

FINAL REPORT
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**VALIDATION OF THERMAL DESTRUCTION OF PATHOGENIC BACTERIA
AND VIRUSES IN RENDERED ANIMAL PRODUCTS**

Principal Investigator(s): Annel Greene, Professor
agreene@clemson.edu
Department of Animal & Veterinary Sciences
250 Poole Agricultural Center
Clemson University
Clemson, SC 29634
Telephone: (864) 656-3123
FAX: (864) 656-3131

Collaborators: Yubo Zhang, Master of Science Graduate Student
yuboz@g.clemson.edu
Department of Food, Nutrition & Culinary Sciences
250 Poole Agricultural Center
Clemson University
Clemson, SC 29634

William C. Bridges, Jr., Professor
wbrdgs@clemson.edu
Department of Applied Economics and Statistics
243 Barre Hall
Clemson University
Clemson, SC 29634
Telephone: (864) 656-3012
FAX: (864) 656-1309

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Lay Summary:

The rendering process is used for handling remnants and whole carcasses from poultry and animal slaughter. A disease outbreak in the animal livestock industry could have serious consequences to the rendering industry. With the current level of media coverage, an outbreak of even a low path disease could receive widespread publicity. Upon such an outbreak, miscommunication and lack of solid data could lead to economic instability in the rendering industry and its markets. It is imperative that the rendering industry have conclusive validation data on the effectiveness of rendering to destroy animal disease pathogens. Additionally, 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. Test procedures have been developed through earlier studies funded by the rendering industry. This project was designed to build on that knowledge and validate the thermal destruction of three disease entities in rendering materials. In further consultation/discussion with the members of the ACREC Research Committee and due to expenses/difficulties in developing procedures based on pathogenic microorganisms, it was decided that the project would begin with validation of thermal destruction of one of the most heat resistant, non-pathogenic microorganism known – *Geobacillus stearothermophilus*. *G. stearothermophilus* is up to 20 times more resistant to heat than the very heat stable bacteria *Clostridium botulinum*. Destruction of *G. stearothermophilus* would be indicative that the known pathogens *Salmonella* and *Clostridium perfringens*, which are both known to be less heat tolerant, should be destroyed under the same conditions. In this experiment, 280°F (137.8°C), 285°F (140.6°C) and 290°F (143.3°C) were used as the temperatures in order to determine the different impact rendering material could have on the heat stable *G. stearothermophilus*. Validation of the minimum time factor has been difficult in the rendering cooker. Based on previous research on validating a time factor as well as validating thermal destruction of avian influenza, times of 0 min (the time to come-up to temperature), 1 min and 2 min were chosen for study. Additionally, since the rendering industry claims processing times between 40 and 90 min for most rendering cookers, an additional study was conducted at 40 min processing time. Four different poultry rendering crax/fat samples from three rendering facilities were used in the study and adjusted to 50% fat. During the project, methodology challenges proved difficult, time-consuming and expensive but the research team was successful in developing new methods that allowed conducting thermal death time studies on the high fat rendering materials. Experimental results indicated that only 2 min at the lowest temperature tested (280°F) destroyed approximately 2 log cfu/g of *G. stearothermophilus*. However, the data also clearly demonstrated that increased temperature caused much greater lethality with approximately 2.2 log cfu/g destroyed after 2 min at 285°F and approximately 4.6 log cfu/g destroyed after 2 min at 290°F.

Objective(s):

To prove that thermal processing of rendered materials destroys pathogenic bacteria and viruses, a two-stage study will be conducted to 1) identify methodologies to verify presence/absence of the virus and 2) determine the thermal dose necessary to destroy three pathogens (to be selected in consultation with the rendering industry representatives and contingent on availability of suitable primers) in rendering materials.

Project Overview:

Abstract

The non-pathogenic, heat stable organism *Geobacillus stearothermophilus* was used during thermal death time testing of rendered poultry materials from three different rendering plants. *G. stearothermophilus* is an endospore-forming, Gram positive, thermophilic bacterium that is commonly used in the canning industry. A modification of the method of Kim and Naylor (1966) was used to prepare a *G. stearothermophilus* spore suspension. Uninoculated and inoculated rendering samples containing 50% fat and rendered crax were prepared. Lecithin and 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) were used during sample enumeration to emulsify the high fat samples and to aid in detection of colonies, respectively. Commercial rendering cookers are reported to process in the temperature range of 240 to 290°F (115.6 to 143.3°C). In this experiment, 280°F (137.8°C), 285°F (140.6°C) and 290°F (143.3°C) were used as the temperatures in order to determine the different impact rendering material could have on the heat stable *G. stearothermophilus*. was used as the temperature in order to determine the maximum impact rendering could have on the heat stable *G. stearothermophilus*. The temperature selected for this experiment was the most rigorous rendering temperature reportedly used in commercial rendering cookers. Uninoculated and inoculated samples included unheated control and 0 min, 1 min and 2 min heat treatment at 290°F (143.3°C). Results of this study indicated that inoculation condition, time, inoculation & time combination, and sample, inoculation & time combination had a strong impact on bacterial populations. Sample & inoculation combination, replications within different sample & inoculation combination, sample difference, and sample and time combination had no significant effect or impact on data. Although *G. stearothermophilus* provided a valid thermal death time indicator for rendering processing, it may not be the best surrogate for use in the rendering industry for environmental studies since thermophilic bacterial colonies were detected in uninoculated controls. This research study is the first known study on use of surrogate bacteria as an indicator organism to validate the thermal treatment for the rendering industry. Further studies need to be done to provide more scientific data and explanation.

Introduction

A by-product is defined as a secondary product obtained during the manufacture of a principal commodity. Animal by-products, also known as animal co-products, can include residual tissues, hides, skins, hair, feathers, hooves, horns, feet, heads, bones, blood, organs, and sometimes whole carcasses (Meeker and Hamilton, 2006). The rendering industry processes raw animal by-product materials into value-added, shelf-stable products. The finished rendered products include fats such as tallow, lard and poultry fat and proteins such as meat and bone meal (bovine and/or porcine) and poultry meal. While the fats are utilized in a variety of products ranging from animal feeds to cosmetics to oleochemicals to biodiesel, rendered proteins are used primarily as animal feed ingredients. Because of their great economic value, the by-products of food animal production have most recently been called "animal co-products" (Meeker, 2006).

Annually, approximately 100 million hogs, 35 million cattle and eight billion chickens are slaughtered and processed in the United States. Of these, on average 51 percent of the live weight of cattle, 56 percent of the live weight of hogs, 63 percent of the live weight of broilers, and 43 percent of the live weight of most fish species can be consumed by human. The remainder of the animal is considered inedible and typically these animal co-products are rendered. As the world's population continues to grow, animal food product volume is increasing. Recently, the trend

towards convenient boneless, skinless, ready-to-eat (RTE), and/or heat-and-eat packaged foods has increased the amount of raw animal tissues which are diverted to the rendering industry (Kinley, 2009). In 2006, approximately 54 billion pounds of raw materials were generated in the United States. Because these organic materials contain about 60 percent water, 20 percent fat and 20 percent protein and mineral, microbial growth and degradation can occur very rapidly under warm conditions. Therefore, raw animal co-products could carry animal viruses and for bacteria such as *Salmonella* species, *Clostridium perfringens*, and *Listeria* species. With the growth of these organisms, raw animal tissues could become a health hazard to both humans and animals without a disinfecting treatment such as rendering (Meeker, 2006).

Throughout human history, animals have been used for various applications and consumption, and after slaughter, inedible residual parts of the animal have been generated. Rendering, as one of the oldest animal processing activities, has been practiced for many centuries. Animal rendering is the converting or recycling process that transforms raw animal tissue from animals and extracts fat by melting. In addition, the modern rendering industry also processes waste cooking fats and oils from restaurants. Approximately 85 percent of rendered products are used as animal feed ingredients. The rendering industry, through their trade organization the National Renderers Association, has reported over 3000 rendering product industrial applications identified in many areas including metallurgy, soap, personal care industries, biofuel, and chemical industries (Meeker and Hamilton, 2006).

There are currently many alternative ways to handle animal co-products such as burial/landfill, composting, anaerobic digestion, and incineration. However, there are many limitations and disadvantages with other methods for disposing of animal carcasses or by-products. For example, disposal of animal by-products into landfills would introduce tremendous risk to both humans and animals due to lack of pathogen control, biosecurity and traceability. In addition, with the volume of animal by-products generated annually, there would be insufficient landfill space to accommodate this material stream.

Another alternative method used to dispose animal by-products is composting. However, during the composting process, it is necessary to balance the carbon and nitrogen ratio as well as moisture content in the raw by-products to ensure proper composting. Volume of raw material would make composting limited to small-scale operations in areas where carbonaceous plant material would be sufficient. This would be especially true in a high mortality event where carcass disposal requirements would be increased. A rendering processing plant can handle very high volumes of raw material per hour and rapidly stabilize the perishable products (Woodgate et al., 2006). With proper wastewater and air emission treatment, rendering can protect groundwater and the environment from contamination due to rotting carcasses.

Rendering has the advantage of adding a thermal treatment to destroy disease-causing microorganisms which potentially could reside in the raw tissues. Rendering also generates a value-added, shelf-stable product. This product stream resulted in a significant economic addition to the animal livestock industry. In 2006, the value of the products sold via the worldwide rendering industry was estimated to be \$6 to 8 billion annually (Swisher, 2006). Considering the biosecurity, processing volume, time, and environmental and economic aspects, rendering offers a reliable animal by-product handling solution (Meeker and Hamilton, 2006).

Rendering Processes

In rendering, through the use of heat, moisture is removed and animal fat melts to allow easy separation. As a result, the rendering process causes both physical and chemical transformation of raw animal tissues. Various types of rendering systems are in use commercially and, in North America, most rendering systems are continuous-flow units. In 2006, there were 273 rendering plants in the United States and 29 in Canada (Bisplinghoff, 2006). These rendering plants annually process more than half of the total weight of material produced by animal agriculture. (Meeker and Hamilton, 2006).

Despite different rendering systems and different types of raw materials being processed, there are several general steps common in all rendering processing. Since these tissues are not processed for human consumption, the processing environments of rendering plants are very different from food processing plants. Uncovered open raw material bins or pits are used to convey these animal co-products into the rendering processing plant. Most American rendering plants use a continuous cooker which is an agitated vessel generally heated through indirect contact by boiler steam to bring the raw material to between 240° and 290°F (115° to 145°C). This temperature is designed to inactivate bacteria, viruses, protozoa and parasites. The range of processing time is reported to be 40-90 minutes (Meeker and Hamilton, 2006). These conditions are not proven for destruction of prions but because of strict guidelines concerning ruminant to ruminant feeding, prions should not be encountered in U.S. or Canadian rendering.

Rendering cookers are used to evaporate moisture and free fat from protein and bone components. The majority of the moisture is removed in the cooking process (Meeker and Hamilton, 2006). Once the materials have been fully heated and processed, free fat will be drained by passing the heated material over a screen. Remaining solid materials are conveyed to a screw press where additional fat is removed. “Cracklings” or “crax,” which includes protein, minerals and residual fat, exit the screw press and are conveyed to a storage bin for cooling. After cooling, the crax are ground to particles resembling the size of corn meal. The ground meal is conveyed to storage silos for subsequent shipment. Fat is centrifuged to remove residual particulate and stored in insulated and/or heated silos. Because molten fat can be pumped, the renderers maintain the fat at temperatures to maintain the fat in a liquid form.

In the early rendering industry, open kettles were used to cook the fat over a wood fire or hot coal (Anderson, 2006). Several major types of rendering processing are used in North American rendering facilities, including wet, dry, batch and continuous rendering. (Ockerman and Hansen, 1988; Anderson, 2006). Wet rendering is similar to an autoclave system in which steam is injected directly in contact with the product. Vertical digesters are used in wet rendering systems to inject steam into the material through perforated plates. There are many disadvantages involved in wet rendering, including long cooking time, labor-intensity, large amount of meal lost during processing and high moisture; however, the advantages include production of high quality of tallow (Ockerman and Hansen, 1988). The finished products of wet rendering tend to contain high moisture which may or may not be removed during further process.

In the modern rendering industry, dry rendering systems are most commonly used. Both batch rendering and continuous rendering can be used to accomplish the dry rendering process. In the

batch rendering system, materials are loaded into the cooking vessel, exposed to a thermal process to cook and dry the materials and, subsequently, fat is separated (Anderson, 2006; Kinley, 2009). Batch systems usually have little loss of material during process. Batch cooker systems have an advantage of allowing processing material in the same vessel, thereby allowing different rendering products to be separated in different cookers. Batch rendering also has many disadvantages including the inability to rapidly and continuously process large volumes of materials, the high probability to produce darker tallow, and the use of large amounts of energy resulting in an expensive cost to operate (Ockerman and Hansen, 1988).

European rendering cookers are pressurized whereas North American rendering cookers are not. Pressure cooking was initially designed to make the bones softer and easier to handle. Due to cooker designs which enhance particle size reduction, the pressure-cooking step has been eliminated in North American rendering. However, European rendering facilities still use pressurized technology as part of their bovine spongiform encephalopathy (BSE) reduction programs.

Continuous rendering systems are used nearly exclusively in North American rendering facilities. In this process, an initial grinding step is dependent on the starting material. Larger animals such as cattle and hogs are ground into smaller, more uniform particles. Smaller animals such as poultry are not ground prior to cooking. The materials are transferred into a continuous cooker and heated to 240 to 290°F (115 to 145°C). In the most common type of dry rendering continuous cookers used in North America, steam is used to heat the internal metal components of the rendering cooker but is not actually added into the rendering product. Water from the condensed steam is conveyed out of the cooker back to the steam generator.

After cooking, the molten fat and protein/bone material are separated through a screen drainer. An auger conveyor is typically used after the separation of liquid fat and solid material to move the material to the screw press. The screw will further reduce the fat content of the solid material (Ockerman and Hansen, 1988; Anderson, 2006; Kinley, 2009).

Microbial Aspects of Rendering

Many pathogenic bacteria species survive and exist in animals and especially in digestive tracts. These can include *Staphylococcus* species, *Listeria* species, *Bacillus* species, *Clostridium* species, *Mycobacterium* species, *Enterobacteriaceae*, *Pseudomonas* species, *Aeromonas* species, *Plesiomonas shigelloides*, and *Vibrio* species (Jay, 2005). Smith et al. (2001) reported 23 percent of cattle fecal samples were contaminated with *E. coli* O157:H7. Swanenburg et al. (2001) reported 47 percent of swine were contaminated with *Salmonella*. Therefore, the incoming raw rendering materials from animals could serve as a reservoir for many pathogenic bacteria, which, if not destroyed, could lead to the contamination of animal feed. As a significant source of proteins and fat used in the animal feed industry, rendering products play a crucial part of animal feed safety.

There is a concerted effort among food safety experts to create a "farm-to-fork" model for breaking the cycle of potential pathogens entering the food chain. Because animal feed is the initial source of the livestock "farm-to-fork" model, considerable study has been directed toward ensuring animal feed safety. The emphasis on animal feed safety was highlighted by the emergence of

variant Creutzfeldt-Jakob disease (CJD) which was transmitted from beef to humans. CJD is a transmissible spongiform encephalopathy caused by prion-contaminated cattle feed in the United Kingdom (Brown and Will, 2001).

Early investigations into the role of animal feed transmission of pathogens centered on *Salmonella* and other *Enterobacteriaceae* in finished feed and rendered products (Gordon and Tucker, 1965; Loken, 1968). Crump et al. (2002) reported *Salmonella* in animal feeds could lead to foodborne outbreaks. In 2010, an egg recall due to *Salmonella enteritidis* 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 (http://rendermagazine.com/articles/2010_issues/2010_october/2010_10_newsline)

Recognizing potential for problems with *Salmonella* contamination, the rendering industry established the Animal Protein Producers Industry (APPI) in 1984 "to promote and heighten the production and manufacture of safe animal." In 2004, APPI affiliated with the National Renderers Association established a rendering industry Code of Practice in order to strengthen the biosecurity of rendering processing plants and rendering products.

In a rendered product environmental investigation, Troutt et al. (2001) examined nine rendering facilities and 14 processing lines located in six states in the midwestern area of the U.S. No *Salmonella* was found in crax samples or in the rendering processing environment. *Clostridium* species including one sample that contained *C. perfringens* were found in eight crax samples. No most probable number testing was conducted during the study. Troutt et al. (2001) reported another investigation among 17 rendering facilities and 21 processing lines in seven states in the midwestern U.S. area. *Clostridium* species including *C. perfringens*, *Listeria* species including *Listeria monocytogenes*, *Campylobacter* species including *C. jejuni* and *Salmonella* species were found in the raw rendering materials. *Clostridium* species (except *C. perfringens*) were found in crax samples. No pathogenic bacteria were found in the final rendered products.

Recently, a research survey was conducted to determine the prevalence of *Salmonella* and enterococci in rendering products from 12 rendering companies (Kinley et al., 2010). In rendered meal samples collected from all over the United States, a low contamination rate of *Salmonella* (8.6%) and no *Escherichia coli* were detected. This report suggested improved rendering product safety as compared to a previous analysis conducted by FDA and indicated that the rendering industry has made progress toward controlling pathogenic bacteria and improving the safety of animal by-products. Jones (2011) conducted research of *Salmonella* in animal feed, and reported that *Salmonella* cells were present in low numbers in animal feed.

High temperature in the cooking process will reduce the number of microorganisms in the raw perishable animal tissues, however, studies have indicated that not all of the microorganisms are inactivated or killed during the rendering process or recontamination post-process can occur (Crump et al., 2002).

In the North American rendering industry, thermal processing is used to kill and inactivate microorganisms that are in raw tissues; however, excessive temperature/time can reduce the nutritional quality and palatability of rendered fats and proteins. Proper temperature/time and

other factors are crucial for killing potential pathogenic bacteria that harbored in raw animal tissues and maintain the quality of the products (Meeker and Hamilton, 2006).

Bacterial Thermal Death Time

Any thermal inactivation of microorganisms is dependent on time/temperature control. Thermal death time (TDT) is defined as the time needed to kill or reduce a given number of organism at a specific temperature (Jay, 2005; Teixeira, 2006). D value is defined as decimal reduction time, which indicates the time needed to kill 90% of a particular organism at a specific temperature. D value is associated with bacterial death rate. D_0 refers to the D value at 250°F (121.1°C). The z value reflects the temperature needed to traverse one log on the thermal destruction curve. The F value is a very useful reference in designing thermal processing, and it equals the time needed to kill a specific number of microorganisms at 250°F (121.1°C). F_0 is a universal standard value to show the capacity of a heat process. The 12-D concept is used as a lethality time required for the canning industry, and refers to the time required to destroy 12 logs of *C. botulinum* (Jay, 2005; Teixeira, 2006).

In the food industry, numerous research studies have been conducted regarding the different factors affecting the thermal lethality of pathogens. Many factors including cooking methods, food formulation and composition, product types have been identified which affect thermal death times of pathogenic bacteria (Senhaji and Loncin, 1977; Schoeni et al., 1991; Juneja et al., 1994; Ahmed et al., 1995; Kotrola and Conner, 1997; Veeramuthu et al., 1998; Doyle and Mazotta, 2000). For instance, Murphy et al. (2002) and Murphy et al. (2004) demonstrated that *Salmonella* and *Listeria innocua* had significantly different thermal inactivation D and z values among several different formulated commercial products such as chicken breast meat, chicken patties, chicken tenders, franks, beef patties, blended beef and turkey patties.

Raw rendering materials have many unique characteristics which are different from commercial meat products used for human consumption. In general, raw rendering materials contain approximately 60% moisture, 20% fat and 20% meat and bone (Meeker, 2006). In order to thermally process in a rendering cooker, these raw materials are deposited into hot rendered fat. Therefore, the fat content in the cooking matrix can be greater than 60% fat. Senhaji and Loncin (1977) studied the effect of oil/water matrices on the heat resistance of *Bacillus subtilis* spores and *Pseudomonas fluorescens*. They reported that increasing oil content conferred heat resistance to the microorganisms during thermal trials at 85°C, 95°C and 105°C. Ramirez-Lopez (2006) studied the thermal resistance of bacteria using ground beef as a model media for raw rendering materials and concluded that greater than 96°C is necessary to destroy heat-resistant organisms isolated from rendering materials. Glenn (2006) measured microbial loads in raw poultry rendering materials and discovered difficulties in enumerating bacteria by traditional aqueous buffer dilution methods due to the high fat content of the rendering material. Since fat and water are not miscible, large particles of fat floating in dilution buffers made it difficult to enumerate bacteria and determine the effect of thermal processing (Glenn, 2006). Leaphart et al. (2012) studied thermal death time avian influenza in rendering materials using sub-particles of the virus and reported complete destruction of the RNA at 110°C for 15 sec. The thermal death time of most pathogens in rendering materials has not been determined.

Emulsifiers

Emulsifiers allow the mixing of two immiscible phases. Commonly used to allow mixing of fat and water, emulsifiers consist of molecules which have portions attracted to water and portions attracted to fats. These portions can be hydrophilic or hydrophobic and lipophilic or lipophobic (Whitehurst, 2004).

Lecithin, which consists of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidic acid, is a popular emulsifier used in the food industry. Common sources of lecithin are soybeans, egg yolk, and sunflower (Szuhaj and List, 1985; Whitehurst, 2004). According to FDA Guidance for Industry (2006), lecithin is considered generally recognized as safe (GRAS). Weete et al. (1994) indicated that lecithin had improved water/oil emulsification after heat treatment. In their study, lecithin was preheated to 180°C for 90 min and then was mixed with water/oil phases which also had been pre-heated to 60°C. The researchers measured properties following mixing for 1 min and cooling. Viscosity, discontinuous phase-holding capacity, stability and water retention were reported to be greatly improved in thermalized lecithin versus non-thermalized lecithin.

Geobacillus stearothermophilus

Geobacillus stearothermophilus is an endospore-forming, Gram positive thermophilic bacterium. The organism is ubiquitous with soil being a reservoir. *G. stearothermophilus* bacterial spores, with a D₁₂₁ between 4.0 to 5.0 minutes and z-value between 7.8 to 12.2°C, are capable of withstanding extreme heat such as found in the thermal processing and handling during food production (Abdul Ghani, 2001). *G. stearothermophilus* was initially isolated by Donk in 1917 from canned corn which had been heat treated at 118°C for 75 minutes. Donk named the organism *Bacillus stearothermophilus* (Donk, 1920). However, the genus name was changed in 2001 to *Geobacillus* (Nazina et al., 2001). With up to 20 times more heat resistance than the heat stable *C. botulinum*, inactivation of *G. stearothermophilus* would indicate the destruction of other microorganisms and especially vegetative cells (Abdul Ghani, 2001; Iciek, 2008). As a challenge organism in food industries such as canning, *G. stearothermophilus* is commonly used to indicate effectiveness of the sterilization cycle.

Microorganisms of Potential Concern in Rendering

Clostridium perfringens is a spore-forming, Gram-positive bacillus that is frequently associated with foodborne illnesses in the United States (Shandera et al., 1983; Bean and Griffin, 1990; Granum, 1990). *C. perfringens* can grow at temperatures between 10 and 52°C (FDA-CFSAN, 1998) but has a higher growth rate between 30 and 47°C (Craven, 1980; Juneja et al., 1994). The spore form of *C. perfringens* is more resistant to heat treatment than vegetative cells and can survive normal processing conditions during food processing conditions. *C. perfringens* spores can be activated by sub-lethal heat and also can withstand heating treatment at 100°C for up to 1 hour (Collee et al., 1961; Barnes et al., 1963; Huang, 2003). Therefore, heat-resistant *C. perfringens* spores could become hazardous to public health without proper food treatment and maintenance. *C. perfringens* is one of the organisms of concern to the rendering industry (Meeker, 2006).

Salmonella is a member of the family *Enterobacteriaceae*, and is commonly correlated with foodborne disease (Jay, 2005). *Salmonella* are Gram-negative, rod-shaped, motile, non-spore-forming bacilli which can ferment glucose, maltose, and mannitol (Jay, 2005). These organisms

are not highly resistant to physical or chemical agents, but are able to multiply over a wide variety of conditions (Franco, 1997). The optimal growth temperature for *Salmonella* is 37°C and growth is faster in moist conditions (Franco, 1997). *Salmonella* can be destroyed at 55°C for 1 hour or at 60°C for 15 to 20 minutes (Franco, 1997). In the United States, approximately 2 to 4 million cases of salmonellosis occur annually (HHS and FDA, 2009). Many of these *Salmonella* spp. outbreaks are associated with consumption of animal products (Shacher and Yaron, 2006). *Salmonella* species have been reported in many mammals, fish, reptiles, amphibians, arthropods, insects and also birds (D'Aoust et al., 2001). In 2010, FDA identified eight strains of *Salmonella* serotypes that are pathogenic to animal and listed those organisms as concern for potential for transmission through animal feeds. The organisms of concern associated with poultry were *Salmonella* Pullorum, *Salmonella* Gallinarum, or *Salmonella* Enteritidis. The organism of concern for swine was *Salmonella* Choleraesuis and for sheep was *Salmonella* Abortusovis. For horses, the pathogenic *Salmonella* serotype was identified as *Salmonella* Abortusequi and for cattle *Salmonella* Newport or *Salmonella* Dublin were listed as pathogenic. Certain researchers demonstrated that animal feed is not an important source of *Salmonella* spp. contamination in poultry industries (Brunton, 1990; Cox and Bailey, 1991), whereas Crump et al. (2002) claimed that animal feeds were a source of contamination and could lead to transmission of *Salmonella* to humans. In order to reduce *Salmonella*, the rendering industry created the Animal Protein Producers Industry (APPI) to promote biosecurity in rendered animal feeds. In a study on *Salmonella* contamination in rendered animal co-products, Franco (2005) reported 25% of weekly samples collected from rendering processing plants were contaminated with *Salmonella*. One of the major reasons for the contamination of rendering products with *Salmonella* is considered to be cross-contamination from the raw animal tissue during processing (Ockerman and Hansen, 1988). Other sources for *Salmonella* contamination such as including environmental surfaces and equipment have been studied. Kusumaningrum et al. (2003) demonstrated *Salmonella* could survive and adhere to stainless steel. Although there is little stainless steel in a rendering plant, the adherence to surfaces is a valid concern. Jones and Bradshaw (1996) reported *Salmonella* spp. are capable of producing biofilms on environmental surfaces. It is crucial to prove that temperature/time and other factors are reliable and efficient enough to kill *Salmonella* that harbored in raw animal tissues. However, few articles or research has been done so far to further prove the hypothesis.

Thermal Death Time Studies

Numerous researchers have reported various methods for determining thermal death time. David and Merson (1990) conducted inactivation of *G. stearothermophilus* using a modified resistometer, which had computer control to monitor the time and temperature. In this study, a spore suspension was carried by piston into a steam heated chamber.

Abraham et al. (1990) used capillary tubes (75 mm length, 1.15 mm inside diameter and 0.2 mm glass thickness) filled with *G. stearothermophilus* spore suspension during heat treatment in an oil bath. Capillary tubes were crushed into a recovery medium which contains bio-trypticase, Bacto-soytone, dextrose, NaCl, bromcresol purple. Ocio et al. (1995) used ring marked micro-haematocrit capillary tubes (0.7 mm inner diameter, 1.5 mm outer diameter and 75 mm length) containing the microbial suspension. These tubes were heated in an oil bath after sealing.

Abdul Ghani et al. (2001) reported using a three dimensional pouch (0.12 m width, 0.04 m height,

0.22 m length) to expose samples to 121°C for measuring the heat resistance of *G. stearothermophilus*. After heat treatment, spore agar was used to enumerate the spores. Rajan et al. (2005) used custom-fabricated aluminum tubes and an oil bath to determine thermal inactivation of *Bacillus stearothermophilus* spores in egg patties. Ramirez-Lopez (2006) conducted a thermal treatment in ground beef using pyrex tubes in a heating block to simulate the heating process during rendering. Byrne et al. (2006) used custom-made thermospacers and bags (Deli bags and plastic bags) were used in studying thermal death time. Thermospacers consisted of an acrylic base with vertical dividers to ensure all sample bags were evenly heated. Schubert and Beaudet (2010) used blind-end stainless steel tubes (1.27 cm internal diameter, 10.16 cm long, 0.025 cm wall thickness) and a high-temperature silicone oil bath to determine the lethality rate of bacillus spores when exposed to dry heat.

In past studies, it has proven difficult to determine the exact time and temperature that is applied to rendered materials during cooking (Greene, 2011). Modern rendering cookers used in the U.S. and Canada are large, continuous processing units (<http://www.dupps.com/cookdry.html>). Because moving internal parts would entangle thermocouple wires and because the temperatures used in the cookers are above the failure point of battery-operated wireless devices, validation of the inside temperatures of rendering cookers has been challenging (Greene, 2011). In recent studies conducted in this laboratory, temperatures and flow rates of rendering materials after exiting the cooker have been measured. It has been determined that temperatures in a 2 min time frame post-cooker can be reliably measured. Therefore, the purpose of this study is to determine the thermal death time of the heat stable organism *G. stearothermophilus* in rendering materials within a 2 min time frame. For purposes of this study, the temperature maximum typically used in rendering was chosen to simulate the maximum lethality that can be validated through rendering cookers. Due to the processing time within the cooker which is reported by the renderers to be between 40 and 90 minutes at 240 to 290°F, additional lethality will obviously be incurred.

Further Studies: Surrogate Microorganisms in Rendering Processing Environments

Surrogate organisms, also known as indicator organisms or marker organisms, are a group of non-pathogenic organisms utilized to indicate the potential of food product quality issues and/or safety complications. Since the non-pathogenic, surrogate organisms behave similarly to pathogenic organisms when exposed to the same treatment or conditions, surrogate organisms are applied to a given food at certain levels or a specific condition to assess and determine the existing food product quality, as well to improve product shelf life. (Liu and Schaffner, 2007).

Surrogate bacteria are commonly used in many industrial areas. *Bacillus subtilis* and *Clostridium sporogenes* have been used as indicator organisms to detect the potential for conditions conducive to survival of *Clostridium botulinum* (Ananta et al., 2001). Additionally, generic *E. coli* (Duffy et al., 2000; Leenanon and Drake, 2001) and *Enterococcus faecium* (Franz et al., 2001) have been used in studies related to *E. coli* O157:[H7]. *Listeria innocua* (Goff and Slade, 1990; Piyasena and McKellar, 1999; Sabanadesan et al., 2000), *Lactobacillus delbrueckii* subspecies (Siegumfeldt et al., 2000), and *Leuconostoc mesenteroides* (Kalchayanand et al., 2002) have been used in studies of *L. monocytogenes*. *Enterobacteria aerogenes* (Montville et al., 2001), *Lactobacillus bulgaricus*, *Lactococcus lactis*, *Streptococcus thermophilus*, *L. innocua* (Siegumfeldt et al., 2000) have all been used as surrogates for *Salmonella* species.

In the rendering industry, a surrogate organism would be beneficial for thermal death time studies as well as plant environmental quality studies to measure air flow, and product microbial contamination, etc. The following study was designed to investigate thermal death time of the highly heat-resistant bacteria *Geobacillus stearothermophilus* in rendered animal co-products under processing conditions that can be verifiable within the rendering plant. Since *G. stearothermophilus* is more heat resistant than any known bacterial pathogen, thermal destruction of this non-pathogen surrogate should yield validation data for rendering processing as a method of destroying pathogenic organisms that may be inherent in raw animal tissues. The second part of this study is to determine the suitability for using *G. stearothermophilus* as a general microbial surrogate for a variety of uses in the rendering industry.

Observations from Previous Thermal Work in Rendered Animal Co-Products

Previous studies (Glenn, 2006; Greene, 2011) have indicated a heat resistant background flora in processed rendering materials. The nature of this background flora has not been ascertained but it was shown to survive the rigorous thermal treatment of rendering as well as post-process laboratory thermal trials. However, since this organism(s) seems persistent in all rendering materials and no disease has been reported due to the rendered products, it is assumed this is a non-pathogenic, thermophilic, spore-forming organism.

MATERIALS AND METHODS

Rendering Sample Preparation

Cooked poultry rendering samples (poultry fat and crax) were collected from three rendering companies in the southeastern United States. Samples were transported to the laboratory and labeled Plant 1A, Plant 2, Plant 3 and Plant 1B. Crax samples were ground in a food processor (Robot Coupe Model R2 Ultra, Ridgeland, MS) for approximately 10 min to reduce particle size. Each crax sample was analyzed on three separate days for ash, crude fat, crude protein, and dry matter content by the Clemson University Agricultural Service Laboratory. All samples were stored at -20°C until needed in experimentation.

Spore Preparation

A *Geobacillus stearothermophilus* (ATCC 7953) spore suspension was prepared by a modification of the method of Kim and Naylor (1966). *G. stearothermophilus* was incubated for 10 h at 55°C in TYG broth containing 1% w/v tryptone (Bacto® Tryptone, Difco, Detroit, MI), 0.5% w/v yeast extract (MP Biomedicals, LLC, Solon, Ohio), 0.2% K₂HPO₄ (Mallinckrodt Specialty Chemicals, Paris, KY), and 0.5% w/v glucose (Mallinckrodt Baker, Philipsburg, NJ) which had been pH adjusted to 7.2 and autoclaved prior to use. Broth cultures were spread-plated onto twelve 150 mm x 15 mm Petri dishes (Cat No. 25384-326, VWR Scientific Products, Suwanee, GA) containing sporulation agar comprised of 0.8% w/v nutrient broth (Difco, Detroit, MI), 0.4% w/v yeast extract (MP Biomedicals, LLC, Solon, Ohio), 10 mg/L MnCl₂·4H₂O (MP Biomedicals, LLC, Solon, Ohio), and 2.0% w/v agar (Difco, Detroit, MI) and pH adjusted to 7.2. Prepared plates were incubated upright at 55°C for 10 h and then turned upside down for another 18 to 26 h incubation. Spores were washed from agar surfaces using sterile TYG broth, centrifuged at 8,500 x g for 12 min (GSA rotor in a, DuPont RC5C Sorvall Instruments Centrifuge, DuPont Company, Newtown, CT), and the pellet was re-suspended in

cold sterile distilled deionized water (ddH₂O) containing 1% w/v filter sterilized lysozyme (L6876, Sigma Chemical, St. Louis, MO). After overnight storage under refrigeration, the spore suspension was centrifuged and washed four times in cold sterile ddH₂O. Spores were re-suspended in cold sterile ddH₂O and stored under refrigeration until used. The final spore suspension concentration was determined by plating. Over the course of the study, the spore suspension was re-made three times on three different dates; the spore content was adjusted to similar concentration after enumeration and calculation of dilution.

Preparation of Crax/Fat Samples for Thermal Trials

A crax/fat sample (50% fat) was mixed in a disinfected food processor jar (Model 72500R/Type FC08 Food Chopper, Hamilton Beach/Proctor Silex, Inc., Southern Pines, NC). The food processor jar was disinfected by soaking in Antibac B™ (Diversey Corporation, Cincinnati, OH) dissolved in ddH₂O (0.6 g per l) for approximately 1 min, followed by rinsing six times with sterile ddH₂O. Uninoculated samples were removed aseptically and 3 g samples were measured into each sterile stainless steel tube (locally manufactured, 8.5 cm long, 1.6 cm OD, 1.3 cm ID). Three ml of *G. stearothersophilus* spores were added to the remaining blended 75 g rendering sample which inoculated the sample with approximately 10⁶ spores/g. The mixture was blended in the food processor jar and frequently scraped down with a sterile spatula and re-blended to ensure even distribution of the spores. The inoculated samples were measured (3 g each) into sterile stainless steel tubes.

Thermal Trials

Sample tubes were heated in an analog dry block heater (Model# 12621-108, VWR International, Suwanee, GA) equipped with Model#13259-162 heating blocks (VWR International, Suwanee, GA). The block heater was heated to treatment temperatures as measured by thermometers inserted into the blocks. Sample tubes containing either uninoculated or inoculated crax/fat samples were placed in the hot blocks. Thermal trials were conducted at 280°F (137.8°C), 285°F (140.6°C) and 290°F (143.3°C). Additional sample tubes containing crax/fat mix were used for measuring temperature. Upon reaching an internal temperature equaling the treatment temperature of 280°F (137.8°C), 285°F (140.6°C) and 290°F (143.3°C) for each trial, uninoculated and inoculated tubes were heated for 0 min, 1 min and 2 min. Each test procedure comprised a total of 12 uninoculated and 12 inoculated tubes. After thermal treatment, tubes were immediately placed on ice.

Samples were enumerated by plating in duplicate on BHI + 0.1% w/v yeast extract (BHI/ye) agar and incubating at 55°C for 36 h. Because of the high fat content of the samples, previous experiments in this laboratory indicated plating with aqueous diluents can lead to significant error. Standard Class O phosphate/magnesium chloride (PO₄/MgCl₂) dilution buffer (Wehr and Frank, 2004) was used but in order to distribute the high fat sample in the dilution buffer, lecithin (AA36486-A1, Alfa Aesar, Ward Hill, MA) was added as an emulsifier at the rate of 2 g per 100 ml in the first dilution bottle and 0.5 g per 100 ml in the second dilution bottle. These bottles were pre-warmed to

Table 1. Time and temperatures used for thermal treatment of each sample.

Plant 1A	Plant 2	Plant 3	Plant 1B
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Inoculated tubes

Without heat	3 replicates	3 replicates	3 replicates	3 replicates
0 min†	3 replicates	3 replicates	3 replicates	3 replicates
1 min†	3 replicates	3 replicates	3 replicates	3 replicates
2 min†	3 replicates	3 replicates	3 replicates	3 replicates

Uninoculated tubes

Without heat	3 replicates	3 replicates	3 replicates	3 replicates
0 min†	3 replicates	3 replicates	3 replicates	3 replicates
1 min†	3 replicates	3 replicates	3 replicates	3 replicates
2 min†	3 replicates	3 replicates	3 replicates	3 replicates

† timing began after each treatment temperature was achieved

32°C. Subsequent dilution bottles were the standard PO₄/MgCl₂ solution. Rate of lecithin usage was determined in preliminary experiments to select the lowest lecithin rate for most efficient fat emulsification. Preliminary studies also were conducted to ensure lecithin did not affect growth of *G. stearothermophilus*. All prepared dilution bottles were sterilized by autoclaving. Controls included enumeration of the spores using lecithin-modified buffer versus standard PO₄/MgCl₂ buffer. Media and diluent sterility controls also were conducted.

Extended Thermal Treatment Trials

Since these thermal trials indicated the presence of a background flora in rendering samples similar to previous studies in this laboratory, further studies were conducted using same four samples from three different facilities at 280°F (137.8°C) for 40 min after the come-up time. Samples were plated using 10⁻¹ as the lowest dilution in order to have more accurate bacteria count. In order to obtain the 10⁻¹ dilution, 1 g of the crax/fat sample was transfer into 9 ml of 0.5% w/v lecithin tube in order to enumerate the high fat. After thorough mixing on a vortex, the entire crax, fat, and lecithin buffer sample was distributed evenly to three Petri dishes to make 10⁻¹ dilution. After incubation, bacterial colonies were counted on all three Petri dishes to obtain data for the 10⁻¹ dilution. The remainder of the plating procedures were conducted the same as above for the other dilutions.

Enumeration

After incubation, plates were flooded with approximately 1.4 ml of 0.5% w/v 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (EMD TX1510-1, VWR Scientific Products, Suwanee, GA) and incubated at 55°C for 90 to 120 min. Red colonies were enumerated. In order to study effectiveness of TTC for distinguishing bacteria colonies from lecithin droplets, a preliminary study was conducted to compare flooding of plates after incubation with 1% TTC versus use of agar containing 1% TTC.

Statistical Analysis

Data were analyzed by first making a graphical display of how cfu/g changed over time in uninoculated versus inoculated samples. These data were analyzed once before graphing without pooling data. (The lowest limit of reporting was "less than 3.0×10^1 ") A statistical model was then developed which included the overall mean and considerations for treatment (i.e - inoculation condition - yes/no), time (unheated, 0 min, 1 min and 2 min), specific combinations of treatment and time, plant to plant variation and overall error. Analysis of variance (ANOVA) was used on the model to determine if time and/or treatment had any significant effect on cfu/g. Then the Fisher's Least Significant Differences (LSD) t test was used to determine specific differences among time and/or treatment combinations. Statistical Analysis Software (SAS[®] Version 9.1.3, Cary, NC) was used in the analysis.

RESULTS AND DISCUSSION

Preliminary tests

Four cooked poultry rendering samples (poultry fat and crax) were collected from three rendering companies in the southeastern United States. Samples were transported to the laboratory and labeled Plant 1A, Plant 2, Plant 3 and Plant 1B. Plant 1A and Plant 1B were obtained from the same processing plant on separate days. Crax samples were analyzed on three separate days for ash, crude fat, crude protein, and dry matter content by the Clemson University Agricultural Service Laboratory. All samples were stored at -20°C until needed in experimentation.

Spore Preparation

A *Geobacillus stearothermophilus* (ATCC 7953) spore suspension was prepared by a modification of the method of Kim and Naylor (1966). Upon preparation of the spore suspension, clumps of spores were noted. The suspension was sonicated (Vibra-Cell VC-50, Sonics and Materials, Inc. Danbury, CT) for approximately 20 sec to reduce clump size. Visible clumps were still evident after sonication.

For making the first spore suspension, spores were suspended in cold sterile ddH₂O and stored under refrigeration until used. The final spore suspension concentration was determined by plating. The mean spore concentration for the first spore suspension was 2.4×10^8 cfu/ml with a standard error of $0.6 \pm \times 10^8$ cfu/ml. The first spore suspension was used for all the inoculated samples with 290 °F treatment.

For the second spore suspension, due to the visible clumps, the suspension was filtered (Whatman Filter papers, Grade qualitative 4, 20-25 μm) to minimize the amount of visible clumps. The mean spore concentration for the second spore suspension was 2.2×10^8 cfu/ml with a standard error of $0.7 \pm \times 10^8$ cfu/ml. The second spore suspension was used for all the inoculated samples with 280°F and 285°F.

Preparation of Crax/Fat Samples for Thermal Trials

Crax samples were analyzed for fat, protein, ash and moisture content. The mean fat, protein and ash \pm standard error are reported in Table 2. The mean percent moisture content \pm standard error of samples from Plant 1A was 4.6 ± 1.0 , from Plant 2 was 3.2 ± 0.2 , from Plant 3 was 6.0 ± 0.4 and from Plant 1B was 6.1 ± 3.0 . Due to limitation of laboratory for measuring fat content and since the fat samples were known to be practically 100% fat (as per rendering plant information), fat samples were assumed to be 100% fat.

Table 2. Mean percent fat, protein and ash content \pm standard error in crax samples (dry matter basis).

	Mean Percent Fat \pm Standard Error	Mean Percent Protein \pm Standard Error	Mean Percent Ash \pm Standard Error
Plant 1A	46.8 ± 2.7	43.0 ± 1.9	5.7 ± 0.8
Plant 2	12.2 ± 0.2	71.8 ± 2.2	18.3 ± 2.4
Plant 3	13.9 ± 0.4	70.0 ± 1.5	10.9 ± 0.2
Plant 1B	49.0 ± 3.2	42.4 ± 4.6	6.1 ± 2.0

Based on the chemical analysis, samples of fat and crax from each processing plant were re-mixed to yield a 50% fat product which is indicative of typical fat/crax ratios found in a rendering cooker.

Thermal Trials

A preliminary trial was conducted to determine the amount of sample to test during thermal trials. The stainless steel tubes held only a small volume (less than 10 g). In order to conduct microbial enumeration tests, it was imperative that at least 1 g of sample would be available after thermal treatment. Preliminary tests were conducted on 2, 3, and 5 g samples taking into account the potential for thermal expansion during heating. After the preliminary trials, a 3 g sample size was selected for thermal trials.

Commercial rendering cookers are reported to process in the temperature range of 240 to 290°F (115.6 to 143.3°C) (Anderson, 2006). Research in this laboratory has been conducted in an attempt to validate the temperature treatment and time applied in a commercial rendering cooker. However, this task proved to be difficult. The moving impeller inside a cooker prevented suspension of wired thermocouples into the unit. Wireless transmitting thermal data-loggers were unable to transmit a signal out of the thick steel walls of the cooker and in addition the temperatures used in the rendering cooker are at the failure point of most battery-operated electronic devices. Therefore, it has been impossible to validate the temperature and time within the rendering cooker to ensure there are no cold spots. Consequently, a method of measuring temperature and time similar to a dairy processing plant holding tube was devised using the post-cooker draining system for separating crax from free fat. In this validation hypothesis, the engineer at the rendering cooker manufacturing company ascertained that the absolute fastest any rendering material could exit this draining unit was 1.9 min. In experiments in this laboratory, the temperature of materials inside this draining unit were measured and correlated with exit temperature of the cooker. Therefore,

this experiment was designed to study thermal death time under the conditions that could be validated in a rendering cooker system using the draining unit. In the study, the extremely heat resistant sporeformer *G. stearothermophilus* was selected as a surrogate. *G. stearothermophilus* is a commonly used indicator organism in the canning industry due to its high heat resistance. It is reported to be up to 20 times more heat resistant as the dangerous pathogen *Clostridium botulinum* which itself is much more heat resistant than *Salmonella*. Because it was not known if *G. stearothermophilus* could be killed under rendering conditions, in this study, the most rigorous rendering temperature (290°F [143.3°C]) was selected and a high inoculation rate was selected. Due to the 1.9 min known time factor that rendering material temperature could be validated, experimental treatment times of unheated, 0 min, 1 min and 2 min were selected. The timed treatments began upon sample core temperature reaching (290°F [143.3°C]). The commercial rendering cooker acts as a large frying unit in which hot fat is pre-heated and raw materials are deposited into this fat. The hot fat treatment results in formation of steam which is removed and the treatment cooks the rendering materials. It is known that during the 40 to 90 min at 240 to 290°F (115.6 to 143.3°C), there will be significant thermal lethality on microorganisms. However, due to the unique design of the rendering cooker, it is difficult to validate the time/temperature within the cooker and ensure there are no cold spots. However, since this is a large volume of very hot fat which has a high heat capacity, it is unlikely there are significant cold spots within the cooker. But with current technology, these researchers have been unable to determine these factors. Therefore, the use of an external "holding tube" idea similar to the time/temperature measurement used in the dairy industry offers a method of validating post-cooker time/temperature which may be sufficient to destroy even the most heat resistant organisms notwithstanding the lethality applied within the cooker.

In the literature, there are several methods described for conducting thermal death time trials. The analog dry block heater (Model# 12621-108, VWR International, Suwanee, GA) equipped with Model# 13259-162 heating blocks (VWR International, Suwanee, GA) was selected over an oil bath due to ease of removing tubes after thermal treatment. Previous work in this laboratory indicated difficulties in removing stainless steel tubes from a hot oil bath. The analog unit was selected because of its temperature maximum was within the parameters of this study.

Three ml of the *G. stearothermophilus* spore suspension were added to 75 g of crax/fat samples to generate 6.98 log cfu/g spores in the inoculated samples. Samples (uninoculated and inoculated) were measured aseptically into sterile stainless steel tubes. Loose fitting Kim-Kap™ Closures (Catalog # 60825-801, VWR Scientific Products, Inc., Suwanee, GA) were selected for covering the tubes to allow steam release during heating. Preliminary tests on stainless steel tubes were conducted to test the effect of stainless steel on *G. stearothermophilus* growth. One ml of unheated *G. stearothermophilus* spore suspension was transferred into stainless steel tubes and plated using the same enumeration procedures. The concentration of spores was 1.8×10^8 cfu/ml. Since the original spore concentration was measured to be 2.4×10^8 , these results indicated the stainless steel tubes had no obvious negative effect on *G. stearothermophilus*. An additional preliminary trial was conducted using 1 ml of *G. stearothermophilus* in the stainless steel tubes (no crax or fat) and heated at 290°F for 0, 1 and 2 min. Enumeration results indicated there were 5×10^0 cfu/ml, 1×10^1 cfu/ml and 1×10^0 cfu/ml recovered, respectively, which indicated that there was a 7 to 8 log reduction in spore survival in the suspension in water just during the come-up time. This preliminary test was conducted only once and the low residual concentration indicated few *G.*

stearothermophilus survived after the heat treatment. More control tests need to be done on the effect of stainless steel tubes and the thermal death time studies with *G. stearothermophilus* under various temperatures.

The block heater was heated to 290°F (143.3°C) as measured by thermometers inserted into the blocks. When the desired 290°F (143.3°C) was achieved, sample tubes containing uninoculated and inoculated crax/fat samples were randomly placed in the hot blocks. Additional sample tubes containing crax/fat mix were used for measuring temperature. Upon reaching an internal temperature of 143.3°C (290°F) in approximately 2 to 4 min, timing began. Uninoculated and inoculated tubes were heated for 0 min, 1 min and 2 min as shown in Table 1. Each test procedure comprised a total of 12 uninoculated and 12 inoculated tubes. After thermal treatment, tubes were removed using heat resistant gloves and tubes were immediately placed on ice and maintained on ice until bacterial enumeration was completed.

The above procedure was repeated for 280°F (137.8°C) and 285°F (140.6°C).

Enumeration

According to previous research conducted in this laboratory on bacterial enumeration from high fat raw rendering materials, it was noted that standard class O phosphate and magnesium chloride buffer does not sufficiently distribute fat particles to allow accurate enumeration of high fat materials. In general, fat and water are not miscible. A preliminary test was conducted using various levels of lecithin (AA36486-A1, Alfa Aesar, Ward Hill, MA) were added to emulsify the fat in the aqueous buffer. This test was conducted to determine the optimum amount of lecithin for emulsifying the amount of fat within the rendering samples. In order to ensure the lecithin could distribute fat particles even after autoclaving, lecithin buffers were prepared and emulsifying ability was compared with and without autoclaving. It was determined that autoclaved lecithin buffer retained the capacity to emulsify the 50% fat in each rendering sample.

A thermal death time trial was conducted in triplicate with fat and crax from Plant 1A and the following samples were plated using 2% w/v lecithin in the first dilution bottle and 0.5% w/v lecithin in all subsequent dilution bottles:

- Uninoculated, unheated plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 0 min plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 1 min plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 2 min plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, unheated plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, 0 min plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, 1 min plated duplicate from 10^{-2} to 10^{-11}
- Spore control (not in rendering material) plated using 2% w/v lecithin in the first bottle and 0.5% lecithin in all subsequent bottles
- Spore control (not in rendering material) plated using standard class O phosphate and magnesium chloride buffer
- Sterility controls on 2% w/v lecithin buffer, 0.5% w/v lecithin buffer and standard class O phosphate and magnesium chloride buffer
- Sterility controls on microbial media

Plates were incubated at 55°C for 36 hours. Results of this study indicated contamination issues due to a malfunctioning autoclave. Results were discarded.

A second thermal death time trial was conducted in triplicate with fat and crax from Plant 1A and the following samples were plated using 2% w/v lecithin in the first dilution bottle and 0.5% w/v lecithin in all subsequent dilution bottles:

- Uninoculated, unheated plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 0 min plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 1 min plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 2 min plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, unheated plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, 0 min plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, 1 min plated duplicate from 10^{-2} to 10^{-11}
- Spore control (not in rendering material) plated using 2% w/v lecithin in the first bottle and 0.5% lecithin in all subsequent bottles
- Spore control (not in rendering material) plated using standard class O phosphate and magnesium chloride buffer
- Sterility controls on 2% w/v lecithin buffer, 0.5% w/v lecithin buffer and standard class O phosphate and magnesium chloride buffer
- Sterility controls on microbial media

Plates were incubated at 55°C for 36 hours. Results of this study indicated difficulties in enumerating bacteria due to inability to distinguish between bacterial colonies and lecithin droplets. Results were discarded and a preliminary study was initiated using the 2% w/v lecithin in the first dilution bottle (pre-warmed to 35°C) and 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer in all subsequent dilution bottles. In order to minimize any potential deleterious effects of lecithin, samples were transferred immediately through the 2% w/v lecithin and the 0.5% w/v lecithin buffers and immediately plated. Results of the preliminary study indicated this sequence was effective in emulsifying the samples and there were no deleterious effects to the *G. stearothermophilus* noted.

A third thermal death time trial was conducted in triplicate with fat and crax from Plant 1A and the following samples were plated using 2% w/v lecithin in the first dilution bottle and 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:

- Uninoculated, unheated plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 0 min plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 1 min plated duplicate from 10^{-2} to 10^{-11}
- Uninoculated, 2 min plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, unheated plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, 0 min plated duplicate from 10^{-2} to 10^{-11}
- Inoculated, 1 min plated duplicate from 10^{-2} to 10^{-11}
- Spore control (not in rendering material) plated using 2% w/v lecithin in the first bottle, 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:

- Spore control (not in rendering material) plated using standard class O phosphate and magnesium chloride buffer
- Sterility controls on 2% w/v lecithin buffer, 0.5% w/v lecithin buffer and standard class O phosphate and magnesium chloride buffer
- Sterility controls on microbial media

Plates were incubated at 55°C for 36 hours. Results of this study indicated difficulties in enumerating bacteria due to inability to distinguish between bacterial colonies and lecithin droplets. Plates were retained at room temperature while a preliminary experiment using 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (EMD TX1510-1, VWR Scientific Products, Suwanee, GA) could be conducted. During this preliminary study, experiments were conducting on flooding plates after incubation with approximately 1.4 ml of 0.5% w/v 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (EMD TX1510-1, VWR Scientific Products, Suwanee, GA) and incubated at 55°C for 90 to 120 min. Red colonies were enumerated.

In addition, another preliminary study was conducted to determine if adding 10 ml per L of a 0.5% w/v solution of TTC in sterile ddH₂O to microbial media and incubating would be as effective as flooding pre-incubated plates with 1.4 ml of a 0.5% w/v solution of TTC for enumerating colonies. A preliminary thermal trial was conducted in duplicate heating using *G. stearothermophilus* spores to 290°F (143.3°C) for 0 min and 1 min and subsequently examining growth in TTC agar versus BHI/ye agar (with subsequent flooding with TTC) with and without passage through rendering materials. Results indicated that flooding the plates was just as effective as adding the TTC to the microbial media for detection of bacterial colonies and there was less concern about the possibility of effect of TTC on the growth of *G. stearothermophilus* during incubation. No deleterious effects were noted from either adding TTC to the growth media or treating plates after incubation with TTC. Upon determining the flooding method worked for distinguishing *G. stearothermophilus* colonies from lecithin droplets, the stored plates from the third study were examined using the TTC method. However, since the plates had been stored at room temperature for more than 3 days, the results of this study were discarded but the TTC procedure was validated and used in subsequent trials.

A fourth thermal death time trial was conducted in triplicate with fat and crax from Plant 1A and the following samples were plated using 2% w/v lecithin in the first dilution bottle and 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:

- Uninoculated, unheated plated duplicate from 10⁻² to 10⁻¹¹
- Uninoculated, 0 min plated duplicate from 10⁻² to 10⁻¹¹
- Uninoculated, 1 min plated duplicate from 10⁻² to 10⁻¹¹
- Uninoculated, 2 min plated duplicate from 10⁻² to 10⁻¹¹
- Inoculated, unheated plated duplicate from 10⁻² to 10⁻¹¹
- Inoculated, 0 min plated duplicate from 10⁻² to 10⁻¹¹
- Inoculated, 1 min plated duplicate from 10⁻² to 10⁻¹¹
- Spore control (not in rendering material) plated using 2% w/v lecithin in the first bottle, 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:

- Spore control (not in rendering material) plated using standard class O phosphate and magnesium chloride buffer
- Sterility controls on 2% w/v lecithin buffer, 0.5% w/v lecithin buffer and standard class O phosphate and magnesium chloride buffer
- Sterility controls on microbial media

After incubation at 55°C for 36 hours, plates were flooded with 1.4 ml of a 0.5% w/v solution of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (EMD TX1510-1, VWR Scientific Products, Suwanee, GA) in ddH₂O and incubated at 55°C for 90 to 120 min. Red colonies were enumerated. Results of this study indicated that lower dilutions than 10⁻² would be beneficial for determining thermal death. The lowest limit of reporting was "less than 3.0 x 10¹" but it was apparent that the higher dilutions were not necessary. Mean log cfu/g results for uninoculated versus inoculated samples for Plant 1A are shown in Figure 1.

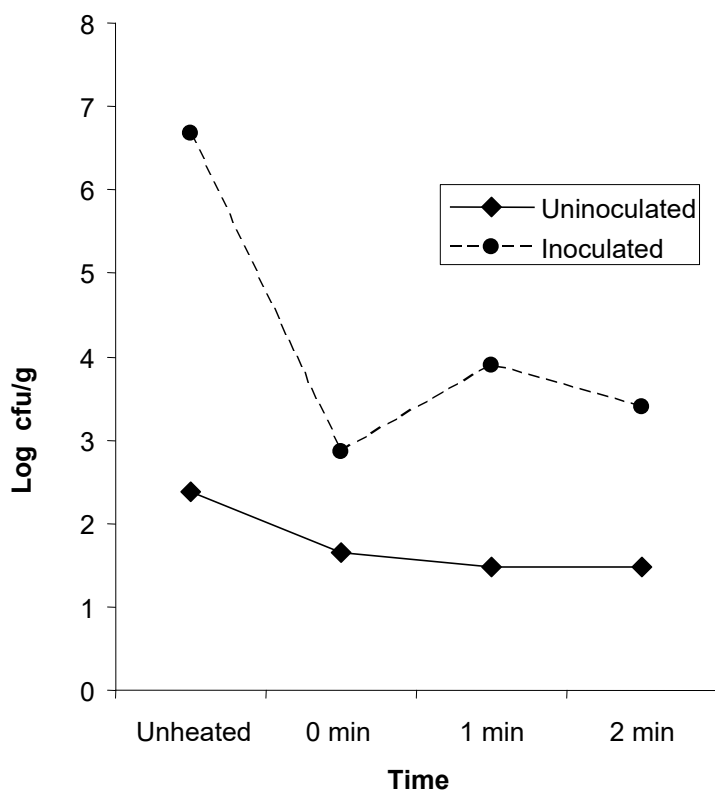


Figure 1. Mean log cfu/g for uninoculated versus inoculated samples from Plant 1A.

A fifth thermal death time trial was conducted in triplicate with fat and crax from Plant 2 and the following samples were plated using 2% w/v lecithin in the first dilution bottle and 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:

- Uninoculated, unheated plated duplicate from 10⁻¹ to 10⁻⁷
- Uninoculated, 0 min plated duplicate from 10⁻¹ to 10⁻⁷

- Uninoculated, 1 min plated duplicate from 10^{-1} to 10^{-7}
- Uninoculated, 2 min plated duplicate from 10^{-1} to 10^{-7}
- Inoculated, unheated plated duplicate from 10^{-1} to 10^{-9}
- Inoculated, 0 min plated duplicate from 10^{-1} to 10^{-7}
- Inoculated, 1 min plated duplicate from 10^{-1} to 10^{-7} Spore control (not in rendering material) plated using 2% w/v lecithin in the first bottle, 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:
- Spore control (not in rendering material) plated using standard class O phosphate and magnesium chloride buffer
- Sterility controls on 2% w/v lecithin buffer, 0.5% w/v lecithin buffer and standard class O phosphate and magnesium chloride buffer
- Sterility controls on microbial media

In order to create a 10^{-1} dilution, a 1 g sample was transferred into the 99 ml dilution blank containing 2% w/v lecithin. From this bottle, 10 ml were transferred into three labeled plates. After incubation, all three plates were counted and total of all three plates represented the 10^{-1} dilution. After incubation at 55°C for 36 hours, plates were flooded with 1.4 ml of a 0.5% w/v solution of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (EMD TX1510-1, VWR Scientific Products, Suwanee, GA) in ddH₂O and incubated at 55°C for 90 to 120 min. Red colonies were enumerated. Mean log cfu/g results for uninoculated versus inoculated samples for Plant 2 are shown in Figure 2.

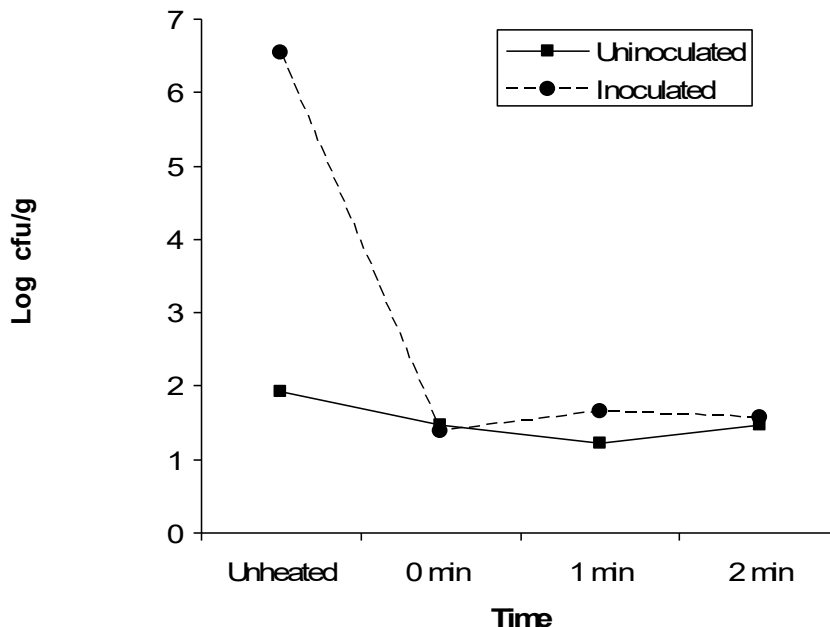


Figure 2. Mean log cfu/g for uninoculated versus inoculated samples from Plant 2.

A sixth thermal death time trial was conducted in triplicate with fat and crax from Plant 3 and the following samples were plated using 2% w/v lecithin in the first dilution bottle and 0.5% w/v

lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:

- Uninoculated, unheated plated duplicate from 10^{-1} to 10^{-7}
- Uninoculated, 0 min plated duplicate from 10^{-1} to 10^{-7}
- Uninoculated, 1 min plated duplicate from 10^{-1} to 10^{-7}
- Uninoculated, 2 min plated duplicate from 10^{-1} to 10^{-7}
- Inoculated, unheated plated duplicate from 10^{-1} to 10^{-9}
- Inoculated, 0 min plated duplicate from 10^{-1} to 10^{-7}
- Inoculated, 1 min plated duplicate from 10^{-1} to 10^{-7}
- Spore control (not in rendering material) plated using 2% w/v lecithin in the first bottle, 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:
- Spore control (not in rendering material) plated using standard class O phosphate and magnesium chloride buffer
- Sterility controls on 2% w/v lecithin buffer, 0.5% w/v lecithin buffer and standard class O phosphate and magnesium chloride buffer
- Sterility controls on microbial media

In order to create a 10^{-1} dilution, a 1 g sample was transferred into the 99 ml dilution blank containing 2% w/v lecithin. From this bottle, 10 ml were transferred into three labeled plates. After incubation, all three plates were counted and total of all three plates represented the 10^{-1} dilution. After incubation at 55°C for 36 hours, plates were flooded with 1.4 ml of a 0.5% w/v solution of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (EMD TX1510-1, VWR Scientific Products, Suwanee, GA) in ddH₂O and incubated at 55°C for 90 to 120 min. Red colonies were enumerated. Mean log cfu/g results for uninoculated versus inoculated samples for Plant 3 are shown in Figure 3.

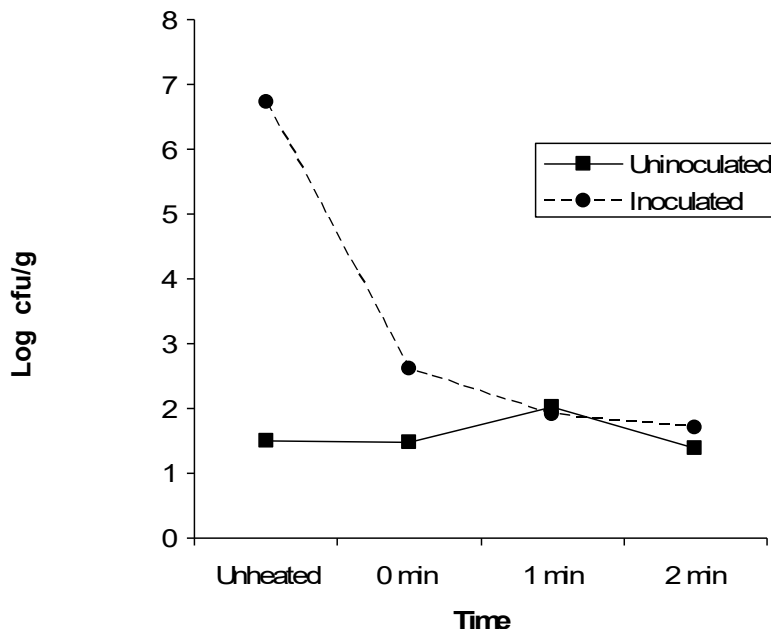


Figure 3. Mean log cfu/g for uninoculated versus inoculated samples from Plant 3.

A seventh thermal death time trial was conducted in triplicate with fat and crax from Plant 1B and the following samples were plated using 2% w/v lecithin in the first dilution bottle and 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:

- Uninoculated, unheated plated duplicate from 10^{-1} to 10^{-7}
- Uninoculated, 0 min plated duplicate from 10^{-1} to 10^{-7}
- Uninoculated, 1 min plated duplicate from 10^{-1} to 10^{-7}
- Uninoculated, 2 min plated duplicate from 10^{-1} to 10^{-7}
- Inoculated, unheated plated duplicate from 10^{-1} to 10^{-9}
- Inoculated, 0 min plated duplicate from 10^{-1} to 10^{-7}
- Inoculated, 1 min plated duplicate from 10^{-1} to 10^{-7}
- Spore control (not in rendering material) plated using 2% w/v lecithin in the first bottle, 0.5% w/v lecithin in the second dilution bottle and standard class O phosphate and magnesium chloride buffer was used in all subsequent dilution bottles:
- Spore control (not in rendering material) plated using standard class O phosphate and magnesium chloride buffer
- Sterility controls on 2% w/v lecithin buffer, 0.5% w/v lecithin buffer and standard class O phosphate and magnesium chloride buffer
- Sterility controls on microbial media

In order to create a 10^{-1} dilution, a 1 g sample was transferred into the 99 ml dilution blank containing 2% w/v lecithin. From this bottle, 10 ml were transferred into three labeled plates. After incubation, all three plates were counted and total of all three plates represented the 10^{-1} dilution as stated previously. After incubation at 55°C for 36 hours, plates were flooded with 1.4 ml of a 0.5% w/v solution of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (EMD TX1510-1, VWR Scientific Products, Suwanee, GA) in ddH₂O and incubated at 55°C for 90 to 120 min. Red colonies were enumerated. Mean log cfu/g results for uninoculated versus inoculated samples for Plant 1B are shown in Figure 4.

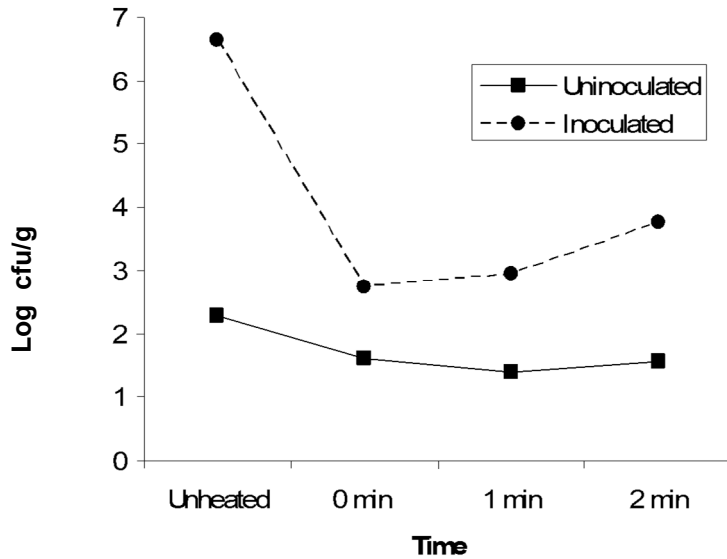


Figure 4. Mean log cfu/g for uninoculated versus inoculated samples from Plant 1B.

Figure 5 is a graphical representation of how mean log cfu/g changed over time in uninoculated versus inoculated rendering materials. From Figure 1, it is evident that thermophilic bacteria were inherent in uninoculated rendering materials from all three processing plants and in all four samples. The trend line indicated that mean log cfu/g was drastically reduced in the come-up time on the inoculated sample and less so on the uninoculated sample. From 0 min to 2 min, the mean log cfu/g remained relatively similar for the inoculated sample and for the uninoculated sample.

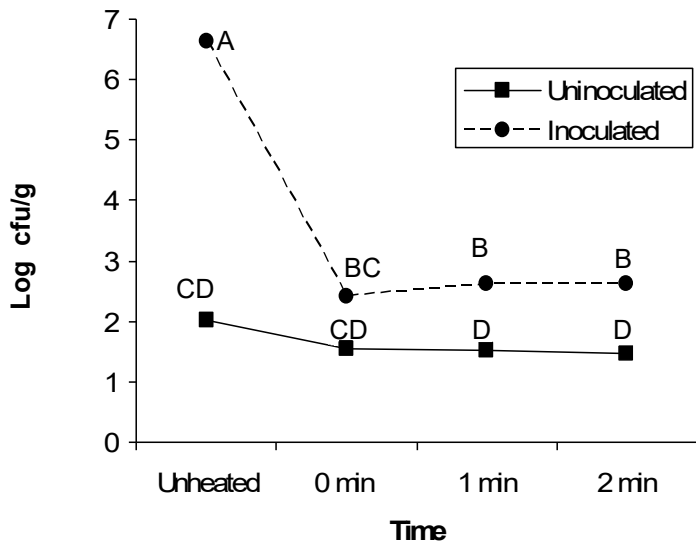


Figure 5. Overall mean log cfu/g ± standard error of uninoculated and inoculated fat/crax samples from all plants over time (unheated, 0 min, 1 min and 2 min).

^{A,B,C,D} Means followed by different letters are significantly different at $P > 0.05$.

Several preliminary tests were conducted to determine whether use of lecithin in the diluent had any potential effect on the growth of *G. stearothermophilus*. Control tests on lecithin buffer and standard class O phosphate and magnesium chloride buffer were conducted by enumerating *G.*

stearothermophilus spore suspension. The average bacteria concentration in lecithin buffer and standard class O phosphate and magnesium chloride buffer control tests were 1.96×10^8 cfu/ml and 1.87×10^8 cfu/ml respectively. Based on the data from each control tests on every thermal death time trial, lecithin did not cause a visible effect on *G. stearothermophilus* growth. As mentioned in the previous procedures, preliminary experiments were conducted using 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) and no noticeable deleterious effect on *G. stearothermophilus* growth or any difference between using TTC agar or pouring TTC onto the plates.

Statistical Analysis

For the initial study on thermal death at 290°F, a statistical model of uninoculated versus inoculated was developed and included factors for the overall mean and considerations for treatment (i.e - inoculation condition - yes/no), time (unheated, 0 min, 1 min and 2 min), specific combinations of treatment and time, plant to plant variation and overall error:

$$\log(\text{cfu}) = \mu + I + T + I*T + P + E$$

where μ = overall mean

- I = inoculation condition (yes or no?),
- T = time (unheated, 0 min, 1 min, 2 min)
- I*T = specific combinations of inoculation condition and time
- P = plant to plant variation
- E = overall error

Using analysis of variance (ANOVA) the following were tested:

- 1) Whether overall did inoculation condition have an effect;
- 2) Whether there is a difference within each samples;
- 3) Whether overall did each sample and inoculation combinations have an effect;
- 4) Whether overall did replication within different sample and inoculation combinations have an effect;
- 5) Whether overall did time have an effect;
- 6) Whether overall did time and inoculation combinations have an effect;
- 7) Whether overall did time and sample have an effect;
- 8) Whether overall did time, inoculation and sample have an effect.

Table 3. List of F value and p value of each factor from SAS analysis.

Source	F value	Pr>F
Inoculation	47.16	0.0063
Sample	2.48	0.3425
Sample*Inoculation	2.49	0.1567
Rep (Sample*Inoculation)	0.71	0.7681
Sample*Inoculation*time	2.10	0.0483
Time	76.9	<0.0001
Inoculation*Time	22.4	0.0002
Sample*Time	0.47	0.8593

Based on the valued in the ANOVA table and SAS results, at the 0.05 significance level:

1. Inoculation condition (yes or no) had a strong impact on cfu/g;
2. No signification variance or difference was found among each sample;

3. Sample and inoculation combination had no significant effect or impact;
4. Replications within different sample and inoculation combination did not have significant effect or impact;
5. Sample, inoculation and time combination had significant effect;
6. Time had a highly significant effect on reducing bacterial numbers;
7. Inoculation and time interaction was significant;
8. Sample and time combination did not have a significant effect.

Then the Fisher's Least Significant Differences (LSD) t test was used to determine specific differences among time and/or treatment combinations. Statistical Analysis Software (SAS[®] Version 9.1.3, Cary, NC) was used in the analysis.

Based on the LSD t test results, at the 0.05 significance level, overall means for inoculated and uninoculated samples were significantly different ($P > 0.05$) and had an effect or impact on the data. Among the overall means for all of the inoculated and uninoculated samples, unheated samples were significantly different ($P > 0.05$) from 0 min, 1 min and 2 min samples. For the treatment & time combination analysis of overall means, unheated inoculated samples were different from other treatments, and there were no significant differences ($P < 0.05$) noted among heated inoculated samples.

No significant difference ($P < 0.05$) was found among each sample and each replication in this experiment, which may suggest that these four rendering samples had no major impact on the data though they had different chemical analysis. Inoculation, time and their combination all showed significant effects. However, there were no significant differences ($P < 0.05$) among heated inoculated samples, which included 0 min, 1 min and 2 min. According to Figure 5 and Table 3, the data and the trend line of the inoculated samples indicated that mean log cfu/g was drastically reduced during the come-up time (4.24 log cfu/g), but showed no appreciable change after that. It is highly possible that a population or populations of thermophilic microorganisms survived and could not be destructed at 290°F for 2 min after the come-up time. The existence of these thermophilic microorganisms can be explained by presence of thermophilic microorganisms in the unheated and uninoculated rendering samples. The data indicated that uninoculated samples had no significant difference ($P < 0.05$) among all the uninoculated samples, which could support the fact that thermophilic microorganisms survived in the four rendering samples and could not be killed under established time/temperature conditions in this experiment.

The trend line from Figure 5 and data from Table 3 indicated that mean log cfu/g was drastically reduced in the come-up time (before 0 min) on the inoculated samples. The come-up time to reach 290°F was estimated to be approximately 1-2 min after inserting tubes into the pre-heated heating block but the exact come-up time was not measured. Due to the fact that there were no samples collected between unheated control and 0 min (come-up time) in this experiment, no further information on thermal death can be derived from this data. D is the time required to reduce the microbial population by 90% (or one log) at a specified temperature. In this experiment, the temperature used was 290°F. However, since there was a greater than 4 log reduction in the bacterial population and the exact time for the come-up time is not known, it is not possible to accurately estimate the D value from this data. Future studies need to be done to acquire sufficient data to allow calculation of accurate D-values for *G. stearothermophilus* in rendering materials.

To evaluate across all three temperatures (280°F, 285°F and 290°F), a second statistical model for log (cfu) was developed and included factors for the overall mean and considerations for treatment (i.e - inoculation condition -yes/no), time (unheated, 0 min, 1 min and 2 min), temp, interactions of treatment, time, temperature, sample to samples variation and overall error. The form of the model was:

$$\text{Log (cfu)} = \mu + \text{sample} + \text{trt} + T + \text{Temp} + \text{trt} * T + \text{temp} * T + \text{trt} * \text{Temp} + \text{trt} * T * \text{Temp} + E$$

where μ =overall mean

sample=samples from different rendering facilities

trt=Inoculated or unionculated treatment

T = time (unheated, 0 min, 1 min, 2 min)

Temp=temperatures (290°F, 285°F and 280°F)

E = overall error

An analysis of variance (ANOVA) was used to determine if trt, time, temperature, or their interactions had significant effects on log (cfu). If an effect was found to be significant, then the Fisher's Least Significant Differences (LSD) t test was used to determine specific differences among specific time, treatment, and/or temperatures. JMP[®] software from Statistical Analysis Software (SAS[®] Version 9.1.3, Cary, NC) was used in the analysis. All hypothesis tests were evaluated with $\alpha=0.05$. Based on the ANOVA (Table 4), temperature, time, treatment and the interactions all had a significant effect on log (cfu).

Table 4. List of F value and p value for all the samples of each factor from SAS analysis.

Effect Tests Source	DF	F Ratio	Prob > F
sample	3	11.3019	<.0001*
temp	2	10.0868	<.0001*
trt	1	1453.578	<.0001*
time	3	86.2478	<.0001*
temp*trt	2	69.7712	<.0001*
temp*time	6	6.9345	<.0001*
trt*time	3	69.4735	<.0001*
temp*trt*time	6	2.5967	0.0198*

Samples 1 & 2 were treated at temperature 290°F, and samples 3 & 4 used temperature 280°F, 285°F and 290°F. There was a significant interaction of temperature, time and treatment using the ANOVA, then the analysis was split into two parts. First, the effects of time and treatment were evaluated for sample 1 & 2; and then the effect of time, treatment and temperature were evaluated for samples 3 & 4.

For sample 1 & 2, there was a significant difference between absent heat and 0 min, 1 min & 2 min heat treatment. From Table 3 and Figure 1, there was also significant difference between the inoculated samples and the uninoculated samples. For the additional times, there was no difference between inoculated and the uninoculated treatment.

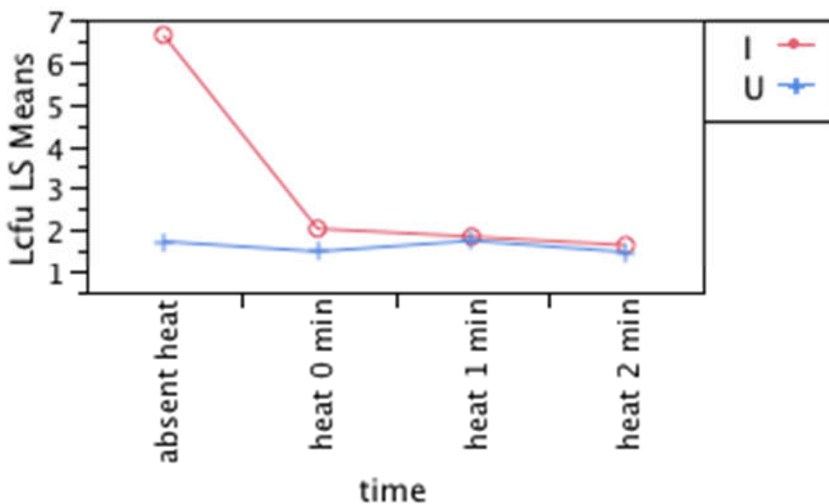


Figure 6. Plot of the log (cfu) least square means for the inoculated (I) and uninoculated (U) treatments at the different heating times for samples 1 & 2 at 290 °F.

Table 5. Results of Fisher's LSD test comparing treatment and time least square means for samples 1 & 2.

Trt	Time	Least Sq Mean	Letters*
Inoculated	absent heat	6.6363442	A
Inoculated	heat 0 min	2.0324653	B
Inoculated	heat 1 min	1.8428479	B
Uninoculated	heat 1 min	1.7465047	B
Uninoculated	absent heat	1.7279796	B
Inoculated	heat 2 min	1.6387941	B
Uninoculated	heat 0 min	1.4882791	B
Uninoculated	heat 2 min	1.4771213	B

* Means with similar letters not significantly different.

For samples 3 & 4 (Figure 7 and Table 6), there was a significant difference between absent heat and 0 min, 1 min & 2 min of heat treatment. In the inoculated treatment, at all temperatures, there were no significant difference among 0 min, 1 min and 2 min of heat treatment. There was a significant difference between 290 °F and 280 °F & 285 °F. At 0, 1 and 2 min, the inoculated treatment was significantly different from all the uninoculated treatment. There was no significant difference among all the uninoculated treatments at 0 min, 1 min and 2 min. No significant differences were found among the inoculated treatment at 0 min, 1 min and 2 min.

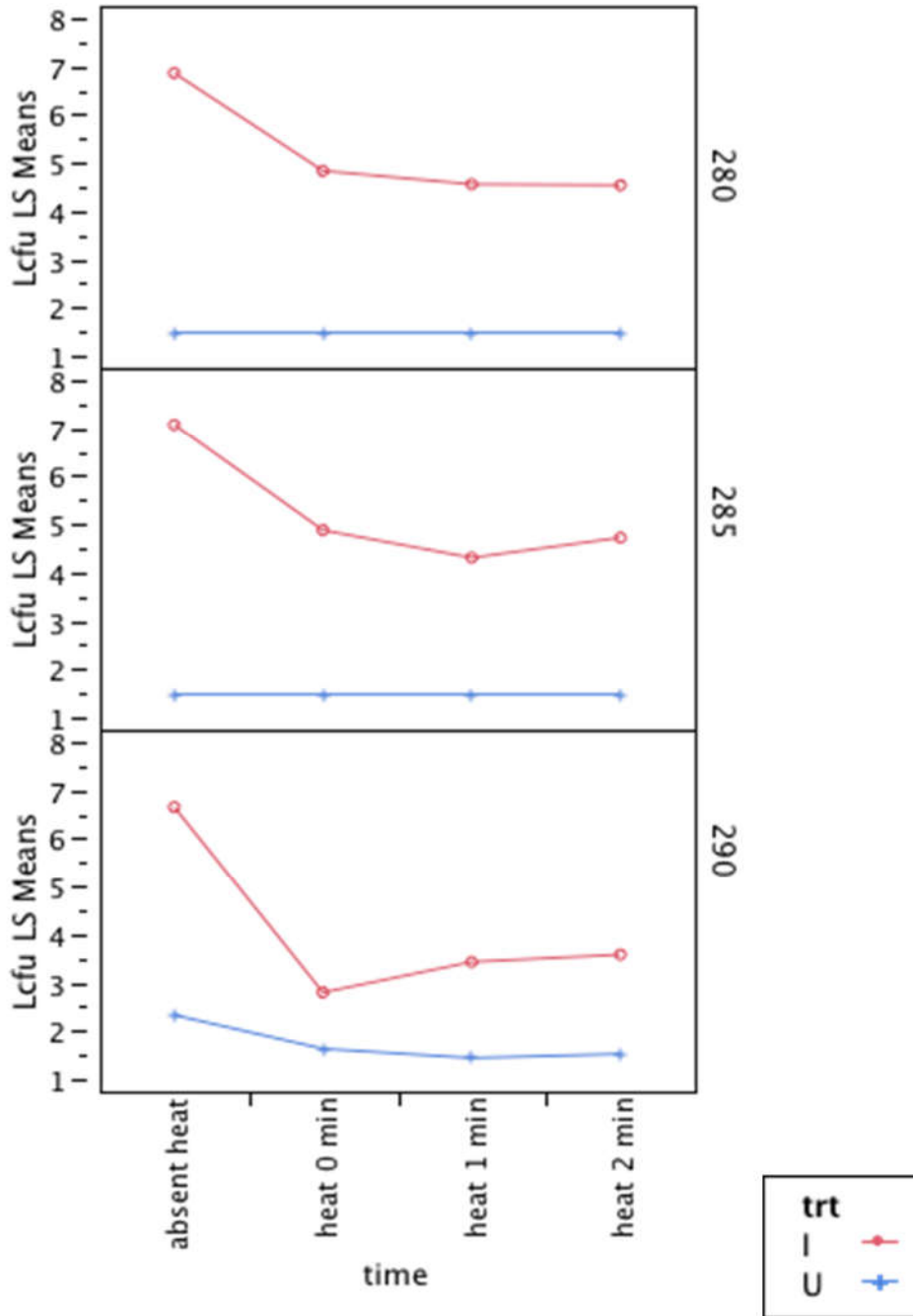


Figure 7. Plot of the log (cfu) least square means for the inoculated (I) and uninoculated (U) treatments at the different heating times and temperatures for samples 3 & 4.

Table 6. Results of Fisher's LSD test comparing treatment, time, and temperature least square means for samples 3 & 4.

Trt	Time	Temperature (°F)	Least Sq Mean	Letters*						
Inoculated	absent heat	285	7.0723783	A						
Inoculated	absent heat	280	6.8635070	A						
Inoculated	absent heat	290	6.6532467	A						
Inoculated	heat 0 min	285	4.8734473		B					
Inoculated	heat 0 min	280	4.8345470		B					
Inoculated	heat 2 min	285	4.7199506		B					
Inoculated	heat 1 min	280	4.5558668		B					
Inoculated	heat 2 min	280	4.5373113		B	C				
Inoculated	heat 1 min	285	4.3047235		B	C	D			
Inoculated	heat 2 min	290	3.5791840			C	D	E		
Inoculated	heat 1 min	290	3.4280845				D	E		
Inoculated	heat 0 min	290	2.7953894					E	F	
Uninoculated	absent heat	290	2.3262838						F	G
Uninoculated	heat 0 min	290	1.6305910							G
Uninoculated	heat 2 min	290	1.5226215							G
Uninoculated	heat 2 min	280	1.4771213							G
Uninoculated	absent heat	280	1.4771213							G
Uninoculated	heat 0 min	280	1.4771213							G
Uninoculated	heat 1 min	280	1.4771213							G
Uninoculated	heat 0 min	285	1.4771213							G
Uninoculated	heat 1 min	285	1.4771213							G
Uninoculated	absent heat	285	1.4771213							G
Uninoculated	heat 2 min	285	1.4771213							G
Uninoculated	heat 1 min	290	1.4381074							G

* Means with similar letters not significantly different.

Since the effect of time was significant for the inoculated treatment in both analyses (in samples 1 & 2 and in samples 3 & 4) it was concluded that time lead to a significant decrease in log (cfu) in rendering mixture in this project. The overall pattern is seen in Figure 8.

LS Means Plot

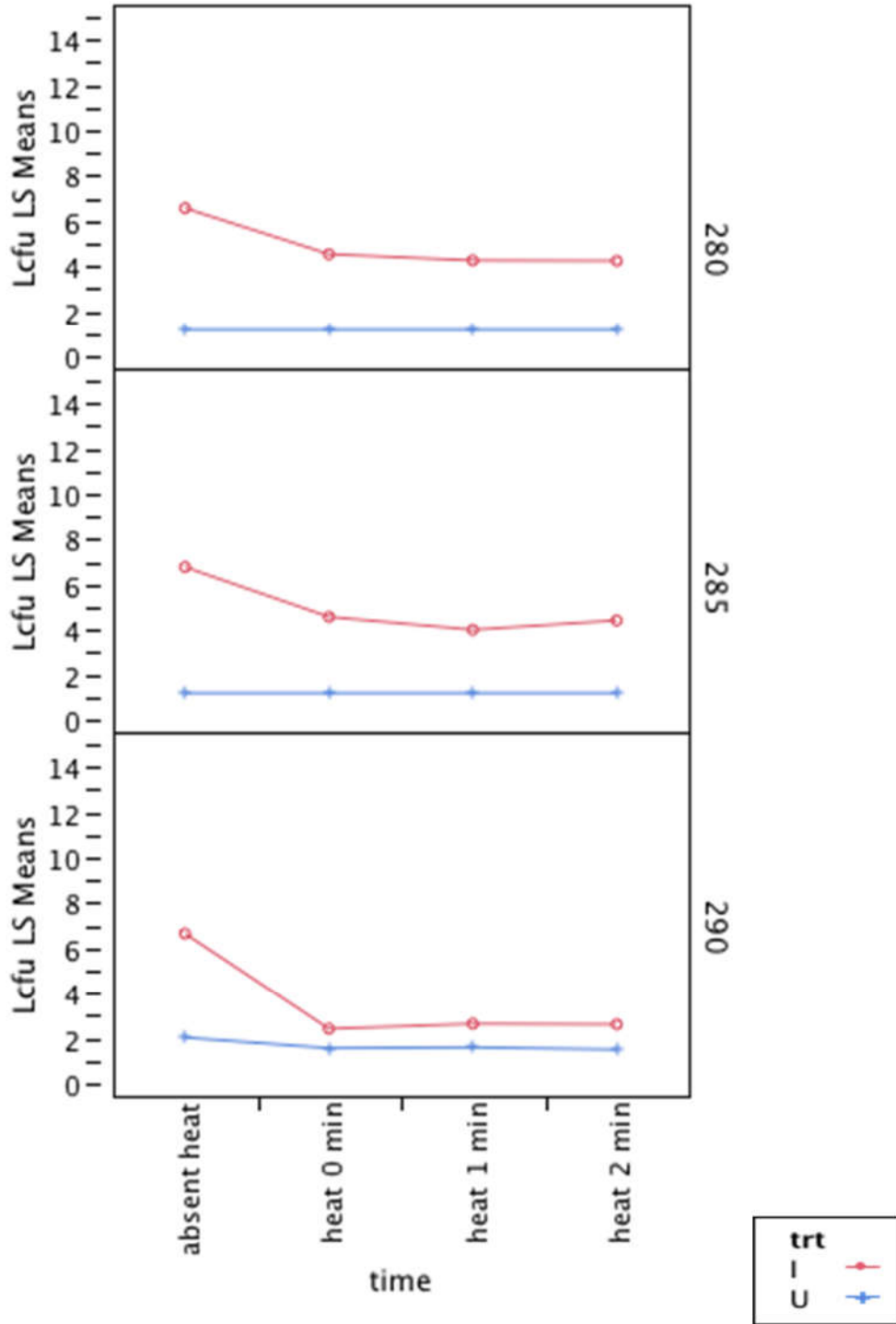


Figure 8. Plot of the log (cfu) least square means for the inoculated (I) and uninoculated (U) treatments at the different heating times and temperatures for all the samples.

One of the reasons for the sample effect is probably caused by the fact that four samples from three different rendering facilities were used for 290°F (143.3°C) treatment, and two samples from one rendering facilities that were collected on different dates were used for 280°F (137.8°C) and 285°F (140.6°C) treatments. Sample 1 and 2, which were from two different facilities and were treated at 290°F (143.3°C), showed no significant difference on samples. Samples 3 and 4, which were from the same rendering facilities, also showed no significant difference on samples.

Another possibility is that the samples from one facility had higher initial bacteria colonies in the uninoculated controls. Due to the variance between every rendering facility and their products, it is highly possible that some of the samples have higher heat resistant bacteria, which could be *G. stearothermophilus* or other unknown heat resistant bacteria. This hypothesis may also further explain that a population or populations of thermophilic microorganisms survived and could not be destroyed at 290°F for 2 min after the come-up time. The existence of these thermophilic microorganisms can be explained by presence of thermophilic microorganisms in the unheated and uninoculated rendering samples. Further study is needed to better understand the rendering microbial environment.

In order to further study the effect of time and existence of a background of heat-resistant bacteria in the products, samples were treated at 280°F for 40 min treatment (Figure 9 and Table 7). In this preliminary test, for each sample, the inoculated treatment was conducted in triplicate and the uninoculated treatment was conducted in duplicate due to limitations of the heating block which could only hold 24 tubes in each run. Results indicated there was a significant difference ($P>0.05$) between absent heat, 0 min & 1 min & 2 min heat treatment, and the 40 min heat treatment across all inoculated and uninoculated samples.. There was no difference among 0 min, 1 min and 2 min heat treatment across all inoculated and uninoculated samples. For the time & treatment combination in the inoculated samples, there was a significant difference ($P>0.05$) between absent heat & 0 min & 1 min & 2 min heat treatment, and the 40 min heat treatment. There was no significant difference ($P<0.05$) among 0 min, 1 min and 2 min heat treatment for the inoculated samples for the time & treatment combination. For the uninoculated samples, there was no significant difference ($P<0.05$) among the absent heat, 0 min & 1 min and 2 min heat treatment. There was a significant difference ($P>0.05$) between 40 min heat treatment and absent heat & 0 min & 1 min & 2 min heat treatment. However, presence of a heat resistant population persisting in the uninoculated indicated a background population of unknown identity. At 40 min treatment at 280°F, the population was reduced to the limit of detection (10^{-1} cfu/g).

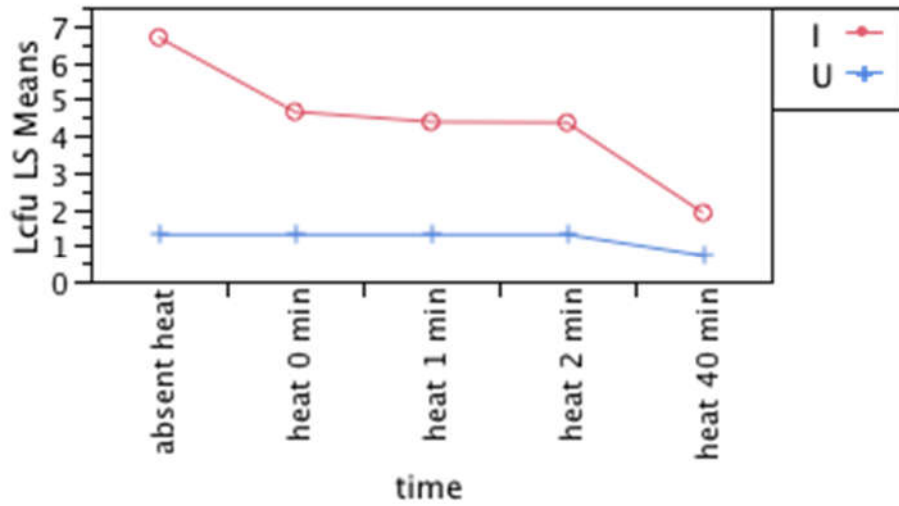


Figure 9. Least square means plot of all the samples at 280 °F for 40 min treatment.

Table 7. LS Means Differences Student's t tests results for all the samples at 280 °F for 40 min treatment. ($\alpha=0.050$ $t=2.00758$)

Trt	Time	Least Sq Mean	Letters
Inoculated	absent heat	6.6763966	A
Inoculated	heat 0 min	4.6474365	B
Inoculated	heat 1 min	4.3687563	B
Inoculated	heat 2 min	4.3502008	B
Inoculated	heat 40 min	1.8874057	C
Uninoculated	absent heat	1.2900108	D
Uninoculated	heat 1 min	1.2900108	D
Uninoculated	heat 0 min	1.2900108	D
Uninoculated	heat 2 min	1.2900108	D
Uninoculated	heat 40 min	0.7357200	E

* Means with similar letters not significantly different.

CONCLUSIONS

Based on results of this study, the spore suspension preparation method modified from Kim and Naylor (1966) worked sufficiently to prepare *Geobacillus stearothermophilus* spore suspension. A sonicator was used for 20 sec to try to dissolve the visible clumps; however, visible clumps were

still evident after treatment. Future experimentation could include using glass beads with agitation to suspend visible clumps to make sure the spore suspension is evenly distributed.

Crax samples were analyzed for fat, protein, ash and moisture content. According to the chemical analysis results, Plant 1 samples had higher fat content and lower protein and ash content as compared to Plants 2 and 3. Based on the chemical analysis, samples of fat and crax from each processing plant were re-mixed to yield a 50% fat product. Future research needs to be conducted to determine how chemical compositional differences would have an effect on thermal death.

A preliminary test suggested that stainless steel tubes did not have a noticeable effect on *G. stearothermophilus* growth. The methodology used in this study employed use of lecithin as emulsifier to assist in accurate serial dilution of bacterial populations in the high fat rendering samples. More research on recovering other pathogenic bacteria from high fat environments using lecithin are underway in this laboratory.

In this experiment, 290°F (143.3°C) was used as the processing temperature to determine the maximum impact rendering could have on the very heat stable *G. stearothermophilus*. The temperature selected is the most rigorous rendering temperature reportedly used in commercial rendering cookers. Commercial rendering cookers are reported to process in the temperature range of 240 to 290°F (115.6 to 143.3°C). Therefore, additional thermal trials at different temperatures of 280°F and 285°F were used to mimic the impact of the most common rendering temperatures on *G. stearothermophilus*.

In a previous study on validating rendering cooker temperatures, 1.9 min was determined as the minimum time that rendering material temperature could be validated. Therefore 2 minutes was selected as the longest time during this experiment. Rendering processing is reported to be 40 to 90 min at 240 to 290°F (115.6 to 143.3°C) which would be expected to induce considerable additional lethality during rendering processing.

According to several preliminary tests using various levels of lecithin (AA36486-A1, Alfa Aesar, Ward Hill, MA) to emulsify the fat in the aqueous buffer and its subsequent effect on *G. stearothermophilus*, using TTC to indicate bacteria colonies and its subsequent effect on *G. stearothermophilus*, and based on a previous study of lecithin in the same laboratory, lecithin appears to be promising to enumerate high fat samples with *G. stearothermophilus*. TTC also appears to be a useful indicator to distinguish bacterial colonies from other non-bacteria particles. Further studies need to be conducted to test whether lecithin and TTC will have any deleterious effect on other bacteria such as *Salmonella* or *C. perfringens*. Future studies could include comparing other potential emulsifiers which might better distribute fat particles.

This study was designed as an initial study to determine the effectiveness of rendering processing conditions on one of the most heat resistant microorganism known, *G. stearothermophilus*. This study began with this organism for a number of reasons. First, a number of procedures had to be developed and tested and due to cost and safety, it is better to conduct initial procedure development on a non-pathogenic microorganism rather than on a pathogen. The initial procedure development has been conducted with this study and, although validation of procedures will be necessary and some further procedure development will be needed for thermal death time studies

of *Salmonella* and *C. perfringens*, knowledge derived in this study will be of great benefit. Secondly, it was hoped that rendering temperatures would completely destroy the very heat resistant *G. stearothersophilus* making need to validate destruction of the heat labile *Salmonella* and the much less heat resistant *C. perfringens* either not necessary or much less rigorous. Indeed, the fact that only 2 min at the lowest temperature tested (280°F) destroyed approximately 2 log cfu/g of *G. stearothersophilus* indicated good lethality. However, the data also clearly demonstrated that increased temperature caused much greater lethality with approximately 2.2 log cfu/g destroyed after 2 min at 285°F and approximately 4.6 log cfu/g destroyed after 2 min at 290°F.

Based on the data of unheated, uninoculated samples from each trial, there were background thermophilic microorganisms existing in the crax/fat samples. Future research should be conducted to speciate these background thermophilic organisms and to determine whether there are also thermophilic microorganisms present in other rendering materials from other rendering facilities and in other types of rendering products including swine, beef and fish. Additionally, it would be helpful to conduct future studies to determine whether the presence of these thermophilic microorganisms in the samples collected from the rendering plants is due to insufficient time/temperature during rendering cooking or due to the post-contamination after the cooking process.

The graph of inoculated samples after heat treatment indicated thermophilic microorganisms survived after treatment at 290°F for 2 min after the come-up time. The number of log cfu/g of these surviving thermophilic microorganisms among inoculated samples was slightly higher than uninoculated samples. Further studies should be performed to determine the species of these surviving thermophilic microorganisms as well as their thermal death time.

The presence of other inherent thermophilic microorganisms indicates *G. stearothersophilus* would be a poor surrogate bacteria for rendering environmental research since absence of the surrogate organism in the background environment is a crucial criteria for selecting surrogate microorganisms. Although these background microorganisms might not be *G. stearothersophilus*, the high heat resistance of *G. stearothersophilus* and growth under similar thermophilic conditions would make it difficult to distinguish *G. stearothersophilus* from other thermophilic microorganisms. The search for a good surrogate microorganism to assess thermal death time and environmental microbiology in rendered products should continue with studies on non-thermophilic microorganisms which are not present in the background environment, and are easy to recover, enumerate and identify from other existing microorganisms. These potential surrogates must be safe to humans, animals and the environment, and must not be capable of establishing itself with existing thermophilic or other microorganisms and causing potential hazards. Additionally, studies on distance of spread within a rendering plant from a source location such as raw receiving should be conducted to assist in industry needs for sanitation programs.

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Impacts and Significance:

There is a concerted effort among food safety experts for breaking the cycle of potential pathogens entering the food chain. Rendering plays an important role in the “farm-to-fork” model for animal feed microbial safety and ultimately human and pet food safety. Many pathogenic bacteria species survive and exist in animals and especially in digestive tracts. These include *Staphylococcus* species, *Listeria* species, *Bacillus* species, *Clostridium* species, *Mycobacterium* species, *Enterobacteriaceae*, *Pseudomonas* species, *Aeromonas* species, *Plesiomonas shigelloides*, and *Vibrio* species and consequently considerable study is needed toward ensuring animal feed safety.

Geobacillus stearothermophilus is an endospore-forming, Gram positive thermophilic bacterium. *G. stearothermophilus* bacterial spores are capable of withstanding extreme heat such as found in the thermal processing and handling during food production and, therefore, the organism is commonly used to indicate effectiveness of food processing procedures. This project is the first project that used *G. stearothermophilus* to validate the processing efficiency of rendering. With up to 20 times more heat resistance than the heat stable *C. botulinum*, inactivation of *G. stearothermophilus* would indicate the destruction of other microorganisms and especially vegetative cells.

In this experiment, temperatures selected were indicative of rendering temperatures reportedly used in commercial rendering cookers. Indeed, the fact that only 2 min at the lowest temperature tested (280°F) destroyed approximately 2 log cfu/g of *G. stearothermophilus* indicated good lethality. However, the data also clearly demonstrated that increased temperature caused much greater lethality with approximately 2.2 log cfu/g destroyed after 2 min at 285°F and approximately 4.6 log cfu/g destroyed after 2 min at 290°F.

The significance of this study for the rendering industry is that rendering at 280°F, 285°F or 290°F for only 2 min causes a significant reduction in the population of the thermophilic *G. stearothermophilus* and increasing temperature more than from 280°F to 290°F more than doubles the thermal lethality within the same time span. Further processing for 40 min at 280°F caused even greater population destruction and clearly indicates that rendering is very effective in reducing populations of even the very heat resistant *G. stearothermophilus*. Demonstrating that *G. stearothermophilus* spores can be reduced by rendering indicates that less heat resistant pathogenic organisms such as *Salmonella* and *C. perfringens* will be more profoundly affected by rendering thermal conditions. It was hoped in the planning of this study that *G. stearothermophilus* would be destroyed by rendering conditions at 2 min or less. However, *G. stearothermophilus* was reduced to the level of detection (10^{-1} cfu/g) after 40 min treatment at 280°F. Since the rendering industry reports they thermally treat materials for a minimum of 40 min, destruction of the highly heat resistant *G. stearothermophilus* lends further credence to the verification that thermal lethality will be sufficient to destroy the heat labile pathogen *Salmonella* and the less heat

resistant *C. perfringens*. However, as the only experimentally verifiable time factor for a rendering cooker has been approximately 2 min, it is necessary to continue the experiments to validate thermal destruction of *Salmonella* and *C. perfringens* under these thermal conditions for up to 2 min.

A further significance of this study has been development of procedures for thermal death time studies. These procedures will be used in further studies on thermal death time validation of *Salmonella* and *C. perfringens*.

Publications:

Zhang, Y., A. Leaphart, W. C. Bridges, Jr. and A. K. Greene. 2011. Surrogate bacteria in rendering processing. Poster #198-52 presented at IFT Meeting, New Orleans, LA. June 13, 2011.

Zhang, Y., W.C. Bridges, Jr. and A.K. Greene. 2012. Validation of thermal destruction of *Geobacillus stearothermophilus* in rendered animal products as an indicator of thermal lethality. In preparation for submission to Journal of Food Science.

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Future Work:

Further research needs to be conducted with more rendering samples from more rendering plants/processes including the evaporative type of rendering cooker. Based on the data of unheated, uninoculated samples from each trial, there were background thermophilic microorganisms existing in the crax/fat samples. Future research should be conducted to speciate these background thermophilic organisms and to determine whether there are also thermophilic microorganisms present in other rendering materials from other rendering facilities and in other types of rendering products including fish, swine, and beef. Additionally, it would be helpful to determine in future studies whether the presence of these thermophilic microorganisms is due to insufficient time/temperature during rendering cooking or due to the post-contamination after the cooking process.