FOODBORNE PATHOGENS AND DISEASE
Volume 10, Number 5, 2013

DOI: 10.1089/fpd.2012.1355

Pathogenic Psychrotolerant Sporeformers: An Emerging Challenge for Low-Temperature Storage of Minimally Processed Foods

Sarah M. Markland,[1] Daniel F. Farkas,[2] Kalmia E. Kniel,[1] and Dallas G. Hoover[1]

Abstract

Sporeforming bacteria are a significant problem in the food industry as they are ubiquitous in nature and capable of resisting inactivation by heat and chemical treatments designed to inactivate them. Beyond spoilage issues, psychrotolerant sporeformers are becoming increasingly recognized as a potential hazard given the ever-expanding demand for refrigerated processed foods with extended shelf-life. In these products, the sporeforming pathogens of concern are *Bacillus cereus, Bacillus weihenstephanensis,* and *Clostridium botulinum* type E. This review article examines the foods, conditions, and organisms responsible for the food safety issue caused by the germination and outgrowth of psychrotolerant sporeforming pathogens in minimally processed refrigerated foods.

Introduction

Bacterial spores

No other life form is as difficult to eliminate as a bacterial endospore. Spores have evolved into resilient survival packages withstanding just about any stress agent or conditions imaginable over extremely long periods of time. Normally the success of any sterilization procedure is judged by its ability to inactivate the last remaining spore. In foods, ingestion of spores themselves is generally of no concern unless the spores are capable of germinating in the digestive tract. Otherwise, intact resting spores harbor no measurable pathogenic activity. This makes the understanding of conditions for spore germination and outgrowth in foods during storage and consumption of fundamental importance. This is of particular importance due to increased demand for minimally processed foods and refrigerated processed foods of extended durability, where psychrotolerant sporeformers can be a problem. These types of foods include those that are able to be refrigerated for extended periods of time without spoilage and are not heated prior to consumption. If these foods are contaminated with psychrotolerant sporeformers, these spores may have the ability to germinate and even grow at refrigeration temperatures.

Spores of *Bacillus* and *Clostridium* species are of considerable concern in the food industry, due to their common occurrence, robustness, and in some cases the toxins that can be synthesized upon germination and outgrowth (Setlow, 2003; Coleman *et al.*, 2007; De Vries *et al.*, 2004). Spore formation is triggered by nutrient depletion, causing a vegetative cell to be transformed into a dormant spore; however, the spore is still responsive to specific agents in the environment. When the environment becomes favorable again with available nutrients, spores can revert back to their active state.

The process of germination is triggered by germinant compounds that bind to receptors in the inner membrane of the spore (Fig. 1). These nutrient germinants include certain amino acids, sugars, and purine nucleosides (Setlow, 2003). There are also several non-nutrient germinants, including dodecyclamine (DPA), sublethal heat treatment, high pressure, specific peptidoglycan fragments, and bryostatin, an activator of serine/threonine protein kinases (Wei *et al.*, 2010).

Spores can survive for long periods of time in food products, particularly in foods where nutrient content is low or nonexistent (Coleman *et al.*, 2007). When these spores germinate, foodborne illness can occur (Setlow, 2003). It would be ideal to be able to trigger the germination of all spores present in the food product prior or during any preservation treatment in order to eliminate them since spores are much more susceptible to inactivation after they have germinated (Ghosh and Setlow, 2009b). Although this strategy seems simple, germination rates vary, and a small percentage of spores germinate extremely slowly or not at all after exposure to germinants (Ghosh and Setlow, 2009a; Wei *et al.*, 2010). Such spores are known as superdormant spores. Tyndallization, whereby low-acid foods are heated and cooled several times

[1]Department of Animal and Food Sciences, University of Delaware, Newark, Delaware.
[2]Department of Food Science and Technology, University of Oregon, Corvallis, Oregon.

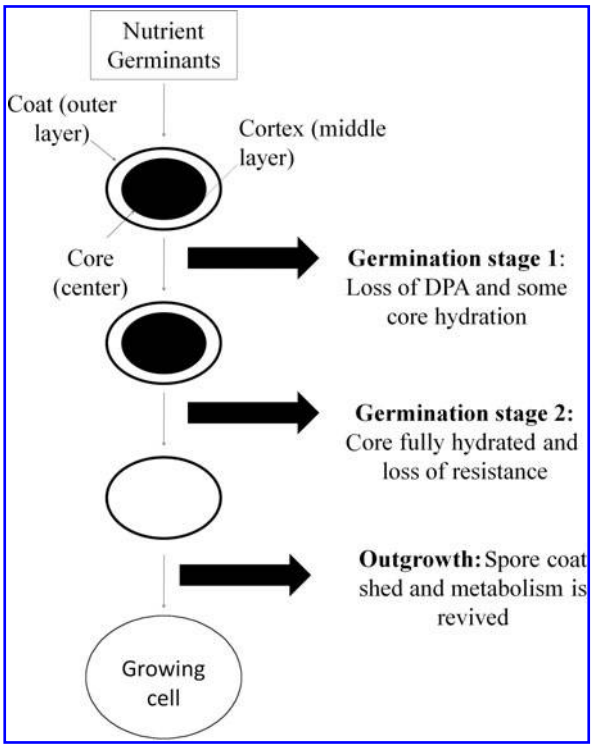

FIG. 1. Events in germination triggered by nutrients. DPA, dodecyclamine. (Adapted from Black *et al*., 2007).

over a period of days to germinate and inactivate spores, is based on this concept and is no longer used due to risks presented with superdormant spores.

Superdormant spores

Superdormant spores are spores that either fail to germinate or germinate very slowly (Ghosh and Setlow, 2009a). Superdormancy has been recognized as a characteristic in *Bacillus* species, and has also been found to occur in *Clostridium difficile* spores where it is more likely to occur as spores age (Rodriguez-Palacios and Lejeune, 2011). The study of superdormant spores has been facilitated by a simple isolation method developed by Ghosh and Setlow (2009a) for *B. subtilis*, *B. megaterium*, and *B. cereus.* This method, called buoyant density centrifugation, separates dormant spores from germinating spores and debris by density adjustment of the centrifuging fluid with multiple cycles of heat shock and germination.

It appears that the physiological state for superdormancy is similar for all *Bacillus* species (Ghosh and Setlow, 2009a) and more than likely similar with regard to *Clostridium* species. Recent studies provide evidence that superdormancy is a result of a reduced level of germinant receptors in the inner membrane (Ghosh and Setlow, 2009b; Wei *et al*., 2010; Zhang *et al*., 2010).

Sublethal heat treatment prior to germination reduces the yield of superdormant spores; however, superdormant spores still show a higher temperature optimum for heat activation than the remainder of the spore population (Ghosh and Setlow, 2009b). It also appears that superdormant spores have greater wet-heat resistances and lower core water contents (Ghosh *et al*., 2009). Superdormant spores germinate poorly in the presence of nutrient germinants as compared to other germinants such as dodecylamine or calcium dipicolinic acid. This is not surprising, since germination by dodecylamine or calcium dipicolinic acid does not require nutrient binding by receptors, nor does it require prior heat activation (Ghosh and Setlow, 2009b; Wei *et al.*, 2010).

A number of factors increase the rate of spore germination (Ghosh and Setlow, 2009b; Zhang *et al*., 2010). These include heat activation and an increased level of germinant receptors. Different germinant receptors within an individual spore are suspected to interact through aggregation. This could potentially amplify signals from large numbers of germinant receptors. The lack of these nutrient receptors may inhibit the amplification of this germination signal and may explain why higher yields of superdormant spores are observed with *Bacillus* strains that lack one or more germinant receptors (Ghosh and Setlow, 2009b). Heterogeneity in a spore population, resulting in varying rates of germination among individual spores, may also be due to adaptation of a particular bacterial species. Spores that germinate more slowly or at a reduced rate than the majority of the population are more likely to survive environmental changes where the majority of germinating spores are inactivated, thus increasing the likelihood of survival for the entire population (Ghosh and Setlow, 2009b).

Currently it is difficult to assess the food safety importance of superdormant spores in *B. cereus, B. weihenstephanensis*, and *C. botulinum* type E; however, one can assume that temperatures at or below 3°C used in chilled foods substantially reduce the frequency of spore germination. The phenomenon of superdormancy can reduce the rate even more. Therefore, a greater percentage of superdormancy in spore populations would help reduce the risk of foodborne disease from these bacteria in chilled foods.

Pathogenic Psychrotolerant Sporeformers

Bacillus cereus

B. cereus is a motile Gram-positive sporeforming bacterium that is a well-established foodborne pathogen (Chorin *et al*., 1997; Kotiranta *et al.,* 2000; De Vries *et al.,* 2004). It is found throughout nature but is most commonly isolated from soil and plants (Valero *et al*., 2003; Priest *et al*., 2004). Foodborne illnesses caused by *B. cereus* are directly related to the production of two toxin types: An emetic-type enterotoxin, and a group of several diarrheogenic-type enterotoxins (Chorin *et al*., 1997; De Vries *et al*., 2004). The emetic-type toxin, also known as cereulide, is a thermostable, cyclic peptide. The emetic-type toxin is not able to survive the acidic conditions of the host gastrointestinal environment and therefore is unable to contribute to diarrheal illness (Ceuppens *et al*., 2012). The enterotoxins responsible for the diarrheogenic symptoms are hemolysin, nonhemolytic enterotoxin, and cytotoxin (Ehling-Schulz *et al*., 2005; Lucking *et al*., 2009). The cell wall of vegetative *B. cereus* is also covered by proteins (called the S-layer) that play a role in cell adhesion and attribute to virulence of the organism (Kotiranta *et al*., 2000). Since the emetic toxin of *B. cereus* is heat stable, it can remain stable after cooking or heating. In a study reported by Rajkovic *et al*. (2008), cereulide toxin was demonstrated to remain stable at temperatures as high as 150°C at a pH as high as 10.6. The authors of this study also reported that highly alkaline conditions are needed to

achieve cereulide inactivation, and when alkaline buffer was removed, toxin activity could be recovered. Cereulide production does not occur until a stationary-growth phase is achieved; therefore, relatively high counts of vegetative cells or spores able to germinate in foods are usually required for cereulide intoxication to occur (Thorsen *et al.,* 2009).

Cereulide toxin is absorbed from the gut into the bloodstream and induces emetic-like symptoms including nausea and vomiting through stimulation of the vagus nerve (Jääskeläinen *et al.,* 2003). Ingestion of approximately $\leq 8\,\mu g/kg$ body weight of cereulide toxin within a food product is required to cause illness in humans (Jääskeläinen *et al*., 2003). Cereulide toxin affects the mitochondria by acting as a potassium ion channel–former (Mikkola, 1999) and causes apoptosis of human natural killer cells (Paananen *et al*., 2002). Mesophilic strains of *B. cereus* can only produce cereulide at temperatures above 10–15°C (Thorsen *et al*., 2009). This may explain why the toxin is mostly associated with foods that are improperly cooled and stored, such as rice and pasta dishes.

The diarrheogenic toxins are heat labile and can be destroyed by heat. These toxins are produced during the exponential-growth phase (Fermanian *et al*., 1994). Since spores of *B. cereus* are capable of surviving heat treatment and the acidic environment of the stomach, diarrheal-like symptoms can occur when spores of *B. cereus* are consumed in raw or minimally processed foods. Upon entering the small intestine, spores can germinate, outgrow, and multiply, enabling the production of the diarrheogenic toxin. According to an *in vitro* gastrointestinal transit study by Ceuppens *et al*. (2012), no vegetative cells were able to survive passage through the gastrointestinal system, but spores were able to very successfully survive the passage, indicating the importance of the physiological state of *B. cereus* cells in foods in order to cause foodborne illness. Depending on the amount of bacteria present in the food product, sometimes both sets of symptoms (emesis and diarrhea) can develop, causing what is known as "two-bucket disease." Symptoms from either *B. cereus* toxin should resolve within 24–48 h of onset; however, in extreme cases and in immunocompromised individuals, emetic intoxication can lead to liver failure and ultimately death.

The foods most frequently recognized with *B. cereus* intoxication are milk, vegetables, rice, potatoes, grains, cereals (including batters, mixes, and breadings), spices, and various sauces (Doona and Feeherry, 2007). *B. cereus* is not nutritionally fastidious, which is why the bacterium can replicate in soil and the low-nutrient foods including rice and pasta (Kotiranta *et al*., 2000). The reservoir for *B. cereus* is the soil, where transmission of the organism can occur through various vectors (Abee *et al*., 2011).

It is estimated that there are approximately 63,000 illnesses and 20 hospitalizations caused by *B. cereus* every year in the United States (Scallan *et al*., 2011). Foodborne illness caused by *B. cereus* is often underreported; however, recalls, illnesses, and even deaths caused by the organism have been documented within the past few years. According to *The Sydney Morning Herald*, an 81-year-old man died on January 12, 2007 after eating contaminated asparagus sauce prepared at a local restaurant. The article reported that the sauce was left at room temperature for more than 4 h after being refrigerated, and was found to contain $9.8\,\log_{10}$ colony-forming units/g of *B. cereus*. Naranjo *et al*. (2011) described the presumed cause of death of a healthy 20-year-old male in Brussels, Belgium following the ingestion of pasta contaminated with emetic strains of *B. cereus.* High levels of cereulide ($14.8\,\mu g/g$) were found in the leftover spaghetti. *B. cereus* counts of 9.5×10^7 colony-forming units/g were found in the pasta but not in the tomato sauce. The authors emphasized the need for adequate refrigeration of prepared foods because the emetic toxin is preformed in the food and not inactivated by heat treatment. Toxin production is closely linked to temperature (Finlay *et al*., 2000) and not strictly correlated with bacterial counts.

The *Bacillus cereus* group comprises seven species: *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. cytotoxicus.* The members of the *B. cereus* group are nearly impossible to distinguish phenotypically from each other (Kotiranta *et al*., 2000; Guinebretière *et al*., 2008). It has even been suggested through genetic evidence that members of the *B. cereus* group may actually represent one single species (Priest *et al*., 2004; Ehling-Schulz *et al.,* 2005; Auger *et al*., 2009). Because of the inability to separate these species genetically, they have been distributed between seven distinct phylogenic groups (Guinebretière *et al*., 2008; 2010). It has been found that there is a clear correlation between these phylogenic groups and adaptation to temperature, pH, and water activity, which can be used to predict the risk of a particular species to cause foodborne illness (Carlin *et al*., 2013). Several epidemiological studies have been performed comparing the genetic sequences of a variety environmental and food *B. cereus* isolates. The authors of these studies reported that psychrotolerant *B. cereus* isolates were more genetically similar to other psychrotolerant species, including *B. mycoides,* than to mesophilic *B. cereus* species, which were identified to be more genetically similar to *B. thuringiensis* (Schraft *et al*., 1996; Daffonchio *et al*., 2000; Sorokin *et al*., 2006; Guinebretière *et al*., 2008). Therefore, it was suggested that the taxonomy of the *B. cereus* group be revised. In 1998, a new species named *Bacillus weihenstephanensis* was proposed to accommodate the psychrotolerant strains of *B. cereus* (Lechner *et al*., 1998).

Bacillus weihenstephanensis

B. weihenstephanensis is differentiated from *B. cereus* by its ability to grow aerobically at 7°C in liquid culture and the absence of ability to grow at 43°C (Lechner *et al*., 1998). Genetically, *B. weihenstephensis* can be differentiated by the presence of the 16S rDNA signature sequence ^{1003}TCTAGAGATAGA and the signature sequence of the major cold-shock gene *cspA*, ^{4}ACAGTT (Lechner *et al*., 1998). Other research shows that not all strains of psychrotolerant *B. cereus* can be classified as *B. weihenstephanensis* and that there is an intermediate form between the two species (Stenfors *et al*., 2001). These intermediate forms produce both mesophilic and psychrotolerant genetic (polymerase chain reaction) products; for example, *B. cereus* strains identified as mesophilic by polymerase chain reaction demonstrated the ability to grow at 6°C, and other strains containing the *cspA* signature sequence were able to grow at 43°C (Stenfors *et al*., 2001). According to Guinebretière *et al*. (2008), *B. weihenstephanensis* belongs to *B. cereus* phylogenic group VI, which was shown to have a minimal growth temperature of 5°C. Specific guidelines need to be established for researchers to be able to distinguish mesophilic and psychrotolerant species of *B. cereus* and *B. weihenstephanensis*.

According to Guinebretière *et al*. (2010), only those species included in *B. cereus* phylogenic groups III, IV, and VII have been implicated as the cause of foodborne illness. These species include *B. cereus*, *B. thuringiensis*, and *B. anthracis*, which have a minimum growth temperature of ≥10°C. To assess whether *B. weihenstephanensis* may serve as a hazard in refrigerated food products, it must first be determined whether this species can produce toxin, and if so, at what temperatures cereulide toxin can be produced. This is especially important since this toxin is heat stable and cannot be destroyed during cooking and food processing. In a study where 93 strains of *B. weihenstephanensis* were screened for the presence of genes responsible for toxin production including *ces*B *cytK*-1 and *cytK*-2, none of these strains were found to contain any of these genes (Guinebretière *et al*., 2010). *B. weihenstephanensis* strain isolated from whole liquid egg product was able to produce toxin in the food at 6, 8, and 10°C, but not at 4°C; however, the isolate did not contain the *cesB* gene, which encodes for cereulide production (Baron *et al*., 2007). Environmental isolates of *B. weihenstephanensis* were able to produce the emetic toxin at temperatures as low as 8°C in food; however, toxin was not produced at levels great enough to cause illness (Thorsen *et al*., 2006). Strains of *B. weihenstephanensis* have been found to contain the gene responsible for cereulide production (*cesB*) (Thorsen *et al*., 2006; 2009), although *B. weihenstephanensis* has not been demonstrated to be able to produce cereulide at recommended refrigeration temperatures of 4°C or below. Temperature abuse can often occur during food shipping, distribution, and storage. Because *B. weihenstephanensis* has demonstrated the ability to grow at 6°C and produce toxin at 8°C, this bacteria should be identified as a potential hazard for refrigerated foods that are commonly subject to temperature abuse.

Because of their potential to grow in refrigerated food products, their ability to produce toxins, and their implications in foodborne outbreaks, psychrotolerant species of *B. cereus,* including *B. weihenstephanensis,* have been of concern in the food industry (Baron *et al*., 2007). *B. weihenstephanensis* is a known causative agent of spoilage in white liquid egg products but can also cause spoilage in pasteurized milk. The *B. weihenstephanensis* strain isolated from the spoiled whole liquid egg product also demonstrated the ability to adhere to surfaces and form biofilms. These films can form on processing equipment commonly used in egg-breaking facilities including stainless steel, model hydrophilic materials (glass), and model hydrophobic materials (polytetrafluoroethylene) (Baron *et al*., 2007).

Clostridium botulinum *type E*

C. botulinum is a Gram-positive, anaerobic bacterium that produces the most potent natural neurotoxin known. It is classified as a category A terrorism agent (CDC, 2003; Yule *et al*., 2006; Peck, 2010). There are seven types of *C. botulinum* that make up the proteolytic and nonproteolytic groups, which are distinguished on the basis of antigenically distinct toxins (A–G). Four types cause disease in humans. These types are A, B, E, and F (Telzak *et al*., 1990; Peck *et al*., 2010). Foodborne intoxication is caused by consumption of food containing amounts as small as 30–100 ng of preformed botulinal neurotoxin (Peck *et al*., 2010). Each year in the United States, there are approximately 55 cases, 42 hospitalizations, and 9 deaths that occur due to foodborne botulism (Scallan *et al*., 2011).

Type E neurotoxin is produced by the nonproteolytic, psychrotrophic form of *C. botulinum*, which has the ability to secrete neurotoxin at temperatures as low as 3°C (Peck, 2010). It has been demonstrated that environmental factors and pretreatments can affect lag time duration and variability of nonproteolytic *C. botulinum* more than germination rates (Stringer *et al.,* 2009). Lowering incubation temperature has a proportionally greater affect on outgrowth and doubling time of nonproteolytic *C. botulinum* rather than germination rates of spores (Stringer *et al.,* 2009), which indicates that historical treatment and growth conditions of nonproteolytic spores may help determine the risk of *C. botulinum* germination and outgrowth at refrigeration temperatures. Proteolytic varieties of *C. botulinum* are mesophiles producing neurotoxins A, B, or F and demonstrate little genetic similarity to the nonproteolytic types (Sebaihia *et al*., 2007; Peck, 2010). Because nonproteolytic strains of *C. botulinum* are psychotrophs, they are able to derive their energy through the degradation of sugars and produce neurotoxins at reduced temperatures (Peck, 2010). The nonproteolytic strains of *C. botulinum* are reportedly the main hazard associated with minimally heated refrigerated foods (Peck *et al*., 2010). Botulism causes flaccid paralysis, respiratory failure, and ultimately death, depending on the amount of toxin exposure (Telzak *et al*., 1990).

C. botulinum type E is the most prevalent form of botulism associated with marine life. Home-canned, raw or fermented, dried, and vacuum-packaged seafoods are most commonly associated with outbreaks (Telzak *et al*., 1989; Peck, 2010). Probably the botulism outbreak most relevant for comparison to contemporary minimally processed refrigerated foods was the 2006 outbreak associated with temperature abuse of pasteurized organic carrot juice. The carrot juice was low-acid (pH 6.0), low-sugar, and low-salt. It was flash-pasteurized, clean-filled, and sealed in 1-L bottles prior to refrigerated storage. Pasteurization will not inactivate spores of *C. botulinum* or other sporeforming bacteria. After purchase, it appears consumers did not properly refrigerate the juice, allowing for eventual production of neurotoxin type A in the product. Toxin-positive bottles of carrot juice gave no indication of clostridial growth. The juice smelled normal and no gas was produced (CDC, 2006).

Challenges for the Food Industry

Minimally processed foods

The increase in demand by consumers for convenient food products of premium sensory quality, including ready-to-eat, cooked, or chilled foods, and minimally processed foods, has led to the development of food products known as refrigerated processed foods of extended durability (Nissen *et al*., 2002). These products are normally low-acid foods refrigerated at close to freezing to maintain wholesomeness and safety (i.e., a national cold chain). They are often globally sourced. Fluid products usually are pasteurized. Produce and other raw foods commonly rely on surface cleaning/washing, modified or controlled atmosphere packaging, and other traditional hurdle approaches to ensure integrity and shelf-life.

Low-temperature storage

For mesophilic sporeforming species, temperatures below 15°C are generally thought to prevent spores from germinating.

This is why in a laboratory setting spore crops are suspended in water and stored under refrigeration with the assumption that the spore crop concentration will remain stable until use. Low temperatures and limited nutrients prevent germination; however, in the case of psychrotolerant sporeforming species, temperatures at or above 6°C (Lechner *et al.*, 1998) may allow for spore germination (albeit slowly) with outgrowth and perhaps permit cell multiplication in nutrient-rich environments. This possibility is why psychrotolerant sporeforming species, such as *B. weihenstephanensis*, which can potentially germinate and grow in refrigerated foods stored above optimum refrigeration temperatures of 4°C, are of possible concern. Such would be the case with minimally processed foods not heated or significantly heat processed prior to eating.

Influence of sporulation temperature on rates of germination and outgrowth. Spores populations of mesophilic *B. cereus* isolates were shown to germinate more rapidly in 10 mM of L-alanine when sporulated at 37°C than populations sporulated at 20°C (Raso *et al*., 1998); however, Gounina-Allouane *et al*. (2008) found the opposite with psychrotolerant *B. cereus* spores sporulated at 15 and 20°C. These spores were more easily germinated than spores sporulated at 37°C. Several previous studies demonstrated similar patterns with other sporeforming species. Spores of psychrotolerant strains of *B. subtilis* and psychrotolerant *C. botulinum* type E were more easily germinated with nutrients when sporulation occurred at lower temperatures (Cortezzo and Setlow, 2005; Evans *et al*., 1997). These results may indicate that spores sporulated at temperatures close to 37°C may have lower rates of germination and have higher percentages of superdormant spores. Heat activation of spores prior to germination increases spore germination and reduces overall yields of superdormant spores (Ghosh and Setlow, 2009a; Garcia *et al*., 2010; Wei *et al*., 2010); however, the mechanism by which heat activation affects germination is not well understood. Further investigation on the relationship of sporulation temperature to spore germination and superdormancy is needed. Understanding these mechanisms may also lead to further understanding of spore germination and outgrowth in refrigerated food products. In addition to affecting the rate of spore germination, sporulation temperature has been shown to affect spore size. In a study by Garcia *et al.* (2010), spores of *B. weihenstephanensis* sporulated at 12, 20, and 30°C were sized by scanning electron microscopy. Spores sporulated at 12 and 30°C were of similar size at approximately 1.5 μm, while spores sporulated at 20°C were slightly larger at approximately 1.8 μm. It was suggested that sporulation temperature may also affect the degree by which spores are germinated or inactivated through food-processing methods. The authors observed that as much as 99% of *B. weihenstephanensis* spore populations sporulated at 30°C germinated using 150 MPa of pressure, whereas only 50 and 15% germinated when sporulated at 12 and 20°C, respectively (Garcia *et al*., 2010).

Research Needs and Conclusions

The botulism outbreak involving Bolthouse™ carrot juice demonstrated the somewhat tenuous nature of nonsterile, low-acid products that rely on strict maintenance of the cold chain until the product is fully consumed. In this instance, anaerobic conditions and elevated temperatures allowed one or more spores of *C. botulinum* to germinate, replicate, and synthesize neurotoxin. Given the innate abilities of *B. cereus, B. weihenstephanensis*, and *C. botulinum* type E to conduct metabolic activities at lower temperatures than other bacteria, and the inherent resistance of their spores to inactivation, a high level of vigilance for these pathogens in nonsterile refrigerated foods carries merit. History has witnessed many emerging pathogens over the last 40 years become established issues in our food manufacturing and distribution systems. With continued demands for foods with minimal processing and reduced use of preservatives, there is clearly a need for additional hurdles that will protect these foods against temperature abuse during storage, distribution, and while in the hands of the consumer. These hurdles must meet the criteria of minimal processing and be perceived as generally regarded as safe preservatives.

Disclosure Statement

No competing financial interests exist.

References

Abee T, Groot MN, Tempelaars M, Zwietering M, Moezelaar R, Van Der Voort M. Germination and outgrowth of spores of *Bacillus cereus* group members: Diversity and role of germinant receptors. Food Microbiol 2011;28:199–208.

Al-Holy MA, Lin M, Rasco BA. Inactivation of *Bacillus cereus* by high hydrostatic pressure. In: *High Pressure Processing of Foods.* Doona CJ, Feeherry FE (eds.). Ames, IA: Blackwell, 2007, pp. 41–68.

Auger S, Ramarao N, Fouet A, Aymerich S, Gohar M. Biofilm formation and cell surface properties among pathogenic and nonpathogenic strains of the *Bacillus cereus* group. Appl Environ Microbiol 2009;75:6616–6618.

Baron F, Cochet MF, Grosset N, Madec MN, Briandet R, Dessaigne S, Chevalier S, Gautier M, Jan S. Isolation and characterization of a psychrotolerant toxin producer, *Bacillus weihenstephanensis* in liquid egg products. J Food Prot 2007;70:2782–2791.

Black EP, Setlow P, Hocking AD, Stewart CM, Kelly AL, Hoover DG. Response of spores to high-pressure processing. Comp Rev Food Sci Food Saf 2007;6:103–119.

Carlin F, Albagnac C, Rida A, Guinebretière ML, Couvert O, Nguyen-the C. Variation of cardinal growth parameters and growth limits according to phylogenetic affiliation in the *Bacillus cereus* Group: Consequences for risk assessment. Food Microbiol 2013;33:69–76.

[CDC] Centers for Disease Control and Prevention. Outbreak of a botulism type E associated with eating a beached whale—Western Alaska, July 2002. MMWR 2003;52:24–26.

Ceuppens S, Uyttendaele M, Hamelink S, Boon N, Van de Wiele T. Inactivation of *Bacillus cereus* vegetative cells by gastric acid and bile during in vitro gastrointestinal transit. Gut Pathol 2012;4:11.

Chorin E, Thuault D, Cleret JJ, Bourgeois CM. Modelling *Bacillus cereus* growth. Int J Food Microbiol 1997;38:229–234.

Coleman WH, Chen D, Li Y, Cowan A, Setlow P. How moist heat kills spores of *Bacillus subtilis.* J Bacteriol 2007;189:8458–8466.

Cortezzo DE, Setlow P. Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J Appl Microbiol 2005;98:606–617.

Daffonchio D, Cherif A, Borin S. Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the *Bacillus cereus* group. Appl Environ Microbiol 2000;66:5460–5468.

De Vries YP, Van Der Voort M, Wijman J, Van Schaik W, Hornstra LM, De Vos VM, Abee T. Progress in food-related research focusing on *Bacillus cereus.* Microb Environ 2004;19:265–269.

Ehling-Schulz M, Svensson M, Guinebretiere MH, Lindback LT, Andersson M, Schulz A, Fricker M, Christiansson A, Granum PE, Martlbauer E, Nguyen-The C, Salkinoja-Salonen M, Scherer S. Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. J Microbiol 2005;151:183–197.

Evans RI, Russell NJ, Gould GW, McClure PJ. The germinability of spores of a psychrotolerant, non-proteolytic strain of *Clostridium botulinum* is influenced by their formation and storage temperature. J Appl Microbiol 2007;83:273–280.

Fermanian C, Fremy JM, Claisse M. Effect of temperature on the vegetative growth of type and field strains of *Bacillus cereus*. Lett Appl Microbiol 1994;19:414–418.

Finlay WJJ, Logan NA, Sutherland AD. *Bacillus cereus* produces most emetic toxin at lower temperatures. Lett Appl Microbiol 2000;31:385–389.

Garcia D, van der Voort M, Abee T. Comparative analysis of *Bacillus weihenstephanensis* KBAB4 spores obtained at different temperatures. Int J Food Microbiol 2010;140:146–153.

Ghosh S, Setlow P. Isolation and characterization of superdormant spores of *Bacillus* species. J Bacteriol 2009a;191:1787–1797.

Ghosh S, Setlow P. The preparation, germination properties and stability of superdormant spores of *Bacillus cereus*. J Appl Microbiol 2009b;108:582–595.

Ghosh S, Zhang P, Li Y, Setlow P. Superdormant spores of *Bacillus* species have elevated wet-heat resistance and temperature requirements for heat activation. J Bacteriol 2009;191:3822–3831.

Gounina-Allouane R, Broussolle V, Carlin F. Influence of the sporulation temperature on the impact of the nutrients inosine and L-alanine on *Bacillus cereus* spore germination. Food Microbiol 2008;25:202–206.

Guinebretière ML, Thompson FL, Sorokin A, Normand P, Dawynd P, Ehling-Schulz M, Svensson B, Sanchis V, Nguyen-The C, Heyndrick M, De-Vos P. Ecological diversification in the *Bacillus cereus* group. Environ Microbiol 2008;10:851–865.

Guinebretière MH, Velge P, Couvert O, Carlin F, Debuyser ML, Nguyen-The C. Ability of Bacillus cereus group strains to cause food poisoning varies according to phylogenic affiliation (groups I to VII) rather than species affiliation. J Clin Microbiol 2010;48:3388–3391.

Jääskeläinen EL, Teplova V, Andersson MA, Andersson LC, Tammela P, Andersson MC, Pirhonen TI, Saris NE, Vuorela P, Salkinoja-Salonen MS. In vitro assay for human toxicity of cereulide, the emetic mitochondrial toxin produced by food poisoning *Bacillus cereus.* Toxicol In Vitro 2003;17:737–744.

Kotiranta A, Lounatmaa K, Haaplasalo M. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect 2000;2:189–198.

Lechner S, Mayr R, Francis KP, Prub BM, Kaplan T, Weibner-Gunkel E, Stewart GASB, Scherer S. *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int J Syst Bacteriol 1998;48:922–931.

Lucking G, Dommel MK, Scherer S, Fouet A, Ehling-Schulz M. Cereulide synthesis in emetic *Bacillus cereus* is controlled by the transition state regulator AbrB, but not by the virulence regulator PlcR. J Microbiol 2009;155:922–931.

Mikkola R, Saris NE, Grigoriev PV, Andersson MA, Salkinoja-Salonen MS. Ionophoretic properties and mitochondrial effects of cereulide, the emetic toxin of *Bacillus cereus*. Eur J Biochem 1999;263:112–117.

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

Nissen H, Rosnes JT, Brendehaug J, Kleiberg GH. Safety evaluation of sous vide-processed ready meals. Lett Appl Microbiol 2002;35:433–438.

Paananen A, Mikkola R, Sarneva T, Matikainen S, Hess M, Andersson M, Julkunen I, Salkinoja-Salonen MS, Timonen T. Inhibition of human natural killer cell activity by cereulide, an emetic toxin from *Bacillus cereus*. Clin Exp Immunol 2002;129:420–428.

Peck MW. *Clostridium botulinum*. In: *Pathogens and Toxins in Foods: Challenges and Interventions*. Juneja VK, Sofos JN (eds.). Washington, DC: ASM, 2010, pp. 31–48.

Priest FG, Barker M, Baillie LWJ, Holmes EC, Maiden MCJ. Population structure and evolution of the *Bacillus cereus* group. J Bacteriol 2004;186:7959–7970.

Rajkovic A, Uyttendaele M, Vermeulen A, Andjelkovic M, Fitz-James I, in't Veld P, Denon Q, Verhe R, Debevere J. Heat resistance of *Bacillus cereus* emetic toxin, cereulide. Lett Appl Microbiol 2008;46:536–541.

Raso J, Gongora-Nieto MM, Barbosa-Canovas V, Swanson B. Influence of several environmental factors on the initiation of germination and inactivation of *Bacillus cereus* by high hydrostatic pressure. Int J Food Microbiol 1998;44:125–132.

Rodriguez-Palacios A, Lejeune JT. Moist-heat resistance, spore aging, and superdormancy in *Clostridium difficile*. Appl Environ Microbiol 2011;77:3085–3091.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States—Major pathogens. Emerg Infect Dis 2011;17:7–15.

Schraft H, Steele M, McNab B, Odumeru J, Griffiths MW. Epidemiological typing of *Bacillus* spp. isolated from food. J Appl Environ Microbiol 1996;62:4229–4232.

Sebaihia M, Peck MW, Minton NP, Thomson NR, Holden MTG, Mitchell WJ, Carter AT, Bentley SD, Mason DR, Crossman L, Paul CJ, Ivens A, Wells-Bennik MJH, Davis IJ, Cerdeno-Tarraga AM, Churcher C, Guail MA, Chillingworth T, Feltwell T, Fraser A, Goodhead I, Hance Z, Jagels K, Larke N, Maddison M, Moule S, Mungall K, Norbertezak H, Rabbinowitsch E, Sanders ME, Simmonds M, White B, Whithead S, Parkhill J. Genome sequence of proteolytic (Group I) *Clostridium botulinum* strain Hall A and comparative analysis of the clostridial genomes. Genome Res 2007;17:1082–1092.

Setlow P. Spore germination. Curr Opin Microbiol 2003;6:550–556.

Sorokin A, Candelon B, Guilloux K, Galleron N, Wacherow-Kouzova N, Ehrlich SD, Bourguet D, Sanchis V. Multiple-locus sequence typing analysis of *Bacillus cereus* and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of psychrotrophic strains. Appl Environ Microbiol 2006;72:1569–1578.

Stenfors LP, Granum PE. Psychrotolerant species from the *Bacillus cereus* group are not necessarily *Bacillus weihenstephanensis*. FEMS Microbiol Lett 2001;197:233–238.

Stringer SC, Webb MD, Peck MW. Contrasting effects of heat treatment and incubation temperature on germination and outgrowth of individual spores of nonproteolytic *Clostridium botulinum* bacteria. Appl Environ Microbiol 2009;75:2712–2719.

Telzak EE, Bell EP, Kautter DA, Crowell L, Budnick LD, Morse DL, Schultz S. An international outbreak of type E botulism due to uneviscerated fish. J Infect Dis 1990;161:340–342.

Thorsen L, Hansen BM, Nielsen KF, Hendriksen NB, Phipps RK, Budde BB. Characterization of emetic *Bacillus weihenstephanensis*, a new cereulide-producing bacterium. Appl Environ Microbiol 2006;72:5118–5121.

Thorsen L, Budde BB, Henrichsen L, Martinussen T, Jakobsen M. Cereulide formation by *Bacillus weihenstephanensis* and mesophilic emetic *Bacillus cereus* at temperature abuse depends on preincubation conditions. Int J Food Microbiol 2009;134:133–139.

Valero M, Fernandez PS, Salmeron MC. Influence of pH and temperature on growth of *Bacillus cereus* in vegetable substrates. Int J Food Microbiol 2003;82:71–79.

Wei J, Shah IM, Ghosh S, Dworkin J, Hoover DG, Setlow P. Superdormant spores of *Bacillus* species germinate normally with high pressure, peptidoglycan fragments, and bryostatin. J Bacteriol 2010;192:1455–1458.

Yule AM, Barker IK, Austin JW, Moccia RD. Toxicity of *Clostridium botulinum* type E neurotoxin to great lakes fish: Implications for avian botulism. J Wild Dis 2006;42:479–493.

Zhang P, Garner W, Yi X, Yu J, Li Y, Setlow P. Factors affecting the variability in time between addition of nutrient germinants and rapid dipicolinic acid release during germination of spores of *Bacillus* species. J Bacteriol 2010;192:3608–3619.

Address correspondence to:
Dallas G. Hoover, PhD
Department of Animal and Food Sciences
University of Delaware
531 S. College Ave.
044 Townsend Hall
Newark, DE 19716

E-mail: dgh@udel.edu

This article has been cited by:

1. A. Charnot-Katsikas, V. Tesic, S. Boonlayangoor, C. Bethel, K. M. Frank. 2013. Prospective Evaluation of the VITEK(R) MS for the Routine Identification ofBacteria and Yeast in the Clinical Microbiology Laboratory: Assessment ofAccuracy of Identification and Turnaround Time. *Journal of Medical Microbiology* . [CrossRef]