0% of outbreaks). The following food vehicles were also reported to be implicated in strong-evidence outbreaks caused by *B. cereus*: 'poultry meat and products thereof' (5.3% of outbreaks), 'vegetables and juices and other products thereof' (4.6% of outbreaks) and 'fish and fish products' (3.4% of outbreaks). Additional implicated food vehicles ('crustaceans, shellfish and molluscs', 'eggs and egg products', various dairy products, 'herbs and spices', 'bakery products', 'sweets and chocolate', 'canned food products' and drinks) each accounted for less than 3% of the outbreaks caused by *B. cereus*. For 113 outbreaks (27.4%) no detail on the implicated food was reported (indicated as either 'unknown' or categorised as 'other foods').

One strong-evidence outbreak where *B. subtilis* was indicated as causative agent was reported in 2010 as being associated with the consumption of mixed food. In addition, *Bacillus* spp. was indicated as causative agent for two strong-evidence outbreaks reported in 2012 and 2009 as being associated with the consumption of 'vegetables and juices and other products thereof' and 'other foods', respectively.

Further details on the number of strong-evidence outbreaks caused by *B. cereus* and *Bacillus* spp., human cases and hospitalisations by implicated food vehicle and reporting year can be found in Tables A.1 and A.2 of Appendix A.

In addition, 714 weak-evidence[7] outbreaks due to *B. cereus* involving 6,089 human cases, 415 hospitalisations and 4 deaths were reported. Furthermore, 149 weak-evidence outbreaks were also reported due to unspecified *Bacillus* involving 1,798 human cases, 116 hospitalisations and no deaths (EFSA, 2014).

2.1.2. Data obtained via consultation with PRAS network

A questionnaire (Appendix B) was distributed in autumn 2015 to 31 countries (28 EU MSs, Iceland, Norway and Switzerland) via the Pesticide Steering Network and the Pesticide Monitoring Network to retrieve any available data on:

1) testing/monitoring on the occurrence and levels of *B. thuringiensis* in fruits, vegetables and/or other crops eligible to be treated with *B. thuringiensis;*
2) additional studies on survival, persistence and multiplication of *B. thuringiensis* strains/toxins in the environment and in food (e.g. vegetable and fruits) after application on crop fields;
3) additional information on natural background levels of *B. thuringiensis* in the environment (e.g. groundwater, water reservoirs, crop fields (non-treated), etc.).

Answers were received from 16 countries (Austria, Belgium, Croatia, Denmark, France, Germany, Greece, Hungary, Iceland, Lithuania, the Netherlands, Romania, Slovenia, Spain, Sweden and the United Kingdom).

[7] Food-borne outbreaks are classified as either strong- or weak-evidence outbreaks based on the evidence implicating a suspect food vehicle, as described in the EFSA's updated technical specifications for harmonised reporting of food-borne outbreak (EFSA, 2014).

[8] Directive 203/99/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. OJ L 325, 12.12.2003, p. 31–40.

[9] Mixed foods are meals composed of various foods, for example paella, risotto and curries. This category also includes miscellaneous foodstuffs served in one plate.

[10] A buffet meal is a meal at which guests serve themselves from various dishes displayed on a large table.

Some data on laboratory or greenhouse studies were reported, but the conclusion from the received answers is that practically no *B. thuringiensis* monitoring data exist in the EU. Only one country reported the results of the analysis of seven food samples covering a period from 2000 until autumn 2015. No data were reported in response to questions 2 and 3 by any of the countries.

2.1.3. Data obtained via European Commission on microbiological criteria or guideline microbiological limits for *Bacillus cereus* or *Bacillus* spp.

A questionnaire (Appendix C) was distributed in autumn 2015 to 31 countries (28 EU MSs, Iceland, Norway and Switzerland) via DG SANTE's Microbiological Criteria Working Group to retrieve any available data on microbiological criteria or recommendations for *Bacillus* spp., *B. cereus* or *B. thuringiensis* for any type of food.

Answers were received from 25 MSs (Austria, Belgium, Croatia, the Czech Republic, Cyprus, Denmark, Estonia, Finland, France, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, the Netherlands, Norway, Poland, Portugal, Slovenia, Slovakia, Spain, Sweden, Switzerland and the United Kingdom). Some countries (17 out of 25) provided details for microbiological criteria or recommendations for *Bacillus* spp., *B. cereus* or *B. thuringiensis* in place for specific types of food such as the sampling stage, sampling plan and parameters *m*, *M*, *c*[11] and *n*[12] (Appendix D). Most of this information refers to ready-to-eat (RTE) foods, dried infant formulae, dried dietary foods and baby food.

It should be noted that Commission Regulation (EC) No 1441/2007[13] of 5 December 2007 amending Regulation (EC) 2073/2005[14] on microbiological criteria for foodstuffs establishes one Process Hygiene Criterion for presumptive *Bacillus cereus* (EN/ISO 7932) for dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age at the end of the manufacturing process with the following parameters: $m = 50$ CFU/g, $M = 500$ CFU/g, $c = 1$, $n = 5$. This microbiological criterion is applied at the EU level.

2.1.4. Data obtained via technical consultation with International Biocontrol Manufacturers Association (IBMA)

A questionnaire (Appendix E) was distributed in autumn 2015 to the International Biocontrol Manufacturers Association (IBMA) to retrieve any additional available data on the: (i) production process for biopesticides containing *B. thuringiensis*; (ii) strain identification methods used during the production of biopesticides containing *B. thuringiensis*; (iii) genetic stability of the *B. thuringiensis* strains; and (iv) testing/monitoring on *B. thuringiensis* in the environment and in food.

Answers were received and discussed during a technical consultation organised with five nominated IBMA representatives. Annex A contains all relevant information which was considered useful by the Working Group (WG) to answer the ToRs and was not available from other sources. This information represents solely the views of the IBMA and does not represent the views of this WG.

2.2. Methodologies

Due to lack of available data, it is not possible to conduct a quantitative evaluation of the risk to public health arising from the presence of *B. thuringiensis* in food. Therefore, only a qualitative evaluation has been done, considering both the relevant scientific literature and the information from the background documents provided by the European Commission describing the specific cases in the alleged food poisoning due to *B. thuringiensis* in 2012 and mentioned in Section 1.1.

In order to reply to the ToRs, the Panel agreed with the Commission to proceed with different methods, according mostly to the availability of data. An evaluation of the occurrence and levels of *B. thuringiensis* in foods (ToR 5) has been carried out, through an extensive literature review. The detailed protocol for the literature review on the occurrence and levels of *B. thuringiensis* in foods is included in Appendix F. As far as the other ToRs are concerned, it was agreed, due to limited time and resources, to carry out a qualitative evaluation, by means of literature reviews based on the knowledge and expertise of the WG members. In these cases the experts in the WG selected relevant references starting from review papers, books chapters, non peer-review papers known by the experts

[11] *c* = number of sample units giving values over *m* or between *m* and *M*.
[12] *n* = number of units comprising the sample.
[13] Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. OJ L 322, 7.12.2007, p. 12–29.
[14] OJ L 338, 22.12.2005, p. 1–26.

themselves or retrieved through non-systematic searches, and increasing the number of papers through 'footnote chasing' until reaching a coverage of the subject considered sufficient by the WG (White et al., 1992).

In order to obtain additional information on the monitoring schemes for *B. cereus* and *B. thuringiensis* on crops and food at the MS level, questionnaires were prepared and distributed to Commission contact points, as described in Sections 2.1.2 and 2.1.3. These consultations clarified that no monitoring plans are applied, apart from the ones prescribed by EU legislation (*B. cereus* in dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age), and from some guideline limits applied in some MSs for national monitoring. Also, due to the lack of data arising from EU-wide monitoring schemes, the WG searched for this kind of information in the scientific literature, as described in the previous paragraph. Another consultation was organised with IBMA (see Section 2.1.4), and some data obtained through this consultation and considered useful from the WG are reported in specific chapters of this Opinion.

In addition, data on food-borne outbreaks caused by *Bacillus* spp. were retrieved from EFSA's zoonoses database, which includes data reported by the EU MSs and non-MSs in accordance with the zoonoses Directive 2003/99/EC. In order to provide a general overview of the reported data, EU-level (including non-MSs) information on *Bacillus* food-borne outbreaks reported during the period 2007–2014 are summarised by implicated food vehicle in Section 2.1.1, as well as in Appendix A. However, these data were not used to answer the ToRs because no information on the characterisation of the *B. cereus* strains nor on its levels on the suspected implicated foods was available. It is also important to note that, although the reporting of food-borne outbreaks is mandatory, as laid down in the zoonoses Directive, the national information reported to EFSA is not always complete leading to possible inconsistencies with the food-borne outbreak data derived from other sources (e.g. literature, national reports, etc.).

3. Assessment

3.1. The genus *Bacillus*

The genus *Bacillus* (Kingdom Bacteria; Phylum Firmicutes; Class Bacilli; Order Bacillales; Family Bacillaceae) was established in 1872 by Ferdinand Cohn; it is one of the most diverse genera in the class Bacilli and includes aerobic and facultative anaerobic, rod-shaped, Gram-positive spore-forming bacteria with G + C contents ranging from 32% to 69%, and currently the largest genus within the family Bacillaceae, presently consisting of at least 226 species. However, new strains are constantly added as new species, as well as being reclassified into new genera. For example, in the past 3 years, 10 existing species were reclassified into other genera and 39 new species were added to the genus (Mandic-Mulec et al., 2015). Recently, Maughan and Van der Auwera (2011), using 16S rRNA gene sequences and the conventional cut-off of 97% identity, reported the existence of 116 species of *Bacillus*, but 16S rRNA gene sequencing does not always allow species to be discriminated. The species in the genus *Bacillus* are mostly saprophytes widely distributed in the environment, and commonly isolated from soil, air, water, plants and animals (Drobniewski, 1993).

Several *Bacillus* species, such as *B. licheniformis* (Salkinoja-Salonen et al., 1999), *B. pumilus* (From et al., 2007), *B. brevis* and to a lesser extent *B. subtilis* (From et al., 2005), have been related to cases of food-borne disease associated with human illness (Logan et al., 2011; Logan, 2012). However, the majority of *Bacillus* strains have rarely been associated with diseases in humans or other animals. The principal exceptions to this are the members of the *B. cereus* group (including especially *B. cereus*, *B. thuringiensis* and *B. anthracis*) that contain strains of key medical and economic importance.

At the time of writing this opinion, the *Bacillus cereus* group, also known as *B. cereus sensu lato*, is a subdivision of the *Bacillus* genus that consists of eight formally recognised species: *B. cereus sensu stricto* (or *B. cereus* as it is usually called), *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus* and the recently validated new species *B. toyonensis* (Jimènez et al., 2013; Oren and Garrity, 2014).

Throughout the rest of this report we will use the terminology '*Bacillus cereus* group' to refer to the subdivision of the *Bacillus* genus composed of the eight validated and closely related species mentioned above (see also Glossary).

Some members of this group can cause diverse diseases while others appear to be non-pathogenic; *B. cereus sensu stricto* is an opportunistic human pathogen involved in food-related infection

outbreaks and considered as an important food contaminant. *Bacillus anthracis* is the causative agent of anthrax, an acute fatal disease in animals and in humans also used as a bioterrorism agent; *B. thuringiensis* is a well-known insect pathogen used as a biocontrol agent against some insect pests; *B. weihenstephanensis* is a psychrotolerant species which includes most of the psychrotolerant strains of the *B. cereus* group; it can grow at < 7°C but not at 43°C (this characteristic forms the basis of its differentiation from *B. cereus sensu stricto*) (Lechner et al., 1998). *Bacillus weihenstephanensis* can also be identified using rRNA or cold shock protein-targeted PCR (Lechner et al., 1998). *Bacillus mycoides and B. pseudomycoides* form rhizoidal colonies on agar plates and cannot be distinguished by physiological or morphological characteristics but they can be differentiated based on fatty acid composition and 16S rRNA (Nakamura and Jackson, 1995; Nakamura, 1998). *Bacillus mycoides* is also a psychrotolerant member of the *B. cereus* group (Guinebretière et al., 2008). *Bacillus weihenstephanensis*, *B. mycoides* and *B. pseudomycoides* have not been described as food poisoning agents, but their toxigenic potential remains uncertain (Stenfors et al., 2002). Some *B. cereus* group strains other than *B. weihenstephanensis* and *B. mycoides* are also able to grow at refrigeration temperatures. *Bacillus cytotoxicus* is a new species of the *B. cereus* group established in 2013 on the basis of presence of the cytK-1 gene, its thermotolerance (growth at up to 50–53°C), DNA–DNA hybridisation and multilocus sequence typing (MLST) and a smaller chromosome size of 4.1 Mb instead of 5.3–5.9 Mb for the other members of the group (Guinebretière et al., 2013). *Bacillus toyonensis* is represented by a single *B. cereus* group strain isolated from soil in Japan, and which is used as Toyocerin® powder (a product of heat-stable spores) in animal feed (Jimènez et al., 2013). *Bacillus toyonensis* has been distinguished from other *B. cereus* group type species by pairwise calculations of the average nucleotide identity (ANI) (Jimènez et al., 2013). The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) adopted in 2014 a Scientific Opinion on the safety and efficacy of Toyocerin® (*B. toyonensis*) as a feed additive for chickens for fattening, weaned piglets, pigs for fattening, sows for reproduction, cattle for fattening and calves for rearing and for rabbits for fattening (EFSA FEEDAP Panel, 2014). The FEEDAP Panel concluded that *B. toyonensis* poses a risk for the spread of genes coding for resistance to tetracycline and chloramphenicol, antibiotics of human and veterinary importance, and has the capacity to elaborate functional toxins and thus, to pose a risk to humans exposed to the organism.

One of the key characteristics of the *B. cereus* group, and of most members of the family Bacillaceae, is their ability to form endospores that provide a high level of resistance to heat, radiation, chemicals and desiccation, allowing these bacteria to survive adverse conditions for a prolonged period of time. These Gram-positive bacteria are ubiquitously distributed in nature and commonly isolated from foods, especially of plant origin (Slater and Murray, 2002). They are frequently found in milk and milk products, rice, vegetables, spices, egg and RTE foods (Kramer and Gilbert, 1989) and can survive pasteurisation or cooking, and then germinate and outgrow to reach hazardous levels when they are stored at room temperature (Andersson et al., 1995).

The most noteworthy and most frequently studied species within the *B. cereus* group are *B. cereus sensu stricto*, *B. anthracis* and *B. thuringiensis*. Several studies have suggested that *B. thuringiensis* and *B. anthracis* should be considered as varieties of *B. cereus sensu stricto* (Daffonchio et al., 2000; Helgason et al., 2000b; Bavykin et al., 2004). In fact, genotypically and phenotypically *B. anthracis* and *B. thuringiensis* essentially differ from *B. cereus sensu stricto* by the presence of plasmid-encoded factors (specific insecticidal toxins for *B. thuringiensis* and the three anthrax toxin subunits and poly-D-glutamic acid capsule components for *B. anthracis*). Other than these specific plasmid-encoded genes, the genomes of the three species, *B. anthracis*, *B. thuringiensis* and *B. cereus,* are very similar. The genetic determinants required for non-species-specific aspects of infection (a range of enterotoxins and other extracellular virulence factors on the chromosome) seem to be common to all the bacteria of the *B. cereus* group, as discussed in Section 3.1. Moreover, horizontal transfer of plasmid-encoded genes is widespread among *B. cereus* group strains, not only in soil and the rhizosphere (Saile and Koehler, 2006) but also in insects (Thomas et al., 2000), rats (Wilcks et al., 2008) and foodstuffs (Van der Auwera et al., 2007; Modrie et al., 2010).

3.1.1. Taxonomy and nomenclature of the *Bacillus cereus* group

For the identification and classification of *B. thuringiensis* strains, H serotyping, based on the immunological reaction to the bacterial flagellar antigen flagellin, was first established as a typing method (de Barjac and Frachon, 1990). Today, among the 3,500 *B. thuringiensis* isolates analysed, *B. thuringiensis* strains are classified into more than 69 H serotypes (Lecadet et al., 1999) and

13 sub-antigenic groups, giving 82 serological varieties (serovars). Based on this phenotypic characteristic, *B. thuringiensis* strains allocated to given serovars were given names that are still in use today (e.g. serovars (= var.): *kurstaki* (H3a, 3b, 3c), *galleriae* (H5a, 5b), *aizawai* (H7) *israelensis* (H14), etc.), although these only reflect one characteristic of the species (flagellin amino acid sequence) that has very little value in predicting other phenotypic characteristic or for classifying them phylogenetically (Xu and Côté, 2008).

Interestingly (but not unexpectedly), *B. cereus sensu stricto* strains, isolated from soil, food, dairies as well as from patients with *B. cereus* infections, can also be classified in the same H-serotyping system as *B. thuringiensis* (Helgason et al., 1998, 2000a). The type strain *B. cereus* ATCC 14579 has the H-antigen 6 (serovar *entomocidus*) (Helgason et al., 1998).

Bacillus cereus group bacteria have highly similar 16S and 23S rRNA sequences indicating that they have diverged from a common evolutionary lineage (Ash et al., 1991; Ash and Collins, 1992). Comparisons of genome sequence similarity between the *B. cereus* group species have revealed very high similarity in terms of nucleotide sequence identity and gene and operon organisation, which highlights the close relationship between these bacteria, making their identification to species level difficult. Enumeration of viable presumptive *B. cereus* following the EN ISO 7932 (ISO, 2004), the International Standard microbiological method used for the detection of *B. cereus*, does not distinguish between the *B. cereus* group members. Other phylogenetic studies using multilocus enzyme electrophoresis (MEE), amplified fragment length polymorphism (AFLP) or MLST (Helgason et al., 2000a; Ticknor et al., 2001; Vilas-Boas et al., 2002; Sorokin et al., 2006; Tourasse et al., 2011) also failed to discriminate between *B. cereus* group members at the species level.

Thus, the current taxonomy of the *B. cereus* group and the status of separate species for the different bacteria that constitute the *B. cereus* group mainly rely, for historic reasons, on phenotypic characteristics, established before the era of genome sequencing, and without the knowledge that important traits are plasmid-borne. In addition, the economic and medical importance of the individual species and in particular the separate species status of *B. thuringiensis* is clearly relevant to the use of *B. thuringiensis* as a biopesticide. In this opinion, biopesticides are defined as biological pesticides with a microorganism as the active ingredient. Other common designations for biopesticides are: bioinsecticides, microbial plant protection products (MPPPs), microbial pest control agents (MPCAs) and microbial control agents (MCAs). Nevertheless, despite the multiple species names, which are attributed to phenotypes mostly conferred by mobile genetic elements, there is a growing consensus in the research community that all these organisms could be considered members of a single species as demonstrated by many molecular studies (Priest et al., 2004; Tourasse et al., 2006; Rasko et al., 2007). Already in 1952 and 1973, two studies concluded that *B. cereus* should be considered as the parent species of the *B. cereus* group and that the other species of the *B. cereus* group should be considered as subspecies (Smith et al., 1952; Gordon et al., 1973; Kolstø et al., 2009), a recommendation that was never adopted. Moreover, *B. thuringiensis* strains display a similar repertoire of the potential virulence genes on the chromosome as *B. cereus sensu stricto* strains and it has been shown that these genes are actively expressed also in *B. thuringiensis* strains. These features clearly question the relevance of the taxonomic segregation of the *B. cereus* group into separate species. Due to practical reasons and traditions, the established species names within the *B. cereus* group have been kept.

3.1.2. The *Bacillus cereus* group from a genomic perspective

The *B. cereus* group contains eight species (see above), well separated from the other *Bacillus* species (Kolstø et al., 2009). The chromosomes of most *B. cereus* group strains are between 5.3 and 5.9 Mb (Kolstø et al., 2009), although some are considerably smaller, like *B. cytotoxicus* strains with a chromosome of only 4.1 Mb. The bacterial genome includes both the chromosome and extrachromosomal elements like plasmids and phages. The *B. cereus* group members usually have one or more plasmids (up to 12) of sizes from 2 to 600 kb (see Section 3.1.3 on plasmids). The phylogenetic relationship between the bacteria is based on genes located on the chromosomes, where the major part of the general virulence factors is also located, while important genes for specific virulence (like anthrax toxin genes and cereulide toxin synthesis genes) or for useful products like biopesticides (*cry* genes) are located on plasmids. Thus, the genes coding for key features distinguishing the *B. thuringiensis* species are located on plasmids rather than on the chromosome, and if these plasmids are lost, *B. thuringiensis* strains will lose their species-specific traits. Various typing methods have been used to study the genetic relationship between species and strains

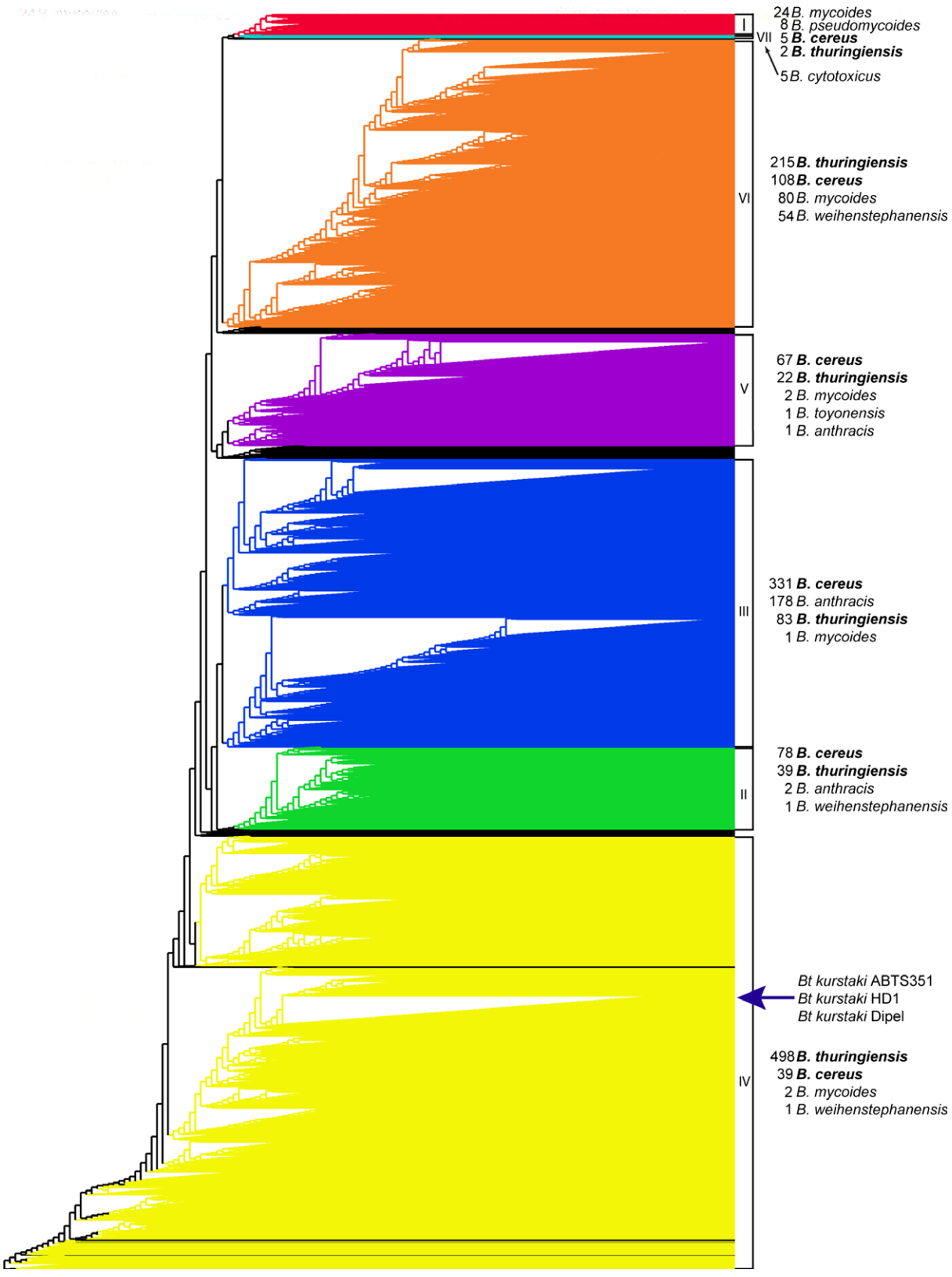
belonging to the *B. cereus* group. The most common are AFLP (Ticknor et al., 2001; Hill et al., 2004; Guinebretière et al., 2008), MLST (Helgason et al., 2004; Ko et al., 2004; Priest et al., 2004; Sorokin et al., 2006; Tourasse et al., 2006) and MEE (Helgason et al., 2000a,b). The first two methods compare the strains on the DNA level, while the third method compares the protein sequence of highly conserved genes, like 'house keeping genes'.

Bacillus cereus group strains show growth characteristics ranging from psychrotrophic to nearly thermophilic, and their genetic backgrounds seem to be strongly determined by an ecological adaptation associated with particular thermal niches (Carlin et al., 2010). A collection of 425 well-characterised *B. cereus* group strains derived from very different ecological niches has been distributed into seven major phylogenetic groups (I–VII), by using both genetic and phenotypic criteria (Guinebretière et al., 2008). The potential of strains for causing food poisoning appeared to correlate with certain phylogenetic groups (Guinebretière et al., 2010). A procedure to assign *B. cereus* group strains to one of these seven genetic groups using the sequence of the *panC* gene is described in Guinebretière et al. (2010) and an online tool has been developed which is available at https://www.tools.symprevius.org/Bcereus/english.php. Interestingly, a supertree based on MLST and AFLP data of 2,143 *B. cereus* group strains revealed that isolates from food and dairy sources can share identical genotypes with strains from environment and clinical samples (Tourasse et al., 2011) and confirmed the seven major phylogenetic subdivisions of the *B. cereus* group as defined by Guinebretière et al. (2008).

The *B. cereus* group are among the most heavily sequenced bacteria, particularly *B. anthracis, B. cereus sensu stricto* and *B. thuringiensis*. Around 300 strains are fully sequenced and their sequences are published and/or are available in public databases (like NCBI databases). Draft sequences are available for several hundred additional strains. In addition, a high number of *B. cereus* group strains have been analysed by phylogenetic typing, primarily using MLST or AFLP, or MEE. The phylogenetic relationship of the 3,193 analysed *B. cereus* group strains (including all fully sequenced and publicly available genomes) are now available in the HyperCat database (updated November 2015) at http://mlstoslo.uio.no/. For details of the methods, see Kolstø et al. (2009), Tourasse et al. (2011) Tourasse and Kolstø (2008a) and Tourasse et al. (2010).

In Figure 1, the genetic relationship of the strains in the updated HyperCat database is shown, and the seven phylogenetic groups (I–VII) (Guinebretière et al., 2008) are marked with different colours. The genetic relationship between strains within each group is stronger than between any two strains from different groups. The phylogenetic group VII only contains five isolates (all *B. cytotoxicus*), while all the other six phylogenetic groups (I–VI) have both *B. cereus sensu stricto* and *B. thuringiensis* strains (Figure 1). These six groups (I–VI) also contain *B. cereus* group isolates where the specific species of the strains has not been determined (data not shown). The phylogenetic group IV is the largest group, with 498 *B. thuringiensis* and 316 *B. cereus sensu stricto* strains (Figure 1). A great part of these isolates in group IV is from the soil, grassland and leaves from European countries, and from USA and Asia. Still there are also *B. cereus* group strains isolated from dairies and food and from hospitals and from patients in group IV, although most of the *B. cereus sensu stricto* isolates from patients are located in group III, where almost all *B. anthracis* strains are located (Figure 1). Interestingly, in the phylogenetic group IV, only one *B. weihenstephanensis* and three *B. mycoides* isolates were identified in addition to the 498 *B. thuringiensis* and 316 *B. cereus sensu stricto* strains. The rest of the *B. weihenstephanensis* strains were found in the phylogenetic group VI, together with almost all the *B. mycoides* strains (Figure 1). The figure clearly shows that strains belonging to the *B. cereus* group are genetically intermingled, and specifically that *B. cereus sensu stricto* and *B. thuringiensis* belong to the same phylogenetic groups.

The sequences of three of the *B. thuringiensis* strains used as biopesticide within the EU countries – *B. thuringiensis kurstaki* ABTS351*, B. thuringiensis kurstaki* HD1 and*B. thuringiensis kurstaki* Dipel – are available in the public databases and are thus included in the http://mlstoslo.uio.no/ database, and these strains are located very close to each other in the phylogenetic group IV, marked in Figure 1. These strains are closely related, from a genetic perspective, to both *B. cereus sensu stricto* and *B. thuringiensis* strains. Most likely, the other known commercially used biopesticide strains are found in the same phylogenetic group IV, and this could be confirmed once the relevant data of the strains are available and included in the http://mlstoslo.uio.no/ database.



Note the presence of both *B. cereus* and *B. thuringiensis* in groups II–VI. Strains from each group are given different colours, those outside any group are in black.

Figure 1: Phylogenetic relationship between 3,193 *Bacillus cereus* group isolates (http://mlstoslo.uio.no/) based on MLST and AFLP typing data

The phylogenetic intermixing of *B. cereus* and *B. thuringiensis* strains (Helgason et al., 1998, 2000a,b, 2004; Guinebretière et al., 2008; Kolstø et al., 2009; Tourasse et al., 2011) combined with information that some of the main phenotypic traits of the *B. cereus* group species are carried on plasmids have often raised the debate on species definition of the *B. cereus* group species. The picture is even more complicated as a few *B. cereus* group strains with *B. anthracis* virulence plasmid(s) have been isolated, both from humans in USA (Hoffmaster et al., 2004; Wright et al., 2011) and from great apes in Africa (Klee et al., 2006). The human cases have primarily been otherwise healthy welders who acquired acute infection resembling inhalation anthrax, but the strains clearly had a *B. cereus* chromosome and one plasmid that were highly similar to the *B. anthracis* pXO1 plasmid, named pBCXO1, and another large plasmid. The strains in Africa have been isolated from dead animals (gorilla and chimpanzee) in both Cameroon and Cote d'Ivoire, and the strains have both pXO1 and pXO2-like plasmids (named pBCXO1 and pBCXO2), and were fully virulent like *B. anthracis.* The name *B. cereus* biovar (bv) *anthracis* has recently been suggested for these strains from animals in Africa (Brezillon et al., 2015). The chromosomes of the identified strains from humans in USA and from dead animals in Africa are not identical, but they all map to the phylogenetic group III, where almost all the *B. anthracis* strains are also located (Figure 1).

Two scientific reports on genomic insights into the *B. cereus* group have been recently published (Liu et al., 2015; Zhu et al., 2015). Zhu et al. (2015) highlight the increase in genome complexity in the highly entomopathogenic strains, including several large plasmids and the presence of insecticidal toxicity-related genes. The authors also point to the importance of the gene expression levels of the insecticidal toxicity-related genes as well of the expression of the regulators as key factors for various *B. thuringiensis* strains, in addition to the more traditional *cry* and *cyt* genes. In the second scientific report, Liu et al. (2015) present a phylogenetic and taxonomic status of 224 *B. cereus* group genomes, based on genome sequence analysis, and grouped these bacteria in 30 clusters. The separation of the species *B. thuringiensis* and *B. cereus* was not clear, but the authors suggest to keep for the time being these two species as separate for the reason of taxonomic conservation. Taken together, these two reports clearly show the complexity of this group of bacteria, with the presence of a large number of plasmids of up to total 1 Mb DNA with a high number of genes of unknown functions. Among these plasmids are virulence plasmids that can be transferred or lost.

3.1.3. Plasmids and other extrachromosomal genetic elements in *Bacillus cereus* group

Most strains of the *B. cereus* group harbour extrachromosomal genetic elements (also referred to as 'plasmids'). Most of these molecules are circular dsDNA although a few linear dsDNA molecules (see below) have also been identified (Verheust et al., 2003). They were originally described in *B. thuringiensis* strains by González and Carlton (1980).

The sizes of the plasmids vary considerably, from ca. 2 kb to more than 500 kb, and their numbers can reach more than 12 distinct extrachromosomal molecules (Reyes-Ramírez and Ibarra, 2008). It is also generally accepted that two types of plasmids can be recognised based on their sizes:

- The small plasmids (< 15–20 kb) that display high copy numbers and replicate using the Sigma-replication mechanism (also called RCR for rolling circle replication) (Andrup et al., 2003);
- The larger ones (> 20 kb) whose copy numbers are lower and can be no more than 1 copy/chromosome. Most of them replicate using Theta-replication, much like the chromosome (Wilcks et al., 1999; Tang et al., 2006; Li et al., 2009; Pomerantsev et al., 2009).

Of note are the linear molecules that replicate by a protein-prime replication mechanism (Berjón-Otero et al., 2015). These extrachromosomal molecules were found to correspond to the prophage state of temperate phages belonging to the Tectivirus group (Verheust et al., 2003, 2005; Gillis and Mahillon, 2014b). They were found mainly, but not exclusively, in *B. thuringiensis* strains.

A few cases of hybrid plasmid-phage molecules have also been reported in strains of both *B. cereus* and *B. thuringiensis* (Smeesters et al., 2011; Swanson et al., 2012).

Concerning *B. thuringiensis*, the NCBI database reports (as of 1 January 2016) 28 fully annotated genomes, including those of the biopesticidal serovar *kurstaki* strains HD-1 (Day et al., 2014) and HD73 (Liu et al., 2013) and serovar *israelensis* strain HD-789 (Doggett et al., 2013). Among these 28 strains, the number of extrachromosomal molecules varies from 1 to 15. Of note is strain Bt407 that harbours the smallest plasmid (2,062 bp, named BTB_2P) and one of the two largest plasmids

(501,911 bp, named BTB_502p) (Sheppard et al., 2013). The largest element is found in strain *B. thuringiensis* HS18-1 and contains 509,170 bp, almost 10% of its host chromosome (Li et al., 2015).

The functions associated with the *B. cereus* group plasmids are still largely unknown, with the notable exceptions of the genes coding for the entomopathogenic toxins of *B. thuringiensis* (e.g. the 'Cyt' cytolysins and the δ-endotoxins, also called 'Cry' proteins for crystal) active against insect larvae, the genetic determinants of the cereulide toxin found in the emetic strains of *B. cereus* (Hoton et al., 2005; Ehling-Schulz et al., 2006a) and the genes associated with the anthrax toxins (Lethal and Oedema toxins) and the capsule of *B. anthracis* (Moayeri et al., 2015). Of note are the pBCXO1 and pBCXO2 plasmids found in strains referred to as '*B. cereus* biovar *anthracis*'. These plasmids are very similar to the pXO1 and pXO2 virulent plasmids of *B. anthracis*, but reside in strains more related to other *B. cereus* than to *B. anthracis* strains. These *B. cereus* biovar *anthracis* strains have been isolated in Côte d'Ivoire and Cameroon from chimpanzees and a gorilla that died from anthrax-like disease (Klee et al., 2010; Brezillon et al., 2015).

Other plasmid-borne elements include:

- Antimicrobial resistance genes, e.g. tetracycline resistance in plasmid pBC16 (Palva et al., 1990) and heavy metal resistance genes, e.g. mercury resistance (Belliveau and Trevors, 1990).
- Genetic determinants of NRPS (non-ribosomal peptide synthetases) antagonistic molecules active on other bacteria, fungi, protozoa or other (micro-)organisms (Zhao et al., 2007; Kevany et al., 2009).
- The genes of bacteriocins such as Thuricin Bn1 (Ugras et al., 2013), entomocin 110 (Cherif et al., 2008) or Bacthuricin F4 (Ben Fguira et al., 2014).
- The presence of numerous MGE (mobile genetic elements), including IS (insertion sequences) (Léonard et al., 1997), MICs (mobile insertion cassettes) (Chen et al., 1999), Tn (transposons) (Mahillon and Lereclus, 1988; Baum, 1994; Van der Auwera and Mahillon, 2005), or group I and group II introns (Tourasse and Kolstø, 2008b).

Some of the small plasmids are said to be 'cryptic' as they do not encode any noticeable function besides their replication (and mobilisation) cassettes. They are also assumed to behave as potential reservoirs for future gene acquisition and/or transfer (Andrup et al., 2003; Amadio et al., 2009).

Based on their incompatibility (two plasmids using the same type of replication origins are said to be 'incompatible' as they cannot reside in the same strain because of their competition for replication), several plasmids could be classified in known Sigma or Theta-replicative plasmid families.

Another feature of the *B. cereus* group plasmids is their ability to be readily exchanged 'horizontally', i.e. to be transferred from cell to cell through a process known as bacterial conjugation. These genetic exchanges have been extensively characterised under laboratory conditions, including in food matrices (Van der Auwera et al., 2007; Modrie et al., 2010), and also in more real-life conditions such as in insect larvae or river water (Thomas et al., 2000, 2001).

Three types of plasmids are recognised:

- The 'conjugative' plasmids able to mediate their own transfer. At least two types of conjugative processes have now been described: the Type IV-like Secretion System (T4LSS) used by plasmids such as pAW63 from *B. thuringiensis* serovar *kurstaki* (Wilcks et al., 1998; Van der Auwera et al., 2005, 2008; Van der Auwera and Mahillon, 2008) and the unique and quite efficient system associated with pXO16 from *B. thuringiensis* serovar *israelensis* (Jensen et al., 1995; Timmery et al., 2009; Makart et al., 2015).
- The 'mobilisable' plasmids that require the presence of another co-resident conjugative plasmid to be transferred horizontally. These elements harbour a 'mobilisation cassette' consisting of a *mob* gene acting on an origin of transfer (*oriT*) (Andrup et al., 1995).
- The 'non-mobilisable' plasmids, that are typically not able to move (they do not harbour the mobilisation cassette) (Andrup et al., 1996).

In contrast to other Bacilli, no efficient natural competence transformation has yet been reported for strains of the *B. cereus* group. The only case of artificial 'natural competence' was recently reported in *B. cereus* strain ATCC14579 after the heterologous expression of the *Bacillus subtilis comK* (competence K) gene (Mironczuk et al., 2008). However, artificial transformation methods have been described: protoplasts (Fischer et al., 1984) and electroporation (Belliveau and Trevors, 1989; Turgeon et al., 2006; Groot et al., 2008) can be used to introduce DNA molecules into these bacteria. Consequently, several plasmids from *B. cereus* and *B. thuringiensis* have been designed for biotechnology purposes:

- Shuttle cloning vectors between the Gram-negative *Escherichia coli* and Gram-positive bacilli (Baum et al., 1990; Arantes and Lereclus, 1991; Mesrati et al., 2005);
- BAC (bacterial artificial chromosome) vectors for the cloning of large DNA fragments in bacilli (Liu et al., 2009);
- Numerous expression vectors, including reporter genes such as β-galactosidase, the GFP (green fluorescent protein) of the luciferase system;
- Thermo-sensitive vectors, e.g. pRN5101 (Biswas et al., 1993) used for integrative recombination and gene knockout.

Plasmids from other Gram-positive bacteria (e.g. the staphylococcal plasmids pC194, pE194 and pUB110) have also been used to develop vectors for members of the *B. cereus* group. Finally, a trans-gramic conjugation system between *E. coli* and bacilli allows mobilising shuttle vectors into members of the *B. cereus* group (for cloning or gene expression purposes) (Cataldi et al., 1990).

As a final note on the plasmids and extrachromosomal genetic elements present among members of the *B. cereus* group, it is worth mentioning the existence of numerous phages, either virulent or temperate, some of which may contribute to efficient horizontal transfers via generalised phage transduction (Lecadet et al., 1980) among strains (Gillis and Mahillon, 2014a).

3.1.4. Summarising remarks (Section 3.1)

The *B. cereus* group, also known as *B. cereus sensu lato*, is a subdivision of the *Bacillus* genus that consists of eight formally recognised species: *B. cereus sensu stricto* (or *B. cereus* as it is usually called), *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus* and the recently validated new species *B. toyonensis.* However, the current taxonomy of the *B. cereus* group and the status of separate species for the different bacteria that constitute the *B. cereus* group, for historic reasons, mainly rely on phenotypic characteristics established before the era of genome sequencing and without the knowledge of important traits being plasmid-borne. In addition, the economic and medical importance of the individual species and in particular the separate species status of *B. thuringiensis* is clearly relevant to the use of *B. thuringiensis* as a biopesticide.

Bacillus thuringiensis strains display a similar repertoire of the potential virulence genes on the chromosome as *B. cereus sensu stricto* strains and it has been shown that these genes can also be actively expressed in *B. thuringiensis* strains. These features clearly question the relevance of the taxonomic segregation of *B. cereus* group into separate species. The phylogenetic relationship between the bacteria is based on genes located on the chromosomes, while important genes for virulence (like the cereulide toxin synthesis genes) or for useful products like biopesticides (*cry* genes) are located on plasmids. Thus, the genes coding for key features of *B. thuringiensis* are located on mobile elements rather than on the chromosome.

The extrachromosomal genetic elements in the *B. cereus* group members vary considerably in number and size from strain to strain and may represent more than 15% of the entire genome (e.g. strain *B. thuringiensis* HS18-1). This is particularly true in the case of the *B. thuringiensis* strains which often contain a plethora of plasmids, although no particular plasmid type or profile has been attributed (and can be used) to differentiate this species from the other members of the *B. cereus* group.

In addition, several of these extrachromosomal molecules participate in horizontal gene transfers via phage transduction or plasmid conjugation. Although these genetic transfers have been documented under laboratory conditions, their extent and biological implications (e.g. in modifying the bacterial virulence spectra) are, however, difficult to assess.

The fact that the most pronounced properties of some of the species within this group are due to genes present on mobile genetic elements like plasmids make the taxonomy of the *B. cereus* group very challenging, particularly regarding *B. thuringiensis* and *B. cereus* where the species in general are so closely related, and a strain of one of the two species may be more closely related to a strain in the other species. The two species have the same repertoire of chromosomal determinants of potential virulence factors, although this may vary from one strain to another. Thus, despite the multiple species names which are attributed to phenotypes mostly conferred by mobile genetic elements, there is a growing consensus in the research community that all these organisms could be considered members of a single species.

3.2. Pathogenicity and contributing virulence factors in the *Bacillus cereus* group

3.2.1. Insect pathogenicity of *Bacillus thuringiensis*

The commercial *B. thuringiensis* products are powders containing a mixture of dried spores and toxin crystals. Presently there are over 400 *B. thuringiensis*-based formulations that have been registered in the market, and most of them contain insecticidal proteins and viable spores, though the spores are inactivated in some products (Ali et al., 2010). *Bacillus thuringiensis* has been used successfully to control insect pests in agriculture (e.g. vegetable cultivation, tree fruit and nut crops), forestry and mosquito control (Marrone, 1994). Agriculture accounts for more than 60% of the market for *B. thuringiensis* bioinsecticides. Globally, sales of *B. thuringiensis* in 2010 were estimated at US$ 210 million. Geographically, 35% of all sales are in North America, 35% in Asia and Australasia, 8% in Europe, 14% in Latin America and 3% in Africa and the Middle East (Glare et al., 2012). Forests and fruit and vegetable crops account for 80% of *B. thuringiensis* bioinsecticide use. In 2001, in the US, more than 20,000 ha of brassica and tomato crops (corresponding to 60% of the total area under brassicas and 40% of the area under tomatoes), together with 40,000 ha of vines (10% of the entire area under vines), 35,000 ha of almond orchards and 23,000 ha of apple orchards (18% and 13% of the area under these trees) were treated with *B. thuringiensis* (Walker et al., 2003). In California, *B. thuringiensis* applications (which include *aizawai* and *kurstaki* strains) increased significantly from 2002 to 2009 for crops such as broccoli, cabbage, cauliflower, corn, leaf lettuce, tomatoes and strawberries (CDPR, 2009).

Between 2013 and 2014, in Western Europe, a total of 454,210 ha were treated with *B. thuringiensis*-based insecticides, among them: 122,000 ha of vegetable crops, 91,000 ha of pome/stone fruit, 86,000 ha of vine/grapes and 25,700 ha of forest (see Annex A for the detail of treated hectares by crop group and by country). Spain and Italy were the largest *B. thuringiensis* users in Europe representing 37.9% and 34.8% of areas treated, respectively. Combined with France (7.9%) and the Netherlands (7.6%), these four countries represent over 88% of *B. thuringiensis* usage in Western Europe. During the same period, worldwide, it is estimated that a total of 34,901,300 ha of crop land were treated by *B. thuringiensis*-based insecticides. Global usage was dominated by raw crops, specifically soya bean and cotton. Soya bean alone accounted for 22,058,100 ha of the treated area followed by vegetables (4,498,600 ha) and cotton (2,924,900 ha) (see Annex A for details).

Bacillus thuringiensis sprays are chosen by organic farmers to meet guidelines for using strictly non-synthetic materials (Sanchis, 2011). An additional use of *B. thuringiensis* is in the protection of stored commodities (e.g. wheat) from pest infestation. Some *B. thuringiensis* products also play a major role in the microbial control of forest pests such as the gypsy moth (*Lymantria dispar*) and the spruce budworm (*Choristoneura fumiferana*) in North America. For example, in Canada, between 1980 and 1999, almost 6 million hectares of forest were treated by aerial spraying with products based on *B. thuringiensis*. It is also estimated that 1.8 million hectares of forest in Europe were treated with *B. thuringiensis*-based products between 1990 and 1998 (van Frankenhuyzen, 2000). *Bacillus thuringiensis* is also widely used in the urban and the peri-domestic control of mosquitoes in many countries all over the world and for the rural control of blackfly larvae in Africa (both vectors of tropical diseases, such as malaria, onchocerciasis and dengue fever). For example, *B. thuringiensis*-based formulations active against dipteran larvae are used in France by l'Entente Interdépartementale pour la Démoustication (EID), and in Germany by the Kommunale Aktionsgemeinshaft zur Bekämpfung der Schnakenplage Ludwigshafen (KABS), for mosquito control measures in several French regions and along the Rhine valley, respectively (Sanchis and Bourguet, 2008). *Bacillus thuringiensis* has also become a key source of genes for transgenic expression in plants to make genetically modified (GM) crops that are resistant to target insects. The adoption by farmers of these transgenic plants engineered with *cry* genes has been dramatic, with 75.9 million hectares of transgenic *cry* containing crops (Bt maize, Bt rice, Bt cotton, Bt soy) planted in 2013 representing over 40% of all GM crops planted that year (James, 2013).

The species *B. thuringiensis* is characterised by the production of crystalline inclusions in parallel with spore formation. The insecticidal proteins in the crystalline bodies produced during sporulation have been shown to contain mainly two types of insecticidal proteins: the Cry proteins (for crystal) also known as δ-endotoxins, and the Cyt (for cytolytic) proteins. The Cry proteins constitute the largest group of insecticidal proteins produced by *B. thuringiensis* whereas the Cyt proteins constitute a

smaller and distinct group of insecticidal proteins that can also be present in the crystal inclusions along with *cry* genes in *B. thuringiensis* subsp. *israelensis*. The Cry and Cyt proteins are unrelated structurally. Cry proteins are active against larvae of very diverse insect orders as well as, in some cases, against species from other invertebrate phyla (mites, nematodes) or even against human cancer cells. The Cyt proteins are active against several dipteran larvae, particularly mosquitoes and black flies. Additionally, some *B. thuringiensis* isolates can also synthesise other insecticidal proteins during the vegetative growth phase; these are subsequently secreted into the culture medium and have been designated as vegetative insecticidal proteins (VIPs) that have insecticidal activity against lepidopteran or coleopteran insect species. There are currently 73 families of crystal (Cry) toxins comprising a total of 732 toxins, three families of cytotoxic (Cyt) proteins including 38 different toxins and 125 VIPs belonging to four different families (Lacey et al., 2015). A website which hosts details of all the *cry, cyt and vip* cloned genes has been established at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/ and is frequently updated as new genes are discovered. The product of each individual *cry* gene generally has a restricted spectrum of activity, limited to the larval stages of a small number of species.

The mode of action of Cry proteins has been extensively studied and reviewed (Bravo et al., 2007; Vachon et al., 2012). In brief, the toxicity of the Cry proteins is explained by the formation of transmembrane pores or ion channels that lead to osmotic cell lysis. Pathogenesis begins with the ingestion of the *B. thuringiensis* crystal, which is solubilised by the alkaline pH of the insect midgut. The Cry proteins in the crystals are inactive protoxins, which are converted through proteolytic cleavage, by the insect midgut proteases, into active toxins (Lecadet and Martouret, 1967) consisting of the amino-terminal part of the protoxin (Choma et al., 1991). Following their activation, the Cry toxins can pass through the peritrophic membrane, a chitinous sheath, thought to provide protection against physical abrasion of the midgut epithelium (Richards and Richards, 1977). Activated Cry proteins then bind to specific receptors on the apical brush border of the midgut microvilli in susceptible insects (Hofmann et al., 1988a,b; Van Rie et al., 1990). Following binding, the toxins rapidly and irreversibly insert into the cell membrane and aggregate, which results in the formation of pores which leads to epithelial cell lysis as a result of selective cation permeability (English and Slatin, 1992). The specificity of Cry toxins is determined by their potential to bind to the surface proteins that are located in the microvilli of larval midgut cells (Bravo et al., 2011). The specific receptors of some of the Cry proteins have been identified and shown to be membrane aminopeptidases (Knight et al., 1994), proteins of the cadherin family (Vadlamudi et al., 1995) and glycosylphosphatidyl-inositol (GPI)-anchored alkaline phosphatases (Bravo et al., 2011). Currently, 38 different aminopeptidases have been reported for 12 different lepidopterans (Pigott and Ellar, 2007). At the physiological level, the lysis of the epithelial cells leads to paralysis of the insect's digestive system and it quickly stops eating. Alone, this effect of the Cry toxins can cause the death of the susceptible insects within 1–3 days after the ingestion of the crystals. However, generally the insects also ingest *B. thuringiensis* spores along with the crystals. The result is that, when *B. thuringiensis* sprays are used, septicaemia due to the germination of the spores and the development of the bacteria that is almost always associated with the toxaemia develops, and this may amplify the toxic effect of the Cry toxins. This specific mode of action, including solubilisation and activation from pro-toxin to toxin in the gut and binding to specific receptors, dictates that the activity of specific Cry-toxins are restricted to specific orders of insect larvae; this also means that activity of these toxins in the gut of mammals including humans is very unlikely, which is also demonstrated by feeding studies with Bt crops expressing different activated Cry-toxins, as no effects have been recorded (Koch et al., 2015). Cyt toxins need also to be solubilised and activated in the gut under basic conditions this is unlikely to occur in humans; however, their action is not dependent on specific receptors, and the activated Cyt-toxins have been shown to be cytotoxic to mammalian cultured cell lines and to cause haemolysis of a number of different erythrocytes (Thomas and Ellar, 1983). VIP toxins which are not phylogenetically related to Cry-toxins are also dependent on specific receptors on the gut surface for their activity, so their activity is also limited to specific orders of insects (Chakroun et al., 2016).

The use of *B. thuringiensis* as a biopesticide in the EU is restricted to the strains *Bacillus thuringiensis* subsp. *aizawai* strains ABTS-1857 and GC-91, *Bacillus thuringiensis* subsp. *israelensis* (serotype H-14) strain AM65-52, *Bacillus thuringiensis* subsp. *kurstaki* strains ABTS 351, PB 54, SA 11, SA12 and EG 2348 and *Bacillus thuringiensis* subsp. *tenebrionis* strain NB 176 (TM 14 1).

Bacillus thuringiensis subsp. *aizawai*, strains ABTS-1857 and GC-91 (the active ingredients of XenTari WG and Agree 50 WP) harbour a combination of three to four active insecticidal cry toxins (Cry1Aa, Cry1Ab, Cry1Ca and Cry1Da) that are especially active against lepidopteran larvae that feed on stored grains (see Annex A).

Bacillus thuringiensis israelensis AM65-52 (the active ingredients of VectoBac) harbours four Cry proteins (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and two Cyt proteins (Cyt1Aa and Cyt2Ba) that are effective against dipteran larvae including blackflies and mosquitoes.

Bacillus thuringiensis subsp*. kurstaki,* strains ABTS 351, PB 54, SA 11, SA 12 and EG 2348 (the active ingredients of DiPel WG, Belthirul or Delfin WG) harbours various combinations of four active insecticidal toxins (Cry1Aa, Cry1Ab, Cry1AC and Cry2Aa) that are effective in controlling many leaf-feeding lepidopteran larvae that are important crop pests or forest pest defoliators (see Annex A).

Bacillus thuringiensis subsp*. tenebrionis* strain NB-176 (the active ingredients of Novodor SC) harbours the Cry3Aa insecticidal toxins active against the Colorado potato beetle larvae, *Leptinotarsa decemlineata* (see Annex A).

3.2.2. Pathogenicity of *Bacillus cereus* group for humans

The *B. cereus* group organisms can cause a variety of diseases in humans. *Bacillus anthracis* is the causative agent of the fatal animal and human disease anthrax (Moayeri et al., 2015) while *B. cereus* is well known as an opportunistic food-borne pathogen causing two forms of gastrointestinal (GI) diseases: emesis and diarrhoea (Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008). Besides its food poisoning potential, *B. cereus* is also increasingly recognised as a nosocomial pathogen causing life-threatening infections in immunocompromised patients but there are also case reports available of *B. cereus* infections in immunocompetent patients. Among the highest risk groups are neonates or patients with indwelling catheters. In addition, *B. cereus* is frequently isolated from severe eye infections as well as from traumatic and surgical wound infections. The spectrum of *B. cereus*-related non-GI related diseases include fulminant septicaemia, meningitis and brain abscesses, pneumonia and endocarditis, endophthalmitis and soft tissue infections (Bottone, 2010).

The *B. cereus* group produces various virulence factors, which may act synergistically within the host. Several toxins have been associated with two distinct forms of food poisoning, emetic and diarrhoeal syndrome. Information about the role and importance of the known toxins and putative virulence factors, such as enzymes and proteases, in non-GI diseases is largely unknown.

The panoply of toxins and virulence factors found in the *B. cereus* group is broadly distributed among the different members of the group (Prüss et al., 1999), except the species defining toxins, namely the anthrax toxin, which is restricted to *B. anthracis* and a few *B. cereus* strains, and the insecticidal crystal-forming toxins (Cry toxins) characteristic of *B. thuringiensis*.

As *B. cereus* and *B. thuringiensis* strains are genetically intermingled (Kolstø et al., 2009), these species are usually not differentiated in routine clinical diagnostics or food microbiology. Thus, the actual contribution of the two species to GI and non-GI diseases is currently unknown. A study at retail level revealed a high presence of *B. thuringiensis* among the randomly tested *B. cereus* group isolates from foodstuffs (31 out of 40) (Rosenquist et al., 2005), although reports on *B. thuringiensis* related food poisonings are rarely found (McIntyre et al., 2008). As *B. thuringiensis* is capable of producing the same diarrhoeal enterotoxins as *B. cereus* (for details, see below), cases of food-borne infections with *B. thuringiensis* may have been overlooked. There are some reports from clinical infections linked to *B. thuringiensis* such as bacteraemia, wound infections and periodontitis (Helgason et al., 2000a; Kuroki et al., 2009) but in general, the significance of *B. thuringiensis* as a causative agent for human disease is far from understood (WHO, 2009). Thus, much more data from systematic investigation would be necessary to decipher the role of *B. thuringiensis* in food-borne as well as in clinical infections.

3.2.2.1. Emetic toxin (cereulide)

The cereulide toxin is responsible for the emetic type of *B. cereus* food poisoning (Agata et al., 1995). Cereulide is commonly preformed in food resulting in rapid onset of vomiting (15 min to 6 h) after uptake of the contaminated food. Normally this disease is self-limiting, but occasionally, more severe intoxications are reported requiring hospitalisation and intensive medical care, including liver transplantations (Messelhäusser et al., 2014; Tschiedel et al., 2015). Sometimes fatalities have been reported in connection with cereulide food-borne intoxications (Dierick et al., 2005; Naranjo et al., 2011). Based on data from animal trials, the minimal intoxication dose is estimated to be in the range of 8–10 μg/kg body weight. However, further research will be necessary to define the actual emetic dose for humans. In recent food-borne outbreaks, concentrations in food remnants ranging from 2 to 6 μg/g food have been reported (Messelhäusser et al., 2014; Delbrassinne et al., 2015; Marxen et al., 2015b). Due to its acid and heat stability, cereulide is usually not inactivated once it is formed in the

normal food-processing environment (Rajkovic et al., 2008). Moreover, because of its small size (1.2 kDa), cereulide cannot be removed by filtration or bactofugation,[15] which may explain why often only low levels of bacteria, but significant amount of cereulide toxin, are detected (Messelhäusser et al., 2014). Moreover, as cereulide is resistant to cleavage by pepsin and trypsin, it will not be inactivated during stomach passage in the host.

Chemically, cereulide is a depsipeptide toxin that is produced non-ribosomally by the cereulide peptide synthetase Ces, which belongs to the family of non-ribosomal peptide synthetases (NRPS). The *ces* gene cluster is localised on the 270-kb megaplasmid pCER270 that shares its backbone with the anthrax toxin plasmid pXO1 (Ehling-Schulz et al., 2005b, 2006a; Rasko et al., 2007). Different levels of regulation are involved in the control of cereulide toxin production (Ehling-Schulz et al., 2015; Lücking et al., 2015) and toxin production capacity is highly strain-dependent. Toxin production under standard laboratory conditions may vary from 7 up to 80 μg/mL (Stark et al., 2013). In the past, quantification of cereulide in outbreak situations (Delbrassinne et al., 2011) has been hampered by the lack of appropriate methods. The development of a stable isotope mass spectrometry (MS)-based dilution assay (SIDA) and the availability of a commercial isotope labelled cereulide to be used as internal standard (Bauer et al., 2010; Biesta-Peters et al., 2010) paved the way for an EU initiative (CEN action) to set up an ISO standard for quantitative detection of cereulide in foods (EN ISO 18465) (ISO, online). Recently, 18 different isoforms of the emetic toxin have been described with highly variable cytotoxic potential, ranging from non-toxic to 10-fold more toxic than the classical cereulide (Marxen et al., 2015a). By employing the MS-based assay, which was established for the simultaneous quantification of the major iso-cereulides, the highly toxic iso-cereulide A was detected in food implicated in recent food-borne outbreaks in amounts which suggest it is a key toxic component (Marxen et al., 2015b).

Generally, the capacity for cereulide production is linked to a lineage of closely related *B. cereus* strains (Ehling-Schulz et al., 2005a). The gene cluster encoding the cereulide synthetase genes has been detected in a few *B. weihenstephanensis* strains suggesting a horizontal transferability of the *ces* genes (Mei et al., 2014). To date, the *ces* genes have not been found in *B. thuringiensis* (Thorsen et al., 2006), even though a systematic assessment was performed on more than 200 strains (Hoton et al., 2009). Recently, two cereulide-like depsipeptides have been isolated from a *Paenibacillus tundrae* strain, which have been shown *in vitro* to be highly toxic to mammalian cells (Rasimus et al., 2012), indicating that there might be other, yet to explore, cereulide toxin-like depsipeptides existing outside the *B. cereus* group.

Several severe *B. cereus* intoxication cases with rhabdomyolysis and/or liver failure (including five deaths) have been reported, linked to the production of cereulide in food. In 1976, a 11-year-old boy died about 15 h after eating Chinese noodles (Takabe and Oya, 1976). The cause of death was considered to be heart failure resulting from myocardial fatty degeneration. *Bacillus cereus* was isolated and identified as the causative agent of this food poisoning. In 1997, a 17-year-old boy and his father had GI symptoms, including nausea and emesis, followed by liver damage and rhabdomyolysis. They ate spaghetti with homemade pesto 30 min before. The food had been prepared for 4 days and refrigerated, although on several occasions, it had been left at room temperature for one or more hours before being reheated in a pan. The father made a complete recovery but his son died of fulminant liver failure (Mahler et al., 1997). In August 2003, in Belgium, five children of a family became sick after eating pasta salad. The pasta salad was prepared on a Friday and taken to a picnic on the next day. Leftovers had been stored in the fridge until the following Monday evening when they were served for supper to the children. The youngest girl died (Dierick et al., 2005). In 2008, two healthy children and their mother became sick 30 min after eating reheated fried rice. The rice had been cooked the day before and kept at room temperature. The 1-year-old boy died and his 2-year-old sister recovered after plasma exchange (Shiota et al., 2010). The same year, in France, a 15-year-old boy became very sick after he had eaten pasta. The pasta had been cooked 4 days before and stored in a refrigerator. The patient eventually recovered after several days in an intensive care unit (Saleh et al., 2012). In Brussels, a 20-year-old student became sick after eating a meal of spaghetti with tomato sauce. The meal had been prepared 5 days before and left at room temperature. He died a few hours after eating the pasta (Naranjo et al., 2011).

[15] Bactofugation: a process to separate microorganisms from milk using a special centrifuge.

3.2.2.2. Enterotoxins

Two different protein toxin complexes, the non-haemolytic enterotoxin (NHE) and the haemolysin BL (HBL), as well as the single protein cytotoxin K (CytK) (Beecher et al., 1995; Lund and Granum, 1996; Lund et al., 2000) have been linked to the diarrhoeal type of *B. cereus* food poisoning, which is characterised by abdominal pain and watery diarrhoea. In contrast to the plasmid-encoded cereulide toxin synthetase genes, the chromosomal-encoded enterotoxin *hbl, cytK* and *nhe* genes show considerable molecular diversity (Ehling-Schulz et al., 2006b), which may influence their toxic activity. It has been shown that besides the original CytK (renamed to CytK-1), a second form, designated CytK2, with much lower cytotoxic potential than CytK1 exists. The diarrhoeal syndrome is thought to be the result of toxico-infections. In contrast to the cereulide toxins, the enterotoxins are heat labile and are enzymatically digested during the stomach passage while *B. cereus* spores (and also some vegetative cells) survive GI passage (Ceuppens et al., 2012). Thus, it is generally assumed that diarrhoea is induced by enterotoxin production after outgrowth of spores in close proximity or direct contact to the intestinal epithelium cells (Granum and Lund, 1997; Berthold-Pluta et al., 2015). To define the minimum infectious dose is difficult because the enterotoxigenic potential of *B. cereus* strains is highly variable (Guinebretière et al., 2002; Jessberger et al., 2015). Commonly 10^5–10^8 *B. cereus* cells or spores per gram are found in foods associated with the diarrhoeal syndrome, although occasionally lower amounts have been reported from food-borne *B. cereus* infections (EFSA, 2005). Nearly all *B. cereus* strains harbour the *nhe* genes, while *hbl* and *cytK* are detected in about 30–70% of isolates (Guinebretière et al., 2002; Ehling-Schulz et al., 2005a; Castiaux et al., 2015). While *hbl* and *cytK* are frequently exchanged, *nhe* shows a more clonal inheritance (Böhm et al., 2015). Nevertheless, a few strains (4 out of 142) have been reported to possess a second copy of the *nhe* operon, which may have arisen from two recent independent horizontal gene transfer events. Notable, in one of the four strains carrying the second *nhe* operon, the *nhe* copy is located on a mega plasmid (Böhm et al., 2015). In contrast to the emetic toxin cereulide, the enterotoxins are also frequently found in other *B. cereus* group members, including *B. thuringiensis* (Prüss et al., 1999; Gaviria Rivera et al., 2000; Swiecicka et al., 2006; Melnick et al., 2012).

The chromosomally encoded enterotoxins are comparatively well characterised at the molecular level, but their mode of action is largely unknown and their contribution to the overall enterotoxicity of *B. cereus* is still unclear (Schoeni and Wong, 2005; Fagerlund et al., 2008; Doll et al., 2013; Castiaux et al., 2015; Jessberger et al., 2015). The tripartite enterotoxins NHE and HBL belong to the family of AB toxins. Although they show high structural similarities and both require sequential binding of their components to the host cell, their mode of action seems to be different as they cannot complement each other (Lindbäck et al., 2010; Sastalla et al., 2013). NHE was first identified from a strain isolated from a large food-poisoning outbreak in Norway (Lund and Granum, 1996). By cell culture assays, it has been shown that it is a cytolytic against epithelial cells and erythrocytes due to osmotic lysis induced by pore formation in the host cell membrane (Fagerlund et al., 2008). Depending on the cell line, all three enterotoxin components, or only two, are necessary for cytotoxic activity (Lund and Granum, 1997; Haug et al., 2010). HBL possesses haemolytic and dermonecrotic activities and has been reported to contribute to non-GI-related *B. cereus* infections (Beecher and Wong, 1994, 2000). HBL as well as NHE are organised in operons that are each transcribed from a central promoter, which is positively regulated by the pleiotropic regulator PlcR as well by other transcriptional regulators (Stenfors Arnesen et al., 2008). The global regulator PlcR is perhaps the most important general virulence factor of the *B. cereus* group, regulating the expression of a range of the more specific and secreted virulence factors of these bacteria, including at least 45 genes (Gohar et al., 2008) including the enterotoxins and phospholipases. PlcR is a member of a group of quorum-sensing proteins termed the RNPP family, and a signal peptide PapR is necessary for the binding of PlcR to the DNA during the regulation. PapR is synthesised as a larger peptide, secreted, processed and re-enters the bacterial cell before binding to PlcR and contributing to the binding of PlcR to the DNA sequence to be regulated – thus the transcriptional regulation by PlcR also responds to the environment. However, the transcriptional control of enterotoxins only partially explains the huge differences in enterotoxic capacities of *B. cereus* strains. Recently, it has been shown that enterotoxin expression is more complex than expected, involving not only transcriptional but also post-transcriptional and post-translational regulatory mechanisms (Jessberger et al., 2015).

CytK is a β-barrel pore-forming toxin related to the *Staphylococcus aureus* α-toxin, which was isolated cloned and sequenced from an unusual strain (strain NVH 391/98) (Lund et al., 2000). This strain was responsible for a large, severe, food-borne outbreak of diarrhoeal disease in France in 1998,

in which several elderly people had bloody diarrhoea after consumption of vegetable puree containing 3.2×10^5 *B. cereus* per gram, three of whom died (Lund et al., 2000). Several other similar strains have since been isolated, also mostly from food poisoning cases. In 2004, Fagerlund and coworkers isolated, from *B. cereus* strain NVH 1230/88, a variant of CytK (CytK-2) with an amino acid sequence 89% similar to that of the first CytK (now designated as CytK-1) originally isolated from strain NVH 391-98. The CytK-2 protein variant from *B. cereus* strain NVH 1230/88 is only about a fifth as toxic to human intestinal Caco2 cells and Vero cells as the CytK-1 variant (Fagerlund et al., 2004), questioning its contribution to the diarrhoeal syndrome caused by *B. cereus*. Indeed, recent studies did not support any essential contribution of CytK-2 to enterotoxigenicity of *B. cereus* (Castiaux et al., 2015; Jessberger et al., 2015).

The original CytK (CytK-1), which causes severe necrotic enteritis, is only found in very few strains that form a robust and well separated cluster in the *B. cereus* group different from the *B. cereus* strains harbouring the CytK-2 variant (see above) and have recently been reclassified to the new species '*Bacillus cytotoxicus*' (Guinebretière et al., 2013).

The presence of enterotoxins in *B. thuringiensis* strains used in the EU as PPPs has been studied in some detail. Damgaard (1995) isolated *B. thuringiensis* from a number of different PPPs and found that the strains, including *B. thuringiensis* ABTS-1857, GC-91, ABTS 351, AM65-52 and NB 176, are able to produce enterotoxins, as quantified by the 3M™ Tecra™ *Bacillus* diarrhoeal enterotoxin visual immunoassay kit. Additionally Hansen and Hendriksen (2001) found by PCR analysis that strains ABTS 351 and AM65-52 possessed all the genes for NHE and HBL and that they expressed NHE. This was further demonstrated for strains GC-91, AM65-52, ABTS 351 and NB 176 by Hansen et al. (2011) who found that these strains possessed at least one of the genes for NHE, HBL and CytK2. They also showed that NHE, HBL and CytK were expressed by AM65-52 *in vitro* and *in vivo* in a Caco2 cell model and that NHE was expressed by ABTS 351 *in vitro* and in the wax moth *Galleria mellonella*, in both cases analysed by quantitative reverse transcriptase PCR. AM65-52 or ABTS 351 (here a strain identical to HD1, which is the origin to ABTS 351) seem, in contradiction to this, not to produce enterotoxins in the gut of rats, although they do germinate (Wilcks et al., 2006). Hansen et al. (2011) also compared the cytotoxicity of GC-91, AM65-52, ABTS 351 and NB 176 with the activity of four *B. cereus* strains isolated from cases of GI disease. Three different mammalian cell lines were investigated for the comparisons; they included measurements of trans-epithelial electrical resistance, adhesion, invasion and morphological changes in the cells and in addition to this macrophage engulfment. The results show that all eight bacterial strains affected the cells. No clear difference between the *B. thuringiensis* strains and the *B. cereus* strains was evident; GC-91 and AM65-52 affected the cells to a lesser extent that the *B. cereus* strains, while ABTS 351 affected the cells to the same extent as the *B. cereus* strains, while NB 176 affected them to a greater extent than the *B. cereus* strains.

3.2.2.3. Additional virulence factors

In addition to the toxins described above, the strains of the *B. cereus* group produce phospholipases, sphingomyelinases, haemolysins and metalloproteinases, which may represent additional virulence factors (Schoeni and Wong, 2005). However, the actual role of these putative virulence factors in different *B. cereus*-associated disease is largely unexplored. For instance, sphingomyelinase (SMase) has been reported to synergistically interact with NHE as well as with HBL (Beecher and Wong, 2000; Doll et al., 2013) and results from *in vivo* experiments suggest that contribution of SMase to *B. cereus* virulence have been underestimated in the past (Oda et al., 2012; Doll et al., 2013).

A good example comes from *B. cereus* strains producing large amounts of SMase. Such strains have been isolated from patients with sepsis and endophthalmitis, and were lethal to mice (Oda et al., 2012). The results from an *in vivo* study using *B. cereus* mutants foster the hypothesis that SMase complements enterotoxin-induced cytotoxicity also in the human host and may play, due to its haemolytic activity, a significant, yet unexplored, role in the development of septicaemia (Oda et al., 2010; Doll et al., 2013). Haemolysin II has been reported to induce apoptosis in macrophages and InhA1 has been shown to be pivotal for escape of *B. cereus* from macrophages (Guillemet et al., 2010; Tran et al., 2011). Another potential virulence factor secreted by *B. cereus* is the metalloprotease NprA, putatively involved in immune-evasion and tissue degradation of *B. anthracis* (Gohar et al., 2005; Chung et al., 2006).

As most of the aforementioned potential virulence factors are broadly distributed among the different members of the *B. cereus* group, including *B. thuringiensis* and *B. anthracis*, it is therefore not possible to draw any firm conclusion about the pathogenic potential of a certain strain based on the sole presence or absence of potential virulence factors. In addition, it must be kept in mind that

complex regulatory networks gear the expression of *B. cereus* toxins and other virulence factors (Gohar et al., 2008; Stenfors Arnesen et al., 2008; Frenzel et al., 2012; Ehling-Schulz et al., 2015).

The pathogenicity of *B. cereus* strains is also partly dependent on other phenotypic characteristics such as their ability to colonise and persist in the host and subsequently to invade tissues. This encompasses mechanisms for colonisation (such as motility, adhesion to epithelial cells, biofilm formation) and overcoming primary defence mechanisms (such as antimicrobial peptide (AMP) resistance) (Kamar et al., 2013).

3.2.2.4. The antimicrobial resistance issues/status for the *Bacillus cereus* group

Members of the *B. cereus* group display resistance to antibiotics. The most common resistance is against β-lactam antibiotics (e.g. penicillin, ampicillin, oxacillin) due to the presence of β-lactamase genes in almost all strains of all species of the group, with the notable exception of *B. anthracis*. Although this species carries both *bla1* and *bla2* β-lactamase genes, their expression is repressed, which results in its susceptibility to β-lactams (Chen et al., 2003). Most *B. cereus* group members are also resistant to co-trimoxazole (Ombui et al., 1996; Rather et al., 2012), albeit with variable levels depending on the strains. Similarly, resistance to fosfomycin is widespread among these bacteria. Interestingly, the corresponding gene has been found, in some strains, located on a MIC (De Palmenaer et al., 2004) or on the *B. anthracis*-specific Gamma phage (Schuch and Fischetti, 2006), indicating its potential mobility.

Resistance to other antimicrobials is variable and strain-dependent (Turnbull et al., 2004; Luna et al., 2007; Chaves et al., 2011; Chon et al., 2012; Ikeda et al., 2015). The four most regularly found resistances are to clindamycin (lincosamide) (up to 60% of the strains), tetracycline (10–33%) and levofloxacin (fluoroquinolone) (ca. 10%). Resistances to vancomycin and erythromycin are uncommon. Finally, apparently resistance to ciprofloxacin, chloramphenicol, gentamicin, rifampicin, streptomycin or kanamycin has not been recorded in *B. cereus sensu stricto* or *B. thuringiensis*.

An interesting observation has recently been made on the behaviour of certain strains of *B. cereus* towards certain antimicrobials: exposure to aminoglycoside antimicrobials induces a phenotype switching of emetic *B. cereus* subpopulations to a slow-growing small colony variant (SCV) state that escapes the antimicrobial activity through a slow-growing lifestyle (Frenzel et al., 2015).

The genes responsible for these antimicrobial resistances are generally located on the chromosome, although some are included on potentially mobile elements (MIC and phage for fosfomycin) or on mobile elements such as plasmids. As early as 1978, Bernhard et al. (1978) described pBC16, a small *B. cereus* plasmid bearing resistance to tetracycline. Similarly, the plasmid-associated *tetA* and *tetB* genes have been reported in a food-borne *B. cereus* strain (Rather et al., 2012).

As mentioned in Section 3.1.3, bacteria of the *B. cereus* group have the potential to exchange genetic material through conjugation and transduction, with high efficiency when the genetic determinants (e.g. antimicrobial resistance genes) are located on conjugative or mobilisable plasmids. Although instances of horizontal gene transfer have been extensively described under laboratory conditions (and some controlled environmental conditions), the frequency and efficiency in nature is still largely unknown.

3.2.3. Human outbreaks described as associated with *Bacillus thuringiensis*

3.2.3.1. Literature review of food-borne outbreaks described as associated with *Bacillus thuringiensis*

Investigations which connect *B. thuringiensis* to food poisoning are restricted to two, based on the literature search performed on the occurrence of *B. thuringiensis* in food which is described in Section 2.2. Jackson et al. (1995) found bacterial isolates presumptively identified as *B. cereus* from four persons during the investigation of a gastroenteritis outbreak in a chronic care institution in Canada. The symptoms were nausea, vomiting and watery diarrhoea. Phage typing confirmed all the clinical *Bacillus* isolates to be phage type 2. The consumed food was not directly analysed; however, *B. cereus* of this phage type was not identified in the onion powder used as a spice of the food. All 10 clinical isolates (from the stool) were subsequently identified as *B. thuringiensis*, as demonstrated by the formation of crystals and observed by staining and microscopy. This suggests, but does not prove, the involvement of *B. thuringiensis* in the gastroenteritis, as *B. cereus* (later identified as *B. thuringiensis*) was the only pathogenic bacteria isolated from the stool of three of the individuals. In the fourth individual, Norovirus was identified as well. Additionally, Norovirus was found in one individual negative for the presence of *B. cereus* or other pathogens.

McIntyre et al. (2008) re-identified 155 *B. cereus*-like isolates collected from food or clinical specimens in 39 food-borne outbreaks between 1991 and 2005 in British Columbia, Canada. The re-identification was based on molecular and phenotypic typing methods. They identified *B. cereus* in 23 outbreaks, *B. thuringiensis* in four, *B. mycoides* in one outbreak and mixed strains of *Bacillus* in 11 outbreaks. *Bacillus thuringiensis* may therefore have been involved in four of the outbreaks including 14 ill individuals. The organisms were isolated from the food specimens but none of the clinical specimens were positive for *B. cereus* group isolates. In total, 20 isolates from five food specimens were tested and identified as *B. thuringiensis.* The symptoms of the ill individuals included nausea, diarrhoea, abdominal cramps, vomiting, fever and headache. The methods for identification of *B. thuringiensis* included the presence of insecticidal crystal protein genes (*cry1* and *cry2*) by PCR and presence of crystals by transmission electron microscopy. In the cases related to *B. thuringiensis*, the bacteria were all isolated from the consumed food, and were not isolated from vomit or faecal material of any of the patients.

None of the *B. thuringiensis* isolates examined in these two investigations were characterised further than to species level, so their phylogenetic relations within the *B. cereus* group are unknown. The possible involvement of strains used as biopesticides was not investigated.

3.2.3.2. Alleged food-borne outbreak in one Member State

The specific alleged food-borne outbreak, where three members of a family of five became ill with nausea and diarrhoea on 28 July 2012 is summarised in Section 1.1. The family ate cheese noodles and salad, and only those who ate the salad became ill. Official laboratories were involved and performed microbiological testing of the food as well as of the salads in the store.

The only bacteria that were found to be present in the salad above the generally accepted level were *B. cereus* group bacteria, identified as *B. thuringiensis*. Bacteria like *E. coli, Clostridium perfringens, Salmonella* spp., coagulase-positive *S. aureus or Listeria* spp. could not be detected nor Norovirus. The package containing the salad contained information advising that the product be washed before consumption. It is difficult to know whether the consumed salad was washed, and likewise there is no mention of bacterial analysis of the patients' faeces.

The quantification of *B. cereus* on salad samples resulted in levels ranging from 2.5×10^4 to 1.4×10^5 CFU/g; colonies were subsequently confirmed to be *B. thuringiensis*. The leftovers of cheese noodles were also tested for the presence of *B. cereus*, with levels reported as 6×10^3 CFU/g.

A general problem when *Bacillus cereus* group bacteria are identified in a food-borne outbreak is that routinely it is presumed to be *B. cereus* and not *B. thuringiensis*. As *B. cereus s.s.* and *B. thuringiensis* may contain the same chromosomal determinants for potential virulence factors, it is important to realise that a *Bacillus* food poisoning event might be caused by *B. thuringiensis* as well as by *B. cereus*. Therefore, testing (by microscopy) the presence of the parasporal crystals containing Cry (and Cyt) toxins during sporulation is an important test, which is normally not done in routine food microbiology diagnostics. Usually, routine food microbiology laboratories follow the ISO 7932 standard (ISO, 2004) for diagnostics of *B. cereus* group bacteria ('presumptive *B. cereus*'), which does not allow discrimination of different members of the *B. cereus* group. However, it should be kept in mind that the crystals may not always be easy to see (see also Section 3.3.3). In this particular case, testing showed that the salad contained *B. cereus* group organisms, and further testing showed the presence of crystals, which are characteristic of *B. thuringiensis* (as shown by confidential documents made available by the European Commission which are listed under Section 1.3 and under section 'Documentation provided to EFSA').

For subtyping of the *B. cereus* group organisms isolated from the salad samples, Fourier transform infrared (FTIR) spectroscopy was employed. FTIR is a vibrational spectroscopic technique allowing the identification of microorganisms at different taxonomic levels (Naumann et al., 1991; Wenning and Scherer, 2013). The entire chemical and biochemical composition of whole microbial cells are recorded by the absorbance of mid-infrared light by the molecules present in the cells. Due to its discriminatory power, FTIR has been shown to be suitable for typing of *B. cereus* group organisms at a sub-species level and for use in contamination route analysis (Ehling-Schulz et al., 2005a, 2011).

It is possible that the salad contained both *B. cereus* and *B. thuringiensis*, and therefore a synergistic effect in triggering the symptoms cannot be excluded. The fact that the five isolates, picked after plating from each salad sample and subsequently analysed by FTIR all clustered to the XenTari strain used as biopesticide in the salad field, as well as that all isolates were positive for parasporal crystals of the same appearance, indicate that a high proportion of the bacteria were *B. thuringiensis* that were indistinguishable from XenTari. Isolated strains were tested by means of an immunologic

assay for the expression of potential enterotoxins and resulted positive. Although FTIR has a high resolution power that may even exceed that of molecular typing methods, such as MLST, AFLP or PFGE (Ehling-Schulz et al., 2005a; Johler et al., 2016), it would have been useful to also have information about molecular typing, such as MLST or AFLP. The latter method(s) are currently the gold standard methods for subtyping of *B. cereus* group organisms (Ehling-Schulz and Messelhausser, 2013). The strains from both the salad in the household of the patients, as well as from the sealed salad bags used for further investigation and the XenTari strain used as biopesticide could have been analysed by molecular methods.

There is no information on the time lapse between the spraying and the harvesting of the salad (postharvest interval). This seems to be a critical point, and should be a point for consideration for further routine improvements concerning the use of biopesticides on food.

3.2.4. Summarising remarks (Section 3.2)

The species *B. thuringiensis* is characterised by the production of crystal inclusions in parallel with spore formation. The insecticidal proteins in the crystal bodies have been shown to contain mainly two types of insecticidal proteins. The taxonomy and classification of *B. thuringiensis* genes refer to the most common type as *cry* (for crystal) genes, along with the *cyt* (for cytolytic) genes present in *B. thuringiensis* subsp. *israelensis*. The commercial *B. thuringiensis* products are powders containing a mixture of dried spores and toxin crystals. Presently, there are over 400 *B. thuringiensis* -based formulations that have been registered in the market and most of them contain insecticidal proteins and viable spores.

The panoply of potential toxins and virulence factors found in the *B. cereus* group is broadly distributed among different members of the group (Prüss et al., 1999), and it is therefore not possible to draw any firm conclusion about the pathogenic potential of a certain strain based only on the presence of potential virulence factors. Some species contain additional toxins defining the species, namely the anthrax toxins restricted to *B. anthracis* and a few *B. cereus* strains, and the insecticidal crystal-forming toxins (Cry and the Cyt toxins) characteristic of *B. thuringiensis*.

As *B. cereus* and *B. thuringiensis* strains are genetically intermingled (Kolstø et al., 2009), these species are usually not differentiated in routine clinical diagnostics or food microbiology. Thus, the actual contribution of the two species to GI and non-GI diseases is currently unknown.

Between 10^5 and 10^8 *B. cereus* cells or spores/g are commonly found in foods associated with the diarrhoeal syndrome although occasionally lower levels have been reported from food-borne *B. cereus* infections. Nearly all *B. cereus* strains harbour the *nhe* genes, while *hbl* and *cytK* are detected in about 30–70% of isolates. In contrast to the emetic toxin cereulide, these potential enterotoxins are also frequently found in other *B. cereus* group members, including *B. thuringiensis.* So far the *ces* gene has not been found in *B. thuringiensis* (Thorsen et al., 2006), even though a systematic assessment was performed on more than 200 strains (Hoton et al., 2009).

Two papers report on the involvement of *B. thuringiensis* in human outbreaks. The first is a description of one outbreak, while the other is a reassessment of isolates from 39 outbreaks. In this paper, it is suggested that *B. thuringiensis* might have been the cause of the infection in four of the outbreaks.

In the alleged food poisoning outbreak described in the background section of this Opinion, it appears that the only bacteria that were found above the generally accepted level were *B. cereus* group bacteria, identified as *B. thuringiensis* in the salad samples. The *B. thuringiensis* isolated from the salad were characterised by FTIR spectroscopy and could not be discriminated from *B. thuringiensis* subsp. *aizawai* (XenTari) which had been sprayed on the salad on the field.

It is not clear if those persons who consumed the salad also ate cheese noodles and therefore a synergistic effect between *B. thuringiensis* and another *B. cereus* group strain cannot be excluded. It cannot be excluded that another *B. cereus* group strain was also present at low levels in the salad, although such a strain was not detected in any of the samples.

3.3. Methods to detect, enumerate and differentiate members of *Bacillus cereus* group

Because the major features differentiating *B. thuringiensis* from other members of the *B. cereus* group are based on the phenotypes of the strains (i.e. the presence of toxin crystals within the bacterial sporangium), most (all) of the routine methods described so far are unable to correctly and/

or reliably detect and identify/discriminate the *B. thuringiensis* strains from other members of the *B. cereus* group. As indicated below, some methods can give more taxonomic information than others, but confirmation tests (e.g. PCR) are always required and might not always be discriminatory.

3.3.1. Identification methods for *Bacillus cereus* group

The 'classical' methods used to identify members of the *B. cereus* group include: (i) growth on selective media (MYP and NGKG); (ii) Gram staining and sporulation tests; and (iii) biochemical galleries (e.g. API). These classical and conventional methods are detailed in the following sections, together with more recent and/or molecular approaches.

3.3.1.1. Growth on selective media

The MYP (mannitol, egg yolk and polymyxin) medium was recommended by Mossel et al. (1967) for the enumeration of *B. cereus* group in food matrices. The principle is based on the lack of mannitol fermentation by *B. cereus* and the production of lecithinase by most *B. cereus* group strains. The addition of the antibiotic polymyxin B eliminates most of the contamination by other microbes. The positive colonies appear pink and are surrounded by a halo of precipitate. A variation of this medium, PEMBA (polymyxin egg yolk mannitol bromothymol blue agar) was developed by Holbrook and Anderson (1980) and give rise to *B. cereus* colonies surrounded by a white-blue halo. The presumptive colony forming unit (CFU) should then be confirmed by a haemolytic activity on Sheep blood agar or by biochemical tests (see below). All these media contain chromogenic substances (often a brand secret) that, in the presence of enzymatic activities (e.g. phospholipases or beta-glucosidases) specific to members of the *B. cereus* groups, release a coloured substance that stains the positive CFU into a particular colour. However, as all these tests rely on the activity of enzymes that are under control of the pleiotropic regulator PlcR, mutations in the PlcR regulator or the enzymes could lead to false-negative results (Fricker et al., 2008).

Another conventional method (NGKG) is based on the medium of Kim and Goepfert (1971) agar supplemented with NaCl and glycine (Kim and Goepfert, 1971).

Currently (December 2015), four methods have been validated for the enumeration of *B. cereus* in food products: ISO 7932:2004 (ISO, 2004) and 21871:2006 (ISO, 2006), AFNOR BKR-/23/06-02/10 and AFNOR ARES-10/10-07/10.

3.3.1.2. ISO 7932:2004

Based on the MYP medium, this official reference ISO standard specifies a horizontal method (applicable to products intended for human consumption and the feeding of animals, and environmental samples in the area of food production and food handling) for the enumeration of viable presumptive *B. cereus* by means of the colony-count technique at 30°C. It is based on the typical appearance of the colonies on MYP agar and a confirmation for haemolysis on sheep-blood agar. It is considered 'presumptive' as the confirmatory stage does not enable the distinction of *B. cereus* from other closely related but less commonly encountered species, e.g. *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis* or *B. mycoides*. An additional motility test may help to differentiate *B. cereus* from *B. anthracis* in cases where the presence of the latter is suspected. The method is applicable to products intended for human consumption and the feeding of animals, and environmental samples in the area of food production and food handling.

3.3.1.3. ISO 21871:2006

This standard specifies a horizontal method for the detection or the enumeration of low numbers of viable presumptive *B. cereus* by means of the most probable number (MPN) technique.

3.3.1.4. AFNOR BKR-23/06-02/10 and AFNOR ARES-10/10-07/10

These two methods are based on the Compass® *Bacillus cereus* Agar and BACARA® (Bacillus Cereus Rapid Agar), respectively. Note that they have been validated as 'alternative' methods that can be used by routine laboratories as a substitute for ISO 7932:2004.

3.3.1.5. Gram staining and sporulation tests

In the process of identifying members of the *B. cereus* group, Gram stain is often performed to confirm the Gram-positive status of the bacterium. Similarly, the presence of spores is also investigated

by visual observation under the microscope (presence of refringent bodies under phase-contrast light or green-stained bodies after malachite staining).

3.3.1.6. Biochemical galleries

In routine laboratory testing, the API50CH and API20 galleries allow the identification of an isolate to the genus/species level. In the case of the *B. cereus* group members, these tests cannot differentiate at the species level.

3.3.1.7. PCR, qPCR and RT-qPCR

For molecular typing of members of the *B. cereus* group various PCR-based methods have been proposed based on sequences of single genes like *gyrB*, *groEL motB*, or variable regions of the 16S and 23S rRNA (Chen and Tsen, 2002; Chang et al., 2003; Bavykin et al., 2004; La Duc et al., 2004; Park et al., 2007; Oliwa-Stasiak et al., 2010). The results of these approaches suggest that there is a high level of sequence homology among the strains and the methods have failed to reveal consistent differences between isolates that will enable accurate differentiation among *B. cereus* group species. For example, the multiplex PCR method proposed by Park et al. (2007) for identification of *B. cereus* group individual bacterial species has been proved to be useful only for identification of the entire *B. cereus* group (Forminska et al., 2012). PCR-based methods have been developed to detect virulent plasmid pXO1, encoding protective antigen (*pag*), lethal factor (*lef*) and oedema factor (*cya*), and virulent plasmid pXO2, encoding capsule protein (*cap*) to differentiate fully virulent *B. anthracis* strains from the other species (Ramisse et al., 1996; Bell et al., 2002). However, there is difficulty in distinguishing plasmid-cured *B. anthracis* or near-neighbour *B. anthracis*-like species. Members of the *B. cereus* group can be detected using primers specific to their 16S rRNA and/or to housekeeping genes with conventional PCR, real-time PCR (qPCR) (Martínez-Blanch et al., 2010; Fernández-No et al., 2011; Dzieciol et al., 2013; Cremonesi et al., 2014) or quantitative reverse-transcription PCR (RT-qPCR) (Reiter et al., 2011). Some of the methods allow discrimination between the emetic and the non-emetic strains of *B. cereus* (Fricker et al., 2007; Dzieciol et al., 2013; Ueda et al., 2013). In addition, PCR and real-time PCR-based systems have been developed for the identification of various combinations of toxin genes within the *B. cereus* group (Guinebretière et al., 2002; Ehling-Schulz et al., 2006b; Wehrle et al., 2010).

3.3.2. Methods for identification of toxins (excluding Cry toxins) produced by *Bacillus cereus* group

Over the years, several publications have suggested a number of potential enterotoxins are responsible for the diarrhoeal syndrome associated with *B. cereus* food-borne infections (see Section 3.2.2). The three most frequently reported putative toxins are HBL, NHE and CytK. It is however important to emphasise that no definitive demonstration has been provided of the role of these components (alone or in combination) in the diarrhoeal syndrome. Yet, several immunological methods have been developed to detect the presence of (some of) these potential enterotoxins, either in the supernatant of a *B. cereus* liquid culture or in the contaminated food products themselves. These methods are, at best, semi-quantitative, but they generally detect only part of the components of the enterotoxin complexes.

3.3.2.1. Immunoassays

Two commercial immunoassays for the detection of *B. cereus* enterotoxins have been available for several decades. Both are screening tests for indicating the presence of *B. cereus* enterotoxins both in food or food-related samples, and in enrichment cultures of *B. cereus* isolates.

- The 3M™ Tecra™ *Bacillus* diarrhoeal enterotoxin visual immunoassay kit. This immunological ELISA test specifically targets the NheAB subunits of the NHE complex, with a reported sensitivity of > 1 ng/mL of prepared sample.
- The *B. cereus* enterotoxin reversed passive latex agglutination kit. The antibodies of this test recognise the HblC (L2) component of the Hbl complex, with a reported sensitivity between 2 and 4 ng/mL of the extract.

3.3.2.2. Immunochromatographic tests

The Duopath® Cereus Enterotoxin (Merck) is a gold labelled immuno sorbent assay (GLISA) for the qualitative detection of the two major *B. cereus* enterotoxin complexes NHE and HBL (both the NheB

and HblC components are targeted). Its detection limit is said to be 1 CFU/g or mL of food sample, and was also reported to range between 6 and 20 ng/mL of enterotoxin in sample (Krause et al., 2010). However, as NHE is expressed by most strains belonging to the *B. cereus* group (including *B. thuringiensis*) and due to the qualitative nature of this assay, it does not allow firm conclusions on the toxigenic potential of a certain strain to be drawn.

3.3.2.3. Cereulide detection using the boar sperm assay

In 1998, the group of Salkinoja-Salonen (Andersson et al., 1998) described a simple but reliable technique for the detection of cereulide. This method is based on the activity of the emetic toxin on the boar sperm cells: in the presence of cereulide, spermatozoa lose their motility within a couple of minutes. Although it is not specific to cereulide (other toxins can act on the sperm cells), this sensitive, inexpensive and rapid bioassay has been widely used. More recent variations have also been reported that permit semi-quantitative analysis of cereulide in various matrices (Rajkovic et al., 2007).

3.3.2.4. Cereulide detection via cytotoxicity

The human epithelial type 2 (Hep-2)-based cytotoxicity assay can be used as an alternative bioassay for the semi-quantitative analysis of the cereulide toxin from heat-inactivated bacterial or food samples (Finlay et al., 1999; Frenzel et al., 2011). Generally, valinomycin is used as an external calibrator for this assay. However, a comparative study of purified cereulide and valinomycin revealed that the latter is not an appropriate surrogate for cereulide quantification as it behaves differently in the Hep-2 cytotoxicity test (Biesta-Peters et al., 2010).

3.3.2.5. Cereulide detection and quantification by liquid chromatography mass spectrometry

Several liquid chromatograph mass spectrometry (HPLC-MS)-based systems have been developed to analyse the cereulide toxin directly (Häggblom et al., 2002). Usually, valinomycin was used as a surrogate standard compound for quantification. However, as indicated above, since it is not co-eluting with cereulide, it has been shown to be unsuitable for accurate analysis of complex samples as it does not allow counterbalancing matrix effects. Thus, a LC-MS stable isotope dilution analysis (SIDA), using stable isotope labelled cereulide as internal standard, was established (Bauer et al., 2010), which provided the basis of the development of an SIDA-based ISO method for the quantification of cereulide (ISO, online) (under development). Very recently, 17 different isoforms of cereulide, including one highly toxic variant, showing about eightfold higher cytotoxicity than the classical cereulide, have been described (Marxen et al., 2015a). Using a modified LC-MS approach, it was shown that these cereulide variants are produced in foods connected to food-borne outbreaks (Marxen et al., 2015b). However, further studies are needed to decipher the role and contribution of these variants to *B. cereus* intoxications.

3.3.2.6. Enterotoxin detection using the vascular permeability reaction

The rabbit vascular permeability reaction (VPR) was originally tested on culture filtrates of *B. cereus* by Glatz et al. (1974) as an alternative test to replace the 'rabbit ligated intestinal loop' that shows a fluid accumulation when subjected to injection of enterotoxins. The VPR is performed by intradermal injection of the toxin(s), in guinea pig or rabbit skin, and the observation of the release of plasma proteins (via Evans blue dye) in the dermal tissues due to altered vascular permeability. Although this assay has been used by several authors (Portnoy et al., 1976; DeBuono et al., 1988; Christiansson et al., 1989; Granum et al., 1993), its significance as an appropriate indicator of enteropathogenicity, as well as its correlation with the activity of the rabbit ligated intestinal loop have been questioned (Hostacká et al., 1992).

3.3.2.7. Enterotoxin detection via cytotoxicity assays on human and animal cell lines

The most frequently used cells to test *B. cereus* group isolates for their enterotoxin activity are Vero cells as they are easy to grow and to maintain (Ehling-Schulz et al., 2011). However, various other cell lines have also been employed for determination of the cytotoxicity of enterotoxigenic *B. cereus* group strains. Recently, a comparative test of different cell lines carried out by Jessberger et al. (2014) revealed that different cell lines show different susceptibilities towards the different *B. cereus* enterotoxins. Primary endothelial cells (HUVEC) were found to be highly susceptible to NHE, whereas Hep-G2 (human hepatocellular carcinoma cells), Vero (*Cercopithecus aethiops* kidney cells) and A549 (human epithelial lung carcinoma cells) were most sensitive to a combination of NHE and HBL. Thus, results for overall cytotoxicity of enterotoxigenic strains achieved by these methods will

depend on the presence and combination of enterotoxins and the chosen cell line, questioning the suitability of such tests for routine diagnostics.

3.3.3. Differentiation between *Bacillus cereus* and *Bacillus thuringiensis*

As already indicated, the primary definition of a *B. thuringiensis* strain is its capacity to be active against invertebrates. This activity is mainly (but not exclusively) related to the production, during sporulation, of a parasporal crystal containing the Cyt and Cry toxins. For some strains (e.g. HD1-Dipel) the crystal inclusions are easily observable with a phase-contrast light microscope where they appear as bipyramidal-shaped, diamond-shaped or spherical-shaped structures located next to the spore (Figures 2 and 3a). However, for other strains, such as those of serovar *israelensis*, the crystals are tinier (Figure 3b) and might be confused with other internal bodies, such as PHB (polyhydroxybutyrate) granules (Figure 3c). This discrimination method between *B. cereus* and *B. thuringiensis* is therefore not always reliable and should always be considered as presumptive, especially in the cases of *B. thuringiensis* strains containing tiny and irregular crystals.

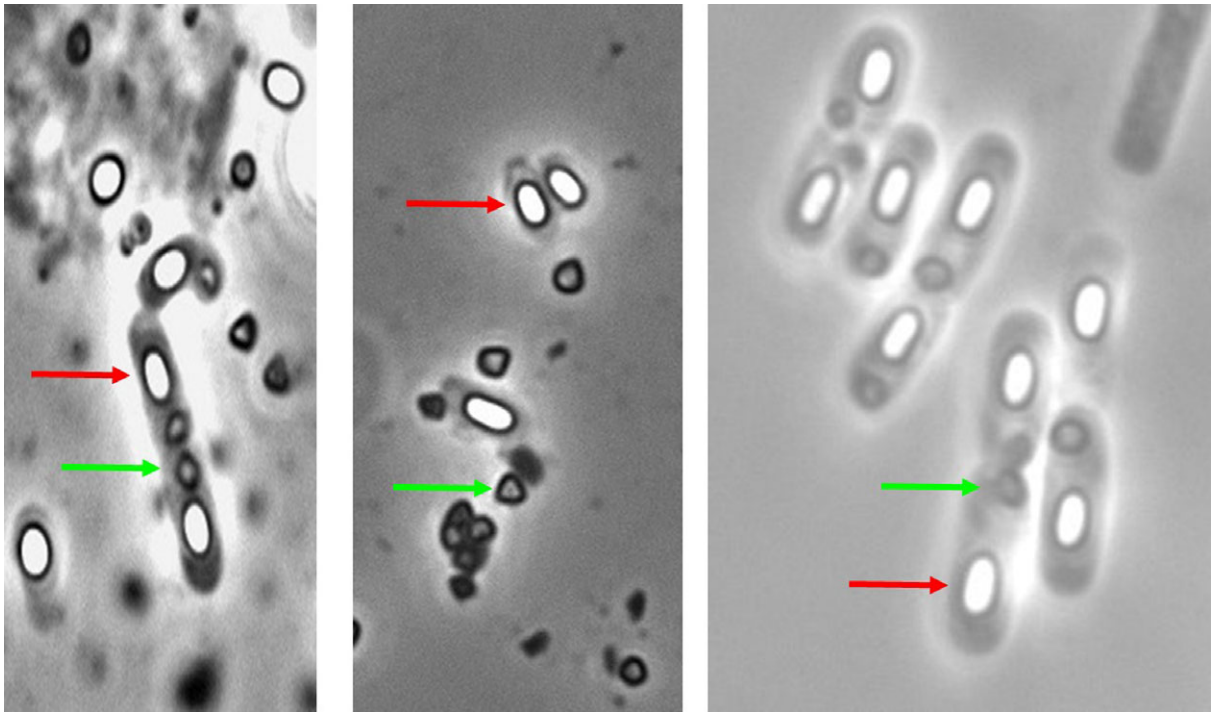


Figure 2: Photomicrographs of *Bacillus thuringiensis* strains viewed by phase-contrast microscopy showing the parasporal crystals (green arrows) of insecticidal toxin, which are less phase-bright than the spores (red arrows)

Another, often complementary, approach involves the detection by PCR of genes coding for the δ-endotoxins and/or cytolysin present in the crystals. Although several studies have provided degenerated primers able to detect multiple genes (Ben-Dov et al., 1997, 1999; Thammasittirong and Attathom, 2008; Noguera and Ibarra, 2010), any negative result should be considered with great care as the diversity of these genes is known to be enormous (see Section 3.3.1) and could easily escape detection by PCR.

Finally, it should be emphasised that some *B. thuringiensis* strains contain parasporal crystals but do not display any entomopathogenic activity, at least on the insect larvae that have been tested. Similarly, some of the *cry* genes identified (i.e. through genome sequencing) have so far no identified insect or nematode targets.

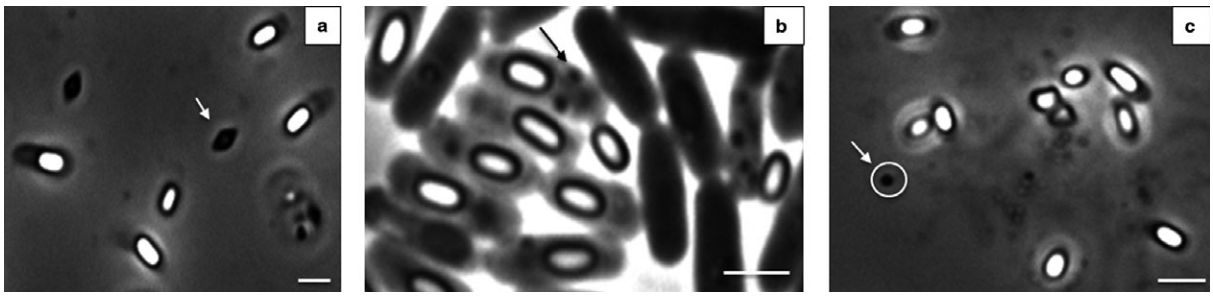


Figure 3: Phase contrast microscopy of sporulated cultures of bacilli. (a) *Bacillus thuringiensis* serovar *kurstaki* strain HD1; (b) *B. thuringiensis* serovar *israelensis* strain ATCC35646 sporangia (note the crystals inside the mother cell); (c) *Bacillus cereus* reference strain ATCC14579. The arrows indicate the δ-endotoxin parasporal crystals in 'a' (bi-pyramidal shape) and 'b' (irregular shape). For 'c', the arrow is pointing to an inclusion that can be easily confused with crystals. White bars represent the 2 μm scale

Currently no ISO and/or other standardised method to reliably differentiate between strains of *B. cereus* and *B. thuringiensis* is available.

3.3.4. Identification of *Bacillus thuringiensis* strains

Historically, facing the huge diversity of the *B. thuringiensis* strains and the limitation of appropriate alternative methods (e.g. biochemical tests), microbiologists turned to the flagellar (H) serotyping to address the issue of strain identification. The Pasteur Institute was particularly involved in this task (de Barjac and Bonnefoi, 1967) as it became one of the most important reference laboratories of *B. thuringiensis*, not only by hosting a huge collection of *B. thuringiensis* isolates but also by acting as reference centre for serotyping (new) strains into serovars (e.g. *thuringiensis* or *israelensis*) and serotypes (e.g. H1, H3, H14). As already mentioned (Section 3.2), several hundred serovars/serotypes were recorded and maintained (Lecadet et al., 1999). Unfortunately, this activity was terminated about 15 years ago and was not taken over by any other reference laboratory. Attempts have been tried to substitute this serological approach by molecular-based strategies, using PCR targeting flagellin genes (Xu and Côté, 2006, 2008) or RAPD-PCR (Gaviria Rivera and Priest, 2003). Although they can partially replace serotyping, they cannot be used as reliable and accurate alternatives (Soufiane et al., 2007).

As reported in previous sections, toxin crystals are formed during sporulation and are located inside the sporangium (mother cell). Eventually, the crystals will be released outside the cells. For the strains used to control lepidopteran insects, the large size and typical bipyramidal shape of their parasporal crystals are relatively easy to detect under a phase-contrast microscope (e.g. strain HD1, Figure 3a). In contrast, strains of the anti-dipteran serovar *israelensis* are notoriously difficult to identify because of the small size and irregular form of their complex crystals (Figure 3b). It is also important to note that the absence of (visible) crystals does not rule out that the strain is a *B. thuringiensis*.

At this moment, the only way to unambiguously recognise a specific strain of *B. thuringiensis* is to identify it through whole genome sequence (WGS). Recent advances in WGS provide the tools, at a reasonable price, to fully characterise and identify any specific *B. thuringiensis* strain. Moreover, bioinformatics studies can also provide appropriate genetic markers to be used for rapid and unmistakable strain identification.

3.3.5. Summarising remarks (Section 3.3)

The major features differentiating *B. thuringiensis* from other members of the *B. cereus* group are based on the phenotypes of the strains (i.e. the presence of toxin crystals within the bacterial sporangium), and routine methods do not discriminate *B. thuringiensis* from other *B. cereus* group strains. The primary definition of a *B. thuringiensis* strain is its capacity to be active against invertebrates. This activity is mainly (but not exclusively) related to the production, during sporulation, of a parasporal crystal containing the Cyt and Cry toxins. For some strains, this crystal is easily observable with a phase-contrast light microscope where it appears as a bipyramidal, diamond or spherical structure located next to the spore; for other strains, the crystals are tinier and might be confused with other internal bodies.

The discrimination method based on microscopic examination is therefore not always reliable and should always be considered as presumptive, especially in the cases of *B. thuringiensis* strains containing tiny and irregular crystals.

Different methods to identify the presence of potential enterotoxins are available, such as immunoassays, cytotoxicity assays and molecular assays.

None of the available discrimination methods have been standardised.

At this moment, the only way to unambiguously recognise a specific strain of *B. thuringiensis* is to identify it through WGS.

3.4. Reservoirs, natural background prevalence and levels of *Bacillus thuringiensis* in the environment

3.4.1. Natural occurrence in the environment

The *B. cereus* group is ubiquitous in the environment and has been isolated from most materials investigated, including soils, plant materials, sediments, water, invertebrates and mammals; spores may be spread passively and are thus found everywhere (Jensen et al., 2003; Stenfors Arnesen et al., 2008; Ceuppens et al., 2013). Furthermore, *B. cereus* has been found to have a worldwide distribution and is described from samples from all continents including Antarctica (Stenfors Arnesen et al., 2008; Prabhakar and Bishop, 2011). Collier et al. (2005), Hendriksen et al. (2006) and Raymond et al. (2010) quantified the number of organisms in English and Danish cultivated soils, they reported around 2×10^4, 2×10^5 and 5×10^5 CFU/g, respectively. Similar concentrations were also found by Chatterjee et al. (2007) in rice fields in India and by Brillard et al. (2015) in an agricultural soil in France. In a more comprehensive investigation, Hendriksen (2011) found the density to vary between 4×10^4 and 2×10^5 with a mean of 1×10^5 CFU/g in 17 Danish cultivated soils, sampled across the country. The concentration found in soil of natural terrestrial soil ecosystems, such as forests, grassland and dunes, may differ from these numbers; however, this cannot be substantiated by any studies. Collier et al. (2005) found that the distribution of *B. cereus* on broad-leaved dock followed a log-normal distribution with a maximum density of 2×10^4 CFU/g. Hendriksen (2011) found that the density on curly kale also followed this distribution across 17 sites in Denmark, with a maximum density of 6×10^4 CFU/g, and with a mean density of 3×10^2 CFU/g. He further found a statistically significant positive relationship between the density on the individual leaf and the amount of soil on the leaf. *Bacillus cereus* has also been reported to be endophytic in potato tubers (Hoornstra et al., 2013) and in other agronomic crops (Melnick et al., 2012). Knowledge of the distribution of the different species or phylogenetic groups within the *B. cereus* group in the environment is limited; von Stetten et al. (1999) found that the annual mean temperature is an important parameter for the distribution of psychrotolerant *B. cereus* (notably *B. weihenstephanensis*) and mesophilic *B. cereus*, as psychrotolerant isolates were dominant in alpine soils, while only mesophilic isolates were found in soil from a tropical locality. In a temperate locality, the distribution of psychrotolerant and mesophilic isolates was equal. This is in accordance with data from Denmark, where psychrotolerant isolates are dominant in soils as well as on leaves of curly kale, albeit with differences between different localities (Hendriksen et al., 2006; Hendriksen, 2011). The 'natural' occurrence of *B. thuringiensis*, here defined as occurrence in habitats and materials, which have not been treated with biopesticides based on *B. thuringiensis*, is ubiquitous as for *B. cereus* (Glare and O'Callaghan, 2000). The densities vary between 0% and 50% of the *B. cereus* density (Hansen et al., 1996), although cases where they constitute up to 100% on leaves have been found (Collier et al., 2005; Raymond et al., 2010). The occurrence of *B. cereus* group in the environment is notably associated with spores, as demonstrated by the lack of statistical differences between densities estimated before and after heat treatment of samples, although *B. cereus* has been found to germinate and multiply in soil extracts (Vilain et al., 2006). Vegetative cells have been found in invertebrates (Swiecicka and Mahillon, 2006), on the surface of plant roots and in mammals (Margulis et al., 1998; Jensen et al., 2003; Hendriksen and Hansen, 2006; Swiecicka and Mahillon, 2006; Ceuppens et al., 2013), suggesting germination and growth connected to eukaryotic organisms.

3.4.2. Fate of *Bacillus thuringiensis* after application

The fate of *B. thuringiensis* after application as a MPCA has been investigated in soils and on broad-leaved plants; most of the studies have been performed with *B. thuringiensis* serotype *kurstaki*

strains, which are used for control of lepidopteran larvae. According to Hansen et al. (1996), half-lives of spores in soils between 100 and 200 days were experimentally determined; this is in accordance with Pedersen et al. (1995) who determined half-lives to be 120 days in the soil of a cabbage field with 20% remaining after 336 days. Hendriksen and Hansen (2002) and Hendriksen and Carstensen (2013) found that spores were still present in this soil after 13 years, most likely due to germination and growth. However, Vettori et al. (2003) found that the number of spores remained constant through 28 months and persisted for 7 years in soil under cork oak in Sardinia; Smith and Barry (1998) also found *B. thuringiensis* to persist for 2 years in the Rocky Mountains after aerial application. Similarly, Van Cuyk et al. (2011) found persistence for 4 years in an urban environment. A reduction in pH to acidic values has been shown to reduce the persistence of *B. thuringiensis* spores (Saleh et al., 1970a,b). Similar effects were also reported by Petras and Casida (1985).

When *B. thuringiensis* spores are applied to leaves, the half-lives, estimated in the field, are notably shorter than in soil, and values between 16 and 38 h have been reported on different vegetables and tree leaves (Hansen et al., 1996). Pedersen et al. (1995) reported a half-life of 16 h and that spores were undetectable after 28 days on white cabbage in a field in Denmark, while Madsen et al. (2011) were able to detect between 100 and 1,000 spores/g leaf 60 days after application, also on white cabbage. The first experiments took place from June to August while the second took place from August to October. In an experiment where *B. thuringiensis* spores were spread on curly kale in a field in Denmark four times, with 2 weeks between the applications, from August to November it was found that the rate of disappearance decreased from the first to the last application. This resulted in a decrease to detection level within 30 days after the first application, a level which was not reached for the last application in 120 days (Hendriksen, 2011). In fields sprayed with 'DiPel' containing *B. thuringiensis* serotype *kurstaki,* 2×10^4 spores/g were found in broccoli 1 week after the spraying and 8×10^3 and 2×10^3 spores/g in celery 1 and 2 months after the spraying, respectively (Madsen et al., 2011). In an experiment performed in a glasshouse in China, it was found that initial half-lives in cotton, amaranth and rice were approximately 120 h with a decrease to about 5% of the applied number in 15 days (Wang et al., 2014).

Based on these results, a structural model for the fate of *B. thuringiensis* on vegetables has been proposed (Madsen et al., 2011). The basis for the model is that the density of a bacterial population on a leaf at a specific time can be described by a simple equation proposed by Andrews and Harris (2000):

$$\text{Population density} = \mathrm{I} + \mathrm{G} - \mathrm{E} - \mathrm{D}$$

where I is immigration (viz. the number of the living bacteria immigrating to the leaf), G is growth (viz. the increase in number of the bacteria by divisions), E is emigration (a physical loss or elimination of living bacteria) and D is death (viz. bacteria entering from a living to an inactivated state).

During the application of microbiological pesticides based on *B. thuringiensis*, high numbers of the bacterium are sprayed as endospores on the leaves; therefore, the immigration in this specific situation and at this time point is determined by the application density. Other kinds of immigration of *B. thuringiensis* have been documented, such as dispersal from the soil to leaves, most likely caused by rain-mediated splashing of soil (Pedersen et al., 1995) and dispersal from seeds and soil during germination and growth of the plant (Bizzarri and Bishop, 2008). However, both kinds of immigration resulted in only relatively low densities of the leaf surfaces. So, by the use of *B. thuringiensis* as a spray, immigration is predominantly determined by the large number of bacteria applied.

The growth of endospores is initially dependent on the germination of the spore, followed by divisions of the vegetative cell. On leaves, *B. thuringiensis* occurs mainly as spores (Hansen et al., 1996), but Bizzarri and Bishop (2008) reported that vegetative cells also occur in low densities on leaf surfaces. However, it has not been documented that this occurrence of vegetative cells adds significantly to the density of *B. thuringiensis* on leaves, as the concentration of nutrients of the leaf surface is insufficient to mediate growth of *B. thuringiensis* (Maduell et al., 2008). It has also been shown that *B. thuringiensis* germinates and multiplies in target hosts and in their faecal material; however, this seems only in very limited extent to add to the density of *B. thuringiensis* on leaves (Raymond et al., 2010). The conclusion is that germination and growth of *B. thuringiensis* on leaves adds little to the density occurring on the leaves.

Emigration will primarily be affected by rain, which is able to wash the spores from the leaves. It has been documented that rain to a significant extent reduces the activity of *B. thuringiensis* on leaves (Burges and Jones, 1998). Additionally, it has been shown experimentally that the density of *B. thuringiensis* spores decreases as a function of the amount of rain in an experiment with artificial

rain (Svestka and Vankova, 1976). So, rain is able to significantly decrease the density of *B. thuringiensis* on leaves, depending on the amount of rain, duration and frequency.

Endospores are in general very resistant to extreme stresses, e.g. desiccation, high temperatures and ultraviolet (UV) radiation (Nicholson et al., 2000). Nevertheless, sunlight significantly decreases the survivability of *B. thuringiensis* spores (Burges and Jones, 1998), and it has been shown that the spores of a number of *B. thuringiensis* subspecies are susceptible to UV radiation (Glare and O'Callaghan, 2000). Additionally, it has been shown that *B. thuringiensis* spores are susceptible to visible light; especially blue-light with wavelengths around 400 nm (Griego and Spence, 1978). Moreover, the viability of the spores is dependent of the UV intensity and its duration. Contrary to this, high temperatures and desiccation seem not to affect the viability of *B. thuringiensis* to a significant extent (Glare and O'Callaghan, 2000). In conclusion, sunlight affects the death of *B. thuringiensis* on leaves and therefore decreases density on leaves; this reduction is dependent on the intensity and the duration of the sunlight. As a consequence, the exposure of the individual spore to sunlight affects its viability. This exposure is dependent on location on the plant, e.g. on the dorsal or the ventral side of a leaf. Greenhouse crops are less exposed to UV radiation.

With the above-mentioned general model for the density of bacteria on leaves as a starting point and interpretation of the different factors controlling the density of *B. thuringiensis* on leaves, it is possible to revise the general equation in this specific case to:

$$\text{Population density} = I_u + G_0 - E_r - D_s$$

where I_u is the density of *B. thuringiensis* spores on the leaf obtained by spraying, G_0 is the limited growth, E_r is the emigration of spores affected by rain and D_s is the inactivation of the spores primarily affected by sunlight.

Taking into account knowledge about the fate of *B. thuringiensis* spores on leaves after spraying (i.e. the decay initially occurs rapidly and tails off thereafter), and that the decay follows the general exponential model for decay, that growth is very limited and that the spores are either directly or indirectly exposed to sunlight, it is possible to revise the model to:

$$\text{Population density} = I_u - k_1 \,\text{Exp}(I_1) - k_2 \,\text{Exp}(I_2) - E_r$$

where I_u is the density of spores on the plant obtained by the spraying, I_1 is the fraction of the density of spores directly exposed to sunlight on the plant and I_2 is the density of spores indirectly exposed. The ratio between I_1 and I_2 is determined by the plant species and its morphology, its age and the coverage in the field. k_1 is the decay constant for the directly exposed spores and k_2 for the indirectly exposed spores. The two decay constants are dependent on factors such as number of sun-hours, time of the year, latitude, height above water level, shadow and reflections. Additionally, the formulation of the product can be of importance, as it might contain sunlight protectants (Burges and Jones, 1998), and decay will be affected if the plants are grown in a glasshouse. E_r is a function which is difficult to predict, as it is dependent on factors such as rainfall, its occurrence, amount and duration. This function might also be dependent on the formulation of the product, which might contain adhesive components (Burges and Jones, 1998); further, the species, morphology and the coverage of the plant might have importance for the rain-mediated emigration.

It is not possible with today's knowledge to estimate the different parameters of this explanatory model. The model can be used for a general designation of factors of importance for fate of *B. thuringiensis* on vegetables after its use as a biopesticide, and as a starting point for experiments aimed at elucidating the model and for estimation of the different parameters used in the model.

The fate of *B. thuringiensis* on a specific crop after application is hard to predict, as it is dependent on many factors including the crop, the climatic conditions and the cultivation practice. It is not possible with today's knowledge to estimate the number of *B. thuringiensis* present on a specific crop at a specific locality after a certain period of time.

3.4.3. *Bacillus thuringiensis* transfer from the environment to foodstuffs

As there is little or no information on the transfer of *B. thuringiensis* from the environment to foodstuffs, it is not possible to estimate rates of transfer. Factors of importance for transfer to foodstuffs include rain-mediated splashing of soil with *B. thuringiensis* as shown by Pedersen et al. (1995), epiphytic and endophytic colonisation of seedlings from soil (Bizzarri and Bishop, 2008), Raymond et al., 2010) and the presence of target organisms on the plant (Raymond et al., 2010),

although *B. thuringiensis* multiplication in frass seems to be negligible (Prabhakar and Bishop, 2011). Long-distance transport between fields and even countries and continents might be affected by air (Kellogg and Griffin, 2006), as endospores can survive aerial transport and *B. thuringiensis* has been isolated from dust (Glare and O'Callaghan, 2000). Further transport of *B. thuringiensis* to lakes and the sea might be affected by transport by streams and rivers by mass flow of soils from fields after storm events, as endospores survive in aquatic systems.

3.4.4. Summarising remarks (Section 3.4)

Bacillus thuringiensis is ubiquitous and has a worldwide distribution, so it can be isolated from most materials including soils, plants, sediments, water, invertebrates and mammals.

In soils, between 0% and 50% of the *B. cereus* group isolates are *B. thuringiensis*, which may reach levels up to 5×10^5 CFU/g of cultivated soil. The natural occurrence of the *B. cereus* group on plants is most likely log-normal distributed and can vary between 0 and 6×10^4 CFU/g, with a mean density around 1×10^2 CFU/g. Systematic studies on the fate of *B. thuringiensis* after application are lacking; however, some papers report half-lives of *B. thuringiensis* in soil after application vary between 100 and 200 days. Survival is much shorter on plant surfaces where reports of half-lives after application vary between 16 and 38 h. However, long-term survival up to 13 years of low numbers of organisms has been reported. Available data only relate to leaves and information on survival in greenhouses is restricted to one publication.

The fate of *B. thuringiensis* on a specific crop after an application is hard to predict, as it is dependent on many factors including the crop, the climatic conditions and the cultivation practice. It is not possible to estimate the number of *B. thuringiensis* present on a specific crop at a specific locality after a certain period of time.

Factors of importance for transfer to foodstuffs include rain-mediated splashing of soil, epiphytic and endophytic colonisation of seedlings from soil and the presence of target organisms on the plant. Long-distance spread may be mediated by air and water. Little information is available on such transfer and this lack of information makes it impossible to estimate rates of transfer.

3.5. Occurrence and levels of *Bacillus* spp., and specifically of *Bacillus thuringiensis*, in food

3.5.1. Occurrence and levels of *Bacillus* spp. in food

The food-borne pathogen *B. cereus* is widespread in the environment. As a result, diverse raw food ingredients such as vegetables, potatoes, milk, herbs and spices are often contaminated with *B. cereus* group spores. For example, the cultivation soil of courgettes contained 4.1×10^4 *B. cereus* group spores per gram soil, which constituted an important source of *B. cereus* contamination in the finished food product, courgette puree (Guinebretière and Nguyen-Thé, 2003).

In a previous scientific opinion (EFSA, 2005), the BIOHAZ Panel concluded that *B. cereus* is ubiquitous and only heat treatments used for canning of low acid foods (e.g. 121°C for ca. 3 min) will ensure complete destruction of spores of *B. cereus*. Spores are present in almost all categories of foods before storage, generally in numbers too low to cause food-borne poisoning, whereas risk for human health could arise from unusually high initial contamination of foods but, more usually, from multiplication of *B. cereus* after temperature abuse.

Following the observation that published reports of *B. cereus* food-borne poisoning cases show that 10^5–10^6 cells or spores per gram of food can cause food-borne poisoning and that in rare cases, 10^3 cells or spores per gram of food has caused illness, the same Opinion recommended 10^3–10^5 CFU/g as the maximum limit at consumption to be used as a target for food business operators to verify their Hazard Analysis and Critical Control Points system (HACCP) and that could be considered as microbiological criteria to test the acceptability of a process. Such levels have been confirmed in different publications. The minimal level required to provoke both types of diseases was estimated to be around 10^5–10^8 CFU/g of ingested food (Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008).

In a more recent publication (Cadel Six et al., 2012), 55 reported food-borne outbreaks classified in a single profile of *B. cereus* group strains were investigated by the Anses Laboratory for Food Safety, Maisons-Alfort, France. The contaminated foods implicated in the outbreaks contained *B. cereus* group levels between 10^2 and 3.2×10^7 CFU/g. Comparable levels (from less than 10^2 to 6×10^7 CFU/g)

have also been reported from food-borne outbreaks associated with emetic *B. cereus* in Germany (Messelhäusser et al., 2014).

All strains of *B. cereus* may not be equivalent in their ability to cause food poisoning due to variation in toxin production, which may be related to the composition of the food (Notermans and Batt, 1998). Moreover, differences in the pathogenic potential are also correlated with the growth domains of the *B. cereus* genetic groups. The more psychrotrophic strains are able to grow at 5°C, seem to be less heat resistant and seem to have a low ability to cause gastroenteritis (Afchain et al., 2008). All these factors can be responsible for the large variation in the estimated infectious doses, which makes a valid dose–response relationship hard to establish (Notermans and Batt, 1998).

As described in Section 3.2.3, *B. thuringiensis* was identified among strains of *B. cereus* group isolated from food poisoning cases (McIntyre et al., 2008), demonstrating the possibility that some cases of food-borne illness attributed to *B. cereus sensu stricto* are actually caused by *B. thuringiensis*. Indeed, testing of commercial *B. thuringiensis*-based insecticides (Bactimos, DiPel, Florbac FC, Foray 48B, Novodor FC, Turex, VecTobac, XenTari) has shown that enterotoxins are present in these strains and are produced at relevant levels. The production of diarrhoeal enterotoxin varied by a factor of more than 100 among the different strains tested (Damgaard, 1995). Fletcher and Logan (1999) and Tayabali and Seligy (2000) also found that strains of *B. thuringiensis* were positive in the commercial tests for enterotoxin and in a cytotoxicity assay. In another investigation, it has been shown that *B. thuringiensis* strains are indistinguishable from the commercial strains in the microbial bioinsecticides present on fresh fruit vegetables in Danish retail shops to a level which may exceed 10^4 CFU/g (Frederiksen et al., 2006). *Bacillus thuringiensis* strains have also been isolated from cabbage for human consumption (Hendriksen and Hansen, 2006) as well as from RTE foods (Rosenquist et al., 2005). Rosenquist et al. (2005) found that most RTE food investigated contained *B. cereus* group organisms at a level below 1,000 CFU/g, while between 0% and 3.1% (mean 0.5%) of the different groups of food contained above 10,000 CFU/g. They investigated 40 randomly selected strains for the ability to produce crystals and for the presence of selected *cry* genes by PCR, and found that 77.5% of the strains could be identified as *B. thuringiensis*; this suggests that the majority of the *B. cereus*-like organisms found in this selection of RTE food are *B. thuringiensis*. As some of these isolated strains were indistinguishable from commercial *B. thuringiensis* subsp. *kurstaki* HD-1, the authors suggest that these strains might be residuals of biopesticides applied in the field (Frederiksen et al., 2006; Hendriksen and Hansen, 2006). For all the food products mentioned in these publications, however, the use of *B. thuringiensis* as biopesticide is suspected but not demonstrated.

3.5.2. Occurrence and levels of *Bacillus thuringiensis* in food

As mentioned in Section 2.2, an extensive literature search was performed in order to obtain information on the presence and levels of *B. thuringiensis* in food. The protocol applied for this review is described in detail in Appendix F. The terms used in the search string were selected in order to cover a very broad range of food matrices, at any stage of the food chain. After screening a total of 4,903 references for relevance by looking at title and abstract, 137 references underwent a full text evaluation and out of these, only 80 references met eligibility criteria (Appendix G). The results for the screening process are detailed in the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram (Appendix G). A summary of the information extracted from relevant papers is reported in Table H.1 (Appendix H).

Information on the presence and levels of *B. thuringiensis* in food extracted from the papers included in the extensive literature search is difficult to summarise because very heterogeneous types of food (raw and cooked) have been analysed and in most of the cases, studies are focused on ethnic traditional foods that are not very well known in most of the EU countries. Additionally, the methodologies and techniques used to determine the presence and levels of *B. thuringiensis* in food samples are very diverse and in general, none of the analytical methodologies available and used in the selected research studies can be classified as 100% reliable, and thus, there are many uncertainties regarding the obtained results. Nevertheless, after summarising the obtained results, it has been observed that fruits and vegetables have been the most commonly analysed commodities among the raw foods, and studies focused on both identification of *B. cereus*-like organisms isolated from foods and the detection of *B. thuringiensis* in food samples. In most of these studies, *B. cereus* group organisms were isolated from fruits and vegetables, and differentiation between *B. cereus* and *B. thuringiensis* strains has been performed using conventional culture methods and molecular techniques. Several studies have also focused on the evaluation of legumes and grain. In these

studies, *B. thuringiensis* was identified in a relatively high number of samples, mostly from rice, maize and beans. This is also in agreement with results obtained in RTE meals containing pasta and rice. In these studies, several isolates were identified as *B. thuringiensis*, and numerous samples were positive for the presence of this bacterium. Milk and dairy products have been also included in several studies and the presence of *B. thuringiensis* was demonstrated through the identification of isolated microorganisms. On the other hand, enumeration of *B. thuringiensis* levels in food samples has been carried out in only a very limited number of studies. *Bacillus thuringiensis* levels in cauliflower were reported to be between 2.62 and 3 log CFU/g, while much lower levels (3.6–4.5 CFU/g or mL) were found in some dairy products. Levels of *B. thuringiensis* spore-formers in legumes were close to 20 CFU/g while spore levels in spices were between 3 and 240 MPN/g. A couple of studies enumerated the *B. thuringiensis* levels in wine and vinegar and reported levels between 1 and 3 log CFU/mL. However, reported data of the *B. thuringiensis* levels should be interpreted with caution due to the limitations in the analytical methods used.

In general, the information concerning the detection of *B. thuringiensis* in foods is quite scarce, and few studies were specifically aimed at this evaluation. Among these, Rosenquist et al. (2005) analysed 48,901 samples of RTE foods sampled at retail in Denmark, for the presence and levels of *B. cereus*-like bacteria. The products analysed were fresh fruits and vegetables, heat-treated products such as ready-prepared dishes, sauces, meat, pasta and rice, and products with both fresh and heat-treated ingredients, such as sandwiches, pasta salad, vegetable/meat/fish mayonnaise, and desserts including ice cream and cream-cakes. Enumeration of *B. cereus*-like organisms showed that 98.7% of the products had counts below 10^3 CFU/g, 0.7% were in the range 10^3–10^4 CFU/g and 0.5% of the samples had counts above 10^4 CFU/g. The high counts were most frequently found in fresh cucumbers and tomatoes, heat-treated rice, cake custard, and in desserts containing milk and rice. Forty isolated strains were randomly selected and analysed for the production of parasporal crystals; 28 of the strains produced visible crystals and were therefore classified as *B. thuringiensis;* three additional strains were identified as *B. thuringiensis* through molecular methods. All 40 strains examined had genes or components for toxins involved in human diarrhoeal disease; genes for the emetic toxin were only found in one strain identified as *B. cereus*. Eventually, by comparing the genotypic (based on detection of genes and components for enterotoxins, insecticide toxin and emetic toxin) and phenotypic (intracellular crystal presence) profiles of the food isolates with the profiles of the commercial biopesticides, it was observed that five strains isolated from sausage, pasta, red peppers and cauliflower stowage grouped together with the commercial Dipel strain according to the content of *cry* genes and enterotoxin genes and proteins. The authors conclude that a large proportion of the *B. cereus*-like organisms present in foods may be *B. thuringiensis*. Further studies are needed to clarify the genetic relationship of the isolated strains to commercial *B. thuringiensis*.

The same research group, in a different paper (Frederiksen et al., 2006), further characterised 128 of the strains isolated in the previous study. Fifty of these were classified as *B. thuringiensis* because of their content of either crystal proteins visualised by phase-contract microscopy (38 strains) or *cry* genes as detected by PCR (12 strains). Moreover, plasmid profiling divided these 50 strains into two groups, one group of 14 isolates indistinguishable from the *B. thuringiensis* subsp. *kurstaki* strain used in some commercial products, and 9 isolates indistinguishable from the *B. thuringiensis* subsp. *aizawai* strain of another commercial product. A statistically significantly high proportion of these isolates were from tomatoes, cucumbers and peppers. Twenty-seven isolates were grouped by plasmid profiling as non-commercial *B. thuringiensis* strains.

Twenty-nine of the papers considered relevant and from which data were extracted reported the identification of *B. thuringiensis* among strains isolated from different food matrices. In these papers the sampling strategy is not reported, so the percentage of positive samples is not retrievable. From these papers, *B. thuringiensis* appears to be present in different food items, such as fresh vegetables, milk and dairy products, spices, pasta and bread, fermented traditional foods, spoiled seafood products, spoiled fruit pulp, rice, green tea and whey protein concentrate. Studies were performed in different countries, both in Europe (Italy, Denmark, Finland) and outside Europe (Africa, Asia, United States), using different confirmation methods (microscopic examination, 16s rRNA sequencing, Riboprinter, matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF), PCR-based techniques).

The range of growth temperature of *B. cereus* group strains was investigated by Guinebretière et al. (2008). They found that temperature growth range of the individual strains was related to which of the seven phylogenetic groups presented in Section 3.1.2 it belongs. This relationship is shown in Table 1. From the table it appears that growth range differs between strains and that strains exist which are able to grow at refrigeration temperatures and others at temperatures as high as 50°C.

Table 1: Temperature (in °C), pH and water activity growth range for the *Bacillus cereus* group

Phylogenetic group of *B. cereus* (see Section 3.1.2)	Temperature growth range (in °C)	pH (ICMSF, 1996)			Water activity (ICMSF, 1996)
		Minimum	Optimum	Maximum	Minimum
All	NA	5.0	6.0–7.0	8.8	0.93
I	10–43	NA	NA	NA	NA
II	7–40	NA	NA	NA	NA
III	15–45	NA	NA	NA	NA
IV	10–45	NA	NA	NA	NA
V	8–40	NA	NA	NA	NA
VI	5–37	NA	NA	NA	NA
VII	20–50	NA	NA	NA	NA

NA: not applicable.

Most strains able to produce the emetic toxin are not able to multiply at temperatures below 10°C; however, some strains able to grow at 5°C have been identified (Carlin et al., 2006; Thorsen et al., 2006; Hoton et al., 2009). When incubated at temperatures between 24 and 37°C, they are able to multiply at pH 5, 7 and 8 without any difference in growth kinetic between emetic toxin-producing strains and other strains of diarrhoeal and food-environmental origin (Carlin et al., 2006).

3.5.3. Summarising remarks (Section 3.5)

Bacillus cereus group strains are widespread in the environment and can be isolated from soil and vegetation. From the soil, they can be transferred to various associated items, including plants and a variety of raw materials used for food processing. Their spores can survive the intense processing of dehydrated foods and subsequently contaminate diverse foodstuffs via dehydrated ingredients as well as cleanrooms used by many industries.

Most cases of food-borne outbreaks caused by the *B. cereus* group have been associated with concentrations above 10^5 CFU/g. However, cases of both emetic and diarrhoeal illness have been reported involving between 10^3 and 10^5 CFU/g of *B. cereus.*

The levels of the *B. cereus* group posing a health risk to consumers are highly strain-dependent due to the highly diverse pathogenic potential. The possibility of multiplication in foods after storage and/or handling must be taken into account when defining safe levels for human consumption, as well as the composition of the food, which can affect toxin production. All these factors can be responsible for the large variation in the estimated infectious dose, which makes a valid dose–response relationship hard to establish.

The occurrence of *Bacillus* species in raw materials used for food processing or in prepared foods such as soups, sauces, puddings, milk, meat and vegetables, is generally below 10^5 cells/g or mL food. This indicates that *B. cereus* group vegetative cells or spores must have the opportunity to multiply in the food chain environment to enable them to cause food spoilage or poisoning.

Taking the enterotoxigenic potential into account, together with the fact that *B. thuringiensis* cannot be distinguished from *B. cereus* at the chromosomal level, the levels of *B. cereus* that can be considered as a risk for consumers are also likely to be valid for *B. thuringiensis*. There is, however, no evidence that *B. thuringiensis* has the genetic determinants for the emetic toxin cereulide.

Few papers describe the isolation of *B. thuringiensis* from food matrices. According to the scientific literature, *B. thuringiensis* strains have been isolated from a range of foods in different countries worldwide.

In one paper (Rosenquist et al. (2005), 70% (28/40) of the *B. cereus* group strains isolated from food matrices contained visible crystals and were therefore identified as *B. thuringiensis*.

Bacillus thuringiensis strains isolated from foods can in some cases be related to the use of biopesticides containing *B. thuringiensis*, but in most cases this possible relation has not been investigated.

The levels of *B. thuringiensis* reported in food are very variable, in most cases below 10^3 CFU/g.

3.6. Control options to manage the contamination of foods with *Bacillus* spp. and their toxins

Control measures designed to inactivate *Bacillus* spp. and their toxins in foods have to take into account the ability of spores to survive industrial heat treatment processes and that some bacterial spores are able to proliferate under conditions generally presumed to prevent growth, such as low temperatures (Esteban et al., 2015). Among the *Bacillus* spp. toxins, enterotoxins, which are responsible for the diarrhoeal syndrome, are heat-labile and produced during vegetative growth of *B. cereus* in the small intestine (Stenfors Arnesen et al., 2008). Cereulide, which is responsible for the emetic syndrome, is a small heat- and acid-stable cyclic dodecadepsipeptide toxin (Frenzel et al., 2011). The heat and chemical stability of cereulide has been proved to be remarkable, even at highly alkaline pH values (7.0) and different temperatures (121°C, 2 h) (Rajkovic et al., 2008). Control options can be applied at primary production and postharvest. Conventional treatments to inactivate *Bacillus* spp. in the food chain are postharvest treatments such as heat treatment, high pressure and irradiation. Although these physical processes are usually effective, they can cause changes in the sensory and nutritional attributes of foods that may not be so desirable as well as loss of functional properties of food components (Techathuvanan et al., 2014). Therefore, alternatives to these conventional treatments have been recently evaluated to fulfil consumer demands of fresh and microbiologically safe products.

3.6.1. Control options at primary production

3.6.1.1. Pre-harvest interval

Bacillus thuringiensis is considered to be sensitive to different environmental factors affecting its fate in the field (see Section 3.4.2). Therefore, the time interval between commercial application and harvest might represent a control measure. Establishment of a pre-harvest interval is very challenging due to the possible impact of multiple interacting environmental factors (e.g. UV, weather conditions), as well as the crop and the cultivation practice and the climatic conditions rather than the action of a single factor that contributes significantly to the fate of *B. thuringiensis*.

3.6.1.2. Use of doses recommended by the manufacturer

Commercial formulations of *B. thuringiensis* used as biopesticide should be applied according to label directions, using the doses and pre-harvest interval recommended by the manufacturer. Almost any product can be harmful if used improperly or under inappropriate conditions.

3.6.2. Control options at postharvest

3.6.2.1. Temperature of storage

A wide variety of finished food products, such as cooked chilled foods (refrigerated processed foods of extended durability, REPFEDs) (Valero et al., 2002), vegetable purees, legume products and sauces (Rusul and Yaacob, 1995), rice (Sarrías et al., 2002), fish and seafood (Wijnands et al., 2006; Rahmati and Labbe, 2008), milk and dairy products (Andersson et al., 1995; Reyes et al., 2007), are considered as high-risk food products for *B. cereus* food poisoning. This is because these products commonly contain low numbers of *B. cereus* spores that are not inactivated by the drying or heat treatment (e.g. pasteurisation) applied to these products (see below) during the processes designed for their production (Brown, 2000; Silva et al., 2013; Ačai et al., 2014). Germination and outgrowth of spores in the finished food products during storage is possible, when the food intrinsic properties, packaging, storage conditions and minimal growth temperature of the *Bacillus* spp. strains are suitable for outgrowth of the activated spores. The storage temperature of the final food product is the major factor influencing the number and type of *Bacillus* spp. present at the end of shelf life. For example, storage of zucchini puree at 4°C for 21 days did not result in any detectable *B. cereus* ($< 5.1 \times 10^1$ CFU/g), while storage at 10°C for 21 days resulted in 4.1×10^4 CFU/g of mesophilic and psychrotolerant soil strains and storage at room temperature for 5 days resulted in 2.6×10^6 CFU/g (Guinebretière and Nguyen-Thé, 2003). Similarly, several commercial purees of broccoli, carrot, courgette, leek, potato and split pea, pasteurised in their final packaging, have been analysed at two periods and at different storage temperatures for the presence of *Bacillus* spp. *Bacillus cereus* was isolated under all storage conditions, but mostly from products stored at abuse temperature

(Carlin et al., 2000). Milk and milk products are also among the main foods of concern with respect to *B. cereus* contamination, mainly because pasteurisation activates the spores (heat activation) and they can start germinating. The increase in numbers of *B. cereus* in milk and milk products is very important when the storage temperature is elevated by just 2°C, from 6°C to 8°C. In order to fulfil the packaging stage legal requirements for milk in Sweden (< 10^4/mL after 6 days at 8°C), no more than 10 spores/100 mL are allowed at the packaging stage (Andersson et al., 1995). Therefore, for *B. cereus*, the most effective control measure is to assure that the rate and extent of cooling to < 4°C is rapid to prevent potential spore germination and growth of vegetative cells. If the cooling rate is not rapid, sufficient reheating before consumption would be required to kill large numbers of vegetative cells (Juneja, 2003). Thus, the common factor in most cases of food-borne illness is the consumption of raw or cooked foods which have not been stored at temperatures < 4°C or above 60°C, resulting in growth in cell numbers above 10^6 CFU/g or mL of food.

3.6.2.2. Heat treatments

Thermal inactivation of *Bacillus* spp. has been studied extensively. Estimation of the inactivation rate for a specific condition based on the reported heat parameters is a difficult task, as one has to select representative conditions, and data obtained exactly at the required representative conditions are generally not available (van Asselt and Zwietering, 2006). Some factors highlighted as relevant to the heat resistance of a bacterial strain are: strain variations, presence of salt or acid, growth phase of the cells and experimental conditions (Doyle et al., 2001). Nevertheless, van Asselt and Zwietering (2006) stated that the variability due to most factors reported to have an effect on the D value[16] is smaller than the variability of published D values. Thus, spores of *B. cereus* have been reported to have a broad range of heat resistance, creating a challenge for producers trying to develop consistent heat treatment processes (EFSA, 2005). In the previous EFSA opinion (2005) on *B. cereus* and other *Bacillus* spp. in foodstuffs, D values were provided for different set of *Bacillus* spp. strains, showing differences in the D value from a few minutes to > 100 min when a heat treatment was applied at 90°C and pH 7. It is generally accepted that the vegetative cells of *Bacillus* spp. are readily destroyed by both batch and pasteurisation. However, there is ample evidence to indicate that the spores of *Bacillus* spp. are very heat resistant and readily survive any heat treatments in the normal pasteurisation range. The pasteurisation heat treatment is sufficient to heat-activate the fast-germinating spores of *Bacillus* spp. but not the slow-germinating spores (FSANZ, 2006). The elimination of background microbiota by pasteurisation and the induction of spore germination might lead to a more abundant development of germinated spores compared to the non-heated product. Similarly, pasteurisation inactivates diarrheagenic toxins produced by *Bacillus* spp., but not the emetic toxin. Thus, it should be taken into account that pasteurisation or cooking would not eliminate *Bacillus* spp. spores and only heat treatments used for canning of low acid foods will ensure a complete destruction of spores of *B. cereus* (e.g. 121°C for ca 3 min). Gentler preservation methods which combine mild thermal treatments and immediate storage under refrigeration to preserve foods have been demanded by consumers interested by more convenient, fresher and more natural foods with a high organoleptic quality (Fernández et al., 1999). It should be considered that although typical strains of *Bacillus* spp. do not normally grow at refrigerated temperatures (< 10°C), psychrotolerant or psychrotrophic strains have been isolated from foods stored at refrigeration temperatures (van Netten et al., 1990; Väisänen et al., 1991). Therefore, foods subjected to gentler heat treatments will occasionally carry spores of *Bacillus* spp. (EFSA, 2005). Additionally, in an attempt to reduce the detrimental effects of over-processing on food quality, milder heat treatments have been combined with natural products such as essential oils and organic acids, although the potential of these combined treatments is still uncertain. Recently, Esteban et al. (2015) demonstrated that heat resistance of spores of *Bacillus* spp. was only slightly reduced when thymol was present in the heating medium. Similar results have been previously reported by other authors when adding antimicrobial compounds in the medium during the heating process (Tremoulet et al., 2002; Lekogo et al., 2010; Haberbeck et al., 2012; Esteban et al., 2015).

[16] D value (decimal reduction time) = the time required, at a given temperature, to reduce the number of vegetative cells or spores of a given microorganism to 10% of the initial number; it is usually quoted in minutes. The temperature (°C) at which the D value is determined may be indicated by a subscript, e.g. D_{112}.

3.6.2.3. High hydrostatic pressure

High hydrostatic pressure (HHP) has been proved to be effective against vegetative cells and spores, although very high pressures are needed to inactivate spores (Knorr, 1995; EFSA, 2005). High pressure has the potential to inactivate microorganisms and certain enzymes and to modify the functional properties of some food constituents, while reducing the impact on the nutritional and sensory properties of foods (Moerman, 2005). However, it has been reported that inactivation of microbial spores and enzymes by HHP alone is not feasible (Evelyn and Silva, 2015). Since the 1960s, scientific reports have been published reporting the efficacy of HHP to kill thermally resistant spore-forming microorganisms, taking into account that spores are first germinated through these pressures (Moerman, 2005). Pressures between 300 and 400 MPa have been proved to be necessary to inactivate spore-formers while lower pressures were insufficient and had also the drawback of inducing germination (Sale et al., 1970; Russell, 1982). On the other hand, higher pressures (> 400 MPa) were too high to allow spore germination (Hoover, 1993). It has also been highlighted that using these HHP treatments, there is also an 'un-germinated' fraction that has been defined as a 'super-dormant fraction' that under temperature abuse could rapidly germinate and multiply. Thus, in most cases, a combination of HHP with moderate or elevated temperatures is required for the inactivation of bacterial spores. An extensive literature is available regarding the high-pressure sensitivity of several *Bacillus* spp. strains subjected to different temperature–high pressure treatments (Moerman et al., 2001; Moerman, 2005; Reineke et al., 2013; Evelyn and Silva, 2015; Luu-Thi et al., 2015). Some authors reported that combinations of high pressure (300–700 MPa) and high temperature (45–100°C) are needed to inactivate the most resistant bacterial spores (Evelyn and Silva, 2015). This treatment combines a preheated step at a moderate initial temperature and subsequent pressure treatment for a few minutes at 500–800 MPa (Luu-Thi et al., 2015).

Recently, the efficacy of HHP treatments (100–900 MPa), applied as pulses or continuously, and in combination with heat and antimicrobial agents or an additional control hurdle such as the addition of natural compounds (e.g. plant essential oils), to inactivate spores of *Bacillus* spp. has been investigated (Palhano et al., 2004; Evelyn and Silva, 2015). However, as previously mentioned for the heat treatments alone, different resistances to combined treatments have been reported and the kinetics are rarely modelled, which hinders the implementation of these treatments (Evelyn and Silva, 2015). Some studies showed that the use of these combined treatments is not always successful. Luu-Thi et al. (2015) reported that spore inactivation by high pressure high temperature (HPHT) (600 MPa for 5 min) was less than 1 log unit when applied in a temperature range between 50 and 70°C. However, the efficacy of treatment gradually increased at higher temperatures, up to 5 log units when combined with a temperature of 100°C. On the other hand, when the pressure treatment (600 MPa for 5 min) was applied in the presence of 5 mM of carvacrol, spore germination was strongly inhibited at mild temperatures (65–70°C) but unaffected at 95–100°C. Spore germination was completely inhibited at ≤ 65°C, while at 95–100°C, carvacrol had no effect on HPHT inactivation. Other organic compounds have been shown to have similar effects on spore germination (van Melis et al., 2011, 2012).

3.6.2.4. Pulsed light

Pulsed light is a method of food preservation that involves the use of intense and short duration pulses of broad-spectrum 'white light'. The spectrum of light for pulsed light treatment includes wavelengths in the UV to the near-infrared region (USFDA/CFSAN, 2002).This technology has been proposed as an alternative to conventional heat preservation processes to ensure the microbial quality and safety of food products. However, as with many other non-thermal technologies, the main challenge for the application of this technology in the food industry is the inactivation of bacterial spores (Artíguez and Martínez de Marañón, 2015). Additionally, in common with other technologies based on light, several factors may interfere with the disinfection capacity of pulsed light (PL), including light absorption by microorganisms, suspended solids and presence of absorbing compounds among others (Selma et al., 2008). Artíguez and Martínez de Marañón (2015) have recently demonstrated that the inactivation effectiveness would not only depend on the physiological state of the cells but also on their exposure to the incident light, which could be influenced by cell population density among other factors (e.g. presence of particles in the solution). These authors concluded that more research is needed to determine the efficacy of PL treatments in complex food systems, in particular in fluids with limited light transmittance.

3.6.2.5. Irradiation

Many studies have shown that irradiation is a powerful process for inactivating various types of microorganisms and that bacterial spores are generally the most resistant to inactivation (Russell et al., 1999; Dauphin et al., 2008). Several research studies have demonstrated the efficacy of gamma irradiation to inactivate vegetative cells and spores of *Bacillus* spp. when doses of about 15–22 kGy were applied (Horne et al., 1959; Dang et al., 2001). Vegetative cells are inactivated using much lower radiation doses (1.6 kGy) as previously highlighted in the previous EFSA opinion on *Bacillus* spp. (EFSA, 2005). More recently, Sun et al. (2013) reported that in the radiation dosage range of 10–15 kGy, no viable spore formation and no significant reduction in the efficiency of *B. thuringiensis* against lepidopteran larvae were observed, demonstrating that the use of gamma radiation is effective to inactivate the spores of engineered *B. thuringiensis* strains while preserving toxicity against the target insect larvae.

3.6.2.6. Antimicrobial compounds

Several previously reported studies have focused on non-therapeutic antimicrobial compounds to suppress *B. cereus* growth in food, including bacteriocins, terpenoid substances, organic acids and chlorine-based disinfectants among others (Russell and Gould, 2003; Valero and Salmerón, 2003; Galvez et al., 2007; Morente et al., 2010; Ter Beek and Brul, 2010; Nam et al., 2014). Long-chain polyphosphates have been shown to have antimicrobial activity and recently, their capacity to inhibit cereulide synthesis has been evaluated (Frenzel et al., 2011). Results showed the potential of polyphosphate formulations to reduce the risk of cereulide synthesis in foods.

Chlorine-based disinfectants such as sodium hypochlorite, chlorine dioxide and electrolysed water are widely used to disinfect water, mostly because they are powerful oxidising agents (Gómez-López et al., 2013). The efficacy of several chlorine-based disinfectants against vegetative cells and spores of *Bacillus* spp. has been recently considered by several authors (Nam et al., 2014; Forghani et al., 2015). High doses and long contact times are usually required to completely inactivate *Bacillus* spp. spores. For example, Nam et al. (2014) reported that doses of about 115 ppm of gaseous chlorine dioxide applied for up to 1 h were needed to inactivate *Bacillus* spp. spores. On the other hand, the use of slightly acidic electrolysed water in combination with ultrasound using a free chlorine concentration of 20–22 ppm for 1 min reduced *B. cereus* vegetative cells for about 1 log CFU/mL.

3.6.3. Summarising remarks (Section 3.6)

Control options for managing the risk caused by *Bacillus* spp. and their toxins can be divided between pre- and postharvest intervention strategies.

In the case of primary production, commercial formulations of *B. thuringiensis* used as biopesticide should be applied according to label directions, using the doses and the time interval between commercial application and harvest recommended by the manufacturer.

Foods associated with food-borne outbreaks implicating the *B. cereus* group have often been heat-treated and/or insufficiently cooled after preparation and therefore kept at a temperature at which *B. cereus* group bacteria can multiply (temperatures between 4°C and 55°C).

At postharvest, the main management option for controlling *B. cereus* group strains in the food chain is to maintain the foods refrigerated at $\leq$ 7°C (and preferably at $\leq$ 4°C). Other potentially efficient control measures include heat treatment, HHP, pulsed light, irradiation and chemical sanitisers. Most of these treatments are relatively efficient against vegetative cells but most measures fail to inactivate spores and so far no commonly used control option used in the food industry can inactivate cereulide toxins. Combinations of high pressure and high temperature are needed to inactivate the most resistant bacterial spores.

4. Conclusions

4.1. General conclusions

The *B. cereus* group is a set of ubiquitous rod-shaped Gram-positive soil bacteria with eight species that are genetically very similar but have highly specialised lifestyles. It includes the opportunistic pathogen *B. cereus sensu stricto*, which is frequently implicated in cases of food poisoning, the entomopathogen *B. thuringiensis*, from which a number of selected strains are widely used as biopesticides, and the causative agent of anthrax *B. anthracis*.

The main distinguishing phenotypic features of *B. thuringiensis*, *B. anthracis* and emetic *B. cereus*, including their respective virulence properties, are directly associated with large plasmids. Therefore, despite the multiple species names, which are attributed to phenotypes mostly conferred by mobile genetic elements, there is a growing consensus in the research community that all these organisms could be considered members of a single species as demonstrated by many molecular studies.

All *B. cereus* group strains display a similar repertoire of potential virulence genes. These genes, coding for the more general virulence factors, including enterotoxin genes and the global regulator PlcR, are located on the chromosome, and it has been shown that these genes are actively expressed in *B. thuringiensis* strains. Therefore, when a *B. thuringiensis* strain loses its *cry* gene-containing plasmid(s), the strain will appear as a *B. cereus sensu stricto* strain. These features clearly question the relevance of the taxonomic segregation of *B. cereus* group strains into separate species.

The current taxonomy of the *B. cereus* group and the status of separate species for the different bacteria that constitute the *B. cereus* group, for historic reasons, mainly rely on phenotypic characteristics, established before the era of genome sequencing, and without the knowledge of important traits being plasmid-borne.

The economic and medical importance of the individual species and in particular the separate species status of *B. thuringiensis* are clearly relevant to the use of *B. thuringiensis* as a biopesticide.

4.2. Answer to Term of Reference 1

ToR 1. Provide an update of information available on pathogenicity, and contributing virulence factors, in the genus Bacillus *(with the exclusion of* B. anthracis*). Specifically to evaluate the risk to public health arising from the presence of* B. thuringiensis *in food (including in the evaluation information from the specific case of illness described above).*

Bacillus cereus group produces various virulence factors, which may act synergistically within the host. Several toxins have been associated with two distinct forms of food poisoning, emetic and diarrhoeal syndrome.

The panoply of potential toxins and virulence factors, found in the *B. cereus* group is broadly distributed among the different members of the group, including *B. thuringiensis,* and it is not possible to draw any firm conclusion about the pathogenic potential of a certain strain based on the sole presence of potential virulence factors.

Nearly all *B. cereus* strains harbour the *nhe* genes, while *hbl* and *cytK* are detected in about 30–70% of isolates. In contrast to the emetic toxin cereulide, these potential enterotoxins are also frequently found in other *B. cereus* group members, including *B. thuringiensis.* To date, the *ces* genes have not been found in *B. thuringiensis.*

Two papers report on the involvement of *B. thuringiensis* in food-borne outbreaks. The first is a description of one outbreak, while the other is a reassessment of isolates from 39 outbreaks. In this paper, it was concluded that *B. thuringiensis* might have been the cause of the infection in four of the outbreaks.

In the alleged food poisoning outbreak described in the background section of this Opinion, it appears that the only bacteria that were found above the generally accepted level were *B. cereus* group bacteria, identified as *B. thuringiensis* in the salad samples. The *B. thuringiensis* isolates from the salad were characterised by FTIR spectroscopy and could not be discriminated from *B. thuringiensis* subsp. *aizawai* (XenTari) isolates which had been sprayed on the salad on the field.

It is not clear if people eating salad also ate cheese noodles and therefore an additive effect between *B. thuringiensis* and *B. cereus* cannot be excluded. It cannot be excluded that *B. cereus* was present at low levels in the salad, although it was not detected.

4.3. Answer to Term of Reference 2

ToR 2. Review the microbiological methods available to distinguish between the members of the B. cereus *group, to identify different* B. thuringiensis *strains, and the methods to identify the presence of toxins produced by these microorganisms.*

The major features differentiating *B. thuringiensis* from other members of the *B. cereus* group are based on the phenotypes of the strains (i.e. the presence of toxin crystals within the bacterial sporangium), and routine methods do not discriminate *B. thuringiensis* from other *B. cereus* group strains.

The primary definition of a *B. thuringiensis* strain is its capacity to be active against invertebrates. This activity is mainly, but not exclusively, related to the production, during sporulation, of a parasporal crystal containing the Cyt and Cry toxins. For some strains, this crystal is easily observable with a phase-contrast light microscope where it appears as a bipyramidal-shaped structure located next to the spore; for other strains, the crystals could appear as diamond or spherical structures and in some strains they are smaller and might be confused with other internal bodies.

The discrimination method based on microscopic examination is therefore not reliable and should always be considered as presumptive, especially in the cases of *B. thuringiensis* strains containing tiny and irregular crystals.

Different methods to identify the presence of potential enterotoxins are available, such as immunoassays, cytotoxicity assays and molecular assays.

Currently, no ISO and/or other standardised methods to reliably differentiate between strains of *B. cereus* and *B. thuringiensis* are available.

At this moment, the only way to unambiguously recognise a specific strain of *B. thuringiensis* (including those used commercially as biopesticides) is to identify it through WGS.

4.4. Answer to Term of Reference 3

ToR 3. Review existing data on natural background prevalence and levels of B. thuringiensis *in the environment (e.g. soil), and rates of transfer to foodstuffs, including conditions under which this transfer may take place.*

Bacillus thuringiensis is ubiquitous and has a worldwide distribution, so it can be isolated from most materials including soils, plants, sediments, water, invertebrates and mammals.

In soils, between 0% and 50% of the *B. cereus* group isolates are *B. thuringiensis*, which may reach levels up to 5×10^5 CFU/g in cultivated soils. The natural occurrence of *B. cereus* group organisms on plants is most likely log-normal distributed and might vary between 0 and 6×10^4 CFU/g, with a mean density around 1×10^2 CFU/g.

Systematic studies on the fate of *B. thuringiensis* after application are lacking; however, some papers report half-lives of *B. thuringiensis* in soil after application has been found to vary between 100 and 200 days. Survival is much shorter on plant surfaces where reports of half-lives after application vary between 16 and 38 h. However, long-term survival, up to 13 years, of low numbers of organisms has been reported. Available data only relate to leaves and information on survival in greenhouses.

The fate of *B. thuringiensis* on a specific crop after an application is hard to predict, as it is dependent on many factors including the crop, the climatic conditions and the cultivation practice. It is not possible to estimate the number of *B. thuringiensis* present on a specific crop at a specific locality after a certain period of time.

Factors of importance for transfer to foodstuffs include rain-mediated splashing of soil, epiphytic and endophytic colonisation of seedlings from soil and the presence of target organisms on the plant. Long-term spread might be mediated by air and water. Little information is available on such transfer and this lack of information makes it impossible to estimate rates of transfer.

4.5. Answer to Term of Reference 4

ToR 4. Indicate, if possible, the maximum levels (number) of Bacillus*, and specifically of* B. thuringiensis*, in food that could be regarded as safe for human consumption.*

Bacillus cereus group strains are widespread in the environment and can be isolated from soil and vegetation. From the soil, they can be transferred to various associated items, including plants and a variety of raw materials used for food processing. Their spores can survive the intense processing of dehydrated foods and subsequently contaminate diverse foodstuffs via dehydrated ingredients as well as cleanrooms used by many industries.

Most cases of food-borne outbreaks caused by the *B. cereus* group have been associated with concentrations above 10^5 CFU/g. However, cases of both emetic and diarrhoeal illness have been reported involving between 10^3 and 10^5 CFU/g of *B. cereus.* Recently, in some food-borne outbreaks associated with emetic *B. cereus,* the level of contamination of food ranged from less than 10^2 CFU/g to 6×10^7 CFU/g.

The levels of *B. cereus* group posing a health risk to consumers are highly strain-dependent due to the highly diverse pathogenic potential. The possibility of multiplication in foods after storage and/or handling must be taken into account when defining safe levels for human consumption, as well as the

composition of the food, which can affect toxin production. All these factors can be responsible for the large variation in the estimated infectious dose, which makes a valid dose–response relationship hard to establish.

The occurrence of *Bacillus* species in raw materials used for food processing or in prepared foods such as soups, sauces, puddings, milk, meat and vegetables, is generally low and thus vegetative cells or spores must be able to multiply in the food chain environment to enable them to cause food spoilage or poisoning.

Taking the enterotoxigenic potential into account, as well as the fact that *B. thuringiensis* cannot be distinguished from *B. cereus* at the chromosomal level, the levels of enterotoxigenic *B. cereus* that can be considered as a risk for consumers are also likely to be valid for *B. thuringiensis*.

There is, however, no evidence that *B. thuringiensis* has the genetic determinants for the emetic toxin cereulide, which is considered the cause of food-borne outbreaks related to numbers of *B. cereus* group strains in implicated foods as low as 10^2 CFU/g.

4.6. Answer to Term of Reference 5

ToR 5. Evaluate what would be the B. thuringiensis *levels in food, at all stages of the food chain, if this microorganism was applied as PPP.*

Bacillus thuringiensis is ubiquitous and has a worldwide distribution, so it can be isolated from most materials including soils, plants, sediments, water, invertebrates and mammals. The natural occurrence of *B. cereus* group on plants varies between 0 and 6×10^4 CFU/g, with a mean density around 1×10^2 CFU/g.

Few papers describe the isolation of *B. thuringiensis* from food matrices. According to the scientific literature, *B. thuringiensis* strains have been isolated from a range of foods, in different countries worldwide.

In one study, 70% (28/40) of the *B. cereus* group strains isolated from food matrices contained visible crystals and were therefore identified as *B. thuringiensis*.

Bacillus thuringiensis strains isolated from foods can in some cases be related to the use of biopesticides containing *B. thuringiensis*, but in most cases this possible relation has not been investigated.

The levels of *B. thuringiensis* reported in the most diverse kinds of food are very variable, in most cases below 10^3 CFU/g.

4.7. Answer to Term of Reference 6

ToR 6. Provide an update on specific control options, to manage the risk caused by B. cereus, B. thuringiensis, *and other* Bacillus *spp. and their toxins.*

Control options aimed at managing the risk caused by *Bacillus* spp. and their toxins can be divided into pre- and postharvest intervention strategies.

In the case of primary production, commercial formulations of *B. thuringiensis* used as biopesticide should be applied according to label directions, using the doses and the time intervals between commercial application and harvest recommended by the manufacturer.

At postharvest, the main management option for controlling *B. cereus* group strains in the food chain is to maintain the foods refrigerated at ≤ 7°C (and preferably at ≤ 4°C).

Other efficient control measures include heat treatment, HHP, pulsed light, irradiation and the use of antimicrobial compounds alone and/or in combination. Most of these treatments are efficient against vegetative cells but some of them fail to inactivate the most resistant spores and so far no commonly used control option used in the food industry can inactivate cereulide toxins.

In the case of the most common postharvest control option, heat treatment, only those treatments used for canning of low acid foods will ensure a complete destruction of spores of *B. cereus* (e.g. 121°C for ca 3 min).

Therefore, in most of the cases, postharvest treatments have to be followed by adequate refrigeration to prevent potential spore germination and growth of most vegetative cells, although some psychrotolerant or psychrotrophic *Bacillus* strains may multiply slowly at refrigeration temperatures.

Foods associated with food-borne outbreaks implicating the *B. cereus* group have often been heat-treated and insufficiently cooled after preparation and therefore kept at a temperature at which *B. cereus* group can grow (temperatures between 4°C and 55°C).

5. Recommendations

The Biological Hazards (BIOHAZ) Panel recommends to:

Obtain information through whole genome sequencing in order to provide unambiguous identification of strains used as biopesticides and assist further safety assessment. These strains and respective sequences should be available for laboratories specialised in the *B. cereus* group.

In cases of food-borne outbreaks associated with the *B. cereus* group, characterise strains in detail allowing discrimination of *B. thuringiensis* from *B. cereus,* as well as the identification of strains related to commercial *B. thuringiensis* used as biopesticides.

Maintain *B. cereus* group food-borne outbreak strains in accessible culture collections preferentially managed by reference laboratories.

Identify markers for commercial *B. thuringiensis* strains to allow regular monitoring and easy differentiation in suspect outbreak situations.

Promote field studies after application of *B. thuringiensis* biopesticides in order to inform the possible establishment of pre-harvest intervals.

Develop research on dose–response and behavioural characteristics of *B. cereus* group strains and specifically of *B. thuringiensis*, to facilitate risk characterisation.

Develop studies to monitor and characterise the factors that lead to/favour the transfer of the *B. cereus* group and specifically *B. thuringiensis* from the environment to foodstuffs and identify the routes and critical steps of contamination in the food industry.

Documentation provided to EFSA

Documents listed in the Mandate letter shared with the WG via CIRCABC (Communication and Information Resource Centre for Administrations, Businesses and Citizens) Pesticides:

1) Data and comments uploaded on CIRCABC for the SCOFCAH pesticides residues of 24–25 February 2014, point A.15.
2) Data and comments uploaded on CIRCABC for the SCOFCAH pesticides residues of 12–13 June 2014, point B.08.00.
3) Data and comments uploaded on CIRCABC for the PAFF (Standing Committee on Plants, Animals, Food and Feed) pesticides residues of 22–23 September 2014, point A.08.03.
4) Data and comments uploaded on CIRCABC for the PAFF pesticides residues of 24–25 November 2014, point A.15.03.
5) Data and comments uploaded on CIRCABC for the PAFF pesticides residues of 12–13 February 2015, point A.13.03.
6) Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. EFSA Journal 2014;12(5):3665, 10 pp. doi:10.2903/j.efsa.2014.3665

References

Ačai P, Valík L and Liptáková D, 2014. Quantitative risk assessment of *Bacillus cereus* in pasteurised milk produced in the Slovak Republic. Czech Journal of Food Sciences, 32, 122–131.

Afchain AL, Carlin F, Nguyen-the C and Albert I, 2008. Improving quantitative exposure assessment by considering genetic diversity of *B. cereus* in cooked, pasteurised and chilled foods. International Journal of Food Microbiology, 128, 165–173.

Agata N, Ohta M, Mori M and Isobe M, 1995. A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. FEMS Microbiology Letters, 129, 17–20.

Ali S, Zafar Y, Ali GM and Nazir F, 2010. *Bacillus thuringiensis* and its application in agriculture. African Journal of Biotechnology, 9, 2022–2031.

Amadio AF, Benintende GB and Zandomeni RO, 2009. Complete sequence of three plasmids from *Bacillus thuringiensis* INTA-FR7-4 environmental isolate and comparison with related plasmids from the *Bacillus cereus* group. Plasmid, 62, 172–182.

Andersson A, Ronner U and Granum PE, 1995. What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? International Journal of Food Microbiology, 28, 145–155.

Andersson MA, Mikkola R, Helin J, Andersson MC and Salkinoja-Salonen M, 1998. A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. Applied and Environmental Microbiology, 64, 1338–1343.

Andrews JH and Harris RF, 2000. The ecology and biogeography of microorganisms on plant surfaces. Annual Review of Phytopathology, 38, 145–180.

Andrup L, Bendixen HH and Jensen GB, 1995. Mobilization of *Bacillus thuringiensis* plasmid pTX14-3. Plasmid, 33, 159–167.
Andrup L, Jorgensen O, Wilcks A, Smidt L and Jensen GB, 1996. Mobilization of "nonmobilizable" plasmids by the aggregation-mediated conjugation system of *Bacillus thuringiensis*. Plasmid, 36, 75–85.
Andrup L, Jensen GB, Wilcks A, Smidt L, Hoflack L and Mahillon J, 2003. The patchwork nature of rolling-circle plasmids: comparison of six plasmids from two distinct *Bacillus thuringiensis* serotypes. Plasmid, 49, 205–232.
Arantes O and Lereclus D, 1991. Construction of cloning vectors for *Bacillus thuringiensis*. Gene, 108, 115–119.
Artíguez ML and Martínez de Marañón I, 2015. Inactivation of spores and vegetative cells of *Bacillus subtilis* and *Geobacillus stearothermophilus* by pulsed light. Innovative Food Science and Emerging Technologies, 28, 52–58.
Ash C and Collins MD, 1992. Comparative analysis of 23S ribosomal RNA gene sequences of Bacillus anthracis and emetic Bacillus cereus determined by PCR-direct sequencing. FEMS Microbiology Letters, 73, 75–80.
Ash C, Farrow JA, Dorsch M, Stackebrandt E and Collins MD, 1991. Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. International Journal of Systematic Bacteriology, 41, 343–346.
van Asselt ED and Zwietering MH, 2006. A systematic approach to determine global thermal inactivation parameters for various food pathogens. International Journal of Food Microbiology, 107, 73–82.
de Barjac H and Bonnefoi A, 1967. Classification of strains of *Bacillus thuringiensis*. C R Acad Sci Hebd Seances Acad Sci D, 264, 1811–1813.
de Barjac H and Frachon E, 1990. Classification of *Bacillus thuringiensis* strains. Entomophaga, 35, 233–240.
Bauer T, Stark T, Hofmann T and Ehling-Schulz M, 2010. Development of a stable isotope dilution analysis for the quantification of the *Bacillus cereus* toxin cereulide in foods. Journal of Agricultural and Food Chemistry, 58, 1420–1428.
Baum JA, 1994. Tn5401, a new class II transposable element from *Bacillus thuringiensis*. Journal of Bacteriology, 176, 2835–2845.
Baum JA, Coyle DM, Gilbert MP, Jany CS and Gawron-Burke C, 1990. Novel cloning vectors for *Bacillus thuringiensis*. Applied and Environmental Microbiology, 56, 3420–3428.
Bavykin SG, Lysov YP, Zakhariev V, Kelly JJ, Jackman J, Stahl DA and Cherni A, 2004. Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of *Bacillus cereus* group microorganisms. Journal of Clinical Microbiology, 42, 3711–3730.
Beecher DJ and Wong AC, 1994. Improved purification and characterization of hemolysin BL, a hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. Infection and Immunity, 62, 980–986.
Beecher DJ and Wong AC, 2000. Cooperative, synergistic and antagonistic haemolytic interactions between haemolysin BL, phosphatidylcholine phospholipase C and sphingomyelinase from *Bacillus cereus*. Microbiology, 146(Pt 12), 3033–3039.
Beecher DJ, Schoeni JL and Wong AC, 1995. Enterotoxic activity of hemolysin BL from *Bacillus cereus*. Infection and Immunity, 63, 4423–4428.
Bell CA, Uhl JR, Hadfield TL, David JC, Meyer RF, Smith TF and Cockerill FR 3rd, 2002. Detection of *Bacillus anthracis* DNA by LightCycler PCR. Journal of Clinical Microbiology, 40, 2897–2902.
Belliveau BH and Trevors JT, 1989. Transformation of *Bacillus cereus* vegetative cells by electroporation. Applied and Environmental Microbiology, 55, 1649–1652.
Belliveau BH and Trevors JT, 1990. Mercury resistance determined by a self-transmissible plasmid in *Bacillus cereus* 5. Biology of Metals, 3, 188–196.
Ben Fguira I, Fourati Z, Kamoun F, Tounsi S and Jaoua S, 2014. Isolation of the *Bacillus thuringiensis* plasmid carrying Bacthuricin F4 coding genes and evidence of its conjugative transfer. Journal of Infection in Developing Countries, 8, 727–732.
Ben-Dov E, Zaritsky A, Dahan E, Barak Z, Sinai R, Manasherob R, Khamraev A, Troitskaya E, Dubitsky A, Berezina N and Margalith Y, 1997. Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. Applied and Environmental Microbiology, 63, 4883–4890.
Ben-Dov E, Wang Q, Zaritsky A, Manasherob R, Barak Z, Schneider B, Khamraev A, Baizhanov M, Glupov V and Margalith Y, 1999. Multiplex PCR screening to detect *cry9* genes in *Bacillus thuringiensis* strains. Applied and Environmental Microbiology, 65, 3714–3716.
Berjón-Otero M, Villar L, de Vega M, Salas M and Redrejo-Rodríguez M, 2015. DNA polymerase from temperate phage Bam35 is endowed with processive polymerization and abasic sites translesion synthesis capacity. Proceedings of the National Academy of Sciences of the United States of America, 112, E3476–E3484.
Bernhard K, Schrempf H and Goebel W, 1978. Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. Journal of Bacteriology, 133, 897–903.
Berthold-Pluta A, Pluta A and Garbowska M, 2015. The effect of selected factors on the survival of *Bacillus cereus* in the human gastrointestinal tract. Microbial Pathogenesis, 82, 7–14.
Biesta-Peters EG, Reij MW, Blaauw RH, In 't Veld PH, Rajkovic A, Ehling-Schulz M and Abee T, 2010. Quantification of the emetic toxin cereulide in food products by liquid chromatography-mass spectrometry using synthetic cereulide as a standard. Applied and Environmental Microbiology, 76, 7466–7472.

Biswas I, Gruss A, Ehrlich SD and Maguin E, 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. Journal of Bacteriology, 175, 3628–3635.
Bizzarri MF and Bishop AH, 2008. The ecology of *Bacillus thuringiensis* on the Phylloplane: colonization from soil, plasmid transfer, and interaction with larvae of *Pieris brassicae*. Microbial Ecology, 56, 133–139.
Böhm ME, Huptas C, Krey VM and Scherer S, 2015. Massive horizontal gene transfer, strictly vertical inheritance and ancient duplications differentially shape the evolution of *Bacillus cereus* enterotoxin operons hbl, cytK and nhe. BMC Evolutionary Biology, 15, 246.
Bottone EJ, 2010. *Bacillus cereus*, a volatile human pathogen. Clinical Microbiology Reviews, 23, 382–398.
Bravo A, Gill SS and Soberon M, 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon, 49, 423–435.
Bravo A, Likitvivatanavong S, Gill SS and Soberon M, 2011. *Bacillus thuringiensis*: a story of a successful bioinsecticide. Insect Biochemistry and Molecular Biology, 41, 423–431.
Brezillon C, Haustant M, Dupke S, Corre JP, Lander A, Franz T, Monot M, Couture-Tosi E, Jouvion G, Leendertz FH, Grunow R, Mock ME, Klee SR and Goossens PL, 2015. Capsules, toxins and AtxA as virulence factors of emerging *Bacillus cereus* biovar anthracis. PLoS Neglected Tropical Diseases, 9, e0003455.
Brillard J, Dupont CM, Berge O, Dargaignaratz C, Oriol-Gagnier S, Doussan C, Broussolle V, Gillon M, Clavel T and Bérard A, 2015. The water cycle, a potential source of the bacterial pathogen *Bacillus cereus*. BioMed Research International, 2015, 356928.
Brown KL, 2000. Control of bacterial spores. British Medical Bulletin, 56, 158–171.
Burges HD and Jones KA, 1998. Formulation of bacteria, viruses, and protozoa to control insects. In: Burges HD (ed.). *Formulation of Microbial Pesticides: Beneficial Microorganisms, Nematodes and Seed Treatments*. Kluwer Academic Publishers, Dordrecht, the Netherlands. pp. 33–127.
Cadel Six S, De Buyser ML, Vignaud ML, Dao TT, Messio S, Oairaud S, Hennekinne JA, Pihier N and Brisabois A, 2012. Toxi-infections alimentaires collectives à *Bacillus cereus*: bilan de la caractérisation des souches de 2006 à 2010. BEH – Bulletin Epidémiologique Hebdomadaire, Hors-série, 9 mai 2012, 45–49.
Carlin F, Guinebretière M-H, Choma C, Pasqualini R, Braconnier A and Nguyen-thé C, 2000. Spore-forming bacteria in commercial cooked, pasteurised and chilled vegetable purées. Food Microbiology, 17, 153–165.
Carlin F, Fricker M, Pielaat A, Heisterkamp S, Shaheen R, Salkinoja Salonen M, Svensson B, Nguyen-the C and 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, 132–138.
Carlin F, Brillard J, Broussolle V, Clavel T, Duport C, Jobin M, Guinebretière M-H, Auger S, Sorokine A and Nguyen-Thé C, 2010. Adaptation of *Bacillus cereus*, an ubiquitous worldwide-distributed foodborne pathogen, to a changing environment. Food Research International, 43, 1885–1894.
Castiaux V, Liu X, Delbrassinne L and Mahillon J, 2015. Is Cytotoxin K from *Bacillus cereus* a bona fide enterotoxin? International Journal of Food Microbiology, 211, 79–85.
Cataldi A, Labruyère E and Mock M, 1990. Construction and characterization of a protective antigen-deficient *Bacillus anthracis* strain. Molecular Microbiology, 4, 1111–1117.
CDPR (California Department of Pesticide Regulation), 2009. Pesticide Use Reporting - 2009 Summary Data. Available online: http://www.cdpr.ca.gov/docs/pur/pur09rep/09_pur.htm
Ceuppens S, Uyttendaele M, Drieskens K, Heyndrickx M, Rajkovic A, Boon N and Van de Wiele T, 2012. Survival and germination of *Bacillus cereus* spores without outgrowth or enterotoxin production during *in vitro* simulation of gastrointestinal transit. Applied and Environmental Microbiology, 78, 7698–7705.
Ceuppens S, Boon N and Uyttendaele M, 2013. Diversity of *Bacillus cereus* group strains is reflected in their broad range of pathogenicity and diverse ecological lifestyles. FEMS Microbiology Ecology, 84, 433–450.
Chakroun M, Banyuls N, Bel Y, Escriche B and Ferre J, 2016. Bacterial Vegetative Insecticidal Proteins (Vip) from Entomopathogenic Bacteria. Microbiology and Molecular Biology Reviews, 80, 329–350.
Chang YH, Shangkuan YH, Lin HC and Liu HW, 2003. PCR assay of the *groEL* gene for detection and differentiation of *Bacillus cereus* group cells. Applied and Environmental Microbiology, 69, 4502–4510.
Chatterjee SN, Bhattacharya T, Dangar TK and Chandra G, 2007. Ecology and diversity of *Bacillus thuringiensis* in soil environment. African Journal of Biotechnology, 6, 1587–1591.
Chaves JQ, Pires ES and Vivoni AM, 2011. Genetic diversity, antimicrobial resistance and toxigenic profiles of *Bacillus cereus* isolated from food in Brazil over three decades. International Journal of Food Microbiology, 147, 12–16.
Chen ML and Tsen HY, 2002. Discrimination of *Bacillus cereus* and *Bacillus thuringiensis* with 16S rRNA and *gyrB* gene based PCR primers and sequencing of their annealing sites. Journal of Applied Microbiology, 92, 912–919.
Chen Y, Braathen P, Leonard C and Mahillon J, 1999. MIC231, a naturally occurring mobile insertion cassette from *Bacillus cereus*. Molecular Microbiology, 32, 657–668.
Chen Y, Succi J, Tenover FC and Koehler TM, 2003. Beta-lactamase genes of the penicillin-susceptible *Bacillus anthracis* Sterne strain. Journal of Bacteriology, 185, 823–830.
Cherif A, Rezgui W, Raddadi N, Daffonchio D and Boudabous A, 2008. Characterization and partial purification of entomocin 110, a newly identified bacteriocin from *Bacillus thuringiensis* subsp. Entomocidus HD110. Microbiological Research, 163, 684–692.

Choma CT, Surewicz WK and Kaplan H, 1991. The toxic moiety of the *Bacillus thuringiensis* protoxin undergoes a conformational change upon activation. Biochemical and Biophysical Research Communications, 179, 933–938.
Chon JW, Kim JH, Lee SJ, Hyeon JY and Seo KH, 2012. Toxin profile, antibiotic resistance, and phenotypic and molecular characterization of *Bacillus cereus* in Sunsik. Food Microbiology, 32, 217–222.
Christiansson A, Naidu AS, Nilsson I, Wadstrom T and Pettersson HE, 1989. Toxin production by *Bacillus cereus* dairy isolates in milk at low temperatures. Applied and Environmental Microbiology, 55, 2595–2600.
Chung MC, Popova TG, Millis BA, Mukherjee DV, Zhou W, Liotta LA, Petricoin EF, Chandhoke V, Bailey C and Popov SG, 2006. Secreted neutral metalloproteases of *Bacillus anthracis* as candidate pathogenic factors. Journal of Biological Chemistry, 281, 31408–31418.
Collier FA, Elliot SL and Ellis RJ, 2005. Spatial variation in *Bacillus thuringiensis/cereus* populations within the phyllosphere of broad-leaved dock (*Rumex obtusifolius*) and surrounding habitats. FEMS Microbiology Ecology, 54, 417–425.
Cremonesi P, Pisani LF, Lecchi C, Ceciliani F, Martino P, Bonastre AS, Karus A, Balzaretti C and Castiglioni B, 2014. Development of 23 individual TaqMan(R) real-time PCR assays for identifying common foodborne pathogens using a single set of amplification conditions. Food Microbiology, 43, 35–40.
Daffonchio D, Cherif A and Borin S, 2000. Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the "*Bacillus cereus* group". Applied and Environmental Microbiology, 66, 5460–5468.
Damgaard PH, 1995. Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides. FEMS Immunology and Medical Microbiology, 12, 245–250.
Dang JL, Heroux K, Kearney J, Arasteh A, Gostomski M and Emanuel PA, 2001. *Bacillus* spore inactivation methods affect detection assays. Applied and Environmental Microbiology, 67, 3665–3670.
Dauphin LA, Newton BR, Rasmussen MV, Meyer RF and Bowen MD, 2008. Gamma irradiation can be used to inactivate *Bacillus anthracis* spores without compromising the sensitivity of diagnostic assays. Applied and Environmental Microbiology, 74, 4427–4433.
Day M, Ibrahim M, Dyer D and Bulla L Jr, 2014. Genome Sequence of *Bacillus thuringiensis* subsp. kurstaki Strain HD-1. Genome Announcements, 2, e00613–e00614.
De Palmenaer D, Vermeiren C and Mahillon J, 2004. IS*231*-MIC*231* elements from *Bacillus cereus sensu lato* are modular. Molecular Microbiology, 53, 457–467.
DeBuono BA, Brondum J, Kramer JM, Gilbert RJ and Opal SM, 1988. Plasmid, serotypic, and enterotoxin analysis of *Bacillus cereus* in an outbreak setting. Journal of Clinical Microbiology, 26, 1571–1574.
Delbrassinne L, Andjelkovic M, Rajkovic A, Bottledoorn N, Mahillon J and Van Loco J, 2011. Follow-up of the *Bacillus cereus* emetic toxin production in penne pasta under household conditions using liquid chromatography coupled with mass spectrometry. Food Microbiology, 28, 1105–1109.
Delbrassinne L, Botteldoorn N, Andjelkovic M, Dierick K and 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, 84–87.
Dierick K, Van Coillie E, Swiecicka I, Meyfroidt G, Devlieger H, Meulemans A, Hoedemaekers G, Fourie L, Heyndrickx M and Mahillon J, 2005. Fatal family outbreak of *Bacillus cereus*-associated food poisoning. Journal of Clinical Microbiology, 43, 4277–4279.
Doggett NA, Stubben CJ, Chertkov O, Bruce DC, Detter JC, Johnson SL and Han CS, 2013. Complete genome sequence of *Bacillus thuringiensis* serovar *israelensis* strain HD-789. Genome Announcements, 1(6).
Doll VM, Ehling-Schulz M and Vogelmann R, 2013. Concerted action of sphingomyelinase and non-hemolytic enterotoxin in pathogenic *Bacillus cereus*. PLoS ONE, 8, e61404.
Doyle ME, Mazzotta AS, Wang T, Wiseman DW and Scott VN, 2001. Heat resistance of *Listeria monocytogenes*. Journal of Food Protection, 64, 410–429.
Drobniewski FA, 1993. *Bacillus cereus* and related species. Clinical Microbiology Reviews, 6, 324–338.
Dzieciol M, Fricker M, Wagner M, Hein I and 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, 176–185.
EFSA (European Food Safety Authority), 2005. Opinion of the Scientific Panel on biological hazards (BIOHAZ) on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs. EFSA Journal 2005;3(4):175, 48 pp. doi:10.2903/j.efsa.2005.2175
EFSA (European Food Safety Authority), 2014. Update of the technical specifications for harmonised reporting of food-borne outbreaks through the European Union reporting system in accordance with Directive 2003/99/EC. EFSA Journal 2014;12(3):3598, 25 pp. doi:10.2903/j.efsa.2014.3598
EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014a. Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and Norovirus in berries). EFSA Journal 2014;12(6):3706, 95 pp. doi:10.2903/j.efsa.2014.3706
EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014b. Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and Norovirus in leafy greens eaten raw as salads). EFSA Journal 2014;12(3):3600, 118 pp. doi:10.2903/j.efsa.2014.3600

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014c. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and Norovirus in tomatoes). EFSA Journal 2014;12(10):3832, 75 pp. doi:10.2903/j.efsa.2014.3832

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014d. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* in melons). EFSA Journal 2014;12(10):3831, 77 pp. doi:10.2903/j.efsa.2014.3831

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014e. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella*, *Yersinia*, *Shigella* and Norovirus in bulb and stem vegetables, and carrots). EFSA Journal 2014;12(12):3937, 91 pp. doi:10.2903/j.efsa.2014.3937

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014. Scientific Opinion on the safety and efficacy of Toyocerin® (*Bacillus toyonensis*) as a feed additive for chickens for fattening, weaned piglets, pigs for fattening, sows for reproduction, cattle for fattening and calves for rearing and for rabbits. EFSA Journal 2014;12(7):3766, 17 pp. doi:10.2903/j.efsa.2014.3766

Ehling-Schulz M and Messelhausser U, 2013. *Bacillus* "next generation" diagnostics: moving from detection toward subtyping and risk-related strain profiling. Frontiers in Microbiology, 4, 32.

Ehling-Schulz M, Fricker M and Scherer S, 2004. *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. Molecular Nutrition and Food Research, 48, 479–487.

Ehling-Schulz M, Svensson B, Guinebretiere MH, Lindbäck T, Andersson M, Schulz A, Fricker M, Christiansson A, Granum PE, Märtlbauer E, Nguyen-Thé C, Salkinoja-Salonen M and Scherer S, 2005a. Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiology, 151, 183–197.

Ehling-Schulz M, Vukov N, Schulz A, Shaheen R, Andersson M, Märtlbauer E and Scherer S, 2005b. Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. Applied and Environmental Microbiology, 71, 105–113.

Ehling-Schulz M, Fricker M, Grallert H, Rieck P, Wagner M and Scherer S, 2006a. Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. BMC Microbiology, 6, 20.

Ehling-Schulz M, Guinebretiere MH, Monthan A, Berge O, Fricker M and Svensson B, 2006b. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiology Letters, 260, 232–240.

Ehling-Schulz M, Messelhäusser U and Granum PE, 2011. *Bacillus cereus* in milk and dairy production. In: Hoorfar J (ed.). *Rapid Detection, Characterization and Enumeration of Food-Borne Pathogens*. ASM Press, Washington, DC. pp. 275–289.

Ehling-Schulz M, Frenzel E and Gohar M, 2015. Food-bacteria interplay: pathometabolism of emetic *Bacillus cereus*. Frontiers in Microbiology, 6, 704.

English L and Slatin SL, 1992. Mode of action of delta-endotoxins from *Bacillus thuringiensis*: a comparison with other bacterial toxins. Insect Biochemistry and Molecular Biology, 22, 1–7.

Esteban MD, Conesa R, Huertas JP and Palop A, 2015. Effect of thymol in heating and recovery media on the isothermal and non-isothermal heat resistance of *Bacillus* spores. Food Microbiology, 48, 35–40.

Evelyn and Silva FVM, 2015. High pressure processing of milk: modeling the inactivation of psychrotrophic Bacillus cereus spores at 38–70°C. Journal of Food Engineering, 165, 141–148.

Fagerlund A, Ween O, Lund T, Hardy SP and Granum PE, 2004. Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. Microbiology, 150, 2689–2697.

Fagerlund A, Lindbäck T, Storset AK, Granum PE and Hardy SP, 2008. *Bacillus cereus* Nhe is a pore-forming toxin with structural and functional properties similar to the ClyA (HlyE, SheA) family of haemolysins, able to induce osmotic lysis in epithelia. Microbiology, 154, 693–704.

Fernández A, Ocio MJ, Fernández PS, Rodrigo M and Martínez A, 1999. Application of nonlinear regression analysis to the estimation of kinetic parameters for two enterotoxic strains of *Bacillus cereus* spores. Food Microbiology, 16, 607–613.

Fernández-No IC, Guarddon M, Böhme K, Cepeda A, Calo-Mata P and Barros-Velázquez J, 2011. Detection and quantification of spoilage and pathogenic *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis* by real-time PCR. Food Microbiology, 28, 605–610.

Finlay WJ, Logan NA and Sutherland AD, 1999. Semiautomated metabolic staining assay for *Bacillus cereus* emetic toxin. Applied and Environmental Microbiology, 65, 1811–1812.

Fischer HM, Luthy P and Schweitzer S, 1984. Introduction of plasmid pC194 into *Bacillus thuringiensis* by protoplast transformation and plasmid transfer. Archives of Microbiology, 139, 213–217.

Fletcher P and Logan NA, 1999. Improved cytotoxicity assay for *Bacillus cereus* diarrhoeal enterotoxin. Letters in Applied Microbiology, 28, 394–400.

Forghani F, Eskandari M and Oh D-H, 2015. Application of slightly acidic electrolyzed water and ultrasound for microbial decontamination of kashk. Food Science and Biotechnology, 24, 1011–1016.

Forminska K, Zasada AA and Jagielski M, 2012. Evaluation of multiplex PCR to identify the species of microorganisms from *Bacillus cereus* group. Medycyna Doświwiadczalna i Mikrobiologia, 64, 101–108.

van Frankenhuyzen K, 2000. Application of *Bacillus thuringiensis* in forestry. In: Charles J-F (ed.). *Entomopathogenic Bacteria: From Laboratory to Field Application*. Delécluse A and Roux CN-L, Springer, Netherlands, Dordrecht. pp. 371–382.

Frederiksen K, Rosenquist H, Jorgensen K and 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, 3435–3440.

Frenzel E, Letzel T, Scherer S and Ehling-Schulz M, 2011. Inhibition of cereulide toxin synthesis by emetic *Bacillus cereus* via long-chain polyphosphates. Applied and Environmental Microbiology, 77, 1475–1482.

Frenzel E, Doll V, Pauthner M, Lucking G, Scherer S and Ehling-Schulz M, 2012. CodY orchestrates the expression of virulence determinants in emetic *Bacillus cereus* by impacting key regulatory circuits. Molecular Microbiology, 85, 67–88.

Frenzel E, Kranzler M, Stark TD, Hofmann T and Ehling-Schulz M, 2015. The endospore-forming pathogen *Bacillus cereus* exploits a small colony variant-based diversification strategy in response to aminoglycoside exposure. MBio, 6, e01172–e01115.

Fricker M, Messelhäusser U, Busch U, Scherer S and Ehling-Schulz M, 2007. Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. Applied and Environmental Microbiology, 73, 1892–1898.

Fricker M, Reissbrodt R and 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, 27–34.

From C, Pukall R, Schumann P, Hormazábal V and Granum PE, 2005. Toxin-producing ability among *Bacillus* spp. outside the *Bacillus cereus* group. Applied and Environmental Microbiology, 71, 1178–1183.

From C, Hormazabal V and Granum PE, 2007. Food poisoning associated with pumilacidin-producing *Bacillus pumilus* in rice. International Journal of Food Microbiology, 115, 319–324.

FSANZ (Food Standards Australia New Zealand), 2006. A risk profile of dairy products in Australia. Available online: https://www.foodstandards.gov.au/code/proposals/documents/P296%20Dairy%20PPPS%20FAR%20Attach%202%20FINAL%20-%20mr.pdf

Galvez A, Abriouel H, Lopez RL and Ben Omar N, 2007. Bacteriocin-based strategies for food biopreservation. International Journal of Food Microbiology, 120, 51–70.

Gaviria Rivera AM and Priest FG, 2003. Molecular typing of *Bacillus thuringiensis* serovars by RAPD-PCR. Systematic and Applied Microbiology, 26, 254–261.

Gaviria Rivera AM, Granum PE and Priest FG, 2000. Common occurrence of enterotoxin genes and enterotoxicity in *Bacillus thuringiensis*. FEMS Microbiology Letters, 190, 151–155.

Gillis A and Mahillon J, 2014a. Phages preying on *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*: past, present and future. Viruses, 6, 2623–2672.

Gillis A and Mahillon J, 2014b. Prevalence, genetic diversity, and host range of tectiviruses among members of the *Bacillus cereus* group. Applied and Environmental Microbiology, 80, 4138–4152.

Glare TR and O'Callaghan M, 2000. *Bacillus thuringiensis*: Biology, Ecology and Safety. Wiley, Chichester, New York. 350 pp.

Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Kohl J, Marrone P, Morin L and Stewart A, 2012. Have biopesticides come of age? Trends in Biotechnology, 30, 250–258.

Glatz BA, Spira WM and Goepfert JM, 1974. Alteration of vascular permeability in rabbits by culture filtrates of *Bacillus cereus* and related species. Infection and Immunity, 10, 299–303.

Gohar M, Gilois N, Graveline R, Garreau C, Sanchis V and Lereclus D, 2005. A comparative study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. Proteomics, 5, 3696–3711.

Gohar M, Faegri K, Perchat S, Ravnum S, Økstad OA, Gominet M, Kolstø AB and Lereclus D, 2008. The PlcR virulence regulon of *Bacillus cereus*. PLoS ONE, 3, e2793.

Gómez-López VM, Marín A, Medina-Martínez MS, Gil MI and Allende A, 2013. Generation of trihalomethanes with chlorine-based sanitizers and impact on microbial, nutritional and sensory quality of baby spinach. Postharvest Biology and Technology, 85, 210–217.

González JM Jr and Carlton BC, 1980. Patterns of plasmid DNA in crystalliferous and acrystalliferous strains of *Bacillus thuringiensis*. Plasmid, 3, 92–98.

Gordon RE, Haynes WC and Pang CH-N, 1973. The genus *Bacillus*. Agricultural Research Service, United States Department of Agriculture, Washington, DC.

Granum PE and Lund T, 1997. *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Letters, 157, 223–228.

Granum PE, Brynestad S and Kramer JM, 1993. Analysis of enterotoxin production by *Bacillus cereus* from dairy products, food poisoning incidents and non-gastrointestinal infections. International Journal of Food Microbiology, 17, 269–279.

Griego VM and Spence KD, 1978. Inactivation of *Bacillus thuringiensis* spores by ultraviolet and visible light. Applied and Environmental Microbiology, 35, 906–910.

Groot MN, Nieboer F and Abee T, 2008. Enhanced transformation efficiency of recalcitrant *Bacillus cereus* and *Bacillus weihenstephanensis* isolates upon *in vitro* methylation of plasmid DNA. Applied and Environmental Microbiology, 74, 7817–7820.

Guillemet E, Cadot C, Tran SL, Guinebretière MH, Lereclus D and Ramarao N, 2010. The InhA metalloproteases of *Bacillus cereus* contribute concomitantly to virulence. Journal of Bacteriology, 192, 286–294.

Guinebretière MH and Nguyen-Thé C, 2003. Sources of *Bacillus cereus* contamination in a pasteurized zucchini puree processing line, differentiated by two PCR-based methods. FEMS Microbiology Ecology, 43, 207–215.

Guinebretière MH, Broussolle V and Nguyen-Thé C, 2002. Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. Journal of Clinical Microbiology, 40, 3053–3056.

Guinebretière MH, Thompson FL, Sorokin A, Normand P, Dawyndt P, Ehling-Schulz M, Svensson B, Sanchis V, Nguyen-Thé C, Heyndrickx M and De Vos P, 2008. Ecological diversification in the *Bacillus cereus* Group. Environmental Microbiology, 10, 851–865.

Guinebretière MH, Velge P, Couvert O, Carlin F, Debuyser ML and Nguyen-Thé 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, 3388–3391.

Guinebretière MH, Auger S, Galleron N, Contzen M, De Sarrau B, De Buyser ML, Lamberet G, Fagerlund A, Granum PE, Lereclus D, De Vos P, Nguyen-Thé C and 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.

Haberbeck LU, da Silva Alberto, Riehl C, de Cássia Martins Salomão B and Falcão de Aragão GM, 2012. *Bacillus coagulans* spore inactivation through the application of oregano essential oil and heat. LWT – Food Science and Technology, 46, 267–273.

Häggblom MM, Apetroaie C, Andersson MA and Salkinoja-Salonen MS, 2002. Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus*, produced under various conditions. Applied and Environmental Microbiology, 68, 2479–2483.

Hansen BM and Hendriksen NB, 2001. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Applied and Environmental Microbiology, 67, 185–189.

Hansen BM, Damgaard PH, Eilenberg J and Pedersen JC, 1996. *Bacillus thuringiensis*. Ecology and Environmental Effects if its Use for Microbial Pest Control. Miljøprojekt 316. Miljøstyrelsen, Denmark.

Hansen BM, Thorsen L, Nielsen-LeRoux C, Wilcks A and Hendriksen NB, 2011. *New Experimental Approaches for Human Risk Assessment of Microbial Pest Control Agents – Exemplified by the Bacterium Bacillus thuringiensis*. Bekæmpelsesmiddel forskning fra Miljøstyrelsen 136. Miljøstyrelsen, Denmark.

Haug TM, Sand SL, Sand O, Phung D, Granum PE and Hardy SP, 2010. Formation of very large conductance channels by *Bacillus cereus* Nhe in Vero and GH(4) cells identifies NheA + B as the inherent pore-forming structure. The Journal of Membrane Biology, 237, 1–11.

Helgason E, Caugant DA, Lecadet MM, Chen Y, Mahillon J, Lovgren A, Hegna I, Kvaløy K and Kolstø AB, 1998. Genetic diversity of *Bacillus cereus/B. thuringiensis* isolates from natural sources. Current Microbiology, 37, 80–87.

Helgason E, Caugant DA, Olsen I and Kolstø AB, 2000a. Genetic structure of population of *Bacillus cereus* and *B. thuringiensis* isolates associated with periodontitis and other human infections. Journal of Clinical Microbiology, 38, 1615–1622.

Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I and Kolstø AB, 2000b. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*-one species on the basis of genetic evidence. Applied and Environmental Microbiology, 66, 2627–2630.

Helgason E, Tourasse NJ, Meisal R, Caugant DA and Kolstø AB, 2004. Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. Applied and Environmental Microbiology, 70, 191–201.

Hendriksen NB, 2011. Report of the second biopesticide steering group seminar on the fate in the environment of microbial control agents and their effects on non-target organisms. OECD Environment Health and Safety publications, Series on pesticides, No 64.

Hendriksen NB and Carstensen J, 2013. Long-term survival of *Bacillus thuringiensis* subsp. *kurstaki* in a field trial. Canadian Journal of Microbiology, 59, 34–38.

Hendriksen NB and Hansen BM, 2002. Long-term survival and germination of *Bacillus thuringiensis* var. *kurstaki* in a field trial. Canadian Journal of Microbiology, 48, 256–261.

Hendriksen NB and Hansen BM, 2006. Detection of *Bacillus thuringiensis kurstaki* HD1 on cabbage for human consumption. FEMS Microbiology Letters, 257, 106–111.

Hendriksen NB, Hansen BM and Johansen JE, 2006. Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam. Antonie van Leeuwenhoek, 89, 239–249.

Hill KK, Ticknor LO, Okinaka RT, Asay M, Blair H, Bliss KA, Laker M, Pardington PE, Richardson AP, Tonks M, Beecher DJ, Kemp JD, Kolstø AB, Wong AC, Keim P and Jackson PJ, 2004. Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis* isolates. Applied and Environmental Microbiology, 70, 1068–1080.

Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, Marston CK, De BK, Sacchi CT, Fitzgerald C, Mayer LW, Maiden MC, Priest FG, Barker M, Jiang L, Cer RZ, Rilstone J, Peterson SN, Weyant RS, Galloway DR, Read TD, Popovic T and Fraser CM, 2004. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proceedings of the National Academy of Sciences of the United States of America, 101, 8449–8454.

Hofmann C, Luthy P, Hutter R and Pliska V, 1988a. Binding of the delta endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). European Journal of Biochemistry/ FEBS, 173, 85–91.

Hofmann C, Vanderbruggen H, Hofte H, Van Rie J, Jansens S and Van Mellaert H, 1988b. Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proceedings of the National Academy of Sciences of the United States of America, 85, 7844–7848.

Holbrook R and Anderson JM, 1980. An improved selective and diagnostic medium for the isolation and enumeration of *Bacillus cereus* in foods. Canadian Journal of Microbiology, 26, 753–759.

Hoornstra D, Andersson MA, Teplova VV, Mikkola R, Uotila LM, Andersson LC, Roivainen M, Gahmberg CG and Salkinoja-Salonen MS, 2013. Potato crop as a source of emetic *Bacillus cereus* and cereulide-induced mammalian cell toxicity. Applied and Environmental Microbiology, 79, 3534–3543.

Hoover DG, 1993. Pressure effects on biological systems. Food Technology, 47, 150–155.

Horne T, Turner GC and Willis AT, 1959. Inactivation of spores of *Bacillus anthracis* by gamma-radiation. Nature, 183, 475–476.

Hostacká A, Kosiarová A, Majtán V and Kohútová S, 1992. Toxic properties of *Bacillus cereus* strains isolated from different foodstuffs. Zentralblatt für Bakteriologie: International Journal of Medical Microbiology, 276, 303–312.

Hoton FM, Andrup L, Swiecicka I and Mahillon J, 2005. The cereulide genetic determinants of emetic *Bacillus cereus* are plasmid-borne. Microbiology, 151, 2121–2124.

Hoton FM, Fornelos N, N'Guessan E, Hu X, Swiecicka I, Dierick K, Jääskeläinen E, Salkinoja-Salonen M and Mahillon J, 2009. Family portrait of *Bacillus cereus* and *Bacillus weihenstephanensis* cereulide-producing strains. Environmental Microbiology Reports, 1, 177–183.

ICMSF (International Commission on Microbiological Specifications of Foods), 1996. *Microorganisms in Foods. 5: Characteristics of Microbial Pathogens*. Kluwer Academic/Plenum Publishers. London, UK. pp. 20–35.

Ikeda M, Yagihara Y, Tatsuno K, Okazaki M, Okugawa S and Moriya K, 2015. Clinical characteristics and antimicrobial susceptibility of *Bacillus cereus* blood stream infections. Annals of Clinical Microbiology and Antimicrobials, 14, 43.

ISO (International Organization for Standardization), 2004. ISO 7932:2004 Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of presumptive *Bacillus cereus* – Colony-count technique at 30 degrees C. Last reviewed in 2015. Abstract Available online: http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=38219

ISO (International Organization for Standardization), 2006. ISO 21871:2006 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. Last reviewed in 2010. Abstract Available online: http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=36015

ISO (International Organization for Standardization), online. ISO/DIS 18465 Microbiology of the food chain - Quantitative analysis of emetic toxin (cereulide) using LC-MS/MS. Under development. Info Available online: http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=62512

Jackson SG, Goodbrand RB, Ahmed R and Kasatiya S, 1995. *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. Letters in Applied Microbiology, 21, 103–105.

James C, 2013. *Global Status of Commercialized Biotech/GM Crops: 2013*. International Service for the Acquisition of Agri-biotech Applications (ISAAA), Ithaca, NY. Available online: http://www.isaaa.org

Jensen GB, Wilcks A, Petersen SS, Damgaard J, Baum JA and Andrup L, 1995. The genetic basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large conjugative plasmid pXO16. Journal of Bacteriology, 177, 2914–2917.

Jensen GB, Hansen BM, Eilenberg J and Mahillon J, 2003. The hidden lifestyles of *Bacillus cereus* and relatives. Environmental Microbiology, 5, 631–640.

Jessberger N, Dietrich R, Bock S, Didier A and 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 VM, Rademacher C, Bohm ME, Mohr AK, Ehling-Schulz M, Scherer S and Martlbauer E, 2015. From genome to toxicity: a combinatory approach highlights the complexity of enterotoxin production in *Bacillus cereus*. Frontiers in Microbiology, 6, 560.

Jimènez G, Urdiain M, Cifuentes A, Lopez-Lopez A, Blanch AR, Tamames J, Kampfer P, Kolsto AB, Ramon D, Martinez JF, Codoner FM and 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, 383–391.

Johler S, Stephan R, Althaus D, Ehling-Schulz M and Grunert T, 2016. High-resolution subtyping of *Staphylococcus aureus* strains by means of Fourier-transform infrared spectroscopy. Systematic and Applied Microbiology, 39, 189–194.

Juneja VK, 2003. *Sous-vide* processed foods: safety hazards and control of microbial risks. In: Novak JS, Sapers GM, Juneja VK (ed.). *Microbial Safety of Minimally Processed Foods*. CRC Press Inc., Boca Raton, USA. pp. 97–124.

Kamar R, Gohar M, Jehanno I, Rejasse A, Kallassy M, Lereclus D, Sanchis V and Ramarao N, 2013. Pathogenic potential of *Bacillus cereus* strains as revealed by phenotypic analysis. Journal of Clinical Microbiology, 51, 320–323.

Kellogg CA and Griffin DW, 2006. Aerobiology and the global transport of desert dust. Trends in Ecology and Evolution, 21, 638–644.

Kevany BM, Rasko DA and Thomas MG, 2009. Characterization of the complete zwittermicin a biosynthesis gene cluster from *Bacillus cereus*. Applied and Environmental Microbiology, 75, 1144–1155.

Kim HU and Goepfert JM, 1971. Enumeration and identification of *Bacillus cereus* in foods. I. 24-hour presumptive test medium. Applied Microbiology, 22, 581–587.

Klee SR, Ozel M, Appel B, Boesch C, Ellerbrok H, Jacob D, Holland G, Leendertz FH, Pauli G, Grunow R and Nattermann H, 2006. Characterization of Bacillus anthracis-like bacteria isolated from wild great apes from Cote d'Ivoire and Cameroon. Journal of Bacteriology, 188, 5333–5344.

Klee SR, Brzuszkiewicz EB, Nattermann H, Bruggemann H, Dupke S, Wollherr A, Franz T, Pauli G, Appel B, Liebl W, Couacy-Hymann E, Boesch C, Meyer FD, Leendertz FH, Ellerbrok H, Gottschalk G, Grunow R and Liesegang H, 2010. The genome of a Bacillus isolate causing anthrax in chimpanzees combines chromosomal properties of *B. cereus* with *B. anthracis* virulence plasmids. PLoS ONE, 5, e10986.

Knight PJ, Crickmore N and Ellar DJ, 1994. The receptor for *Bacillus thuringiensis* CrylA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. Molecular Microbiology, 11, 429–436.

Knorr D, 1995. Hydrostatic pressure treatment of food: microbiology. In: Gould GW (ed.). *New Methods of Food Preservation*. Springer US, Boston, MA. pp. 159–175.

Ko KS, Kim JW, Kim JM, Kim W, Chung SI, Kim IJ and Kook YH, 2004. Population structure of the *Bacillus cereus* group as determined by sequence analysis of six housekeeping genes and the *plcR* Gene. Infection and Immunity, 72, 5253–5261.

Koch MS, Ward JM, Levine SL, Baum JA, Vicini JL and Hammond BG, 2015. The food and environmental safety of Bt crops. Frontiers in Plant Science, 6, 283.

Kolstø AB, Tourasse NJ and Økstad OA, 2009. What sets *Bacillus anthracis* apart from other *Bacillus* species? Annual Review of Microbiology, 63, 451–476.

Kramer JM and Gilbert RJ, 1989. *Bacillus cereus* and other *Bacillus* species. In: Doyle MP (ed.). *Foodborne Bacterial Pathogens*. Marcel Dekker Inc, New York, USA. pp. 21–70.

Krause N, Moravek M, Dietrich R, Wehrle E, Slaghuis J and Märtlbauer E, 2010. Performance characteristics of the Duopath(R) cereus enterotoxins assay for rapid detection of enterotoxigenic *Bacillus cereus* strains. International Journal of Food Microbiology, 144, 322–326.

Kuroki R, Kawakami K, Qin L, Kaji C, Watanabe K, Kimura Y, Ishiguro C, Tanimura S, Tsuchiya Y, Hamaguchi I, Sakakura M, Sakabe S, Tsuji K, Inoue M and Watanabe H, 2009. Nosocomial bacteremia caused by biofilm-forming *Bacillus cereus* and *Bacillus thuringiensis*. Internal medicine, 48, 791–796.

La Duc MT, Satomi M, Agata N and Venkateswaran K, 2004. gyrB as a phylogenetic discriminator for members of the *Bacillus anthracis-cereus-thuringiensis* group. Journal of Microbiological Methods, 56, 383–394.

Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M and Goettel MS, 2015. Insect pathogens as biological control agents: back to the future. Journal of Invertebrate Pathology, 132, 1–41.

Lecadet MM and Martouret D, 1967. Enzymatic hydrolysis of the crystals of *Bacillus thuringiensis* by the proteases of *Pieris brassicae* II. Toxicity of the different fractions of the hydrolysate for larvae of *Pieris brassicae*. Journal of Invertebrate Pathology, 9, 322–330.

Lecadet MM, Blondel MO and Ribier J, 1980. Generalized transduction in *Bacillus thuringiensis* var. *berliner* 1715 using bacteriophage CP-54Ber. Journal of General Microbiology, 121, 203–212.

Lecadet MM, Frachon E, Dumanoir VC, Ripouteau H, Hamon S, Laurent P and Thiéry I, 1999. Updating the H-antigen classification of *Bacillus thuringiensis*. Journal of Applied Microbiology, 86, 660–672.

Lechner S, Mayr R, Francis KP, Pruss BM, Kaplan T, Wiessner-Gunkel E, Stewart GS and Scherer S, 1998. *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. International Journal of Systematic Bacteriology, 48 (Pt 4), 1373–1382.

Lekogo BM, Coroller L, Mathot AG, Mafart P and Leguerinel I, 2010. Modelling the influence of palmitic, palmitoleic, stearic and oleic acids on apparent heat resistance of spores of *Bacillus cereus* NTCC 11145 and *Clostridium sporogenes* Pasteur 79.3. International Journal of Food Microbiology, 141, 242–247.

Léonard C, Chen Y and Mahillon J, 1997. Diversity and differential distribution of IS231, IS232 and IS240 among *Bacillus cereus,* Bacillus thuringiensis and Bacillus mycoides. Microbiology, 143(Pt 8), 2537–2547.

Li MS, Roh JY, Tao X, Yu ZN, Liu ZD, Liu Q, Xu HG, Shim HJ, Kim YS, Wang Y, Choi JY and Je YH, 2009. Cloning and molecular characterization of a novel rolling-circle replicating plasmid, p K1S–1, from Bacillus thuringiensis subsp. kurstaki K1. Journal of Microbiology, 47, 466–472.

Li Q, Zou T, Ai P, Pan L, Fu C, Li P and Zheng A, 2015. Complete genome sequence of *Bacillus thuringiensis* HS18-1. Journal of Biotechnology, 214, 61–62.

Lindbäck T, Hardy SP, Dietrich R, Sødring M, Didier A, Moravek M, Fagerlund A, Bock S, Nielsen C, Casteel M, Granum PE and Märtlbauer E, 2010. Cytotoxicity of the *Bacillus cereus* Nhe enterotoxin requires specific binding order of its three exoprotein components. Infection and Immunity, 78, 3813–3821.

Liu X, Peng D, Luo Y, Ruan L, Yu Z and Sun M, 2009. Construction of an *Escherichia coli* to *Bacillus thuringiensis* shuttle vector for large DNA fragments. Applied Microbiology and Biotechnology, 82, 765–772.

Liu G, Song L, Shu C, Wang P, Deng C, Peng Q, Lereclus D, Wang X, Huang D, Zhang J and Song F, 2013. Complete genome sequence of *Bacillus thuringiensis* subsp. *kurstaki* strain HD73. Genome Announcements, 1, e0008013.

Liu Y, Lai Q, Göker M, Meier-Kolthoff JP, Wang M, Sun Y, Wang L and Shao Z, 2015. Genomic insights into the taxonomic status of the *Bacillus cereus* group. Scientific Reports, 5, 14082.

Logan NA, 2012. *Bacillus* and relatives in foodborne illness. Journal of Applied Microbiology, 112, 417–429.

Logan NA, Hoffmaster A, Shadomy SV and Stauffer K, 2011. *Bacillus* and related genera. In: Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW (ed.). *Manual of Clinical Microbiology*. 10th, American Society for Microbiology, Washington, DC. pp. 381–402.

Lücking G, Frenzel E, Rütschle A, Marxen S, Stark TD, Hofmann T, Scherer S and Ehling-Schulz M, 2015. Ces locus embedded proteins control the non-ribosomal synthesis of the cereulide toxin in emetic *Bacillus cereus* on multiple levels. Frontiers in Microbiology, 6, 1101.

Luna VA, King DS, Gulledge J, Cannons AC, Amuso PT and Cattani J, 2007. Susceptibility of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre automated microbroth dilution and Etest agar gradient diffusion methods. The Journal of Antimicrobial Chemotherapy, 60, 555–567.

Lund T and Granum PE, 1996. Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. FEMS Microbiology Letters, 141, 151–156.

Lund T and Granum PE, 1997. Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. Microbiology, 143(Pt 10), 3329–3336.

Lund T, De Buyser ML and Granum PE, 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Molecular Microbiology, 38, 254–261.

Luu-Thi H, Corthouts J, Passaris I, Grauwet T, Aertsen A, Hendrickx M and Michiels CW, 2015. Carvacrol suppresses high pressure high temperature inactivation of *Bacillus cereus* spores. International Journal of Food Microbiology, 197, 45–52.

Madsen AM, Hansen VM, Meyling NV, Hendriksen NB, Winding A, Kock KT and Eilenberg J 2011. Human eksponering for mikrobiologiske bekæmpelsesmidler, deres naturligt forekommende slægtninge og andre mikroorganismer Bekæmpelsesmiddelforskning fra Miljøstyrelsen Nr. 132, 93 pp.

Maduell P, Armengol G, Llagostera M, Orduz S and Lindow S, 2008. *B. thuringiensis* is a poor colonist of leaf surfaces. Microbial Ecology, 55, 212–219.

Mahillon J and Lereclus D, 1988. Structural and functional analysis of Tn4430: identification of an integrase-like protein involved in the co-integrate-resolution process. EMBO Journal, 7, 1515–1526.

Mahler H, Pasi A, Kramer JM, Schulte P, Scoging AC, Bar W and Krahenbuhl S, 1997. Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. The New England Journal of Medicine, 336, 1142–1148.

Makart L, Gillis A and Mahillon J, 2015. pXO16 from *Bacillus thuringiensis* serovar *israelensis*: almost 350 kb of *terra incognita*. Plasmid, 80, 8–15.

Mandic-Mulec I, Stefanic P and van Elsas JD, 2015. Ecology of *Bacillaceae*. Microbiology Spectrum, 3, TBS-0017-2013.

Margulis L, Jorgensen JZ, Dolan S, Kolchinsky R, Rainey FA and Lo SC, 1998. The Arthromitus stage of *Bacillus cereus*: intestinal symbionts of animals. Proceedings of the National Academy of Sciences of the United States of America, 95, 1236–1241.

Marrone PG, 1994. Present and future use of *Bacillus thuringiensis* in integrated pest management systems: an industrial perspective. Biocontrol Science and Technology, 4, 517–526.

Martínez-Blanch JF, Sánchez G, Garay E and 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, 1480–1485.

Marxen S, Stark TD, Frenzel E, Rutschle A, Lucking G, Purstinger G, Pohl EE, Scherer S, Ehling-Schulz M and Hofmann T, 2015a. Chemodiversity of cereulide, the emetic toxin of *Bacillus cereus*. Analytical and Bioanalytical Chemistry, 407, 2439–2453.

Marxen S, Stark TD, Rutschle A, Lucking G, Frenzel E, Scherer S, Ehling-Schulz M and Hofmann T, 2015b. Multiparametric quantitation of the *Bacillus cereus* toxins cereulide and isocereulides A-G in foods. Journal of Agricultural and Food Chemistry, 63, 8307–8313.

Maughan H and Van der Auwera G, 2011. *Bacillus* taxonomy in the genomic era finds phenotypes to be essential though often misleading. Infection, Genetics and Evolution, 11, 789–797.

McIntyre L, Bernard K, Beniac D, Isaac-Renton JL and Naseby DC, 2008. Identification of *Bacillus cereus* group species associated with food poisoning outbreaks in British Columbia, Canada. Applied and Environmental Microbiology, 74, 7451–7453.

Mei X, Xu K, Yang L, Yuan Z, Mahillon J and Hu X, 2014. The genetic diversity of cereulide biosynthesis gene cluster indicates a composite transposon Tnces in emetic *Bacillus weihenstephanensis*. BMC Microbiology, 14, 149.

van Melis CC, Nierop Groot MN and Abee T, 2011. Impact of sorbic acid on germinant receptor-dependent and -independent germination pathways in *Bacillus cereus*. Applied and Environmental Microbiology, 77, 2552–2554.

van Melis CC, Almeida CB, Kort R, Groot MN and Abee T, 2012. Germination inhibition of *Bacillus cereus* spores: impact of the lipophilic character of inhibiting compounds. International Journal of Food Microbiology, 160, 124–130.

Melnick RL, Testen AL, Poleatewich AM, Backman PA and Bailey BA, 2012. Detection and expression of enterotoxin genes in endophytic strains of *Bacillus cereus*. Letters in Applied Microbiology, 54, 468–474.

Mesrati LA, Karray MD, Tounsi S and Jaoua S, 2005. Construction of a new high-copy number shuttle vector of *Bacillus thuringiensis*. Letters in Applied Microbiology, 41, 361–366.

Messelhäusser U, Frenzel E, Blöchinger C, Zucker R, Kämpf P and 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, 2014, 465603.

Mironczuk AM, Kovács ÁT and Kuipers OP, 2008. Induction of natural competence in *Bacillus cereus* ATCC14579. Microbial Biotechnology, 1, 226–235.

Moayeri M, Leppla SH, Vrentas C, Pomerantsev AP and Liu S, 2015. Anthrax Pathogenesis. Annual Review of Microbiology, 69, 185–208.

Modrie P, Beuls E and Mahillon J, 2010. Differential transfer dynamics of pAW63 plasmid among members of the *Bacillus cereus* group in food microcosms. Journal of Applied Microbiology, 108, 888–897.

Moerman F, 2005. High hydrostatic pressure inactivation of vegetative microorganisms, aerobic and anaerobic spores in pork Marengo, a low acidic particulate food product. Meat Science, 69, 225–232.

Moerman F, Mertens B, Demey L and Huyghebaert A, 2001. Reduction of *Bacillus subtilis*, *Bacillus stearothermophilus* and *Streptococcus faecalis* in meat batters by temperature-high hydrostatic pressure pasteurization. Meat Science, 59, 115–125.

Moher D, Liberati A, Tetzlaff J, Altman DG and Group P, 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Medicine, 6, e1000097.

Morente EO, Abriouel H, Lopez RL, Ben Omar N and Galvez A, 2010. Antibacterial activity of carvacrol and 2-nitro-1-propanol against single and mixed populations of foodborne pathogenic bacteria in corn flour dough. Food Microbiology, 27, 274–279.

Mossel DA, Koopman MJ and Jongerius E, 1967. Enumeration of *Bacillus cereus* in foods. Applied Microbiology, 15, 650–653.

Nakamura LK, 1998. *Bacillus pseudomycoides* sp. nov. International Journal of Systematic Bacteriology, 48 Pt 3, 1031–1035.

Nakamura LK and Jackson MA, 1995. Clarification of the taxonomy of *Bacillus mycoides*. International Journal of Systematic and Evolutionary Microbiology, 45, 46–49.

Nam H, Seo H-S, Bang J, Kim H, Beuchat LR and Ryu J-H, 2014. Efficacy of gaseous chlorine dioxide in inactivating *Bacillus cereus* spores attached to and in a biofilm on stainless steel. International Journal of Food Microbiology, 188, 122–127.

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

Naumann D, Helm D and Labischinski H, 1991. Microbiological characterizations by FT-IR spectroscopy. Nature, 351, 81–82.

van Netten P, van De Moosdijk A, van Hoensel P, Mossel DA and Perales I, 1990. Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. The Journal of Applied Bacteriology, 69, 73–79.

Nicholson WL, Munakata N, Horneck G, Melosh HJ and Setlow P, 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiology and Molecular Biology Reviews, 64, 548–572.

Noguera PA and Ibarra JE, 2010. Detection of new cry genes of *Bacillus thuringiensis* by use of a novel PCR primer system. Applied and Environmental Microbiology, 76, 6150–6155.

Notermans S and Batt CA, 1998. A risk assessment approach for food-borne Bacillus cereus and its toxins. Symposium Series Society for Applied Microbiology, 27, 51S–61S.

Oda M, Takahashi M, Matsuno T, Uoo K, Nagahama M and Sakurai J, 2010. Hemolysis induced by *Bacillus cereus* sphingomyelinase. Biochimica et Biophysica Acta, 1798, 1073–1080.

Oda M, Hashimoto M, Takahashi M, Ohmae Y, Seike S, Kato R, Fujita A, Tsuge H, Nagahama M, Ochi S, Sasahara T, Hayashi S, Hirai Y and Sakurai J, 2012. Role of sphingomyelinase in infectious diseases caused by *Bacillus cereus*. PLoS ONE, 7, e38054.

Oliwa-Stasiak K, Molnar CI, Arshak K, Bartoszcze M and Adley CC, 2010. Development of a PCR assay for identification of the *Bacillus cereus* group species. Journal of Applied Microbiology, 108, 266–273.

Ombui JN, Mathenge JM, Kimotho AM, Macharia JK and Nduhiu G, 1996. Frequency of antimicrobial resistance and plasmid profiles of *Bacillus cereus* strains isolated from milk. East African Medical Journal, 73, 380–384.

Oren A and Garrity GM, 2014. List of new names and new combinations previously effectively, but not validly, published. International Journal of Systematic and Evolutionary Microbiology, 64, 1–5.

Palhano FL, Vilches TTB, Santos RB, Orlando MTD, Ventura JA and Fernandes PMB, 2004. Inactivation of *Colletotrichum gloeosporioides* spores by high hydrostatic pressure combined with citral or lemongrass essential oil. International Journal of Food Microbiology, 95, 61–66.

Palva A, Vigren G, Simonen M, Rintala H and Laamanen P, 1990. Nucleotide sequence of the tetracycline resistance gene of pBC16 from *Bacillus cereus*. Nucleic Acids Research, 18, 1635.

Park SH, Kim HJ, Kim JH, Kim TW and Kim HY, 2007. Simultaneous detection and identification of *Bacillus cereus* group bacteria using multiplex PCR. Journal of Microbiology and Biotechnology, 17, 1177–1182.

Pedersen JC, Hansen BM, Damgaard PH and Eilenberg J, 1995. Dispersal of *Bacillus thuringiensis* var. *kurstaki* in an experimental cabbage field. Canadian Journal of Microbiology, 41, 118–125.

Petras SF and Casida LE, 1985. Survival of *Bacillus thuringiensis* spores in soil. Applied and Environmental Microbiology, 50, 1496–1501.
Pigott CR and Ellar DJ, 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiology and Molecular Biology Reviews, 71, 255–281.
Pomerantsev AP, Camp A and Leppla SH, 2009. A new minimal replicon of *Bacillus anthracis* plasmid pXO1. Journal of Bacteriology, 191, 5134–5146.
Portnoy BL, Goepfert JM and Harmon SM, 1976. An outbreak of *Bacillus cereus* food poisoning resulting from contaminated vegetable sprouts. American Journal of Epidemiology, 103, 589–594.
Prabhakar A and Bishop AH, 2011. Invertebrate pathogenicity and toxin-producing potential of strains of *Bacillus thuringiensis* endemic to Antarctica. Journal of Invertebrate Pathology, 107, 132–138.
Priest FG, Barker M, Baillie LW, Holmes EC and Maiden MC, 2004. Population structure and evolution of the *Bacillus cereus* group. Journal of Bacteriology, 186, 7959–7970.
Prüss BM, Dietrich R, Nibler B, Märtlbauer E and Scherer S, 1999. The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. Applied and Environmental Microbiology, 65, 5436–5442.
Rahmati T and Labbe R, 2008. Levels and toxigenicity of *Bacillus cereus* and *Clostridium perfringens* from retail seafood. Journal of Food Protection, 71, 1178–1185.
Rajkovic A, Uyttendaele M and Debevere J, 2007. Computer aided boar semen motility analysis for cereulide detection in different food matrices. International Journal of Food Microbiology, 114, 92–99.
Rajkovic A, Uyttendaele M, Vermeulen A, Andjelkovic M, Fitz-James I, In 't Veld P, Denon Q, Verhe R and Debevere J, 2008. Heat resistance of *Bacillus cereus* emetic toxin, cereulide. Letters in Applied Microbiology, 46, 536–541.
Ramisse V, Patra G, Garrigue H, Guesdon JL and Mock M, 1996. Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. FEMS Microbiology Letters, 145, 9–16.
Rasimus S, Mikkola R, Andersson MA, Teplova VV, Venediktova N, Ek-Kommonen C and Salkinoja-Salonen M, 2012. Psychrotolerant *Paenibacillus tundrae* isolates from barley grains produce new cereulide-like depsipeptides (paenilide and homopaenilide) that are highly toxic to mammalian cells. Applied and Environmental Microbiology, 78, 3732–3743.
Rasko DA, Rosovitz MJ, Økstad OA, Fouts DE, Jiang L, Cer RZ, Kolstø AB, Gill SR and Ravel J, 2007. Complete sequence analysis of novel plasmids from emetic and periodontal *Bacillus cereus* isolates reveals a common evolutionary history among the *B. cereus*-group plasmids, including *Bacillus anthracis* pXO1. Journal of Bacteriology, 189, 52–64.
Rather MA, Aulakh RS, Gill JP, Mir AQ and Hassan MN, 2012. Detection and sequencing of plasmid encoded tetracycline resistance determinants (tetA and tetB) from food-borne *Bacillus cereus* isolates. Asian Pacific Journal of Tropical Medicine, 5, 709–712.
Raymond B, Wyres KL, Sheppard SK, Ellis RJ and Bonsall MB, 2010. Environmental factors determining the epidemiology and population genetic structure of the *Bacillus cereus* group in the field. PLoS Pathogens, 6, e1000905.
Reineke K, Schlumbach K, Baier D, Mathys A and Knorr D, 2013. The release of dipicolinic acid–the rate-limiting step of *Bacillus endospore* inactivation during the high pressure thermal sterilization process. International Journal of Food Microbiology, 162, 55–63.
Reiter L, Kolstø AB and Piehler AP, 2011. Reference genes for quantitative, reverse-transcription PCR in *Bacillus cereus* group strains throughout the bacterial life cycle. Journal of Microbiological Methods, 86, 210–217.
Reyes JE, Bastias JM, Gutierrez MR and Rodriguez Mde L, 2007. Prevalence of *Bacillus cereus* in dried milk products used by Chilean school feeding program. Food Microbiology, 24, 1–6.
Reyes-Ramírez A and Ibarra JE, 2008. Plasmid patterns of *Bacillus thuringiensis* type strains. Applied and Environmental Microbiology, 74, 125–129.
Richards AG and Richards PA, 1977. The peritrophic membranes of insects. Annual Review of Entomology, 22, 219–240.
Rosenquist H, Smidt L, Andersen SR, Jensen GB and Wilcks A, 2005. Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. FEMS Microbiology Letters, 250, 129–136.
Russell AD (ed.), 1982. *Inactivation of Bacterial Spores by Hydrostatic Pressure*. Academic Press, London and New York. 259 pp.
Russell NJ and Gould GW, 2003. Major preservation technologies. In: Russell NJ and Gould GW (eds.). *Food Preservatives*. Springer, New York, USA. pp. 14–24.
Russell AD, Hugo WB and Ayliffe GAJ, 1999. *Principles and Practice of Disinfection, Preservation and Sterilization*. 3rd Edition. In: Russell AD (ed.). Blackwell Science Ltd., Malden, MA.
Rusul G and Yaacob NH, 1995. Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. International Journal of Food Microbiology, 25, 131–139.
Saile E and Koehler TM, 2006. *Bacillus anthracis* multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. Applied and Environmental Microbiology, 72, 3168–3174.
Sale AJ, Gould GW and Hamilton WA, 1970. Inactivation of bacterial spores by hydrostatic pressure. Journal of General Microbiology, 60, 323–334.

Saleh SM, Harris RF and Allen ON, 1970a. Fate of *Bacillus thuringiensis* in soil: effect of soil pH and organic amendment. Canadian Journal of Microbiology, 16, 677–680.
Saleh SM, Harris RF and Allen ON, 1970b. Recovery of *Bacillus thuringiensis* var. *thuringiensis* from field soils. Journal of Invertebrate Pathology, 15, 55–59.
Saleh M, Al Nakib M, Doloy A, Jacqmin S, Ghiglione S, Verroust N, Poyart C and Ozier Y, 2012. *Bacillus cereus*, an unusual cause of fulminant liver failure: diagnosis may prevent liver transplantation. Journal of Medical Microbiology, 61, 743–745.
Salkinoja-Salonen MS, Vuorio R, Andersson MA, Kampfer P, Andersson MC, Honkanen-Buzalski T and Scoging AC, 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Applied and Environmental Microbiology, 65, 4637–4645.
Sanchis V, 2011. From microbial sprays to insect-resistant transgenic plants: history of the biopesticide *Bacillus thuringiensis*. A review. Agronomy for Sustainable Development, 31, 217–231.
Sanchis V and Bourguet D, 2008. *Bacillus thuringiensis*: applications in agriculture and insect resistance management: a review. Agronomy for Sustainable Development, 28, 11–20.
Sarrías JA, Valero M and Salmerón MC, 2002. Enumeration, isolation and characterization of *Bacillus cereus* strains from Spanish raw rice. Food Microbiology, 19, 589–595.
Sastalla I, Fattah R, Coppage N, Nandy P, Crown D, Pomerantsev AP and Leppla SH, 2013. The *Bacillus cereus* Hbl and Nhe tripartite enterotoxin components assemble sequentially on the surface of target cells and are not interchangeable. PLoS ONE, 8, e76955.
Schoeni JL and Wong AC, 2005. *Bacillus cereus* food poisoning and its toxins. Journal of Food Protection, 68, 636–648.
Schuch R and Fischetti VA, 2006. Detailed genomic analysis of the Wbeta and gamma phages infecting *Bacillus anthracis*: implications for evolution of environmental fitness and antibiotic resistance. Journal of Bacteriology, 188, 3037–3051.
Selma MV, Allende A, Lopez-Galvez F, Conesa MA and Gil MI, 2008. Disinfection potential of ozone, ultraviolet-C and their combination in wash water for the fresh-cut vegetable industry. Food Microbiology, 25, 809–814.
Sheppard AE, Poehlein A, Rosenstiel P, Liesegang H and Schulenburg H, 2013. Complete genome sequence of *Bacillus thuringiensis* strain 407 Cry. Genome Announcements, 1.
Shiota M, Saitou K, Mizumoto H, Matsusaka M, Agata N, Nakayama M, Kage M, Tatsumi S, Okamoto A, Yamaguchi S, Ohta M and Hata D, 2010. Rapid detoxification of cereulide in *Bacillus cereus* food poisoning. Pediatrics, 125, e951–e955.
Silva MP, Pereira CA, Junqueira JC and A.O.C. J, 2013. Methods of destroying bacterial spores. In: Méndez-Vilas A (ed.). *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education*. Formatex Research Center, Spain. pp. 490–496.
Slater TB and Murray PR, 2002. *Medical Microbiology*. 4th Edition. Mosby, St. Louis, MO, London.
Smeesters PR, Drèze PA, Bousbata S, Parikka KJ, Timmery S, Hu X, Perez-Morga D, Deghorain M, Toussaint A, Mahillon J and Van Melderen L, 2011. Characterization of a novel temperate phage originating from a cereulide-producing *Bacillus cereus* strain. Research in Microbiology, 162, 446–459.
Smith RA and Barry JW, 1998. Environmental persistence of *Bacillus thuringiensis* spores following aerial application. Journal of Invertebrate Pathology, 71, 263–267.
Smith NR, Gordon RE and Clark FE, 1952. *Aerobic Spore Forming Bacteria*. United States Department of Agriculture, Washington, DC.
Sorokin A, Candelon B, Guilloux K, Galleron N, Wackerow-Kouzova N, Ehrlich SD, Bourguet D and Sanchis V, 2006. Multiple-locus sequence typing analysis of *Bacillus cereus* and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of psychrotrophic strains. Applied and Environmental Microbiology, 72, 1569–1578.
Soufiane B, Xu D and Côté JC, 2007. Flagellin (FliC) protein sequence diversity among *Bacillus thuringiensis* does not correlate with H serotype diversity. Antonie van Leeuwenhoek, 92, 449–461.
Stark T, Marxen S, Rutschle A, Lucking G, Scherer S, Ehling-Schulz M and Hofmann T, 2013. Mass spectrometric profiling of *Bacillus cereus* strains and quantitation of the emetic toxin cereulide by means of stable isotope dilution analysis and HEp-2 bioassay. Analytical and Bioanalytical Chemistry, 405, 191–201.
Stenfors Arnesen LP, Fagerlund A and Granum PE, 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Reviews, 32, 579–606.
Stenfors LP, Mayr R, Scherer S and Granum PE, 2002. Pathogenic potential of fifty *Bacillus weihenstephanensis* strains. FEMS Microbiology Letters, 215, 47–51.
von Stetten F, Mayr R and Scherer S, 1999. Climatic influence on mesophilic *Bacillus cereus* and psychrotolerant *Bacillus weihenstephanensis* populations in tropical, temperate and alpine soil. Environmental Microbiology, 1, 503–515.
Sun S, Fan J, Cheng Z, Cai Y, Li G and Pang Y, 2013. The effect of gamma sterilization on the insecticidal toxicity of engineered and conventional *Bacillus thuringiensis* strains. Journal of Economic Entomology, 106, 36–42.
Svestka M and Vankova J, 1976. Effects of artificial rain on the persistence of spores of *Bacillus thuringiensis* Berl. Lesnictvi, 22, 829–838.
Swanson MM, Reavy B, Makarova KS, Cock PJ, Hopkins DW, Torrance L, Koonin EV and Taliansky M, 2012. Novel bacteriophages containing a genome of another bacteriophage within their genomes. PLoS ONE, 7, e40683.

Swiecicka I and Mahillon J, 2006. Diversity of commensal *Bacillus cereus sensu lato* isolated from the common sow bug *(Porcellio scaber*, Isopoda). FEMS Microbiology Ecology, 56, 132–140.
Swiecicka I, Van der Auwera GA and Mahillon J, 2006. Hemolytic and nonhemolytic enterotoxin genes are broadly distributed among *Bacillus thuringiensis* isolated from wild mammals. Microbial Ecology, 52, 544–551.
Takabe F and Oya M, 1976. An autopsy case of food poisoning associated with *Bacillus cereus*. Forensic Science, 7, 97–101.
Tang M, Bideshi DK, Park HW and Federici BA, 2006. Minireplicon from pBtoxis of *Bacillus thuringiensis* subsp. *israelensis*. Applied and Environmental Microbiology, 72, 6948–6954.
Tayabali AF and Seligy VL, 2000. Human cell exposure assays of *Bacillus thuringiensis* commercial insecticides: production of *Bacillus cereus*-like cytolytic effects from outgrowth of spores. Environmental Health Perspectives, 108, 919–930.
Techathuvanan C, Reyes F, David JR and Davidson PM, 2014. Efficacy of commercial natural antimicrobials alone and in combinations against pathogenic and spoilage microorganisms. Journal of Food Protection, 77, 269–275.
Ter Beek A and Brul S, 2010. To kill or not to kill *Bacilli*: opportunities for food biotechnology. Current Opinion in Biotechnology, 21, 168–174.
Thammasittirong A and 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, 121–126.
Thomas WE and Ellar DJ, 1983. *Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. Journal of Cell Science, 60, 181–197.
Thomas DJ, Morgan JA, Whipps JM and Saunders JR, 2000. Plasmid transfer between the *Bacillus thuringiensis* subspecies *kurstaki* and tenebrionis in laboratory culture and soil and in lepidopteran and coleopteran larvae. Applied and Environmental Microbiology, 66, 118–124.
Thomas DJ, Morgan JA, Whipps JM and Saunders JR, 2001. Plasmid transfer between *Bacillus thuringiensis* subsp. *israelensis* strains in laboratory culture, river water, and dipteran larvae. Applied and Environmental Microbiology, 67, 330–338.
Thorsen L, Hansen BM, Nielsen KF, Hendriksen NB, Phipps RK and Budde BB, 2006. Characterization of emetic *Bacillus weihenstephanensis,* a new cereulide-producing bacterium. Applied and Environmental Microbiology, 72, 5118–5121.
Ticknor LO, Kolsto AB, Hill KK, Keim P, Laker MT, Tonks M and Jackson PJ, 2001. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. Applied and Environmental Microbiology, 67, 4863–4873.
Timmery S, Modrie P, Minet O and Mahillon J, 2009. Plasmid capture by the *Bacillus thuringiensis* conjugative plasmid pXO16. Journal of Bacteriology, 191, 2197–2205.
Tourasse NJ and Kolstø AB, 2008a. SuperCAT: a supertree database for combined and integrative multilocus sequence typing analysis of the *Bacillus cereus* group of bacteria (including *B. cereus*, *B. anthracis* and *B. thuringiensis*). Nucleic Acids Research, 36, D461–D468.
Tourasse NJ and Kolstø AB, 2008b. Survey of group I and group II introns in 29 sequenced genomes of the *Bacillus cereus* group: insights into their spread and evolution. Nucleic Acids Research, 36, 4529–4548.
Tourasse NJ, Helgason E, Økstad OA, Hegna IK and Kolstø AB, 2006. The *Bacillus cereus* group: novel aspects of population structure and genome dynamics. Journal of Applied Microbiology, 101, 579–593.
Tourasse NJ, Økstad OA and Kolstø AB, 2010. HyperCAT: an extension of the SuperCAT database for global multi-scheme and multi-data type phylogenetic analysis of the *Bacillus cereus* group population. Database (Oxford), 2010, baq017. doi: 10.1093/database/baq017
Tourasse NJ, Helgason E, Klevan A, Sylvestre P, Moya M, Haustant M, Økstad OA, Fouet A, Mock M and Kolstø AB, 2011. Extended and global phylogenetic view of the *Bacillus cereus* group population by combination of MLST, AFLP, and MLEE genotyping data. Food Microbiology, 28, 236–244.
Tran SL, Guillemet E, Ngo-Camus M, Clybouw C, Puhar A, Moris A, Gohar M, Lereclus D and Ramarao N, 2011. Haemolysin II is a *Bacillus cereus* virulence factor that induces apoptosis of macrophages. Cellular Microbiology, 13, 92–108.
Tremoulet F, Rabier P and Gas G, 2002. Inhibition of B*acillus stearothermophilus* spores in a liquid medium by free fatty acids with and without heat: possible mechanisms for the microbiological stability of canned fat-duck liver. Journal of Food Science, 67, 1144–1148.
Tschiedel E, Rath PM, Steinmann J, Becker H, Dietrich R, Paul A, Felderhoff-Muser U and Dohna-Schwake C, 2015. Lifesaving liver transplantation for multi-organ failure caused by *Bacillus cereus* food poisoning. Pediatric Transplantation, 19, E11–E14.
Turgeon N, Laflamme C, Ho J and Duchaine C, 2006. Elaboration of an electroporation protocol for *Bacillus cereus* ATCC 14579. Journal of Microbiological Methods, 67, 543–548.
Turnbull PC, Sirianni NM, LeBron CI, Samaan MN, Sutton FN, Reyes AE and Peruski LF Jr, 2004. MICs of selected antibiotics for *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* from a range of clinical and environmental sources as determined by the Etest. Journal of Clinical Microbiology, 42, 3626–3634.
Ueda S, Yamaguchi M, Iwase M and Kuwabara Y, 2013. Detection of emetic *Bacillus cereus* by real-time PCR in foods. Biocontrol Science, 18, 227–232.

Ugras S, Sezen K, Kati H and Demirbag Z, 2013. Purification and characterization of the bacteriocin Thuricin Bn1 produced by *Bacillus thuringiensis* subsp. *kurstaki* Bn1 isolated from a hazelnut pest. Journal of Microbiology and Biotechnology, 23, 167–176.

USFDA/CFSAN (United States Food and Drug Administration/Center for Food Safety and Applied Nutrition), 2002. Kinetics of Microbial Inactivation for Alternative Food Processing Technologies – Pulsed Light Technology. Available online: http://www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm103058.htm

Vachon V, Laprade R and Schwartz J-L, 2012. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. Journal of Invertebrate Pathology, 111, 1–12.

Vadlamudi RK, Weber E, Ji I, Ji TH and Bulla LA Jr, 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. The Journal of Biological Chemistry, 270, 5490–5494.

Väisänen OM, Mwaisumo NJ and Salkinoja-Salonen MS, 1991. Differentiation of dairy strains of the *Bacillus cereus* group by phage typing, minimum growth temperature, and fatty acid analysis. The Journal of Applied Bacteriology, 70, 315–324.

Valero M and Salmerón MC, 2003. Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. International Journal of Food Microbiology, 85, 73–81.

Valero M, Hernández-Herrero LA, Fernández PS and Salmerón MC, 2002. Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. Food Microbiology, 19, 491–499.

Van Cuyk S, Deshpande A, Hollander A, Duval N, Ticknor L, Layshock J, Gallegos-Graves L and Omberg KM, 2011. Persistence of *Bacillus thuringiensis* subsp. *kurstaki* in urban environments following spraying. Applied and Environmental Microbiology, 77, 7954–7961.

Van der Auwera G and Mahillon J, 2005. TnXO1, a germination-associated class II transposon from *Bacillus anthracis*. Plasmid, 53, 251–257.

Van der Auwera G and Mahillon J, 2008. Transcriptional analysis of the conjugative plasmid pAW63 from *Bacillus thuringiensis*. Plasmid, 60, 190–199.

Van der Auwera GA, Andrup L and Mahillon J, 2005. Conjugative plasmid pAW63 brings new insights into the genesis of the *Bacillus anthracis* virulence plasmid pXO2 and of the *Bacillus thuringiensis* plasmid pBT9727. BMC Genomics, 6, 103.

Van der Auwera GA, Timmery S, Hoton F and Mahillon J, 2007. Plasmid exchanges among members of the *Bacillus cereus* group in foodstuffs. International Journal of Food Microbiology, 113, 164–172.

Van der Auwera GA, Timmery S and Mahillon J, 2008. Self-transfer and mobilisation capabilities of the pXO2-like plasmid pBT9727 from *Bacillus thuringiensis* subsp. *konkukian* 97-27. Plasmid, 59, 134–138.

Van Rie J, Jansens S, Hofte H, Degheele D and Van Mellaert H, 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Applied and Environmental Microbiology, 56, 1378–1385.

Verheust C, Jensen G and Mahillon J, 2003. pGIL01, a linear tectiviral plasmid prophage originating from *Bacillus thuringiensis* serovar *israelensis*. Microbiology, 149, 2083–2092.

Verheust C, Fornelos N and Mahillon J, 2005. GIL16, a new gram-positive tectiviral phage related to the *Bacillus thuringiensis* GIL01 and the *Bacillus cereus* pBClin15 elements. Journal of Bacteriology, 187, 1966–1973.

Vettori C, Paffetti D, Saxena D, Stotzky G and Giannini R, 2003. Persistence of toxins and cells of *Bacillus thuringiensis* subsp. *kurstaki* introduced in sprays to Sardinia soils. Soil Biology and Biochemistry, 35, 1635–1642.

Vilain S, Luo Y, Hildreth MB and Brozel VS, 2006. Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. Applied and Environmental Microbiology, 72, 4970–4977.

Vilas-Boas G, Sanchis V, Lereclus D, Lemos MV and Bourguet D, 2002. Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. Applied and Environmental Microbiology, 68, 1414–1424.

Walker K, Mendelsohn M, Matten S, Alphin M and Ave D, 2003. The role of microbial Bt products in US crop protection. In: Metz M (ed.). *Bacillus thuringiensis*: A Cornerstone of Modern Agriculture. Food Products Press, Binghamton, USA. pp. 31–51.

Wang X, Xue Y, Han M, Bu Y and Liu C, 2014. The ecological roles of *Bacillus thuringiensis* within phyllosphere environments. Chemosphere, 108, 258–264.

Wehrle E, Didier A, Moravek M, Dietrich R and 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, 124–130.

Wenning M and Scherer S, 2013. Identification of microorganisms by FTIR spectroscopy: perspectives and limitations of the method. Applied Microbiology and Biotechnology, 97, 7111–7120.

White HD, Bates MJ and Wilson P, 1992. *For Information Specialists: Interpretations of Reference and Bibliographic Work*. Ablex Publishing Corporation, Norwood, New Jersey, USA.

WHO (World Health Organization), 2009. *Microbial Pest Control Agent. Bacillus thuringiensis*. Environmental Health Criteria 217 125 pp.

Wijnands LM, Dufrenne JB, Rombouts FM, in 't Veld PH and van Leusden FM FM, 2006. Prevalence of potentially pathogenic Bacillus cereus in food commodities in The Netherlands. Journal of Food Protection, 69, 2587–2594.

Wilcks A, Jayaswal N, Lereclus D and Andrup L, 1998. Characterization of plasmid pAW63, a second self-transmissible plasmid in *Bacillus thuringiensis* subsp. *kurstaki* HD73. Microbiology, 144 (Pt 5), 1263–1270.

Wilcks A, Smidt L, Okstad OA, Kolsto AB, Mahillon J and Andrup L, 1999. Replication mechanism and sequence analysis of the replicon of pAW63, a conjugative plasmid from *Bacillus thuringiensis*. Journal of Bacteriology, 181, 3193–3200.

Wilcks A, Hansen BM, Hendriksen NB and Licht TR, 2006. Fate and effect of ingested *Bacillus cereus* spores and vegetative cells in the intestinal tract of human-flora-associated rats. FEMS Immunology and Medical Microbiology, 46, 70–77.

Wilcks A, Smidt L, Bahl MI, Hansen BM, Andrup L, Hendriksen NB and Licht TR, 2008. Germination and conjugation of *Bacillus thuringiensis* subsp. *israelensis* in the intestine of gnotobiotic rats. Journal of Applied Microbiology, 104, 1252–1259.

Wright AM, Beres SB, Consamus EN, Long SW, Flores AR, Barrios R, Richter GS, Oh SY, Garufi G, Maier H, Drews AL, Stockbauer KE, Cernoch P, Schneewind O, Olsen RJ and Musser JM, 2011. Rapidly progressive, fatal, inhalation anthrax-like infection in a human: case report, pathogen genome sequencing, pathology, and coordinated response. Archives of Pathology and Laboratory Medicine, 135, 1447–1459.

Xu D and Côté JC, 2006. Sequence diversity of the *Bacillus thuringiensis* and *B. cereus sensu lato* flagellin (H antigen) protein: comparison with H serotype diversity. Applied and Environmental Microbiology, 72, 4653–4662.

Xu D and Côté JC, 2008. Sequence diversity of *Bacillus thuringiensis* flagellin (H antigen) protein at the intra-H serotype level. Applied and Environmental Microbiology, 74, 5524–5532.

Zhao C, Luo Y, Song C, Liu Z, Chen S, Yu Z and Sun M, 2007. Identification of three Zwittermicin A biosynthesis-related genes from *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520. Archives of Microbiology, 187, 313–319.

Zhu L, Peng D, Wang Y, Ye W, Zheng J, Zhao C, Han D, Geng C, Ruan L, He J, Yu Z and Sun M, 2015. Genomic and transcriptomic insights into the efficient entomopathogenicity of *Bacillus thuringiensis*. Scientific Reports, 5, 14129.

Abbreviations

AFLP	amplified fragment length polymorphism
AMP	antimicrobial peptide
ANI	average nucleotide identity
BAC	bacterial artificial chromosome
CFU	colony forming unit
CytK	cytotoxin K
FEEDAP Panel	EFSA Panel on Additives and Products or Substances used in Animal Feed
FTIR	Fourier transform infrared
GFP	green fluorescent protein
GI	gastrointestinal
GLISA	gold labelled immuno sorbent assay
GM	genetically modified
GPI	glycosylphosphatidyl-inositol
HACCP	Hazard Analysis and Critical Control Points system
HBL	haemolysin BL
Hep-2	human epithelial type 2
HHP	high hydrostatic pressure
HPHT	high pressure high temperature
HPLC-MS	high performance liquid chromatography-mass spectrometry
HUVEC	human umbilical vein endothelial cell
IBMA	International Biocontrol Manufacturers Association
IS	insertion sequence
ISO	International Organization for Standardization
MALDI-TOF	matrix-assisted laser desorption ionisation-time of flight
MCA	microbial control agent
MEE	multilocus enzyme electrophoresis
MGE	mobile genetic element
MIC	mobile insertion cassette
MLST	multilocus sequence typing
MPCA	microbial pest control agent
MPN	most probable number
MPPP	microbial plant protection product
MRL	maximum residue limit
MS	mass spectrometry
MS	Member State

MYP	mannitol, egg yolk and polymyxin
NHE	non-haemolytic enterotoxin
NRPS	non-ribosomal peptide synthetases
PCR	polymerase chain reaction
PEMBA	polymyxin egg yolk mannitol bromothymol blue agar
PFGE	pulsed field gel electrophoresis
PHB	polyhydroxybutyrate
PL	pulsed light
PPP	plant protection product
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
REPFED	refrigerated processed food of extended durability
RCR	rolling circle replication
SCV	small colony variant
SIDA	stable isotope mass spectrometry (MS)-based dilution assay
SMase	sphingomyelinase
ToR	Term of Reference
UV	ultraviolet
VIP	vegetative insecticidal protein
VPR	vascular permeability reaction
WG	Working Group
WGS	whole genome sequence

Glossary

***Bacillus cereus* group**	A subdivision of the *Bacillus* genus that consists of eight formally recognised species: *B. cereus sensu stricto* (or *B. cereus* as it is usually called), *B. anthracis, B. thuringiensis, B. weihenstephanensis, B. mycoides, B. pseudomycoides,* B. cytotoxicus and *B. toyonensis*. It is also often designated as *B. cereus sensu lato* or *B. cereus* complex in the literature.
***Bacillus thuringiensis* (Bt)**	A naturally occurring bacterium present in the environment that is a member of the *B. cereus* group of Gram-positive spore-forming bacteria and that it is closely related to *B. cereus sensu stricto* and *B. anthracis;* the three organisms differ mainly in their plasmids. The defining feature of *B. thuringiensis* is its ability to produce, during sporulation, proteinaceous crystals composed of insecticidal proteins, called δ-endotoxins or Cry proteins, toxic to certain types of insects. When ingested by a target insect larva, the proteins produced by *B. thuringiensis* kill the insect by disturbing the digestive system.
Biopesticide	Terminology used in this opinion for biological pesticides where a microorganism is the active ingredient. Other common designations for biopesticides are: bioinsecticides, microbial plant protection products (MPPPs), microbial pest control agents (MPCAs) and microbial control agents (MCAs).
Endophytic bacteria	Bacteria that live within a plant without causing apparent disease.
Epiphytic bacteria	Bacteria which live on the surface of plants; they do not harm the plants.
Genome	The entire DNA content that is present within one cell of an organism. This includes both the genes and the non-coding sequences of the DNA with regulatory and architectural functions. This entails all of an organism's chromosomal DNA as well as DNA contained on any extra chromosomal heritable determinant (such as plasmids or phages) that can or not replicate with different degrees of autonomy.
Genotype	The set of genes of an organism or the actual sequence of nucleotides of its DNA; it is distinct from its physical characteristics, or phenotype.
Minimal processing	Any action applied to the initial product (e.g. cleaning, coring, peeling, chopping, cutting, slicing or dicing, freezing and washing) and which is not included in the definition of processing according to Regulation (EC) No 852/2004[17] (e.g. heating, smoking, curing, maturing, drying, marinating, extraction, extrusion or a combination of those processes). Minimal processing may occur at harvest as well as on farm postharvest and at processing.
Peritrophic membrane or peritrophic matrix	A semi-permeable, non-cellular structure which surrounds the food bolus in an organism's midgut and that is continuously secreted at the anterior end of the midgut. Although they are often found in the midgut of many insects, peritrophic matrixes are also found in other phyla. The peritrophic matrix serves several functions, including improvement of digestion, protection against mechanical and chemical damage and as a barrier to infection by pathogens.
Phenotype	The observable properties or characteristics of a genotype, in a given individual. The phenotype is produced by all the genes that are expressed, in a given condition, in combination with the effects of the environment.
Species	A taxonomic subdivision of a genus. A group of closely related and morphologically similar individuals that, actually or potentially, interbreed. The concept of a bacterial species is less definitive than for higher organisms. It must be emphasised that there is no 'official' classification of bacteria and that some species have greater phenotypic and genetic diversity than others. A bacterial species may be regarded as a group of strains that have many features in common and exhibit a particular level of DNA homology and that differ significantly from another group of strains.

[17] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. OJ L 139, 30.4.2004, p. 1–54.

Spore	A dormant cellular form, derived from a bacterial or fungal cell, that is devoid of metabolic activity and that can give rise to a vegetative cell upon germination; it is dehydrated and can survive for prolonged periods of time under drastic environmental conditions.
Sporulation	A developmental or differentiation process that leads to the development of spores, which are dormant cells containing the DNA of the organism and are often resistant to desiccation and other harsh environmental conditions.
Strain	A subdivision of species. A group of organisms of the same species that are identical to each other and that differ genetically from other organisms of the same species. A genetically homogeneous population of organisms, at a subspecies level, that can be differentiated by biochemical, pathogenic or other taxonomic features.
Subspecies	A subdivision of the species based on minor but consistent phenotypic variations within the species or on genetically determined clusters of strains within species.
Taxonomy	The scientific classification of living organisms that is based on their relatedness; it includes the rules, principles and practices for the systematic grouping, ordering and naming of living organisms.

Appendix A – *Bacillus cereus* and *Bacillus* spp. food-borne outbreak data in the European Union Member States (MSs) and European non-MSs (2007–2014)

Table A.1: Reported strong-evidence food-borne outbreaks by implicated food vehicle where *Bacillus cereus* was implicated in reporting countries in accordance with Directive 2003/99/EC, from 2007 to 2014

Food vehicle	Year	Total number of food-borne outbreaks	Human cases	Hospitalisations
Bakery products	2008	2	25	2
	2010	1	8	0
	2011	4	29	0
	2012	1	5	0
Total – Bakery products		8	67	2
Poultry meat and products thereof	2007	7	95	1
	2008	1	10	0
	2009	6	63	8
	2011	3	47	0
	2012	3	46	0
	2014	2	5	0
Total – Poultry meat and products thereof		22	266	9
Red meat and products thereof	2007	8	40	0
	2008	4	175	3
	2009	5	59	0
	2011	4	98	0
	2012	5	256	1
	2013	6	145	0
	2014	2	27	0
Total – Red meat and products thereof		33	752	4
Mixed food or buffet meals	2007	12	83	0
	2008	18	307	32
	2009	17	401	12
	2010	10	349	3
	2011	10	94	16
	2012	13	216	5
	2013	19	306	81
	2014	15	319	32
Total – Mixed food or buffet meals		114	2,075	181
Cheese, milk or dairy products	2007	4	23	0
	2008	2	5	0
	2009	1	20	NR
	2010	1	2	0
	2011	1	3	3
	2012	1	2	0
	2013	1	10	0
Total – Cheese, milk and dairy products		11	65	3
Canned food products	2010	1	62	0
Total – Canned food products		1	62	0

Food vehicle	Year	Total number of food-borne outbreaks	Human cases	Hospitalisations
Cereal products including rice and seeds/ pulses (nuts, almonds)	2007	5	62	0
	2008	6	165	2
	2009	7	79	0
	2010	7	28	0
	2011	6	37	4
	2012	5	63	10
	2013	5	27	0
	2014	4	32	0
Total – Cereal products including rice and seeds/pulses (nuts, almonds)		45	493	16
Crustaceans, shellfish, molluscs and products thereof	2007	3	36	2
	2008	1	2	0
	2009	3	25	0
	2010	1	2	0
	2011	1	2	NR
	2013	1	2	0
	2014	2	8	0
Total – Crustaceans, shellfish, molluscs and products thereof		12	77	2
Fish and fish products	2007	5	172	0
	2008	1	2	0
	2009	1	2	0
	2012	5	80	3
	2013	2	15	0
Total – Fish and fish products		14	271	3
Eggs and egg products	2007	1	4	0
	2009	2	19	3
	2011	2	15	0
	2012	1	3	3
	2013	2	19	0
Total – Eggs and egg products		8	60	6
Sweets and chocolate	2013	1	2	NR
	2014	1	8	0
Total – Sweets and chocolate		2	10	0
Vegetables and juices and other products thereof	2008	2	7	0
	2009	2	14	NR
	2010	2	4	0
	2011	4	189	0
	2012	1	4	0
	2013	6	64	0
	2014	2	238	15
Total – Vegetables and juices and other products thereof		19	520	15
Herbs and spices	2007	2	149	0
	2009	2	9	0
	2011	4	78	0
	2013	2	6	0
Total – Herbs and spices		10	242	0

Food vehicle	Year	Total number of food-borne outbreaks	Human cases	Hospitalisations
Drinks, including bottled water	2013	1	7	0
Total – Drinks, including bottled water		1	7	0
Other foods	2007	31	241	23
	2008	3	306	2
	2009	13	269	53
	2010	2	22	0
	2011	8	81	6
	2012	2	12	0
	2013	8	90	2
	2014	11	219	22
Total – Other foods		78	1,240	108
Unknown	2007	28	303	3
	2008	5	128	0
	2009	2	19	NR
Total – Unknown		35	450	3
Total outbreaks		413	6,657	352

NR: not reported.

Table A.2: Reported outbreaks by implicated food vehicle where *Bacillus* other than *B. cereus* was implicated in reporting countries in accordance with Directive 2003/99/EC, from 2007 to 2014

Bacillus* other than *B. cereus	**Food vehicle**	**Year**	**Total number of food-borne outbreaks**	**Human cases**	**Hospitalisations**
Bacillus – *Bacillus* spp., unspecified	Other foods	2009	1	120	50
	Vegetables and juices and other products thereof	2012	1	NR	NR
	Cheese	2012	1	33	2
Total – *Bacillus* spp., unspecified			3	153	52
Bacillus – *B. subtilis*	Mixed food	2010	1	84	0
Total – *B. subtilis*			1	84	0
Total outbreaks			4	237	52

NR: not reported.

Appendix B – Questionnaire on testing/monitoring on the occurrence of *Bacillus thuringiensis* in fruits, vegetables and/or other crops eligible to be treated with *Bacillus thuringiensis*

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-Q-2015-00254)

Draft questions to be answered by the Pesticides Steering Network and the Pesticide Monitoring Network

- Are you aware of any data available at your Member State level regarding:
  - testing/monitoring on the occurrence and levels of *Bacillus thuringiensis* in fruits, vegetables and/or other crops eligible to be treated with *B. thuringiensis*?
  - additional studies on survival, persistence and multiplication of *B. thuringiensis* strains/toxins in the environment and in food (e.g. vegetable and fruits) after application on crop fields?
  - additional information on natural background levels of *B. thuringiensis* in the environment (e.g. groundwater, water reservoirs, crop fields (non-treated), etc.)?
- If any data is available, please provide the methodologies used for analysis, the sampling plans and results.

Appendix C – Questionnaire on microbiological criteria or recommendations for *Bacillus* spp., *Bacillus cereus* or *Bacillus thuringiensis* for any type of food

EFSA questionnaire related to the 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-Q-2015-00254)

Date of submission: (date field)
Member State: (free type)
Contact point: (free type)

1) Are there any microbiological criteria or recommendations for *Bacillus* spp., *Bacillus cereus* or *Bacillus thuringiensis* defined and/or in use in your country for any type of food?

   (Yes/NO answer)

2) In case you replied yes to the previous question, please provide all available details for all relevant microbiological criteria indicating in each case the:

   - specific type of food, e.g. vegetables, fruits, salads, aromatic plants and herbs, cereals, dried fruits and vegetables, rice, RTE foods (cooked and non-cooked) (Free type question)
   - sampling stage (or point of application) (Free type question)
   - specific sampling plan, e.g. indicating the parameters *m, M, c* and *n* (if applicable). (Free type question)

Appendix D – Replies provided by countries to the questionnaire on microbiological criteria or guideline microbiological limits for *Bacillus cereus* or *Bacillus* spp.

Table D.1: Summary table of the replies provided by countries to the questionnaire on microbiological criteria or guideline microbiological limits for *Bacillus cereus*, *Bacillus* spp. or aerobic spore-forming bacteria in foods

Member State	Specific type of food	Sampling stage or point of application	Specific sampling plan indicating the parameters *m*, *M*, *c*[(c)] and *n*[(d)] (if applicable) or guideline limits
Austria	RTE food including spices[(a)], presumptive *Bacillus cereus* > 10,000 CFU/g, unsafe – unfit for human consumption		
	RTE food including spices[(a)], presumptive *Bacillus cereus* > 100,000 CFU/g, unsafe – injurious to health		
	Dried infant formulae intended for infants below 3 months of age[(a)], presumptive *Bacillus cereus* > 5,000 CFU/g, unsafe – unfit for human consumption		
Belgium[(b)]	RTE foods (cooked)	HORECA/distribution	*m* = 1,000 CFU/g, *M* = 10,000 CFU/g, *c* = 2, *n* = 5
	RTE foods (cooked)	Kindergarten/processing	*m* = 100 CFU/g, *M* = 1,000 CFU/g, *c* = 2, *n* = 5
	Pasteurised milk/spices/gelatine/ meat substitutes	Processing/distribution	*m* = 1,000 CFU/g, *M* = 10,000 CFU/g, *c* = 2, *n* = 5
	Prepared milk in baby bottles	Maternity dep. hospital	*m* = 50 CFU/g, *M* = 500 CFU/g, *c* = 1, *n* = 5
Croatia	Dairy milk desserts	Production process	*m* = 500 CFU/g, *M* = 1,000 CFU/g, *c* = 2, *n* = 5
	Dehydrated baby food which is not cooked before use	Production process	*m* = 10 CFU/g, *M* = 100 CFU/g, *c* = 1, *n* = 5
Czech Republic	Foods intended for direct consumption	Ready for consumption	Maximum acceptable limit: 10,000 CFU/g
	Foods not intended for direct consumption	As sold	Maximum acceptable limit: 100,000 CFU/g
	Foods for infants and small children	As sold	Maximum acceptable limit: 100 CFU/g
	Delicatessen foods and titbits Tolerable limits	As sold and prepared for consumption	*m* = 100, *M* = 1,000 *c* = 2, *n* = 5
	Milk desserts, creams and custards Tolerable limits	As prepared for consumption	*m* = 100, *M* = 1,000 *c* = 1, *n* = 2
Denmark	Dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age	End of the manufacturing process	*m* = 50 CFU/g, *M* = 500 CFU/g *c* = 1, *n* = 5
Finland	The health hazard related to *B. cereus* and other *Bacillus* findings is evaluated and considered case by case. In order to help local authorities and FBOs in this work the central authority has given a guideline: RTE food: *B. cereus* > 10,000 CFU/g Fresh vegetables (including chopped): *B. cereus* > 100,000 CFU/g Dried herbs and spices: *B. cereus* > 100,000 CFU/g		

Member State	Specific type of food	Sampling stage or point of application	Specific sampling plan indicating the parameters *m*, *M*, *c*[(c)] and *n*[(d)] (if applicable) or guideline limits
France	Dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age (see guide to alert management: http://agriculture.gouv.fr/ministere/note-de-service-dgalmusn2009-8188-du-07072009)	Stages of manufacturing and marketing	Process Hygiene Criterion: 50 CFU/g Alert threshold: 1,000 CFU/g
Greece	Dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age	End of the manufacturing process	m = 50 CFU/g, M = 500 CFU/g, c = 1, n = 5
Hungary	Baby food	At the end of manufacturing process	*B. cereus* not detected (Process Hygiene Criterion) n and weight of subsample not defined
	Canned or semi-canned food	At the end of manufacturing process	Aerobic spore-forming bacteria M = 1,000 CFU/g (Process Hygiene Criterion)
Ireland	RTE foods (cooked and non-cooked)	When placed on the market	These are guideline limits rather than criteria with associated sampling plans. The limits for *B. cereus* and other pathogenic *Bacillus* spp. are as follows: 'Satisfactory' < 10^3 CFU/g; 'Borderline' 10^3 to ≤ 10^5 CFU/g; and 'Unsatisfactory' > 10^5 CFU/g. For more details, please see the following link: https://www.fsai.ie/publications_GN3_microbiological_limits/
Lithuania	Bakery confectionery with filling	End of the manufacturing process	m = 1,000 CFU/g, M = 10,000 CFU/g, c = 0, n = 5
	Meals and RTE culinary speciality	End of the manufacturing process	m = 1,000 CFU/g, M = 10,000 CFU/g, c = 2, n = 5
Netherlands	All foods	All stages	No sampling plan, limit is 100,000. Limit is applicable to (presumptive) *B. cereus* (according to ISO 7932)
Poland	Dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age (presumptive *Bacillus cereus* – according to Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs)	End of the manufacturing process	m = 50 CFU/g, M = 500 CFU/g, c = 1, n = 5

Member State	Specific type of food	Sampling stage or point of application	Specific sampling plan indicating the parameters *m*, *M*, *c*[(c)] and *n*[(d)] (if applicable) or guideline limits
Slovakia	Soft pastry with filling, without filling	FBO (Process Hygiene Criterion)	*m* = 1,000 CFU/g, *M* = 10,000 CFU/g, *c* = 2, *n* = 5
	RTE for infants and young children in public catering establishments – dried food for infants and young children after restore	FBO (Process Hygiene Criterion)	*m* = 0 CFU/g, *c* = 0, *n* = 5
Sweden	There are no microbiological criteria for *Bacillus* in the Swedish national legislation. The National Food Agency (NFA) has not given detailed national guidance on sampling (such as specific sampling plan) either, but *Bacillus*, especially *B. cereus*, is dealt with more generally in different guidances. The NFA notes that high levels of *B. cereus* can pose a risk especially in certain foods, and for readymade food it is recommended that competent authorities consider levels around and above 100,000 CFU/g as unsatisfactory in official controls, taking into account the different factors affecting the infective dose. In some cases (such as when patients show emetic symptoms), also lower levels should be taken into account. Additional information can be found in Swedish at the National Food Agency website www.livsmedelsverket.se		
Switzerland	Dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age	End of the manufacturing process	*m* = 50 CFU/g, *M* = 500 CFU/g, *c* = 1, *n* = 5
	Heat-treated RTE foods, in its natural state or reheated	Retail, during shelf-life	1,000 CFU/g
United Kingdom	RTE foods placed on the market guidance only, not laid down in legislation. Reported, if present, as presumptive *Bacillus* spp./*Bacillus cereus* > 100,000 CFU/g = unsatisfactory 1,000–100,000 CFU/g = borderline < 1,000 CFU/g = satisfactory		

RTE: ready-to-eat.
(a): Products placed on the market during their shelf life.
(b): These are not legal limits, but indicative values for hygiene used to evaluate the results of official controls. These values can be used by FBOs for self-checking. When the results exceed 100,000 CFU/g, toxins are analysed. Legal limits (Process Hygiene Criterion) from Commission Regulation (EC) No 2073/2005 are also applied.
(c): *c* = number of sample units giving values over *m* or between *m* and *M*.
(d): *n* = number of units comprising the sample.

Appendix E – Questionnaire provided to the International Biocontrol Manufacturers Association (IBMA) on biopesticides containing *Bacillus thuringiensis* provided

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-Q-2015-00254)

Draft questions to be clarified with *IBMA* (International Biocontrol Producers Association)

- **Please provide any additional data** you may have regarding the production process (e.g. any change subsequent to the peer-review by EFSA) for microbial plant protection products (MPPPs) (= bioinsecticides) containing *Bacillus thuringiensis*.
- Provide an overview of the most commonly produced formulations of MPPPs containing *Bacillus thuringiensis* (differentiated at strain level) in the EU.
- Which countries are the major users of MPPPs containing *Bacillus thuringiensis* in the EU?
- On which plants or crops are MPPP containing *Bacillus thuringiensis* most commonly applied in the EU and worldwide?
- **Please provide any additional data** you may have (e.g. any change subsequent to the peer-review by EFSA) regarding the strain identification methods used during production of MPPPs containing *Bacillus thuringiensis*.
  - What are the quality control practices of the batch inoculum starter cultures as well as the final product?
  - How are the starter strains kept and reisolated to make new batches of starter cultures?
  - Are the inoculum starter cultures further characterised, e.g. according to MLST classification schemes?
- **Please provide any additional data** you may have (e.g. any change subsequent to the peer-review by EFSA) regarding the genetic stability of the *Bacillus thuringiensis* strains?
- Are you aware of any data available from stakeholders (e.g. the producers or producer associations) doing regular testing/monitoring on the:
  - natural background levels of *Bacillus thuringiensis* in the environment (e.g. groundwater, water reservoirs, crop fields (non-treated), etc.)?
  - survival, persistence and multiplication of *Bacillus thuringiensis* strains/toxins in the environment and in food (e.g. vegetable and fruits) after application on crop fields?
  - occurrence and levels of *Bacillus thuringiensis* in fruits, vegetables and other crops eligible to be treated with *Bacillus thuringiensis*?
- If any testing/monitoring is performed, please provide the results and describe the sampling plans?

Appendix F – Protocol for the extensive literature review on the occurrence and levels of *Bacillus thuringiensis* in food

This literature review protocol will be used in the context of the EFSA mandate on risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs (EFSA-Q-2015-00254).

F.1. Problem formulation

F.1.1. Objective of the literature review

This literature review protocol aims at identifying in a systematic way relevant available scientific information on the occurrence and levels of *Bacillus thuringiensis* (*Bt*) in food, at all stages of the food chain. This literature review will allow retrieving relevant data to support the answer to ToR 5 of the mandate on risks for public health related to the presence of *B. cereus* and other *Bacillus* spp. including *B. thuringiensis* in foodstuffs.

F.1.2. Identification of the review questions

The question concerns the 'evaluation of the occurrence and enumeration of the number of *B. thuringiensis* levels in food, at all stages of the food chain'.

The following sub-questions have been identified:

1) Presence of *Bacillus thuringiensis* in food items

In the population of food items, what are the items where the presence of *Bacillus thuringiensis* has been detected worldwide?

2) Enumeration of *Bacillus thuringiensis* in food items

Which level of *Bacillus thuringiensis* has been detected in each food item?

F.1.3. Target population

Food items produced/imported for human consumption worldwide in any stage of the food chain.

F.1.4. Presence and levels of *B. thuringiensis* in foods

Presence and enumeration of *B. thuringiensis* (i.e. levels in CFU/g or CFU/mL).

F.2. Eligibility criteria for study selection

The selection of studies relevant to questions 1 and 2 will be performed using the eligibility criteria described below in Table F.1:

Table F.1: Eligibility criteria for questions 1 and 2

Study design	No restrictions will be applied regarding type of study design or the geographical location of study, time, type of food matrix, analytical method used and minimum number of samples.
	For Question 1, the study must report the detection of *B. thuringiensis* in food items either by microbiological detection or enumeration methods.
	For Question 2, the studies must have detected the presence of *B. thuringiensis* in specific food items AND quantified the number of microorganisms (CFU/g or CFU/mL). *Studies not reporting a quantitative estimate of the presence (e.g. CFU/g or CFU/mL) will not be selected for Question 2, but will be retained for Question 1.*
Study characteristics	No exclusion will be based on study characteristics
Population	Food items produced/imported in the EU including processed food
Outcome	• Presence of *B. thuringiensis* in food items in all stages of the food chain. • Levels of *B. thuringiensis* (number of CFU/g or CFU/mL) in food items in all stages of the food chain.

Language	English, French, Spanish and Italian
Time	Web of science Core Collection: from date of inception until 15/12/2015 CABI: CAB Abstracts: 1910 until 15/12/2015 MEDLINE: 1950 until 21/12/2015
Publication type	Papers published in peer-reviewed scientific journals (including primary research studies as well as secondary research studies, i.e. narrative and systematic reviews). In case systematic reviews are retrieved, it will be ensured that the results from cited primary studies will be de-duplicated.

F.3. Literature searches

The literature search will be conducted using a range of relevant information sources (Table F.2) to identify evidence on the presence and levels of *B. thuringiensis* in food, at all stages of the food chain.

Table F.2: Proposed information sources to be searched

Database	Interface
Web of Science Core Collection (excluding Social Sciences Citation Index and Arts and Humanities Citation Index)	Web of Science, Thomson Reuters
CABI: CAB Abstracts	Web of Science, Thomson Reuters
MEDLINE	Web of Science, Thomson Reuters

After discussion at WG level and considering the available resources, it was decided not to search the grey literature. It is known that potentially relevant papers could have been published in languages other than English. However, due to resource limitation, the searches will be limited to English, French, Italian and Spanish.

A search strategy to identify studies in indexed records in: (i) Web of Science Core Collection (excluding Social Sciences Citation Index and Arts and Humanities Citation Index) and (ii) CABI: CAB Abstracts is presented in Table F.3.

Table F.3: Draft search strategy for Web of Science Core Collection (excluding Social Sciences Citation Index and Arts and Humanities Citation Index) and CABI: CAB Abstracts

Set	String	Results
#1	**TOPIC:** (food OR vegetable OR fruit OR crop OR fresh produce OR leafy greens OR meal OR salad OR cereal OR spice OR herb OR seed OR pulses OR berry OR berries OR sprout OR mushroom OR potato OR potatoe OR nut OR coconut OR honey OR cocoa OR chocolate OR coffee OR tea OR ready-to-eat OR ready to eat OR RTE OR soup OR sauce OR dressing OR oil OR meat OR milk OR dairy OR cheese OR egg OR shellfish OR mollusc OR crustacean OR surimi OR snail OR fish OR fishery product OR gastropod OR bakery product OR sweet OR confectionery product OR infant formula OR beverage OR water OR juice OR tuber OR leafy brassica OR fungi OR legume)	5,746,921 (CABI: CAB Abstracts) 4,679,136 (Web of Science CC)
#2	**TOPIC:** (thuringiensis OR thuringensis OR turingensis OR turingiensis)	18,534 (CABI: CAB Abstracts) 13,114 (Web of Science CC)
#3	**TOPIC:** (prevalence OR occurrence OR incidence OR enumeration OR quantification OR monitoring OR presence OR detection OR count OR level OR isolation OR identification)	2,733,595 (CABI: CAB Abstracts) 8,806,340 (Web of Science CC)
#4	#1 AND #2 AND #3	3,748 (CABI: CAB Abstracts) 2,333 (Web of Science CC)
#5	#1 AND #2 AND #3 Refined by: LANGUAGES: (ENGLISH OR FRENCH OR SPANISH OR ITALIAN) #1 AND #2 AND #3 Refined by: LANGUAGES: (ENGLISH OR FRENCH OR SPANISH OR ITALIAN)	3,570 (CABI: CAB Abstracts) 2,315 (Web of Science CC)

A draft search strategy to identify studies in indexed records in MEDLINE is presented in Table F.4.

Table F.4: Draft search strategy for MEDLINE

Set	String	Results
#1	**TOPIC:** (food) OR MeSH HEADING: (Food) OR (MeSH HEADING:exp: ((Anti-Allergic Agents)) AND MeSH HEADING:exp: ((Vaccines)))	452,079 (MEDLINE)
#2	**TOPIC:** (vegetable OR fruit OR crop OR fresh produce OR leafy greens OR meal OR salad OR cereal OR spice OR herb OR seed OR pulses OR berry OR berries OR sprout OR mushroom OR potato OR potatoe OR nut OR coconut OR honey OR cocoa OR chocolate OR coffee OR tea OR ready-to-eat OR ready to eat OR RTE OR soup OR sauce OR dressing OR oil OR meat OR milk OR dairy OR cheese OR egg OR shellfish OR mollusc OR crustacean OR surimi OR snail OR fish OR fishery product OR gastropod OR bakery product OR sweet OR confectionery product OR infant formula OR beverage OR water OR juice OR tuber OR leafy brassica OR fungi OR legume)	1,894,026 (MEDLINE)
#3	**TOPIC:** (thuringiensis OR thuringensis OR turingensis OR turingiensis)	7,112 (MEDLINE)
#4	**TOPIC:** (prevalence OR occurrence OR incidence OR enumeration OR quantification OR monitoring OR presence OR detection OR count OR level OR isolation OR identification)	6,989,882 (MEDLINE)
#5	#1 OR #2	2,151,975 (MEDLINE)
#6	#5 AND #4 AND #3	1,108 (MEDLINE)
#7	#5 AND #4 AND #3 Refined by: LANGUAGES: (ENGLISH OR FRENCH OR SPANISH OR ITALIAN)	1,067 (MEDLINE)

References obtained from electronic databases will be electronically imported in Endnote X7® (Thomson Reuters©, 2014) bibliographic management software and de-duplicated. Once all references are obtained, they will be uploaded into DistillerSR® (Evidence Partners©, Canada, 2012) an internet-based systematic review software package, which will then be used for another check for duplicates (using the Duplicate detection feature) and for the screening and data extraction.

F.4. Study selection process and article evaluation

Studies to be included in the review will be selected by a two-step selection procedure.

1) **Screening of title and abstract** to identify potentially relevant studies that will be included for full text screening applying the selection criteria described in Section F.2. If the information contained in the title or abstract is not relevant to the research objectives, the article is not selected for full text assessment.
   Articles that will be excluded during screening of title and abstract will be stored in Distiller SR. This step will be conducted in duplicate by WG members and EFSA staff, in case of doubts or divergences between the reviewers, the full article will be screened (i.e. it will go to Step 2).
2) **Screening of full article** to assess whether the article is relevant to the risk assessment.
   This step will be conducted by WG members and EFSA staff in duplicate for the references retrieved. Possible divergences will be discussed between reviewers; in addition, it will be considered if these would highlight the need for amendments to the inclusion/exclusion criteria in the protocol.
   During the screening process, studies will be categorised into two groups corresponding to the two sub-questions which are the objectives of this literature review (i.e. all studies will be included in the assessment of Question 1, only studies where a measure of the number of microorganisms are provided will be included in the assessment of Question 2).

The results of the different phases of the study selection process will be reported in a flowchart as recommended in the PRISMA statement on preferred reporting items for systematic reviews and meta-analyses (Moher et al., 2009).

F.5. Data extraction from included studies

One reviewer per study will extract data from studies that have passed screening for relevance. At least 5% of these studies will be cross-checked, at the start of the data extraction step, by another reviewer in order to identify and correct any potential problem in the data extraction process.

Data extraction forms will be designed in DistillerSR®. Initial forms will be designed and piloted on several papers and modified as required.

Data will not be extracted from figures.

Data to extract

- List of food items in which presence/enumeration of *B. thuringiensis* has been tested. The specific food items will be extracted by filling in a free text field.
- Sampling stage of the food chain (if available, main stages to be extracted in a harmonised way according to the following categories: (i) harvested, (ii) minimally processed and (iii) processed foods).
- Place of origin of food items (country).

For each combination of food item/sampling stage of the food chain:

- Presence/absence of the microorganisms.
- Microorganism enumeration (e.g. CFU/g or CFU/mL food item in specific sampling stage of the food chain) if available.
- Measurement units (CFU/g or CFU/mL).
- Detection/enumeration method used.
- Previous application of *B. thuringiensis* as microbial plant protection product (MPPP)/ bioinsecticide/biopesticide.

F.5.1. Data collection and predefined values

F.5.1.1. General manuscript-level characteristics

- Author
- ID
- Title

F.5.1.2. Food item category

- Please specify, free text field

F.5.1.3. Sampling stage of the food chain

- Harvested foods (e.g. tomato)
- Minimally processed foods (e.g. fresh cut leafy greens)
- Processed foods (e.g. cheese)
- Other (please specify, free text field) (e.g. raw milk or raw meat)
- Don't know

F.5.1.4. Place of origin of food items (country)

- All countries in the world (specified individually)
- Not reported
- Other (if needed could cover e.g. a continent, region, etc.) (please specify, free text field)

F.5.1.5. Presence of *Bt*

- Analysis on food samples
  - Number of tested samples
  - Number of *B. thuringiensis*-positive samples
- Analysis on previously isolated strains from food(s), i.e. when study regards analysis of strains from specific culture collections
  - *In this case no extraction of 5.1.6., 5.1.7. or 5.1.10*
  - Number of analysed *B. thuringiensis* strains (isolates)

F.5.1.6. Enumeration (levels/counts) of *Bt*

- Indicate *B. thuringiensis* levels
- Not carried out or not reported

F.5.1.7. Measurement units for outcome

- CFU/g
- CFU/mL
- None
- Other (please specify, free text field)

F.5.1.8. Detection/enumeration method(s) used

- Conventional microbiological methods (e.g. culture)
- Molecular methods (e.g. PCR)
  Note: allow optional free text comment field for each answer
- Other (please specify, free text field)
- Not reported

F.5.1.9. Characterisation method(s) used

- Microscopic examination
- Molecular methods (e.g. PCR)
  Note: allow optional free text comment field for each answer
- Other (please specify, free text field)
- Not reported

F.5.1.10. Previous application of *B. thuringiensis* as biopesticide

- Yes
- No
- Not reported

F.6. Appraisal of the study quality

It was decided not to perform any appraisal of the study quality due to limitations in time and resources.

F.7. Analysis of data/presentation of results

The overall results will be presented in tabular format.

F.8. Evidence becoming available after deadline for retrieving evidence

Considering the time and resources available, the literature review (see Section F.3.) will not be repeated.

F.9. Human resources, software and timelines for performing the extensive literature review (ELR)

Tasks for performing the extensive literature review will be allocated among EFSA staff and WG experts as shown in Table F.5:

Provisional deadlines are given below, subject to changes depending on the volume of data retrieved.

Table F.5: Task allocation for performing the literature review

What	Who	Software	By when (provisional)
Search process	EFSA staff	Endnote	**18/12/2015**
Screening of title and abstracts	Four reviewers, two in parallel	DistillerSR	**18/1/2016**
Referee in case of doubt or divergences on title/abstract screening	Within pairs resolving divergences	NA	**18–21/1/2016**
Screening of full text	Four reviewers	DistillerSR	**15/2/2016**
Data extraction	Two reviewers	DistillerSR	**15/2/2016**
Analysis of data/presentation of results	Working Group	NA	**16/3/2016**

NA: not applicable.

Appendix G – PRISMA flow chart for the extensive literature review on the occurrence and levels of *Bacillus thuringiensis* and the list of references used for qualitative synthesis

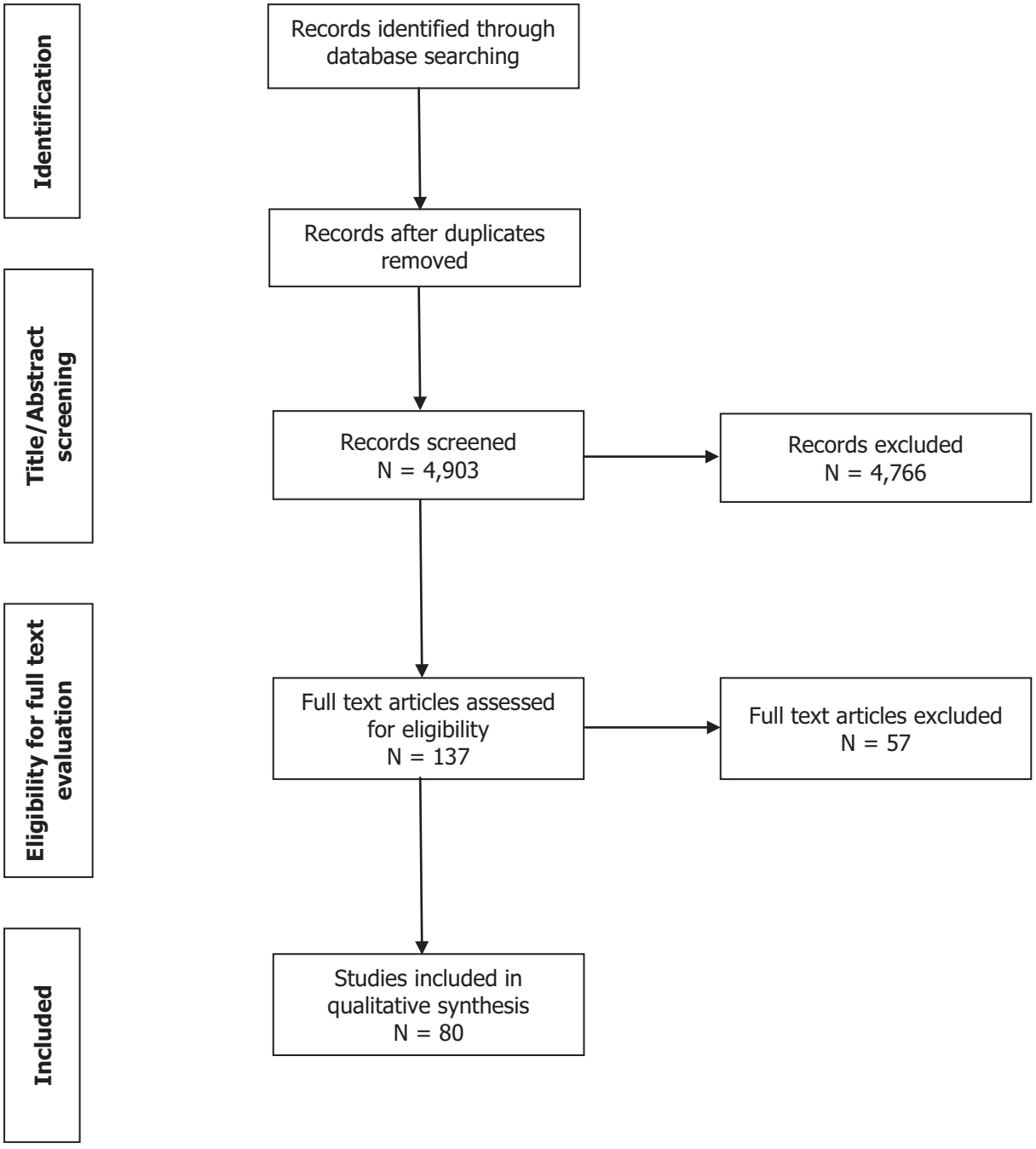


Figure G.1: PRISMA flow chart adapted from Moher et al. (2009)

G.1. List of references meeting eligibility criteria for full text evaluation and which were used for qualitative synthesis

Adewumi GA, Oguntoyinbo FA, Keisam S, Romi W and Jeyaram K, 2013. Combination of culture-independent and culture-dependent molecular methods for the determination of bacterial community of iru, a fermented *Parkia biglobosa* seeds. Frontiers in Microbiology, 4, 436.

Adewumi GA, Oguntoyinbo FA, Romi W, Singh TA and Jeyaram K, 2014. Genome subtyping of autochthonous *Bacillus* species isolated from iru, a fermented *Parkia biglobosa* seed. Food Biotechnology, 28, 250–268.

Alper M, Gunes H, Tatlipinar A, Col B, Civelek HS, Ozkan C and Poyraz B, 2014. Distribution, occurrence of *cry* genes, and lepidopteran toxicity of native *Bacillus thuringiensis* isolated from fig tree environments in Aydin Province. Turkish Journal of Agriculture and Forestry, 38, 898–907.

Amin WF and Shaker EM, 2011. Incidence and antibiotic resistance of psychrotolerant *Bacillus cereus* group in ice cream samples, with special reference to *Bacillus thuringiensis*. Assiut Veterinary Medical Journal, 57, 157–168.

Ankolekar C and Labbé RG, 2010. Physical characteristics of spores of food-associated isolates of the *Bacillus cereus* group. Applied and Environmental Microbiology, 76, 982–984.

Ankolekar C, Rahmati T and Labbé RG, 2009. Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in U.S. rice. International Journal of Food Microbiology, 128, 460–466.

Apaydin Ö, Yenidunya AF, Harsa S and Gunes H, 2005. Isolation and characterization of *Bacillus thuringiensis* strains from different grain habitats in Turkey. World Journal of Microbiology & Biotechnology, 21, 285–292.

Bae S, Fleet GH and Heard GM, 2004. Occurrence and significance of Bacillus thuringiensis on wine grapes. International Journal of Food Microbiology, 94, 301–312.

Bartoszewicz M, Hansen BM and Swiecicka I, 2008. The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. Food Microbiology, 25, 588–596.

Barus T, Kristani A and Yulandi A, 2013. Diversity of amylase-producing *Bacillus* spp. from "tape" (fermented cassava). Hayati Journal of Biosciences, 20, 94–98.

Cantoni C, Stella S, Cocolin L, Comi G, Marchese R and Ripamonti B, 2002. Identificazione di ceppi di *Bacillus* spp. isolati da varie tipologie di alimenti. Industrie Alimentari, 41, 268–273.

de Carvalho AA, Costa ED, Mantovani HC and Vanetti MC, 2007. Effect of bovicin HC5 on growth and spore germination of *Bacillus cereus* and *Bacillus thuringiensis* isolated from spoiled mango pulp. Journal of Applied Microbiology, 102, 1000–1009.

Chadare FJ, Jonkman J, Wolkers-Rooijackers J, Nout MJR, Hounhouigan JD and Zwietering MH, 2011. Microbiota of Tayohounta, a fermented baobab flavour food of Benin. African Journal of Biotechnology, 10, 15607–15615.

Chaves JQ, Cavados CF and Vivoni AM, 2012. Molecular and toxigenic characterization of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from commercial ground roasted coffee. Journal of Food Protection, 75, 518–522.

Choo E, Jang S, Kim K, Lee K, Heu S and Ryu S, 2007. Prevalence and genetic diversity of *Bacillus cereus* in dried red pepper in Korea. Journal of Food Protection, 70, 917–922.

Cinar C, Apaydin O, Yenidunya AF, Harsa S and Gunes H, 2008. Isolation and characterization of *Bacillus thuringiensis* strains from olive-related habitats in Turkey. Journal of Applied Microbiology, 104, 515–525.

Damgaard PH, Hansen BM, Pedersen JC and Eilenberg J, 1997. Natural occurrence of *Bacillus thuringiensis* on cabbage foliage and in insects associated with cabbage crops. Journal of Applied Microbiology, 82, 253–258.

Damgaard PH, Larsen HD, Hansen BM, Bresciani J and Jorgensen K, 1996. Enterotoxin-producing strains of *Bacillus thuringiensis* isolated from food. Letters in applied microbiology, 23, 146–150.

Damgaard PH, Smits PH, Hansen BM, Pedersen JC and Eilenberg J, 1994. Natural occurrence of *Bacillus thuringiensis* on cauliflower and grass foliage. Bulletin OILB/SROP, 17, 262–266.

De Clerck E and De Vos P, 2002. Study of the bacterial load in a gelatine production process focussed on *Bacillus* and related endospore forming genera. Systematic and Applied Microbiology, 25, 611–617.

De Santis EP, Foddai A, Virdis S, Marongiu P, Pilo AL and Scarano C, 2008. Toxin gene pattern in *Bacillus cereus* group strains isolated from sheep ricotta cheese. Veterinary research communications, 32 (Suppl. 1), S323–326.

Dréan P, McAuley CM, Moore SC, Fegan N and Fox EM, 2015. Characterization of the spore-forming *Bacillus cereus sensu lato* group and *Clostridium perfringens* bacteria isolated from the Australian dairy farm environment. BMC Microbiology, 15.

Femi-Ola TO, Falegan CR and Adebule OM, 2014. Isolation, characterization and distribution of *Bacillus* strains in some Nigerian fermented food and drinks. International Journal of Agriculture Innovations and Research, 2, 717–719.

Fernández-No IC, Böhme K, Caamaño-Antelo S, Barros-Velázquez J and Calo-Mata P, 2015. Identification of single nucleotide polymorphisms (SNPs) in the 16S rRNA gene of foodborne *Bacillus* spp. Food Microbiology, 46, 239–245.

Fernández-No IC, Böhme K, Díaz-Bao M, Cepeda A, Barros-Velázquez J and Calo-Mata P, 2013. Characterisation and profiling of *Bacillus subtilis*, *Bacillus cereus* and *Bacillus licheniformis* by MALDI-TOF mass fingerprinting. Food Microbiology, 33, 235–242.

Forghani F, Kim JB and Oh DH, 2014. Enterotoxigenic profiling of emetic toxin- and enterotoxin-producing *Bacillus cereus*, isolated from food, environmental, and clinical samples by multiplex PCR. Journal of Food Science, 79, M2288–M2293.

Frederiksen K, Rosenquist H, Jorgensen K and 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, 3435–3440.

te Giffel MC, Beumer RR, Klijn N, Wagendorp A and Rombouts FM, 1997. Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on variable regions of 16S rRNA. FEMS Microbiology Letters, 146, 47–51.

Hariram U and Labbé R, 2015. Spore prevalence and toxigenicity of *Bacillus cereus* and *Bacillus thuringiensis* isolates from U.S. retail spices. Journal of Food Protection, 78, 590–596.
Harmon SM, Kautter DA and Lancette G, 1991. Lipid globule staining to aid in differentiating *Bacillus* species. Journal of the Association of Official Analytical Chemists, 74, 649–651.
Hatzikamari M, Yiangou M, Tzanetakis N and Litopoulou-Tzanetaki E, 2007. Changes in numbers and kinds of bacteria during a chickpea submerged fermentation used as a leavening agent for bread production. International Journal of Food Microbiology, 116, 37–43.
Hendriksen NB and Hansen BM, 2006. Detection of *Bacillus thuringiensis kurstaki* HD1 on cabbage for human consumption. FEMS Microbiology Letters, 257, 106–111.
Hosseini H, Hippe B, Denner E, Kollegger E and Haslberger A, 2012. Isolation, identification and monitoring of contaminant bacteria in Iranian Kefir type drink by 16S rDNA sequencing. Food Control, 25, 784–788.
Hsu HH, Chuang TC, Lin HC, Huang YR, Lin CM, Kung HF and Tsai YH, 2009. Histamine content and histamine-forming bacteria in dried milkfish (*Chanos chanos*) products. Food Chemistry, 114, 933–938.
Jackson SG, Goodbrand RB, Ahmed R and Kasatiya S, 1995. *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. Letters in applied microbiology, 21, 103–105.
Jang JH, Lee NA, Woo GJ and Park JH, 2006. Prevalence of *Bacillus cereus* group in rice and distribution of enterotoxin genes. Food Science and Biotechnology, 15, 232–237.
Jara S, Maduell P and Orduz S, 2006. Diversity of *Bacillus thuringiensis* strains in the maize and bean phylloplane and their respective soils in Colombia. Journal of Applied Microbiology, 101, 117–124.
Kaur S and Singh A, 2000. Natural occurrence of *Bacillus thuringiensis* in leguminous phylloplanes in the New Delhi region of India. World Journal of Microbiology & Biotechnology, 16, 679–682.
Kaynar P and Beyatli Y, 2008. Protein profiles and biochemical characterizations of *Bacillus* spp. strains isolated from fishes. Fresenius Environmental Bulletin, 17, 1316–1321.
Kim B, Bang J, Kim H, Kim Y, Kim BS, Beuchat LR and Ryu JH, 2014. *Bacillus cereus* and *Bacillus thuringiensis* spores in Korean rice: prevalence and toxin production as affected by production area and degree of milling. Food Microbiology, 42, 89–94.
Kim YR, Czajka J and Batt CA, 2000. Development of a fluorogenic probe-based PCR assay for detection of *Bacillus cereus* in nonfat dry milk. Applied and Environmental Microbiology, 66, 1453–1459.
Kitnamorti T, Rathinam X and Subramaniam S, 2011. Novel isolation and characterization techniques for *Bacillus thuringiensis* strains from the cabbage growing area in Cameron Highlands Malaysia. African Journal of Microbiology Research, 5, 3343–3350.
Kumar TDK, Murali HS and Batra HV, 2010. Multiplex PCR assay for the detection of enterotoxic *Bacillus cereus* group strains and its application in food matrices. Indian journal of microbiology, 50, 165–171.
Li P, Li S, Cheng L and Luo L, 2014. Analyzing the relation between the microbial diversity of DaQu and the turbidity spoilage of traditional Chinese vinegar. Applied microbiology and biotechnology, 98, 6073–6084.
Manzano M, Giusto C, Iacumin L, Cantoni C and Comi G, 2009. Molecular methods to evaluate biodiversity in *Bacillus cereus* and *Bacillus thuringiensis* strains from different origins. Food Microbiology, 26, 259–264.
Martinez-Blanch JF, Sanchez G, Garay E and 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, 15–21.
Martinez-Blanch JF, Sanchez G, Garay E and Aznar R, 2011. Evaluation of phenotypic and PCR-based approaches for routine analysis of *Bacillus cereus* group foodborne isolates. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 99, 697–709.
Matarante A, Baruzzi F, Cocconcelli PS and Morea M, 2004. Genotyping and toxigenic potential of *Bacillus subtilis* and *Bacillus pumilus* strains occurring in industrial and artisanal cured sausages. Applied and Environmental Microbiology, 70, 5168–5176.
McGaughey WH, Dicke EB, Finney KF, Bolte LC and Shogren MD, 1980. Spores in dockage and mill fractions of wheat treated with *Bacillus thuringiensis*. Journal of Economic Entomology, 73, 775–778.
McIntyre L, Bernard K, Beniac D, Isaac-Renton JL and Naseby DC, 2008. Identification of *Bacillus cereus* group species associated with food poisoning outbreaks in British Columbia, Canada. Applied and Environmental Microbiology, 74, 7451–7453.
Miguel PSB, Delvaux JC, de Oliveira MNV, Monteiro LCP, de Souza Freitas F, Costa MD, Tótola MR, de Moraes CA and Borges AC, 2013. Diversity of endophytic bacteria in the fruits of *Coffea canephora*. African Journal of Microbiology Research, 7, 586–594.
Mitra S, Mukhopadhyay BC and Biswas SR, 2011. Potential application of the nisin Z preparation of *Lactococcus lactis* W8 in preservation of milk. Letters in Applied Microbiology, 53, 98–105.
Molva C, Sudagidan M and Okuklu B, 2009. Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey. Food Control, 20, 829–834.
Ngamwongsatit P, Buasri W, Pianariyanon P, Pulsrikarn C, Ohba M, Assavanig A and Panbangred W, 2008. Broad distribution of enterotoxin genes (*hblCDA*, *nheABC*, *cytK*, and *entFM*) among *Bacillus thuringiensis* and *Bacillus cereus* as shown by novel primers. International Journal of Food Microbiology, 121, 352–356.
Nissen H, Holo H, Axelsson L and Blom H, 2001. Characterization and growth of *Bacillus* spp. in heat-treated cream with and without nisin. Journal of Applied Microbiology, 90, 530–534.

Nour MA, Tohami M and Khater AAK, 2002. Identification and characterization of *Bacillus cereus* group isolated from different sources. Egyptian Journal of Dairy Science, 30, 1–14.

Oguntoyinbo FA, Huch M, Cho GS, Schillinger U, Holzapfel WH, Sanni AI and Franz CM, 2010. Diversity of *Bacillus* species isolated from Okpehe, a traditional fermented soup condiment from Nigeria. Journal of Food Protection, 73, 870–878.

Ouattara HG, Reverchon S, Niamke SL and Nasser W, 2011. Molecular identification and pectate lyase production by *Bacillus* strains involved in cocoa fermentation. Food Microbiology, 28, 1–8.

Padonou SW, Nielsen DS, Hounhouigan JD, Thorsen L, Nago MC and Jakobsen M, 2009. The microbiota of Lafun, an African traditional cassava food product. International Journal of Food Microbiology, 133, 22–30.

Phelps RJ and McKillip JL, 2002. Enterotoxin production in natural isolates of *Bacillaceae* outside the *Bacillus cereus* group. Applied and Environmental Microbiology, 68, 3147–3151.

Pirttijärvi TS, Ahonen LM, Maunuksela LM and Salkinoja-Salonen MS, 1998. *Bacillus cereus* in a whey process. International Journal of Food Microbiology, 44, 31–41.

Pirttijärvi TS, Andersson MA, Scoging AC and Salkinoja-Salonen MS, 1999. Evaluation of methods for recognising strains of the *Bacillus cereus* group with food poisoning potential among industrial and environmental contaminants. Systematic and Applied Microbiology, 22, 133–144.

Rabinovitch L, de Jesus FF, Cavados CF, Zahner V, Momen H, da Silva MH, Dumanoir VC, Frachon E and Lecadet MM, 1995. *Bacillus thuringiensis* subsp. *oswaldocruzi* and *Bacillus thuringiensis* subsp. *brasiliensis*, two novel Brazilian strains which determine new serotype H38 and H39, respectively. Memorias do Instituto Oswaldo Cruz, 90, 41–42.

Rai AK, Tamang JP and Palni U, 2010. Microbiological studies of ethnic meat products of the Eastern Himalayas. Meat science, 85, 560–567.

Rather MA, Aulakh RS, Gill JPS, Verma R and Rao TS, 2011. Enterotoxigenic profile of *Bacillus cereus* strains isolated from raw and pasteurized milk. Indian Journal of Animal Sciences, 81, 448–452.

de Rijk TC, van Dam RC, Zomer P, Boers EA, de Waard P and Mol HG, 2013. Development and validation of a confirmative LC-MS/MS method for the determination of beta-exotoxin thuringiensin in plant protection products and selected greenhouse crops. Analytical and Bioanalytical Chemistry, 405, 1631–1639.

Roman-Blanco C, Sanz-Gomez J, Lopez-Diaz T-M, Otero A and Garcia-Lopez M-L, 1999. Numbers and species of *Bacillus* during the manufacture and ripening of Castellano cheese. Milchwissenschaft, 54, 385–388.

Rosenquist H, Smidt L, Andersen SR, Jensen GB and Wilcks A, 2005. Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. FEMS Microbiology Letters, 250, 129–136.

Rusul G and Yaacob NH, 1995. Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. International Journal of Food Microbiology, 25, 131–139.

Sandra A, Afsah-Hejri L, Tunung R, Tuan Zainazor TC, Tang JYH, Ghazali FM, Nakaguchi Y, Nishibuchi M and Son R, 2012. *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat cooked rice in Malaysia. International Food Research Journal, 19, 829–836.

Sarkar PK, Hasenack B and Nout MJR, 2002. Diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala). International Journal of Food Microbiology, 77, 175–186.

Seifinejad A, Jouzani GRS, Hosseinzadeh A and Abdmishani C, 2008. Characterization of Lepidoptera-active *cry* and *vip* genes in Iranian *Bacillus thuringiensis* strain collection. Biological Control, 44, 216–226.

Souza A, Cruz JC, Sousa NR, Procópio ARL and Silva GF, 2014. Endophytic bacteria from banana cultivars and their antifungal activity. Genetics and molecular research, 13, 8661–8670.

Thorsen L, Abdelgadir WS, Rønsbo MH, Abban S, Hamad SH, Nielsen DS and Jakobsen M, 2011. Identification and safety evaluation of *Bacillus* species occurring in high numbers during spontaneous fermentations to produce Gergoush, a traditional Sudanese bread snack. International Journal of Food Microbiology, 146, 244–252.

Thorsen L, Kando CK, Sawadogo H, Larsen N, Diawara B, Ouédraogo GA, Hendriksen NB and Jespersen L, 2015. Characteristics and phylogeny of *Bacillus cereus* strains isolated from Maari, a traditional West African food condiment. International Journal of Food Microbiology, 196, 70–78.

Väisänen OM, Mwaisumo NJ and Salkinoja-Salonen MS, 1991. Differentiation of dairy strains of the *Bacillus cereus* group by phage typing, minimum growth temperature, and fatty acid analysis. Journal of Applied Bacteriology, 70, 315–324.

Vidal-Quist JC, Castañera P and González-Cabrera J, 2009. Diversity of *Bacillus thuringiensis* strains isolated from citrus orchards in Spain and evaluation of their insecticidal activity against *Ceratitis capitata*. Journal of Microbiology and Biotechnology, 19, 749–759.

Zhou G, Liu H, He J, Yuan Y and Yuan Z, 2008a. The occurrence of *Bacillus cereus*, *B. thuringiensis* and *B. mycoides* in Chinese pasteurized full fat milk. International Journal of Food Microbiology, 121, 195–200.

Zhou G, Yan J, Dasheng Z, Zhou X and Yuan Z, 2008b. The residual occurrences of *Bacillus thuringiensis* biopesticides in food and beverages. International Journal of Food Microbiology, 127, 68–72.

Zhou G, Zheng D, Dou L, Cai Q and Yuan Z, 2010. Occurrence of psychrotolerant *Bacillus cereus* group strains in ice creams. International Journal of Food Microbiology, 137, 143–146.

Appendix H – Summary of the information extracted from papers meeting the eligibility criteria for the full text screening in the scope of the extensive literature review on occurrence and levels of *Bacillus thuringiensis* in food

Table H.1: Summary of the data extracted from the literature review on the occurrence of *B. thuringiensis* in food

Food	Number of samples	Number of *B. cereus* group isolates	Number of *B. thuringiensis*-positive samples or number of *B. thuringiensis* isolates[f]	*B. thuringiensis* levels	Detection methods[d]	Reference[g]
Raw fresh vegetables						
Cabbage and sprouts						
Cabbage foliage	63	NA	34 samples/ 321 isolates	NA	C	Damgaard et al. (1997)
Cabbage leaves	30	NA	8	NA	C, GE	Kitnamorti et al. (2011)
Cabbage, cauliflower, Chinese cabbage, sprouts	10 batches	NA	26 isolates	NA	C	Hendriksen and Hansen (2006)
Broccoli	10 batches	NA	48 isolates	NA	C	Hendriksen and Hansen (2006)
Cauliflower						
Cauliflower stowage[a]	NA	40[a]	1 isolate	NA	C, M[e]	Rosenquist et al. (2005)
Cauliflower leaves	NA	NA	20 isolates	80–1,700 CFU/ cm^2 leaf	C, M	Damgaard et al. (1994)
Tomato[b]	44[b]	NA	0 isolates	NA	C, LC	de Rijk et al. (2013)
Cucumber[b]	44[b]	NA	0 isolates	NA	C, LC	de Rijk et al. (2013)
Pepper						
Red pepper[a]	NA	40[a]	2 isolates	NA	C, M[e]	Rosenquist et al. (2005)
Bell pepper[b]	44[b]	NA	0 isolates	NA	C, LC	de Rijk et al. (2013)
Leafy greens						
Dill[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Spinach[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Spinach stowage[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Salad[a]	NA	40[a]	2 isolates	NA	C, M	Rosenquist et al. (2005)
Parsley[a]	NA	40[a]	2 isolates	NA	C, M	Rosenquist et al. (2005)
Eggplant[b]	44[b]	NA	0 isolates	NA	C, LC	de Rijk et al. (2013)
Leek[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Carrots[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)

Food	Number of samples	Number of *B. cereus* group isolates	Number of *B. thuringiensis*-positive samples or number of *B. thuringiensis* isolates[f]	*B. thuringiensis* levels	Detection methods[d]	Reference[g]
Processed vegetables						
Dried red pepper	140	NA	19 samples	NA	C, M	Choo et al. (2007)
Olive pomace	13	NA	3 samples	NA	C	Cinar et al. (2008)
Fig spread[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Fruits						
Figs	130	NA	103 samples/ 376 isolates	NA	C, M	Alper et al. (2014)
Grapes	NA	NA	NA	2–6 log CFU/g	M	Bae et al. (2004)
Citrus (damaged fallen fruits)	27	NA	19 samples	NA	M	Vidal-Quist et al. (2009)
Strawberry[b]	44[b]	NA	0 isolates	NA	C, LC	de Rijk et al. (2013)
Legumes and grains and bread						
Raw rice	178		11 samples	Up to 23 CFU/g	C	Ankolekar et al. (2009)
Grain	13		3 samples	NA	C	Apaydin et al. (2005)
Raw rice	136		11 isolates	NA	C, M	Jang et al. (2006)
Unhusked rice samples	31		6 isolates	NA	C, M	Jang et al. (2006)
Brown rice	26		1 isolate	NA	C, M	Jang et al. (2006)
Kimbap	44		1 isolate	NA	C, M	Jang et al. (2006)
Japanese vinegard rice delicacies	35		3 isolates	NA	C, M	Jang et al. (2006)
Maize leaves	48		27 samples/ 135 isolates	0.46 spores/cm^2	C, M	Jara et al. (2006)
Bean leaves	48		22 samples/ 82 isolates	1.5 spores/cm^2	C, M	Jara et al. (2006)
Rice	189		13 samples	2.0–11.2 CFU/g	C	Kim et al. (2014)
Rough rice	NA		1 sample	11.2 CFU/g	C	Kim et al. (2014)
Brown rice	NA		6 samples	2.0 CFU/g	C	Kim et al. (2014)
White rice	NA		6 samples	2.0–10.0 CFU/g	C	Kim et al. (2014)
Chickpea leaves	5		36 isolates	NA	C	Kaur and Singh (2000)
Pigeon pea leaves	5		40 isolates	NA	C	Kaur and Singh (2000)
Pea leaves	5		21 isolates	NA	C	Kaur and Singh (2000)
Mung bean leaves	5		15 isolates	NA	C	Kaur and Singh (2000)
Baby maize[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)

Food	Number of samples	Number of *B. cereus* group isolates	Number of *B. thuringiensis*-positive samples or number of *B. thuringiensis* isolates[f]	*B. thuringiensis* levels	Detection methods[d]	Reference[g]
Spices						
Chives, garlic, paprika powder, cardamom seed, cayenne powder, chicken spices, jaifal powder (nutmeg), anardana powder, garlic powder among others	247		11 isolates	Spore levels: 3–240 MPN/g	C, M	Hariram and Labbé (2015)
Onion powder	NA	10	NA	NA	C, P	Jackson et al. (1995)
Milk and dairy products						
Pasteurised milk	32	NA	NA	17 ± 3 CFU/mL	C, M	Hosseini et al. (2012)
Milk (raw and pasteurised)	160	NA	6 samples	NA	C, M	Rather et al. (2011)
Milk	44	111	10 isolates	4–18 spores/L	C	Bartoszewicz et al. (2008)
Raw milk[c]	27 mixed	46	4 isolates	NA	C, PFGE	Dréan et al. (2015)
Pasteurised milk	10	NA	1 isolate	NA	C, M	Mitra et al. (2011)
Milk (pasteurised full fat milk)	54	102	6 samples/ 9 isolates	3.0–11.0 MPN/mL	C, M	Zhou et al. (2008a)
Milk (non-fat dry milk)	43	NA	2 isolates	93 and 1,100 CFU/g	C, M	Kim et al., 2000
Cheese	100	NA	22 isolates	NA	C	Molva et al. (2009)
Hard ripened Castellano cheese	NA	158	4 isolates	NA	C, B	Roman-Blanco et al. (1999)
Sheep ricotta cheese	66	NA	29 isolates	NA	V	De Santis et al. (2008)
Heat-treated cream	10	40	0 isolates	NA	C	Nissen et al. (2001)
Ice cream	150	NA	11 samples	NA	C, M	Amin and Shaker et al. (2011)
Ice cream	40	NA	1 sample	3.6 CFU/g	C, M	Zhou et al. (2008b)
Fermented foods						
Iru (fermented condiment from African locust bean seeds)	16	NA	4 isolates	NA	C, M	Adewumi et al. (2013)

Food	Number of samples	Number of *B. cereus* group isolates	Number of *B. thuringiensis*-positive samples or number of *B. thuringiensis* isolates[f]	*B. thuringiensis* levels	Detection methods[d]	Reference[g]
Tape (fermented cassava)	NA	26 amylase-producing *Bacillus* spp.	2 isolates	NA	C, M	Barus et al. (2013)
Tayohounta (fermented baobab seed kernels)	3 producers		1 producer	NA	C, M	Chadare et al. (2011)
Nigerian fermented foods	NA	22	NA	NA	C	Femi-Ola et al. (2014)
Yoghurt	NA	NA	1 isolate	NA	C	Femi-Ola et al. (2014)
Ogi	NA	NA	0 isolates	NA	C	Femi-Ola et al. (2014)
Burukutu	NA	NA	0 isolates	NA	C	Femi-Ola et al. (2014)
Garri	NA	NA	0 isolates	NA	C	Femi-Ola et al. (2014)
Palmwine	NA	NA	1 isolate	NA	C	Femi-Ola et al. (2014)
Iranian Kefir type drink	32	NA	NA	30 ± 10 CFU/mL	C, M	Hosseini et al. (2012)
Fermented coarsely ground chickpea (4 experiments of fermentation)	NA	59	7 isolates	NA	C, B, SP	Hatzikamari et al. (2007)
Lafun (fermented cassava product)	NA	9	0 isolates	NA	C, M	Padonou et al. (2009)
Maari (fermented condiment from baobab tree seeds)	NA	NA	*B. thuringiensis* isolated, but unknown numbers	NA	C, M	Thorsen et al. (2015)
Indian Kinema (fermented soybeans)	33	83	2 isolates	NA	C, M	Sarkar et al. (2002)
African Soumbala (fermented locust beans)	14	43	4 isolates	NA	C, M	Sarkar et al. (2002)
Meat and fish						
Dried milkfish	32	NA	2 isolates	NA	C, M	Hsu et al. (2009)
Various fresh fishes	NA	NA	NA	NA	C, B, SP	Kaynar and Beyatli (2008)
Whiting	NA	NA	2 isolates	NA	C, B, SP	Kaynar and Beyatli (2008)
Anchovy	NA	NA	1 isolate	NA	C, B, SP	Kaynar and Beyatli (2008)

Food	Number of samples	Number of *B. cereus* group isolates	Number of *B. thuringiensis*-positive samples or number of *B. thuringiensis* isolates[f]	*B. thuringiensis* levels	Detection methods[d]	Reference[g]
Cured ready-to-eat sausages	8	NA	1 isolate	NA	M	Matarante et al. (2004)
Himalayan meat (sausage-like) from beef	17	NA	1 isolate	NA	C, B	Rai et al. (2010)
Himalayan air dried or smoked chevon or buffalo meat product	6	NA	1 isolate	NA	C, B	Rai et al. (2010)
Raw sausage[a]	1,666	40[a]	1 isolate	NA	C, M[e]	Rosenquist et al. (2005)
Kebab[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Beverages						
Roasted ground coffee beans	30	NA	8 samples	10–100 CFU/g	C	Chaves et al. (2012)
Coffee fruits	63	NA	2 isolates	NA	C, M	Miguel et al. (2013)
Green tea	2	NA	2 samples/ 9 isolates	ND (not determined) or < 1	C, M	Zhou et al. (2008b)
Wine and grape juice	21	NA	NA	50–5,000 CFU/mL	C, M	Bae et al. (2004)
Wine	9	NA	NA	50–5,000 CFU/mL	C, M	Bae et al. (2004)
Grape juice	12	NA	NA	50 CFU/mL	C, M	Bae et al. (2004)
Chinese vinegar	6	NA	6	About 3 log CFU/mL	C, M	Li et al. (2014)
Soft ice[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Sweets						
Honey[a]	NA	40[a]	2 isolates	NA	C, M	Rosenquist et al. (2005)
Strawberry tart[a]	NA	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Ready-to-eat (RTE) foods						
Bread[a]	53	40[a]	1 isolate	NA	C, M	Rosenquist et al. (2005)
Milk and rice-based dishes	40	NA	1 sample	NA	C, M	Kumar et al. (2010)
Milk	20	NA	NA	NA	C, M	Kumar et al. (2010)
Rice-based dishes	20	NA	NA	NA	C, M	Kumar et al. (2010)
Pasta[a]	2,216	40[a]	9 isolates	NA	C, M[e]	Rosenquist et al. (2005)
Pasta salad[a]	593	40[a]	1 isolate	NA	C, M[e]	Rosenquist et al. (2005)

Food	Number of samples	Number of *B. cereus* group isolates	Number of *B. thuringiensis*-positive samples or number of *B. thuringiensis* isolates[f]	*B. thuringiensis* levels	Detection methods[d]	Reference[g]
Meals	58	459	70 isolates	NA	C, B	Rusul and Yaacob (1995)
Rice noodles	3	NA	NA	NA	C, B	Rusul and Yaacob (1995)
Wet wheat noodles	2	NA	NA	NA	C, B	Rusul and Yaacob (1995)
Dried wheat noodles	10	NA	NA	NA	C, B	Rusul and Yaacob (1995)
Spices	8	NA	NA	NA	C, B	Rusul and Yaacob (1995)
Grains	4	NA	NA	NA	C, B	Rusul and Yaacob (1995)
Legumes	11	NA	NA	NA	C, B	Rusul and Yaacob (1995)
Legume products	3	NA	NA	NA	C, B	Rusul and Yaacob (1995)
Variety of cooked foods	17	NA	NA	NA	C, B	Rusul and Yaacob (1995)
Cooked rice	115	NA	28 samples	NA	MPN, M	Sandra et al. (2012)
Nasi lemak (rice cooked with coconut milk)	54	NA	19 samples	> 1,100 MPN/g (maximum)	MPN, M	Sandra et al. (2012)
Nasi briyani (Persian rice)	20	NA	6 samples	93 MPN/g (maximum)	MPN, M	Sandra et al. (2012)
Nasi ayam (chicken rice)	20	NA	2 samples	3.6 MPN/g (maximum)	MPN, M	Sandra et al. (2012)
Nasi putih (white rice)	21	NA	1 sample	9.2 MPN/g (maximum)	MPN, M	Sandra et al. (2012)
Raw rice	25	NA	0 samples	NA	MPN, M	Sandra et al. (2012)
Keladi halus wangi	5	NA	0 samples	< 3 MPN/g (maximum)	MPN, M	Sandra et al. (2012)
Keladi wangi	5	NA	0 samples	< 3 MPN/g (maximum)	MPN, M	Sandra et al. (2012)
Kanowit halus wangi	5	NA	0 samples	< 3 MPN/g (maximum)	MPN, M	Sandra et al. (2012)
Lansam halus wangi	5	NA	0 samples	< 3 MPN/g (maximum)	MPN, M	Sandra et al. (2012)
Bario	5	NA	0 samples	< 3 MPN/g (maximum)	MPN, M	Sandra et al. (2012)

Food	Number of samples	Number of *B. cereus* group isolates	Number of *B. thuringiensis*-positive samples or number of *B. thuringiensis* isolates[f]	*B. thuringiensis* levels	Detection methods[d]	Reference[g]
Legumes used for production of Gergoush (traditional fermented Sudanese bread snack)	NA	180	62 isolates	NA	C, M	Thorsen et al. (2011)
Chickpeas	NA	45	6 isolates	NA	C, M	Thorsen et al. (2011)
Faba beans	NA	45	5 isolates	NA	C, M	Thorsen et al. (2011)
Lentils	NA	45	6 isolates	NA	C, M	Thorsen et al. (2011)
White beans	NA	45	45 isolates	NA	C, M	Thorsen et al. (2011)

NA: not available; CFU: colony forming unit(s).
(a): Study which evaluated 40 randomly selected isolates obtained from different food commodities. 31 out of 40 were classified as *B. thuringiensis*.
(b): Study which evaluated 44 different samples of different fruits and vegetables.
(c): 27 samples of soil, faeces, feed (grain), raw milk and milk filter. Number of milk samples not specified.
(d): Detection Method: C = culture + microscopy; M = molecular methods; LC = LC-MS (liquid chromatography-tandem mass spectrometry); SP = SDS-PAGE analysis; P = phage typing, tissue culture testing (cytotoxicity); PFGE = pulsed field gel electrophoresis typing; B = biochemical, MPN = most probable number, V = Vitek system.
(e): By comparing the profiles of the food isolates with the profiles of the commercial biopesticides it was observed that five strains isolated from sausage, pasta, red pepper (x2), and cauliflower stowage had profiles similar to the commercial Dipel strain.
(f): Numbers refer to *B. thuringiensis*-positive samples or number of *B. thuringiensis* isolates depending on what was available and indicated under the columns 'number of samples' and 'number of *B. cereus* group isolates'.
(g): Details for all references cited in Table H.1 (Appendix H) are available under the list of references meeting eligibility criteria for full text evaluation in Appendix G.

Annex A – Information received from the International Biocontrol Manufacturers Association (IBMA) on 18 January 2016 (Prepared by Valent BioSciences, Certis USA and CBC (Europe))

Annex A can be found in the online version of this output ('Supporting information' section): http://dx.doi.org/10.2903/j.efsa.2016.4524