EVALUATION OF ALTERNATIVE COOKING AND COOLING PROCEDURES FOR LARGE, INTACT MEAT PRODUCTS TO ACHIEVE LETHALITY AND STABILIZATION MICROBIOLOGICAL PERFORMANCE STANDARDS

A Thesis

by

ASHLEY NICOLE HANEKLAUS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2009

Major Subject: Animal Science

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Approved by:

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	Kerri B. Harris
Committee Member,	R. Daniel Lineberger
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ABSTRACT

Evaluation of Alternative Cooking and Cooling Procedures for Large, Intact Meat Products to Achieve Lethality and Stabilization Microbiological Performance Standards.

(May 2009)

Ashley Nicole Haneklaus, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Jeffrey W. Savell
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This study was conducted to determine if alternative heating times and slower cooling times, other than those defined by FSIS, could be utilized and still comply with FSIS performance standards. Large (10.43 to 12.25 kg), cured bone-in hams (n = 190) and large (≥ 9.07 kg), uncured beef inside rounds (n = 180) were utilized in a two-phase study. Phase 1 of the study investigated the effect of alternative lethality parameters on toxin production of *Staphylococcus aureus* and log reduction of *Salmonella* Typhimurium and coliforms. Both the hams and roast beef were subjected to 1 of 10 treatments defined by varying final internal product temperatures (48.9ºC, 54.4ºC, 60.0ºC, 65.6ºC, or 71.1ºC) and smokehouse relative humidities (50% or 90%). Phase 2 investigated the effect of alternative stabilization parameters on log growth of C*lostridium perfringens.* Stabilization treatments extended the times taken to reduce internal product temperature from 54.4°C to 26.7°C and from 26.7°C to 7.2ºC (ham) or 4.5ºC (beef), independently. Further, a control treatment following current FSIS, Appendix B guidelines was conducted for ham, and a "worst case" scenario was assessed for both products. The "worst case" treatment evaluated the effects of cooling

products at room temperature (approximately 22.8°C) in place of normal cooling procedures in a temperature controlled environment. Results of the study showed at least a 6.5-$\log_{10}$ reduction in *S.* Typhimurium across all lethality treatments for both products. Further, coliform counts also were reduced significantly, and *S. aureus* toxin kits returned negative results for toxin production for all treatments of ham and roast beef. Stabilization showed less than 1-log growth of *C. perfringens* for any treatment, with the exception of the "worst case" scenario for roast beef. As expected, > 1 log growth of *C. perfringens* was found for uncured roast beef maintained at room temperature for cooling. This study supports that there are multiple time and temperature combinations, other than those currently provided by FSIS, which may be utilized for cooking and cooling large roast beef and bone-in ham products while still meeting FSIS lethality and stabilization microbiological performance standards.

DEDICATION

I dedicate this work to my family. Completing the project would have proven much more difficult without their love and support.

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CHAPTER I

INTRODUCTION

During the production of ready-to-eat and partially cooked meat and poultry products, establishments must meet microbiological performance standards set in place by the United States Department of Agriculture, Food Safety Inspection Service (USDA-FSIS). These standards, found in Chapter 9 of the Code of Federal Regulations (CFR), "set forth levels of pathogen reduction and limits on pathogen growth that official meat and poultry establishments must achieve in order to produce unadulterated products" (*27*). More specifically, a 6.5-log reduction of *Salmonella* must be achieved in ready-to-eat beef products to obtain adequate lethality and no more than 1-log growth of *Clostridium perfringens* may occur during product stabilization (*9, 10, 27).*

In January of 1999, USDA-FSIS published compliance guidelines for meeting lethality and stabilization performance standards for some ready-to-eat and partially cooked meat and poultry products (*9, 10*). Then, on February 27, 2001, FSIS published a proposed rule in the Federal Register that suggested these standards be extended to all ready-to-eat and partially heat-treated meat and poultry products (*27*). These compliance guidelines contain time and temperature recommendations for cooking and cooling

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This thesis follows the style of *Journal of Food Protection*.

procedures that produce products which meet the performance standards. However, achieving FSIS lethality and stabilization microbiological performance standards for cooking and cooling procedures proves to be challenging for processors when manufacturing large, whole-muscle meat products. Failing to satisfy the FSIS "safe harbor" compliance guideline processing parameters for cooking and cooling processes may result in lack of compliance with the performance standards, and as a result, a deviation from a critical limit will occur and corrective actions must be performed on all products associated with the deviation. By examining effects of slower heating and longer cooling times, alternative times that meet the lethality and stabilization performance standards may be achieved. This change in acceptable cooking and cooling parameters will reduce the incidence of deviations and the false assumption of unsafe product.

"Appendix A Compliance Guidelines for Meeting Lethality Performance Standards for Certain Meat and Poultry Products" (*9*) provides times and temperatures that have been validated to comply with the performance standard requirements of a 6.5-$\log_{10}$ reduction of *Salmonella* in ready-to-eat beef products and 7-$\log_{10}$ reduction in ready-to-eat poultry products. Appendix A lists the minimum internal temperature that must be reached during thermal processing, and the length of time it must be maintained to achieve lethality. In addition to achieving lethality through effective time and temperatures, several studies suggest that maintaining a high relative humidity during the cooking process ensures adequate lethality. Injecting steam during the cooking process has been used to destroy *Salmonella* on the surface of beef (*2, 11*). The importance of maintaining a high relative humidity during thermal processing in order to

ensure sufficient destruction of *Salmonella* is addressed in the FSIS compliance guidelines for lethality (*27*). These guidelines recommend using a sealed oven or steam injection to raise the relative humidity above 90% during the cooking process.

"Appendix B Compliance Guidelines for Cooling Heat-Treated Meat and Poultry Products (Stabilization)" (*10*) states that the entire cooling process should allow no more than 1-$\log_{10}$ total growth of *Clostridium perfringens*. These guidelines state that *Clostridium perfringens* can be used alone in an inoculation study to test the performance standards of a cooling process because controlling the outgrowth of *Clostridium perfringens* spores to one log or less also would prevent outgrowth of *Clostridium botulinum* spores. Spores and vegetative cells of *Clostridium perfringens* are present on raw meat. The cooking process of ready-to-eat products will kill the vegetative cells, but may activate the spores to germinate. During the cooling process, germinated spores will grow until the product reaches a cool enough temperature to prevent such outgrowth. The chilling process is a critical step in controlling *Clostridium perfringens*. According to the compliance guidelines for cooling, the most rapid growth for clostridia is between 54.4°C and 26.7°C (*10*). Excessive dwell time in this range is hazardous, and thus product should be cooled as rapidly as possible.

Processors are permitted to utilize customized lethality and stabilization processing parameters. However, individuals choosing to design a customized process must conduct or have access to adequate validation research (*9, 10*). Smaller processors may not have the financial or scientific ability to conduct credible "in-house" validation

research. Currently, there is a lack of such research available to processors with a need for alternative processing schedules other than those outlined by FSIS (*18*).

In an effort to address this issue, this study was designed in two phases. Phase 1 addressed alternative relative humidities and final internal product temperatures for lethality treatments of cured, bone-in hams, and uncured beef inside rounds for roast beef. This phase analyzed the effect of various lethality treatments on *Staphylococcus aureus* toxin production and log reductions of *Salmonella* Typhimurium and coliforms. Phase 2 utilized the same raw materials and investigated lengthening current stabilization guidelines to determine the effectiveness of longer cooling treatments on the inhibition of *Clostridium perfringens* spore outgrowth.

The results of this study demonstrated the ability to produce products that comply with the performance standards without following the current guidelines provided by FSIS. These data may be used as validation research by meat processors, potentially allowing industry increased flexibility associated with heating and cooling large, whole-muscle cuts while still achieving the required performance standards.

CHAPTER II

REVIEW OF LITERATURE

Raw meat and poultry are common sources of foodborne illness due the inherent presence of pathogenic organisms. Outbreak of such foodborne illnesses can be caused by product mishandling in all stages of preparation of raw meat products. Cross-contamination can occur during harvest, fabrication, food service handling, commercial processing of ready-to-eat products, and consumer preparation. Research has estimated that foodborne diseases cause approximately 76 million illnesses of which a reported 325,000 hospitalizations and 5,000 deaths occurred. Known pathogens including *Salmonella* Typhimurium*, Escherichia coli* O157:H7*, Clostridium perfringens,* and *Staphylococcus aureus,* are responsible for 14 million of those illnesses, causing 60,000 hospitalizations, and 1,800 deaths. Because more than 200 known diseases caused by pathogenic organisms are transferred through food, processing activities at any stage, must be conducted with strict adherence to current food safety guidelines and regulations (*19*).

Salmonella. *Salmonella* is a Gram-negative, non-sporeforming, rod-shaped, motile bacterium (*28*). Salmonellae are able to grow on a wide variety of growth media and food products. Ideal pH for optimum growth is near neutrality, if a pH greater than 9.0 or less than 4.0 is achieved, Salmonellae cannot survive. The lowest temperature for which growth has been reported for *S.* Typhimurium is 6.2°C with the upper limit for growth being 45°C (*13).* Symptoms of a salmonellosis infection include nausea,

vomiting, abdominal cramps, diarrhea, fever, and headache, and usually persist for 2 to 3 d (*13, 28*). The mortality rate associated with *Salmonella* averages 4.1%, this ranges from 5.8% under one year of age to 15% over the age of 50 (*13*). The majority of reported cases of salmonellosis occurred in children under 5 years of age (*8*). Among the different species of *Salmonella, S.* Typhimurium has been reported most frequently since 1993 (*6, 19*) and *S.* Choleraesuis has produced the highest mortality rate (*13*). Annually, an estimated 1.4 million cases of foodborne illness are reportedly attributed to salmonellosis infection (*6*). In 2007, *Salmonella* was responsible for 364 outbreak-associated infections, with *S.* Typhimurium proving partially responsible. A variety of other subspecies also contributed to large peanut butter, frozen pot pie, and puffed vegetable snack outbreaks, in addition to, an incident traced back to pet turtles (*7*). Other recorded food sources associated with previous *Salmonella* infections or outbreaks include but are certainly not limited to raw ground meats, poultry, eggs, milk and dairy products, fish, frog legs, coconut, cake mixes, and chocolate (*28*). Regarding food safety of commercially processing cooked, ready-to eat meat products, careful adherence to USDA-FSIS "Appendix A: Compliance Guidelines for Meeting Lethality Performance Standards for Certain Meat and Poultry Products," will ensure safe product. Reaching a final internal product temperature of 70°C or higher will achieve instant lethality of *Salmonella* (*9*).

Coliforms. Coliforms, similar to *Salmonella,* are Gram-negative, non-sporeforming rods, also of the family Enterobacteriaceae. Coliforms have been found to grow at temperatures as low as -2°C and as high as 50°C, and at a pH higher than 4.4 to

9.0. *Escherichia, Citrobacter, Enterobacter,* and *Klebsiella* comprise the vast majority of organisms represented by the coliform group. The primary habitat of the coliform *Escherichia coli* is the intestinal tract of most-warm blooded animals. Because *E. coli* is more indicative of fecal contamination than other genera mentioned, coliforms are typically utilized to determine presence or possible presence of *E. coli.* Since 1895, coliforms have been used as indicator organisms to detect presence of *E. coli* as a fecal contaminant in water, similar to the present day use of generic *E.* coli testing for fecal indication in food processing facilities as part of the USDA-FSIS Pathogen Reduction, and HACCP Final Rule (*13*, *26*). One organism of particular food safety concern is *E. coli* O157:H7 which is classified as an enterohemorrhagic *E. coli* (EHEC), sometimes also referred to as a Shiga toxin-producing *E. coli* (STEC). Healthy cattle harbor this organism normally, and have been found to be the most common animal carrier of the pathogen (*8*). *E. coli* O157:H7 only affects the large intestine of humans, producing high numbers of Shiga toxins, and causing severe, bloody diarrhea. Estimates show that between 2 and 7% of individuals infected with *E. coli* O157:H7 will develop Hemolytic Uremic Syndrome (HUS). HUS usually affects children and has three components, hemolytic anemia, thrombocytopenia, and acute renal failure (*13*). In the elderly, thrombocytopenic purpura (TTP) can develop from the combination of HUS, fever, and neurologic symptoms, resulting in a 50% mortality rate (*28*). For the span of 1998 to 2002, the CDC reported that multistate outbreaks caused by *E. coli* O157:H7 failed to decline due to several issues with the pathogen found in raw ground beef and fresh produce (*6*). A report released in 2007 further reiterated that large multistate outbreaks

related to raw ground beef were not allowing a decline in the prevalence of *E. coli* O157:H7 related illnesses. Despite efforts by the beef industry to implement microbiological interventions to reduce ground beef contamination, 21 recalls were issued in 2007, related to contaminated ground beef (*7)*. Generic *E. coli* testing in meat processing plants (*26*), and adequate thermal processing (*9*) are both examples of interventions that can assist in reducing the occurrence of meat related *E. coli* O157:H7 illnesses and outbreaks.

Staphylococcus aureus. Staphylococci are Gram-positive, mesophilic, spherical, bacteria, with some strains capable of producing a heat-stable toxin, responsible for staphylococcal enteritis in humans. Humans are common carriers of the organism and thus, outbreaks are usually traced back to food preparers (*13, 28*). *S. aureus* can grow at temperatures ranging from 6.7°C to 47.7°C, while enterotoxin production occurs from 10°C to 46°C. Growth and toxin production at these temperatures are dependent upon other ideal environmental parameters, including pH, water activity, and salt concentrations. An optimal pH range for *S. aureus* growth is 6.0 to 7.0, however, growth is possible from 4.0 to 9.8. *S. aureus* is unique for having an ability to grow at very low water activity, with 0.83 being the lowest reported and 0.86 being recognized as the minimum (*13*). The minimum quantity of enterotoxin necessary to cause illness is 20 ng, and symptoms of staphylococcal intoxication include nausea, vomiting, severe abdominal cramps, diarrhea, headache, prostration, and sometimes a fall in body temperature (*13, 28*). Onset of symptoms usually occurs quickly, ranging from 1 to 6 hours from the time of consumption of contaminated foods. Staphylococcal food

poisoning has been estimated to cause 185,060 outbreak-related cases per year in the United States, 100% of which were due to foodborne transmission. However, *S. aureus* intoxication is not routinely reported because of spontaneous onset of symptoms. Due to lack of reporting, projected number of cases per year are be approximated at 10 times the reported number of outbreak-related illnesses (*19*). From 1993 to 1997, the CDC reported that 68% of outbreaks could not be identified by causative organism. However, 428 cases, or 30% were described as having an incubation period of 1 to 7 hours, and were presumably labeled as *S. aureus* intoxications (*5*). In a similar report presenting data for 1998 to 2002, the overall number of outbreaks caused by all foodborne pathogens increased, while number of cases per outbreak decreased; however, the same assumptions for *S. aureus* intoxication cases were not presented for this time period (*6*). Complications and deaths associated with *S. aureus* are very rare, however, like other foodborne pathogens, infants and the elderly are more susceptible than the general population. Further, illness can be prevented by maintaining foods at temperatures below 7.2°C and above 60°C (*28*).

Clostridium perfringens. *Clostridium perfringens* is an anaerobic, Gram-positive, spore-forming rod, which is widely distributed in the environment. *C. perfringens* can be found in the intestines of humans, domestic, and feral animals, in addition to, dirt, dust, spices, and foods (*13, 28*). Humans can be carriers of the heat-sensitive strains of the organism, especially after falling victim to the infection. Research has shown that 20 to 30% of healthy hospital personnel and their families are carriers, as well as, 50 to 80% of individuals two weeks post infection. Symptoms of

infection include severe abdominal cramps and diarrhea, whereas nausea and vomiting are rare. Onset of symptoms usually occurs within 8 to 22 h of consumption of the contaminated food. Symptoms typically cease after 24 h, and are rarely reported due to the prolonged onset, and short duration of the infection (*13, 28*). There are five classifications of *C. perfringens* recognized based on specific production of exotoxins: types A, B, C, D, and E. Of these, types B, D, and E affect domestic animals and are rarely found in humans. However, type A is the most common strain and is most frequently associated with foodborne illness. Type C has been reported to produce an enterotoxin that causes a more serious food-poisoning syndrome (*13*). The syndrome is known as necrotic enteritis or pig-bel disease, although rare, necrotic enteritis is usually fatal. Fatalities are due to impending infection and necrosis of the intestines which ultimately causes death by septicemia (*28*). Similar to *S. aureus, C. perfringens* is rarely reported. Total cases of *C. perfringens* infections are estimated to be 38 times that of the actual number of reported cases each year (*19*).

Because *C. perfringens* is mesophilic, ideal growth temperatures range from 37°C to 45°C, with the most rapid growth occurring at 45°C (*12, 13*), assuming an ideal pH for growth is maintained within the range of 5.5 to 8.0. Optimum sporulation temperature in a research setting is 37°C to 40°C. Strains of *C. perfringens* vary in levels of heat resistance. Normally, infection is caused by foods that were temperature abused. Vegetative cells of *C.perfringens* are usually eliminated during adequate thermal processing, but endospores can survive, and depending on the cooling processes, spores can germinate and grow. Foodborne illness is usually associated with the

consumption of left-over meat products. Typically, these products are cooled inadequately and in some cases, improperly re-warmed causing sporulation (*13*). One report describes an outbreak related to corned beef served to several individuals on St. Patrick's Day in 1993. A deli prepared a large quantity of the beef in advance, in anticipation of a large crowd. Beginning on March 12, the product was cooked, cooled at room temperature, and then refrigerated. On March 16 and 17, the beef was moved from the refrigerator to a warmer (48.8°C), from which it was served. To prevent future outbreaks, the deli was advised to quickly cool all meat portions, not to be immediately consumed, on ice. Further, before serving any leftover cooled product, it must be reheated to a minimum internal temperature of 74°C (*4*). For commercially processed, cooked, ready-to-eat meat and poultry products, USDA-FSIS released "Appendix B: Compliance Guidelines for Cooling Heat-Treated Meat and Poultry Products." Cooling of product should take place as quickly as possible to avoid excessive dwell time between 54.4°C and 26.7°C because this range contains the optimal temperatures for both cell growth and sporulation of clostridia (*10*). Specific "safe harbor" processing guidelines are provided in Appendix B for both cured and uncured products, in an effort to aid processors in producing safe, cooked, ready-to-eat meat products which meet FSIS microbiological performance standards.

Hazard Analysis Critical Control Point (HACCP) System. HACCP is a science-based, proactive system focused on preventing food safety hazards before, during, and after entry into a processing facility (*13, 26, 27)*. All official meat and poultry establishments must operate with a validated HACCP plan in place as part of a

conscious effort to decrease outbreaks related to foodborne illness (*26).* Seven basic principles comprise the HACCP system, the first of which requires all establishments to conduct a hazard analysis. The purpose of conducting this analysis is to identify hazards that are reasonably likely to occur at various steps in a production process. Once all hazards are identified, preventative measures to control those hazards must be established. The second principle identifies a critical control point, as each step in a process, where absence of a control leads to an unacceptable health risk (*13, 26)*. Principle three requires processing facilities to establish critical limits. A critical limit is described as, "a maximum or minimum value to which a physical, biological, or chemical hazard must be controlled at a critical control point to prevent, eliminate or reduce to an acceptable level the occurrence of identified food safety hazard" (*26, 27*). Critical limits are most commonly based on processing parameters, for example, pH, water activity, humidity, time, and temperature. Most importantly, establishments must set critical limits to comply with FSIS regulations, which include performance standards (*26, 27*).

Performance standards were established by FSIS in an effort to ensure microbiological safety of meat and poultry products by satisfying necessary processing parameters. These standards are designed to be, "objective, measurable pathogen reduction standards that can be met by official establishments and compliance with which can be determined through Agency inspection" (*27*). HACCP systems rely on science-based controls thus making performance standards a fundamental part of HACCP systems. Performance standards outline necessary control levels for meat and

poultry processing, allowing establishments to maintain effective HACCP plans, and FSIS to maintain oversight (*26, 27*). One objective of FSIS in publishing Appendix A and B compliance guidelines was to provide processors with innovative and flexible processing schedules based on validated scientific research (*27*). These compliance guidelines produce product that meet the FSIS microbiological performance standards.

In the event that a processing facility fails to satisfy a critical limit, which was set forth in their HACCP plan, a deviation has occurred. The fourth principle of HACCP requires ongoing monitoring of critical control points and critical limits for efficacy. In the event that a deviation from a critical limit is found, corrective actions must be taken to correct the problem. Principle five requires the implementation of corrective actions, in the event that a deviation occurs. This would include deviating from the parameters found in compliance guidelines and associated performance standards (*27*). Correcting a deviation can become very labor intensive, requiring multiple steps to ensure that safety has been restored to the system. As part of corrective actions, the cause of the deviation must be identified and eliminated. After implementing a corrective action, the CCP must be under control, and measures to prevent recurrence of the problem must be established. Any product subject to the deviation must be dealt with in such a manner that no product "injurious to health or otherwise adulterated…enters commerce" (*26).* The final two principles of HACCP first require ongoing verification procedures to ensure that the system is working properly. Secondly, record-keeping of all other steps of the HACCP system must be maintained. The importance of record-keeping is for

establishments to have the ability to prove safety of their product by proper implementation of their HACCP system (*13*).

Validation. As set forth in 9 CFR 417.4, establishments are required to evaluate the effectiveness of their HACCP plans in controlling the food safety hazards that were identified while conducting a hazard analysis. Validating a HACCP plan determines if a HACCP plan and its associated processing controls are being properly implemented, and are properly controlling hazards. During the validation process, an establishment will repeatedly test CCP's, critical limits, monitoring, corrective actions and record keeping procedures to determine effectiveness (*26*). Validation activities also include the identification or development of scientific data sufficient to support processing methods and critical limits. For plants which desire the use of new processing methods, "extensive scientific and in-plant validation of its [the establishment's] HACCP plan under commercial operating conditions" must first be completed (*26*). Therefore, there is a need for the ongoing development of innovative scientific data, sufficient to offer meat production parameters other than those published in Appendix A and B. Although such data would be beneficial to establishments of all sizes, such data would be especially valuable to smaller processing facilities which may lack the scientific and financial means to conduct in-plant studies adequate for validation activities.

CHAPTER III

LETHALITY

Materials and Methods. Eighty bone-in hams (IMPS # 401A) (*20)*, weighing between 10.43 and 12.25 kg, and eighty boneless beef inside rounds (IMPS # 168) (*20)*, weighing greater than 9.07 kg, were purchased from a commercial processing facility and shipped frozen to the Rosenthal Meat Science and Technology Center (RMSTC) at Texas A&M University (College Station, TX). Eight hams and eight inside rounds were assigned randomly to each of the ten cooking treatments. For both ham and roast beef, each lethality treatment (n = 8) was conducted twice, with each run (n = 4) taking place on separate days.

Prior to each treatment, frozen hams were removed from the freezer (-40ºC) and were allowed to thaw for 7 days at approximately 1.1ºC. Each thawed ham was weighed and trimmed free of intermuscular fat and connective tissue required to expose the *M. gracilis* and *M. semimembranosus* muscles. Trimming of the hams allowed for an increase in uniformity between products and a fresh lean surface for microorganism application and attachment during inoculation. During the weighing and trimming process, each ham was assigned an individual identification number and an associated treatment group (run). Following trimming, each ham was re-weighed to assess compliance with the weight parameters set forth in the proposal for this experiment; this weight is referred to as the "trimmed weight." Using a curing pump with a four-needle hand-valve injector (Koch Supplies, North Kansas City, MO), hams were stitch pumped

to 20% of their raw, trimmed weights with a brine solution consisting of 2% sodium chloride (Morton International, Chicago, IL), 2% sucrose (Imperial Sugar Company, Sugarland, TX), 200 ppm sodium nitrite, 540 ppm sodium erythorbate, and 5000 ppm of sodium tripolyphosphate (REO Spice & Seasoning, Huntsville, TX). Brine was mixed in fifty-pound batches (1 batch per run) utilizing a 61 cm hand whisk (Kesco Supply, Bryan, TX). While stirring, the ingredients were introduced into cold tap water in the following order: sodium tripolyphosphate, sodium chloride, sucrose, sodium nitrite, sodium erythorbate. Pumped hams were weighed to verify initial brine retention (≥ 20% of initial raw, trimmed ham weight), placed in gondolas (by run), covered with plastic, and allowed to equilibrate at approximately 1.1°C for 12 to 15 h prior to thermal processing. Post-equilibration, each ham was re-weighed to determine final brine retention. Least squares means for brine retention and product weights are shown in Table 3.1.

Table 3.1. Least squares means of weight (kg) and brine retention (%) for lethality treated hams

	Weight Classification				Brine Retention	
	Raw	Trimmed	Pumped	Post-Equilibration	Pre-Equilibration	Post-Equilibration
Mean	10.8	10.6	13.2	11.9	23.0	15.5
Minimum	9.7	9.4	12.0	11.0	16.0	9.8
Maximum	12.0	12.1	14.8	13.5	32.4	21.4
[1]SEM	0.06	0.07	0.06	0.05	0.00	0.00

[1]SEM = is the standard error of the least squares means.

Hams were placed on smokehouse truck racks by run (one run/truck/ smokehouse). After inoculation (detailed procedures to follow), two thermocouple probes (D605; Dickson Data, Addison, IL) attached to a single data logger (SM-325; Dickson Data) were inserted into each ham. One probe was inserted into the geometric

center of the ham for internal product temperature assessment, and the other probe was inserted directly below the surface of the ham for external temperature assessment. Each data logger recorded internal and external temperatures of each ham at 10 min intervals. The heaviest ham of each run contained the fore-stated data logger probes and two smokehouse control probes (Type J; Alkar, Lodi, WI), inserted into the geometric center of the ham. The ham containing the smokehouse control probes dictated the smokehouse processing program and was considered the "temperature control" ham for each run.

Prior to each treatment, frozen beef inside rounds were removed from the freezer (-40ºC) and were allowed to thaw for 5 days at approximately 1.1ºC. Each thawed inside round was weighed and trimmed free of intermuscular fat and connective tissue required to expose the *M. gracilis* and *M. semimembranosus* muscles. Trimming of the rounds allowed for an increase in uniformity between products and a fresh lean surface for organism application and attachment during inoculation. During the weighing and trimming process, each inside round was assigned an individual identification number and an associated treatment group (run). Following trimming, each inside round was re-weighed to assess compliance with the fore stated weight parameters (10.43 to 12.25 kg for hams, and ≥ 9.07 kg for beef), this weight is referred to as the "trimmed weight" (Table 3.2).

Table 3.2. Least squares means of weights (kg) for lethality treated roast beef

	Weight Classification	
	Raw	Trimmed
Mean weight	10.2	8.7
Minimum weight	8.2	7.2
Maximum weight	13.5	11.8
[1]SEM	0.13	0.10

[1]SEM = is the standard error of the least squares means.

Inside rounds were placed on smokehouse truck racks by run (one run/truck/ smokehouse). After inoculation, two thermocouple probes (D605; Dickson Data, Addison, IL) attached to a single data logger (Dickson Data) were inserted into each roast. One probe was inserted into the geometric center of the roast for internal product temperature assessment and the other probe was inserted directly below the surface of the roast for external temperature assessment. Each data logger recorded internal and external temperatures of each roast at 10 min intervals. The heaviest roast of each run contained the fore-stated data logger probes and two smokehouse control probes (Alkar), inserted into the geometric center of the roast. The roast containing the smokehouse control probes dictated the smokehouse processing program and was considered the "temperature control" roast for each run.

Eighty hams and eighty beef inside rounds were designated for the lethality phase of the experiment, allowing eight hams and eight roast beef for each treatment. Both hams and roast beef were placed in a smokehouse (Model 1000; Alkar, Lodi, WI) and subjected to thermal processing schedules with varying final internal temperatures. The treatments consisted of cooking hams and roast beef at either 50% or 90% relative humidity. Steam humidity was injected into the smokehouse to achieve and maintain the appropriate levels of relative humidity. Hams and roasts were removed from the

smokehouse for sampling when the internal product temperatures reached 48.9ºC, 54.4ºC, 60.0ºC, 65.6ºC, or 71.1ºC, as determined by treatment designation. The ten treatments for each product type were derived from cooking the product to one of five internal temperatures at either 50% or 90% humidity (Table 3.3).

Table 3.3. Final internal temperature (°C) and relative humidity (%) parameters by treatment for lethality

	Treatment Number									
	1	2	3	4	5	6	7	8	9	10
Temperature	48.9	54.4	60.0	65.6	71.1	48.9	54.4	60.0	65.6	71.1
Humidity	90.0	90.0	90.0	90.0	90.0	50.0	50.0	50.0	50.0	50.0

The microbiological analyses taken after each cooking treatment demonstrated which treatments met the FSIS lethality microbiological performance standards by producing at least a 6.5-$\log_{10}$ reduction of *Salmonella.*

The bacterial strain utilized for *Salmonella* Typhimurium was a Rifampicin resistant (rif) mutant derived from the parent strain of *Salmonella enterica* serovar Typhimurium ATCC® 13311. For coliform inoculation, a collection of five individual strains including *Citrobacter freundii* (ATCC® 8090); *Escherichia coli* (ATCC® 11775); *E. coli* (ATCC® 35128); *Enterobacter aerogenes* (ATCC® 306121) and *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC® 31488) were identified as the basis for the coliform cocktail to be used in this research. The *Staphylococcus* strain was derived from a toxin-producing strain of *Staphylococcus aureus* subsp *aureus* (ATCC® 13565) isolated from ham implicated in an outbreak.

All strains were maintained at –80°C in cryocare vials (Key Scientific Products, Round Rock, TX), and stock working cultures were prepared by transferring one bead

from frozen cryocare vials to Tryptic Soy Agar (TSA, BD Diagnostic Systems, Sparks, MD) slants for propagation. Slants were incubated at 35°C for 24 h and stock cultures were kept at room temperature (25°C) and transferred every 2 to 3 weeks. Rif *S.* Typhimurium was confirmed by streaking cultures onto rif TSA and incubating at 35°C for 24 h. Rif TSA was prepared by adding a solution of 0.1 g of rifampicin (Sigma-Aldrich Inc., St. Louis, MO) dissolved in 5 ml methanol (EM Science, Gibbstown, NJ) to 1 ml of autoclaved and cooled (55°C) TSA. All isolates (rif *S.* Typhimurium, individual coliforms and *S. aureus*) were confirmed using conventional biochemical tests as well as VITEK (bioMerieux, Hazelwood, MO).

Two days prior to inoculation, one loop of rif *S.* Typhimurium stock culture was transferred to Tryptic Soy Broth (TSB, Difco) and incubated at 35°C for 20 to 24 h. One ml of culture growth from TSB then was transferred aseptically to a NUNC EasYFlask™ (VWR, Suwannee, GA) containing TSA. Sterile glass beads then were added to each flask in order to evenly distribute the inoculum. The flasks then were incubated at 35°C for 20 to 24 h. Phosphate buffered saline (PBS) (2 to 3 ml) (pH 7.4) (MD Biosciences, Inc., San Diego, CA) was added to each and carefully shaken. The culture then was transferred to a Falcon™ conical centrifuge tube (Becton Dickinson and Co., Franklin Lakes, NJ), and cells were harvested by centrifugation at 1,620 x *g* for 15 min. The supernatant was discarded and the pellet re-suspended in 5 ml of PBS. The prepared inoculum contained approximately 10.8 and 10.7-$\log_{10}$ CFU/ml of rif *S.* Typhimurium for ham and roast beef, respectively. The inoculum was stored at room

temperature (25°C) throughout the inoculation procedure and was used within 2 h after preparation.

Coliform preparation was conducted by individually culturing each of the five individual coliform strains in TSB at 35°C for 18 h for two consecutive days. One ml of culture growth from TSB then was aseptically transferred to a NUNC EasYFlask™ containing TSA. Sterile glass beads then were added to each flask in order to evenly distribute the inoculum. The flasks then were incubated at 35°C for 20 to 24 h. Phosphate buffered saline (2 to 3 ml) (pH 7.4) was added to each and carefully shaken. The culture then was transferred to a Falcon™ conical centrifuge tube, and cells were harvested by centrifugation at 1,620 x *g* for 15 min. The supernatant was discarded, and the pellet re-suspended in 5 ml of PBS. The mixed strain cocktail was prepared by mixing equal volumes of each re-suspended culture, and the final concentration of each organism in the cocktail was approximately 10.9 and 10.5-$\log_{10}$ CFU/ml for hams and roast beef, respectively. The culture preparation was stored at room temperature (25°C) during the inoculation procedure and used within 2 h after preparation.

Staphylococcus aureus preparation was conducted by aseptically transferring a loopful of culture from a 24 h TSA slant to 10 ml of sterile Brain Heart Infusion (BHI, Difco) broth and incubated at 35°C for 24 h. The culture was removed from the incubator and vortexed. The culture then was transferred to a Falcon™ conical centrifuge tube, and cells were harvested by centrifugation at 1,620 x *g* for 15 min. The supernatant was discarded and the pellet was re-suspended in 10 ml of PBS. This rinsing step was carried out three times. The prepared inoculum contained

approximately 8.9 and 8.7-$\log_{10}$ CFU/ml for hams and roast beef, respectively. Inoculum was stored at room temperature (25°C) during the inoculation procedure and used within 2 h after preparation.

Surfaces of either hams or beef were delineated with metal pins to differentiate areas for individual organism inoculation. Approximately 100 cm^2 was inoculated with the bacterial suspension of either *S*. Typhimurium or the coliform cocktail with a sterile disposable spreader (VWR). Approximately 200 cm^2 was inoculated with the bacterial suspension of *S. aureus* using a sterile disposable spreader. The initial inoculum concentration of each organism on the ham surface was approximately 5.8, 8.0, 7.8-$\log_{10}$ CFU/cm^2 for *S. aureus,* coliforms and *S*. Typhimurium, respectively. The initial inoculum concentration of each organism on the roast beef was approximately 6.1, 8.2, and 8.5-$\log_{10}$ CFU/cm^2 for *S. aureus,* coliforms and *S*. Typhimurium, respectively. The inoculation area was contained well within the boundaries established with the pins (> 3 cm) to prevent run off. Each inoculated ham or roast beef was allowed a 15 to 30 min dwell time for proper attachment of the microorgansims. An initial sample was taken to provide a baseline data point for which post-treatment lethality could be compared.

Prior to thermal processing, representative samples were removed from each of the inoculated areas before cooking by excising one 10-cm^2 (2 mm in depth) area using a sterile template, disposable surgical blades and forceps, and placing the sample into a sterile stomacher bag. The uncooked samples were packed in an insulated cooler with refrigerant packs and transported from the RMSTC smokehouse area to the Food Microbiology Laboratory located in the adjacent building for analysis. Post thermal

processing, after the designated final internal product temperature was achieved, the hams or roast beef were removed from the smokehouse and a 10-cm^2 area (2 mm in depth) was immediately excised from each inoculated area using a sterile template, disposable surgical blades and forceps, placed into a sterile Whirlpak® (VWR) bag, and immersed in an ice slurry to prevent continued rise in product temperature. Post-lethality samples were transported from the RMSTC smokehouse area to the Food Microbiology Laboratory located in the adjacent building for analysis. For Staphylococcal enterotoxin production assay, approximately 50 g of lean was excised from the surface of either the ham or roast beef, placed in a Whirlpak® bag, and immersed in an ice slurry. These samples were transported to the Food Microbiology Lab for further analysis.

To each stomacher and Whirlpak® bag containing the 10-cm^2 sample, 100 ml of sterile 0.1% peptone (Difco) diluent was added. The samples were pummeled for 1 min using a Stomacher-400 (Tekmar Company, Cincinnati, OH). Counts of rifampicin-resistant *S.* Typhimurium were determined by plating appropriate dilutions of the sample onto plated rif-TSA and incubating for 24 h at 35°C. Plates containing 25 to 250 colonies typical of *S*. Typhimurium were selected. This count was reported as number of rif *S*. Typhimurium/cm^2 of sample tested. Coliform counts were determined by plating onto 3M™ Petrifilm™ *E. coli*/Coliform Count plates (3M, St. Paul, MN) and incubating at 35°C for 24 h. Plates containing 15 to 150 colonies typical of coliforms were selected. This count was reported as number of coliforms/cm^2 of sample tested. *S. aureus* count was completed by plating appropriate serial dilutions on Baird-Parker agar

(Difco) supplemented with Egg Yolk Tellurite (Difco). Plates were incubated at 35°C for 45 to 48 h. Plates containing 20 to 200 colonies typical of *S*. aureus were selected. This count is reported as number of *S*. *aureus*/cm^2 of sample tested. Appropriate negative controls were taken and plated onto rif-TSA, Petrifilm™ and Baird Parker agar to indicate background flora (if present at each sampling date).

Staphylococcal enterotoxin production in ham and roast beef was determined following the AOAC (Association of Official Analytical Chemists) Official Method 993.06 – Polyvalent Enzyme Immunoassay Method (TECRA SET VIA) for the detection of Staphylococcal enterotoxins A, B, C_1, C_2, C_3, D and E in food and food-related samples.

Data were analyzed using PROC GLM of SAS (SAS Institute, Inc., Cary, NC). Least squares means were generated for main effects and separated using PDIFF option when appropriate with an alpha-level ($P < 0.05$).

Results. The initial $\log_{10}$ CFU/cm^2 concentration of *S.* Typhimurium for all treatments was sufficient to produce a 6.5-$\log_{10}$ reduction as shown by Table 3.4.

Table 3.4. Least squares means of initial $\log_{10}$ (CFU/ cm^2) concentration of inoculum by organism for all lethality treatments

	Ham			Roast Beef		
	Salmonella	Coliforms	*S. aureus*	*Salmonella*	Coliforms	*S. aureus*
Mean Initial concentration	7.8	8.0	5.8	8.5	8.2	6.1
Minimum initial concentration	6.6	6.9	4.9	7.5	7.7	5.2
Maximum initial concentration	8.6	8.7	6.7	9.4	9.4	6.8
[1]SEM	0.04	0.04	0.03	0.04	0.03	0.04

[1]SEM = is the standard error of the least squares means.

All lethality treatments applied to ham and roast beef produced post-lethality samples with < 1 CFU/cm^2 of *S*. Typhimurium, *S.* aureus vegetative cells, and coliforms.

Therefore, all internal temperature and relative humidity combinations yielded product that met FSIS lethality performance standards. Further, all toxin test kits returned negative results for *S. aureus* toxin production. In some cases, it may appear that a 6.5-$\log_{10}$ reduction in *S.* Typhimurium was not achieved. For purposes of statistical analysis, raw plate counts of < 1 CFU/cm^2 were represented as a log value of 0.7. Therefore, a minimum reduction value of 5.9-$\log_{10}$ CFU/cm^2 for *S.* Typhimurium appears misleading, as shown in Table 3.5. If 0.7-$\log_{10}$ CFU/ cm^2 is added to 5.9-$\log_{10}$ CFU/cm^2, a net reduction of 6.6-$\log_{10}$ CFU/cm^2 of *S.* Typhimurium is observed.

Table 3.5. Least squares means of $\log_{10}$ (CFU/ cm^2) reduction by organism for all lethality treatments

	Ham			Roast Beef		
	Salmonella	Coliforms	*S. aureus*	*Salmonella*	Coliforms	*S. aureus*
Mean reduction	7.1	6.4	5.7	7.8	7.5	5.4
Minimum reduction	5.9	5.4	4.7	6.8	7.0	4.5
Maximum reduction	7.9	7.2	6.5	8.7	8.7	6.1
[1]SEM	0.04	0.04	0.04	0.04	0.03	0.04

[1]SEM = is the standard error of the least squares means.

Discussion. Processors are currently suffering from a lack of research related to the effects of thermal processing on pathogenic microorganisms in cured pork products. Additionally, with increasing carcass weights, large, whole-muscle meat products, like hams and roast beef, are becoming more prevalent and have proven more difficult to thermally process according to current "safe harbor" guidelines offered in Appendix A. Current guidelines primarily focus on cooked beef, corned beef, and roast beef, justifying the use of large, uncured roast beef, and the need for lethality data on large, cured hams.

The results presented here agree with those of previous studies *(2, 3, 11, 18)* with regards to the introduction of steam humidity into a cooking cycle for various lengths of time. Goodfellow and Brown (*11)* found that introducing high humidity or steam for a minimum of 30 min during the cooking process produced sufficient lethality of *Salmonella.* Studies also have produced data supporting the use of steam for a minimum of 10 min (*2),* and reducing the relative humidity to 30% for 100% of the cooking cycle (*18)* while still achieving a 6.5-$\log_{10}$ reduction of *Salmonella*. However, this study employed the use of steam to achieve 50% or 90% relative humidity, "for at least 25% of the total cooking time, but in no case less than 1 h" (*9).* This application of humidity produced complete lethality across all treatments for *S.* Typhimurium, *S. aureus,* and coliforms for both hams and roast beef. Further, this study utilized a lower final internal product temperature (48.9ºC) than previous research (*2, 3, 11, 18*) while still achieving a 6.5-$\log_{10}$ reduction.

Customized processes for establishments producing ready-to-eat beef products must achieve an appropriate reduction of *Salmonella*, in addition to, "an appropriate reduction of other pathogens of concern and their associated toxins" *(9).* These results suggest that alternative temperature and humidity parameters may be utilized to offer processors increased flexibility of processing cycles, while still maintaining safe, ready-to-eat ham or roast beef.

CHAPTER IV

STABILIZATION

Materials and Methods. One-hundred-and-ten bone-in hams (IMPS # 401A) (*20)*, weighing between 10.43 and 12.25 kg, and one-hundred boneless beef inside rounds (IMPS # 168) (*20)*, weighing greater than 9.07 kg, were purchased from a commercial processing facility and shipped frozen to the Rosenthal Meat Science and Technology Center (RMSTC) at Texas A&M University (College Station, TX). Ten hams were assigned randomly to each of the eleven ham cooling treatments and ten inside rounds were randomly assigned to each of the ten roast beef cooling treatments. For both ham and roast beef, each stabilization treatment (n = 10) for either ham or roast beef, was conducted twice, with each run (n = 5) taking place on separate days.

Preparation of each ham took place before treatment application. For each treatment group, frozen hams were removed from the freezer (-40ºC) and were allowed to thaw for 7 days at approximately 1.1ºC. Each thawed ham was weighed and trimmed free of intermuscular fat and connective tissue required to expose the *M. gracilis* and *M. semimembranosus* muscles. Trimming of the hams allowed for an increase in uniformity between products and a fresh lean surface for microorganism attachment during inoculation. During the weighing and trimming process, each ham was assigned an individual identification number and an associated treatment group (run). Following trimming, each ham was re-weighed to assess compliance with the weight parameters set forth in the proposal for this experiment; this weight is referred to as the “trimmed

weight." Using a curing pump with a four-needle hand-valve injector (Koch Supplies, North Kansas City, MO), hams were stitch pumped to 20% of their raw, trimmed weights with a brine solution consisting of 2% sodium chloride (Morton International, Chicago, IL), 2% sucrose (Imperial Sugar Company, Sugarland, TX), 200 ppm sodium nitrite, 540 ppm sodium erythorbate, and 5000 ppm of sodium tripolyphosphate (REO Spice & Seasoning, Huntsville, TX). Brine was mixed in fifty-pound batches (1 batch per run) utilizing a 61 cm hand whisk (Kesco Supply, Bryan, TX). While stirring, the ingredients were introduced into cold tap water in the following order: sodium tripolyphosphate, sodium chloride, sucrose, sodium nitrite, sodium erythorbate. Pumped hams were weighed to verify initial brine retention (≥ 20% of initial raw trimmed ham weight), placed in gondolas (by run), covered with plastic, and allowed to equilibrate at approximately 1.1°C for 12 to 15 h prior to thermal processing. Post-equilibration, each ham was re-weighed to determine final brine retention. Least squares means for brine retention and product weights are shown in Table 4.1.

Table 4.1. Least squares means of weight (kg) and brine retention (%) for stabilization treated hams

	Weight Classification				Brine Retention	
	Raw	Trimmed	Pumped	Post-Equilibration	Pre-Equilibration	Post-Equilibration
Mean	10.8	10.8	13.1	11.9	23.3	14.8
Minimum	9.4	8.9	11.5	10.4	12.5	11.4
Maximum	12.1	12.1	14.9	13.3	27.4	20.5
[1]SEM	0.06	0.01	0.07	0.06	0.00	0.00

[1]SEM = is the standard error of the least squares means.

Hams were placed on smokehouse truck racks by run (one run/truck/smokehouse). After inoculation, two thermocouple probes (D605; Dickson Data, Addison, IL) attached to a single data logger (SM-325; Dickson Data) were inserted into each ham. One probe was

inserted into the geometric center of the ham for internal product temperature assessment, and the other probe was inserted directly below the surface of the ham for external temperature assessment. Each data logger recorded internal and external temperatures of each ham at 10 min intervals. The heaviest ham of each run contained the fore-stated data logger probes and two smokehouse control probes (Type J; Alkar, Lodi, WI), inserted into the geometric center of the ham. The ham containing the smokehouse control probes dictated the smokehouse processing program and was considered the "temperature control" ham for each run.

Preparation of each inside round took place before treatment application. For each treatment group, frozen inside rounds were removed from the freezer (-40ºC) and were allowed to thaw for 5 days at approximately 1.1ºC. Each thawed inside round was weighed and trimmed free of intermuscular fat and connective tissue required to expose the *M. gracilis* and *M. semimembranosus* muscles. Trimming of the rounds allowed for an increase in uniformity between products and a fresh lean surface for organism attachment during inoculation. During the weighing and trimming process, each inside round was assigned an individual identification number and an associated treatment group (run). Following trimming, each inside round was re-weighed to assess compliance with the fore stated weight parameters (10.43 to 12.25 kg for hams, and ≥ 9.07 kg for beef)., this weight is referred to as the "trimmed weight" (Table 4.2).

Table 4.2. Least squares means of weights (kg) for stabilization treated roast beef

	Weight Classification	
	Raw	Trimmed
Mean weight	10.1	8.7
Minimum weight	8.2	6.8
Maximum weight	13.2	11.4
[1]SEM	0.14	0.11

[1]SEM = is the standard error of the least squares means.

Inside rounds were placed on smokehouse truck racks by run (one run/truck/ smokehouse). After inoculation, two thermocouple probes (Dickson Data) attached to a single data logger (Dickson Data) were inserted into each roast. One probe was inserted into the geometric center of the roast for internal product temperature assessment and the other probe was inserted directly below the surface of the roast for external temperature assessment. Each data logger recorded internal and external temperatures of each roast at 10 min intervals. The heaviest roast of each run contained the fore-stated data logger probes and two smokehouse control probes (Alkar), inserted into the geometric center of the roast. The roast containing the smokehouse control probes dictated the smokehouse processing program and was considered the "temperature control" roast for each run.

Following preparation and inoculation (detailed procedures to follow), the hams and beef inside rounds were thermally processed, in a smokehouse (Model 1000, Alkar, Lodi, WI), to an internal temperature of 64.4ºC for a minimum of 107 s to achieve lethality as suggested by Appendix A (*9*). After thermal processing, the products underwent one of the assigned cooling treatments (Tables 4.3 and 4.4).

Ham cooling treatments included a control as defined by Appendix B, which recommends that the maximum internal temperature be reduced from 54.4 to 26.7ºC in 5 h and from 26.7 to 7.2ºC in 10 h (15 h total cooling time) (*10*).

Table 4.3. Time parameters (h) by treatment for ham stabilization

	Treatment Number										
	1	2	3	4	5	6	7	8	9	10	11
54.4°C to 26.7°C	5.0	6.0	7.0	8.0	9.0	5.0	5.0	5.0	5.0	9.0	*
26.7°C to 7.2°C	10.0	10.0	10.0	10.0	10.0	11.0	12.0	13.0	14.0	14.0	n/a
Total h	15.0	16.0	17.0	18.0	19.0	16.0	17.0	18.0	19.0	23.0	*

* denotes an unspecified number of hours due to a "worst case" treatment.

Both ham and roast beef cooling treatments included a "worst case" scenario as defined by removing the products from the smokehouse upon completion of thermal processing and reducing the temperature from 54.4 to 26.7ºC by allowing the products to equilibrate at room temperature (approximately 22.8ºC). For all stabilization treatments, samples were taken from each ham or roast, and plate counts were used to determine log growth of *Clostridium perfringens*, once desired time and temperature were reached.

Table 4.4. Time parameters (h) by treatment for roast beef stabilization

	Treatment Number									
	1	2	3	4	5	6	7	8	9	10
54.4°C to 26.7°C	*	2.0	2.5	3.0	3.5	2.0	2.0	2.0	2.0	3.5
26.7°C to 4.5°C	n/a	5.0	5.0	5.0	5.0	5.5	6.0	6.5	7.0	7.0
Total h	*	7.0	7.5	8.0	8.5	7.5	8.0	8.5	9.0	10.5

* denotes an unspecified amount of hours due to a "worst case" treatment.

The microbiological analyses taken after each cooling treatment demonstrated which treatments met the FSIS stabilization microbiological performance standards.

Three bacterial strains of *Clostridium perfringens* were utilized for inoculation and analyses during the stabilization phase of the study. Specifically, a cocktail of *C. perfringens* ATCC® 12916, ATCC® 12917 and ATCC® 14809 were used.

C. perfringens spore suspension was prepared according to the procedures described by Juneja et al. (*16*). Stock culture of *C. perfringens* was maintained in

Cooked Meat Medium (Difco), and 0.1 ml of stock culture was transferred into 2 tubes containing 10 ml of freshly autoclaved Fluid Thioglycollate Medium (FTG). Inoculated tubes were heated in a water bath at 75°C for 20 min and then allowed to cool down to 37°C in an ice bath. An uninoculated tube of FTG was used to monitor the temperature of the inoculated tubes. Heat-shocked cultures were incubated at 37°C for 16 h. From each tube, 1 ml of inoculated FTG was transferred to 10 ml of freshly autoclaved FTG tempered to 37°C. The second inoculate was then incubated at 37°C for 4 h. The transfer and re-incubation was repeated a second time. From the final incubated tubes, 10 ml of FTG was transferred to 100 ml of Modified Duncan-Strong sporulation medium (mDS) and tempered to 37°C. Inoculated mDS was incubated at 37°C for 16 and 40 h. After 16 and 40 h of incubation, 5 ml of each mDS media was transferred to sterile tubes. A smear of 0.01 ml of culture was prepared by using a sterile calibrated loop and spreading the culture over a 1-cm^2 surface (a 1-cm^2 template under a microscope was used). The smear was fixed by flaming 3 times and applying a stain using Schaffer's spore stain method (*21)*. After flaming, the smear was flooded with 5% aqueous malachite green solution and heated to steaming for 2 min. Finally, tap water was used to rinse the smear, and 0.5% aqueous safranin solution was applied for 30 s. Excess stain was rinsed off with tap water and the smear was allowed to air dry. Once dry, the stained samples were observed under a microscope (DME; Leica, Buffalo, NY) using 10x magnification to verify spore presence and estimate the count of spores present. Spore count estimation was conducted by adding a drop of immersion oil to the smear slide and switching to the oil immersion lens (100 x). Spores were counted from at least

10 microscopic fields and averaged. Spores/ml were estimated by using microscopic factor (MF). MF for the Leica microscope (DME; Leica, Buffalo, NY) is 390,000 (spores/ml = spores per microscopic field x MF). At least 10 spores, per microscopic field, are required to achieve approximately 10^6 spores/ml. Final spore suspension was prepared by centrifuging the incubated mDS media at 3000 rpm for 15 min and washing cells twice with 50 ml of distilled water. Suspensions were reconstituted in 20 ml of distilled water and maintained at 4°C until used (within 1 month).

Spores were enumerated by diluting 1 ml of spore suspension in 9 ml of 0.1% peptone water. The culture aliquot was heated in a water bath at 75C for 20 min and allowed to cool down to 37°C in an ice bath. Decimal dilutions were prepared with 0.1% peptone water, and plates were poured using a double-layer pouring plate method with freshly autoclaved TSC agar. Approximately 5 ml of TSC at 45 to 50°C was poured into each petri dish, spread evenly, and allowed to solidify. One ml of the desired dilution was dispensed onto the solidified TSC agar and combined with approximately 12 ml of 45 to 50°C TSC, and the mixture was allowed to solidify. The third agar layer was applied by pouring approximately 3 to 5 ml of 45 to 50°C TSC over the second layer as an overlay. Plates were incubated under anaerobic conditions using an AnaeroGen gas pack (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 24 h. After incubation, plates with 20 to 200 black colonies were selected for counting. Spore suspensions were maintained at 4°C until used (no longer than 15 days).

After suspension, preparation of spores from each bacterial strain was performed, and a cocktail of spores from the three individual strains was created. On the day of

inoculation, equal volumes of spores from each individual strain were mixed to create a final cocktail concentration of 10^7-$\log_{10}$ CFU/ml of *C. perfringens*.

For inoculation of ham and roast beef, a core and cheesecloth method was used. Cheesecloth was prepared by cutting cheesecloth sheets into 40 x 7 cm strips and overlaying two strips to form a cross. Ten cheesecloth pairs were each separated with white paper, and each set of ten pairs was wrapped in a white paper envelope for autoclaving. Cheesecloth packages were autoclaved at 121°C for 15 min.

Following aseptic procedures, four cores were removed from each ham or roast using a 3.3 cm autoclaved corer (5 cores were taken from the ham or roast used as the control). Each core was removed and a 2.5 cm long portion was cut from the internal end of each core. One uninoculated 2.5 cm portion from each inoculation day was placed in a sterile stomacher bag as the negative control. All other 2.5 cm long core portions were inoculated by injecting 0.1 ml of 10^7 *C. perfringens* spore suspension into the center of each core. Each inoculated 2.5 cm core was wrapped in the center of a cheesecloth pair, introduced back into the original ham or roast, and covered with the remaining core portion. One extra core portion per run (day) was inoculated and immediately placed in a sterile stomacher bag as a positive control. The stomacher bags containing the positive and negative controls were placed in an ice chest with refrigerant packs and transported to the Food Microbiology Lab for further analysis.

Samples were taken when the internal temperature reached 54.4°C and 7.2°C for ham, and 54.4°C and 4.5°C for roast beef. Utilizing aseptic techniques, two cores were removed from each ham or roast by pulling the cheesecloth strips. Uninoculated,

external core portions were returned to the hams or roasts. Each pair of core samples was placed in sterile stomacher bags. Each stomacher bag was placed into a WhirlPak® bag, immersed in an ice slurry (for 54.4°C samples) or in an ice chest with refrigerant packs (for 7.2°C and 4.5°C samples), and transported to the Food Microbiology Lab for further analysis.

Stomacher bags were removed from the WhirlPak® bags. From each stomacher bag, meat cores were unwrapped using flame-sterilized forceps. Forceps were flamed by dipping the tool in 95% alcohol and passing through a flame. The two unwrapped meat cores from each bag were placed into a previously tared, sterile stomacher bag and weighed. Nine times the sample weight was added in volume of 0.1% peptone water. Samples were pummeled for 1.5 min using a Stomacher-400 (Tekmar Company, Cincinnati, OH). Appropriate serial dilutions were made and plated onto TSC agar, using the double-layer pouring technique described previously. Plates were incubated under previously described anaerobic conditions at 37°C for 24 h. Plates containing 20 to 200 black colonies were counted. This number was reported as number of *C. perfringens*/g of sample tested.

Data were analyzed using PROC GLM of SAS (SAS Institute, Inc., Cary, NC). Least squares means were generated for main effects and separated using PDIFF option when appropriate with an alpha-level ($P < 0.05$).

Results. Microbiologically significant spore outgrowth is reported as any *C. perfringens* growth greater than 1 $\log_{10}$ (CFU/g). All ham stabilization treatments returned post-stabilization samples with < 1 log growth of *C. perfringens*. Therefore, as

reported in Table 4.5, no significant growth of *C. perfringens* was seen across the ham stabilization treatments.

Table 4.5. Least squares means for treatment effect on $\log_{10}$ (CFU/g) growth of *C. perfringens* spores after stabilization for all hams

Treatment Number	$\log_{10}$ (CFU/g)
1	-0.3^{a}
2	-0.5^{ab}
3	-0.3^{ab}
4	-0.2^{a}
5	-0.2^{a}
6	-0.2^{a}
7	-0.3^{ab}
8	-0.6^{ab}
9	-0.3^{a}
10	-0.1^{ab}
11	-0.9^{b}
^{1}SEM	0.12

Least squares means within a column with different letters (*a*-*d*) differ ($P < 0.05$)
^{1}SEM = Standard error of the least squares means

As expected, the roast beef stabilization phase of this experiment returned post-stabilization samples with < 1 log growth *C. perfringens* on all treatments except treatment 1 (Table 4.6). Treatment 1, defined as the "worst case" scenario for roast beef, differed ($P < 0.05$) from all other roast beef stabilization treatments.

Table 4.6. Least squares means for treatment effect on $\log_{10}$ (CFU/g) growth of *C. perfringens* spores after stabilization for all roast beef

Treatment Number	$\log_{10}$ (CFU/g)
1	1.9^{a}
2	-0.1^{d}
3	0.1^{cd}
4	0.4^{bcd}
5	0.9^{b}
6	0.1^{d}
7	0.2^{bcd}
8	0.3^{bcd}
9	0.3^{bcd}
10	0.9^{bc}
11	*
[1]SEM	0.18

*denotes no data, roast beef underwent 10 treatments
Least squares means within a column with different letters (*a-d*) differ ($P < 0.05$)
[1]SEM = Standard error of the least squares means

As displayed in Table 4.6, treatments 5 and 10 are nearing significant levels of spore outgrowth, with 0.9-$\log_{10}$ (CFU/g) growth achieved by both treatments. Upon review of the stabilization treatment structure for roast beef, treatments 5 and 10 vary greatly in overall treatment length; however, both required 3.5 h from 54.4ºC to 26.7ºC. Due to the excessive dwell time which occurred from 54.4ºC to 26.7ºC for both treatments, inference can be made that a 3.5 h cool down from 54.4ºC to 26.7ºC and any length of time thereafter may be in danger of violating microbiological performance standards for stabilization. Therefore, these data support the use of all roast beef stabilization treatments except 1, 5, and 10 to ensure definite product safety. However, treatments 5 and 10 may be utilized while still meeting microbiological performance standards, but some caution should be exercised since these treatments have a significantly lower margin of safety.

Discussion. Results of this study regarding cured ham products confirm the findings of several other studies based on the effect of curing agents on microorganisms.

Several researchers have investigated the effect of various curing ingredients on outgrowth of *C.perfringens* spores. Sodium citrate (*25*), sodium chloride (*29*), sodium nitrite (*24*), and sodium pyrophosphate (*14*) have each been shown to have significant bacteriostatic activity on spore outgrowth and in turn, can safely lengthen cooling times of cured pork products up to 21 h (*25, 29*). However, these data support the use of cooling treatments for cured, bone-in hams up to a maximum length of 23 h, while actually displaying a reduction in *C perfringens* spores (-0.3 and -0.1-$\log_{10}$, respectively). Although extensive research has been conducted regarding the effects of cooling processes on outgrowth of *C. perfringens* in several meat products, including turkey (*17)*, chili (*1*), ground beef (*15*), cured pork and cured beef (*25, 29*), fewer studies have been conducted utilizing uncured, whole-muscle roast beef (*22, 23*). One study reported complete absence of *C. perfringens* spore germination in cooked beef samples, if cooled in 15 h or less (*15*), which is much longer than the current 6.5 h (54.4°C to 26.7°C in 1.5 h and 26.7°C to 4.5°C in 5 h) cooling time currently outlined in Appendix B (*10).* However, no designation was made between time taken to cool samples from 54.4°C to 26.7°C and 26.7°C to 4.5°C during that 15 h. The findings from this study substantiate the use of cooling times up to 10.5 h for uncured beef, under standard refrigerated conditions (approximately 1.1ºC). However, based on data from this study, the time taken to cool the product from 54.4°C to 26.7°C should not exceed 3.5 h in any cooling scenario (Appendix B states that this portion of the cooling cycle must not exceed 1.5 h). Data from this study clearly exhibit the production of safe product

utilizing cooling parameters other than those offered in Appendix B for large, cured ham, and uncured roast beef products.

CHAPTER V

SUMMARY AND CONCLUSIONS

Data from this study support product safety with alternative heating times and humidities than those defined in Appendix A and slower cooling times than those defined in Appendix B for both cured bone-in ham and uncured roast beef. The identification of slower cooking and cooling times that meet the FSIS lethality and stabilization microbiological performance standards will permit the processing industry to explore more accommodating processing procedures. This will allow extended processing times to be utilized without the concern of producing an unsafe product. In turn, processing deviations, associated corrective actions, and resulting product disposal may be greatly minimized. The results demonstrated that industry may have increased flexibility associated with heating and cooling large, whole-muscle cuts while still complying with the required performance standards.

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