COMPARING THE MANNITOL-EGG YOLK-POLYMYXIN AGAR PLATING METHOD TO THE THREE TUBE MOST PROBABLE NUMBER METHOD FOR ENUMERATION OF *BACILLUS CEREUS* SPORES IN RAW AND HIGH-TEMPERATURE-SHORT-TIME PASTEURIZED MILK

by

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Abstract

The Food and Drug Administration's Bacteriological Analytical Manual recommends two enumeration methods for *Bacillus* spp.: 1) standard plating method using mannitol-egg yolk-polymyxin (MYP) agar and 2) most probable number (MPN) method with tryptic soy broth supplemented with 0.1% polymyxin sulfate. Preliminary research evaluated three inoculum preparation methods using EZ-Spore™ *B. cereus* pellets. Two methods involved EZ-Spore™ *B. cereus* pellets that were dissolved in deionized (DI) water, grown in brain heart infusion broth with manganese sulfate, and then heated to produce spores. The third inoculum preparation method of dissolving EZ-Spore™ pellets only in DI water was the most efficient due to 100% spores being present in the inoculum. Preliminary research also determined that MPN method recovered greater ($p<0.05$) *B. cereus* populations than MYP method in inoculated ultra-high temperature pasteurized skim and 2% milk. The objective of the main study was to compare the MYP and MPN method for detection and enumeration of *B. cereus* in raw and high-temperature-short-time pasteurized skim, 2%, and whole milk at 4 °C for 96 h. Milk samples were inoculated with *B. cereus* EZ-Spores™ dissolved in DI water and sampled at 0, 48, and 96 h after inoculation. No differences ($p>0.05$) were observed among sampling times so data was pooled for overall mean values for each treatment. The overall *B. cereus* population mean of pooled sampling times for MPN method (2.59 log CFU/mL) was greater ($p<0.05$) than MYP plating method (1.89 log CFU/mL). *B. cereus* populations ranged from 3.40 log CFU/mL to 2.40 log CFU/mL for inoculated milk treatments for MYP and MPN methods, which is well below the necessary level for toxin production. Even though MPN method enumerated more *B. cereus*, the MYP method should be used by industry for enumeration of *B. cereus* due to its ease of use and rapid turnover time (2 d compared to 5 d with MPN). However, MPN method should be used for

validation research due to its greater populations recovered. EZ-Spore™ *B. cereus* pellets were found to be an acceptable spore inoculum for validation research because the inoculum consists of 100% spores and does not contain vegetative cells.

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CHAPTER 1 - INTRODUCTION

Milk is an important staple of the average American's diet. Total milk production for the United States (U.S.) reached 47 billion pounds in 2003 with an annual per capita consumption rate of 594 pounds of dairy products (FAO 2006). The large consumption rate is due to the use of milk as an ingredient in other food products as well as to consumption based on the vast nutritional benefits linked to dairy products (Miller and others 2007; Chandon and Kilara 2008).

With the high consumption rate of milk and dairy products in the United States also comes the great risk of illness if intentional or unintentional contamination occurs in the U.S. milk supply. Since the attacks of September 11th, 2001 and the "Amerithrax" incident in October 2001, the American public has become highly aware of the devastation that terrorism and bioterrorism can cause (FBI 2008). Wein and Liu (2005) published a risk assessment that focused on the potential impact of intentional contamination of the U.S. dairy supply with the spore-forming pathogen *Clostridium botulinum*. They concluded that of all food products, milk may be at the greatest risk due to its rapid distribution and high consumption rates (both in the U.S. and worldwide). Dr. David Acheson, former Director of the Food and Drug Administration's (FDA) Center for Food Safety and Applied Nutrition (CFSAN), further testified that the dairy industry is of great concern as a potential bioterrorism target (FDA 2007).

Another spore-forming pathogen, *Bacillus cereus*, is widely known to be part of the natural microflora of milk (Jay and others 2005). *Bacillus cereus* has also been found to grow at temperatures as low as 4 °C (Larsen and Jorgensen 1999; Jay and others 2005).

Bacillus spp. can be found in both spore and vegetative form in raw and high-temperature-short-time (HTST) pasteurized milk. Research has shown that less than 1 log

CFU/mL of *Bacillus* spores are destroyed during heating to 72 °C and 78 °C for 35 min (Novak and others 2005).

To determine the effect of processing procedures on controlling pathogens, food products are often inoculated with high levels of a microorganism ranging from 4 to 7 log CFU/mL or g. Preparing an inoculum for validation research involves determining the amount of cells or spores initially needed and the optimum growth phase of the cells prior to incorporating them into a food product (Montville and others 2005; de Siano and others 2006; Rice and others 2005; Rose and others 2005).

Recently, Microbiologics® (Saint Cloud, Minn.) released a new product of lyophilized spore pellets that contains 4.6 log CFU per pellet of *B. cereus* EZ-Spore™ (Microbiologics 2008). These pellets could make inoculum preparation more efficient and consistent as cells would be in the spore form rather than vegetative cells. However, there is limited research on the use of *B. cereus* EZ-Spore™ pellets as an inoculum for milk products or optimum methods and media to produce growth from these pellets for higher levels prior to inoculation.

Once microorganisms have been inoculated into a food matrix and a validation study has been completed, detection of viable and injured cells is critical to determining actual populations. The National Center for Food Protection and Defense (NCFPD) describes detection methodologies as an important area of research in food defense. With effective detection methods, the contaminant can be identified, a recall or hold can be initiated, and deleterious public health effects can be limited. It is also very important to determine the type of microorganism in the food because microorganisms have differing resistance to antimicrobial agents and other microbial intervention strategies.

FDA's Bacteriological Analytical Manual (BAM) documents specific methods that should be used for detection and enumeration of specific bacteria (FDA 2006). The BAM states that two different techniques can be used for detection of *Bacillus* spp. in food: 1) a standard plating method using mannitol-egg yolk-polymyxin (MYP) agar and 2) a three tube most probable number (MPN) method using tryptic soy broth supplemented with 0.1% polymyxin sulfate (TSB-P) (FDA 2006). Neither of these methods is recommended by FDA over the other for detection and enumeration of *B. cereus*.

The objectives of the preliminary research were to determine the feasibility of *B. cereus* EZ-Spores™ as an inoculum in ultra-high temperature (UHT) pasteurized skim and 2% milk and to evaluate the efficacy of three different spore growth media: 1) brain heart infusion broth supplemented with 0.1% manganese sulfate heptahydrate (BHI + $MnSO_4 \cdot 7H_2O$), 2) twice the recommended BHI supplemented with 0.1% manganese sulfate heptahydrate (2xBHI + $MnSO_4 \cdot 7H_2O$), and 3) deionized water to increase EZ-Spore™ populations prior to inoculation into UHT skim and 2% milk. The preliminary research also evaluated the three tube MPN and MYP plating methods' effectiveness in recovering and enumerating *B. cereus* in inoculated UHT skim and 2% milk.

The objectives of the main study were: 1) to determine the efficacy of the standard MYP plating method and MPN method in detecting and enumerating *B. cereus* spores and vegetative cells in raw and high-temperature-short-time (HTST) pasteurized milk and 2) to determine the effectiveness of EZ-Spore™ pellets as an inoculum for milk validation research. A final objective was to determine if the fat content of the milk had any effect on the detection or enumeration of *B. cereus*.

CHAPTER 2 - REVIEW OF LITERATURE

1. Introduction

Food defense is an important and re-emerging field of research in the food industry. While the airline attacks of September 11, 2001, and the October 2001 letters to Congress that contained anthrax toxin brought terrorism to a forefront, the United States (U.S.) has been dealing with the possibility of a terrorist attack on the food supply for years (Miller and others 2002; CDC 2003; Roth and others 2008).

The Dalles, Oregon, was the site of a terrorist attack on the food supply in September 1984. The Rajneesh cult inoculated salad bars at local restaurants with *Salmonella* Typhimurium so that the population would not be able to vote in the local elections. By the end of the attack, more than 750 people had been hospitalized. However, an entire year passed before a defected member of the cult described the incident to the Federal Bureau of Investigation (FBI), which revealed that the outbreak was an act of terrorism (Miller and others 2002).

In late December 2002, 36 people became ill after purchasing ground beef at a Michigan supermarket. Following an investigation by the U.S. Department of Agriculture (USDA), it was determined that a disgruntled employee had intentionally contaminated over 200 pounds of product with the insecticide Black Leaf 40, an ingredient used for the production of nicotine (CDC 2003).

In 2007, the Food and Drug Administration (FDA) launched an investigation into the cause of unexplained deaths of several cats and dogs. The FDA determined that the chemical melamine had been added to wheat gluten to falsify the protein content. Consumption of melamine by animals can result in renal failure leading to illness or even death. This outbreak prompted studies on the detection of melamine in food for human consumption by using methods

such as enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC), or gas chromatography combined with mass spectroscopy (GC/MS) (Lin and others 2008 and Ibens 2009).

In 2008, FDA reported that melamine had been found in infant formulas in China (FDA 2009b). One study estimates that over 290,000 Chinese children were affected by this incident; more than 50,000 were hospitalized and at least 6 died (Ingelfinger 2008). Melamine was added to milk to artificially elevate the protein levels so that the product would appear to meet proper concentrations as required by customers (Roth and others 2008). Although this event was for economic gain, not meant to harm consumers, it is still categorized under food defense because it was an intentional contamination that caused deleterious effects to public health (Roth and others 2008).

Based on these incidents and others, the U.S. government and academic institutions have begun and continue research and education efforts in the area of food defense. The National Center for Food Protection and Defense (NCFPD) was founded in 2004 as a Department of Homeland Security (DHS) Center of Excellence to research the vulnerabilities of the nation's food supply to an intentional attack (NCFPD 2006b). The Center is composed of researchers and investigators from food industry companies, governmental agencies, and academic institutions.

The Center has defined eight categories of food defense research needs: 1) supply chain and information management; 2) public health response and epidemiology; 3) economic analysis; 4) detection and diagnostics; 5) inactivation and food processing; 6) decontamination and disposal; 7) risk communication; and 8) education programs (NCFPD 2006b). Detection is an important research area because without effective detection methods, a contaminant may go unrecognized in a food and can cause serious illness to those who consume it. However, the

biological contaminant must be differentiated to the species level for proper detection to occur. With effective detection methods, the contaminant can be identified, a recall or hold can be initiated, and deleterious public health effects can be limited. It is also critical to determine the contaminant so that public health officials will know what treatments to administer to those affected. However, detection of contaminants can be difficult due to interference from the food system. Also, the onset of illness could take days to manifest and the food may have been destroyed by that time (FDA 2001).

2. Food Defense

The term "food defense" is sometimes used interchangeably with "food security." Currently, the World Health Organization (WHO) recognizes the original definition of food security as access to food (WHO 2008). However, after the attacks of September 11th, 2001, the term "security" has changed dramatically in the U.S. The Department of Homeland Security has used the term "security" as synonymous with defense to refer to the protection of the food supply from intentional contamination. USDA's Food Safety and Inspection Service (FSIS) officially uses the term "defense" to describe "protection of food products from intentional adulteration by biological, chemical, physical, or radiological agents" (FSIS 2008). The FDA defines food defense as "tampering or [performing] other malicious, criminal, or terrorist actions" on the food supply (FDA 2008).

Since the attacks on September 11th, 2001, the U.S. government has adopted new policies to strengthen the protection of the food system. Many of these policies were to grant FDA powers similar to those held by USDA for importing, detaining, and regulating the transport of food (Strongin 2002).

2.1 Public Health Security and Bioterrorism Preparedness and Response Act

In 2002, Congress enacted and the FDA implemented the 2002 Public Health Security and Bioterrorism Preparedness and Response Act (also known as the Bioterrorism Act). Title III encompasses four main points of this act: 1) requirement of a manufacturing, processing, packaging or holding facility of food to be registered with FDA; 2) advanced notice to FDA of any food being imported into the U.S.; 3) authorization of FDA to detain an article of food with credible evidence; and 4) documentation of food when it is received, released, or transported (FDA 2005a). Prior to the 2002 Bioterrorism Act, USDA had these powers, but FDA did not (GAO 1999). Implementation of this act has allowed FDA to become more proactive in the protection of the food supply from both intentional and unintentional contamination. It also will allow the agency to respond more quickly to a foodborne outbreak (Strongin 2002).

While increased authoritative powers are beneficial for the safety and defense of the food supply, there are further complications in regards to implementation of this Act. In 2002, when the Act became effective, FDA staffed 770 inspectors for 57,000 inspected establishments and 132 ports while USDA employed 7,600 inspectors for 6,500 plants as well as another 5,000 inspectors and veterinarians working at ports, research laboratories, and crop fields. It is also estimated that FDA facilities are inspected every 5 to 10 years while USDA inspects most facilities every shift (Strongin 2002).

2.2 Homeland Security Presidential Directive 9

In January 2004, President George W. Bush signed Homeland Security Presidential Directive 9 (HSPD-9), which established "a national policy to defend the agriculture and food system against terrorist attacks." This directive also placed "major disasters and other emergencies" under the definition of food defense (OPS 2004).

Section 4 (e) of HSPD-9 states that it is "the policy of the U.S. to protect the agriculture and food system from terrorist attacks, major disasters, and other emergencies by enhancing screening procedures for domestic and imported products." The phrase "enhancing screening procedures" can mean many things, from increasing the number of inspectors to the development of better detection methods for determining the type of contaminant in a food or animal product (OPS 2004).

3. Milk

Milk is an extremely nutritious food that is important to the human diet, which also makes it a vulnerable source for an attack on the food system. Typical cow's milk is composed of 87.3% water, 3.4% protein, 3.7% fat, and 4.8% lactose. Milk contributes 19% of protein, 72% of calcium, 26% of riboflavin, 22% of vitamin A, and 20% of vitamin B_{12} to the average recommended daily intake of the American diet (Chandon and Kilara 2008). Milk also contains citrate, chloride, magnesium, inorganic phosphorus, potassium, calciferol, tocopherol, phylloquinone, thamine, riboflavin, ascorbic acid, niacin, biotin, pantothenic acid, and folic acid, as well as many other trace elements. Fat concentration can be changed during processing to produce skim, ½%, 1%, 2%, or whole milk (Chandon and Kilara 2008). Milk is also used in the production of other foods such as cheese, ice cream, and yogurt. Highly nutritious foods such as milk and dairy products are rapidly consumed in large quantities, especially when they have many known health benefits (Putnam and Allshouse 2003).

3.1 Nutrition and milk

During the past twenty years, numerous studies have assessed the health benefits of milk and other dairy products. Whole milk contains a moderate amount of fat (~4%) and research has found that moderate consumption of whole milk could reduce the risk of coronary heart disease

(Chandon and Kilara 2008). In fact, consuming 3 to 4 servings of dairy products per day can lower the risk of coronary heart disease by 10% and stroke by 20% (Chandon and Kilara 2008). Because milk and other dairy products contain a high concentration of calcium, dairy products have been shown to be able to protect against colon cancer as well (Chandon and Kilara 2008). Other studies have shown that milk consumption can reduce the risk of breast and prostate cancer, too (Chandon and Kilara 2008). Some of the better-known health benefits of consuming dairy products also include a reduced incidence of osteoporosis and increased tooth strength due to the presence of calcium and vitamin D in milk (Miller and others 2007; Chandon and Kilara 2008).

3.2 Milk consumption trends

Putnam and Allshouse (2003) observed that as more health benefits have been linked to milk and dairy products, the consumption rate of low-fat fluid milk has increased significantly. Total milk production in the U.S. reached 47 billion pounds in 2003 with an annual per capita consumption rate of 594 pounds of dairy products. Fluid milk is consumed at a rate of 20 billion gallons annually in the U.S. and 20 gallons annually by each person worldwide (FAO 2006). Raw milk is also consumed by a large portion of consumers, although many more people consume pasteurized milk. In a study of dairy consumption in the state of New York, 45% of those surveyed had consumed raw milk at least once in the past year (Kaylegian and others 2008).

3.3 Milk as a food for intentional contamination

Milk can be an excellent medium to sustain microbial growth for several different reasons. First, it has a very neutral pH (approximately 6.8), which will not inhibit germination or cell growth (Miller and others 2007). It is a great source of the proteins, sugars, and vitamins

that microorganisms need to reproduce and grow. Milk does not contain any natural antimicrobials that would inhibit or kill microorganisms that might be present. Bacterial populations in raw milk may be as high as 5 log CFU/mL before commingling and 5.4 log CFU/mL after commingling (FDA 2005b). However, the high-temperature-short-time pasteurization process destroys most microorganisms, excluding spore-formers and other thermo-tolerant microbes. The shelf life of milk can vary depending on the microorganisms present after pasteurization. Schaffner and others (2003) found that shelf life is based mostly on whether the pasteurized milk contains psychrotrophic or mesophilic microorganisms. They observed that pasteurized milk containing psychrotrophic microorganisms had a shelf life of approximately 14 d, while milk contaminated with mesophiles had a shelf life of 19 d. Carey and others (2005) also determined the typical shelf life of pasteurized milk to be at least 14 d.

Many different microorganisms are naturally present and can grow in dairy products (Jay and others 2005). Raw milk can contain species of *Enterococcus, Lactococcus, Streptococcus, Leuconostoc, Lactobacillus, Microbacterium, Proteus, Pseudomonas, Bacillus,* and *Listeria*. Pasteurized milk typically contains species of *Streptococcus, Lactobacillus,* and *Bacillus*. In fact, Shehata and Collins (1972) found that as much as 36% of raw milk samples in one study contained psychrotrophic *Bacillus* spp. Milk has also been linked to the foodborne pathogens that cause human illnesses such as campylobacteriosis, salmonellosis, listeriosis, Q fever, and hemorrhagic colitis. *Mycobacterium paratuberculosis* has been found in raw milk, which has been linked to Crohn's disease in humans (Jay and others 2005).

Milk is considered by many to be a major target for a bioterrorist attack. Wein and Lui (2005) stated that milk may be at great risk due to its rapid distribution and high consumption rates (both in the U.S. and worldwide). Wein and Lui (2005) discovered that in the absence of

detection, botulism toxin in milk could injure or kill as many as 100,000 people if 1 g of toxin were introduced into 586,000 gallons of milk.

In addition, Dr. David Acheson, former Director of the Food and Drug Administration's (FDA) Center for Food Safety and Applied Nutrition (CFSAN), reported that the dairy industry is of great concern as a potential bioterrorism target (FDA 2007). Spore-forming bacteria such as *Clostridium botulinum* and *Bacillus anthracis* are the microorganisms that would most likely be used for an attack on the milk supply (FDA 2007).

4. Spore-Forming Bacteria

Food products containing spore-forming bacteria are more difficult to decontaminate due to their resistance to heat, acid, and other standard microbial intervention strategies. A spore coat forms around certain bacteria to protect them from adverse conditions that could harm the vegetative cell. A spore coat is a multilayer protein shell composed of three different layers that have varying purposes. The inner layer is an amorphous undercoat in contact with the cortex (outer cell wall of the vegetative cell). The intermediate level is a very laminar layer while the outer level is a very thick, electrodense, striated layer (Ricca and others 2004).

The spore coat is responsible for the bacterium's resistance against acids, enzymes, radiation, and other intervention strategies. However, the thick peptidoglycan layer, called cortex, gives the bacterium its resistance to heat. Specifically, dipicolinic acid (DPA) in the cortex allows for the high heat resistance. Novak and others (2005) found that both *B. anthracis* and *B. cereus* are only reduced by less than 1 log CFU/mL when inoculated into skim milk that is heated and held at 72 °C and 78 °C for 35 min. Therefore, once milk has been contaminated with spore-forming bacteria, such as *B. anthracis* or *B. cereus*, decontamination is potentially difficult (Ricca and others 2004). Bacterial spores also contain a polar charge on the exterior of

the spore coat (Singleton 2004). This is potentially important because spores will attach to the fat cells rather than the free water of the milk, which could cause insulation or shielding by the fat.

4.1 Bacillus anthracis

Bacillus anthracis is a potential "Category A" threat agent that could be intentionally added to the milk supply (FDA 2003). This bacterium has the potential to survive milk pasteurization because of its ability to form an endospore and resist heat treatments and because it can grow after pasteurization due to absence of competitive microflora (Hanson and others 2005). In fact, Bowen and Turnbull (1992) found that *B. anthracis* spores survive in both unpasteurized (populations remained constant) and pasteurized milk (populations increased 1 log CFU/mL at 37 °C and decreased 1.5 log CFU/mL at 5-9 °C for 48 h). Montville and others (2005) also determined that *B. anthracis* is resistant (D-value=138 min) to heating milk to temperatures similar to high-temperature-short-time pasteurization of 72 °C for 15 sec.

4.1.1 History of Bacillus anthracis

The anthrax toxin has been very important in the history of the human race. It is believed to have been responsible for the Sixth Plague in Egypt, which is referenced in the Bible. Starting in the 1500s and continuing for several hundred years, anthrax was known as an agricultural disease. Humans became sick after coming into contact with animal wool (Woolsorter's disease) and hides that harbored *B. anthracis* cells. In 1881, Louis Pasteur developed the first effective live bacterial vaccine against anthrax (Akula and others 2005).

During the Cold War, both the U.S. and Russia conducted extensive research on *B. anthracis* and the anthrax toxin as biological weapons (Miller and others 2002). Many studies were done by both sides to determine the most effective method of infection as well as

environmental resistance to adverse conditions. In 1979, the large city of Sverdlovsk, Russia, began seeing cases of anthrax poisoning. The Russian government denied American reports that Sverdlovsk was the site of a Russian bioweapons facility. Sixty-eight victims eventually died from the accident. Most importantly, animals more than 50 km away from the town became sick from anthrax poisoning. Later, others became interested in the use of anthrax as a weapon, and by 1990, Saddam Hussein had developed a large stockpile of anthrax toxin in Iraq that could be used against enemies (Miller and others 2002).

4.1.2 Characteristics of Bacillus anthracis

B. anthracis is a Gram-positive, non-motile, spore-forming bacterium that is 1-1.5 μm in diameter and 3-10 μm long. Its name originates from the Greek word "anthrakis," which means coal, because of the black, cutaneous papule that anthrax causes (Claus and Berkley 1986). It does not form a spore in living tissues, but instead sporulates in food or tissues that have been exposed to air because of its need for oxygen for growth and sporulation. Spores can survive very harsh environmental conditions, even surviving decades in certain soils. *B. anthracis* can grow at temperatures between 7-48 °C and in a pH range of 4.9-9.3 (Jay and others 2005). Clery-Barraud and others (2004) found that *B. anthracis* spores suspended in deionized (DI) water are only reduced by about 1 log CFU/mL when held at 20 °C and treated by high hydrostatic pressure of 280 and 400 MPa for 350 min.

4.1.3 Anthrax toxin

Anthrax toxin causes a zoonotic disease, meaning it is pathogenic to both humans and animals. Anthrax is highly pathogenic through three main forms of contraction: inhalation, cutaneous infection, and ingestion (Anderson and others 2006). While the most common form is through the skin and the most lethal form occurs through inhalation, ingestion of the toxin in a

food terrorism scenario is still a serious concern because an infection contracted in that manner can also be quite lethal.

Cutaneous anthrax typically causes small papules to appear, which then progress into vesicles. Finally, the vesicle ruptures and causes an ulcer with a necrotic center. If left untreated, 20% of cutaneous anthrax can progress to septicemia. Inhalational anthrax is caused by the inhalation of 4,000 spores. While being transported to the mediastinal and peribronchial lymph nodes, the spores germinate and produce toxin. Inhalational anthrax closely resembles pneumonia, but is characterized by a widening of the mediastinum and pleural effusion. Ingestion of anthrax toxin causes mesenteric lymphadentitis by the toxin breaching the mucosal lining (Akula and others 2005).

The American Medical Association (AMA) states that the mortality rate for anthrax poisoning can be as high as 60% from ingestion of the toxin (AMA 2005). Research has shown that the anthrax toxin is produced when the bacterial concentration reaches 10^6 CFU/mL or g in food (Murray and others 1995).

4.2 Bacillus cereus

Bacillus cereus is a Gram-positive, spore-forming bacterium that is commonly found in milk and other dairy products, but unlike *B. anthracis*, it is motile. *Bacillus cereus* can cause foodborne illness through the production of toxins that cause gastroenteritis. The diarrheal toxin is caused by a high molecular weight, heat-labile enterotoxin produced in the intestines when bacterial concentrations reach 7 log CFU/g or mL in food (Jay and others 2005). Typical symptoms develop within 8-16 h after ingestion of food and include nausea, abdominal pains, tenesmus, and diarrhea. Symptoms usually persist for 6-12 h. Diarrheal toxin is commonly associated with meat and milk products (Jay and others 2005; FDA 2002).

The emetic syndrome is produced by a low molecular weight, heat-stable toxin produced in the food product when bacterial concentrations are 6 log CFU/g or mL in food (Jay and others 2005; FDA 2002). Symptoms of emetic toxin usually begin within 2-5 h of ingestion and include vomiting and stomach pain, which resemble staphylococcal toxin food poisoning. These symptoms generally last for less than 24 h (FDA 2009a). The emetic toxin typically occurs in starchy products (Jay and others 2005; FDA 2009a).

Bacillus cereus has been found to grow at temperatures as low as 4 °C (Larsen and Jorgensen 1999; Jay and others 2005). The psychrotrophic bacterium can grow and produce toxin while stored at refrigeration temperatures (Larsen and Jorgensen 1999). Christiansson and others (1989) reported that 28% of *B. cereus* naturally found in milk could grow and produce toxin in skim milk after 7 days at 8 °C. However, no naturally occurring foodborne outbreaks have been reported in milk due to *B. cereus* (Christiansson and others 1989). The reason for this is still not understood.

4.3 Destruction of Spore-forming Bacteria

Typical HTST milk pasteurization conditions of 72 °C for 15 sec has been shown to reduce *B. cereus* by less than 1 log CFU/mL in milk (Novak and others 2005). D-values of approximately 246 min and 12.2 min for *B. cereus* in skim milk at 72 °C and 100 °C were determined by Novak and others (2005).

B. cereus has also been shown to grow at a pH range of 4.9-9.3 (Jay and others 2005). Browne and Dowds (2002) observed only a 1 log CFU/mL reduction of *B. cereus* spores that were exposed to low concentrations of organic acids (pH 5.0) for 20 min. A contact time of 60 min with nisin (5.3 μg/mL) also caused reductions of only 1 log CFU/mL of *B. cereus* spores (Pol and Smid 1999).

Salt solutions of 10% reduced *B. cereus* spores by approximately 1 log CFU/mL with 20 min of contact time (Browne and Dowds 2001). However, research by den Besten and others (2006) has shown that when *B. cereus* spores were exposed to a 12% NaCl solution for 20 min, 2 log CFU/mL reductions occurred.

Bacterial spores can be very difficult to destroy in food due to their very resistant spore coat. Black and others (2008) determined that when 2% fluid milk is intentionally contaminated with *B. cereus* and *B. anthracis* spores, the spores can be resistant to hydrogen peroxide, Clorox® bleach, and other household cleaning supplies (including ammonia, rubbing alcohol, Pine Sol®, and Tilex® Mold and Mildew remover) that were used as potential decontaminants when added to milk. Hydrogen peroxide at a concentration of 3% caused a <1 log CFU/mL reduction after 2 h of exposure and only a 2.5 log CFU/mL reduction was observed after 2 h of exposure to Clorox bleach (6%). Other products studied had less of an effect on the spores. Rose and others (2005) found that 0.8 mg of free available chlorine/mL reduced *B. anthracis* spores by 2 log CFU/mL. They also determined that chlorine can be used as a decontaminant for *B. anthracis* in water at levels of 0.8 mg/mL of free chlorine. However, Rice and others (2005) determined that a 5 log CFU/mL reduction can be achieved by increasing the free available chlorine concentration to 2 mg/mL. This research has only been proved in simple matrices such as water or broth and has not been proven successful in a complex food matrix.

High hydrostatic pressure (~400 MPa) along with high temperature (~75 °C) has been shown to reduce *B. anthracis* spores in water by >8 log CFU/mL (Clery-Barraud 2004). However, this technology has not been used on a food matrix either.

The thermal resistance of spore-forming bacteria is well-documented. This resistance has been attributed in the past to dipicolinic acid, but has more recently been found to be caused by

small, acid-soluble proteins that aid the cortex in reducing the water content of the cell (Jay and others 2005). Montville and others (2005) determined that *B. anthracis* can have a D-value between 1.5-6.7 min at 90 °C in ultra-high temperature (UHT) pasteurized 2% milk while *B. cereus* can have a D-value as high as 12.8 min at 90 °C in UHT 2% pasteurized milk.

4.4 Detection of Bacillus spp.

Many different methods of detection have been used for *Bacillus* spp. Typically, the plating method on mannitol-egg yolk-polymyxin (MYP) agar is used for enumeration (Mossel and others 1967; Hanson and others 2005; Huck and others 2007; Valero and others 2007; Wong and others 1988; Crielly and others 1994) and confirmation is usually performed with modified Voges-Proskauer (VP) broth (Mossel and others 1967) or with the presence or absence of rhizoid growth on nutrient agar (Hanson and others 2005; Byrne and others 2006). Several researchers have used polymerase chain reaction or pulse field gel electrophoresis for further genetic sequencing of *Bacillus* spp. (Durak and others 2006; He and others 2008; Nakano and others 2004; Perdue and others 2003; Ralyea and others 1998).

While many researchers have used the plating method on MYP agar (FDA 2006), the Bacteriological Analytical Manual (BAM) recommends that either MYP or the most probable number (MPN) method be used for enumeration of *Bacillus* spp. The Standard Methods for the Examination of Dairy Products (Wehr and Frank 2004) recommend the use of an aerobic plate count agar for *Bacillus* spp., but other bacteria can grow on this medium as well. Another complication for detection and enumeration is that *Bacillus* spp. can be in both a vegetative and spore form. Visual evidence of sporulation can be observed through the use of a phase contrast microscope (Montville and others 2005) or using malachite green stain under general microscopy (Bischof and others 2007).

5. Surrogates for Research

Since studies with pathogenic microorganisms can be extremely dangerous, researchers use surrogates that have very similar growth and resistance characteristics as the more hazardous microorganisms, but do not have the added danger to researchers. Surrogates are extremely important to validation research because they can be used by companies or universities with pilot plants to conduct research with a lower level of risk (Sommers and others 2008).

5.1 Surrogates for Bacillus anthracis

Many investigations have been conducted to identify surrogates for *B. anthracis*. Naturally, the surrogate must be a spore-forming microorganism in order to simulate the resistance and growth properties of *B. anthracis*. Researchers have conducted studies on *B. cereus*, *B. mycoides*, *B. subtilis*, and *B. thuringiensis* (Montville and others 2005; de Siano and others 2006; Rice and others 2005; Rose and others 2005). Some of these studies have involved growth modeling while others investigated comparative resistance of these bacteria to heat, acid, or pressure. The research concluded that *B. cereus* (ATCC 4342 and 9819), *B. subtilis* (ATCC 6633), and *B. thuringiensis* (ATCC 35646) are good surrogates while other *B. subtilis* strains are not as good, but also adequate. Researchers have found that twelve strains (total) of *B. cereus* and *B. thuringiensis* shared similar genetic sequences to that of the anthrax toxin producing gene in *B. anthracis* (Helgason and others 2000; Hu and others 2006).

5.2 Gaps in Bacillus spp. research

Research has focused on identifying *Bacillus* spp. surrogates that will have similar characteristics as *B. anthracis*. Most studies comparing *B. anthracis* versus *B. cereus* were conducted in broth systems (peptone or tryptic soy broth solutions) (Montville and others 2005; de Siano and others 2006; Rice and others 2005; Rose and others 2005) instead of food matrices.

These studies generally determined the efficacy of chlorination (Rice and others 2005; Rose and others 2005) and thermal processing for reducing *B. anthracis* populations (Montville and others 2005). However, little research has focused on combinations of methods of destruction.

6. Spore inoculum preparation

Several methods have been used to produce spore inoculums for scientific research. The lawn method, which utilizes colonies on agar, is used by several researchers (Black and others 2008; Bowen and Turnbull 1992; Johnson and others 1984) to produce spores. Others (Byrne and others 2006; Montville and others 2005) have utilized broth solutions such as brain heart infusion broth or tryptic soy broth for the growth of vegetative cells; sporulation is then caused by shocking the bacteria with chemicals, extreme temperatures, or by changing the pH.

Recently, Microbiologics® (Saint Cloud, Minn.) released a new product of lyophilized spore pellets called EZ-Spores™, which contain 4.6 log of *B. cereus* per pellet according to the company's guarantee (Microbiologics 2008). The ability to simply dissolve the pellet in distilled water to form the inoculum makes spore inoculum preparation easier. However, limited research has been performed using EZ-Spores™ in food matrices.

7. Further Research

Further research is needed to determine the efficacy of using the EZ-Spore™ pellets in a food matrix for validation research. Furthermore, these pellets should also be researched to determine if they will be a suitable surrogate for *B. anthracis*. The strain (ATCC 11778) of *B. cereus* used in the EZ-Spore™ pellets has not been determined as a strain that can grow at psychrotrophic temperatures, so this should also be determined through research in broth systems as well as food matrices.

CHAPTER 3 - INOCULATION AND RECOVERY OF *BACILLUS CEREUS* SPORES INOCULATED WITH EZ-SPORE™ PELLETS IN ULTRA-HIGH TEMPERATURE PASTEURIZED MILK: PRELIMINARY STUDY 1

1. Introduction

Bacillus spp. can be found in both spore and vegetative form in raw and high-temperature-short-time (HTST) pasteurized milk, but has been found to resist high heat treatments that are slightly less than those used in ultra-high temperature (UHT) pasteurization of milk (Wong and others 1988). It has been hypothesized that *Bacillus* spp. are the cause of sweet curdling in milk and other dairy products (Christiansson and others 1989). If *Bacillus cereus* survives during pasteurization of milk and outgrowth occurs, *B. cereus* levels may be high enough to potentially cause foodborne illness.

Therefore, detection and enumeration of *Bacillus* spp. in milk products is important when determining the potential source linked to a foodborne outbreak. The Food and Drug Administration's (FDA) Bacteriological Analytical Manual (BAM) recommends two different methods for enumerating *Bacillus* spp.: 1) standard plating method using mannitol-egg yolk-polymyxin (MYP) agar and 2) most probable number (MPN) method with tryptic soy broth supplemented with polymyxin sulfate (TSB-P) (FDA 2006).

To determine the effect of processing procedures on controlling pathogens, food products are often inoculated with high levels of a microorganism ranging from 4 to 7 log CFU/mL or g (Getty and others 2000). Preparing an inoculum for validation research involves determining the amount of cells initially needed and the optimum growth phase of the cells prior to incorporating them into a food product.

Preparation of a spore inoculum can be achieved through several different methods including the lawn method (Johnson and others 1982) or the broth method (Byrne and others 2006; Montville and others 2005). Typically, *B. cereus* spores are produced in brain heart infusion (BHI) broth supplemented with manganese sulfate heptahydrate ($MnSO_4 \cdot 7H_2O$) to increase populations to levels of 4 to 7 logs (de Siano and others 2006). Doubling the concentration of BHI could potentially promote further growth to even higher levels by providing a more nutrient-rich environment.

Recently, Microbiologics® (Saint Cloud, Minn.) released a new product of lyophilized spore pellets (*B. cereus* EZ-Spore™), which contains 4.6 log CFU per pellet of *B. cereus* (Microbiologics 2009). These pellets could make inoculum preparation more efficient and consistent as cells would be in spore form versus a vegetative cell form, as well as there being a consistent spore concentration in each pellet. Furthermore, deionized (DI) water is recommended for dissolving lyophilized EZ-Spores™ into solution prior to inoculation into a food matrix (Microbiologics 2009). However, there is limited research on the use of *B. cereus* EZ-Spore™ pellets as an inoculum for food, especially milk products.

UHT milk is an optimum food matrix for initially evaluating *B. cereus* EZ-Spore™ pellets as an inoculum. UHT is shelf-stable and contains no background flora that would compete with detection and enumeration of *B. cereus* spores. It also can be commercially purchased at varying fat contents.

Therefore, the first objective of this study was to determine the feasibility of using *B. cereus* EZ-Spores™ as an inoculum in ultra-high temperature pasteurized skim and 2% milk. A secondary objective was to evaluate the ability of three different spore production media: 1) BHI broth supplemented with manganese sulfate heptahydrate (BHI + $MnSO_4 \cdot 7H_2O$), 2) twice the

recommended BHI broth supplemented with manganese sulfate heptahydrate (2xBHI + $MnSO_4 \cdot 7H_2O$), and 3) deionized water (DI) to increase EZ-Spore™ populations prior to inoculation into UHT milk. The third objective was to compare the three tube MPN and MYP plating method to recover and enumerate *B. cereus* in inoculated UHT skim and 2% milk.

2. Materials and Methods

2.1 Experimental Design

Phase 1 consisted of inoculating UHT skim milk with *B. cereus* ATCC 11778 EZ-Spore™ pellets that had been grown in BHI + 0.1% $MnSO_4 \cdot 7H_2O$, whereas in Phase 2, UHT 2% milk was inoculated with EZ-Spore™ pellets that were grown in 2xBHI + 0.1% $MnSO_4$. For Phase 3, EZ-Spore™ pellets were dissolved in DI water prior to inoculating into UHT skim milk. For each phase, a replication consisted of two packages of the same lot of milk with duplicate enumerations for each package. Three replications were completed for each phase.

2.2 Inoculum Preparation

Phase 1: Inoculum was prepared using *B. cereus* ATCC 11778 EZ-Spore™ technology (Microbiologics, St. Cloud, Minn.). The EZ-Spore™ pellet was prepared according to manufacturer's instructions (Microbiologics 2009) as follows: one pellet was aseptically removed from the package and transferred to a sterile test tube containing 4 mL of sterile DI water heated to 35 °C. The pellet mixture was vortexed for 5 s every 10 min over a 30 min period to allow the lyophilized pellet to dissolve.

One mL of the dissolved pellet mixture was aseptically transferred to 9 mL of BHI broth (Becton Dickinson, Franklin Lakes, N.J.) supplemented with 0.1% manganese sulfate heptahydrate (BHI + $MnSO_4 \cdot 7H_2O$), which was added prior to autoclaving (Remel, Lenexa, Kans.) (FDA 2006). Inoculum was prepared in duplicate and incubated at 35 °C for 72 h.

Following incubation, two inoculum pellet mixtures in BHI broth were aseptically combined in a sterile 50 mL beaker and 5 mL of this combined inoculum was transferred into 100 mL UHT skim milk samples in duplicate. To determine the initial inoculum level, 1 mL of inoculum was serially diluted and enumerated on MYP agar (Difco, Detroit, Mich.) and by MPN method using TSB supplemented with 0.1% polymyxin sulfate (TSB-P) (Difco, Detroit, Mich.).

Phase 2: The inoculum was again prepared using *B. cereus* ATCC 11778 EZ-Spore™ technology. The pellet was aseptically removed from the package and transferred to a sterile test tube containing 4 mL of sterile DI water heated to 35 °C. The pellet mixture was vortexed for 5 s every 10 min over 30 min to allow the lyophilized pellet to dissolve. One mL of the dissolved pellet mixture was aseptically transferred to 9 mL of twice the recommended brain heart infusion broth (74 g of dehydrated BHI per L of DI water instead of 37 g of dehydrated BHI per L of DI water) supplemented with 0.1% manganese sulfate heptahydrate (2xBHI + $MnSO_4 \cdot 7H_2O$) that was added prior to autoclaving (FDA 2006). Inoculum was prepared in duplicate and incubated at 35 °C for 72 h. Following incubation, two inoculum pellet mixtures from BHI were aseptically combined in a sterile 50 mL beaker and 5 mL of this combined inoculum was transferred into 100 mL UHT 2% milk samples in duplicate. To determine the initial inoculum level, 1 mL of inoculum was serially diluted and enumerated using the MPN and MYP methods.

Phase 3: The last portion of this preliminary study involved the comparison of the EZ-Spores™ (Microbiologics, St. Cloud, Minn.) dissolved in DI water as an inoculum and to see if it yielded similar populations as inoculums grown in BHI + $MnSO_4 \cdot 7H_2O$ and 2xBHI + $MnSO_4 \cdot 7H_2O$. The EZ-Spore™ pellet was prepared according to manufacturer's instructions (Microbiologics 2006) as follows: one pellet was aseptically removed from the package and transferred to a sterile test tube containing 4 mL of sterile DI water heated to 35 °C. The pellet

mixture was vortexed for 5 s every 10 min over 30 min to allow the lyophilized pellet to dissolve. Following this, the final inoculum for each of the milk samples was prepared by combining two of the dissolved pellet solutions together into a sterile test tube for a combined total of 10 mL of inoculum. Five mL of this combined inoculum was transferred into 100 mL UHT skim milk samples in duplicate. To determine the initial inoculum level, 1 mL of inoculum was serially diluted and enumerated using the MPN and MYP methods.

2.3 Milk Sample Preparation, Inoculation, and Sampling

Ultra-high temperature pasteurized skim and 2% milk samples were purchased at a local grocery store over a period of three weeks. UHT pasteurized milk is considered a shelf stable product and was utilized so that background microflora would not interfere with detection and enumeration of *B. cereus*. Different fat concentrations (skim and 2%) were used to determine if fat concentration has any effect on detection and enumeration of *B. cereus*.

Each replication consisted of duplicate packages from the same lot and different lots were used. Each package of UHT skim or 2% milk was aseptically opened and 100 mL of milk was aseptically transferred to a sterile 250 mL round bottom boiling flask (Pyrex, Lowell, Mass.) that contained a small x-shaped magnetic stir bar (VWR International, West Chester, Pa.).

Prior to inoculation, milk was stored at 4 °C to remove the need for temperature adjustment. Following the addition of the 5 mL of inoculum, flasks were then covered with sterile aluminum foil and stored at 4 °C over a 3 h sampling period. To determine population levels, sampling was performed at 0, 45, 90, 135, and 180 min after inoculation for Phases 1 and 3 and at 0, 60, 120, and 180 min after inoculation for Phase 2. After a sample was removed at each time point, the flask was immediately placed back in the refrigerator (4 °C) for storage until the next sampling period. Although, different sampling times were utilized, no differences in

populations were observed due to the short storage time. No growth was expected in the 3 h storage time. The multiple sampling times were used to gain more data points for each replication of the experiment.

2.4 Enumeration Procedure

For all phases, three tube MPN and MYP methods were used for enumeration of *B. cereus* in UHT pasteurized milk (FDA 2006). Inoculated milk samples were removed from refrigeration and stirred for 30 s on a stirring plate to allow for proper mixing prior to sampling. Three 1 mL samples were removed from each boiling flask containing milk and placed in three separate MPN test tubes containing 9 mL of TSB with 0.1% polymyxin sulfate (TSB-P). Flasks containing inoculated milk samples were returned to refrigeration until the next sampling time.

Each MPN test tube was vortexed for 3 to 4 s on high speed and then shaken vigorously based on the BAM recommendation (FDA 2001) prior to serial dilution. During each sampling time, 1 mL of the milk sample was transferred into one test tube containing 9 mL of 0.1% peptone (Becton Dickinson, Franklin Lakes, N.J.). This procedure was repeated twice more to perform the three tube MPN method. Following this single dilution, 1 mL was removed from the peptone dilution blank and serially diluted for each of the three MPN tubes (containing 9 mL of TSB-P) to the 10^{-7} dilution. MPN tubes were incubated at 35 °C for 48 h. Sample tubes were removed from the incubator and the turbidity of the dilutions was recorded. *B. cereus* populations were then determined using the three tube MPN chart (FDA 2006).

The MYP plating method involved serial dilution of the milk samples seven times (to the 10^{-7} level) before plating. A small amount (0.1 mL) of each dilution was placed on each plate and then spread-plated. One mL of the UHT milk was placed over four plates (0.25 mL per

plate) for the non-diluted sample to allow for a detection limit of 1 CFU/mL. MYP plates were then incubated at 35 °C for 48 h and colonies were counted.

2.5 Spore confirmation

Confirmation of spore production for all phases was performed using malachite green dye and a counterstain of 0.25%. Safranin O (Remel, Lenexa, KS) was utilized to visualize vegetative Gram positive cells under general microscopy (ASM 2002). Ten fields were evaluated and a percentage was calculated from these 10 fields to determine the amount of cells that were in spore form.

2.6 Statistical Analysis

Mean and standard deviations of populations, as well as levels of significance were determined using LS Means in SAS Version 9.0 (SAS Institute, Cary, N.C.). The fixed effects for statistical analysis were media type, replications, and replication by media type. The random effect for this study was sampling time. Differences ($p<0.05$) were determined for sampling times by media type and media type by type of milk.

3. Results and Discussion

3.1 Initial Inoculum Populations

For Phase 1, mean *B. cereus* EZ-Spore™ populations for the initial inoculum grown in BHI + $MnSO_4 \cdot 7H_2O$ were 3.2 and 3.1 log CFU/mL as determined by the MPN and MYP plating method, respectively. However, for Phase 2, mean *B. cereus* EZ-Spore™ populations for the initial inoculum grown in 2xBHI + $MnSO_4 \cdot 7H_2O$ were 4.9 and 3.4 log CFU/mL on MPN and MYP, respectively. Mean *B. cereus* EZ-Spore™ populations dissolved in DI water for Phase 3 were 3.0 and 2.8 log CFU/mL as determined by the MPN and MYP plating method, respectively.

Furthermore, the initial inoculum level for 2xBHI + $MnSO_4 \cdot 7H_2O$ was greater ($p<0.05$) than BHI + $MnSO_4 \cdot 7H_2O$ and DI water for the MPN method. No differences ($p>0.05$) were observed among media and DI water for initial inoculum populations using MYP plating method for all three phases. These results are as expected because double concentration of BHI would cause the bacteria to grow at a greater rate in the presence of a growth inhibitor such as $MnSO_4 \cdot 7H_2O$. Brain heart infusion is used as a growth medium for a pure inoculum and is quite useful to rapidly grow bacterial cultures (Liu and Ream 2008, Byrne and others 2006; Montville and others 2005).

Although, EZ-Spore™ populations produced in DI water were fairly low at 3.0 log CFU/mL, the level was sufficient enough for detection and enumeration throughout the sampling time. When EZ-Spore™ pellets are dissolved in DI water and then inoculated into a food matrix, the cells are in spore form versus vegetative form. By growing *B. cereus* spp. in a nutrient broth such as BHI, there may also be the possibility for the presence of vegetative cells since BHI is used to grow vegetative cell cultures (Liu and Ream 2008). This hypothesis was validated by the staining results that were obtained in this experiment. Phase 1 (BHI + $MnSO_4 \cdot 7H_2O$) produced no spores and Phase 2 (2xBHI + $MnSO_4 \cdot 7H_2O$) produced only 8% spores in the inoculum as compared to the 100% for Phase 3 (DI water). This means that only EZ-Spore™ pellets dissolved in DI water were actually spores, whereas the other two media (BHI + $MnSO_4 \cdot 7H_2O$ and 2xBHI + $MnSO_4 \cdot 7H_2O$) produced vegetative cells and some spores.

3.2 Phase 1: Bacillus cereus Populations and Confirmation of Spore Presence in UHT Skim Milk

Control samples (non-inoculated milk) determined that no *B. cereus* was present in the UHT skim milk prior to inoculation for either enumeration method. For Phase 1, *B. cereus*

inoculum (from EZ-Spore™ pellets dissolved in DI water) was grown in BHI + $MnSO_4 \cdot 7H_2O$ and inoculated into UHT skim milk. Mean *B. cereus* populations in UHT skim milk enumerated by MPN method ranged from 3.8 to 4.4 log CFU/mL during 3 h of storage at 4 °C (Table 1). The MYP populations were lower ($p<0.05$) than the MPN method at each sampling period with populations ranging from 2.1 to 2.4 log CFU/mL in UHT skim milk. However, no growth ($p>0.05$) was observed for either enumeration method during 3 h of storage at 4 °C. This was expected because the 3 h time period at 4 °C would not be sufficient for significant growth of *B. cereus*.

Table 1. Phase 1: *Bacillus cereus* mean[a] populations and standard deviations in UHT skim milk inoculated with EZ-Spore™ pellets grown in BHI + MnSO4•7H2O[b] and enumerated using the three tube MPN and MYP plating method (n=6).

Time (min after inoculation)	MPN[c] (log CFU/mL)	MYP[d] (log CFU/mL)
0	3.8±0.2[ex]	2.1±0.2[ey]
45	4.0±0.6[ex]	2.2±0.2[ey]
90	4.4±0.7[ex]	2.3±0.1[ey]
135	4.3±0.1[ex]	2.4±0.0[ey]
180	4.2±0.7[ex]	2.4±0.0[ey]

[a]Mean populations were determined for 3 replications.
[b]BHI + $MnSO_4 \cdot 7H_2O$ = brain heart infusion broth (BHI) supplemented with manganese sulfate heptahydrate produced from EZ-Spore™ pellets dissolved in DI water.
[c]MPN = most probable number.
[d]MYP = mannitol-egg yolk-polymyxin.
[e]Means with different superscripts within a column are not significantly different ($p>0.05$).
[xy]Means with different superscripts within a row are not significantly different ($p>0.05$).

3.3 Phase 2: Bacillus cereus Populations and Spore Confirmation in UHT 2% Milk

Control samples (non-inoculated milk) determined that no *B. cereus* was present in the UHT 2% milk prior to inoculation using either MYP or MPN enumeration method. Phase 2 of this study consisted of EZ-Spore™ inoculum produced in 2xBHI + $MnSO_4 \cdot 7H_2O$ that was added to UHT 2% milk. Mean *B. cereus* populations enumerated by MPN method were approximately 4.7 log CFU/mL during 3 h storage (4 °C) of UHT 2% milk (Table 2). The MYP populations

were approximately 2.3 log CFU/mL during the 3 h of storage and were lower ($p<0.05$) than the MPN method during all sampling periods. Again, no growth ($p>0.05$) was observed for either enumeration method during the 3 h of storage (4 °C) of UHT 2% milk. The MPN populations were also greater ($p<0.05$) in Phase 2 than in Phase 1 because of the greater concentration of BHI in the initial inoculum production media.

Table 2. Phase 2: *Bacillus cereus* mean[a] populations and standard deviations in UHT 2% milk inoculated with EZ-Spore™ pellets grown in 2xBHI + MnSO4•7H2O[b] and enumerated using the three tube MPN and MYP plating method (n=6).

Time (min after inoculation)	MPN[c] (log CFU/mL)	MYP[d] (log CFU/mL)
0	4.8 ± 0.6^{ex}	2.2 ± 0.2^{ey}
60	4.8 ± 0.4^{ex}	2.3 ± 0.1^{ey}
120	4.6 ± 0.8^{ex}	2.4 ± 0.1^{ey}
180	4.6 ± 0.8^{ex}	2.4 ± 0.1^{ey}

[a]Mean populations were determined for 3 replications.
[b]2xBHI + $MnSO_4 \cdot 7H_2O$ = twice the recommended brain heart infusion broth (BHI) supplemented with manganese sulfate heptahydrate produced from EZ-Spore™ pellets dissolved in DI water.
[c]MPN = most probable number.
[d]MYP = mannitol-egg yolk-polymyxin.
[e]Means with different superscripts within a column are not significantly different ($p>0.05$).
[xy]Means with different superscripts within a row are not significantly different ($p>0.05$).

3.4 Phase 3: Bacillus cereus Populations and Spore Confirmation in UHT Skim Milk

The inoculum for Phase 3 was prepared by simply dissolving EZ-Spore™ pellets in DI water followed by inoculation into UHT skim milk. Mean *B. cereus* populations using the MPN method ranged from 3.5 to 3.8 log CFU/mL during 3 h storage of UHT skim milk at 4 °C (Table 3). Populations enumerated on MYP were lower ($p<0.05$) than the MPN method with populations ranging from 2.5 to 3.1 log CFU/mL for the UHT skim milk. Again, no growth ($p>0.05$) was observed for either enumeration method during 3 h storage of UHT skim milk at 4 °C.

Table 3. Phase 3: *Bacillus cereus* mean populations and standard deviations of UHT skim milk inoculated with EZ-Spore™ pellets[a] and enumerated using the three tube MPN and MYP plating (n=6).

Time (min after inoculation)	MPN[b] (log CFU/mL)	MYP[c] (log CFU/mL)
0	3.8±0.2[dx]	2.5±0.3[dy]
45	3.8±0.2[dx]	2.7±0.2[dy]
90	3.5±0.2[dx]	2.9±0.2[dy]
135	3.5±0.2[dx]	3.1±0.5[dy]
180	3.5±0.2[dx]	3.1±0.4[dy]

[a]EZ-Spore™ pellets dissolved in sterile 35 °C DI water.
[b]MPN = most probable number
[c]MYP = mannitol-egg yolk-polymyxin
[d]Means with different superscripts within a column are not significantly different ($p>0.05$).
[xy]Means with different superscripts within a row are not significantly different ($p>0.05$).

Bacillus cereus populations in UHT skim and 2% milk using either recovery method did not change ($p>0.05$) throughout 3 h of storage for all three phases. One would not expect growth to occur in this short of a time period. *Bacillus cereus* can grow at refrigeration temperatures (Larsen and Jorgensen 1999). Research has shown that *B. cereus* in milk can grow more than 1 log CFU/mL during a week's storage at a refrigeration temperature of 8 °C (Christiansson and others 1989). The consistency of our results throughout the different sampling periods demonstrates the accuracy and reliability of MPN and MYP to detect and recover *B. cereus* in UHT milk samples.

A significant difference was observed between the two enumeration (MPN and MYP) methods at all sampling times for all phases with MPN method recovering more ($p<0.05$) *B. cereus* than the MYP plating method. As much as a 2.0 log CFU/mL difference was observed between the MPN and MYP method for Phases 1 (BHI + $MnSO_4 \cdot 7H_2O$) and 2 (2xBHI + $MnSO_4 \cdot 7H_2O$) of this study while Phase 3 (DI water) observed up to a 1.2 log CFU/mL difference. This effect might be due to the fact that the MPN method is more of an enrichment

procedure since it is involves the use of tryptic soy broth (TSB) (a typical enrichment medium). The MPN tubes contain TSB, a medium typically used to grow pure cultures and polymyxin sulfate is used as a selective agent for *Bacillu*s spp. In contrast, the MYP plates do not contain additional ingredients that would provide nutrient for recovery or enrichment, as is evident by TSB's use to grow an inoculum (Benkerroum and others 2004).

4. Conclusions

Results from this preliminary study show that *B. cereus* vegetative cells and spores in UHT skim and 2% milk are detected and recovered more ($p<0.05$) via the three tube MPN method than on MYP. The method of dissolving EZ-Spore™ pellets only in DI water for preparation of an inoculums was validated since the populations were similar to other growth methods (BHI + $MnSO_4 \cdot 7H_2O$ and 2x BHI + $MnSO_4 \cdot 7H_2O$). It is the preferred inoculum preparation method because it also guarantees that the inoculum is composed of spores and not of vegetative cells.

Results from this study show that *B. cereus* can be recovered and enumerated in UHT skim and 2% milk using the MPN method recommended by FDA. However, further research is needed to determine if *B. cereus* can be recovered and enumerated in raw and high-temperature-short-time (HTST) pasteurized milk. While the constituents are the same in both UHT and HTST milk, the latter contains background microflora, which could cause potential issues concerning enumeration.

CHAPTER 4 - PRELIMINARY STUDY 2: COMPARING EZ-SPORE™ PELLETS TO OTHER *BACILLUS CEREUS* GROWTH AND SPORE PRODUCTION METHODS FOR INOCULUM

1. Introduction

Food products are often inoculated with high levels of a microorganism ranging from 4 to 7 log CFU/mL or g to determine the effect of processing procedures on controlling pathogens (Getty and others 2000). Preparing an inoculum for validation research involves determining the amount of cells initially needed and the optimum growth phase of the cells prior to incorporating them into a food product. Generally, microorganisms are grown in tryptic soy broth (TSB) or brain heart infusion (BHI) broth prior to inoculation into food matrices (Benkerroum and others 2004; Liu and Ream 2008). These growth media allow for optimum levels of microorganisms for conducting validation research.

Recently, Microbiologics® (Saint Cloud, Minn.) released a new product of lyophilized spore pellets that contains 4.6 log CFU per pellet of *Bacillus cereus* EZ-Spore™ (Microbiologics 2009). These pellets could make inoculum preparation more efficient and consistent as cells would be in spore form versus a vegetative cell form; an additional advantage is that the pellets provide a known specific concentration of spores. However, there is limited research on optimum media for growth of *B. cereus* from EZ-Spore™ pellets and what conditions are needed for sporulation. Deionized (DI) water is the recommended solvent for dissolving lyophilized EZ-Spores™ into solution prior to inoculation into a food matrix (Microbiologics 2009).

Typically, *B. cereus* spores are produced in BHI broth supplemented with manganese sulfate heptahydrate ($MnSO_4 \cdot 7H_2O$) to increase populations to levels of 4 to 7 log (de Siano and

others 2006). Manganese sulfate heptahydrate is used to cause sporulation of the bacteria by creating a difficult environment for vegetative cells to survive (Black and others 2008). Our preliminary research demonstrated that an inoculum prepared by dissolving EZ-Spore™ pellets in DI water and then growing them in a medium of double the concentration of BHI plus $MnSO_4 \cdot 7H_2O$ increased the initial population by approximately 1.5 log CFU/mL when compared to an inoculum of only EZ-Spore™ pellets dissolved in DI water. However, the inoculum with BHI plus $MnSO_4 \cdot 7H_2O$ only produced vegetative cells and not spores.

Yang and others (2008) have also shown that by heating *B. cereus* to 60 °C, vegetative cells are able to go from a vegetative cell to spore form. Therefore, the objective of this study was to compare populations of *B. cereus* EZ-Spore™ pellets dissolved in DI water and then grown in BHI supplemented with manganese sulfate heptahydrate ($MnSO_4 \cdot 7H_2O$) or twice the BHI supplemented (2xBHI + $MnSO_4 \cdot 7H_2O$) and heated shocked for 12 min to 50 °C or 12 min to 60 °C to the EZ-Spores™ populations produced by only dissolving in DI water.

2. Materials and Methods

2.1 Experimental Design

One EZ-Spore™ pellet was used for each sample, with duplicate samples being used for each replication in both of the growth media at both temperatures. Duplicate MPN tubes were used for enumeration of populations for each sample. The experiment was replicated three times (n=6) for each media at each temperature. All averages and standard deviations were determined using Microsoft Excel 2007 (Redmond, Wash.).

2.2 Media Preparation

The first medium, BHI broth supplemented with manganese sulfate heptahydrate ($MnSO_4 \cdot 7H_2O$), was produced by suspending 37 g of dehydrated BHI broth (Becton Dickinson, Franklin Lakes, N.J.) per liter of DI water and supplementing with 0.1% $MnSO_4 \cdot 7H_2O$ (Remel, Lenexa, Kans.) prior to autoclaving. The second medium, 2xBHI + $MnSO_4 \cdot 7H_2O$, was produced by using twice the concentration of dehydrated BHI broth (74 g dehydrated BHI per L of DI water) supplemented with 0.1% $MnSO_4 \cdot 7H_2O$, which was added prior to autoclaving.

2.3 Culture Preparation and Spore Production

Bacillus cereus (ATCC 11778) EZ-Spore™ (Microbiologics, St. Cloud, Minn.) pellets were prepared according to instructions from the manufacturer (Microbiologics 2006). One pellet was aseptically removed from the package and transferred to a sterile test tube containing 4 mL of sterile DI water heated to 35 °C. The pellet mixture was vortexed for 5 s every 10 min over a 30 min time period to allow the lyophilized pellet to dissolve.

An amount of 1 mL of the dissolved pellet mixture was then transferred to a tube containing 9 mL of BHI + $MnSO_4 \cdot 7H_2O$ or 2xBHI + $MnSO_4 \cdot 7H_2O$. Lastly, pellet mixtures in BHI + $MnSO_4 \cdot 7H_2O$ were incubated at 37 °C for 24 h while pellet mixtures in the 2xBHI + $MnSO_4 \cdot 7H_2O$ medium were stored at 37 °C for 48 h. Twenty-four hours is the typical period used to grow cultures; however, manganese sulfate heptahydrate slows bacterial growth so incubation time was extended to 48 h. This extended incubation period was used to determine if a longer incubation time would aid in a higher spore population.

Following incubation, test tubes with the two media-containing cultures were removed from the incubator and heated in a water bath (Model N-12, Precision Scientific Co., Chennai, India). Both cultures were heated gradually (1 °C increase every 2 min from 37 °C to 50 °C and

60 °C for 12 min) to cause sporulation of the vegetative cells (Yang and others 2008). Temperature was monitored using a mercury thermometer that was inserted into a non-inoculated sample of each media (BHI + $MnSO_4 \cdot 7H_2O$ or 2xBHI + $MnSO_4 \cdot 7H_2O$).

2.4 Enumeration and Determination of Spore Concentration

Following heating for spore production, cultures in each media were enumerated using MPN method for *B. cereus*, which uses tryptic soy broth (TSB) (Becton Dickinson, Franklin Lakes, N.J.) with 0.1% polymyxin sulfate (MP Biomedicals, Solon, Ohio) (TSB-P) (FDA 2001). Three 1 mL samples were added to the first MPN tubes (9 mL tubes) and serially diluted four times. Test tubes were then incubated at 37 °C for 72 h. After 72 h incubation, MPN tubes were removed and enumerated according to the three-tube MPN chart (FDA 2006).

2.5 Spore confirmation

Confirmation of spore production was performed using malachite green. A counterstain of 0.25% Safranin O (Remel, Lenexa, Kans.) was used to visualize vegetative Gram positive cells under general microscopy (ASM 2002). Ten fields were evaluated and a percentage was calculated from these 10 fields to determine the amount of cells that were in spore form.

3. Results and Discussion

Results showed that 2xBHI + $MnSO_4 \cdot 7H_2O$ is able to produce more *B. cereus* spores and/or vegetative cells when combined with heating at 60 °C for 12 min (Table 4). The medium 2xBHI + $MnSO_4 \cdot 7H_2O$ achieved 3.1 log CFU/mL at 60 °C for 12 min while populations were 2.6 log CFU/mL for *B. cereus* produced in BHI + $MnSO_4 \cdot 7H_2O$ and heated to the same temperature (Table 4). Concentrations for samples grown in BHI + $MnSO_4 \cdot 7H_2O$ and 2xBHI + $MnSO_4 \cdot 7H_2O$ at 50 °C for 12 min were 2.5 and 2.4 log CFU/mL, respectively.

From our previous studies, inoculum of an EZ-Spore™ pellet dissolved in DI water was 3.0 log CFU/mL with the MPN method. EZ-Spore™ cultures dissolved in DI water displayed spores with no vegetative cells using malachite green stain under microscopy, whereas no spores were observed by heating the EZ-Spore™ pellet mixture in either BHI + $MnSO_4 \cdot 7H_2O$ and 2xBHI + $MnSO_4 \cdot 7H_2O$ to 50 °C for 12 min or by heating cultures grown in BHI + $MnSO_4 \cdot 7H_2O$ to 60 °C for 12 min (Table 5). The staining procedure did show that spores were produced in BHI + $MnSO_4 \cdot 7H_2O$ and 2xBHI + $MnSO_4 \cdot 7H_2O$ when heated to 60 °C for 12 min; however, they were not found in high quantities (approximately 42% and 44%, respectively (Table 5)). This may be due to the 2xBHI + $MnSO_4 \cdot 7H_2O$ medium allowing cells to be in a nutrient rich environment prior to heating, which makes them sporulate more easily instead of being damaged or destroyed by heating because $MnSO_4 \cdot 7H_2O$ aids in sporulation. It was observed that a lower temperature (50 °C) for 12 min does not provide adequate heating to cause sporulation of vegetative cells.

Table 4. MPN[a] method mean[b] and standard deviations of *B. cereus* populations (log CFU/mL) grown in various media and heated to 50 or 60 °C for 12 min (n=6).

	50 °C for 12 min	**60 °C for 12 min**
BHI+ $MnSO_4 \cdot 7H_2O$[c]	2.5±0.4 log CFU/mL	2.6 ±0.3 log CFU/mL
2xBHI + $MnSO_4 \cdot 7H_2O$[d]	2.4±0.0 log CFU/mL	3.1±0.5 log CFU/mL

[a]MPN=most probable number.
[b]Compare populations to 3.0±0.2 log CFU/mL for EZ-Spore™ dissolved in DI water.
[c]BHI + $MnSO_4 \cdot 7H_2O$ = brain heart infusion broth (BHI) supplemented with manganese sulfate heptahydrate produced from EZ-Spore™ pellets dissolved in DI water.
[d]2xBHI + $MnSO_4 \cdot 7H_2O$ = twice the recommended brain heart infusion broth (BHI) supplemented with manganese sulfate heptahydrate produced from EZ-Spore™ pellets dissolved in DI water.

Table 5. Percentage[a] of inoculum in spore form based on four inoculum preparation methods[b] (n=30).

	50 °C for 12 min	60 °C for 12 min
BHI+ $MnSO_4 \cdot 7H_2O$[c]	0%	42%
2xBHI + $MnSO_4 \cdot 7H_2O$[d]	0%	44%

[a]Percentage is based on the average of ten fields of view per replication.
[b]Compare populations to 100% spores for EZ-Spore™ dissolved in DI water.
[c]BHI + $MnSO_4 \cdot 7H_2O$ = brain heart infusion broth (BHI) supplemented with manganese sulfate heptahydrate produced from EZ-Spore™ pellets dissolved in DI water.
[d]2xBHI + $MnSO_4 \cdot 7H_2O$ = twice the recommended brain heart infusion broth (BHI) supplemented with manganese sulfate heptahydrate produced from EZ-Spore™ pellets dissolved in DI water.

4. Conclusions

While spores were observed in BHI + $MnSO_4 \cdot 7H_2O$ and 2xBHI + $MnSO_4 \cdot 7H_2O$ when heated to 60°C for 12 min based on microscopy using malachite green stain, the bacteria that were heated caused some bacteria to sporulate while others stayed in the vegetative cell form. While the inoculum produced in 2xBHI + $MnSO_4 \cdot 7H_2O$ and heated to 60 °C for 12 min showed similar populations to EZ-Spore™ pellets dissolved in DI water, the inoculum did not produce a high concentration of spores. Furthermore, the heating might have damaged some of the cells, which would make for an inadequate inoculum for validation research. EZ-Spore™ pellets also allow the researcher to know the cells in the inoculum are in spore form without having to grow the culture and sporulate the cells for a period of time prior to inoculation into a food matrix.

Results of this study confirm that dissolving EZ-Spore™ pellets in DI water to prepare an inoculum provides a consistent level (3.0±0.2 log CFU/mL) of *B. cereus* spores for validation research. Manufacturers of EZ-Spore™ pellets recommended preparation procedure of dissolving pellets in DI water allows researchers to know that the inoculum contains spores rather than vegetative cells. This procedure is also less time consuming and more cost effective

as there is not additional media preparation or extended incubation of cultures (Microbiologics 2006).

CHAPTER 5 - DETECTION AND RECOVERY OF *BACILLUS CEREUS* SPORES IN RAW AND HIGH-TEMPERATURE-SHORT-TIME PASTEURIZED MILK

1. Introduction

Bacillus cereus has been linked to foodborne outbreaks in many different foods. The diarrheal syndrome is associated with meat and dairy products while the emetic toxin is linked to starchy products such as cereals and rice (Jay and others 2005). In fact, Shehata and Collins (1972) found that as much as 36% of raw milk samples in one study contained psychrotrophic *Bacillus* spp. *Bacillus* spp. can be found in both the spore and vegetative form in raw and high-temperature-short-time (HTST) pasteurized milk. Bacterial spores also contain a polar charge on the exterior of the spore coat (Singleton 2004). This is potentially important because spores will attach to the fat cells rather than the free water of the milk, which could cause insulation or shielding by the fat. It has been hypothesized that *Bacillus* spp. are the cause of sweet curdling in milk and other dairy products (Larsen and Jorgensen 1999). Studies have also shown that *B. cereus* has the potential to produce toxin in milk at 4 °C during a two-week shelf life test (Larsen and Jorgensen 1999; Christiansson and others 1989). Christiansson and others (1989) reported that 28% of *B. cereus* naturally found in milk could grow and produce toxin in skim milk after 7 days at 8 °C. However, no naturally occurring foodborne outbreaks have been reported in milk due to *B. cereus*. A risk assessment performed by Wein and Liu (2005) determined that a spore-forming pathogenic microorganism, *Clostridium botulinum*, could have devastating effects on the U.S. dairy supply and public health of consumers if the microorganism or toxin were to be intentionally introduced into the dairy supply.

The U.S. federal government has highly prioritized the defense of the food supply. Following the attacks of September 11, 2001, the Public Health Security and Bioterrorism Preparedness and Response Act was implemented by the Food and Drug Administration to grant FDA more powers to protect the food supply (GAO 1999; Strongin 2002). In 2004, President George W. Bush signed Homeland Security Presidential Directive 9, which established "a national policy to defend the agriculture and food system against terrorist attacks" (OPS 2004).

In 2004, the National Center for Food Protection and Defense (NCFPD) was created at the University of Minnesota as a Center of Excellence under the Department of Homeland Security. The NCFPD divides food defense research into eight areas, one of which is detection and diagnostics (NCFPD 2006b). Detection is important in the event of a bioterrorist attack for several reasons. First, in order to treat those who become ill, the microorganism must be detected and typed for correct treatment to be administered. Second, the microorganism must also be detected and typed from its original agricultural or food source so that companies may initiate recalls and decontaminate and dispose of the food appropriately. Third, spore-forming bacteria need more extreme measures for their destruction than other vegetative foodborne pathogens such at *Salmonella* spp. and *Listeria monocytogenes*, and spore formers may have differing resistance to antimicrobial agents and other microbial intervention strategies (Jay and others 2005).

The Food and Drug Administration's (FDA) Bacteriological Analytical Manual (BAM) (FDA 2000) sets specific recommendations that describe procedures and media needed for detection, enumeration, and determination of various bacteria in foods. The BAM recommends two different methods for detection and enumeration of *Bacillus* spp.: 1) standard plating method using mannitol-egg yolk-polymyxin (MYP) agar, a selective medium and 2) most probable

number (MPN) method using tryptic soy broth supplemented with 0.1% polymyxin sulfate (TSB-P), an enrichment medium. However, FDA does not specifically recommend either of the methods over the other for detection and enumeration of *Bacillus* spp. for any specific foods matrixes. Thus, either method for the detection and enumeration of *B. cereus* could be used.

To determine the ability of a microorganism to grow or survive during food processing and storage, an inoculum of a specific organism is introduced into food. Preparation of a spore inoculum can be achieved through several different methods. The lawn method, which utilizes colonies on agar to produce spores, has been used by several researchers (Black and others 2008; Bowen and Turnbull 1992; Johnson and others 1982). Other scientists (Byrne and others 2006; Montville and others 2005) utilize broth solutions such as brain heart infusion broth or tryptic soy broth for the growth of vegetative cells and then cause sporulation by shocking the bacteria with chemicals, extreme temperatures, or by pH change.

Recently, Microbiologics® (Saint Cloud, Minn.) released a new product of lyophilized spore pellets called EZ-Spores™, which contain 4.6 log CFU of *B. cereus* per pellet. The company markets these pellets by stating that the "EZ-Spore™ preparations provide a guaranteed concentration of 4.6 log CFU per pellet" of spores (Microbiologics 2008). Preliminary studies were performed 1) to ascertain the ability of the FDA recommended media (MYP and MPN) to detect spores in ultra-high temperature (UHT) pasteurized milk and 2) to determine the best method for producing a spore inoculum with EZ-Spores™ using different media and heating methods.

Results of the first preliminary study showed that the MPN method had greater ability ($p<0.001$) than the MYP method at detecting *B. cereus* spores and vegetative cells in UHT skim and 2% milk. Results from a second study showed that dissolving EZ-Spore™ pellets in

deionized (DI) water consistently resulted in an inoculum containing only spores. EZ-Spore™ pellets dissolved in DI water and then grown in brain heart infusion broth supplemented with manganese sulfate heptahydrate (BHI + $MnSO_4 \cdot 7H_2O$) or twice the recommended brain heart infusion broth supplemented with manganese sulfate heptahydrate (2xBHI + $MnSO_4 \cdot 7H_2O$) and heated (to cause sporulation) to 60 °C for 12 min contained *B. cereus* populations of 2.6 log CFU/ml and 3.1 log CFU/mL, respectively, by the MPN method. EZ-Spore™ pellets that were only dissolved in DI water had similar populations of 3.0 log CFU/mL by MPN method. However, staining with malachite green confirmed that inoculum grown in BHI + $MnSO_4 \cdot 7H_2O$ and 2xBHI + $MnSO_4 \cdot 7H_2O$ and heated to 60 °C for 12 min contained >40% spores while staining the EZ-Spore™ pellets that were only dissolved in DI water showed 100% spores.

While our research demonstrated the ability of EZ-Spore™ pellets dissolved in sterile DI water to produce an inoculum that contains only spores and that both MYP and MPN media were able to detect and enumerate *B. cereus* in UHT skim and 2% milk, limited research has focused on the potential use of EZ-Spore™ pellets as an inoculum for raw and pasteurized milk. It has been determined that *B. cereus* can survive the pasteurization process used for milk (Hanson and others 2005).

Therefore, the objective of this study was to compare the results of the MYP or MPN methods for detection and enumeration of *B. cereus* in raw and high-temperature-short-time (HTST) pasteurized skim, 2%, and whole milk. A secondary objective was to determine the ability of *B. cereus* to maintain similar populations at refrigeration temperatures (4 °C) in raw and HTST pasteurized milk over a 96 h storage time. The different types of milk were used to determine if a significant difference in *B. cereus* populations was observed in the different media due to background microflora or varying levels of fat.

2. Materials and Methods

2.1 Experimental Design

Nine treatments included: raw milk (inoculated with *B. cereus* EZ-Spores™ pellets (ATCC 11778) and non-inoculated); HTST pasteurized skim, 2%, and whole milk (inoculated with *B. cereus* EZ-Spores™ pellets and non-inoculated); and TSB (Difco, Detroit, Mich.) inoculated with *B. cereus* EZ-Spores™ pellets. The TSB was added to simulate a nutrient rich system so that spores can germinate and the vegetative form can grow. Raw milk samples were obtained from the Kansas State University dairy farm while HTST pasteurized samples were purchased from the Kansas State University processing facility at Call Hall. The raw samples were collected immediately prior to processing by the dairy plant personnel while all HTST milk samples were collected directly after processing. All milk samples were immediately stored at 4 °C after they were obtained. Every milk sample was enumerated in duplicate for each replication. Three replications of each experiment were completed.

2.2 Preparation of Spore Inoculum

The EZ-Spore™ pellets (Microbiologics, St. Cloud, Minn.) were prepared according to instructions from the manufacturer (Microbiologics 2008). One pellet was aseptically removed from the package and transferred to a sterile test tube containing 5 mL of sterile deionized (DI) water heated to 35 °C. The pellets were vortexed for 5 s every 10 min over a 30 min period to allow the lyophilized pellet to dissolve. Following this, the final inoculum for each of the milk samples was prepared by combining two of the dissolved pellet solutions together into a sterile test tube for a combined total of 10 mL of inoculum. Following inoculation, 1 mL of inoculum was serially diluted and enumerated using the spread plate technique (0.1 mL per plate) on

mannitol-egg yolk-polymyxin (MYP) agar (Difco, Detroit, Mich.). It was also enumerated using the most probable number (MPN) method using TSB-P (Difco, Detroit, Mich.).

2.3 Milk Sample Preparation

Raw or HTST milk (100 mL) was aseptically transferred from a half-gallon (1.89 L) jug to a sterile 250 mL (Pyrex, Lowell, Mass.) round bottom boiling flask containing an x-shaped stir bar (VWR International, West Chester, Pa.). Milk was then inoculated with 5 mL of *B. cereus* inoculum and placed in a refrigerator (4 °C) for 15 min to allow for milk to equilibrate before the first sample was taken. The milk was refrigerated at 4 °C throughout the process except for the times of sampling.

2.4 Sampling and Enumeration

All milk treatments and the TSB treatment were removed from the refrigerator and the flasks were placed on a stir plate for 30 s to allow for even distribution of spores and other constituents prior to sampling. Two 1 mL samples were removed and serially diluted four times (to the 10^{-4} level) in a 0.1% peptone solution for enumeration by MYP and three 1 mL samples were removed and serially diluted four times for the three tube MPN method. The boiling flasks were returned to the refrigerator until the next sampling time. Each test tube was vortexed for 3-4 s on high speed before being transferred to the next dilution. Samples were plated (1 mL per Petrifilm™) on APC Petrifilm™ at four different dilution levels with duplicate plates for each dilution.

Samples were stored at 4 ˚C for 96 h for each of the five milk samples and enumerated at 0, 48, and 96 h (times represent time after inoculation) to simulate the short holding period of milk from producers. APC Petrifilm™ (3M, St. Paul, Minn.) was incubated at 35 °C for 24 h

while the MYP plates were stored at 35 °C for 48 h and the MPN tubes were incubated at 35 °C for 72 h (Beuchat et al. 1998; FDA 2006).

2.5 Enumeration and Confirmation of Bacteria

Three different types of media were used for this study. Aerobic plate count (APC) Petrifilm™ was used for the enumeration of all aerobic mesophilic microorganisms in milk based on the recommendation by the "Standard Methods for the Examination of Dairy Products" (Wehr and Frank 2004). A direct plating method using MYP agar and a three tube MPN method was used for enumerating *B. cereus* in milk samples (FDA 2006).

Populations for the MYP method were determined by counting all typical *B. cereus* colonies (pink with crater morphology). All varying types of colonies on MYP were then confirmed using BBL Gram-Positive crystal (BD, Franklin Lakes, N.J.), which contains 29 biochemical tests for Gram-positive bacteria in a miniaturized kit. BBL Gram-Positive crystals™ were inoculated per manufacturer instructions and incubated for 24 h at 35 °C (BD 2009).

The confirmation of bacteria in the MPN tubes was performed by spreading 0.5 mL of the MPN diluent onto MYP. The MYP was then incubated for 24 h at 35 °C and then all colonies of different morphology that grow on the MYP plates were transferred to the BBL Gram-Positive crystal™ for confirmation. BBL Gram-Positive Crystals™ were inoculated per manufacturer instructions and incubated for 24 h at 35 °C (BD 2009). Enumeration of the MPN tubes for *B. cereus* populations was determined by using the 3-tube MPN chart (FDA 2006). Only the tubes confirmed to have *B. cereus* were used for enumeration. Confirmation was performed using the BBL Gram-Positive Crystals™ ID book, which can confirm over 1,000 Gram positive taxa.

2.7 Statistical Analysis

This experiment was a split plot with a half block design. Data were analyzed using PROC MIXED in SAS Version 9.0 (SAS Institute, Cary, N.C.). Levels of significance were not determined for sampling times, but were found for media versus milk. Therefore, the data for all sampling times was pooled together and the fixed effects for statistical analysis were media and milk. The random effect for this experiment was time. Differences ($p < 0.05$) were tested for sampling times by media type and media type by milk type using LS Means in SAS Version 9.0. Due to inconsistency in one replication for the APC Petrifilm counts, only data from two replications were analyzed.

3. Results and Discussion

Combined *B. cereus* EZ-Spore™ populations for the initial inoculum were 3.49 and 4.13 log CFU/mL by MPN and MYP standard plating methods, respectively, when simply suspended in sterile DI water. While the MYP plating method enumerated more *B. cereus* from the inoculum than the MPN method, no difference ($p>0.05$) was detected.

For both inoculated and non-inoculated milk treatments and TSB treatments, no differences ($p>0.05$) were observed in *B. cereus* populations among all sampling times (0, 48, and 96 h). Therefore, data at 0, 48, and 96 h for each treatment were pooled to eliminate time as a variable. Christiansson and others (1989) reported that 28% of *B. cereus* naturally found in milk could grow and produce toxin in skim milk after 7 d at 8 °C. However, our study held the milk at 4 °C, which could be the reason for the lack of growth over the 96 h (5 d) storage time. The USDA Pathogen Modeling Program has shown that it would take *B. cereus* (in vegetative form) 360.9 h (approximately 15 d) to increase by three logs at 5 °C (USDA 2006).

Bacillus cereus populations in inoculated milk treatments ranged from 3.15 to 2.37 log CFU/mL using the MYP plating method while the non-inoculated treatments ranged from 1.16 to 0.15 log CFU/mL for the same enumeration method (Figure 1). *B. cereus* populations for the three tube MPN method, inoculated and non-inoculated treatments ranged from 3.43 to 1.20 log CFU/mL and 3.48 to 1.71 log CFU/mL, respectively. As expected, means were slightly greater for the MPN method on both inoculated and non-inoculated milk treatments compared to means for the MYP plating method. However, the inoculated TSB results showed that the MYP plating method enumerated greater populations than the MPN method, which is not what would be expected. The only difference ($p<0.05$) found due to fat content was between inoculated whole and inoculated skim milk using the MPN method. The inoculated whole milk treatment averaged 3.40 log CFU/mL while inoculated skim milk averaged 2.76 log CFU/mL of *B. cereus* for the combined sampling times. However, no research has reported that milk with higher fat content has a greater *B. cereus* population than low fat milk or that fat concentration has any effect on recovery rate of spores or vegetative cells (Black and others 2008; Novak and others 2005). Singleton (2004) reported that bacterial spores contain polar charge on the exterior that which could lead to attachment to hydrophobic substances (milk fat). This effect was not observed in our research.

B. cereus mean populations ranged from 3.11 to 2.72 log CFU/mL for inoculated treatments during the 96 h of refrigerated storage using the MYP plating method while *B. cereus* mean populations ranged from 2.91 to 2.72 log CFU/ml for inoculated treatments using the MPN enumeration method (Table 6 and Table 7). In contrast, mean *B. cereus* populations from non-inoculated treatments ranged from 0.87 to 0.67 log CFU/mL and 2.83 to 2.21 log CFU/mL during the 96 h storage time for the MYP and MPN methods, respectively (Tables 6 and 7).

These results are expected because the inoculated treatments should have greater populations of *B. cereus* than the non-inoculated treatments. A study by Shehata and Collins (1972) found that 36% of raw milk samples contained psychrotrophic *Bacillus* spp.

The overall mean *B. cereus* population for the three-tube MPN method (2.59 log CFU/mL) for all milk treatments was greater ($p<0.05$) than the MYP plating method (1.89 log CFU/mL). The MPN method is more of an enrichment procedure since it is involves the use of TSB (a typical enrichment medium). The MPN tubes contain TSB, a medium typically used to grow pure cultures and polymyxin sulfate is used as a selective agent for *Bacillu*s spp. In contrast, the MYP plates do not contain additional ingredients that would provide nutrient for recovery or enrichment, as is evident by TSB's use to grow an inoculum (Benkerroum and others 2004). Therefore, the enrichment medium in MPN may have allowed for additional recovery and enumeration of *B. cereu*s in milk.

This significant difference may also be due to the fact that one can count *B. cereus* colonies specifically on MYP but cannot differentiate in the MPN tubes without the aid of confirmation testing since the MPN tubes are enumerated based on presence or absence of turbidity. However, if the MPN method is performed correctly in conjunction with confirmation methods, then the MPN method results in greater counts. Thus, the MPN method should be the preferred method for validation research involving HTST milk.

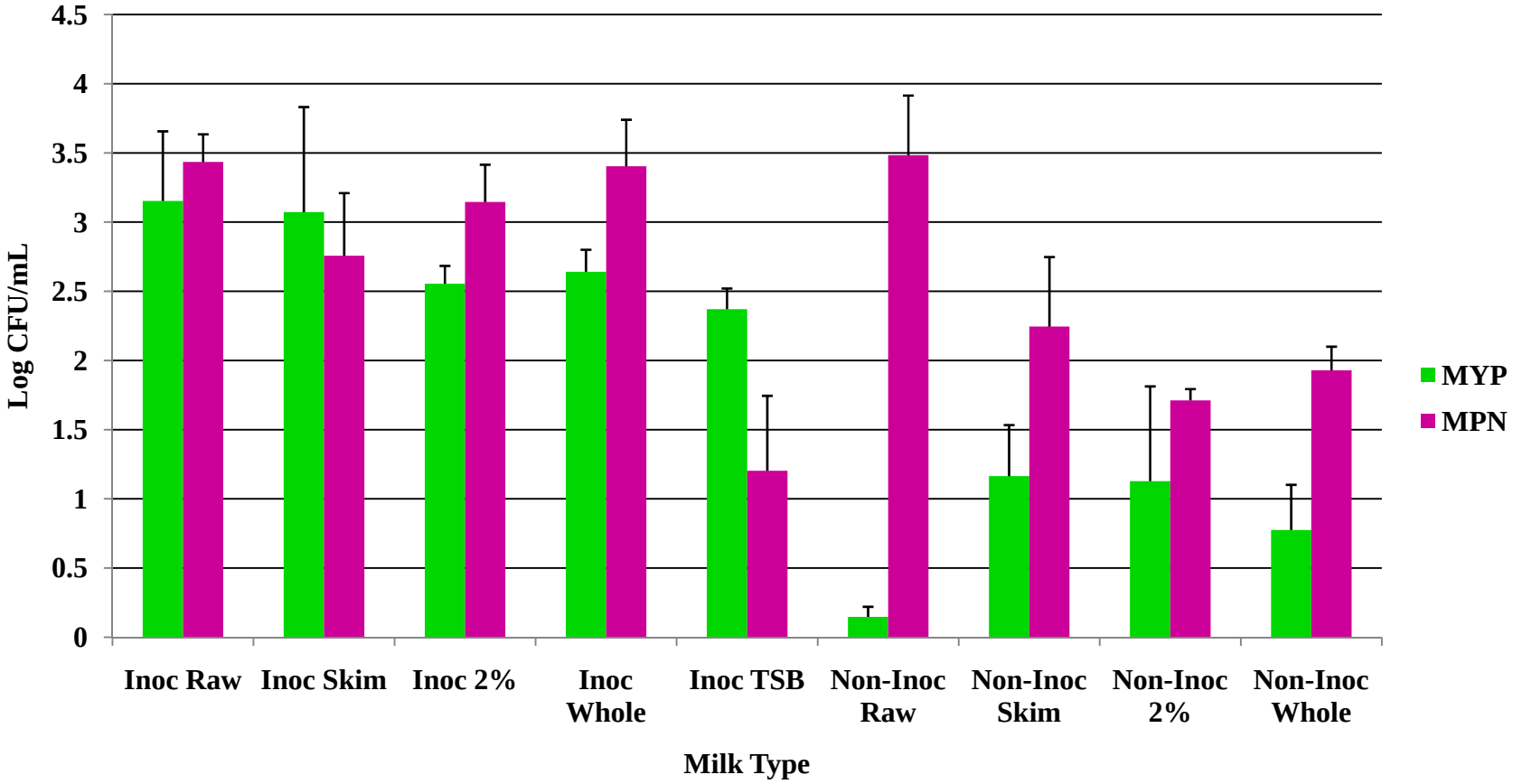
All MPN tubes that were used for enumeration in this study tested positive for *B. cereus* during confirmation. Other microorganisms confirmed with BBL Gram positive crystals and found on MYP included *Streptococcus agalactiae*, *S. salivarius*, *S. uberis, S. pyrogenes, S. avium, Enterococcus faecalis, E. faecium, B. subtilis,* and *Oerskovia* spp. However, all of these colonies were distinguishable from *B. cereus* on MYP and were not enumerated for *B. cereus*

populations. When confirming turbid MPN tubes by BBL Gram positive crystals, *B. cereus* was detected in addition to *B. subtilis*, *B. megaterium*, and *S. agalactiae*.

For both inoculated and non-inoculated milk treatments and TSB treatments, no differences ($p>0.05$) were observed in total aerobic populations among all sampling times (0, 48, and 96 h). Therefore, data at 0, 48, and 96 h for each treatment were pooled to eliminate time as a variable (Figure 2). This is probably due to microorganisms' inability to rapidly grow at refrigeration temperatures (4 °C). Also, the 96 h may not be enough time for the bacteria to show significant growth, especially since the shelf life of HTST milk is about two weeks. The 96 h period was used to simulate the time after pasteurization, as well as shipping time and time at the store. Other studies have reported that milk needs to be held for 7 to 9 d for significant growth to occur (Schaffner and others 2003; Carey and others 2005).

Total aerobic populations for inoculated milk sample populations were between 5.02 to 2.40 log CFU/mL, while non-inoculated milk populations ranged from 4.24 and 1.21 log CFU/mL during storage (pooled data) (Table 8). No difference ($p>0.05$) was observed due to fat level in the different milk treatments. Bacterial populations in Class 1 raw milk may be as high as 5 log CFU/mL before commingling and 5.4 log CFU/mL after commingling and must be below 20,000 CFU/mL following pasteurization (FDA 2005b). The populations from this study show that this milk (non-inoculated) meets this criteria.

Figure 1. Mean[a] and standard error[b] of *Bacillus cereus* populations (log CFU/mL) from mannitol-egg yolk-polymyxin (MYP) plating and three-tube most probable number (MPN) methods for inoculated[c] and non-inoculated raw and high-temperature-short-time pasteurized milk of various fat contents as well as tryptic soy broth (TSB) (n=18).



[a]Mean populations at 0, 48, and 96 h for each treatment were pooled due to no significance ($p>0.05$) among sampling times.
[b]Standard error at 0, 48, and 96 h for each treatment were pooled due to no significance ($p>0.05$) among sampling times.
[c]Inoculated with dissolved EZ-Spore™ pellets. Initial inoculum 3.49 and 4.13 log CFU/mL by MPN and MYP standard plating methods, respectively.

Table 6. Mean and standard deviations of *Bacillus cereus* populations (log CFU/mL) in varying mediums during 96 h of storage at 4 °C and enumerated on mannitol-egg yolk-polymyxin agar (n=6).

Sampling Time	Inoculated Milk Samples				Growth Medium	Non-Inoculated Milk Samples			
	Raw	Skim Milk	2% Fat Milk	Whole Milk	Tryptic soy broth	Raw	Skim Milk	2% Fat Milk	Whole Milk
0 h	4.20±1.14[a]	3.40±0.86[a]	2.68±0.17[a]	2.67±0.26[a]	2.60±0.17[a]	0.10±0.00[a]	1.07±0.59[a]	1.64±1.47[ab]	0.78±0.42[a]
48 h	2.49±0.13[b]	2.36±0.21[a]	2.52±0.10[a]	2.46±0.07[a]	2.39±0.08[ab]	0.03±0.04[a]	1.33±0.18[a]	0.97±0.35[b]	0.67±0.29[a]
96 h	2.78±0.25[b]	3.46±1.21[a]	2.47±0.12[a]	2.80±0.15[a]	2.12±0.20[b]	0.19±0.18[a]	1.07±0.34[a]	2.80±0.24[a]	0.87±0.27[a]

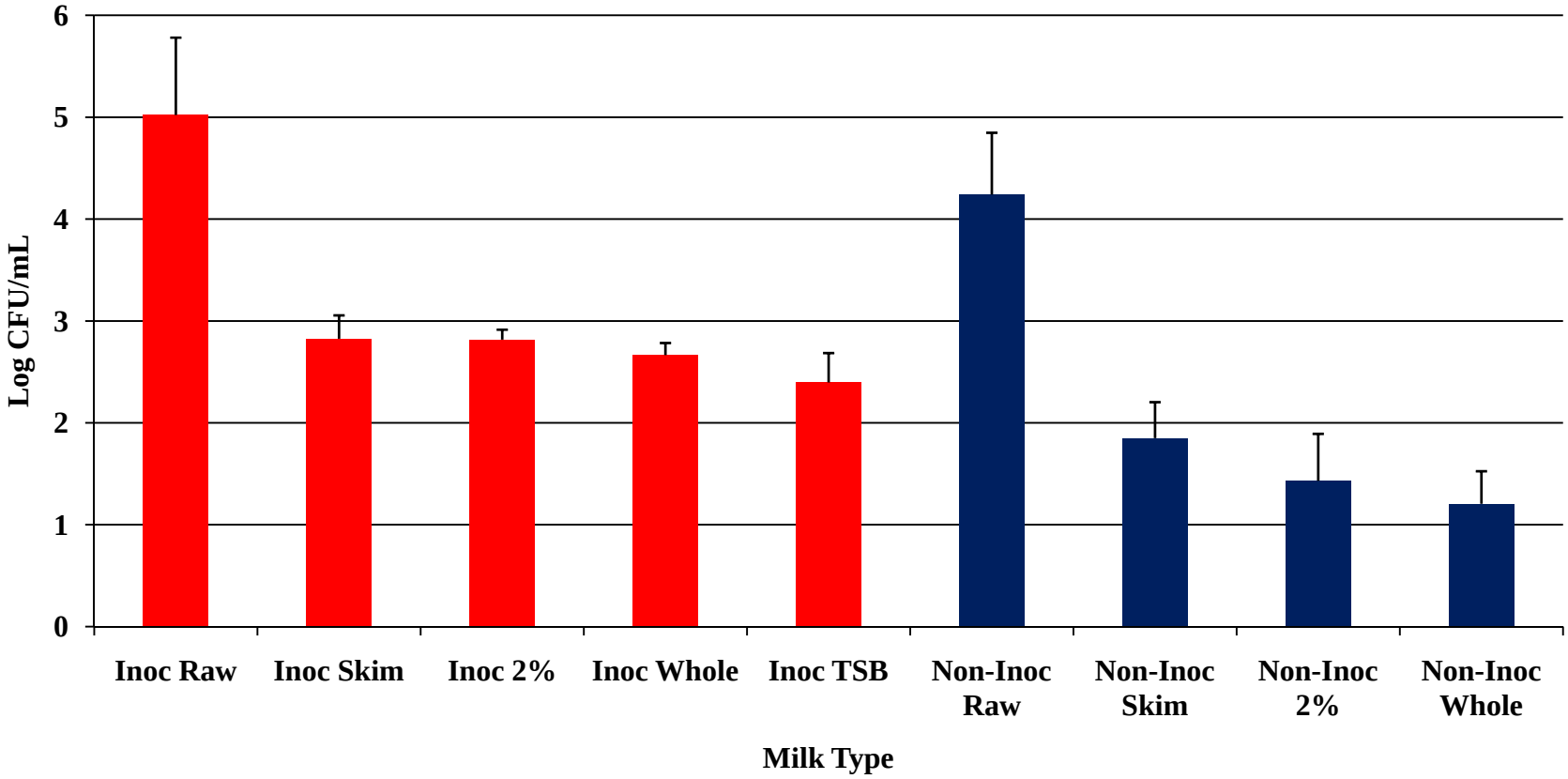
[ab]Means with similar superscripts within a column are not significantly different (p>0.05).

Table 7. **Mean and standard deviations *Bacillus cereus* populations (log CFU/mL) in varying mediums during 96 h of storage at 4 °C and enumerated with the most probable number method (n=6).**

Sampling Time	Inoculated Milk Samples				Growth Medium	Non- Inoculated Milk Samples			
	Raw	Skim Milk	2% Fat Milk	Whole Milk	Tryptic soy broth	Raw	Skim Milk	2% Fat Milk	Whole Milk
0 h	3.36±0.02[a]	2.66±0.63[a]	3.05±0.41[a]	3.48±0.36[a]	1.52±0.04[a]	3.83±0.28[a]	2.31±0.22[a]	2.66±0.00[a]	2.52±0.14[a]
48 h	3.36±0.22[a]	2.80±0.37[a]	3.14±0.15[a]	3.14±0.29[a]	1.17±0.73[a]	3.27±0.59[a]	1.89±0.45[a]	1.76±0.21[b]	1.90±0.22[b]
96 h	3.58±0.36[a]	2.81±0.36[a]	3.25±0.25[a]	3.58±0.36[a]	1.33±0.85[a]	3.35±0.42[a]	2.53±0.84[a]	1.59±0.04[b]	1.33±0.15[b]

[ab]Means with similar superscripts within a column are not significantly different (p>0.05).

Figure 2. Mean[a] and standard error[b] aerobic plate count populations (log CFU/mL) on APC Petrifilm™ for inoculated[c] and non-inoculated raw and high-temperature-short-time pasteurized milk of various fat contents as well as tryptic soy broth (TSB) (n=12).



[a]Mean populations at 0, 48, and 96 h for each treatment were pooled due to no significance ($p>0.05$) among sampling times.
[b]Standard error at 0, 48, and 96 h for each treatment were pooled due to no significance ($p>0.05$) among sampling times.
[c]Inoculated with dissolved EZ-Spore™ pellets. Initial inoculum 3.49 and 4.13 log CFU/mL by MPN and MYP standard plating methods, respectively.

Table 8. Mean and standard deviations aerobic plate count populations (log CFU/mL) in varying mediums during 96 h of storage at 4 °C and enumerated with the APC Petrifilm™ method (n=4).

Sampling Time	Inoculated Milk Samples				Growth Medium	Non- Inoculated Milk Samples			
	Raw	Skim Milk	2% Fat Milk	Whole Milk	Tryptic soy broth	Raw	Skim Milk	2% Fat Milk	Whole Milk
0 h	5.45±1.55[a]	2.74±0.09[a]	2.76±0.03[a]	2.49±0.31[a]	2.82±0.02[a]	4.90±1.00[a]	1.68±0.21[a]	1.77±0.34[a]	1.12±0.33[a]
48 h	3.70±0.03[a]	2.94±0.20[a]	2.80±0.10[a]	2.72±0.02[a]	2.10±0.55[a]	4.18±0.41[a]	1.92±0.40[a]	1.32±0.57[a]	1.00±0.15[a]
96 h	4.58±0.70[a]	3.03±0.40[a]	2.88±0.17[a]	2.79±0.02[a]	2.27±0.29[a]	4.39±0.41[a]	1.96±0.44[a]	1.70±0.47[a]	1.49±0.48[a]

[ab]Means with similar superscripts within a column are not significantly different ($p>0.05$).

4. Conclusions

Results from previous research show that the EZ-Spore™ pellets dissolved in deionized water can provide a *B. cereus* spore inoculum for validation research that would be at a reasonable log CFU/mL level consisting of spores. However, results from this study demonstrate that further research is needed to determine if this strain is a representative strain that can be used for psychrotrophic *B. cereus*. The EZ-Spore™ pellets were useful in the preparation of a spore inoculum for inoculation into a milk matrix. They produce an inoculum that could allow for detection of bacterial growth as well as reduction, and all cells were spores.

Neither MYP plating nor three-tube MPN method were selective for *B. cereus* alone. Both methods allowed for growth of other types of bacteria. However, both methods can still differentiate other species of *Bacillus* as well as other microorganisms in conjunction with proper confirmation methods.

While both media are expensive, the MYP method is more practical for using in the food industry than MPN for several reasons. The first is that it is less labor intensive and the standard plating method is typically used in the food industry. It also requires less time for microbiological testing because when combined with confirmation testing, the MYP method requires two days while the MPN method requires five days. By the time confirmation is obtained using the MPN method (approximately 5 d), the milk will have already been distributed to consumers and perhaps consumed.

However, populations using MPN were statistically greater than MYP for raw and HTST milk. The enrichment properties in MPN allow for recovery and detection of injured cells, which are extremely important to enumerate when conducting validation research. So for validation

research, the MPN method should be used instead of the MYP method due to its greater population recovery.

As expected, the APC Petrifilm™ results showed that this recommended method (Wehr and Frank 2004) does not allow for detection of *B. cereus* spores. The APC Petrifilm™ method would not aid in detection of *B. cereus* and it could still pass through standards set by the PMO (FDA 2005b). *B. cereus* can also survive the pasteurization process and low levels would be allowed into the food supply (Hanson and others 2005).

CHAPTER 6 - CONCLUSIONS

Research performed on ultra-high temperature (UHT) pasteurized, raw, and high-temperature-short-time (HTST) pasteurized milk all showed that the most probable number (MPN) method enumerated greater ($p<0.05$) populations of *Bacillus cereus* in milk than the mannitol-egg yolk-polymyxin (MYP) agar method. However, culture confirmation is critical to correctly determine populations for both methods.

For inoculated and uninoculated milk treatments and TSB treatments, no differences ($p>0.05$) were observed in *B. cereus* populations among all sampling times (0, 48, and 96 h) at 4 °C. In addition, no differences were found for total aerobic plate populations on the APC Petrifilm™ for the same treatments among all sampling times (0, 48, and 96 h) at 4 °C.

EZ-Spore™ pellets did consistently provide the inoculum population that was expected (approximately 3.0 log CFU/mL). Our research has shown that EZ-Spore™ pellets do produce a consistent inoculum level and that the inoculum is composed of 100% spores. However, further research is needed to determine if this strain is a good representative strain for other *B. cereus* strains with regards to heat-resistance and ability to survive or even grow at refrigerated temperatures (4 °C).

The MYP method should be used by the dairy industry for *B. cereus* testing. The MYP method is less labor intensive and is a standard plating method that is typically used in the food industry. It also requires less time for microbiological testing because when combined with confirmation testing, the MYP method requires two days while the MPN method requires five days. By the time that the enumeration and confirmation is completed using the MPN method, milk will have already been distributed to consumers and partially consumed.

However, *B. cereus* populations in inoculated UHT skim and 2%, raw, and HTST skim, 2%, and whole milk using MPN for enumeration were greater ($p<0.05$) than MYP. The enrichment properties in MPN allow for recovery and detection of potentially injured cells, which are extremely important to enumerate. Therefore, when conducting validation research the MPN method should be used instead of the MYP method due to its greater population recovery.

CHAPTER 7 - REFERENCES

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APPENDIX A

Below is the SAS statistical analysis program (SAS Version 9.0, SAS Institute, Inc., Cary, NC) and data that was used to determine differences among milk and TSB treatments APC counts on APC Petrifilm. The first column is a code where the number represents the replication. The second column represents the time of the sample (0=0 h, 48=48 h, and 96=96 h). The third column represents the milk and TSB treatment (R=Inoculated Raw milk, NR=Non-inoculated Raw milk, 0=Inoculated skim milk, N0=Non-inoculated skim milk, 2=Inoculated 2% milk, N2%=Non-inoculated 2% milk, 4=Inoculated whole milk, N4=Non-inoculated whole milk, and TSB=Tryptic soy broth). The fourth column represents the medium (APC=APC Petrifilm). The fifth column represents the log CFU/mL average count of the duplicate Petrifilm. The period represents missing data.

SAS Statistical Analysis Program

```
Data labels;
input Sample Time Milk $ Media $ logcfu;
datalines;
2    0    R    APC  .
3    0    R    APC  6.999565488
2    0    NR   APC  3.829303773
3    0    NR   APC  .
2    0    0    APC  2.86923172
3    0    0    APC  2.619093331
2    0    N0   APC  1.602059991
3    0    N0   APC  1.439332694
2    0    2    APC  2.781755375
3    0    2    APC  2.792391689
2    0    N2   APC  1.322219295
3    0    N2   APC  1.703291378
2    0    4    APC  2.799340549
3    0    4    APC  1.995635195
2    0    N4   APC  0.602059991
3    0    N4   APC  1.311753861
2    0    T    APC  .
3    0    T    APC  2.841046465
2    48   R    APC  3.67669361
3    48   R    APC  3.73239376
2    48   NR   APC  3.767155866
3    48   NR   APC  3.926856709
2    48   0    APC  2.806179974
```

```
3	48	0	APC	2.744292983
2	48	N0	APC	2.51851394
3	48	N0	APC	1.380211242
2	48	2	APC	2.703291378
3	48	2	APC	2.959041392
2	48	N2	APC	1.352182518
3	48	N2	APC	2.11058971
2	48	4	APC	2.703291378
3	48	4	APC	2.698970004
2	48	N4	APC	0.77815125
3	48	N4	APC	1.021189299
2	48	T	APC	.
3	48	T	APC	1.550228353
2	96	R	APC	3.880813592
3	96	R	APC	5.276461804
2	96	NR	APC	3.73239376
3	96	NR	APC	4.638489257
2	96	0	APC	2.658011397
3	96	0	APC	2.748188027
2	96	N0	APC	2.40654018
3	96	N0	APC	1.51851394
2	96	2	APC	2.694605199
3	96	2	APC	3.146128036
2	96	N2	APC	1.230448921
3	96	N2	APC	2.16879202
2	96	4	APC	2.792391689
3	96	4	APC	2.748188027
2	96	N4	APC	0.77815125
3	96	N4	APC	2.139879086
2	96	T	APC	.
3	96	T	APC	1.973127854
;
run;
proc glm data=Labels;
class Sample Time Milk Media;
model logcfu=Milk;
run;
proc mixed data=Labels;
class Sample Time Milk Media;
model logcfu=Milk;
lsmeans Milk/pdiff;
run;

Data labels;
input Sample Time Milk $ Media $ logcfu;
datalines;
```

APPENDIX B

Below is the SAS statistical analysis program (SAS Version 9.0, SAS Institute, Inc., Cary, NC) and data that was used to determine differences among milk and TSB treatments *B. cereus* populations. The first column is a code where the number represents the replication. The second column represents the time of the sample (0=0 h, 48=48 h, and 96=96 h). The third column represents the milk and TSB treatment (R=Inoculated Raw milk, NR=Non-inoculated Raw milk, 0=Inoculated skim milk, N0=Non-inoculated skim milk, 2=Inoculated 2% milk, N2%=Non-inoculated 2% milk, 4=Inoculated whole milk, N4=Non-inoculated whole milk, and TSB=Tryptic soy broth). The fourth column represents the medium (MYP=Mannitol-egg yolk-polymyxin agar standard plating method and MPN=Three tube most probable number method). The fifth column represents the log CFU/mL average count of the duplicate MYP plates or MPN tubes. Periods represent missing data.

SAS Statistical Analysis Program

```
Data labels;
input Sample Time Milk $ Media $ logcfu;
datalines;
1       0       R       MYP     .
2       0       R       MYP     3.06069784
3       0       R       MYP     5.33243846
1       0       NR      MYP     0.1
2       0       NR      MYP     0.1
3       0       NR      MYP     .
1       0       0       MYP     4.759667845
2       0       0       MYP     3.021189299
3       0       0       MYP     2.408239965
1       0       N0      MYP     1.653212514
2       0       N0      MYP     0.477121255
3       0       N0      MYP     .
1       0       2       MYP     2.462397998
2       0       2       MYP     2.929418926
3       0       2       MYP     2.638489257
1       0       N2      MYP     3.975431809
2       0       N2      MYP     0.1
3       0       N2      MYP     0.954242509
1       0       4       MYP     2.666517981
2       0       4       MYP     3.041392685
3       0       4       MYP     2.298853076
```

1	0	N4	MYP	1.204119983
2	0	N4	MYP	0.1
3	0	N4	MYP	1.041392685
1	0	T	MYP	2.771587481
2	0	T	MYP	.
3	0	T	MYP	2.432969291
1	48	R	MYP	.
2	48	R	MYP	2.614897216
3	48	R	MYP	2.357934847
1	48	NR	MYP	0.1
2	48	NR	MYP	0.1
3	48	NR	MYP	0.1
1	48	0	MYP	2.037426498
2	48	0	MYP	2.627365857
3	48	0	MYP	2.424881637
1	48	N0	MYP	1.612783857
2	48	N0	MYP	1.255272505
3	48	N0	MYP	1.113943352
1	48	2	MYP	2.525044807
2	48	2	MYP	2.658964843
3	48	2	MYP	2.374748346
1	48	N2	MYP	1.531478917
2	48	N2	MYP	0.77815125
3	48	N2	MYP	0.602059991
1	48	4	MYP	2.440909082
2	48	4	MYP	2.562292864
3	48	4	MYP	2.369215857
1	48	N4	MYP	1.113943352
2	48	N4	MYP	0.301029996
3	48	N4	MYP	0.602059991
1	48	T	MYP	2.465382851
2	48	T	MYP	.
3	48	T	MYP	2.309630167
1	96	R	MYP	.
2	96	R	MYP	3.021189299
3	96	R	MYP	2.530199698
1	96	NR	MYP	0.1
2	96	NR	MYP	0.1
3	96	NR	MYP	0.477121255
1	96	0	MYP	5.423245874
2	96	0	MYP	2.40654018
3	96	0	MYP	2.540329475
1	96	N0	MYP	1.556302501
2	96	N0	MYP	1.041392685
3	96	N0	MYP	0.602059991
1	96	2	MYP	2.276461804

2	96	2	MYP	2.606381365
3	96	2	MYP	2.517195898
1	96	N2	MYP	.
2	96	N2	MYP	0.301029996
3	96	N2	MYP	0.77815125
1	96	4	MYP	2.602059991
2	96	4	MYP	2.77815125
3	96	4	MYP	3.011570444
1	96	N4	MYP	1.230448921
2	96	N4	MYP	0.477121255
3	96	N4	MYP	0.903089987
1	96	T	MYP	2.324282455
2	96	T	MYP	.
3	96	T	MYP	1.919078092
1	0	R	MPN	3.380211242
2	0	R	MPN	3.322219295
3	0	R	MPN	3.380211242
1	0	NR	MPN	3.380211242
2	0	NR	MPN	4.041392685
3	0	NR	MPN	4.079181246
1	0	0	MPN	2.361727836
2	0	0	MPN	3.662757832
3	0	0	MPN	1.968482949
1	0	N0	MPN	1.963787827
2	0	N0	MPN	2.380211242
3	0	N0	MPN	2.579783597
1	0	2	MPN	3.380211242
2	0	2	MPN	2.380211242
3	0	2	MPN	3.380211242
1	0	N2	MPN	0.1
2	0	N2	MPN	2.662757832
3	0	N2	MPN	2.662757832
1	0	4	MPN	3.380211242
2	0	4	MPN	3.041392685
3	0	4	MPN	4.041392685
1	0	N4	MPN	0.1
2	0	N4	MPN	2.380211242
3	0	N4	MPN	2.662757832
1	0	T	MPN	0.1
2	0	T	MPN	1.477121255
3	0	T	MPN	1.556302501
1	48	R	MPN	3.380211242
2	48	R	MPN	3.662757832
3	48	R	MPN	3.041392685
1	48	NR	MPN	3.380211242
2	48	NR	MPN	4.041392685

3	48	NR	MPN	2.380211242
1	48	0	MPN	2.633468456
2	48	0	MPN	2.380211242
3	48	0	MPN	3.380211242
1	48	N0	MPN	2.361727836
2	48	N0	MPN	1.176091259
3	48	N0	MPN	2.146128036
1	48	2	MPN	3.380211242
2	48	2	MPN	3.041392685
3	48	2	MPN	3
1	48	N2	MPN	1.556302501
2	48	N2	MPN	1.968482949
3	48	N2	MPN	1.633468456
1	48	4	MPN	3.380211242
2	48	4	MPN	3.380211242
3	48	4	MPN	2.662757832
1	48	N4	MPN	1.556302501
2	48	N4	MPN	2.176091259
3	48	N4	MPN	1.968482949
1	48	T	MPN	1.963787827
2	48	T	MPN	0.1
3	48	T	MPN	1.544068044
1	96	R	MPN	3.662757832
2	96	R	MPN	4.041392685
3	96	R	MPN	3.041392685
1	96	NR	MPN	2.968482949
2	96	NR	MPN	4.041392685
3	96	NR	MPN	3.041392685
1	96	0	MPN	3.380211242
2	96	0	MPN	2.662757832
3	96	0	MPN	2.380211242
1	96	N0	MPN	3.380211242
2	96	N0	MPN	3.041392685
3	96	N0	MPN	1.176091259
1	96	2	MPN	3.662757832
2	96	2	MPN	3.041392685
3	96	2	MPN	3.041392685
1	96	N2	MPN	1.556302501
2	96	N2	MPN	1.633468456
3	96	N2	MPN	1.633468456
1	96	4	MPN	3.662757832
2	96	4	MPN	4.041392685
3	96	4	MPN	3.041392685
1	96	N4	MPN	1.963787827
2	96	N4	MPN	2.176091259
3	96	N4	MPN	2.380211242

```
1       96      T       MPN   2.361727836
2       96      T       MPN   0.1
3       96      T       MPN   1.62324929
;
run;
proc glm data=Labels;
class Sample Time Milk Media;
model logcfu=Milk Media;
run;
proc mixed data=Labels;
class Sample Time Milk Media;
model logcfu=Milk Media Milk*Media;
lsmeans Milk Media Milk*Media/pdiff;
run;
```