EVALUATING THE IMPACT OF COOLING METHODS ON BIOSAFETY LEVEL I *ESCHERICHIA COLI* AND *BACILLUS CEREUS* POPULATIONS IN FOUR FOOD PRODUCTS

by

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B.S., Kansas State University, 2011

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Approved by:

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Abstract

Food preparation in institutional settings is often carried out in large quantities. Food is cooked and then cooled and stored for later service. Improper or "slow" cooling has been identified by the United States Food and Drug Administration (US FDA) as a contributing factor in foodborne illness outbreaks. This study was designed to test the efficacy of cooling technique combinations on controlling microbial growth within pre-cooked taco meat, chili con carne with beans, low sodium marinara sauce, and brown rice food products. These products were cooked to 73.8°C (165°F) and then portioned to 2 and 3-inch depths in steam table pans. Food product was allowed to cool to 60°C ± 5°C (140°F ± 5°F) before inoculation with surrogate *Escherichia coli* (*E. coli*) or Biosafety Level I (BSL I) *Bacillus cereus* (*B. cereus*). Pans were uncovered or covered with one or two layers of aluminum foil to allow or restrict air exposure and then placed in a -20°C (-4°F) commercial walk-in freezer or situated in ice water baths in a commercial walk-in refrigerator 4°C (39.2°F). Food products were sampled over a 24-hour period (0, 4, 8, 12, and 24 hour time points) for enumeration of microbial populations.

Conclusions from the cooling temperature data in this study revealed uncovered pans and pans stored in the freezer at 2-inch food product depths cooled most rapidly. However, few cooling methods achieved the two-step US FDA Food Code requirement for pre-cooked taco meat, chili con carne with beans, and brown rice products and none of the cooling methods tested achieved the US FDA food code requirement for low sodium marinara sauce. Surrogate *E. coli* and BSL I *B. cereus* microbial population data revealed pre-cooked taco meat, chili con carne with beans, and brown rice products all exhibited a certain degree of overall population decline during the 24-hour cooling period. However, a small recovery of surrogate *E. coli* population was observed in the low sodium marinara sauce product as well as 2-inch product depths of the

chili con carne with beans product. This observed growth was less than 0.50 $\log_{10}$ CFU/g, indicating low risk for microbial proliferation from the cooling methods tested. It is possible that the surrogate *E. coli* and BSL I *B. cereus* population changes observed were not the result of cooling failure or risk, but rather due to natural variations within the food products. These results indicate all 12 cooling methods tested were low risk and therefore effective at controlling *E. coli* and *B. cereus* microbial populations within the four food products.

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Acknowledgements

To my advisor, Dr. Sara Gragg, thank you for investing your time and energy in mentoring a non-traditional student like myself. Your style of leadership and your trust in my ability to problem solve and think critically have helped me build confidence in myself as a student and as a researcher. Thank you for the consistent and constructive feedback and the time you spent in the lab, working with me on long project days. Your dedication to my growth and education has been unwavering and I can't tell you enough how appreciative I am to have worked with a mentor like you. I would also like to thank the other members of my committee, Dr. Randall Phebus and Dr. Paola Paez, for their constant support and guidance during my thesis work. Dr. Randall Phebus, thank you for being an honest and practical sounding board. I appreciate your ability to critically evaluate and provide constructive feedback. To Dr. Paola Paez, thank you for your dedication to this project. I truly value all those early mornings you spent with me in the kitchen, teaching me what I could not have learned in a laboratory. I admire your drive to accomplish tasks and solve problems that arise. Your perspective on this research was unique and taught me to think about the different aspects and angles of a research question. I am very grateful to have had the opportunity to work with you.

To those fellow students Jacob Jenott, Tracee Watkins, and Danny Unruh, thank you for finding ways to set aside time and energy to devote to assisting me in my thesis research. This project would not have been completed without all of your hard work. I also want to thank the Center of Excellence for Food Safety Research in Child Nutrition Programs at Kansas State University for their partnership and support in this research, especially Kerri Cole and Ellen Thomas for not only working with us in the kitchen but also guiding the design of the study and providing valuable and essential feedback. I owe another big thank you to Bryan Severns for his

leadership in the kitchen at the Olathe campus and his input on the design of this study. Dr. Dallas Johnson, I thank you for all the time you devoted to the statistical analysis of these data. You were integral in our ability to produce results that are meaningful. I also owe a sincere thank you to Dr. Brian Lubbers who mentored me in my formative career experience in diagnostic microbiology. That experience provided me a unique knowledge base and a true appreciation for my future studies. I have enjoyed working with you and learning from you over years. I cannot thank you enough for choosing to invest in my education by connecting me with this unique and challenging learning experience. You have been the one to provide me with two of the most significant life-changing opportunities I've ever taken advantage of and I owe you a great deal of thanks for that. I am incredibly grateful. Lastly, I want to thank my incredible support system of family and friends for showing me love, patience, and kindness during a stressful yet rewarding time in my life. Thank you for keeping me grounded and supporting me through my studies.

Chapter 1 - INTRODUCTION

Foodborne illness affects 48 million people each year, resulting in 128,000 hospitalizations and 3,000 deaths (*39*). Of these illnesses, only 20% can be traced back and attributed to 31 major foodborne pathogens (*132*). Of those pathogens, *Bacillus cereus (B. cereus)* causes more than 63,000 illnesses annually, and 100% of these illnesses are foodborne in origin (*132*). Exposure to *Escherichia coli* (*E. coli*) O157:H7 results in a similar number of illnesses annually, but with over 46% of cases resulting in hospitalization and 0.5% in death (*132*).

Escherichia coli O157:H7 and *B. cereus* do not rank among the top pathogens for numbers of hospitalizations, illnesses, or deaths (*132*). However, it is critical to emphasize these pathogens when considering those at risk for severe illness and life-threatening complications, including young children. Children, especially those under the age of five, have underdeveloped immune systems that may not be equipped to handle a pathogenic infection (*24*). An underdeveloped immune system compounded with a child's low body weight makes even a small amount of pathogen a significant risk (*24*). Therefore, children under the age of five account for almost 30% of all deaths from foodborne illness (*171*).

Escherichia coli O157:H7 is a critical concern for young children (age 1-9 years) as they experience an infection rate of 8.2 per 100,000 which is four times the infection rate of adults aged 20-29 (*24*). Children also have a higher likelihood of developing chronic sequelae like hemolytic uremic syndrome (HUS) (*24*). The World Health Organization has identified HUS as the most common cause of acute renal failure in children (*146, 170*). Long-term complications from an *E. coli* O157:H7 infection can affect children in many ways, including decreased neuromotor skills, increased risk of hypertension, and chronic renal issues that have been

documented in over a third of cases in children (*22*, *124*). The ability of *E. coli* to survive improper holding temperatures is a critical concern for school nutrition programs. A school associated outbreak of *E. coli* O157:H7 in Japan sickened over 6,000 school children because dishes served at one school were held for serving at a temperature just 5°C lower than other schools that did not report illness (*89*).

When compared to other pathogens, *B. cereus* generally causes mild and self-limiting symptoms of diarrhea or vomiting, contributing to the fact that it is a markedly underreported foodborne illness (*9*, *74*, *75*, *76*, *77*). However, there have been documented cases that suggest certain strains are more virulent than others are and may cause life-threatening illness. The emetic strain produces a heat stable toxin that has been implicated in cases of fulminant liver failure resulting in the deaths of several young people (including a 7-year-old girl in 2003) (*56*, *106*, *112*). The three documented cases resulted from the ingestion of a variety of contaminated pasta dishes (*56, 106, 112*). Another high-risk food product for this pathogen is fried rice, a dish commonly served in schools and daycares that has been identified as the leading cause of emetic-type *B. cereus* food poisoning in United States schools (*16, 143*). Milk is another product of concern, as spores of *B. cereus* may survive pasteurization and some species have the ability to then germinate and cause vegetative cell growth at low temperatures (*15, 49*). In fact, milk was implicated in 11 outbreaks and over 1,600 illnesses in United States schools from 1973-1997 (*55*).

Large outbreaks of foodborne illness are prevalent in environments where food is cooked in large batches, a common practice for restaurants and institutional settings (*72, 102, 107, 110*). Outbreaks can be seasonal in school settings and also peak in the fall during November, perhaps correlating with thanksgiving style meals or leftovers (*31*). Outbreaks, illnesses, and

hospitalizations in school settings peak in the spring during the month of March (*31*). A 2013 Centers for Disease Control and Prevention (CDC) surveillance summary reviewed outbreak data collected during the years 1998-2008 and concluded that schools were associated with the largest number of outbreaks (286) and illnesses (17,266) compared to other institutional settings like daycares, workplace cafeterias, and prisons or jails (*72*). The risk for large outbreaks is considerable in schools, as The National School Lunch program serves over 31 million children each day, with a total of 224 billion lunches served since the beginning of the formal program in 1946 (*150*).

The large population of children served at school, combined with their classification as an at-risk population, make proper food preparation practices especially critical in a school lunch setting. Many factors during food preparation may lead to an outbreak, and the US FDA has consistently identified time/temperature control as a critical point where control is necessary to prevent foodborne illness (*155*, *157*, *158*). A recent survey of school food service managers concluded that cooling is an intrinsic aspect of food preparation for school nutrition programs, with 78% of managers reporting cooling leftovers to reheat for service at another meal (*98*). Though it may be a common practice in the school lunch setting, improper or "slow" cooling has been identified as a major contributing factor for school associated foodborne illness (*123*, *163*). To address factors like improper cooling, schools are now required to utilize a food safety program based on the principles of Hazard Analysis and Critical Control Point (HACCP) as stated in the *Child Nutrition and WIC Reauthorization Act* of 2004 (*1*). The FDA Food Code was also updated to reflect this concern, requiring cooked food products to be cooled from 57ºC (135ºF) to 21.1ºC (70°F) within 2 hours and from 57ºC (135ºF) to 5ºC (41°F) or less within a total of 6 hours (*152*, *156*).

To meet these guidelines, the FDA has suggested certain methods to effectively cool food products in the required amount of time, such as storing food in shallow pans, in smaller or thinner portions, and using rapid cooling equipment (*156*). Previous studies have evaluated the cooling of food products with several methods including refrigerators, ice baths, freezers, and blast chillers (*98*, *117*, *118*, *129*). Food products selected for this study were among those identified as most commonly served in schools by a survey of school foodservice managers and included taco meat filling, chili, marinara sauce, and rice (*98*). These studies provided the information necessary to design this experiment to reflect common cooling methods used in school nutrition programs with relevant food products. The blast chiller, a form of rapid cooling equipment, was one of the few cooling methods that produced results meeting the FDA Food Code requirement (*117*). However, this may represent a financial barrier for schools as a blast chiller represents a significant investment, in fact, only 8% of schools nationwide own and use them (*98*). Another common barrier is a lack of adequate freezer space; schools report an average of just 20% free or open space for storage (*129*). According to these studies, few cooling techniques meet the requirements of the 2013 FDA Food Code. Thus, the focus of research should be to scientifically characterize and validate cooling methods that are both feasible and effective at preventing pathogen growth, in regard to meals prepared in school nutrition program settings, as it is critical to public health.

The primary objective of this project was to assess 12 cooling methods and their effect on surrogate *E. coli* and *B. cereus* populations in four food products commonly served by school nutrition programs. A variety of cooling variables were tested in order to validate methods that effectively control pathogenic microbial populations. These cooling methods were also chosen to

simulate those that are already commonly used in school nutrition programs. The experimental design was created to reflect these goals.

One multifaceted objective was necessary to complete this study: the enumeration of surrogate *E. coli* and *B. cereus* populations within four food products during a 24-hour cooling period. The hypothesis for this project was that a majority of the cooling methods would be effective in controlling surrogate *E. coli* and *B. cereus* population growth within all four food products. It was also hypothesized that the most effective method for rapid cooling would be the uncovered 2-inch food product depths cooled in the -20°C (-4°F) freezer for all four food products.

Chapter 2 - REVIEW OF THE LITERATURE

Escherichia coli O157:H7

Background & General Properties

Escherichia coli is classified as a Gram negative with rod morphology and it is a facultatively anaerobic microorganism (*103, 108*). This bacteria was first characterized in 1885 by Dr. Theodor Escherich, a pediatrician and researcher who studied the microorganisms that inhabit the feces of infants and neonates (*81*). He termed the microorganisms he found the "bacterium coli commune" after the colon, due to the colonization of this specific section of the intestines (*62, 81*). It has been suggested by further research that just a few hours after the birth of an infant, *E. coli* has already successfully colonized the gastrointestinal tract (*57, 93, 113*). Almost a century after Dr. Escherich's discovery, the first clinical reports of bloody diarrhea from the novel *E. coli* O157:H7 serotype were associated with outbreaks in Michigan and Oregon in 1982, prompting the food safety community to recognize it as a significant foodborne pathogen (*103, 128, 164*).

The non-pathogenic species of *E. coli* are known as commensal organisms meaning they routinely colonize the intestines or gut of mammals including humans (*80, 113*). In fact, it is the most abundant facultative anaerobe found in the intestines of humans, despite competition from other microorganisms (*93*). The ability to compete successfully make a serotype like *E. coli* O157:H7 especially virulent since the strain has evolved and acquired pathogenicity through various exposures and genetic evolution (*93, 103*). Common symptoms of *E. coli* O157:H7 infection are bloody diarrhea, stomach cramps, and vomiting lasting 5-7 days (*32*). However, certain populations are at a higher risk for more severe illness and sequelae. The virulence of *E.*

coli O157:H7 and its documented presence in a variety of food products from spinach to fruit juices to ground beef contributes to its significance as a source of foodborne illness (*28*, *30*, *33*).

Classification

As a Gram negative microorganism, *E. coli* is encapsulated in an outer layer of primary surface polysaccharides that contain virulence determinants (*166*). Identification based on somatic antigen (O), flagellar antigen (H), and capsular antigen (K) date back to methods developed in the 1940's (*94*). Kauffman's 1947 publication titled *The Serology of the Coli Group* described the development of unique serotyping for *E. coli* that built upon techniques discovered for *Salmonella* classification in 1929 (*65*). Kauffman began by analyzing K antigens that indicate the thermolabile (L antigen), thermostable (A antigen), or binding (B antigen) qualities of the capsule surrounding the O antigen (*94*). He then worked to classify groups of O and H antigens as well. As of 2013, 174 O antigen groups and 53 H antigen groups are recognized utilizing the Kauffman classification scheme (*52*). However, two alternate identification methods have recently been developed to identify *E. coli* that may be difficult to classify because of cross reactivity of antigens or other factors that make them unable to be serotyped (*52*). These methods include pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) which are considered the gold standard for identification of previously untypeable *E. coli* (*52*, *103*).

There are six recognized pathotypes for *E. coli* most often associated with diarrheal illness in the human population. These pathotypes include *E. coli* that are enteroaggregative (EAggEC), enteropathogenic (EPEC), diffuse-adhering (DAEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohemmorrhagic (EHEC) (*49*). These pathotypes are often distinguished by modes of attachment, toxins produced, and severity of illness. *Escherichia coli* O157:H7 is the most frequently isolated EHEC from those sickened in the United States, Japan,

and the United Kingdom (*103*). Therefore, this review will focus on *E. coli* O157:H7 as a Shiga toxin-producing EHEC.

Mechanisms of Pathogenicity and Illness

It has been proposed that there are four stages recognized for bacterial infection of a host: (i) colonization of a mucosal site, (ii) evasion of host defenses, (iii) multiplication, and (iv) host damage (*110*). *Escherichia coli* O157:H7 possesses several virulence factors that enable it to successfully infect humans as described above, including acid tolerance and highly effective methods of host cell attachment (*103*). Pathogenicity islands within the genome allow for the acquisition of these pathogenic mechanisms through mobile genetic elements including bacteriophages, transposons, insertion sequences, and plasmids (*52, 103*).

As a member of the EHEC group, a particular bacteriophage gives *E. coli* O157:H7 the ability to produce cytotoxins like Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) (*52, 103*). It has been hypothesized that the acquisition of these bacteriophages is one of the main steps in the evolutionary process of an EPEC serotype, O55:H7, to the more virulent *E. coli* O157:H7 EHEC (*167*). The Shiga toxin produced by *E. coli* O157:H7 contains one A subunit and five B subunits (*103*). Once fimbriae (*lpf*1 and *lpf*2) facilitate the superficial attachment of *E. coli* O157:H7 to the extracellular matrix of an epithelial cell in the intestine, the B subunits of the Shiga toxin can facilitate binding to the host cell at specific receptor sites in order for the A subunit to internalize into the cytoplasm of the host cell, inhibiting protein synthesis and causing cell death (*52, 103*). The *eae* gene encodes for intimin which also facilitates the intimate attachment to the host cell via translocated intimin receptors (Tir) (*52, 130*). These *eae* genes are commonly identified in serotypes of *E. coli* that cause an attaching and effacing infection like *E. coli* O157:H7 (*52*). Hemolysin (*ehx*) is another genetic element that contributes to pathogenicity and it is housed on

the O157 plasmid (*102*). Hemolysin is considered cytotoxic to epithelial cells and causes pore formation (*103*).

The ability to survive acidic conditions contributes to the pathogenicity of EHEC pathotypes of *E. coli*. An *rpoS* regulation system produces protective proteins that allow *E. coli* O157:H7 to become acid tolerant (*47*). This system allows *E. coli* O157:H7 to evade the acidity of the stomach, facilitating a successful colonization of the intestines and subsequent illness within a human host (*17*). Acid tolerance may also contribute to a lower infective dose for pathogens like *Shigella* species, *E. coli* O157:H7, and *Salmonella* (*17*). Cells that are starved or in stationary phase show the greatest acid tolerance, which might represent several growth scenarios resulting from cross contamination or improper holding temperatures (*47*).

These genetic elements make *E. coli* O157:H7 a virulent pathogen that sickens an estimated 63,000 people annually in the United States (*132*). Infection usually results in diarrhea and stomach cramps, but may also lead to hemorrhagic colitis and a severe sequelae known as hemolytic uremic syndrome (HUS) (*32, 103*). HUS develops in 5-10% of cases after symptoms of hemorrhagic colitis, and it occurs more frequently in children and the elderly, resulting in acute renal failure (*14, 103*). *Escherichia coli* that produce Stx1 may cause an infection leading to HUS but Stx2 production causes more severe human disease (*52*). Current research and past evidence suggest Shiga toxin-producing *E. coli* (STEC) causes all, or almost all, of post-diarrheal HUS cases in developed countries like the United States (*14, 103*).

Reservoirs and Modes of Transmission

Escherichia coli infections are most commonly contracted via the fecal-oral route, often by consuming contaminated food or water (*52, 103*). It is well known that ruminants, especially cattle, are the major reservoir for *E. coli* O157:H7 and it is the exposure to their feces that causes

a high risk for human infection (*52, 59, 80, 103*). Some theorize a prevalence of up to 60% of cattle may shed the microorganism in the summer months, whereas an average estimate is 10 to 25% for other seasons (*80*). Research indicates a correlation between the summer months and a seasonal increase in *E. coli* shedding from cattle, contamination of carcasses, and an increase in the incidence of human STEC infections (*134*). An increase in shedding during the summer months can be especially critical to public health when considering cattle known as "high shedders" or "super shedders" that may have populations of $>10^4$ Colony Forming Units (CFU) g^{-1} detectable in their feces (*46, 64, 122*).

The prevalence of *E. coli* O157:H7 shedding in the feces of cattle has lead to the microorganism's ubiquitous status in agricultural environments, however, its presence has also been well documented during carcass processing. Data show that once cattle reach an abattoir, up to 75% of hides test positive for *E. coli* O157:H7; meanwhile, carcasses at pre-evisceration test positive up to 43.4% and up to 3.8% of samples test positive post-evisceration (*10, 11, 19*). In fact, beef was implicated in 20% of *E. coli* O157:H7 foodborne outbreaks occurring from 2003-2012 (*85*). However, leafy vegetables and fruits have continued to grow in significance since the late 1990s and now account for 9% of recent *E.coli* O157:H7 outbreaks (*85*). Sprouts, melon, lettuce, and apple cider or juice are some of the most commonly implicated products in the produce or "raw food" category (*85, 125*). Contaminated irrigation water, contaminated manure, or exposure to equipment during processing may be to blame for produce-associated *E. coli* O157:H7 outbreaks (*85, 125*). It is important to note that evidence suggests products consumed raw such as vegetables and fruits result in higher hospitalization rates (*85).*

There have been many documented outbreaks of pathogenic *E. coli* associated with food products like beef and produce; however, another vehicle of transmission for *E. coli* is water

(*169*, *170*). Human infections from a waterborne source accounted for 4-9% of *E. coli* O157:H7 outbreaks from 1982-2012, and the majority of those outbreaks were caused by exposure to contaminated recreational water (*85*, *125*). It is possible that recreational water may become contaminated with pathogenic *E. coli* from improperly treated or improperly disposed waste water of various facilities including slaughterhouses and hospitals (*13*, *68*, *97*). Over 20% of waste water samples from slaughter facilities may test positive for *E. coli* O157:H7 and antibiotic resistant *E. coli* can be detected in hospital sewage at populations as high as 1×10^5 CFU/mL (*13*, *68*, *97*). Drinking water may pose a risk to humans as well, especially if potable water is supplied by systems that are smaller in size and may be unprotected from contamination or not properly maintained (*119*). Unchlorinated water resulted in an outbreak in a small Wyoming town in 1998 where 157 people fell ill after drinking water from the municipal supply (*119*).

Person-to-person contact has also been identified by the World Health Organization (WHO) as an important mode of transmission for *E. coli* O157:H7 (*170*). An estimated 10-14% of *E. coli* O157:H7 outbreaks resulted from person-to-person transmission from 1982-2012 (*85*, *125*). Outbreaks from this route of transmission peak in the summer months from June to August and a vast majority of person-to-person outbreaks (80%) occurred in child care centers (*125*). Up to 60% of person-to-person outbreaks occur in children younger than five years of age (*85*).

Major Outbreaks and Illness

There have been a number of major outbreaks in the United States that have contributed to the status of *E. coli* O157:H7 as a significant pathogen. Beef and ground beef products have been implicated in some of the largest outbreaks including the most prolific outbreak in United States history in the early 1990's. From mid-November in 1992 through the end of February in

1993, laboratories confirmed more than 500 cases of *E. coli* O157:H7 foodborne illness from individuals who reported symptoms after consuming undercooked hamburger patties served at a fast food chain (*30*). Four children died in this outbreak, and at least one child was exposed secondarily through a daycare setting (*30*). The 11 lots of contaminated meat used for making the fast food hamburgers were traced back to five different slaughter facilities in the United States and one in Canada (*30*). This prompted the FDA to revise the Model Food Code for Restaurants regarding cooking temperature, and in 1994, *E. coli* O157:H7 became a nationally notifiable infection (*125*). In the years since, outbreaks have decreased in size as a result of increased awareness, increased reporting, and improvements in the accuracy of testing and detection (*125*). Although outbreaks are decreasing in size, ground beef products continue to be implicated in *E. coli* O157:H7 infections, including a 2007 outbreak from ground beef patties that sickened 40 consumers and prompted a recall of 21.7 million pounds of product (*36*). However, in more recent research, other beef products like steak are becoming more frequently associated with *E. coli* O157:H7 infection, perhaps as a result of contamination via tenderizing and marination processes (*85*). Recent outbreak data confirms this trend, as assorted beef products were implicated in another multistate outbreak in 2009 which resulted in 23 illnesses (*35*).

In the last 30 years produce has also become a significant food product associated with *E. coli* O157:H7 contamination. In 2006, 205 people were sickened in 26 different states from contaminated spinach (*33, 151*). In this outbreak, 31 people developed hemolytic uremic syndrome and 3 died (*33*). Although the FDA could not confirm the exact cause of the contamination, environmental risks were cited in a 2007 report including the risk of fecal contamination of irrigation wells and surface waterways from cattle or wild pigs (*151*). This was a novel outbreak and prompted the FDA to launch programs like the “Leafy Greens” initiative

and an official guidance document for processors titled “Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables” (*151*). It has been hypothesized that high cattle density in or around farms where leafy greens are harvested may be to blame for cross contamination from feces (*85*). Another large outbreak associated with lettuce occurred in 2006 (*34*). In this outbreak, multiple states reported 77 *E. coli* O157:H7 infections, including 51 hospitalizaitons (*34*). Epidemiological studies concluded that the most likely culprit was shredded lettuce consumed at a fast food restaurant (*138*). Other produce-associated outbreaks have resulted from contaminated sprouts. A recent outbreak in 2016 resulted in 11 people falling ill in Minnesota and Wisconsin after they bought *E. coli* O157:H7 contaminated sprouts at grocery stores and consumed them (*40*).

Novel food products that might not traditionally be associated with this pathogen have also been implicated in outbreaks. In 1996, unpasteurized, fresh-pressed apple cider sickened 66 (*28*). The apples used in the cider may have become contaminated after being washed in contaminated well water at the mill where they were then pressed and the juice sold unpasteurized (*28*). Children as young as one and two years old fell ill in this outbreak, prompting the FDA to encourage consumers to boil unpasteurized cider or juice before consumption or to consume pasteurized juice products instead (*28*). Gouda cheese was implicated in an outbreak that occurred in 2010 that resulted in 38 illnesses (*37*). The FDA eventually seized over 100,000 pounds of cheese made with unpasteurized milk from the producers implicated in the outbreak (*148*). Another novel outbreak of *E. coli* O157:H7 occurred in 2011 from in-shell hazelnuts in which eight people were infected (*38*).

Bacillus cereus

Background & General Properties

The *Bacillus* family was first identified in 1872 by Ferdinand Cohn and was later characterized into three groups based on spore morphology (*50, 135*). In fact, the *Bacillus* group were differentiated from other Gram-positive microorganisms based upon their spore forming abilities (*69*). Historically, it had been determined that species within the *Bacillus cereus sensu lato* group were nearly identical and later research concluded some species within the group share over 99% genetic similarity to one another (*86, 131*). However in 1887, *Bacillus cereus sensu stricto* was identified by two scientists from London after they isolated the bacteria from air samples of a cow shed (*66*). They preliminarily characterized and differentiated *Bacillus cereus sensu stricto* from seven other species within the *Bacillus cereus* group based on phenotypic observations of both cellular morphology and colonies grown on gelatin plates (*66, 131*). *Bacillus cereus sensu stricto*, hereon referred to as *Bacillus cereus*, is an aerobic, Gram-positive rod with a centrally located spore (*20*). It is ubiquitous in the environment and often found in spore form in soil, sediment, and other organic matter (*9*). These spores are highly resistant to stressors like heat and dehydration, making *B. cereus* a hardy pathogen (*9*).

Its ubiquity in the environment lends *B. cereus* the ability to inhabit food processing environments as well because of its spore forming capabilities and effective establishment of biofilms (*9, 63, 99, 161*). This pathogenic microorganism can cause either diarrheal or emetic illness dependent upon which toxin is produced and when it is produced (*20*). Individuals who have eaten contaminated food will experience emetic symptoms shortly after consuming contaminated food, usually within 30 minutes to an hour, whereas, diarrheal symptoms may take up to six hours to exhibit (*9*). The entirety of the illness is short in duration and lasts, on average,

12-24 hours (though longer periods have been reported) (*9*). While hospitalization and death are rare, *B. cereus* infections result in an estimated 63,400 illnesses annually, similar to estimated annual illnesses from Shiga toxin-producing *E. coli* O157:H7 (*132*). It is interesting also to note that *B. cereus* foodborne illness is also considered highly underreported because of the mild and self-limiting symptoms (*9*, 69, *74*, *75*, *77*, *132*). However, several severe cases have resulted in death from fulminant liver failure in children and young adults (*56*, *105*, *112*).

Classification

The *Bacillus cereus* group consists of eight species: *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis* (*131*). These species share very similar genetic components, most notably, *B. cereus* is nearly identical to *B. anthracis* genetically and phenotypically (*131*). Although species like *B. athracis* or *B. cereus* can be highly toxic, causing illness and even death, others have beneficial uses (*131*). *Bacillus thuringiensis* has intrinsic insecticidal properties and is used as a pest control mechanism to protect crops while other *Bacillus cereus* strains can be utilized as probiotics (*9*, *58*, *131*).

Bacillus cereus can be identified based on many criteria, including core gene sequences, toxin production, hemolysis, motility, and spore morphology (*9*, *69*, *131*). Genome sequencing is often considered the gold standard for identification of many other bacteria; however, several species within the *B. cereus* group are almost entirely genetically homogenous, making it difficult to distinguish between species based on sequencing alone (*9*, *69*, *74*, *75*, *76*, *77*, *131*). However, amplification of specific virulence genes, specifically those located on the plasmid, can be an effective method to identify the presence of *B. cereus* and differentiate it from other *B. cereus* species (*9*, *131*). On the other hand, multilocus sequence typing (MLST) has been used to

target both virulence genes and housekeeping genes in order to provide a relatively accurate phylogenetic tree for *B. cereus* (*9*). Other distinguishing traits considered in identification and characterization include the evaluation of colony phenotypes, as *B. cereus* has a unique wax-like or matte appearance (*69, 74, 75, 131*). *B. cereus* also has a unique inability to ferment mannitol but actively produces lecithinase, resulting in characteristic colony phenotypes on selective agar (*9*). Some *B. cereus* species also prefer different growth temperatures and an increase in the identification of psychrotrophic species growing at temperatures as low as 7-10°C have been noted in recent years (*131*).

Mechanisms of Pathogenicity and Illness

Bacillus cereus has the ability to produce two different toxins that result in emetic or diarrheal types of foodborne illness (*9, 20, 74, 75, 76, 77*). Although each toxin is produced during the vegetative growth phase, the mechanism by which each toxin affects the host is unique. Cereulide is the toxin produced by *B. cereus* in the vegetative state within the food product and results in a foodborne intoxication with rapid illness onset (*9, 20*). On the other hand, the enterotoxin responsible for delayed onset diarrheal symptoms is produced by vegetative cells of *B. cereus* within the gastrointestinal tract, and this illness is considered a toxicoinfection (*9, 20, 77*). Genes regulating for virulence and toxin production are located on both the plasmid and the chromosome (*9, 20, 76*). These genes or operons encode for mechanisms like pore formation, hemolytic activity, and cytotoxicity (*9, 20, 76*).

Cereulide is a ring structured or cyclic peptide regulated by the cereulide synthetase (*ces)* gene encoded on the plasmid (*9, 20, 74*). It is resistant to high temperatures, acidity, and proteolysis, which facilitates its survival in the conditions of the gut (*9, 75*). Cereulide inhibits the activity of mitochondria and damages hepatocytes in the liver (*74*). The cellular damage and

other pathological changes brought on by the toxin lasts for several weeks after the infection (*75, 76*). In fact, the toxic effect cereulide has on liver cells has been implicated in several cases where those sickened by the emetic strain of *B. cereus* have died from fulminant liver failure (*56, 105, 106, 112*).

The diarrheal illness caused by *B. cereus* can be attributed to the production of three pore-forming toxins: hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe) and cytotoxin K (CytK) (*9, 20, 73*). Hbl and Nhe are classified as three component proteins where each component is responsible for binding and lytic functions (*74*). The gene regulation for Nhe and Hbl are primarily carried out by chromosomally located operons (*9*). Aside from pore formation, exposure to Hbl also results in dermonecrotic activity and fluid accumulation (*9, 20*). CytK is classified as a ß-barrel toxin and is responsible for similar pathological changes like pore formation and necrotic activity (*9, 20*). Cytotoxin K belongs to a family of toxins that include those produced by *Clostiridum perfrignens* and *Staphylococcus aureus* (*9*).

Although these toxins are responsible for generally mild and self-limiting emetic and diarrheal illness, there are also many other types of non-gastrointestinal infections. *Bacillus cereus* species may cause eye infections, respiratory illness, endocarditis, and gas gangrene-like infections of wounds (*20*). It is also important to note that food production facilities are not the only unique environments susceptible to *B. cereus* contamination, as illustrated by several significant outbreaks of hospital-associated infections involving contaminated linens and medical equipment (*130, 162*).

Reservoirs and Modes of Transmission

There are several foods associated with *Bacillus cereus* outbreaks including rice, grains, milk, cereal, potatoes, and vegetables (*131*). However, different foods may be responsible for

different illnesses caused by the pathogen (*9*). It is well known that emetic illness is most associated with rice and pasta whereas the diarrheal illness is associated with soups and meat products (*9*, *69*). Certain food products provide a better environment for the distinct strains of *B. cereus.* The emetic strain seems to be associated with starch-rich foods whereas the diarrheal strain is associated with proteinaceous foods (*9*). Therefore, the diets of communities may make one strain more prevalent than another in certain geographic areas (*9*). For example, the emetic illness is more common in Japan and the United Kingdom, but the diarrheal illness is more common in Northern Europe and North America (*9*, *76*).

In particular, the dairy industry faces an increased risk for *B. cereus* contamination (*7*, *48*, *49*). Milk may become contaminated by the exposure of the udder to soil and, if sold unpasteurized, could pose an even greater risk for infection (*75*). Similar to *E. coli* O157:H7, seasonal peaks occur for *B. cereus* in the summer months when cattle are grazed outdoors rather than indoors, resulting in higher rates of contamination (*48*). Pasteurized food products may present a particular challenge as it allows the non-competitive *B. cereus* spores to survive and, if product is temperature abused, germinate and allow vegetative cells to proliferate in an environment with little competition (*75*). Hydrophobic spores of *B. cereus* possess proteinaceous appendages that can facilitate attachment to many surfaces, especially pipelines in dairy production environments, where biofilms are readily formed and bacteria can flourish (*7*, *140*).

The ubiquitous presence of *B. cereus* in soil leads to plant origin foods being at high risk for contamination, and processing these foods may result in cross contamination to other products in a production environment (*75*). Even low water activity foods ($a_w \leq 0.92$) or foods with a high pH (>9.3) are not considered low risk for *B. cereus* contamination because of the resistance of hardy spores; in fact, outgrowth can occur in these products (*75*). It is clear that the

ubiquitous nature of *B. cereus* combined with its adaptive ability to survive heat processing in a resistant spore form make it a pathogen of concern rather than a low risk contaminant.

Major Outbreaks

Bacillus cereus has been implicated in many outbreaks of foodborne illness. The diarrheal strain was implicated as early as 1948 in a hospital-associated outbreak in Oslo, Norway (*82*). The most notable in literature occurred in France in 1998 during which 44 elderly patients at a nursing home experienced severe diarrhea after consuming contaminated vegetable puree (*104*). Six patients experienced bloody diarrhea and three died, giving the outbreak a mortality rate of 6.8% (*45*). This was a novel outbreak in that bloody diarrhea is not a common symptom with *B. cereus* foodborne illness and the severity of this outbreak was attributed to cytotoxin K produced by the strain (*104*).

Fried rice is another well documented food product that has been implicated in *B. cereus* foodborne illness since 1971 (*111*). In 1993, an outbreak in Virginia took place at two separate day care facilities where twelve children and two day care workers became ill after eating chicken fried rice that was improperly cooled and then served without reheating (*29*). *Bacillus cereus* was isolated from rice samples at $> 10^6$ CFU/g (*29*). Rice and fried rice products are often implicated in outbreaks as uncooked rice harbor spores of *B. cereus* that can survive the cooking process and proliferate when food is cooled improperly (*29*).

Several unique outbreaks have occurred in hospital settings as well. In 2006, a hospital located in Japan discovered a significant increase in positive *B. cereus* blood cultures, especially during the month of August, when a total of 15 patients tested positive (*130*). Eleven patients developed bacteremia and an investigation concluded a washing machine used for bed linens was highly contaminated with *B. cereus* (*130*). Patients had been exposed through bed sheets and

even through intravenous fluid lines (*130*). Another hospital-associated outbreak was documented in the Netherlands in 1998 in which a neonatal intensive care unit exposed infants to the pathogen via balloons utilized for manual ventilation (*162*). After *B. cereus* was isolated from the blood samples of three neonates, including one who died, further testing of tracheal aspirate samples revealed 35 total neonates that tested positive (*162*).

Non-O157 Shiga toxin-producing *Escherichia coli*

There are also serotypes of Shiga toxin-producing *E. coli* other than O157:H7 that contribute to foodborne illness. In 2011, the USDA Food Safety Inspection Service (FSIS) declared six additional serogroups as adulterants in non-intact beef products, referred to as "The Big Six" including O26, O103, O45, O111, O121 and O145 (*160*). The motivation to identify these serogroups as adulterants is most likely due to increased surveillance and identification as well as the 112,752 illnesses non-O157 STEC is estimated to cause annually (*132*).

Non-O157 STEC serotypes possess the same ability as O157:H7 serotype to produce Shiga toxins Stx1 and Stx2 as well as the ability to facilitate attachment via the *eae* gene (*172*). Between 1983 and 2002, stool samples were collected from over 900 patients with non-O157 infections, and 70% were attributed to six serogroups including O26, O111, O103, O121, O45, and O145 (*21*). The isolates were analyzed by PCR, and results showed 61% of isolates had *Stx1*, 22% had S*tx2*, and 17% had both stx1 and S*tx2*; S*tx2* production was associated with higher risk of HUS (*21*). An epidemiological study utilized results from cases reported via FoodNet sites from 2000-2010 to compare non-O157 STEC with O157:H7 STEC illnesses (*71*). A laboratory survey on reported cases provided O antigen identification for 1,708 isolates and "The Big Six" serogroups accounted for 83% of non-O157 illnesses over the ten year period (*71*). Of the isolates from this study, the Shiga toxin type was reported for 74% of isolates, 74% of which had

just S*tx1* as opposed to 17% that had just S*tx2*, while just 9% had both S*tx1* and S*tx2* (*71*). Again, HUS was more frequently associated with S*tx2* production (*71*). The similarity in virulence factors leads to findings of clonality in certain serogroups including O26, O111, and O103 from MLST testing, furthering the evolutionary theory that S*tx* genes encoded on bacteriophages may be transferred between certain EPEC and EHEC pathotypes (*61*).

Reservoirs and modes of transmission for non-O157 STEC are similar to those known for O157:H7 and include food and water, as well as person-to-person and animal-to-person contact (*133*). International travel may also play a significant factor in the transmission of non-O157:H7 as one study concluded that individuals were five times more likely to report international travel around the time of illness (*71*). Person-to-person contact has been implicated in 39% of outbreaks of non-O157:H7 STEC as opposed to just 14% of O157:H7 STEC, suggesting person-to-person could be a more significant form of transmission for non-O157 STEC (*125*). Food commodities implicated in outbreaks of O157:H7 STEC and non-O157 STEC also differ in that 41% of foodborne outbreaks were attributed to beef and 21% to produce for O157:H7 STEC infection, but for non-O157 STEC, 35% of foodborne outbreaks implicated dairy, leafy vegetables, and fruits or nuts while less than 6% were attributed to beef (*104*). Although beef is a product that has been found to harbor non-O157 STEC, the outbreak information suggests beef products have not been as highly implicated in foodborne illness outbreaks of non-O157 STEC as opposed to O157:H7 STEC (*104*, *133*, *165*). In fact, two different sprout outbreaks in recent years have been attributed to non-O157 STEC serogroups. A retail restaurant serving sprouts contaminated with *E. coli* O26 as a part of sandwiches and other dishes sickened 29 people in 11 different states from December of 2011 to March of 2012 (*42*). In 2014, another sprout outbreak caused 19 people in six states to fall ill and was again associated with deli restaurants serving

sprouts, this time contaminated with *E. coli* O121 (*41*). *E. coli* O26 caused another outbreak in 2015 at a Mexican grill style restaurant and although the contamination could not be traced back to a specific food product, 60 people fell ill, of which 22 were hospitalized (*44*). Lastly, a unique outbreak was attributed to flour contaminated with *E. coli* O121 and O126 in 2016, during which 63 people in 24 states were affected by foodborne illness from the product (*43*).

Prediction Modeling Programs for Foodborne Pathogens

There are several programs available to model specific pathogens including the USDA Pathogen Modeling Program (PMP) and the ComBase Predictor modeling program which are available online (*51, 154*). Both programs offer a fairly comprehensive database of results from studies conducted with foodborne pathogens under different growth or inactivation scenarios whether within food products or selected broths. If either database does not contain the combination of pathogen, food product, and growth or inactivation scenario needed, predictive modeling is offered by both the PMP and ComBase programs. Each program offers modeling based on conditions intrinsic to food products or broth conditions including the initial population of pathogen, % NaCl or a_w, and pH as well as external conditions like holding temperature.

The ComBase Predictor offers growth, thermal inactivation, and non-thermal survival prediction methods. The thermal inactivation and non-thermal survival methods are limited to certain pathogens but provide modeling based on the results of published research. There are also static and dynamic versions of the growth model for constant holding temperature modeling, or to correlate a series temperatures and time points during the growth process. The PMP allows for similar modeling based on the results of published research including cooling, growth, heat inactivation, survival, and transfer models for certain pathogens. However, the PMP only allows for static condition predictive modeling regarding growth. The limitations for these predictive

models are especially apparent for studies regarding cooling as a variety of temperatures outside growth conditions are expected and cooling modeling is often carried out for a select few pathogens. Other limitations include the lower limits of 4.5 pH and 0.5% NaCl which are not completely accurate for the modeling of low sodium or acidic products.

In summary, both programs are limited in their ability to model the survival or growth of BSL I *E. coli* and *B. cereus* in the food products evaluated in the research described herein. The lack of cooling modeling for the microorganisms tested compounded with the lower limits of temperature and % NaCl result in only a nominal amount of reliability for predictions made by ComBase and PMP. Perhaps the research described herein could be used to inform databases and programs such as ComBase and PMP to further the understanding of how surrogates and pathogens behave in food products during cooling.

The Use of BSL I Surrogate *Escherichia coli* and Pathogenic *Bacillus cereus*

Bacillus cereus Isolates

The research activities described in subsequent chapters utilized two *Bacillus cereus* strains with a Biosafety Level I (BSL I) status designation (ATCC® 11778 and ATCC®14579). Both isolates were originally obtained from air samples taken within a cow shed (*55*). Although given a BSL I designations, PCR testing identified that these two *Bacillus cereus* reference strains possess virulence genes including those regulating hemolysis and cytotoxin K production (Oltuszak-Walczak). The application of the ATCC®14579 strain, as designated by ATCC®, is specifically for food testing (*5*). The ATCC® 11778 strain (FDA strain PCI 213) is designated under the International Organization for Standardization (ISO) 6888-3:2003 "Microbiology of Food and animal Feeding Stuffs" (*6*). Therefore, these strains were selected to model true pathogen behavior in the brown rice product during cooling. Although many studies exist for

inactivation and some interventions, the use of research databases did not reveal a representative study under cooling conditions where either BSL I *B. cereus* ATCC® strain (ATCC®11778 and 14579) were utilized.

Escherichia coli Isolates

The research activities described in subsequent chapters utilized four *Escherichia coli* strains (ATCC® BAA-1427, BAA-1429, BAA-1430, and BAA-1431) from The ATCC® Non-pathogenic *Escherichia coli* Surrogate Indicators Panel (ATCC® MP-26™) to serve as surrogates for Shiga toxin-producing *Escherichia coli* (STEC) (*4*). All four strains were originally isolated from cattle hides and each are recommended by the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) for use in research when evaluating changes in microbial populations in the food processing environment during validation studies (*4*). The *E. coli* Reference Center of Pennsylvania State University has confirmed that these strains lack virulence factor genes (*4*). Therefore, these strains are categorized as Biosafety Level I (BSL I). Therefore, these four strains were selected to model pathogenic STEC activity during cooling in the pre-cooked taco meat, low sodium marinara sauce, and chili con carne with beans products.

The use of research databases revealed two representative studies under which the five ATCC® surrogate *E. coli* strains (ATCC® BAA-1427, BAA-1428, BAA-1429, BAA-1430, and BAA-1431) were utilized under cooling conditions. One study examined the five ATCC® surrogate *E. coli* strains in uncooked, irradiated beef patty product and compared the survival or growth rates to that of O157:H7 STEC (*95*). Frozen, irradiated beef patties were stored at 4°C (39.2°F) and sampled on days 0, 4, 7, and 14 of cooling; the results indicated none of the ATCC® surrogate *E. coli* strains were statistically different ($P > 0.10$) in population under the cooling conditions when compared with *E. coli* O157:H7 (*94*). Another study evaluated the ability of the

five ATCC® surrogate *E. coli* strains to model *Salmonella enterica* populations under 4°C (39.2°F) refrigeration and -20°C (-4°F) freezer conditions by inoculating irradiated, frozen ground beef (*116*). Sampling took place during a 21 or 90 day period and results indicated the five ATCC® surrogate *E. coli* strains were comparable in population, or slightly greater than *S. enterica* under the cold storage conditions (*116*). These results indicate the five ATCC® surrogate *E. coli* strains provided a margin of safety and could be used for modeling pathogens like *S. enterica* under similar conditions (*116*). Many other studies exist for inactivation and interventions, but these two studies provide the strongest evidence for the efficacy of ATCC® *E. coli* surrogates under cooling conditions.

Cold Holding: Time/Temperature Control

Introduction

The US FDA has consistently identified time/temperature control, specifically cold holding, as a major factor contributing to the incidence of foodborne illness (*156*, *157*, *158*). Schools in particular may struggle with this critical control point for several reasons including: limited cooling capacity in freezers or refrigerators, a lack of funding for more effective cooling equipment, or the limitations that come with a short workday for school lunch program employees (*129*). As part of the 2000 Retail Food Program Database of Foodborne Illness Risk Factors report, the FDA reported a high percentage of observations indicated as "out of compliance" (39.5%) for schools regarding proper holding time/temperature procedures (*158*). Although schools have shown statistically significant improvement over the past ten years when it comes to cold holding and proper holding time/temperature (*155*, *158*), there will always be a certain amount of risk associated with school nutrition programs, as they often utilize foods classified as Potentially Hazardous (PHF) that requires Time Control for Safety (TCS) (*115*, *152*,

156). Potentially hazardous food includes sliced fruits, lettuce, meat, poultry, milk, and other dairy products will become more frequently served by school nutrition programs to comply with the Healthy, Hunger-Free Kids Act of 2010, which requires more diversified options for fruit, vegetables, and lower sodium food options (*147*). Even pre-cooked foods are subject to TCS, requiring a heating step to 57.2°C (135°F) (*115*). Therefore, school nutrition programs face risk with these pre-cooked food products as well.

Cooling Practices in U.S. Schools

Holding time/temperature and cold holding is a critical aspect of food safety for school nutrition programs. A survey of 411 school food service managers by Krishnamurthy et al (*98*) revealed that 78% of respondents cool leftovers to reheat for service at another meal in their school lunch operations. The survey also pointed to a financial barrier for schools regarding blast chillers, which effectively cool food according to FDA Food Code standards (*117*). Only 8% of respondents across the U.S. reported having access to a blast chiller (*94*). This low percentage is most likely because of the high cost of this piece of equipment. Other cooling risk factors identified via survey responses include 18% who did not monitor temperature over time, 12% who did not use appropriate thermometers, and 30% who did not own an ice machine. School nutrition managers also reported an average walk-in refrigerator/freezer capacity of just 20% free or open space (*129*).

Over a ten-year period from 2000-2010, the Centers for Disease Control and Prevention (CDC) collected data via the Foodborne Disease Outbreak Surveillance System regarding foodborne illness outbreaks in school settings (*163*). A 2015 analysis of the data revealed the second leading contributing factor for the proliferation of pathogens within a food product was that no attempt was made to control the temperature of the implicated food or the length of time

the food was out of temperature control (*163*). The analysis also concluded that the third leading contributing factor for proliferation to be improper cold holding as a result of malfunctioning refrigeration equipment (*163*). Of 105 food safety errors reported in the proliferation category for this analysis, the two factors above accounted for a total of 38% of those errors (*163*). These factors are of particular issue in school environments because, often, only breakfast and lunch meals are served, and employees leave work for the day shortly after beginning the cooling process (*129*).

It is clear from these studies that there are various barriers and issues faced by school nutrition programs regarding their ability to implement food safety protocols. They include obvious factors like lack of cold storage space or errors where temperature control was not even attempted. They also provide insight to more nuanced issues and passive errors such as not being attentive to equipment malfunction and the inability to afford equipment maintenance. Research into effective and feasible cooling is critical in order to offer alternatives considering the various limitations in the school nutrition program setting.

Time/Temperature Control Studies

Several studies have been conducted to validate cooling methods used by school nutrition programs. One foundational study published in 2005 evaluated refrigerator, chill stick, and blast chiller methods of cooling chili (*117*). The blast chiller met the 2001 FDA Food Code requirement for 2 and 3-inch food product depths but the other methods did not (*117*). The chill stick method was also evaluated in this study and is considered a large plastic reservoir that is filled with water and subsequently frozen to later be used as a way to cool food without watering down the product (*117*). The chill stick method in the refrigerator was just ten minutes out of compliance (*117*). However, the chill stick method may not be a feasible cooling technique as it

is most effective as an active cooling method, requiring more labor and time from employees (*117*).

Another study contributing to knowledge of effective cooling techniques was published in 2013, concluding that the density and nature of food products pose different challenges to proper cooling (*118*). In this study, cooked beef taco meat was cooled using three different methods including storage in a walk-in refrigerator, with or without an ice bath, and storage in a walk-in freezer (*118*). The only method for the cooked taco meat that met FDA Food Code standards was when the food was cooled in a walk-in freezer at a 2-inch product depth (*118*). Two methods of cooling for the steamed rice product met the FDA Food Code requirement, including storage in a walk-in refrigerator, with and without an ice bath, for 2-inch product depths (*118*). Because the 2-inch product depth of rice cooled in the refrigerator according to the FDA Food Code requirement and cooked taco meat did not, the results provide evidence that the density and composition of each food had a definitive impact on which cooling method was effective (*118*). For this reason, a standard cooling guideline for food products is difficult to achieve when many different types of food products and methods are involved in the cooling process as they often are in school nutrition lunch programs.

Another cooling method study was published in 2013 with similar results (*129*). In this study, chili con carne with beans and meatless tomato sauce were cooled at 2 and 3-inch product depths in a walk-in freezer, walk in refrigerator with or without ice bath, and cooled with a chill stick (*129*). The only cooling method for both products that met both FDA Food Code standards was the 2-inch food product depth stored in the walk-in freezer (*129*). Similar to the other cooling studies already referenced above, this study concluded that the freezer cooling method was the most effective, but only at 2-inch food product depths (*117*, *118*, *129*).

All three studies concluded that none of the 3-inch product depth pans met the 2001 or 2009 FDA Food Code time and temperature requirement. However, even 2-inch product depth pans in the refrigerator did not meet FDA code standards in these studies for most products, excluding the steamed rice. Attempting to reduce heated food products to a depth that would meet time and temperature cooling standards for refrigerator methods may not be practical in a school setting because of a lack of space in a school lunch setting (*129*). Although blast chillers cool food products to meet the FDA Food Code requirement, the cost of the equipment does not make it a feasible option for most schools (*117*). Summarily, few methods have been validated that cool food products at a pace to meet the FDA Food Code requirement, signaling a critical need to continue research into validating cost effective and safe cooling techniques.

Post-process Contamination

Introduction

Foodborne illness may have many origins and there are certain factors that facilitate the spread of pathogens. Hand hygiene and cross contamination are two significant factors identified by the CDC (*25*). According to FDA Good Manufacturing Practices (GMPs), post-process contamination is defined as an incident after processing when a finished food product becomes adulterated, whether with pathogens, chemicals, allergens, or foreign objects, making the processed product unsafe to eat (*159*). Post-process contamination within production facilities has led to several outbreaks of Salmonellosis from peanut butter spreads in the United States (*26*, *27*). *Listeria monocytogenes* and *Staphylococcus aureus* are often implicated in post-process contamination in restaurant or other food service settings as well, especially in foods that are ready-to-eat or require more hand preparation (*3*, *91*, *92*). Several factors can reduce the safety of

finished food products, two of the most significant being poor hand hygiene and cross contamination.

Hand Hygiene and School Associated Outbreaks

Poor hand hygiene facilitates the spread of many viral and bacterial borne illnesses. It is a major contributing factor in outbreaks of norovirus, up to 54% of which can be attributed to infected workers' bare hand contact with ready-to-eat food (*82*). Poor hand hygiene has been linked to outbreaks of *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* as well (*53*, *67*, *92*). When hands are still wet after washing, the ability of bacteria, even at low populations, to transfer easily between hands and surfaces makes proper hand hygiene critical (*87*). Although studies have shown that hand hygiene is a simple way to reduce diarrheal illness by up to 35%, this poor hygiene practice continues to be a significant factor in foodborne illness (*60*, *67*, *169*).

School nutrition programs face challenges and barriers for hand hygiene that affect food handlers in other food production environments as well, such as time pressure (*78*). Inadequate staffing can lead to increased time pressure and infected employees coming to work despite illness (*55*, *78*, *144*, *145*). One study evaluated school associated outbreaks of foodborne illness documented from 1973-1997 and concluded that 57% were caused by contamination from a food handler (*55*). Another study in support of these findings evaluated outbreaks in schools during 1998-2008 and concluded that enhancing effective handwashing was the top recommendation for preventing future outbreaks in schools (*101*). Handwashing is not only critical for food handlers, but for school age students as well since an estimated 20% of *E. coli* O157:H7 infections are the result of secondary spread (*136*).

Several significant school associated outbreaks have occurred as a result of poor hand hygiene. In 1990, an outbreak of Staphylococcal food poisoning was traced back to one specific

food handler who unwrapped hams (*127*). A nasopharyngeal sample taken from a worker tested positive for the same phage and plasmid profile of the *Staphylococcus* strain isolated from those infected (*127*). In another school associated outbreak, *Campylobacter jejuni* sickened 27 who ate at a "Grandparents Luncheon" held by a school in Kansas (*120*). This outbreak was traced to a food handler at a central kitchen who was suffering a diarrheal illness (*120*). Pulse- field gel electrophoresis identified the strain from the food handler as indistinguishable from those isolated from case patients (*120*). In 1989, Japan suffered a massive outbreak of norovirus from contamination caused by an ill food handler in a centralized kitchen, resulting in over 3,000 students and 117 teachers reporting illness from seven different elementary schools (*96*).

Cross Contamination and School Associated Outbreaks

Cross contamination is defined as a transfer of harmful substances or disease causing microorganisms to food via food contact surfaces, hands, even sponges and utensils (*149*). Cross contamination results from many food handling scenarios, such as cutting or slicing, during food preparation. For example, cutting utensils used when preparing raw animal meat that may be improperly sanitized and then used to cut produce (*89, 125, 126*). The handling of money around ready-to-eat foods may also pose a risk for contamination, especially if paper bills are dirty or damaged (*100, 109*). It is estimated that 13% of coins and 42% of paper money in the United States are contaminated with pathogenic bacteria (*2*). However, more commonly implicated in cross contamination are food contact surfaces which pose a particularly high risk if not sanitized correctly (*137, 141*). For instance, cutting boards, countertops, and non-food contact surfaces (faucet handles and sink drains) may be primary culprits for contamination (*137, 141*).

It is common for raw foods like meats and vegetables to be prepared in one kitchen. Therefore, cross contamination is a likely risk during food preparation (*89, 125, 126*). Cross

contamination in a restaurant setting was modeled by four different outbreaks that occurred in Oregon and Washington in 1993 from salad bar items contaminated with *E. coli* O157:H7 (*89*). The conclusion of an epidemiological investigation was that multiple events of cross contamination from raw beef products to salad bar food items had led to the illnesses, resulting in 39 culture confirmed cases and 15 hospitalizations (*89*). Processing equipment can also play a role in cross contamination and *Listeria* species are often implicated in these outbreaks because of their biofilm forming abilities (*90*). Dairies may provide a particularly nutritive environment for pathogens like *Listeria* as dairy foods are proteinaceous and surfaces within a dairy production environment may create the perfect niches for biofilms (*139, 168*). A processing environment cross contamination resulted in an outbreak that took place in 1994 when *Listeria monocytogenes* sickened 54 individuals after they consumed contaminated chocolate milk at a picnic in Illinois (*54*). An investigation revealed that contamination most likely occurred when milk had leaked into an insulation jacket around a holding tank and was able to re-enter the product when the tank was drained at a dairy processing facility (*54*).

Cross contamination occurs in school settings for similar reasons, including contaminated sink drains and work surfaces which were implicated in an outbreak involving over 1,400 students in Italy (*12*). *L. monocytogenes* was detected at a population greater than 10^6 CFU/g in a corn and tuna salad prepared by a food caterer that served both primary schools and a university (*12*). Corn and tuna samples by themselves tested as sterile from unopened cans and the positive results from the work surface and sink drain make cross-contamination a likely suspect in this outbreak (*12*). In 2001, an outbreak of *Salmonella enteritidis* in Japan sickened 163 school age children who ate dessert buns that became contaminated after unpasteurized liquid and shelled eggs were utilized in the same production facility (*108*). It was theorized that equipment and bins

previously used during the production of cream puffs were not properly sanitized but were subsequently used in the production of the ready-to-eat dessert buns (*108*). Large outbreaks seem to occur in Japan where the food preparation system for elementary schools follows a menu distributed by a centralized kitchen, meaning schools in different districts within a large city receive the same menu prepared in once central kitchen (*110*). Produce for these menus often come from farms around the city, one such farm contributed to a massive *E. coli* O157:H7 outbreak after it produced and shipped contaminated white radish sprouts to a centralized kitchen serving schools and childcare centers in Sakai city in 1999 (*110*). The white radish sprouts were included in several dishes as an uncooked ingredient that was then consumed by thousands of children, resulting in over 9,000 illnesses and 398 hospitalizations, making it the largest outbreak in Japan's history (*110*).

Chapter 3 - OBJECTIVE ONE: EVALUATING THE IMPACT OF COOLING METHODS ON BIOSAFETY LEVEL I SURROGATE AND PATHOGEN POPULATIONS IN FOUR FOOD PRODUCTS

Introduction

Improper or "slow" cooling has been identified as the third leading factor in school associated foodborne illness and a considerable risk for improper cooling is also present for other institutional settings as well (*123*). According to a 2013 Morbidity and Mortality Weekly Report, the CDC concluded that school settings were associated with the largest number of foodborne outbreaks (286) and illnesses (17,266) when compared with other institutions like daycares, workplace cafeterias, and prisons or jails (*1*). However, the nature of large outbreaks and number of illnesses may be attributed to the fact that the National School Lunch Program provides meals to over 31 million children each day in the United States (*150*). Because many school nutrition program directors report cooling leftover food for later service (*98*) and slow cooling is a public health risk, the Food and Drug Administration Food Code was updated in 2009, requiring food products to be cooled to 21.1°C (70°F) within 2 hours of cooking and down to 5°C (41°F) within a total of 6 hours (*156*). Several studies have been conducted to evaluate cooling techniques commonly used in school nutrition programs for various food products and have concluded that very few techniques meet the FDA Food Code requirement (*98*, *117*, *118*, *129*). This study was designed to evaluate *B. cereus* and surrogate *E. coli* microbial populations in food products during a 24-hour cooling period as a follow up to these published cooling studies.

Experimental Design

Pre-cooked taco meat, chili con carne with beans, marinara sauce, and brown rice were evaluated in this study. Food products were re-heated or cooked, portioned to 2 and 3-inch depths in steam table pans, then allowed to cool to 60°C ± 5°C (140°F ± 5°F) before inoculation with a Biosafety Level I (BSL I) *Bacillus cereus* (*B. cereus*) or surrogate *Escherichia coli* (*E. coli*) at a target concentration of 4 $\log_{10}$ CFU/g. Pre-cooked taco meat, chili con carne with beans, and marinara sauce were inoculated with a cocktail of four ATCC® strains of surrogate *E. coli* to model Shiga toxin-producing *E. coli* (STEC). Brown rice was inoculated with a cocktail of two ATCC® strains of BSL I *B. cereus*. After inoculation, each pan was covered with one of three methods and then cooled in either a 4°C (39.2°F) walk-in refrigerator situated in an ice bath or in a -20°C (-4°F) walk-in freezer. Food products were then sampled at time points including 0, 4, 8, 12, and 24 hours of cooling. At these time points, samples were collected, serially diluted, and plated in order to enumerate microbial population within the food products at each time point. Three replications of all experimental methods were completed for each of the four food products.

Materials and Methods

Bacterial Strains and Propagation

Escherichia coli

Four *Escherichia coli* strains were chosen from The ATCC® Non-pathogenic *Escherichia coli* Surrogate Indicators Panel (ATCC® MP-26™) to serve as surrogates for Shiga toxin-producing *Escherichia coli* (STEC) (*4*). The four strains that were utilized in a cocktail included ATCC® BAA-1427, BAA-1429, BAA-1430, and BAA-1431. All four strains were originally isolated from cattle hides and each are recommended by the United States Department of

Agriculture Food Safety Inspection Service (USDA-FSIS) for use in research when evaluating changes in microbial populations in the food processing environment during validation studies (*4*). According to ATCC® instructions, isolates were rehydrated in tryptic soy broth (TSB; BD Difco™ from Fisher Scientific, Franklin Lakes, NJ) and incubated separately at 37°C (98.6°F)for 24 hours. Rehydrated cultures were then dispensed in 1 mL portions to microcentrifuge tubes with 10% glycerol (Fisher Scientific, Lenexa, KS) added. The microcentrifuge tubes were then stored at -80°C until later use.

Developing and Assessing Acid Tolerance

The first repetition with the marinara sauce product revealed lower than expected survival rates of surrogate *E. coli*. A 5 $\log_{10}$ CFU/g inoculum of *E. coli* surrogate cocktail was prepared using methods described in the Inoculation Procedure section of this paper, however, colony enumeration from time point testing revealed inconsistent results over a 24-hour period ranging from poor to no surrogate survival from the first experimental repetition of the marinara sauce product. Therefore, a hypothesis was developed: if the *E. coli* surrogates could not survive as effectively at low pH like *E. coli* O157:H7 then there would be a lower than expected rate of survival in the marinara sauce product. The level of acidity was hypothesized to have a negative impact on surrogate survival in the food product. The first step to assess this hypothesis involved utilizing a benchtop pH meter (Education pH meter; Fisher Scientific, Lenexa, KS) to accurately measure the acidity of the marinara sauce. Once the meter was calibrated, the pH of the uninoculated, room temperature sauce product measured 4.18. To further evaluate these hypotheses, preliminary testing was performed by conducting a small study to compare the survival of three microorganisms in the marinara sauce product: *E. coli* surrogate cocktail, *Salmonella enterica subsp. enterica* serovar Typhimurium (ATCC® 14028), and *Escherichia coli*

O157:H7. Each microorganism was prepared in two different growth mediums for inoculum: TSB + 1% glucose and Buffered Peptone Water (BPW; BD Difco™ Fischer Scientific, Franklin Lakes, NJ). It was hypothesized that the TSB + 1% glucose (Fisher Scientific, Lenexa, KS) would foster an increased acid tolerance after incubation for 18 hours, a hypothesis previously tested by Buchanan, et. al. in 1996 (*23*).

Acid Habituation Preliminary Study Results

Salmonella enterica subsp. enterica serovar Typhimurium (ATCC® 14028), *E. coli* O157:H7, and the cocktail of four ATCC® *E. coli* surrogates were each grown for 24 hours at 37°C (98.6°F). Each microorganism was grown in both TSB + 1% glucose and BPW. Six 500 - mL glass bottles were each filled with 100 mL of marinara sauce after it had been heated to 73.8°C (165°F) in a commercial tilt skillet (Cleveland Tilt Skillet). The sauce was allowed to cool to 60°C ± 5°C (140°F ± 5°F), at which time, 1 mL of inoculum was added to each bottle of sauce to achieve a 10^5 CFU/g distribution of each pathogen grown in each medium. Samples were obtained at time points 0, 4, and 8 hours. 25 gram samples were diluted with 225 mL BPW and serially diluted and plated on MacConkey Agar (MAC; Remel, Lenexa, KS). MAC plates were incubated at 37°C (98.6°F) for 18-24 hours at which point colonies were enumerated.

The pH of the ATCC® *E. coli* surrogate cocktail grown in TSB + 1% glucose was 4.68. During the 8 hour cooling period, the ATCC® *E. coli* surrogate cocktail inoculum grown in TSB + 1% glucose provided increased population survival of 0.23 $\log_{10}$ CFU/g over the ATCC® *E. coli* surrogate cocktail grown in BPW. *Salmonella* serovar Typhimurium survival was improved when grown in TSB + 1% glucose by an average of 1.56 $\log_{10}$ CFU/g compared to survival when grown in BPW. *Escherichia coli* O157:H7 survival was nearly identical when grown in TSB + 1% glucose as in BPW, with only 0.07 $\log_{10}$ CFU/g difference. This acid habituation method was

chosen to prepare inoculum for the marinara sauce product because of the moderately improved survival of the ATCC® *Escherichia coli* surrogate cocktail when grown in TSB + 1% glucose.

Bacillus cereus

Two *Bacillus cereus* strains of Biosafety Level I status were utilized in a cocktail (ATCC® 11778 and ATCC®14579). Both isolates were originally obtained from air samples taken within a cow shed (*55*). Each strain was propagated from a freeze-dried state according to ATCC® instructions. Under aseptic conditions, the two ATCC® isolates were rehydrated in Nutrient Broth (BD Difco™ from Fisher Scientific, Frankland Lakes, NJ) and incubated separately at 30°C for 24 hours. They were then dispensed in 1 mL portions to micro centrifuge tubes with 10% glycerol added. The microcentrifuge tubes were stored at -80°C until later use.

To enumerate populations of the originally rehydrated ATCC® frozen suspensions, micro centrifuge tubes of each strain were thawed and 1 mL was serially diluted in BPW and plated on Mannitol Egg Yolk Polymyxin B agar (MEP; Remel, Lenexa, KS) for enumeration after incubation at 30°C for 24-48 hours. For the ATCC®11778 and 14579 strain, a 7.07 $\log_{10}$ CFU/mL and a 7.49 $\log_{10}$ CFU/mL population was detected, respectively.

Spore Harvesting and Enumeration

In order to harvest spores for inoculum preparation, a procedure outlined by Grande et al. was performed (*73*). A frozen microcentrifuge tube of each ATCC® *B. cereus* strain (ATCC® 11778 and ATCC®14579) was thawed and 1 mL of each strain was added to its own test tube containing 9 mL Brain Heart Infusion Broth (BHI; Fisher Scientific, Lenexa, KS). The strains grew separately by incubating at 30°C (86°F) for 24 hours. After incubation, 100 µl of each strain grown in BHI broth was spread plated onto Nutrient Agar (Fisher Scientific, Lenexa, KS) supplemented with 0.05 g/l manganese sulfate (Acros Organics™ from Fisher Scientific, Geel,

Belgium). Plates were incubated for four days at 37°C (98.6°F) to obtain spores from an estimated 90-95% of cells (*73*). Spores and vegetative cells were directly harvested from the plates with sterile loops and then deposited directly into sterile distilled water at approximately 3 mL per plate. These 3 mL spore + vegetative cell suspensions were added to a 25 mL centrifuge tube and centrifuged at 5,000 x *g* for 15 minutes at 4°C (39.2°F). The resulting pellet was washed with sterile distilled water and re-suspended for a second, identical centrifugation and washing. The final pellet was re-suspended in 25 mL of sterile distilled water. This suspension was then aliquoted in 5 mL amounts to conical tubes (MIDSCI, St. Louis, MO) and stored at -20°C (-4°F) until later use.

Preliminary enumeration testing was performed to evaluate the population of the vegetative + spore populations within the harvested suspensions. A conical tube of vegetative + spore suspension was thawed to room temperature (20°C) and a 1 mL aliquot was serially diluted in BPW and plated on MEP agar plates that were incubated at 30°C for 24-48 hours. The enumeration revealed a pre-heat shock population of the harvested spore + vegetative cell suspension to be 8.06 $\log_{10}$ CFU/mL.

However, it was critical to assess the approximate spore population after the heat shock as that step would be performed the day of inoculation. Therefore, a conical tube of spore + vegetative cell suspension in distilled water was thawed to room temperature (20°C) and subsequently heat shocked at 80°C for 10 minutes to simulate inoculum preparation, which was designed to mimic the cooking process. A 1 mL aliquot of the heat-shocked suspension was then serially diluted in BPW and plated on MEP agar plates that were incubated at 30°C for 24-48 hours. The population post-heat shock was found to be 8.63 $\log_{10}$ CFU/mL. This was considered to be primarily spores. A phase contrast microscope was utilized to visually confirm populations

pre- and post-heat shock. The pre-heat shock suspension had very high populations of both vegetative cells and spores. Post-heat shock, there were very few vegetative cells and a large population of spores apparent (See Appendices F, Figures F-1 and F-2). The larger population post-heat shock may be due to the fact that spore suspensions were not cooled on ice immediately after heating, cooling heated suspensions on ice has been suggested in some research in order to stabilize spores and prevent germination (*140*).

To ensure that vegetative cells were eliminated as a source of error and to obtain a true spore enumeration, a Brightline Hemocytometer was utilized along with a protocol published by the biology department of Massachusetts Institute of Technology (*6*). Frozen spore + vegetative cell suspension in distilled water was thawed and 20 microliters were dispensed for dilution in 120 microliters of 0.4% trypan blue (dilution factor of 7). After vortexing the spore + vegetative suspension in trypan blue, 10 microliters of the resulting suspension was injected into each well of the hemocytometer and spores were enumerated under the 20x objective magnification of a phase contrast microscope. Spores appeared small, round, and clear indicating they were viable cells as they did not take up the trypan blue solution. Vegetative cells were rod shaped and pre-heat shock appeared clear while post-heat shock, they appeared blue. Pre-heat shock, the hemocytometer testing revealed a spore population of approximately 5.41 $\log_{10}$ CFU/mL spores. Post-heat shock, small, round, and clear spores were prevalent with very few vegetative cells apparent. Post-heat shock, the population of spores was 5.31 $\log_{10}$ CFU/mL spores. These results support the utilization of several enumeration methods including a hemocytometer in order to yield more accurate spore enumeration than performing the plating method alone.

A spore stain was also conducted to evaluate spore + vegetative cell suspensions pre- and post-heat shock, following the Schaeffer-Fulton method for staining endospores (*88*). The stain

reflected a similar number of spores pre- and post-heat shock but much fewer vegetative cells post-heat shock. A preliminary test was carried out to monitor the population of spores vs. vegetative cells within the brown rice product. A 2 and 3-inch product depth of brown rice was prepared according to the procedures outlined in the Product Preparation section, allowed to cool to 60°C ± 5°C (140 ± 5°F), and then inoculated with a post-heat shock inoculum of 10^5 CFU/mL spores. Over a 3-hour period, the 2 and 3-inch pans of brown rice were stored in the 4°C (39.2°F) walk-in refrigerator and 25 gram samples were collected each hour. Sampling procedures were identical to those in the Sampling section, with dilutions plated on MEP agar that were then incubated at 30°C for 24-48 hours. Enumeration from MEP agar plates revealed a slight 0.03 $\log_{10}$ CFU/g population increase for 2-inch product depths over the 3-hour cooling period and a 1.56 $\log_{10}$ CFU/g decrease in populations in the 3-inch product depths. An endospore stain was conducted from the BPW homogenate of diluted rice samples at each hour. The endospore stains from time 0 hour revealed a high spore population and few vegetative cells. The endospore stains from time 1, 2, and 3 hours revealed a decreasing spore population and a slight increase in vegetative cell population. These results indicate the sublethal heat shock of 80°C (176°F) in the laboratory successfully mimicked the scenario of the cooking process. These results provide confidence that on the day of the experimental run, the food product was inoculated with 10^5 CFU/mL of *B. cereus* spores which would then germinate with possible vegetative growth during the cooling process.

Product Preparation

All food products and ingredients were ordered from a foodservice product distributor and were chosen to meet National School Lunch Program Nutrition Standards 7 CFR Parts 210 and 220 (*149*). Pre-cooked, frozen taco meat was stored in a commercial refrigerator at 4°C

(39.2°F) for several days prior to an experimental run in order to thaw properly for reheating. Pre-cooked taco meat was packaged in 5 lb. bags that were placed in 2-inch steam table pans and heated in commercial steamers (Electrolux Air-o-Steam Touchline Combi Oven, Pordenone, Italy; Cleveland SteamChef Electric Countertop Steamer, Cleveland, Ohio) to 73.8°C (165°F). Canned, low sodium marinara sauce was cooked to 73.8°C (165°F) in a commercial tilt skillet (Cleveland Tilt Skillet). Chili was prepared according to a recipe (see Appendices F, Figure F-3) used by a school nutrition program and was cooked to 73.8°C (165°F) in the same commercial tilt skillet. For the brown rice product, water was heated to 190°F in the commercial tilt skillet and was then added to uncooked brown rice measured in 2 ½ and 4-inch counter pans. Pans were then covered with a layer of plastic wrap and a layer of aluminum foil and placed in a commercial grade convection oven (Garland Master 200) at 350°F for 35 minutes. After the food products were re-heated or cooked, they were then portioned to 2 and 3-inch food product depths in 2 ½ and 4 inch counter pans. The product was stirred and allowed to cool to 60°C ± 5°C (140°F ± 5°F) for inoculation.

Inoculation Procedure

The day prior to inoculation of pre-cooked taco meat and chili products, a microcentrifuge tube of each frozen ATCC® *Escherichia coli* strain (ATCC® BAA 1427, BAA 1429, BAA 1430, BAA 1431) was thawed and grown separately in four large centrifuge tubes with 25 mL of BPW. These cultures were incubated at 37°C (98.6°F) for 18-24 hours. For the marinara sauce product, each ATCC® *Escherichia coli* surrogate was grown separately at 37°C (98.6°F) for 18-24 hours in 25 mL of TSB + 1% glucose in order to prepare acid-adapted cultures according to Buchanan et al. (*20*). The following day, the 25 mL culture tubes were centrifuged at 5,000 x *g* for 15 minutes at 4°C (39.2°F). The supernatant was discarded and the

pellets were then re-suspended in 25 mL of 0.1% Peptone Water (PW; BD Bacto™ Fischer Scientific, Franklin Lakes, NJ) after which all four strains were combined in a sterile 100 mL container, resulting in 100 mL of cocktail. This 100 mL cocktail in PW was estimated to be 10^9 CFU/mL and was utilized to prepare inoculum for all samples. Inoculum for each pan was prepared based on the weight of food product within each pan in order to achieve a target concentration of 1.0 x 10^4 CFU/g, with the liquid of the inoculum comprising no more than 1% of the food product (*114*).

On the day of inoculation of the brown rice food product, six conical tubes of 5 mL frozen spore suspension were removed and allowed to completely thaw for 45-60 minutes at room temperature (20°C). The thawed tubes were then placed in an 80°C bead bath and heat shocked for 10 minutes to simulate the cooking process and subsequent sublethal heat-induced germination of spores. After the spore suspensions were allowed to cool to room temperature, tubes were thoroughly vortexed and inoculum was prepared from these tubes of 10^5-10^6 CFU/mL suspensions. Inoculum for each pan was prepared by diluting the heat shocked spore suspensions in 0.1% PW based on the weight of food product within each pan to achieve a 10^4 - 10^5 CFU/g spore inoculation, such that the inoculum comprised no more than 1% of the total food product (*114*).

The temperature of all products was monitored using a Taylor 9842FDA waterproof digital thermometer (Taylor; Las Cruces, NM) and all food products were stirred and allowed to cool to 60°C ± 5°C (140°F ± 5°F) prior to inoculation. After liquid inoculum was added to each pan, food was stirred thoroughly (~2 minutes per pan) to obtain an even distribution of bacterial cells. Inoculation times were recorded for each pan upon completion of stirring and time points at 0, 4, 8, 12, and 24 hours were set accordingly.

Treatments and Cooling

After the food products were inoculated and time point 0 samples obtained, each pan was fitted with a Lascar EL-USB-2- LCD USB temperature data logger (Lascar; Erie, PA) in the center of the pan to track the temperature of the food product every 60 seconds for the next 24 hours. To ensure the probe of the data logger was centered, a placement system using a ruler, clips, and a straw was used (see Appendices F, Figure F-4). Pans were then prepared with three treatments: uncovered, covered with a single layer of aluminum foil over the top of the pan to allow for air exposure, or covered twice to restrict air exposure with one layer of plastic wrap or aluminum foil directly over the top of the food product and another layer of aluminum foil over the top of the pan. Each cover method was applied to both a 2 and 3-inch food product depth pan. Each cover method and product depth combination treatment were also prepared in duplicate, with one pan being stored in the 4°C (39.2°F) walk-in refrigerator with an ice bath and the other duplicate pan stored in the -20°C (-4°F) walk-in freezer (see Appendices F, Figures F-5 and F-6). Pans in the refrigerator were also situated in ice baths as suggested in the FDA Food Code (*134*, *136*). The ice baths were prepared by filling 3 and 6-inch steam table pans ¾ of the way full with ice (for use with the 2 and 3-inch food product depth pans, respectively). It is important to note that once the ice within the ice bath had melted, it was not replaced with fresh ice. A total of twelve pans were stored for cooling and sampling. To avoid food products becoming completely frozen and unable to be sampled, pans in the freezer were transferred to the refrigerator immediately after the 8 hour time point.

Sampling

After inoculation, composite samples were collected from each pan at five time points: 0, 4, 8, 12, and 24 hours. At each time point, a representative composite sample was obtained by

using a spoon to gather food from four to five different areas within each pan. These sampling points were randomly selected and the food was taken from under the food surface in order to collect from the interior of the pan where the food was likely the warmest and, therefore, most at risk for microbial growth. This composite sample was homogenized by hand mixing, after which, a 25 gram aggregate sample was removed and deposited in a sterile stomacher bag for further testing. This aggregated 25 gram sample was then diluted 1:10 with 225 mL of BPW and stomached for one minute at 230 rpm (Stomacher® 400 Circulator; Seward, Bohemia, NY). Serial dilutions of the samples were then carried out in tubes with 9 mL of BPW, after which the appropriate dilutions were spread plated onto MacConkey agar and Mannitol Egg Yolk Polymyxin B agar to enumerate *E. coli* and *B. cereus* populations, respectively. The MacConkey plates were incubated 37°C (98.6°F) for 18-24 hours while the Mannitol Egg Yolk Polymyxin B plates were incubated at 30°C for 24-48 hours.

Statistical Analysis

E. coli and *B. cereus* population data and temperature data were analyzed using a compound symmetry covariance structure, a compound symmetry with heterogeneous time variances structure, or an unstructured covariance matrix combined with a PROC MIXED procedure in SAS. This was considered a four factor repeated measures experiment and it was analyzed accordingly. A Type III test for fixed effects was carried out as well. Because three repetitions were carried out for the testing of cooling methods for the four food products, least square means of microbial populations were obtained and used to compare the significance of variables and variable interactions at a significance level of $P \leq 0.05$. For the cooling curves, the average of 5 temperature values near each time point was utilized to reduce variability. The

significance of variables and variable interactions for temperature data was also observed at a significance level of $P \leq 0.05$.

Results and Discussion

Temperature Data Analysis

Temperature data for the pre-cooked taco meat, chili con carne with beans, low sodium marinara sauce, and brown rice products were in agreement with previously published findings (*117*, *118*, *129*). The significance of variables and their effect on the cooling process at each of the five time points (0, 4, 8, 12, and 24 hours) is discussed for each product in the sections below. In this section, if a variable or variable interaction is described as significant, it is implied that $P \leq 0.05$ as mentioned in the statistical analysis section above. Ambient temperature data for the -20°C (-4°F) walk-in freezer and the 4°C (39.2°F) walk-in refrigerator can be found in Appendix F, Figure F-11.

Achieving FDA Food Code Standards

Pre-Cooked Taco Meat

No variable was significant at time point 0 hours. Treatment, treatment by product depth, and cover were significant at time point 4 and 8 hours. The freezer treatment cooled 2-inch product depths more rapidly than the refrigerator during the first 8 hours of cooling. The 3-inch product depths cooled more rapidly in the refrigerator for the first 4 hours but by time point 8, 3-inch product depths were at lower temperatures in the freezer. The 2-inch product depth in the refrigerator cooled less rapidly than the 3-inch product depth in the refrigerator during the first 8 hours of cooling. The cover significance was observed for uncovered pans which cooled more rapidly than single or double covered pans during the first 8 hours. Treatment in the refrigerator or freezer was the only significant factor for cooling at the 12 and 24-hour time points. The pans

removed from the freezer and placed in the refrigerator after the 8-hour time point (as discussed in the Treatments and Cooling section) continued to remain at a lower temperature than those stored in the refrigerator with an ice bath.

A previous study by Olds, et al. concluded that the only method that met the two step FDA Food Code requirement for beef taco meat was storing the product at 2-inch product depths in the freezer (*118*). The results of this study indicate uncovered 3-inch product depths stored in ice baths in the refrigerator also meet FDA Food Code as shown in Table 1. Appendix A contains cooling curve graphs to help illustrate the conclusions in this section.

Table 1 Pre-Cooked Taco Meat Cooling Methods that Achieved FDA Food Code Requirements

Table 1: Pre-Cooked Taco Meat Cooling Methods that Met FDA Food Code Requirement

Treatment	57°C to 21°C 2 hours	Limits Lower	Limits Upper	57°C to 5°C 6 hours	Limits Lower	Limits Upper	Both Requirements
2-inch Refrigerated ice bath Single cover	30.12°C	23.81°C	36.45°C	15.65°C	9.78°C	21.52°C	
2-inch Refrigerated ice bath Double cover	36.57°C	30.26°C	42.89°C	23.68°C	17.82°C	29.56°C	
2-inch Refrigerated ice bath Uncovered	26.51°C	20.20°C	32.83°C	11.33°C	5.46°C	17.20°C	
3-inch Refrigerated ice bath Single cover	25.32°C	18.99°C	31.63°C	10.41°C	4.54°C	16.28°C	
3-inch Refrigerated ice bath Double cover	29.15°C	22.83°C	35.47°C	18.15°C	12.28°C	24.02°C	

3-inch Refrigerated ice bath Uncovered*	5.28°C ✓	-1.04°C	11.59°C	2.24°C ✓	-4.86°C	9.34°C	✓
2-inch, freezer Single cover	25.46°C	19.14°C	31.78°C	4.94°C ✓	-0.93°C	10.82°C	
2-inch, freezer Double cover	31.85°C	25.53°C	38.17°C	4.17°C ✓	-1.71°C	10.04°C	
2-inch, freezer Uncovered*	19.78°C ✓	13.46°C	26.09°C	-3.56°C ✓	-9.43°C	2.32°C	✓
3-inch, freezer Single cover	34.32°C	27.99°C	40.63°C	9.61°C	3.74°C	15.48°C	
3-inch, freezer Double cover	37.48°C	31.16°C	43.80°C	13.98°C	8.11°C	19.85°C	
3-inch, freezer Uncovered	24.06°C	17.73°C	30.37°C	-1.39°C ✓	-7.26°C	4.48°C	

*Indicates cooling treatment achieved both FDA Food Code Requirements

Chili Con Carne with Beans

No variable was significant at the 0 hour time point. At the 4 hour time point, product depth, treatment by product depth, and cover were significant for the cooling of this product. The pans stored in the freezer at 2-inch product depths cooled more quickly than those in the refrigerator during the first 4 hours of cooling, but the 3-inch product depths cooled more quickly in the refrigerator than in the freezer during this time. The uncovered pans cooled more rapidly during the first 4 hours than single or double covered pans. At the 8 and 12 hour time point, treatment, treatment by product depth, and cover were significant. During these hours, the 2-inch product depths in the freezer cooled most rapidly while the 2-inch product depth in the refrigerator cooled at a slower rate than the 3-inch product depth in the refrigerator. At the 24 hour time point, treatment and product depth by cover were significant. Pans in the refrigerator at the 24 hour time point were cooler by a small but statistically significant amount. The product

depth by cover significance was observed for 3-inch product depths in the refrigerator that were recorded as the lowest in temperature at the 24 hour time point.

Two previous studies published results on the cooling of chili products. Olds, et al. concluded the blast chiller was the only cooling method that met both FDA Food Code requirements (*117*). Roberts, et al. concluded only 2-inch product depths cooled in the freezer met both FDA Food Code requirements for this product (*129*). The results from this study indicate three cooling methods met both FDA Food Code requirements as shown in Table 2. Appendix B contains cooling curve graphs to help illustrate these conclusions.

Table 2 Chili Con Carne with Beans Cooling Methods that Achieved FDA Food Code Requirements

Table 2: Chili Con Carne with Beans Cooling Methods that Met FDA Food Code Requirement

Treatment	57°C to 21°C 2 hours	Limits Lower	Limits Upper	57°C to 5°C 6 hours	Limits Lower	Limits Upper	Both Requirements
2-inch Refrigerated ice bath Single cover	16.79°C ✓	10.39°C	23.20°C	5.65°C	-0.77°C	12.07°C	
2-inch Refrigerated ice bath Double cover	30.18°C	23.78°C	36.59°C	12.61°C	6.19°C	19.03°C	
2-inch Refrigerated ice bath Uncovered	14.72°C ✓	8.32°C	21.13°C	4.70°C ✓	-1.72°C	11.12°C	✓
3-inch Refrigerated ice bath Single cover	23.33°C°C	16.93°C	29.74°C	6.76°C	0.34°C	13.18°C	
3-inch Refrigerated ice bath Double cover	27.79°C	21.39°C	34.20°C	10.13°C	3.70°C	16.56°C	

3-inch Refrigerated ice bath Uncovered*	13.24°C ✓	6.83°C	19.64°C	2.90°C ✓	-3.52°C	9.33°C	✓
2-inch, freezer Single cover	29.96°C	23.56°C	36.37°C	1.83°C ✓	-4.59°C	8.26°C	
2-inch, freezer Double cover	30.74°C	24.33°C	37.14°C	2.68°C ✓	-3.74°C	9.10°C	
2-inch, freezer Uncovered*	15.89°C ✓	9.48°C	22.29°C	-3.22°C ✓	-9.64°C	3.20°C	✓
3-inch, freezer Single cover	36.98°C	30.58°C	43.39°C	12.32°C	5.89°C	18.74°C	
3-inch, freezer Double cover	38.22°C	31.82°C	44.63°C	15.72°C	9.30°C	22.14°C	
3-inch, freezer Uncovered	29.85°C	23.44°C	36.26°C	4.72°C ✓	-1.70°C	11.14°C	

*Indicates cooling treatment achieved both FDA Food Code Requirements

Low Sodium Marinara Sauce

At time point 0 and 4 hours, product depth was significant as 3-inch product depths were observed at a significantly higher temperature than 2-inch product depths. Treatment and product depth were significant at the 8 hour time point. The freezer cooled pans to lower temperatures at this time point and 3-inch product depths continued to be significantly higher in temperature than 2-inch product depths. Treatment was significant for the 12 and 24 hour time point with the freezer cooling pans to lower temperatures than the refrigerator.

None of the cooling methods tested met either FDA Food Code requirement for this food product, as indicated in Table 3. However, in a previous study, Roberts, et al. concluded 2-inch product depths cooled in the freezer met both FDA Food Code requirements for this product. Appendix C contains cooling curve graphs to help illustrate these conclusions.

Table 3 Low Sodium Marinara Sauce Cooling Methods that Achieved FDA Food Code Requirements

Table 3: Low Sodium Marinara Sauce Cooling Methods that Met FDA Food Code Requirement

Treatment	57°C to 21°C 2 hours	Limits		57°C to 5°C 6 hours	Limits		Both Requirements
		Lower	Upper		Lower	Upper	
2-inch Refrigerated ice bath Single cover	29.29°C	17.78°C	40.80°C	12.82°C	3.23°C	22.39°C	
2-inch Refrigerated ice bath Double cover	30.00°C	18.49°C	41.51°C	15.39°C	5.81°C	24.97°C	
2-inch Refrigerated ice bath Uncovered	17.07°C ✓	5.56°C	28.58°C	7.33°C	-2.24°C	16.91°C	
3-inch Refrigerated ice bath Single cover	32.52°C	21.01°C	44.03°C	15.94°C	6.36°C	25.52°C	
3-inch Refrigerated ice bath Double cover	24.48°C	12.97°C	35.99°C	14.32°C	2.64°C	25.99°C	
3-inch Refrigerated ice bath Uncovered*	26.24°C	14.73°C	37.76°C	11.14°C	1.57°C	20.73°C	
2-inch, freezer Single cover	28.54°C	17.03°C	40.04°C	-0.41°C ✓	-9.98°C	9.17°C	
2-inch, freezer Double cover	28.30°C	14.20°C	42.4°C	2.59°C ✓	-9.08°C	14.28°C	
2-inch, freezer Uncovered*	22.11°C	10.60°C	33.62°C	-6.44°C ✓	-1.02°C	3.13°C	
3-inch, freezer Single cover	34.57°C	23.07°C	46.08°C	9.03°C	-0.54°C	18.62°C	
3-inch, freezer Double cover	42.50°C	30.99°C	54.01°C	17.54°C	7.96°C	27.12°C	

3-inch, freezer Uncovered	32.67°C	30.99°C	54.01°C	4.53°C ✓	-5.04°C	14.12°C	

*Indicates cooling treatment achieved both FDA Food Code Requirements

Brown Rice

At time point 0 hours, product depth and treatment by cover was significant. At this time point, 3-inch product depths were significantly higher in temperature than 2-inch product depths. Treatment by cover significance could be due to the lower temperature of uncovered pans situated in ice water baths. Product depth and cover were significant at time point 4 hours with 3-inch product depths significantly higher in temperature and uncovered pans at a significantly lower temperature. Cover was significant at time point 8 and 12 hours with uncovered pans at lower temperatures than single or double covered pans. Treatment and product depth by cover were significant at the 24 hour time point. Pans in the refrigerator were lower in temperature than pans in the freezer. Uncovered 3-inch product depths were lowest in temperature.

A previous study by Olds, et al. concluded 2-inch product depths cooled in a refrigerator with an ice water bath was the only cooling method that would meet both FDA Food Code requirements for a steamed rice product (*118*). The results of this study indicate four cooling methods met both FDA Food Code requirements for this food product as shown in Table 4. Appendix D contains cooling curve graphs to help illustrate these conclusions.

Table 4 Brown Rice: Cooling Methods that Achieved FDA Food Code Requirements

Table 4: Brown Rice Cooling Methods that Met FDA Food Code Requirement

	57°C to 21°C	**Limits**		**57°C to 5°C**	**Limits**		
Treatment	**2 hours**	**Lower**	**Upper**	**6 hours**	**Lower**	**Upper**	**Both Requirements**

2-inch Refrigerated ice bath Single cover	13.65°C ✓	6.37°C	20.93°C	6.18°C	-0.77°C	12.07°C	
2-inch Refrigerated ice bath Double cover	20.94°C ✓	13.67°C	28.22°C	8.43°C	6.19°C	19.03°C	
2-inch Refrigerated ice bath Uncovered	9.46°C ✓	2.18°C	16.74°C	4.06°C ✓	-1.72°C	11.13°C	✓
3-inch Refrigerated ice bath Single cover	20.02°C ✓	12.74°C	27.29°C	9.06°C	0.34°C	13.18°C	
3-inch Refrigerated ice bath Double cover	24.20°C	16.92°C	31.48°C	9.74°C	3.70°C	16.56°C	
3-inch Refrigerated ice bath Uncovered*	8.94°C ✓	1.66°C	16.22°C	1.76°C ✓	-3.52°C	9.33°C	✓
2-inch, freezer Single cover	20.32°C ✓	13.03°C	27.59°C	1.37°C ✓	-4.59°C	8.26°C	✓
2-inch, freezer Double cover	28.86°C	19.94°C	37.77°C	13.21°C	-3.74°C	9.10°C	
2-inch, freezer Uncovered*	10.68°C ✓	3.40°C	17.96°C	0.96°C ✓	-9.64°C	3.2°C	✓
3-inch, freezer Single cover	30.22°C	22.94°C	37.50°C	4.72°C ✓	5.89°C	18.74°C	
3-inch, freezer Double cover	30.98°C	23.70°C	38.26°C	6.76°C	9.30°C	22.14°C	
3-inch, freezer Uncovered	28.33°C	21.05°C	35.61°C	1.04°C ✓	-1.70°C	11.14°C	

*Indicates cooling treatment achieved both FDA Food Code Requirements

Summary of Temperature Data Findings

Treatment, product depth, treatment by product depth, and cover were often significant in the cooling of these food products. In general, the freezer cooled more consistently to lower

temperatures, 2-inch product depths cooled more quickly than 3-inch product depths, and uncovered pans cooled most rapidly. Although not statistically significant, it is interesting to note that for all four products, the 3-inch product depths stored in the freezer cooled less effectively in the first four hours than 3-inch product depths in the refrigerator with an ice bath. However, at 4 to 5 hours, the ice had mostly melted, leading to stagnated cooling that did not necessarily facilitate the transition to lower temperatures, but rather held food products at a steady temperature. The freezer, however, continued to cool to lower temperatures at a steady rate.

In general, the temperature data results reflect similar conclusions to previously established research (*117*, *118*, *129*). The results of this study also add to those conclusions on cooling methods already validated to achieve FDA Food Code requirements for three of the four food products. This study identified several refrigerator and ice bath cooling combinations that achieved FDA Food Code which previous studies had not been able to validate for pre-cooked taco meat, chili con carne with beans, and brown rice (*117*, *118*, *129*). These conclusions are unique in that other studies indicated chili and taco meat products may be too dense for refrigerator and ice bath methods to effectively cool to FDA Food Code requirements (*117, 118, 129*). This may be due to the composition of the ice water baths, as this study utilized ice filling the pans to ¾ full with no water added. Perhaps this allowed more rapid cooling than if an ice water bath contained added water as they may have been in others studies. There were some cooling methods that came close to meeting the two-step FDA Food Code requirement including the 2-inch product depths, covered with one layer of aluminum foil in the refrigerator for the chili con carne with beans and brown rice products. This cooling method met the first step of the requirement for each food product but missed meeting the second step of the requirement by 0.65°C and 1.18°C, respectively. For the pre-cooked taco meat, the 3-inch product depth,

uncovered in the freezer met the first step of the requirement but missed the first step of the requirement by 3.06°C.

On the other hand, one study identified a method that achieved requirements that this study was unable to validate and that method was for the marinara sauce product. In 2013, Roberts, et al. concluded that 2-inch product depths of tomato sauce cooled in the freezer met both FDA Food Code requirements (*129*). In this study, the uncovered 2-inch product depth in the freezer missed achieving the first step of the FDA Food Code requirement by 1.11°C while successfully meeting the second time and temperature step.

Temperature differences like these can be attributed to several variations between studies including the facilitation of the cooling of food products to 60°C ± 5°C (140°F ± 5°F) before placement in the freezer or refrigerator, how often the refrigerator or freezer door is opened during the cooling period, and whether water is used as an additive in the ice bath method. The two main studies referenced evaluated the cooling of chili, meatless tomato sauce, beef taco meat, and steamed rice; in these studies, the freezer and refrigerator were not opened once the cooling process had begun (*118*, *129*). In order to access the food products for microbiological sampling at the five time points for this study, the -20°C (-4°F) walk-in freezer and 4°C (39.2°F) walk-in refrigerator were opened after the cooling process had begun (See Appendices F, Figure F-12 for Ambient Temperature Data). It must also be taken into consideration that food products went directly from heating to cooling in the two previous studies, whereas this study facilitated the cooling of food products to 60°C ± 5°C (140°F ± 5°F) before placement in the -20°C (-4°F) walk-in freezer and 4°C (39.2°F) walk-in refrigerator (*118*, *129*). The four food products were also left uncovered in the previous cooling studies, which may have influenced the observed differences between cooling results (*118*, *129*).

The variables mentioned above that may influence the variations in temperature data conclusions from these studies are akin to real life variables in cooling protocols and food preparation settings of school nutrition programs. That is why it is critical to identify and scientifically validate a variety of cooling methods. Doing so provides school nutrition programs with flexibility and a number of cooling options so they may identify and validate feasible cooling methods that meet FDA Food Code requirements within their unique food preparation settings.

Microbiological Data Analysis

Microbiological population data over the 24-hour cooling period are discussed in this section for the four food products: pre-cooked taco meat, chili con carne with beans, low sodium marinara sauce, and brown rice. The significance ($P<0.05$) of depth, cover, and treatment variables and their effect on microbial populations during the 24-hour cooling process is also detailed for each product in the sections below. If a variable or variable interaction is described as significant in this section, it is understood that significance is $P< .05$ as mentioned in the statistical analysis section.

Pre-cooked Taco Meat

Time ($P=0.0022$) was the only one significant factor for pre-cooked taco meat. The most significant decrease in *E. coli* population occurred between time point 0 and 4 hours (-0.31 $\log_{10}$ CFU/g) and overall, between time point 0 and 24 hours *E. coli* populations decreased 0.20 $\log_{10}$ CFU/g. The marginal decrease in population during this time may be due to variations of populations within the food product. No statistically significant difference ($P>0.05$) in *E. coli* population was observed for cover (two layers, one layer, uncovered), treatment (refrigerator vs. freezer), or product depth variables and there were no significant variable interactions. The lack

of these effects combined with the slight but significant decrease in *E. coli* population over time demonstrates an effective control for the cooling methods evaluated. In Appendices A, Figure A-1 and Figure A-2 represent $\log_{10}$ CFU/g population data analyzed over time alone, as time alone was the only significant variable.

Chili con carne with beans

Microbiological data revealed no statistically significant difference ($P>0.05$) in *E. coli* populations for cover (two layers, one layer, uncovered), treatment (refrigerator vs. freezer), or product depth variables. However, time ($P=0.0015$) and the product depth by time ($P=0.0197$) interaction, were significant for this product. Populations did increase in the 2-inch product depths between 0 and 24 hours (0.11 $\log_{10}$ CFU/g) whereas they decreased in the 3-inch product depths between 0 and 24 hours (-0.15 $\log_{10}$ CFU/g). The temperatures of 2 and 3-inch product depths were very similar at inoculation and the populations at time 0 for 2 and 3-inch product depths were also very similar. However, temperature data indicates that product depth was significant in the first 4 hours of the cooling process as 3-inch pans cooled less rapidly and were 10°F hotter than 2-inch pans at the 4 hour time point. The retention of heat in 3-inch pans may have resulted in pockets of lethal temperature, which led to a small but significant population decline of 0.28 $\log_{10}$ CFU/g during the first 4 hours of cooling. Therefore, the *E. coli* population in 3-inch product depths at time point 4 hours were interpreted as statistically different in comparison to slightly larger populations in 2-inch product depths or even 3-inch product depths at other time points. It is also plausible that the variation in populations is the result of non-uniform inoculation throughout the product. However, these population differences were well under 0.5 $\log_{10}$ CFU/g and it is possible that a difference of this size in population was simply the result of natural variation in populations throughout the food product. These results indicate

all cooling method variables suppressed growth to the same degree, suggesting all the cooling methods evaluated were effective at controlling *E. coli* populations in the chili con carne with beans product. In Appendices A, Figure A-3 and Figure A-4 represent $\log_{10}$ CFU/g population data analyzed by product depth and time because the product depth by time variable interaction was significant. Surrogate *E. coli* $\log_{10}$ CFU/g population data was not analyzed by time alone due to the time variable being included in the product depth by time interaction.

Marinara sauce

Product depth ($P<0.0001$) and time ($P=.0312$) were statistically significant for marinara sauce. The difference in *E. coli* populations between 2-inch (4.20 $\log_{10}$ CFU/g) and 3-inch (3.79 $\log_{10}$ CFU/g) pans, overall, were 0.40 $\log_{10}$ CFU/g. Temperature data also suggests product depth was significant within the first four hours of cooling. It is possible the significance of product depth was influenced by 3-inch food product depths that may have facilitated the retention of pockets of lethal temperature, which may have reduced some of the bacterial population at inoculation. The heat combined with the acidity may have also injured the cells, causing them to lag and then recover. Temperature data indicates product depth was significant at inoculation, or time 0 hour, with 3-inch product depths being significantly higher in temperature than 2-inch product depths. Therefore, the *E. coli* population in 3-inch product depths were considered statistically significantly less than 2-inch product depths.

Though time was statistically significant, 0.21 $\log_{10}$ CFU/g was the largest increase in populations occurring between the 0 and 8 hour time points, which is not considered a noteworthy impact, microbiologically. This slight difference is more likely due to natural variation within the product and the properties of the food product. It is possible that *E. coli* populations that were initially injured as a result of heat and/or acidity were able to make a slight

recovery during the first 8 hours of cooling. The recovery of *E. coli* O157:H7 cells after sub lethal heat treatment has been well documented (*142*). The cells go through periods of recovery, regaining their ability to grow and divide during the first 9 hours after being subjected to sub lethal heat conditions (*142*). It is important to note the results from the preliminary study indicate 0.23 $\log_{10}$ CFU/g was an expected population recovery for the acid habituated surrogate *E. coli* in this product. The recovery of microbial populations during the first 8 hours of cooling was nearly identical to the preliminary study findings. Therefore, it is likely that these results indicate the slight increase in populations for the marinara sauce product were a natural consequence of acid habituation rather than the result of a cooling failure or risk. No statistically significant difference ($P>0.05$) in populations were observed for cover (covered two layers, covered one layer, uncovered) or treatment (refrigerator vs. freezer) variables and no interaction combinations tested were significant. These results indicate all cooling method variables suppressed growth to the same degree, suggesting all the cooling methods evaluated were effective at controlling *E. coli* populations in marinara sauce. In Appendices A, Figure A-5 and Figure A-6 represent $\log_{10}$ CFU/g population data analyzed by time alone, as time alone was a significant variable. Figure A-7 represents $\log_{10}$ CFU/g population data analyzed by product depth alone, as product depth alone was a significant variable.

Brown Rice

Microbiological data revealed two factors were significant for the brown rice product including time ($P<.0001$) and product depth ($P=0.0235$). Significant two way variable interactions include treatment by time ($P=0.0026$) and product depth by time ($P=0.0268$). Treatment by time was significant and demonstrated a population decrease of 0.37 $\log_{10}$ CFU/g between time point 0 and 24 hour when food product depths were stored in the freezer. The ice

bath in the refrigerator proved less effective with a population decrease of 0.09 $\log_{10}$ CFU/g between time points 0 through 24 hours. Product depth by time significance was observed because populations did decrease overall in both 2 and 3-inch product depths between time points 0 and 24 hours (-0.21 $\log_{10}$ CFU/g and -0.25 $\log_{10}$ CFU/g, respectively). *Bacillus cereus* populations at time 0 were slightly, but significantly, different as the 3-inch product depths were observed at a 0.29 $\log_{10}$ CFU/g higher population than the 2-inch product depths at inoculation. This difference in population may be attributed to an uneven distribution of inoculum due to the absorbency of the brown rice product as has been encountered in a previous study (Gilbert 1974). Therefore, the *B. cereus* populations in 3-inch product depths were interpreted as statistically different in comparison to populations in 2-inch product depths or even 3-inch product depths at other time points. No statistically significant difference ($P>0.05$) in *B. cereus* population was observed for the cover (two layers, one layer, uncovered) variable and the slight decrease in *B. cereus* populations from significant factors prove cooling techniques tested were effective at controlling *B. cereus* populations. These results reflect the findings of a similar study conducted in 1974, where rice was cooked and inoculated with various *B. cereus* cultures, then cooled at a range of different temperatures (*70*). Three different *B. cereus* strain populations declined in the cooked rice over a 24-hour cooling period at 4°C (39.2°F) (*70*). The strains utilized in this study were either from isolates obtained from samples of feces from foodborne outbreaks involving fried rice or an isolate from uncooked rice (*70*).

In Appendices A, Figure A-8 and Figure A-9 represent $\log_{10}$ CFU/g population data analyzed by treatment and time, as treatment by time was a significant variable interaction. Figure A-10 and Figure A-11 represent $\log_{10}$ CFU/g population data analyzed by product product depth and time, as product depth by time was a significant variable interaction. *B. cereus* $\log_{10}$

CFU/g population data was not analyzed by time alone or by product depth alone due to the time variable and product depth variable being included in the product product depth by time interaction.

ComBase and PMP Growth Predictions

To evaluate the microorganisms and temperature conditions modeled in this study, two online programs were utilized: the USDA Pathogen Modeling Program (PMP) and the ComBase Predictor modeling program (*51*, *154*). Neither the PMP nor the ComBase programs had data from previous research regarding the specific microorganism, food products, or cooling conditions evaluated in this study. For *E. coli* and *B. cereus*, data for broth cultures and food products were available but they were not similar in % NaCl or pH at cold holding temperatures. In fact, only broth models were available for *B. cereus* at cold holding temperatures. Therefore, modeling was carried out based on conditions that were intrinsic to the food products tested including initial population level, % NaCl or a_w, and pH as well as external conditions like holding temperature. Nutrition labels from the food products provided information to calculate % NaCl based on weight (See Appendices F, Figures F-7 through F-10).

The ComBase Predictor offers a non-thermal survival prediction method, but this method only allows for modeling of *Listeria monoctyogenes* and *Salmonella* and not for modeling of the microorganisms evaluated in this study. Therefore, the growth prediction method was chosen as the most appropriate predictor model. The dynamic version of the model correlates temperatures with time points during the "growth" process. This was an advantage over the static model, as temperatures changed frequently during the cooling process in this study. However, this model was not ideal as the temperature ranges for each pathogen were limited to growth conditions, for *B. cereus* 5-34°C (41-93°F) and *E. coli* 10-42°C (50-107°F). Therefore, temperature data

gathered from this project fell out of range for modeling during a majority of the 24-hour cooling period. The dynamic model was carried out for food products stored in the refrigerator as more time points fell within modeling range than those for products in the freezer. The ComBase program was also limited as far as pH input with the lower limit being 4.5, so modeling for the marinara sauce product at a pH of 4.18 was not possible.

As or the PMP, there were models for cooling conditions regarding *Clostridium botulinum* and *Clostridium perfringens*; however, there were no cooling models for the microorganisms evaluated in this study. The PMP did not contain a bacteria-specific model for the food products tested in this study, so a bacteria-specific, broth-based model was chosen. Unlike the ComBase program, the PMP allowed for a modeling scenario including initial population level and pH but had the advantage of allowing a lower temperature limit to be selected at 5°C (41°F). However, the limitations of the PMP included the absence of a dynamic model as well as the problematic lower limit of 0.5 % NaCl. This lower limit resulted in limited prediction potential as the food products evaluated in this study were between 0-0.45% NaCl.

The following information was input in ComBase to run the modeling program for the pre-cooked taco meat product: initial level = 4.52-$\log_{10}$, temperatures of food product in the refrigerator from time points 2, 4, 6, and 8 hours of cooling that were within modeling limits, 0.45% NaCl, and two pH scenarios at 5 and 6. Two pH scenarios were run to model a worst-case scenario as the final pH of the pre-cooked taco meat product was unknown. At a pH of 5 and 6, *E. coli* was predicted by the ComBase dynamic model to grow by 0.11-$\log_{10}$ and 0.49-$\log_{10}$, respectively, over the 8 hour period. The PMP was also run to model growth in this product, with identical input information, but at a lower temperature of 5°C and at the lower limit of 0.5% NaCl. This model reported a 0.01 $\log_{10}$ (CFU/mL)/h growth rate in an aerobic, broth-based

scenario. The model predicted that over a 24-hour period, the population would increase by 0.17-$\log_{10}$ and 0.26-$\log_{10}$ for pH 5 and 6, respectively. The results from the microbiological data for the pre-cooked taco meat in this study showed a decrease of 0.16 $\log_{10}$ CFU/g between time point 0 and 8 hours.

For the low sodium marinara sauce product, there was limited prediction potential as the pH for the product (4.18) fell below the lower limit in ComBase (4.5). The following information was used as input for ComBase to run the modeling program for the low sodium marinara sauce product: initial level = 3.86-$\log_{10}$, temperatures of food product in the refrigerator from time points 1, 2, 4, and 6 hours of cooling that were within modeling limits, 0.16% NaCl, and pH = 4.5. Under these conditions, *E. coli* was predicted by the ComBase dynamic model to grow by 0.15-$\log_{10}$ over the 6 hour period. The PMP was also run to model growth in this product, but at a lower temperature of 5°C, at the lower limit of 0.5% NaCl, and at the lower limit of pH 4.5. This model predicted a 0.01 $\log_{10}$ (CFU/mL)/h growth rate. At 24-hours, the population was predicted to grow by 0.12-$\log_{10}$. The results from the microbiological data for the low sodium marinara sauce in this study showed an increase of 0.15 $\log_{10}$ CFU/g between time point 0 and 4 hours which was very similar to the ComBase and PMP predictions.

The following information was input in ComBase to run the modeling program for the chili con carne with beans product: initial level = 4.56-$\log_{10}$, temperatures of food product in the refrigerator from time points 1, 2, and 4 hours of cooling that were within modeling limits, 0.17% NaCl, and two pH scenarios at 5 and 6. Two pH scenarios were run to model a worst-case scenario as the final pH of the chili con carn with beans product was unknown. *E. coli* was predicted by the ComBase dynamic model to grow by 0.05-$\log_{10}$ and 0.09-$\log_{10}$, at a pH of 5 and 6 respectively. The PMP was also run to model growth in this product, but at a lower

temperature of 5°C and at the lower limit of 0.5% NaCl. This model reported a 0.01 log_{10} (CFU/mL)/h growth rate. At 24-hours, the population was predicted to grow by 0.17-log_{10} and 0.26-log_{10} at a pH of 5 and 6, respectively. The results from the microbiological data for the chili con carne with beans product in this study showed a decrease of 0.12 log10 CFU/g between time point 0 and 4 hours.

The following information was input in ComBase to run the modeling program for the brown rice product: initial level = 4.48-log_{10}, temperatures of food product in the refrigerator from time points 1, 2, 4, and 6 hours of cooling that were within modeling limits, 0% NaCl, and pH= 6. *B. cereus* was predicted by the ComBase dynamic model to remain at the same population over the 6 hour period. The PMP was also run to model growth in this product, but at a lower temperature of 5°C and at the lower limit of 0.5% NaCl. This model reported a 0.04 log_{10} (CFU/mL)/h growth rate. At 24-hours, the population was predicted to grow by 0.60-log_{10}. The results from the microbiological data for the brown rice product in this study showed a decrease of 0.16 log_{10} CFU/g between time point 0 and 4 hours.

Summary of Microbiology Data Findings

The microbiological data suggests that all 12 cooling methods were effective at controlling microbial populations. The pre-cooked taco meat and brown rice products exhibited a certain degree of overall population decline over the 24 hour cooling period, indicating that microorganism populations were effectively controlled by the cooling methods tested. The small recovery of the microbial population in the low sodium marinara sauce product and 2-inch product depths of chili con carne with beans were less than 0.50 log_{10} CFU/g. In fact, the variation in microbial populations for all the cooling variable combinations tested was lower than 0.5 log_{10} CFU/g.

According to the 2013 FDA Food Code section "Holding Cold Food Without Temperature Control", food kept without temperature control should meet the performance standard of no more than 1 $\log_{10}$ growth of *Clostridium perfringens* and *Bacillus cereus* (*152*). Experts agreed this was reasonable and even somewhat conservative (*156*). This is based on the knowledge that 10^5 - 10^7 CFU/g of vegetative cells within food products lead to a production of enterotoxin within the intestines, however, levels of spores in raw food products is relatively low at 10-1000 CFU/g (*152, 156*). The FDA Food Code also states that when held at ambient temperatures (75°F) for 4 hours, the performance standard is no more than 1 $\log_{10}$ CFU/g growth of *Listeria monoctyogenes* even though the infectious dose is not known (*152, 156*). Results of the USDA Pathogen Modeling Program in 1999 suggested safe time and temperatures resulted in 3 $\log_{10}$ growth in broth cultures and even some food related studies, however, these 1 $\log_{10}$ parameters were set after more exploratory studies were conducted on pathogens in defined food products (*152, 156*). These parameters far exceed the population changes observed in this study.

Future Research

The *E. coli* surrogates used were intended to model O157:H7 STEC and were utilized in three food products. Therefore, the results of this research can only provide an indication of the suspected behavior of O157:H7 STEC in these food products. Future research could evaluate variables like replacing or removing ice baths after several hours of cooling in order to validate more refrigerator cooling methods. Cover methods utilizing plastic wrap instead of aluminum foil could also be investigated. According to the survey in Krishnamurthy, et al. 2011, a large percentage (48.7%) of school foodservice managers reported leaving food uncovered in the refrigerator for cooling but then covering at a later time (*98*). This may correlate with the end of a work shift and could be a cooling variable worthy of investigation as well. Lastly, spore

forming bacteria like *Clostridium perfringens* should be investigated in proteinaceous foods like chili and taco meat, as rapid cooling is imperative for controlling this microorganism (*152*, *156*).

Chapter 4 - SUMMARY AND CONCLUSIONS

The microbiological data from this project builds upon previous studies that evaluated cooling methods used in school lunch settings in order to meet the two-step FDA Food Code cooling requirement (*117, 118, 129*). This study was designed to include variables to properly model cooling techniques that may be utilized by school nutrition programs. Time points were also planned to reflect a passive cooling process that often takes place within a school lunch setting.

Temperature data results from this study agreed with previously established research as well as establishing new understanding of microbial populations within specific food products. The variables tested included cover method, treatment with 4°C (39.2°F) refrigerator + ice bath or -20°C (-4°F) freezer, and product depth. These variables were all significant for the cooling of food products, but had limited or no impact on the control of microbial populations. None of these methods significantly improved control of microbial populations, but rather, controlled populations in an equal manner. Therefore, all 12 cooling combinations tested could be utilized for the food products evaluated despite the inability of some methods to meet FDA Food Code requirements with regard to temperature. However, it is critical to follow certain recommendations to facilitate rapid cooling including leaving pans uncovered when possible or covering with just one layer of aluminum foil or plastic food wrap, replacing or removing ice baths after several hours of cooling, and cooling foods at 2-inch product depths in the freezer whenever possible. It may be beneficial to store 3-inch product depths with the refrigerator + ice bath method based on the conclusion that this method cooled 3-inch product depths more rapidly than the freezer during the first four hours of the cooling process.

Cooling food properly will continue to serve as a significant step in preventing the proliferation of microbial populations within food products. However, the results of this research suggest microbial populations within food products were stable during the cooling process despite inability to achieve time and temperature limit requirements. Overall, this research identified and scientifically validated several economical cooling methods. These data may provide school nutrition programs, restaurants, and other food preparation settings with an increased flexibility to explore and validate cooling methods that meet FDA Food Code requirements within their unique food preparation settings. A variety of options for economical cooling methods may have a positive impact for institutional food service settings that may be limited by financial challenges, staffing, or cooling equipment capacity. Although the results of this research suggest a certain degree of flexibility in regards to time and temperature limits would mostly likely not raise the risk of microbial proliferation in food products, it is important to note that that this study is not exhaustive with regard to potential foodborne pathogen and food product combinations that could be explored in future experimentation.

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Appendix A - Microbial Population Data

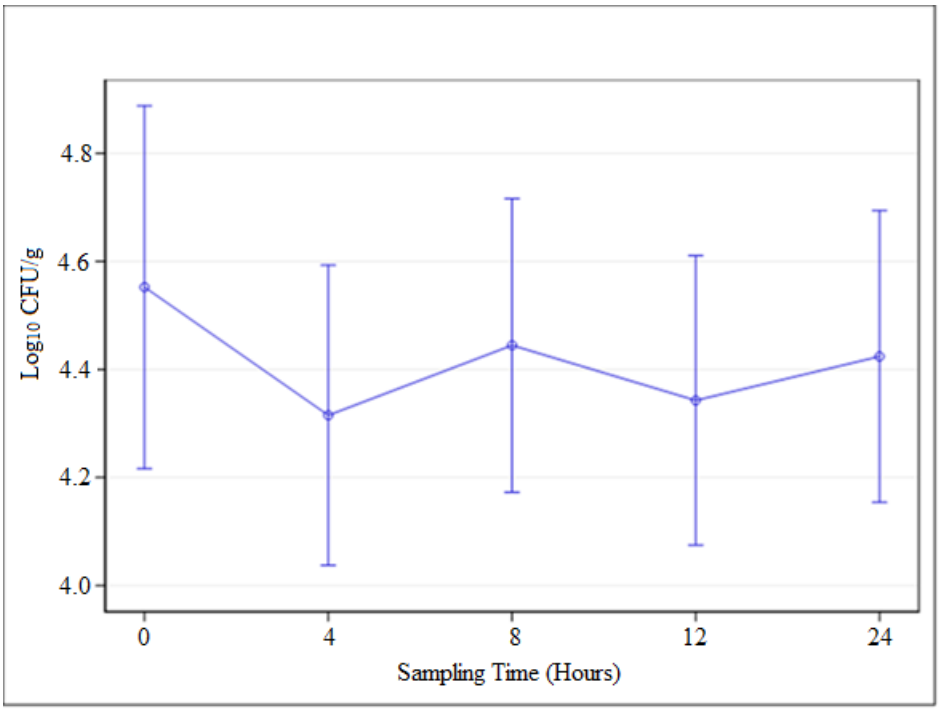


Figure A-1: Least Squares Means of Surrogate *E. coli* Populations (Log_{10} CFU/g) in Pre-Cooked Taco Meat Analyzed by Time with 95% Confidence Limits

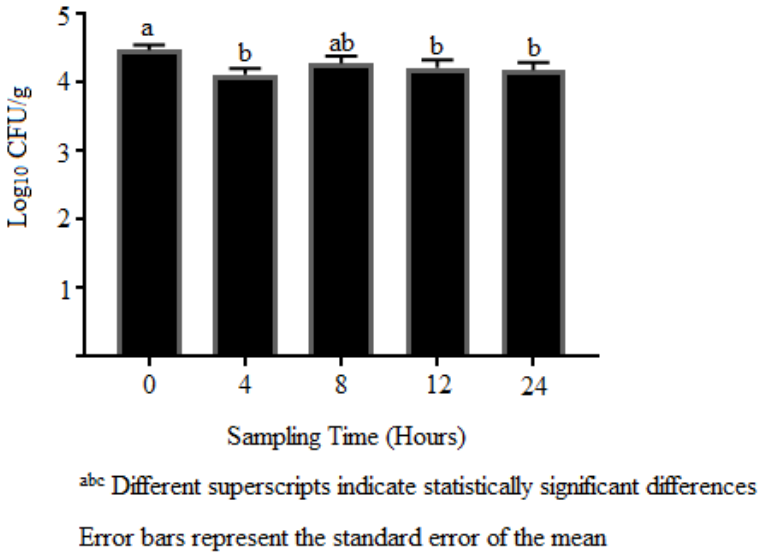


Figure A-2: Surrogate *E. coli* Populations (Log_{10} CFU/g) in Pre-Cooked Taco Meat Analyzed by Time

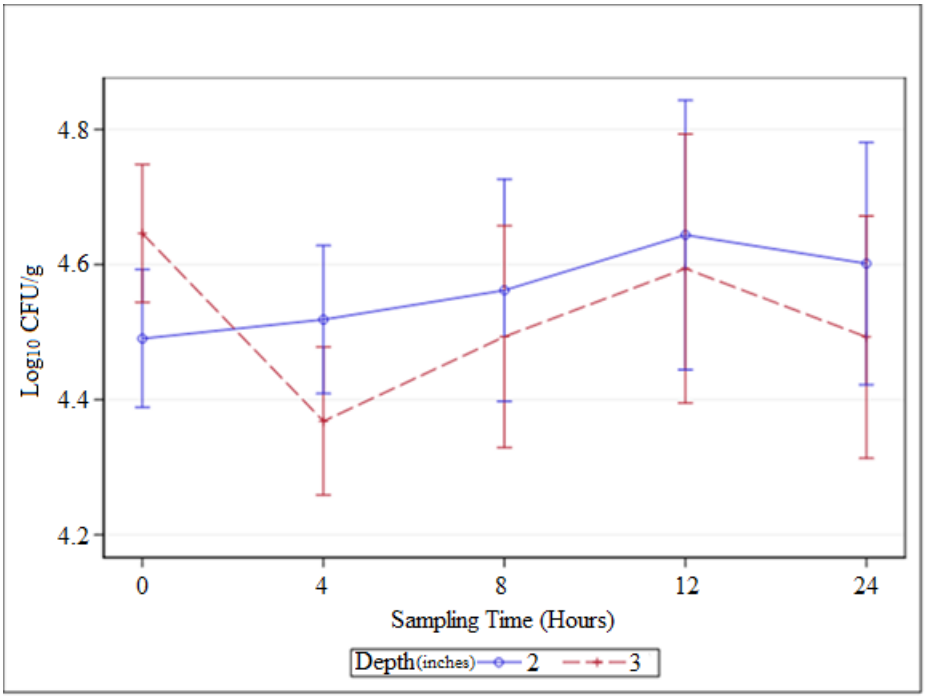
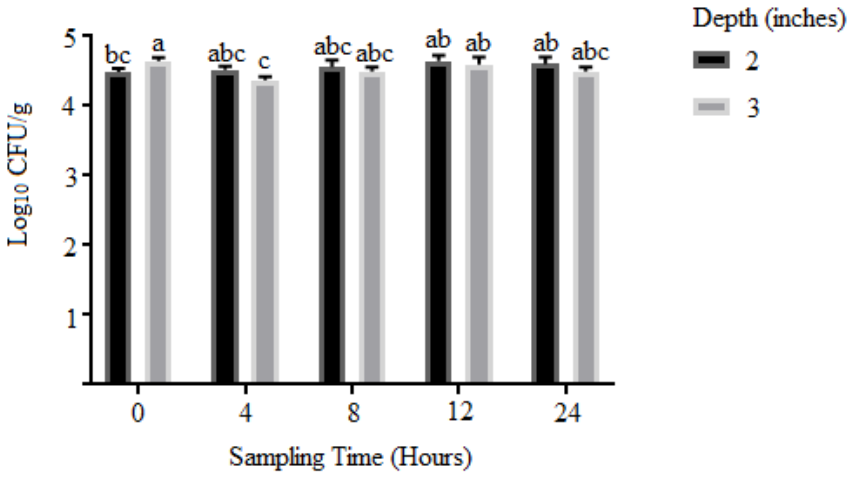


Figure A-3: Least Squares Means of Surrogate *E. coli* Populations (Log_{10} CFU/g) in Chili Con Carne with Beans Analyzed by Product Depth and Time with 95% Confidence Intervals



abc Different superscripts indicate statistically significant differences

Error bars represent the standard error of the mean

Figure A-4: Surrogate *E. coli* Populations (Log_{10} CFU/g) in Chili Con Carne with Beans Analyzed by Product Depth and Time

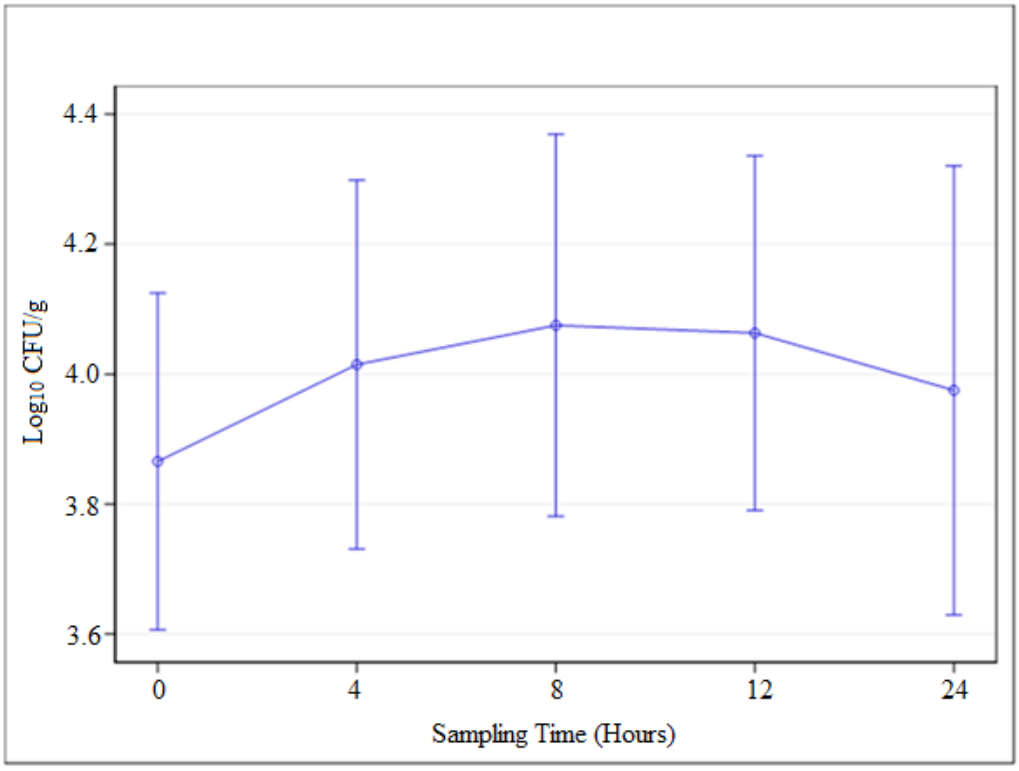


Figure A-5: Least Squares Means of Surrogate *E. coli* Populations (Log_{10} CFU/g) in Low Sodium Marinara Sauce Analyzed by Time with 95% Confidence Intervals

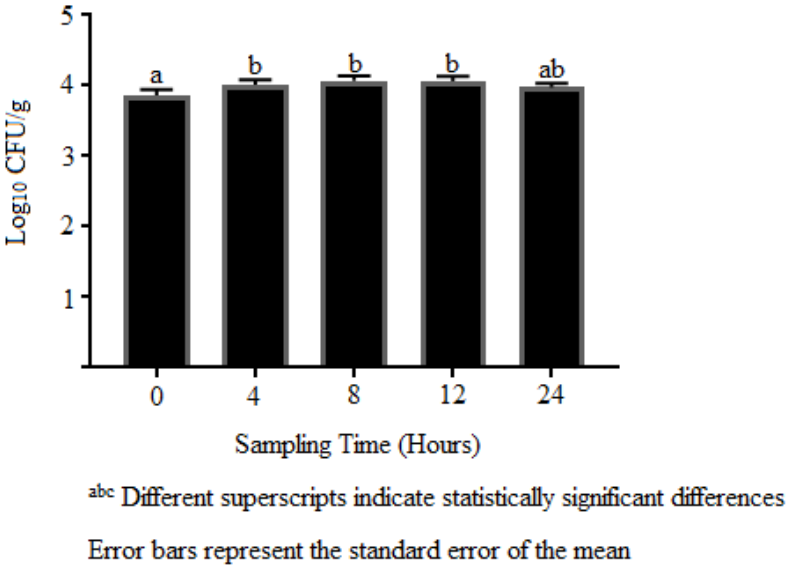
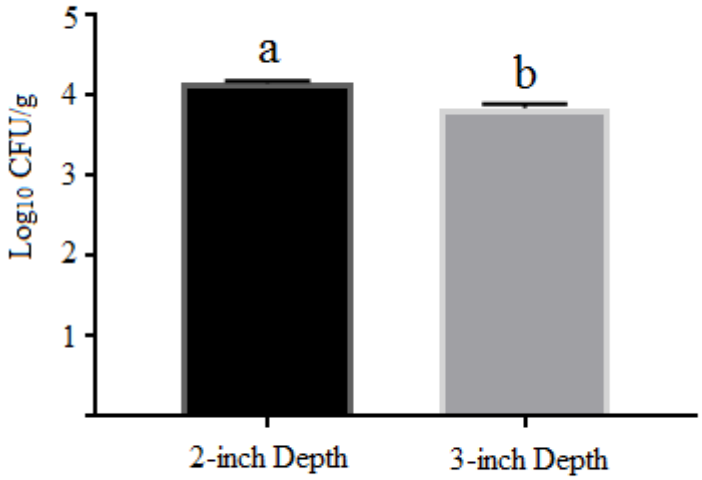


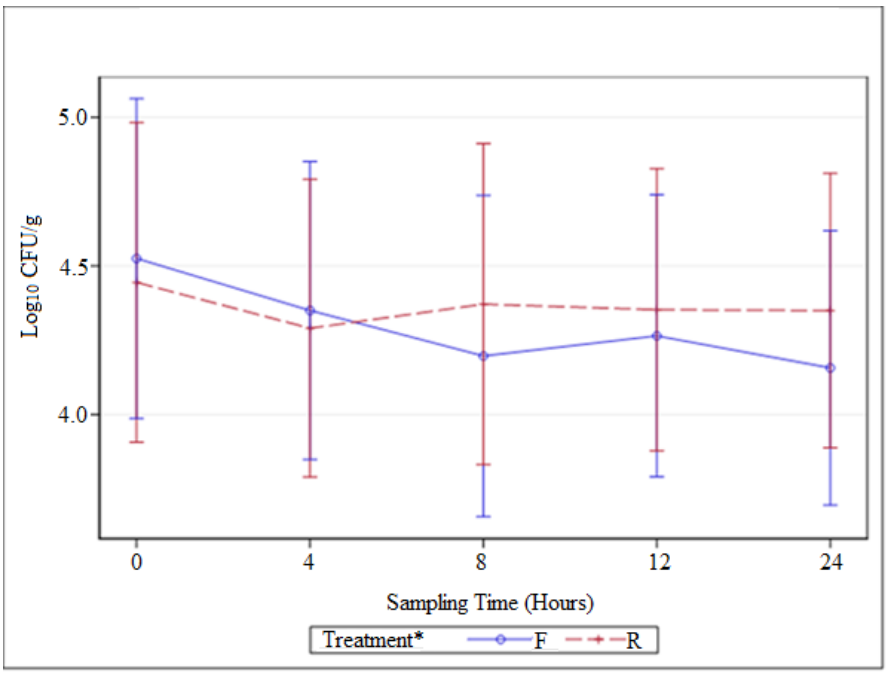
Figure A-6: Least Squares Means of Surrogate *E. coli* Populations (Log_{10} CFU/g) in Low Sodium Marinara Sauce Analyzed by Time with 95% Confidence Intervals



[abc] Different superscripts indicate statistically significant differences

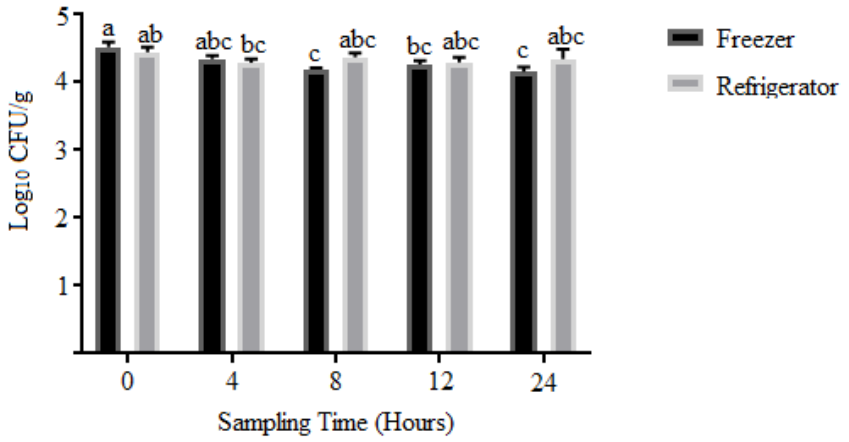
Error bars represent the standard error of the mean

Figure A-7: Surrogate *E. coli* Populations (Log_{10} CFU/g) in Low Sodium Marinara Sauce Analyzed by Product Depth



*F represents the freezer (-20°C) treatment and R represents the refrigerator (4°C) + ice bath treatment

Figure A-8: Least Squares Means of *B. cereus* Populations (Log_{10} CFU/g) in Brown Rice Analyzed by Treatment and Time with 95% Confidence Intervals



[abc] Different superscripts indicate statistically significant differences

Error bars represent the standard error of the mean

Figure A-9: *B. cereus* Populations (Log_{10} CFU/g) in Brown Rice Analyzed by Treatment and Time

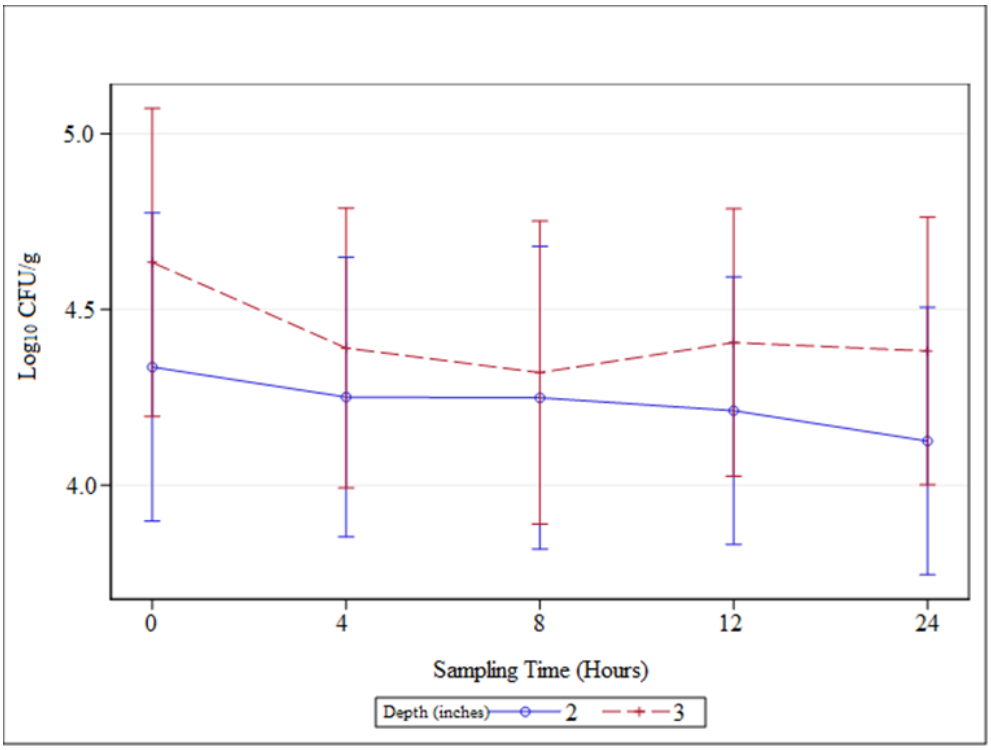
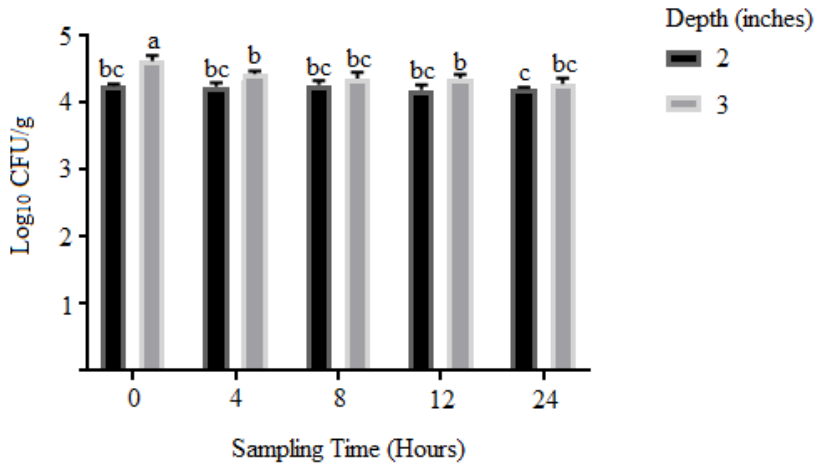


Figure A-10: Least Squares Means of *B. cereus* Populations (Log_{10} CFU/g) in Brown Rice Analyzed by Product Depth and Time with 95% Confidence Intervals



[abc] Different superscripts indicate statistically significant differences

Error bars represent the standard error of the mean

Figure A-11: *B. cereus* Populations (Log_{10} CFU/g) in Brown Rice Analyzed by Product Depth and Time

Appendix B - Pre-Cooked Taco Meat Cooling Curves

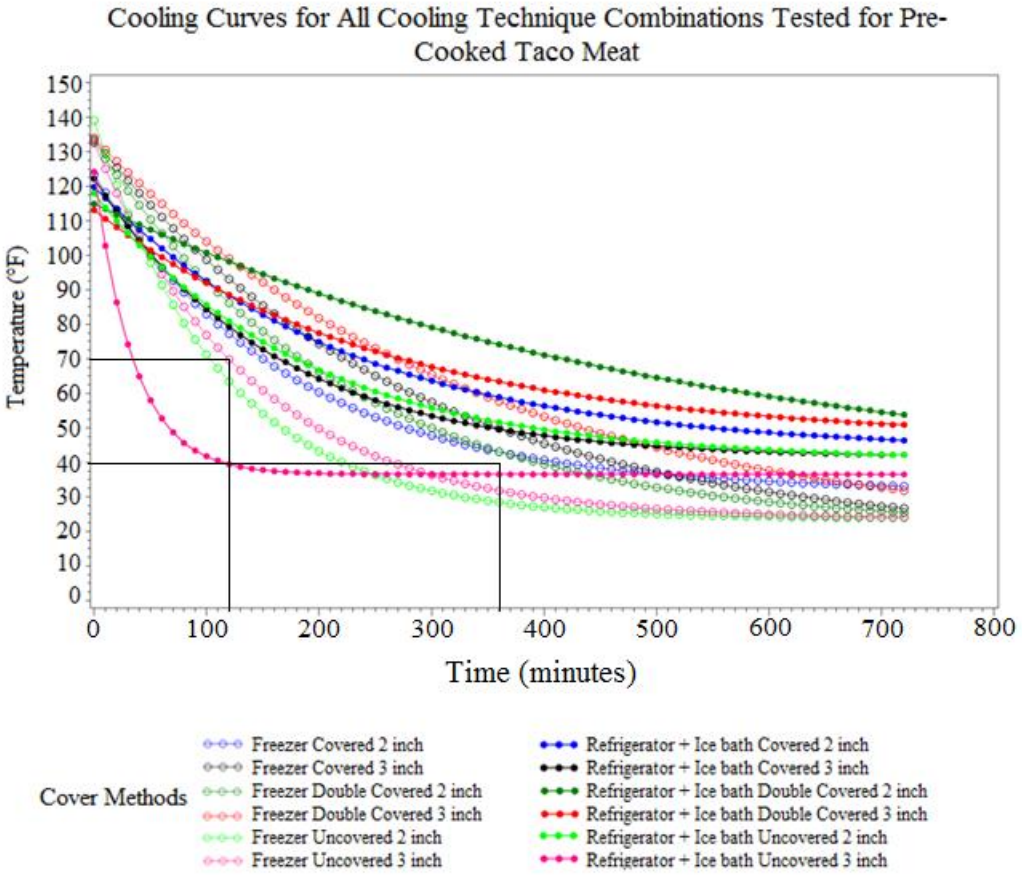


Figure B-1: Cooling Curves for all Cooling Technique Combinations Tested for Pre-Cooked Taco Meat

The cooling curves in this graph represent all 12 cooling treatment combinations tested for the pre-cooked taco meat product. Treatment combinations are referenced by color patterns shown in the Cover Methods key at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual for the cooling effects of all 12 treatments tested for this product.

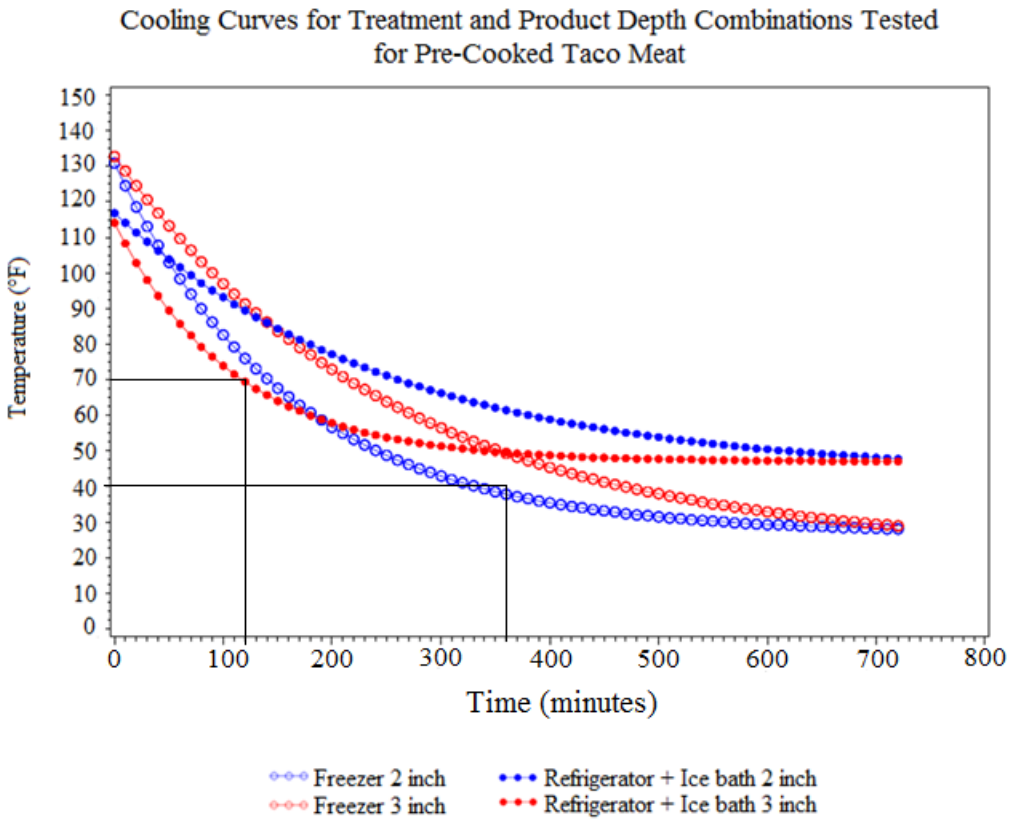


Figure B-2: Cooling Curves for Treatment and Product Depth Combinations Tested for Pre-Cooked Taco Meat

The cooling curves in this graph represent each treatment and product depth combination tested for the pre-cooked taco meat product. Treatment combinations are referenced by color patterns at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual to support the significance of treatment and product depth on the cooling of this product.

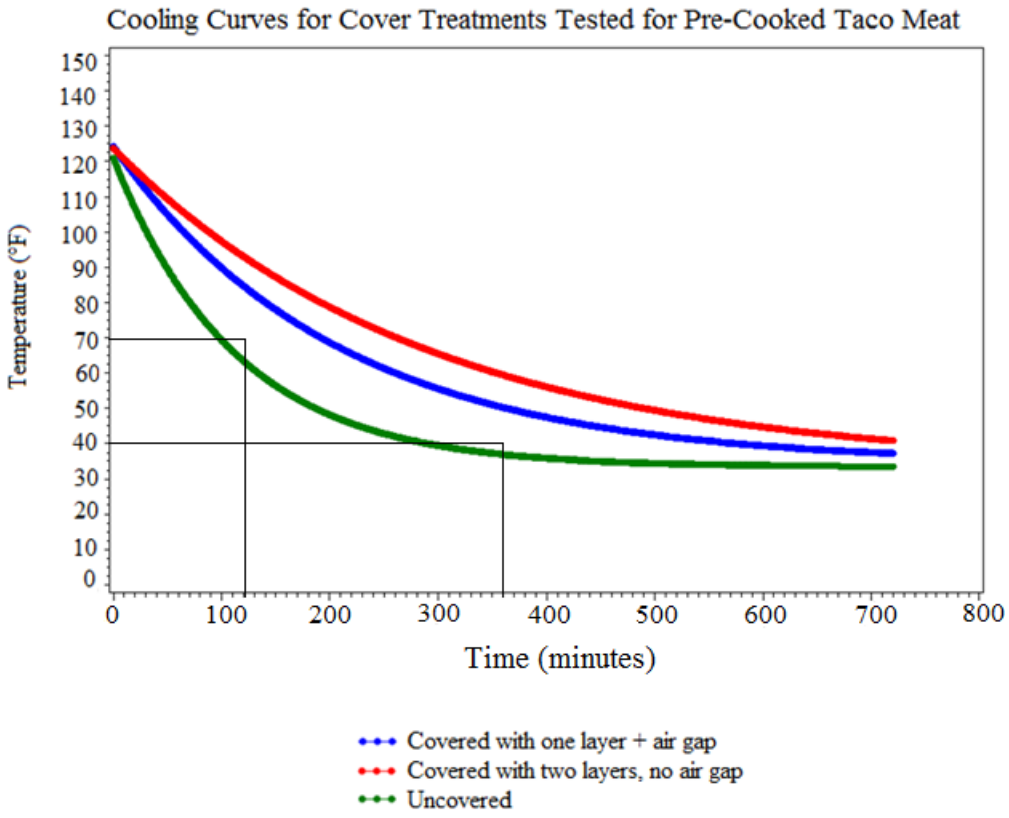


Figure B-3: Cooling Curves for Cover Treatments Tested For Pre-Cooked Taco Meat

The cooling curves in this graph represent each cover method tested for the pre-cooked taco meat product. Cover methods are referenced by color patterns at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual to support the significance of cover method on the cooling of this product.

Appendix C - Chili Con Carne with Beans Cooling Curves

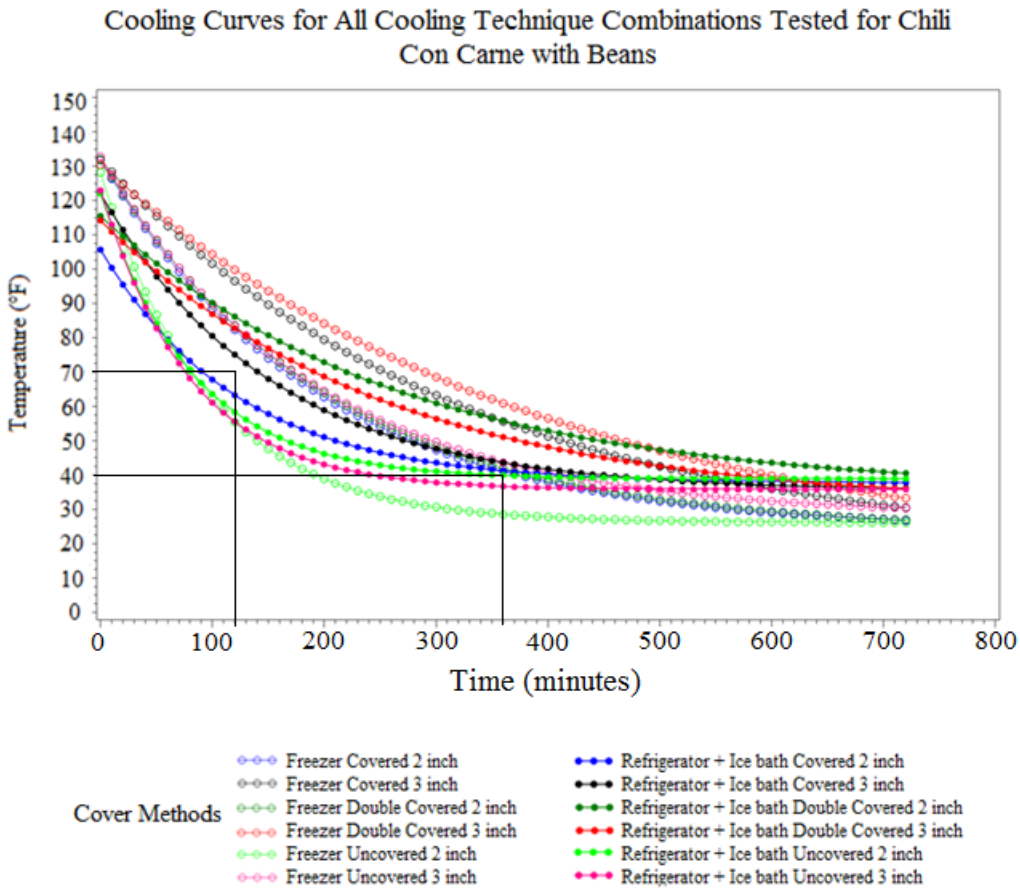


Figure C-1: Cooling Curves for all Cooling Technique Combinations Tested for Chili Con Carne with Beans

The cooling curves in this graph represent all 12 cooling treatment combinations tested for the chili con carne with beans product. Treatment combinations are referenced by color patterns shown in the Cover Methods key at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual for the cooling effects of all 12 treatments tested for this product.

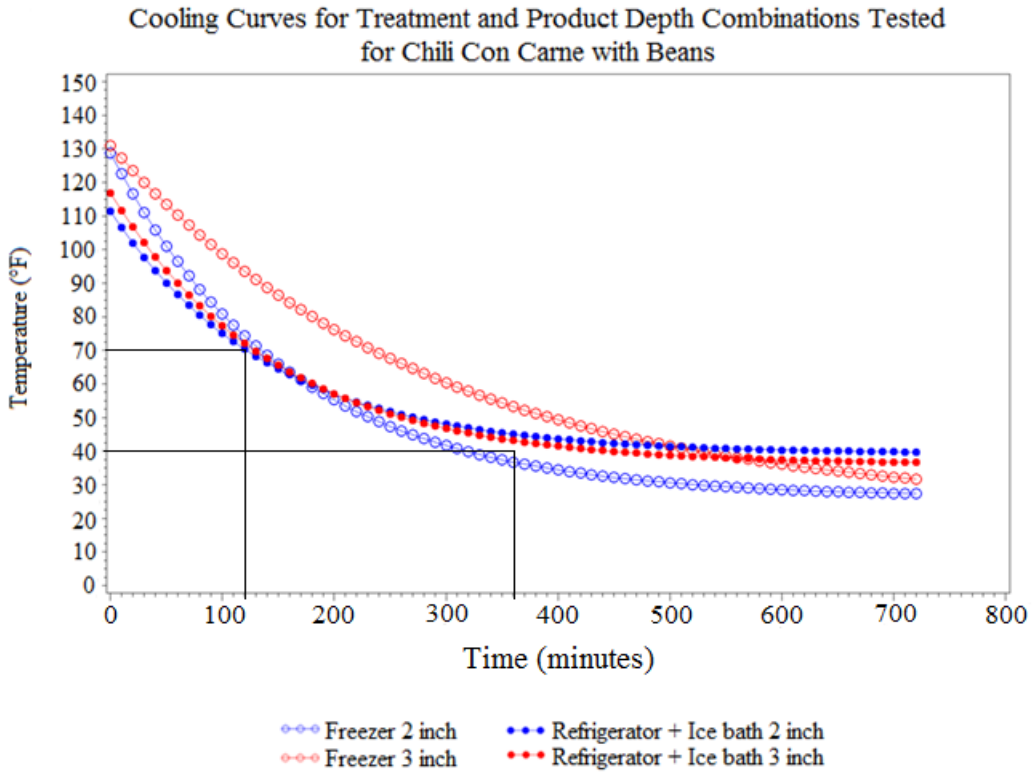


Figure C-2: Cooling Curves for Treatment and Product Depth Combinations Tested for Chili Con Carne with Beans

The cooling curves in this graph represent each treatment and product depth combination tested for the chili con carne with beans product. Treatment combinations are referenced by color patterns at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual to support the significance of treatment and product depth on the cooling of this product.

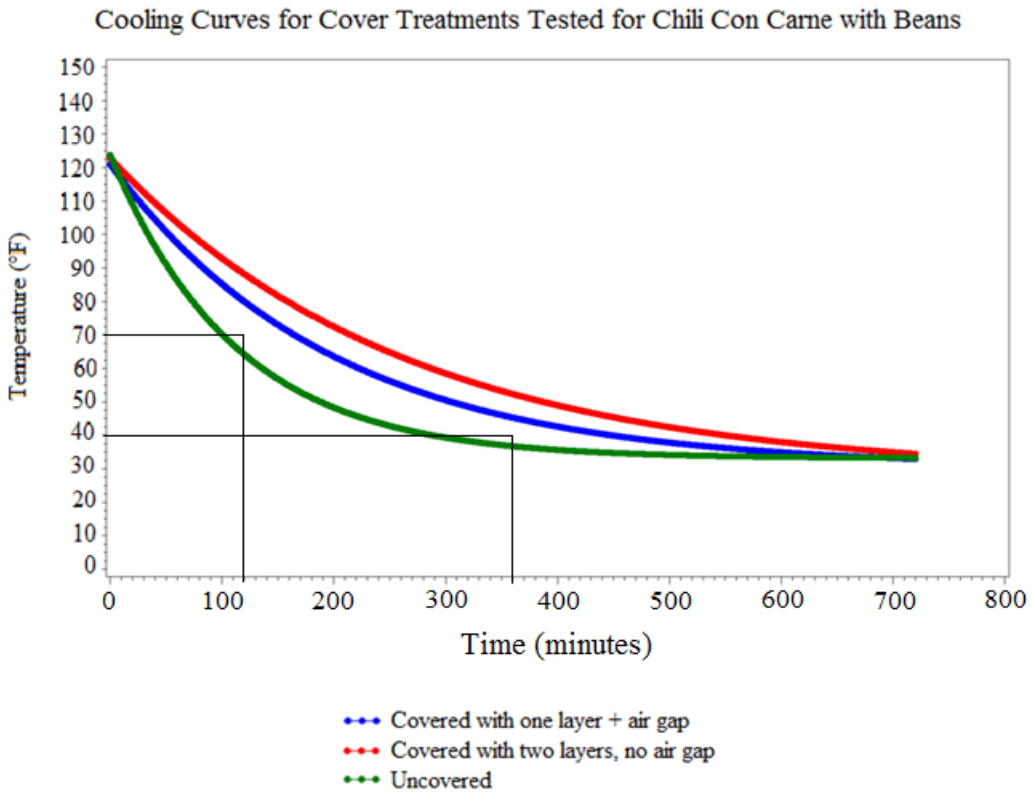


Figure C-3: Cooling Curves for Cover Treatments Tested For Chili Con Carne with Beans

The cooling curves in this graph represent each cover method tested for the low sodium marinara sauce products. Cover methods are referenced by color patterns at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual to support the significance of cover method on the cooling of this product.

Appendix D - Low Sodium Marinara Sauce Cooling Curves

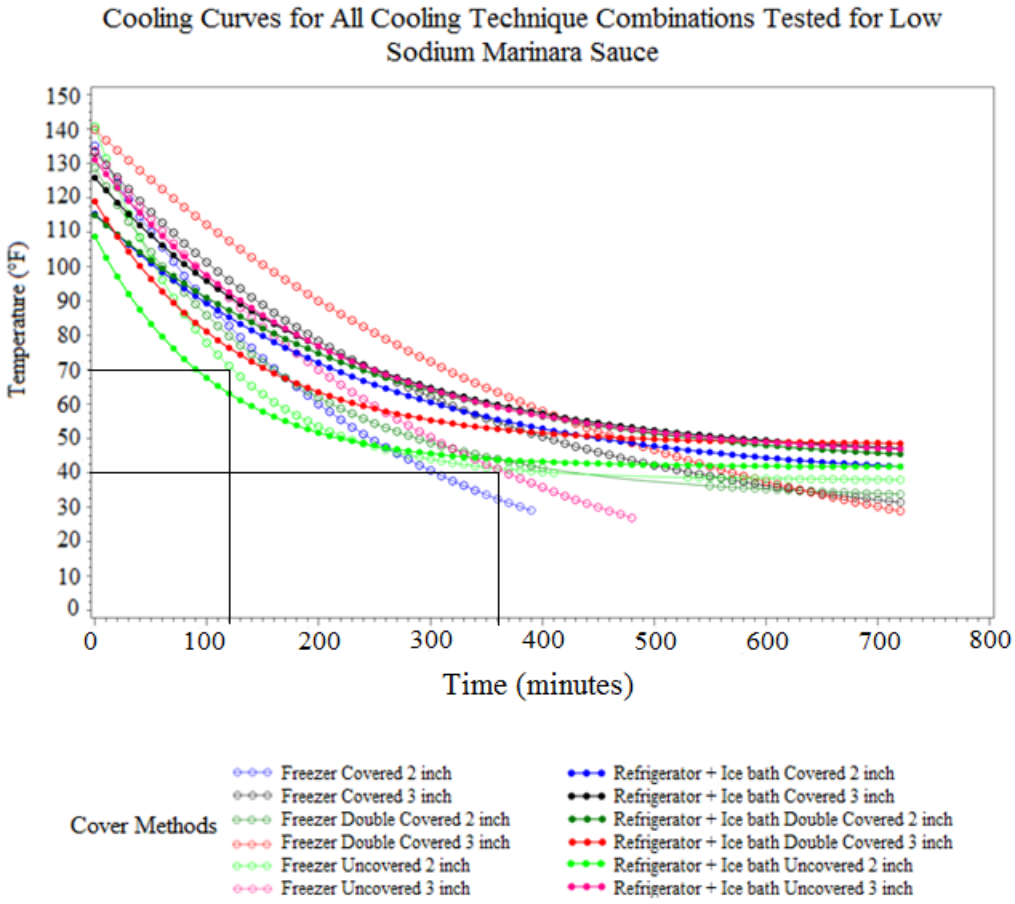


Figure D-1: Cooling Curves for all Cooling Technique Combinations Tested for Low Sodium Marinara Sauce

The cooling curves in this graph represent all 12 cooling treatment combinations tested for the low sodium marinara sauce product. Treatment combinations are referenced by color patterns shown in the Cover Methods key at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual for the cooling effects of all 12 treatments tested for this product.

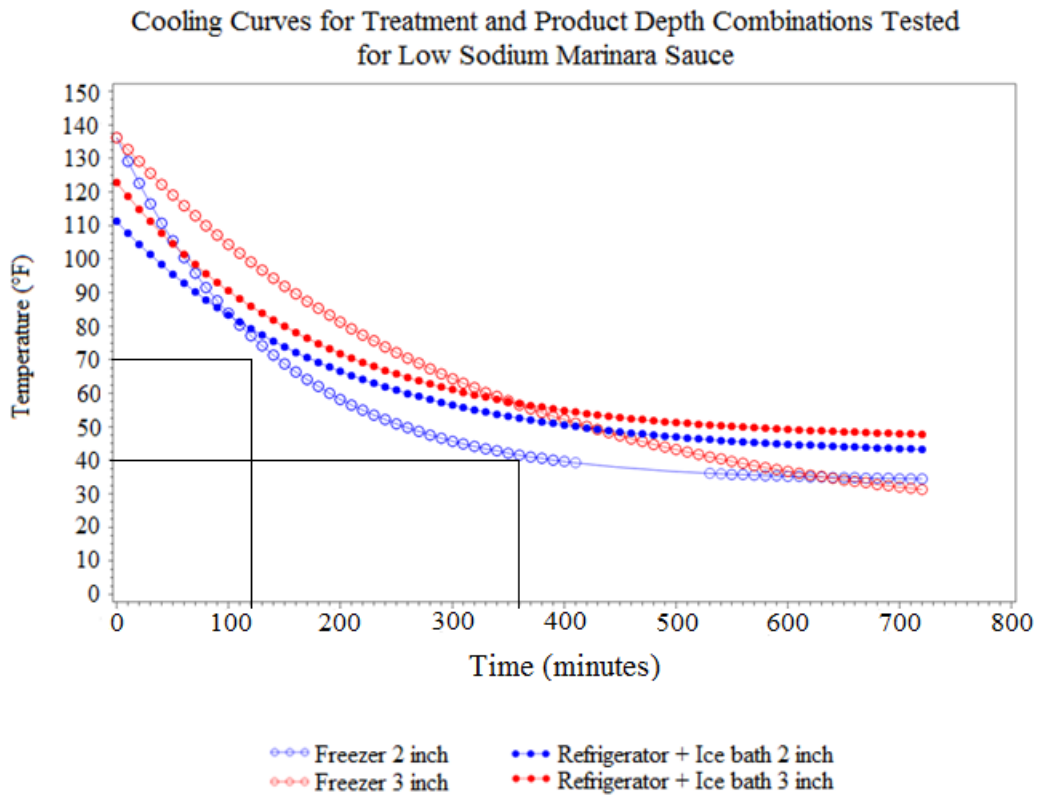


Figure D-2: Cooling Curves for Treatment and Product Depth Combinations Tested for Low Sodium Marinara Sauce

The cooling curves in this graph represent each treatment and product depth combination tested for the low sodium marinara sauce product. Treatment combinations are referenced by color patterns at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual to support the significance of treatment and product depth on the cooling of this product.

Appendix E - Brown Rice Cooling Curves

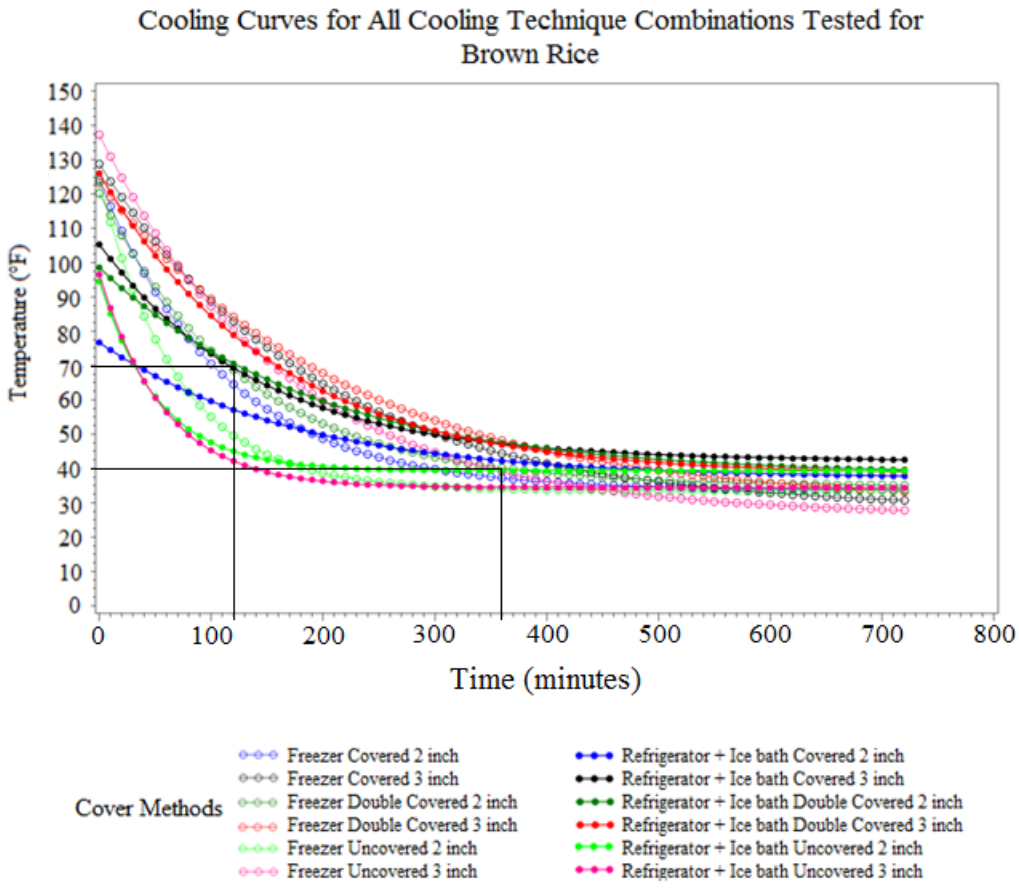


Figure E-1: Cooling Curves for all Cooling Technique Combinations Tested for Brown Rice

The cooling curves in this graph represent all 12 cooling treatment combinations tested for the brown rice product. Treatment combinations are referenced by color patterns shown in the Cover Methods key at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual for the cooling effects of all 12 treatments tested for this product.

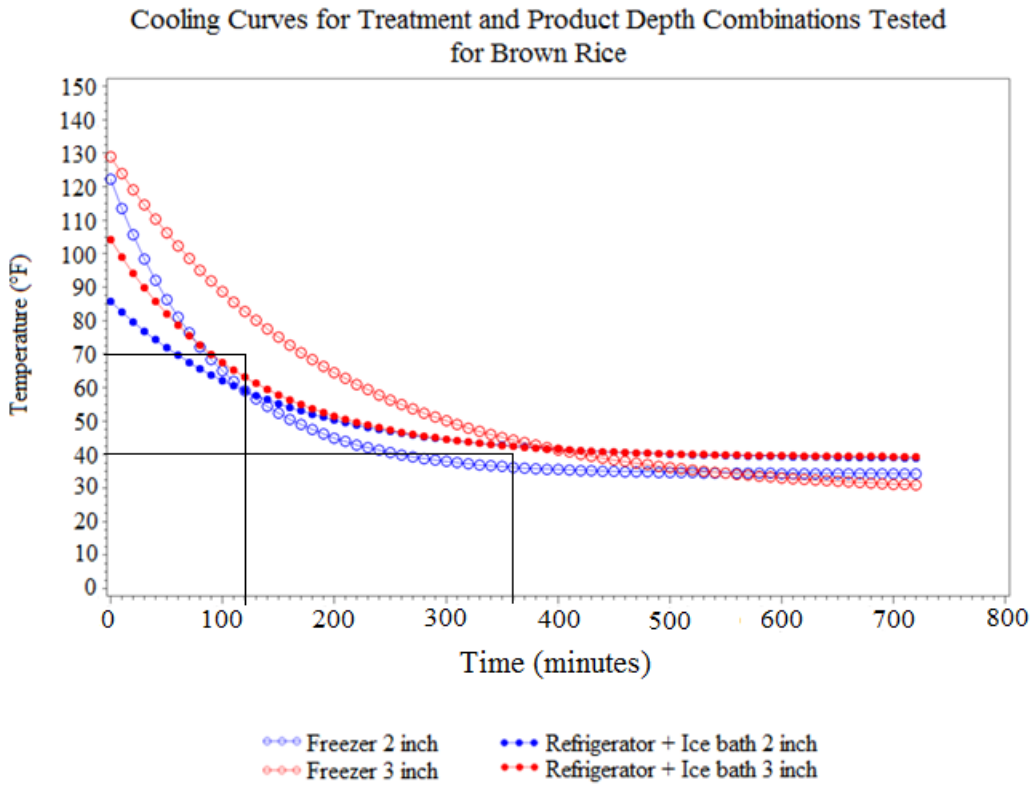


Figure E-2: Cooling Curves for Treatment and Product Depth Combinations Tested for Brown Rice

The cooling curves in this graph represent each treatment and product depth combination tested for the brown rice product. Treatment combinations are referenced by color patterns at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual to support the significance of treatment and product depth on the cooling of this product.

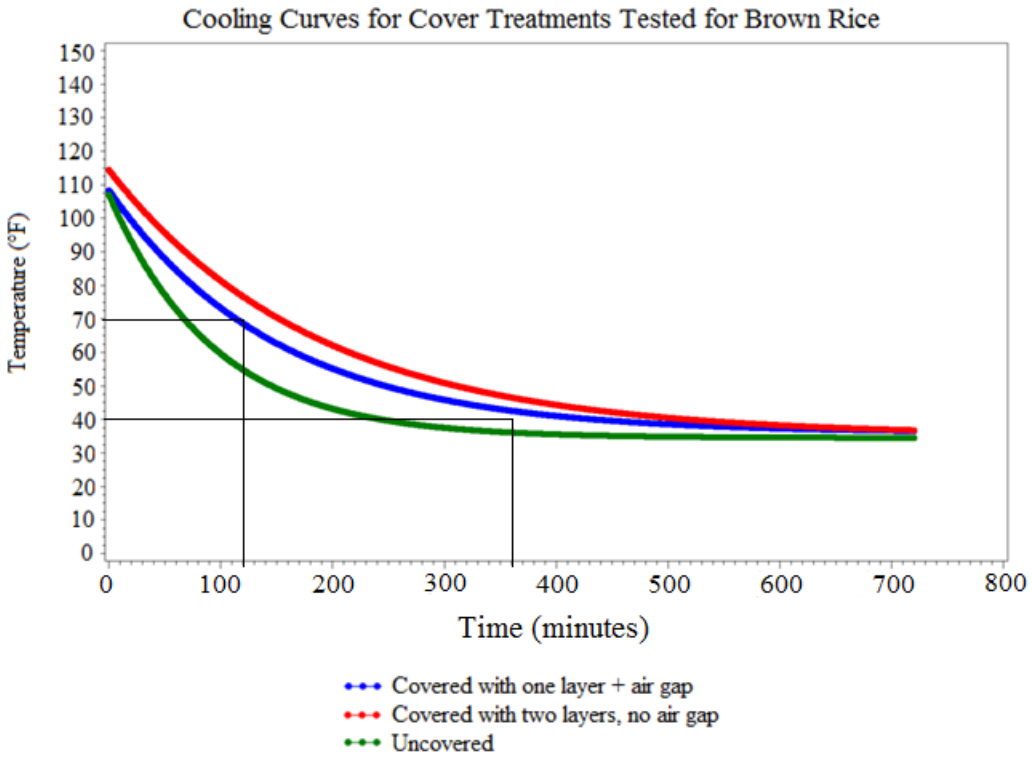


Figure E-3: Cooling Curves for Cover Treatments Tested For Brown Rice

The cooling curves in this graph represent each cover method tested for the brown rice product. Cover methods are referenced by color patterns at the bottom of the graph. Black lines represent the two FDA Food Code time and temperature requirements. This figure was included to provide a visual to support the significance of cover method on the cooling of this product.

Appendix F - Additional Figures

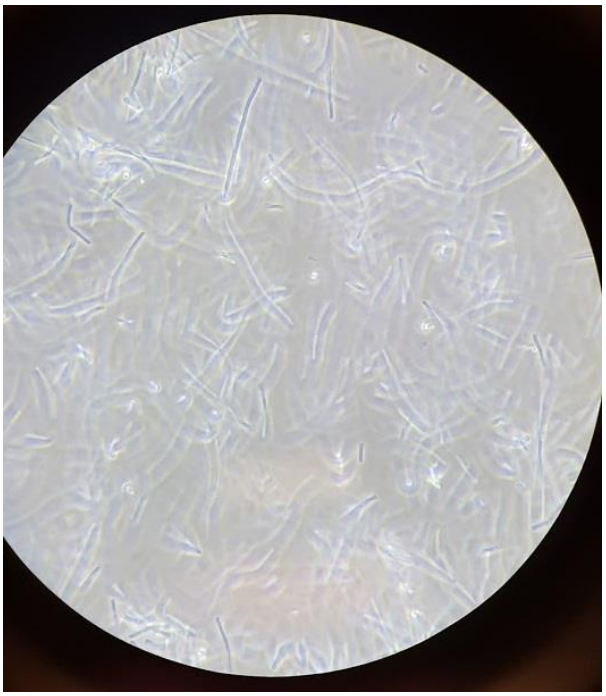


Figure F-1: *B. cereus* Spores and Vegetative Cells Pre-Heat Shock Viewed Under 100x Magnification of a Phase Contrast Microscope

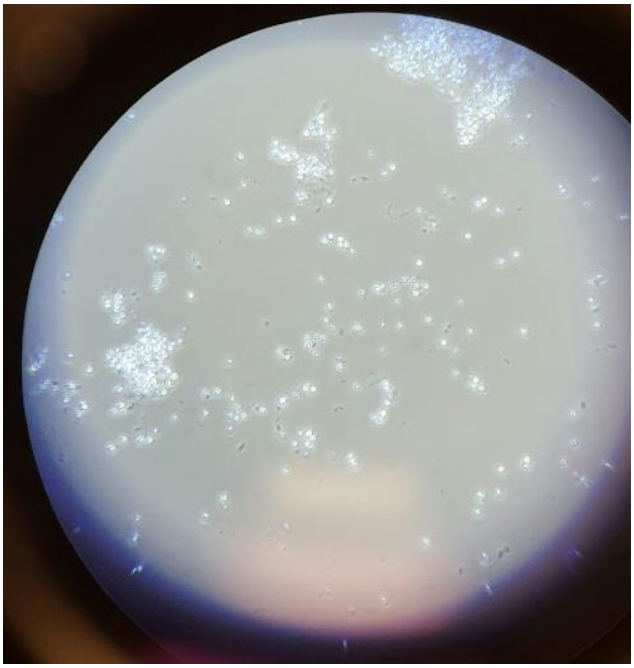


Figure F-2: Primarily *B. cereus* Spores Post-Heat Shock Viewed Under 100x Magnification of a Phase Contrast Microscope

RECIPE NAME: Chili DATE: 8/1/15

INGREDIENTS	100 SERVINGS		SERVINGS	NOTES
Beef Crumbles, cooked, frozen	5 lb.			
Dry Onions	2 ½ cups			
Garlic, powder	1 ¼ tsp.			
Tomatoes, canned, crushed	2 - #10 cans			
Tomato Puree	1 - #10 can			
Water	1 gal.+1 qt.			
Chili Powder	1 ½ cups			
Cumin, ground	5 TBL			
Pepper, Black	5 tsp.			
Sugar	¾ cup			
Beans, chili (do not drain)	3 - #10 cans			

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DIRECTIONS:

1. Thaw beef crumbles under refrigeration.
2. Combine beef crumbles, canned tomatoes, puree, beans, dry ingredients, and water. Do not use mixer.
3. Cook for approximately 45 – 60 minutes or until flavors are blended and internal temperature reaches 165 degrees.
4. **CCP. Heat to 165 degrees for 15 seconds.**
5. **CCP. Hold for hot service 150 degrees or higher.**
6. **Process 2 same day cook or Process 3 if left over**

Note: Frozen beef can be thawed by placing sealed bag in the steamer or boiling water for 10 – 15 minutes. Open bag carefully to avoid being burned. Immediately combine with other ingredients and continue the cooking process.

Serving Size: 8 oz. ladle	Provides: 1.75 oz. Meat/Meat Alternate	Yield: 100 servings
		(Weight or Volume)
		servings:
		(Weight or Volume)
	(Component Requirements)	

Figure F-3: School Lunch Recipe for Chili Con Carne with Beans

Figure F-4: Ruler and Binder Clip System Implemented to Stabilize Data Logger Probe in the Center of the Pan and Food Product

A1	2 in	Ice bath	Covered	Air
A2	2 in	Ice bath	Covered	No Air
A3	2 in	Ice bath	Uncovered	Air
A4	3 in	Ice bath	Covered	Air
A5	3 in	Ice bath	Covered	No Air
A6	3 in	Ice bath	Uncovered	Air
B1	2 in	Freezer	Covered	Air
B2	2 in	Freezer	Covered	No Air
B3	2 in	Freezer	Uncovered	Air
B4	3 in	Freezer	Covered	Air
B5	3 in	Freezer	Covered	No Air
B6	3 in	Freezer	Uncovered	Air

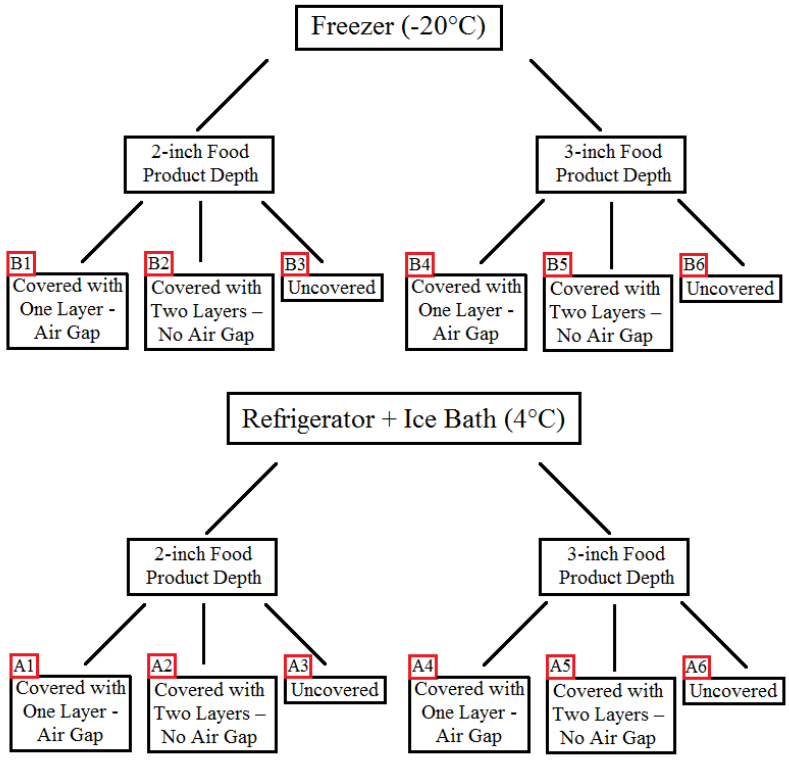


Figure F-5: Key and Diagram Describing All 12 Cooling Treatments Evaluated

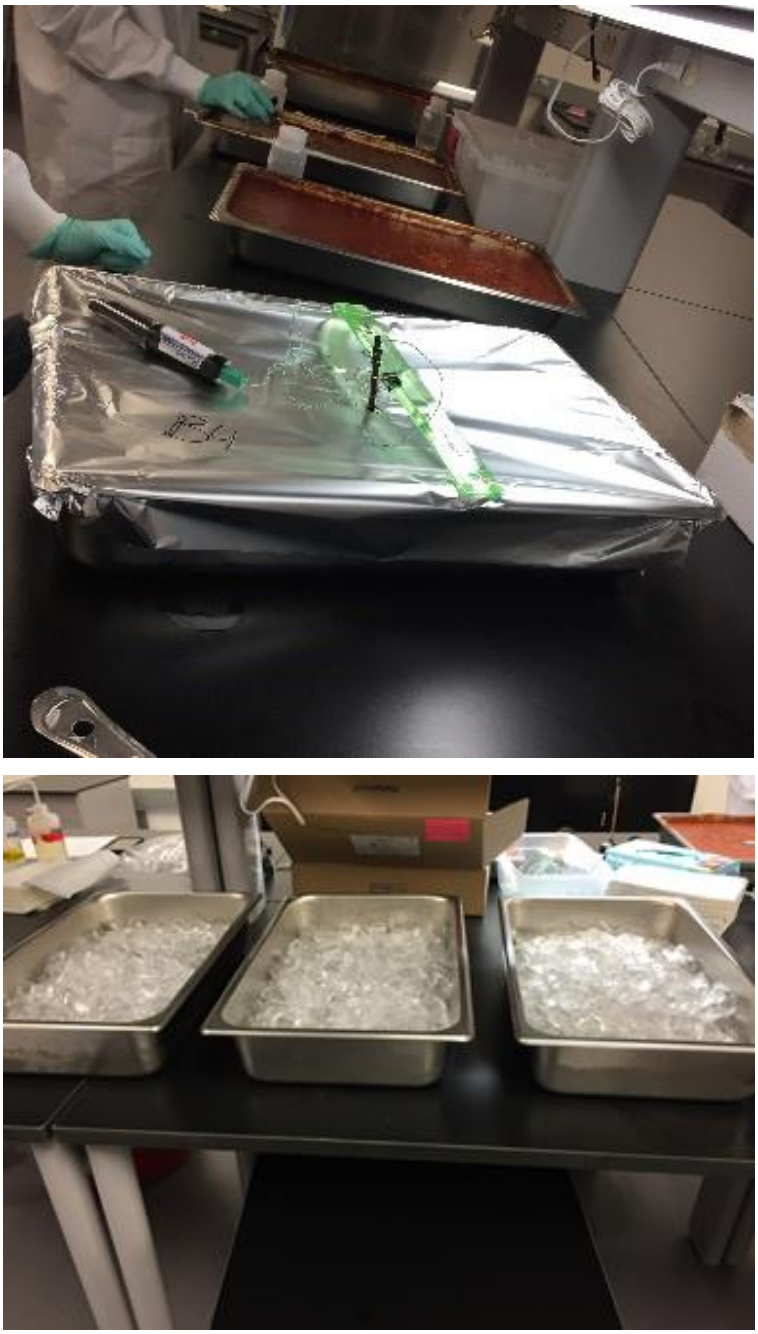


Figure F-6: Examples of Cover and Cooling Treatments Applied to Pans

Figure F-7: Nutrition Label for Pre-Cooked Taco Meat Product

Figure F-8: Nutrition Label for Low Sodium Marinara Sauce Product

School Lunch Chili

Nutrition Facts

100 servings per container

Serving size **1 cup (260.34g)**

Amount per serving

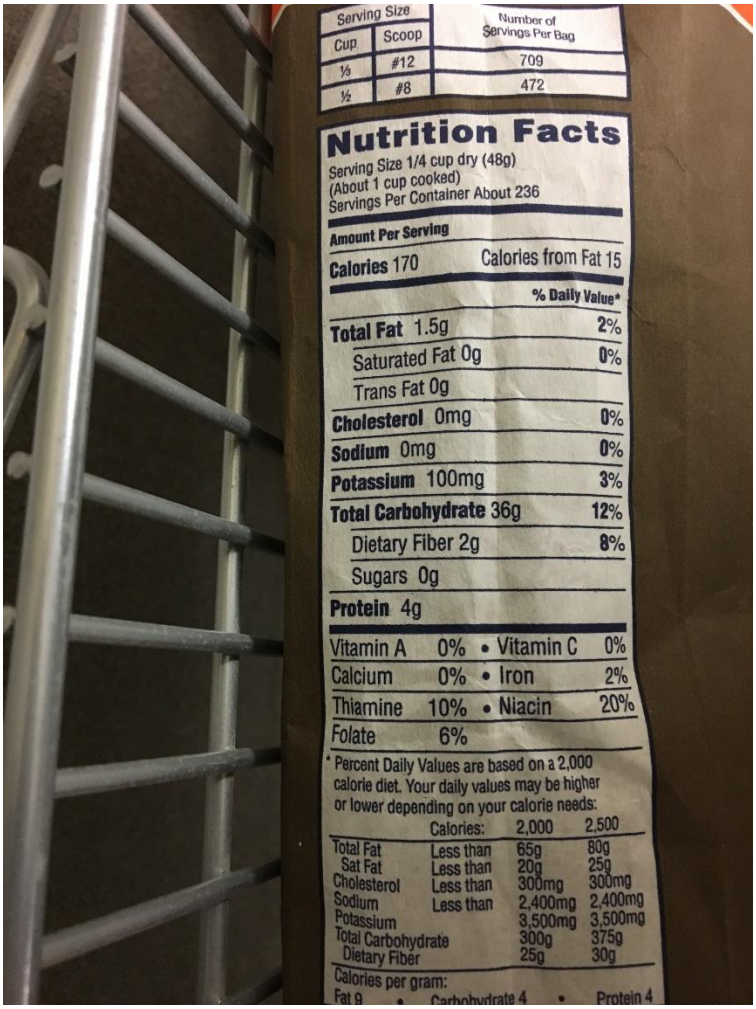
Calories **180**

	% Daily Value*
Total Fat 4.5g	**6%**
Saturated Fat 1.5g	**8%**
Trans Fat 0g	
Cholesterol 20mg	**7%**
Sodium 440mg	**19%**
Total Carbohydrate 25g	**9%**
Dietary Fiber 7g	**25%**
Total Sugars 8g	
Includes 2g of Added Sugars	**4%**
Protein 13g	**26%**
Vitamin D 0mcg	**0%**
Calcium 74mg	**6%**
Iron 4mg	**20%**
Potassium 720mg	**15%**

*The % Daily Values (DV) tells you how much a nutrient in a serving contributes to a daily diet. 2000 calories a day is used for general nutrition advice

Ingredients: Red Kidney Beans, Crushed Tomatoes, Water, Tomatoe Puree, 85/15 Ground Beef, Onion Flakes, Chili Powder, Sugar, Ground Cumin, Ground Black Pepper, Garlic Powder

Figure F-9: Nutrition Label for Chili Con Carne with Beans Product



Serving Size		Number of Servings Per Bag
Cup	Scoop	
⅓	#12	709
½	#8	472

Nutrition Facts

Serving Size 1/4 cup dry (48g)
(About 1 cup cooked)
Servings Per Container About 236

Amount Per Serving	
Calories 170	Calories from Fat 15
	% Daily Value*
Total Fat 1.5g	2%
Saturated Fat 0g	0%
Trans Fat 0g	
Cholesterol 0mg	0%
Sodium 0mg	0%
Potassium 100mg	3%
Total Carbohydrate 36g	12%
Dietary Fiber 2g	8%
Sugars 0g	
Protein 4g	

Vitamin A	0%	• Vitamin C	0%
Calcium	0%	• Iron	2%
Thiamine	10%	• Niacin	20%
Folate	6%		

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Potassium		3,500mg	3,500mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

Figure F-10: Nutrition Label for Brown Rice Product

Ambient Temperature Data for -20°C Walk-in Freezer and 4°C Walk-in Refrigerator			
	Average Temperature (°C)	Standard Deviation (°C)	Mean Kinetic Temperature (°C)
Freezer Inside	-21.27	±0.43	-20.19
Freezer Outside	-21.05	±0.24	-20.72
Refrigerator Inside	4.64	±0.24	4.68
Refrigerator Outside	5.21	±0.14	5.22

*Inside describes temperature data loggers that were placed on the shelves along the back wall of the refrigerator or freezer
*Outside describes temperature data loggers that were placed near the doorway of the freezer or refrigerator

Figure F-11: Ambient Temperature Data for -20°C Walk-in Freezer and 4°C Walk-in Refrigerator