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VALIDATION OF THERMAL DESTRUCTION OF PATHOGENIC BACTERIA IN RENDERED ANIMAL PRODUCTS

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Lay Summary:

The thermal death of four pathogenic strains of *Salmonella* recognized by the FDA as hazardous in animal feeds (*Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD)) was not a straight line decrease. After periods of appearing to be destroyed, some cultures reappeared at later treatment times. In thermal treatments up to 420 s at 240ºF (115.6ºC), SC was last detected at 120 s, SE at 120 s, SN at 300 s and SD at 360 s in inoculated beef rendering materials. In thermal treatments up to 420 s at 240ºF (115.6ºC), SC, SE, SN, and SD were last detected at 360 s, respectively, in inoculated poultry rendering materials. Controls indicated thermally resistant strains in the background of both beef and poultry rendering materials which when tested using standard FDA Bacteriological Analytical Manual (BAM) techniques indicated *Salmonella*. Hypotheses to explain the results of this study include: 1) thermally resistant sub-particles such as bone or tissue protected bacteria from thermal treatment; 2) presence of thermally resistant species in the background of rendering samples caused false positive results on BAM procedures; or 3) presence of thermally resistant *Salmonella*. Further research will need to be conducted at 240ºF (115.6ºC) for longer time intervals to ensure that SC, SE, SN and SD are destroyed and to identify the impact of particles on thermal conductivity through the rendering matrices. Additionally, future experimentation will be needed to verify that the microorganisms identified are indeed *Salmonella* or other another microorganism(s) cross-reacting as *Salmonella*.

Objective (s):

a) Thermal death time studies will be conducted to determine thermal death time for a cocktail of *Salmonella* in poultry rendering materials at 240°F.
b) Thermal death time studies will be conducted to determine thermal death time for a cocktail of *Salmonella* in beef rendering materials at 240°F.

Project Overview:

CHAPTER 1

VALIDATION OF THERMAL DESTRUCTION OF *SALMONELLA* IN RENDERED BEEF PRODUCTS

Abstract

Animal rendering is a process that converts inedible animal tissue into stable, value-added materials. The North American rendering industry annually recycles over 61 billion pounds of residual animal by-products. Approximately 85% of rendered products are used as animal feed ingredients. Therefore, it is vital that the rendering industry has conclusive validation data on the thermal lethality of rendering thermal processing to destroy animal disease pathogens in finished products. The high fat, bone and protein content of rendering materials leaves the industry with no comparable thermal death time values from the human food industry or any other industry. The objective of this study was to determine thermal death time values for beef rendering materials containing 50% fat content for four pathogenic *Salmonella* recognized by FDA as hazardous for

animal feeds (*Salmonella* Choleraesuis (SC), *Salmonella* Enteritidis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD)). In the study, each serotype appeared to have unique thermal death time characteristics. With increasing thermal treatment time, reduction in the population of each serotype of *Salmonella* was not a straight line decrease. In fact, on most of the cultures, after failing to detect the cultures after certain time treatments, the culture were later detected after longer thermal treatments. In thermal treatments up to 420 s at 240ºF (115.6ºC), SC was last detected at 120 s, SE at 120 s, SN at 300 s and SD at 360 s. However, uninoculated controls indicated thermally resistant strains in the background which testing indicated were *Salmonella*. The presence of *Salmonella* or organisms detected as *Salmonella* was noted up to 360 s of treatment in the uninoculated samples. Further research will be needed to verify that these organisms are *Salmonella* or some other organism that is cross-reacting. In rendering materials, bone and tissue fragments can vary greatly across samples. In this study, a large range of particle sizes was present in the beef rendering materials.

Introduction

The United States and Canadian rendering industry annually recycles over 61 billion pounds of residual animal by-products into animal feeds, fats and proteins to prevent waste of these materials (Meeker and Hamilton, 2006). Validating thermal lethality of rendering processes is crucial to the livestock and pet food industry and to the FDA to ensure destruction of bacterial pathogens in products. A disease outbreak in the animal livestock industry could have serious negative consequences to the rendering industry and to the entire food animal chain, including consumers.

The high temperatures used in the rendering cooking process reduce the number of microorganisms in raw perishable animal tissues. The continuous cooking process is reported to be 40 to 90 min at 240 to 290ºF (115.6 to 143.3ºC) (Meeker and Hamilton, 2006). Crax is a solid material composed of protein, minerals, and residual fat that is discharged from the screw press during the rendering process and is typically further ground into meat and bone meal (Meeker and Hamilton, 2006). Meat and bone meal is frequently used in animal feeds and pet foods. Marginal processing conditions could result in survival of residual microorganisms in this protein rich product (Crump et al., 2002).

Thermal death time (TDT) is a factor of time, temperature, material matrix and organism (Heldman and Hartel, 1998). TDT is defined as the time needed to reduce a given number of organisms at a specific temperature in a specific matrix (Jay, 2005; Teixeira, 2006). Decimal reduction time (D value) specifies the time required for a one $\log_{10}$ reduction of a particular organism at a specific temperature. The larger the D value at a given temperature, the higher the thermal resistance of the microbial population (Heldman and Hartel, 1998). The high fat, bone and protein content of rendering materials leaves the rendering industry with no comparable thermal death time values from the human food industry or any other industry. The objective of this study was to determine the TDT and D values for beef rendering materials containing 50% fat content for four pathogenic *Salmonella* recognized by FDA as hazardous for animal feeds (*Salmonella* Choleraesuis, *Salmonella* Enteritidis, *Salmonella* Newport, and *Salmonella* Dublin) (FDA, 2010; FDA, 2013) at 240ºF (115.6ºC).

Materials and Methods

Rendering Sample Preparation

Samples of beef crax and beef tallow were obtained from a midwestern rendering company on three separate days. The crax samples were submitted in duplicate to the Clemson University Agricultural Service Laboratory for ash, fat, and moisture content analysis. The crax and 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 (ddH_2O) (0.6 g per L) for approximately 2 min, followed by rinsing 5 times with sterile ddH_2O. 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 was used to scrape material from the sides during pauses in processing. All samples were stored under refrigeration until needed for experimentation.

Salmonella Preparation

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 obtained 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 from collaborator Dr. Xiuping Jiang at Clemson University.

A preliminary study was conducted to determine the optimal media conditions for *Salmonella* growth. Trypticase soy broth (TSB) (90000-050, VWR Scientific Products, Suwanee, GA), TSB with the addition of 0.1% (wt/vol) yeast extract (MP Biomedicals, LLC, Solon, Ohio), and brain heart infusion broth (BHI) (211059, VWR Scientific Products, Suwanee, GA) were tested. TSB with the addition of 0.1% (wt/vol) yeast extract was chosen as the best media based highest cell densities determined from optical density measurements (μQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, VT) at 600 nm and dilution plating in duplicate onto onto bismuth sulfite agar (90003-904, VWR Scientific Products, Suwanee, GA), Hektoen enteric agar (9004-054, VWR Scientific Products), xylose lysine deoxycholate (XLD) (90003-996, VWR Scientific Products), and trypticase soy agar (TSA) (90000-050, VWR Scientific Products).

As a preliminary study, each individual *Salmonella* serotype was plated onto bismuth sulfite agar, Hektoen enteric agar, XLD, and TSA. Enumeration data indicated use of XLD and TSA as the preferred agar media for enumerating SC, SE, SN, and SD.

A preliminary goal of this experiment was to obtain concentrated bacterial slurry of each serotype to use in inoculating beef rendering materials for thermal processing. The average concentrations of *Salmonella* cultures in broth for SC, SE, SN, and SD after 24 h incubation at 35ºC were 8.66±0.02, 8.56±0.03, 8.80±0.06, and 8.65±0.03 $\log_{10}$ cfu/g, respectively. Preliminary experiments were conducted to determine the volume of culture as well as concentration rate necessary. Enumeration on XLD and TSA verified that 5 L of a 24 h *Salmonella* culture grown in TSB with 0.1% (wt/vol) yeast extract and then concentrated by centrifugation was optimal.

Centrifugation was conducted 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) and the supernatant was discarded after autoclaving. The pellet was resuspended in 5 mL sterile TSB. In preliminary studies conducted 3 times in duplicate (n=6), the average bacterial concentrations after centrifugation and resuspension for SC, SE, SN, and SD were determined. This procedure was used to prepare the bacterial cultures used throughout the experiment.

Each slurry of *Salmonella*, prepared as above, was inoculated into beef rendering material at the rate of 100 µL culture per 1 g sample. In a preliminary study, two methods were conducted. The mean bacterial counts of each concentrated bacterial slurry and the inoculated samples were determined. Method 1 was the serial dilution of each bacterial slurry and each inoculated sample to 10^{-14} utilizing the standard Class O phosphate/magnesium chloride dilution buffer (Wehr and Frank, 2004). Method 2 was the serial dilution of each bacterial slurry and sample to 10^{-14} using pre-warmed (32ºC) modified Class O phosphate/magnesium chloride diluent. Controls included media and uninoculated beef rendering samples. Each experiment was conducted 3 times in duplicate (n=6).

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 were capped (60825-801, VWR International, Suwanee, GA) and autoclaved. Beef rendering samples (50% fat) were aseptically transferred (1 g) into sixteen sterile tubes. 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. 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 individual culture (100 µL) was directly pipetted into 1 g of the heated rendering samples. After culture inoculation, the sample was pipetted up and down approximately four times to thoroughly mix. Upon inoculation and mixing, time measurements (0, 15, 30, 60, 90, 120, 180, 240, and 300 s) were started on the thermal treatment. After preliminary experiments on SN and SD indicated longer thermal treatment was needed, additional trials were included for the time treatments of 0, 90, 240, 300, 360 and 420 s for these cultures. Samples were placed on ice immediately after thermal treatment. Additional sample tubes containing beef rendering used for unheated controls were placed on ice until used for plating. All samples were processed for microbial content immediately after conclusion of heat treatments.

A preliminary experiment was conducted to validate the use of 1 g of sample pre-enriched in 5 mL of sterile universal pre-enrichment broth (UPB) (95021-036, VWR Scientific Products, Suwanee, GA) in comparison to 1 g of sample pre-enriched in 9 mL of UPB as recommended by the FDA Bacteriological Analytical Manual (BAM) (Andrews et al., 2011). The stainless steel tubes used in this experiment would not hold the 1 g of sample pre-enriched plus 9 mL of UPB. Results indicated that the 1:5 ratio of sample to pre-enrichment broth was as effective as the 1:9 ratio of sample to pre-enrichment broth. Therefore, this procedure was used throughout the experiment.

Once 5 mL of sterile UPB 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 for 30 s. Each UPB diluted sample (0.1 mL) was directly pipetted onto XLD and TSA plates and spread using an alcohol-flamed bent glass rod. As a control, each *Salmonella* slurry was serially diluted to 10^{-12} in the standard Class O phosphate/magnesium chloride dilution buffer and either 1.0 mL or 0.1 mL was spread plated onto XLD and TSA. Media and dilution buffer controls also were conducted. All plates were incubated overnight at 35ºC. In this experimental design, XLD selected for *Salmonella* spp. while TSA measured total aerobic, mesophilic bacterial counts. This included any background bacteria and, in the test samples, background bacteria plus inoculated *Salmonella.* For each inoculated or uninoculated beef rendering sample, dilutions were carried out such that the direct plating on XLD and TSA had a lower detection limit of 1.4 $\log_{10}$ cfu/g.

Because the direct plate counting method had a lower detection limit of 1.4 $\log_{10}$ cfu/g, an additional experiment was conducted in accordance with the FDA BAM procedures to detect as low as 1 cfu/g (Andrews et al., 2011). The remaining UPB diluted sample in the stainless steel tube was incubated overnight at 35ºC and then vortexed (Super Mixer, 1290, Labline Instruments, Inc., Melrose Park, IL) on the fast setting for approximately 30 s. The sample was aseptically pipetted (0.1 mL) to Rappaport-Vassiliadis (RV) pre-enrichment broth (10 mL) (95039-382, VWR Scientific Products, Suwanee, GA). The same sample was aseptically pipetted (1 mL) to tetrathionate broth (TT) (10 mL) (90000-008, VWR Scientific Products, Suwanee, GA). Controls included the concentrated bacterial slurry and sterile media. The samples and control broth were incubated overnight at 42ºC. A 3 mm inoculation loop 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. As per FDA BAM, positive samples obtained from the RV and TT pre-enrichments were validated using two confirmation tests (Feng, 2001). Latex agglutination tests (FT0203, Thermo Fisher Scientific, Waltham, MA 02454) and ChromAgar™ (90006-158, VWR Scientific Products, Suwanee, GA) were conducted using each *Salmonella* culture as a control (BD Diagnostics, 2008; Oxoid Limited, 2013). In order to analyze the data, when duplicate results from the pre-enriched samples were both negative the data was reported as 0.0 (Fig. 3.1). 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 (Fig. 3.1).

Bone Particle Size Determination

To determine the variation in bone particle size in the processed 50% fat rendering material used, 10 g of the rendering sample was sized through a series of sieves (57333-965, VWR Scientific Products, Suwanee, GA) equipped with eight different standard mesh sizes (25, 35, 45, 60, 80, 120, 170 and 230 μm). Samples were measured into the upper sieve and processed using 100 mL of hexane (AAAL13233-AU, VWR Scientific Products, Suwanee, GA) to dissolve fat and assist in particle separation. The hexane fraction was washed through the sieve column 10 times. Each fraction of particle size was reported as a percentage of the total weight of the rendering sample. Each trial was repeated 10 times per day for 3 days (n=30).

Determination of Estimated D Values

The direct plate count of each concentrated *Salmonella* slurry and the time at which each culture was destroyed were compared on graphs. In a preliminary experiment, percent recoveries of *Salmonella* from inoculated beef samples were calculated for each recoverable *Salmonella* population density. Due to the experimental design, the actual population count from beef rendering material was not conducted. However, the total count in each bacterial slurry was measured. This population count was used in estimated D value calculations. The final time the population was no longer detected in each RV and TT pre-enrichment as validated by the two confirmation tests was used as the thermal death time. These data were graphed and the slope of the line was used to calculate the estimated D value.

Results

Analysis of beef rendering materials indicated fat content ranged from 9.9% to 13.8%, ash content was 20.6% to 33.5% and moisture content was 2.1% to 3.35%. 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.

Preliminary results indicated that the average concentrations of the culture slurries of SC, SE, SN, and SD (n=6 for each culture) ± standard error were 12.60±0.15, 12.12±0.01, 12.28±0.03, and 12.16±0.15 $\log_{10}$ cfu/g, respectively. Average bacterial counts ± standard error on XLD from inoculated beef rendering samples were 10.60±0.269, 10.67±0.08, 10.76±0.04, and 10.65±0.08 $\log_{10}$ cfu/g, respectively (Table 3.1).

All *Salmonella* counts were conducted in a two-step process. Enumeration on XLD had a lower detection limit of 1.4 $\log_{10}$ cfu/g. With the exception of SD, under all treatment conditions, SC, SE, and SN were reduced to below the lower detection limit across all thermal treatment times in inoculated beef samples. SD was detected until 60 s (Fig. 3.2). To check for experimental error, day 1, day 2 and 3 rendering samples were re-tested to add additional data points. Data shown in Fig. 3.2 represent n=42 for SD. The presence of *Salmonella* noted at 0 and 30 s represented only 1 out of 24 samples and 1 out of 42 samples, respectively.

In uninoculated beef samples, SC, SE, and SN were reduced to or below the lower detection limit across all thermal treatments. However, SD was detected at 60 s in the uninoculated beef samples (Fig. 3.3). To check for experimental error, day1, day 2 and 3 rendering samples were re-tested to add additional data points. Data shown in Fig. 3.3 represents n=24, except at 0, 90, 240, 300, 360 and 420 s for SD. Two out of the 24 samples were determined to be positive in uninoculated beef at 60 s. A similar result was noted at 0 s with 1 positive out of 42 samples. The uninoculated SD control sample had *Salmonella* present for longer thermal treatment than the inoculated sample indicating the presence of a background culture of either thermally resistant *Salmonella* or a thermally resistant microorganism(s) that is detected as *Salmonella* using current methodology.

Enumeration on TSA had an upper detection limit of 4.3 $\log_{10}$ cfu/g. Under all treatment conditions, bacterial plate counts on TSA for SC, SE, SN, and SD inoculated beef samples were above the upper detection limit after all thermal treatments (Fig. 3.4). In uninoculated beef samples used as controls for the SC, SE, SN, and SD experiments, plate counts on TSA were above the upper detection limit after all thermal treatments in uninoculated beef samples (Fig. 3.5).

Pre-enrichment results on RV and TT were confirmed using both latex agglutination and ChromAgar™; the following results are reported as confirmed findings. The unheated, inoculated

controls plated on XLD after pre-enrichment in RV and TT were significantly higher ($P<0.05$) than the heated, inoculated samples (this control is indicated as unheated on Fig. 3.6, 3.7, 3.8 and 3.9). In general, *Salmonella* serotypes in heated, inoculated samples declined with longer thermal treatment (Fig. 3.6 and 3.8). The number of positive samples for *Salmonella* for each inoculated and uninoculated samples in either RV or TT validated by the two confirmation tests are shown in Tables 3.4 and 3.5. Some samples that were reported as present had high standard errors.

In the SC samples, *Salmonella* was reduced to 0 at all time intervals after 0 s in RV and TT with the exception of reappearing at 120 s in TT (Fig. 3.8). Populations of *Salmonella* in the SE inoculated samples were reduced but not completely eliminated at 0 s in both RV and TT pre-enrichments (Fig. 3.6 and 3.8). For SE samples pre-enriched in RV, *Salmonella* levels were reduced to 0 at 30, 60, 180, 360, and 420 s but were noted at all other times (Fig. 3.6). *Salmonella* was present in SE inoculated samples at every time interval until eliminated at 180 s and afterwards in TT pre-enrichments (Fig. 3.8). Although populations were reduced, *Salmonella* was not eliminated until 360 s on SN inoculated samples in RV pre-enrichments and until 300 s on TT pre-enrichments (Fig 3.6 and 3.8). In the SN and SD experiments, a population of *Salmonella* appeared to be present in both inoculated and uninoculated samples and appeared to be more thermally resistant than *Salmonella* detected on the SC and SE experiments (Fig. 3.6, 3.7, 3.8, and 3.9). In the heated, inoculated samples, SD was reduced to 0 at 30 s and 60 s, was present at 90 s, was killed at 120 s and 180 s, and was present at 240 s in both RV and TT pre-enrichments (Fig. 3.6 and 3.8). At 360 and 420 s, SD was reduced to 0 in RV pre-enrichments (Fig. 3.6). At 360 s SD was present but at 420 s was reduced to 0 in TT pre-enrichments (Fig. 3.8). Since 420 s was the maximum time tested, future studies should include longer treatment times (Fig. 3.6 and 3.8).

Variations were noted in *Salmonella* populations in heated uninoculated samples (Fig. 3.7 and 3.9). *Salmonella* was not detected at any thermal treatment time in the SC experiments using RV enrichment but was detected at 90 s only in TT pre-enrichments (Fig. 3.7 and 3.9). *Salmonella* was not detected in the SE experiments at 0, 15, 30, 60, 240, 300, 360, and 420 s in RV pre-enrichments but was detected at 90, 120, and 180 s (Fig. 3.7). In TT, *Salmonella* was present in the SE experiments in all thermal treatment times up to 180 s and was absent at 240 and 300 s (Fig. 3.9). In the SN experiments, *Salmonella* was reported as in heated, uninoculated samples until 300 s in RV (Fig 3.7). Also in the SN experiments, *Salmonella* was present in heated uninoculated samples until 240 s in TT (Fig. 3.9). In the SD experiments in RV, *Salmonella* was not detected at 0, 15, 120, 360, and 420 s in the heated, uninoculated samples (Fig. 3.7). In TT during the SD study, *Salmonella* was not detected at 15, 30, 120, and 420 s (Fig. 3.9).

The estimated D values for *Salmonella* in beef rendering samples containing 50% fat at 115.6ºC pre-enriched in RV and validated by two confirmation tests were calculated. SC and SE had D values of 0.01 and 0.29 min, respectively, while SN and SD had longer D values of 0.58 and 0.60 min (Table 3.2). The estimated D values for *Salmonella* serotypes in beef rendering samples containing 50% fat at 115.6ºC pre-enriched in TT and validated by two confirmation tests also were determined. SC and SE had D values of 0.30 and 0.29 min, respectively, while SN and SD had D values of 0.49 and 0.70 min, respectively (Table. 3.3).

In the sieve separation experiment, each particle size fraction was indicated as a percentage of the total weight of the rendering sample. The largest fraction of particles collected was collected on the 25 μm mesh sieve and represented 56.6 ± 1.5% of the original sample. Sieves 35, 45, 60, 80, 120, 170 and 230 μm collected 3.7± 0.3%, 4.5± 0.1%, 4.2 ± 0.3%, 3.7± 0.2%, 3.9 ± 0.8%, 4.70 ± 0.81% and 5.4 ± 0.5%, respectively (Fig. 3.10).

Discussion

Due to the large number of samples plated per day, a preliminary experiment was conducted to determine the percent recoveries ± standard error for each *Salmonella* culture from beef rendering. The purpose of the preliminary study was to reduce plating of each inoculated, unheated sample through extended dilutions during the study. However, more accurate data would be obtained if plating of each inoculated, unheated sample had been conducted. In future experiments, this control should be included.

Enumeration on XLD indicated that SC, SE and SN were reduced to below the detection limit after the initial thermal treatment in inoculated rendering samples. Similarly, in the uninoculated samples, SC, SE, and SN were reduced to below the detection limit after the initial thermal treatment. SD, however, was detected at 30 s in the inoculated samples and at 60 s in the uninoculated samples indicating the presence of a thermally resistant bacterial strain in the background of the samples. *Salmonella* was detected as present in both inoculated and uninoculated SD samples after thermal treatment (Fig. 3.2 and 3.3). It should be noted that a positive *Salmonella* result from current methodology on either inoculated or uninoculated was not validated by genetic analysis or serotyping which would be necessary for confirmation in this study. Other explanations for differences in recovery of *Salmonella* could be due to variation in particle size distribution in the sample. SD or background organisms appearing to be *Salmonella* in the samples may have been entrapped in a bone particle or in fat. A particle size distribution test was conducted and showed great variability among sizes of bone fragments. Due to the nature of rendering material collection, *Salmonella* could be present in the porous structure of bone. Additionally, *Salmonella* could have been coated in fat or tissue allowing for a protective effect due to slower thermal conductivity of particles, fat and tissue. The samples in this study were randomly placed in the heating block and, therefore, sampling error was not considered a cause for the observed variability.

Enumeration on TSA for both inoculated and uninoculated samples indicated the presence of the bacteria in the background of the rendering samples. The mean bacterial counts of all samples, under all thermal treatments, were above the detection limit of 4.3 $\log_{10}$ cfu/g. Glenn (2006) conducted a study on the bacterial loads in raw rendering materials, but the current study was focused on the bacterial loads in finished rendered materials. A wide variety of heat resistant or post-process contaminating bacteria could be present in the rendering materials; therefore, the presence of 4.3 $\log_{10}$ cfu/g in the rendering samples is not unexpected.

From the preliminary study, it was determined approximately 10 $\log_{10}$ cfu/g of each *Salmonella* culture could be recovered from inoculated rendering samples. This concentration exceeds the detection limit of the direct plating method utilized to enumerate on TSA. The presence of bacteria after 420 s of thermal treatment at 115.6ºC on TSA indicated the presence of heat resistant bacteria in the background of the rendering samples. Autoclaving requires exposure to 121ºC at 15 psi of pressure for a minimum of 15 min to kill most bacteria (Laroussi and Leipold 2004). Bacterial endospores are very heat resistant and there have been cases where endospores have not been killed under autoclave conditions (Tuominen et al. 1994). Therefore, the thermally-resistant bacteria in the background of rendering materials could potentially be spore-forming bacteria. The design of this experiment did not allow for further analysis of these heat-resistant bacteria. However, future experiments will isolate and identify these bacterial species through genetic analysis or serotyping.

Results of RV and TT pre-enrichments indicated variation in recovery amongst cultures identifying as *Salmonella* in the SC, SE, SN, and SD inoculated and uninoculated samples. In inoculated and uninoculated samples pre-enriched in RV, the presence of SC or organisms appearing to be *Salmonella* declined after the application of heat. In TT, the presence of SC or microbes appearing to be *Salmonella* followed a similar trend as the RV pre-enriched samples. However, *Salmonella* were detected in both inoculated and uninoculated samples at 90 and 120 s in TT. In RV, SE or bacteria detected as *Salmonella* were present in both inoculated and uninoculated samples at 90 and 120 s. However, in TT, SE or organisms presenting as *Salmonella* were detected in both inoculated and uninoculated samples at 0, 15, 30, 60, 90, and 120 s. The presence of *Salmonella* or organisms detected as *Salmonella* at 90 s and 120 s may be background bacteria. The presence of SN or organisms detected as *Salmonella* were present at 0, 15, 30, 60, 90, 120, 180, 240 s in both RV pre-enriched inoculated and uninoculated samples. SN or *Salmonella*-like bacterial species were detected in TT until 300 s in inoculated and uninoculated samples. Positive results in inoculated samples may be due to background organisms. SD or organisms detected as *Salmonella* were present in both inoculated and uninoculated samples pre-enriched in RV at 60, 90, 240 and 300 s. In TT, SD or organism detected as *Salmonella* were present at 0, 90, 240, 300 and 360 s in inoculated and uninoculated samples. Again, positive results in inoculated samples may be due to background organisms. Another explanation for the results of this study could be that *Salmonella* species may have been entrapped in bone particles or in fat. In comparing the presence of *Salmonella* in inoculated samples pre-enriched in either RV or TT, the presence of *Salmonella* or a *Salmonella*-like organism appeared to follow similar trends across all experiments.

The presence of a thermally resistant organism reacting as *Salmonella* has been well-noted in the rendering samples 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 bacterial strain(s) may have acquired thermal resistance and/or *Salmonella*-like characteristics through repetitive cycles of animal feed, animals and rendering. Inedible animal tissues including the gastrointestinal tract and its inherent microorganisms would be rendered and the cycle through animal feed to animal to slaughter to rendering could hypothetically repeat. Potentially these conditions could select for thermally resistant microorganisms. Since this hypothesis has not been tested, it is vital that this unknown strain or strains is isolated in future experimentation to determine its identity and characteristics.

Preliminary estimated D values were calculated. SN and SD appeared to have longer D values than SC and SE. As a general rule of thumb, with increase in temperature, the thermal lethality increases (Earle and Earle 1983). Liu et al. (1969) reported D values for *Salmonella* senftenberg 775 W were highly variable between 10 to 115 min at 70ºC in meat and bone meal. Lui et al. (1969) conducted their study in meal and the current study was conducted in cooked beef rendered products containing 50% fat content. Similar to the Lui et al. (1969) study, the D values of this study were variable and high which could potentially be due to the thermally resistant background organism(s).

Further research needs to be conducted at 240ºF (115.6ºC) for longer time intervals to ensure that SC, SE, SN and SD are destroyed. It should be noted the results of this study were obtained from the lower end of the cooking temperatures utilized in the rendering industry. Many rendering facilities process materials at higher temperatures close to 280ºF (137.8ºC) to 290ºF (143.3ºC) for 40 to 90 min in order to produce microbiologically safe products (Meeker and Hamilton 2006). However, the industry also employs a different type of cooker known as a Carver-

Greenfield unit. These units operate at lower temperatures, typically closer to 240ºF (115.6ºC). Carver-Greenfield units operate under vacuum to process the materials at this lower temperature (Meeker and Hamilton 2006).

It was necessary to grind rendering materials for transfer into stainless steel tubes. Factors for comparing data to typical bone particle sizes will necessary for future experiments. Thermal conductivity studies on larger bone particles could provide further understanding of thermal lethality in rendering materials.

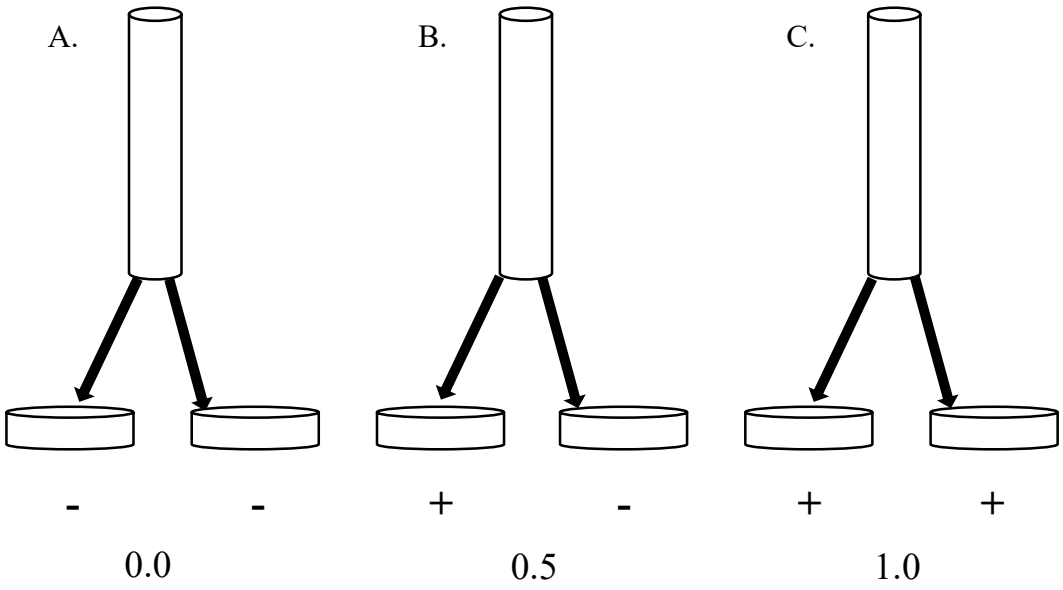

Figure 3.1. Method utilized to report RV and TT pre-enrichments results on XLD validated by two confirmation tests at each thermal treatment. If both plates were negative, the result was assigned a 0 (A). If one was positive and one was negative, the result was assigned a 0.5 (B). If both were positive, the result was assigned a 1.0 (C).

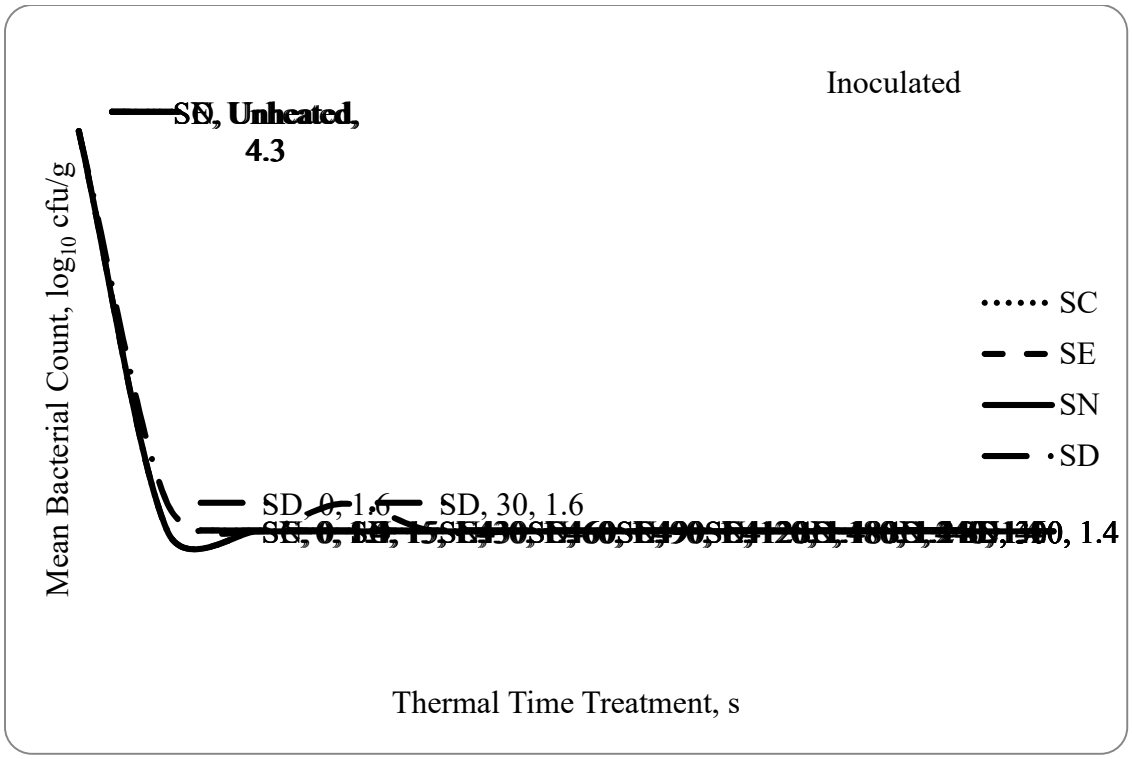

Figure 3.2. Enumeration of *Salmonella* on XLD from beef rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]The lower limit of detection is 1.4 $\log_{10}$ cfu/g of *Salmonella* (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

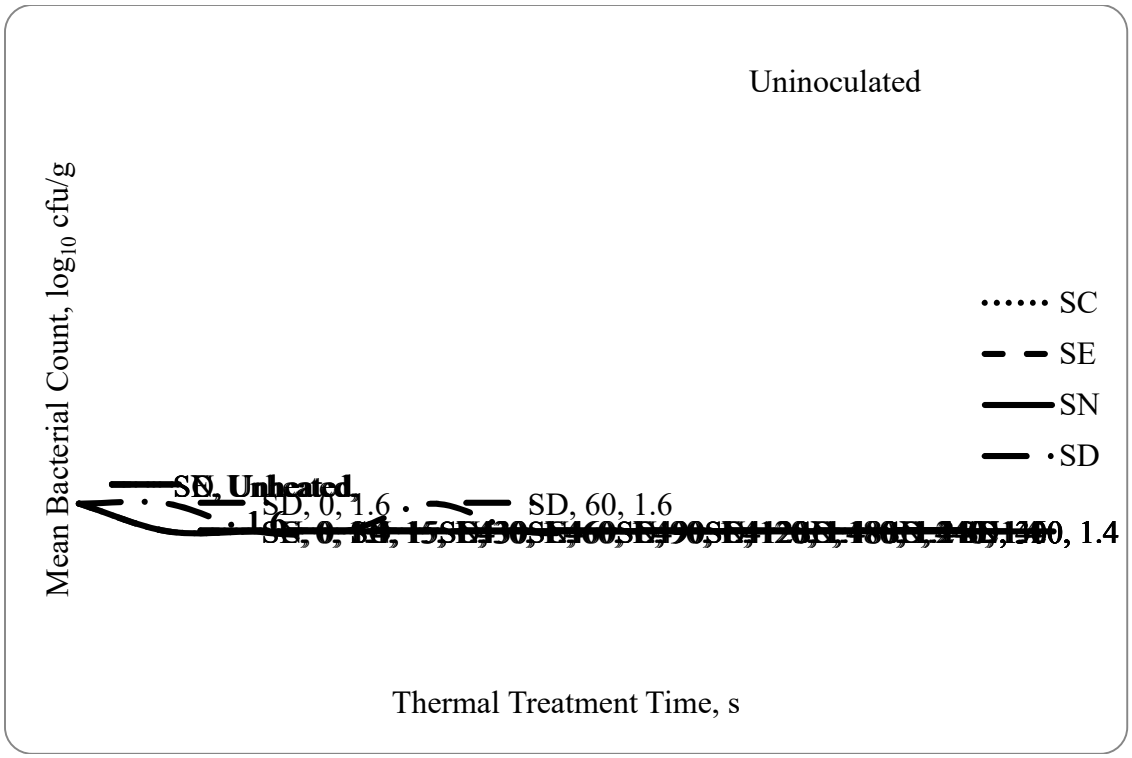

Figure 3.3. Enumeration of *Salmonella* on XLD from uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]The lower limit of detection is 1.4 $\log_{10}$ cfu/g of *Salmonella* (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

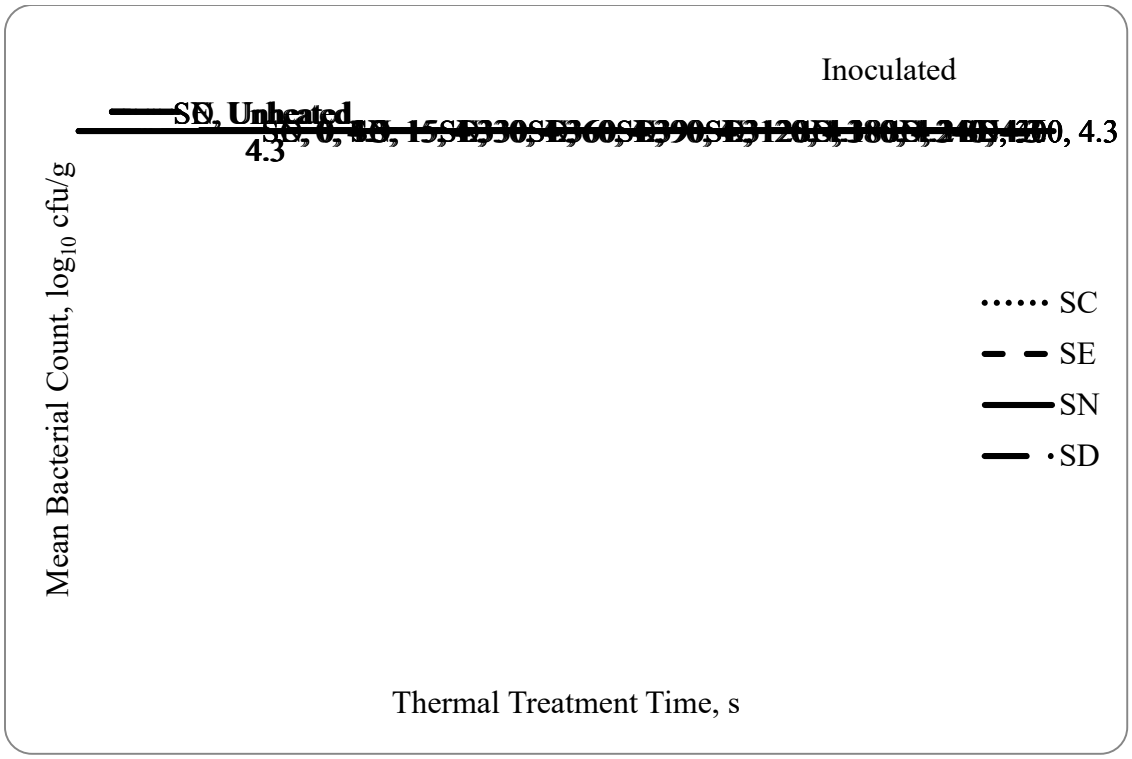

Figure 3.4. Enumeration of total bacteria on TSA from beef rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]The lower limit of detection is 1.4 $\log_{10}$ cfu/g of *Salmonella* (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

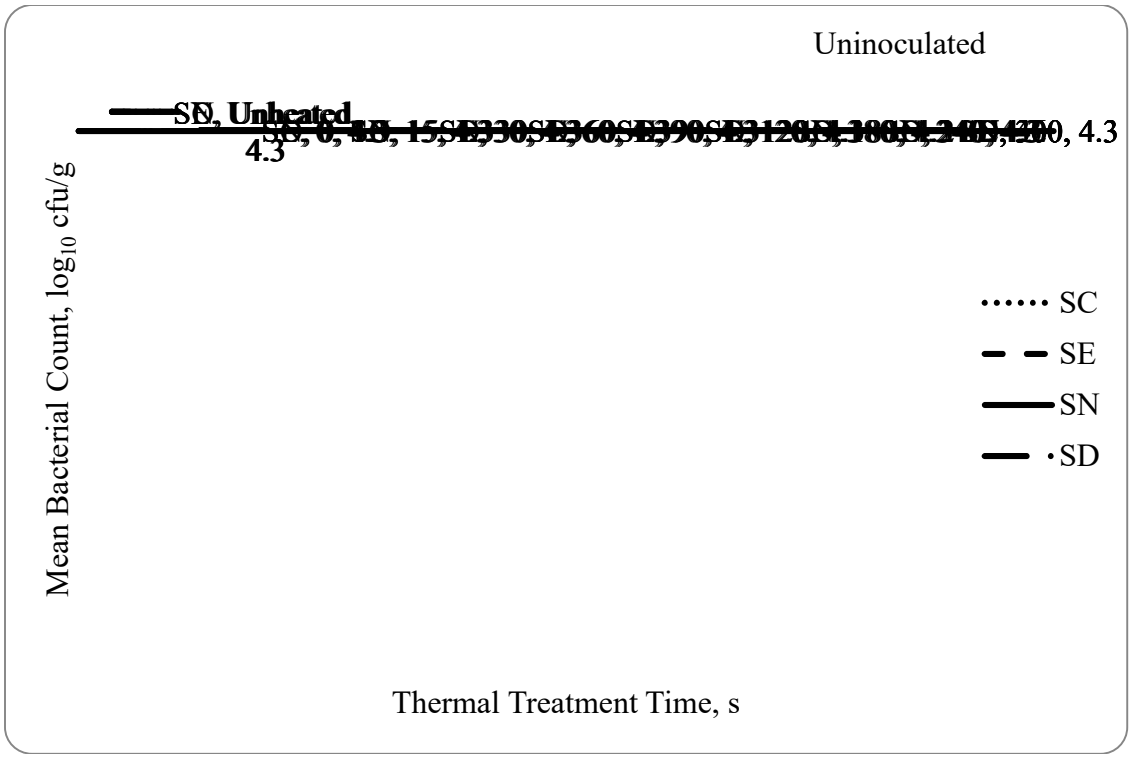

Figure 3.5. Enumeration of total bacteria on TSA from uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]The lower limit of detection is 1.4 $\log_{10}$ cfu/g of *Salmonella* (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

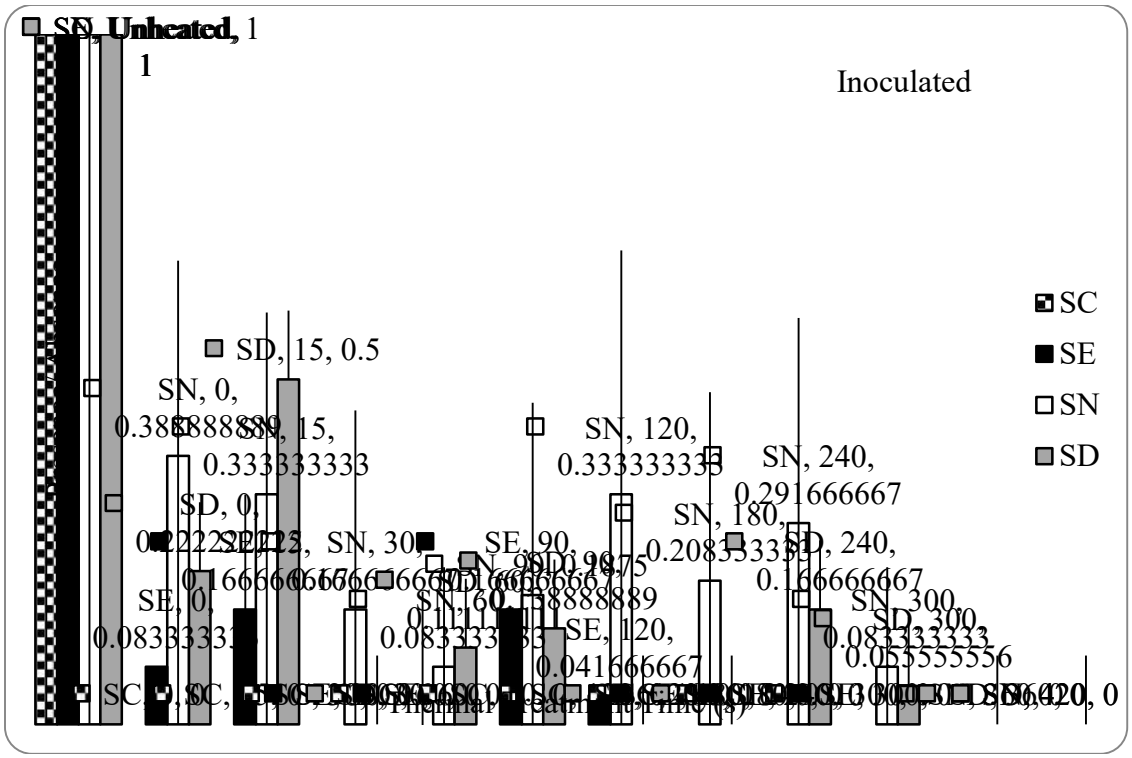

Figure 3.6. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, RV pre-enriched beef rendering samples (50% fat).[1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

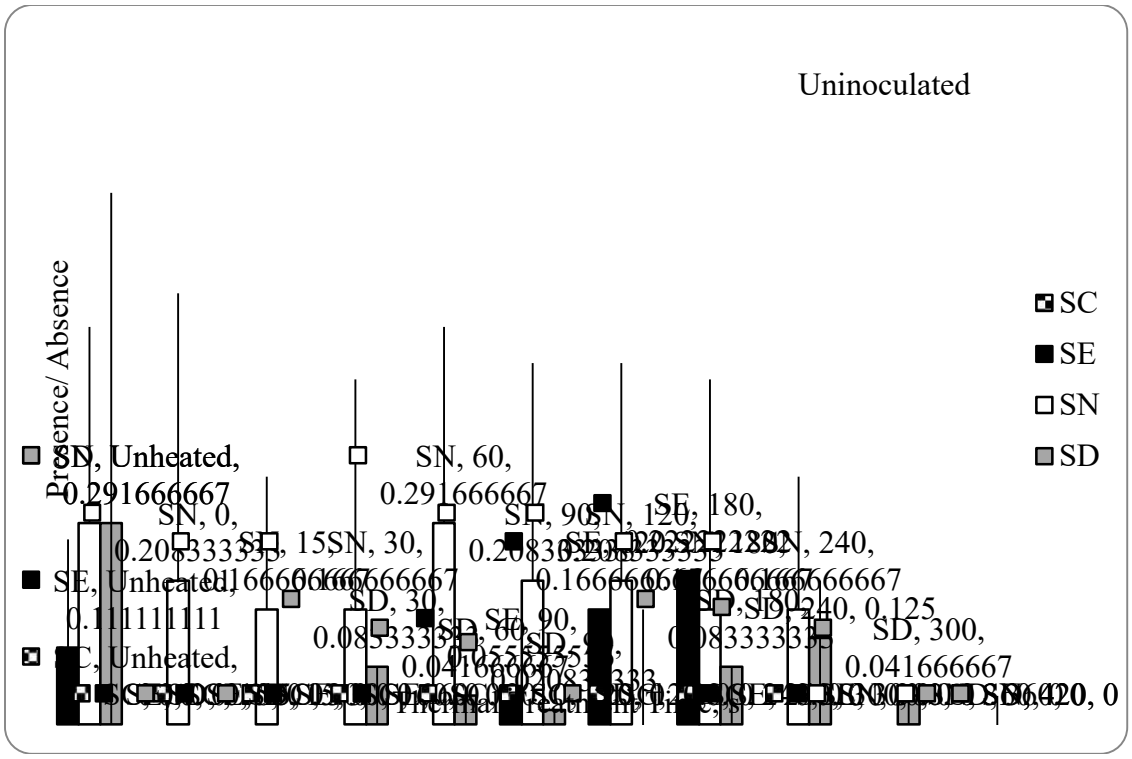

Figure 3.7. Presence or absence ± standard deviation of *Salmonella* for each RV pre-enriched, uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s)

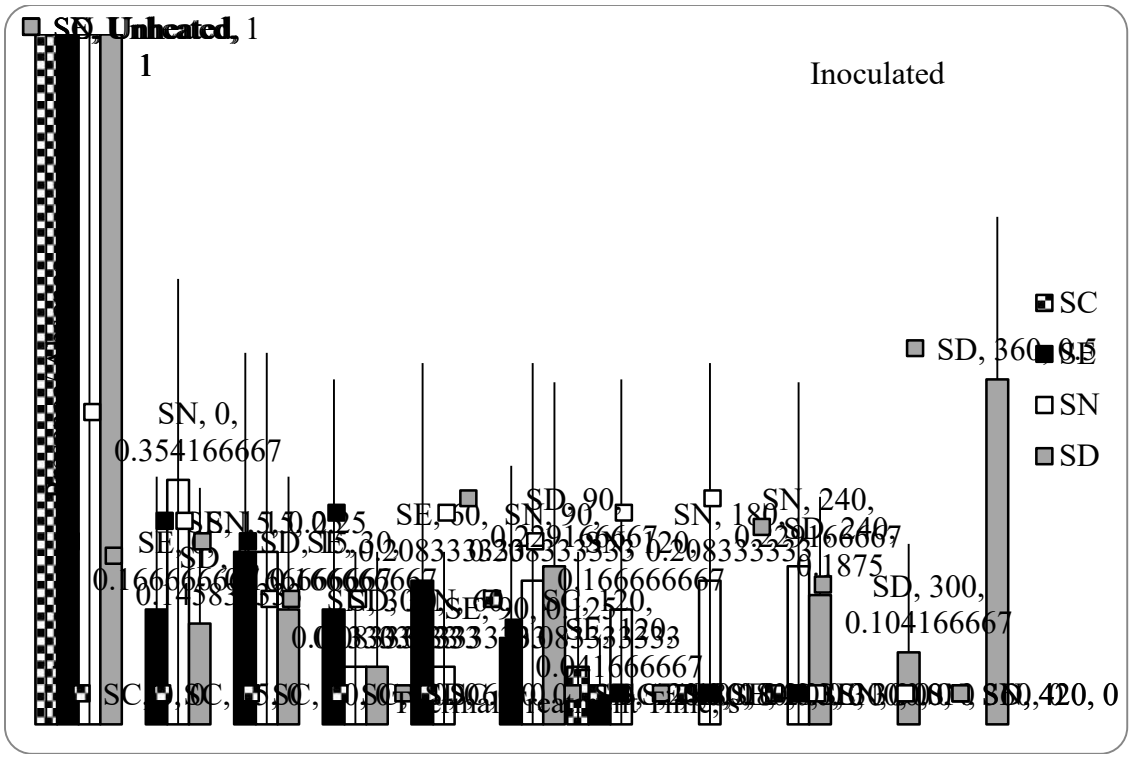

Figure 3.8. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, TT pre-enriched beef rendering samples (50% fat).[1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

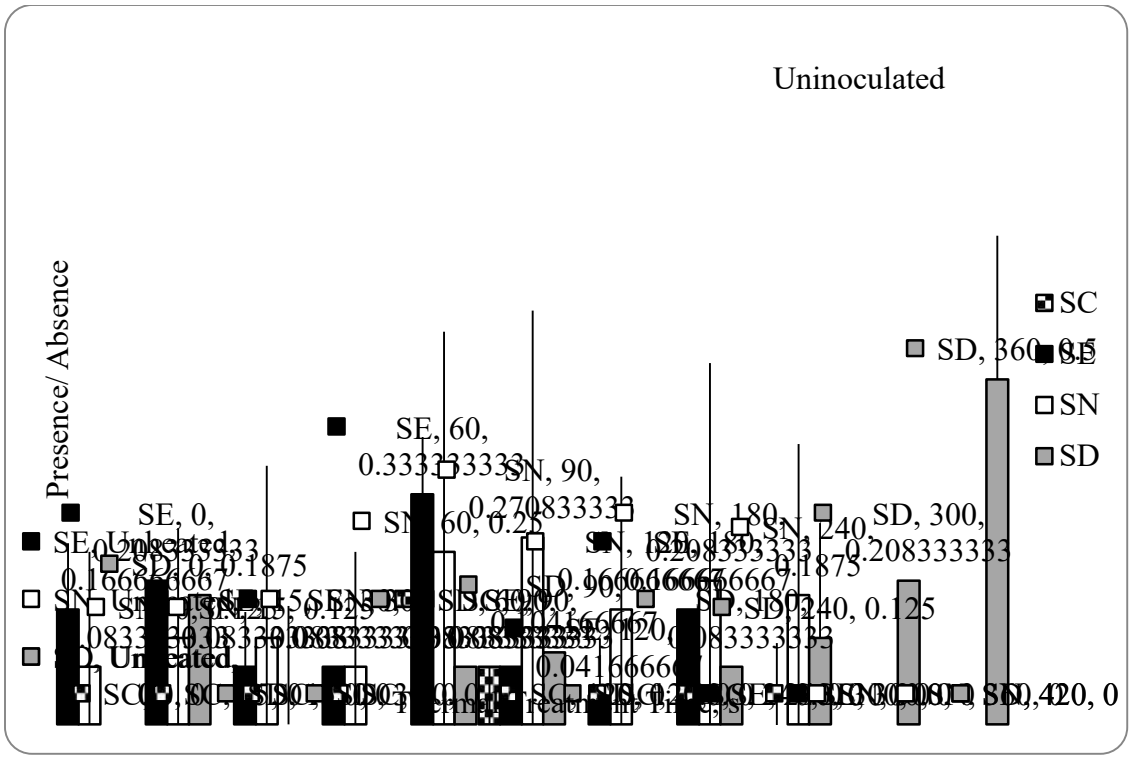

Figure 3.9. Presence or absence ± standard deviation of *Salmonella* for each TT pre-enriched, uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]A count of 0 represent the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

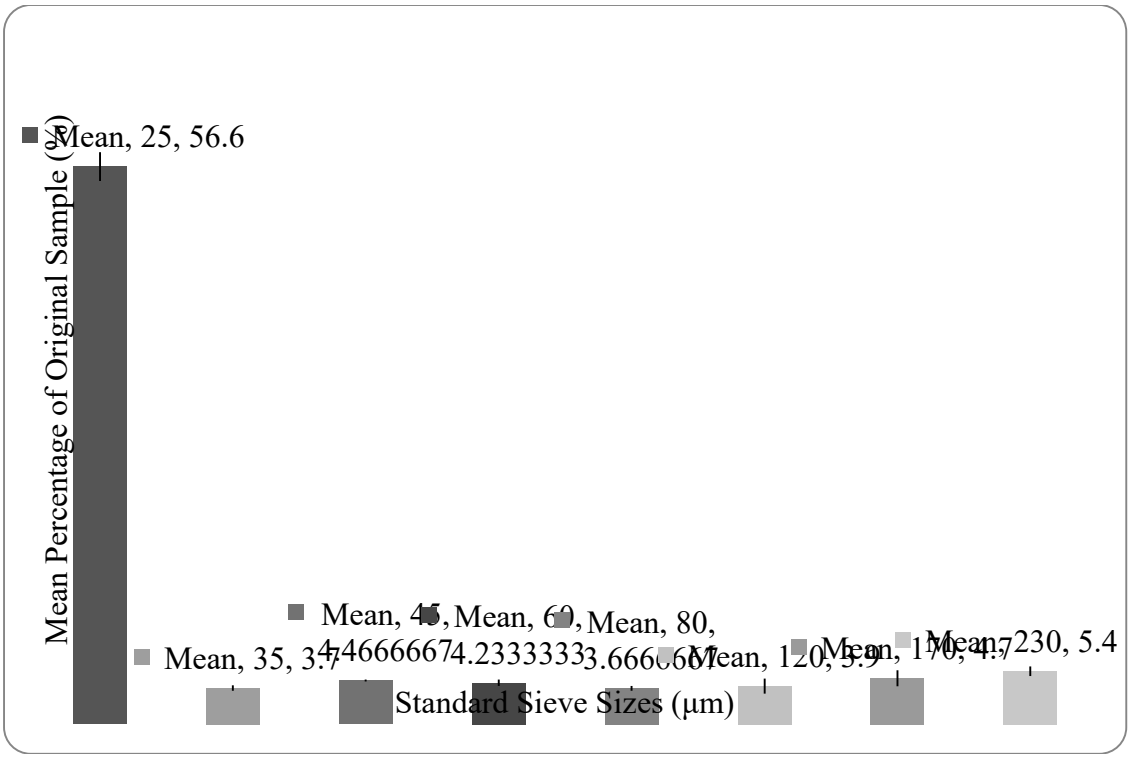

Figure 3.10. Mean percent particle size distribution ± standard error of beef rendering samples collected from a rendering plant on three different days (n=30). Each fraction of particle size was indicated as a percentage of the total weight of the rendering sample. The error bars indicate standard error for each data point.

Table 3.1. *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD) after inoculation into beef rendering materials and plated onto XLD (n=6).

Serotype	Average Broth Culture, $\log_{10}$ cfu/g ± standard error	Average in Beef Samples, $\log_{10}$ cfu/g ± standard error
SC	12.60±0.15	10.60±0.29
SE	12.12±0.01	10.67±0.08
SN	12.28±0.03	10.76±0.04
SD	12.16±0.15	10.65±0.08

Table 3.2. Estimated D values for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) in beef rendering samples (50% fat) at 115.6ºC pre-enriched in RV and validated by two confirmation tests.

Serotype	Estimated D Value, min
SC	0.01
SE	0.29
SN	0.58
SD	0.60

Table 3.3. Estimated D values for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) in beef rendering samples (50% fat) at 115.6ºC pre-enriched in TT and validated by two confirmation tests.

Serotype	Estimated D Value, min
SC	0.30
SE	0.29
SN	0.49
SD	0.70

Table 3.4. Number of samples positive for *Salmonella* in *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD) inoculated beef rendering samples (50% fat) after pre-enrichment in RV or TT broth (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

Serotypes	Thermal Treatment Time, s	RV Number of Positive Samples	TT Number of Positive Samples
SC	Unheated	24 out of 24 samples	24 out of 24 samples
	0	0 out of 24 samples	0 out of 24 samples
	15	0 out of 24 samples	0 out of 24 samples
	30	0 out of 24 samples	0 out of 24 samples
	60	0 out of 24 samples	0 out of 24 samples
	90	0 out of 24 samples	0 out of 24 samples
	120	0 out of 24 samples	2 out of 24 samples
	180	0 out of 24 samples	0 out of 24 samples
	240	0 out of 24 samples	0 out of 24 samples
	300	0 out of 24 samples	0 out of 24 samples
SE	Unheated	24 out of 24 samples	24 out of 24 samples
	0	2 out of 24 samples	4 out of 24 samples
	15	4 out of 24 samples	6 out of 24 samples
	30	0 out of 24 samples	4 out of 24 samples
	60	0 out of 24 samples	5 out of 24 samples
	90	4 out of 24 samples	3 out of 24 samples
	120	1 out of 24 samples	1 out of 24 samples
	180	0 out of 24 samples	0 out of 24 samples
	240	0 out of 24 samples	0 out of 24 samples
	300	0 out of 24 samples	0 out of 24 samples
SN	Unheated	42 out of 42 samples	42 out of 42 samples
	0	16 out of 42 samples	14 out of 42 samples
	15	8 out of 24 samples	6 out of 24 samples
	30	4 out of 24 samples	2 out of 24 samples
	60	2 out of 24 samples	2 out of 24 samples
	90	8 out of 42 samples	9 out of 42 samples
	120	8 out of 24 samples	4 out of 24 samples
	180	5 out of 24 samples	5 out of 24 samples
	240	12 out of 42 samples	10 out of 42 samples
	300	3 out of 42 samples	0 out of 42 samples
	360	0 out of 42 samples	0 out of 42 samples
	420	0 out of 42 samples	0 out of 42 samples
SD	Unheated	42 out of 42 samples	42 out of 42 samples
	0	9 out of 42 samples	6 out of 42 samples
	15	12 out of 24 samples	4 out of 24 samples
	30	0 out of 24 samples	2 out of 24 samples
	60	3 out of 24 samples	0 out of 42 samples
	90	6 out of 42 samples	10 out of 42 samples

120	0 out of 24 samples	0 out of 24 samples
180	0 out of 24 samples	0 out of 24 samples
240	7 out of 42 samples	8 out of 42 samples
300	3 out of 42 samples	4 out of 42 samples
360	0 out of 42 samples	21 out of 42 samples
420	0 out of 42 samples	0 out of 42 samples

Table 3.5. Number of samples positive for *Salmonella* in uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD) after pre-enrichment in RV or TT broth (n=24, except for SN and SD n=42 at 0, 90, 240, 300, 360 and 420 s).

Serotype	Thermal Treatment Time, s	RV	TT
		Number of Positive Samples	Number of Positive Samples
SC	Unheated	0 out of 24 samples	0 out of 24 samples
	0	0 out of 24 samples	0 out of 24 samples
	15	0 out of 24 samples	0 out of 24 samples
	30	0 out of 24 samples	0 out of 24 samples
	60	0 out of 24 samples	0 out of 24 samples
	90	0 out of 24 samples	2 out of 24 samples
	120	0 out of 24 samples	0 out of 24 samples
	180	0 out of 24 samples	0 out of 24 samples
	240	0 out of 24 samples	0 out of 24 samples
	300	0 out of 24 samples	0 out of 24 samples
SE	Unheated	3 out of 24 samples	4 out of 24 samples
	0	0 out of 24 samples	5 out of 24 samples
	15	0 out of 24 samples	2 out of 24 samples
	30	0 out of 24 samples	2 out of 24 samples
	60	0 out of 24 samples	8 out of 24 samples
	90	1 out of 24 samples	2 out of 24 samples
	120	4 out of 24 samples	1 out of 24 samples
	180	5 out of 24 samples	4 out of 24 samples
	240	0 out of 24 samples	0 out of 24 samples
	300	0 out of 24 samples	0 out of 24 samples
SN	Unheated	12 out of 42 samples	3 out of 42 samples
	0	9 out of 42 samples	5 out of 42 samples
	15	4 out of 24 samples	3 out of 24 samples
	30	4 out of 24 samples	2 out of 24 samples
	60	7 out of 24 samples	6 out of 24 samples
	90	9 out of 42 samples	11 out of 42 samples
	120	5 out of 24 samples	4 out of 24 samples
	180	4 out of 24 samples	5 out of 24 samples
	240	7 out of 42 samples	8 out of 42 samples
	300	0 out of 42 samples	0 out of 42 samples
	360	0 out of 42 samples	0 out of 42 samples
	420	0 out of 42 samples	0 out of 42 samples
SD	Unheated	12 out of 42 samples	0 out of 42 samples
	0	0 out of 42 samples	8 out of 42 samples
	15	0 out of 24 samples	0 out of 24 samples
	30	2 out of 24 samples	0 out of 24 samples
	60	1 out of 24 samples	2 out of 24 samples

90	1 out of 42 samples	4 out of 42 samples
120	0 out of 24 samples	0 out of 42 samples
180	2 out of 24 samples	2 out of 24 samples
240	5 out of 42 samples	5 out of 42 samples
300	2 out of 42 samples	9 out of 42 samples
360	0 out of 42 samples	21 out of 42 samples
420	0 out of 42 samples	0 out of 42 samples

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CHAPTER 2

VALIDATION OF THERMAL DESTRUCTION OF *SALMONELLA* IN RENDERED POULTRY PRODUCTS

Abstract

Only a portion of a food animal is considered edible by humans. The remainder of the animal tissue is considered inedible and typically rendered into animal co-products. Rendering recycles the residual animal tissue from food animals into stable, value-added materials for use primarily in animal feeds. Therefore, the rendering industry must have validation data on the thermal lethality of rendering thermal process to ensure the destruction of animal disease pathogens in finished products. The unique high fat, bone and protein content of rendering materials leaves the industry with no comparable thermal death time values from the human food industry or any other industry. The objective of this study is to determine thermal death time values for poultry rendering materials containing 50% fat content for four pathogenic *Salmonella* recognized by FDA as hazardous for animal feeds (*Salmonella* Choleraesuis (SC), *Salmonella* Enteritidis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD)). Recoverability of *Salmonella* varied after pre-enrichment in either RV or TT broth. Levels of *Salmonella* in the samples did not exhibit a straight line decrease with increasing thermal treatment times. In thermal treatment trials extended up to 420 s at 240ºF (115.6ºC), *Salmonella* were detected in the SC, SE, SN and SD samples at 360 s. Thermally resistant *Salmonella* or *Salmonella*-like strains in the background were detected up to 360 s of treatment in uninoculated controls. Future experiments will be needed to validate whether these organisms are *Salmonella*.

Introduction

Rendered animal products can potentially be contaminated with *Salmonella* spp. Approximately 85% of rendered products are used as animal feed ingredients which can potentially transmit *Salmonella* to humans through the food chain (Crump et al. 2002). Loken et al. (1968) tested 1,395 rendered products from seven different plants and detected the presence of *Salmonella* in 241 (17%) of the samples. The study also tested the plant via environmental swabs, and *Salmonella* was isolated from 359 out of 1901 (19%) of the swabs. In a study conducted in 1977, *Salmonella* was detected in 81% of the meat meal and 40% of the feather meal produced over a four mo period in Ontario feed mills (Hacking et al. 1977). In 1993 and 1994, FDA conducted two separate studies examining rendered animal feed products for the presence of *Salmonella enterica* and determined 56% and 25% of the samples, respectively, were positive (McChesney et al., 1995; Crump et al., 2002). Troutt et al. (2001) examined 17 rendering facilities located in seven midwestern states of the United States. No *Salmonella* was found in crax samples or in the rendering processing environment. However, the finished rendered products contained 12 serovars of *Salmonella*. Franco (2005) reported *Salmonella* cells were present in low numbers in animal feed after analyzing approximately 200 rendered animal protein meal samples over a 12 mo period. Kinley et al. (2009) examined products from 12 rendering facilities in the United States and detected 13 *Salmonella* serovars. In 2010, Kinley et al. determined the prevalence of *Salmonella* and *Enterococcus* spp. in poultry meal or feather meal from 12 United States rendering companies. *Enterococcus* spp. were detected in 81.3% of the samples and accounted for up to 54% of the total bacterial counts in some samples. *Salmonella* was only detected in 8.7% of the samples.

To ensure the microbiological safety of rendering products, rendering facilities utilize thermal processing for 40 to 90 min at 240 to 290ºF (115.6 to 143.3ºC) (Meeker and Hamilton, 2006). Marginal processing conditions potentially could result in microbial survival (Crump et al., 2002). Thermal death time (TDT) is a factor of time, temperature, material matrix and organism (Heldman and Hartel, 1998). Decimal reduction time (D value) indicates the time required for a one $\log_{10}$ reduction of a particular organism at a specific temperature (Heldman and Hartel, 1998). TDT of *Salmonella* has been investigated in food products (Murphy et al., 2000; D'Aoust, 2001; Murphy et al., 2004; Bucher et al., 2008), but few studies have been conducted in rendered animal products. Franco (1997 and 2005) conducted surveys of *Salmonella* in rendered animal co-products and suggested rendering processes destroy *Salmonella*. Ramirez-Lopez (2006) studied TDT of a single unknown isolate from animal co-products. However, data has never been generated on TDT of *Salmonella* in rendered poultry materials. Since this factor must consider the parameters of matrix, temperature and organism, it was necessary to conduct validation in the actual rendering material matrices. The objective of this study was to determine the TDT and D values for four pathogenic *Salmonella* recognized by FDA as hazardous for animal feeds (*Salmonella* Choleraesuis*, Salmonella* Enteritidis*, Salmonella* Newport and *Salmonella* Dublin) in poultry rendering materials containing 50% fat content (FDA, 2010; FDA, 2013) at 240ºF (115.6ºC).

Materials and Methods

Rendering Sample Preparation

Samples of poultry crax and poultry fat were obtained from a southeastern rendering company on three separate days. Crax is a solid material composed of protein, minerals, and residual fat that is discharged from the screw press during the rendering process and is typically further ground into meat and bone meal (Meeker and Hamilton, 2006). The crax samples were submitted in duplicate to Clemson University Agricultural Service Laboratory for ash, fat, and moisture content analysis. The fat and crax samples were 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 (ddH_2O) (0.6 g per L) for approximately 2 min, followed by rinsing 5 times with sterile ddH_2O. 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 was used to scrape material from the sides during pauses in processing. All samples were stored under refrigeration until needed for experimentation.

Salmonella Preparation

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 obtained 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 from collaborator Dr. Xiuping Jiang at Clemson University.

A preliminary study was conducted to determine the optimal media conditions for *Salmonella* growth. Trypticase soy broth (TSB) (90000-050, VWR Scientific Products, Suwanee, GA), TSB with the addition of 0.1% (wt/vol) yeast extract (MP Biomedicals, LLC, Solon, Ohio), and brain heart infusion broth (BHI) (211059, VWR Scientific Products, Suwanee, GA) were tested. TSB with the addition of 0.1% (wt/vol) yeast extract was chosen as the best media based highest cell densities determined from optical density measurements (μQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, VT) at 600 nm and dilution plating in duplicate onto onto bismuth sulfite agar (90003-904, VWR Scientific Products, Suwanee, GA), Hektoen enteric agar (9004-054, VWR Scientific Products), xylose lysine deoxycholate (XLD) (90003-996, VWR Scientific Products), and trypticase soy agar (TSA) (90000-050, VWR Scientific Products).

As a preliminary study, each individual *Salmonella* serotype was plated onto bismuth sulfite agar, Hektoen enteric agar, XLD, and TSA. Enumeration data indicated use of XLD and TSA as the preferred agar media for enumerating SC, SE, SN, and SD.

A preliminary goal of this experiment was to obtain concentrated bacterial slurry to use in inoculating poultry rendering materials for thermal processing. The average concentrations of cells in broth for SC, SE, SN, and SD after 24 h incubation at 35ºC were 8.66±0.02, 8.56±0.03, 8.80±0.06, and 8.65±0.03 $\log_{10}$ cfu/g, respectively. Preliminary experiments were conducted to determine the volume of culture as well as concentration rate necessary. Enumeration on XLD and TSA verified that 5 L of a 24 h *Salmonella* culture grown in TSB with 0.1% (wt/vol) yeast extract and then concentrated by centrifugation was optimal. Centrifugation was conducted 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) and the supernatant was discarded after autoclaving. The pellet was resuspended in 5 mL sterile TSB. In preliminary studies conducted 3 times in duplicate (n=6), the average bacterial slurry concentrations for SC, SE, SN, and SD were 12.60±0.15, 12.12±0.01, 12.28±0.03, and 12.16±0.15 $\log_{10}$ cfu/g, respectively. This procedure was used to prepare the bacterial cultures used throughout the experiment.

Each *Salmonella* slurry, prepared as above, was inoculated into poultry rendering material at the rate of 100 μL culture per 1 g sample. A preliminary study was conducted to determine the difference in mean bacterial counts of the inoculated samples versus the bacterial slurry in TSB with 0.1% (wt/vol) yeast extract using two different methods. Method 1 was the serial dilution of each broth culture as well as each inoculated sample to 10^{-14} utilizing the standard Class O phosphate/magnesium chloride dilution buffer (Wehr and Frank, 2004). Method 2 was the serial dilution of each broth culture and sample to 10^{-14} using pre-warmed (32ºC) modified Class O phosphate/magnesium chloride diluent. Controls included media and uninoculated poultry rendering samples. Each experiment was conducted 3 times in duplicate (n=6).

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. These tubes were capped (60825-801, VWR International, Suwanee, GA) and autoclaved. Poultry rendering samples (50% fat) were aseptically transferred (1 g) into sixteen sterile tubes. 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. 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 individual culture (100 µL) was directly pipetted into 1 g of the heated rendering samples. After culture inoculation, the sample was pipetted up and down approximately 4 times to thoroughly mix. Upon inoculation and mixing, time measurements (0, 15, 30, 60, 90, 120, 180, 240, 300, 360, and 420 s) began on the thermal treatment. Samples were placed on ice immediately after thermal treatment. Additional sample tubes containing poultry rendering used for unheated controls were placed on ice until utilized for plating. All samples were processed for microbial content immediately after conclusion of heat treatments.

A preliminary experiment was conducted to validate the use of 1 g of sample pre-enriched in 5 mL of sterile universal pre-enrichment broth (UPB) (95021-036, VWR Scientific Products, Suwanee, GA) in comparison to 1 g of sample pre-enriched in 9 mL I of UPB as per recommendations in the FDA Bacteriological Analytical Manual (BAM) (Andrews et al., 2011). The stainless steel tubes used in this experiment would not hold 1 g of sample pre-enriched in 9 mL of UPB. Results indicated that the 1:5 ratio of sample to pre-enrichment broth was as effective as the 1:9 ratio of sample to pre-enrichment broth. Therefore, this procedure was used throughout the experiment.

Once 5 mL of sterile UPB 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 for 30 s. Each UPB diluted sample (0.1 mL) was directly pipetted onto XLD and TSA plates and spread using an alcohol-flamed bent glass rod. As a control, each *Salmonella* slurry was serially diluted to 10^{-12} in the standard Class O phosphate/magnesium chloride dilution buffer and either 1.0 mL or 0.1 mL was spread plated onto XLD and TSA. Media and dilution buffer controls also were conducted. All plates were incubated overnight at 35ºC. In this experimental design, XLD selected for *Salmonella* spp. while TSA measured total bacterial counts (aerobic, mesophilic), which included any background bacteria and in test samples background bacteria plus inoculated *Salmonella.* For each inoculate or uninoculated poultry rendering sample, dilutions were carried out such that the direct plating on XLD and TSA had a lower detection limit of 1.4 $\log_{10}$ cfu/g.

Because the direct plate counting method had a lower detection limit of 1.4 $\log_{10}$ cfu/g, an additional experiment was conducted in accordance with FDA Bacteriological Analytical Manual (BAM) procedures; this second experiment had a detection limit of 1 cfu/g (Andrews et al., 2011). The remaining UPB diluted sample in the stainless steel tube was incubated overnight at 35ºC and then vortexed (Super Mixer, 1290, Labline Instruments, Inc., Melrose Park, IL) on the fast setting for approximately 30 s. The sample (0.1 mL) was aseptically pipetted to Rappaport-Vassiliadis (RV) pre-enrichment broth (10 mL) (95039-382, VWR Scientific Products, Suwanee, GA). The same sample (1 mL) was aseptically pipetted to 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 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.

As per FDA BAM recommendations to validate positive samples obtained from the RV and TT pre-enrichments, two confirmation tests were conducted (Feng, 2001). Latex agglutination tests (FT0203**,** Thermo Fisher Scientific, Waltham, MA 02454) and ChromAgar™ (90006-158, VWR Scientific Products, Suwanee, GA) were conducted using the each *Salmonella* culture as a

control (BD Diagnostics, 2008; Oxoid Limited, 2013). In order to analyze the data, when duplicate results from the pre-enriched samples were both negative the data was reported as 0.0 (Figure 4.1). 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 (Figure 4.1).

Determination of Estimated D Values

The direct plate count of each concentrated *Salmonella* slurry and the time at which each culture was destroyed were compared on a graph. In a preliminary experiment, percent recoveries of *Salmonella* from inoculated poultry samples were calculated for each recoverable *Salmonella* population density. Due to the experimental design, the actual population count from poultry rendering material was not conducted. However, the total count in each bacterial slurry was measured. This population count was used in estimated D value calculations. The final time the population was no longer detected was used as the thermal death time. These data were graphed and the slope of the line was used to calculate the estimated the D value.

Results

Analysis of poultry rendering materials indicated mean fat content was 15.97±1.13%, mean ash content was 10.55±1.14% and mean moisture content was 3.73±0.33%. 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.

Preliminary results indicated that the average concentration of the culture slurries of SC, SE, SN, and SD were 12.60±0.15, 12.12±0.01, 12.28±0.03 and 12.16±0.15 $\log_{10}$ cfu/g, respectively. The mean bacteria counts ± standard error on XLD from inoculated poultry rendering materials were 10.47±0.20 15, 10.59±0.23, 10.43±0.22 and 10.40±0.13 $\log_{10}$ cfu/g, respectively (Table 4.1).

All *Salmonella* counts were conducted in a two-step process. Enumeration on XLD had a lower detection limit of 1.4 $\log_{10}$ cfu/g. Under all treatment conditions, SC, SE, SN and SD were reduced to or below the lower detection limit after initial thermal treatment (0 s) in inoculated poultry samples (Figure 4.2). *Salmonella* levels were reduced to or below lower detection limit during after initial thermal treatment (0 s) in uninoculated poultry control samples (Figure 4.3).

Enumeration on TSA had an upper detection limit of 4.3 $\log_{10}$ cfu/g. Under all treatment conditions, total bacterial counts in the SC, SE, SN, and SD trials were above the upper detection limit after all thermal treatments in inoculated poultry samples (Figure 4.4). Total bacterial counts were above the upper detection limit after all thermal treatments in all uninoculated poultry samples (Figure 4.5).

Pre-enrichment results on RV and TT were confirmed using both latex agglutination and ChromAgar™; the following results are reported as confirmed findings. In general, *Salmonella* serotypes in heated inoculated samples declined with longer thermal treatment (Figure 4.6 and 4.8). The positive counts for *Salmonella* in each inoculated and uninoculated sample in either RV or TT validated by the two confirmation tests are shown in Tables 4.4 and 4.5. Populations of *Salmonella* in the SC inoculated samples were reduced, but did not appear to be eliminated until 360 s in RV pre-enrichments. Although populations were reduced, *Salmonella* levels did not appear to be destroyed until 420 s in TT pre-enriched, SC inoculated samples (Figure 4.6 and 4.8). In RV, *Salmonella* in the SE inoculated samples was present at every time interval until it appeared to be eliminated at 420 s (Figure 4.6). Populations of *Salmonella* in the SE inoculated samples in

TT were reduced to 0 at 90 s, were present at 120, 180, 240, 300 and 360 s, and appeared to be killed at 420 s (Figure 4.8). Levels of *Salmonella* in the SN inoculated samples were reduced to 0 at 120 s, were present at 180 s, but were eliminated at 240 s in RV (Figure 4.6). In TT, *Salmonella* populations were reduced to 0 at 120 s, but were present again until 420 s in SN inoculated samples (Figure 4.8). For SD samples pre-enriched in RV, *Salmonella* levels decreased until reaching 0 at 90, 120, 180 and 240 s, but *Salmonella* was present at 300 s on RV pre-enrichments. *Salmonella* appeared to be eliminated at 360 s and thereafter (Figure 4.6). In TT, *Salmonella* was reduced to 0 at 300 s, was present at 360 s and appeared to be killed at 420 s in SD inoculated samples (Figure 4.8). Since 420 s was the maximum time tested, future studies should include longer treatment times (Figure 4.6 and 4.8).

Variations were noted in *Salmonella* populations in heated uninoculated samples (Figure 4.7 and 4.9). *Salmonella* was detected at 0, 60, 90, 120, 180, 240 and 360 s in RV pre-enrichments for SC uninoculated samples but was not detected at 15, 30, 300, and 420 s (Figure 4.7). In TT, *Salmonella* levels in the uninoculated controls for SC were not reduced to 0 until 420 s (Figure 4.9). In RV and TT pre-enrichments for the uninoculated SE samples, populations of *Salmonella* were present in all thermal treatment times up to 420 s (Figure 4.7 and 4.9). For the uninoculated SN samples, *Salmonella* was present at 15, 30, 60, 90, 180, 240, and 300 s in RV (Figure 4.7). In TT, *Salmonella* was not detected in uninoculated SN controls at 0, 120, 360, and 420 s (Figure 4.9). Levels of *Salmonella* in uninoculated SD samples were not detected at 60, 90, 120, 180, 360 and 420 s in RV (Figure 4.7). In TT, *Salmonella* was not detected at 0 s and 420 s but was present at all other thermal treatment times in the uninoculated SD samples (Figure 4.9).

The estimated D values for *Salmonella* in poultry rendering samples containing 50% fat at 115.6ºC pre-enriched in RV and validated by two confirmation tests were calculated. SC, SE, SN, and SD had D values of 0.60, 0.67, 0.39, and 0.58 min, respectively (Table 4.2). The estimated D values for *Salmonella* serotypes in poultry rendering samples containing 50% fat at 115.6ºC pre-enriched in TT and validated by two confirmation tests also were determined. SC, SE, SN, and SD had D values of 0.70, 0.67, 0.67, and 0.67 min, respectively (Table 4.3).

Discussion

Since large numbers of samples were plated per day, a preliminary experiment was conducted to determine the percent recoveries ± standard error for each *Salmonella* culture from poultry rendering instead of conducting a full dilution series on each day of plating. The preliminary study allowed for the reduction of plating of each inoculated, unheated sample through extended dilutions during the study. However, future experiments should be designed to conduct the plating of each inoculated, unheated sample to obtain more accurate data.

Enumeration on XLD indicated that SC, SE, SN, and SD were reduced to below the detection limit after the initial thermal treatment in inoculated and uninoculated rendering samples (Figures 4.2 and 4.3). The presence of the bacteria in the background of the rendering samples was indicated through enumeration on TSA for both inoculated and uninoculated samples (Figures 4.4 and 4.5). The mean bacterial counts of all samples, under all thermal treatments, were above the detection limit of 4.3 $\log_{10}$ cfu/g. The current study enumerated total bacterial content in finished rendered materials. However, Glenn (2006) conducted a study on the bacterial loads in raw rendering materials and detected high levels of microbial content. Diverse populations of non-pathogenic and pathogenic heat-resistant bacteria could be contaminants in rendering materials due to either survival of the rendering cooking process or post-process contamination. Therefore,

the presence of 4.3 $\log_{10}$ cfu/g in the rendering samples is not unexpected. After thermal treatments of 420 s at 115.6ºC, bacterial populations were still present as measured on TSA indicating the presence of heat resistant bacteria in the background of the rendering samples. Autoclaving requires exposure to 121ºC at 15 psi of pressure for a minimum of 15 min to kill most bacteria (Laroussi and Leipold 2004). Bacterial endospores are very heat resistant and in certain cases are not killed under autoclave conditions (Tuominen et al. 1994). Therefore, the thermally-resistant bacteria in the background of rendering materials could potentially be spore-forming bacteria. The design of this experiment did not allow for further analysis of these heat-resistant bacteria. However, future experiments are needed to isolate and identify these bacterial species through genetic analysis or serotyping.

Results of RV and TT pre-enrichments indicated variation in recovery of *Salmonella* amongst SC, SE, SN, and SD inoculated and uninoculated samples. SC or organisms detected as *Salmonella* were present in both inoculated and uninoculated samples pre-enriched in RV and TT but it appeared more frequently in TT pre-enriched samples. In RV and TT, SE or bacteria detected as *Salmonella* were present in both inoculated and uninoculated samples at all thermal treatment times up to 420 s, except in inoculated samples pre-enriched in TT at 90 s. The presence of SN or organisms detected as *Salmonella* peaked at 90 s, decreased to 0 at 120 s, and re-emerged at 180 s in both RV and TT pre-enriched inoculated samples. SN or a *Salmonella*-like bacterial species was detected in uninoculated samples pre-enriched in TT at 90 s, not detected at 120 s, and detected again at 180 s. This trend was also observed in uninoculated samples pre-enriched in RV. SD or organisms detected as *Salmonella* were present in both inoculated and uninoculated samples pre-enriched in RV and TT but it appeared more frequently in TT pre-enriched samples. Positive results in inoculated samples may be due to background organisms. It should be noted that a positive *Salmonella* result from current methodology on either inoculated or uninoculated was not validated by genetic analysis or serotyping which would be necessary for confirmation in this study.

Due to the nature of rendering material collection, *Salmonella* could be present in the porous structure of bone. Additionally, *Salmonella* could have been coated in fat or tissue allowing for a protective effect due to slower thermal conductivity of bone particles, fat and/or tissue. The samples in this study were randomly placed in the heating block and therefore, this factor was not considered a cause for the observed variability.

The presence of a thermally resistant organism reacting as *Salmonella* has been well-noted in the rendering samples in this study. The rendering process recycles inedible animal tissue to produce products that can be used in animal feed. Therefore, it can be hypothesized that an unknown bacterial strain(s) may have acquired thermal resistance and/or *Salmonella*-like characteristics through repetitive cycles of rendered animal feed to animals to rendering. In this hypothesis, inedible animal tissues including the gastrointestinal tract and its inherent microbes would be rendered and the cycle through animal feed to animal to slaughter to rendering would repeat. These conditions potentially could select for thermally resistant microbes. Since this hypothesis has not been tested, it is vital that this unknown strain(s) is isolated in future experimentation to determine its identity and characteristics.

Preliminary estimated D values were calculated. In general, with increase in temperature, thermal lethality increases (Earle and Earle 1983). Liu et al. (1969) reported D values for *Salmonella* senftenberg 775 W were highly variable between 10 to 115 min at 70ºC in meat and bone meal. Lui et al. (1969) conducted their study in meal and the current study was conducted in cooked poultry rendered products containing 50% fat content. Similar to the Lui et al. (1969) study,

the D values of this study were variable and high which could potentially be due to the thermally resistant background organism(s).

Further research needs to be conducted at 115.6ºC for longer time intervals to ensure that SC, SE, SN and SD are destroyed. It should be noted the results of this study were obtained from the lower end of the cooking temperatures utilized in the rendering industry. Many rendering facilities process materials at higher temperatures closes to 280ºF (137.8ºC) to 290ºF (143.3ºC) for 40 to 90 min in order to produce microbiologically safe products (Meeker and Hamilton 2006). However, the industry also employs a different type of cooker known as a Carver-Greenfield unit. These units operate under vacuum at lower temperatures, typically closer to 240ºF (115.6ºC) to process the materials (Meeker and Hamilton 2006).

It was necessary to grind rendering materials for transfer into stainless steel tubes. Factors for comparing data to typical bone particle sizes will be necessary for future experiments. Thermal conductivity studies on large bone particles could provide further understanding of thermal lethality in rendering materials.

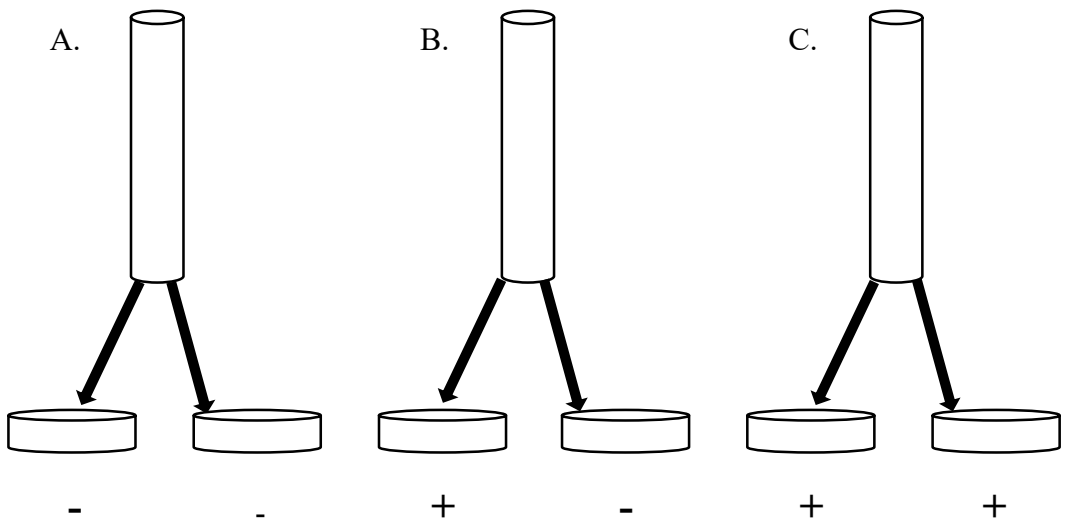

Figure 4. 0.0 ıod utilized to report F 0.5 TT pre-enrichments ı 1.0 ɔn XLD confirmed by two confirmation tests at each thermal treatment. The result was assigned a 0 if both plates were negative (A). The result was assigned a 0.5 if one was positive and one was negative (B). The result was assigned a 1.0 if both were positive (C).

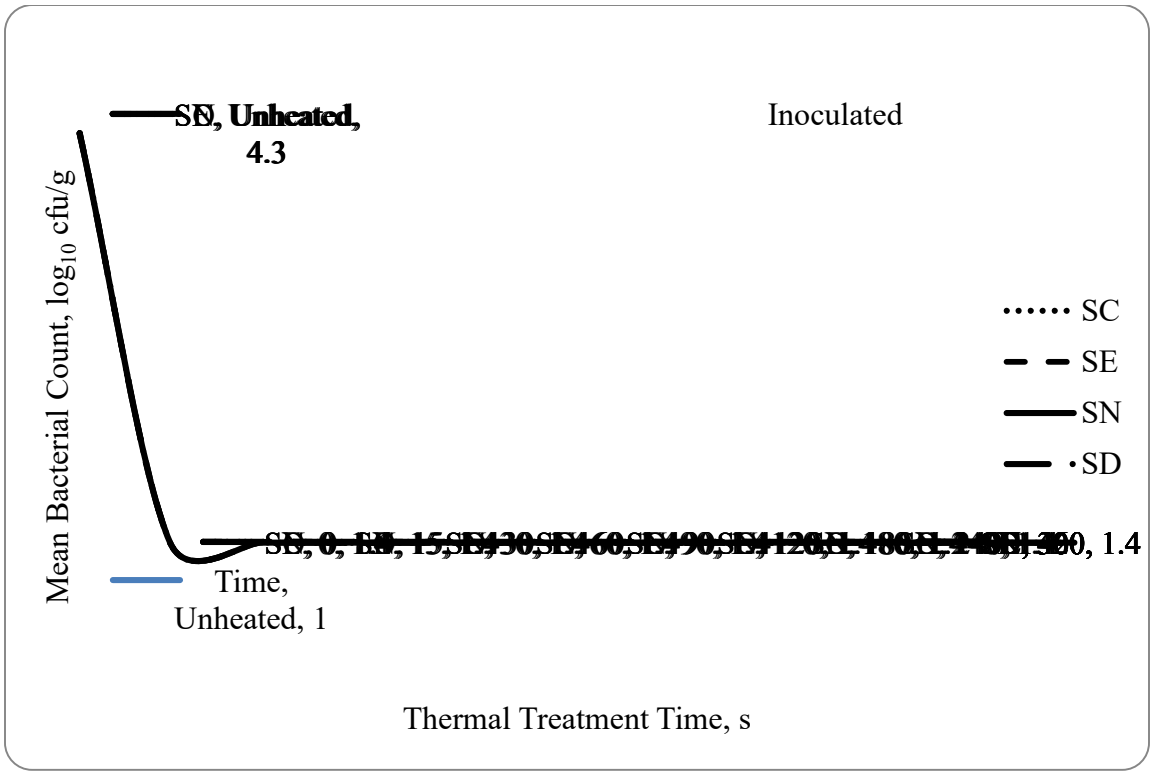

Figure 4.2. Enumeration of *Salmonella* on XLD from poultry rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]The lower limit of detection is 1.4 $\log_{10}$ cfu/g of *Salmonella* (n=24).

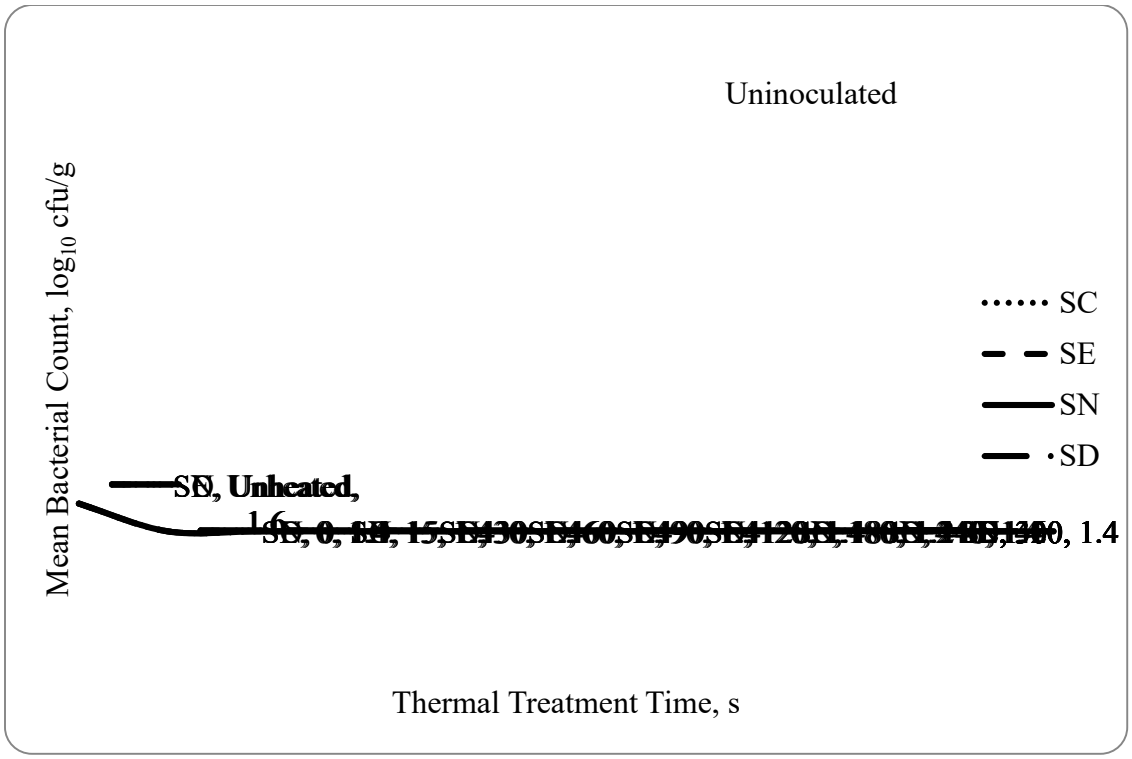

Figure 4.3. Enumeration of *Salmonella* on XLD from uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]The lower limit of detection is 1.4 $\log_{10}$ cfu/g of *Salmonella* (n=24).

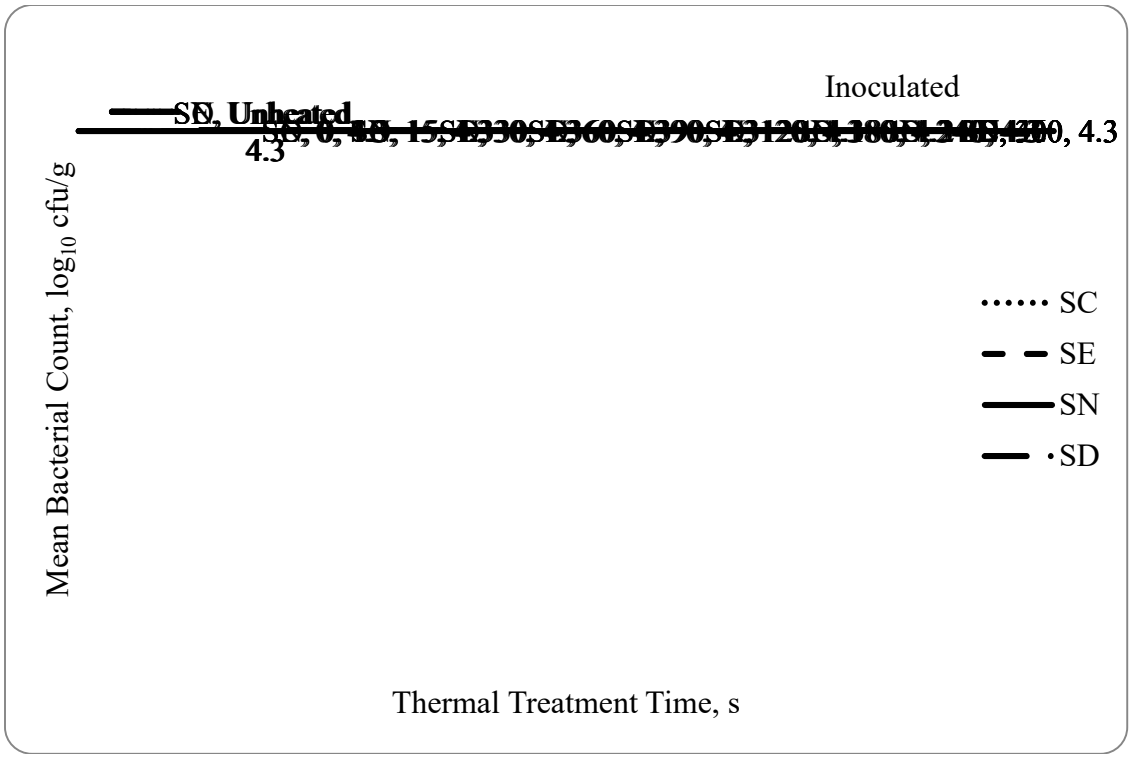

Figure 4.4. Enumeration of total bacteria on TSA from poultry rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]The lower limit of detection is 1.4 $\log_{10}$ cfu/g of *Salmonella* (n=24).

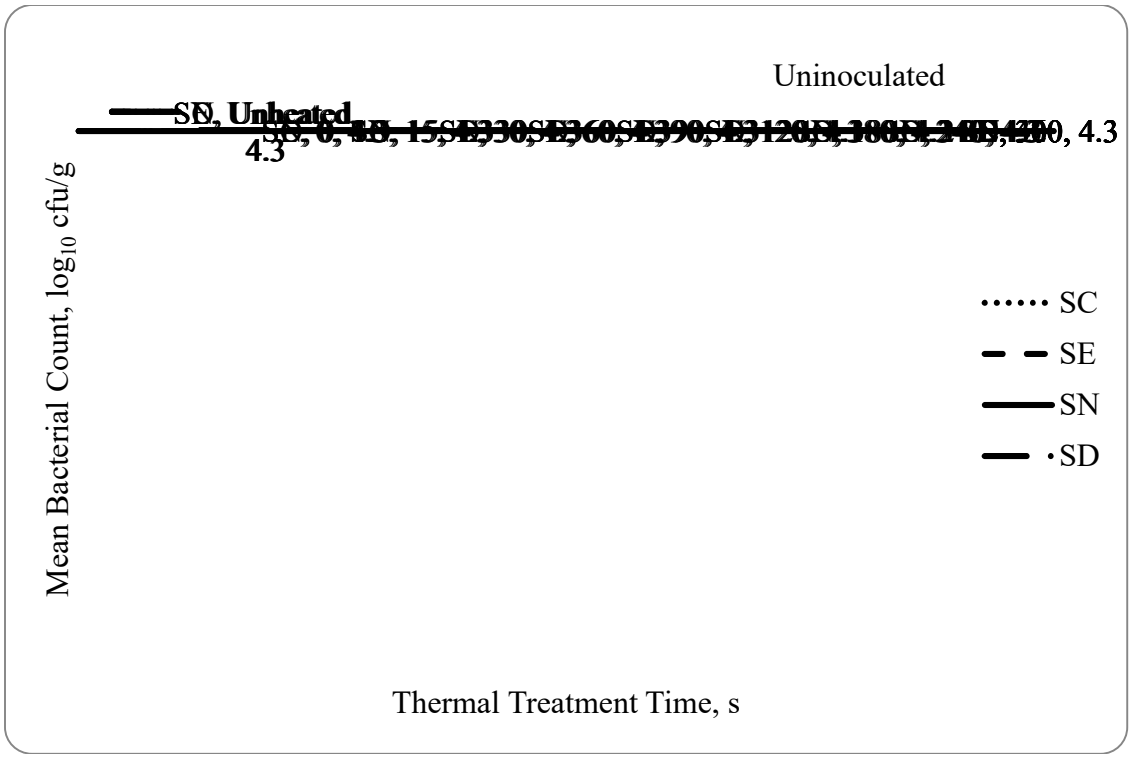

Figure 4.5. Enumeration of total bacteria on TSA from uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]The lower limit of detection is 1.4 $\log_{10}$ cfu/g of *Salmonella* (n=24).

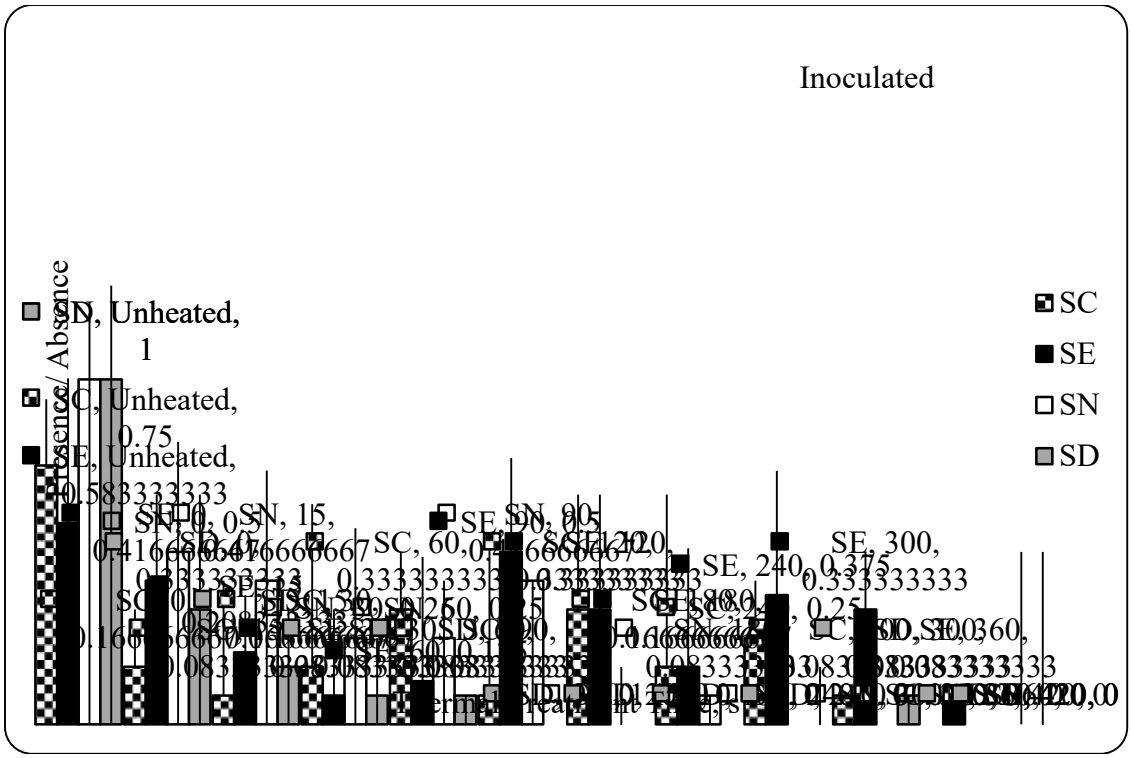

Figure 4.6. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, RV pre-enriched poultry rendering samples (50% fat).[1]

[1]A count of 0 represents the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=24).

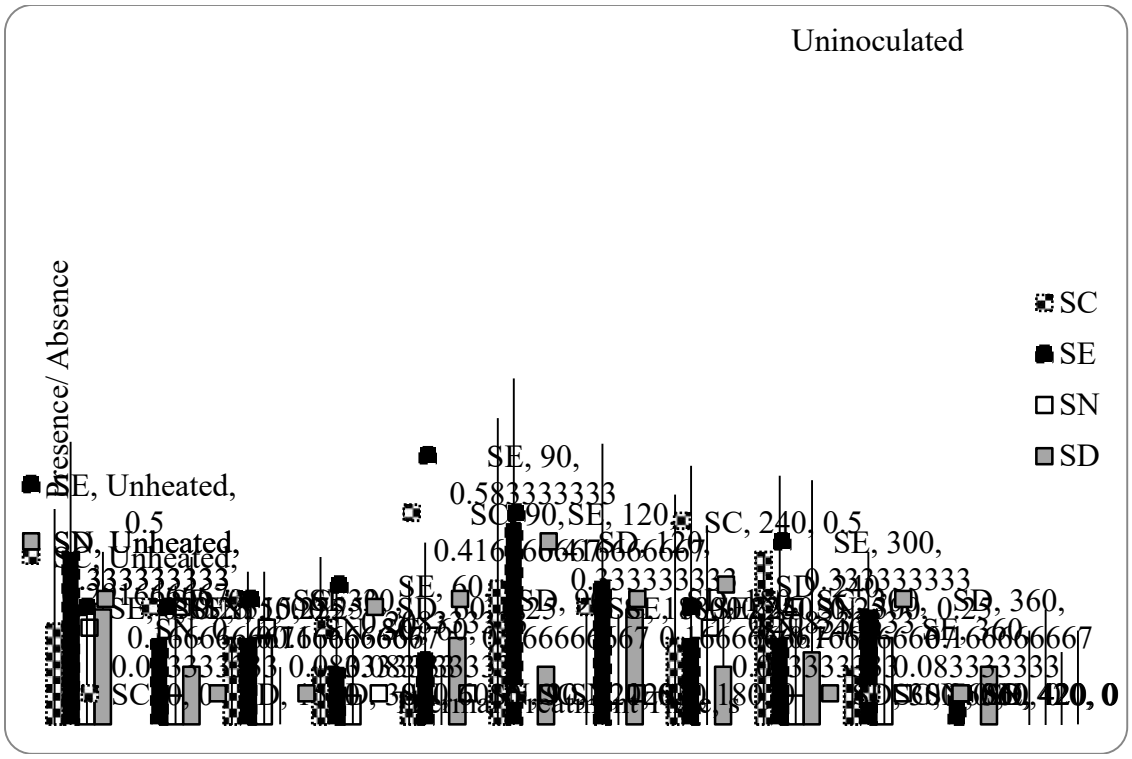

Figure 4.7. Presence or absence ± standard deviation of *Salmonella* for each RV pre-enriched, uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]A count of 0 represents the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=24).

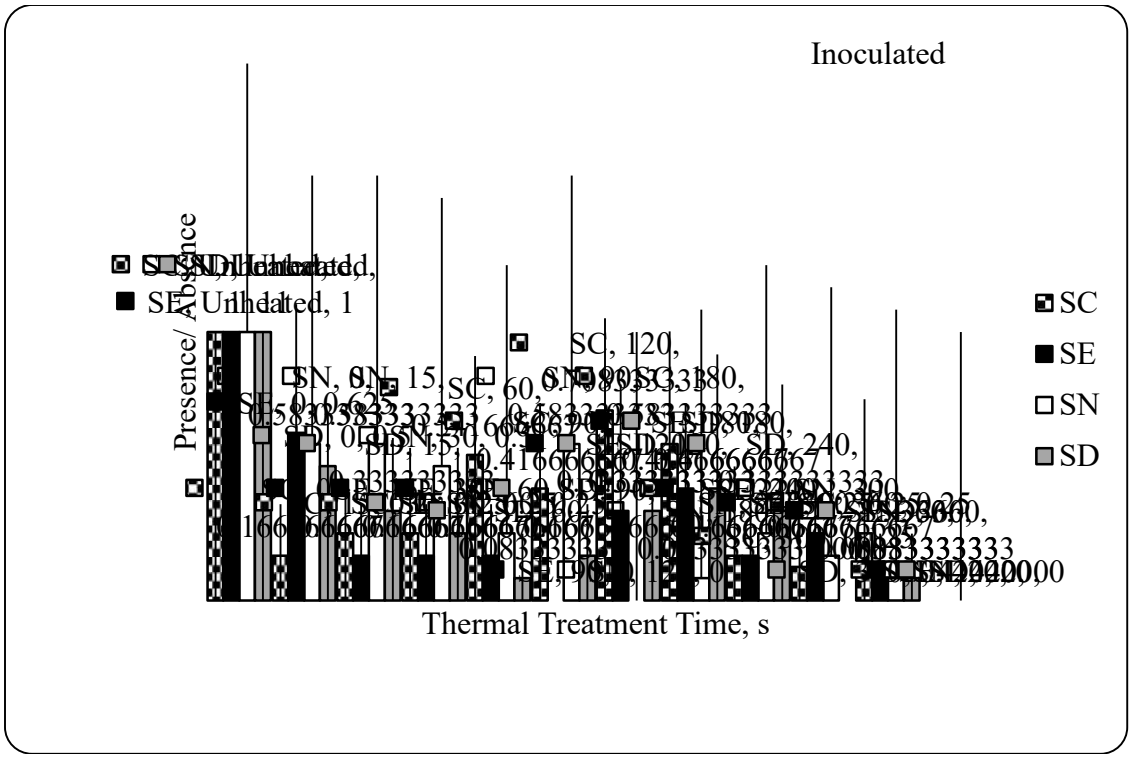

Figure 4.8. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, TT pre-enriched poultry rendering samples (50% fat).[1]

[1]A count of 0 represents the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=24).

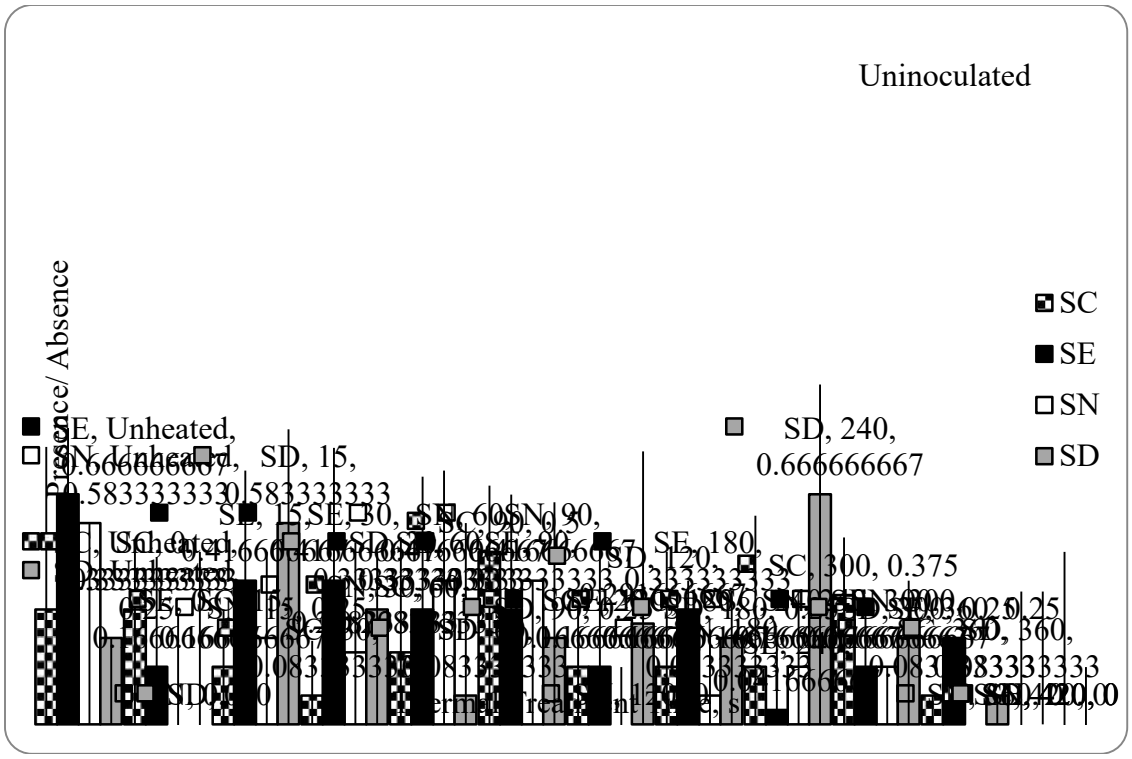

Figure 4.9. Presence or absence ± standard deviation of *Salmonella* for each TT pre-enriched, uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[1]A count of 0 represents the absence of *Salmonella*, while a count of 1 represents the presence of *Salmonella* (n=24).

Table 4.1. *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD) after inoculation into poultry rendering materials and plated onto XLD (n=6).

Serotype	Average Broth Culture, log_{10} cfu/g ± standard error	Average in Poultry Samples, log_{10} cfu/g ± standard error
SC	12.60±0.15	10.47±0.20
SE	12.12±0.01	10.59±0.23
SN	12.28±0.03	10.43±0.22
SD	12.16±0.15	10.40±0.13

Table 4.2. Estimated D values for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) in poultry rendering samples (50% fat) at 115.6ºC pre-enriched in RV and validated by two confirmation tests.

Serotype	Estimated D Value, min
SC	0.60
SE	0.67
SN	0.39
SD	0.58

Table 4.3. Estimated D values for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) in poultry rendering samples (50% fat) at 115.6ºC pre-enriched in TT and validated by two confirmation tests.

Serotype	Estimated D Value, min
SC	0.70
SE	0.67
SN	0.67
SD	0.67

Table 4.4. Number of samples positive for *Salmonella* in *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD) inoculated poultry rendering samples (50% fat) after pre-enrichment in RV or TT broth (n=24).

		RV	TT
Serotype	Thermal Treatment Time, s	Number of Positive Samples	Number of Positive Samples
SC	Unheated	18 out of 24 samples	24 out of 24 samples
	0	4 out of 24 samples	4 out of 24 samples
	15	2 out of 24 samples	6 out of 24 samples
	30	4 out of 24 samples	6 out of 24 samples
	60	8 out of 24 samples	13 out of 24 samples
	90	2 out of 24 samples	10 out of 24 samples
	120	8 out of 24 samples	17 out of 24 samples
	180	4 out of 24 samples	14 out of 24 samples
	240	6 out of 24 samples	4 out of 24 samples
	300	2 out of 24 samples	3 out of 24 samples
	360	0 out of 24 samples	6 out of 24 samples
	420	0 out of 24 samples	0 out of 24 samples
SE	Unheated	14 out of 24 samples	24 out of 24 samples
	0	10 out of 24 samples	15 out of 24 samples
	15	5 out of 24 samples	4 out of 24 samples
	30	2 out of 24 samples	4 out of 24 samples
	60	3 out of 24 samples	4 out of 24 samples
	90	12 out of 24 samples	0 out of 24 samples
	120	8 out of 24 samples	8 out of 24 samples
	180	4 out of 24 samples	10 out of 24 samples
	240	9 out of 24 samples	4 out of 24 samples
	300	8 out of 24 samples	6 out of 24 samples
	360	2 out of 24 samples	2 out of 24 samples
	420	0 out of 24 samples	0 out of 24 samples
SN	Unheated	24 out of 24 samples	24 out of 24 samples
	0	12 out of 24 samples	14 out of 24 samples
	15	10 out of 24 samples	14 out of 24 samples
	30	6 out of 24 samples	12 out of 24 samples
	60	6 out of 24 samples	6 out of 24 samples
	90	10 out of 24 samples	14 out of 24 samples
	120	0 out of 24 samples	0 out of 24 samples
	180	2 out of 24 samples	2 out of 24 samples
	240	0 out of 24 samples	6 out of 24 samples
	300	0 out of 24 samples	4 out of 24 samples
	360	0 out of 24 samples	2 out of 24 samples
	420	0 out of 24 samples	0 out of 24 samples
SD	Unheated	24 out of 24 samples	24 out of 24 samples
	0	8 out of 24 samples	12 out of 24 samples
	15	4 out of 24 samples	8 out of 24 samples

30	2 out of 24 samples	6 out of 24 samples
60	2 out of 24 samples	2 out of 24 samples
90	0 out of 24 samples	4 out of 24 samples
120	0 out of 24 samples	8 out of 24 samples
180	0 out of 24 samples	10 out of 24 samples
240	0 out of 24 samples	8 out of 24 samples
300	2 out of 24 samples	0 out of 24 samples
360	0 out of 24 samples	2 out of 24 samples
420	0 out of 24 samples	0 out of 24 samples

Table 4.5. Number of samples positive for *Salmonella* in uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN) and *Salmonella* Dublin (SD) after pre-enrichment in RV or TT broth (n=24).

		RV	TT
Serotype	Thermal Treatment Time, s	Number of Positive Samples	Number of Positive Samples
SC	Unheated	8 out of 24 samples	8 out of 24 samples
	0	4 out of 24 samples	8 out of 24 samples
	15	0 out of 24 samples	4 out of 24 samples
	30	0 out of 24 samples	2 out of 24 samples
	60	6 out of 24 samples	5 out of 24 samples
	90	4 out of 24 samples	12 out of 24 samples
	120	8 out of 24 samples	4 out of 24 samples
	180	4 out of 24 samples	4 out of 24 samples
	240	5 out of 24 samples	4 out of 24 samples
	300	0 out of 24 samples	9 out of 24 samples
	360	4 out of 24 samples	2 out of 24 samples
	420	0 out of 24 samples	0 out of 24 samples
SE	Unheated	12 out of 24 samples	16 out of 24 samples
	0	6 out of 24 samples	4 out of 24 samples
	15	6 out of 24 samples	10 out of 24 samples
	30	4 out of 24 samples	10 out of 24 samples
	60	5 out of 24 samples	8 out of 24 samples 9
	90	14 out of 24 samples	8 out of 24 samples
	120	10 out of 24 samples	4 out of 24 samples
	180	6 out of 24 samples	8 out of 24 samples
	240	6 out of 24 samples	1 out of 24 samples
	300	8 out of 24 samples	4 out of 24 samples
	360	2 out of 24 samples	7 out of 24 samples
	420	0 out of 24 samples	0 out of 24 samples
SN	Unheated	7 out of 24 samples	14 out of 24 samples
	0	0 out of 24 samples	0 out of 24 samples
	15	6 out of 24 samples	6 out of 24 samples
	30	4 out of 24 samples	5 out of 24 samples
	60	2 out of 24 samples	10 out of 24 samples
	90	10 out of 24 samples	10 out of 24 samples
	120	0 out of 24 samples	0 out of 24 samples
	180	6 out of 24 samples	2 out of 24 samples
	240	12 out of 24 samples	4 out of 24 samples
	300	4 out of 24 samples	4 out of 24 samples
	360	0 out of 24 samples	0 out of 24 samples
	420	0 out of 24 samples	0 out of 24 samples
SD	Unheated	8 out of 24 samples	6 out of 24 samples
	0	2 out of 24 samples	0 out of 24 samples
	15	6 out of 24 samples	14 out of 24 samples

30	2 out of 24 samples	8 out of 24 samples
60	0 out of 24 samples	2 out of 24 samples
90	0 out of 24 samples	6 out of 24 samples
120	0 out of 24 samples	7 out of 24 samples
180	0 out of 24 samples	6 out of 24 samples
240	2 out of 24 samples	16 out of 24 samples
300	6 out of 24 samples	6 out of 24 samples
360	0 out of 24 samples	2 out of 24 samples
420	0 out of 24 samples	0 out of 24 samples

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Impacts and Significance:
Validation of thermal death time of *Salmonella* species recognized as of importance to animal feeds will give the rendering industry evidence of the safety of animal co-products.

Publications:
Two refereed journal articles are in preparation for submission to the Journal of Poultry Science and the Journal of Animal Science.

Outside funding:
Three proposals were submitted to USDA for funding of the thermal death time of pathogenic microorganisms in rendered products. Reviews were dismal including from one USDA reviewer who claimed there is no reason to do this work: "However, currently rendered animal products are not fed to food animals in teh United States. Therefore, finding Salmonella in offals and controlling it will not have an ultimate animal health and U.S. Agriculture significance [sic]." We were further discouraged by the USDA program administrator from applying in the future for any funding related to rendered animal products. We encourage members of the rendering industry to include educational programs that somehow can be directed toward USDA program administrators during the annual June Fly-In.

Future Work:
Work is continuing on validation of thermal death time of *Salmonella* in rendered products at different temperatures.

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