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Dedication

I wish to dedicate this thesis to my family of Food Science colleagues. It was by God's great blessing that I was able to meet them and share with them 2 of the greatest years of my life so far. I would not have been able to write this thesis, or make it through graduate school without them.

Abstract

Process cheese sauce is typically prepared by combining one or more natural cheeses of the same type or different varieties, mixing with emulsifying salts and other dairy ingredients, and thermal processing to temperatures above 90°C. The processing renders the product free of all viable organisms and is considered commercially sterile. Despite the safety of process cheese sauce during processing, its low acid and high moisture environment are conducive to support the growth of bacteria contaminated by the consumer. Spore-forming bacteria such as *Bacillus cereus* and *Clostridium perfringens* are particularly problematic because their spores state can survive temperatures above the required reheating temperature of 60°C and the sub-lethal heating could induce germination in the cheese sauce medium if the product is mishandled.

The goal of this project was to assess the survival and germination potential of spore-forming bacteria in process cheese sauce through typical temperature-abuse schemes that could happen in a food service setting. Commercial cheese sauce was inoculated with cocktails of *B. cereus* and *C. perfringens* at 10^4 CFU/g and held at temperatures of 4, 12, 23, 37, and 45°C for short-term and long-term storage. Cyclic temperature scenarios typical of commercial buffet restaurants were also evaluated, as well as the use of a common antimicrobial nisin for *B. cereus*. Three strains of each bacterium were combined and tested in each sample and bacterial counts were determined by direct plating on PEMB agar for *B. cereus* and anaerobic blood agar for *C. perfringens*.

Bacillus cereus germinated well in the cheese sauce medium. At 37°C the vegetative cell population reached 8 log CFU/g after 24 hours, but took 48 hours to reach the same

level when the antimicrobial nisin was added. At temperatures of 23°C and 45°C, vegetative cell populations reached 8 log CFU/g within 48 hours and at 12°C reached 7 log CFU/g after 1 month. When incubated at 4°C no growth was observed even after 2 months. Almost no increase in *B. cereus* spore count was observed at any temperature, except at 23°C.

The *B. cereus* inoculated cheese sauce was subjected to cyclic temperature abuse schemes that simulated a buffet restaurant schedule. One scenario depicted samples that were not properly heated (below 60°C) on the buffet line. Short incubation times at abusive temperatures did not influence germination, but after 24 hours of fluctuating temperatures vegetative cells began to grow. However, when the contaminated sample was reheated to temperatures above 60°C vegetative cells actually began to decrease.

The cheese sauce matrix did not support germination and growth of *C. perfringens* at any temperature. Even during a worst case scenario where the cheese sauce was allowed to cool from 75°C to 4°C in a 9 hour period, vegetative cell and spore counts declined below a detectable level.

In almost all experiments the vegetative cell population level of bacteria was either maintained or increased in the cheese sauce, while the spore population was maintained. If the process cheese sauce were contaminated by the consumer the potential for an outbreak may exist if left at temperatures favorable for bacterial growth.

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1.1 Process Cheese Sauce

1.1.1 History/background

Process cheese sauce is a subset of pasteurized process cheese which is defined by the United States Code of Federal Regulations (CFR) as a food made by combining one or more natural cheeses of either the same type or different varieties and mixing at a heat of at least 150°F for no less than 30 seconds. All cheese can be used in the making of process cheese except for cream cheese, neufchatel cheese, cottage cheese, low-fat cottage cheese, cottage cheese dry curd, cook cheese, hard grating cheese, semisoft part-skim cheese, part-skim spiced cheese, and skim milk cheeses. The melted cheese is combined with other dairy and non-dairy products to certain levels specified by 21CFR133.169 and to varying percentages of moisture and fat based on the final application of the process cheese (CFR 2011).

Pasteurized process cheese is broken down into three different kinds of products: pasteurized process cheese food, pasteurized process cheese spread, or pasteurized process cheese product or sauce. Categorizing the products is based off of the standards of identity set in 21CFR 133—that is by how much fat, moisture, percentage of natural cheese, and other ingredients are included in the homogenous mixture. The category for process cheese sauce is the least strict on standards and is what a product is considered when it does not fall into any of the other categories, meaning it would have <50% cheese, <20% fat, >60% moisture, or a substantial level of nondairy ingredients (Glass and Doyle 2005).

Process cheese has been suggested to have started in Europe in the mid 1890's with the intention to increase the shelf life of natural cheese. Natural cheeses vary in their shelf life but typically stay in good quality for a couple of weeks. Even when vacuum-packed and stored at refrigeration temperatures, cheeses provide a great environment for microorganisms to grow, particularly yeasts and molds. Because cheese was as popular in Europe then as it is today, there was an increased demand to assure the stability of natural cheese for a longer period of time or even to make a new style of cheese that was slightly milder in flavor. Germany was one of the first to advance this style of cheese when they worked to improve the length of time their exported soft cheeses such as Camembert, Brie, and Limburger could maintain good quality by heating them in metal cans (Tamime 2011).

Other countries worked to make cheese more shelf-stable but the most successful was in Switzerland when Walter Gerber and Fritz Stettler melted Swiss cheese with sodium citrate, or emulsifying salts, to make fondue in 1911. Once the commercialization of emulsifying salts occurred, more and more producers started making and perfecting the manufacture of process cheese. It wasn't until 1916 that process cheese was introduced to America by J. L. Kraft. In an attempt to salvage the natural cheese that did not meet the standards of identity set by the Code of Federal Regulations, or re-work cheese, Kraft combined citrates and phosphates, as the emulsifying salts, and natural cheese in a can and then thermally processed it (Kapoor and Metzger 2008). The quality of process cheese in the early 1900s was lacking until the 1930s when better emulsifying salts became more common in the market (Tamime 2011).

1.1.2 Manufacturing of Process Cheese Sauce

As stated previously, process cheese sauce is typically made from a mixture of one or more natural cheeses, emulsifying salts, milk proteins, dairy fat ingredients, colorings, preservatives, and other non-dairy ingredients (Zehren and Nusbaum 2000). A typical formulation for process cheese can be seen in Table 1.

Table 1: A typical industrial formulation for process cheese sauce adapted from Berger et al. (1989)

Ingredient	Weight (Kg)	Percentage %
Young cheddar	19.5	75
Medium cheddar	3.63	14
Milk fat	0.18	0.7
Di-sodium phosphate	0.45	1.7
Tri-sodium phosphate	0.18	0.7
Table salt	0.14	0.5
Water	1.91	7.4
Total	25.54	100

The selection and amount of ingredients is based on the final application for the cheese sauce because of the effect that different salts and varying moistures can have on the body and texture of the sauce. The factor which has the biggest effect on the overall outcome of the process cheese is its meltability most commonly measured with the Schreiber melt test. The principle ingredient, raw cheese, has the most decisive impact on the overall meltability of the final product as well as the overall appearance, taste, consistency, texture and shelf-life (Berger et al. 1989). Natural cheese, the most important ingredient and hardest variable to control, is chosen by its type and age and dictates the rest of the recipe. Re-work cheese is used as much as possible, but is

contingent on the stock a company has, which is not always readily available, and widely deviates in its percentages of fat, moisture, and other characteristics. The amount and type of other ingredients change from batch to batch based on the type and age of natural cheese used and availability of re-work cheeses, and is often controlled by an automated system. Because of the variation in the base cheese from day to day, the amounts of other ingredients are altered in order to make the most consistent product possible (Kapoor and Metzger 2008). The pH of the raw cheese is the most important variable to determine the amount of other ingredients used. Other important variables are found using analytical tests to determine the amount of casein content and above all the total amount of protein and protein structure. This information is used to determine the overall meltability of the cheese used as a raw ingredient. The higher the casein content in the natural cheese, which is usually between 90 and 95% for a typical rennet cheese aged only a couple days, the more stable the process cheese will be (Berger et al. 1989).

Another major ingredient is the emulsifying salt, which is a compound of monovalent cations and polyvalent cations. The emulsifying salt is used to break the calcium-phosphate bonds in the natural cheese and to adjust the pH of the mixture so that more calcium is available to facilitate bonding and strengthen the fat-protein and protein-protein network (Purna, Pollard and Metzger 2006). Currently there are 13 different types of emulsifying salts, approved for use in process cheese sauce, although only two are highly used, either by themselves or in conjugation (Purna et al. 2006). Trisodium citrate (TSC) is most popular in loaf-style process cheese and disodium phosphate (DSP) is used for slice applications or in spreads. While several studies have been done on the effects of

these and other emulsifying salts, the results cannot be standardized because the variability in processing conditions and raw material used play such an important role and are different for every batch of cheese. What can be determined from these studies however, is that the salts play a role in the number and size of fat globules in the mixture which effect the physiochemical properties, especially the meltability, of the final application (Purna et al. 2006). Salts also play a role as an antimicrobial ingredient. The spore-forming bacteria, that cause the biggest concern in the safety of the process cheese sauce, have shown a lack of resistance to certain organic acid salts such as propionic, lactic, pyruvic, acetic, and citric acids (Sanchez-Plata et al. 2005).

The last of the major ingredients important for cheese sauce quality are the lactose and whey content of the process cheese sauce. Non-fat dried milk and dried whey are added to the formulation to affect the sensory and physical characteristics of the cheese sauce. If too much is added, then lactose crystals are formed resulting in the availability of free reducing sugars that increase Maillard browning and thus create a different flavor profile. The lactose also promotes creaming and affects the overall flavor and consistency so its contents are kept below 4% in the final product (Berger et al. 1989). The dried whey contributes β-lactoglobulins and α-lactalbumin to the recipe that include a free sulfhydryl group in their structure. When subjected to extreme temperatures, whey proteins denature easily creating opportunities to form disulfide bonds with other β-lactoglobulins which will form a stronger matrix in the mixture decreasing its meltability and so high amounts would not be favorable for cheese sauces (Kapoor and Metzger 2008).

Most processing plants have an automated system that will vary ingredients to increase or decrease certain properties of the product. The natural cheese used will have an effect on the product's pH, total calcium content and intact casein content; emulsifying salts will affect the pH and availability of calcium; and the dairy ingredients will affect the whey and lactose levels in the product (Kapoor and Metzger 2008, Berger et al. 1989). The overall pH of the product plays a decisive role on the consistency and texture: below pH of 5.4 will be distinctively firmer, whereas a higher pH will produce a softer more spreadable texture (Berger et al. 1989, Zehren and Nusbaum 2000). The two specs that have to be consistent are that of percent moisture and percent fat, because of CFR standards of identity, but the other functional properties will vary in content. The amount and type of additional ingredients will change the pH, total calcium content, intact casein content, lactose content, and whey content (Kapoor and Metzger 2008), therefore changing the physiochemical properties and end-use applications of the process cheese sauce.

Processing conditions play a huge role in the consistency and quality of the final product, specifically the cook time and temperature. An example of a commercial process flow diagram can be seen in Figure 1.

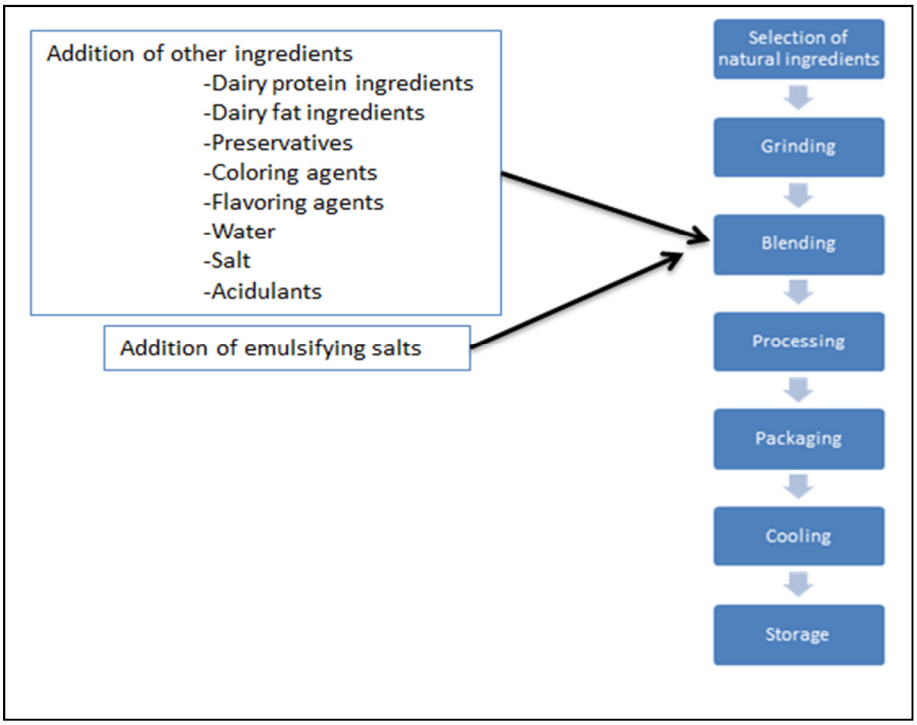

Figure 1: A commercial process flow diagram in the manufacturing of process cheese sauce adapted from Kapoor and Metzger 2008

An extensive study was performed at North Carolina State University that evaluated different processing conditions and their effect on the final meltability of the process cheese (Glenn et al. 2003). A processing scenario that included three different cook temperatures, three mixing rates, and six different cook times was used and evaluated by the Schreiber melt test. Mechanical history, thermal history, and strain history were other variables considered during the evaluation of the melt quality of the cheese tested. That study's conclusions were that thermal (time-temperature effect) and strain (time-shear effect) histories are of the most important variables to consider for the quality of the final product (Glenn et al. 2003). Even though these two values by themselves cannot be used to calculate the final melt, certain correlations were observed. In addition to thermal and

strain histories, it is also noted that as the cook time, cook temperature, and mixing time were increasing during the make, the meltability of the cheese decreased (Kapoor and Metzger 2008).

1.1.3 Relevance of Process Cheese Sauce

Process cheese has been growing in popularity in the United States ever since Kraft introduced it in the 1900s because of its price, convenience, and usefulness. It is a very versatile food product because of the different textures and styles in which it is marketed. According to the International Dairy Federation (IDF) from the period from 1995 to 2004 the United States produced more process cheese (between 543,000 to 668,000 tons annually) than any of the 30 countries who are IDF members (Tamime 2011). It can be as a slice, loaf, sauce, shredded, cubed, or spread form. The scope of the present thesis will only include the sauce form. According to Kapoor and Metzger (2008) the usage of process cheese sauce is only around 0.1% compared to the popularity of the sliced form which is around 74.0% Process cheese sauce is usually made as a lower cost option or as a substitute for vegetarians because it contains so little natural cheese. Process cheese sauce is used as a complement to a variety of other foods including chips, vegetables, and pasta dishes. It is also a target of many food trends in the U.S. including low-fat and low-sodium formulations.

1.2 Safety Concerns for Process Cheese Sauce

1.2.1 Microbial Growth

By definition process cheese sauce is a low acid canned food (LACF) because it has a pH of greater than 4.5 and a water activity ($a_{w)}$ of greater than 0.85. Because it is packaged in a hermetically sealed metal can and is thermally processed to temperatures of approximately 121°C for two and a half to three minutes, process cheese sauce is considered commercially sterile (Glass and Doyle 2005). For these reasons, process cheese sauce is not considered a high risk food product, but because the spores of bacteria such as *Clostridium botulinum*, *Clostridium perfringens*, and *Bacillus cereus* can survive the high temperatures of thermal processing, there is still potential for foodborne illness. Group I *C. botulinum* toxins are inhibited at water activity (a_w) values of less than 0.94 and group II when the a_w is below 0.97. Other spore forming bacteria have similar levels of inhibition.

Clostridium perfringens does not grow below an a_w range of 0.95-0.97 (Jay, Loessner and Golden 2005), and *Bacillus cereus* below 0.90. The level of water activity for process cheese spreads (standard level 0.94-0.96) and slices (0.91-0.93) can be low enough to inhibit growth, but process cheese sauce has the highest water activity (0.95 – 0.99) which is more favorable for spore-forming bacteria (Glass and Doyle 2005). Other factors that make process cheese sauce a good growing environment for foodborne illness causing spore formers is its low acid content (pH higher than 4.5) and its moisture level (>60%). Processing temperatures and times are used as a critical control point (CCP) in order to achieve the necessary 12-log destruction of *C. botulinum* spores. However, the

longer the process cheese is held above 90°C the greater negative impact on the quality of the product, and the more the flavor is negatively affected. For this reason, processing temperatures are often set between 85-100°C and rely on certain additives in the cheese sauce to help control the microbial activity which will be discussed later (Glass and Doyle 2005).

1.2.2 History of Outbreaks

Process cheese sauce has rarely caused outbreaks because it is subjected to thermal processing which renders it "commercially sterile". However, a few outbreaks have occurred in the past. The recorded outbreaks caused by process cheese include one fatal case of *Clostridium botulinum* that happened in 1951 from Liederkranz Brand canned cheese spread in California, and another in 1974 in Argentina from a different brand of process cheese. Neither product could be considered sterile because they had not been thermally processed, and the outbreaks were caused by the high pH and water activity in the cheese, creating an environment conducive for the organism to grow (Briozzo et al. 1983, Meyer and Eddie 1951).

Less than 1% of all botulism outbreaks reported since 1899 have included dairy products as the vehicle for intoxication. Process cheese sauce specifically has very few reported outbreaks, but most are caused by thermally processed cheese that was contaminated and temperature abused at the retail level. Townes et al (1996) did a retrospective cohort study in 1996 of customers from a deli in southern Georgia that became sick with *C. botulinum* poisoning. A totally of eight customers were affected by the toxin, five of which were hospitalized and one died. Upon investigation, *C. botulinum*

type A was found in the cheese sauce can in 400 mouse minimum lethal doses per gram and reached a maximum of 20,000 mouse minimum lethal doses per gram after being incubated at room temperature (22°C) for 15 days. The packaging had the clear standard instructions of "refrigerate after opening" printed on the side, and everything suggested that the can had been made and processed according to FDA regulations. There was nothing found in the investigation of the deli that suggested the cheese was left unrefrigerated, but other cheese sauce cans from the same lot were tested without signs of *C. botulinum*. The source of the contamination was never found but assumed to have happened once the can was already open (Townes et al. 1996). Only two other outbreaks have been reported from process cheese sauce; one in 1951 and one in 1993 in Texas. The Georgia outbreak of botulism was the first ever reported in process cheese sauce that came from a can, and had therefore undergone a high-temperature thermal processing.

1.2.3 Significance of Outbreaks in the Industry

As stated earlier process cheese sauce is considered as a "low-acid canned food" and is commercially sterile. The CFR defines commercial sterility by heat as rendering the food completely free of microorganisms capable of reproducing in the food under normal nonrefrigerated conditions of storage and distribution; and viable microorganisms (including spores) of public health significance (21CFR113.3 2011 ed). Spore formers are problematic because they can survive the high temperatures of the canning process. *C. botulinum* is the most heat resistant of the three species and the principal aim when setting the processing temperatures of 121°C for two and a half to three minutes to assure

a 12-log inactivation of *C. botulinum* (IJ. 1987). Most of the outbreaks from spore formers stem from misuse by the food preparer at restaurants and home settings.

Irving Pflug wrote a review of the chances of acquiring *Clostridium botulinum* intoxication from canned foods. He found that most of the problems were due to human error during process manufacture and distribution. The amount of spores that survive the processing should be viewed as a variable to the kind of organism and processing conditions, rather than a constant (Pflug 2010). Because *Clostridium botulinum* is the organism by which the canning process uses to determine its time and temperatures, it is the organism that has been most studied for validating canning processes. From 1971 to 2009 there is not a documented outbreak of *C. botulinum* in cheese sauce, or any other dairy product, in America. Understanding the ability of spores to survive the canning process is essential for any kind of canned food, especially when the environment in process cheese sauce is so conducive for *C. botulinum* growth (Pflug 2010).

There are as many as 10 steps identified by Pflug for the most common cause of error resulting in improper processing, ranging from inadequate records to faulty devices and improper handling of temperature deviation. Since the FDA started the low-acid canned food (LACF) regulations there has been a considerable decrease in outbreaks of canned food, but they are still not eliminated. The highest percentage of canned-food outbreaks comes from home canning, where human error is much more pronounced, than commercially canning processes. From 1970 to 1973 there were 30 foodborne outbreaks of botulism reported and only three of the 30 outbreaks were from commercial products (Merson et al. 1974). From the years 1980-1989, a total of 69 (87.3%) of the reported

outbreaks were from home canning, and from 1990-1996 the number diminished to 40 (71.4%) outbreaks (CDC 1998).

The chances of survival of *C. botulinum* through the thermal process and causing illness in a person are relatively small. Pflug estimated this probability to be in total 1.0×10^{-8} to 1.0×10^{-10}. First, the spore has to survive the thermal process intended to destroy it without being so damaged that it cannot outgrow. Next, depending on the food product in question, it must outlast the other outgrowth of competing microorganisms. Because of the outgrowth of microbes that produce gas as a by-product, the can will usually bloat or be visually defective that a human will discard the can before it can be consumed to cause illness. Overall the spore needs to survive processing, germinate, outgrow, produce toxin, and be consumed before it can lead to human sickness which is why the chances seem so remote (Pflug 2010). Despite the small chances for illness, it still occurs because we cannot yet destroy every cell that will be present in the food, and this is where the consumer plays a much larger role in outbreaks than does the canning industry. It has also been theorized that heat-injured spores will germinate faster and easier after processing upon temperature abuse (Cronin and Wilkinson 2008a).

Evaluating the processing plants ability to deliver a commercially sterile product is only one part of the equation to be assessed. Once the product is delivered to a commercial setting, the ability for microbial growth is still possible because of human error in proper treatment and storage of food. Pflug et al (1981) did a study of swelled cans at the consumer level over a period of 17 months. A total of 1,104 cans were collected from supermarkets in Minnesota where 314 were determined to have had

significant damage to the can itself that caused microorganisms to grow and produce gas that made the can swell. The remaining 790 cans were determined to have swelled up because of microbial issues prior to reaching the retail outlet. A microbial analysis was performed on the 790 cans where it was discovered that a majority (91.6%) of the cans contained expected leaker spoilage organisms, a few (0.5%) were thermophiles, and the rest (7.9%) contained pure cultures of resistant, mesophilic spore-formers. None of the organisms that were recovered were *Clostridium botulinum* or even toxigenic, but were still indicators that the food had been through under-processing (Pflug, Davidson and Holcomb 1981).

The National Outbreak Reporting System (NORS) has a Food Outbreak Online Database (FOOD) that includes all the recorded outbreaks by the CDC and this system can be searched by microorganism, year, state, and location of consumption. Using the FOOD system, Table 2 was created to show all the known outbreaks of *Clostridium botulinum*, *Clostridium perfringens*, or *Bacillus cereus* that were associated with some kind of cheese product. These data are a good estimation of the number of outbreaks involving cheese but are not exact or a number of reasons: i) *B. cereus* is often underreported or reported to be *C. perfringens*, ii) *B. cereus* is not a required reportable organism iii) These numbers only represent the outbreaks documented by the CDC and do not account for all outbreaks.

Table 2: Outbreaks from *Clostridia* and *Bacillus* species involving a cheese product from 1998-2006 obtained from the Centers for Disease Control's Food Outbreak Online Database.

Bacteria	Etiology Status	Location of Consumption	Total Ill	Food Vehicle	Contaminated Ingredients
Bacillus cereus	Suspected	Restaurant	2	Blue cheese dressing	Spices
Clostridium perfringens	Suspected	Restaurant	12	Pizza, steak, unspecified	Beef; Cheese
Clostridium perfringens	Suspected	Restaurant	73	Burrito, cheese	
Clostridium perfringens	Suspected	Restaurant	32	Cream sauce, tortellini, cheese	
Clostridium perfringens	Suspected	Restaurant	2	Pizza, cheese	
Clostridium perfringens	Suspected	School	52	Burrito, bean	Cheese; legume
Bacillus cereus	Suspected	Restaurant	3	cheese sauce; potato, mashed	
Clostridium perfringens	Confirmed	Other	269	cheese sauce; tortellini, cheese	
Bacillus cereus	Confirmed	Private home	4	pizza, cheese; pizza, meat and vegetable	
Bacillus cereus	Suspected	Restaurant - other or unknown type	3	cheese fondue	
Clostridium perfringens	Confirmed	Private home	4	gravy, chicken; macaroni and cheese; rice, yellow	Cheese; chicken; pasta; rice; sauces/ dressings
Bacillus cereus	Confirmed	School	47	macaroni and cheese	
Bacillus cereus	Confirmed	School	100	macaroni and cheese	

1.2.4 Mechanism of Sporulation and Germination

Sporulation is the process of used by some species of bacteria in which vegetative cell becomes a spore. Spores of bacteria are formed when there is an applied environmental stress on the vegetative cell which can be heat, nutritional depletion, radiation, or chemical. For foodborne illness, the species of clostridia and bacilli are the most problematic spore formers. *Bacillus subtilius* is the species that has been most thoroughly studied for understanding sporulation. There is still a lot of information that is unknown about the mechanisms of sporulation and germination, particularly the molecular changes by which spores are destroyed (Mafart et al. 2010, Jay et al. 2005).

Once the cell of spore-forming bacteria is under stress it starts the sporulation process, seen in Figure 2. First, a small forespore is formed when the chromosomes in the bacterial cell separate into two Z-rings that move to the opposite poles of the cell. Instead of dividing in the binary fission style, as happens during vegetative growth, the chromosomes make an axial filament that extends from pole to pole. Proteins known as RacA anchor the origin of replication of the chromosomes to opposite poles of the cell to the protein DivIVA. One Z-ring forms the septum separating it from the larger mother cell while the other Z-ring disappears. This septum traps part of the chromosome from the axial filament into its own segment of the cell and the rest of the chromosome is pulled into the septum by DNA translocase so that the forespore has a complete chromosome set in it. The larger mother cell then completely surrounds the spore, now an endospore, and helps to nourish the spore for the rest of its development. Both the mother cell and spore have full genomes but they are not identical since some genes that are

expressed in the spore are not expressed in the mother cell (Losick 1992, Errington 2003, Piggot and Coote 1976).

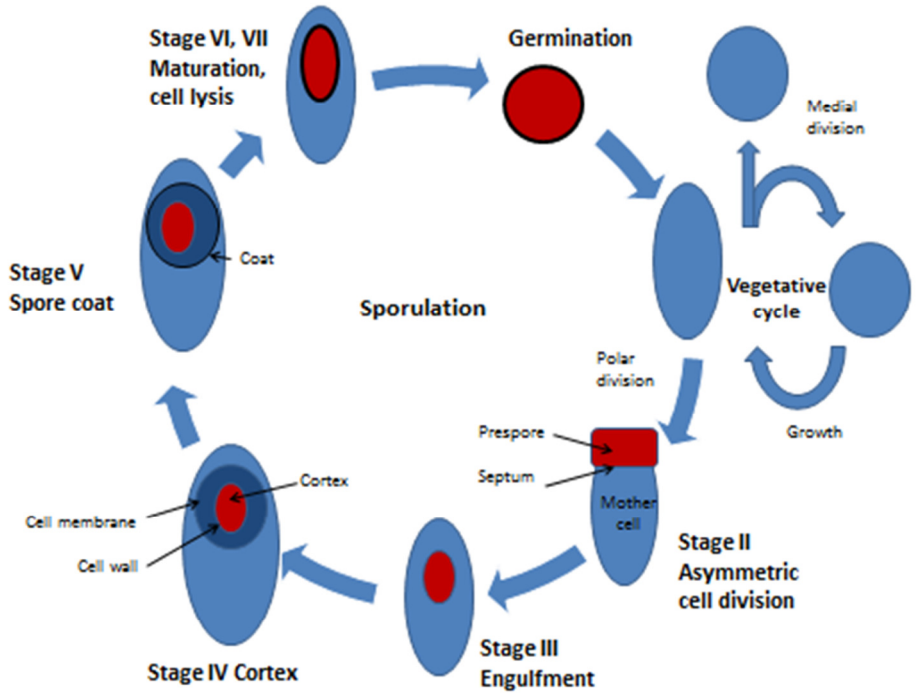

Figure 2: The sporulation process adapted from (Errington 2003)

The spore has seven layers: the exosporangium, coat, outer membrane, cortex, germ cell wall, inner membrane, and core which can be seen in Figure 3.

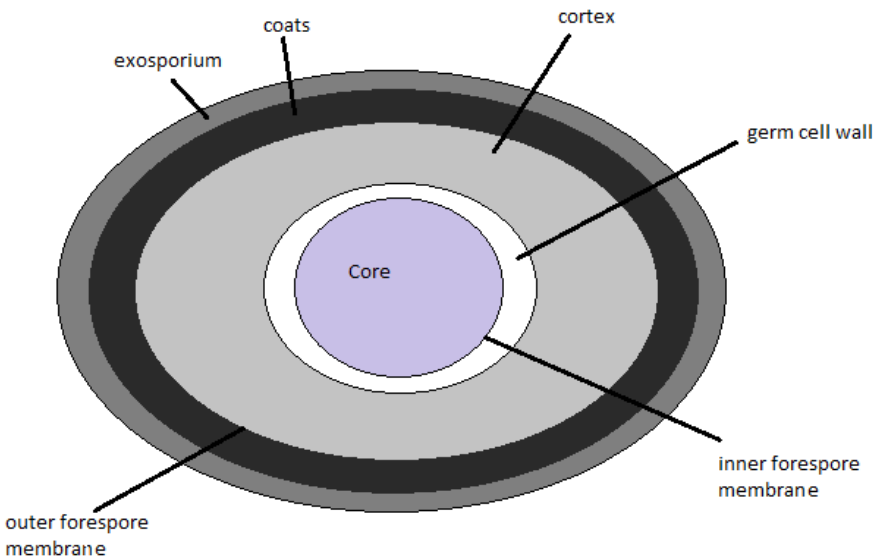

Figure 3: Structure of spore developed from Montville and Mathews (2008) (sizes not drawn to scale)

During sporulation the spore stores a large percentage of dipicolinic acid (DPA) and small acid-soluble proteins (SASP) which make it unique from the other stages of the cell. This is supported by the fact that the concentrations of DPA and SASP are significantly decreased minutes after the germination process begins. There are some spores found to lack DPA, but cannot retain their dormancy and are defective during germination (Piggot and Coote 1976). Spores are completely dormant which means there is no metabolism occurring within the cell, and can stay dormant for years. Another factor thought to help the spore stay dormant for so long is how dehydrated the core is which is regulated by the spore cortex. There is a strong correlation between the extent of dehydration of the core and the ability to withstand high temperatures. It is thought that because there is so little water activity, certain metabolic reactions cannot develop and as the core becomes more dehydrated, its heat resistance increases (Losick, Youngman and Piggot 1986).

The core contains the cell's DNA, proteins, DPA and divalent cations. The makeup of the core is unique to spores and is responsible for the properties that allow it to turn off metabolism and survive an extreme environment created by heat, radiation, or chemicals (Montville and Mathews 2008, Losick et al. 1986, Piggot and Coote 1976). The contents of the core, namely the DPA and acid-soluble proteins are the first to be depleted upon germination.

The resistance to heat is the most well-known characteristic attributed to spores and the reason they pose such a threat to the food industry, canning in particular. There is still a lot that is unknown related to controlling and destroying spores in food except that

scientists have found that it is not due to DNA damage (Setlow 2006), though there is a theory that protein denaturation plays a big role in thermally killed spores (Setlow 1998). Because the *Clostridium* species is also anaerobic the conditions set forth during processing make a suitable environment for these organisms to grow, though some species sporulate easier than others. *Clostridium perfringens*, for example, requires specific conditions optimal to form spores and thus can be limited by its surroundings. In order for *C. perfringens* to sporulate, the optimal conditions require a temperature between 27 and 50°C, pH values between 6.0 and 8.0, and water activity greater than 0.96 (Kang et al. 1969, Labbe and Duncan 1974, Rey, Walker and Rohrbaugh 1975). It is well documented that the heat resistance of a spore is directly correlated with its growth temperature, but inactivation of spores is still a matter of debate.

Controlling sporulation studies in the lab is difficult and even if the exact method is duplicated with a different strain of the same species, often times the results of heat resistance will still vary. Every species and strain is different, and the way they react in different food matrixes is dissimilar (Blackburn 2006). The methods for control and deactivation used in the industry currently in addition to heat-inactivation are irradiation and high hydrostatic pressure (HHP). Though spores are still more resistant to irradiation than are vegetative cells, it has still been found that radiation doses of 10-50 kGy have inactivated spores in food with typical D-values (Blackburn 2006). *B. cereus* spores were more susceptible to irradiation than *C. perfringens* but for both bacteria their vegetative cells were more susceptible than the spores (Monk, Beuchat and Doyle 1994). With HHP the best results of spore inactivation are obtained in combination to mild heat treatments,

low pH, or an antimicrobial such as nisin. Again there is much variation between species and strains but it is generally regarded for *Clostridium* and *Bacillus* that pressures ranging from 500 to 800 MPa at temperatures between 60 to 80°C are required for inactivation of spores where the higher the temperature, the more lethal the effects (Blackburn 2006).

Proper cleaning of processing equipment is also an important part of controlling spore formers in food systems. The spore coat keeps most chemicals from penetrating, but a higher concentration and longer contact time with certain chemicals can have a sporicidal effect (Bloomfield and Arthur 1994). Compounds such as glutaraldehyde, alkali, P-propiolactone, ethylene and propylene oxides in solution, iodine, hydrogen peroxide, chloramines and peroxy acids have shown to be the most effective chemical agents that cause destruction in spores, while formaldehyde, methyl bromide, silver compounds, chlorine and bromine can be used but require a longer contact time in order to be affective (Roberts 1969, Russel 1990, Waites 1985).

1.3 *Bacillus cereus*

1.3.1 Microorganism Characteristics

The taxonomy of the genus *Bacillus* is relatively complex. The first division is by optimum growth temperatures; thermophilic or mesophilic. *Bacillus* species typically associated with foodborne illness are mesophilic and they are further classified into three types based on shape. Within the first type, group 1A and 1B are divided based on vegetative cell size and whether or not the cytoplasm contains lipid globules (Granum and Baird-Parker 2000). *Bacillus cereus* is in group 1A, the large cell sub-group (cell width $\geq$1µm), which includes most of the clinically significant species of the genus

Bacillus, along with *B. megaterium*, *B.anthracis*, and *B. thuringiensis*. The spore that is formed by the large cell sub-group does not swell the sporangia like some of the other sub-groups (Drobniewski 1993).

Bacillus cereus is an aerobic or facultative anaerobic, Gram positive, spore-forming rod. Its public health importance has been recently more recognized because of its ubiquitous nature. It can be found in soil, plants, multiple food sources, and animal origins. The first studies of *B. cereus* as a causative foodborne illness did not occur until the 1950s when S. Hauge (Hauge 1955) performed an experiment by consuming vanilla sauce with a high count of *B. cereus* cells. *B. cereus* causes two different types of foodborne illnesses in humans based on which kind of toxin is ingested; an emetic syndrome, and diarrheal syndrome. *Bacillus cereus* currently has 42 serotypes which are distinguished based on their flagellar antigens. Serotypes 1, 3, and 8 are part of the emetic illness and serotypes 1, 2, 6, 8, 10, and 19 are associated with the diarrheal illness (Granum and Baird-Parker 2000). Each form of illness has distinct differences in their mechanisms and ways they cause illness.

The emetic syndrome is characterized by nausea, vomiting, and malaise, and needs between 100,000 and 100,000,000 cells per gram to produce sufficient emetic toxin in the food before a person ingests it (Labbe and Garcia 2001). This illness has an incubation period of 0.5 to 5 hours and is usually implicated in foods such as rice, pasta, and noodles. The toxin is a cyclic peptide that is most likely produced enzymatically. Its mode of action is the stimulation of the vagus afferent nerve by binding to a specific receptor, but the biosynthetic pathway of the toxin has only been hypothesized. The toxin

produced is not antigenic but is resistant to heat (90 minutes at 121°C), pH, and proteolysis (Granum and Baird-Parker 2000).

The diarrheal syndrome is caused by at least two enterotoxins and causes symptoms of abdominal pain, watery diarrhea, and sometimes nausea. The diarrheal syndrome needs a total cell count of 100,000- 10,000,000 and is usually involved with food products such as meats, soups, vegetables, puddings, sauces, and milk or milk products (Labbe and Garcia 2001). There is some speculation that the enterotoxin could be on the food before it is ingested, but for this to happen the *B. cereus* population would have to be 10^6 g^{-1} (Christiansson 1993, Kramer and Gilbert 1989) and the enterotoxin level would have to be sufficiently high to survive the pH of the stomach and retain enough activity to make someone sick (Granum 1994, Granum et al. 1993). This relatively high infectious dose and longer incubation period (8-16 hours) suggest that enterotoxins are not pre-formed on the food, but are released once inside the small intestines during vegetative growth. The mode of action of the enterotoxin is unclear and a multi-unit enterotoxin has been suggested, and the most recent analysis of the toxin supports a three component enterotoxin theory (Lund and Granum 1996).

Labbe and Garcia discussed the metabolic indicators of *B. cereus* that are used in the plating method for creating a selective and differential agar known as mannitol egg yolk polymyxin (MYP) which is also the AOAC plating technique (Labbe and Garcia 2001). This agar plays on the ability of *B. cereus'* enzyme to produce turbidity surrounding a colony that is grown on this medium, and its resistance to antimicrobial polymyxin B. A process that is known as the "egg yolk reaction" could be because of a single enzyme,

such as the phospholipase C, or by a much more complicated mechanism that has yet to be understood (Labbe and Garcia 2001).

1.3.2 *B. cereus* Foodborne Illness Incidence

Until recently *B. cereus* has not produced a significant amount of foodborne illnesses each year and was considered to be a minor foodborne disease. From 1970 to the 1980s it accounted for only about 1-3% of total foodborne illnesses in the United States and United Kingdom. It has been more problematic in other countries like the Netherlands and Canada where 22% and 7% of outbreaks respectively are caused by *B. cereus* (Kramer et al. 1982), but has implications of becoming more of a threat since it has been reported to account for over 30% of the illnesses in Europe (Montville and Mathews 2008). The most recent estimates by the CDC suggest that *B. cereus* is becoming more problematic and accounting for nearly 63,000 cases annually in the United States (Elaine Scallan 2011). These estimates are still thought to be low due to underreporting and misdiagnosis because the symptoms are so similar to the symptoms of other illness, and it is not bacteria that state Departments of Health are required to report by Pulsenet or Foodnet. The diarrheal type is often reported as *Clostridium perfringens* and the emetic syndrome is reported as *Staphylococcus aureus*. The California Department of Food and Agriculture (CDFA), Animal Health and Food Safety Services Division made a best guess estimate in 2007 of the number of annual foodborne illnesses caused by *B. cereus* seen in Table 3.

Table 3: Estimated foodborne illness caused by *B. cereus* adapted from CDFA

Agent	Cases	Percent	Deaths	Percent
B. cereus	27,360	0.2	0	0
Total bacterial	4,175,565	30.2	1,297	71.1
Total foodborne	13,814,924	100	1,809	100

The dairy industry in particular is taking more notice of the organism because of its affinity to grow in dairy products and the recent development of psychrotrophic strains in the dairy industry. It is also a common contaminant in raw milk. In fact, 31% of 167 milk products sampled in Australia were found to contain *B. cereus* (Coghill and Juffs 1979). The spores of *B. cereus* were found in great numbers in the soil at farms, roughly in a population of 10^5-10^6 CFU/g, which is often found on the cow's udder and teat. If the cow is not cleaned properly before it is milked, cross contamination is almost certain to happen and spores are difficult to eliminate. *B. cereus* spores adhere easily to the stainless steel processing equipment and stay attached even after cleaning because they are highly hydrophobic, have morphology with long appendages, and a low spore surface charge (Lopez-Pedemonte et al. 2003, Andersson, Ronner and Granum 1995). Its growth patterns are consistent with other mesophilic spore formers with a minimum growth range of around 10-12°C and a maximum range of 48-50°C with an ideal growth temperature range of 30-40°C (Labbe and Garcia 2001).

1.3.3 Importance of *B. cereus* in the Industry

Because of the hardiness of *B. cereus* spores and its ubiquitous nature, it can be a major problem in the food industry. Thermal inactivation of spore species in general is

non-linear because of the great diversity in strains and the variety of resistance between species. Cronin and Wilkinson (2008) studied the response of *Bacillus cereus* to heat treatment and low temperature storage to understand the effect of sub-lethal heat damage to the viability of spores. Using strain NCTC 7464, bacterial cultures were placed in tubes of phosphate-buffered saline (PBS) to levels of 10^6 CFU/mL and were heated for 0, 10, or 20 minutes to a temperature of 80, 85, 90, or 95°C. Each treatment was plated directly after heating, and then plated again after one month at 4°C. They concluded that some injury to endospore is caused by damage to the permeability barriers of the cell because of the high heat suggesting intracellular damage plays a role in killing spores.

Other findings were that storage of the spores exerted deleterious effects causing a 99% loss of viability seen by the high propidium iodine (PI) permeability and high esterase activity of the spores after one month of storage. The most important conclusion from this study was that non-viable or injured spores germinate more rapidly than intact spores. This difference implied that, when food is thermally processed, the spores would not be destroyed but they would have an enhanced ability of germinating once temperature abused (Cronin and Wilkinson 2008a). That study was not performed in a food matrix, and may only partially explains how spore forming bacteria act with sub-lethal heating schemes.

Bacillus cereus has been reported to affect many different food sources, the main problems have been caused by rice, spices, meats, eggs, and dairy products. *B. cereus* spores in rice has been a big concern for Chinese restaurants when rice is boiled in bulk and then portions are saved and stored at room temperature (Andersson et al. 1995). In

the dairy industry, the problem stems mostly from distribution temperatures. The emergence of psychrotrophic strains of *B. cereus* has allowed the cells to stay viable and even grow at low temperatures. Milk is typically stored at approximately 4°C, but during distribution temperatures it may increase to 8°C which would allow germination of some sporeformers. Temperature abuse at restaurants and at home is also common. A study of the growth of *B. cereus* in pasteurized milk showed significant changes in the population of the bacterium with only 2 degrees of difference in the holding temperatures (Figure 4) (Andersson et al. 1995). If milk is temperature abused during transportation to a cheese processing plant, then the problem will persist through the rest of the cheese make. Pasteurizing the milk will only active the spores and kill off any competing flora making germination a higher possibility.

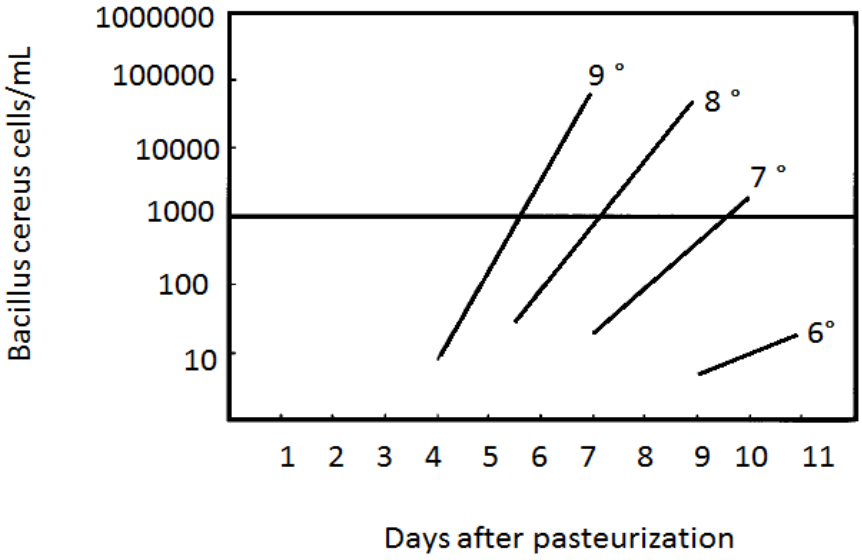

Figure 4: Growth rate of *Bacillus cereus* in milk stored at different temperatures. Figure was re-drawn from Andersson et al. 1995

1.3.4 Controlling *Bacillus cereus* in Cheese

The mechanism for growth of *B. cereus* in process cheese sauce is different than that of *Clostridia* species because it is aerobic. However, some cells are facultative anaerobes and because of the ability to form spores, the problem can still exist. In fact, the opportunity for illness could be increased because of the inherent nature of food products to be temperature abused at a commercial setting in an oxygen rich environment. Not much has been studied on the post processing contamination of process cheese sauce. Because most processing lines use aseptic packaging and a hot-fill canning or retort system, the introduction of bacterial spores is negligible. Even when using a common packaging paper inoculated with *B. thuringiensis*, a surrogate of *B. cereus,* to levels 100 to 1,000 times higher than typical counts on commercial paper, only 0.001 to 0.03% were transferred to the processed food (EKMAN et al. 2009).

The effects of high hydrostatic pressure (HHP) have been studied for inactivation of *B. cereus* spores in cheese. Lopez et al (2003) evaluated model cheeses made from raw-milk as the matrix to test HHP and its ability to inactivate *B. cereus* spores. Using a pressurization cycle of 60 MPa at 30°C for 120 minutes to achieve a vegetative cell destruction cycle of 300 or 400 MPa at 30°C for 15 minutes, less than 1 log CFU/g viable spore reduction was observed. However, the highest inactivation of more than 1 log CFU/g was achieved with addition of nisin in the model cheeses (Lopez-Pedemonte et al. 2003). In a second study on miniature cheese the efficacy of a germinative cycle of a 60 MPa at 30°C for 210 minutes was determined. Using HHP cycles of 300, 400, and 500 MPa at 30°C for 15 minutes with or without the conjunction of a germinative cycle, it

was determined that the germinative treatments and the 500 MPa cycle resulted in the highest lethality of more than a 2-log CFU/mL reduction of *B. cereus* (Lopez et al. 2003).

1.4 *Clostridium perfringens*

1.4.1 Microorganism Characteristics

Clostridium perfringens is an anaerobic, Gram positive, spore-forming rod that was first recognized as a food borne illness in 1953 and was referred as *Clostridium welchii* until the 1970s (Labbe 2000). Among the bacteria in the clostridial group, *C. perfringens* is the most abundant toxin-producing species (Labbe and Garcia 2001). *C. perfringens* is a mesophilic organism that grows optimally at 37°C to 45°C, but has been seen to grow at refrigeration temperatures as low as 6°C by (Johnson 1990). It is a very oxygen tolerant bacterium with an oxygen-reduction potential of -45 mV or lower where growth has been observed at an initial E_h of +320 mV. As long as the environment is conducive enough for initial growth, then *C. perfringens* can modify the E_h of its surroundings by producing reducing compounds, like ferredoxin, to improve the conditions to a more favorable level (Montville and Mathews 2008). The optimum pH for growth is typically between 6.0 and 7.0 but growth has been reported from 5.0 to 9.0. A minimum a_w level between 0.95 is also required (Jay et al. 2005). This microorganism is also quite notable for its very fast grow, as it is capable of doubling in number in less than five minutes (Montville and Mathews 2008).

1.4.2 Toxin Production and Mechanism

The *Clostridium perfringens* species can be divided into five different types of strains,

A, B, C, D and E, by the types of toxin that is produced; alpha, beta, epsilon, and iota. All five strains produce the alpha toxin which have deadly effects in humans (gas gangrene) and in animals (necrotic enteritis). It can also produce a number of proteins or hydrolytic enzymes such as lecithinase, hemolysins, hyaluronidase, collagenase, deoxyribonuclease (DNAse), and amylases (Labbe and Garcia 2001). All five types of *C. perfingens* may be lethal but only types A and C are pathogenic for humans, types B and D have only been isolated from the intestines of animals, and type E has rarely been isolated (Jong 2003, Labbe and Garcia 2001).

Type A strain, the only important strain associated with foodborne illness, can be differentiated from other type A strains, as well as the other four types by the lack of volatile fatty acid production, specifically propionic acid. Types C and D have also been associated with enterotoxin production but have not been isolated in any food borne illness cases. *Clostridium perfringens* food poisoning is a toxicoinfection and is caused by the production of an enterotoxin (CPE) that accumulates in large quantities intracellularly during sporulation. Cells are lysed in the stomach and the enterotoxin is processed to a more active form in the intestines by trypsin. There are two separate phases involved in the enterotoxin-mediated cytotoxicity. The first step involves complexing with specific protein receptors in the membrane of epithelial cells. This step is accompanied by a conformation change in the enterotoxin and causes loss of permeability in the cells and loss of metabolite and ions. The second step details morphologic damage and more intense permeability impairment and loss of larger solutes (Rood and Cole 1991). The enterotoxin produced by *C. perfringens* is distinct from other

intestinal toxins and could be considered as a new class of toxin (McClane, Hanna and Wnek 1988).

Not all types of *C. perfringens* are entertoxin positive (CPE+), even though they may still carry the gene, and those that are CPE- will not cause harm when the cells are ingested. Most wild types of *C. perfringens* have been found to be CPE- which suggests that processing conditions have an effect on the cell so that the gene coding for the enterotoxin can be changed. Uemura and Skjelkvble performed a study that showed repetitive heat shock on spores at 75°C for 20 minutes could convert a cell that is CPE- to a CPE+ cell. This suggests that most foodborne illness caused by *C. perfringens* is due to practices in the industry, because the strains could not initially produce the entertoxin that causes illness. The most effective way to minimize the conversion of CPE- cells to CPE+ cells is by careful and complete cleaning systems of processing equipment (Skjelkvale, Stringer and Smart 1979).

1.4.3 *Clostridium perfringens* Foodborne Illness Incidence

The most recent estimations ranks *C. perfringens* as one of the top five organisms responsible for causing foodborne disease annually in the United States, accounting for an estimated 965,958 illnesses a year, according to Scallan et al (2011). The symptoms of *Clostridium perfringens* food poisoning are most commonly watery diarrhea and stomach cramps that occur quickly after ingesting the bacteria. Because the toxin is released upon sporulation in the intestines, symptoms will start to appear very shortly within 8 to 12 hours. With the exception of elderly and infants, whose symptoms can persist for one or two weeks, symptoms typically last no longer than 24 hours. Because of its susceptibility

to stomach acids, a relatively large infectious dose (more than 10^6) of vegetative cells must be ingested in order to get sick (Montville and Mathews 2008).

C. perfringens is a very ubiquitous organism that can be found in soils (approximately 10^3-10^4 CFU/g), dust, food (nearly 50% of raw meat), spices, and in the intestines of animals and humans (Jay et al. 2005, Labbe and Garcia 2001). Protein rich foods are particularly susceptible for *C. perfringens* growth because it can only synthesize seven of the necessary 20 amino acids. As a result of this growth requirement, most of the outbreaks due to *Clostridium perfringens* are caused by raw meats, poultry, and meat products such as gravies, but it can still play a significant role in poultry, fish, vegetables, dairy products, dehydrated foods, spices, pasta, and other foods exposed to soil. Most often outbreaks occur because of inadequate reheating of pre-cooked food. Incidence are high when food is cooked in large quantities and subjected to long periods of slow cooling and non-refrigerator storage (ICMSF 1996).

There are two main factors that allow *C. perfringens* to survive so readily in foods. First it has the ability to grow at temperatures ranging from 15°C-50°C but has a comparatively high optimum range of 43°C-46°C compared to other mesophilic organisms. Other mesophilic organisms grow between 25°C-40°C so *C. perfringens* can start growing before its competitors when food is improperly cooled down (Montville and Mathews 2008). The second factor is its ability to form spores. The spores produced by *C. perfringens* can survive the high thermal processing conditions and grow with little or no oxygen making it much more difficult to eliminate in the environment with typical treatments. *C. perfringens* does not adhere to stainless steel as well as *B. cereus* does,

and, because it is anaerobic, it is much less of a problem to the industry as long as the disinfecting process is done carefully. After standard cleaning of equipment: rinse, caustic wash, rinse, acidic wash, and final rinse, the addition of hypochlorite with an adjusted pH of 8 will successful eliminate the spores (Granum and Magnussen 1987).

1.4.4 *Clostridium perfringens'* Sporulation

C. perfringens forms spores in the intestines of humans and produces an enterotoxin that causes food poisoning. While sporulation in the intestines happens relatively easily, inducing its *C. perfringens* sporulation in the lab often proves difficult because of the variability of the strains. Many studies have been done on the efficacy of sporulating media for *C. perfringens* (Duncan and Strong, Labbe et al, Jong, Juneja et al, among others). The method used most for sporulating *C. perfringens* in the lab was originally reported by Duncan and Strong (Duncan and Strong 1968). The study was aimed to develop a new media that would induce sporulation of *C. perfringens* better than the standard media used previously, SEC media and Ellner media.

Duncan and Strong used five strains: ATCC 3634, NCTC 8238, NCTC 10240, 214a and T-65, the latter two strains had been isolated by the Wisconsin Department of Health from meat sources. A small volume amount of the stock spore suspension was transferred to fluid thioglycollate media, heat shocked at 75°C for 20 min and then incubated at 37°C for 20 hours. Subsequent inoculations were done in fluid thioglycollate and fresh sporulation media was used before transferring cells to the different sporulation media tested. A basal medium was made combining different compounds from the SEC and Ellner's media in the attempt of formulating a more effective sporulation media e.

The basal media consisted of (as percentages) 2.0 trypicase, 0.25 l-arginine, 0.1 lactose, 0.2 yeast extract, 1.0 proteose peptone, 0.3 soluble starch, 0.1 sodium thioglycollate, and 0.5 $Na_2HPO_4 \cdot 7H_2O$ which was modified one ingredient at a time to determine its effect on sporulation of strains.

The results of Duncan and Strong's experiment showed that this basal media was more successful than SEC media in producing spores by all five strains, but when compared to the Ellner's media only two of the five strains sporulated better. As for the basal medium that was created, starch played the biggest role in increasing the concentration of spores. When starch was omitted, the turbidity was decreased and very few spores were produced, and when the concentration was increased to 0.5% from 0.1%, sporulation increased in all five strains. Other ingredients that increased sporulation were yeast extract and proteose peptone. The latter ingredient was tested by Perkins as well who reported that, not only concentration, but type of peptone was important (Perkins 1965). This theory is also supported by Hsieh and Labbé who tested seven strains against 32 different sources of peptone (Hsieh and Labbe 2007). The two sources that produced the highest number of spores were potato peptone and Peptone no. 3. They also tested the carbohydrate source coming from raffinose against starch and saw that raffinose produced more spores than starch. From the initial results, 49 more strains were tested with the potato peptone and Peptone no. 3 source against the control (proteose peptone) using raffinose as the carbohydrate source. Of the 49 strains, 23 sporulated best in the potato peptone, some at a 25% higher concentration than the control source.

1.4.5 *Clostridium perfringens* in the Dairy and Food Industry

Clostridium perfringens is most often found in vehicles of meat or poultry, but there are some documented outbreaks involved with thermally processed cheese as a suspected vehicle, and reports of spores in raw milk and cheese products (Johnson, Nelson and Johnson 1990). Despite the fact that it does not adhere to surfaces like *Bacillus cereus* does, and the strict requirements needed for growth, *C. perfringens* is still a major problem in the food industry. In 1999 the Food Safety Inspection Service (FSIS) put out Directive 7111.1 which converted previous regulation to performance standards in cooking and cooling guidelines of meat and poultry. The Directive did not detail the process of killing or stabilizing bacteria populations, but established criteria for time and temperature to achieve a specific log reduction (U.S. Department of Agriculture FSIS Directive 7111.1). Even with these performance standards for retail establishments and HACCP plans for manufacturers in place, *C. perfringens* continues to cause large amounts of illness annually.

Lin and Labbe (2002) collected commercial food samples from retail outlets to test for the presence of *C. perfringens* and its enterotoxin (*cpe*) and alpha toxin (*cpa*) genes. Of the 131 samples tested, 39 (30%) identified a population from 3 to 292 CFU/g but none were positive for *cpe* or *cpa* that would cause foodborne illness (Lin and Labbe 2002). A follow up study was performed with more advanced methodology by Wen and McClane in 2004 who found that 1.4% of roughly 900 samples of retail foods contained *cpe*. Their conclusions also suggested that superior heat resistance of *C. perfringens* is not an evolutionarily acquired trait, but rather an intrinsic trait of a chromosomal *cpe*

gene (Wen and McClane 2004). A study was conducted in 2003 to test the FSIS Directive on *C. perfringens* in raw and cooked meat samples. For meats cooked to 73.9°C only two of 197 samples had detectable amount of spores, chilled samples had approximately 2 log CFU/g when chilled for six hours, and 3 log CFU/g when chilled for 24 hours. Samples that were held at refrigeration temperatures decreased in population by at least 2.5 CFU/g (Kalinowski et al. 2003).

The biggest reason attributed to causation of outbreaks comes from temperature abuse -- mostly improper cooling, and insufficient reheating of the contaminated food. The most typical location for an outbreak has been in the food service – hospitals, restaurants, factories, prisons, schools, and catered events – because of the need to cook food in bulk well before the consumption of the product (Labbe 2000). There are few studies conducted on temperature abuse involving cheese, or process cheese products, but there have been studies on the thermal inactivation of spores and how varying temperatures affect the ability of clostridia to germinate or sporulate. As was discussed earlier, vegetative cells are destroyed commercially by high thermal processing, but even these temperatures do not guarantee the product will be free of spores.

Most studies of temperature abuse for *Clostridium perfringens* have been performed on meats and meat products such as gravies. Sanchez-Plata et al performed a study on the abusive cooling temperatures of roast beef and their effects on the germination potential and outgrowth of *C. perfringens* (Sanchez-Plata et al. 2005). The beef product started at 54.5°C and was cooled to 7.2°C to mirror industry cooling schedules of the product. Roast beef inoculated with an initial population of at least 3 log

CFU/g of *Clostridium perfringens* was allowed to cool exponentially in 9, 12, 15, 18, and 21 hours and tested after heat shock, during the cool schedule, and while being stored at 10°C for up to 60 days. The organism increased its population by 2.00, 3.44, 4.04, 4.86, and 5.72 log CFU/g respective to the cooling temperature length. Sanchez-Plata et al concluded that given abusive temperatures, *C. perfringens* would germinate and cause outgrowth in food products. Another similar study by Juneja, Marmer, and Miller tested the difference of aerobic and anaerobic packing of cooked beef on *C. perfringens* growth during static and cyclic temperature storage. During the temperature abuse trials, shorter times of temperature abuse (6 hours) had no effect on cell outgrowth, but when the meat was held at abuse temperatures for 24 hours or more an increase of more than 6 log CFUs/g was observed (Juneja, Marmer and Miller 1993, USDA 1999).

1.4.6 Methods of Controlling *Clostridium perfringens*

Spores of *Clostridium perfringens* can be classified as either "heat resistant" or "heat sensitive" based on their D values at 95°C. Heat resistant spores typically have a D value range between 17.6 and 63 minutes while heat sensitive is between 1.3 and 2.8 minutes. At thermal processing temperatures (65.5°C for 30 seconds), vegetative cells die, but heat resistant spores are still able to survive (Labbe and Garcia 2001). There are different factors that are associated with varying amounts of heat resistant spores, which can be used as means for control. Irradiation has been shown to make spores more susceptible to heat (Labbe and Garcia 2001), but in general *C. perfringens* is particularly radiation resistant. Its vegetative cells are more resistant to irradiation than other pathogens as no significant difference in population has been reported after a dose of 1.75 kGy (Monk et

al. 1994).

The effect of long-chain polyphosphates has also been explored as a method for controlling clostridia species. Two strains isolated from spoiled process cheese spread were used to test the inhibitory effects of varying concentrations of a commercial brand of polyphosphates (Bekaplus FS) on *Clostridium sporogenes* and *C. cochlearium*, closely related species to *C. perfringens*. By adding 1.5% polyphosphate, total inhibition of *Clostridium sporogenes* was achieved, and 0.75% polyphosphate had the same effect on *Clostridium cochlearium*. When combined with heat (5 minutes at 98°C) a concentration of 0.75% polyphosphate was enough to be sporicidal for *C. sporogenes*. These findings suggested that the use of polyphosphates could be used as a strategy for growth and survival control of clostridia species in process cheese spread (Borch and Lycken 2007).

Some salts are used as a means to control growth and survival of *C. perfringens* which is supported by the lack of outbreaks (only 2 from 1978 to 1992) in different kinds of cured meats (Labbe 2000). Though the mechanism is still somewhat unknown, the use of sodium chloride and sodium nitrites aid in the restriction of spore germination and outgrowth, as well as contributing to flavor. Labbe and Duncan performed a study testing four different mechanisms by which heat-injured spores could be affected by the presence of nitrites in cured meats. Only one of the four, the inhibition of outgrowth, was successful with the entire spore population when adding concentrations of 0.02 and 0.01% nitrites to the suspension. Levels between 60 and 2560 ppm are typical when used commercially to inhibit growth, but when levels of nitrites were too high, nitrite induced germination followed (Labbe and Duncan 1969). In general, sodium chloride levels of 7-

8% are used to inhibit the growth of most strains of *C. perfringens*, but when sodium chloride is paired in conjunction with nitrites the former only needed 3-6% while the latter concentration could be decreased from 300 to 25 µg/mL (Roberts and Derrick 1978).

It may be very difficult to completely eliminate *C. perfringens* from the food supply, since most outbreaks are a cause of mishandling after cooking, but the best way to control the outgrowth and germination of the bacterium is to keep the food product stored at refrigeration temperatures (<8°C) and reheated to sufficiently high temperatures (>60°C). Vegetative cells of *C. perfringens* have been well documented to be sensitive to storage at low temperatures, losing close to 90% of viability when held at 4°C for 60 minutes (Traci and Duncan 1974). Even more than 90% of cell populations, were reduced when held at frozen storage temperatures ranging from -17.7°C to -29°C for a period of time greater than 28 days (Traci and Duncan 1974, Strong, Weiss and Higgins 1966, Harmon and Kauter 1970). FSIS ascertains that as long as food, particularly meat, is cooled from 54.5°C to 26.6°C in one and a half hours and 26.6°C to 4.4°C in five hours it is within compliance standards for safe consumption (Sanchez-Plata et al. 2005).

1.5 Antimicrobials in Process Cheese Sauce

1.5.1 Nisin

Antimicrobials and food preservatives have been used in cheese production within the last century when inhibitory microorganisms were found to delay the activity of starter cultures causing certain batches of cheese to be defective. It was not until 1951 that Hirsch et al considered the use of nisin as an ingredient to control clostridial species in

cheese (Hirsch et al. 1951). The utilization of nisin as an antimicrobial and food preservative was reviewed by Delves-Broughton in the early 90s (Delves-Broughton 1990). Nisin is a polypeptide bacteriocin made by lactococci that consists of 34 amino acid residues and acts most effectively on gram positive organisms such as *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Micrococcus*. However it is most useful against all spore-forming species of *Clostridium* and *Bacillus* having a greater impact on the spores of the species rather than the vegetative cells. Nisin attacks the cell in the germination phase at the pre-emergent swelling by inactivating the sulphydryl groups causing the cytoplasmic membrane to leak essential materials, or in extreme cases, to lyse the cells (Delves-Broughton 1990).

Nisin is a common preservative in process cheese and process cheese spread, but not as many studies have been done on process cheese sauce. It is stable in acidic environments and can survive the typically heat used in the manufacturing of dairy foods. The amount of nisin in the product is usually between 200 and 500 IU nisin/g (Fowler 1979) but levels in the actual product decrease during processing because of heat and pH, and during storage time. Nisin has a higher retention of activity when the pH is more acidic. When it reaches a pH of 5.0 and 6.0, the pH of most process cheese sauces, it has 35% and 14.5% retention of activity respectfully. Because the formulation for process cheese sauce has higher moisture, lower fat and added flavors compared to process cheese and process cheese spread, higher amounts of nisin should be added to compensate (Fowler 1979).

Nisin is produced naturally by *Lactococcus lactis* subsp. *lactic* and is destroyed by

enzymes in the human stomach, so it is generally recognized as safe (GRAS) by the FDA. Using nisin in conjunction with the regulated time and temperature for the canning process is important because even after heating to 121°C some heat resistant spores can still remain in the food. The longer a food is held at such extreme temperatures the more it affects the organoleptic properties of that food. Because food is only held to the specific regulations set by the FDA, the addition of nisin in food is a safe way to help ensure the existence of spores in cans is at the lowest possible level.

Zottola et al studied the effect of cheddar cheese that contains nisin as an antimicrobial for other foods (Zottola et al. 1994). Starter strains of lactococci were used during the cheddar cheese manufacturing that produced the bacteriocin naturally. The goal was to make a high enough quantity of nisin in the cheddar cheese so that when it was used in other products, such as process cheese spread, the antimicrobial would lower the amount of *L. monocytogenes*, *S. aureus*, and *C. sporogenes* added to the product. Seven different cheese varieties were made through the course of the experiment with different compositions of solids, fat, moisture, pH, and IU Nisin/g with the intent of maximizing spoilage potential by not adding traditional inhibitors used commercially. The nisin content in the pasteurized process cheese spread (PCS) came from the cheddar cheese that was used to manufacture it, and it was compared to PCS that was made from the control cheddar cheese without nisin. The cheese was incubated at 37°C and 22°C for up to 6 months and all batches containing nisin showed significantly longer shelf-life than there moisture counterpart without nisin. The high moisture spreads spoiled faster than the lower moisture spreads, and the spreads incubated at 37°C spoiled faster than

those at 22°C. The cheeses were inoculated with approximately 2000 spores per gram of spread, and the PCS was considered spoiled by off odors and gas production. All eight batches of the higher moisture PCS without nisin incubated at 22°C spoiled after 14 days, whereas the PCS containing nisin at the same temperature did not spoil until the 87th day. At 37°C the lower moisture PCS without nisin spoiled before 24 days, and the PCS containing nisin did not spoil during the 90 day incubation period tested (Zottola et al. 1994).

Another study was done on the effect of different antimicrobials in raw milk cheese by Gadotti at the University of Minnesota. The addition of nisin, caprylic acid, trans-cinnamaldehyde, monolaurin, carvacrol, eugenol, levulinic acid, orange-terpenes, d-limonene, eucalyptol, thymol, and clove essential oils were evaluated in their ability to inhibit the growth of *Salmonella*, *L. monocytogenes*, *and E. coli* O157:H7 in queso fresco. The findings showed that nisin, caprylic acid, and cinnamaldehyde were the only compounds to show a significant level of inhibition of the three organisms. When testing different concentrations of the compounds, a level of nisin at 0.5g/kg was most effective (Gadotti 2011).

1.5.2 Additional Commercial Antimicrobials

Other antimicrobials have been tested on the effectiveness in process cheese spreads. Borch and Lycken studied the effects of long-chain polyphosphate on *Clostridium sporogenes* and *Clostridium cochlearium*. At 1.5% and 0.75% polyphosphate, total inhibition of *C. sporogenes* and *C. cochlearium* spores was observed respectfully, and when combined with a heat treatment of 5 min at 98°C, a 0.75% polyphosphate level was

sufficient to inhibit sporulation for both organisms (Borch and Lycken 2007). Also the use of organic acid salts was shown to inhibit *Clostridium perfringens* germination and outgrowth in roast beef despite abusive cooling schedules (Sanchez-Plata et al. 2005).

Some organic acid salts are used to control or inhibit the growth of pathogens in food as a secondary measure of safety to sufficient heating. In dairy spreads where the fat content is sufficiently low (below 20%) the solution is viewed as an "oil-in-water" mixture where microbes have trouble growing from the lack of nutrients in the aqueous phase. Once dairy fats and other dairy ingredients are added, the safety of the product is compromised and preservatives such as salts are often used to help with stability. Benzoic acid or sorbic acid are commonly used in their salt form in the concentrations of 0.1% with a fat content greater than 60% or 0.2% with a lower fat content. In low-acid foods, where the pH is less than 5.0, sorbic acid is undissociated making it more effective so sorbic acid is a well-known antimicrobial in these conditions (Zijl and Klapwijk 2000).

In the meat industry a number of organic acid salts are used for flavor enhancers as well as microbial inhibitors. Sodium and potassium lactate used in the meat and poultry industry when controlling *Listeria monocytogenes* (Juneja and Thippareddi 2004), and sodium citrate and citric acid are the most favored generally recognized as safe (GRAS) ingredients for other pathogens like *C. perfringens* (Sanchez-Plata et al. 2005). Sodium and potassium lactate are added at 4.8% of weight while less than 0.25% of sodium acetate and diacetate are suggested for flavor enhancement and microbial inhibition in processed meat products (Juneja and Thippareddi 2004). Much of the data available for spore heat resistance and antimicrobial organic acids salts are for *Clostridia*

species and *Bacillus stearothermophilus*. What has been observed in these studies is that the strength of antimicrobial effects is in the order of acetic > lactic > malic > tartaric (Leguerinel and Mafart 2001).

Introduction

The thermal processing associated with process cheese sauce makes the product commercially sterile and thus safe before it is distributed to the consumer. The safety concerns materialize once in the hands of the consumer where it could be contaminated through destruction of the primary package or by poor sanitary practices in a restaurant setting. The dairy industry is becoming pro-active and taking precautionary steps for the safe consumption of process cheese sauce. By determining the survival and germination potential of common spore-forming bacteria that could contaminate the product, certain guidelines could be developed to minimize a foodborne risk.

While other pathogens have the same avenue for contamination that spore-forming bacteria do, their potential for infection is reduced because the high temperatures reached in typical cooking and heating in food service (greater than 60°C) are sufficient to kill vegetative cells. Spores on the other hand can survive the sub-lethal heat and remain in the cheese sauce during holding time on self-serving food service stations and germinate more rapidly in storage due to the heat activation of the spores. *Bacillus cereus* and *Clostridium perfringens* are two ubiquitous spore-forming organisms that are capable of contaminating and surviving in the cheese sauce environment. *Bacillus cereus* surveillance has increased recently because of its ability to grow at psychrotrophic and thermophilic temperatures, and *Clostridium perfringens* is among the top 5 pathogens causing foodborne illness annually.

The objectives of this study were to:

1. Determine the growth of spore forming bacteria at different temperatures in cheese sauce.

Temperatures were chosen based on their relevance to the restaurant industry and the organism. Two different refrigerator temperatures; one of a properly working refrigerator (4°C) and one of on old refrigerator (12°C), room temperature (23°C), and two temperatures in the upper growth range of the organisms (37°C and 45°C) were used to evaluate survival and growth potential of each bacteria in the process cheese sauce.

2. Identify the effects of repeated heating and cooling on germination capacity of spore forming bacteria in cheese sauce.

Different scenarios were designed to simulate buffet restaurant heating and cooling schemes including good and improper handling practices.

3. Determine the survival of thermophilic *Bacillus cereus* and *C. perfringens* in cheese sauce.

Cheese sauce was held in storage at 4, 12, and 23°C for 2 months to evaluate how long-term storage affected survival and growth potential.

4. Assess the efficacy of antimicrobials to develop the methods to control this spore formers in cheese sauce

Based on the results of the first 3 objectives, nisin was added to the process cheese sauce to evaluate how well it inhibited growth in a worst case scenario temperature abuse for the samples.

Our hypotheses for this experiment are as follows:

H_0: Traditional conditions of the supply chain of process cheese sauce would not support germination of *B. cereus* and *C. perfringens.*

H_a: Traditional conditions of the supply chain of process cheese sauce would support germination of *B. cereus* and *C. perfringens.*

Chapter 2

Materials and Methods

2.1 Bacterial Strains

The strains used for this project are listed in Table 4 below. Because of the variability of sporulation in *Clostridium perfringens* in the lab, outbreak strains used were provided by the University of Minnesota's Veterinary and Biomedical Sciences (VBS) at the College of Veterinary Medicine and were held at -55°C in a storage freezer and then converted to a working culture before they were harvested for spores. All the *Clostridium perfringens* strains were type A and isolated from cellulitis lesions in turkeys in Minnesota (Thachil et al. 2010).

Table 4: List of *Bacillus cereus* and *Clostridium perfringens* strains used in this study and their origin

Organism	Strain	Source
Bacillus cereus	14579	ATCC
	9139	ATCC
	10987	ATCC
Clostridium perfringens	UMNCP- 01	VBS
	UMNCP- 02	VBS
	UMNCP- 03	VBS
	UMNCP- 04	VBS
	UMNCP- 05	VBS
	UMNCP- 06	VBS
	UMNCP- 07	VBS
	UMNCP- 08	VBS
	UMNCP- 09	VBS
	UMNCP- 10	VBS
	3624	ATCC

2.2 Process Cheese Sauce

Two brands of commercial process cheese sauce were used in this study and supplied by two different companies. Cheese sauce brand A contained 9.0% natural cheese and was sealed in hermetic aluminum cans containing 250 grams of product per can. Cheese sauce brand B contained 33% natural cheese and was contained in large aseptically packaged plastic pouches with 1,000 grams of product per bag. The formulation for brand A can be seen in Table 5. Measurements of pH were determined before inoculation using an Oakton pH electrode (Oakton Instruments, Vernon Hills, Il) and water activity will be determined using Aqua lab a_w meter (Decagon Devices, Inc., Pullman, Wa).

Table 5: Approximate formula for cheese sauce brand A provided by the company

Ingredient	**Approximate amount (%)**
Skim milk	50-55
Water	10-15
Cheddar cheese (cultured milk, salt, enzymes)	10-15
Partially hydrogenated soybean oil	5-10
Modified Food Starch- Corn	4-9
Salt	1.0-2.5
Sodium phosphate	0.5-1.5
Lactic acid	0.05-0.5
Mono and diglycerides	0.05-0.5
Annatto & oleoresin paprika color	0.05-0.5
Spices	0.01-0.05

2.3 Spore Harvesting Method

2.3.1 *Bacillus cereus*

Spores were grown from pure stock culture and were first streaked onto tryptic soy agar (TSA; Neogen, Inc., Lansing, MI) media then incubated at 37°C for 24 hours. Single colonies from each strain were transferred from TSA to separate tryptic soy broth (TSB; Neogen, Inc.) tubes and incubated at 37°C for another 24 hours. One hundred µL of TSB cultures were spread plated onto sporulation media and incubated at 37°C for 48 hours, followed by additional 24 hours at room temperature. The biomass was re-suspended by pouring sterile distilled water onto the plates and gently scraping the colonies off of the media with a loop and transferred to sterile centrifuge tubes by serological pipettes. A total of 30 ml of water were used to re-suspend the biomass for each strain in centrifuge tubes. The tubes were incubated at 37°C for 72 hours. The spore suspensions were centrifuged at 4°C at 8,000 × g for 10 min using a RC-5 Superspeed refrigerated centrifuge (Dupont Company, Willington Delaware). The supernatants were removed, 30 ml sterile water was added to the tubes to wash the spores, and they were centrifuged again at the same speed. The supernatant was removed and washed for a second time and 30 ml were added to each tube for the last time. The spores were stained using malachite green and viewed under a phase-contrast microscope to check for successful harvesting and stored at 4°C for no more than 2 weeks.

2.3.2 *Clostridium perfringens*

The sporulation of *C. perfringens* was based on the methods developed by Duncan and Strong (Duncan and Strong 1968), but slightly modified according to recommendations by Dr. Ron Labbe (Hsieh and Labbe 2007, Juneja et al. 1993). All incubations took place at 37°C in an anaerobic glove box. Multiple strains were tested for

their ability to sporulate and the three that sporulated best were used in the cocktail for the inoculum. Cells were taken from the prepared stock solutions for each strain and 1 mL was placed in 9 mL of DS media (0.4% yeast extract, 1.5% proteose peptone, 0.4% soluble starch, 0.1% sodium thioglycolate, and 1.0% $Na_2HPO_4 \cdot 7H_2O$) (Duncan and Strong 1968) and incubated for 24 hours. Volumes of 1 mL of the stock solution/DS media mixtures were transferred to 9 mL of fluid thioglycolate (FTG) medium (formula/L; enzymatic digest of casein 15 g, yeast extract 5 g, dextrose 5.5 g, l-cystine 0.5 g, sodium chloride 2.5 g, sodium thioglycollate 0.5 g, resazurin 0.001 g) and incubated for 24 hours. FTG media (1 mL) were spread plated onto potato peptone media (potato decoction 200 mL--infusion from 200 g potato--, yeast extract 1.0 g, peptone 5.0 g, agar 30.0 g, distilled water 800.0 mL) and incubated for 48 hours, and the rest of the FTG was kept to supply vegetative cells for inoculum. The biomass on the potato peptone agar was re-suspended in sterile distilled water using a loop to gently scrape the spores from the agar surface and combined with the vegetative cells from the FTG in a sterilized centrifuge tube. The mixture was then centrifuged at 4°C at 8,000 × g for 10 min. The supernatants were removed and spores were washed and stored as described for *B. cereus* before.

2.4 Cheese preparation for inoculation

Cheese was held at room temperature in the storage containers in which they were supplied, either in cans or vacuum-sealed bag. Cheese sauce was distributed aseptically into stomacher bags by single use adult tongue depressors next to a flame. The control

bags had 100 grams of cheese sauce and the treatment bags had 99 grams of cheese sauce.

2.5 Inoculation of cheese sauce

One trial consisted of 2 separate treatment bags and 2 separate control bags, for a total of 4 bags per trial. Each trial was run in duplicate and the results were averaged.

2.5.1 *Bacillus cereus*

Once the spores were harvested 5 mL from each strain was taken from their respective centrifuge tube and placed into separate, sterile 50 mL centrifuge tubes. The tubes were placed in a hot water bath for 30 minutes at 75°C and 2 mL of each suspension was combined in a single tube. The cocktail was serially diluted in phosphate buffered saline (PBS) to achieve an overall population of 10^6 CFU/ mL before it was placed into treatment stomacher bags containing 99 g of cheese sauce to attain a population of 10^4 CFU/g. The populations of the inocula were determined by spread plating at the beginning of each trial to assure the bacteria population was at the correct level. Volumes of 1 mL of spore suspensions were combined with 99 grams of cheese sauce using a serological pipette. After the bags had been inoculated they were stomached for 1 minute in a stomacher lab blender 4,000 (Seward Laboratory Systems Inc, Port St. Lucie Fl.)

2.5.2 *Clostridium perfringens*

Because of the difficulty in acquiring *C. perfringens* spores, the spore suspension was not heat shocked before inoculation of treatment bags of cheese sauce. Inoculation of cheese bags followed the same steps as those used for *B. cereus*.

2.6 Sampling of cheese sauce

Cheese sauce samples (1 g) were taken with tongue depressors from the treatment and control bags and placed in centrifuge tubes and mixed with 9 mL of PBS in 50 mL sterile centrifuge tube. The sampling scheme for each temperature and organism is shown in Table 5. Samples were serially diluted using PBS according to incubation temperature and number of hours after inoculation and then spread plated on selective and differential agar to obtain a number of colonies between 15 to 300 per plate. Negative controls (non-inoculated) were spread plated at the limit of detection which was 10^{-2} . Samples were first spread plated for total cell count to find the amount of vegetative cells and then heat shocked and plated again for spore population. *Bacillus cereus* samples were heat shocked at 75°C for 30 minutes and *Clostridium perfringens* was heat shocked at 75°C for 20 minutes.

Table 6: Sampling scheme for cheese sauce inoculated with *Bacillus cereus* and *Clostridium perfringens*

Organism	Temperature (°C)	Sampling Time
Bacillus cereus	4	0, 3, 6, 15, 28, 56 (days)
	12	0, 1, 2, 3, 4, 5, 28, 56 (days)
	23	0, 1, 2, 3, 6, 28 (days)
	37	0, 2, 4, 6, 24, 48, 72, 144 (hours)
	45	0, 3, 6, 24, 48, 72, 144 (hours)
Clostridium perfringens	4	0, 5, 8, 15, 35 (days)
	12	0, 1, 2, 3, 7, 28 (days)
	23	0, 0.5, 1, 2, 3, 7, 28
	37	0, 0.17, 0.33, 1, 2, 6

2.7 Temperature Abuse Trials

The sampling schemes for the temperature abuse trials were set up to mimic conditions that could apply to those of a hypothetical food service buffet restaurant. The time and temperature the cheese sauce was heated would resemble the time the product would spend on the buffet line. A worst case scenario was chosen for the two organisms based on other temperature abuse trials found in the literature (Juneja et al. 1993, Sanchez-Plata et al. 2005, Cronin and Wilkinson 2008a, Lopez et al. 2003). The openings of the inoculated stomacher bags were folded over and secured using tape but not sealed, and were placed directly in a water bath to best simulate conditions on a buffet line. Inoculation and sampling during temperature abuse trials was performed in the same manner as written above for the static temperature trials.

2.7.1 *Bacillus cereus*

Three scenarios were generated based on common mistakes found in the restaurant industry. Scenario 1 was based on a worker leaving the cheese sauce out at room temperature for longer than 2 hours (the longest recommended time for food to be left out at room temperature according to the USDA) in between transferring it the hot buffet line and the refrigerator. Scenario 1 is also characterized by a buffet line that is heated to insufficient temperatures according to restaurant guidelines (above 60°C). Scenario 2 was based on good handling practices and allowing no time between the hot line and refrigerator, but still with insufficient heating while the inoculated product was on the

simulated buffet line. Scenario 3 was similar to scenario 1 except that the cheese sauce was heated to the proper temperature while on the buffet line. The sampling scheme for each scenario is displayed in Table 6.

Table 7: Different time temperature incubation scenarios in *B. cereus* trials simulating temperature abuse

Scenario 1		**Scenario 2**		**Scenario 3**	
Time (h)	**Temperature (°C)**	**Time (h)**	**Temperature (°C)**	**Time (h)**	**Temperature (°C)**
0	23	0	23	0	23
0 to 3	45	0 to 6	45	0 to 4	60
3 to 6	23	6 to 24	4	4 to 6	23
6 to 22	4	24 to 28	45	6 to 22	4
22 to 24	23			22 to 24	23
24 to 28	45			24 to 28	60

2.7.2 *Clostridium perfringens*

For *C. perfringens* most commercial outbreaks reported stemmed from improper cooling of the food product (Juneja et al. 1993, Sanchez-Plata et al. 2005), so cooling time after being heated in the hot water bath was longer than it was for *B. cereus*. The scenario used was to simulate a situation where the cheese sauce would sit on the buffet for the duration of the dinner shift while being heated properly (60°C/140°F). Once the busiest time of the evening was over, a worker turns off the heating mechanism for the cheese and allows it to sit on the line for the rest of the night until the very end of clean-up when it is finally put into a walk-in cooler to chill overnight and the whole process is repeated the following day. The sampling scheme for the *C. perfringens* temperature

abuse trial (scenario 4) can be seen in Table 7 below. Samples were plated in the same manner as the static temperature trials and incubated anaerobically in a glove box.

Table 8: Sampling scheme for *C. perfringens* trial simulating temperature abuse

Scenario 4	
Time (hours)	**Temperature (°C)**
0	23
4	75
8	28
23	4
26	23
30	60
33	23

2.8 Antimicrobial Treatment Experiment

The temperature and organism used for the antimicrobial trial was based on the results from the single temperature trials. Because *C. perfringens* did not grow in the cheese sauce medium only *B. cereus* was used. The temperature where *B. cereus* had the most rapid and extensive growth was used which was 37°C in this case. The same sampling schedule was used as in Table 6, but the treatment bags contained 0.5 g/kg as Nisaplin® (which contains 2.5% nisin; Danisco, Inc., Copenhagen, DK) based on the work done by Gadotti (Gadotti 2011).

2.9 Bacterial Enumeration

Samples were plated on selective agar for enumeration. *Bacillus cereus* samples were plated on mannitol egg yolk polymyxin B (MEYP; Neogen, Inc.) agar according to Mossel, Koopman, and Jongerius (Mossel, Koopman and Jongerius 1967). The bacterium cannot dissimilate mannitol and the polymyxin B serves as the selective agent to produce

colonies that appear rough and dry with a bright pink background surrounded by an egg yolk precipitate. *Clostridium perfringens* was plated on anaerobic blood agar (TSA containing per liter: yeast extract, 5.0 g; hemin, 0.005 g; vitamin K1, 0.01 g; L-cystine, 0.4 g; 5% defibrinated sheep blood) using the standard method stated by the ATCC (Thachil et al. 2010). Colonies were differentiated by their hemolytic reactions as seen by the double zone beta – hemolysis (Systems 2007). Spread plates were incubated at 30°C for 24 h for *B. cereus* and 37°C for 48 h in an anaerobic glove box for *C. perfringens*. Final counts were calculated based on the dilution factors. Controls were prepared by streaking the stock culture onto the selective media for comparison.

2.10 Statistical Analysis

The count of bacterial strains for each individual sample was an average of two plate counts per treatment bag, two treatment bags per trial, and two trials for each experiment for a total of 8 values per data point. This value was transformed to $\log_{10}$ CFU/g scale. Positive samples detected only by enrichment were assigned a value of 1 CFU/g to be included into the calculation. The results for the two independent experiments of each treatment were used to calculate the mean and standard deviation at each sample point per treatment. The average data points at each sampling time were plotted or tabulated against a time course where the differences among control and treatment groups could be visualized and standard deviation error bars displayed. Counts of vegetative cells and spores from each bacterium in the logarithm form were analyzed using a typical ANOVA test where $p<0.05$.

Chapter 3

Results

3.1 Growth of *Bacillus cereus* in Cheese Sauce at Different Temperatures

A series of experiments were used to determine if *B. cereus* grew at different temperatures in cheese sauce. The detectable limit for all trials was 100 CFU/g and un-inoculated control bags were tested in each trial to rule out the possibility of natural *Bacillus* contamination. None of those negative controls yielded any bacterial growth and the data was not included in any of the graphs. The pH of the cheese sauce was found to range from 5.8-5.96 and the a_w measured 0.99.

B. cereus was the first bacterium evaluated because of its relatively simple growth requirements. Figure 5 shows the count of vegetative cells of *B. cereus* incubated at five different temperatures in brand A cheese sauce for a short incubation time. At 4°C *B. cereus* count remain constant during the experiments, and at 12°C the population remained at the inoculum level until day four when it increased to less than 1 log CFU/g. *B. cereus* grew much better when held at or above room temperature (23°C). *B. cereus* grew faster at 37°C as its count reached almost 8 log CFU/g in 24 h. The only significant difference in the growth plots is after 24 hours when the population at 37°C is much higher than the other four temperatures. Cells incubated at the three highest temperatures attained population levels of more than 7 log CFU/g. The growth at 37°C had the shortest lag time, but reached its stationary phase first and began dying after three days whereas 23 and 45°C reached their stationary phase at three days and continued to a higher population closer to 8 log CFU/g.

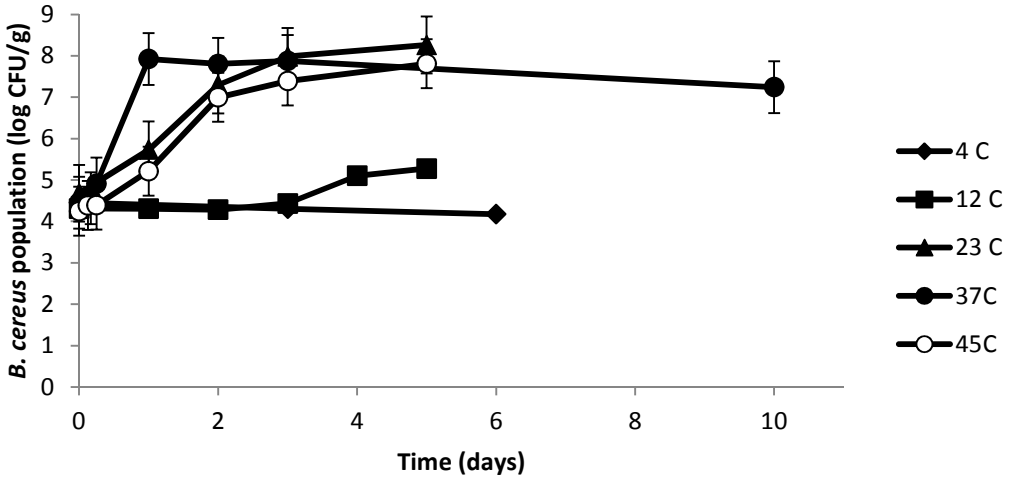

Figure 5: Effect of temperature on the growth of *Bacillus cereus'* total cell count in cheese sauce brand A. Each plot represents an average of two separate trials run in duplicate.

After samples were plated, dilutions were heated for 30 minutes at 75°C to kill off any vegetative cells and determine spore counts. The amount of *B. cereus* spores at different temperatures is depicted in Figure 6. Sporulation was not supported in cheese sauce except at 23°C where the population level was significantly higher than it was at all the other temperatures and increased by more than 2 log CFU/g. At the other temperatures the spore population level did not increase more than 1 log CFU/g. As the population of vegetative cells began to plateau in the stationary phase at 3 days, the population of spores increased suggesting sporulation. It can be seen in the figures that the population of spores appeared to decline initially but then recovered and at two temperatures increased past the initial level of inoculation.

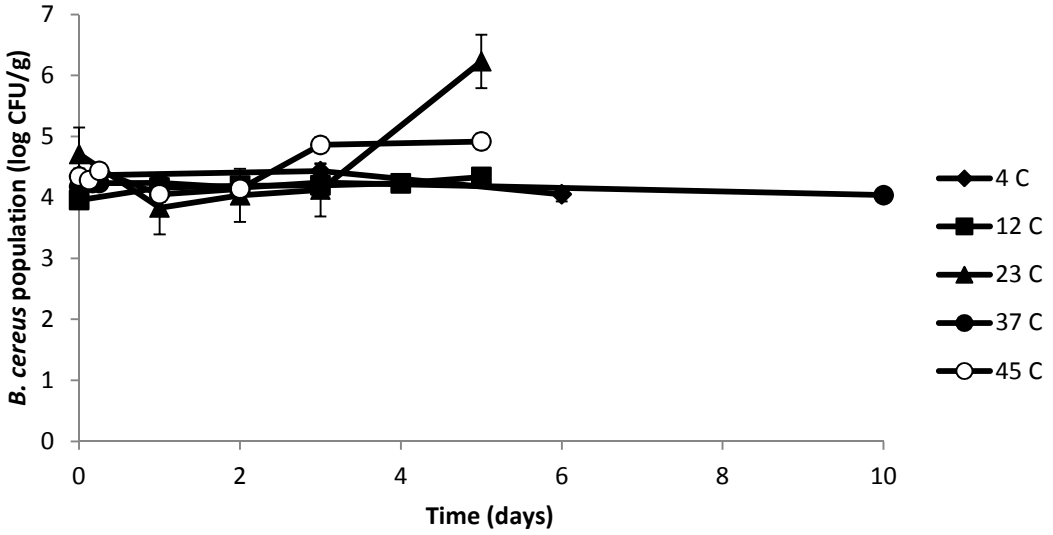

Figure 6: Effect of temperature on *Bacillus cereus'* spore counts in cheese sauce brand A. Samples were plated after heating for 30 min at 75°C. Each plot represents an average of two separate trials run in duplicate.

Bacillus cereus growth and survival were also tested in another process cheese sauce brand to determine if a different commercial formulation would change the pattern of growth. The process cheese sauce brand B had a higher content of natural cheese, 33% as compared to 9%. The trial run at 45°C was included to serve as an upper limit of growth for the mesophilic organism with brand A, but it was not included with brand B because 37°C was a more optimum temperature of growth. The count of vegetative cells of *B. cereus* when cheese sauce samples of brand B were incubated at four temperatures is depicted in Figure 7.

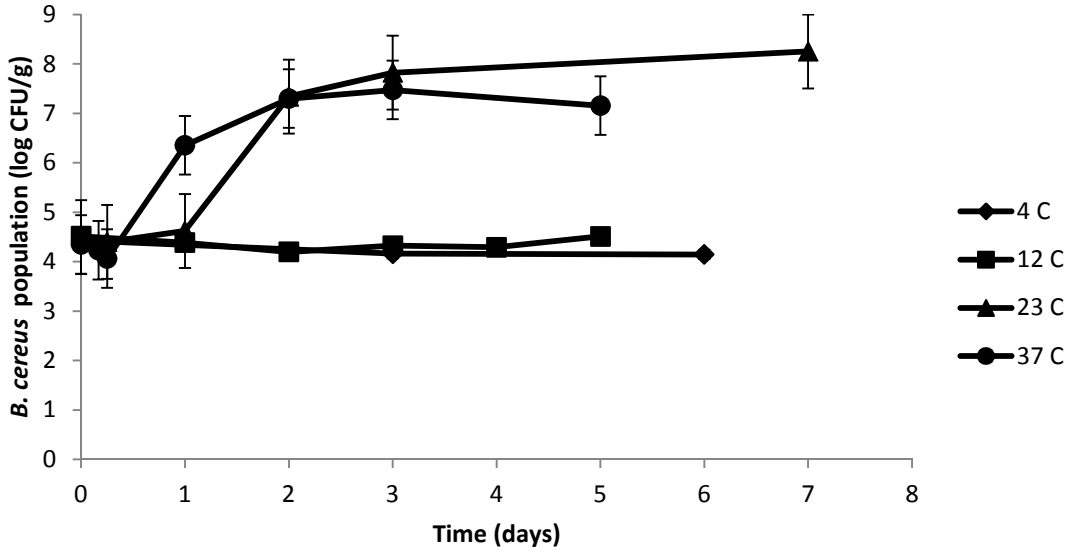

Figure 7: Effect of temperature on the count of *Bacillus cereus'* total cell count in cheese sauce brand B. Each plot represents an average of two separate trials run in duplicate.

The growth was very similar to that observed in brand A. At day one, the count at 37°C was significantly higher than those at other temperatures but after two days, the samples at 23°C had similar levels of approx. 7 log CFU/g. Just like in brand A, *B. cereus* growth at 23°C also surpassed the growth at 37°C by day two, and reached stationary phase with a maximum population of 8 log CFUs/g. The growth at 4°C and 12°C was not supported as well in brand B as in brand A because as counts remained at almost the initial inoculation during the experiments. The spore counts of *B. cereus* in brand B were also similar to those in brand A (Figure 8), but unlike with brand A, the spores at 23°C did not increase in population. None of the spores in cheese sauce brand B increased by more

than 1 log CFU/g.

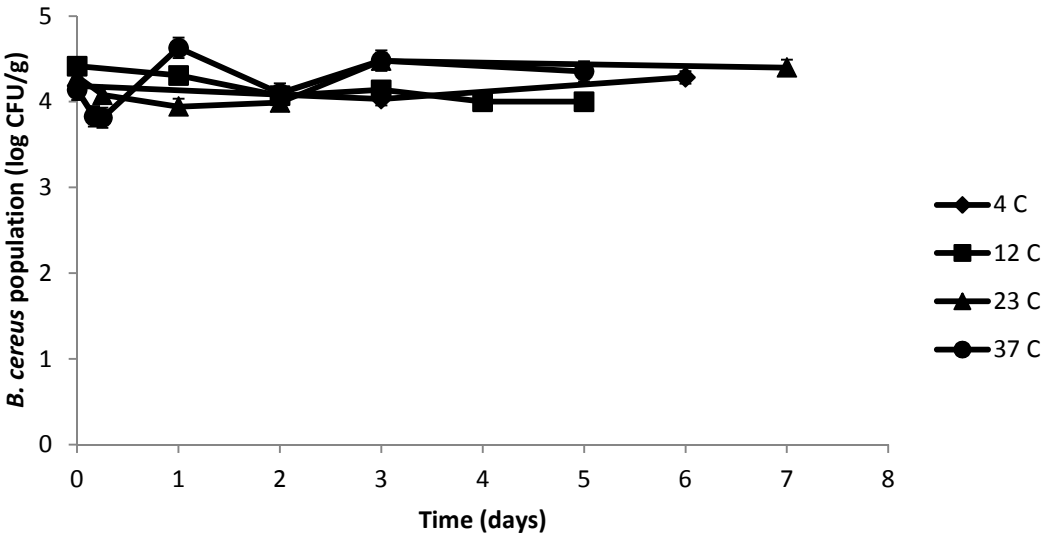

Figure 8: Effect of temperature on *Bacillus cereus'* spore counts in cheese sauce brand B. Samples were plated after heating for 30 min at 75°C. Each plot represents an average of two separate trials run in duplicate.

3.2. Growth of *Clostridium perfringens* in Cheese Sauce at Different Temperatures

Experiments conducted with *Clostridium perfringens* inoculated cheese sauces resulted in different growth patterns than *B. cereus*. Only brand A was used for *C. perfringen's* trials. Growth of *C. perfringens* was not supported in cheese sauce brand A at any temperature of incubation (Figure 9). At almost any temperature the direct bacterial remained almost unchanged at approximately 4 log CFU/g with the exception of samples incubated at 12°C which increased its population almost 0.5 log CFU/g, but was significantly different to the count at 23°C at day seven. The spore count remained at

almost the same levels as the initial inoculation level for all temperatures, with only non-significant differences (Figure 10).

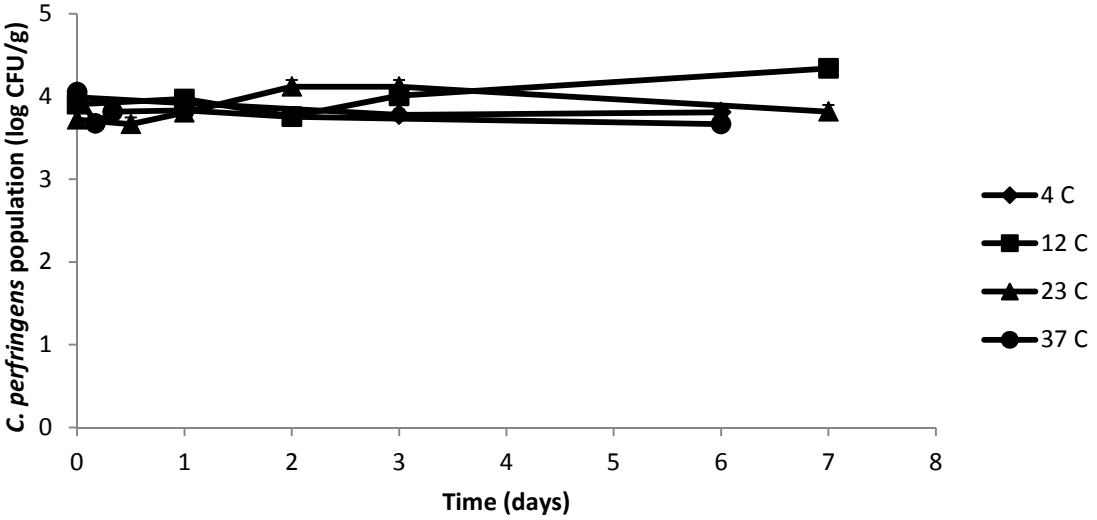

Figure 9: Effect of temperature on the counts of *Clostridium perfringens'* total cell count in cheese sauce brand A. Each plot represents an average of two separate trials run in duplicate.

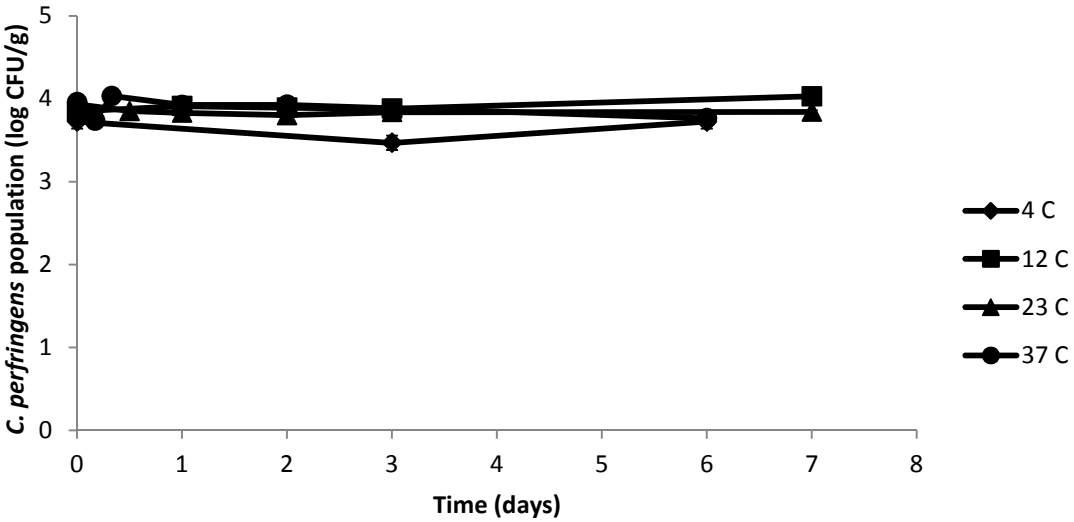

Figure 10: Effect of temperature count of *Clostridium perfringens'* spores in cheese sauce brand A. Samples were plated after heating for 30 min at 75°C. Each plot represents an average of two separate trials run in duplicate.

3.3 Temperature Abuse Trials

A total of 5 different temperature abuse scenarios were used to evaluate the effects of a mishandled process cheese sauce product at a commercial level that was contaminated upon arrival. Four of the scenarios included *B. cereus* and the last trial used *C. perfringens*.

3.3.1 Scenario 1

The first scenario (Figure 11 and Figure 12) represented a case in which an employee would leave the inoculated cheese sauce product out at room temperature after being removed from the buffet line that was not heated properly (below 60°C) and again allowing the product to sit at room temperature before being placed on the line the following day. The growth in cheese sauce brand A is displayed in Figure 11, and cheese sauce brand B in Figure 12. Times where the product was held at room temperature was no less than 2 hours which the U.S. Department of Health and Human Services recommends be the longest amount a time a food should remain on the counter without heating or refrigeration. The count of *B. cereus* vegetative cells did not increase until the second period where the product was incubated at room temperature. The vegetative cells continued to increase by 1 log CFU/g to a level greater than 4.5-log CFU/g as the cheese sauce was heated at 45°C, a hypothetical abuse temperature on the simulated buffet line.

Fluctuating temperatures did not appear to have an affect the bacteria's spore count. The level of spores decreased throughout the scenario but by less than 1-log CFU/g. In brand B, the growth during the last stages of the experiments was only 0.5 log CFU/g.

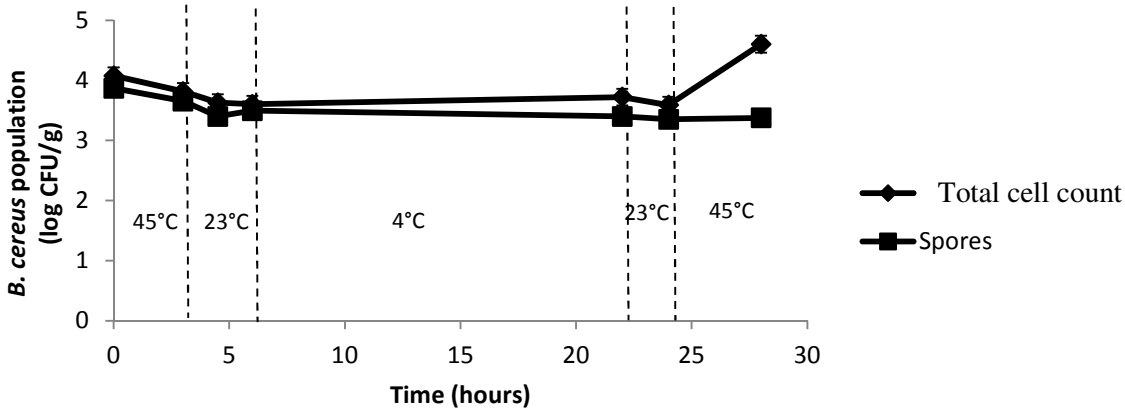

Figure 11: Effect of fluctuating temperature on the survival and germination potential of *Bacillus cereus* in cheese sauce brand A. The scenario represents a commercial setting in which cheese sauce is left at room temperature before and after refrigeration.

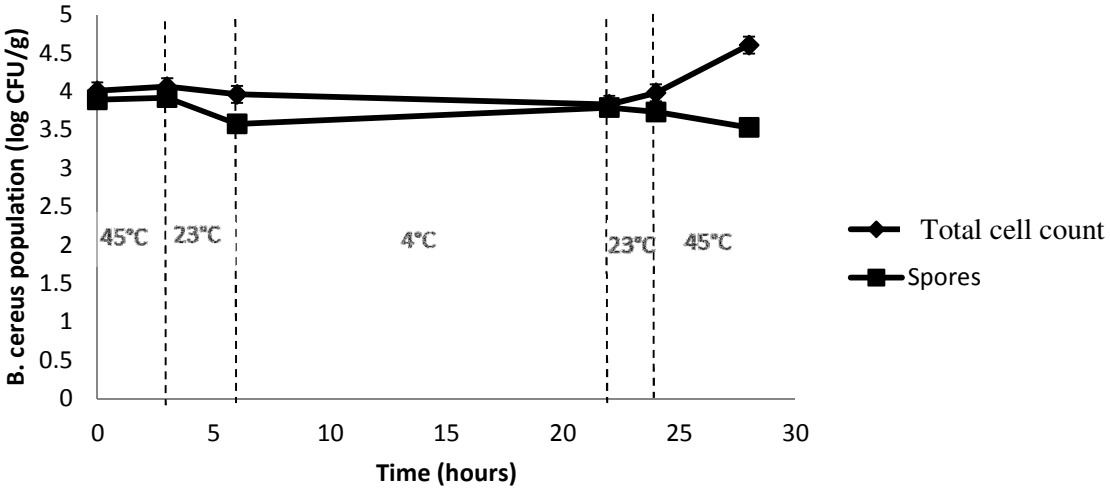

Figure 12: Effect of fluctuating temperature on the survival and germination potential of *Bacillus cereus* in cheese sauce brand B. The scenario represents a commercial setting in which cheese sauce is left at room temperature before and after refrigeration.

3.3.2 Scenario 2

The second scenario, represented an inoculated cheese sauce that was properly refrigerated, in that there was no holding time at room temperature between heating and refrigeration, but still with a temperature (45°C) on the simulated buffet line that was insufficient to kill vegetative cells (Figure 13). The vegetative cell counts followed a trend similar to that of the first scenario during most of the incubation periods, where there was no noticeable growth observed except for a half log increase during the second heating period. The spore count in this scenario was also unaffected, as it was in the first scenario, and declined less than 0.5 log CFU/g during the experiments.

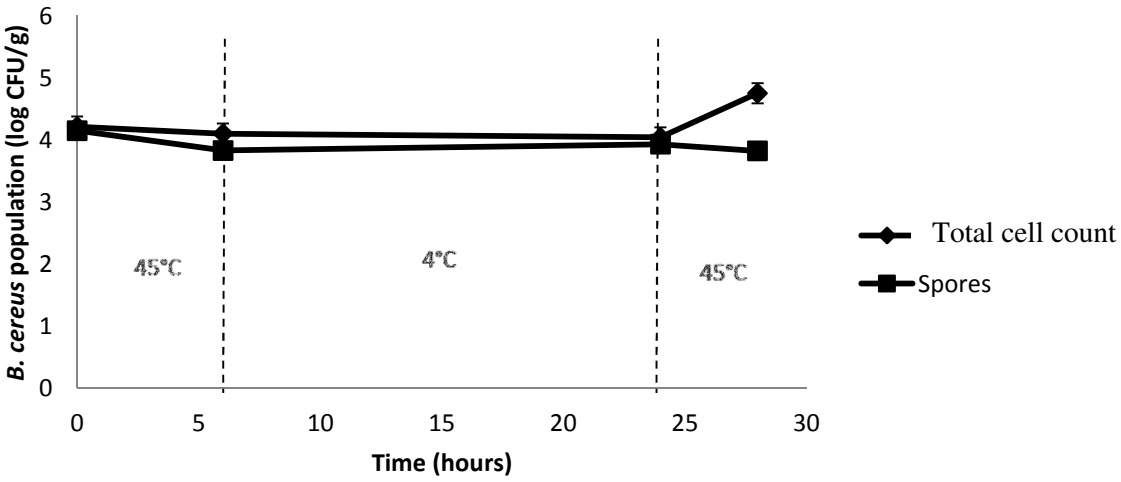

Figure 13: Effect of fluctuating temperatures on growth and survival of *Bacillus cereus* in cheese sauce brand A. This scenario represents cheese sauce at a buffet that is refrigerated properly but is heated insufficiently on the line.

3.3.3 Scenario 3

The third scenario (Figure 14) was similar to scenario 1, but the cheese sauce was heated to a regulated temperature on the simulated buffet line (60°C). In contrast to the results from that of the first scenario, no significant change in vegetative cell counts were measured. The counts remained at the inoculation level for a few hours and then gradually declined. The testing period was not long enough for the vegetative cells to decline more than half a log CFU/g. The spore count was also very different from that of the other scenarios. The two areas of the graph when the population declined was during the holding time at 23°C and increased by 1 log CFU/g during the incubation at 4°C. By the end of the 28 hour temperature abuse trial the overall spore population was slightly less than the original inoculation level.

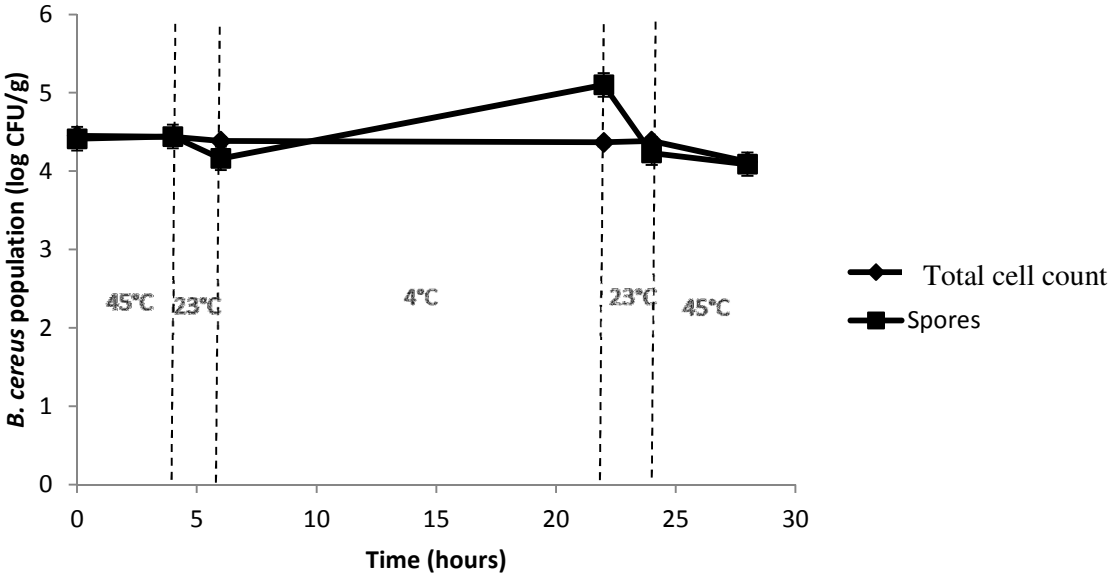

Figure 14: Effect of fluctuating temperature on the survival and germination potential of *Bacillus cereus* in cheese sauce brand A. The scenario represents a commercial setting in which cheese sauce is left at room temperature before and after refrigeration but heated to the proper temperature on the buffet line.

3.3.4 Scenario 4

Scenario four (Figure 15) was a worst case scenario of temperature abuse for *C. perfringens* in cheese sauce brand A. The cheese sauce inoculated with *C. perfringens* was heated to 65°C and allowed to cool to 4°C in approximately 9 hours. The fluctuating temperatures and improper cooling scheme were not enough to induce *C. perfringens* growth in the cheese sauce medium, or to support sporulation or germination. The vegetative cells survived the first holding time at 65°C but then declined quickly when

held at 23°C. After that initial period, both spore and cell counts reached undetecable levels for the reminder of the experiments.

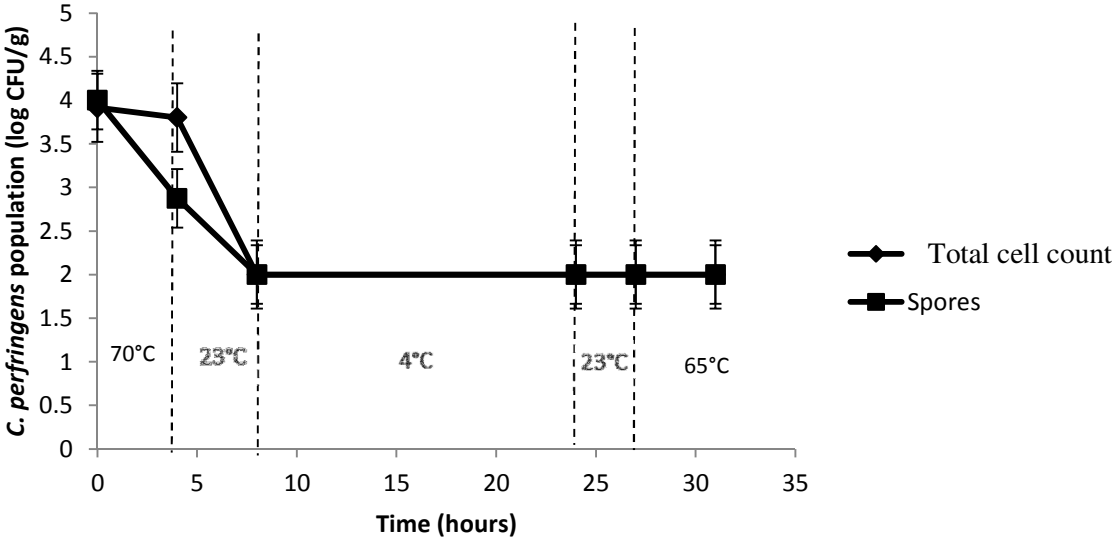

Figure 15: Effect of fluctuating temperature on the survival and germination potential of *Clostridium perfringens* in cheese sauce brand A. The scenario represents a worst case commercial scenario in which cheese sauce is improperly cooled before refrigeration. The detetion limit was 2.0 log CFU/g.

3.4 Bacteria Survival during Long-Term Storage

The effect of long term storage on the survival and growth of *Bacillus cereus* and *Clostridiuim perfringens* was studied at 4, 12, and 23°C. The experiments at higher temperatures were not conducted for more than 30 days because the extensive deterioration resulting in physical and chemical changes. Visual detection of quality problems included dried out/hard cheese, discoloration, growth of mold, appreance of

translucent liquid, and foul odor. A series of trials was run to determine the survival of mixed trains of *C. perfringens* and *B. cereus*.

3.4.1 *Bacillus cereus*

In both brands, rapid growth of vegetative cells at 23°C was observed during the first 2 days of the experiments and reached a maximum cell count after 5 days. At 12°C, slow growth was observed and depending on the cheese sauce brand, the count increased from 1 to 3 log CFU/g after 30 days. In both cases, the final cell density was approx. 7 log CFU/g. Vegetative cell growth was not significant at 4°C. The growth of *B. cereus* during long term storage in cheese sauce brand A is depicted in Figure 16. The treatment bags were no longer tested once visual and olfactory defects were apparent. During the one month the cheese sauce was held at 23°C the population of cells began to decline. While the growth of *B. cereus* at 12°C was not very rapid, when held for two months the population eventually reached a level of 7 log CFU/g.

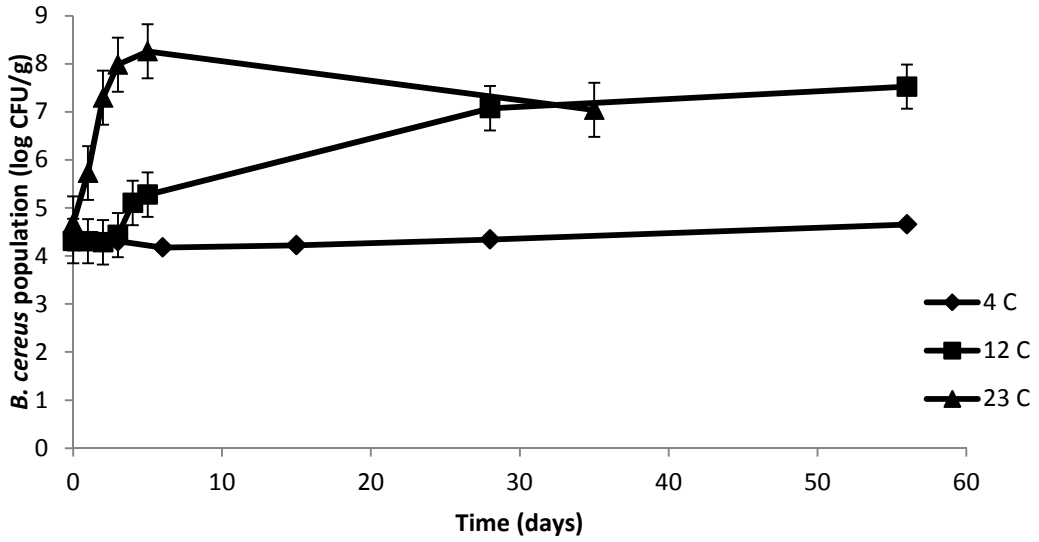

Figure 16: Effect of long term storage on the counts of *Bacillus cereus'* total cells in cheese sauce brand A. Each plot represents an average of two separate trials run in duplicate.

The experiments run in the second brand of cheese sauce (Figure 17) produced similar results where the cell counts at 12°C reached a level of 7 log CFU/g and the numbers at 23°C began to decline after 10 days. The cell count at 4°C increased to higher levels than in brand A, but still less than 1 log CFU/g.

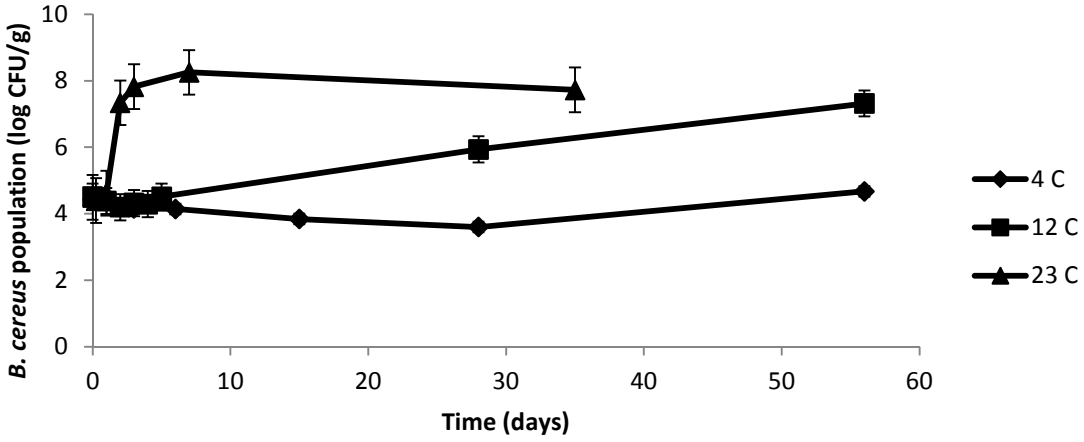

Figure 17: Effect of long term storage on the counts of *Bacillus cereus'* total cells in cheese sauce brand B. Each plot represents an average of two separate trials run in duplicate.

The spore counts remained constant in cheese sauce kept at 4°C temperatures (Figure 18 and Figure 19). The spore population at 23°C increased by almost 2 log CFU/g in cheese sauce brand A samples after 5 days, but this change was not observed with brand

B samples. At 12°C, no significant change was observed after 30 days, but by 60 days 1 log CFU/g more spores were determined in both brands.

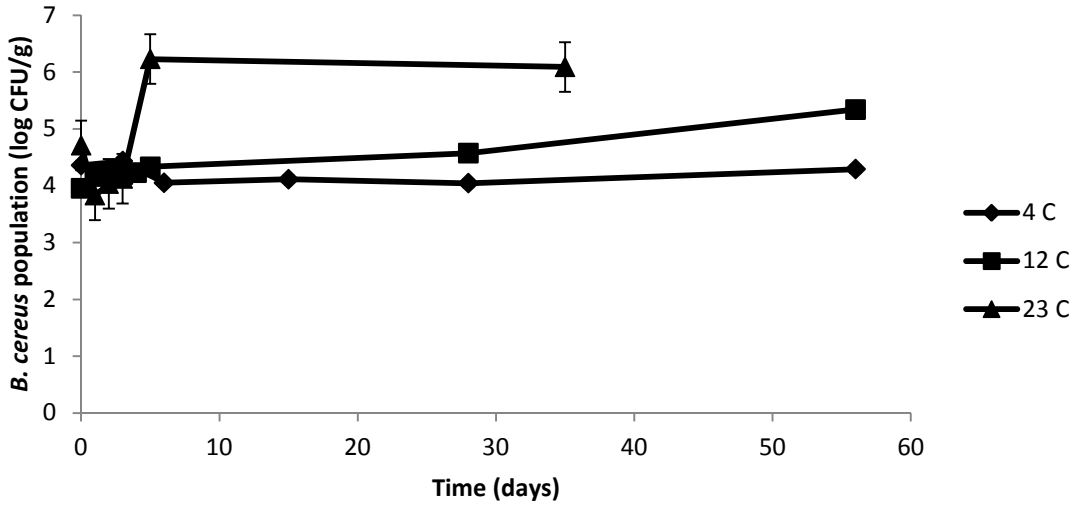

Figure 18: Effect of long-term storage on *Bacillus cereus'* spore counts in cheese sauce brand A. Samples were plated after heating for 30 min at 75°C. Each plot represents an average of two separate trials run in duplicate.

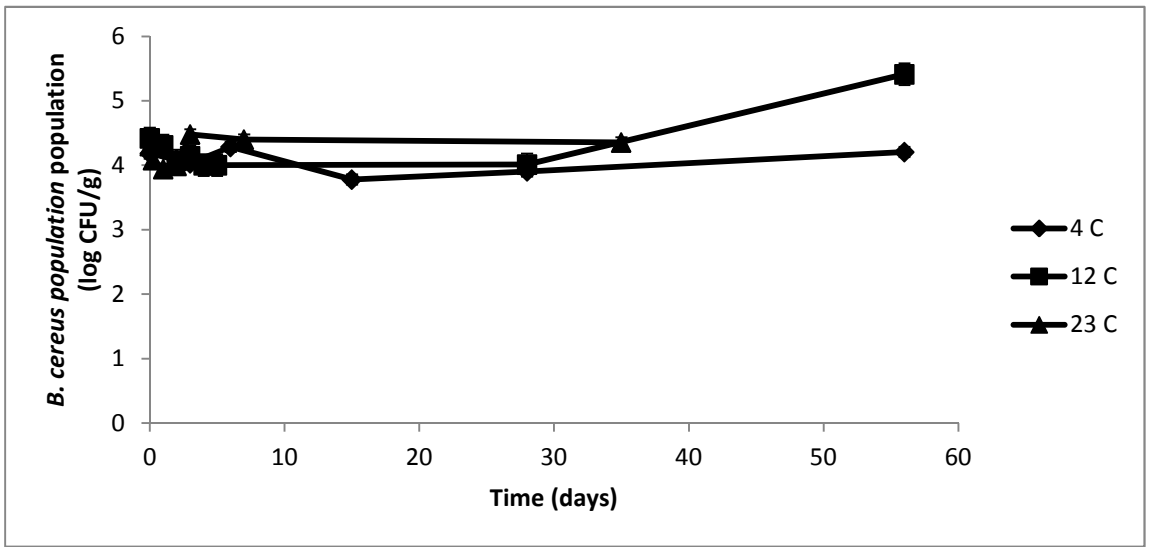

Figure 19: Effect of long-term storage on *Bacillus cereus'* spore counts in cheese sauce brand B. Samples were plated after heating for 30 min at 75°C. Each plot represents an average of two separate trials run in duplicate.

3.4.2 *Clostridium perfringens*

Similar to short term incubation, the *C. perfringens* was not capable of growing during long term storage in cheese sauce (Figure 20 and Figure 21). For the same reasons as stated earlier, storage at 37°C was not tested over a long term period due to extensive product deterioration. Vegetative cells and spore counts were very similar throughout the experiments, but there was some variability during the experiments. At 23°C, the cell count increased by more than 1 log CFU/g after 30 days.

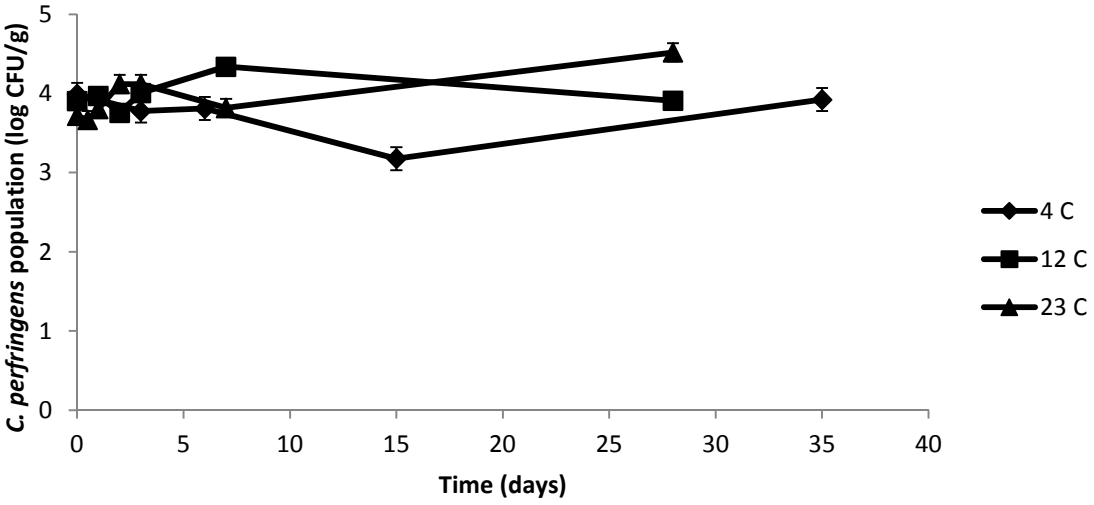

Figure 20: Effect of long-term storage on the growth of *Clostridium perfringens'* total cell counts in cheese sauce brand A. Each plot represents an average of two separate trials run in duplicate.

Sporulation (Figure 21) was not supported during long-term storage of *C. perfringens*. None of the counts at the three temperatures changed significantly in spore population during the one month incubation period.

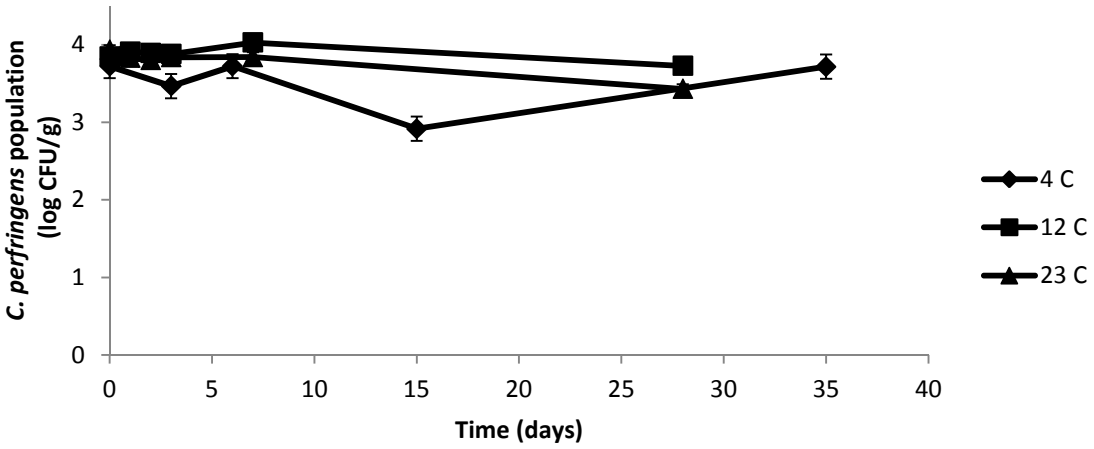

Figure 21: Effect of long-term storage *Clostridium perfringens*' spore counts in cheese sauce brand A. Samples were plated after heating for 30 min at 75°C. Each plot represents an average of two separate trials run in duplicate.

3.5 Addition of Nisin to Cheese Sauce

Since *C. perfringens* did not grow on cheese sauce, it was not used to test the effect on nisin. The worst case scenario of *B. cereus* growth was used to test nisin as an antimicrobial based on the results of objective 1. For this reason 37°C was used for testing the effects of this antimicrobial (Figure 22) in cheese sauce brand A with the addition of 0.5 g/kg Nisaplin. The growth of vegetative cells in control samples reached

maximum cell densities of 8 log CFU/g after 24 h, but nisin-treated samples had less than 6 log CFU/g counts at the same time. After 2 and 3 days, nisin-treated cheese sauce had more than 7 log CFU/g, a level slightly less than controls, but not statistically significant. The levels of spores in control samples were largely constant during the 10 day trial, but a slight increase of less than 1 log CFU/g was observed in the nisin treated samples by day 2.

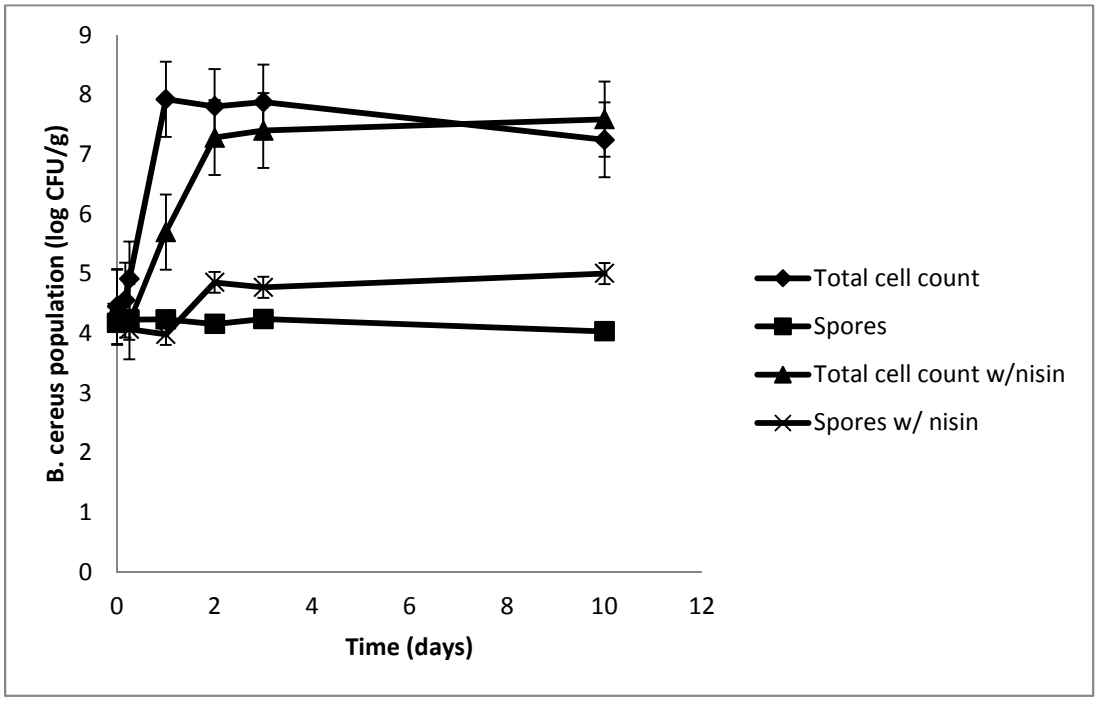

Figure 22: Effect of nisin on the growth and germination of Bacillus cereus' vegetative cells and spores in cheese sauce brand A at 37°C. Spores were determined by plating after heating for 30 min at 75°C

Chapter 4

Discussion

The objective of the study was to determine how typical food service handling conditions of process cheese sauce would affect the potential for germination and sporulation of two of the most important spore-forming foodborne pathogens, *Bacillus cereus* and *Clostridium perfringens*. This goal was accomplished by inoculating process cheese sauce and subjecting it to relatively high temperatures considered in the range of "abuse temperatures" to simulate a traditional restaurant buffet setting. Because of the high heat associated with the processing of process cheese sauce, the product is commercially sterile when delivered to the consumer; once opened it can be subjected to contamination. The cheese sauce is heated in restaurants to temperatures greater than 60°C which is sufficient to kill off vegetative cells but do not normally inactivate spore forming bacteria. With the competing flora eliminated the sub-lethal heat is enough to activate spores, and induce germination which would enhance the potential for illness (Lovdal et al. 2011).

To date research of spore-forming bacteria in cheese, especially process cheese sauce is relatively limited. Most reports are reviews on how processing affects meltability with brief references to microbial safety. There are studies, however, on these organisms in different food matrices and how temperature affects their survival. The main reason why there has been a lack of research to assess the potential of process cheese sauce as a vehicle for foodborne disease is the lack of reported illnesses to date. This project, however, was initiated by the interest of Minnesota cheese producers to anticipate any potential problems.

4.1 Bacterial Spore Observations

At all temperatures of incubation, *C. perfringens'* spore populations remained relatively constant (Figure 10) as none increased by substantial amounts (0.5 log CFU/g or more) compared with the initial inoculum. The same pattern was observed in *B. cereus* spores at all temperatures. The largest increase in spore counts was seen after cheese sauce was stored at 23°C, as the spore population increased by almost 2 log CFU/g. Spore counts in cheese sauce stored at 45°C also increased but only by approximately 1 log CFU/g. In some instances in which the spore count increased, this change was preceded by a slight decline in viable spores.

This phenomenon of spore populations decreasing before increasing to a maximum count has been previously described by Shoemaker and Pierson (1976) and named the "Phoenix phenomenon" after the mythological bird that is reborn from its ashes. The Phoenix phenomenon is associated with an injury-repair state of the spores and vegetative cells (Sanchez-Plata et al. 2005). After the spores are heat shocked their plate count decreases until they can adapt to the environment and sporulate beyond the initial inoculation level. Shoemaker worked to explain this phenomenon using different media and growing conditions for *C. perfringens*. The study observed that the initial decrease disappeared when optimal growth conditions (pre-reduced media, pre-reduced veal diluent, and strict anaerobic conditions) for the bacteria were present and concluded that the drop in cell population was the recovery time the spores needed after being heat shocked (Shoemaker and Pierson 1976). The cheese sauce matrix did not present an optimal environment to support the high recovery yield of *C. perfringens* or *B. cereus*

spores in our experiments, and significant increases in spore population, however, were not observed.

Some spore populations did not recover well until after a longer storage time (greater than 1 month). *B. cereus* vegetative cells in cheese sauce stored at 12°C did not show any significant increase until after 2 months of storage. This result suggests that the age of the bacteria was an additional stress at relatively low temperatures and induced sporulation. Similar findings have been observed in previous research. Collado et al (2003) reported findings where ageing spores reacted similarly to heat activated spores. When spores were newly harvested, they were easier to deactivate and showed a higher reduction number than spores that had been stored at 4°C for 22 days. Research by Collado concluded that aged spores, or spores that have been in storage were less likely to germinate (Collado et al. 2003).

4.2 *Bacillus cereus*

In our observations, *B. cereus* vegetative cells grew as much as 8 log CFU/g with variations according to different incubation temperatures. At temperatures of 23°C and above, the population increased by 4 log CFU/g, and at 12°C by 3 log CFU/g. No significant growth was observed at 4°C. This is in accordance with data reported in literature: *Bacillus cereus* is not normally capable of growing between 10°C and 12°C and it has an ideal growth temperature range between 30°C and 40°C (Labbe and Garcia 2001). For growth near the maximum range (45°C), the lag time was longer than when

held in the optimum temperature range (37°C), but after only two days reached levels that would be sufficiently high to cause illness if ingested.

Because the spores were grown at 37°C they may not have had a markedly high heat resistance. Spore strains are variable in their resistance to heat, but it has been reported that the higher temperature spores are formed the more resistant to heat they would be (Carlin et al. 2010). Though there are reports of thermophilic strains of *B. cereus* that will grow at temperatures as high as 75°C (Drobniewski 1993), for the cocktail of strains used in this experiment, the growth was most easily achieved when incubated at 37°C.

A complete growth curve was observed after less than 10 days of testing cheese sauce stored at 37°C. *B. cereus* had the shortest lag time and achieved a maximum cell density that was significantly higher than those observed at any other storage temperature after 1 day, but began to die after 3 days. Because spores were harvested at this temperature they were better able to grow quickly when stored at this temperature

At a temperature of 23°C is not considered to be in the optimum growth temperature for *B. cereus* in process cheese sauce, but vegetative cells yielded a population of 7 log CFU/g after 2 days and 8 log CFU/g after 3 days. High water activity and a relatively neutral pH provided favorable growth conditions despite being stored at a less than optimum growth temperature. Room temperature was the only storage temperature able to induce extensive sporulation. This may be especially significant for restaurant settings where food could be left out at room temperature. The toxin produced by *B. cereus* is produced between 25°C and 35°C so leaving process cheese sauce at room temperature

would cause even a relatively low amount to increase beyond the infectious dose of 10^5 CFU/g and cause illness (ICMSF 1996).

When *B. cereus* was held at lower temperatures for this experiment, growth was not observed. The contaminated process cheese sauce stored at 4°C had significantly less viable cells than any other temperature, and did not show any increase above the initial inoculum, even after 2 months. The same result was observed when cooked rice was spiked with *B. cereus* spores and held at 4°C (Cronin and Wilkinson 2008b). There are several studies on the development of psychrotropic strains of *B. cereus* in the environment: in liquid eggs, growth was identical at 6°C to growth at optimum temperatures (Jan et al. 2011), foodborne outbreak strains of *B. cereus* were isolated from food in Spain and the Netherlands and showed growth between 4-37°C (Van Netten et al. 2008), and Kramer and Gilbert reported growth at temperatures as low at 5°C (Kramer et al. 1982). However, strains used for this study were not as capable of growing at low temperatures.

The vegetative cell population was maintained when cheese was held at 4°C. The population of spores increased gradually after one month of storage, though still less than 1 log CFU/g. When Cronin et al (2008) performed a study on the effect of storage at 4°C for 1 month on *B. cereus*, however, their results showed a loss of viability in *B. cereus* cells. Cronin also showed that when the spores were injured by heat shock they were more prone to germinate rapidly. These findings are inconsistent with the finding in process cheese sauce because the spores that were heat shocked before inoculation did not germinate when held at 4°C for 1 month. The spores in Cronin's experiment,

however, were heated to 80°C and above for 10 or 20 minutes and stored in PBS, whereas the spores in this experiment were only heated to 75°C but for a time of 30 minutes and were in a cheese matrix with a stronger nutrient profile that helped to keep the level of spores and vegetative cells at a constant level (Cronin and Wilkinson 2008a).

When cheese sauce was held at 12°C, a growth of 1.2 log CFU/g was observed after 5 days and after 1 month of storage in cheese sauce the population increased to more than 7 log CFU/g. This result was expected because *B. cereus* strains are capable of growth at moderately low temperatures. In the genetic analysis of *B. cereus* by Afchain and Carlin (2008) who found that 5 of the 7 genetic groups are able to grow at temperature ranges that include 12°C, however only 10-15% of outbreak strains belong to the most cold tolerant groups (Guinebretiere et al. 2008, Afchain et al. 2008). When *B. cereus* was isolated from pasteurized milk, Andersson et al (1995) showed that growth increased greatly with only a 1 degree change in temperatures above 6°C (Andersson et al. 1995). The formula for cheese sauce used in our experiment contained 50 to 55% skim milk, and explains why *B. cereus* adapted so well to the environment at a temperature in the minimum range requirement.

Fluctuating temperatures did not affect the growth of *B. cereus* vegetative cells, or support sporulation. When the cheese sauce was subjected to periods of storage at room temperature followed by higher temperatures (45°C water bath) for no longer than 6 hours at a time, there was no significant growth; it was not until after more than 24 hours that the vegetative cell population grew by 1 log CFU/g. However, when the sample was

heated above 60°C, germination was not induced, even after periods of room temperature holding.

It is well documented that most foodborne outbreaks related to *B. cereus* contamination are a result of time/temperature abuse that promoted the growth of initially low levels of *B. cereus* to increase to infectious amounts (ICMSF 1996). Few studies have examined the effect of dynamic temperatures on the growth of pathogens in food, and none have studied a cheese matrix. One study of note was performed by Juneja et al. (1993) using *C. perfringens* in a meat medium. Samples were subjected to a cyclic temperature abuse scheme similar to what could occur in ground meat sold in a commercial grocery store. Samples that were moved from refrigeration temperatures to storage at 28°C for 24 hours 4 days before plating, had at least a 6 log CFU/g increase in the vegetative cell population. However, when samples were moved to 28°C for only 6 hours, all samples had less than 3 log CFU/g increases. Juneja et al. reported that when food was subjected to a short period of temperature abuse, similar to that observed in a typical buffet , it was not sufficient to promote the growth of *C. perfringens* (Juneja et al. 1993). These findings are mirrored in the observations of *B. cereus* in the present study. Germination began after more than 24 hours of a cyclic temperature abuse scenario. It is possible that when several short time intervals of abusive temperatures were compounded, it stimulated growth patterns similar to those observed when product is held at abusive static temperatures. This emphasizes the importance of proper storage conditions required by process cheese sauce; these findings are especially important for food handlers.

For all scenarios of fluctuating temperatures, if the population of vegetative cells and spores of *B. cereus* did not increase, the counts were maintained at initial inoculum levels, even when the cheese sauce was properly heated and cooled (Figure 11-14). If the contaminated product were to come in contact with another food medium better able to support growth, there would be a greater potential for the stable population of *B. cereus* to germinate and cause illness. The previously mentioned outbreak in Georgia from process cheese sauce contaminated with *C. botulinum* was traced back to consumers who ate a barbeque baked potato that also contained meat (Townes et al. 1996). Though it was not directly related with the outbreak, it could be possible that the meat from the baked potato promoted the growth of *C. botulinum* when cross contaminated with the cheese sauce.

Meat and process cheese sauce are often combined in restaurants, and because meat is a known vehicle for *B. cereus* contamination it could induce the growth of the surviving population in cheese sauce if combined. In our study, when the cheese sauce was heated to only 45°C in the water bath, the population of vegetative cells increased. This is indicative of the potential of *B. cereus* to germinate in cheese sauce set on the buffet line. However, when cheese sauce was heated to sufficiently high temperatures (above 60°C) the population of vegetative cells declined slightly. These data show the importance of properly heating food to avoid potential contamination.

Nisin is a well-known antimicrobial often used in the dairy industry. Many studies have been performed that have demonstrated the the efficacy of nisin on the inhibition of pathogenic growth in various types of cheese and cheese products. The results of the

experiments in which nisin was added to contaminated cheese sauce showed that nisin did not entirely inhibit growth of *B. cereus*, but only delayed it. After 24 hours, the growth of *B. cereus* in samples without nisin was significantly higher than the sample with nisin, but after 48 hours of storage at 37°C there was not a significant difference in the population of vegetative cells. These results are consistent with a study conducted by Beuchat (1997) but differ from those reported by Zottola and coworkers (1994). Beuchat showed that growth of *B. cereus* in gravy was slowed initially, but that after 3 days of storage at15°C and 9 days of storage at 8°C the activity of nisin was lost and growth of vegetative cells was observed (Beuchat et al. 1997). The study by Zottola et al. on Cheddar cheese containing nisin and the resulting effect of this cheese when used to manufacture cheese spreads showed that the nisin significantly extended the shelf life of the cheese spreads (Zottola et al. 1994).

The antimicrobial trial performed in our study was designed to simulate a worst-case scenario for process cheese sauce. Although nisin is a well-established inhibitor of the growth of Gram positive organisms, especially those in the *Clostridium* and *Bacillus* genus, the antimicrobial only delayed growth of *B. cereus* in process cheese sauce at 37°C instead of complete inhibition. Delves-Broughton mentioned that as moisture levels increase, a higher amount of nisin is needed to achieve consistent levels of inhibition of pathogen growth (Delves-Broughton 1990). The process cheese sauce used in this study had very high moisture levels. This may be the reason the observed effect of nisin was lower than the effect in process cheese spread in the study by Zottola and colleagues. For future studies nisin could be combined with other antimicrobials for a greater effect.

When the same amount of nisin (0.5 g/kg) was combined with caprylic acid by Gadotti, it was shown to inhibit growth of *L. monocytogenes* in queso fresco (Gadotti 2011).

4.3 *Clostridium perfringens*

The behavior of *Clostridium perfringens* in cheese sauce was very different from that of *Bacillus cereus*. None of the scenarios of static or fluctuating temperatures was enough to support the growth of this anaerobic bacterium. These results are inconsistent with the known behaviors of *C. perfringens*. In a medium with high water activity of 0.99 and a pH ranging from 5.6-5.9, *C. perfringens* growth was expected to be supported when stored at the temperature to which it was subjected. *Clostridium perfringens* has not often been isolated from cheese, likely because cheese is not the most suitable medium for growth of *C. perfringens*. The population of both vegetative cells and spores was maintained near the level initial level of inoculum in the majority of trials. As with *B. cereus* these data suggest that if contaminated, cheese sauce when combined with another food product that could better support growth of the organism (such as a meat product), then a threat could still arise.

Our observations showed that the most probable temperature to allow growth of *C. perfringens* would be achieved when cheese sauce was stored at 23°C. Populations increased the most after storage for 1 month, but the growth was less than 1 log CFU/g; given more time populations may have continued to grow. Though natural cheese is considered to provide anaerobic conditions, the liquid matrix of process cheese sauce does not provide the same environment once the hermetic seal is broken. The storage environment for cheese sauce in a commercial setting would be aerobic, and despite the

ability of *C. perfringens* to grow in a wide range and change the oxidation/reduction potential (E_h) in a food, the E_h must first start in at an acceptable level for growth (Jay et al. 2005). It has been reported that *C. perfringens* is able to grow even when stored aerobically. Juneja et al. (1993) showed the effects of storage temperatures of 4, 8, 12, 15, 28, 37, and 42°C on the ability of *C. perfringens* to survive/grow in a cooked beef medium when stored both aerobically and anaerobically. Little or no growth of *C. perfringens* was observed in foods stored at lower temperatures. The bacterium population in the meat stored aerobically at 4°C and 12°C continually declined from the starting inoculum of 3 log CFU/g, but when stored at higher temperatures (28°C and 37°C) the population reached levels near 6 log CFU/g (Juneja et al. 1993). The cooked beef, unlike the results observed in process cheese sauce, provided a sufficient amount of nutrients to support the growth of bacteria even when stored aerobically.

Because *C. perfringens* only synthesizes 7 of the necessary amino acids (AA) it requires a growth medium rich in amino acids, which is why it is so often found in meats and meat products. Red meat is typically contains between 26 and 36 g protein/100 g, whereas process cheese contains 13.2 g protein/100 g (Williams 2007, Berger et al. 1989). When broken down into the availability of lysine and methionine – the most limiting AAs—casein has 8.2% and 3.2% respectively and meat has 1.52% and 0.64% respectively (Berger et al. 1989). However the amount of casein in the process cheese sauce is minimal. The protein content of process cheese sauce is dependent on the raw materials used and traditionally displays a profile similar to the raw cheese it is made with.

Cheese typically has a relatively high protein content and a high concentration of essential amino acids (Berger et al. 1989). However, the process cheese sauce used in experiments with *C. perfringens* was made with only 9% real cheese, 50% skim milk (3.39 g protein/ 100 g) and 10-15% water. This formulation may not provide a significant amount of amino acids for the bacterium to utilize while trying to grow. In an evaluation of free amino acids in cheese, methionine and lysine were 2 of the 4 amino acids present in the highest amounts (~7.0 and 13.0 mg/g product respectively), but because there is such a low level of cheese in process cheese sauce the small amounts of methionine and lysine in milk may have been the limiting factor for growth of *C. perfringens* (Puchades, Lemieux and Simard 1989).

It is frequently reported that foodborne illness outbreaks attributed to contamination by *C. perfringens* arise from food products that are cooled improperly. The FSIS regulations state that meat must be cooled from 54.58°C (130.8°F) to 26.68°C (80.8°F) within 1.5 h and from 26.6 to 4.48°C (408°F) within 5 h in order to keep the microbial populations at safe levels. Sanchez-Plata et al. studied abusive cooling curves in meat samples spiked with *C. perfringens.* They concluded that leaving samples to cool for a period of 9 hours was enough to promote an increase in the bacteria population to a level unsafe for consumption (Sanchez-Plata et al. 2005). When spiked cheese sauce was cooled from 75°C to 4°C in roughly a 9 hour period simulating worst case scenario, no growth was observed. Vegetative cell populations were maintained during storage in a heated water bath (70°C) but then declined quickly during the cooling period and did not recover, while spore levels declined instantly and never recovered under these conditions.

Byrne, Dunn, and Bolton reported that vegetative cell inactivation requires a temperature between 50-65°C and a temperature between 90-100°C to inactivate spores (Byrne, Dunne and Bolton 2006) so the heated water bath was likely not hot enough to inactivate the spores. The growth medium of cheese was simply insufficient to sustain growth of *C. perfringens* throughout the experiment.

Chapter 5

Conclusion

The thermal processing of process cheese sauce renders the product free of any viable organism. However, once the product is distributed to consumer there are many critical points in which it may be contaminated before it is served at a food service setting such as a buffet or a self-serving station. Because the product is heated to temperatures above 60°C, the only organisms that can survive the high heat are spore-forming bacteria, and two of the three most problematic and ubiquitous of this type of bacteria are *Clostridium perfringens* and *Bacillus cereus*.

While there are few reported outbreaks in process cheese sauce, all reported cases have been due to temperature abuse at restaurant settings. With a water activity of 0.99 and a pH values of approx. 5.6, the conditions could be considered conducive to support germination of some spore-forming bacteria that could be contaminated by the consumer. Process cheese sauce is typically made using one or multiple types of natural cheeses as raw ingredients. The cheese is then combined with salts and other ingredients then heated to 121°C for 2.5-3.0 minutes to achieve commercial sterility.

The current study evaluated different temperature scenarios that could be seen in a commercial setting, both static and cyclic, and their effect on the spore-forming bacteria *Bacillus cereus* and *Clostridium perfringens*. The aerobic *Bacillus cereus* grew very well in 2 different formulations of process cheese sauce. At temperatures close to the optimum temperature range of growth, 23, 37, and 45°C, the count of *B. cereus* increased to populations of 8 log CFU/g between within the first 3 days. When held at 12°C, *B. cereus* did not reach a population of 7 log CFU/g until 1 month of storage, and growth at 4°C

was not observed until after 2 months of storage. Spore populations were maintained at all temperatures, and an increase was observed at 23°C and 12°C in the cheese sauce containing a lower percentage of natural cheese, but not in the brand with higher natural cheese. For these 2 temperatures, the vegetative cell populations began to decline as the spore population increased suggesting sporulation during long term storage (around 1 month). Short periods of temperature abuse during cyclic scenarios did not support a large increase of vegetative cells or sporulation. However, when multiple periods of temperature abuse were combined, over time, an increase in vegetative cells was observed. Adding a common GRAS antimicrobial, nisin, at a level of 0.5 g/kg did not stop the growth of *B. cereus*, but was enough to delay growth of vegetative cells for a period of 24 hours.

Contrary to the results observed with *B. cereus*, *C. perfringens'* growth was not supported by process cheese sauce. Under the aerobic conditions of a typical restaurant buffet line and with a lack of proper amino acids in the matrix, the environment proved to be inhibitory for the anaerobic species. Even in a worst case scenario where the spores were heat activated and allowed to cool over a period of 9 hours, cell levels declined below a detectable limit. During static holding, the population of spores and vegetative cells were maintained and could still pose a threat if combined with another food product, such as meat, that has a more favorable environment for *C. perfringens*.

Considering the results this study, process cheese sauce should not be held at abusive temperatures (above 4°C) after the product has been opened to ensure that any spores that made it through processing do not germinate. When held on the buffet line, the cheese

should be heated to greater than 65°C and cooled down as quickly as possible before placing it into the refrigerator, once it is removed from the heating unit. Future studies could go more in-depth into the use of other antimicrobials and combining multiple antimicrobials to inhibit growth and even inactivate spores, even if the process cheese sauce is left at mild temperatures for more than 24 hours. Another opportunity includes testing how the organisms react when inoculated cheese sauce is combined with a meat sauce.

Chapter 6

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