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Thermal Death Time of *Salmonella* Species in Beef and Poultry Rendering Materials

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Lay Summary:
Pathogenic microbial contamination of animal feed ingredients is a concern for animal health as well as for humans who may handle contaminated animal feed. For livestock and horse feeds, the U.S. Food and Drug Administration (FDA) list eight specific strains of *Salmonella* that can cause disease in the animal after consumption of contaminated feed. In pet food ingredients, FDA has proposed zero tolerance for *Salmonella*. Since rendered animal products include recycled animal digestive tracts and contents, it is important to validate the thermal lethality of rendering processes to destroy bacteria in these animal feed or pet food ingredients.

In 2013, a thermal death time (TDT) study was conducted in this laboratory with *Salmonella* Enteritidis (SE), *Salmonella* Newport (SN), *Salmonella* Choleraesuis (SC), and *Salmonella* Dublin (SD) (four of the eight pathogenic strains identified by the FDA as hazardous in animal feeds) in commercially cooked rendered poultry and beef products. In that study, the uninoculated and inoculated samples were treated at 240°F (115.6°C) for up to 420 seconds (Hayes, 2013). Cooked materials used in the study had been collected from commercial rendering plants post-cooker and aseptically re-mixed to 50% fat content. The inoculated populations of 10.4 logarithmic colony forming units per gram (log cfu/g) *Salmonella* were reduced by approximately 8 to 9 log immediately after inoculation into the hot rendering materials. It is hypothesized the inoculated populations may have been further destroyed but the limit of detection of the first stage of the *Salmonella* test prevented further validation. Therefore, a second stage test was conducted to determine a lower detection limit of present or absent. In the second stage of the *Salmonella* test, *Salmonella* were still detected in up to 71 percent of the poultry samples and 50 percent of the beef samples seemingly indicating the added *Salmonella* were not all destroyed. However, further study has shown there is a heat resistant background biota existing in the rendered materials that is identified as *Salmonella* and there currently is no realistic way possible to distinguish between the added *Salmonella* strains and the background *Salmonella*. The only way to confirm the isolates were not the added strains of *Salmonella* would have been to do serotyping or conducting polymerase chain reaction (PCR) testing on each isolated colony to rule it out. However, serotyping was not a practical solution. Serotyping costs $40 per sample (http://www.aphis.usda.gov/animal_health/lab_info_services/downloads/AmesDiagnosticTestingCatalog.pdf). In the poultry study, there were approximately 3200 isolates derived from the confirmation tests that were tagged as *Salmonella*. In order to serotype all of these samples to determine if these were the added strains of *Salmonella*, would have cost $128,000. In the second study on beef rendering products, there were approximately 4400 isolates derived from the confirmation tests that were tagged as *Salmonella*. In order to serotype all of these samples to determine if these were the added strains of *Salmonella*, would have cost an additional $176,000. NP Analytical Laboratories charges $160 per sample for complete serological identification of Salmonella (https://www.npal.com/_Pricing.aspx?svtpid=2) which would be a cost of four times that of the USDA APHIS laboratory charges. PCR is not as expensive at approximately $18 per sample but is very time consuming and was still cost prohibitive for this purpose. Through enrichment, there are known duplicates of the same strains grown in the second presence/absence test. Doing serotyping or PCR on only a subset of the isolates would still leave the question of "did the test actually validate complete destruction of the added strains?" Consequently, serotyping and PCR were not feasible as ways of ruling out the residual *Salmonella* in the background and distinguishing them from the added *Salmonella*.

In the currently reported study, 245°F (118.3°C) was used as the thermal cooking temperature for up to 600 seconds under similar methodology. Based on knowledge learned in the previous study, additional plating was conducted to expand the level of detection of added *Salmonella*. Immediately upon inoculation of approximately 11 to 12 log cfu/g of each *Salmonella* strain into the hot rendering samples, the thermal lethality was sufficient to destroy at least 9 to 10 log cfu/g of the added *Salmonella* strains which is a significant thermal reduction. However, since the FDA routinely considers 12 log reduction as the gold standard for food processing, we would like to validate as large of population reduction as possible in rendering processes. We believe the thermal treatment applied in these experiments kills more of the added *Salmonella* population but is not shown by the results of these experiments because of 1) limitations of the test procedure and 2) inability to distinguish between the added *Salmonella* strains and the background bacteria which are identified on these microbiological tests as *Salmonella*.

The *Salmonella* enumeration test procedure had a lower limit of detection of 1.78 $\log_{10}$ cfu/g of *Salmonella*. The reason there is a lower detection limit is due to the need to dilution plate these samples. The amount of material that could be heated in the tubes was limited due to equipment capabilities. However, the main reason for not doing direct plating of sample in order to get a lower detection limit of 0 cfu/g of *Salmonella* was because in preliminary tests using direct plating of the rendering material, it was difficult to impossible to differentiate between the large number of protein and bone particles versus bacterial colonies in 1 gram of direct plated rendering material. Thus, direct plating of materials made accurate enumeration impossible on the *Salmonella* selective agars. It may have been possible to use something like Dynabeads® to selectively bind to the *Salmonella.* However, preliminary tests conducted in this laboratory on rendering materials more than 8 years ago indicated that Dynabeads® would not work in the high fat materials.

Therefore, the test procedure above had a lower detection limit corresponding to the observed results and this procedure could not reveal if all of the added *Salmonella* were killed. Therefore, the second stage test recommended by the FDA BAM was conducted. This second stage test has limitations but was necessary to detect lower levels of the *Salmonella* in the samples and to determine if the heat treatment was sufficient to kill all added *Salmonella*. The results of the second stage were presence/absence. Results obtained in the second stage of the *Salmonella* test indicated *Salmonella* were still alive in up to 50 percent of the poultry samples and 23.8 percent of the beef samples. Cooking times even up to 600 seconds were not sufficient to completely destroy all *Salmonella* bacteria identified in the cooked rendering samples. The residual *Salmonella* are believed due to a background biota which identifies as *Salmonella* by FDA BAM procedures. However, as above, the only way to confirm the isolates were not the added strains of *Salmonella* would have been to do serotyping or conducting PCR. In these poultry and beef studies, there were approximately 2000 isolates and 1500 isolates, respectively derived from the confirmation tests that were tagged as *Salmonella.* Again, serotyping and PCR were not feasible as ways of ruling out the residual *Salmonella* in the background and distinguishing them from the added *Salmonella* due to cost and shear volume of isolates. Clearly, a better way of ruling out the background biota identified as *Salmonella* and allowing a way to validate that the added *Salmonella* was completely destroyed is needed.

Since that 240°F or 245°F processing temperatures could not destroy these micro-organisms, commercially cooked rendered poultry and beef samples were irradiated for a total of 3.498 Mrad (1325 minutes @ 2640 R/minute) (equivalent to 34.98 kGy). The irradiation was done in an attempt to destroy the background bacteria in the beef and poultry samples and to obtain more accurate thermal death time results that could be attributed only to the added strains of *Salmonella*. This level of irradiation treatment is considered a high dose equivalent to two times the FDA sterilization dosage. However, results indicated the background resistant biota identified as *Salmonella* **survived the irradiation treatment**. Therefore, the background biota in the rendering materials was shown to be both heat resistant and radiation resistant and identified by standard FDA BAM testing as *Salmonella*. And once again, the background biota interfered with the ability to validate the total destruction of the added strains of *Salmonella*.

To further elucidate if this background biota was actually *Salmonella*, colony-PCR was performed on selected colony isolates that had survived the thermal treatments in both the 240°F and 245°F thermal death time studies above (from inoculated and uninoculated test samples) and on isolates from the irradiated samples (controls and thermally treated at 240°F). The selected isolates had all been identified as positive for *Salmonella* via the standard FDA BAM identification tests and confirmed as *Salmonella* on both latex agglutination and ChromAgar™ confirmation tests. Gram stain and morphological characteristics were recorded to ensure the purity of the colony and also identify the bacterial colony morphology. Samples were submitted to the Clemson University Genomic Institute after colony PCR was performed, and data was analyzed through the BLASTn program on the NCBI website. Bacterial identity was selected from the top 10 BLAST nucleiotide database match with max identity greater than 99%. Using colony PCR, all of the bacterial colonies from the poultry rendering samples were identified as *Salmonella*. Several pathogenic *Salmonella* serotypes were listed on both inoculated and uninoculated samples. From SC inoculated samples heat treated for 360 seconds, *Salmonella* Pullorum, *S.* Dublin, *S.* Typhimurium and *S.* Enteritidis isolates were identified. From SE inoculated samples heat treated for 600 seconds, *S.* Tennessee, *S.* Abony, *S.* Newport, *S.* Bovismorbificans, *S.* Thompson, *S.* Typhimurium and *S.* enteritidis were identified. From SE uninoculated samples heat treated for 240 seconds, *S.* Anatum, *S.* Abony, *S.* Typhimurium, *S.* Thompson and *S.* Heidelberg isolates were identified.

Results of the thermal death time studies have indicated a 9 to 10 log reduction in added strains of *Salmonella. This data is very encouraging concerning thermal death time of these pathogens but reporting this data without also reporting the presence of the heat resistant, irradiation resistant background biota that appears to be Salmonella would be ethically wrong.* The FDA BAM tests, the two confirmation tests and the PCR tests report these isolates as *Salmonella* on literally thousands of test samples. A thermally resistant biota has been noted in all of the test samples used in this laboratory for the past 12 years. This raises concerns if the 113 years of recycling rendering materials through animal guts may have resulted in development of an extremely resistant biota of *Salmonella* (and potentially other genera/species of bacteria not investigated in this study) that is cycling through animal gastrointestinal tracts to rendering processing and back again. If so, this raises further questions as to why no disease manifestations have been noted in animals fed these materials. If these are indeed pathogenic *Salmonella*, then why have they not caused disease outbreaks? Or could this biota cause subclinical disease in animals? Or could these be defective *Salmonella* bacteria incapable of

causing disease? Or could these be other species mimicking *Salmonella* by picking up *Salmonella* genes? How are these organisms surviving such intense thermal treatments and even sterilizing irradiation? We do not know the answers to these questions. Unlike on the popular CSI television shows, laboratory tests cannot always definitively answer all questions. Laboratory tests are a tool to provide answers and are limited in the scope of information that can be derived by each test. Although there could be other explanations for these results being positive for *Salmonella*, we do not have the ability to answer further questions concerning this biota without conducting more extensive testing. At this point, we can only say there is an extraordinarily heat resistant and irradiation resistant biota in the rendering samples that appears to be *Salmonella*. This also means there is still no economically feasible way to remove the background biota so an accurate measure of the thermal death time of the added *Salmonella* strains can completed.

The researchers believe fully sequencing the genomes of these bacterial isolates is necessary to determine if these isolates are actually *Salmonella* or if they are other genera which have picked up genes and capabilities similar to *Salmonella*. Genomic sequencing would provide further information as to if the isolates have the ability to be pathogenic, more information about the scope of their capabilities (resistance), the mechanisms by which they are exhibiting this extreme resistance, whether they could produce commercially beneficial proteolytic and lipolytic enzymes, and if these background *Salmonella* pose a potential danger or not to the current or future safety of rendered animal products. This research was proposed to ACREC but not funded. The researchers are currently writing a USDA Exploratory grant proposal in an attempt to further elucidate these answers.

As a side project, several of the isolated thermally resistant bacteria were tested and were shown to have potent protease and lipase activity. Could this background biota contribute lipases that promote release of free fatty acids in rendering materials? Could these heat resistant organisms produce unique heat-resistant enzymes that may have commercial potential? Further research is needed in this area.

Conclusions:
The reported data estimates that 240°F and 245°F will instantaneously reduce populations of approximately 11 to 12 log each of *Salmonella* Dublin, *Salmonella* Newport, *Salmonella* choleraesuis and *Salmonella* enteritidis by 9 to 10 log or more in 50% fat rendered poultry and beef materials but the limitations of testing procedures and the presence of a background biota of an extremely thermally resistant bacteria which identifies as *Salmonella* prevent concluding the thermal treatment completely eliminates the added *Salmonella* strains.

Objective (s):
a) Thermal death time studies will be conducted to determine thermal death time for *Salmonella* choleraesuis, *Salmonella* Dublin, *Salmonella* enteritidus and *Salmonella* Newport in poultry rendering materials.
b) Thermal death time studies will be conducted to determine thermal death time for *Salmonella* choleraesuis, *Salmonella* Dublin, *Salmonella* enteritidus and *Salmonella* Newport in beef rendering materials.

Project Overview:

Validation of Thermal Destruction of *Salmonella* in Rendered Poultry Products at 245 ºF Processing Temperature

Abstract:
In this study, 245ºF (118.3ºC) was used as the thermal cooking temperature for up to 600 seconds holding time after the rendering material reached 245ºF internally. Poultry crax was analyzed for the fat content and then adjusted with poultry fat to 50% fat content. Samples were heat treated and tested for *Salmonella* content using the FDA BAM method. Results were confirmed by using latex agglutination, ChromAgar™ and, on a small subset of the isolates, colony PCR technique. Each *Salmonella* serotype thermal death time test was conducted separately and the results indicated that each serotype appeared to have unique thermal death time characteristics in the rendered products. Variable recoverability was noted among the different *Salmonella* serotypes. *S.* Choleraesuis was last detected at 420 seconds of thermal treatment, *S.* Enteritidis at 600 seconds, *S.* Newport at 540 seconds and *S.* Dublin at 600 seconds. Uninoculated *Salmonella* controls were also conducted, and the results indicated that thermally resistant strains in the background which testing indicated as *Salmonella* were positive up to 600 seconds. Further thermal death time with processing temperature higher than 245ºF and processing time longer than 600 seconds is needed to fully validate the thermal lethality of the rendering facilities. Also the mechanisms for the thermally resistant *Salmonella* or the background bacteria that react as *Salmonella* should be investigated in order to control and eliminate pathogenic *Salmonella* in the animal feed ingredients.

Introduction

The rendering industry processes raw animal by-product materials into value-added, shelf-stable products. Inedible animal by-products comprise a wide variety of products including blood, bones, meat trimmings, fat tissues, horns, hoofs and internal organs (Nollet and Toldra 2011). Commercial rendering cookers are reported to process in the temperature range of 240 to 290°F (115.6 to 143.3°C) for 40-90 minutes in order to thoroughly cook the raw animal by-products (Meeker and Hamilton 2006). Four different pathogenic serotypes of *Salmonella* (*Salmonella* Choleraesuis, *Salmonella* Enteritidis, *Salmonella* Newport and *Salmonella* Dublin) were used in this experiment to validate the thermal lethality of rendering processing time and temperature at 245°F. Hayes (2013) conducted the thermal death time study with the same four pathogenic serotypes of *Salmonella* at 240°F (115.6°C) for up to 420 seconds and *Salmonella* were still detected using the FDA Bacteriological Analytical Manual (BAM) *Salmonella* testing method.

Material and Methods

Rendering Samples Preparation

Samples of cooked poultry crax and poultry fat were collected from a rendering company in the United States on three separate days. All samples were stored at -20°C until needed in the experiments. The poultry crax samples were analyzed on two separate days by the Clemson University Agricultural Service Laboratory for ash, fat, and moisture content analysis. The crax and fat samples were mixed in a disinfected stainless steel food processor jar (Robot Coupe Model R2 Ultra, Ridgeland, MS) to produce 50% fat samples according to the crax fat content.

The food processor jar with blade and lid were disinfected by soaking 2 minutes in Antibac B™ (0.6 g per L, Diversey Corporation, Cincinnatti, OH) dissolved in sterile distilled deionized water (ddH2O), and rinsing for 5 times with sterile ddH2O. Poultry crax and fat were mixed for 10 minutes on the pulse setting in the disinfected food processor to reduce the size particles. A sterile stainless steel spatula was used during mixing and processing to aseptically scrape material from the sides. All mixed samples were stored in the sealed food processor jar under refrigeration until needed for experimentation.

Salmonella Preparation

In this study, four pathogenic *Salmonella* serotypes recognized by FDA as hazardous for animal feeds (*Salmonella* Choleraesuis (FDA 8326) (SC), *Salmonella* Enteritidis (USDA H4386) (SE), *Salmonella* Newport (USDA H1073) (SN) and *Salmonella* Dublin (FDA 23742) (SD)) were collected for this study (FDA, 2010; FDA, 2013). SE and SN were obtained from Dr. Vijay Jejuna of the USDA Agricultural Research Service, Microbial Food Safety Research Unit, 600 East Mermaid Lane, Room 2129, Wyndmoor, PA 19038. SC and SD were obtained from the food microbiology culture collection of collaborator Dr. Xiuping Jiang at Clemson University.

Procedures using selective media and pre-enrichment delineated in the FDA Bacteriological Analytical Manual (BAM) for identification of *Salmonella* (Andrews et al., 2011) and in the previous study by Hayes (2013) were used in this study. Trypticase soy broth (TSB) (90000-050, VWR Scientific Products, Suwanee, GA) with the addition of 0.1% (wt/vol) yeast extract (MP Biomedicals, LLC, Solon, Ohio) was selected for growth of *Salmonella* serotypes grown for inoculation into the rendering materials.

A preliminary trial was conducting by inoculating each *Salmonella* serotype strain into 5 L TSB with the addition of 0.1% (wt/vol) yeast extract for 24 h incubation at 35ºC. After incubation, all 5 L of TSB broth were centrifuged at 7,000 x g for 7 min (GSA rotor, DuPont RC5C Sorvall Instruments Centrifuge, DuPont Company, Newtown, CT) at 4ºC in several sterile centrifuge bottles (47735-696, VWR Scientific Products, Suwanee, GA). The pellet was resuspended in 5 mL sterile TSB by vortexing (Super Mixer, 1290, Labline Instruments, Inc., Melrose Park, IL) on the fast setting for approximately 5 min. After centriguation and resuspension, the average bacterial concentrations for each *Salmonella* serotype were determined. For *Salmonella* concentration determination, xylose lysine deoxycholate (XLD) (90003-996, VWR Scientific Products), and trypticase soy agar (TSA) (90000-050, VWR Scientific Products) were used with the standard Class O phosphate/magnesium chloride dilution buffer (Wehr and Frank, 2004) for serial dilution. Spread plating technique was used for serial dilution on XLD and TSA agar plates, and all the plates were incubated for 24 h at 35ºC. The average concentrations of *Salmonella* cultures in broth for SC, SE, SN, and SD after 24 h incubation at 35ºC were 12.34±0.02, 11.34±0.02, 12.30±0.08, and 11.7±0.03 $\log_{10}$ cfu/g respectively. The preliminary results proved that TSA and XLD enumeration gave similar *Salmonella* concentration after 24 h incubation at 35ºC, and also suggested that 5L of a 24 h *Salmonella* culture grown in TSB with 0.1% (wt/vol) yeast extract and then concentrated by centrifugation would result in a higher production of *Salmonella* concentration. This procedure was used and repeated each time to freshly prepare *Salmonella* cultures before the thermal death time trials.

Selective Media Preliminary Experiment

Background:
In order to conserve resources and keep the cost of conducting this study within the budget provided by the grant, the following preliminary experiment was conducted.

To determine the best media for enumerating the *Salmonella* species used in the thermal death time trials, each individual *Salmonella* serotype was plated onto bismuth sulfite agar, Hektoen enteric agar, and XLD as selective media for measuring populations of *Salmonella*.

Materials and Methods:
Each individual *Salmonella* serotype was dilution plated onto bismuth sulfite agar (90003-904, VWR Scientific Products, Suwanee, GA), Hektoen enteric agar (9004-054, VWR Scientific Products, Suwanee, GA), and xylose lysine deoxycholate (XLD) agar (90003-996, VWR Scientific Products, Suwanee, GA).

Dilutions were made and samples plated to the 10^{-9} dilution with standard Class O phosphate/magnesium chloride buffer (Wehr and Frank, 2004) and plated in duplicate onto bismuth sulfite agar, Hektoen enteric agar, XLD. Media and diluent sterility controls were included.

Results/ Conclusions:
All three media resulted in the exact same enumeration data for each sample. However, it was easier to distinguish colonies from particles on XLD agar. Therefore, this preliminary experiment indicated use of XLD as the preferred selective agar media for enumerating SC, SE, SN and SD in rendered animal products.

Thermal Death Time Trials

Stainless steel sample tubes (8.5 cm length, 1.6 cm outer diameter, 1.3 cm inner diameter, custom manufactured by a local company) with plastic caps (60825-801, VWR International, Suwanee, GA) were autoclaved. One gram of poultry rendering crax/fat samples (50% fat content) was aseptically weighed into the sterile tubes by using sterile spatulas. Four of the tubes were randomly selected as inner tube temperature controls using dial thermometers (61159-409, VWR Scientific Products, Suwanee, GA). This experiment had both heated and unheated control tubes. For the heated control ones, the capped tubes were placed in an analog dry block heater (Model #12621-108, VWR International, Suwanee, GA) equipped with Model #13259-162 heating blocks (VWR International, Suwanee, GA). The analog dry block heaters were adjusted to 118ºC for thermal trials. On average of 27 min heating time, the tubes were heated to an internal treatment temperature of 118.3°C. Each individual culture (100 µL) was directly pipetted into 1 g of the inoculated heated rendering samples when the internal temperature was reached. After pipetting *Salmonella* culture into the inoculated heated tubes, the sample was pipetted up and down approximately four times to thoroughly mix the rendering sample with the *Salmonella* slurry. Upon inoculation and mixing, time measurements (0, 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540 and 600s) were started as thermal treatment time. Samples were

placed on ice immediately after thermal treatment. Uninoculated samples were also placed on ice after thermal treatment time was reached. For each thermal treatment time, samples were conducted in triplicate tubes to increase the accuracy of the results. Unheated control samples tubes were placed on ice until used for plating.

Universal pre-enrichment broth (UPB) (95021-036, VWR Scientific Products, Suwanee, GA) was selected based on previous experimental results by Hayes (2013) and the FDA BAM. A preliminary test was conducted to determine different volume of UPB pre-enrichment that the stainless steel can hold. The stainless steel tubes used in this experiment were not designed to hold 1 g of sample pre-enriched plus 9 mL or 7ml of UPB. The preliminary result suggested that 5 ml of UPB pre-enrichment was optimal for this experiment. Results also indicated that the 1:5 ratio of sample to pre-enrichment broth was not significantly different from the 1:9 ratio of sample to pre-enrichment broth. After thermal treatment, 5 ml of the UPB pre-enrichment was aseptically pipetted into each tube.

The wooden shaft of a sterile cotton-tipped applicator (89133-814, VWR Scientific Products, Suwanee, GA) was used to thoroughly mix the sample with UPB for approximately 30 seconds after UPB had been transferred into each tube. After evenly mixing the pre-enrichment broth with the rendered samples, 0.1 mL of the mixture was directly pipetted onto duplicate XLD and spread immediately using a bent glass rod flame-sterilized after dipping in ethyl alcohol. Several controls were also conducted, including culture controls, media controls, buffer controls, unheated uninoculated sample controls, unheated inoculated sample controls and heated uninoculated controls. The *Salmonella* stock culture was serially diluted by using the standard Class O phosphate/magnesium chloride dilution buffer and spread plating technique by pipetting either 1.0 ml or 0.1 ml of culture on XLD plates and spread with sterile glass rod for even distribution. Stainless steel tubes containing UPB pre-enrichment were incubated for 24 hr at 35ºC for injured *Salmonella* recovery. All XLD plates were incubated overnight for 24 hr at 35ºC.

In order to recover the injured *Salmonella* to obtain more accurate results, additional pre-enrichment steps were conducted in order to have a lower detection limit than the direct plating method. Rappaport-Vassiliadis (RV) pre-enrichment broth (10 mL) (95039-382, VWR Scientific Products, Suwanee, GA) and tetrathionate pre-enrichment broth (TT) (10 mL) (90000-008, VWR Scientific Products, Suwanee, GA) were used based on FDA BAM procedures. RV and TT pre-enrichment broth were made before use and 10 ml was aseptically transferred into sterile glass tubes, respectively. Each stainless steel tube containing UPB broth and sample was vortexed on the fast setting for approximately 30 s to thoroughly mix the sample and the UPB pre-enrichment broth again. The sample was aseptically pipetted (0.1 mL) to RV pre-enrichment broth. The same sample was also aseptically pipetted (1 mL) to TT pre-enrichment broth. RV and TT media controls and also *Salmonella* culture controls in RV and TT were conducted. The samples and control broth were incubated overnight at 42ºC. After the incubation period, all the glass tubes containing either RV or TT broth were vortexed on the fast setting for approximately 30 s to thoroughly mix. An inoculation loop (3mm) of each pre-enriched sample and control was streaked onto XLD agar plates. All XLD plates were incubated overnight at 35ºC. After the incubation period, results would indicate either the presence or absence of *Salmonella* in the samples. Positive samples obtained from the RV or TT pre-enrichments were validated using two

confirmation tests provided by FDA BAM (Feng 2001). Latex agglutination tests (FT0203, Thermo Fisher Scientific, Waltham, MA 02454) and ChromAgar™ (90006-158, VWR Scientific Products, Suwanee, GA) were conducted to confirm the presence of *Salmonella* using each *Salmonella* culture as a control (BD Diagnostics 2008; Oxoid Limited 2008).

Bacterial Identification via Colony PCR and 16S rRNA

After latex agglutination and ChromAgar™ tests, Gram stain and bacterial identification via colony PCR and 16S rRNA were used for selected colonies testing as positive for *Salmonella*. Individual colonies were selected from plates. A small amount of an isolated bacterial colony and 50µl of Promega nuclease-free water (VWR, PAP1195, West Chester PA) were added to a sterile PCR reaction tube. Tubes were placed into boiling water for 10 min. After boiling for 10 min, 12.5µl of the boiled mixture was added to a sterile PCR reaction tube as a DNA template. After determining the concentrations and purities of nucleic acid concentration using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), 1µl of forward oligonucleotide primer (8F, 5'AGAGTTTGATCMTGGCTCAG 3', Integrated DNA Technologies, Coralville, IA), 1µl of reverse oligonucleotide primer (1492R, 5'GGYTACCTTGTTACGACTT 3‟, Integrated DNA Technologies, Coralville, IA) and 10µl of GoTaq® Green Master Mix (VWR, PAM7122, West Chester PA) were added to the PCR reaction tube. The thermal cycle program for this experiment was 94°C for 30 s, 50.6°C for 30 s, 72°C for 1 min, repeat for 30 cycles, and a final incubation at 4°C. After the thermal cycle program finished, Promega PCR Clean Up kit (VWR, PAA9281, West Chester PA) was used for PCR purification. The 16S rRNA sequencing was completed by the Clemson University Genomic Institute. Sequences data were analyzed with National Center for Biotechnology Information (NCBI) BLAST database (Altschul et al 1997).

Determination of Estimated D Values

Estimated D value calculations were based on the direct plate count and the percent recoveries of each *Salmonella* culture. These data were graphed and the slope of the line was used to calculate the estimated D value. Each *Salmonella* culture was compared on graphs, and the percent recoveries of each *Salmonella* culture from inoculated poultry samples were compared on graphs to show the actual inoculated *Salmonella* recover rate. Latex agglutination tests and ChromAgar™ were both used as confirmation tests when the final time the population was no longer detected in each RV and TT pre-enrichment.

Results

Analysis of poultry rendering materials indicated average fat content ranged from 44.3% to 49.7%, ash content was 4.9% to 6.4% and moisture content was 3.5% to 5.3%. Averaged analysis data for each pair of duplicate samples (Day 1, Day 2, Day 3) were used to prepare 50% fat materials for use in this study. The average concentrations of *Salmonella* cultures in broth for SC, SE, SN, and SD after 24 h incubation at 35ºC were 12.34±0.02, 11.34±0.02, 12.30±0.08, and 11.70±0.03 $\log_{10}$ cfu/g respectively. After inoculating *Salmonella* cultures into the rendered animal samples, the recovered *Salmonella* cultures for SC, SE, SN, and SD were 11.92±0.09, 11.23±0.03, 11.66±0.07, and 11.52±0.02 $\log_{10}$ cfu/g respectively.

All *Salmonella* count results were conducted in both direct plating method and pre-enrichment method. Direct plating method on XLD had a lower detection limit of 1.78 $\log_{10}$ cfu/g. Under the

direct plating method, SC, SE, and SN were reduced to below the lower detection limit across all thermal treatment times in inoculated beef samples indicating at least a 9 log reduction in the inoculated populations. SD was detected until 30 s and SE was present in one poultry sample at 15 s. SD was last present at 30 s, and SN was last present at 0 s (Fig.1). In uninoculated poultry samples, SC, SE, SD and SN were reduced to or below the lower detection limit of the direct plating method across all thermal treatments (Fig. 2).

Pre-enrichment results on RV, TT combined with latex agglutination and ChromAgar ™ were recorded. Pre-enrichment plating method on XLD had a lower detection limit of presence/absence. In general, *Salmonella* serotypes in heated, inoculated samples declined with longer thermal treatment (Fig. 3, 4, 5, and 6). For inoculated samples, different *Salmonella* serotypes tend to have different heat resistance (Fig. 3 and 5). Some inoculated samples that were reported as present had high standard errors than others. For the uninoculated samples, there were some variations shown between each samples during thermal treatment.

Among the inoculated samples, SC was reduced from 0 seconds to up to 300 seconds on RV pre-enrichment. SC was reduced from 0s to up to 420 seconds TT, SC was last appeared on 420s on both RV and TT pre-enrichment. SE inoculated samples were reduced from 0s to up to 600s in RV pre-enrichments except 60 seconds and 90 seconds. For SE samples pre-enriched in TT, *Salmonella* levels were reduced to 0 at 0, 15, 30, 90, 480 and 600 seconds. For SN inoculated samples, it reduced to 0 at 0, 15, 30, 60, 120, 180, 240 and 540 seconds in RV pre-enrichments. In TT pre-enrichments, it reduced to 0 at 90, 240 and 600 seconds, but *Salmonella* was not eliminated at 540 seconds. In the heated, inoculated samples, SD was reduced to 0 at 300, 360, 420 and 540 seconds. It was present at 600 seconds in the RV pre-enrichment but seemingly destroyed at 15, 30, 60, 90, 300, 420, and 540 seconds. It was also present at 600 seconds TT pre-enrichments. In both RV and TT pre-enrichments, SE and SD were present at 600 seconds. SC was present at 420 seconds and SN at 540 seconds (Fig. 3 and 5).

For the SC uninoculated samples, there were no *Salmonella* detected on RV pre-enrichment except for 0s. However, on TT pre-enrichment, there were *Salmonella* positive present except at 480, 540 and 600 seconds. For SE uninoculated samples, there were *Salmonella* positive at all time intervals on RV pre-enrichment. There were *Salmonella* positive at all time intervals except at 60 and 90 seconds on TT pre-enrichment. For SN unionculated samples, there were no *Salmonella* positive except at 0 on RV and TT pre-enrichments. For SD uninoculated samples, there were no *Salmonella* positive except at 120 and 180 seconds for RV pre-enrichment, and there was no *Salmonella* positive for TT pre-enrichment. Variations between RV and TT pre-enrichment and also among different uninoculated samples were noted in *Salmonella* populations (Fig. 4 and 6).

Colony-PCR was performed on selected colony isolates from SC inoculated 360 seconds, SE inoculated 600 seconds and SE uninoculated 240 seconds. *Salmonella* positive colonies were selected from TSA agar plates after Latex agglutination and ChromAgar ™ tested positive as confirmation tests. Gram stain and morphological characteristics were also recorded to ensure the purity of the colony and also identify the bacterial colony. Results were submitted through the Clemson University Genomic Institute after colony PCR was performed, and data was analyzed through the BLASTn program on the NCBI website. Bacterial identity was selected

from the top 10 BLAST nucleiotide database match with max identity greater than 99% (Table.1). All the bacterial colonies from the poultry rendering samples were identified as *Salmonella*. Several pathogenic *Salmonella* serotypes were listed as identified from both inoculated and uninoculated samples. From SC inoculated 360 seconds isolates, *Salmonella* Pullorum, *S.* Dublin, *S.* Typhimurium and *S.* Enteritidis were identified. From SE inoculated 600s isolates, *S.*Tennessee, *S.* Abony, *S.* Newport, *S.* Bovismorbificans, *S.* Thompson, *S.* Typhimurium and *S.* Enteritidis were identified. From SE uninoculated 240 seconds isolates, *S.*Anatum, *S.* Abony, *S.* Typhimurium, *S.* Thompson and *S.* Heidelberg were identified.

The estimated D values for four *Salmonella* serotypes in poultry rendering samples containing 50% fat were determined. At 118.3ºC, SC and SE had D values of 0.67 min and 0.89 min, respectively, while SN and SD had D values of 0.85 and 0.88 min, respectively, from the TT pre-enrichment. SC and SE had D values of 0.59 min and 0.89 min, respectively, while SN and SD had D values of 0.85 and 0.88 min from the RV pre-enrichment.

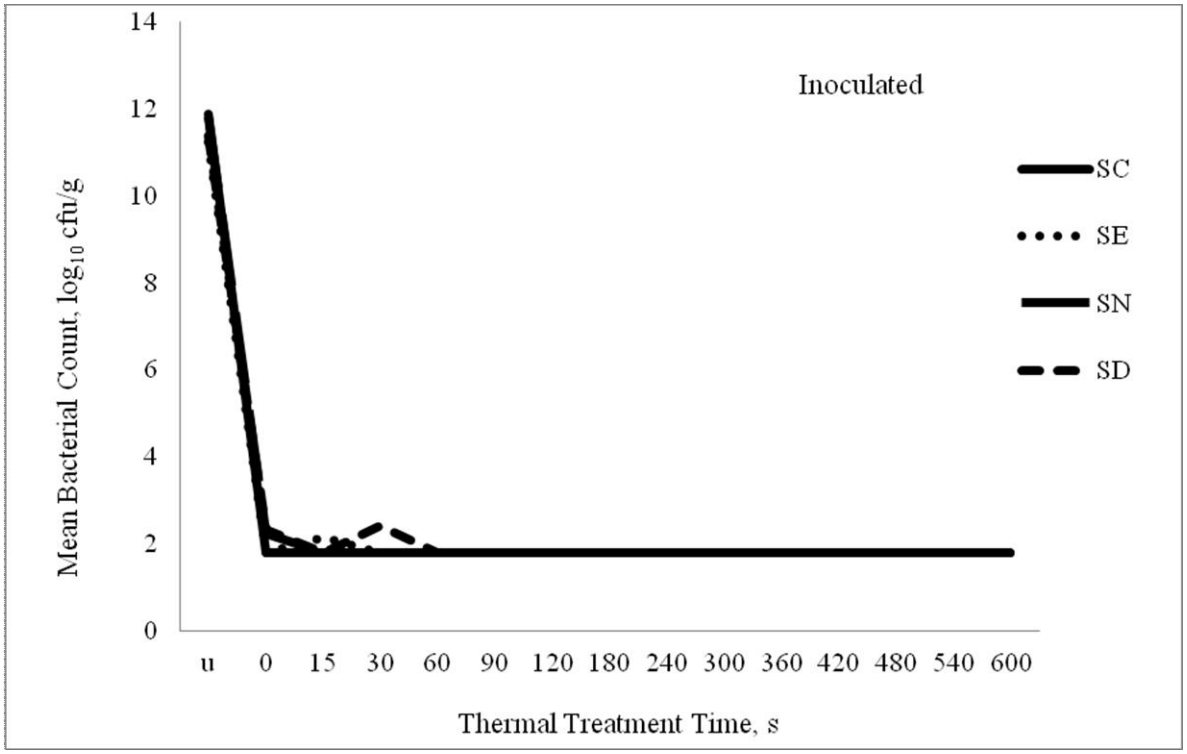

Figure 1. Enumeration of *Salmonella* on XLD from inoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD). The lower limit of detection is 1.78 $\log_{10}$ cfu/g of *Salmonella* (n=18). U means unheated control.

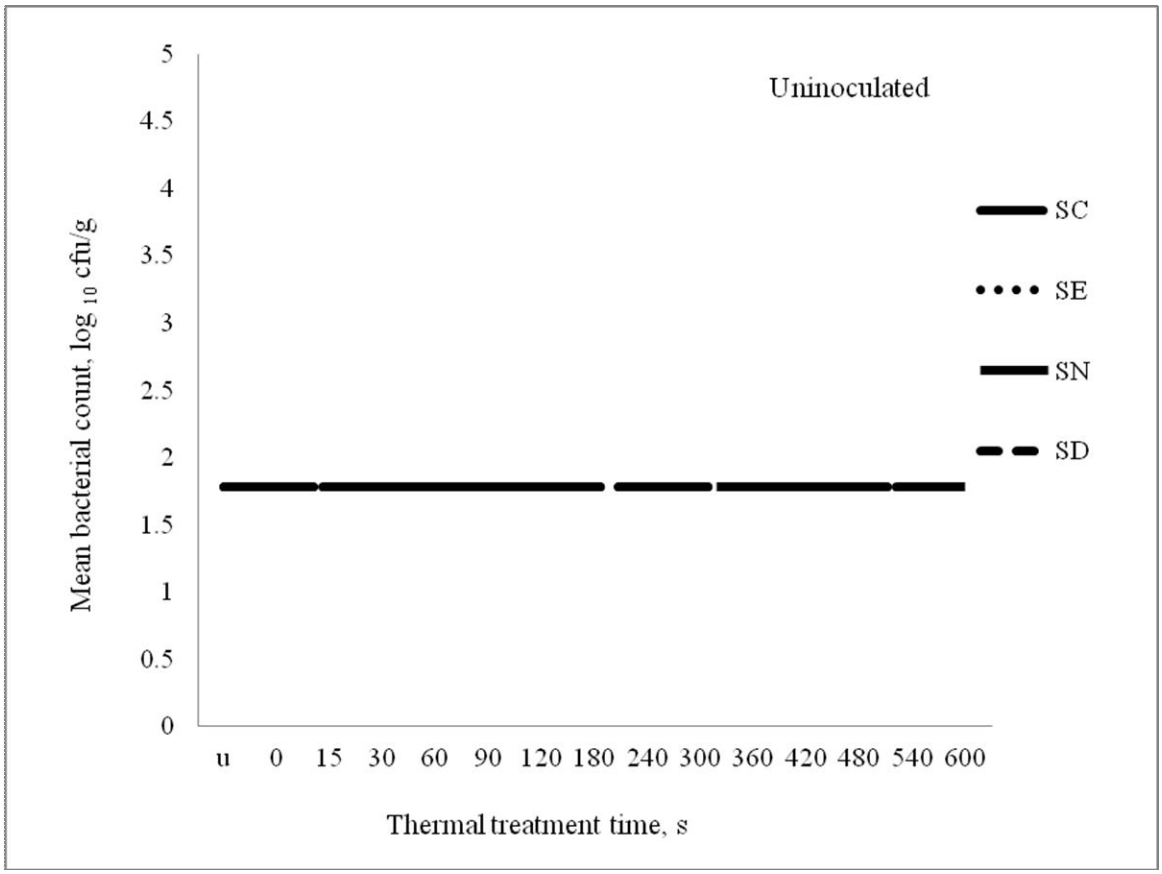

Figure 2. Enumeration of *Salmonella* on XLD from uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD). The lower limit of detection is 1.78 $\log_{10}$ cfu/g of *Salmonella* (n=18). U means unheated control.

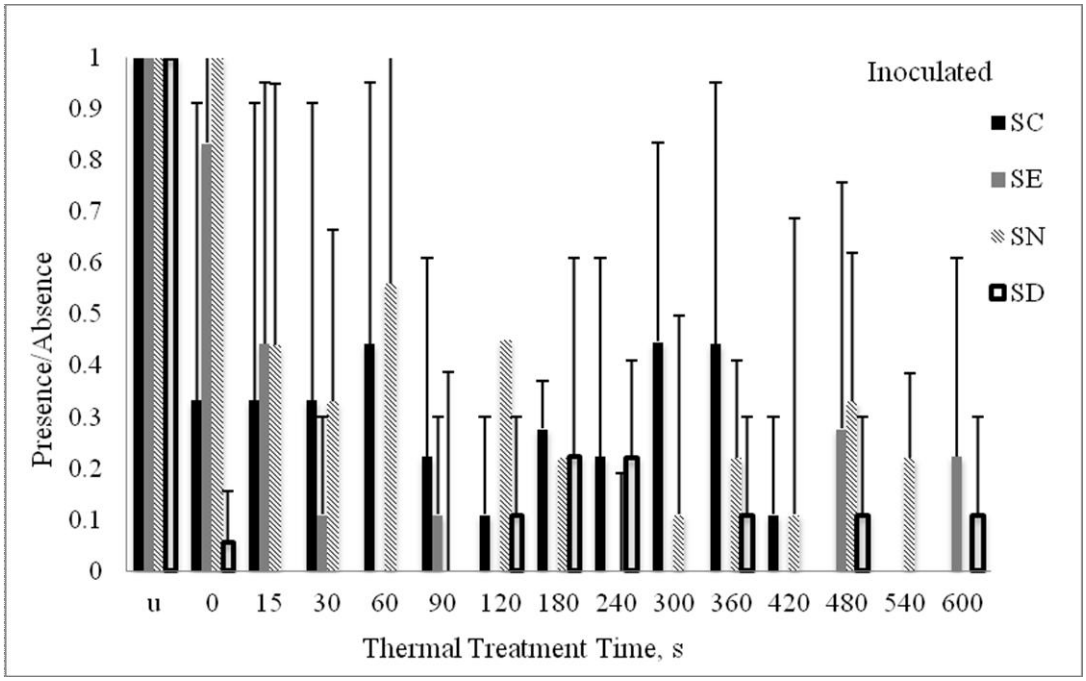

Figure 3. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, TT pre-enriched poultry rendering samples (50% fat). A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=18).

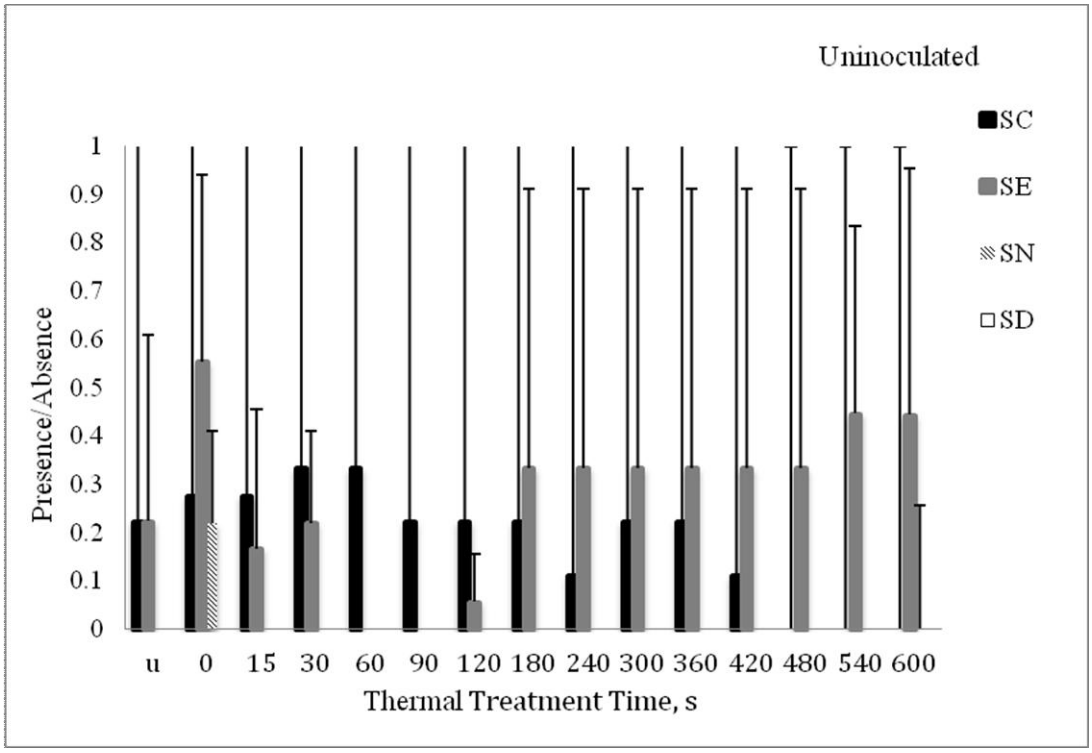

Figure 4. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) uninoculated, TT pre-enriched poultry rendering samples (50% fat). A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=18).

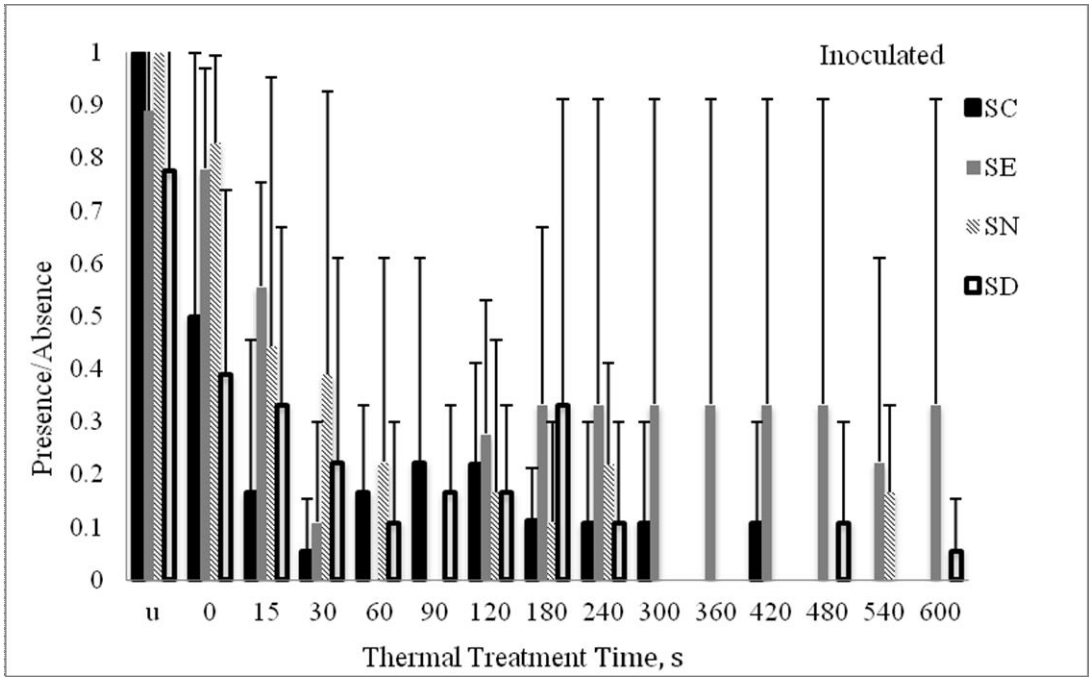

Figure 5. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, RV pre-enriched poultry rendering samples (50% fat). A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=18).

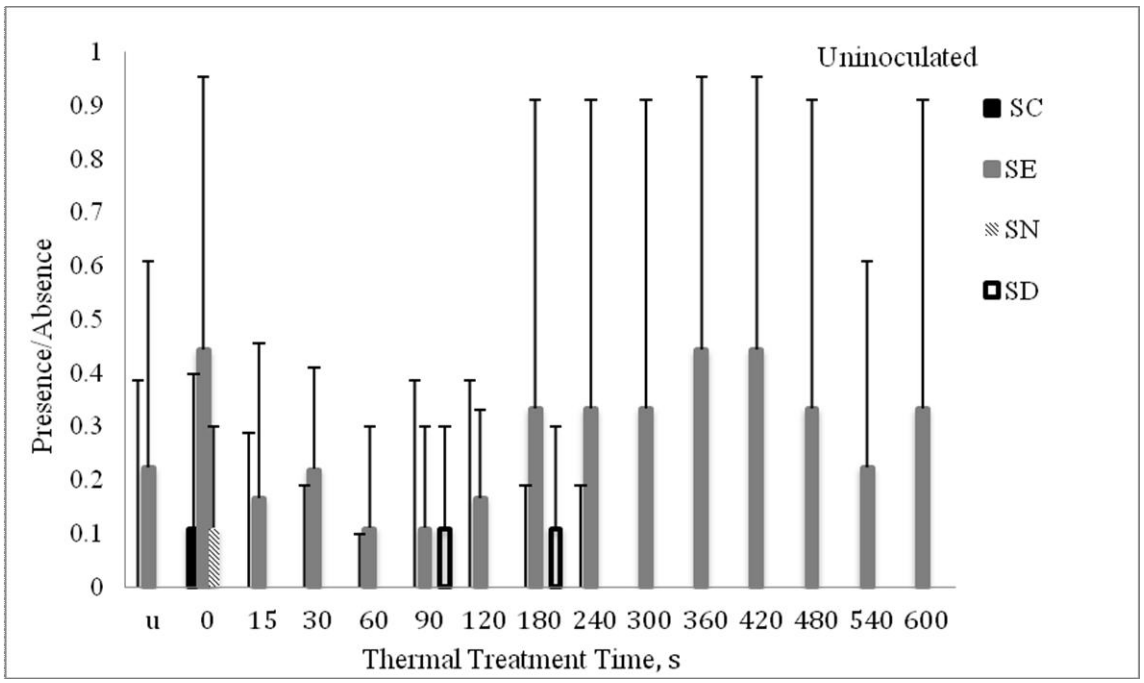

Figure 6. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) uninoculated, RV pre-enriched poultry rendering samples (50% fat). A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=18).

Table.1 Bacterial Species identified from selected *Salmonella* positive colonies in poultry samples using 16S rRNA sequencing Analysis.

Selected *Salmonella* positive colonies	Gram stain and cell morphology	16S rRNA identification (>99% top 10 identify match)
SC inoculated 360s treatment	Gram positive rod	*Salmonella* enterica strain SAA3
		Salmonella enterica strain Z-A14
		Salmonella Dublin
		Salmonella Pullorum
		Salmonella Typhimurium L-3553
		Salmonella Typhimurium VNP20009
		Salmonella Typhimurium 138736
		Salmonella Enteritidis 77-1427
		Salmonella Enteritidis EC20100325
SE inoculated 600s treatment	Gram positive rod	*Salmonella* Typhimurium VNP20009
		Salmonella Typhimurium 138736
		Salmonella Abony 0014
		Salmonella Tennessee
		Salmonella Bovismorbificans 3114
		Salmonella Typhimurium DT2
		Salmonella Thompson RM6836
		Salmonella Typhimurium DT104

Table 1 (continued). Bacterial Species Identified from selected *Salmonella* positive colonies using 16S rRNA sequencing Analysis.

		Salmonella Newport USMARC-S3124.1
		Salmonella Typhimurium DT104
		Salmonella Newport USMARC-S3124.1
		Salmonella Typhimurium 08-1736
SE uninoculated 240s treatment	Gram positive rod	*Salmonella* Typhimurium L-3553
		Salmonella Typhimurium VNP20009
		Salmonella Abony 0014
		Salmonella Anatum ATCC BAA-1592
		Salmonella Heidelberg CFSAN002064
		Salmonella Heidelberg CFSAN002069
		Salmonella Typhimurium DT2
		Salmonella Thompson RM6836
		Salmonella Newport USMARC-S3124.1
		Salmonella Typhimurium 08-1736

TDT Experiment 2: Validation of Thermal Destruction of *Salmonella* in Rendered Beef Products at 245 ºF Processing Temperature

Abstract

Four different pathogenic serotypes of *Salmonella* (*Salmonella* Choleraesuis (SC), *Salmonella* Enteritidis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD)) were used in this experiment to validate the thermal lethality of rendering processing time and temperature. In this study, 245ºF (118.3ºC) was used as the thermal cooking temperature for up to 600 second after the rendering material reached 245ºF internally. Beef crax were analyzed for fat content and then adjusted and mixed with beef tallow to have 50% fat content in the sample material. Both direct plating method and FDA BAM method with two type of per-enrichment were conducted in order to have more accurate results. Results were confirmed by using latex agglutination,

ChromAgar™ and on a select few of the isolates colony PCR technique was used to confirm the results. Each *Salmonella* serotype was conducted separately and the results indicated that each *Salmonella* serotype appeared to have unique thermal death time characteristics in the rendered products. The two types of pre-enrichment resulted in variable recoverability among different *Salmonella* serotypes. After the 600 seconds thermal processing time, SC was last detected at 600 s, SE at 600 s, SN at 540 s and SD at 600 s. Uninoculated *Salmonella* controls were also conducted, and the results showed thermally resistant strains in the background which testing indicated as *Salmonella* survived up to 600 s at 245ºF. Further thermal death time with processing temperature higher than 245ºF and processing time longer than 600 s is needed to fully validate the thermal lethality of the rendering facilities. Also the mechanisms for the thermal resistant *Salmonella* or the background bacteria that reacted as *Salmonella* should be investigated in order to control and eliminate pathogenic *Salmonella* in the animal feed ingredients.

Material and Methods

Rendering Samples Preparation

All the procedures for beef samples were conducted with the same procedure as used for the poultry samples (above). Samples of cooked beef crax and beef tallow were collected from a rendering company in the United States on three separate days. All samples were stored at -20°C until needed in experimentation. The beef crax samples were analyzed on two separate days by the Clemson University Agricultural Service Laboratory for ash, fat, and moisture content analysis. The crax and fat samples were mixed in a disinfected stainless steel food processor jar (Robot Coupe Model R2 Ultra, Ridgeland, MS) to produce 50% fat samples according to the crax fat content. The food processor jar with blade and lid were disinfected by soaking 2 minutes in Antibac B™ (0.6 g per L, Diversey Corporation, Cincinnatti, OH) dissolved in sterile distilled deionized water (ddH2O), and rinsing for 5 times with sterile ddH2O. Beef crax and fat were mixed and processed for 10 min on the pulse setting in the disinfected food processor to reduce the size particles. A sterile stainless steel spatula was used during mixing and processing to push the material down from the sides of the food processor jar. All mixed samples were stored in the sealed food processor jar under refrigeration temperature until needed for experimentation.

Several preliminary studies were conducted with beef samples for pre-enrichment selection and selective media for *Salmonella* isolation and identification as per the FDA Bacteriological Analytical Manual (BAM) (Andrews et al., 2011. Trypticase soy broth (TSB) (90000-050, VWR Scientific Products, Suwanee, GA) with the addition of 0.1% (wt/vol) yeast extract (MP Biomedicals, LLC, Solon, Ohio) was selected for growth of *Salmonella*. Each *Salmonella* serotype strain was inoculated into 5 L TSB with the addition of 0.1% (wt/vol) yeast extract for 24 h incubation at 35ºC. After incubation period, all 5 L of TSB broth were centrifuged at 7,000 x g for 7 min (GSA rotor, DuPont RC5C Sorvall Instruments Centrifuge, DuPont Company, Newtown, CT) at 4ºC in sterile centrifuge bottles (47735-696, VWR Scientific Products, Suwanee, GA). The pellet was resuspended in 5 mL sterile TSB by vortexing (Super Mixer, 1290, Labline Instruments, Inc., Melrose Park, IL) on the fast setting for approximately 5 minutes. After centriguation and resuspension, the average bacterial concentrations for each *Salmonella* serotype were be determined respectively. For *Salmonella* concentration

determination, xylose lysine deoxycholate (XLD) (90003-996, VWR Scientific Products), and trypticase soy agar (TSA) (90000-050, VWR Scientific Products) were used with the standard Class O phosphate/magnesium chloride dilution buffer (Wehr and Frank, 2004) for serial dilution. Spread plating technique was used for serial dilution on XLD and TSA agar plates, and all the plates were incubated for 24 h at 35ºC. The average concentrations of *Salmonella* cultures in broth for SC, SE, SN, and SD after 24 h incubation at 35ºC were 17.5±0.02, 10.8±0.06, 11.82±0.04, and 11.70±0.05 $\log_{10}$ cfu/g respectively. This procedure was repeated to freshly prepare *Salmonella* cultures before the thermal death time trials.

Thermal Death Time Trials

Stainless steel sample tubes (8.5 cm length, 1.6 cm outer diameter, 1.3 cm inner diameter, custom manufactured by a local company) with plastic caps (60825-801, VWR International, Suwanee, GA) were autoclaved. One gram of beef rendering crax/fat samples (50% fat content) was aseptically transferred into sterile tubes by using sterile spatulas. Four of the tubes were randomly selected as inner tube temperature controls using dial thermometers (61159-409, VWR Scientific Products, Suwanee, GA). This experiment had both heated and unheated control tubes. For the heated control ones, the capped tubes were placed in an analog dry block heater (Model #12621-108, VWR International, Suwanee, GA) equipped with Model #13259-162 heating blocks (VWR International, Suwanee, GA). The analog dry block heaters were adjusted to 118ºC for thermal trials. On average of 27 min heating time, the tubes were heated to an internal treatment temperature of 118.3ºC. Each individual culture (100 µL) was directly pipetted into 1 g of the inoculated heated rendering samples when the internal temperature was reached. After pipetting the *Salmonella* culture into the inoculated heated tubes, the sample was pipetted up and down approximately four times to thoroughly mix the rendering sample with the *Salmonella* slurry. Upon inoculation and mixing, time measurements (0, 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540 and 600s) were started as thermal treatment time. Samples were placed on ice immediately after thermal treatment. Uninoculated samples were also placed on ice after thermal treatment time was reached. For each thermal treatment time, samples were conducted in triplicate tubes to increase the accuracy of the results. Unheated control samples tubes were placed on ice until used for plating.

The wooden shaft of a sterile cotton-tipped applicator (89133-814, VWR Scientific Products, Suwanee, GA) was used to thoroughly mix the sample with UPB for approximately 30 seconds after UPB had been transferred into each tube. After evenly mixing the pre-enrichment broth with the rendered samples, 0.1 mL of the mixture was directly pipetted onto duplicate XLD and spread immediately using a bent glass rod flame-sterilized after dipping in ethyl alcohol. Several controls were also conducted, including culture controls, media controls, buffer controls, unheated uninoculated sample controls, unheated inoculated sample controls and heated uninoculated controls. The *Salmonella* stock culture was serially diluted by using the standard Class O phosphate/magnesium chloride dilution buffer and spread plating technique by pipetting either 1.0 ml or 0.1 ml of culture on XLD plates and spread with sterile glass rod for even distribution. Stainless steel tubes containing UPB pre-enrichment were incubated for 24 hr at 35ºC for injured *Salmonella* recovery. All XLD plates were incubated overnight for 24 hr at 35ºC.

In order to recover the injured *Salmonella* to obtain more accurate results, additional pre-

enrichment steps were conducted in order to have a lower detection limit than the direct plating method. Rappaport-Vassiliadis (RV) pre-enrichment broth (10 mL) (95039-382, VWR Scientific Products, Suwanee, GA) and tetrathionate pre-enrichment broth (TT) (10 mL) (90000-008, VWR Scientific Products, Suwanee, GA) were used based on FDA BAM procedures. RV and TT pre-enrichment broth were made before use and 10 ml was aseptically transferred into sterile glass tubes, respectively. Each stainless steel tube containing UPB broth and sample was vortexed on the fast setting for approximately 30 s to thoroughly mix the sample and the UPB pre-enrichment broth again. The sample was aseptically pipetted (0.1 mL) to RV pre-enrichment broth. The same sample was also aseptically pipetted (1 mL) to TT pre-enrichment broth. RV and TT media controls and also *Salmonella* culture controls in RV and TT were conducted. The samples and control broth were incubated overnight at 42ºC. After the incubation period, all the glass tubes containing either RV or TT broth were vortexed on the fast setting for approximately 30 s to thoroughly mix. An inoculation loop (3mm) of each pre-enriched sample and control was streaked onto XLD agar plates. All XLD plates were incubated overnight at 35ºC. After the incubation period, results would indicate either the presence or absence of *Salmonella* in the samples. Positive samples obtained from the RV or TT pre-enrichments were validated using two confirmation tests provided by FDA BAM (Feng 2001). Latex agglutination tests (FT0203, Thermo Fisher Scientific, Waltham, MA 02454) and ChromAgar^{TM} (90006-158, VWR Scientific Products, Suwanee, GA) were conducted to confirm the presence of *Salmonella* using each *Salmonella* culture as a control (BD Diagnostics 2008; Oxoid Limited 2008).

Bacterial Identification via Colony PCR and 16S rRNA

After latex agglutination and ChromAgar™ tests, Gram stain and bacterial identification via colony PCR and 16S rRNA were used for selected colonies testing as positive for *Salmonella*. Individual colonies were selected from plates. A small amount of an isolated bacterial colony and 50µl of Promega nuclease-free water (VWR, PAP1195, West Chester PA) were added to a sterile PCR reaction tube. Tubes were placed into boiling water for 10 min. After boiling for 10 min, 12.5µl of the boiled mixture was added to a sterile PCR reaction tube as a DNA template. After determining the concentrations and purities of nucleic acid concentration using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), 1µl of forward oligonucleotide primer (8F, 5'AGAGTTTGATCMTGGCTCAG 3', Integrated DNA Technologies, Coralville, IA), 1µl of reverse oligonucleotide primer (1492R, 5'GGYTACCTTGTTACGACTT 3‟, Integrated DNA Technologies, Coralville, IA) and 10µl of GoTaq® Green Master Mix (VWR, PAM7122, West Chester PA) were added to the PCR reaction tube. The thermal cycle program for this experiment was 94°C for 30 s, 50.6°C for 30 s, 72°C for 1 min, repeat for 30 cycles, and a final incubation at 4°C. After the thermal cycle program finished, Promega PCR Clean Up kit (VWR, PAA9281, West Chester PA) was used for PCR purification. The 16S rRNA sequencing was completed by the Clemson University Genomic Institute. Sequences data were analyzed with National Center for Biotechnology Information (NCBI) BLAST database (Altschul et al 1997).

Determination of Estimated D Values

Estimated D value calculations were based on the direct plate count and the percent recoveries of each *Salmonella* culture. These data were graphed and the slope of the line was used to calculate the estimated D value. Each *Salmonella* culture was compared on graphs, and the percent recoveries of each *Salmonella* culture from inoculated poultry samples were compared on graphs

to show the actual inoculated *Salmonella* recover rate. Latex agglutination tests and ChromAgar™ were both used as confirmation tests when the final time the population was no longer detected in each RV and TT pre-enrichment.

Results

Analysis of beef rendering materials indicated average fat content ranged from 9.6% to 12.8%, ash content was 17.3% to 29.5% and moisture content was 3.0% to 5.8%. Averaged analysis data for each pair of duplicate samples (Day 1, Day 2, Day 3) were used to prepare 50% fat materials for use in this study. The average concentrations of *Salmonella* cultures in broth for SC, SE, SN, and SD after 24 h incubation at 35ºC were 17.5±0.02, 10.8±0.06, 11.82±0.04, and 11.70±0.05 $\log_{10}$ cfu/g respectively. After inoculating *Salmonella* cultures into the rendered animal samples, the recovered *Salmonella* cultures for SC, SE, SN, and SD were 11.46±0.02 10.4±0.01, 11.54±0.02, and 11.34±0.03 $\log_{10}$ cfu/g respectively.

All *Salmonella* count results were conducted in a both direct plating method and pre-enrichment method. Direct plating method on XLD had a lower detection limit of 1.78 $\log_{10}$ cfu/g. Under the direct plating method, SC, and SD were reduced to below the lower detection limit across all thermal treatment times in inoculated beef samples. SE was detected at 0s. *S.* Newport was last present at 240 s (Fig. 7). In uninoculated beef samples, SC, SE and SN were reduced to or below the lower detection limit across all thermal treatments. SD was detected in the uninoculated unheated samples, which indicated background microorganisms (Fig. 8).

Pre-enrichment results on RV, TT combined with latex agglutination and ChromAgar ™ were recorded (Fig. 9, 10, 11, and 12). Pre-enrichment plating method on XLD had a lower detection limit of presence/absence. For inoculated samples, different *Salmonella* serotypes had different heat resistance (Fig. 9 and 11). Some inoculated samples that were reported as present had high standard errors indicating wide variability among the samples. For the uninoculated samples, there were some variations shown between each samples during thermal treatment.

Colony-PCR was performed on selected colony isolates from SN uninoculated 600 seconds. *Salmonella* positive colonies were selected from TSA agar plates after Latex agglutination and ChromAgar™ confirmation tests. Gram stain and morphological characteristics were also recorded to ensure the purity of the colony and also identify the bacterial colony. Results were submitted through the Clemson University Genomic Institute after colony PCR was performed, and data was analyzed through the BLASTn program on the NCBI website. Bacterial identity was selected from the top 10 BLAST nucleiotide database match with max identity greater than 99% (Table. 2). All the bacterial colonies from the beef rendering samples were identified as *Salmonella*. Several *Salmonella* serotypes have been listed: *Salmonella* Heidelberg, *S.* Weltevreden, *S.* Schwarzengrund, *S.* Arizonae, *S.* Thompson, *S.* Montevideo, *S.* Bredeney, *S.* Typhimurium and *S.* Enteritidis were identified.

The estimated D values for four *Salmonella* serotypes in beef rendering samples containing 50% fat were determined. At 118.3ºC, SC and SE had D values of 0.02 and 0.95 min, respectively, while SN and SD had D values of 0.86 and 0.88 min, respectively, from the TT pre-enrichment. SC and SE had D values of 0.87 min and 0.94 min, respectively, while SN and SD had D values of 0.86 and 0.44 min, respectively, from the RV pre-enrichment.

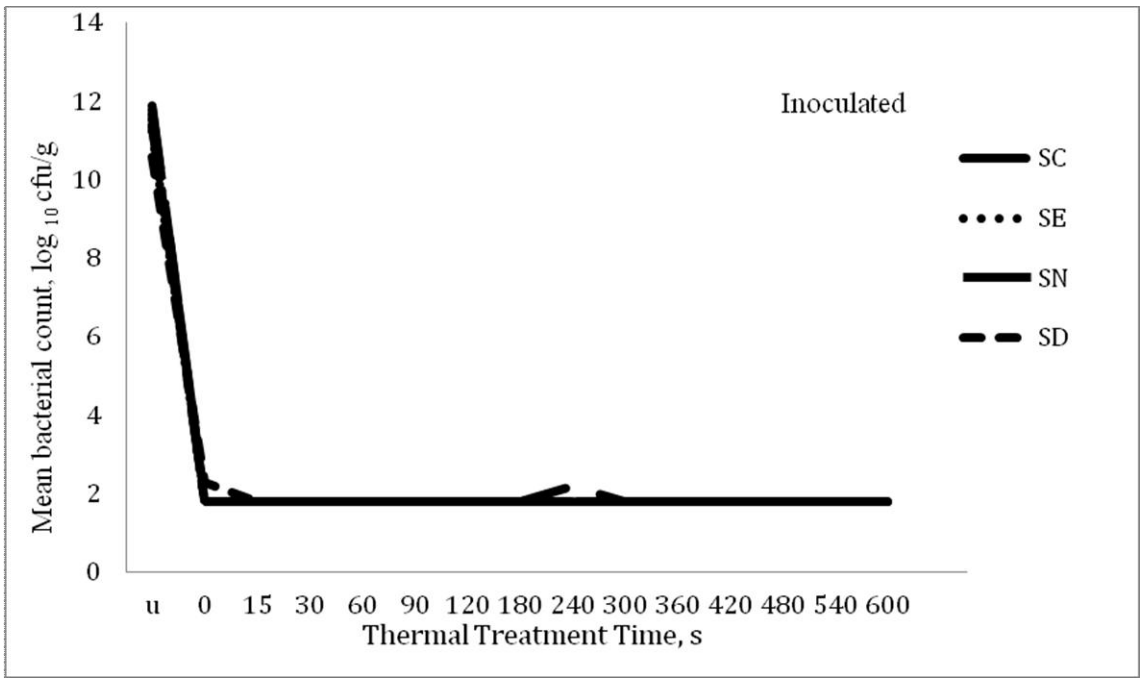

Figure.7. Enumeration of *Salmonella* on XLD from beef rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD). The lower limit of detection is 1.78 $\log_{10}$ cfu/g of *Salmonella* (n=18). U means unheated control.

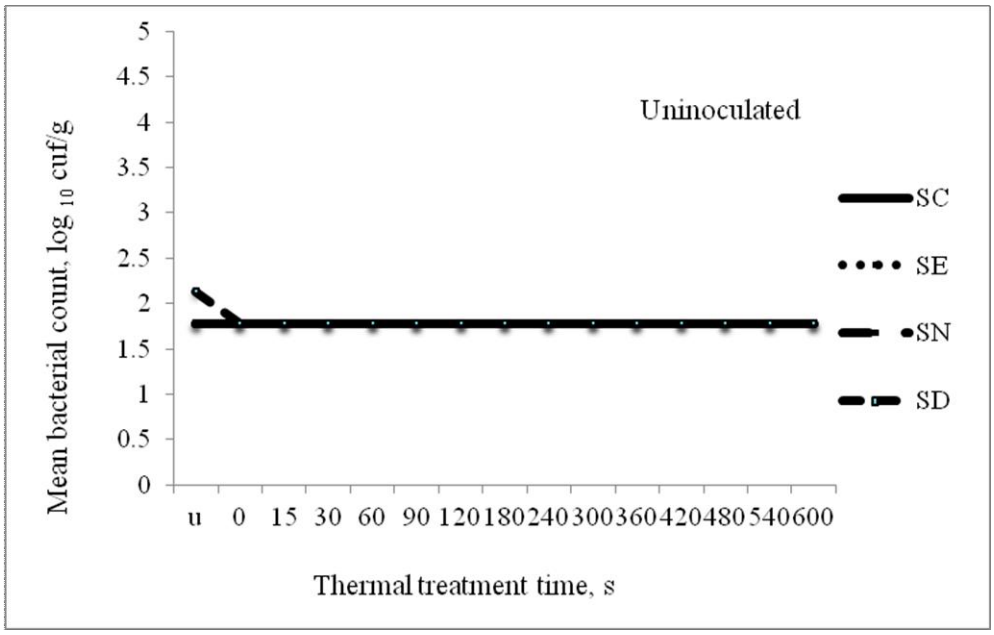

Figure 8. Enumeration of *Salmonella* on XLD from uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD). The lower limit of detection is 1.78 $\log_{10}$ cfu/g of *Salmonella* (n=18). U means unheated control.

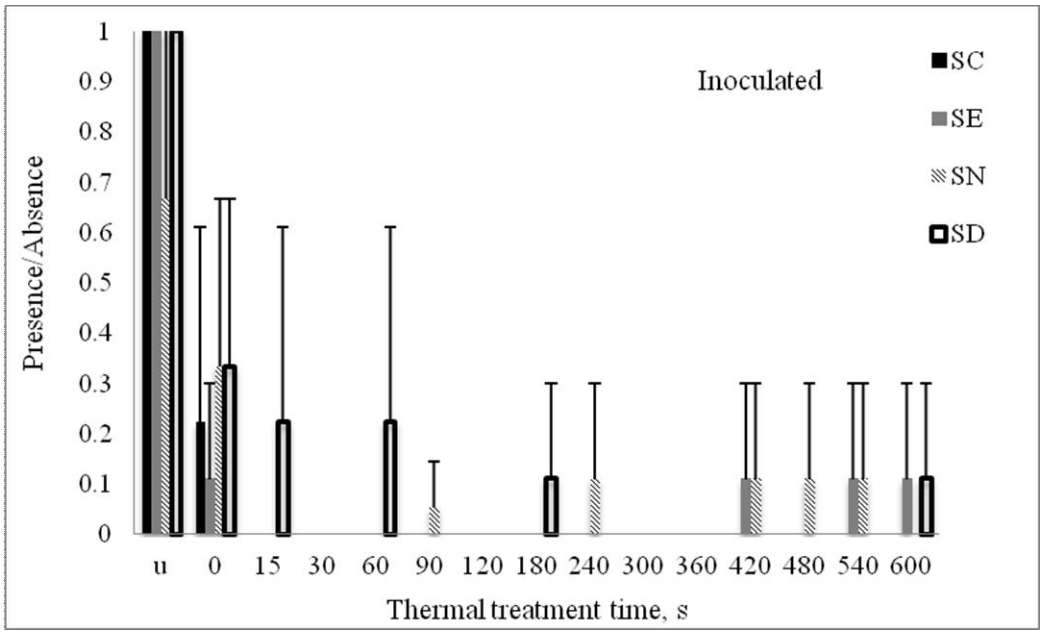

Figure 9. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, TT pre-enriched beef rendering samples (50% fat). A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=18).

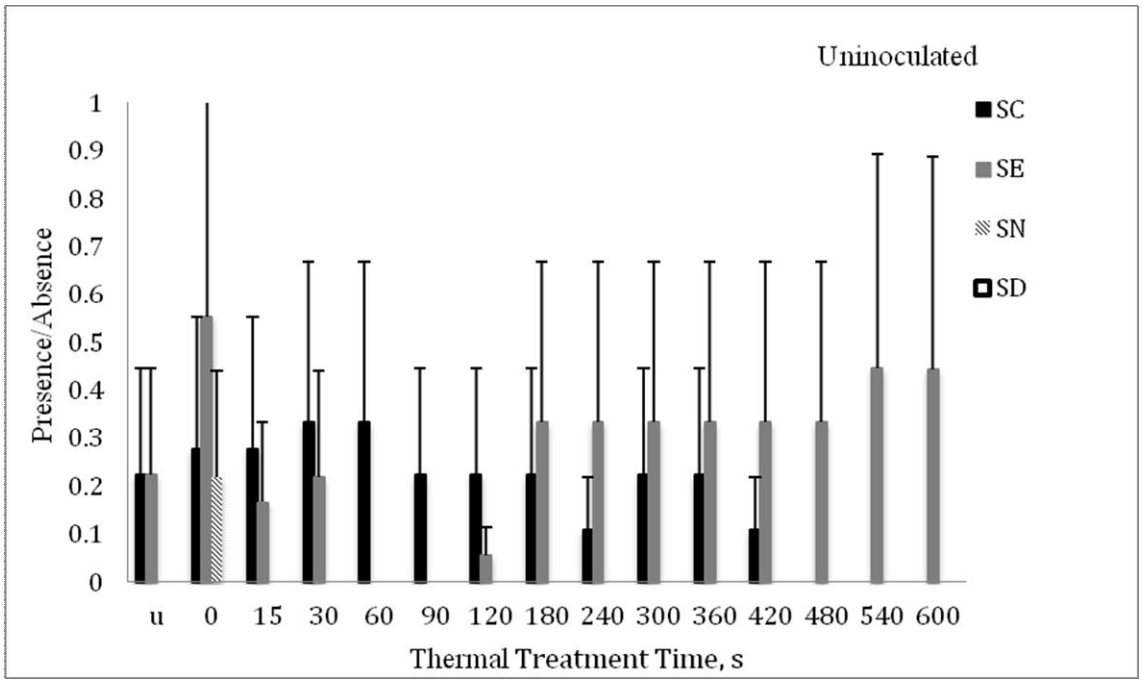

Figure 10. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) uninoculated, TT pre-enriched beef rendering samples (50% fat). A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=18).

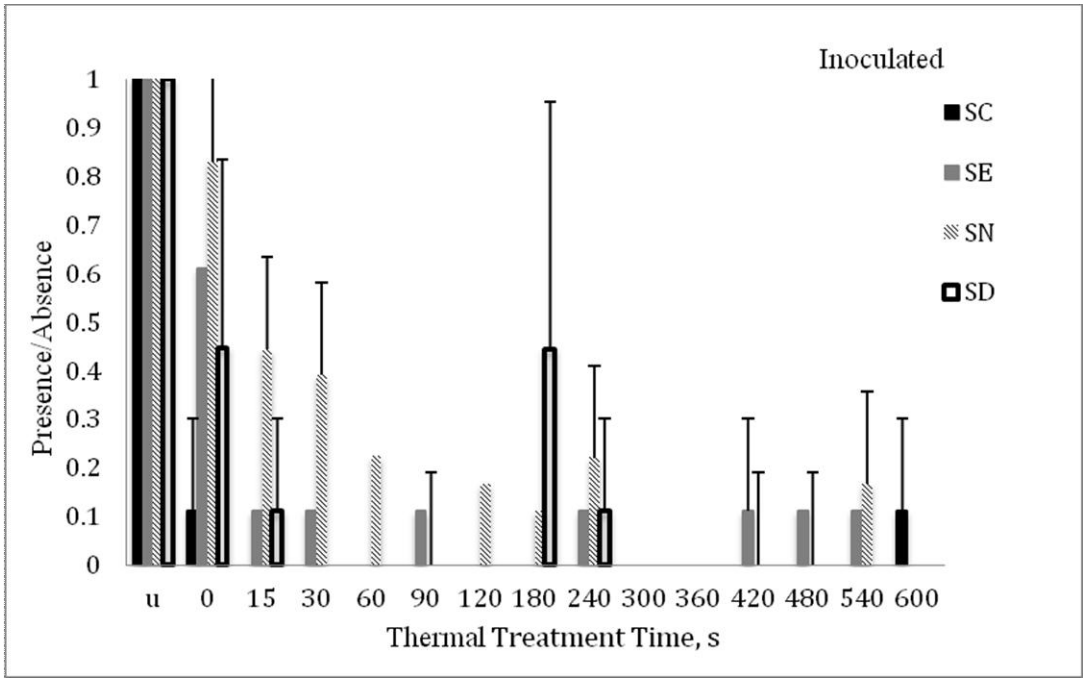

Figure 11. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, RV pre-enriched beef rendering samples (50% fat). A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=18).

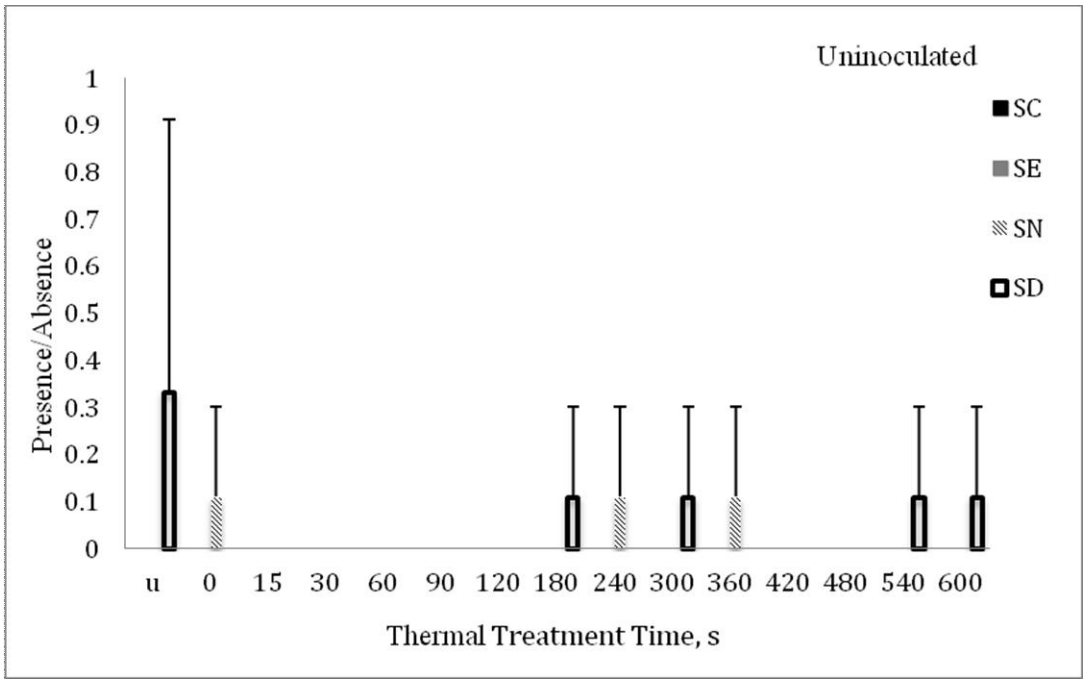

Figure 12. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) uninoculated, RV pre-enriched beef rendering samples (50% fat). A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=18).

Table 2 Bacterial Species Identified from selected *Salmonella* positive colonies in beef samples using 16S rRNA sequencing Analysis.

Selected *Salmonella* positive colonies	Gram stain and cell morphology	16S rRNA identification (>99% top 10 identify match)
SN uninoculated 600s treatment	Gram positive rod	*Salmonella* Weltevreden 2007-60-3289-1
		Salmonella Montevideo507440-20
		Salmonella Bredeney CFSAN001080
		Salmonella Montevideo SM7
		Salmonella Typhimurium CFSAN001921
		Salmonella Heidelberg ATCC 8326
		Salmonella Schwarzengrund CVM1963
		Salmonella Arizonae
		Salmonella Enteritidis E3
		Salmonella Thompson RM6836
		Salmonella Typhimurium DT104

Other Laboratory Experimentation and Standard Protocols Related to the Thermal Death Time Study:

Laboratory Clean-up/Sanitization Protocol

Purpose:
Proper clean-up and sanitization in a microbiology laboratory is essential to reduce contamination potentials and to prevent Salmonella transmission.

Materials and Methods:
During the thermal death time trials for each poultry and beef, countertops, Eppendorf pipettes (89125-294, VWR Scientific Products, Suwanee, GA; 89125-300, VWR Scientific Products, Suwanee, GA; 89125-302, VWR Scientific Products, Suwanee, GA; 89125-306, VWR Scientific Products, Suwanee, GA), vortex) Super Mixer, 1290, Labline Instruments, Inc., Melrose Park, IL), balances (Mettler PL 3000, Mettler Toledo, Columbus, OH; OHaus Navigator NV511, OHaus Corporation, Parsippany, NJ), and analog dry block heater (Model #12621-108, VWR International, Suwanee, GA) equipped with Model #13259-162 heating blocks (VWR International, Suwanee, GA) were thoroughly sanitized with Antibac B™ (Diversey Corporation, Cincinnatti, OH) dissolved in distilled deionized water (ddH2O) (minimum concentration of 0.6 g per L) and allowed to air dry. Antibac B™ is a chlorinated sanitizer with broad-spectrum microbicidal activity. The laboratory environment and equipment were frequently sanitized pre- and post- experimentation with freshly prepared Antibac B™ solutions to avoid contamination issues. The fresh sanitizing solutions were prepared at least daily or more often it the solution became diluted or visibly dirty.

Sterility Testing

Purpose:
The autoclaved stainless steel tubes were subjected to sterility testing to ensure that they were not a source of contamination during the thermal death time trials.

Materials and Methods:
Six stainless steel tubes (8.5 cm length, 1.6 cm outer diameter, 1.3 cm inner diameter; custom manufactured by a local company by boring 304 stainless steel rods) were capped (60825-801, VWR International, Suwanee, GA) and autoclaved (PP202038/D, Primus Sterilizer Co., Omaha, NE) on a 30 minute liquid cycle and allowed to cool. Sterile Universal Pre-enrichment Broth (UPB) (95021-036, VWR Scientific Products, Suwanee, GA) was aseptically pipetted (5 mL) (89130-898, VWR Scientific Products, Suwanee, GA) into each tube and allowed to incubate overnight. After the incubation period, the UPB was streaked in duplicate onto Trypticase Soy Agar (TSA) (90000-050, VWR Scientific Products, Suwanee, GA) plates to determine if any bacterial growth was present in the autoclaved tubes. Media controls were included. The plates were incubated overnight at 35°C and examined.

Results/ Conclusions:
No bacterial growth was present indicating that the autoclaved stainless steel tubes were not a source of contamination during the thermal death time trials.

Airborne Testing

Purpose:

To address the potential of contamination via aerosolized *Salmonella* in the laboratory environment, microbiological air sampling was conducted using settle plating.

Materials and Methods:

Six sterile xylose lysine deoxycholate agar (XLD) plates (90003-996, VWR Scientific Products, Suwanee, GA) were placed in various locations throughout the laboratory. XLD is a selective media commonly used for the detection of *Salmonella* in food products (Andrews et al. 2011). The lids of each of the XLD plates were carefully removed to expose the agar surfaces to the air for approximately 15 minutes. After the agar was exposed to the air, the lids of the XLD plates were carefully re-placed to avoid contamination. Media controls were included. The plates were then incubated overnight at 35°C and enumerated. This experiment was conducted three times throughout the thermal death time trials in each poultry and beef rendering materials.

Results/ Conclusions:

The results indicated *Salmonella* was not present on any of the exposed plates and therefore *Salmonella* was not aerosolized in the laboratory. These results removed aerosolized *Salmonella* as a potential source of contamination during the thermal death time trials of *Salmonella* in each poultry and beef rendering materials.

Media and Buffer Sterility Testing

Purpose:

During the thermal death time trials, numerous controls were used for each experimental trial to ensure the lack of contamination and the proper growth of bacterial cultures.

Materials and Methods:

Triplicate media controls (XLD, TSB, UPB, RV, TT, TSA) (90003-996, VWR Scientific Products, Suwanee, GA; 90000-050, VWR Scientific Products, Suwanee, GA; 95021-036, VWR Scientific Products, Suwanee, GA; 95039-382, VWR Scientific Products, Suwanee, GA; 90000-008, VWR Scientific Products, Suwanee, GA; 90000-050, VWR Scientific Products, Suwanee, GA), Class O phosphate/magnesium chloride buffer controls (BDH-0268-500g, VWR Scientific Products, Suwanee, GA; J364-100g, VWR Scientific Products, Suwanee, GA), unheated and uninoculated rendered material controls, and *Salmonella* culture controls were included in each experimental trial to ensure the lack of contamination and the proper growth of bacterial cultures (Wehr and Frank, 2004).

Results/ Conclusions:

Bacterial growth was not present on any of the media or dilution buffer controls throughout the entire study. The culture controls showed the proper growth of the *Salmonella* cultures used during the thermal death time trials. The uninoculated and unheated rendering materials (50% fat) indicated the presence of an unidentified, heat-resistant, biota present in the background of the samples. These results hold true for each replicate completed at 240°F and 245°F (replicates described below).

Using Irradiated Rendering Materials for TDT Trials

Purpose:

In attempts to determine the thermal death time of *Salmonella* species in rendering materials, each poultry and beef samples were irradiated to remove background biota.

Materials and Methods:

Rendering Sample Preparation

Samples of poultry crax and fat as well as beef crax and tallow were obtained from southeastern and southwestern rendering companies. The crax samples were submitted in duplicate to the Clemson University Agricultural Service Laboratory for ash, fat, and moisture content analysis. The crax and fat/tallow samples were re-mixed to produce 50% fat samples. A food processor bowl, blade and lid were disinfected by rinsing in Antibac B™ (Diversey Corporation, Cincinnatti, OH) dissolved in distilled deionized water (ddH2O) (0.6 g per L) for approximately 2 min, followed by rinsing 5 times with sterile ddH2O. Particle size was reduced by processing for approximately 10 min on the pulse setting in the disinfected food processor (Robot Coupe Model R2 Ultra, Ridgeland, MS) prior to conducting the experiments. A sterile stainless steel spatula (82027-532, VWR Scientific Products, Suwanee, GA) was used to scrape material from the sides during pauses in processing. Each beef and poultry rendering materials (50% fat) (150 g) was aseptically measured into two clean, baby-food glass jars. Each jar was then sent to Max Cichon of the Leach Science Center at Auburn University for irradiation treatment at 3.498 Mrads. Once irradiated, the irradiated samples were returned to the laboratory and stored in the sealed jars at room temperature until needed for experimentation.

Salmonella Preparation

Salmonella Enteritidis (USDA H4386) was obtained for this study from Dr. Vijay Jejuna of the USDA Agricultural Research Service, Microbial Food Safety Research Unit, 600 East Mermaid Lane, Room 2129, Wyndmoor, PA 19038 (FDA, 2010; FDA, 2013). *Salmonella* Enteritidis was grown in 5L of TSB (90000-050, VWR Scientific Products, Suwanee, GA) with 0.1% (wt/vol) yeast extract (0210330390, MP Biomedicals, LLC, Solon, OH) overnight and then concentrated by centrifugation. Centrifugation was conducted at 7,000 x g for 7 min (GSA rotor, DuPont RC5C Sorvall Instruments Centrifuge, DuPont Company, Newtown, CT) at 4°C in sterile centrifuge bottles (47735-696, VWR Scientific Products, Suwanee, GA). The supernatant was discarded after centrifugation. The pellet was resuspended in 5 mL sterile TSB for experimentation. The slurry of *Salmonella* Enteritidis was inoculated into each irradiated poultry and beef rendering materials at the rate of 100 µL culture per 1 g sample.

Thermal Death Time Trials

Stainless steel sample tubes (8.5 cm length, 1.6 cm outer diameter, 1.3 cm inner diameter) were custom manufactured by a local company by boring 304 stainless steel rods. The tubes (108) were capped (60825-801, VWR International, Suwanee, GA) and autoclaved (PP202038/D, Primus Sterilizer Co., Omaha, NE). Irradiated beef and irradiated poultry rendering samples (50% fat) were aseptically transferred (1 g) into sterile tubes. Twenty four of the tubes were placed in an analog dry block heater (Model#12621-108, VWR International, Suwanee, GA)

equipped with Model #13259-162 heating blocks (VWR International, Suwanee, GA) set to 115.6ºC (240ºF). Four of the tubes were randomly selected as temperature controls using dial thermometers (61159-409, VWR Scientific Products, Suwanee, GA). The tubes were heated to an internal treatment temperature of 115.6ºC prior to addition of the cultures. Each thermal trial consisted of multiple rounds consisting of twenty four tubes.

Salmonella Enteriditis (100 µL) was directly pipetted (47745-174, VWR Scientific Products, Suwanee, GA; 89125-306, VWR Scientific Products, Suwanee, GA) into 1 g of either the heated irradiated poultry rendering samples or irradiated beef samples. After culture inoculation, the sample was pipetted up and down approximately four times to thoroughly mix. Upon inoculation and mixing, time measurements (0, 30, 60, 90, 120, 240, 360, and 480 seconds) were started on the thermal treatment. Additional sample tubes containing either the irradiated poultry or beef rendering were used for unheated controls and were placed on ice until used for plating. All samples were processed for bacterial content immediately after conclusion of heat treatments.

Since the stainless steel tubes used in this experiment would not hold 1 g of sample pre-enriched in 9 mL of UPB (95021-036, VWR Scientific Products, Suwanee, GA), a preliminary study was conducted to validate that a 1:5 ratio of sample to pre-enrichment broth was effective. Sterile UPB (5 mL) was aseptically pipetted into each tube and then the wooden shaft of a sterile cotton-tipped applicator (89133-814, VWR Scientific Products, Suwanee, GA) was used to thoroughly mix the sample for 30 s. Each UPB diluted sample (0.1 mL) was directly pipetted (47745-174, VWR Scientific Products, Suwanee, GA; 89125-306, VWR Scientific Products, Suwanee, GA) onto XLD plates and spread using an alcohol-flamed bent glass rod. As a control, the *Salmonella* slurry was serially diluted to 10^{-12} in the standard Class O phosphate/magnesium chloride dilution buffer (BDH-0268-500g, VWR Scientific Products, Suwanee, GA; J364-100g, VWR Scientific Products, Suwanee, GA) and spread plated onto XLD (90003-996, VWR Scientific Products, Suwanee, GA) and TSA (90000-050, VWR Scientific Products, Suwanee, GA) (Wehr and Frank, 2004). Unheated irradiated samples were inoculated with the *Salmonella* slurry and serially diluted to determine the recovery of *Salmonella* Enteriditis from irradiated rendering materials. Media and dilution buffer controls also were conducted. All plates were incubated overnight at 35ºC and enumerated.

The remaining UPB diluted samples in the stainless steel tubes were incubated overnight at 35ºC and then vortexed (Super Mixer, 1290, Labline Instruments, Inc., Melrose Park, IL) on the fast setting for approximately 30 s. Each sample (0.1 mL) was aseptically pipetted into a sterile, capped, glass tubes containing Rappaport-Vassiliadis (RV) pre-enrichment broth (10 mL) (95039-382, VWR Scientific Products, Suwanee, GA). Also, each of the same samples (1 mL) was aseptically pipetted into sterile, capped, glass tubes containing tetrathionate broth (TT) (10 mL) (90000-008, VWR Scientific Products, Suwanee, GA). Controls included bacterial slurry and sterile media. The samples and controls were incubated overnight at 42ºC. A 3 mm inoculation loop (8009-788, VWR Scientific Products, Suwanee, GA) of each pre-enriched sample and control was streaked onto XLD. All plates were incubated overnight at 35ºC. Results indicated the presence or absence of *Salmonella* in the samples. In order to report the data, when duplicate results from the pre-enriched samples were both negative the data was described as 0.0. If one duplicate was positive and one was negative, it was reported as 0.5. If both duplicates were positive, it was reported as 1.0.

Latex agglutination tests (FT0203**,** Thermo Fisher Scientific, Waltham, MA 02454) were conducted on any positive sample from the RV and TT pre-enrichments using the *Salmonella* Enteriditis culture as well as a suspension of non-viable *Salmonella* preserved with 1% formalin included in the kit as controls (BD Diagnostics, 2008; Oxoid Limited, 2013).

Results / Conclusions:

Results on uninoculated irradiated samples indicated the presence of background *Salmonella* remaining in the poultry samples when measured on both TT and RV pre-enrichments (Figures 13 and 14). Pre-enrichment results on RV and TT were confirmed using latex agglutination; the following results are reported as confirmed findings. The number of samples positive for *Salmonella* in each inoculated and uninoculated samples in either RV or TT was validated by a latex agglutination confirmation test.

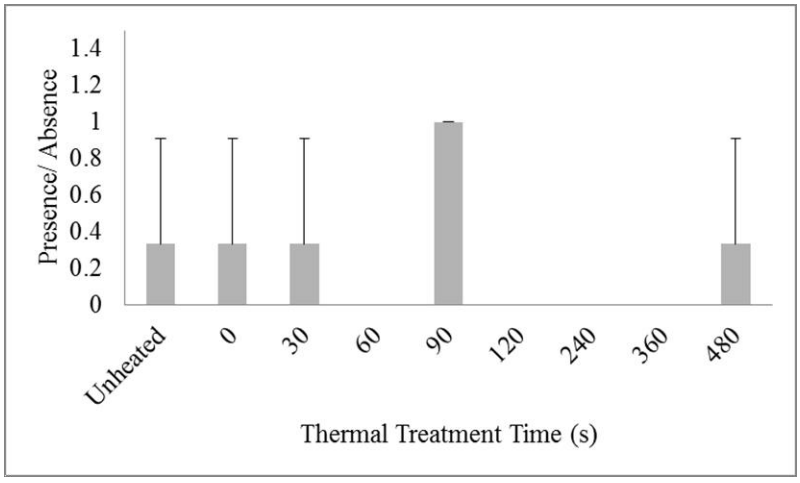

Figure 13. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Enteriditis (SE) in uninoculated, TT pre-enriched, irradiated poultry rendering samples (50% fat). [1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=6).

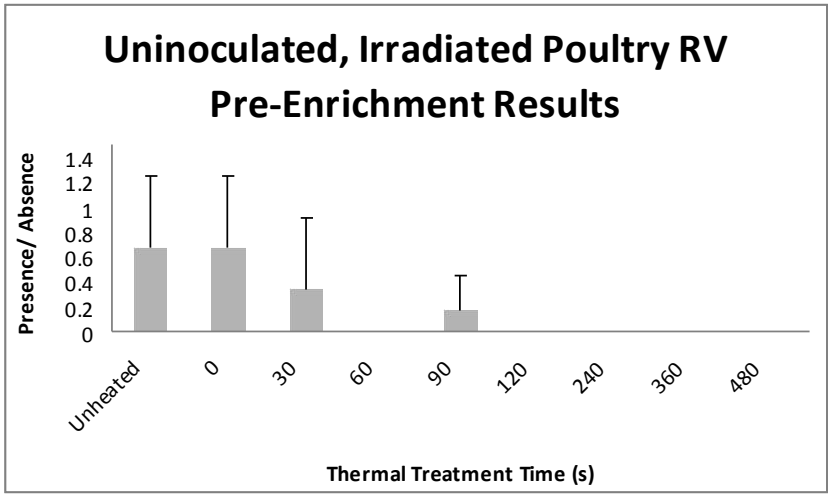

Figure 14. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Enteriditis (SE) in uninoculated, RV pre-enriched, irradiated poultry rendering samples (50% fat). [1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=6).

Results on uninoculated irradiated samples indicated the presence of background *Salmonella* remaining in the beef samples when measured on both TT pre-enrichments (Figure 15) but absence of background *Salmonella* when measured on RV pre-enrichments (Figure16).

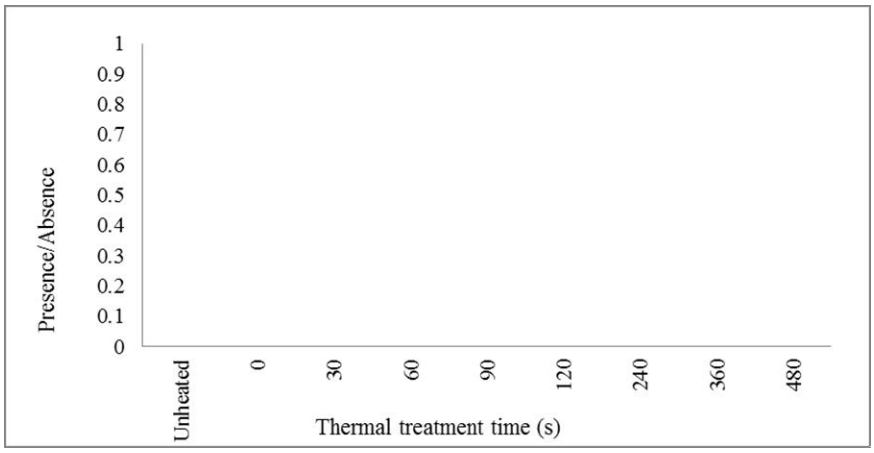

Figure 15. Presence or absence ± standard deviation of *Salmonella* in uninoculated TT pre-enriched, irradiated beef rendering samples (50% fat). [1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=6).

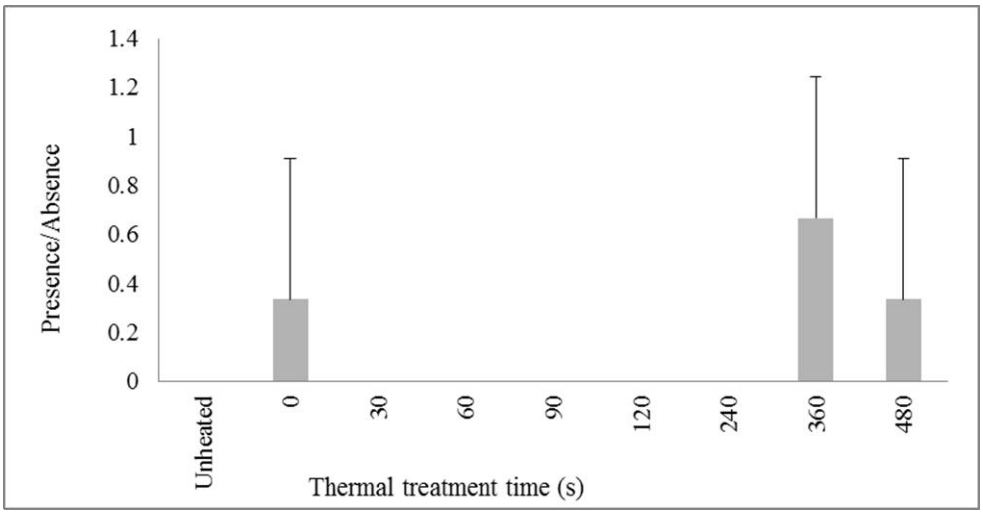

Figure 16. Presence or absence ± standard deviation of *Salmonella* in uninoculated RV pre-enriched, irradiated beef rendering samples (50% fat). [1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=6).

On direct plating on XLD immediately after the thermal trial, under all treatment conditions, *Salmonella* Enteriditis was reduced to or below the lower detection limit after initial thermal treatment (0 seconds) in inoculated, irradiated poultry samples. *Salmonella* levels were reduced to or below lower detection limit during after initial thermal treatment (0 seconds) in uninoculated poultry control samples. After inoculated with a *Salmonella* slurry of $5.30*10^{11}\pm3.54*10^{11}$ cfu/g *Salmonella* recovery levels were at an average of $1.17*10^{11}\pm5.8*10^{8}$ cfu/g in all unheated and inoculated, irradiated poultry samples.

Salmonella was detected at 30 and 90 seconds RV pre-enrichments for SE inoculated poultry samples. In RV, *Salmonella* levels in the uninoculated, poultry controls for SE were detected at 0, 30, and 90 s as well as in the unheated control (Figure 17). Although populations were reduced, *Salmonella* was present at 0, 30, and 120 seconds in TT pre-enriched, SE inoculated, poultry samples. *Salmonella* was also detected at 0, 30, 90, and 480 seconds in TT pre-enrichments for SE uninoculated, poultry samples but was not detected at 60, 120, 240, and 360 seconds as well as the unheated control (Figure 18). Results for the same treatments in beef rendering samples are shown in Figures 19 and 20).

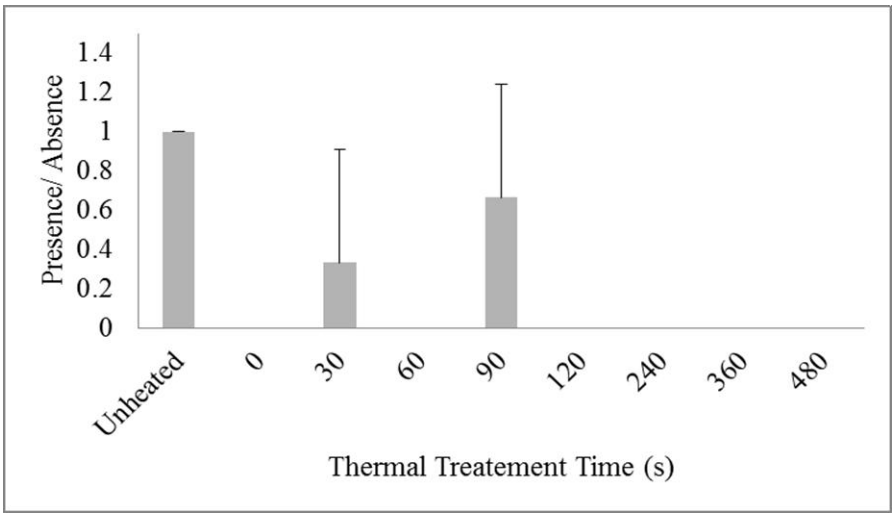

Figure 17. Presence or absence ± standard deviation of *Salmonella* Enteriditis (SE) in inoculated, RV pre-enriched, irradiated poultry rendering samples (50% fat).[1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=6).

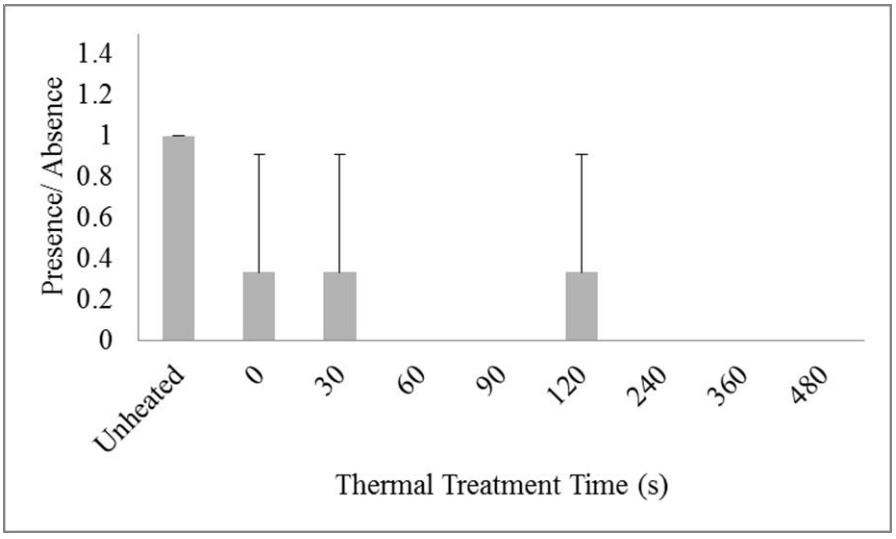

Figure 18. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Enteriditis (SE) in inoculated, TT pre-enriched, irradiated poultry rendering samples (50% fat). [1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=6).

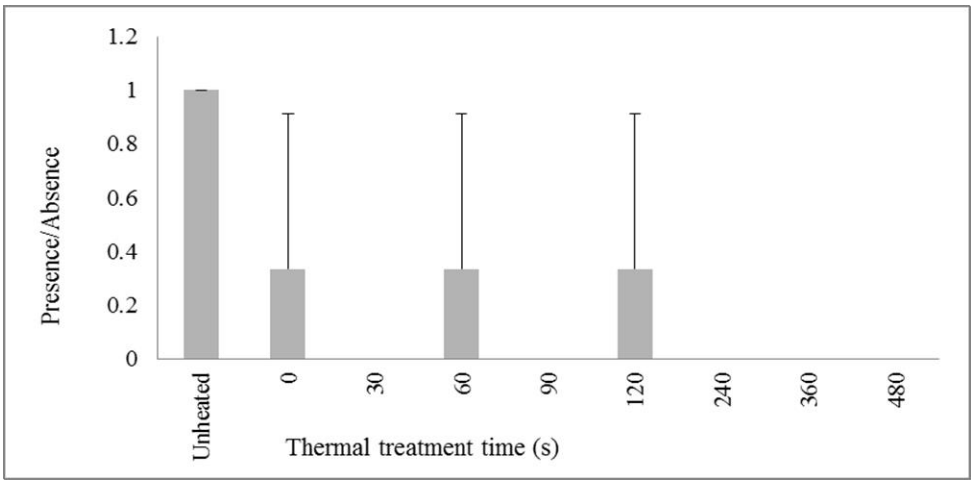

Figure 19. Presence or absence ± standard deviation of *Salmonella* Enteriditis (SE) in inoculated RV pre-enriched, irradiated beef rendering samples (50% fat). [1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=6).

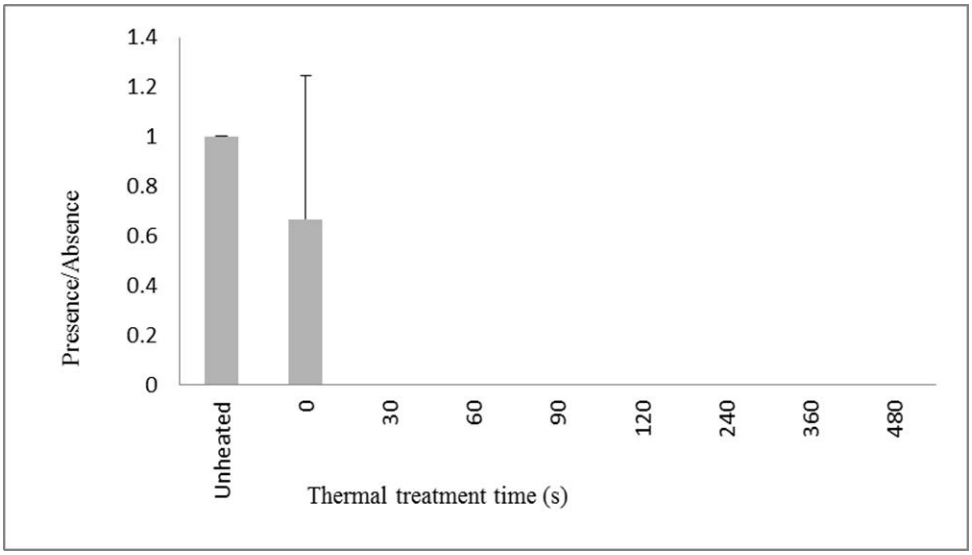

Figure 20. Presence or absence ± standard deviation of *Salmonella* Enteriditis (SE) in inoculated TT pre-enriched, irradiated beef rendering samples (50% fat). [1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=6).

The presence of bacteria after irradiation in the uninoculated poultry rendering samples on XLD indicated *Salmonella*-like resistant bacteria in the background of the samples. Additionally, these irradiation resistant bacteria also appear to be heat resistant indicated by the presence of bacteria after 480 s of thermal treatment at 115.6ºC (240º) on XLD in irradiated poultry samples.

After inoculated with a *Salmonella* slurry of 12.72 ±0.05 $\log_{10}$ cfu/g, *Salmonella* recovery levels were at an average of 11.38 ±0.03 $\log_{10}$ cfu/g in all unheated and inoculated, irradiated beef samples.

All *Salmonella* count results were conducted in a both direct plating method and pre-enrichment method. Under the direct plating method, SE was reduced to below the lower detection limit across all thermal treatment times in inoculated and uninoculated beef samples. Pre-enrichment results on RV, TT combined with latex agglutination results were recorded.

Among the inoculated samples, *Salmonella* positive was present in SE inoculated samples at 0 seconds in TT pre-enrichment, there was no positive *Salmonella* from 0 seconds up to 480 seconds. For RV pre-enrichment, *Salmonella* positive was present at 0, 60 and 120 seconds. For the SE uninoculated samples, there was no *Salmonella* positive present in TT pre-enrichment, however, positive *Salmonella* were presenting at 0, 360 and 480 seconds in RV pre-enrichment. The results indicated that there was some variation between RV and TT recovery rate. *Salmonella* was presented in both uninoculated and inoculated samples. For the uninoculated samples, *Salmonella* from the background microflora was survived during the thermal treatment of up to 480 seconds. The presence of bacteria after irradiation in the uninoculated rendering samples on XLD indicated the presence of *Salmonella*-like, irradiation resistant bacteria in the background of the beef samples. Additionally, these irradiation resistant bacteria also appear to be heat resistant indicated by the presence of bacteria after 480 seconds of thermal treatment at 115.6ºC (240ºF) on XLD.

The presence of an irradiation and heat resistant microbes reacting as *Salmonella* in the rendering samples has been noted in this study. The rendering process recycles inedible animal tissue to produce products that can be used in animal feed. Therefore, it is hypothesized that an unknown, *Salmonella*-like bacterial strain(s) may have acquired thermal and irradiation resistance through the repeating cycle of animal feed to animals to rendering and back to animal feed. The inherent microorganisms of animal digestive tracts would be included in this cycle as well, thereby, creating conditions that could select for resistant microbes. This hypothesis has not been confirmed.

Further Study to Identify the Heat Resistant Background Biota by 16s rRNA Genetic Analysis

Purpose:

Salmonella or *Salmonella*-like organisms were identified from both beef and poultry rendering samples (50% fat). In order to identify the strains isolated during the thermal death time trials at 240°F and 245°F, 16s rRNA genetic analysis was conducted.

Materials and Methods:

Bacterial Identification via Colony PCR and 16S rRNA

Individual colonies indicated as *Salmonella* species were selected from xylose lysine deoxycholate agar (XLD) (90003-996, VWR Scientific Products, Suwanee, GA) plates and streaked for isolation on trypticase soy agar (TSA) plates. The Gram reactions and cell morphologies were recorded. For bacterial identification to be conducted via colony polymerase reaction (PCR), a 3 mm inoculation loopful (50815-028, VWR Scientific Products, Suwanee, GA, USA) of each isolated bacterial colony was aseptically to a sterile PCR reaction tube (20170-012, VWR Scientific Products, Suwanee, GA, USA) along with 50 μL of Promega nuclease-free water (PAP1195, VWR Scientific Products, Suwanee, GA, USA). Each tube was placed into boiling water for 10 min and then placed into a microcentrifuge (5415, Eppendorf®, Hamburg, Germany) for 1 min at 16,000 x g. The supernatant of each boiled mixture was collected and the nucleic concentration was measured using a NanoDrop 2000 spectrophotometer (ND-2000, Thermo Fischer Scientific, Pittsburgh, PA, USA). The nucleic acid concentration of each supernatent was diluted to approximately 10 ng/ ul with sterile, nuclease-free water.

A 12.5 μL sample of each diluted supernatant was used as a DNA template and was added to individual sterile PCR reaction tubes on ice. One μL of 10μM forward oligonucleotide ready-made primer (8F, 5_AGAGTTTGATCMTGGCTCAG 3_, Integrated DNA Technologies, Coralville, IA, USA), 1 μL of 10μM reverse oligonucleotide ready-made primer (1492R, 5_GGYTACCTTGTTACGACTT 3_, Integrated DNA Technologies, , Coralville, IA, USA.) and 10.5 μL of GoTaq® Green Master Mix (PAM7122, VWR Scientific Products, Suwanee, GA, USA) were added to each PCR reaction tube on ice (Hayes et al., 2012).

Each PCR reaction tube containing the DNA template, primers, and GoTaq® Green Master Mix was placed in a thermocycler (iCycler iQ, BioRad Laboratories, Inc., Richmond, CA). The thermal cycle program consisted of 1 cycle of 95°C for 2 min, followed by 30 repeating cycles of 94°C for 30 s, 50.6°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. The cycling program ended by by holding the tubes at 4°C until removal from the thermocycler (Hayes et al., 2012; GoTaq® Green Master Mix Usage Information, 2012). Prior to sequencing, the concentration and 260:280 ratio of the PCR-amplified products was measured using a NanoDrop 2000 spectrophotometer (ND-2000, Thermo Fischer Scientific, Pittsburgh, PA, USA). The PCR-amplified products were observed by gel electrophoresis in 1.5% agarose gels. Ten µl of each PCR product and the HyLadder™ molecular mass marker (Denville Scientific Inc., CB4225-2, Metuchen, NJ) was examined using agarose gel electrophoresis with subsequent ethidium bromide staining (97064-970, VWR Scientific Products, Suwanee, GA). The amplified DNA fragments were visualized by UV illumination.

The PCR purification was accomplished using a Promega PCR Clean Up kit (VWR, PAA9281, West Chester PA). The 16S rRNA sequencing was completed by the Clemson University Genomic Institute. Sequences were analyzed with National Center for Biotechnology Information (NCBI) BLAST nucleotide database (Altschul et al., 1997). The top 10 BLAST nucleotide database results with max identity greater than 99% to indicate possible identity of the

bacterial isolates analyzed. Gram reaction, cellular morphological, and source of isolate are also listed.

Results and Discussion:

The presence of an irradiation and heat resistant microbes reacting as *Salmonella* in the rendering samples has been noted in this study as per FDA BAM *Salmonella* identification procedures. The top 10 BLAST nucleotide database results with max identity greater than 99% to indicate possible identity of the bacterial isolates analyzed (Table 3). Gram reaction, cellular morphological, and source of isolate are also listed. Gram staining is a method to learn more about the nature of the bacterial cell wall. On a Gram negative bacterium, the cell wall is thin whereas on a Gram positive bacterium, the cell wall is thicker. The action of the stain will distinguish between Gram positives and Gram negatives based on cell wall composition.

In this study, upon Gram staining, some of the isolates appeared to be Gram positive whereas the other results indicated the isolates were *Salmonella* which should be Gram negative. Maisnier-Patinm and Richard (1996) noted cell wall thickening in antibiotic resistant variants of Gram negative *Listeria monocytogenes* which made the Gram reaction appear as postive. This phenomenon of cell wall thickening may account for the Gram positive isolates that identified as Salmonella species.

Table 3. PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
1	G+ Rod	Poultry –I (SC)- 360s – 245F	Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica strain Z-A14 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Dublin genome assembly SC50_1,chromosome : I	99
			Salmonella enterica subsp. enterica serovar Pullorum genome assembly S44987_1,chromosome : I	99
			Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	99
			Salmonella enterica subsp. enterica serovar Enteritidis strain CICC 21513 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Enteritidis str. 77-1427, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete genome	99
			Salmonella enterica subsp. enterica serovar Enteritidis str. EC20100325 genome	99

Key

I = Inoculated
U = Uninoculated
SC= Salmonella Choleraesuis
SD = Salmonella Dublin
SE= Salmonella Enteriditis
SN= Salmonella Newport
Source= Poultry or Beef Trial
Serotype Used= Salmonella serotype used during thermal trial
Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
2	G+ Rod	Poultry – I (SE) – 600s – 245F	Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	100
			Salmonella enterica subsp. enterica serovar Abony str. 0014, complete genome	100
			Salmonella enterica subsp. enterica serovar Tennessee str. TXSC_TXSC08-19, complete genome	100
			Salmonella enterica subsp. enterica serovar Bovismorbificans str. 3114 complete Genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium str. DT2, complete genome	100
			Salmonella enterica subsp. enterica serovar Thompson str. RM6836, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome	100
			Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1, complete Genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium str. 08-1736, complete Genome	100

Key

I = Inoculated
U = Uninoculated
SC= Salmonella Choleraesuis
SD = Salmonella Dublin
SE= Salmonella Enteriditis
SN= Salmonella Newport
Source= Poultry or Beef Trial
Serotype Used= Salmonella serotype used during thermal trial
Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
3	G+ Rod	Beef – U (SN) – 600s – 245F	Salmonella enterica subsp. enterica serovar Weltevreden str. 2007-60-3289-1 complete genome, contig 51	99
			Salmonella sp. 'group B' 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Bredeney str. CFSAN001080, complete Genome	99
			Salmonella enterica subsp. enterica serovar Montevideo str. 507440-20, complete Genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium var. 5- str. CFSAN001921, complete genome	99
			Salmonella enterica subsp. enterica serovar Montevideo strain SM7 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Heidelberg strain ATCC 8326 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Schwarzengrund str. CVM19633, complete genome	99
			Salmonella enterica subsp. arizonae serovar 62:z4,z23:--, complete genome	99
			Salmonella enteritidis strain E3 16S ribosomal RNA gene, partial sequence	99

Key

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Source= Poultry or Beef Trial
Serotype Used= Salmonella serotype used during thermal trial
Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
4	G+ Rod	Poultry – U (SE) - 240s – 245F	Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Abony str. 0014, complete genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete genome	100
			Salmonella enterica subsp. enterica serovar Heidelberg str. CFSAN002064, complete genome	100
			Salmonella enterica subsp. enterica serovar Heidelberg str. CFSAN002069, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium str. DT2, complete genome	100
			Salmonella enterica subsp. enterica serovar Thompson str. RM6836, complete genome	100
			Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium str. 08-1736, complete Genome	100

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SN= Salmonella Newport

Source= Poultry or Beef Trial

Serotype Used= Salmonella serotype used during thermal trial

Times= Time exposed to temperature after come-up

Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
C	G- Rod	Poultry-U (SN) - 15 min – 240F	Salmonella enterica subsp. enterica serovar Enteritidis str. EC20110356, complete Genome	99
			Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67, complete Genome	99
			Salmonella enterica subsp. enterica serovar Typhi strain BHUST29 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Typhi strain BHUST13 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica strain Z-A14 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Dublin genome assembly SC50_1 ,chromosome : I	99
			Salmonella enterica subsp. enterica serovar Pullorum genome assembly S44987_1 ,chromosome : I	99
			Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete Genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete Genome	99

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Serotype Used= Salmonella serotype used during thermal trial
Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
L	G- Rod	Irradiated Poultry -U (SE) - 480 s – 240F	Typhi strain BHUST29 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Typhi strain BHUST13 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica strain Z-A14 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Dublin genome assembly SC50_1 ,chromosome : I	99
			Salmonella enterica subsp. enterica serovar Pullorum genome assembly S44987_1 ,chromosome : I	99
			Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	99
			Salmonella enterica subsp. enterica serovar Enteritidis strain CICC 21513 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica strain CICC 21482 16S ribosomal RNA gene, partial sequence	99

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Times= Time exposed to temperature after come-up

Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
M	G- Rod	Irradiated Poultry -U (SE)- 0 s – 240F	Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	99
			Salmonella enterica subsp. enterica serovar Abony str. 0014, complete genome	99
			Salmonella enterica subsp. enterica serovar Heidelberg str. CFSAN002064, complete genome	99
			Salmonella enterica subsp. enterica serovar Heidelberg str. CFSAN002069, complete genome	99
			Salmonella enterica subsp. enterica serovar Thompson str. RM6836, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome	99
			Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium str. 08-1736, complete Genome	99
			Salmonella enterica subsp. enterica Serovar Cubana str. CFSAN002050, complete Genome	99

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Serotype Used= Salmonella serotype used during thermal trial

Times= Time exposed to temperature after come-up

Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
N	G- Rod	Irradiated Poultry-U (SE)-300 s – 240F	Salmonella enterica subsp. enterica serovar Typhi strain BHUST29 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Typhi strain BHUST13 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica strain Z-A14 16S ribosomal RNA gene, partial sequence	99
			Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	99
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	99
			Salmonella enterica subsp. enterica serovar Bredeney str. CFSAN001080, complete Genome	99
			Salmonella enterica subsp. enterica serovar Abaetetuba str. ATCC 35640, complete Genome	99
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete Genome	99

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Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
P	G- Rod	Irradiated Poultry-U (SE)-90 s – 240F	Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	100
			Salmonella enterica subsp. enterica serovar Abony str. 0014, complete genome	100
			Salmonella enterica subsp. enterica serovar Tennessee str. TXSC_TXSC08-19, complete genome	100
			Salmonella enterica subsp. enterica serovar Bovismorbificans str. 3114 complete Genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium str. DT2, complete genome	100
			Salmonella enterica subsp. enterica serovar Thompson str. RM6836, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome	100
			Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1, complete genome	100
			Salmonella enterica subsp. enterica serovarTyphimurium str. 08-1736, complete Genome	100

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Times= Time exposed to temperature after come-up

Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
R	G- Rod	Irradiated Poultry-I (SE)- 120 s–240F	Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome	100
			Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1, complete genome	100
			Salmonella enterica subsp. enterica Serovar Cubana str. CFSAN002050, complete Genome	100
			Salmonella enterica subsp. enterica serovar Bareilly str. CFSAN000189, complete Genome	100
			Salmonella enterica subsp. enterica serovar Newport str. SL254, complete genome	100
			Salmonella enterica subsp. enterica serovar Paratyphi B strain B7 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica subsp. enterica serovar Paratyphi B strain B1 16S ribosomal RNA gene, partial sequence	
			Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	

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Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
U	G- Rod	Irradiated Beef-U (SE)-360 s– 240F	Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome	100
			Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1, complete genome	100
			Salmonella enterica subsp. enterica Serovar Cubana str. CFSAN002050, complete Genome	100
			Salmonella enterica subsp. enterica serovar Bareilly str. CFSAN000189, completegenome	100
			Salmonella enterica subsp. enterica serovar Newport str. SL254, complete genome	100
			Salmonella enterica subsp. enterica serovar Paratyphi B strain B7 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica subsp. enterica serovar Paratyphi B strain B1 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	100

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Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
V	G- Rod	Irradiated Beef- U (SE)- 480 s – 240F	Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain ATCC 13311 16S ribosomal RNA gene, complete sequence	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	100
			Salmonella enterica subsp. enterica serovar Abony str. 0014, complete genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete Genome	100
			Salmonella enterica subsp. enterica serovar Tennessee str. TXSC_TXSC08-19, complete genome	100
			Salmonella enterica subsp. enterica serovar Bovismorbificans str. 3114 complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium str. DT2, complete genome	100
			Salmonella enterica subsp. enterica serovar Thompson str. RM6836, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome	100

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Serotype Used= Salmonella serotype used during thermal trial
Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
W	G- Rod	Irradiated Beef-I (SE)-0 s – 240F	Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica strain Z-A14 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica subsp. enterica serovar Dublin genome assembly SC50_1 ,chromosome : I	100
			Salmonella enterica subsp. enterica serovar Pullorum genome assembly S44987_1 ,chromosome : I	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. 77-1427, complete genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. CDC_2010K_0968, complete genome	100
			Salmonella enterica subsp. enterica serovar Bredeney str. CFSAN001080, complete Genome	100
			Salmonella enterica subsp. enterica serovar Abaetetuba str. ATCC 35640, complete Genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete Genome	100

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Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
X	G- Rod	Irradiated Beef- U (SE)- 0 s – 240F	Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	100
			Salmonella enterica subsp. enterica serovar Abony str. 0014, complete genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete genome	100
			Salmonella enterica subsp. enterica serovar Tennessee str. TXSC_TXSC08-19, complete genome	100
			Salmonella enterica subsp. enterica serovar Bovismorbificans str. 3114 complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium str. DT2, complete genome 1037	100
			Salmonella enterica subsp. enterica serovar Thompson str. RM6836, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome	100

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Serotype Used= Salmonella serotype used during thermal trial
Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
Y	G- Rod	Irradiated Beef-I (SE)- 120 s– 240F	Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome	100
			Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1, complete genome	100
			Salmonella enterica subsp. enterica Serovar Cubana str. CFSAN002050, complete Genome	100
			Salmonella enterica subsp. enterica serovar Bareilly str. CFSAN000189, complete Genome	100
			Salmonella enterica subsp. enterica serovar Newport str. SL254, complete genome	100
			Salmonella enterica subsp. enterica serovar Paratyphi B strain B7 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica subsp. enterica serovar Paratyphi B strain B1 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	100

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Serotype Used= Salmonella serotype used during thermal trial
Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
SE	G- Rod	Control-SE Culture – 240F (this was a pure strain of Salmonella Enteriditis to check to see if the primers were working.)	Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis strain CICC 21513 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. 77-1427, complete genome 1074	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. EC20100325 genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. CDC_2010K_0968, complete Genome	100
			Salmonella enterica subsp. enterica serovar Bredeney str. CFSAN001080, complete genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete genome	100
			Salmonella enterica subsp. enterica serovar Montevideo str. 507440-20, complete genome	100

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Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
16	G- Rod	Poultry – U (SN) 15 min-240F	Salmonella enterica subsp. enterica serovar Enteritidis strain BIM 2 16S ribosomal RNA gene, partial sequence	93
			Salmonella enterica subsp. enterica serovar Enteritidis strain BIM 6 16S ribosomal RNA gene, partial sequence	93
			Salmonella enterica subsp. enterica serovar Enteritidis strain Inspire58 16S ribosomal RNA gene, partial sequence	93
			Salmonella enterica subsp. enterica serovar Enteritidis strain Inspire57 16S ribosomal RNA gene, partial sequence	93
			Salmonella enterica subsp. enterica serovar Typhi strain BHUST29 16S ribosomal RNA gene, partial sequence	93
			Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete genome	93
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	93
			Salmonella enterica subsp. enterica serovar Enteritidis strain CICC 21513 16S ribosomal RNA gene, partial sequence	93
			Salmonella enterica subsp. enterica serovar Enteritidis str. 77-1427, complete genome	93
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete genome	93

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Serotype Used= Salmonella serotype used during thermal trial
Times= Time exposed to temperature after come-up
Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
18	G- Rod	Poultry – I(SN) 15 min-240F	Salmonella enterica subsp. enterica serovar Newlands 16S ribosomal RNA gene, partial sequence	
			Salmonella enterica subsp. enterica serovar Abony str. 0014, complete genome	80
			Salmonella enterica subsp. enterica serovar Abaetetuba str. ATCC 35640, complete Genome	80
			Salmonella enterica subsp. enterica serovar Heidelberg str. CFSAN002064, complete genome	80
			Salmonella enterica subsp. enterica serovar Heidelberg str. CFSAN002069, complete genome	80
			Salmonella sp. Sal1205 16S ribosomal RNA gene, partial sequence	80
			Salmonella enterica strain BAB-210 16S ribosomal RNA gene, partial sequence	80
			Salmonella enterica strain BAB-206 16S ribosomal RNA gene, partial sequence	80
			Salmonella enterica strain BAB-202 16S ribosomal RNA gene, partial sequence	80
			Salmonella enterica subsp. enterica serovar Newport str. USMARC-S3124.1, complete genome	80

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Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
5	G-Rod	Poultry – I(SN) 90 min-240F	Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis strain CICC 21513 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. 77-1427, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. EC20100325 genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. CDC_2010K_0968, complete genome	100
			Salmonella enterica subsp. enterica serovar Abony str. 0014, complete genome	100
			Salmonella enterica subsp. enterica serovar Bredeney str. CFSAN001080, complete Genome	100
			Salmonella enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete genome	100

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Temp = temperature

Table 3 (continued). PCR Results for putative *Salmonella* isolates derived from rendering materials.

Sequence Name	Gram Stain/ Morphology	Source I or U (Serotype Used) - Time -Temp	Top 10 Results for Isolate (Nucleotide Database; Models Excluded)	Identity Match (%)
11	G-Rod	Poultry – U(SN) 40 min- 240F	Salmonella enterica strain SAA3 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica strain Z-A14 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica subsp. enterica serovar Dublin genome assembly SC50_1 ,chromosome : I	100
			Salmonella enterica subsp. enterica serovar Pullorum genome assembly S44987_1 ,chromosome : I	100
			Salmonella enterica subsp. enterica serovar Typhimurium str. L-3553 DNA, complete Genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain VNP20009, complete genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis strain CICC 21513 16S ribosomal RNA gene, partial sequence	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. 77-1427, complete genome	100
			Salmonella enterica subsp. enterica serovar Typhimurium strain 138736, complete Genome	100
			Salmonella enterica subsp. enterica serovar Enteritidis str. EC20100325 genome	100

Key

I = Inoculated

U = Uninoculated

SC= Salmonella Choleraesuis

SD = Salmonella Dublin

SE= Salmonella Enteriditis

SN= Salmonella Newport

Source= Poultry or Beef Trial

Serotype Used= Salmonella serotype used during thermal trial

Times= Time exposed to temperature after come-up

Temp = temperature

Evaluation of Lipolytic and Proteolytic Enzymes from Heat Resistant Background Biota

Purpose:
To determine if there are lipolytic and proteolytic enzymes produced by the heat resistant background biota.

Materials and Methods:
Five each of the heat resistant bacterial isolates collected from the thermal death time trials at 115.6°C (240°F) and 118.3°C (245°F) in each poultry and beef rendering materials (50% fat) were randomly selected from refrigeration storage.

Spirit Blue agar (61001-022, VWR Scientific Products, Suwanee, GA) prepared according to the manufacturer's instructions using lard as the lipid source. The bacterial isolates were spotted onto the agar's surface and incubated overnight at 35°C. Plates were examined for clearing zones around bacterial isolates.

Milk agar preparation begins by autoclaving 900 mL of nutrient agar and 100 mL of rehydrated non-fat milk (20 g non-fat dry milk and 100 mL of distilled, deionized water) in separate flasks. After autoclaving, the nutrient agar and re-hydrated milk were carefully and aseptically mixed and plates were poured. Once agar was solidified, the bacterial isolates were spotted onto the agar's surface and incubated overnight at 35°C. Plates were analyzed for clearing zones around the bacterial isolates.

Results/ Conclusions:
Spirit Blue agar is used to identify bacterial cultures which produce lipolytic enzymes (lipases). Milk agar is used to identify bacterial cultures which product proteolytic enzymes (proteases).

Clearing zones were present around all of the bacterial isolates on both the milk agar plates and the Spirit Blue agar, thereby, indicating the production of proteases and lipases by the unknown, heat resistant bacteria that had been isolated during the thermal death time trials at 115.6°C (240°F) and 118.3°C (245°F).

Figure 21. Spirit Blue agar (left) and Milk Agar (right) indicating lipolytic and proteolytic enzyme production by bacterial isolates identified as *Salmonella* derived from rendered materials.

Serotyping of Isolated Heat Resistant and Radiation Resistant Strains of *Salmonella*

Purpose:

Industry members/guests of the ACREC Research Committee stated that the isolated strains of *Salmonella* derived from heat-treated rendering materials and radiation-treated rendering materials could not be positively identified as *Salmonella* unless the isolates were serotyped.

Methods:

Five isolates identified as *Salmonella* by FDA BAM procedures and by 16S rRNA sequencing were submitted to the National Veterinary Services Laboratories in Ames, Iowa for serotyping. Due to cost considerations, only five of the isolates identified as *Salmonella* in our laboratory testing and 16S rRNA trials were sent for serotyping. The isolates submitted were:

Isolate 4 – Isolate from uninoculated poultry rendering materials (from post-cooker products remixed to 50% fat). Isolate survived heating for 240 seconds at 245ºF in the rendering materials.

Isolate C – Isolate from uninoculated poultry rendering materials (from post-cooker products remixed to 50% fat). Isolate heating for 900 seconds (15 minutes) at 240ºF in the rendering materials.

Isolate L – Isolate from uninoculated poultry rendering materials (from post-cooker products remixed to 50% fat). Isolate survived irradiation for 3.498 Mrads (2X the FDA sterilization dosage) and survived heating for 480 seconds at 240ºF in the rendering materials.

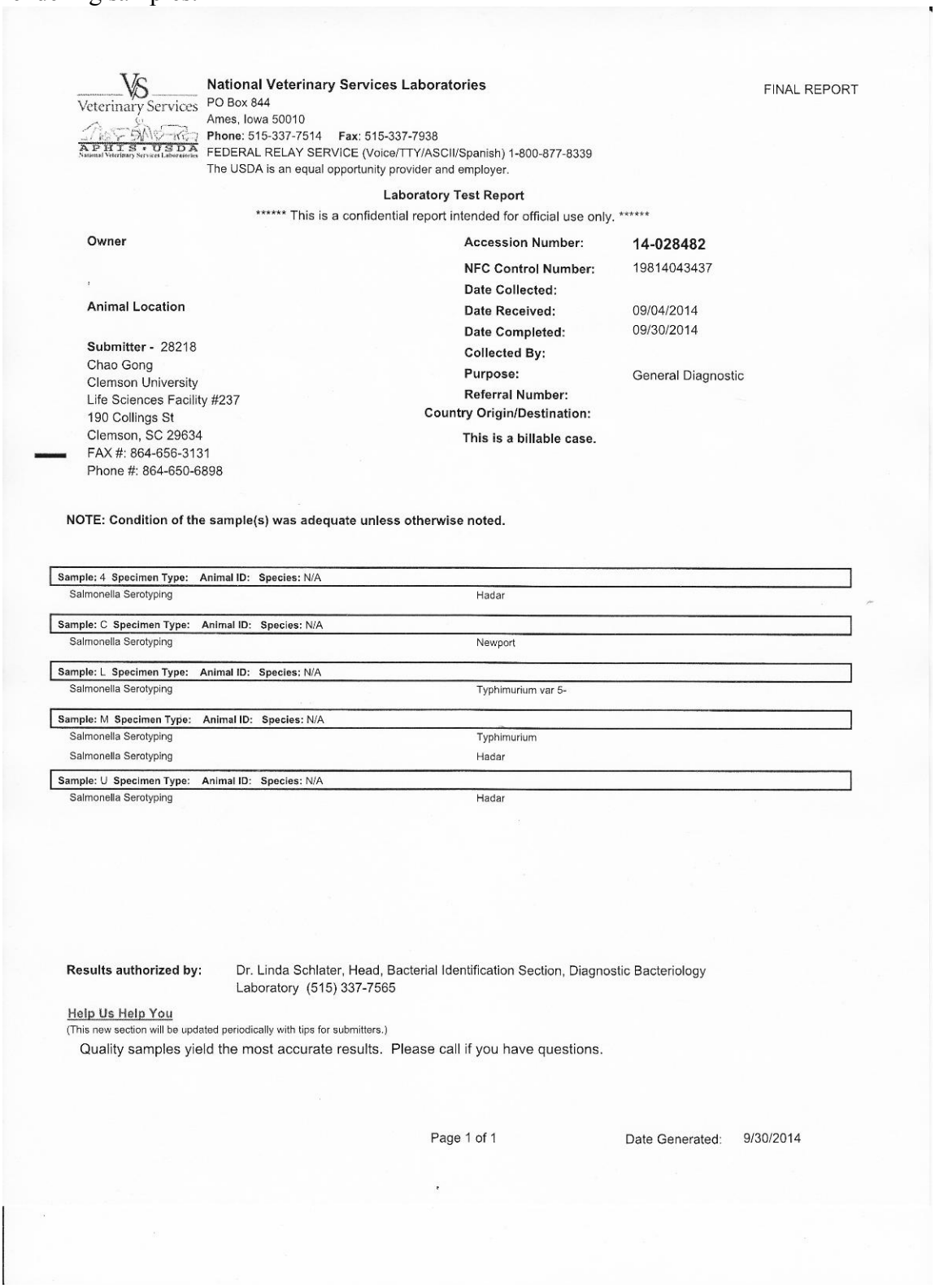
Isolate M – Isolate from uninoculated poultry rendering materials (from post-cooker products remixed to 50% fat). Isolate survived irradiation for 3.498 Mrads (2X the FDA sterilization dosage) (from post-cooker – remixed to 50% fat). Rendering sample was not further heat treated prior to isolation of this strain.

Isolate U – Isolate from uninoculated beef rendering materials (from post-cooker products remixed to 50% fat). Isolate had survived heating for 360 seconds at 240ºF and survived irradiation for 3.498 Mrads (2X the FDA sterilization dosage).

Results:

All tested samples were identified as *Salmonella* (Figure 22).

Figure 22. Results of serotyping isolates derived from heat treated and radiation treated rendering samples.



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FINAL REPORT

Laboratory Test Report

****** This is a confidential report intended for official use only. ******

Owner

Animal Location

Submitter - 28218
Chao Gong
Clemson University
Life Sciences Facility #237
190 Collings St
Clemson, SC 29634
FAX #: 864-656-3131
Phone #: 864-650-6898

Accession Number: **14-028482**
NFC Control Number: 19814043437
Date Collected:
Date Received: 09/04/2014
Date Completed: 09/30/2014
Collected By:
Purpose: General Diagnostic
Referral Number:
Country Origin/Destination:
This is a billable case.

NOTE: Condition of the sample(s) was adequate unless otherwise noted.

Sample: 4 **Specimen Type:** **Animal ID:** **Species:** N/A	
Salmonella Serotyping	Hadar
Sample: C **Specimen Type:** **Animal ID:** **Species:** N/A	
Salmonella Serotyping	Newport
Sample: L **Specimen Type:** **Animal ID:** **Species:** N/A	
Salmonella Serotyping	Typhimurium var 5-
Sample: M **Specimen Type:** **Animal ID:** **Species:** N/A	
Salmonella Serotyping	Typhimurium
Salmonella Serotyping	Hadar
Sample: U **Specimen Type:** **Animal ID:** **Species:** N/A	
Salmonella Serotyping	Hadar

Results authorized by: Dr. Linda Schlater, Head, Bacterial Identification Section, Diagnostic Bacteriology Laboratory (515) 337-7565

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Discussion:

Results of FDA BAM testing and 16S rRNA testing in our laboratory identified all of the heat-resistant and radiation-resistant isolates as *Salmonella*. Serotyping results indicated all submitted samples were *Salmonella* (serotypes *Salmonella* Newport, *Salmonella* Typhimurium, and *Salmonella* Hadar).

Salmonella Newport is on the FDA list of strains not allowed in poultry feed. *Salmonella* Typhimurium causes human illness. *Salmonella* Hadar was reported as the second most prevalent cause of foodborne illness in England and Wales in the 1980s (Rowe et al. 1980). In 2012, *Salmonella* Hadar was implicated in a multi-state foodborne illness outbreak derived from poultry in backyard flocks (http://www.cdc.gov/salmonella/hadar-live-poultry-07-12/). In the summer of 2014, more than 300 people across 23 states became ill from two of the serotypes of *Salmonella* identified in this serotyping study: *Salmonella* Hadar and *Salmonella* Typhimurium (http://www.foodsafetynews.com/2014/08/cdc-300-people-infected-with-salmonella- linked-to-contact-with-live-poultry/#.VEQQn8nSIZw).

The presence of heat-resistant and radiation-resistant strains of these organisms could pose risk to humans. Many questions remain unanswered in this study. Are the isolated heat-resistant and radiation-resistant serotypes carrying unique resistance genetics? Or are these organisms being protected within bone particles? Either way, the results still indicate that pathogenic strains of *Salmonella* are surviving the cooking process in rendered animal products.

Impacts and Significance:
Efforts to reduce or eliminate *Salmonella* in rendered animal products have been underway for more than 60 years. Studies dating back as far as the late 1940s established a correlation between contaminated animal feed and salmonellosis in animals (Hirsch and Sapiro-Hirsh 1958; Gray et al. 1958; Boyer et al. 1958, 1962; Burr 1962; Isa 1963; Jones 1982;).

A 1994 FDA CVM survey presented at the Feed Safety Committee of the United States Animal Health Association, 99th Annual Meeting in Reno, Nevada by McChesney (Li et al. 2012) indicated that animal-derived feed ingredients have higher incidence of *Salmonella* than plant-derived animal feed ingredients. However, a survey reported in 2012 (Li et al.) indicated significant progress has been made in reducing *Salmonella* in animal-derived feed ingredients. Li et al. (2012) reported that *Salmonella* was detected in animal-derived feed ingredients at the rate of 66.1% for the period of 2002 through 2006 and at the rate of 41.3% for the period of 2007 through 2012. Hayes (2013) in our laboratory reported 45.8% *Salmonella* positive samples for rendered poultry products and 18.9% *Salmonella* positive samples for rendered beef products.

The study by Li et al. (2012), as with almost all other reports on *Salmonella* in animal feed and pet food ingredients, was reported as presence/absence of *Salmonella*. Since the finding of *Salmonella* in products is of significance, further enumeration of *Salmonella* is not warranted in most studies. However, because of the need to determine population reduction, our thermal death time study enumerated the actual number of *Salmonella* in the products. Other than our current study, only one other study has been identified that attempted to determine *Salmonella* populations sizes in finished rendered animal products (Franco 2005). Franco (2005) used the most probable number technique to estimate *Salmonella* populations in rendered animal products. The most probable number (MPN) technique is a statistical approximation method that estimates the population. Franco (2005) estimated that the average monthly *Salmonella* MPN/g counts in rendered animal meals ranged from 0.2 to 78.0 MPN/g.

In humans, the infective dose for salmonellosis can be as low as 15 to 20 cells depending on strain differences of the *Salmonella* and age/health of the patient. http://www.fda.gov/food/foodborneillnesscontaminants/causesofillnessbadbugbook/ucm069966.htm In animals, the infective dose can vary depending on strain of *Salmonella* as well as host factors (Lavoie and Hinchcliff 2009; Anderson and Rings 2008). For instance, in 2 week old calves, the infective dose for pathogenic strains of *Salmonella* is approximately 10^5 cells and for older animals the infective dose required for disease manifestation is greater.

In our study, thermal treatment was conducted on added strains of *Salmonella* (*Salmonella* Choleraesuis, *Salmonella* Dublin, *Salmonella* Enteritidis and *Salmonella* Newport) inoculated into previously cooked 50% fat poultry rendering materials and into previously cooked 50% fat beef rendering materials. These strains were grown under normal conditions and not subjected to conditions which would have increased their heat tolerance. The 240°F and 245°F treatment of rendering materials with the added populations of approximately 11 to 12 log each of *Salmonella* Dublin, *Salmonella* Newport, *Salmonella* Choleraesuis and *Salmonella* Enteritidis were nearly instantaneously reduced by 9 to 10 log or more but an extremely thermally resistant bacterial population which identifies as a mixture of *Salmonella* strains remained in the rendering

materials at 0 to 2.2 log cfu *Salmonella*/g (0 to 158 cfu/g *Salmonella*) for beef and 0 to 1.78 log cfu *Salmonella*/g (0 to 60 cfu/g *Salmonella*) for poultry.

The reduction in added *Salmonella* strains during thermal processing is very encouraging in consideration of Franco's report (2005) on typical *Salmonella* population densities in rendered products but the residual resistant *Salmonella* warrant further consideration. There is no known evidence that this background biota in rendered animal products has caused illness or outbreaks in any animals or humans. This may be because the population levels are below infective dose levels or may potentially be due to defects in the *Salmonella* strains themselves. However, the biota exhibits extreme resistance beyond normal for *Salmonella*. The rendering materials were subjected to irradiation in an attempt to destroy the background *Salmonella* but the biota was even resistant to twice the FDA irradiation sterilization dosage. The population size of resistant *Salmonella* remaining in the materials is very low but the extreme thermal and radiation resistance is of concern. Many questions remain as to how these organisms are capable of resisting such rigorous treatments. Have these organisms mutated over time to afford this resistance or are the cells protected in bone or fat within the rendering matrix? We encourage the rendering industry to remain aware of this heat resistant biota and to seek further studies on ways to destroy these *Salmonella* strains. We also encourage the industry to study positive *Salmonella* samples much as Franco (2005) did, but to take the extra step of determining if the positive *Salmonella* are heat resistant strains that survived the rendering cooking process. We hypothesize the rendering process is killing most *Salmonella* but a very low level biota of resistant *Salmonella* is surviving rendering cookers via resistance genes or protection inside bone particles. If this hypothesis is true, then this information will be valuable to the industry to further understand why some positive *Salmonella* samples appear occasionally and to help the industry seek research on different ways to destroy this *Salmonella* biota. It is very likely that destruction of this resistant biota of *Salmonella* coupled with rigorous post-process contamination prevention strategies will lead to *Salmonella*-free rendered animal products. This could make these products comparable to vegetable-based animal feed ingredients in terms of low *Salmonella* incidence and will help the rendering industry remain very competitive in the animal feed ingredient markets.

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8/19/2013 to 8/18/2015
$150,000
Not Funded

Future Work: The isolated heat resistant bacterial biota should be sequenced to determine their entire genome. This will provide valuable insight as to 1) what these organisms really are, 2) what genes these organisms may have acquired including heat resistance genes, irradiation resistance genes and potentially genes from pathogenic bacteria, 3) insights into the mechanisms for this biota's extreme resistance abilities, 4) insights into potential ways to kill these organisms and break the cycle of these bacteria recycling through animal guts to feed, etc., and 5) potential

value-added uses for lipolytic and proteolytic enzymes produced by this biota. Results of this study indicated a 9 to 10 log reduction in added pathogenic serotypes of *Salmonella*. Most likely, the thermal treatment is killing all of the added bacteria. However, until the background biota is eliminated or better a way is found to distinguish background biota strains from the added cultures, there is no easy or economically feasible way to completely evaluate thermal death time in these products.

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