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FINAL REPORT

Evaluation of alternative cooking and cooling procedures for large, intact meat products to achieve lethality and stabilization microbiological performance standards

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FINAL REPORT

TITLE

Evaluation of alternative cooking and cooling procedures for large, intact meat products to achieve lethality and stabilization microbiological performance standards

OBJECTIVES

The goal of this project was to validate the safety of slower cooking and cooling times for large whole-muscle meat products which will meet FSIS lethality and stabilization microbiological performance standards. The specific objectives were:

1. To achieve lethality microbiological performance standards for slower heating times than those defined by Appendix A using alternative heating times and temperatures.
2. To achieve stabilization microbiological performance standards for slower cooling times than those defined by Appendix B using alternative cooling times and temperatures.

EXECUTIVE SUMMARY

This study was conducted to determine if slower heating times than those defined by Appendix A and slower cooling times than those defined by Appendix B could be utilized and still comply with FSIS performance standards. Large (10.43-12.25 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 ham and roast beef were subjected to 1 of 10 treatments defined by varying final internal product temperatures (48.9ºC, 54.5ºC, 60.0ºC, 65.6ºC, or 71.1ºC) and relative humidities (50 or 90%). Phase 2 of the study investigated the effect of alternative stabilization parameters on log growth of C*lostridium perfringens.* Ham stabilization treatments investigated extending the times taken to reduce internal product temperature from 54.5°C to 26.7°C and from 26.7°C to 7.2ºC, independently. Further, a "worst case scenario" and a control defined by current Appendix B guidelines also were assessed. The "worst case" treatment evaluated the effects of cooling product at room temperature (approximately 22.8°C) in place of normal cooling procedures in a temperature controlled environment. Roast beef stabilization treatments investigated extending the times taken to reduce internal product temperature from 54.5°C to 26.7°C and from 26.7°C to 4.5ºC, independently. A "worst case scenario" also was assessed. Results of the study showed at least a 6.5 log reduction in *S.* Typhimurium across all lethality

treatments for both products. Further, coliform counts also were significantly reduced and *S. aureus* toxin kits returned negative results for toxin production across all treatments for both ham and roast beef. Stabilization data failed to show significant growth ($\geq$ 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 reported for uncured roast beef maintained at room temperature for cooling. This study supports product safety with the use of heating times and humidities other than those specified by Appendix A. In addition, safe product may be produced utilizing cooling times much slower than those outlined by Appendix B. The results demonstrate that industry may have increased flexibility associated with heating and cooling large, whole-muscle cuts while still complying with the required performance standards.

INTRODUCTION

In January of 1999, FSIS published guidelines for meeting lethality and stabilization performance standards for some ready-to-eat and partially cooked meat and poultry products (*2, 3*). 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 (*4*). These performance standard guidelines contain time and temperature recommendations for cooking and cooling procedures. Achieving FSIS lethality and stabilization microbiological performance standards for cooking and cooling procedures proves to be challenging for large whole-muscle meat products. Exceeding recommended time limits of the cooking and cooling processes results in a deviation from a critical limit and requires corrective actions to 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 increase in acceptable cooking and cooling times 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" (*2*) 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 (*1, 5*). 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 (*4*). These guidelines recommend using a sealed oven or steam injection to raise the relative humidity above ninety percent during the cooking process.

"Appendix B Compliance Guidelines for Cooling Heat-Treated Meat and Poultry Products (Stabilization)" (*3*) states that the entire process should allow no more than 1-$\log_{10}$ total growth of *Clostridium perfringens* in the finished product. 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 54.5°C and 26.7°C (*3*). Excessive dwell time in this range is hazardous and thus product should be cooled as rapidly as possible.

MATERIALS AND METHODS

This experiment evaluated thermal processing and chilling of both cured bone-in hams and uncured roast beef. Cooking and cooling evaluations were segmented into two experimental phases. Phase 1 entailed product preparation, inoculation, cooking treatments, and microbiological analyses. Phase 2 consisted of inoculation, cooking according to lethality performance standards, cooling treatments, and microbiological analyses. In addition to evaluating various endpoint temperatures, each cooking treatment was performed with high and low relative humidities. During thermal processing, internal and external product temperatures, cooking time, and relative humidity were documented for each treatment. The cooling treatments evaluated extended chilling times during the most critical part of the cooling process, which is 54.5 to 26.7ºC as well as longer cooling times for lowering the temperature from 26.7 to 7.2ºC for cured bone-in hams and 26.7 to 4.5ºC for uncured beef inside rounds.

Raw materials. One-hundred-ninety bone-in hams (IMPS # 401A) (*7)*, weighing between 10.43-12.25 kg, were purchased from a processing facility and shipped to the Rosenthal Meat Science and Technology Center at Texas A&M University. Eighty hams were used for the cooking treatments and one-hundred-and-ten hams were utilized for the cooling treatments. Therefore, eight hams were assigned randomly to each of the ten cooking treatments and ten hams were assigned randomly to each of the eleven cooling treatments.

One-hundred-eighty boneless beef inside rounds (IMPS # 169A) (*7)*, weighing greater than 9.07 kg, were purchased from a processing facility and shipped to the Rosenthal Meat Science and Technology Center at Texas A&M University. Eighty inside rounds were used for roast beef cooking treatments and one-hundred inside rounds were used for roast beef cooling treatments. Therefore, eight inside rounds were assigned randomly to each of the ten cooking treatments, and ten inside rounds were assigned randomly to each of the ten cooling treatments.

Lethality Treatment Structure. 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. Each treatment (n = 8) was conducted twice, with each run (n = 4) taking place on separate days. Both hams and beef roasts were subjected to thermal processing with varying final internal temperatures. The treatments consisted of cooking hams and beef roasts at either 90% or 50% relative humidity. Hams and roasts were removed from the smokehouse for sampling when the internal product temperatures reached 48.9ºC, 54.5ºC, 60.0ºC, 65.6ºC, or 71.1ºC, as determined by treatment designation. Thus, 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 1).

Table 1. 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	54.5	60.0	64.4	65.6	48.9	54.5	60.0	64.4	65.6
Humidity	90.0	90.0	90.0	90.0	90.0	50.0	50.0	50.0	50.0	50.0

Stabilization Treatment Structure. One-hundred-and-ten hams and one-hundred beef inside rounds were utilized for the cooling treatments. This allowed ten hams to be assigned randomly to each of the eleven ham cooling treatments and ten inside rounds to be randomly assigned to each of the ten roast beef cooling treatments. Each treatment (n = 10) was conducted twice, with each run (n = 5) taking place on separate days. Following processing and

inoculation, the ham and beef inside rounds were cooked to an internal temperature of 64.4ºC for a minimum of 107 s to achieve lethality as suggested by Appendix A (*2*). After thermal processing, the products underwent one of the assigned cooling treatments (Tables 2 and 3).

Ham cooling treatments included a control as defined by Appendix B, which recommends that the maximum internal temperature be reduced from 54.5 to 26.7ºC in 5 h and from 26.7 to 7.2ºC in 10 h (15 h total cooling time) (*3*). Once desired time and temperature were reached, samples were taken from each ham, and plate counts were used to determine log growth of *Clostridium perfringens*.

Table 2. Time parameters (in hours) by treatment for ham stabilization

	Treatment Number										
	1	2	3	4	5	6	7	8	9	10	11
54.5°C-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-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 hours	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.

The roast beef cooling treatments included a worst case scenario as defined by removing the roasts from the smokehouse upon completion of thermal processing and reducing the temperature from 54.5 to 26.7ºC by allowing them to equilibration to room temperature conditions. Once desired time and temperature were reached, samples were taken from each beef roast and plate counts were used to determine log growth of *Clostridium perfringens*.

Table 3. Time parameters (in hours) by treatment for roast beef stabilization

	Treatment Number									
	1	2	3	4	5	6	7	8	9	10
54.5°C-26.7°C	*	2.0	2.5	3.0	3.5	2.0	2.0	2.0	2.0	3.5
26.7°C-4.5°C	n/a	5.0	5.0	5.0	5.0	5.5	6.0	6.5	7.0	7.0
Total hours	*	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 cooking and cooling treatment demonstrated which treatments met the FSIS lethality and stabilization microbiological performance standards.

Ham processing. Processing of each ham took place before treatment application. Each ham was weighed and trimmed free of intermuscular fat and connective tissue required to expose the *Gracilis* and *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 ensure compliance with the weight parameters set forth in the proposal for this experiment, and this weight is referred to as the "trimmed weight." Using a curing pump and 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 phosphate (REO Spice & Seasoning, Huntsville, TX). Brine was mixed in fifty-pound batches (1 batch per treatment) utilizing a 24 in hand whisk (Kesco Supply, Bryan, TX). Pumped hams were weighed to verify initial brine retention (≥ 20% of initial raw trimmed ham weight), placed in gondolas (by run), covered, 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. Average brine retention and product weights are shown in Table 4.

Table 4. Simple means of weight (lb) and brine retention (%) for all hams

	Weight Classification				Brine Retention	
	Raw	Trimmed	Pumped	Post-Equilibration	Pre-Equilibration	Post-Equilibration
Mean	23.8	23.4	29.0	26.1	28.0	15.2
Min	20.7	19.6	25.4	22.9	18.0	9.8
Max	26.7	26.6	32.8	29.6	45.8	21.4
SEM	0.09	0.11	0.10	0.09	0.00	0.00

Hams were placed on smokehouse truck racks by run (one run/truck/smokehouse). After inoculation, two thermocouple probes attached to a single data logger (SM-325; Dickson Data,

Addison, IL) 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, inserted into the geometric center of the ham. The ham containing the smokehouse control probes dictated the smokehouse processing program and was considered the "control" ham for each run.

Roast Beef Processing. Processing of each inside round took place before treatment application. Each inside round was weighed and trimmed free of intermuscular fat and connective tissue required to expose the *Gracilis* and *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 ensure compliance with the weight parameters set forth in the proposal for this experiment, and this weight is referred to as the "trimmed weight" (Table 5).

Table 5. Simple means of weights (lb) for all beef roasts

	Weight Classification	
	Raw	Trimmed
Mean weight	22.3	19.1
Min weight	18.0	14.9
Max weight	29.8	26.0
SEM	0.20	0.16

Inside rounds were placed on smokehouse truck racks by run (one run/truck/smokehouse). After inoculation, two thermocouple probes attached to a single data logger (SM-325; Dickson Data, Addison, IL) were inserted into each ham. 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, inserted into the geometric center of the roast. The roast containing the smokehouse control probes dictated the smokehouse processing program and was considered the "control" roast for each run.

Bacterial Strains for Lethality. 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 hr and stock cultures were kept at room temperature (25°C) and transferred every 2-3 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).

Inoculum Preparation for Lethality. 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 20-24 h. One ml of culture growth from TSB then was aseptically transferred to a NUNC EasYFlask™ (VWR, Suwanee, 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 20-24 h. Phosphate buffered saline (2-3 ml) (pH 7.4) (PBS, 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 resuspended in 5 ml of PBS. The prepared inoculum contained approximately 10.8 and 10.7 log 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 20-24 h. Phosphate buffered saline (2-3 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 resuspended in 5 ml of PBS. The mixed strain cocktail was prepared by

mixing equal volumes of each resuspended culture and the final concentration of each organism in the cocktail was approximately 10.9 and 10.5 log CFU/ml for hams and roast beef respectively. The culture preparation was stored at room temperature (25^{o}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^{o}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 resuspended in 10 ml of PBS. This rinsing step was carried out three times. The prepared inoculum contained approximately 8.9 and 8.7 log CFU/ml for hams and roast beef respectively, and was stored at room temperature (25^{o}C) during the inoculation procedure to be used within 2 h after preparation.

Inoculation Procedures for Lethality. Surfaces of either hams or beef were delineated with metal pins to differentiate areas for individual 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 concentration of each organism on the ham surface was approximately 5.8, 8.0, 7.8 log CFU/cm^2 for *S. aureus,* coliforms and *S*. Typhimurium, respectively. The initial concentration of each organism on the roast beef was approximately 6.1, 8.2, and 8.5 log 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 15-30 min dwell time for proper attachment. An initial sample was taken to provide a baseline data point for which post-treatment lethality could be compared.

Microbiological Sampling for Lethality. Prior to thermal processing, representative samples were removed from each of the inoculated areas before cooking by excising 1-10 cm^2 (2 mm in depth) 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 Rosenthal Meat Science and Technology Center 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 internal product temperature. Post-lethality samples were transported from the Rosenthal Meat Science and Technology Center 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.

Microbiological Analysis for Lethality. 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 25-250

colonies typical of *S*. Typhimurium were selected. This count was reported as number of rif *S*. Typimurium/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 15-150 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 45-48 h. Plates containing 20-200 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.

Bacterial Strains for Stabilization. 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.

Media Preparation for Stabilization. Preparation of media for *C. perfringens* began with Fluid Thioglycollate Medium (FTG). Suspension of 29.8 g of BBL Fluid Thioglycollate Medium (BBL 211260) powder in 1 L of distilled water occurred. The solution was stirred and the pH verified and adjusted to 7.1 ± 0.2 if necessary. Adjustment of the pH was conducted with the addition of sodium hydroxide solution (1 N). The flask containing the FTG was covered and

heated to a boil for 1 min. Media was autoclaved at 121°C for 15 min, and stored at 15-30°C following the autoclave procedure.

The Modified Duncan-Strong sporulation medium (DS) (*6*) was prepared on the day of use to maintain integrity. While stirring, 15 g proteose peptone, 4 g yeast extract, 1 g sodium thioglycollate, 10 g $Na_2HPO_47H_2O$, 4 g raffinose, and 100mg caffeine were dissolved into 1 L distilled water. The solution was autoclaved at 121°C for 15 min. After autoclaving, the pH was adjusted to 7.8 ± 0.1 by adding 0.1 ml filter-sterilized 0.66M sodium carbonate per 100 ml of DS. Sodium carbonate solution (0.66 M) was made by dissolving 7 g of sodium bicarbonate into 100 ml of distilled water. Once dissolved, the solution was filtered through a sterile 0.22 μm membrane using a disposable sterile syringe. The filtered solution was collected in sterile tubes and stored at 4°C until needed.

Trypticase Sulfite Cycloserine (TSC) agar (prepared according to BAM specifications, M169) was prepared on the day of use to maintain integrity. TSC base was prepared by dissolving 47 g of dehydrated SFP agar base (Difco 281110) in 900 ml of distilled water. After stirring, the pH was verified and adjusted to 7.6 ± 0.2 with sodium hydroxide solution, when needed. The flask containing the TSC base was covered, agitated and heated until dissolved. Once dissolved, the covered flask was autoclaved at 121°C for 15 min and medium was maintained at 50°C until used. D-cycloserine solution was prepared by dissolving 1 g D-cycloserine (white crystalline powder, Sigma C6880) in 200 ml of distilled water. Solution was filtered through a sterile 0.22 μm membrane using a disposable sterile syringe. The filtered solution was collected in sterile tubes and stored at 4°C until needed. The final agar was prepared by dispensing 20 ml of D-cycloserine solution into 250 ml of TSC base. The final agar was used for pouring plates.

Media preparation was completed by creating solutions necessary for spore identification. A 5% aqueous malachite green solution, 0.5% aqueous safranin solution and 0.1% peptone water were prepared for this purpose. Malachite green solution was prepared by dissolving 5 g of malachite green in 100 ml of distilled water. After allowing the solution to stand for 30 min, it was filtered (Whatman filter paper No. 3). Safranin solution was prepared by dissolving 0.5 g safranin in 100 ml of distilled water. Solution was allowed to stand for 30 min, and then filtered (Whatman filter paper No. 3). Peptone water was prepared by dissolving 1 g bacto peptone in 100 ml of distilled water. Solution was autoclaved at 121°C for 15 min. After autoclaving, final pH of the peptone water was 7.9 ± 0.2.

Inoculum Preparation for Stabilization. C. perfringens spore suspension was prepared according to the procedures described by Juneja et al. (*6*). Stock culture of *C. perfringens* was maintained in Cooked Meat Medium, 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 hr. 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 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 40h 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. 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 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 stained on a light green color. Spores were counted from at least 10 microscopic fields and averaged. Spores/ml were estimated by using microscopic factor (MF). MF for Leica microscope is 390,000 (spores/ml = spores per microscopic field x MF). At least 10 spores had to be present, per microscopic field, 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 75°C 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 Trypticase Sulfite Cycloserine (TSC) agar. Approximately 5 ml of TSC at 45-50°C was poured into each Petri dish, spread evenly, and allowed to solidify. One ml of the desired dilution was added onto the solidified agar and combined with approximately 12 ml of 45-50°C TSC, and the mixture was allowed to solidify. The third agar layer was applied by pouring approximately 3-5 ml of

45-50°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 20-200 black colonies were selected for counting. Spore suspensions were maintained at 4°C until used (no longer than 15 d).

After suspension, preparation for each bacterial strain was performed, and a cocktail of the three individual strains was created. On the day of inoculation, equal volumes of each individual strain were mixed to create a final cocktail concentration of 10^7 log CFU/ml of *C. perfringens.*

Inoculation Procedures for Stabilization. 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 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 portion was inoculated by injecting 0.1 ml of 10^7 of *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.

Microbiological Sampling for Stabilization. The sampling procedures were conducted at 54.5°C and 7.2°C for ham and 54.5°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 cores was placed in sterile stomacher bags. Each stomacher bag was placed into a WhirlPak® bag, immersed in an ice slurry (for 54.5°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.

Microbiological Analysis for Stabilization. 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 absolute ethanol and passing through a flame until the alcohol evaporated. The two unwrapped meat cores from each bag were placed into a previously tared, sterile stomacher bags 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 anaerobic conditions at 37°C for 24 h. Plates containing 20-200 black colonies were counted. This number was reported as number of *C. perfringens*/g of sample tested.

Statistical Methods. 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 ($P < 0.05$). Comparison of simple statistics was generated by using PROC MEANS of SAS.

RESULTS AND DISCUSSION

Lethality. The initial log CFU/cm^2 concentration of *S.* Typhimurium for all treatments was sufficient to produce a 6.5 log reduction as shown by Table 6.

Table 6. Simple means of initial $\log_{10}$ (CFU/ cm^2) concentration of inoculum by organism for all treatments

	Ham			Roast Beef		
	Salmonella	Coliforms	*S. aureus*	*Salmonella*	Coliforms	*S. aureus*
Initial concentration	7.8	8.0	5.8	8.5	8.2	6.1
Min initial concentration	6.6	6.9	4.9	7.5	7.7	5.2
Max initial concentration	8.6	8.7	6.7	9.4	9.43	6.8
SEM	0.04	0.04	0.03	0.04	0.03	0.04

All lethality treatments applied to ham and roast beef produced post-lethality samples with < 1 colony per plate of S. Typhimurium, *S.* aureus, and coliforms. All toxin test kits came back negative for *S. aureus* toxin production. In some cases it may appear that a 6.5 log reduction in *S.* Typhimurium was not achieved. For purposes of statistical analysis, raw plate counts of < 1 were represented as a log value of 0.7. Therefore, a minimum reduction value of 5.9 log CFU/cm^2 appears misleading as shown in Table 7. If 0.7 log CFU/ cm^2 was added to 5.9 log CFU/cm^2, a net reduction of 6.6 log CFU/cm^2 was observed.

Table 7. Simple means of $\log_{10}$ (CFU/ cm^2) reduction by organism for all 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
Min reduction	5.9	5.4	4.7	6.8	7.0	4.5
Max reduction	7.9	7.2	6.5	8.7	8.7	6.1
SEM	0.04	0.04	0.04	0.04	0.03	0.04

Stabilization. Microbiologically significant spore outgrowth is reported as any *C. perfringens* growth greater than 1 log. All ham stabilization treatments returned post-stabilization samples with < 1 log growth of *C. perfringens.* Therefore, as reported in Table 8, no significant growth of *C. perfringens* was seen across ham stabilization treatments.

Table 8. Least-squares means for treatment effect on $\log_{10}$ (CFU/g) growth of *C. perfringens* spores after stabilization

	$\log_{10}$ (CFU/g)	
Treatment Number	Ham	Roast Beef
1	-0.3[a]	1.9[a]
2	-0.5[ab]	-0.1[d]
3	-0.3[ab]	0.1[cd]
4	-0.2[a]	0.4[bcd]
5	-0.2[a]	0.9[b]
6	-0.2[a]	0.1[d]
7	-0.3[ab]	0.2[bcd]
8	-0.6[ab]	0.3[bcd]
9	-0.3[a]	0.3[bcd]
10	-0.1[ab]	0.9[bc]
11	-0.9[b]	*
SEM	0.12	0.18

*denotes no data, roast beef underwent 10 treatments
Least-squares means within a column with different letters (a-d) differ ($P < 0.05$)

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 8). Further, Treatment 1 for roast beef differed ($P < 0.05$) from all other roast beef stabilization treatments.

Conclusions. Data from this study support product safety with alternative heating times and humidities than those defined by Appendix A and slower cooling times than those defined by Appendix B for both cured bone-in ham and uncured roast beef. More extensive data analysis will be presented in the publications associated with this study.

BENEFIT TO THE INDUSTRY

Identifying slower cooking and cooling times that meet the FSIS lethality and stabilization microbiological performance standards will allow the processing industry greater flexibility with processing procedures. This will allow extended processing times to be utilized without the concern of producing an unsafe product.

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