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Eneterotoxigenic Bacillus cereus and Bacillus thuringiensis Spores in U.S. retail Spices

Upasana Hariram
University of Massachusetts Amherst

Eneterotoxigenic *Bacillus cereus* and *Bacillus thuringiensis* Spores in U.S. retail Spices

A Thesis Presented

By

UPASANA HARIRAM

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Approved as to style and content by:

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Ronald Labbe, Chair

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Robert E. Levin, Member

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Lynne A. McLandsborough, Member

_____

Eric Decker, Department Head

Department of Food Science

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ABSTRACT

ENTEROTOXIGENIC *BACILLUS CEREUS* AND *BACILLUS THURINGIENSIS* SPORES IN US RETAIL SPICES

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UPASANA HARIRAM B.E., PES INSTITUTE OF TECHNOLOGY, BANGALORE INDIA

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Ronald Labbe

Bacillus cereus is a ubiquitous organism and a potential foodborne pathogen that can cause two types of gastrointestinal diseases: emesis and diarrhea. The emetic syndrome is caused by a heat and acid stable peptide toxin that is pre-formed in food, while the diarrheal syndrome is associated to two 3-protein, heat labile enterotoxin complexes that are formed in the intestine after ingestion of the organism. There are many reports on the isolation and characterization of *Bacillus cereus* from various foods, however there are no studies on the levels, toxigenicity and physical characteristics of *B. cereus* isolated from U.S. retail spices. A huge part of spices sold in the U.S. are imported from developing nations. Developing nations lack hygienic practices during processing and packaging of spices, due to which there is a high chance of imported spices being contaminated with *B. cereus*. Therefore, the main objective of this thesis work was to characterize *B. cereus* spores from U.S. retail spices. Levels of aerobic spores and *B. cereus* spores were determined. *B. cereus* spores were further analyzed for their enterotoxigenic ability, growth characteristics and physical spore characteristics.

In the 247 spice samples analyzed 77 were found to contain *B. cereus*, while 11 were positive for *B. thuringiensis*. Eighty four of the 88 spices tested possessed either one of the enterotoxin genes.

None of the isolates tested positive for the emetic toxin (*ces*) gene. Seventy five of the *B. cereus* isolates grew at 12 °C, although only two isolates grew well at 9 °C.

Seven selected diarrheal *B. cereus* spore strains had D_{95}-values ranging from 0.64-3.53 min while the two emetic strains had D_{95}-values of 7.04 min and 6.64 min. *B. cereus* grew well in pre-cooked rice. After 48 h, counts of 1.26 X 10^7 and 3.8 X 10^7 *B. cereus*/ 10 g were obtained in pre-cooked rice maintained at 17 °C and 20 °C respectively. At 12 °C, counts did not reach 10^4 CFU/ 10g even after 48 h of incubation. The aerobic mesophilic bacterial population and *B. cereus* population of 0.1% crushed pepper in pre-cooked rice over a period of 48h at temperature 20 °C and 17 °C were also analyzed. Counts of *B. cereus* in pepper rice samples reached a maximum of 1600 MPN/ 10 g and 1100 MPN/ 10 g at 20 °C and 17 °C respectively while the aerobic mesophilic counts per 10 g were 2.4 X 10^8 and 4.4 X 10^6 at these temperatures. The low *B. cereus* counts and high aerobic mesophilic population indicates competition of nutrients in cooked rice by background flora other than *B. cereus*.

The physical spore characteristics of five *B. cereus* and 3 *B. thuringiensis* strains were studied using transmission electron microscopy (TEM). Tubular, whip-like appendages were present in four B. *cereus* and two *B. thuringiensis*, while all seven isolates possessed exosporia.

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CHAPTER 1

INTRODUCTION

Bacillus cereus is an endospore-forming, gram positive, facultative anaerobe that has the potential to cause two types of foodborne diseases: diarrhea and emesis. The organism, commonly isolated from food produces various virulence factors or enterotoxins. The diarrheal disease is associated with foods and has an incubation period of 8-16 h. Symptoms associated to the diarrheal type include abdominal pain and severe watery diarrhea (Kramer and Gilbert, 1989). While the diarrheal type resembles foodborne illness caused by *Clostridium perfringens*, the emetic type is parallel to *Staphylococcus aureus* food poisoning, and is linked to farinaceous foods especially cooked rice. Nausea, vomiting and malaise are the symptoms of the emetic disease which occur rapidly (0.5-5 h) after consumption of contaminated food (Kramer and Gilbert, 1989).

The diarrheal enterotoxins are produced at an optimum temperature of 32 °C to 37 °C and pH of 7.5 during the late exponential growth phase. It consists of two to three protein components that are heat labile and susceptible to protein digestion (Drobniewsky, 1993). The emetic toxin is a heat stable peptide that can withstand temperatures of up to 121°C for 90 minutes. The toxin is resistant to peptide digestion, and is produced during the late log phase or stationary phase of growth at an optimum ranging from 25 °C to 35 °C (Drobniewsky, 1993). Since the symptoms of the diarrheal and emetic syndromes are self-limiting and last for only 24 h, the incidence of gastrointestinal distress is higher than it is reported (Drobniewsky, 1993).

Microorganisms are indigenous to soil and plants in which spices are grown, and the species that survive the drying process make up the initial microflora for spices (ICMSF, 2005). As spices are derived from soil-grown plants, they are considered to be one of the most contaminated food additives. Contamination of spices with bacteria has been linked to environmental and fecal contamination during processing. The use of spices in foods can add to the existing microflora or

could contaminate food (Koohy-Kamaly-Dehkordy et al., 2013). Naeumayr et al., (1983) found that spores make up 50% of mesophilic aerobic plate counts in spices. This is because spores are able to survive the drying process and long storage conditions (ICMSF, 2005). Many foods such as gravies, sauces, or stews can be contaminated as the added spices are store houses for spore forming bacteria, and under favorable conditions, they germinate, proliferate and sometimes produce enterotoxins. (Banerjee & Sarkar, 2003). *Bacillus cereus* in its spore and vegetative form is widely found in soil. Spices contaminated with spores of *B. cereus* used to prepare meat dishes have been attributed to cause several outbreaks of the diarrheal syndrome in Hungary during the 1960's. The spores in spices survive cooking temperatures, germinate when cooled improperly, while the nutrients in meat support growth of the resulting vegetative cells (Kramer and Gilbert, 1989). In 2007, a spice blend used in a cous-cous dish was implicated to have caused an outbreak of *B. cereus* food poisoning inflicting 146 kindergarten and school children in France. Another outbreak in the year 2010 in Denmark occurred due to *B. cereus* contaminated white pepper used to season stew. The stew was temperature abused while being served at a catering setting and affected 112 people. (U.S. FDA, Center for Food Safety and Applied Nutrition, 2013).

Although *B. cereus* has been isolated from various spices at rates ranging from 37% to 100% (Baxter et al., 2006; De Boer et al., 1985; Pafumi, 1986; Kneifel et al., 1993; Banerjee et al., 2003), there have not been any studies that have investigated the toxigenicity of *Bacillus cereus* spore formers in U.S. retail spices. One of the objectives of the current study involves the detection of *B. cereus* spore formers and their toxigenic potential in U.S.-retail spices.

Rice is the most consumed cereal in the world and has been reported as an important vehicle in food poisoning caused by *B. cereus* (Kramer & Gilber, 1989; Granum, 2000). The preparation of many rice dishes requires the addition of spices while cooking and right after cooking which makes it logical to test the growth of *B. cereus* spice-isolates in rice as well as growth of *B. cereus* in rice with selected spices as the inoculum.

CHAPTER 2

LITERATURE REVIEW

2.1 History

In the early 1900's there were many records of food poisoning that were reported to have been caused by a *B. cereus* like organism. Hauge's (1950, 1955) was the first to establish that *B. cereus* is a food borne pathogen after investigating four outbreaks in Norway. He confirmed that the organism was capable of causing watery diarrhea and abdominal pain after he had consumed vanilla sauce containing 92 X 10^6 *B.cereus* per ml (Drobnewiski, 1993).

Twenty years after the diarrheal syndrome was identified, *B. cereus* was established as an agent that is capable of causing an emesis type of gastrointestinal disease that is very similar to staphylococcus food poisoning (Ehling-Shulz et. al., 2004). In 1971 the emetic type of gastroenteritis was identified after many incidents that had been associated with consumption of fried rice from Chinese restaurants and take-away outlets had occurred in the United Kingdom. Between the years 1971 and 1984 as many as 192 episodes involving over a 1000 cases of the emetic syndrome were recorded in the United Kingdom (Kramer and Gilbert, 1989).

2.2 Classification

Bacillus anthracis, B. cereus, Bacillus mycoides, Bacillus thuringiensis, Bacillus psuedomycoides, and Bacillus weihenstephanensis are six closely related species that are members of the *Bacillus cereus* group. DNA hybridization, 16S and 23S rRNA similarity studies of these species suggest that these organisms form a genealogically tight group of organisms (Ash and Collins, 1992; Ash et al., 1991; Montville and Mathews, 2005). These organisms produce large vegetative cells that have a cell diameter of >0.9µm, and produce ellipsoidal spores that do not distend the sporangia (Kramer and Gilbert, 1989). Typical cell dimensions are 3-5µm in length and 1-1.12µm in diameter (Claus and Berkley, 1986; Lechner et al., 1998; Nakamura et al., 1998). *B. thurigiensis* can be differentiated from the other species by the synthesis of an intracellular crystalline toxin that is

toxic to specific insects. The *cry* gene located on a plasmid encodes the crystalline protein, and if lost during sub-culturing, *B. thuringiensis* cannot be differentiated from *B. cereus* (Schraft and Griffiths, 2006). *B. weihenstephanensis* is a psychrotolerant organism that can be differentiated from *B. cereus* by growth between 4°C - 7°C but not at 43°C (Lechner et al., 1998). *B. weihenstephanensis* can be rapidly identified using rRNA analysis or cold shock protein gene-targeted PCR (Lechner et al., 1998). Although, *Bacillus psuedomycoides, B. mycoides* and *B. cereus* are physiologically and biochemically virtually the same, these organisms can be distinguished based on fatty acid analysis and 16S RNA sequencing (Nakamura, 1998).

2.3 *Bacillus cereus* food poisoning

Bacillus cereus causes two types of food borne illness - emesis and diarrhea. The diarrheal syndrome is caused by enterotoxins that are produced in the intestine. These toxins are heat labile and are produced when food contaminated with at least $10^5 – 10^7$ cells or spores is consumed. It takes an incubation period of 8-16 h before the onset of the diarrheal syndrome that is characterized by watery diarrhea, abdominal pain and occasionally nausea and vomiting (Granum, 1994; Arnesen et al., 2008; Logan, 2011). The emetic syndrome is caused by a heat stable toxin that is pre-formed in food; usually farinaceous foods contaminated with levels of 10^5-10^8 cells per gram. The disease is characterized by nausea, vomiting and occasional diarrhea after an incubation period of 0.5-5 h (Arnesen et al., 2008; Logan, 2011).

An infective dose range of 10^4-10^{11} CFU/ g has been isolated in foods implicated in *B. cereus* outbreaks (Kramer and Gilbert, 1989; Granum, 1997; Arnesen et al., 2008). The wide range is partly due to the enterotoxin production potentials of different strains, and the ability of the cells to exist as spores that can survive the acidic barrier in the stomach (Granum, 1997).

2.4 Isolation and identification

B. cereus is isolated by direct plating on two most widely used selective media: MYP (Mannitol-egg-yolk-polymyxin) and PEMBA (Polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue

agar). Both the selective media are based on the same detection principles of inability of *B. cereus* group organisms to ferment mannitol to acids, the expression of lecithinase activity manifesting into precipitation zones around suspect colonies, and resistance to antibiotic polymixin B. Colony characteristics of *B. cereus* on PEMBA and MYP are peacock blue and pink respectively, with a halo of lecithin hydrolysis around the colonies (van Netten and Kramer, 1992; Peng et al., 2001; Fricker et al., 2008). Presumptive positive colonies on the selective media are confirmed by tests recommended by the U.S. Food and Drug administration which include anaerobic utilization of glucose to produce acid, reduction of nitrate, L-tyrosin decomposition, positive Voges-Proskauer, and growth in 0.001% lysozyme (Kramer and Gilbert, 1989). Enrichment in brain heart infusion broth or tryptic soy broth can be used to isolate *B. cereus* from complex foods. For foods with lower that 1000 *B. cereus*/ g, or dried or starchy foods, an MPN procedure using tryptic soy broth and 89 units of polymyxin B/ ml has been suggested followed by confirmation on selective media (Kramer and Gilbert, 1989).

Some of the issues with the recommended selective agars (MYP and PEMBA) are growth of background flora leading to misidentification of colonies, and overlap of precipitation zones that hinder accurate enumeration (Peng et al., 2001; Tallent et al., 2012; Fricker et.al., 2011). These issues have been overcome by the development of a chromogenic agar that uses a synthetic substrate that is specific to the enzymes produced by certain microorganisms (Manafi, 1996). Studies comparing the recommended selective agar (MYP and PEMBA) with chromogenic agar (BCM, BACARA, and CBC) have shown that enumeration, identification, and isolation from food complexes has been easier with the chromogenic agar, and BCM has shown the best overall results (Peng et al., 2001; Tallent et al., 2012; Fricker et.al., 2011). The chromogenic agar BCM contains a synthetic substrate 5-bromo-4-chloro-3-indoxymyoinositol-1-phosphate that is cleaved by phosphatidylinositol phospholipase C (PI-PLC) giving a blue-turquoise color to *B.cereus* colonies. Additionally, the BCM plates can be stored for greater than three months at refrigerated

temperatures compared to the recommended 7-days storage for the MYP plates under refrigeration (Peng et al., 2001).

2.5 Reservoirs

B. cereus is a ubiquitous organism inhabiting a wide variety of environments such as soils, vegetation, natural water bodies, dust and many food types. *B. cereus* has been isolated from different types of foods notably cereals and its derivatives, milk and dairy products, dried or powdered food, spices, vegetables, and meat products (Kramer and Gilbert, 1989). Cooked rice is one of the most important causes of the emetic syndrome and *B.cereus* has been isolated from raw, boiled and fried rice. Additionally, *B. cereus* is present in raw and pasteurized milk (Kramer and Gilbert, 1989). *B. cereus*, in its spore form, can survive cooking temperatures in rice and pasteurization of milk, and with insufficient cooling and absence of competing micro flora the spores can germinate into vegetative cells and produce toxins (Andersson et al., 1995). Some strains of *B. cereus* are psychrotolerant and can survive in milk at refrigerated temperatures. The morphology and hydrophobic nature of spores allows its adhesion to different surfaces which can lead to the formation of biofilms on industry equipment (Andersson et al., 1995). *Bacillus* spores can live in dehydrated foods and when water is added to it, the spores germinate into vegetative cells. Similarly, spices have large number of spores including *B. cereus*, some strains of which can survive cooking temperatures. In the absence of rapid cooling these spores can germinate into vegetative cells that can potentially produce enterotoxins (Kramer and Gilbert, 1989).

2.6 Toxins

B. cereus causes two types of disease, emesis and diarrhea, which are caused by two very different toxins. While the emetic illness is caused by a toxin preformed in food, the diarrheal type is caused by one or more enterotoxins that are produced in the intestine (Kramer and Gilbert, 1989; Granum, 1994).

2.6.1 Emetic Toxin:

The emetic illness is caused by a toxin called cereulide that is stable to heat, acid and trypsin, therefore cannot be digested in food or the gastrointestinal tract. Cereulide is a 1.2kDa circular toxin that consists of three repeats of four amino acids, [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$ (Kramer and Gilbert, 1989). The chemical structure of the dodecadepsipeptide is closely related to the valinomycin toxin produced by *Streptomyces griseus*, thus it acts like a potassium ionophore and inhibits mitochondrial activity (Ehling-Shulz et al., 2004a; Arnesen et al., 2008 et al., 2008).

The expression of cereulide occurs towards the end of logarithmic phase of growth with maximum accumulation at the beginning of the stationary phase of *B. cereus* vegetative cell growth (Ehling-Shulz et.al, 2004a). The amount of cereulide produced varies among strains. Agata et al. (2002) detected cereulide ranging from 10-1280 ng/g in 13 different types of food. One explanation for the variation is that the expression of cereulide depends on various extrinsic factors such as incubation temperatures, oxygen, pH, aeration and presence of certain amino acids. Expression of the emetic toxin occurs between temperatures 12 °C and 37 °C, with maximum production seen at temperatures 12 °C to 22 °C (Finlay et al., 2000). The presence of glucose supports the expression of cereulide, while higher amounts of amino acids (leucine, isoleucine and glutamic acid) prevent its expression (Agata et al., 1999). Static incubation of food has been shown to be better for the production of the cereulide toxin (Shaheen et al., 2006). The synthesis of the cerelulide toxin occurs enzymatically by a non rhibosomal peptide synthetase (Ehling-Shulz et al., 2005).

There are three methods that have been described for *B. cereus* cereulide toxin detection: cytotoxicty assay, boar sperm assay, and LC-MS analysis (Ehling-Schulz et al., 2004a; Arnesen et al., 2008). The cytotoxicity assay is based on the ability of the cereulide toxin to form vacuoles in HEp-2 cells (Finlay et al., 1999). When boar sperm cells are exposed to the cereulide toxin they lose their motility. The ionophoric structure of the cerelulide toxin blocks the oxidative phosphorylation pathway and damages the mitochondria making the sperm cells immotile

(Andersson et al., 1998). The shortcomings of these assays are the low specificity as these assays are sensitive to other mitochondrial toxins, long pre-cultivation times and labor-intensive sample preparations (Ehling-Schulz et al., 2004b). The LC-MS is a more specific technique for the detection of the cereulide toxin; however, this method entails high equipment costs, laborious sample preparations and highly skilled personnel (Ehling-Schulz et al., 2004b). More recently, a new highly specific identification method using PCR specific to the *ces* gene has been described by Ehling-Schulz (2004b, 2005).

2.6.2 Diarrheal Toxin:

Three different toxins that are produced in the intestine during vegetative cell growth can cause the diarrheal syndrome. NHE and HBL are structurally related, both consisting of a 3 protein complex, while Cyt K is a single protein. (Heinriches et al., 1993; Granum and Lund, 1997; Lund et al., 2000). Beecher and Macmillan (1991) discovered the first diarrheal enterotoxin- Hemolysin BL (HBL) which consists of a B protein component (35 kDa) that binds to cells and allows lysis by the second L protein component. The second L protein subunit is made up of two parts L1 (36 KDa) and L2 (40 KDa). The NHE toxin is made up of three proteins A, B and C with molecular masses 39kDa, 45kDa and 105kDa respectively. For maximum cytotoxicity of the multicomponent enterotoxins, all three proteins of the complex are required (Lund and Granum, 1997). The HBL complex is encoded by genes on a single operon, the B, L1 and L2 proteins are encoded by genes *hblA*, *hblD*, and *hblC* respectively. The NHE proteins NheA, NheB and NheC are transcribed from three genes *nheA*, *nheB* and *nheC* respectively, which are also located on a single operon (Ryan et al., 1997; Granum and O'Sullivan, 1999). The NHE toxin is more commonly found in *B. cereus* strains and has been reported to be the main cause for cytotoxicity (Moravek et al., 2006).

Immunoassay kits for the detection of the enterotoxins NHE and HBL toxins are available commercially. These semi-quantitative tests use antibodies that are complementary to specific proteins in the NHE and HBL complex. The L2 component of the HBL toxin is detected by a

BCET-RPLA (Oxoid Ltd, UK) assay kit which is based on the principle of reverse antibody agglutination (Beecher and Wong, 1994). A TECRA-BDE visual immunoassay kit (Tecra International Pty., Australia) detects the NheA component of the NHE protein complex by enzyme linked immune assay (ELISA) sandwich test (Beecher and Wong, 1994).

2.7 Antimicrobial activity of spices

Spices and herbs have not only been used as flavorings in food but have also been used for extending the shelf life of foods, and preventing the spoilage and deterioration of food (Nakatani, 1994). Zaika (1988) listed cinnamon and clove to have a strong inhibitory effect against microorganisms, and listed allspice, oregano, sage, thyme and rosemary to exhibit medium inhibition against microbes. Black pepper, red pepper and ginger were listed to have weak inhibition (Zaika, 1988). Cinnamon and clove were reported to have caused a 0.5-1 log reduction in the viable spore count of *B. subtilis* spores in distilled water (Blank et al., 1988). Shankar and Murthy et al. (1979) demonstrated the inhibitory effect of the oil fraction of turmeric against *B. cereus* and other bacteria. Allium species such as onion and garlic were shown to have an antibacterial effect against a wide variety of gram positive and gram negative species due to its major antimicrobial factor, allicin (Conner, 1993). The flavor, aroma and the antimicrobial components of a spice is contained in its essential oil (Conner, 1993); indicating that the essential oil has a higher inhibitory effect compared to the spice itself.

Some of the important antimicrobial compounds in spices are phenols, alcohols, aldehydes ketones, ethers and hydrocarbons. Eugenol is the antimicrobial factor in clove and allspice as cinnamic aldehyde in cinnamon. These compounds cause cell leakage by interacting with the cell membrane, alter phospholipids and fatty acids, weaken energy meatabolism, upset nutrient uptake and electron transport system, and affect genetic material synthesis (Nychas, 1995). The antimicrobial compounds of essential oils and spices bind to protein and fat molecules in foods, making them less available for microbial inhibition (Raccach, 1984). Additionally, many factors including the

medium, environmental conditions and microorganism being tested, influence the antimicrobial activity of the spice (Zaika, 1988). Gram positive bacteria are more sensitive to spices compared to gram negative species due to the lipids in the cell membrane of gram negative bacteria that protect cells against the antimicrobial activity of spices. In a study that tested the effect of sage, rosemary and allspice on 24-gram positive and 22-gram negative strains, Salmonella Typhimurium required (1.0% sage) 10 times more sage than *B. cereus* (0.1% sage) to cause inhibition in broth. The spores of *B. cereus* also behaved in the same manner (Shelef, 1980).

2.8 Growth and survival conditions of *B. cereus* in foods

B. cereus can grow at temperatures ranging from 4°C to 50°C with optimal growth seen at temperatures ranging from 30°C to 40°C. A study investigating the growth of various emetic toxin strains at different temperatures showed that none of the emetic toxin producing strains grew at temperatures below 10 °C but grew at 48 °C. Fifteen nine percent of the non-emetic producing strains grew at 4°C or 7°C and only 39% grew at 48°C (Carlin et al., 2006).

The reported pH range for vegetative growth of *B. cereus* in media is between 4.5 and 9.3 however growth in food with pH 4.35 has been reported (Kramer and Gilbert, 1989). The optimum pH range for *B. cereus* is 6.0-7.0. Fried rice supports the growth of *B. cereus* and the water activity of different samples of fried rice was reported as 0.912-0.961 (Bryan et al., 1981). The lower limit for water activity for vegetative *B. cereus* growth is 0.912-0.95 (Kramer and Gilbert, 1989). The doubling time for *B. cereus* under optimal conditions of pH, temperature and water activity has been reported as approximately 23 min (ICMSF, 1996).

Studies reporting inhibition of *B. cereus* by chemical preservatives are scarce. Potassium sorbate at 0.39% and Sorbic acid at 0.26% have been reported to have an inhibitory effect on *B. cereus* in rice filling stored at 23 °C. In beef gravy held at 15 °C, 50µg/ml and 5µg/ml of nisin was reported to have an inhibitory effect on *B. cereus* growth and enterotoxin production respectively (Beuchat et al., 1997).

2.9 Heat resistance

One of the major concerns with *B. cereus* in the food and pharmaceutical industry is its ability to produce endospores that are resistant to heat. A lot of attention has been received to the heat resistance of spores in foods. Molin and Snygg, (1967) found *B. cereus* spores in phosphate buffer to have a D_{95} value of 13 min, while in olive and soybean oil D_{121} values of 17.5 and 30 minutes respectively were reported. Additionally, foods with a lower water activity have been shown to bear spores that are more heat resistant (Molin and Snygg, 1967).

B. cereus spores have been shown to have a wide range of heat resistance in phosphate buffer ranging from 1.2 to 36.2 min. Carlin et al., (2006) reported that spores of the emetic *B. cereus* strains are slow germinators and have higher resistance to heat compared to non-emetic *B. cereus* strains. Ankolekar and Labbe, (2009a) reported a significant difference ($P<0.05$) in the $D_{95°C}$ values between six emetic strains (12.7 to 27.9 min) and six diarrheal strains (1.8- 3.5 min). The lower heat resistance found in non-emetic strains could be the reason for cooked rice not being associated with the diarrheal syndrome (Ankolekar and Labbe, 2009a). D-values ranging from 1.4 to 21.2 min were reported for *B. cereus* spores isolated from fresh and minimally processed vegetables (Valero et al., 2002).

Van der Voort and Abee, (2013) showed that heat resistance of *B. cereus* spores was influenced by the sporulation conditions. They reported that spores of two emetic strains were significantly more heat resistant when grown in swarming colonies ($D_{95°C}$ of 18.7 and 19.9 min) and in biofilms ($D_{95°C}$ of 23.0 and 19.3 min), compared to when produced in liquid culture ($D_{95°C}$ of 13.3 and 5.6 min) and in 0.75% agar plates ($D_{95°C}$ of 10.8 and 6.8 min).

2.10 Growth in foods

B. cereus is a ubiquitous organism and due to its endospore forming capability can survive adverse conditions making it impossible to identify raw products not contaminated with *B. cereus* (Schraft and Griffiths, 2006) and can also contaminate other foods such as meat due to cross contamination

(Kramer & Gilbert, 1989). *B. cereus* has been isolated from a variety of foods including barbequed chicken, noodles, cakes, soups, steak, rice, egg, vanilla sauce, salmon, desserts, sprouts, spices etc. indicating that many foods can support the growth of *B. cereus* and production of toxins under favorable conditions.

Many studies have demonstrated the growth of *B. cereus* in rice and milk. *B. cereus* has been demonstrated to have a generation time of 17 h in pasteurized milk maintained at 6 °C and is capable of producing enterotoxins at unfavorable temperatures (te Giffel, 1995). Another study showed rapid growth of *B. cereus* to toxin-producing levels in cream maintained at 23 °C (Feijoo, 1997). Gilbert et. al., (1974) studied the growth of *B. cereus* in boiled rice at temperatures ranging from 5-50 °C and showed growth of *B. cereus* to be optimal at temperatures ranging from 30 to 37 °C. Counts of up to 7 $\log_{10}$ were achieved within 23 h at 30 and 37 °C when samples were inoculated with $\log_{10}$ counts of 1-1.6. Johnson et. al., (1983) demonstrated that there was little difference in the growth rate of diarrheal and emetic strains when grown in rice and TSB, however the maximum counts were higher in rice due to the late onset of the stationary phase. Carlin et. al., (2006) showed no significant difference in the estimated growth kinetics of emetic toxin producing strains, diarrheal strains, and food environment strains tested in Plate Count broth at 24 °C and 37 °C observed at a pH of 5.0, 7.0 and 8.0.

CHAPTER 3

OBJECTIVES

The objectives of this study are:

- To determine the levels of aerobic mesophilic spores in U.S. retail spices
- To determine the spore-levels and toxigenicityof *B. cereus* in U.S. retail spices
- To determine the survival, and growth, of selected enterotoxigenic strains of *B. cereus*
- Determine the physical characteristics of *B. cereus* spores

CHAPTER 4

SPORE PREVALENCE AND TOXIGENICITY OF *B. CEREUS* IN SPICES

4.1 Abstract

B. cereus is an endospore forming, potentially pathogenic, organism that has been isolated from various foods. In its spore state, it can resist cooking and processing steps and eventually germinate and outgrow when conditions are favorable. Recent incidents of foodborne illness with spices as the vehicle of transmission prompted this examination of U.S. retail spices for which there are few reports on the levels of spores of this organism, their growth characteristics of the associated isolates or their toxin producing abilities. Considering that the majority of spices sold in the U.S. are imported from developing countries that do not have sanitary practices in place, *B. cereus*, as a soil-associated organism, is a likely contaminant of imported spices. Therefore, this study focuses on the levels of aerobic-mesophilic spore formers and *B. cereus* spores associated with 247 retail spices purchased from five states in the U.S. Samples contained a wide range of aerobic-mesophilic, bacterial spore-counts that ranged from < 200 to $8.3X10^7$ CFU/g with 19.1% of samples at levels above 10^5/g. Paprika, allspice, peppercorns and mixed spices were some that possessed high levels of aerobic spores (> 10^7CFU/g). Using a novel chromogenic agar, *B. cereus* and *B. thuringiensis* spores were isolated from 77 (31%) and 11 (4%) samples respectively. Levels of *B. cereus* ranged from < 3 MPN/g to 1600 MPN/g. Eighty eight percent of *B. cereus* and 91% of *B. thuringiensis* possessed at least one type of enterotoxin gene: HBL (Hemolysin BL) or non- hemolytic enterotoxin (NHE). None of the 88 isolates obtained in this study possessed the emetic toxin gene (*ces*). Using commercially-available immunological toxin detection kits, the toxigenicity of the isolates was confirmed. The NHE enterotoxin was expressed in 98% *B. cereus* and 91% *B. thuringiensis* isolates that possessed the responsible gene. HBL enterotoxin was detected in 87 % of *B. cereus* and 100 % of *B. thuringiensis* PCR-positive isolates. Fifty-two percent of *B. cereus*

and 54 % of *B. thuringiensis* produced both enterotoxins. Ninety-seven percent of *B. cereus* isolates grew at 12°C though only two grew well at 9°C. The ability of these spice isolates to form spores, produce diarrheal toxins, and grow at moderately-abusive temperatures makes retail spices an important potential vehicle for foodborne illness due to *B. cereus,* in particular that caused by the diarrheal toxin-producing strains.

4.2 Introduction

Levels of aerobic spore-formers of up to 10^6-10^8 CFU/g have been reported in various spices (Baxter and Holzapfel, 1982; ICMSF, 2005; Little et.al., 2003). Of particular concern are spore-forming, foodborne pathogens such as *Bacillus cereus* and *Clostridium perfringens*. *B. cereus* is a potentially pathogenic bacterium that is present ubiquitously in the environment. These spores can survive cooking temperatures and, when exposed to favorable conditions, can germinate and multiply to levels associated with foodborne illness. In some cases *B. cereus* levels of 10^4-10^5 CFU/g have been found in spices (Banerjee and Sarkar, 2003; Baxter and Holzapfel, 1982; de Boer et.al., 1985; Julseth & Deibel, 1974; Knifeil and Berger, 1994; Konuma, 1988; Little, 2003; Pafumi, 1986; Te Giffel, 1996) with one report of levels of 10^8 CFU/g (Antai, 1988). Early surveys of the incidence of *B. cereus* in spices typically reported the distribution and levels of this organism though not their toxin-producing abilities.

Certain strains of *B. cereus* are able to produce one or more enterotoxins or an emetic toxin. *B. cereus* has been identified as the causative agent of two types of foodborne illness. A diarrheal type is characterized by diarrhea and abdominal pain resembling *C. perfringens* foodborne illness and is caused by one or more heat labile enterotoxins, while an emetic type is associated with nausea and vomiting resembling foodborne illness associated with *Staphylococcus aureus* and is due to a heat stable dodecadepsipeptide (Lindback & Granum, 2013). Of the diarrheal toxins, hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE), are regarded as the principle ones involved in foodborne illness and each is composed of three component proteins encoded by *hblA, hblD, and*

hblC in the case of HBL and *nheA*, *nheB*, and *nheC* for NHE (Granum, 2000). Isolates vary in their ability to produce these virulence factors.

In the period 1973-2010, fourteen documented spice-associated foodborne illness outbreaks occurred worldwide. Ten of the 14 outbreaks were caused by *Salmonella* and four by *Bacillus* spp. (U.S. FDA, Center for Food Safety and Applied Nutrition, 2013). Per capita consumption of spices (other than dehydrated onion and garlic) in the U.S. alone increased nearly 300% from 1966 to 2010 (USDA/ERS, 2012), and based on a retail study conducted in July 2009, 78% of U.S. households were found to use spices and herbs beyond salt and pepper (U.S. FDA, Center for Food Safety and Applied Nutrition, 2013). Spices contaminated with spores of *B. cereus* used to prepare meat dishes have been attributed to several outbreaks of the diarrheal syndrome in Hungary during the 1960's.The spores in spices survive cooking temperatures; germinate when cooled improperly, while the nutrients in meat support growth of the resulting vegetative cells (Kramer & Gilbert, 1989). In 2007, a spice blend used in a cous-cous dish was implicated in an outbreak of *B. cereus* food poisoning affecting 146 kindergarten and school children in France. Another outbreak in the year 2010 in Denmark occurred due to *B. cereus* contaminated white pepper used to season stew. The stew was temperature-abused while being served at a catering setting and affected 112 people (U.S. FDA, Center for Food Safety and Applied Nutrition, 2013). Considering that 80% of the 2012 U.S. spice supply was imported (U.S. FDA, Center for Food Safety and Applied Nutrition, 2013) from countries with marginal sanitary practices, there is a high chance of these spices possessing *B. cereus* either due to their ability to survive processing steps (such as drying) or due to environmental contamination. Indeed recent outbreaks due to *Salmonella*-contaminated spices have also highlighted the role of this commodity as a potential vehicle of foodborne illness and prompted this report of the incidence and virulence factors associated with current isolates of *B. cereus* spores from spices from retail outlets in the U.S.

4.3 Materials and Methods

4.3.1 Strains

Isolates in this study were obtained from a total of 247 spice samples from grocery stores, supermarkets, and ethnic food stores in western Massachusetts; south Texas; Milwaukee, Wisconsin; Atlanta, Georgia; and Berkeley, California. Control strains were from our culture collection. *B. cereus* ATCC 14579, (HBL complex), 1230/88 (NHE complex), and F4810/72 (*ces*) strains were kindly provided originally by A. Wong, Univ. of Wisconsin. F4810/72 was originally obtained from an outbreak with cooked rice as the vehicle (Turnbull et.al., 1979). Seafood isolate #10 was another *ces*-positive emetic control used, that was isolated from mussels (Rahmati & Labbe, 2008).

4.3.2 Enumeration of Aerobic Spores

A total of 247 spice samples were obtained from various supermarkets and ethnic food stores located in Western Massachusetts, Texas, Georgia and California. One gram of the spice was diluted in 9 ml of water and homogenized for 60 seconds in a Seward Stomacher 400. The homogenized sample was heated at 75°C for 15 minutes in a water bath to kill any vegetative cells, cooled and diluted. Dilutions were used to determine the aerobic spore count by pour-plating 1ml of each dilution with nutrient agar in duplicate. After the agar solidified a thin overlay of agar was added to each plate to prevent spreaders. Plates were incubated for 48 hours at 32°C. Colonies were counted manually in white light under a magnifying glass. Samples that had less than 20 colonies were analyzed using a 3 tube MPN procedure with tryptic soy broth (TSB). Tubes were incubated at 32°C for 48 hours and then analyzed for turbidity or pellicle formation.

4.3.3 Enumeration of *B. cereus*

One gram of each spice was homogenized with 9 ml of sterile distilled water in a Whirl-Pak stomacher bag. The spice extract was heated for 75°C for 15 minutes in a water bath to kill vegetative cells. The extract was cooled and subsequently diluted to 1:1000. A 3 tube MPN procedure with each of the 9 tubes containing 15ml of TSB with 0.15% polymyxin B was used.

1ml of each dilution was transferred to three sets of MPN tubes of varying dilutions. The inoculated tubes were incubated for 48h at 32°C. The contents of the positive tubes were confirmed by streaking on chromogenic agar (*Bacillus cereus/Bacillus thuringiensis* chromogenic medium, R&F Products, Downers Grove IL) for characteristic turquoise blue colonies (2-7mm) with or without halos. Plates were incubated for 18 to 24 hours at 32°C. Typical blue colonies were aseptically transferred to nutrient agar slants and stored at 4°C for further analysis.

4.3.4 Growth at low temperatures

The ability of *B. cereus* strains from spices to grow at low temperatures was determined. A loop full of each isolate was inoculated in 5ml of TSB and incubated at 32°C. One hundred microliters of the overnight culture was inoculated into 5ml of TSB. The tubes were maintained at 12°C for 14 days in a circulating water bath (Isotemp 1016, Fisher Scientific, Pittsburg, PA), and were analyzed every day for turbidity. Strains that were able to grow at 12°C were then checked for their ability to grow at 7°C using the above described procedure.

4.3.5 Crystal toxin analysis

To distinguish *B. cereus* from *B. thuringiensis* a loopful of each isolate was cultured in 9ml of nutrient broth for 24 hours. The culture (100µl) was spread plated on nutrient agar plates and incubated at 32°C for 3-4 days. Smears with sterile distilled water were prepared and viewed by phase contrast microscopy for the presence bi-pyramid toxin crystals characteristic of *B. thuringiensis* during sporulation. The toxin crystals were found either as free crystals or as parasporal crystal inclusions.

4.3.6 PCR

Strains of *B. cereus* and *B. thuringiensis* were tested for the presence of toxin-encoding genes. Single primer pairs were used to detect genes *nheA* and *nheB* of the NHE complex, genes *hblA* and *hblD* of the HBL complex, *ces* gene, and *hblC* gene (Table 1).

Isolates were cultured in 5ml of TSB and incubated at 20°C on a 150rpm shaker. 100µl of the overnight culture was transferred to 5ml of TSB which was maintained at 32°C at 150 rpm. Cells of each strain were grown until an A_{600} value of 0.6 OD was reached (approximately 3-4 hours). Cell template DNA was extracted by centrifuging 100µl of culture with an equal volume of double distilled water at 13600g for one minute. Cells were washed at least three more times in the same manner. The supernatant was boiled in a water bath for 20 minutes to extract DNA. The boiled supernatant was cooled immediately by placing in ice, and centrifuged for 1 minute at 13,600 g. The supernatant containing the extracted DNA was stored in a freezer.

All PCR reactions were performed using Maxime™ PCR premix tubes (Boca Scientific, Boca Raton, FL). The PCR reaction was carried out in a Techgene thermocycler (Techne Cambridge, UK) with an initial denaturation temperature of 5 minutes followed by 35 cycles of denaturation at 95°C for 40 sec, annealing for 40 sec and extension at 72°C for 1 min 20 sec, followed by a final elongation for 4 min at 72°C. The annealing temperature was set by calculating the melting temperature of both the forward and reverse primers and subtracting 2° from the average of the two melting temperatures. Table 1 summarizes primers used in the study. Results were determined by gel electrophoresis of 10µl of PCR products in a 1.2% agarose gel for 60 min at 70 V and staining with Midori green direct DNA staining dye.

Table 1 Oligonucleotide primers used in the study

Primers	Genes	Amplified Fragment	Sequences 5'→ 3'	T_a Annealing Temperature	Reference
CES F1	*ces*	1271bp	GGTGACACATTATCATATA AGGTG	61°C	Jensen et al., 1993
CES R2			GTAAGCGAACCTGTCTGTA ACAACA		
NA2 F	*nheA and*	766bp	AAGCIGCTCTTCGIATTC	45°C	Ehling- Shulz et al., 2006
NB1 R	*nheB*		ITIGTTGAAATAAGCTGTGG		
HD2 F	*hblD and*	1091bp	GTAAATTAIGATGAICAATT TC	48°C	Ehling- Shulz et al., 2006
HA4 R	*hblA*		AGAATAGGCATTCATAGAT T		
HBLC F	*hblC*	740bp	GATACTAATGTGGCAACTG C	56°C	Guinebretiere et al., 2002
HBLC R			TTGAGACTGCTCGTTAGTT G		

4.3.7 Toxin assay

The presence of NHE enterotoxin in isolates of *B. cereus* and *B. thuringiensis* was determined by the Tecra Visual Immunoassay (VIA) kit (Tecra Diagnostics, Roseville, Australia). The Oxoid Reversed Passive Latex Agglutination (RPLA) kit (Unipath-Oxoid, Columbia, MD) was used to detect the HBL enterotoxin in isolates that were positive for the *hblC* gene. The VIA kit detects the NheA component of the NHE toxin, while the RPLA kit detects the L_2 component (HblC) of the HBL enterotoxin (Beecher and Wong, 1994). The sample preparation and testing were carried out according to the directions provided by the manufacturer. For the VIA kit, an index of < 3 was considered negative. A titer value less than 1:2 was considered negative in the RPLA kit

4.3.8 Antimicrobial activity of spices

Certain spices are known to possess antimicrobial activity. A spice inhibition assay was designed for spice samples that were positive for *B. cereus* but did not grow at the lowest dilution in the MPN test. 1g of the sample was homogenized with 9ml of distilled water. The extract was heated at 75°C for 15 minutes in a water bath to ensure consistency of experiments, and filter sterilized

using a Fisher Scientific 45µm syringe filter to prevent contamination of the assay. Three serial doubling dilutions of the filtrate (up to 1: 40) were prepared and the corresponding isolates were used as test organisms for the assay. A loopful of the test strains were transferred to 9ml of TSB and incubated at 32°C. 100µl of the overnight culture was spread plated on tryptic soy agar plates. Each plate was divided into four quadrants and a 6mm antimicrobial sensitivity disc (Sensi Discs, BBL, Becton Dickinson) was placed in each quadrant, one for each dilution. 25µl of the spice extract dilution was added to the discs, and plates were incubated for 24 h at 32°C for zones of inhibition.

4.3.9 Purification of spores

Overnight culture of selected spice isolates were spread-plated on sporulation medium (nutrient agar and added salts of $CaCl_2$ and $MnSO_4$ at 0.001% each) and incubated at 37° C for 4 to 5 days, and examined under a phase microscope for the presence of mature spores. Sterile spreaders were used to collect the lawn of bacterial spores into chilled, sterile sodium-potassium phosphate buffer (0.05M, pH 7). Samples were centrifuged at 5000 g for 10 min at 4° C to remove vegetative cells and other debris. The precipitate was re-suspended in chilled buffer and the process was repeated (at least four times) until the suspension contained ≥ 95% free spores. Spore samples were stored in phosphate buffer between 2° C to 5° C until used.

4.3.10 Spore morphology by TEM

Samples were adsorbed on to carbon coated colloidal filmed grids for 4-10 min, with the excess being drained to a filter paper. Grids were prepared in the same manner with excess sample drained and air dried. The shadowed grids were placed in a Blazers BA080T evaporator and evacuated to $1X10^{-4}$pa and shadowed at 25 degrees elevation with a 2nm (measured at normal incidence) electron-beam generated platinum carbon. A JEOL 100s transmission electron microscope was used to observe all the preparations, with an accelerating voltage of 80kV.

4.3.11 Statistical Analysis

IBM SPSS Statistics 22 Software was used to run independent sample t-tests to determine significance of observed difference in counts between organic and regular samples as well as between packaged and loose samples.

4.4 Results and Discussion

4.4.1 Aerobic Mesophilic Spores

Table 2 summarizes the total aerobic mesophilic spore counts expressed as the number and percentage of spices. The highest count $8.3X10^7$ CFU/g was found with packaged organic paprika that was purchased in Berkley, California, while the lowest countable aerobic spore count ($2.7X 10^2$ CFU/g) was associated with two spices: paprika and ground cinnamon, both packaged and sold by McCormick. Table 3 lists the spices with high spore counts.

Table 2 Total mesophilic aerobic spore counts in U.S. retail spices

CFU/g	**$>10^7$**	**$>10^6$**	**$>10^5$**	**$>10^4$**	**$>10^3$**	**$>2 X 10^2$**	**$<2 X 10^2$**
Number of Spices	6	15	26	43	41	27	89
Percentage of Spices (N=247)	2.4%	6.1%	10.6%	17.4%	16.6%	10.9%	36.0%

Table 3 Species of spices associated with high mesophilic aerobic spore counts

Number CFU/g	Species of spices and herbs
$>10^7$	Rainbow Peppercorn (blend of black, green, red)
	Allspice
	7 Mixed Spices (blend)
	Basterma Spices (blend)
	Organic Ground Allspice
	Organic Paprika
$>10^6$	Organic Tumeric
	Cayenne Pepper
	New Mexico Chili Powder
	Ancho Chili Powder
	Ginger
	Organic Poultry Seasoning
	Organic Tumeric Root
	Kafta Spices
	Marjoram
	Shwarma Seasoning
	Turmeric powder
	Whole Black Pepper
	Spices for Dopiaza
	Spice Mix for Korma Curry
	Organic Cayenne Pepper

4.4.2 B. cereus and B. thuringiensis

Out of the 247 spices tested *B. cereus* was isolated from 77 (Table 7). Bi- pyramid shaped crystal toxins were detected under the phase microscope confirming the presence of *B. thuringiensis* in 11 spices (Table 8). Levels of *B. cereus* ranged from 3 to 1600 MPN/g with 57% in the 1-10 range, 31% (Table 5) in the 10-100 range and 12% (Table 4) in the 100-1600 range. *B. thuringiensis* ranged from 3.6 to 240 MPN/g with only one spice in the 100-1600 range and the rest evenly distributed between ranges 1 to 10 and 10 to 100. The ratio of *B. cereus* to *B. thuringiensis* in this study is similar to the ratio of *B. cereus* and *B. thuringiensis* isolated from rice (Ankolekar et.al., 2009) and from pasteurized milk (Zhou et al, 2008).

Table 4 Spices with *Bacillus cereus* spores at levels 100-1600/g

Sample no. and spice	MPN/g	*nheA+nheB* genes	NHE toxin index	Growth at 12°C (53°F)
18. Taco Seasoning	240	+	4	+
30. Thyme	460	+	4	+
31. Oriental Five Spices	1100	+	5	-
59. White Pepper	240	+	3	Weak
68. Gourmet Natural seasoning	240	+	4	+
79. Parsley Leaf	240	-	0	+
109. Cardamom	1600	-	0	+
150. Turmeric powder	460	+	4	+
158. Whole Black Pepper	460	+	4	+
242. Organic Ground All Spice	150	+	4	+
138. Fenugreek Seeds	38	+	3	+
149. Kala jeera	43	+	4	+
211. Fennel Powder	43	+	4	+

Table 5 Spices with *B. cereus* spores at levels 10-100/g

Sample no. and spice	MPN/g	*nheA+nheB* genes	NHE toxin Index	Growth at 12°C (53°F)
20. Italian Seasoning Blend	15	+	4	Weak
42. Pure Ground Allspice	36	+	4	+
43. Anise Seed	21	-	0	+
49. Mediterranean Fusion	43	+	4	+
66. All Spice	36	+	5	+
78. Poultry Seasoning	11	+	4	+
80. Pickling Spice	43	+	3	+
96. Oregano	23	-	0	+
108. Chili Powder	23	-	0	+
119. Celery Seed	93	+	3	+
123. Ginger Root	93	+	3	+
135. Kafta Spices	20	+	3	+
164. Rasam Powder	75	+	5	+
182. Onion Powder	23	+	5	+
188. Onion Powder	43	+	4	+
216. Italian Seasoning	23	+	3	+
221. Marjoram Leaf	43	+	5	+
223. Garam Masala	21	+	4	+
238. Organic Anise Ground	43	+	4	-
245. Organic Paprika	23	+	4	+

4.4.3 PCR

For each *B. cereus* and *B. thuringiensis* isolate the presence of the genes of the NHE and HBL enterotoxin complexes, and *ces* gene was determined. Table 6 summarizes the percentage and number of isolates that possessed the corresponding genes. The *ces* gene was absent in all 88 isolates tested but was present in two controls (Fig 2), *B. cereus F4810/72* and *B. cereus* isolate #10 from seafood. The primer pairs used in this study for the detection of HBL and NHE complexes were substituted with inosine at known variable regions due to high degree of polymorphisms reported (Mantynen and Lindstorm, 1998; PrÜß et al., 1999; Hansen et al., 2001). The primer pairs used in the study allows for the simultaneous detection of two genes; NA1 F and NB2 R detect *nhe*A and *nhe*B while HD2F and HA4R detect *hbl*A and *hbl*D. The *nhe* gene has been reported to be the more common gene detected in foodborne and food associated *B. cereus* and *B.thuringiensis* (Guinebretiere et al, 2002; Hansen and Hendriksen, 2001; Moravek et al, 2006; Rivera et al., 2000), which is also true in this study with 82% of *B. cereus* and 72% of *B. thuringiensis* positive for the *nhe* genes. The *hbl* genes (*hbl*A and D) were detected in 72% and 67% of *B. cereus* and *B. thuringiensis* isolates respectively. The gene encoding the *hbl*C gene was detected in 71% and 67% *B. cereus* and *B. thuringiensis* respectively.

Table 6 Enterotoxin genes in *B. cereus* and *B. thuringiensis*

Primers	Genes	Percentage positive in *B. cereus* (N=77)	Percentage positive in *B. thuringiensis* (N=11)
CES F1	*ces*	0	0
CES R2			
NA2 F	*nheA*	82%	72%
	and	(63)	(8)
NB1 R	*nheB*		
HD2 F	*hblD*	72%	67%
	and	(55)	(7)
HA4 R	*hblA*		
HBLC F	*hblC*	71%	67%
HBLC R		(54)	(7)

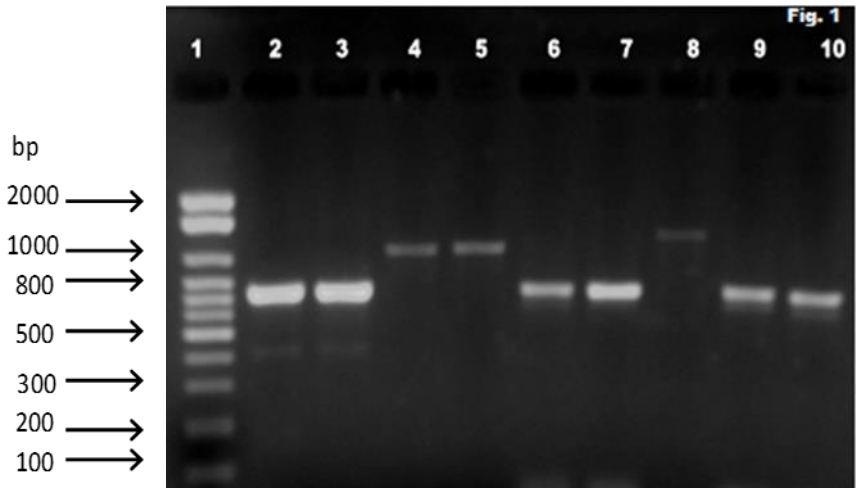

Figure 1 Agarose gel electrophoresis of PCR assay of *Bacillus cereus* isolates.

Lane 1, 2kb Ladder; Lane 2, B. cereus 1230/88 using primer pair NA2 and NB1 (for *nheA* and *B*, 766bp); Lane 3, *B. cereus* spice isolate #3 using primer pair NA2 and NB1; Lane 4, *B. cereus* ATCC 14579 using primer pair HD2 and HA4 (for *hblD* and *hblA*, 1091bp); Lane 5, *B. cereus* spice isolate #23 using primer pair HD2 and HA4; Lane 6, *B. cereus* ATCC 14579 control strain using primer pair HBLC (for *hblC*, 740bp); Lane 7, B. cereus spice isolate #54 using primer pair HBLC; Lane 8, *B. cereus* F4810/72 control strain using primer pair CES (for *ces*, 1271bp); Lane 9, *B. cereus* 1230/88 using primer pair NHEA (for *nheA*, 755bp); Lane 10, *B. cereus* spice isolate #44 using primer pair NHEA. A PCR reaction without template DNA was run for each primer to ensure the absence of non-specific binding (not shown).

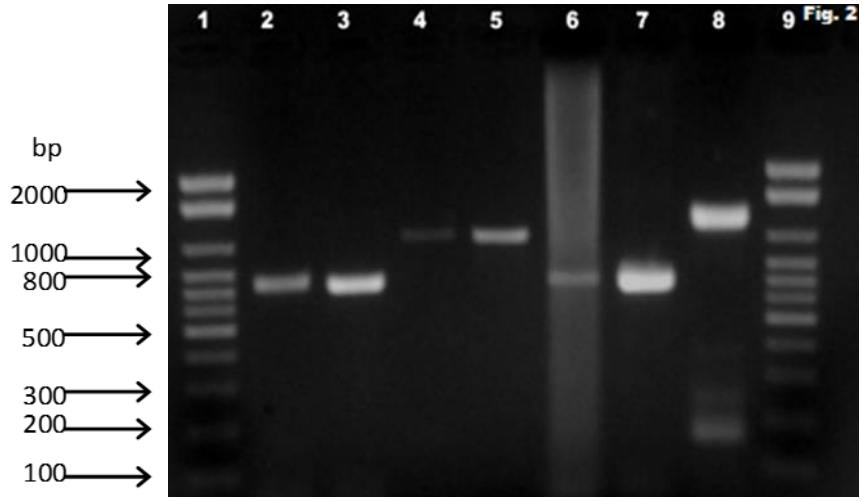

Figure 2 Agarose gel electrophoresis of PCR assay of *B. thuringiensis* isolates.

Lanes 1 and 9, 2kb Ladder; Lane 2, *B. thuringiensis* 1230/88 using primer pair NA2 and NB1 (for *nheA* and *B*, 766bp); Lane 3, *B. thuringiensis* spice isolate #112 using primer pair NA2 and NB1; Lane 4, *B. cereus* ATCC 14579 control strain using primer pair HD2 and HA4 (for *hblD* and *hblA*. 1091bp); Lane 5, *B. thuringiensis* spice isolate #162 using primer pair HD2 and HA4; Lane 6, *B. cereus* ATCC 14579 control strain using primer pair HBLC (for *hblC*, 740bp); Lane 7, *B. thuringiensis* spice isolate #62 using primer pair HBLC; Lane 8, *B. cereus* F4810/72 control strain using primer pair CES (for *ces* 1271bp).

4.4.4 Toxin Assays

The expression of the NHE toxin was detected in 98% of the isolates in which *nheA* and *nheB* were detected by PCR. As mentioned above, the TECRA immunoassay is specific to the *nheA*-coded component of the NHE complex. Two isolates, #44 (*B. cereus*) and #4 (*B. thuringiensis*) were positive for *nheA and B* using the composite primers NA2 and NB1 but were negative by immunoassay for NHE toxin. For both isolates PCR was repeated with primers specific for *nhe*A. A band corresponding to *nheA* was observed by PCR for isolate #44 but not for isolate #4 (not shown). The production of NheA in the absence of PCR detection of *nheA* has been previously reported (Hansen and Hendriksen, 2001) highlighting the polymorphism associated with *nheA.* For the HBL toxin an immunoassay kit was used to detect L_2 (encoded by *hblC*) protein. Although *hblC* was detected by PCR in 55 of 77 (71%) *B. cereus* isolates, its product, HBL enterotoxin, was

expressed in only 48 out of the 55 (87%) of *B. cereus* isolates indicating that the production of toxins not only depends on the presence of toxin gene but also on additional factors that regulate toxin production. Fifty-two percent of *B. cereus* and 54 % of *B. thuringiensis* produced both enterotoxins. In previous studies we identified the production of both enterotoxins by *B. cereus* in 48% and 53% of isolates from seafood and rice respectively (Ankolekar et. al., 2009 & Rahmati and Labbe, 2008).

Table 7 Enterotoxin expression in *B. cereus* and *B. thuringiensis* isolates

Organism	Toxin	Number of Isolates Tested	Percentage positive	Percentage of isolates with high titers
B. cereus	NHE	61	98 %	15 % (>4 Tecra index)
	HBL	56	88.5 %	61 % (≥ 1:128 Oxoid Titer)
B. thuringiensis	NHE	8	88 %	12.5 % (>4 Tecra index)
	HBL	9	89 %	50 % (≥ 1:128 Oxoid Titer)

4.4.5 Growth at Lower Temperature

Seventy five *B. cereus* isolates grew at 12°C out of which two isolates showed weak growth and were slightly turbid at day 14 (Table 7). Among the 11 *B. thuringiensis* isolates 8 grew well at 12°C and one isolate showed weak growth at day 14 (Table 8). Four *B. cereus* isolates and one *B. thuringiensis* isolate grew weakly at 7°C. These 5 isolates were further tested for growth at 9°C. Two *B. cereus* isolates #210 and #56 grew well at 9°C. Ankolekar et al., (2009) reported a *B. mycoides* strain isolated from rice that grew at 7°C and expressed both diarrheal toxins. Additionally, the diarrheal toxins have been shown to be produced at lower temperatures indicating the importance of psychotrophic strains of *B. cereus* (Dufrenne et al., 1995).

Table 8 Summary of *Bacillus cereus* in U.S. retail spices

Sample no. & Spice	MPN/g	12°C	*nheA*+n*heB* genes	NHE Toxin Index [a]	*hblA*+*hblD* genes	*hblC* gene	HBL Toxin Titre[b]
3. Chili Powder[c]	3.6	+	+	3	+	+	1:128
13. Basil	3.6	+	+	3	+	+	1:512
18. Taco Seasoning	240	+	+	4	+	+	1:32
20. Italian Seasoning Blend	15	weak	+	4	-	+	<1:2
23. Turmeric	6.2	+	+	5	+	+	1:256
30. Thyme	460	+	+	4	-	+	1:128
31. Oriental Five Spices	1100	-	+	5	-	-	ND
33. Pumpkin Pie Spice	3.6	+	+	3	-	+	<1:2
38. Italian Seasoning	3.6	+	+	4	+	+	1:256
42. Pure Ground Allspice	36	+	+	4	-	+	<1:2
43. Anise Seed	21	+	-	ND	-	+	<1:2
44. Pure Ground Cinnamon	6.2	+	+	1	+	+	1:16
45. Cajun Spice Seasoning	3	+	+	4	+	+	1:32
49. Mediterranean Fusion	43	+	+	4	+	-	ND
50. Rainbow Peppercorn	6.2	+	+	4	-	+	1:128
54. Nutmeg	3	+	+	4	-	+	1:128

56. Mustard Seed	9.2	+	-	ND	-	-	ND
59. White Pepper	240	weak	+	3	-	-	ND
64. Ancho Chili Powder	3	+	+	4	+	-	ND
66. Allspice	36	+	+	5	+	+	<1:2
68. Gourmet Natural seasoning	240	+	+	4	+	-	ND
71. Oregano Leaf	3.6	+	+	4	+	+	1:32
75. Minced Onion	3.6	+	-	ND	+	+	1:128
77. Pizza Seasoning	9.2	+	+	4	+	-	ND
78. Poultry Seasoning	11	+	+	4	-	+	1:128
79. Parsley Leaf	240	+	-	ND	-	+	1:256
80. Pickling Spice	43	+	+	3	+	+	1:128
81. Pumpkin Pie Spice	3	+	+	4	+	+	1:256
82. Orange Peel	3.6	+	+	3	+	+	1:64
85. Onion soup	7.4	+	+	4	+	+	1:256
87. White Pepper (ground)	3.6	+	+	3	+	+	1:16
89. Onion Powder	3.6	+	+	3	+	+	1:8
96. Oregano	23	+	-	ND	+	-	ND
104. Mexican Oregano	3.6	+	-	ND	-	+	1:256
108. Chili Powder	23	+	-	ND	-	+	<1:2
109. Cardamom	1600	+	-	ND	-	-	ND
111. Allspice	9.4	+	+	4	+	+	1:128
113. Curry Powder	3.6	+	+	4	+	-	ND

117. Chipotle Pepper	3.6	+	+	4	+	+	1:256
119. Celery Seed	93	+	+	3	+	+	1:256
123. Ginger Root	93	+	+	3	+	-	ND
124. Anise Seed	3	-	-	ND	+	+	1:64
128. Star Anise	3.6	+	-	ND	+	+	1:128
131. Arnica Flowers	3.6	+	+	3	+	+	1:256
135. Kafta Spices	20	+	+	3	+	+	1:128
136. 7 Mixed Spices	9.4	+	-	ND	-	-	ND
138. Fenugreek Seeds	38	+	+	3	-	-	1:64
142. Helbe	9.2	+	+	5	-	-	ND
145. Shwarma Seasoning	3.6	+	+	3	-	+	1:32
149. Kala Jeera	43	+	+	4	+	-	ND
150. Turmeric Powder	460	+	+	4	-	-	ND
153. Amchur Powder	3.6	+	+	5	+	+	1:64
158. Whole Black Pepper	460	+	+	4	+	+	1:64
164. Rasam Powder	75	+	+	5	-	+	1:128
177. Ground Allspices	9.2	+	+	4	+	+	1:128
182. Onion Powder	23	+	+	5	+	+	1:32
188. Onion Powder	43	+	+	4	+	+	1:128
191. Ground White Pepper	7.4	+	-	ND	+	+	1:32
197. Ground Coriander Seed	3.6	+	+	4	+	+	1:128
205. Chili Powder	3.6	+	+	4	+	+	1:256
210. Red Anaheim Pepper	7.2	+	+	4	+	+	1:128

211. Fennel Powder	43	+	+	4	+	+	1:128
216. Italian Seasoning	23	+	+	3	-	+	1:4
221. Marjoram Leaf	43	+	+	5	-	+	1:128
223. Garam Masala	21	+	+	4	+	+	1:32
224. Fenugreek Seeds	9.2	+	-	ND	+	+	1:64
225. Fenugreek Seeds	3.6	+	+	5	+	+	1:128
226. Paya Curry Mix	3	+	+	4	+	+	1:256
230. 7 Spices	3	+	-	ND	+	+	1:32
232. Curry Powder	3	+	+	3	+	+	1:64
237. Organic Ground Cinnamon	3	+	+	5	-	+	<1:2
238. Organic Anise Ground	43	-	+	4	-	-	ND
239. Organic Ground Cumin	3	+	+	4	+	-	ND
240. Organic Ground Ginger	9.4	+	+	3	-	-	ND
242. Organic Ground All Spice	150	+	+	4	+	-	ND
245. Organic Paprika	23	+	+	4	+	-	ND
246. Organic Cayenne Pepper	3	+	+	4	-	-	ND

[a] isolates with an index less than 3 were considered negative
[b] isolates with a titer less than 1:2 were considered negative
[c] underlined spices were labeled organic
ND- Not determined

Table 9 Summary of *Bacillus thuringiensis* in U.S. retail spices

Sample no. & Spice	MPN/g	12°C	*nheA+nheB* genes	NHE Toxin Index [a]	*hblA+hblD* genes	*hblC* gene	HBL Toxin Titre [b]
4. Chives	43	+	+	2	-	-	ND
62. Garlic	15	+	-	ND	-	+	1:128
90. Paprika Powder	6.2	+	+	3	+	+	1:64
112. Cardamom Seed[c]	36	+	+	5	+	-	<1:2
114. Cayenne Powder	3.6	-	+	3	+	+	1:16
125. Roasted Paprika Powder	240	-	+	3	+	+	1:128
141. Chicken Spices	6.2	+	-	ND	-	+	1:32
162. Jaifal Powder (Nutmeg)	3	+	+	3	+	+	1:16
167. Anardana Powder	36	weak	+	4	-	+	1:128
195. Garlic Powder	3	+	-	ND	+	-	ND
206. Garlic Powder	11	+	+	3	+	+	1:128

[a] isolates with an index less than 3 were considered negative
[b] isolates with a titer less than 1:2 were considered negative
[c] underlined spices were labeled organic
ND-Not determined

4.4.6 Spore Morphology under TEM

The spore morphology of five *B. cereus* and two *B. thuringiensis* strains were studied by transmission electron microscopy (Table 10 and Figure 4 & 5). Electron micrographs showed that exosporia were present in all the isolates tested. Tubular like appendages were present in four *B. cereus* and two *B. thuringiensis* isolates indicating their ability to adhere to surfaces. The number of appendages (1-10) varied among different strains and protruded from the ends with a whip-like appearance. Ankolekar et al, (2009b) also isolated strains of *B. cereus* from rice that varied in number of appendages.

Table 10 The presence of appendages and exosporium in *B. thuringiensis* and *B. cereus* spore isolates

Isolate Numbers	**Organism**	**Appendages**	**Exosporium**	**Figure**
158	*B. cereus*	1-4	Yes	3
210	*B. cereus*	7-10	Yes	4
66	*B. cereus*	4-6	Yes	5
109	*B. cereus*	None	Yes	6
31	*B. cereus*	1	Yes	7
162	*B. thuringiensis*	7-8	Yes	8
62	*B. thuringiensis*	6-7	Yes	9,10
206	*B. thuringiensis*	None	Yes	11

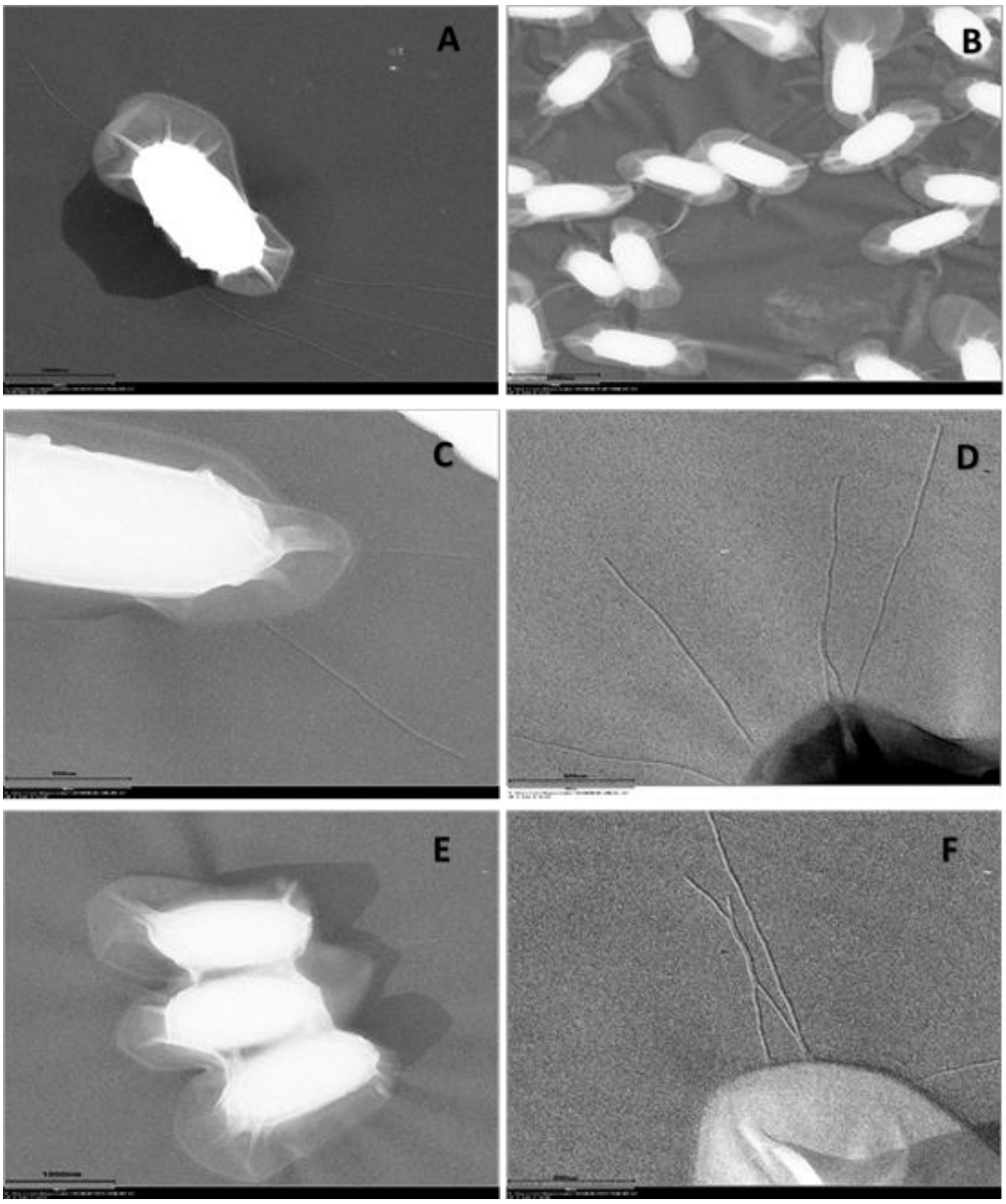

Figure 3 Electron micrographs of *B. cereus* spores

A-Shadowed image of *B. cereus* isolate #158 showing appendages and exosporium; B-Shadowed image of isolate #158 showing spores with exosporia; C & D- Shadowed images of *B. cereus* spore isolate #66 showing exosporium and appendages; E & D- Shadowed images of *B. cereus* isolate #210 showing appendages and exosporia

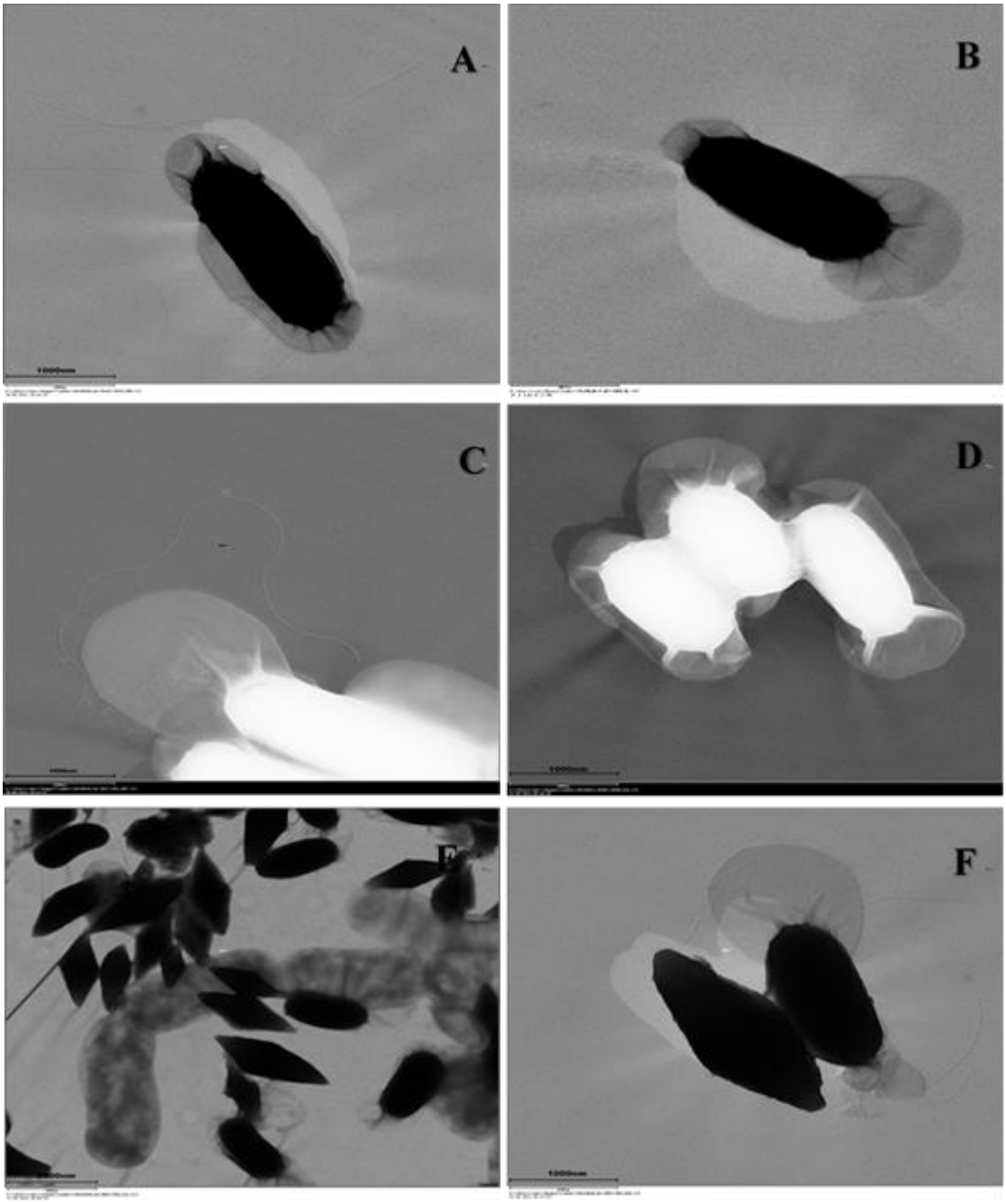

Figure 4 Electron micrographs of *B. cereus* and *B. thuringiensis* spores

A-Shadowed image of *B. thuringiensis* spore isolate #162 showing appendages and exosporium; B-Electron micrograph of *B. cereus* spore isolate #109 showing only exosporium; C-Shadowed image of *B. cereus* spore isolate #31 showing exosporia and whip-like appendages; D-Shadowed image of *B. thuringiensis* spore isolate #206 showing only exosporia E & F- Shadowed images of isolate #62 showing crystal toxins, appendages and exosporium

CHAPTER 5

SPORE SURVIVAL, AND GROWTH OF ENTEROTOXIGENIC *B. CEREUS* STRAINS IN COOKED RICE

5.1 Abstract

Bacillus cereus is a pathogenic organism implicated as a cause of diarrheal and emetic type of foodborne illness. This organism is a gram positive, facultative anaerobe and can produce endospores that are resistant to adverse conditions. We previously found that enterotoxigenic *B. cereus* is widely present in retail spices. In this study, we analyzed the heat resistance of 9 diarrheal spore strains. Seven diarrheal *B. cereus* spore strains had D_{95}-values ranging from 0.64-3.53 min while the two emetic strains had D_{95}-values of 7.04 min and 6.64 min. The ability of selected strains to grow in pre-cooked rice at temperatures 20 °C, 17 °C and 12 °C was determined as well as their toxin expression capability. After 48 h, counts of 1.26 X 10^7 and 3.8 X 10^7 CFU/ 10 g were obtained in cooked rice maintained at 17 °C and 20 °C respectively. At 12 °C, counts did not reach 10^4 CFU/ 10 g even after 48 h of incubation. The growth of aerobic, mesophilic bacterial population and *B. cereus* population of 0.1% crushed pepper in cooked rice over a period of 48h at temperature 20 °C and 17 °C were also analyzed. Counts of *B. cereus* in pepper rice samples reached a maximum of 1600 MPN/ 10 g and 1100 MPN/ 10 g at 20 °C and 17 °C respectively while the aerobic mesophilic counts per ten gram were 2.4 X 10^8 and 4.4 X 10^6 at these temperatures. The low *B. cereus* counts and high aerobic mesophilic population indicates competition for nutrients in cooked rice by background flora other than *B. cereus*.

5.2 Introduction

B. cereus a pathogenic, endospore forming organism is implicated as the cause of two foodborne illnesses, diarrhea and emesis. The emetic type is caused by a 45-kDa cyclic heat-stable and pH-stable peptide that is pre-formed in food. The diarrheal type is caused by three component heat-labile enterotoxins non-hemolytic enterotoxin (NHE) and hemolysin BL (HBL) (Granum, 2000). Foods associated with the emetic type are commonly farinaceous while the diarrheal type is associated with meat, sauces, pudding, vegetables and milk products (Kramer and Gilbert, 1989).

Spices have recently received attention due to cases of foodborne illness caused by *Salmonella*. The U.S. is the largest importer of spices which are obtained from developing nations that have minimal processing steps post-harvest and marginal training in safe handling and sanitation, thus presenting a huge risk of contamination of spices. Between 1973 and 2010 fourteen spice-associated foodborne outbreaks have been documented worldwide. *Salmonella* has been the implicated cause of 10 of the outbreaks and *Bacillus* spp. the cause of the other four (U.S. FDA Center for Food Safety and Nutrition, 2013). In Hungary, from years 1960 to 1966, 88 outbreaks of foodborne illness were caused by *B. cereus*. Further, heavily seasoned meat dishes were the implicated cause of three out of every five outbreaks caused by this organism. It was proposed that Hungarian meat dishes are highly seasoned with dried spices that usually contain large numbers of aerobic spore formers. The spores survive cooking temperatures and due to improper holding conditions germinate and multiply to cause foodborne illness (Geopfert et. al., 1971).

We previously found widespread prevalence of enterotoxigenic *B. cereus* spores in retail spices at levels ranging from <3 MPN/g to 1600 MPN/g (Hariram and Labbe, in press). Since rice is the number one consumed cereal and is commonly associated to *B. cereus* (Ankolekar et. al, 2009a; Dommel et. al., 2009; Gilbert et. al., 1974; Martinez-Blanch et. al., 2009; and Parry and Gilbert et. al., 1980), we determined the growth and enterotoxin expression in cooked rice, and the heat resistance in phosphate buffer of selected spore-strains isolated from spices. Considering spices are

added to dishes during and after cooking, we also determined the kinetics of growth of *B. cereus* and aerobic, mesophilic bacterial population from pepper using cooked rice as the model food.

5.3 Materials and Methods

5.3.1 B. cereus Strains

Control strains ATCC 14579 (HBL complex) and F4810/72 (*ces*-positive, originally obtained from an outbreak associated with cooked rice) (Turnbull et.al., 1979) were kindly provided by A. Wong, Univ. of Wisconsin. Isolate no. 10 from seafood isolated by our laboratory was used as another emetic control (Rahmati and Labbe, 2008). *B. cereus* diarrheal strains no. 182, 221, 66, 158, 226, 23, 210, 109 and 31 used in this study were isolated from retail spices (Table 8). *B. thuringiensis* strains 162, 62 and 206, used in this study were also isolated from spices.

5.3.2 Rice

Nature's Promise white organic rice purchased from a local supermarket (Stop and Shop) was used as the food matrix in this study. An electric rice cooker (model no. ARC-733G, Aroma Housewares Co., San Diego, CA) was used to prepare uninoculated batches of rice from each bag and enumerated right after preparation and also after incubation for 48h at 20 °C for any *B. cereus* viable cells by a 3-tube MPN method using trypic soy broth (TSB). These preliminary experiments showed absence of growth in MPN tubes that were incubated for 48 hours, indicating that any growth in cooked rice is due to the external addition of *B. cereus* or the microbial flora in spice.

5.3.3 Purification of Spores

Overnight culture of selected spice isolates were spread-plated on sporulation medium (nutrient agar and added salts of $CaCl_2$ and $MnSO_4$ at 0.001% each) and incubated at 37° C for 4 to 5 days, and examined under a phase microscope for the presence of mature spores. Sterile spreaders were used to collect the lawn of bacterial spores into chilled, sterile sodium-potassium phosphate buffer (0.05M, pH 7). Samples were centrifuged at 5000 g for 10 min at 4° C to remove vegetative cells and other debris. The precipitate was re-suspended in chilled buffer and the process was repeated

(at least four times) until the suspension contained ≥ 95% free spores. Spore samples were stored in phosphate buffer between 2° C to 5° C until used.

5.3.4 Heat Resistance of Spores

Spore suspensions were thoroughly shaken by hand to disrupt clumps and diluted with chilled sodium-phosphate buffer to an absorbance of 0.1 to 0.2 at 600 nm (~$1X10^7$ CFU/ ml). Two hundred microliters of sample was transferred into 2ml glass ampoules and heat sealed. Ampoules containing spore suspension were bound together using metal wire to ensure removal of two samples at the same time for each time point from a water bath maintained at 95 ° C. To ensure samples were completely immersed in water and to prevent floatation of samples in the water bath, paired-ampoules were placed in between a test tube rack. Paired-ampoules were removed at specific times and immediately cooled in an ice-water bath. Heat-treated spores from each ampoule were serially diluted and enumerated in triplicate by pour plating with nutrient agar supplemented with 0.1% glucose. Plates were covered with a thin overlay with the same media to prevent spreading. Preliminary experiments showed an increase in number of colony forming units from 24h to 48h of incubation in a few samples, thus plates were incubated for 48 h at 32 ° C.

5.3.5 Statistical Methods

Log transformations of spores that survived heat treatment were plotted against time in Microsoft Excel 2013. Two death curves for each sample were plotted, and the negative reciprocal of the slopes (of the log portion) was used to compute the $D_{95\ °C}$-values. To determine significance of observed differences in D-values between emetic and diarrheal strains, an independent sample t-test was run using IBM SPSS Statistics 22 Software.

5.3.6 Growth of *B. cereus* in Rice

Preliminary experiments were conducted to compare the germination and growth rates of selected strains. This allowed the preparation of a cocktail of two strains (isolate no. 221 and 182) with similar growth and germination rates. Each was mixed at equal concentration using 0.1 % peptone as the diluents and heat activated for 10 min at 80 ° C. Two hundred microliters of $1X10^4$cells/ml

inoculum was added to 10 g samples of tempered cooked rice in 30ml beakers to obtain an initial concentration of 200 cells/g. Beakers were maintained at 20 ° C and moderately abusive temperatures of 17 ° C and 12 ° C. Two samples were removed at time points of every four hours, serially diluted in 0.1% peptone, and pour plated in triplicate with NA. A thin overlay of agar was poured on each plate to avoid spreading, and plates were incubated at 32 ° C for 48 h. Two graphs were plotted for each temperature and mean generation times were obtained from the exponential phases of the graphs.

5.3.7 Enterotoxin Production

The production of NHE enterotoxin was detected in the cooked rice samples with a commercial Tecra visual-immunoassay kit (Tecra Diagnostics, Roseville, Australia). One sample beaker of rice inoculated with a heat-activated spore mixture was removed at time points 24, 41, 45 and 48 h. Samples for the immunoassay were prepared using a method previously described (Ankolekar and Labbe, 2009a). In addition to the positive and negative controls provided in the kit, a sample containing only rice was prepared and used as a negative control.

5.3.8 Growth of *B. cereus* in Rice by Addition of Retail Spice Sample

Ground spice sample #158- Whole Black Pepper Corns (Table 8) was inoculated at a level of 0.1 % onto 10 g of cooked rice contained in 30 ml beakers. Beakers were maintained at 20 ° C and 17 ° C. Duplicate samples were removed at intervals of four hours, diluted in 0.1 % peptone and enumerated for *B. cereus* using a chromogenic agar (R & F Laboratories) since the spice contained aerobic spore-formers in addition to *B. cereus*. The aerobic mesophilic bacterial counts were obtained by pour plating with nutrient agar in duplicate and incubating at 32 ° C for 48 h. Mean generation times were computed from the exponential phase of the growth curves plotted in Microsoft Excel 13.

5.4 Results and Discussion

5.4.1 Heat Resistance of Spores

The $D_{95°C}$-values obtained in this study are listed in Table 11. $D_{95°C}$-values of six different diarrheal strains tested ranged from 0.64 min to 3.53 min while the two emetic strains had $D_{95°C}$-values of 7.04 min and 6.62 min. A significant difference in the average heat resistance has been reported between diarrheal and emetic strains of *B. cereus* (Parry and Gilbert, 1977; Ankolekar and Labbe, 2009a). $D_{95°C}$-values of 1.2 to 36.2 min in phosphate buffer were previously reported for *B. cereus* spores (Kramer and Gilbert, 1989). The mean $D_{95°C}$-value of the diarrheal and emetic strains was found to be 1.8 min and 6.8 min respectively. The difference between the heat resistances of diarrheal and emetic type was found to be statistically significant ($p<0.05$).

Table 11 Heat resistance of *B. cereus* spores

Strain	Toxin type	$D_{95°C}$ (min)
ATCC 14579 (control)	Diarrheal	1.36 ± 0.22
182 (spice)	Diarrheal	0.64 ± 0.08
221 (spice)	Diarrheal	2.56 ± 0.19
66 (spice)	Diarrheal	3.53 ± 1.11
158 (spice)	Diarrheal	0.75 ± 0.03
226 (spice)	Diarrheal	2.46 ± 0.04
23 (spice)	Diarrheal	1.20 ± 0.41
F4810/72 (control)	Emetic	7.04 ± 0.51
10 (seafood)	Emetic	6.62 ± 0.73

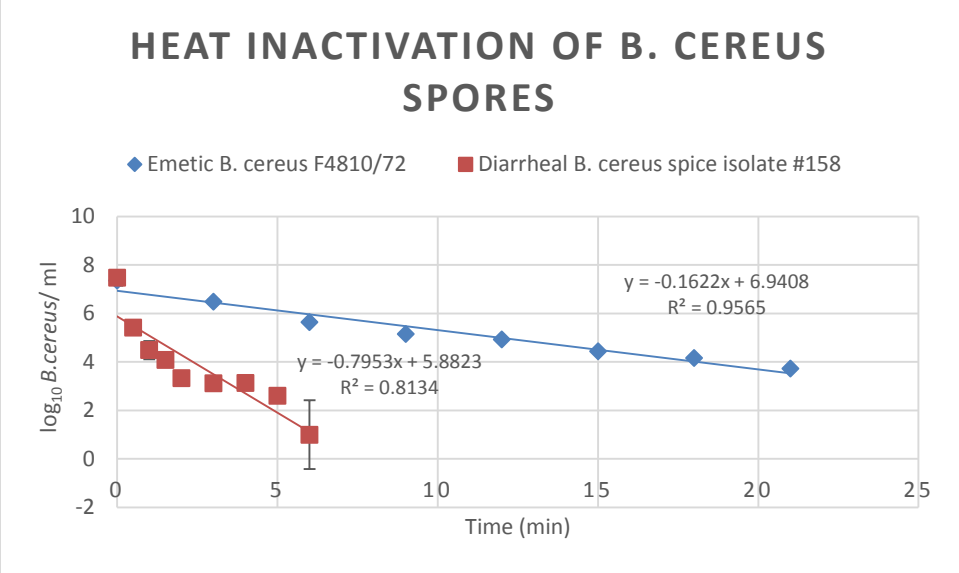

Figure 5 Inactivation of spores of representative diarrheal and emetic *B. cereus* strains. Spores were heated at 95°C in 0.05M, pH 7.0 phosphate buffer

5.4.2 Growth of *B. cereus* in Pre-cooked Rice

Data on growth of a mixture of two heat activated spore strains (isolate no. 221 and 182) of *B. cereus* at 20 °C, 17 °C and 12 °C are shown in the table and Figure below (Table 12, Fig. 6). Viable counts reached 5 $\log_{10}$/ 10 g within 20 h of incubation at 20 °C and within 24 h at 17 °C. At 12 °C, counts of the spore mixture did not reach 4 $\log_{10}$/ 10 g even after incubation for 48 h. Viable counts reached 7 $\log_{10}$/ 10 g within 40 h and 48 h at 20 °C and 17 °C respectively. Mean generation times calculated from the log phase were 85.6 ± 0.1 min and 20 °C and 116.8 ± 1.1 min at 17 °C. Similar mean generation times for *B. cereus* in pre-cooked rice at 20 °C and 17 °C have been reported (Ankolekar and Labbe, 2009; Johnson et. al., 1983).

Table 12 *B. cereus* growth in pre-cooked rice at 20, 17 and 12 °C

Time	Log_{10} counts of *B. cereus* (CFU/ 10 g) at		
	20 °C	17 °C	12 °C
0	1.93 ± 0.07	1.90 ± 0.05	2.18 ± 0.04
9	2.77 ± 0.14	2.02 ± 0.03	ND
13	3.86 ± 0.15	2.99 ± 0.00	2.38 ± 0.11
16	4.46 ± 0.00	3.65 ± 0.05	2.50 ± 0.02
20	5.43 ± 0.08	4.63 ± 0.03	2.53 ± 0.05
24	5.97 ± 0.08	5.21 ± 0.07	2.78 ± 0.36
24	5.72 ± 0.15	5.24 ± 0.28	2.90 ± 0.06
33	6.62 ± 0.10	5.35 ± 0.12	ND
37	6.88 ± 0.05	6.51 ± 0.01	3.45 ± 0.29
40	7.26 ± 0.02	6.58 ± 0.02	3.70 ± 0.05
44	7.52 ± 0.02	6.83 ± 0.11	3.73 ± 0.10
48	7.58 ± 0.01	7.10 ± 0.05	3.96 ± 0.10

Duplicate results for the 24 h point reflect the continuation of the growth experiment on a subsequent day.

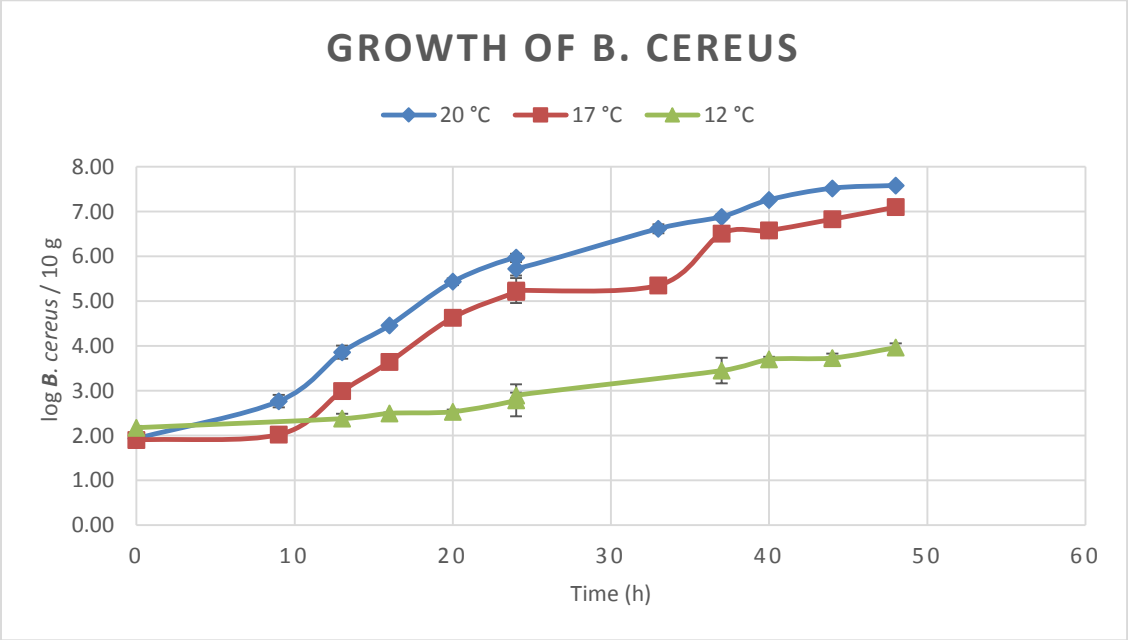

Figure 6. Growth of diarrheal-type *B. cereus* in pre-cooked rice

5.4.3 Pepper-Rice Study

Table 13 shows levels of *B. cereus* and aerobic, mesophilic plate counts in pre-cooked white rice inoculated with 0.1% of crushed peppercorns. The aerobic, mesophilic spore count ($6.5 X10^6$ CFU/ 10 g) and *B. cereus* spore count of the pepper used as the inoculum (460 MPN/ 10 g) were reported in Table 3 and Table 8 respectively. There was variation in the levels of *B. cereus* during the study, e.g. time points 8-40 h shown in Table 13 below. The natural, i.e. uneven distribution of *B. cereus* in pepper can be one reason for the fluctuating counts of *B. cereus* demonstrated in the pepper-rice study. In addition the pepper was not heated before addition to the rice, i.e., *B. cereus* spores therein were not heat-activated (vs the spore inoculum used in Table 12).

Within 48 h the aerobic mesophilic counts of pepper-rice samples reached 8 $\log_{10}$/ 10 g and 6 $\log_{10}$/g at 20 °C and 17 °C respectively. The low *B. cereus* counts (Table 13) could be due to the competing microflora as shown by the high aerobic mesophilic counts that were detected (Table 13). As shown in Table 12 pure cultures of *B. cereus* (as heat-activated spores) are readily able to grow in precooked rice. A separate similar study used 0.1% thyme as the inoculum in pre-cooked rice. The thyme sample (no. 30) contained 460 *B. cereus* spores/g (Table 8) but had aerobic, mesophilic spore count of $1.39 X 10^4$, i.e., 2.5 $\log_{10}$, less than that in pepper. Thyme-rice samples held at 20°C showed higher counts of *B. cereus* ($\log_{10}$ 3.78) and lower aerobic mesophilic plate counts ($\log_{10}$ 5.74) after 40 h (not shown) compared to the pepper-rice results. Thus the $\log_{10}$1.8 difference (reduction) in the aerobic, mesophilic plate counts between thyme and pepper in rice after 40 h of incubation may explain the low *B. cereus* levels at that time and reflect the effect of competing microflora on the levels of *B. cereus* in cooked rice.

Despite the high levels of enterotoxin-positive *B. cereus* in spices the low incidence of confirmed foodborne illnesss due to *B. cereus*-contaminated spice may be due to competing microflora. Of the 247 retail spice samples examined (Table 8), 53% contained aerobic spore counts of $>10^3$/gm

(Table 2). Such high counts can effectively function in a competitive-exclusion manner, minimizing the growth of potential foodborne pathogens.

Table 13 *B. cereus* growth and aerobic mesophilic bacterial growth in pepper-rice samples

Time	**20 °C**			**17 °C**		
	***B. cereus* (MPN/ 10 g)**		**APC (log_{10}CFU/ 10 g)**	**B. cereus (MPN/ 10 g)**		**APC (log_{10}CFU/ 10 g)**
0	7.4	3.6	5.15 ± 0.00	7.4	3.6	5.15 ± 0.01
8	14	9.2	ND	3.6	0	ND
12	15	23	ND	3.6	9.2	ND
16	7.4	3.6	5.28 ± 0.01	23	3.6	ND
20	9.2	9.2	ND	3.6	9.2	5.22 ± 0.16
24	3.6	93	6.04 ± 0.04	9.2	9.2	5.33 ± 0.10
24	15	15	6.33 ± 0.05	14	9.2	5.29 ± 0.00
32	1100	1100	ND	9.2	15	ND
36	1100	20	ND	20	20	5.87 ± 0.15
40	540	1600	7.49 ± 0.00	20	460	ND
44	220	1600	ND	1110	1110	6.40 ± 0.10
48	920	1600	8.39 ± 0.02	1110	460	6.65 ± 0.00

5.4.4 Enterotoxin Expression

Enterotoxin was not detected in pre-cooked rice samples that had log_{10} 5.1 CFU/ 10 g *B. cereus* (after 24 h) however low levels (toxin index of 3) of enterotoxin were detected when counts reached log_{10} 7.2 CFU/ 10 g and log_{10} 6.5 CFU/ 10 g (after 40 h) at 20 °C and 17 °C respectively. Lee et. al., (2006) and van Netten et. al., (1990) reported HBL enterotoxin production in rice cakes (22 °C) and rice meal (17 °C) when levels reached 6.6 log_{10} CFU/g and 7.1 log_{10} CFU/g respectively. Additionally, our lab demonstrated low levels of eneterotoxin production in salmon only when levels reached 8 log_{10} CFU/g and higher (Rahmati and Labbe, 2008). Many reports have demonstrated that growth and enterotoxin production is influenced by pH, carbohydrate sources and, growth rate (Sutherland and Limond, 1993; Rowan and Anderson, 1997; Ouassila et. al., 2006), suggesting that eneterotoxin production is not only dependent on cell numbers but also is affected by pH, water activity, temperature and composition of the food matrix. Although enterotoxin was detected in temperature-abused rice samples, foodborne illness due to *B. cereus* is

believed to result from the production of one or more enterotoxins in the intestine following ingestion of large numbers of *B. cereus* cells from temperature-abused foods (Granum and Lindback, 2013).

5.4.5 Future Work

It will be of interest to continue investigating the role of competing natural microflora in spices on their ability to affect growth of foodborne pathogens. Non-pathogenic, spore-forming microflora may function to limit growth from spores of pathogens such as *B. cereus* and *Clostridium perfringens.*

ADDENDUM

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Research Note

Spore Prevalence and Toxigenicity of *Bacillus cereus* and *Bacillus thuringiensis* Isolates from U.S. Retail Spices

UPASANA HARIRAM AND RONALD LABBÉ*

Department of Food Science, University of Massachusetts, 100 Holdsworth Way, Amherst, Massachusetts 01003, USA

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ABSTRACT

Recent incidents of foodborne illness associated with spices as the vehicle of transmission prompted this examination of U.S. retail spices with regard to *Bacillus cereus*. This study focused on the levels of aerobic-mesophilic spore-forming bacteria and *B cereus* spores associated with 247 retail spices purchased from six states in the United States. Samples contained a wide range of aerobic-mesophilic bacterial spore counts (< 200 to 8.3 × 10^7 CFU/g), with 19.1% of samples at levels above 10^5 CFU/g. For examples, paprika, allspice, peppercorns, and mixed spices had high levels of aerobic spores (>10^7 CFU/g). Using a novel chromogenic agar, *B. cereus* and *B. thuringiensis* spores were isolated from 77 (31%) and 11 (4%) samples, respectively. Levels of *B. cereus* were <3 to 1,600 MPN/g. Eighty-eight percent of *B. cereus* isolates and 91% of *B. thuringiensis* isolates possessed at least one type of enterotoxin gene: HBL (hemolysin BL) or nonhemolytic enterotoxin (NHE). None of the 88 isolates obtained in this study possessed the emetic toxin gene (*ces*). Using commercially available immunological toxin detection kits, the toxigenicity of the isolates was confirmed. The NHE enterotoxin was expressed in 98% of *B. cereus* and 91% of *B. thuringiensis* isolates that possessed the responsible gene. HBL enterotoxin was detected in 87% of *B. cereus* and 100% of *B. thuringiensis* PCR-positive isolates. Fifty-two percent of *B. cereus* and 54% of *B. thuringiensis* isolates produced both enterotoxins. Ninety-seven percent of *B. cereus* isolates grew at 12°C, although only two isolates grew well at 9°C. The ability of these spice isolates to form spores, produce diarrheal toxins, and grow at moderately abusive temperatures makes retail spices an important potential vehicle for foodborne illness caused by *B. cereus* strains, in particular those that produce diarrheal toxins.

Levels of aerobic spore-forming bacteria up to 10^6 to 10^8 CFU/g have been reported in various spices *(4, 14, 20)*. Of particular concern are spore-forming foodborne pathogens such as *Bacillus cereus* and *Clostridium perfringens*. *B. cereus* is a potentially pathogenic bacterium that is present ubiquitously in the environment. Its spores can survive cooking temperatures and, when exposed to favorable conditions, can germinate and multiply to levels associated with foodborne illness. In some cases *B. cereus* levels of 10^4 to 10^5 CFU/g have been found in spices *(3, 4, 7, 15–17, 20, 23, 28)*, with one report of levels of 10^8 CFU/g *(2)*. Early surveys of the incidence of *B. cereus* in spices typically reported the distribution and levels of this organism but not its toxin-producing abilities.

Certain strains of *B. cereus* are able to produce one or more enterotoxins or an emetic toxin. *B. cereus* has been identified as the causative agent of two types of foodborne illness. The diarrheal type is characterized by diarrhea and abdominal pain, resembles illness associated with *C. perfringens* infection, and is caused by one or more heat labile enterotoxins, whereas the emetic type is characterized by nausea and vomiting, resembles illness associated with *Staphylococcus aureus* infection, and is caused by a heat-stable dodecadepsipeptide *(19)*. Of the diarrheal toxins, hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE) are regarded as the most important toxins involved in foodborne illness, and each is composed of three proteins, encoded by *hblA, hblD,* and *hblC* in HBL and by *nheA, nheB,* and *nheC* in NHE *(11)*. Isolates differ in their ability to produce these virulence factors.

From 1973 to 2010, 14 documented spice-associated foodborne illness outbreaks occurred worldwide. Ten of the 14 outbreaks were caused by *Salmonella,* and 4 were caused by *Bacillus* spp. *(32)*. Per capita consumption of spices (other than dehydrated onion and garlic) in the United States increased nearly 300% from 1966 to 2010 *(30)*, and based on a retail study conducted in July 2009, 78% of U.S. households used spices and herbs in addition to salt and pepper *(32)*. Spices contaminated with spores of *B. cereus* and used to prepare meat dishes were attributed to several outbreaks of the diarrheal syndrome in Hungary during the 1960s.The spores in spices survive cooking temperatures and germinate when cooled improperly, and the nutrients in meat support growth of the resulting vegetative cells *(18)*. In 2007, a spice blend used in a couscous dish was implicated in an outbreak of *B. cereus* food poisoning affecting 146 kindergarten and school children in France. Another outbreak in 2010 in Denmark was due to *B. cereus* contamination in white pepper used to season stew. The

* Author for correspondence. Tel: 413-545-1021; Fax: 413-545-1262; E-mail: rlabbe@foodsci.umass.edu.

TABLE 1. *Oligonucleotide primers used in this study*

Primers[a]	Gene(s)	Amplified fragment (bp)	Sequences (5′→3′)	T_a (°C)[b]	Reference
CES F1	*ces*	1,271	GGTGACACATTATCATATAAGGTG	61	*9*
CES R2			GTAAGCGAACCTGTCTGTAACAACA		
NA2 F	*nheA, nheB*	766	AAGCIGCTCTTCGIATTC	45	*8*
NB1 R			ITIGTTGAAATAAGCTGTGG		
HD2 F	*hblD, hblA*	1,091	GTAAATTAIGATGAICAATTTC	48	*8*
HA4 R			AGAATAGGCATTCATAGATT		
NHEA F	*nheA*	755	GTTAGGATCACAATCACCGC	56	*12*
NHEA R			ACGAATGTAATTTGAGTCGC		
HBLC F	*hblC*	740	GATACTAATGTGGCAACTGC	56	*12*
HBLC R			TTGAGACTGCTCGTTAGTTG		

[a] F, forward primer; R, reverse primer; CES, cereulide peptide synthetase; NA2, nonhemolytic enterotoxin A component; NB1, nonhemolytic enterotoxin B component; HD2, hemolytic enterotoxin D component; HA4, hemolytic enterotoxin A component; NHEA, nonhemolytic enterotoxin A component; HBLC, Hemolysin BLC component.
[b] Annealing temperature.

stew was kept at abusive temperatures while being served in a catering setting, and the outbreak affected 112 people *(32)*. Because 80% of the 2012 U.S. spice supply was imported *(32)* from countries with marginal sanitary practices, a high chance exists for these spices to be contaminated with *B. cereus,* either because of the ability of spores to survive processing steps (such as drying) or because of environmental contamination. Recent outbreaks associated with *Salmonella*-contaminated spices also have highlighted the role of spices as a potential vehicle of foodborne illness and prompted this report of the incidence and virulence factors associated with current isolates of *B. cereus* spores from spices from retail outlets in the United States.

MATERIALS AND METHODS

Strains. Isolates in this study were obtained from a total of 247 spice samples from grocery stores, supermarkets, and ethnic food stores in western Massachusetts; southern Texas; Milwaukee, WI; Atlanta, GA; and Berkeley, CA. Control strains were from our culture collection. *B. cereus* strains ATCC 14579 (HBL complex), 1230/88 (NHE complex), and F4810/72 (*ces*) were kindly provided originally by A. Wong (University of Wisconsin, Madison). Strain F4810/72 was originally obtained from an outbreak associated with cooked rice *(29)*. Seafood isolate 10, another *ces*-positive emetic control, was isolated from mussels *(26)*.

Isolation and enumeration. A 10% suspension of each spice was prepared in distilled water, stomached for 60 s, and heated for 15 min at 75°C. Total mesophilic aerobic spore counts were determined by pour plating on nutrient agar. After solidification of the agar, a thin overlay was added to each plate to prevent spreading. Plates were incubated at 32°C for 48 h. Levels of *B. cereus* spores were determined using a three-tube most-probable-number (MPN) method *(31)* with tryptic soy broth (TSB) and 0.015% polymyxin B. After incubation for 48 h at 32°C, culture from positive tubes was streaked onto *B. cereus–B. thuringiensis* chromogenic agar (R&F Products, Downers Grove, IL). Plates were incubated for 18 to 24 h at 32°C, and typical colonies (turquoise blue) were transferred to nutrient agar slants for further analysis. A loopful of each culture was streaked onto nutrient agar plates and incubated at 32°C for 3 to 4 days. The presence of intracellular crystals in sporulating cells was used to distinguish *B. cereus* from *B. thuringiensis*. These bipyramidal crystals, either as free crystals or as parasporal inclusions, were readily visible by phase contrast microscopy.

PCR. Strains of *B. cereus* and *B. thuringiensis* were examined for the presence of toxin-encoding genes. Single primer pairs were used to detect genes *nheA* and *nheB* of the NHE complex, genes *hblA* and *hblD* and separately *hblC* of the HBL complex, and gene *ces* (Table 1). Procedures used for the preparation of template DNA were described previously *(1)*. To ensure that template DNA was extracted from cells in the vegetative state, isolates were cultured in 5 ml of TSB and incubated at 20°C with shaking at 150 rpm. One hundred microliters of the overnight culture was transferred to 5 ml of TSB and incubated at 32°C until the absorbance at 600 nm was 0.4. All PCRs were performed using Maxime PCR premix tubes (Bulldog Bio, Portsmouth, NH). The PCRs were carried out in a thermocycler (Techne, Cambridge, UK) with an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 40 s, annealing at various temperatures (see Table 1 for annealing temperatures for each primer pair) for 40 s, and extension at 72°C for 80 s, followed by a final elongation step at 72°C for 4 min. The presence of reaction products was determined by electrophoresis of 10 μl of the PCR products in a 1.2% agarose gel at 70 V for 60 min. To avoid the use of ethidium bromide, Midori Green (Bulldog Bio) was used as the nucleic acid stain.

Toxin assay. The presence of NHE enterotoxin in isolates of *B. cereus* and *B thuringiensis* was determined with a visual immunoassay (VIA) kit (Tecra Diagnostics, Roseville, Australia). A reversed passive latex agglutination (RPLA) kit (Unipath-Oxoid, Columbia, MD) was used to detect the HBL enterotoxin in isolates that were positive for the *hblC* gene. The VIA kit detects the NheA component of the NHE toxin, and the RPLA kit detects the L_2 component (HblC) of the HBL enterotoxin *(5)*. Sample preparation and testing were carried out according to the directions provided by the manufacturer. For the VIA kit, an index of <3 was considered negative. For the RPLA kit, a titer of $<1{:}2$ was considered negative.

Growth at low temperatures. The ability of *B. cereus* strains isolated from spices to grow at low temperatures was determined as previously described *(1)*.

Statistical analysis. SPSS Statistics 22 software (IBM, Armonk, NY) was used to run independent sample *t* tests to determine the significance of observed differences in pathogen

TABLE 2. *Summary of aerobic mesophilic bacterial spores in U.S. retail spice samples*

Population level (CFU/g)	No. of samples	% samples (n = 247)
$>10^7$	6	2.4
$>10^6$	15	6.1
$>10^5$	26	10.6
$>10^4$	43	17.4
$>10^3$	41	16.6
$>2 \times 10^2$	27	10.9
$<2 \times 10^2$	89	36.0

levels between organic and regular samples and between packaged and loose samples.

RESULTS AND DISCUSSION

Isolation and enumeration. Total aerobic mesophilic spore counts for 247 samples yielded a wide range of levels, from <2.0 to 7.9 log CFU/g (Table 2). Other authors also have reported a wide range in the levels of aerobic spore formers ($<10^2$ to $>10^7$ CFU/g) in spices from different sources and manufacturing processes *(4, 16, 20)*. In the present study, the highest count (8.3×10^7 CFU/g) was found in packaged organic paprika (sample 245, Table 3), and the lowest counts were associated with packaged cinnamon and paprika (samples 192 and 193, not listed). Approximately 19% of samples contained aerobic spore counts $>10^5$ CFU/g (Table 2). Nonorganic paprika, whole black peppercorns, allspice, turmeric, marjoram, and spice blends were other samples containing high levels ($>10^6$ CFU/g) of aerobic mesophilic spores.

Eighty-eight (36%) of the spices yielded *B. cereus* or *B. thuringiensis* with spore levels of <3 to 1,600 MPN/g (Table 3). Eleven of the 88 isolates produced bipyramidal crystal bodies that identified them as *B. thuringiensis,* with spore levels of 3 to 240 MPN/g (Table 4). The ratio of the spore level of *B. cereus* to that of *B. thuringiensis* was similar to that reported in raw rice *(1)* and pasteurized milk *(33)*. In previous studies, *B. cereus* levels up to 53% were found in retail spices *(24)*.

PCR. A PCR assay was used to detect the presence of enterotoxin genes in the *B. cereus* and *B. thuringiensis* isolates. The emetic toxin gene (*ces*) was absent in all 88 isolates tested but was present in two emetic-toxin control strains, *B. cereus* F4810/72 (Figs. 1 and 2) and *B. cereus* isolate 10 from seafood (not shown). The primer pairs used in this study for the detection of HBL and NHE complexes, NA2F and NB1R, were substituted with inosine at known variable regions due to a high degree of polymorphism reported among the enterotoxin genes *(12, 21, 25)*, allowing for the simultaneous detection of two genes, i.e., primer pair NA2F and NB1R detects *nheA* and *nheB,* and primer pair HD2F and HA4R detects *hblD* and *hblA (9). Nhe* has been reported to be the more common enterotoxin gene detected in foodborne and food-associated *B. cereus* and *B. thuringiensis* isolates *(10, 12, 13, 22)*, which was also true in the present study; among the *B. cereus* and *B. thuringiensis* spore isolates, 82 and 72%, respectively, were positive for both *nheA* and *nheB,* and 72 and 67%, respectively, were positive for the *hbl* genes (*hblA* and *hblD*). The gene encoding the HblC component, *hblC,* was detected in 71 and 67% of *B. cereus* and *B. thuringiensis* isolates, respectively (Tables 3 and 4). We previously reported that the *nhe* complex was more common than the *hbl* complex in isolates from seafood and rice *(1, 26)*. Because >99% of *nhe*-positive *B. cereus* strains possess the three-component *nhe* complex *(11)*, a positive PCR result using *nheA* and *nheB* primers would strongly suggest the presence of *nheC*. In previous studies, a PCR assay has revealed the presence of the genes for at least one of the enterotoxins, NHE or HBL, in *B. thuringiensis* isolates *(10, 13, 27)*.

Toxin assays. Expression of the NHE toxin was detected in 98% of the isolates in which *nheA* and *nheB* were detected with the PCR assay. The VIA immunoassay is specific to the *nheA*-coded component of the NHE complex. Two isolates, 44 (*B. cereus*) and 4 (*B. thuringiensis*), were positive for *nheA* and *nheB* when the composite primers NA2 and NB1 were used but were negative by immunoassay for the NHE toxin. For both isolates, when the PCR assay was repeated with primers specific for *nheA* a band corresponding to *nheA* was observed for isolate 44 but not for isolate 4 (not shown). The production of NheA in the absence of PCR detection of *nheA* has been previously reported *(13)*, highlighting the polymorphism associated with *nheA*. For the HBL toxin, the RPLA immunoassay kit was used to detect L_2 protein (encoded by *hblC*). Although *hblC* was detected by PCR in 55 (71%) of 77 *B. cereus* isolates, its product, HBL enterotoxin, was expressed in only 48 (87%) of the 55 *B. cereus* isolates, indicating that the production of toxins depends on both the presence of the toxin gene and additional factors that regulate toxin production. Fifty-two percent of *B. cereus* and 54% of *B. thuringiensis* isolates produced both enterotoxins. In previous studies, we identified the production of both enterotoxins in 48 and 53% of *B. cereus* isolates from seafood and rice, respectively *(1, 26)*.

Organic versus nonorganic. Sixty-six samples were labeled organic; 20 (30.3%) contained *B. cereus* spores, and 2 (3%) contained *B. thuringiensis* spores, with mean counts of 104 and 138 MPN/g, respectively. For the 181 nonorganic samples, 57 (31.5%) contained *B. cereus* spores, and 9 (5%) contained *B. thuringiensis* spores, with mean counts of 74 and 14 MPN/g, respectively. The difference in *B. cereus* spore counts between organic and nonorganic samples was not significant; however, a significant difference in counts of *B. thuringiensis* spores was found between organic and nonorganic samples ($P < 0.05$).

Packaged versus loose. One hundred samples were purchased loose, and 147 were packaged. *B. cereus* spores were found in 36 (36%) of the loose samples, with a mean count of 92 MPN/g, and 41 (28%) of the packaged samples, with a mean count of 72 MPN/g. The observed difference in *B. cereus* spore counts between loose and packaged samples was not significant ($P > 0.05$).

TABLE 3. *Summary of* Bacillus cereus *spores in U.S. retail spice samples*

Sample no.	Level (MPN/g)	Growth at 12°C	*nheA* and *nheB* genes	NHE toxin index[a]	*hblA* and *hblD* genes	*hblC* gene	HBL toxin titer[b]
3. Chili powder[c]	3.6	+	+	3	+	+	1:128
13. Basil[c]	3.6	+	+	3	+	+	1:512
18. Taco seasoning	240	+	+	4	+	+	1:32
20. Italian seasoning blend	15	weak	+	4	−	+	<1:2
23. Turmeric[c]	6.2	+	+	5	+	+	1:256
30. Thyme	460	+	+	4	−	+	1:128
31. Oriental five spices	1,100	−	+	5	−	−	ND[d]
33. Pumpkin pie spice	3.6	+	+	3	−	+	<1:2
38. Italian seasoning	3.6	+	+	4	+	+	1:256
42. Pure ground allspice	36	+	+	4	−	+	<1:2
43. Anise seed	21	+	−	ND	−	+	<1:2
44. Pure ground cinnamon	6.2	+	+	1	+	+	1:16
45. Cajun spice seasoning	3	+	+	4	+	+	1:32
49. Mediterranean fusion	43	+	+	4	+	−	ND
50. Rainbow peppercorn	6.2	+	+	4	−	+	1:128
54. Nutmeg	3	+	+	4	−	+	1:128
56. Mustard seed	9.2	+	−	ND	−	−	ND
59. White pepper	240	weak	+	3	−	−	ND
64. Ancho chili powder	3	+	+	4	+	−	ND
66. Allspice	36	+	+	5	+	+	<1:2
68. Gourmet natural seasoning	240	+	+	4	+	−	ND
71. Oregano leaf[c]	3.6	+	+	4	+	+	1:32
75. Minced onion[c]	3.6	+	−	ND	+	+	1:128
77. Pizza seasoning[c]	9.2	+	+	4	+	−	ND
78. Poultry seasoning[c]	11	+	+	4	−	+	1:128
79. Parsley leaf[c]	240	+	−	ND	−	+	1:256
80. Pickling spice[c]	43	+	+	3	+	+	1:128
81. Pumpkin pie spice[c]	3	+	+	4	+	+	1:256
82. Orange peel[c]	3.6	+	+	3	+	+	1:64
85. Onion soup[c]	7.4	+	+	4	+	+	1:256
87. White pepper (ground)[c]	3.6	+	+	3	+	+	1:16
89. Onion powder[c]	3.6	+	+	3	+	+	1:8
96. Oregano	23	+	−	ND	+	−	ND
104. Mexican oregano	3.6	+	−	ND	−	+	1:256
108. Chili powder[c]	23	+	−	ND	−	+	<1:2
109. Cardamom[c]	1,600	+	−	ND	−	−	ND
111. Allspice[c]	9.4	+	+	4	+	+	1:128
113. Curry powder[c]	3.6	+	+	4	+	−	ND
117. Chipotle pepper[c]	3.6	+	+	4	+	+	1:256
119. Celery seed[c]	93	+	+	3	+	+	1:256
123. Ginger root[c]	93	+	+	3	+	−	ND
124. Anise seed[c]	3	−	−	ND	+	+	1:64
128. Star anise[c]	3.6	+	−	ND	+	+	1:128
131. Arnica flowers	3.6	+	+	3	+	+	1:256
135. Kafta spices	20	+	+	3	+	+	1:128
136. Seven mixed spices	9.4	+	−	ND	−	−	ND
138. Fenugreek seeds	38	+	+	3	−	−	1:64
142. Helbe	9.2	+	+	5	−	−	ND
145. Shwarma seasoning	3.6	+	+	3	−	+	1:32
149. Kala jeera	43	+	+	4	+	−	ND
150. Turmeric powder	460	+	+	4	−	−	ND
153. Amchur powder	3.6	+	+	5	+	+	1:64
158. Whole black pepper	460	+	+	4	+	+	1:64
164. Rasam powder	75	+	+	5	−	+	1:128
177. Ground allspices	9.2	+	+	4	+	+	1:128
182. Onion powder	23	+	+	5	+	+	1:32
188. Onion powder	43	+	+	4	+	+	1:128
191. Ground white pepper	7.4	+	−	ND	+	+	1:32

TABLE 3. *Continued*

Sample no.	Level (MPN/g)	Growth at 12°C	*nheA* and *nheB* genes	NHE toxin index[a]	*hblA* and *hblD* genes	*hblC* gene	HBL toxin titer[b]
197. Ground coriander seed	3.6	+	+	4	+	+	1:128
205. Chili powder	3.6	+	+	4	+	+	1:256
210. Red anaheim pepper[c]	7.2	+	+	4	+	+	1:128
211. Fennel powder[c]	43	+	+	4	+	+	1:128
216. Italian seasoning[c]	23	+	+	3	−	+	1:4
221. Marjoram leaf[c]	43	+	+	5	−	+	1:128
223. Garam masala	21	+	+	4	+	+	1:32
224. Fenugreek seeds[c]	9.2	+	−	ND	+	+	1:64
225. Fenugreek seeds	3.6	+	+	5	+	+	1:128
226. Paya curry mix	3	+	+	4	+	+	1:256
230. Seven spices	3	+	−	ND	+	+	1:32
232. Curry powder	3	+	+	3	+	+	1:64
237. Organic ground cinnamon[c]	3	+	+	5	−	+	<1:2
238. Organic ground anise[c]	43	−	+	4	−	−	ND
239. Organic ground cumin[c]	3	+	+	4	+	−	ND
240. Organic ground ginger[c]	9.4	+	+	3	−	−	ND
242. Organic ground allspice[c]	150	+	+	4	+	−	ND
245. Organic paprika[c]	23	+	+	4	+	−	ND
246. Organic cayenne pepper[c]	3	+	+	4	−	−	ND

[a] Isolates with an index of <3 were considered negative.
[b] Isolates with a titer of <1:2 were considered negative.
[c] Items labeled organic.
[d] ND, not determined.

Growth at low temperatures. Seventy-five (97%) of *B. cereus* isolates grew at 12°C; two of these isolates had weak growth and were slightly turbid at day 14 (Table 3). Among the 11 *B. thuringiensis* isolates, 8 grew well at 12°C and 1 had weak growth at day 14 (Table 3). Four *B. cereus* isolates and one *B. thuringiensis* isolate grew weakly at 7°C. These five isolates were further tested for growth at 9°C. Two *B. cereus* isolates, 210 and 56, grew well at 9°C. In a study of the levels of spores of the *B. cereus* group in rice *(1)*, a *Bacillus mycoides* isolate grew well at 7°C and expressed both diarrheal toxins.

Antimicrobial activity of spices. Certain spices are known to possess antimicrobial activity. To determine whether spice extracts affected growth in the MPN analysis, each spice sample that did not grow in TSB at the lowest dilution in this procedure was tested for antimicrobial activity against the corresponding *B. cereus* isolate (from a

TABLE 4. *Summary of* Bacillus thuringiensis *spores in U.S. retail spice samples*

Sample no.	Level (MPN/g)	Growth at 12°C	*nheA* and *nheB* genes	NHE toxin index[a]	*hblA* and *hblD* genes	*hblC* gene	HBL toxin titer[b]
4. Chives	43	+	+	2	−	−	ND
62. Garlic	15	+	−	ND	−	+	1:128
90. Paprika powder	6.2	+	+	3	+	+	1:64
112. Cardamom seed[c]	36	+	+	5	+	−	<1:2
114. Cayenne powder	3.6	−	+	3	+	+	1:16
125. Roasted paprika powder[c]	240	−	+	3	+	+	1:128
141. Chicken spices	6.2	+	−	ND	−	+	1:32
162. Jaifal powder (nutmeg)	3	+	+	3	+	+	1:16
167. Anardana powder	36	weak	+	4	−	+	1:128
195. Garlic powder	3	+	−	ND	+	−	ND
206. Garlic powder	11	+	+	3	+	+	1:128:

[a] Isolates with an index of <3 were considered negative.
[b] Isolates with a titer of <1:2 were considered negative.
[c] Items labeled organic.
[d] ND, not determined.

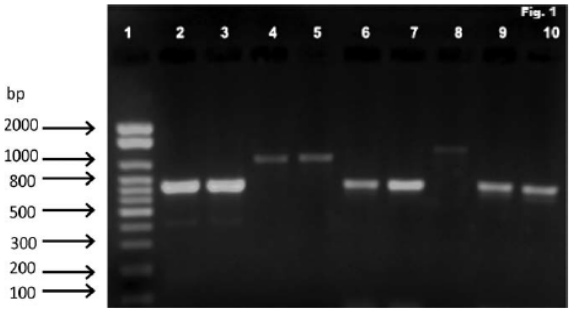

FIGURE 1. *Agarose gel electrophoresis of PCR products from* B. cereus *isolates. Lane 1, 2-kb ladder; lane 2,* B. cereus *1230/88 using primer pair NA2 and NB1 (for* nheA *and* nheB, *766 bp); lane 3,* B. cereus *spice isolate 3 using primer pair NA2 and NB1; lane 4,* B. cereus *ATCC 14579 using primer pair HD2 and HA4 (for* hblD *and* hblA, *1,091 bp); lane 5,* B. cereus *spice isolate 23 using primer pair HD2 and HA4; lane 6,* B. cereus *ATCC 14579 control strain using primer pair HBLC (for* hblC, *740 bp); lane 7,* B. cereus *spice isolate 54 using primer pair HBLC; lane 8,* B. cereus *F4810/72 control strain using primer pair CES (for* ces, *1,271 bp); lane 9,* B. cereus *1230/88 using primer pair NHEA (for* nheA, *755 bp); lane 10,* B. cereus *spice isolate 44 using primer pair NHEA. A PCR without template DNA was run for each primer to ensure the absence of nonspecific binding (not shown).*

positive higher-dilution MPN tube) using a disk assay with the stomached spice sample. In each case, no corresponding inhibitory activity was found. The negative MPN results (at the lowest dilution) were therefore attributed to the statistical nature of the MPN procedure. The flavor, aroma, and antimicrobial components of a spice are contained in its less-water-soluble essential oil *(6)*.

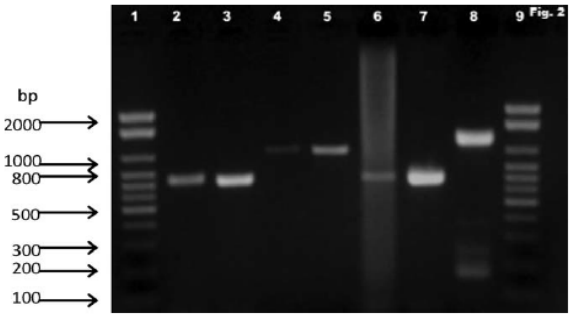

FIGURE 2. *Agarose gel electrophoresis of PCR assay of* B. thuringiensis *isolates. Lanes 1 and 9, 2-kb ladder; lane 2,* B. thuringiensis *1230/88 using primer pair NA2 and NB1 (for* nheA *and* nheB, *766 bp); lane 3,* B. thuringiensis *spice isolate 112 using primer pair NA2 and NB1; lane 4, B. cereus ATCC 14579 control strain using primer pair HD2 and HA4 (for* hblD *and* hblA, *1,091 bp); lane 5,* B. thuringiensis *spice isolate 162 using primer pair HD2 and HA4; lane 6,* B. cereus *ATCC 14579 control strain using primer pair HBLC (for* hblC, *740 bp); lane 7,* B. thuringiensis *spice isolate 62 using primer pair HBLC; lane 8,* B. cereus *F4810/72 control strain using primer pair CES (for* ces, *1,271 bp). A PCR without template DNA was run for each primer to ensure the absence of nonspecific binding (not shown).*

Although *Salmonella* has been the leading cause of outbreaks associated with contaminated spices, *B. cereus* is a potentially pathogenic organism and is a common spore-forming bacterium found in spices *(2, 3, 16)*. Because of the widespread occurrence of *B. cereus* in soil, this pathogen may infect spices and herbs. Spores can survive the varied and multiple steps used in spice processing and may survive food preparation procedures, allowing the spores to germinate and grow (e.g., in temperature-abused foods) to the high levels associated with foodborne illness *(18)*. Because *ces* was not detected in any isolates, the most likely virulence factors associated with spices as a vehicle of foodborne illness due to *B. cereus* are the diarrheal enterotoxins. The ability of certain isolates to grow at 9 and 12°C indicates the psychrotrophic behavior of toxigenic *B. cereus* and its potential for growth in mildly temperature-abused foods. The potential for germination and growth of toxigenic *B. cereus* strains in model foods containing spices spiked with this pathogen should be evaluated.

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