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Validation of Thermal Destruction of Pathogenic Bacteria in Rendered Animal Products

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VALIDATION OF THERMAL DESTRUCTION OF PATHOGENIC BACTERIA IN RENDERED ANIMAL PRODUCTS

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ABSTRACT

This study tested enumeration techniques for high fat food matrices and determined thermal death times in rendering animal products. Standard Class O phosphate/magnesium chloride dilution buffer series (Dilution Series A) and a modified (pre-warmed to 32ºC) lecithin phosphate dilution buffer series (Dilution Series B) were used to enumerate a *Salmonella* cocktail from both poultry and beef rendering materials. Results of this study indicate use of a modified lecithin buffer did not improve *Salmonella* enumeration accuracy from rendering materials. Instead, the results suggested use of xylose lysine deoxycholate agar (XLD) with either buffer system accurately enumerated *Salmonella* from rendering materials.

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*.

DEDICATION

This dissertation is dedicated to my family and many friends. A special dedication in gratitude goes to my grandmother, Ellen Davis, whose words of encouragement helped me to achieve my goals. I dedicate this work to my parents, Ann and Michael Hayes, and my brother, Michael Hayes. Without their love and support, this could not have been possible. I also dedicate this work and give special thanks to my fiancé, Brian Gaines, for being there for me on the tough days. Finally, I would like to dedicate my dissertation to Neko, Bella, Vixen, Hitch, Nokie, Kitty, and Willow, for their hugs and unconditional love.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Rendering is the recycling of residual animal tissue from food animals into stable, value-added materials for animal feeds, chemical feedstocks, and fertilizers. Approximately 50% of a food animal is considered edible; the remainder of the animal tissue is rendered into animal co-products (Meeker and Hamilton 2006). Non-utilization of animal co-products would create major aesthetic and potentially disastrous public health problems since these organic materials are highly perishable and laden with microorganisms, many of which can cause disease in both humans and animals (Meeker and Hamilton 2006).

Approximately 8 billion chickens, 1.6 billion turkeys, 100 million hogs, and 35 million cattle are slaughtered and processed each year in the United States (Meeker and Hamilton 2006; Richardson 2006). However, on average, only 51% of the live weight of cattle, 56% of the live weight of hogs, 63% of the live weight of broilers, and 43% of the live weight of most fish species can be considered edible by Americans and Canadians (Meeker and Hamilton 2006). Due to various dietary practices and taste preferences around the world, the term "edible" may be construed in different ways depending on the region or country. However, non-carcass materials such as liver, tongue, heart, kidney, thymus, stomach, cheeks, head trimmings, blood, lungs, fat, and bones are a source of nutrients and can be consumed by animals if properly processed by the rendering industry (Ockerman and Hansen 2000; Dos Santos 2013). In addition to non-carcass material,

increasing amounts of waste ready-to-eat (RTE) and/or heat-and-eat packaged foods are processed by the rendering industry (Kinley 2009). The rendering industry also processes waste cooking fats and oils from restaurants. The United States and Canadian rendering industry annually recycles over 61 billion pounds of residual products into animal feeds, fats and proteins to prevent waste of these materials and the overfilling of landfills. The rendering facilities predominantly produce meat and bone meal, poultry meal, hydrolyzed feather meal, blood meal, fish meal, and animal fats. In the United States, the rendering industry annually produces approximately 11.2 billion pounds of protein and 10.9 billion pounds of fats. Approximately 85% of rendered products are used as animal feed ingredients for livestock and pets (Meeker and Hamilton 2006). However, the National Renderers Association has reported over 3000 rendering product industrial applications identified in many areas including personal care, biofuel, and chemical industries (Meeker and Hamilton 2006).

Certain provisions are necessary for animal co-products to be effectively used. These requirements include a sufficient volume of animal co-products in a centralized location, a method to commercially process animal co-products into marketable goods, an efficient market to sell products produced from animal co-products, and storage systems for finished animal co-products. Not meeting these requirements leads to under-utilization of animal co-products (Ockerman and Hansen 2000; Clemen 1927).

The Rendering Process

Rendering is a process that involves heat and other procedures to separate water, fat, and protein contained in animal tissues. The temperature and length of time of the

cooking process can impact the quality of the finished product. Despite the type of raw material being processed, the rendering process is comprised of several universal phases (ICMSF 2000; Kinley 2009). Initially all raw material is transported to an area in the rendering plant where, if necessary, it is pre-crushed to reduce size prior to being fed onto a conveyer that transfers it into the cooker. The basic process involves collecting and sizing raw material as needed, heating to remove the water, removing the fat by draining and/or pressing, cooling, milling, and storing. Many variations of these operations have been developed according to the type of raw material, machinery, and the facility (ICMSF 2000). Raw animal materials vary but these materials typically contain approximately 60% water, 20% protein and mineral, and 20% fat before the rendering process (Meeker and Hamilton 2006).

Without barriers and other protections, the aerosols generated during raw material crushing have the potential to spread contaminating microbes in the rendering facility including areas where the finished product is handled (ICMSF 2000; Swingler 1982). The rendering cooking process is reported to be 40 to 90 min at 240 to 290ºF (115.6 to 143.3ºC) (Meeker and Hamilton 2006). Process control is performed and monitored via computers so that time/temperature processes for appropriate moisture loss is achieved. However, the exact time and temperature relationship for thermal death of specific microorganisms has not been established in rendering matrices. It has been demonstrated that the high fat and low water environment of batch dry rendered material will protect bacterial spores against thermal inactivation (Lowry et al. 1979; ICMSF 2000).

Therefore, some marginal processing conditions could result in finished products containing bacterial spores or other heat-resistant biological materials.

In North America, most of the rendering facilities utilize continuous-flow, dry rendering units. Only large animals such as cattle and hogs are crushed or chopped prior to processing, whereas smaller animals such as poultry are not ground prior to cooking. Once in the continuous cooker, steam is utilized to heat the internal metal components of the rendering cooker. In this type of dry continuous cooker unit, the steam transfers heat across metal heating surfaces to the rendering materials. The steam is condensed in a closed loop system so the water will never come in contact with the rendering materials. The condensed water is transported out of the cooker back to the steam generator (Ockerman and Hansen 2000; Kinley 2009).

In order to thermally process in a rendering cooker, raw materials are deposited into hot rendered fat and during the cooking process, moisture is removed by evaporation. After the cooking process, the protein/bone material and molten fat are initially separated by a screen drainer and an auger conveyor that moves the materials to the screw press. The screw press removes additional fat content from solid material (Ockerman and Hansen 2000; Anderson 2006; Kinley 2009). The remaining material known as “cracklings” or “crax” is ground (Ockerman and Hansen 2000). Both the ground processed protein meal and fat are transferred to a storage facility or transported to a consumer (Meeker and Hamilton, 2006). The processed protein meal is either stored in feed bin structures or enclosed silos. Fat is centrifuged to remove residual particulate and stored in insulated and/or heated silos. The renderers maintain the fat at elevated

temperatures to keep it in a liquid form in order to easily pump the fat from the silos. Despite the elevated temperatures utilized during the rendering process, the finished products are susceptible to recontamination from raw materials and the rendering facility environment. In 2000, microbial levels in finished rendered products were reportedly high (ICMSF 2000) but the rendering industry is continuing to make major improvements to reduce microbial levels in finished products. Educational programs such as the Code of Practice Seminar initiated in 2004, use of HAACP, and certification are offered through the American Protein Producers Industry (APPI) audit program to improve microbiological quality of rendered products (Meeker and Hamilton, 2006).

Wet and batch rendering units are used in North American rendering plants in addition to continuous flow dry rendering units (Ockerman and Hansen 2000; Anderson 2006). In a wet rendering facility, steam is injected directly in contact with the product by vertical digesters through perforated plates, which can produce high quality tallow. These inefficient systems are labor intensive, require long cooking times, lose large volumes of meal during processing, and produce high moisture products (Ockerman and Hansen 2000). Batch rendering systems have expensive operation costs and are unable to quickly process large volumes of materials continuously (Ockerman and Hansen 2000). Continuous slurry systems such as the Carver-Greenfield system are utilized in some rendering facilities. These systems produce a more digestible meal and high quality fat. Continuous slurry systems are energy efficient, however, they process at temperatures close to 240ºF (115.6ºC) (Meeker and Hamilton, 2006) thereby possibly not effective at destroying some bacterial species.

Thermal Processing Principles

When microbial populations in food or rendering materials are exposed to elevated temperatures, the microbial population reduction typically occurs in a logarithmic (log_{10}) manner with increasing time at a given constant elevated temperature. Several parameters are utilized to quantify the influence of elevated temperatures on microbial populations. Thermal death time (TDT) or F value is a factor of time, temperature, material matrix and organism (Heldman and Hartel 1998). TDT is defined as the time needed to kill or reduce a given number of organisms at a specific temperature (Jay 2005; Teixeira 2006). TDT can be utilized as a measure of product safety to reduce a microbial population in a product to decrease spoilage microbes and increase shelf-life. Decimal reduction time or D value indicates the time required for a one log_{10} cycle reduction of a particular organism at a specific temperature. Essentially, a large D value at a given temperature indicates an increased thermal resistance of a microbial population in a product (Heldman and Hartel 1998). The 12-D concept is used as a lethality time required for the canning industry and is defined as the time required for destroying 12 log_{10} of *Clostridium botulinum* spores (Jay 2005; Teixeira 2006). The thermal resistance constant or Z value is the parameter used to indicate the temperature increase needed to cause a one log_{10} reduction as shown as the slope on the thermal destruction curve. In most situations, a large Z value would indicate that a microbial culture contains heat resistant vegetative cells or microbial spores (Heldman and Hartel 1998).

Numerous research studies have been conducted in the food industry regarding different factors such as cooking methods, food composition, packaging type and product

type and their impact on the thermal lethality of pathogens. Blackburn et al. (1997) developed and validated thermal inactivation models for *Salmonella* Enteriditis and *Escherichia coli* O157:H7 describing the effect of temperature, pH and sodium chloride concentration on each microbe in whole egg, egg albumen, egg yolk, beef, poultry, apple juice and milk. Orta-Ramirez et al. (1997) demonstrated the temperature dependence of the enzyme triose phosphate isomerase from *E. coli* O157:H7 and *Salmonella senftenberg* indicating this enzyme could potentially be used as a surrogate time-temperature indicator in ground beef products. Juneja et al. (2000) determined beef samples containing between 7 and 24% fat content and inoculated with a *Salmonella* cocktail had varying D and Z values. Murphy et al. (2002) and Murphy et al. (2004) reported *Salmonella* and *Listeria innocua* had significantly different thermal inactivation D and Z values among several different commercial products such as chicken breast meat, chicken patties, chicken tenders, franks, beef patties, blended beef and turkey patties.

Although human food products and their processing systems provide the nearest similarities for studying microbiological population dynamics inherent in rendered animal products, there is not an ideal model found in the food industry to duplicate rendered materials. Procedures used for food microbiological testing are unproven in rendered animal product testing. For instance, after attempting to quantify microbial loads in raw poultry rendering materials, Glenn (2006) discovered difficulties in enumerating bacteria by traditional aqueous buffer dilution methods due to the high fat content of the rendering material. Rendered animal co-products are a combination of various offal tissues, bones and fat (Meeker and Hamilton, 2006), and these materials

have unique compositions not found in any known food product. Therefore, 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. Since thermal death time is a factor of matrix, temperature and organism, it will be necessary to conduct validation in the actual rendering material matrices. The high fat content of rendered products also complicates traditional bacterial enumeration methodology. It is imperative that accurate test methods are developed to detect these pathogens in high fat rendered materials to prevent false positive and false negative results.

Salmonella

Salmonella is a genus of Gram-negative, facultative anaerobic, motile, non-spore-forming bacilli which are classified as members of the *Enterobacteriaceae* family (Wray and Wray 2000). Certain species of *Salmonella* are associated with foodborne disease (Jay 2005). Typically, *Salmonella* are characterized by their ability to ferment glucose into gas and acid on triple sugar iron (TSI) media and will not utilize sucrose or lactose in differential media (Andrews et al. 2011; D'Aoust et al. 1998). However, in some cases, *Salmonella* have demonstrated ability to ferment sucrose and lactose through the use of plasmids (Le Minor et al. 1973; Le Minor et al. 1974).

The optimal growth temperature for *Salmonella* is 37ºC and growth is faster in moist conditions (Franco 1997). These organisms are able to multiply over a wide variety of conditions including extreme temperatures (high and low) and low water activity levels (Franco 1997). Some strains of *Salmonella* have been able to grow in environments as high as 54ºC and some as low as 2ºC (D'Aoust et al. 1975). *Salmonella* can develop

heat resistance by exposure of the cells to temperatures greater than 50ºC between 15 and 30 min resulting in production of heat shock proteins (Humphrey et al. 1993; Mackey and Derrick 1986; Mackey and Derrick 1990).

Rasmussen et al. (1964) reported wet *Salmonella* cells added to dry meat and bone meal were reduced by 8 $\log_{10}$ after the meal was heated for 15 min at 68ºC; however, heating for 1 h at 82ºC was required to kill *Salmonella* cells in naturally contaminated meal. The water activity was not reported in this study. Mossel et al. (1965) observed a rapid 5 $\log_{10}$ reduction immediately after inoculation of a viable *Salmonella* culture containing 10 to 12 $\log_{10}$ concentration of cells. The broth culture was pre-chilled at 4ºC and mixed into dry meat and bone meal which also was pre-chilled at 4ºC. The water activity level of the meat and bone meal was reported as 0.46. Mossel et al. (1965) theorized that the initial rapid decline of the *Salmonella* concentration was due to osmotic shock. After additional storage for 5 days under refrigeration temperatures, a 1 $\log_{10}$ reduction of the Salmonella culture occurred. Mossel et al. (1965) noted that once bacterial cells are within protein protected by lipids, increased resistance seemed to occur. Reinman (1968) indicated a drastic reduction in viable *Salmonella* after meat and bone meal (water activity of 0.9) was heated to 90ºC for a relatively short time.

Genetic mutations in strains of *Salmonella* also can increase heat resistance. Droffner and Yamamoto (1992) determined *Salmonella* Typhimurium was capable of surviving prolonged exposure at 54ºC. The results of this study indicated genetic mutations occurred in the *ttl* gene or the *mth* gene which gives increased heat resistance at temperatures as high as 48ºC and 54ºC, respectively. In addition to these genes, other

environmental factors play a role in the level of heat resistance including the nutrients available in the growth environment, the growth phase of the cells, and the moisture level of the environment from which it was isolated (Goepfert et al. 1970; Kirby and Davies 1990; Ng et al. 1969).

Salmonella and Rendering Co-Products

In the United States, approximately 2 to 4 million cases of human salmonellosis occur annually (FDA 2012). Often these *Salmonella* outbreaks are associated with consumption of animal products (Shacher and Yaron 2006). Crump et al. (2002) claimed that animal feeds were a source of contamination and could lead to transmission of *Salmonella* to humans. Although there are over 2,500 serovars of *Salmonella*, there are very few pathogenic strains which may be found across rendered feed ingredients, farm animals and humans. Knox et al. (1963) established a connection between a *Salmonella* Heidelberg outbreak from contaminated milk and the meat and bone meal used in the feed supplied to the milk-producing cattle. In 2010, an egg recall due to *Salmonella* Enteriditis contamination was initially blamed on rendering materials by the farmer implicated but a thorough investigation proved that rendering products were not the source for this outbreak (Caparella 2010).

In 2010, FDA identified eight *Salmonella* serotypes as pathogenic to animals and listed those serotypes as of concern for potential transmission through animal feeds (FDA, 2010). The organisms of concern associated with poultry are *Salmonella* Pullorum, *Salmonella* Gallinarum, and *Salmonella* Enteritidis. The organism(s) of concern for swine is *Salmonella* Choleraesuis, for sheep is *Salmonella* Abortusovis, for

horses is *Salmonella* Abortusequi and for cattle are *Salmonella* Newport and *Salmonella* Dublin (FDA 2010).

The rendering industry created the Animal Protein Producers Industry (APPI) in 1984 to promote biosecurity in rendered animal feeds and reduce incidence of *Salmonella* (Meeker and Hamilton, 2006). In 2004, the APPI Code of Practice certification program for rendering plants was developed and currently more than 100 rendering plants are certified. The APPI Code of Practice Seminar is an educational series of training courses which teaches rendering plant workers handling and processing procedures to produce safe feed ingredients (Meeker and Hamilton, 2006).

In 1993 and 1994, FDA conducted two separate studies to examine 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. This study also reported that a majority of raw tissue samples entering rendering facilities were positive for *Clostridium*, *Listeria*, and *Salmonella* species. No *Salmonella* was found in crax samples or in the rendering processing environment. The finished rendered products contained 12 serovars of *Salmonella*. Franco (2005) analyzed approximately 200 rendered animal protein meal samples over a 12 mo period for the presence of *Salmonella* species, and reported that *Salmonella* cells were present in low numbers in animal feed at a median level of 0.09 MPN/g. Kinley et al. (2009) examined products from 12 rendering facilities in the United States and detected 13 *Salmonella* serotypes. Kinley et al. (2010) conducted a research survey to

determine the prevalence of *Salmonella* and *Enterococcus* species in rendering products from 12 rendering companies. *Enterococcus* species were detected in 81.3% of the samples. *Salmonella* was detected in 8.7% of the samples. However, 13 serotypes of *Salmonella* including Senftenberg, Oranienburg, Idikan, Johannesburg, IIIa. 42:z4,z23, Banana, Demerara, Putten, Molade, Montevideo, Mbandaka, Livingstone, and Amsterdam were characterized by 16 pulsed-field gel electrophoresis patterns. Each set of pulsed-field gel electrophoresis patterns was compared between product type and rendering plant to demonstrate there was not one particular serotype present in a particular rendering facility over a seven mo period. This study suggested the presence of *Salmonella* in the finished products may be due to post-processing contamination. The results from Franco (2005) and Kinley et al. (2010) indicated the efforts taken by the rendering industry have microbiologically improved its products since the studies conducted by FDA in the 1990s.

Contamination with *Salmonella* species in a rendering facility may be due to cross-contamination from the raw animal tissue during processing (Ockerman and Hansen 2000). Incoming raw rendering materials from animals serve as a reservoir for many pathogenic bacterial species including *Staphylococcus* species, *Listeria* species, *Bacillus* species, *Clostridium* species, *Mycobacterium* species, *Enterobacteriaceae*, *Pseudomonas* species, *Aeromonas* species, *Plesiomonas shigelloides*, and *Vibrio* species which can survive and exist in animals, particularly in their digestive tracts (Jay 2005). Depending on carcass size, raw materials may be ground to reduce particle size prior to the cooking process. Aerosols generated during the grinding process have the potential to

spread contaminating bacteria such as *Salmonella* throughout the rendering plant, including areas where the finished product is handled (ICMSF 2000; Swingler 1982). Jones and Bradshaw (1996) observed the strain *Salmonella* Enteriditis and its capability of producing biofilms on environmental surfaces which could serve as a reservoir for future contamination.

Emulsifiers

Emulsifiers are chemical additives that prevent the separation of two immiscible liquids such as oil and water. Emulsifiers consist of molecules which have hydrophilic or hydrophobic and lipophilic or lipophobic portions. Lecithin consists primarily of phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidyl-inositol and phosphatidic acid in oil (Bueschelberger 2004). According to the FDA Guidance for Industry (2006), lecithins are considered generally recognized as safe (GRAS). They are a class of chemicals that are present in both plant and animal tissue. The main sources of lecithins are soybean and sunflower oils (Szuhaj and List 1985; Bueschelberger 2004). Weete et al. (1994) demonstrated lecithin had improved water/oil emulsification after preheating to 180ºC for 90 min and subsequently mixed with a 60ºC pre-heated water/oil phase. Zhang (2011) observed the effect of various levels of lecithin used to emulsify high fat rendering samples in an aqueous buffer to assist in accurate serial dilution of bacterial populations as well as the impact on the bacteria *Geobacillus stearothermophilus*. Zhang (2011) determined the use of lecithin as an emulsifier in dilution buffers appeared to be a promising method to enumerate high fat samples with *Geobacillus stearothermophilus.*

Conclusion

On January 4, 2011, the Food Safety Modernization Act was signed into law by President Barack Obama. This act expanded the power of the FDA to include regulation of any aspect of food production in order to provide a safe food supply (FDA 2013). In July 2013, the FDA released a compliance guide to inform the rendering industry of current recommendations for pet and animal feed products. Contaminated feed products have the potential to serve as a vehicle which can introduce pathogenic bacteria into the food chain. Currently, the FDA can enforce regulatory actions if pet foods products are contaminated with any serotype of *Salmonella*. Animal feeds contaminated with specific infectious *Salmonella* serotypes can be seized and detained by the FDA. However, animal feed contaminated with non-infectious serotypes of *Salmonella* will be evaluated on a case by case basis by the FDA (FDA 2013). Therefore, conclusive data regarding the validation of thermal lethality of rendering processes is vital to the livestock and pet food industry and to the FDA to ensure thermal destruction of bacterial pathogens in products. A disease outbreak in the animal livestock industry could have serious negative consequences to the rendering industry, to the entire food animal chain, to consumers of animal products, and to pets and their owners.

The specific objectives of this study are to 1) validate methodology for enumerating *Salmonella* in high fat matrices and 2) determine the minimum thermal requirements needed to destroy four pathogenic *Salmonella* serotypes (*Salmonella* Choleraesuis, *Salmonella* Enteritidis, *Salmonella* Newport, and *Salmonella* Dublin) in typical rendering material matrices.

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CHAPTER 2

METHOD VALIDATION FOR ENUMERATING BACTERIA IN HIGH FAT MATRICES

Abstract

The high fat content of rendered animal co-products has complicated traditional bacterial enumeration methodology. Therefore, it is vital that the rendering industry has accurate enumeration methodologies for pathogenic bacteria in finished products. An objective of this study was to examine the use of the standard Class O phosphate/ magnesium chloride dilution series (Dilution Series A) and a modified (pre-warmed to 32ºC) lecithin phosphate dilution buffer series (Dilution Series B) by comparing mean bacterial counts of a *Salmonella* cocktail in poultry and beef rendering materials. The results of this study did not indicate that the use of a modified buffer to improve enumeration of *Salmonella* from poultry and beef rendering materials. Instead, the results suggested that the use of xylose lysine deoxycholate agar (XLD) with either buffer system would produce accurate enumeration data of *Salmonella* from poultry and beef rendering materials.

Introduction

Rendering is the process of converting inedible animal tissue from food animals into granular meals and liquid fats that are used in numerous co-products, including animal feeds, chemical feedstocks, and fertilizers (Meeker and Hamilton 2006). The continuous cooking process used by the rendering industry is reported to be 40 to 90 min at 240 to 290ºF (115.6 to 143.3ºC) (Meeker and Hamilton, 2006). The high temperatures used in the rendering cooking process reduce the number of microorganisms in raw perishable animal tissues. Marginal processing conditions could result in the presence of residual microorganisms in finished products (Crump et al., 2002). Crump et al. (2002) indicated that animal feeds can be a source of contamination of *Salmonella* to humans. Therefore, it is vital to develop accurate enumeration methods for high fat rendering materials.

The high fat content of rendered animal co-products has complicated traditional bacterial enumeration methodology, making it difficult to accurately determine the presence or absence of *Salmonella* in rendering co-products (Glenn 2006). It is hypothesized that this fat content could also entrap the bacterial cells in rendering materials. Therefore, upon serial dilution, the fat globules may not be evenly dispersed throughout dilutions and subsequently not be transferred evenly to plates for enumeration. Inaccurate transference to the microbial media would yield either higher or lower bacterial counts and overall a less accurate method of enumeration. Zhang (2011) determined that the use of lecithin as an emulsifier in dilution buffers appeared to be a promising method to enumerate high fat samples with *Geobacillus stearothermophilus.*

Lecithin consists of complex combinations of phospholipids and is a common emulsifier in the food industry (Bueschelberger 2004).

An objective of the study was to examine the use of the standard Class O phosphate/magnesium chloride dilution buffer series and a modified (pre-warmed to 32ºC) lecithin phosphate dilution buffer series by comparing mean bacterial counts of a *Salmonella* cocktail in each poultry and beef rendering materials, adjusted to 50% fat content.

Materials and Methods

Rendering Sample Preparation

Samples of poultry and beef rendering fat and crax materials were collected on three separate days from rendering plants in the midwestern and southeastern U.S. 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). Crax samples were submitted in duplicate to the Clemson University Agricultural Services Laboratory for ash, fat, and moisture content analysis. The crax and fat samples were re-mixed to produce 50% fat samples. A food processor was 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 a 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* 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 of 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. The media choice was based on 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 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). Bismuth sulfite agar, Hektoen enteric agar and XLD are selective media used for the detection of *Salmonella* in food products (Andrews et al. 2011).

An additional preliminary study was conducted to determine if any combination of SC, SE, SN or SD promoted or inhibited growth. Overnight cultures were adjusted to 0.5 OD at 600 nm. Flasks of sterile TSB with 0.1% yeast extract were inoculated with equal volumes of each *Salmonella* serotype or combinations of the four serotypes. Cultures were incubated overnight at 35ºC and the OD was measured again to determine if growth had increased or stayed the same. The results indicated that no combination of SC, SE, SN, and SD appeared to enhance or inhibit growth.

For the study, each serotype was grown individually in 1 L TSB (90000-050, VWR Scientific Products, Suwanee, GA). Each overnight culture was washed twice by centrifugation at 7,000 x g for 7 min (GSA rotor, DuPont RC5C Sorvall Instruments Centrifuge, DuPont Company, Newtown, CT) and resuspended in sterile physiological (0.85%) saline. Optical density was adjusted to 0.7 (ca. 10^8 cfu/mL) at 600 nm and equal volumes of the four cultures were combined in a sterile flask.

<u>*Salmonella* Enumeration in Rendering Materials</u>

The standard Class O phosphate/magnesium chloride (Wehr and Frank 2004) dilution buffer system (Dilution Series A using diluent a) was compared to a modified dilution system (Dilution Series B) (Fig. 2.1). Dilution Series B was comprised of two modified phosphate/magnesium chloride dilution buffers containing lecithin (AA36486-A1, Alfa Aesar, Ward Hill, MA) at the rate of 2 g per 99 mL (diluent b) and 0.5 g per 99

mL (diluent c), respectively, for dilution of high fat materials followed by use of standard Class O phosphate/magnesium chloride buffer (diluent a) (Fig. 2.1). Dilution Series A and Dilution Series B were prepared, dispensed in 99 mL aliquots into dilution bottles, and autoclaved (Fig. 2.1). Prior to experimentation, Dilution Series A was stored and used at room temperature (Fig. 2.1). Dilution Series B was pre-warmed to 32ºC (Fig. 2.1).

A preliminary experiment was conducted to validate the use of 1 mL of a *Salmonella* cocktail in 20 g of each poultry and beef rendering (50% fat) sample. One mL of crystal violet dye (90008-894, VWR Scientific Products, Suwanee, GA) was added to 20 g of each poultry and beef rendering (50% fat) sample in a sterile Whirl-Pak™ sample bag (11216-409, VWR Scientific Products, Suwanee, GA). Crystal violet dye was used to represent the culture. The mixtures were stomached (Laboratory Blender, Stomacher 400, A.J. Seward and Co. Ltd., London, England) for 2 min on the high setting. Subsamples were observed for color uniformity using a microscope (Carl Zeiss, Photomicroscope III, Oberkochen, West Germany) at 10x and 40x magnification. Results indicated that a 1:20 ratio of culture to sample would allow for even distribution of culture throughout each of the poultry and beef rendering samples.

One mL (ca. 10^8 cfu./mL) of a *Salmonella* cocktail was added to each 20 g poultry and beef rendering sample (50% fat content) in a sterile Whirl-Pak™ sample bag. The mixtures were stomached for 2 min on the high setting. Subsamples of the mixture were diluted using each Dilution Series A and Dilution Series B. Dilutions were carried out to the 10^{-9} dilution (Fig. 2.1) and plated in duplicate onto bismuth sulfite agar, Hektoen

enteric agar, XLD, and TSA. Controls included media and diluent sterility controls as well as uninoculated rendering samples (50% fat). Plates were incubated overnight at 35ºC and enumerated.

Statistical Analysis

The mean bacterial counts of the *Salmonella* cocktail obtained from the culture controls and inoculated samples were converted to $\log_{10}$ cfu/g values ± standard error. The mean bacterial counts of the culture controls diluted in the standard Dilution Series A and the mean bacterial counts of the inoculated samples plated onto the same media were compared using a two-tailed, paired Student's t tests in Microsoft Excel® (Microsoft®, 2010) to determine statistical significance at alpha=0.05.

Results

The analysis of the beef rendering materials (n=6) indicated the average fat content ranged from 9.9% to 13.8%, average ash content ranged from 20.6% to 33.5%, and average moisture content ranged from 2.1% to 3.3%. Averaged analysis data for each pair of duplicate samples (Day 1, Day 2, Day 3) were used to prepare 50% fat materials for this study.

Despite the type of media, the mean bacterial counts obtained from *Salmonella* cocktail culture controls diluted in Dilution Series A were not significantly different ($P<0.05$) from the mean bacterial counts of the *Salmonella* cocktail culture controls diluted in the standard Dilution Series B (Table 2.1). The mean bacterial counts of 6.01±0.28 and 5.77±0.30 $\log_{10}$ cfu/g were obtained from the inoculated poultry rendering samples diluted with each Dilution Series A and Dilution Series B, respectively, and

plated onto bismuth sulfite agar. However, the mean bacterial counts in poultry rendering materials were statistically different than the *Salmonella* culture control when it was diluted with the standard Dilution Series A and plated onto bismuth sulfite agar ($P<0.05$) (Table 2.1).

Enumeration data revealed mean bacterial counts of 7.46±0.99 and 6.48±1.00 $\log_{10}$ cfu/g from the inoculated poultry rendering samples diluted with each Dilution Series A and Dilution Series B, accordingly, and plated onto Hektoen Enteric agar. These mean bacterial counts were not statistically different than the *Salmonella* culture control diluted with the standard Dilution Series A and plated onto Hektoen Enteric agar ($P<0.05$) (Table 2.1).

The mean bacterial counts of 8.14±1.76 and 7.81±1.45 $\log_{10}$ cfu/g obtained from the inoculated poultry rendering samples diluted with each Dilution Series A and Dilution Series B, respectively, and plated onto XLD. These mean bacterial counts were not statistically different than the *Salmonella* culture control diluted with the standard Dilution Series A and plated onto XLD ($P<0.05$) (Table 2.1).

Enumeration of inoculated poultry rendering samples diluted with each Dilution Series A and Dilution Series B revealed the mean bacterial counts of 6.80±0.88 and 7.10±0.85 $\log_{10}$ cfu/g, accordingly, on TSA. These mean bacterial counts were not statistically different than the *Salmonella* culture control diluted with the standard Dilution Series A and plated onto TSA ($P<0.05$) (Table 2.1).

The mean bacterial counts of 7.45±0.99 and 6.47 ±0.99 $\log_{10}$ cfu/g obtained from the inoculated beef rendering samples diluted with each Dilution Series A and Dilution

Series B, respectively, were not statistically different than the *Salmonella* culture control on bismuth sulfite agar ($P<0.05$) (Table 2.1).

Enumeration data revealed the mean bacterial counts of 5.53±0.03 and 5.53 ±0.03 $\log_{10}$ cfu/g from the inoculated beef rendering samples diluted with each Dilution Series A and Dilution Series B, accordingly, and plated on Hektoen Enteric agar. These mean bacterial counts were statistically different than the *Salmonella* culture control on Hektoen Enteric agar ($P<0.05$) (Table 2.1).

Enumeration of inoculated beef rendering samples diluted with each Dilution Series A and Dilution Series B revealed the mean bacterial counts of 7.14±1.67 and 7.14±1.67 $\log_{10}$ cfu/g, respectively, on XLD. These mean bacterial counts were not statistically different than the *Salmonella* culture control on XLD ($P<0.05$) (Table 2.1).

The mean bacterial counts of 5.55±0.06 and 5.51±0.03 $\log_{10}$ cfu/g enumerated from the inoculated beef rendering samples diluted with each Dilution Series A and Dilution Series B, accordingly, on TSA. These mean bacterial counts were statistically different than the *Salmonella* culture control on TSA ($P<0.05$) (Table 2.1).

Discussion

In this study, Dilution Series A and Dilution Series B were used to enumerate the *Salmonella* cocktail. The mean bacterial counts obtained from the culture controls enumerated with each dilution series were not statistically different despite the media used ($P<0.05$) (Table 2.1). There results indicated that the addition of the emulsifier lecithin to the dilution buffer did not inhibit or promote the growth of the *Salmonella* cocktail. The *Salmonella* cocktail enumeration data were compared for Dilution Series A

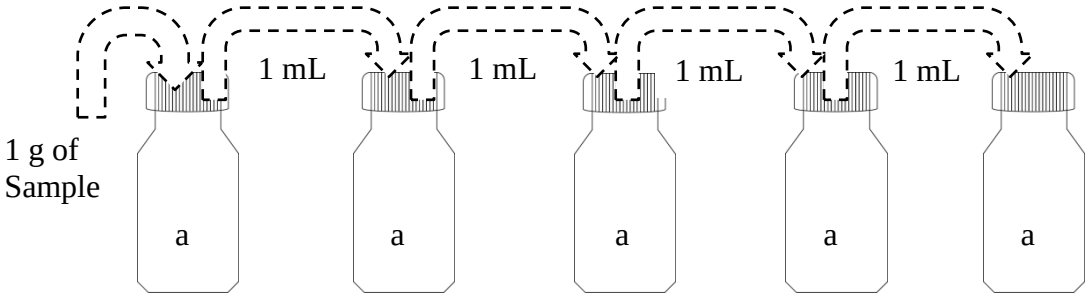
and Dilution Series B in each poultry and beef rendering materials. The mean bacterial counts enumerated from the inoculated poultry rendering samples, serially diluted in each Dilution Series A and Dilution Series B and plated onto bismuth sulfite agar were significantly lower than the mean bacterial counts obtained from the *Salmonella* cocktail culture on bismuth sulfite agar ($P<0.05$) (Table 2.1). An explanation for the lower bacterial counts from the poultry rendering samples is not known, but could include dilution error, presence of free fatty acids, entrapment of the bacteria in bones particles or coating of the bacteria by fat. In previous studies on raw poultry rendering materials, Glenn (2006) determined standard phosphate buffer serial dilutions produced irregular microbial enumeration results. Due to the high fat content of the rendering materials, it was revealed that the immiscibility of fat in the aqueous buffer caused the erroneous results. Glenn (2006) indicated that the fat may have entrapped the bacteria in the rendering materials. Additionally, the fat globules may not have dispersed evenly throughout dilutions due to the use of aqueous buffers and subsequently not transferred accurately to plates for enumeration (Glenn 2006). The 50% fat content in the poultry rendering materials used in this study may have entrapped the bacterial cells leading to lower bacterial counts.

The mean bacterial counts enumerated from the inoculated beef samples diluted with each Dilution Series A and Dilution Series B were significantly lower ($P<0.05$) than *Salmonella* cocktail controls on Hektoen Enteric agar and TSA (Table 2.1). Possible reasons for the lower bacterial counts from the beef rendering samples would be the same as above. Despite the dilution series used, these results suggested that the *Salmonella*

cocktail was accurately enumerated from beef and poultry rendering materials containing 50% fat content on XLD agar. XLD is a selective media used for the detection of *Salmonella*. In this study, XLD appeared to be a better selective media for the SC, SE, SN and SD than Hektoen Enteric or bismuth sulfite. It should also be noted that the black *Salmonella* colonies on the red XLD were easier to distinguish than the black colonies on the light green-yellow bismuth sulfite agar or the green colonies with black centers on the dark green Hektoen enteric agar.

The results of this study did not suggest the use of a modified buffer to improve enumeration of *Salmonella* from poultry and beef rendering materials. Instead, the results suggested that the use of XLD with either buffer system would produce accurate enumeration data of *Salmonella* from poultry and beef rendering materials. This research was a preliminary step toward improving enumeration methods for the detection of pathogenic bacterial species in high fat products.

Dilution Series A: Standard phosphate magnesium chloride buffer system (all bottles at room temperature).



Dilution Series B: Modified lecithin buffer system (all bottles pre-warmed to 32ºC)

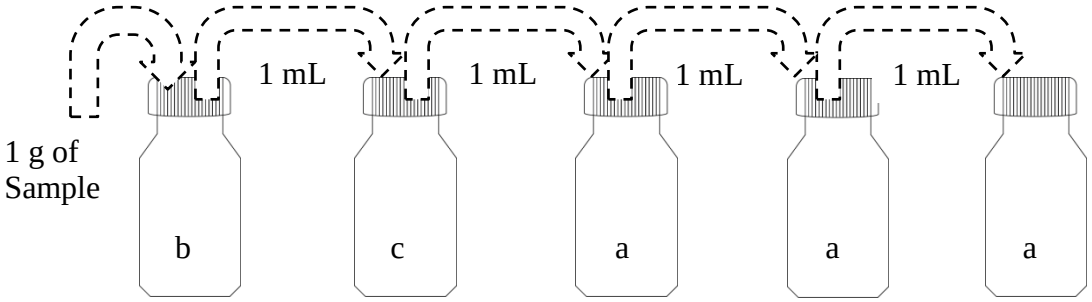


Figure 2.1. Diagram of two buffer systems used for serially diluting rendering materials. Diluent a represents a 99 mL of phosphate/ magnesium chloride buffer. Diluent b represents a 2 g lecithin/99 mL of phosphate/ magnesium chloride buffer. Diluent c represents a 0.5 g lecithin/100 mL of phosphate/ magnesium chloride buffer.

Table 2.1. Comparison of mean bacterial counts from each poultry and beef rendering material using each dilution series to a standard culture control (n=6).

Sample	Dilution Series	Media	Mean Bacterial Count[1] $\log_{10}$ cfu/g ± standard error	P-Value[2]
Salmonella	A	Bismuth Sulfite	7.71±0.10 [aefmn]	-
Salmonella	A	Hektoen Enteric	7.69±0.49 [bghop]	-
Salmonella	A	XLD	8.85±0.89 [cijqr]	-
Salmonella	A	TSA	8.21±0.27 [dklst]	-
Salmonella	B	Bismuth Sulfite	7.45±0.18 [a]	0.45
Salmonella	B	Hektoen Enteric	7.10±0.31 [b]	0.54
Salmonella	B	XLD	9.19±0.64 [c]	0.45
Salmonella	B	TSA	7.91±0.35 [d]	0.33
Poultry	A	Bismuth Sulfite	6.01±0.28 [e]	0.02*
Poultry	B	Bismuth Sulfite	5.77±0.30 [f]	0.03*
Poultry	A	Hektoen Enteric	7.46±0.99 [g]	0.81
Poultry	B	Hektoen Enteric	6.48±1.00 [h]	0.14
Poultry	A	XLD	8.14±1.76 [i]	0.50
Poultry	B	XLD	7.81±1.45 [j]	0.20
Poultry	A	TSA	6.80±0.88 [k]	0.15
Poultry	B	TSA	7.10±0.85 [l]	0.28
Beef	A	Bismuth Sulfite	7.45±0.99 [m]	0.84
Beef	B	Bismuth Sulfite	6.47±0.99 [n]	0.30
Beef	A	Hektoen Enteric	5.53±0.03 [o]	0.05*
Beef	B	Hektoen Enteric	5.53±0.03 [p]	0.05*
Beef	A	XLD	7.14±1.67 [q]	0.21
Beef	B	XLD	7.14±1.67 [r]	0.21
Beef	A	TSA	5.55±0.06 [s]	0.01*
Beef	B	TSA	5.51±0.03 [t]	0.01*

[1] Values with the same superscripts (a-t) indicate the mean bacterial counts compared using two-tailed, paired Student's t tests.

[2]indicates statistical difference at $P<0.05$.

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CHAPTER 3

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.

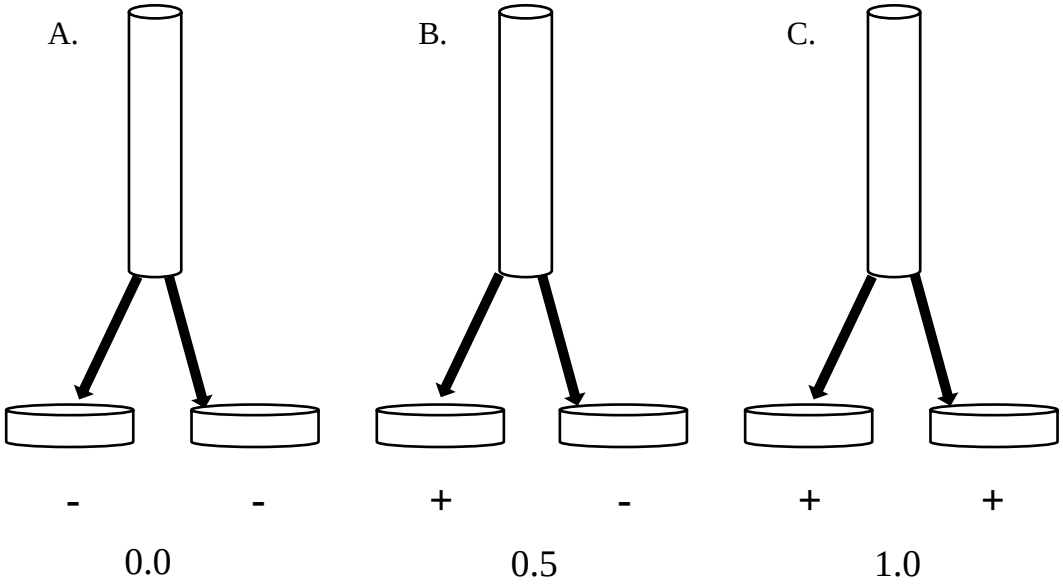

Figure 3.1. Method utilized to report RV and TT pre-enrichments results on XLD validated by two confirmation tests at each thermal treatment. If both plates were negative, the result was assigned a 0 (A). If one was positive and one was negative, the result was assigned a 0.5 (B). If both were positive,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

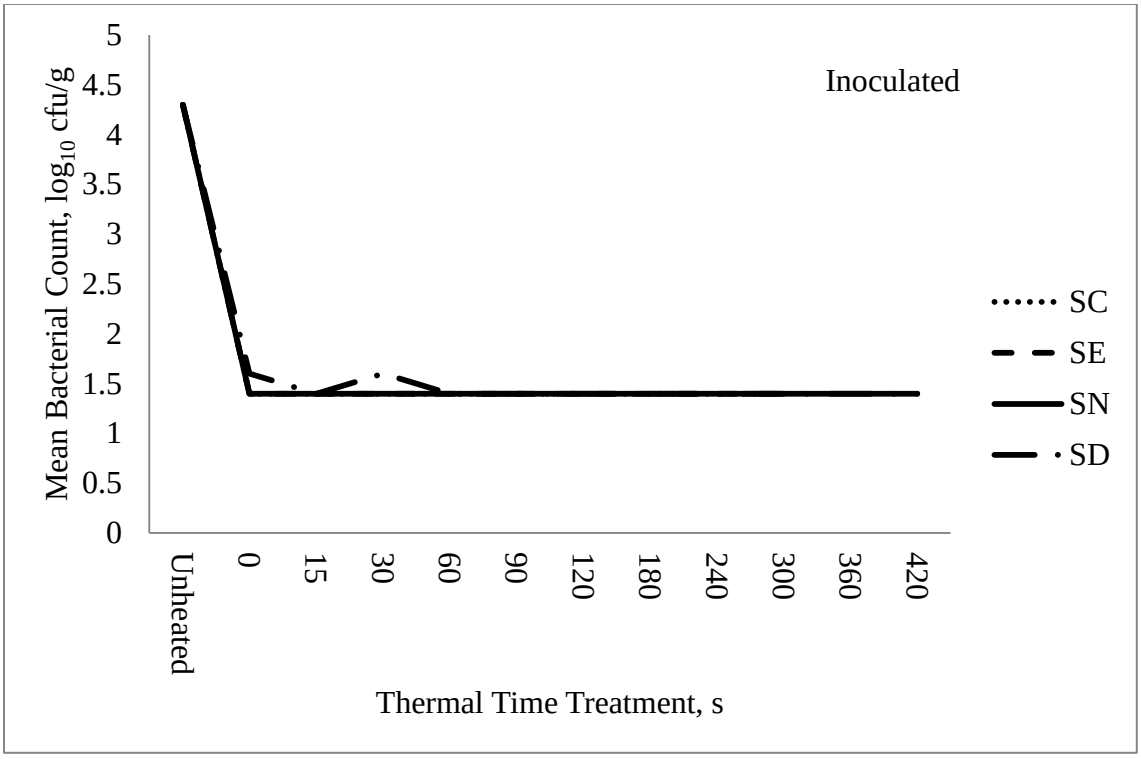

Figure 3.2. Enumeration of *Salmonella* on XLD from beef rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[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).

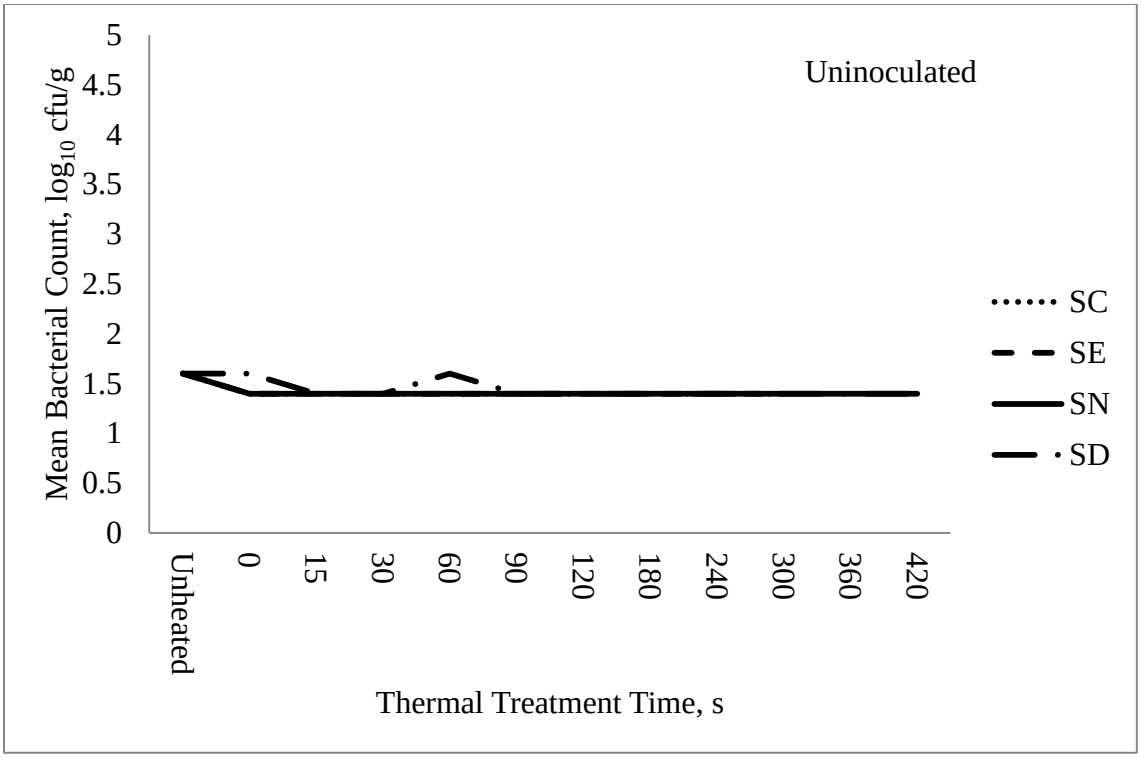

Figure 3.3. Enumeration of *Salmonella* on XLD from uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[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).

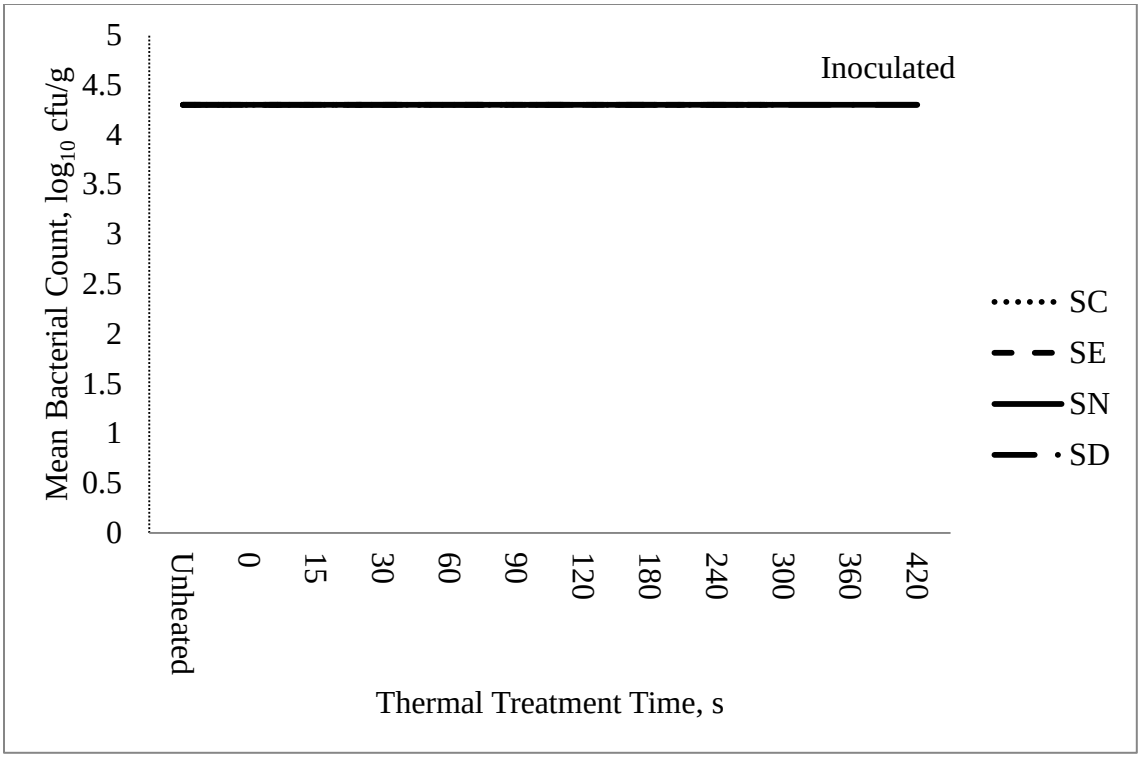

Figure 3.4. Enumeration of total bacteria on TSA from beef rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[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).

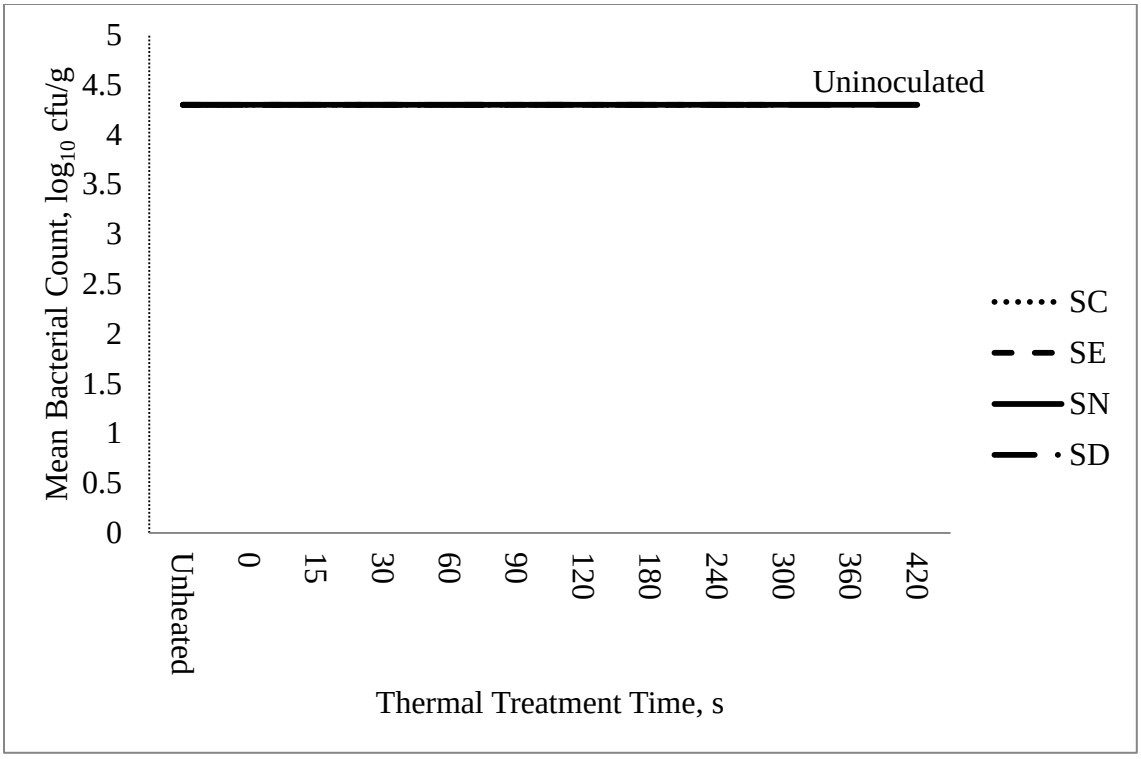

Figure 3.5. Enumeration of total bacteria on TSA from uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[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).

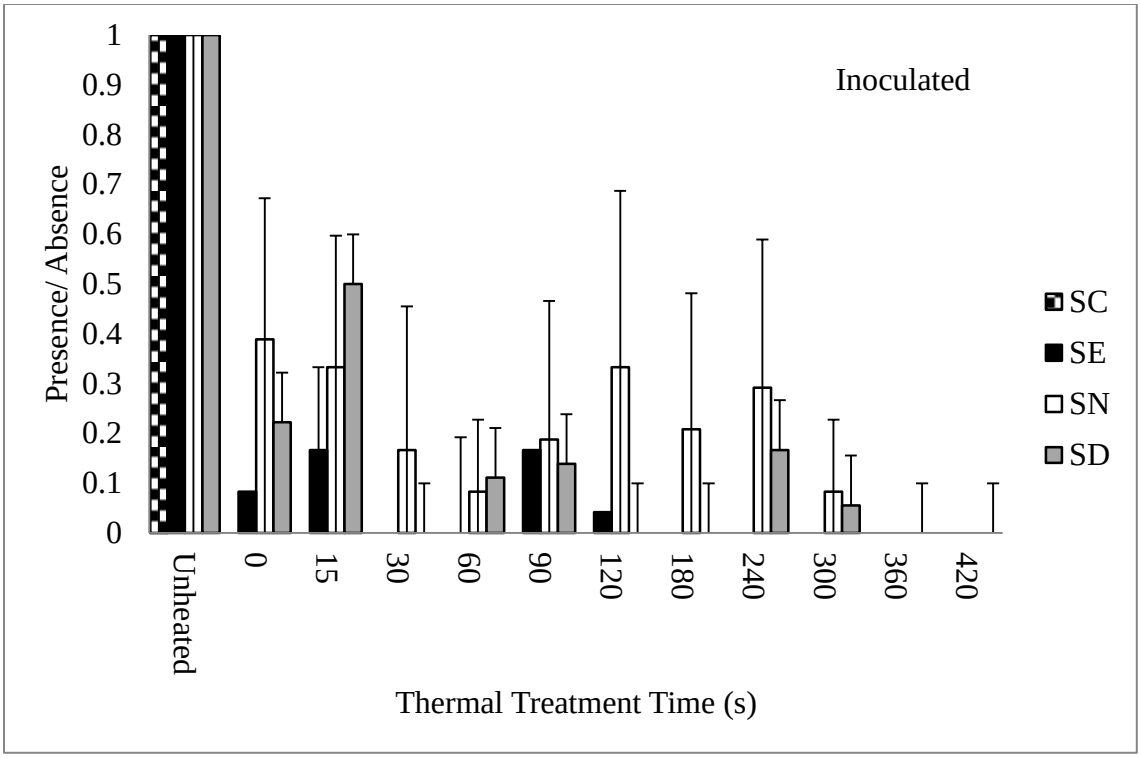

Figure 3.6. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, RV pre-enriched beef rendering samples (50% fat).[1]

[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).

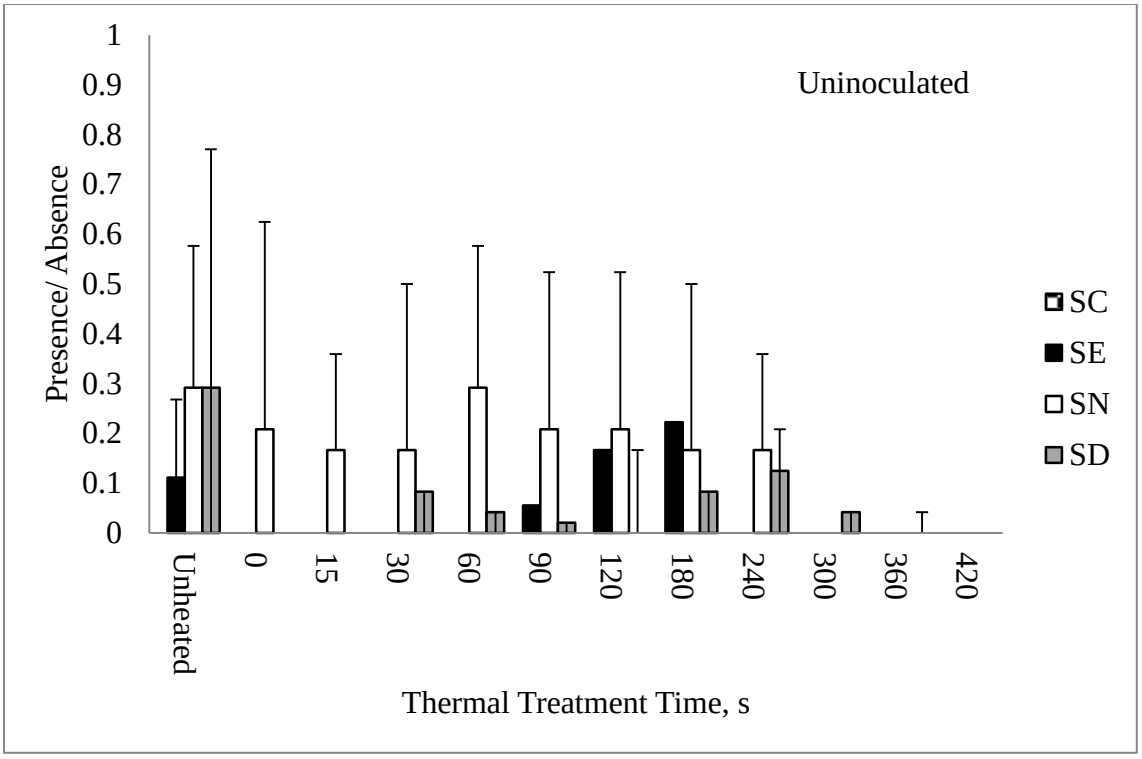

Figure 3.7. Presence or absence ± standard deviation of *Salmonella* for each RV pre-enriched, uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[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)

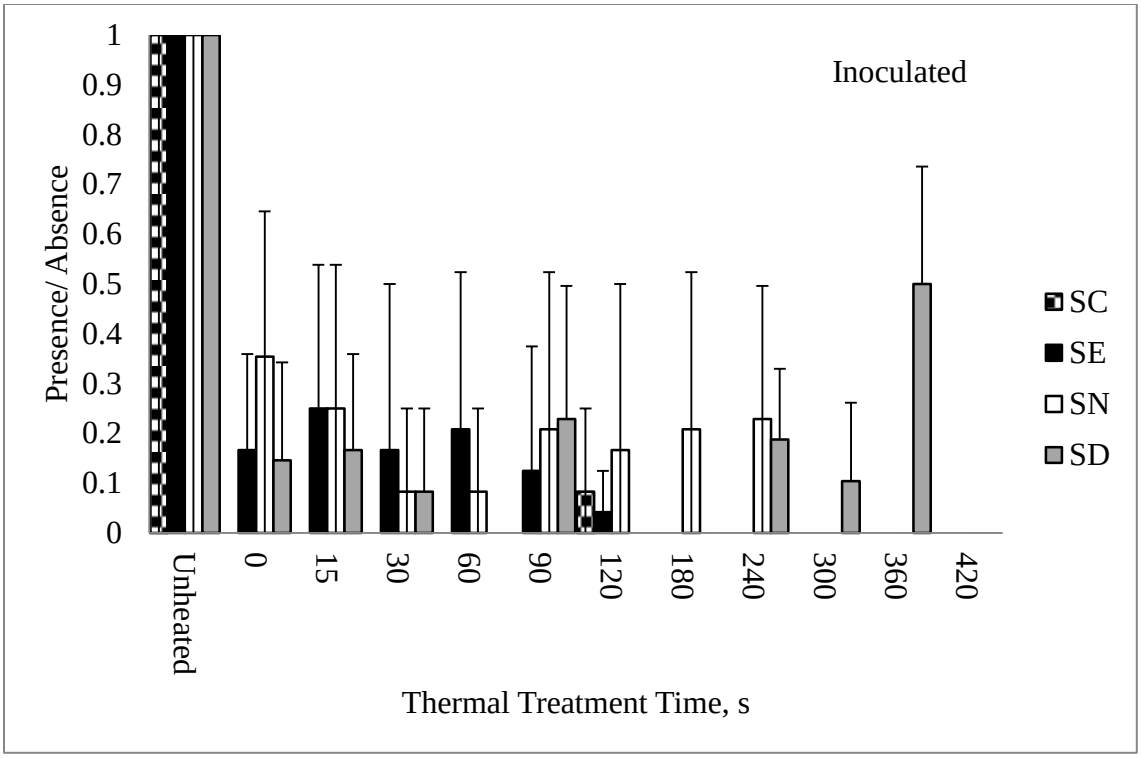

Figure 3.8. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, TT pre-enriched beef rendering samples (50% fat).[1]

[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).

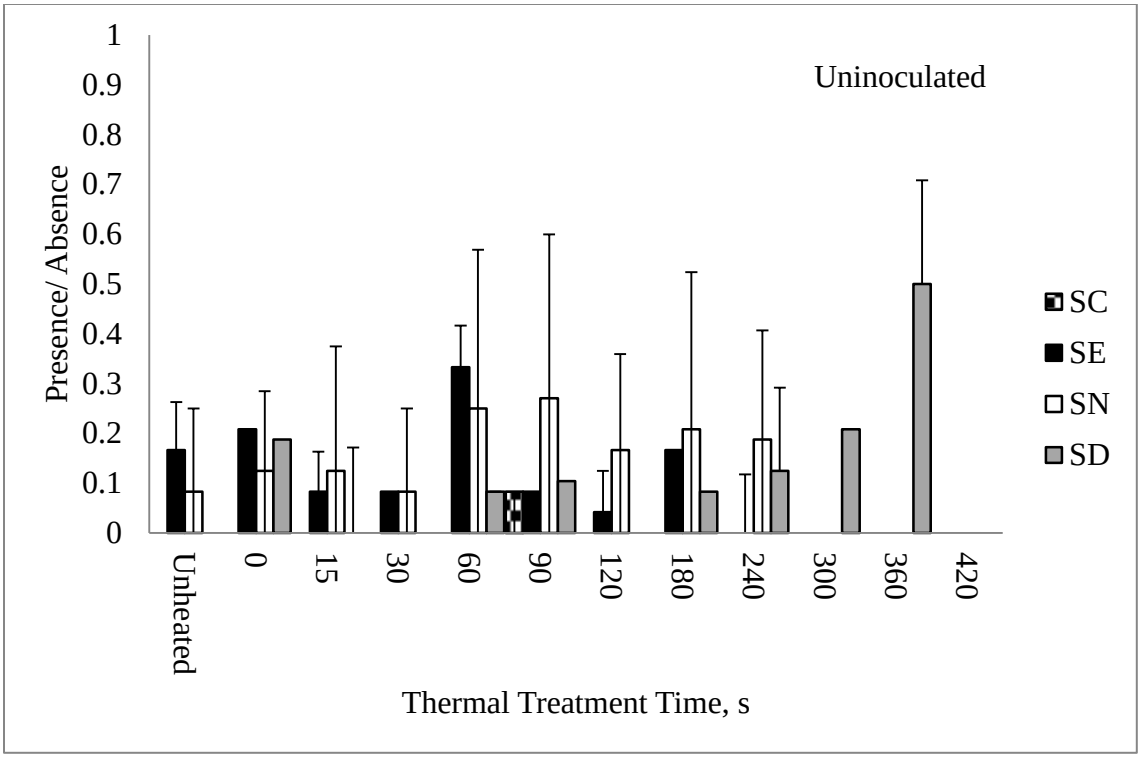

Figure 3.9. Presence or absence ± standard deviation of *Salmonella* for each TT pre-enriched, uninoculated beef rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[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).

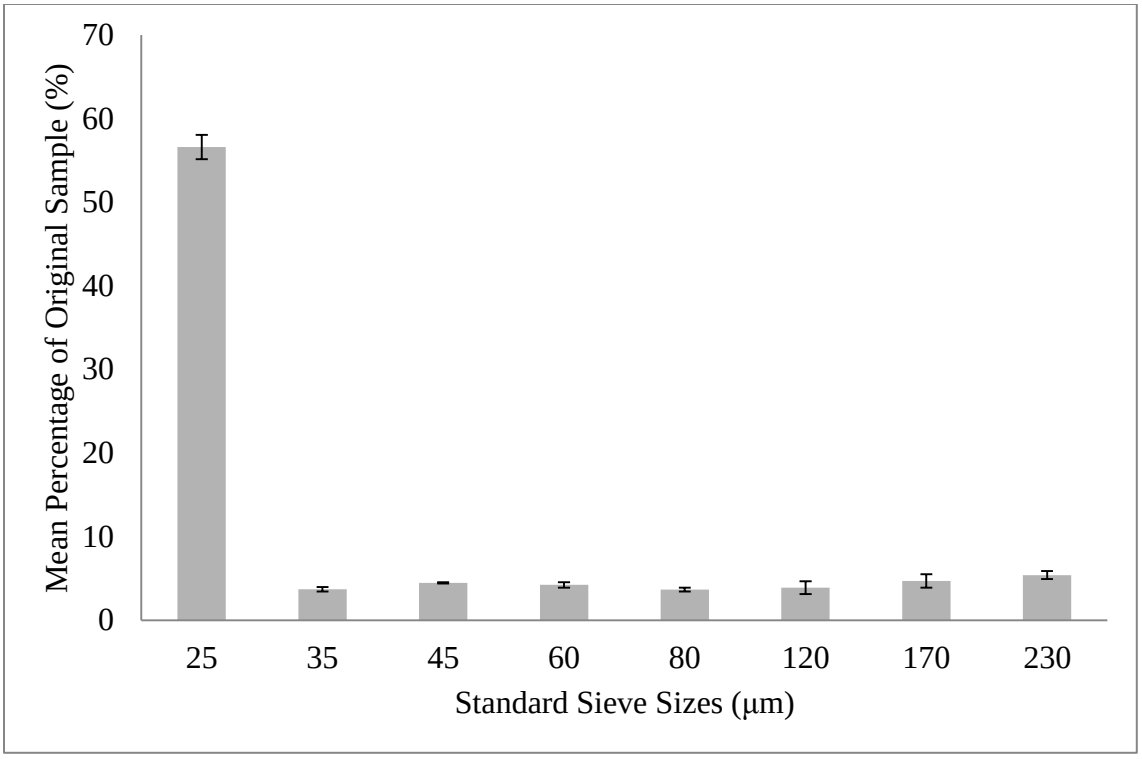

Figure 3.10. Mean percent particle size distribution ± standard error of beef rendering samples collected from a rendering plant on three different days (n=30). Each fraction of particle size was indicated as a percentage of the total weight of the rendering sample. The error bars indicate standard error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

		RV	TT
Serotype	Thermal Treatment Time, s	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 4

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.

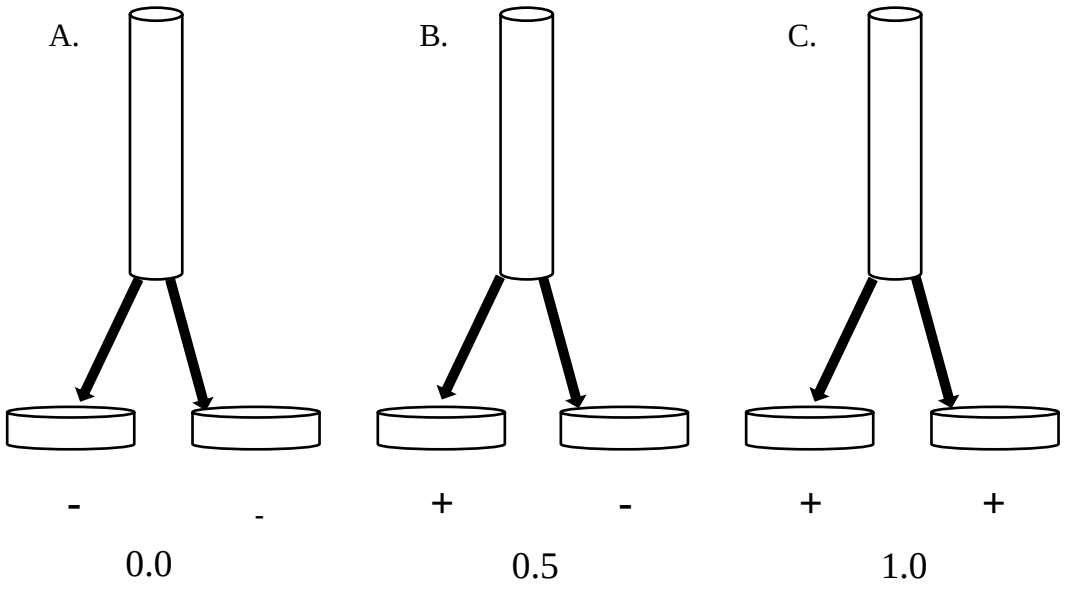

Figure 4.1. Method utilized to report RV and TT pre-enrichments results on XLD confirmed by two confirmation tests at each thermal treatment. The result was assigned a 0 if both plates were negative (A). The result was assigned a 0.5 if one was positive and one was negative (B). The result was assigned a 1.0 if both were positive (C).

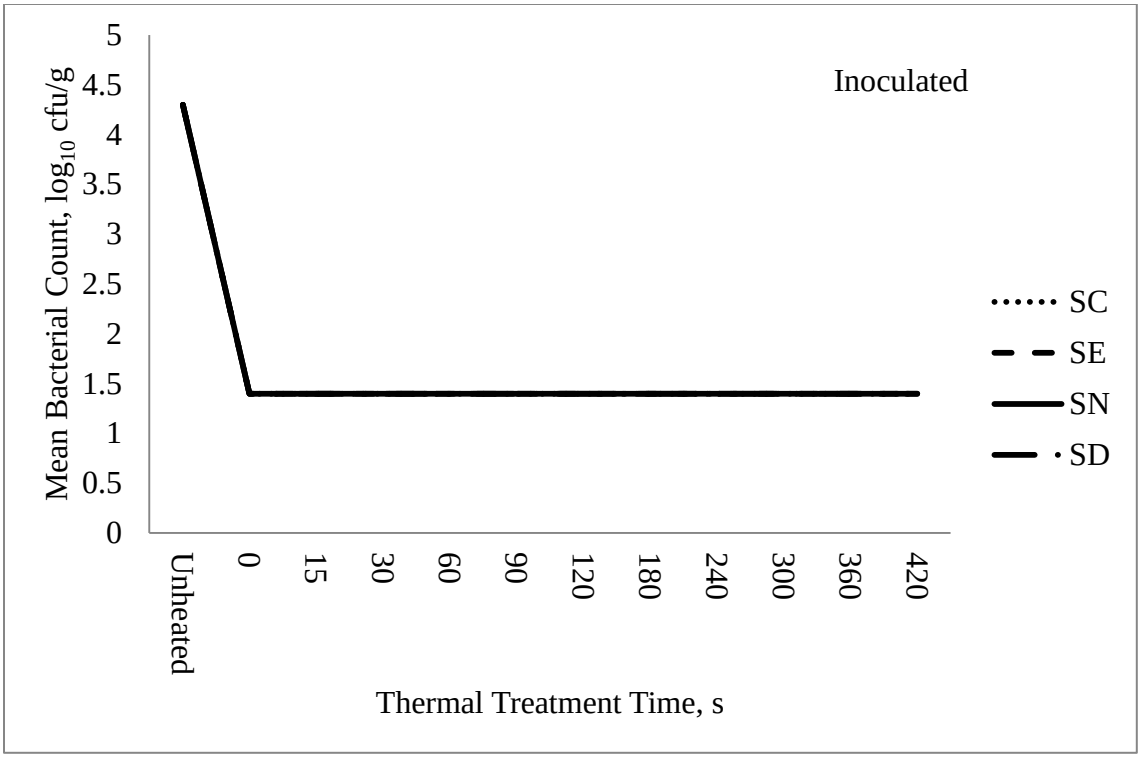

Figure 4.2. Enumeration of *Salmonella* on XLD from poultry rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

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

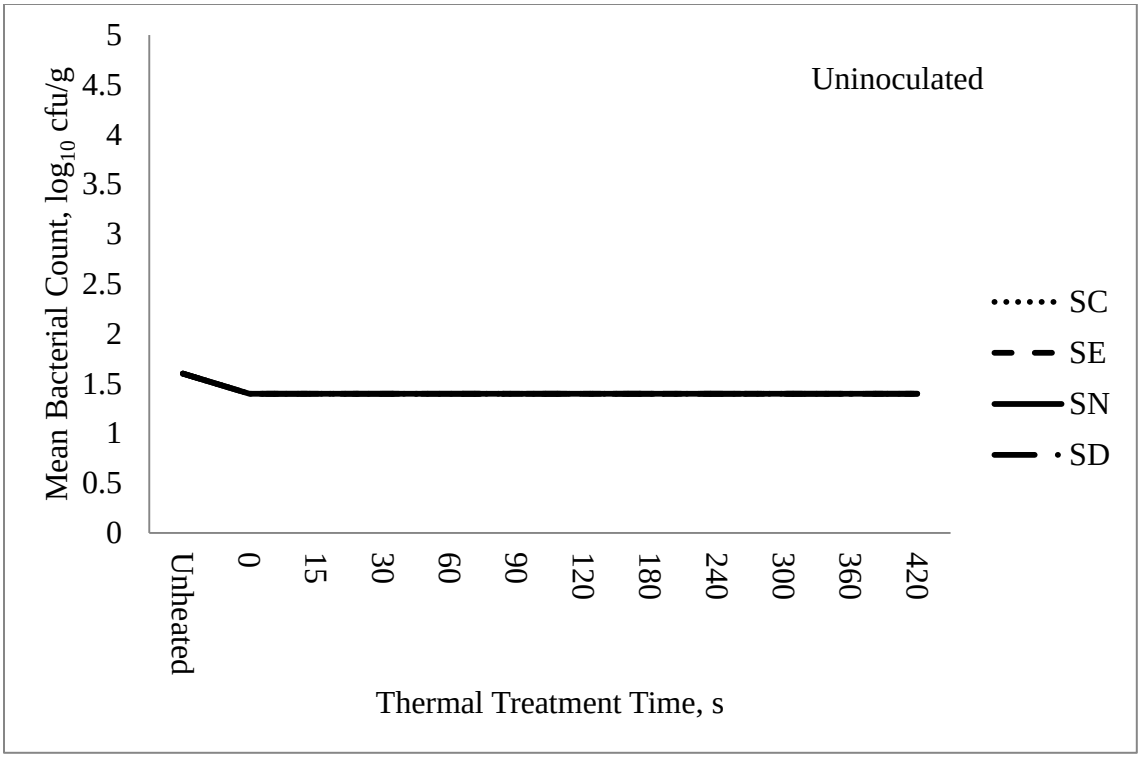

Figure 4.3. Enumeration of *Salmonella* on XLD from uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

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

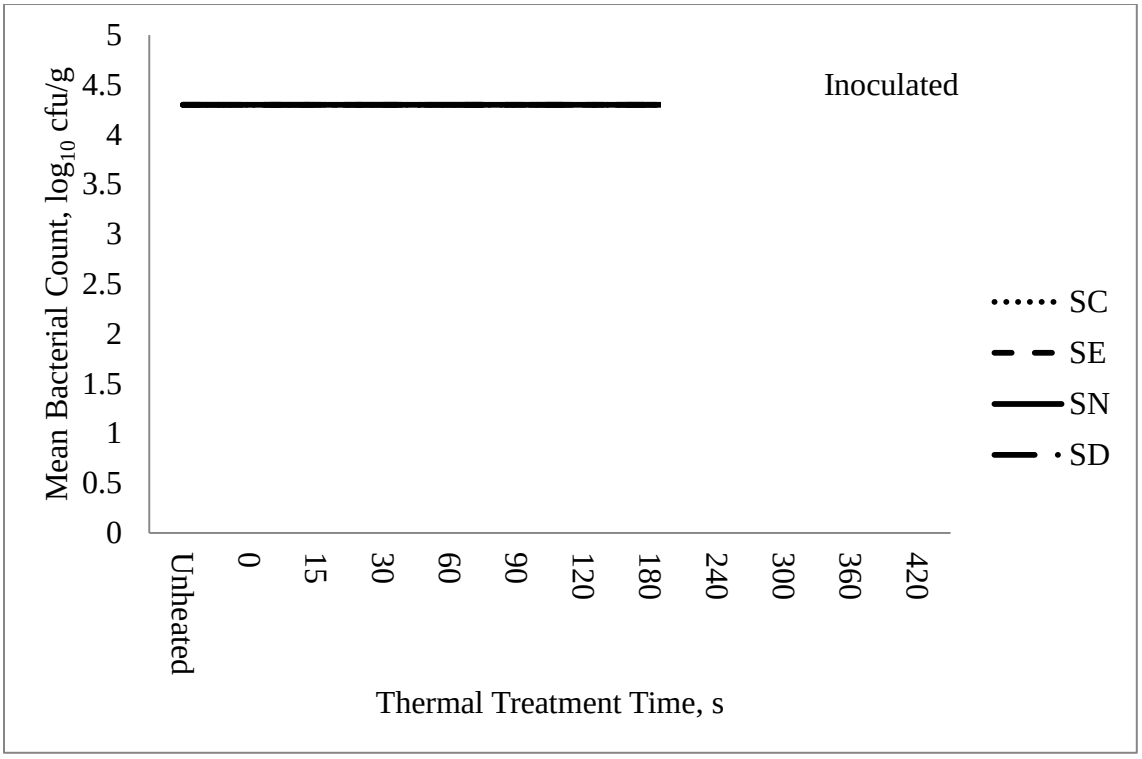

Figure 4.4. Enumeration of total bacteria on TSA from poultry rendering samples (50% fat) inoculated with *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

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

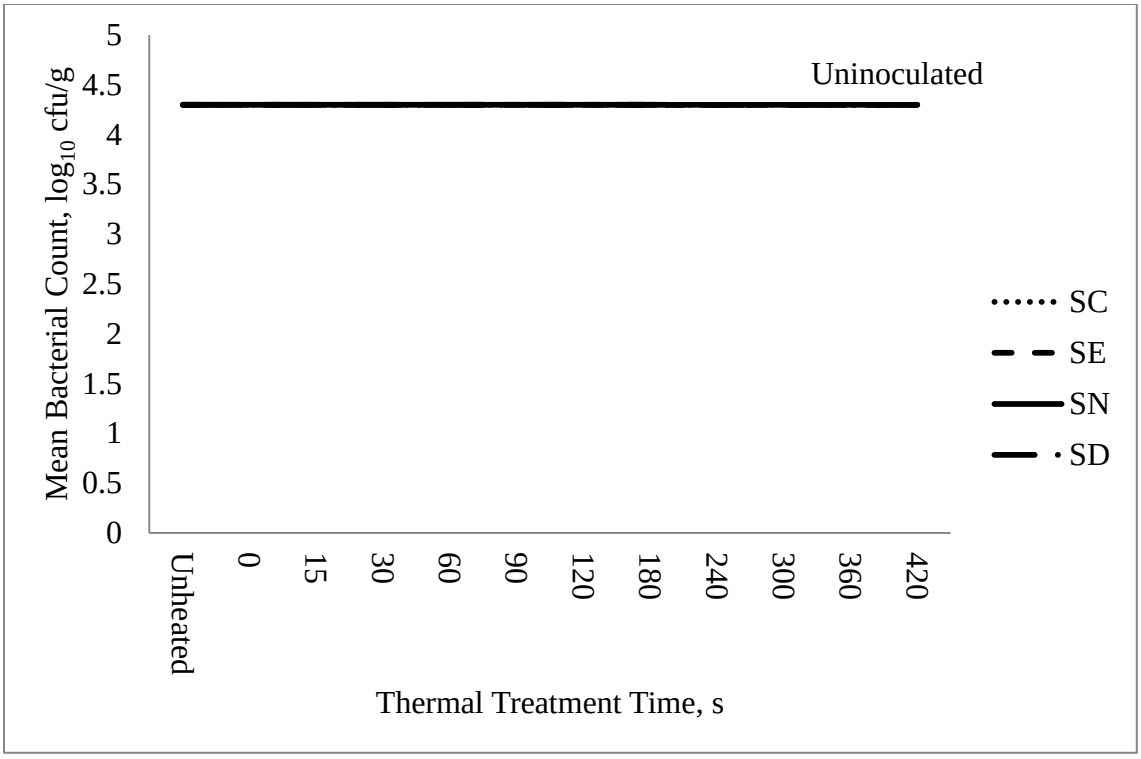

Figure 4.5. Enumeration of total bacteria on TSA from uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

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

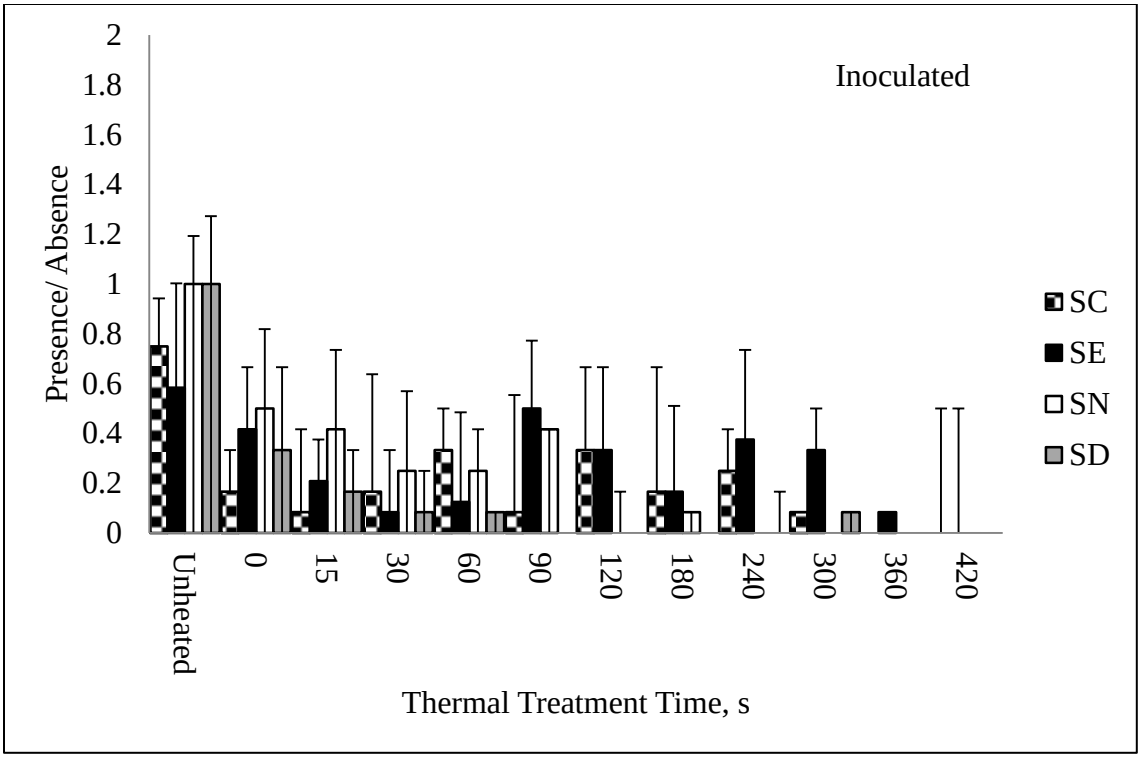

Figure 4.6. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, RV pre-enriched poultry rendering samples (50% fat).[1]

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

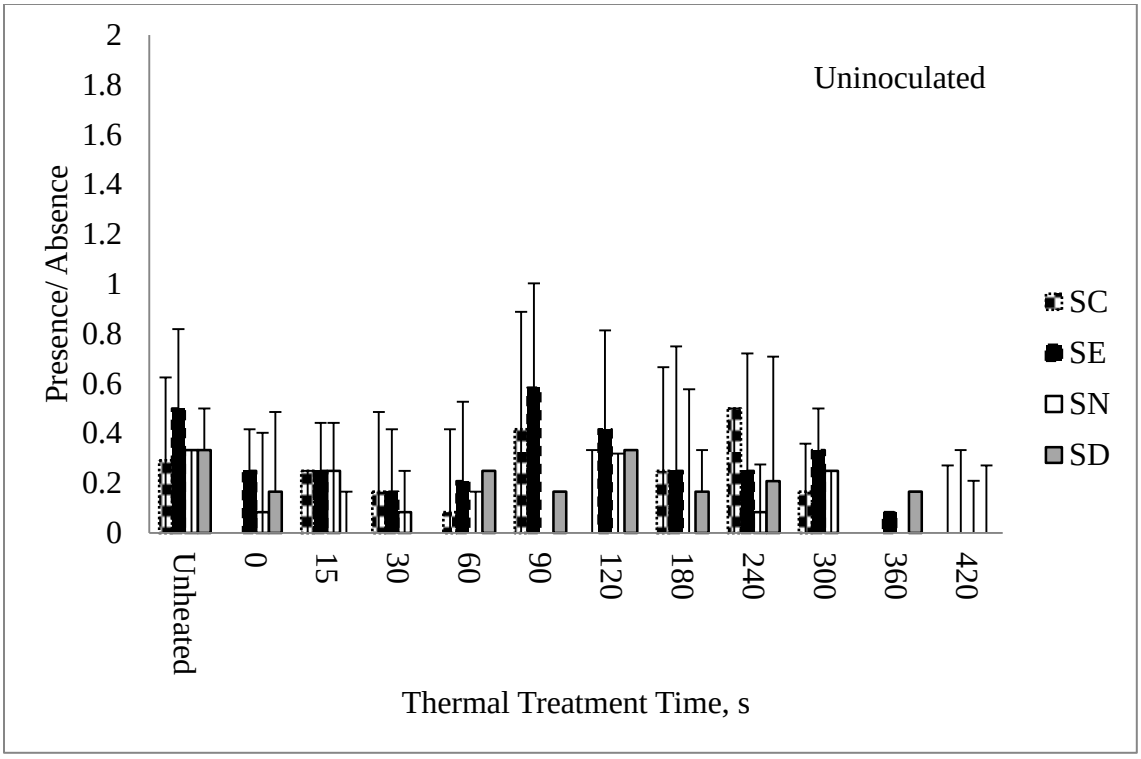

Figure 4.7. Presence or absence ± standard deviation of *Salmonella* for each RV pre-enriched, uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

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

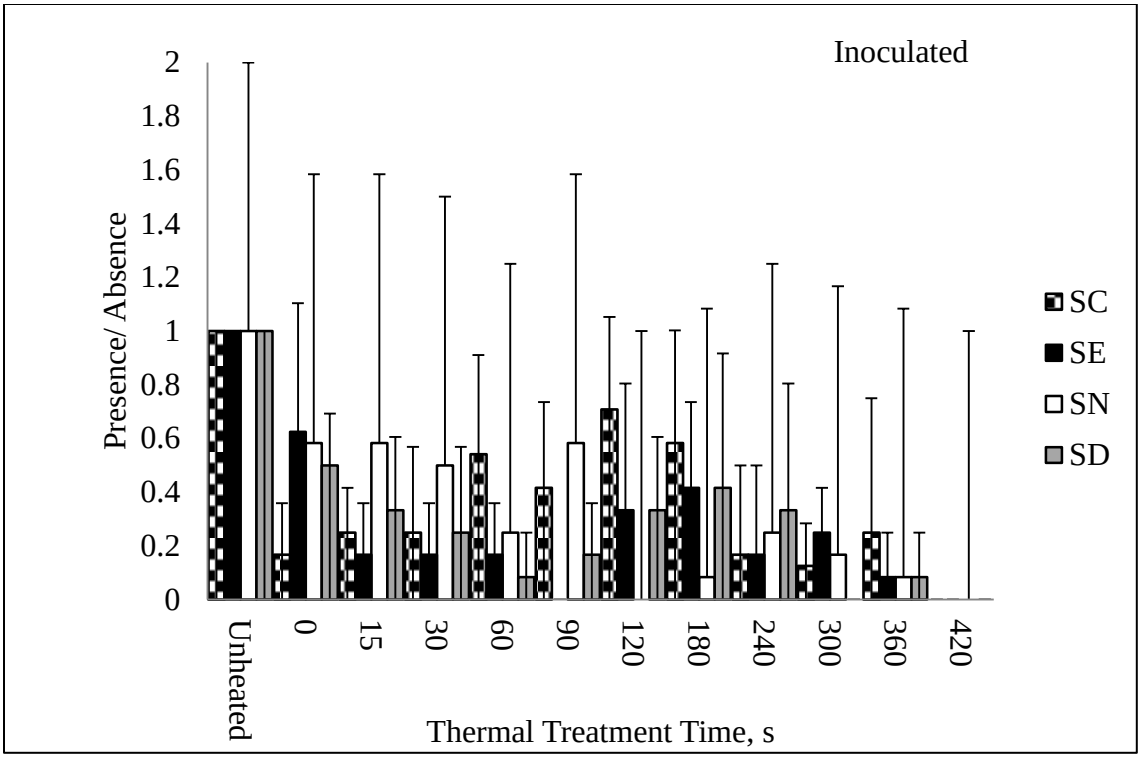

Figure 4.8. Presence or absence ± standard deviation of *Salmonella* for each *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD) inoculated, TT pre-enriched poultry rendering samples (50% fat).[1]

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

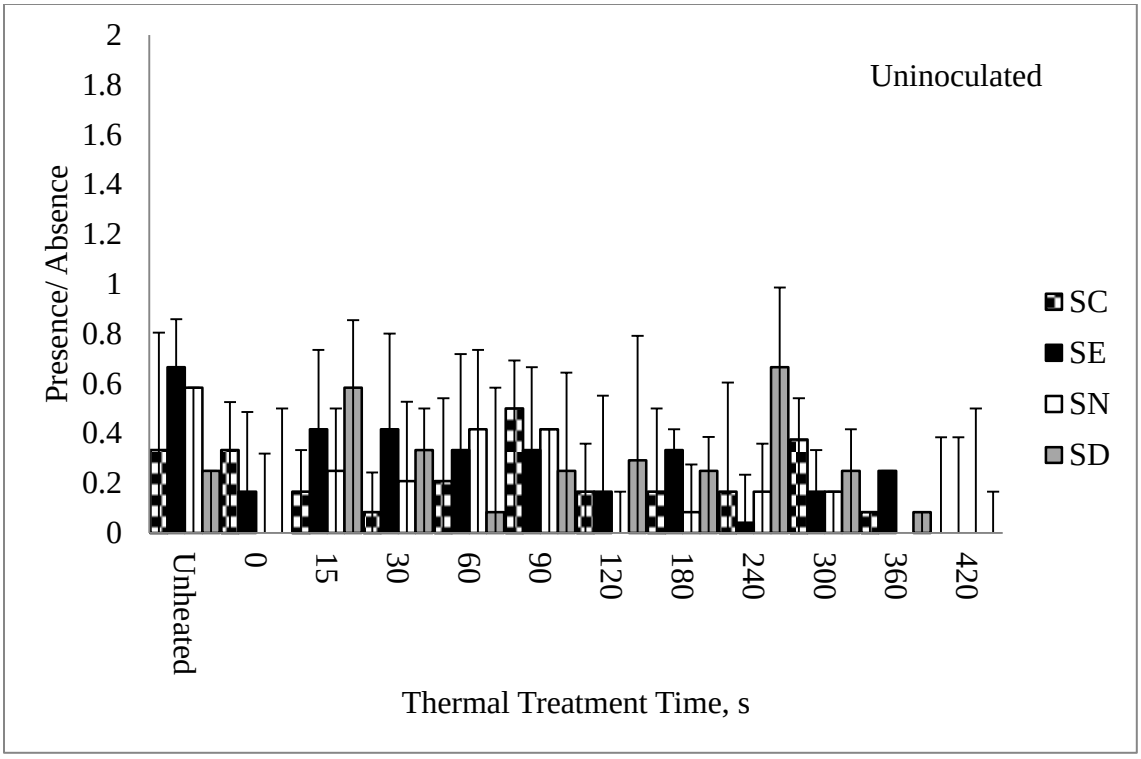

Figure 4.9. Presence or absence ± standard deviation of *Salmonella* for each TT pre-enriched, uninoculated poultry rendering samples (50% fat) for *Salmonella* Choleraesuis (SC), *Salmonella* Enteriditis (SE), *Salmonella* Newport (SN), and *Salmonella* Dublin (SD).[1]

[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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