

Mini-Review

Outbreaks, Germination, and Inactivation of *Bacillus cereus* in Food Products: A Review

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ABSTRACT

Bacillus cereus has been reported as a foodborne pathogen worldwide. Although food processing technologies to inactivate the pathogen have been developed for decades, foodborne outbreaks related to *B. cereus* have occurred. In the present review, foodborne outbreaks, germination, inactivation, and detection of *B. cereus* are discussed, along with inactivation mechanisms. *B. cereus* outbreaks from 2003 to 2016 are reported based on food commodity, number of cases, and consequent illnesses. Germination before sporocidal treatments is highlighted as an effective way to inactivate *B. cereus*, because the resistance of the pathogen increases significantly following sporulation. Several germinants used for *B. cereus* are listed, and their efficacies are compared. Finally, recently used interventions with sporocidal mechanisms are identified, and rapid detection methods that have been developed are discussed. Combining two or more interventions, known as the hurdle technology concept, is suggested to maximize the sporocidal effect. Further study is needed to ensure food safety and to understand germination mechanisms and sporocidal resistance of *B. cereus*.

HIGHLIGHTS

- *Bacillus cereus* has been associated with several foodborne outbreaks.
- Several germinants have been used to induce *Bacillus cereus* germination.
- Resistance of *Bacillus cereus* may depend on the germination method.
- Sporocidal effect of interventions can be maximized by hurdle technology.

Key words: *Bacillus cereus*; Foodborne outbreaks; Germination; Hurdle technology; Sporocidal intervention

Globalization is one of the most popular keywords of modern society. People can travel throughout the world and enjoy local food and drinks at relatively low expense. Moreover, people can indulge in local food from other countries through trade. Despite the availability of these food products, food safety remains an issue of concern. Globalization increases the risk of foodborne illnesses, because disease can spread rapidly (68). In addition, factors such as antibiotic resistance and climate change contribute to these repeated foodborne outbreaks, despite developments in food science and technology (50). In this regard, foodborne illness has become one of the most important issues related to public health worldwide. The Centers for Disease Control and Prevention reported that one in six people acquires a foodborne illness each year, resulting in 128,000 hospitalizations and 3,000 deaths in the United States (11). Bacterial and viral pathogens were the top two causative agents in the United States, related to 39.5 and 46.9% of foodborne illnesses, respectively, in 2017 (12).

Bacterial pathogens were the most prevalent causative agents in South Korea and were related to 62.9% of foodborne illnesses in 2017 (43). It has been reported that some bacterial pathogens, such as *Escherichia coli* O157:H7, *Salmonella*, *Vibrio parahaemolyticus*, *Campylobacter*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus*, are major sources of these outbreaks (56, 60). In this regard, several interventions have been adopted to control microbiological hazards such as *E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, *B. cereus*, and norovirus (34, 35, 40). Among these hazardous microorganisms, *Bacillus* species are found to have high resistance to external physical or chemical treatments via the formation of endospores (52).

Bacillus species, which are ubiquitous in nature, are characterized as gram-positive, rod-shaped, spore-forming bacteria (17). Some *Bacillus* species are obligate aerobes, while most species are facultative anaerobes (4). Two species of *Bacillus* are primarily known to be hazardous: *B. anthracis* and *B. cereus* (7). Anthrax, a fatal disease to the human body, is caused by skin, lung, or gastrointestinal infection by *B. anthracis* (8). In contrast, intoxication by *B.*

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TABLE 1. Foodborne outbreaks by *B. cereus*, 2003 to 2016

Country	Food	Year	Patient information	Case(s)	Reference
Belgium	Pasta salad	2003	5 children (girls: age 7, 9, and 10; boys: age 9 and 14)	4 illnesses and 1 death; severe metabolic acidosis and liver failure by <i>B. cereus</i> were indicated as reasons for the outbreak (death)	15
Belgium	Spaghetti with tomato sauce	2008	1 young adult (age 20)	1 death; a significant amount of <i>B. cereus</i> (>7 log CFU/g) was found in the pasta, while the pathogen was absent from the tomato sauce	49
South Korea	Underground water	2010	193 adults	Attack rate of diarrhea was 20.3%	13
United Kingdom	Navy beans	2012	182 children and 18 adults	Vomiting occurred among children and staff but resolved within a few hours (no hospitalization)	51
United States	Refried beans	2016	Customers of a Mexican fast-food restaurant	179 estimated foodborne illness cases (169 hospitalizations, no deaths)	10

cereus is usually mediated via food products. Foodborne illness can be caused by food contaminated with more than 100,000 CFU/g (5 log CFU/g) of *B. cereus* and is further divided into diarrheal and emetic syndromes (58). Enterotoxins related to the diarrheal syndrome are produced in the gut and are known to be heat sensitive, whereas the toxins related to the emetic syndrome (cereulide) are already present in food and are known to be heat stable (22). It has been reported that various foods may be associated with the diarrheal syndrome, while rice is the major source of the emetic syndrome (2). Thus, *B. cereus* remains a microorganism of concern with respect to food safety, and it is of interest to understand recent attempts to control *B. cereus* contamination and/or growth in food products.

In the present review, recent outbreaks by *B. cereus* (2003 to 2016) and studies about the germination and inactivation of *B. cereus* are discussed to add to the existing body of review literature (1, 9). This article covers several germination agents and sporicidal interventions, as well as a combination of those technologies, also known as hurdle technology. Details regarding recent *B. cereus* outbreaks and data concerning the germination and inactivation of the pathogen may be helpful in controlling *B. cereus* in food products and preparing for potential future outbreaks.

RECENT FOODBORNE OUTBREAKS CAUSED BY *B. CEREUS* (2003 TO 2016)

Foodborne outbreaks caused by *B. cereus* have been reported (Table 1). Various foods, such as pasta salad, spaghetti, tomato sauce, navy beans, refried beans, and water have been indicated as contamination sources of *B. cereus* (10, 13, 15, 49, 51), resulting in hospitalizations and two deaths. Severe metabolic acidosis and liver failure were indicated as causes of death. Most cases were attributed to incorrectly preserved food, such as refrigeration temperatures as high as 14°C (15) or long-term storage of the food at room temperature (49). Food stored at a refrigeration temperature (ca. 4°C) may suffer temperature abuse, leading to increased growth of the pathogen; thus, in many cases, refrigeration is not a fundamental solution for *B. cereus* foodborne outbreak prevention contingent on temperature control of refrigeration systems. In particular, some *B.*

cereus species, known to be psychrotrophs, growing at a refrigeration temperature (6°C), could increase the risk of foodborne outbreaks (24).

From reported outbreaks of *B. cereus*, it can be deduced that several factors, including food storage location, contamination source, and dissemination of foods, may affect the number of illnesses. Outbreaks that occur as a result of restaurant meals or by meal services may be involved in significantly higher numbers of foodborne illnesses than household cases. Moreover, contaminated underground water can lead to large outbreaks by contaminating foods directly or indirectly via utensils. Cross-contamination from a contaminated environment, utensils, or food handlers to the food products can occur during food preparation (23). Kusumaningrum et al. (39) reported that foodborne pathogens remain viable on dry stainless steel surfaces and transfer to foods at rates of 20 to 100%. In this regard, cross-contamination of *B. cereus* can occur because *B. cereus* cells can survive in dry conditions for long periods following sporulation. It is impossible to avoid cross-contamination, but the cross-contamination rate can be reduced by implementing management programs such as hazard analysis and critical control point, good agricultural practices, and good manufacturing practices, because these programs deal with water, food processing, utensils, education, etc.

GERMINATION OF *B. CEREUS* ENDOSPORES

Harsh conditions, often including nutrition limitation, prompt *Bacillus* species to protect themselves via sporulation (3). The resistance of *Bacillus* species to external chemical or physical treatments increases following sporulation because of a multilayer structure (31). A vegetative cell has peptidoglycan in its exterior, while spores have additional structures, including the cortex, spore coats, and exosporium (Fig. 1). Even though peptidoglycan and the cortex look similar, a cortex-lytic enzyme can differentiate the two structures (46). Spores can respond to the surrounding environment based on receptors and may revert to the vegetative state when external conditions become favorable through germination (20).

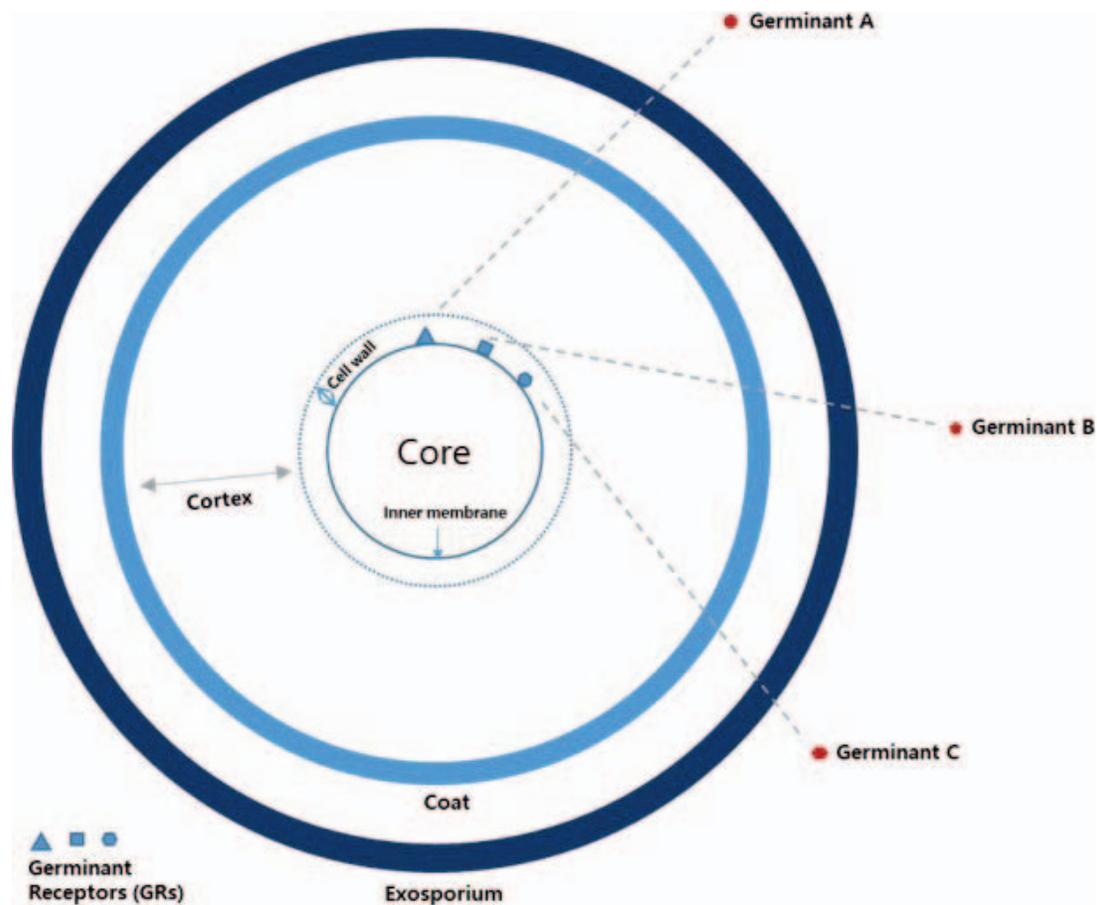


FIGURE 1. Example structure of a *B. cereus* spore.

The general mechanisms of nutrient germinants such as L-alanine on bacterial endospores is described as follows (44). First, the germinant transverse the inner membrane adjacent to the germinant receptor. Some cations and dipicolinic acid are released from the core after the germinant binds to the receptor. At this stage, rehydration is observed in the core, and cortex hydrolysis occurs. Finally, the spore coat collapses, and the cell begins outgrowth. Several germinant agents for *B. cereus* spores have been reported (Table 2). L-Alanine and inosine have been widely used for the germination of *B. cereus* spores. Various other agents, such as disodium 5'-inosinate, adenosine, or other amino acids, can also be used for germination (70). It has been reported that the germination efficacy depends on the bacterial strain and treatment conditions. For example, it was reported that *B. cereus* ATCC 14579 could be germinated not only with the addition of L-alanine and inosine but also by disodium 5'-inosinate, L-cysteine, L-threonine, and L-glutamine (29). A combination of more than two germinants can have a synergistic effect compared with individual treatments; thus, it is supposed that several germinants can bind to multiple receptors simultaneously or sequentially. In this regard, further study is needed to maximize *B. cereus* germination by investigating novel germinants or by combining already reported germinants.

SPORICIDAL TREATMENTS FOR THE INACTIVATION OF *B. CEREUS*

Thermal treatments with or without high pressure have been conventionally used for the inactivation of *B. cereus* spores (57), while several attempts using superheated steam, UV-C irradiation, pulsed electric field, thermosonication, and gaseous chlorine dioxide treatments have also been recently reported (Table 3). Maximum reduction rates of *B. cereus* spores varied from 4- to 7-log CFU depending on the sporicidal treatments and treatment conditions. In addition, biocontrol using bacteriophages is a novel approach for the inactivation of *B. cereus* spores (37).

Treatment conditions in the literature varied based on sample type, treatment method, and *B. cereus* strain. For example, the temperatures of superheated steam were 120 to 180°C and 200 to 300°C for garlic and stainless steel coupon treatments, respectively. In the study focusing on stainless steel coupon treatments, the treatment time or the temperature of the superheated steam could be reduced by combining it with UV-C irradiation. In this regard, researchers investigated the combination of treatments maximizing the sporicidal effect, including a pulsed electric field–nisin combination and a thermosonication treatment. Marco et al. (42) also reported that the addition of olive powder had an additive effect on high hydrostatic pressure processing of *B. cereus* spores. Samples with 2.5% olive powder treated at 400 and 500 MPa showed inhibition of *B.*

TABLE 2. Agents used for germination of *B. cereus*

Strain of <i>B. cereus</i>	Germination agent(s)	Treatment sample ^a	Main result(s)	Reference
ATCC 14579	L-Alanine (Ala), inosine (Ino), disodium 5'-inosinate (IMP), Ala+Ino, Ala+IMP	Distilled water	Combination treatments of Ala and Ino or IMP have a greater germination effect than individual treatments	31
ATCC 10876	Ala, Ino	Germination buffer	Germination in Ala is mediated by more than two receptors including GerL, and germination in Ino required two receptors by <i>gerI</i> and <i>gerQ</i> operons	5
ATCC 14579	20 amino acids, Ino, adenosine	Germination buffer	Ala, L-cysteine, L-threonine, and L-glutamine initiate germination among the 20 amino acids, and Ino and adenosine can trigger germination	29
ATCC 14579 CMCC 3328	Mixture of Ala and Ino	Germination buffer or adhered to stainless steel	Mixture of Ala and Ino was effective for germination both in suspension and adhered to stainless steel	27
ATCC 14579	Ala, Ino	Germination buffer or food products (meat broth and rice water)	The <i>gerR</i> operon has a significant role in germination by Ala and Ino	28
ATCC 14579 ATCC 10987 AH187	20 amino acids, Ino	Germination buffer or food products (rice water, meat broth, and skim milk)	ATCC 10987 spores need heat activation for germination, while ATCC 14579 and AH187 spores germinated without heat activation	67

^a Germination buffer: Tris-HCl+NaCl.

TABLE 3. Sporicidal treatments used for the inactivation of *B. cereus* spores

Strain of <i>B. cereus</i>	Sporicidal treatment(s)	Treatment sample	Main result(s)	Maximum inactivation rate	Reference
ATCC 14579	Superheated steam (120, 150, and 180°C)	Garlic	Treatment of superheated steam with germinant compounds was more effective than independent superheated steam treatment	>5 log reduction	31
ATCC 14579	High hydrostatic pressure (53–690 MPa)	McIlvaine buffer (pH 7)	Sporulation temperature (20, 30, and 37°C) influenced the heat or pressure resistance of <i>B. cereus</i>	>7 log reduction	54
ATCC 7004	Pulsed electric fields with or without nisin	Whole and skim milk	Combined treatments of pulsed electric fields with nisin at a mild temperature were the most effective	>6 log reduction	6
KCTC 1012	High hydrostatic pressure (0.1–600 MPa)	McIlvaine buffer (pH 6, 7, and 8)	Inactivation level of <i>B. cereus</i> was affected by the pH of the sporulation medium	>6 log reduction	53
NZRM 984 ICMP 12442	Pressure-thermal or thermal treatments	Reconstituted skim milk	High pressure treatment combined with thermal processing increased the sporicidal effect compared with independent thermal inactivation	>5 log reduction	58
Bacterial cocktail of ATCC 21366, C1, F4616A/90, F4810/72, and 038-2	Gaseous chlorine dioxide (ClO ₂) treatment	Stainless steel coupons	Even though <i>B. cereus</i> spores in biofilms are more resistant than those not in biofilms, gaseous ClO ₂ treatment was effective for the inactivation of <i>B. cereus</i> spores on the surface of stainless steel	>5 log reduction	48
NZRM 984	Thermosonication or thermal processing	Skim milk and beef slurry	Sporicidal effect of thermosonication was significantly greater than that of thermal processing alone	>4 log reduction	18

TABLE 4. Mechanical mode of action for the inactivation of *B. cereus*

Strain of <i>B. cereus</i>	Bactericidal treatment(s)	Treatment sample	Bactericidal mechanism(s)	Reference
Bacterial cocktail of ATCC 10876, ATCC 13061, and ATCC 14579	Superheated steam (200, 250, and 300°C) and/or UV-C irradiation	Stainless steel coupons	An increased dipicolinic acid ratio by the combination treatment of superheated steam and UV-C irradiation leads to a synergistic sporicidal effect	36
<i>B. cereus</i> T	Wet heat (88°C)	Water	Sporicidal mechanism of wet heat treatment was suggested for the denaturation of one or more key proteins	14
IFR-NL94-25	Carvacrol (1–3 mM)	Buffer containing DiSC ₃ (5)	Carvacrol changed the membrane potential and permeability for cations of <i>B. cereus</i> , leading to cell death	61
ATCC 6464	Atmospheric pressure dielectric-barrier-discharge plasma	Distilled water	Reactive oxygen species damaged internal macromolecules or molecular systems	16
ATCC 14579	<i>B. cereus</i> phage PBC4	SM buffer ^a	Bacteriophage PBC 4 produces endolysin (LysPBC4), has an enzymatic activity, and differentiates the host with a cell wall binding domain	47
ATCC 14579 ATCC 10987	Acidic conditions (pH 4.4–5.4)	Acidification with hydrochloric acid	Excessive radicals, including hydroxyl and peroxy radicals, lead to cell death	45

^a SM buffer, 50 mM Tris-Cl (pH 7.5), 0.1 M NaCl, 8 mM MgSO₄, 7 H₂O.

cerus growth at 32°C when *B. cereus* outgrowth rates at 32 and 20°C were examined under 0, 200, 500, and 500 MPa with or without 1.5 and 2.5% olive powder. Sagong et al. (55) demonstrated the combined effect of ultrasound and surfactants to inactivate *B. cereus* spores on lettuce and carrots. These combination treatments are more effective in inactivating *B. cereus* or in inhibiting the growth of *B. cereus* than the treatments applied individually. However, the treatment conditions may be too harsh for some food commodities, resulting in quality deterioration.

Germination before sporicidal treatment has been reported as an effective way to control *B. cereus* spores, minimizing the deterioration of food quality (21). Various germinants or germination methods have been used, as indicated in the previous section. Spore cells enter the vegetative state during germination, and inactivation of germinated cells is comparably less difficult than inactivation of spores. However, some limitations have been pointed out related to application in the food industry. Germination of spores may be induced in liquid food products, but it is more difficult to apply these methods to semisolid or solid food products. In particular, reported germination methods are difficult to apply to powdered food products, such as red pepper powder and black pepper powder, in which large numbers of heat-resistant spores have been detected (33). Moreover, it was recently reported that the resistance of germinated cells to water-bath heating was significantly higher than that of the original vegetative cells (30). Reductions of *B. cereus* vegetative cells ranged from 4 to 4.5 log CFU/g after 30 to 90 s of heating at 80°C, whereas the reductions of wet heating germinated spores were 1 to 1.5 log CFU/g. Furthermore, the thermal or pressure resistance of *B. cereus* was influenced by the sporulation temperature (54) and the pH of the sporulation medium (53). These results indicate

that the resistance of pathogens can change following germination, which is an important factor to be considered before application of an inactivation technology. In this regard, practical germination and sporicidal methods to control the spores in the various types of food products, including powders, should be investigated further.

MECHANISMS FOR THE INACTIVATION OF *B. CEREUS*

Identifying bactericidal mechanisms for *B. cereus* has been of interest, and several have been proposed (Table 4). It is well known that key proteins are denatured by heat treatments, while an acid-shock effect may lead to cell death by the accumulation of excessive radicals (14, 45). Similar to the acid-shock effect, reactive oxygen species produced from cold plasma treatment damage the internal macromolecules or molecular systems that are critical to cell metabolism (16). It has been reported that reactive oxygen species also play a role in the inactivation of *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* by UV irradiation (32), and a similar mechanism may be suggested for the inactivation of *B. cereus* by UV-C irradiation, even though related investigations are limited. Ultee et al. (61) reported that changes in membrane potential and permeability led to cell death, and Na et al. (47) indicated that endolysin produced by bacteriophage PBC4 showed enzymatic activity for *B. cereus*. Likewise, various targets and mechanisms have been suggested for the inactivation of *B. cereus*.

Multiple inactivation targets for *B. cereus* would be one of the factors contributing to the synergistic effect by the hurdle technology, because several targets may be damaged simultaneously with the combined treatments. Targets for *B. cereus* spores may be more diverse compared with those for

vegetative cells, because the structure of the spore is more complicated, as indicated in Figure 1. Ha and Kang (25) indicated that damage to the ribosome and cell wall, but not to DNA and RNA, accumulated by the combination treatments of UV-C irradiation and near-infrared heating for the inactivation of *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes*. Comprehensive studies concerning the inactivation mechanisms of *B. cereus* spores by combination treatments are limited, even though an increased dipicolinic acid release ratio was observed by the combination treatments of superheated steam and UV-C irradiation (36). Further research is needed to identify the underlying mechanisms of individual or combined treatments for the inactivation of *B. cereus*.

METHODS FOR DETECTING *B. CEREUS*

The U.S. Food and Drug Administration (FDA) reported that *B. cereus* grows at temperatures between 4 and 48°C, with optimal growth temperatures of 28 to 35°C and a pH range of 4.9 to 9.3. The bacteria's salt tolerance was indicated as 7.5% (63). The FDA recommended against holding *B. cereus* outbreak-associated foods, such as meat, poultry, starchy foods (rice and potatoes), pudding, soups, and cooked vegetables, in the danger zone of 5 to 57°C (64). Moreover, it is important to monitor populations of *B. cereus* and temperatures during transportation, processing, and preservation. The *Bacteriological Analytical Manual* published by the FDA suggests mannitol-egg yolk-polymyxin agar as a standard medium for plating *B. cereus* (59). This is part of a conventional method to detect *B. cereus* in food products, but it has a limitation of being time-consuming. Therefore, several rapid detection methods have been developed to detect *B. cereus* in food products.

Various attempts have been made to detect *B. cereus* rapidly. PCR has long been adopted for the detection of *B. cereus*. Hansen and Hendriksen (26) indicated that the enterotoxigenic *B. cereus* and *Bacillus thuringiensis* strains can be detected by PCR using primer sets for the detection of genes encoding the enterotoxin. Lim et al. (41) developed a duplex real-time PCR method to differentiate emetic *B. cereus* from the nonemetic *B. cereus* in food products. Wehrle et al. (69) used a multiplex real-time PCR assay based on SYBR green I to differentiate *Bacillus* groups (*B. cereus*, *B. mycoides*, *B. thuringiensis*, and *B. weihenstephanensis*), while Forghani et al. (19) developed a rapid multiplex real-time PCR assay for the simultaneous detection of *B. cereus*, *L. monocytogenes*, and *S. aureus* in food products. In this regard, PCR can be used effectively for the detection of *B. cereus*. However, it is still difficult to differentiate *B. cereus* from *B. thuringiensis* in food products by PCR. Methods using double-antibody sandwich enzyme-linked immunosorbent assay (71) or endolysin cell wall binding domain from bacteriophage (38) have also been reported. The major limitation of these methods is that their efficiency decreases significantly when applied to complex food matrixes. Further study is needed to increase rapid *B. cereus* detection sensitivity and selectivity in various food products.

REGULATIONS TO CONTROL SPORE-FORMING PATHOGENS IN FOODS

Several regulations have been indicated to ensure microbiological safety in food products. The U.S. Department of Agriculture (USDA) cited the bacterial danger zone as 4.4 to 60°C, in which bacteria can grow rapidly in food products (62). The USDA recommends not leaving food products out of refrigeration for 2 h (or 1 h when the ambient temperature is above 32°C) (62). Moreover, it is recommended to roast meat and poultry at temperatures higher than 162°C (324°F) and to reheat food thoroughly to an internal temperature of 60, 63, 71, and 74°C for ham, beef, ground meat, and poultry, respectively, after prolonged storage to inactivate reintroduced microorganisms (62). The Pasteurized Milk Ordinance indicates that the interaction of pH and water activity (a_w) affects the control of spores in milk and milk products (66). Time-temperature treatments are not needed when the pH is lower than 4.6, but time-temperature treatments are required when pH values are 4.6 to 5.6 and a_w is higher than 0.95 or pH is higher than 5.6 and a_w is higher than 0.92. The Food Safety Modernization Act (FSMA) guidance for the industry indicates that the growth conditions of *B. cereus* are as follows (65): minimum a_w , 0.92; minimum pH, 4.3; maximum pH, 9.3; maximum percentage of water-phase salt, 10; minimum temperature, 4°C; and maximum temperature, 55°C. From these growth conditions, FSMA reported the following time-temperature guidance for the growth and toxin formation conditions by *B. cereus* in food products: 4 to 6°C, 5 days; 7 to 15°C, 1 day; 16 to 21°C, 6 h; and more than 21°C, 3 h.

CONCLUSIONS AND RECOMMENDATIONS

B. cereus is a foodborne pathogen of interest involved in several foodborne outbreaks and deaths, and cases may exceed the number reported based on limitations in reporting systems and data collection. Control of *B. cereus* in food, in groundwater, and on utensils during processing is essential, including preventing cross-contamination. Foodborne illnesses from *B. cereus* can occur even when food samples are pasteurized appropriately because of surviving spores; thus, challenge studies for conventional or alternative inactivation treatments are critical, as well as postprocessing cold storage.

Inducing germination before an intervention treatment is one of the most effective ways to control *B. cereus* spores. Various germinants have been reported, but the resistance of *B. cereus* to intervention treatments following germination may increase and should be studied further. Germinated *B. cereus* can be inactivated effectively by combination treatments, which have received attention as alternative technologies. Several hurdle technologies have been indicated for the inactivation of spores or germinated cells in the present review, while more studies are needed for technologies applied singly or in combination. In addition, understanding of the bactericidal mechanisms by individual or combined interventions and development of rapid detection methods are helpful in providing more meticulous control of *B. cereus* in the food industry.

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