Bacillus cereus bacteria in foodstuffs may cause gastrointestinal diseases

Updated BfR Opinion No. 048/2020 of 30 October 2020[1]

Bacillus (*B.*) *cereus* is the name-giving representative of the *B. cereus* group, which currently includes 17 recognised, closely related species that can only be distinguished from each with great difficulty. Consequently, in food inspections mostly only the so called presumptive *B. cereus* is detected, which means: a bacterium from the *B. cereus* group. The current opinion provides information about health risks from bacteria of the *B. cereus* group in foods and names preventative measures, mainly in order to create a basis for assessing foodstuffs by food control authorities in Germany.

The German Federal Institute for Risk Assessment (BfR) has analysed studies and their own investigation results on the subject and has ascertained that each presumptive *B. cereus* strain can be assumed to be able to form toxins, although the toxin types and amounts of toxins formed vary. These toxins may cause gastrointestinal diseases. Two different types of illness are distinguished; one which is characterised by vomiting (emetic illness), and the other which is accompanied by diarrhoea (diarrhoeal type). These gastrointestinal illnesses can affect people of all ages, are not infectious and rarely last longer than 24 hours. It is very rare for these illnesses to become severe.

Contamination of food by presumptive *B. cereus* cannot be completely avoided. This is because persistent forms of these bacteria (spores), can be transferred to food via soil particles or dust, and also survive extreme conditions such as heat or dehydration for long periods. Initial contamination of food with spores is often very low. However, spores can germinate as a result of improper storage, and bacteria can multiply in food. *B. cereus* grows within a range of 7 to 48 °C. However, the *B. cereus* group also comprises cold tolerant members which can multiply at temperatures of around 4 °C as well thermotolerant members, which can grow even above 50 °C. At low temperatures the microbial growth is, however, significantly slower. Usually, a bacterial count of at least 10^5 colony-forming units per gram (CFU/g) is required to form quantities of toxins in food or the small intestine that cause illness symptoms.

Conventional heat treatments, such as cooking or pasteurisation, do kill bacterial cells, but enable individual spores to survive and germinate. Fast and sufficient cooling (≤ 7 °C) and/or hot holding (≥ 60 °C) is necessary after meals have been treated with heat (at least 70 °C for two minutes throughout the food), to inhibit the germination of spores and consequently, the multiplication of bacteria.

1 Subject of the assessment

Bacillus (*B.*) *cereus* is the name-giving representative of the so-called *B. cereus* group, which currently includes 17 recognised, closely related species, which can only be distinguished from each other with great difficulty. These species include *B. cereus* (*sensu stricto* (*s.s.*)), *B. thuringiensis*, *B. cytotoxicus*, *B. toyonensis*, *B. wiedmannii*, *B. anthracis*, *B. mycoides* and *B. pseudomycoides*, which were already described until 2016. Additionally, nine other species were described by Liu et al. (2017): *B. paranthracis*, *B. pacificus*, *B. tropicus*, *B. albus, B. mobilis, B. luti, B. proteolyticus, B. nitratireducens* und *B. paramycoides*. So far, these new species could only be differentiated from each other, and from the established species of the

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[1] This opinion replaces the opinion No. 035/2019 of the BfR of 16 September 2019

B. cereus group, by means of whole genome sequencing. To date, there is barely any available data about these new representatives beyond actual species descriptions.

Since 2018, the species *B. weihenstephanensis* is regarded as synonymous with *B. mycoides* (Liu et al., 2018; parte et al., 2020). Therefore, all strains previously known as *B. weihenstephanensis* are now classified as *B. mycoides*. The structure of the *B. cereus* group is expected to change significantly in future (Carroll et al., 2020).
So far, during routine food testing individual species are rarely distinguished from each other. However, some laboratories are able to further differentiate species using methods such as microscopy, PCR or FT-IR (Fourier transformation infrared spectroscopy).

In the literature (and this report), the terms "*B. cereus* group", "*B. cereus* (*sensu lato* (*s.l.*))" and "presumptive *B. cereus*" are frequently used synonymously. The term "presumptive *B. cereus*" has also been used in the relevant DIN EN ISO standards[2], with which no clear distinction can be made between individual representatives of the *B. cereus* group.

B. cereus (*s.l.*), with the ability to form toxins, occur in low numbers in many foods. Generally, illnesses in humans after consuming contaminated food occur when these bacteria have been able to multiply to high numbers as a result of incorrect storage of food, and have formed toxins either in the food itself or in the human small intestine.

In order to provide official food control authorities in Germany with a basis for assessing foods contaminated with *B. cereus (s.l.)*, the BfR analysed existing literature and their own research data on bacterial strains from the *B. cereus* group. This opinion summarises essential information on *B. cereus (s.l.)* concerning food, the hazard arising from these bacteria and the key preventative measures. This opinion does not concern the anthrax pathogen (*B. anthracis*) and the hazards it poses.

2   Result

After evaluating the literature and its own research results, the BfR concludes that each *B. cereus (s.l.)* strain is a potential former of enterotoxins, although types and amounts of formed toxins may vary. It is therefore possible that the consumption of foods which contain very high levels of *B. cereus (s.l.)* (from 10^5 colony-forming units per gram (CFU/g)) and/or numbers of *B. cytotoxicus* from 10^4 CFU/g, triggers diarrhoeal illness in humans within a short time period. For strains from the *B. cereus* group which only possess a limited ability to multiply and/or form toxins at human body temperature (*B. mycoides;* synonymous *B. weihenstephanensis*), the probability of causing foodborne diarrhoeal illness is low.
The number of bacteria and with it the risk to health, can only be slightly minimised (if at all) by the thorough washing of plant-based foods with drinking water. Due to the high heat resistance of spores, they can only be completely eliminated when strong heating methods are used, such as the ones used in the canning industry (e.g. 121 degree Celsius (°C), 3 minutes (min)).

However, even at lower *B. cereus* levels (from ca. 10^3 CFU/g) in a food product, a risk of causing illness may emanate from the food should the strain in question possess the ability

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[2] DIN EN ISO 7932:2004, Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of presumptive *Bacillus cereus* - Colony count technique at 30 °C;
DIN EN ISO 10198:2010, Microbiological analysis of milk - Determination of presumptive *Bacillus cereus* - Colony count technique at 37 °C;
DIN EN 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 (MPN) and detection method

to form cereulide and the conditions before consumption of the food support the growth of the strain and the toxin formation. The risk can be minimised if temperatures in which *B. cereus* (*s.l.*) multiplies are avoided when storing meals and drinks containing milk.

If a relevant quantity of cereulide is already present in the food, it can be assumed that the possibility of damage to health cannot be ruled out, as cereulide is extremely stable (> 120 min at 121 °C). Due to the inconsistent information on the possible minimal intoxication dose, from a BfR perspective no universally applicable cereulide limit in foods can be determined, meaning that a legal assessment of a food containing cereulide can only be carried out on a case-by-case basis.

To prevent foodborne infections and food poisoning, the BfR recommends observing the following kitchen hygiene rules when storing and preparing food in the catering industry and other communal catering institutions, as well as in private households:

- Maintain the cold chain for perishable foods.
- Cook dishes thoroughly during preparation, and when rewarming, heat sufficiently to kill any vegetative cells (at least 70 °C for two minutes throughout the food; check the temperature with a meat thermometer in case of doubt); the same applies to rewarming of food in microwaves (make sure heat is distributed equally, stir food at intervals).
- Cool prepared, heated food quickly to ≤ 7 °C (fill larger quantities of food into several shallow bowls) and store in the refrigerator until reheating shortly before consumption
- When holding food hot (e.g. soups, sauces, stews), make sure that a temperature of at least +60 °C is kept throughout the food. Do not store easily perishable foods or heated foods at temperatures between +7 °C and +60 °C for long periods.
- Store the leftovers of cooked dishes in the refrigerator and consume within two to three days.
- Dispose of water used for soaking dried mushrooms carefully and thoroughly clean hands, as well as all objects or working areas which have been in contact with the soaking water or soaked mushrooms.

3 Rationale

3.1 Risk assessment

3.1.1 *Bacillus cereus* and other representatives of the *Bacillus cereus* group (hazard identification)

B. cereus (s.s.) is a ubiquitous gram-positive, facultatively anaerobic, mobile, spore-forming rod-shaped bacterium. Its spores can be found in soil, water, human and animal intestinal tracts, and several foods of animal and particularly plant origin (EFSA BIOHAZ Panel, 2016). Due to its ability to form toxins under certain circumstances, the bacterium is of great significance as a pathogen causing foodborne diseases. Additionally, its growth can cause food spoilage.

Some strains, which have been designated emetic *B. cereus* (*s.s.*), are able to form a heat-stable emetic toxin, known as cereulide (a cyclic peptide), if conditions are favourable. Due to new findings on the phylogenetic relationships within the *B. cereus* group, it is currently assumed that these emetic strains do not belong to the species *B. cereus* (*s.s.*), but rather to

the newly described species *B. paranthracis*. The literature also suggests that *B. paranthracis* be combined with other species (*B. albus*, *B. anthracis*, *B. mobilis*, *B. pacificus*, *B. tropicus* und *B. wiedmannii*) to form the species *B. mosaicus* and to add "biovar Emeticus" to make strains recognizable as cereulide producers (Carroll et al., 2020). For the sake of comprehensibility, the term "emetic *B. cereus*" is used in this report.

It is assumed that cereulide production by "emetic *B. cereus*" begins during the exponential growth phase with very high cell numbers (from 10^5 CFU/g) and continues during the stationary growth phase (Ceuppens et al., 2011; Dommel et al., 2011; Lücking et al., 2009). Suitable conditions are neutral pH values (pH > 5), a medium a_w value[3], low salt concentration and sufficient nutrients (Messelhäußer et al., 2014). Carlin et al. (2013) calculated a minimum a_w value of 0.94 and a minimum pH of 4.6 for the growth of two emetic strains. In growth experiments over a period of 14 days, some of the emetic strains examined in the study also grew at a_w 0.93 and pH 4.3. The formation of cereulide is unlikely in anaerobic conditions (Jääskeläinen et al., 2004). An overview of factors and foods which favour cereulide formation can be found in Messelhäußer et al. (2014) and Rouzeau-Szynalski et al. (2020). Since "emetic *B. cereus* "are mesophilic, the temperature range in which these strains can grow is probably between 10 °C and 48 °C (in various publications growth was only observed from 10 °C or above, Carlin et al. (2013) have calculated a theoretical minimum growth temperature of 7 °C to 8 °C) (Carlin et al., 2006, 2013; Finlay et al., 2000; Guinebretiere et al., 2010). The temperature range in which cereulide can actually be formed is very likely narrower. For example, Kranzler et al. (2016) found no more cereulide formation in a nutrient medium at 43 °C. In contrast, Wang et al. (2014) found cereulide formation in rice that was incubated at 45 °C. The optimal temperature for cereulide formation is between 20 °C and 40 °C (Agata et al., 2002; Häggblom et al., 2002; Rajkovic et al., 2006, Kranzler et al., 2016). Moreover, the cereulides formed by different strains vary with regard to their structure (18 different isocereulides) and quantities (Häggblom et al., 2002; Marxen et al., 2015a; Stark et al., 2013). Further, differences between the cytotoxicity of the various isocereulides have been observed (Marxen et al., 2015).

The *ces* gene cluster, which is necessary for the formation of cereulide, can be found on a large plasmid (Stenfors Arnesen et al., 2008). Literature information on the percentage of emetic strains among food isolates can vary greatly and is mainly within a range of 0 %- 17 %, depending on the food matrix investigated (Biesta-Peters et al., 2016; Ceuppens et al., 2011; Erbslöh, 2007; Messelhäußer et al., 2014; Samapundo et al., 2011; Wehrle et al., 2010).

In contrast to the *ces* gene cluster, the genes for the formation of diarrhoea causing enterotoxins occur in *B. cereus* (*s.s.*) and all other members of the *B. cereus* group. As a result of published studies and its own research results, the BfR concludes that all strains of the *B. cereus* group essentially possess the genetic prerequisites to form enterotoxins (see below for exceptions). To date, three significant enterotoxins/enterotoxin complexes have been identified which can be formed by *B. cereus* (*s.l.*) and are associated with diarrhoeal illness. The "non-haemolytic enterotoxin" (Nhe) consists of the protein components NheA, NheB and NheC (encoded in an operon by the genes *nheA*, *nheB* and *nheC*). Haemolysin BL (Hbl) is composed of the components L2 (HblC), L1 (HblD) and B (HblA) (encoded in an operon by the genes *hblC*, *hblD* and *hblA*). There are two distinct forms of the third toxin, cytotoxin K (CytK): CytK-1, which is exclusively formed by the species *B. cytotoxicus*, and CytK-2, which occurs in different species from the *B. cereus* group. The contribution of CytK-2 to diarrhoeal

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[3] a_w value: activity of water; unit for the presence of water in foods and/or dishes. The larger the a_w value, the more water is available for bacteria to grow/metabolise.

illnesses has not yet been clarified (Fagerlund et al., 2004; Guinebretiere et al., 2013). Enterotoxin genes (*nhe, hbl, cytK-2*) are embedded in the chromosome and can occur in different combinations in all species of the *B. cereus* group. By contrast, the *cytK-1* gene exclusively occurs in the chromosome of *B. cytotoxicus*. Representative studies show that essentially all investigated strains of the *B. cereus* group carry the *nhe* operon, and predominantly in its entirety. Guinebretiere et al. (2010) investigated 391 strains from all species (7) of the *B. cereus* group identified as of 2010 using PCR and southern blotting; the *nhe* genes were detected in all strains, including 38 *ces* positive strains. In Wehrle et al. (2010), 330 of 331 strains from the *B. cereus* group were *nheA* positive, of which 50 were also *ces* positive (real-time PCR). The *nheA* negative strain was a representative of the species *B. cytotoxicus*, which carries an unusual *nhe* variant (Fagerlund et al., 2007). In another study by Wehrle et al. (2009), 176 strains were investigated (probably from the same strain set as Wehrle et al., 2010). In only one of these strains (*B. cytotoxicus*) was the complete *nhe* operon not detected. Moreover, no *nheB* was detected in one strain, and neither *nheB* nor *nheC* were detected in another. Of the *nhe* positive strains, 44 were also found to be *ces* positive. In addition to the *nhe* genes, the formation of the corresponding Nhe toxin components was detected in all strains using ELISA. In a study by Lindbäck et al. (2010), two strains were identified which did not produce any NheC or NheA, as they possess a premature stop codon in the corresponding gene (*nheC* and *nheA* respectively). These results concur with the experiences of the laboratory for spore formers at the BfR. Of all presumptive *B. cereus* isolates examined for *nheA* since 2016, *B. cytotoxicus* and *B. pseudomycoides* isolates were the only ones where no *nheA* was detected (real-time PCR method described by Wehrle et al., 2010). As mentioned above, the missing *nheA* detection in *B. cytotoxicus* is due to an unusual *nhe* variant. This is probably also the case for *B. pseudomycoides*, as it can be assumed that the representatives of this species also carry the *nhe* operon (see Guinebretiere et al., 2010 and Miller et al., 2018).

Even in the case of a lack of PCR detection, the formation of a corresponding toxin cannot be completely ruled out, as many published methods do not detect all sequence variants (Ceuppens et al., 2013). Using whole genome sequencing, Miller et al. (2018) were able to detect toxin genes which could not be detected using PCR.

Regardless of the presence of individual enterotoxin genes, the pathogenicity of *B. cereus* (*s.l.*) is greatly dependent on the strain. To estimate the pathogenic potential of enterotoxin formers, cytotoxicity tests as well as antibody-based methods (ELISA) are used to estimate quantities of toxins formed. Moravek et al. (2006) demonstrated that disease-associated strains have a tendency to produce larger quantities of toxins. In studies by Jessberger et al. (2015, 2017), culture supernatants of *B. cereus* (*s.l.*) strains demonstrated different cytotoxicity values, whereby the groups with different cytotoxicities could not be assigned any specific genotype. Guinebretiere et al. (2008) divided *B. cereus* (*s.l.*) strains into seven phylogenetic groups which could be differentiated using a sequence fragment of the *panC* gene. According to Guinebretiere et al. (2008, 2010), these phylogenetic groups are characterized firstly by different temperature limits for growth, and secondly by different cytotoxicities. According to the authors, strains from group III (growth from 15 °C to 45 °C) are generally the most cytotoxic, followed by strains from group VII (20 °C to 50 °C) (exclusively *B. cytotoxicus*), IV (10 °C to 45 °C) and II (7 °C to 40 °C). Group V (8 °C to 40 °C) is estimated to be less cytotoxic, while groups I[4] (10 °C to 43 °C) and VI (5 °C to 37 °C) are classified as having very low cytotoxicity. However, there is significant heterogeneity with regards to cytotoxi-

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[4] The information that group I is assessed as having low cytotoxicity can be found on the online tool "https://www.tools.symprevius.org/Bcereus/english.php", which is described in the publication by Guinebretiere et al. (2010). However, actual cytotoxicity data on strains from group I (exclusively *B. pseudomycoides*) has not been published.

city within the individual groups. Based on results of Carlin et al. (2013) the temperature limits for growth are likely to be slightly wider. As a result of current publications, it can be assumed that "emetic *B. cereus*" only belong to group III whereas *B. cereus* (*s.s.*) only belongs to group IV (Carroll et al., 2020; Miller et al., 2018).
As a result of *in vitro* studies, it is accepted that the expression of enterotoxin genes is, among others, regulated depending on cell density, and enterotoxins are only produced with high cell densities in the late exponential growth and stationary phases (Ceuppens et al., 2011).

B. cytotoxicus is a thermotolerant member of the *B. cereus* group that can grow at high temperatures. In this context, growth at 50 °C (Guinebretiere et al., 2008), 53 °C (Auger et al., 2008) and 52 °C (Carlin et al., 2013) was observed in laboratory studies. In the last study, a theoretical maximum growth temperature of 55 °C for *B. cytotoxicus* was calculated on the basis of the "Cardinal temperature model with inflection point" (CTMI) (mean of the calculation). Taking into account the 97.5 percentile, the calculated theoretical maximum growth temperature of one strain was 56.8 °C.
The species *B. cytotoxicus* possesses the ability to form the CytK-1 toxin, which demonstrates a significantly higher cytotoxicity than the CytK-2 variant (Fagerlund et al., 2004; Guinebretiere et al., 2013). However, because of different toxin expression levels, not all *B. cytotoxicus* strains are highly cytotoxic. Fagerlund et al. (2007) detected a high cytotoxicity in two *B. cytotoxicus* strains which were isolated from vegetable purée and potato purée respectively in association with diarrhoeal illness, while a third strain (isolated from herbs and spices) only demonstrated a low cytotoxicity. In a study by Heini et al. (2018), only one of nine *B. cytotoxicus* isolates from powdered potato purée was highly cytotoxic (significantly higher than a *B. cereus* (*s.s.*) outbreak reference strain), while the other eight isolates demonstrated low cytotoxicity. As a result of pre-enrichment at 50 °C, *B. cytotoxicus* was mainly detected in powdered potato products or foods made thereof in an investigation by Contzen et al. (2014). Subsequent quantitative investigations of *B. cytotoxicus* revealed a maximum count of 300 CFU/g.

B. thuringiensis is known for its insect pathogenicity, which derives from its so called parasporal crystals formed during sporulation. The genes responsible for the formation of these crystals are plasmid-encoded. Alongside the natural occurrence of *B. thuringiensis*, some strains of this species are used in commercial biological insecticides. Similarly to *B. cereus* (*s.s.*), *B. thuringiensis* can frequently be found in plant-based foods. The presence of the different enterotoxin genes in *B. thuringiensis* is probably similar to *B. cereus* (*s.s.*). By contrast, the *ces* gene cluster for cereulide formation has not yet been detected in *B. thuringiensis* (EFSA BIOHAZ Panel, 2016). The cytotoxicity of *B. thuringiensis* appears to be heavily dependent on each strain. In a study by Johler et al. (2018), 39 *B. thuringiensis* strains were investigated. Of these, nine strains demonstrated a low cytotoxicity (including two biopesticide strains) and 29 strains medium cytotoxicity towards Vero cells (including six biopesticide strains). The majority of these strains (36) were also characterised by a low production of the enzyme sphingomyelinase, which may augment the effect of the enterotoxins. In contrast to these results, a single *B. thuringiensis* strain (food isolate) demonstrated a high cytotoxicity (higher than a *B. cereus* (*s.s.*) outbreak reference strain), combined with high sphingomyelinase production. In a scientific opinion by the EFSA BIOHAZ Panel (2016), it was concluded that the levels of enterotoxigenic *B. cereus* (*s.s.*) in foods which can be considered as a risk for consumers are also likely to be valid for *B. thuringiensis*. In the classification by Guinebretiere et al. (2010), strains with the ability to produce parasporal crystals appear in almost all phylogenetic groups (except groups I and VII). This underlines that the plasmid-encoded characteristic of forming parasporal crystals is not a suitable attribute for

clearly defining a species. Accordingly, the formation of these crystals could already be detected in strains which had been classified as a species other than *B. thuringiensis* due to their phylogeny (Jimenez et al., 2013; Lazarte et al., 2018; Liu et al., 2015; Soufiane and Cote, 2010). The strain, which is used as the basis for describing the species *B. thuringiensis* (type strain), belongs to phylogenetic group IV. The chromosomal DNA sequence of this type strain is so similar to the sequence of the *B. cereus* (*s.s.*) type strain that a separate species status is not justified (Carroll et al., 2020)

B. toyonensis was identified as a new species (Jimenez et al., 2013; Oren and Garrity, 2014) in 2014. The type strain of this species was isolated in Japan in 1966 and was designated *B. cereus* var. *toyoi*. Spores of this strain have been used commercially as probiotic animal feed additives since 1975, particularly in pig and poultry farming. A corresponding product was authorised in the EU in 1994 as an animal feed additive. However, this authorisation has been suspended since 2013 (Implementing Regulation (EU) No 288/2013) as a consequence of assessments by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in 2012 and 2014 (EFSA FEEDAP Panel, 2012, 2014). These assessments state that the strain carries both the antimicrobial resistance genes (*catQ* and *tet(M)*), and the *nhe* and *hbl* operon. The strain is also capable of forming the relevant toxins, albeit less effectively than the *B. cereus* (*s.s.*) control strains used. According to Miller et al. (2018), the species *B. toyonensis* should be assigned to phylogenetic group V.

B. wiedmannii is a psychrotolerant species (growth at 5 °C - 43 °C), which was identified in 2016. The type strain was isolated from raw milk and produces both Nhe and Hbl (Miller et al., 2016). In addition to the *nhe* and *hbl* genes, the *cytK*-2 gene has been detected in *B. wiedmannii* strains. Both the type strain and other strains of this species demonstrated cytotoxicity towards HeLa cells and belong to phylogenetic group II Miller et al. (2016, 2018). In the study by Guinebretiere et al. (2010), strains from this group demonstrated a higher cytotoxicity towards CaCo-2 cells than strains from group V, and a lower cytotoxicity than strains from groups III, IV and VII.

B. mycoides (synonymous *B. weihenstephanensis*) is a psychrotolerant species that already grows at temperatures of around 4 °C. As a result of a reclassification, the species *B. mycoides* also includes all strains that were previously referred to as *B. weihenstephanensis* (Liu et al., 2018; Parte et al., 2020). One of the main distinguishing features between *B. weihenstephanensis* and *B. mycoides* was the rhizoid colony morphology of *B. mycoides*, which is, however, a characteristic that varies with cultivation conditions (Hendriksen and Hansen, 2011). Based on the similarity of the whole genome sequences of the type strains of *B. weihenstephanensis* and *B. mycoides*, both type strains represent only one species, with *B. mycoides* being described first.
It is known that in strains hitherto named *B. weihenstephanensis*, the genes for the formation of enterotoxins (mainly *nhe* and *hbl*) and rarely also for the formation of cereulide may be present (Thorsen et al., 2006). Using two of these emetic strains Guerin et al. (2017b) reported cereulide production already at 8 °C, which, however, significantly increased at higher temperatures (10 °C - 25 °C).

With regards to non-emetic *B. mycoides*/*B. weihenstephanensis* strains, a study by Stenfors et al. (2002) found that 40 of 50 strains tested demonstrated little or no cytotoxicity, while four strains showed medium toxicity. However, six strains also demonstrated a high cytotoxicity, comparable with *B. cereus* (*s.s.*) outbreak strains. In these tests, the strains were grown at 32 °C and Vero cells were used for the cytotoxicity assay. In a publication by Guinebretiere et al. (2010), the investigated *B. mycoides*/*B. weihenstephanensis* strains were assigned to the phylogenetic group VI, which generally demonstrated low cytotoxicity. These strains were

also grown at 32 °C, but CaCo-2 cells were used for the cytotoxicity assay. Ceuppens et al. (2013) reported that at an incubation temperature of 36 °C, psychrotolerant strains (growth at ≥ 7 °C) demonstrate less frequent Nhe formation than mesophilic strains (growth at > 7 °C). This has been explained by a lower growth rate of psychrotolerant strains at higher temperatures. Accordingly, the authors estimate the risk of diarrhoeal illness occurring from these strains as being low, since barely any toxins can be produced at body temperature. Likewise, Miller et al., 2018, also reported that for group VI strains (cultivation at 37 °C), no cytotoxicity towards HeLa cells could be detected.

B. pseudomycoides also demonstrates rhizoid colony morphology, but unlike *B. mycoides*, it is a mesophilic species and, amongst other things, can be distinguished from *B. mycoides* by its lack of a *cspA* gene signature (Francis et al., 1998; Guinebretiere et al., 2008). Both the *nhe* and *hbl* genes have been detected in *B. pseudomycoides* (Guinebretiere et al., 2010; Miller et al., 2018). According to Guinebretiere et al. (2010), *B. pseudomycoides* belongs to phylogenetic group I, to which a low cytotoxicity has been attributed. By contrast, Miller et al. (2018) demonstrated that culture supernatants (cultivation at 37 °C) of *B. pseudomycoides* strains have a high cytotoxic effect on HeLa cells.

3.1.2 Illnesses caused by *Bacillus cereus* (*s.l.*) (hazard characterisation and exposure assessment)

B. cereus (*s.l.*) is mainly transmitted to humans via food. The consumption of food contaminated with *B. cereus* (*s.l.*) can lead to gastrointestinal diseases in humans. It is assumed that in most cases a *B. cereus* (*s.l.*) level of at least 10^5 CFU/g food is necessary to trigger illness. However, cases of illness have also been described in which lower levels of *B. cereus* (*s.l.*) were detected in food (Ceuppens et al., 2013; EFSA BIOHAZ Panel, 2016).
The illnesses of the gastrointestinal tract associated with *B. cereus* (*s.l.*) are not infectious and the symptoms rarely last longer than 24 hours. People of all ages can be affected. In the past, rare cases resulted in death. There are two different types of illness, an emetic illness (vomiting type, intoxication) and a diarrhoeal illness (diarrhoeal type; toxicoinfection).

In the **emetic illness**, an acid, heat and proteolysis stable toxin (cereulide) is ingested, which is formed by vegetative cells in food. As a result of cereulide binding to certain receptors in the gastrointestinal tract, vomiting and nausea results within only six hours of consumption. The symptoms are usually self-limiting within 24 hours. In cases of severe intoxication, cereulide can also cause liver damage and cerebral oedema (Dierick et al., 2005; Shiota et al., 2010). In animal experiments, cereulide-related liver damage was reversible at sub-lethal doses (Yokoyama et al., 1999). It has also been assumed that cereulide has an inhibitory effect on the immune system, due to damaging effects on natural killer cells (Paananen et al., 2002). In rare cases, severe intoxications have also resulted in death (Dierick et al., 2005; Naranjo et al., 2011; Shiota et al., 2010). Intoxication caused by cereulide is frequently associated with the consumption of starchy foods such as rice and pasta. However, cases of emetic illness associated with dairy and meat products have also been reported (Messelhäußer et al., 2014; Rouzeau-Szynalski et al., 2020). The reason for the more frequent occurrence of illnesses in connection with starchy foods could be that the root area or parts of certain crops are a natural habitat for "emetic *B. cereus*". Furthermore, the composition of starchy foods could promote the formation of cereulides, as they might contain a favorable (sufficiently low) content of certain amino acids. It is known that a too high concentration of branched amino acids inhibits cereulide formation (Ehling-Schulz et al., 2015).
In current publications on cereulide concentrations in food, which have been investigated in association with intoxications, values in the range of 0.19-15 µg/g (micrograms per gram) of

food have frequently been reported (Marxen et al., 2015b; Messelhäußer et al., 2014; Rouzeau-Szynalski et al., 2020). A significantly lower concentration of only 0.003 µg/g of food in a suspected sample (rice dish; illness with typical cereulide symptoms) has been described by Biesta-Peters et al. (2016). As a possible reason for the extreme differences in cereulide concentrations measured in association with illness, it is noted that the time between the consumption of a food and the inspection of the food for its cereulide content can vary on a case-by-case basis. Depending on how the food is stored, additional cereulide production (as well as microbial growth) is possible during this period, meaning that the cereulide concentration in the food consumed and the food inspected may vary. In the publication by Biesta-Peters et al. (2016), a risk assessment by the Dutch National Institute for Public Health and the Environment was quoted, which estimated that a value of below 0.0018 µg/g of food (0.03 µg/kg body weight) does not cause an adverse health effect. Based on animal trials, the minimum intoxication dose for humans was estimated to be 8 - 10 µg/kg body weight (EFSA BIOHAZ Panel, 2016).

Information about *B. cereus* levels in foods which have been associated with emetic illnesses varies greatly and ranges from $\leq 10^2$ - 10^7 CFU/g (EFSA BIOHAZ Panel, 2005, 2016; Glasset et al., 2016; Messelhäußer et al., 2014; Rouzeau-Szynalski et al., 2020). One reason for these different levels could be that the quantities of formed toxins depend heavily on strain characteristics and environmental conditions (Agata et al., 2002; Apetroaie-Constantin et al., 2008; Carlin et al., 2006; Rajkovic et al., 2006; Stark et al., 2013). However, the reported low bacterial counts ($< 10^5$) appear to contradict the assumption that cereulide production only begins during the exponential growth phase, at cell numbers of 10^5 CFU/g (see also 3.1.1). A reason for relatively low levels of *B. cereus* found in food associated with illnesses may be that existing vegetative cells in foods have been reduced as a result of processing steps, after cereulide had already been produced. A further reason may be that collected samples were taken before multiplication of *B. cereus* in the food and the subsequent transfer to consumers. It is therefore possible that *B. cereus* levels below 10^5 CFU/g have been determined in these examined foods but the *B. cereus* level at the time of consumption was higher.

Cereulide formed in food is not destroyed by heating at 100 °C for 150 min (pH 8.6-10.6) or by heating at 121 °C for 120 min (pH 7) (Rajkovic et al., 2008).

With the **diarrhoeal illness**, vegetative cells and/or spores of *B. cereus* (*s.l.*) are ingested with the food. Enterotoxins which may have already been produced in the food very likely do not contribute to diarrhoeal symptoms as, for the most part, they are inactivated during gastric passage and in the subsequent intestinal conditions, because the enterotoxins are sensitive against proteinases and low pH values. Enterotoxins are also heat-sensitive and are inactivated by heating at 55 °C for 20 min (Ceuppens et al., 2011, 2013). Vegetative cells are also largely inactivated during gastric passage. The rate of inactivation, however, depends on various factors (e.g. bacterial growth phase, food properties, gastric environment), meaning that vegetative cells may also be involved in causing illness (Berthold-Pluta et al., 2015). Spores survive the gastrointestinal passage for the most part, and can then germinate close to or in direct contact with the small intestine epithelium and subsequently form vegetative cells. It is assumed that spores can attach to enterocytes. In turn, contact with enterocytes promotes spore germination, which results in the cell densities required to trigger the gene regulation cascade for the production of enterotoxins. The ability to interact with enterocytes is likely to be associated with the surface qualities of the spores, which may vary between strains, and may therefore also partly explain the different pathogenicities (Berthold-Pluta et al., 2015; Ceuppens et al., 2012). It is also assumed that the interaction of vegetative cells with enterocytes promotes toxin formation (Jessberger et al., 2017). Enterotoxins form pores in the membranes of enterocytes, consequently leading to osmotic lysis of enterocytes, and

diarrhoeal symptoms. Disease symptoms usually emerge within 8 to 24 hours after consuming the contaminated food and include watery diarrhoea and stomach pain. The illness is generally self-limiting (Messelhäußer and Ehling-Schulz, 2014).

In addition to enterotoxins, *B. cereus* (*s.l.*) also produces other potential virulence factors, such as sphingomyelinases, phospholipases, haemolysins or metalloproteinases, which may augment the effect of the enterotoxins. Information about *B. cereus* levels in foods which have been associated with diarrhoeal illness varies greatly. *B. cereus*-related diarrhoeal outbreaks, which were reported to the EFSA in the period between 2007 and 2014, were mostly associated with foods having *B. cereus* counts of over 10^5 CFU/g. However, there were also outbreaks where *B. cereus* levels of only 10^3 CFU/g were detected (EFSA BIOHAZ Panel, 2016). A publication on foodborne outbreaks in France between 2007 and 2014 reported that the suspected foods contained *B. cereus* levels of 10^2 - 10^9 CFU/g (Glasset et al., 2016). If only the outbreaks in which solely diarrhoeal symptoms occurred are considered, the values range between 10^3 and 10^6 CFU/g, where three out of a total of six such outbreaks were associated with foods which only contained 10^3 to 10^4 CFU/g. Whether or not diarrheal symptoms are triggered depends not only on the *B. cereus* levels, the properties of the bacterial strain and the food ingested, but also on the health status of the consumer (Ceuppens et al., 2013). As previously stated, also the time of sampling influences the bacterial counts determined in foods involved in outbreaks. The levels of bacteria at the time of consumption may be higher or lower.

There is no reliable data in Germany on the frequency of these illnesses caused by *B. cereus* (*s.l.*). Between 2009 and 2015, two to six foodborne outbreaks per year caused by *B. cereus* (*s.l.*) were reported by the competent authorities of the German federal states ("Laender") and the German Armed Forces via BELA[5]. From 2015 to 2017, three to ten cases of outbreaks a year caused by *B. cereus* (*s.l.*) were reported to the EFSA (Rosner et al., 2016, BVL 2017, 2018, 2019). In the period between 2007 and 2014, a total of 1127 *B. cereus*-related outbreaks were reported to the EFSA by European states. However, the actual number of outbreak cases may be significantly higher both in Germany and elsewhere in Europe.

As the species from the *B. cereus* group are usually not distinguished in routine food testing, the contribution of individual species to the emergence of illnesses cannot currently be assessed.

B. cytotoxicus was isolated first in a disease outbreak with diarrhoeal symptoms in 44 patients (three of whom died, and six of whom had bloody diarrhoea) in a nursing home for elderly people in France. The isolate was taken from vegetable purée, which was contaminated with 3 x 10^5 CFU/g presumptive *B. cereus* (Lund et al., 2000). Data on the minimum cell count of *B. cytotoxicus* in a food which is required in order to trigger illness is not available.

To date, there is little information about a connection between the presence of *B. thuringiensis* in foods and diseases in humans (EFSA BIOHAZ Panel, 2016; Jackson et al., 1995; McIntyre et al., 2008).

B. mycoides (synonymous *B. weihenstephanensis*), and *B. pseudomycoides* have not as yet been associated with foodborne illnesses (EFSA BIOHAZ Panel, 2016).

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[5] National System for the Recording of Data for Food Involved in Disease Outbreaks (BELA)

In accordance with the classification of the cytotoxicity of different phylogenetic groups using the *panC* sequence, groups II, III and IV appear to be more frequently associated with illnesses (Glasset et al., 2016).

3.1.3 Heat-inactivation of *Bacillus cereus* (*s.l.*) in foods

Conventional heat treatments, such as cooking or pasteurisation, kill vegetative bacterial cells from the *B. cereus* group, but partially enable spores to survive (EFSA BIOHAZ Panel, 2016; Guerin et al., 2017a). The reduction of competitive microbiota as a result of heat treatment supports the germination of spores and the subsequent growth of the vegetative cells. The heat resistance of spores is heavily dependent on the food matrix in which the spores are found. For example, there is a high resistance of *B. cereus* (*s.l.*) spores in oily foods (van Asselt and Zwietering, 2006). The variability of strains is also very high; because of this, D-values (decimal reduction time) at 90 °C and pH 7 can range between only a few minutes and > 100 min (EFSA BIOHAZ Panel, 2016). In particular, the spores of emetic strains are highly resistant to heat (Ankolekar and Labbe, 2009; Carlin et al., 2006; Hariram and Labbe, 2016; Rajkovic et al., 2006), while the spores of psychrotolerant strains demonstrate a lower resistance to heat (Luu-Thi et al., 2014).
Heat treatment of a food for three minutes at 121 °C would kill all spores (EFSA BIOHAZ Panel, 2005, 2016). The temperature and duration of heat exposure are deciding factors in the inactivation of spores, but not the pressure, as the spores are extremely resistant to high pressure (Nicholson et al., 2000). Temperatures reached during the pasteurisation or cooking of food are not enough to safely inactivate all spores (EFSA BIOHAZ Panel, 2005, 2016). Milder temperature treatments (e.g. 70-80 °C for 10 minutes) may even accelerate the germination and growth of *B. cereus* (*s.l.*) (Samapundo et al., 2014).

3.1.4 Risk characterisation

After evaluating the literature and its own research results, the BfR comes to the conclusion that each *B. cereus* (*s.l.*) strain is a potential former of enterotoxins, although types and amounts of formed toxins may vary. It is therefore possible that the consumption of foods which contain very high numbers of *B. cereus* (*s.l.*) (from 10^5 CFU/g) causes diarrhoeal illness in humans within a short period. For strains from the *B. cereus* group which only possess a limited ability to multiply and/or form toxins at human body temperature (*B. mycoides* (synonymous *B. weihenstephanensis*)), however, the probability of causing foodborne diarrhoeal illness is low.

The BfR further concludes that for *B. cytotoxicus* even a lower number of bacteria in food (from 10^4 CFU/g) may be enough to trigger diarrhoeal illness in humans, due to the higher toxicity of the CytK-1 toxin, even if not all *B. cytotoxicus* strains are highly cytotoxic.

The *B. cereus* (*s.l.*) counts and with it, the risk to health, can only be slightly minimised (if at all) by the thorough washing of plant-based foods with drinking water. Due to the high heat resistance of spores, they can only be eliminated safely when strong heating methods are used, such as the ones used in the canning industry (e.g. 121 °C, 3 min).

However, even at lower *B. cereus* levels (from ca. 10^3 CFU/g) in a food product, a risk of causing illness may emanate from the food should the strain in question possess the ability to form cereulide and the conditions before consumption of the food support the growth of

the strain and toxin formation. The risk can be minimised if temperatures in which *B. cereus* (*s.l.*) multiplies are avoided when storing meals and drinks containing milk.

If a relevant quantity of cereulide is already present in the food, it can be assumed that the possibility of damage to health cannot be ruled out, as cereulide is extremely stable (> 120 min at 121 °C). Due to the varying information on the possible minimal intoxication dose, from a BfR perspective, no universally applicable cereulide limit in foods can be determined, meaning that a legal assessment of a food containing cereulide can only be carried out on a case-by-case basis.

3.2 Further aspects

To protect the population from microbiological hazards in food, the European Commission set out various microbiological criteria for food in Regulation (EC) No 2073/2005. For presumptive *B. cereus* in dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age, Appendix I Chapter 2 Number 2.2.11 of this regulation defines a Process Hygiene Criterion, which food companies must comply with at the end of the production process. If the results are unsatisfactory, food companies must take certain measures (improving production hygiene, preventing recontamination, raw material selection).

4 Risk management options, recommended measures

To prevent foodborne infections and food poisoning, the BfR recommends observing the following kitchen hygiene rules when storing and preparing food in the catering industry and other communal catering institutions as well as private households:

- Comply with the cold chain for perishable foods.
- Cook dishes thoroughly during preparation, and when rewarming, heat sufficiently to kill any vegetative cells (at least 70 °C for two minutes throughout the food; check the temperature with a meat thermometer in case of doubt); the same applies to rewarming of food in microwaves (make sure heat is distributed equally, stir food at intervals).
- Cool prepared, heated food quickly to ≤ 7 °C (fill larger quantities of food into several shallow bowls) and store in the refrigerator until reheating shortly before consumption
- When holding food hot (e.g. soups, sauces, stews), make sure that a temperature of at least +60 °C is kept throughout the food. Do not store easily perishable foods or heated foods at temperatures between +7 °C and +60 °C for long periods.
- Store the leftovers of cooked dishes in the refrigerator and consume within two to three days.
- Dispose water used for soaking dried mushrooms carefully and thoroughly clean hands, as well as all objects or working areas which have been in contact with the soaking water or soaked mushrooms.

Further information on the BfR website

Schutz vor lebensmittelbedingten Erkrankungen durch bakterielle Toxine, from 1 February 2020 (in German only)
https://www.bfr.bund.de/cm/350/verbrauchertipps-schutz-vor-lebensmittelbedingten-erkrankungen-durch-bakterielle-toxine.pdf

Prevention of Foodborne Illness When Keeping Food Hot, from 27 August 2020
https://www.bfr.bund.de/cm/349/prevention-of-foodborne-illness-when-keeping-food-hot.pdf

Recommendations for the hygienic preparation of infant formula in powder form
BfR Opinion No. 040/2012 of 6 November 2012
https://www.bfr.bund.de/cm/349/recommandations-for-the-hygienic-preparation-of-infant-formula-in-powder-form.pdf

BfR "Opinions app"

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