THERMAL LETHALITY VALIDATION FOR HUMAN PATHOGENIC *SALMONELLA* AND THE *SALMONELLA* SURROGATE *ENTEROCOCCUS FAECIUM* ON CHICKEN FEATHERS AND BLOOD

A Thesis

by

CALEB WONG-DE LA ROSA

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	T. Matthew Taylor
Committee Members,	Wesley N. Osburn
	Gary R. Acuff
Head of Department,	G. Cliff Lamb

December 2019

Major Subject: Animal Science

Copyright 2019 Caleb Wong-De La Rosa

ABSTRACT

With the passage of the FDA Food Modernization Act (FSMA) in 2011 and its implementing rules, food safety preventive controls may be developed and used to prevent food safety hazards from being transmitted in finished products, including for covered members of the animal foods/feeds producing industry (21CFR§507). The following study was designed to provide both short- and long-term benefits to the poultry rendering industry by providing data to describe and validate the lethality of high heat processing to *S. enterica* on poultry carcass offal (blood and feathers), generate scientific data allowing the comparison of thermal lethality to *Salmonella* with the non-pathogen *Enterococcus faecium* NRRL B-2354 to determine differences in lethality by application of heat, and determine the utility of *E. faecium* NRRL B-2354 for in-plant validation of high heat processing during the rendering of chicken by-products.

Samples of *Salmonella* or surrogate-inoculated chicken blood and feathers were loaded into metallic vessels and submerged into distilled water tempered to 180, 190, or 200°F for 0, 0.5, 1, 2, 3, 4, or 5 min and 190, 200, or 210°F for 0, 3, 6, 9, 12, 15, or 18 min, respectively. In addition, a cumulative thermality for samples of *Salmonella and E. faecium* tempered to 200°F for 5 min and 300°F for 18 min, respectively, was completed. Statistical analysis determined there was only one significant difference in chicken blood-obtained D-values of the pathogen and surrogate, with the interaction of microorganism x temperature (*P*=0.0006) but effect interactions of microorganism x model (*P*=0.7340) and microorganism x temperature x model (*P*=0.3535) were not statistically different. Also, effect interactions for microorganism x temperature (*P*=0.4609), microorganism x model (*P*=0.5371), and microorganism x temperature x model (*P*=0.8527) were not statistically significant for determination of pathogen or surrogate D-values in chicken feathers. Data generated indicate significant lethality to *Salmonella* cocktail, 7.4±0.03 $\log_{10}$ CFU/ml in

blood and 8.6±0.02 log_{10} CFU/g in feathers, respectively. *E. faecium* NRRL B-2354 was inactivated during thermal processing to 7.8±0.04 log_{10} CFU/ml in blood and 8.6±0.07 log_{10} CFU/g in feathers.

DEDICATION

This work is dedicated to my parents and brother who have always supported me through my journey to obtain this dream.

ACKNOWLEDGEMENTS

I would like to thank my committee chair Dr. T. Matthew Taylor for accepting me into the graduate program and for teaching, guiding, and supporting me throughout the completion of my degree. I would also like to thank my committee members Dr. Wes N. Osburn and Dr. Gary R. Acuff for their guidance and support throughout the course of this research. Thanks also to Kourtney Daniels, Lokesh Motanam, and Sam Annor for their help when I had to travel to collect samples and the department faculty and staff for helping me through the course of this challenge. I would especially like to thank Karla Solis Salazar who helped me in my experiments as well in our time as classmates. Thanks to Glenn and Gloria Lea for giving me a home in my last semester when I couldn’t afford a place to stay; I will always be grateful for your help.

To my parents and brother who have always supported me and encourage me to face the challenges that life will always put in my way. Finally, I would like to thank God for blessing me with this opportunity He provided and that I was able to complete through His grace.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a thesis committee consisting of Associate Professors T. Matthew Taylor and Wes N. Osburn of the Department of Animal Science and Professor Gary R. Acuff (Retired) of the Department of Nutrition and Food Science.

Chicken blood and feathers were gathered from a commercial rendering establishment located in the United States. Graduate study was supported by a scholarship of The Consejo Nacional de Ciencia y Tecnologia – CONACYT (the Mexican National Council for Science and Technology).

This work was also made possible by a grant of the U.S. Poultry & Egg Association.

NOMENCLATURE

atm	atmosphere(s); 1.0 atm = 101.33 kPa
BSA	Bismuth sulfite agar
CDC	Center for Disease Control and Prevention
FDA	U.S. Food and Drug Administration
FSMA	FDA Food Safety Modernization Act
GHPs	Good Hygienic Practices
GMPs	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Point
PFGE	Pulsed-Field Gel Electrophoresis
RF	Radiofrequency
NRRL	Northern Regional Research Laboratory
PW	Peptone Water
KFSA	Kenner fecal *Streptococcus* agar
TTC	Triphenyltetrazolium Chloride
TSA	Tryptic soy agar
psi	Pounds of force per square inch
TDP	3’3’-Thiodipropionic acid

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

CONTRIBUTORS AND FUNDING SOURCES vi

NOMENCLATURE vii

TABLE OF CONTENTS viii

LIST OF FIGURES x

LIST OF TABLES xi

1. INTRODUCTION: LITERATURE REVIEW 1

1.1. Rendering industry 1
1.2. Microbiological safety of rendered products 4
1.3. *Salmonella* and its significance as a human pathogen in animal feed components 6
1.4. *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* 9
1.5. Thermal death time 13

2. MATERIALS AND METHODS 18

2.1. Microorganisms and inoculum preparation 18
2.1.1. Sample inoculation with *Salmonella* cocktail or *E. faecium* NRRL B-2354 19
2.2. Sample thermality processing 20
2.2.1. *Salmonella* cocktail and *E. faecium* NRRL B-2354 D-value in chicken blood 21
2.2.2. *Salmonella* cocktail *and E. faecium* NRRL B-2354 D-values in chicken feathers 24
2.3. Cumulative thermal lethality for *Salmonella* and *E. faecium* NRRL B-2354 in chicken blood and feathers 25
2.4. Statistical analysis 27

3. RESULTS AND DISCUSION 29

3.1. *Salmonella* and *E. faecium* NRRL B-2354 D-values on chicken blood 29
3.2. *Salmonella* and *E. faecium* NRRL B-2354 D-values on chicken feathers 33
3.3. Cumulative thermal lethality for *Salmonella* and *E. faecium* NRRL B-2354 in chicken blood and feathers 37

4. CONCLUSION 39

REFERENCES 41

APPENDIX A 53

APPENDIX B 54

APPENDIX C 55

APPENDIX D 56

APPENDIX E 57

LIST OF FIGURES

Page

Figure 1. Metal vessels used to hold sample materials during thermal rendering experiments of chicken feathers or chicken blood. ... 20

Figure 2. Metal vessels immersed in distilled water in a stainless-steel cooktop on a Precision™ induction cooktop. ... 21

Figure 3. Metal vessels spaced sufficiently in order for sample separation. ... 22

Figure 4. Metal vessels in ice-laden cold water (0°C) to halt heat transfer. ... 23

Figure 5. Metal vessels immersed in peanut oil in a stainless-steel cooktop on a Precision™ induction cooktop. ... 26

LIST OF TABLES

Page

Table 1. REML variance component estimates for replications in blood D-value....................... 30

Table 2. Analysis of variance of *Salmonella* and *E. faecium* for chicken blood D-values........... 30

Table 3. Least squares means for D-values in chicken blood for the interaction of microorganism x heating temperature.. 31

Table 4. Least squares means for D-values in chicken blood for the interaction of microorganism x D-value determination model.. 32

Table 5. Least squares means for D-values in chicken blood for the interaction of microorganism x temperature x model.. 33

Table 6. REML variance component estimates for replications in feathers D-values................. 34

Table 7. Analysis of variance of *Salmonella* and *E. faecium* D-values for chicken feathers....... 34

Table 8. Least squares means for D-values in chicken feathers for the interaction of microorganism x heating temperature.. 35

Table 9. Least squares means for D-values in chicken feathers for the interaction of microorganism x D-value determination model.. 36

Table 10. Least squares means for D-values in chicken feathers for the interaction of microorganism x temperature x model.. 37

Table 11. Inactivation of *Salmonella* in blood and feathers during cooking under conditions used in commercial rendering 38

Table 12. Inactivation of *E. faecium* NRRL B-2354 in blood and feathers during cooking under conditions used in commercial rendering... 38

1. INTRODUCTION: LITERATURE REVIEW

1.1. Rendering industry

An animal by-product is defined as a secondary product obtained during the manufacture of a principal commodity (Meeker and Hamilton, 2006). About one-third to one-half of each animal produced for meat, milk, and/or eggs is not consumed by humans (Meeker and Hamilton, 2006). Approximately 100 million hogs, 35 million cattle, and eight billion chickens are slaughtered in the United States annually (Zhang, 2011). Carcass-recovered inedible by-products such as hides, hair, feathers, hooves, horns, bones, toe nails, blood, glands, fat tissues, and shells are essential raw materials for the rendering industry.

Rendering of animal products has been used historically to convert or recycle inedible or edible raw animal tissues such as liver, tongue, heart, stomach, cheeks, head trimmings, blood, fat, hides, feathers, bones, and shells and transform them into useful products for the animal and human food industries (Zhang, 2011). The temperature and length of time of the cooking process is critical; these are the primary determinants of the microbiological safety and quality of rendered products. Therefore, all rendering system technologies include the collection and sanitary transport of raw material to a facility where it is first ground into a consistent particle size and then conveyed to a cooking vessel, either a continuous-flow or batch configuration. The majority of tissue processed comes from slaughterhouses but may also include restaurant grease and butcher shop trimmings (Clemen, 1978). Rendering involves crushing animal by-products (e.g. internal organs), heating them to drive off the water (which can be as high as 65 percent by weight) and then separating the residue into fat (generally called tallow) and solids (known as greaves). During the rendering process the temperature remains at 100°C for the majority of the cycle, gradually rising to approximately 120°C once the bulk of the water has evaporated (Ramirez-Lopez, 2006). In

Europe, regulations require a period of heating under pressure, where the objective is to ensure that products are sterilized. However, dry lipid environments protect bacterial spores against thermal inactivation (Senhaji, 1977). Conditions for spore survival are favored by the water being driven off the rendering material during the process. Consequently, the rendering process simultaneously dries the material and separates the fat from the bone and protein. The rendering process results in different fat commodities (yellow grease, white grease, bleachable tallow, etc.) and differing protein meal (meat & bone meal, poultry by-product meal, etc.) products. The rendering industry often also handles other by-products, such as blood, feathers, and hair, but does so with modifications from the main rendering process.

Dry rendering can be performed through either batch or continuous processing. For example, a batch system of high pressure and temperature is designed to operate at a temperature of at least 80°C with a pressure of 12 atm, for at least 40 min. (Anderson, 2006). The batch cooker can function as a cooker, dryer, and hydrolyzer for raw material. Although the batch system operates under increased pressure, most modern particle reduction technologies have eliminated the need for it. It is still used in Europe as a means to further reduce risk of bovine spongiform encephalopathy (BSE)-causing prions (Meeker and Hamilton, 2006). Pressure cooking is needed to break down the bonds in the keratin proteins found in feathers to improve digestibility and product quality (Zhang, 2011).

A typical continuous processing system with each sequential cooker being responsible for a specific aspect of the rendering process begins with raw material that is transferred into a grinder where the material is ground (Kinley, 2009). The ground particles are then transferred into a continuous cooker where they are heated to 115-145°C for 40-90 min under 43.5 psi (Meeker and Hamilton, 2006). Once the material has been adequately cooked, the liquid fat and non-fat solid

material are separated using a drainer conveyor. The solid material is fed into a screw press to reduce the fat content from 25 down to 10 to 12 percent. The solid material is then combined to form the finished meal (Ockerman and Hansen, 1988). Feeds are primarily aimed at satisfying an animal's nutrition needs for maintenance, activity, production and reproduction. However, farmed livestock are reared to produce meat, milk and eggs for human consumption; feeds for such animals must also satisfy the requirements of the ultimate consumers of all products of animal origin. Therefore, animal feed is recognized as being part of the human food chain, and any consideration of feed safety needs to assess both the hazards for the animals that eat it and those that may affect the human consumer of animal products (Fink-Gremmels, 2012).

The U.S. Food and Drug Administration (FDA) is responsible for animal feed and pet food safety is responsible for addressing specific identified food safety hazards in its product manufacturing processes. Therefore, the FDA established the Animal Feed Safety System (AFSS); initiative to analyze the feed safety regulatory system and developed recommendations to address 'gaps' in the current system. Also, in 2011 the FDA FSMA became law, bringing additional changes to the rules applying to feed safety such as, application of food safety preventive controls (FSPCs) in order to prevent, eliminate, or reduce to an acceptable level the presence of food safety hazards, including pathogenic microorganisms like *Salmonella* spp.

Feathers are cooked using pressure to break the protein bonds of keratin, which results in a feather meal that is easily digestible by other species of livestock (Meeker and Hamilton, 2006). In addition to other animal by-products, blood from slaughtered animals can be used to produce a blood meal that can be incorporated into the feeding systems of livestock and companion animals. Meeker and Hamilton (2006) described blood meal as flash-dried, produced from clean, fresh animal blood, exclusive of extraneous material such as hair, stomach belchings, and urine, except

as might occur unavoidably even within a facility applying good manufacturing practices (GMPs). Blood is treated by removing a large amount of moisture by a mechanical dewatering process. The semi-solid blood mass is then transferred to a rapid drying facility where the more tightly bound water is removed. Blood products are the richest natural sources of both protein and the amino acid lysine to the feed industry. Hence, nutritionists are interested in blood meal due to its properties as a source of high rumen-bypassing protein that has been highlighted in research findings in dairy, feedlot, and range cattle (Meeker and Hamilton, 2006; Ockerman and Hansen, 1988).

1.2. **Microbiological safety of rendered products**

A pathogen commonly found in animal feeds is *Salmonella enterica,* which has the potential to produce infection and disease in animals and in humans, and must be regarded as a hazard within the meaning of the Food Safety Modernization Act. The rendering industry, besides ensuring high nutrition-useful products for livestock feed as well as foods for humans and companion animals, must ensure microbiological safety of its products. Under section 402(a)(1) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 342(a)(1), *Salmonella*-contamination causes feed, feed ingredients, or pet food to be considered adulterated. *Salmonella* is one of the major microbial hazards in finished feeds (Ockerman and Hansen, 1988). Animal feed has been found to be contaminated with a variety of pathogens such as *Salmonella* spp., *Escherichia coli* O157:H7, and *Enterococcus* spp. (Cox et al., 1983; Davies and Wray, 1997; Davis et al. 2003). Animal by-products are a large source of protein in the diets of animals fed on manufactured feed products. Fish meal, blood meal, feather meal, and meat and bone meals are common types of animal protein meals used in the U.S. and worldwide (Fink-Gremmels, 2012). Contamination can occur during production of these, and in turn pathogens can be carried forward to the feed (Gabis,

1991; Nesse et al. 2003). Hacking et al. (1978) examined samples of meat and feather meal for *Salmonella* and found them to be contaminated at prevalences of 81% (n = 21) and 40% (n = 15), respectively. Boyer et al. (1958) established a link between *Salmonella* serotypes (*S*. Thomasville, *S*. Tennessee, *S*. Cubana, *S*. Kentucky, *S*. Bareilly, *S*. Thompson, *S*. Senftenberg, *S*. Illinois, and *S*. Montevideo) recovered from feed ingredients and animal feeds known to be capable of causing disease not only in poultry but also in humans (Hinshaw and McNeil, 1948; Bruner, 1956). Watkins et al. (1959) a year later recovered 28 different serotypes of *Salmonella* from 37 of 200 (18.5%) samples of poultry and other animal by-products used in feeds.

Two years later, Pomeroy et al. (1961) reported a comprehensive study in which 43 different serotypes of *Salmonella* were recovered from 170 of 980 samples (18%) of by-products of animal origin used in animal feeds from 22 states across the United States. More recently, Kinley et al. (2010) determined the frequency of bacterial contamination in poultry meal or feather meal and analyzed *Salmonella* and enterococci isolates. *Enterococcus* spp. were detected in 81.3% of samples, and *Salmonella* was detected in 8.7% of poultry meal. A total of 13 distinguishable serotypes of *Salmonella,* including *S*. Amsterdam, Senftenberg, Oranienburg, Idikan, Johannesburg, IIIa, 42: z4, z23, Banana, Demerara, Putten, Molade, Montevideo, Mbandaka, and Livingston were identified by 16 differing PFGE patterns. Kinley et al. (2010) drew a comparison between each individual set of PFGE pattern types and what type of product they were isolated from, as well as where and when they were collected. They determined there was no single pattern type present in a particular plant environment over the extended period of sampling period (7 months). This indicated the likely source of contamination was the incoming raw chicken offal (feather meal, meat meal, meat and bone meal (MBM), meat and bone meal from poultry (MBM-P), poultry meal, and blood meal). In a similar study, Hofacre et al. (2001) isolated *Salmonella* and

various coliform bacteria at prevalences of 14% and 23%, respectively, from meat and bone meal samples (n = 43) at two poultry companies feed mills. The authors observed that blended protein meal (rendered fish, cattle and/or poultry) samples had lower *Salmonella* and coliform percentages – 5% and 16%, respectively – compared to meat and bone meals. Isa et al. (1963) collected feed ingredient samples and tested them for the presence of *Salmonella*. The researchers recovered *Salmonella* spp. in 31% of meat meal samples (n = 84). Several *Salmonella* spp. were recovered from fish meal, bone meal, and blood meal at 9.1% (n = 11), 60% (n = 10), and 15.4% (n = 13), respectively. The *Salmonella* serovars identified included *S*. Bredeney, *S*. Senftenberg, *S*. Montevideo, S. Kentucky, and *S*. Heidelberg.

1.3. ***Salmonella* and its significance as a human pathogen in animal feed components**

In the United States, the CDC estimates that *Salmonella* causes about 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths in the United States annually. *Salmonella* is a genus of Gram-negative, facultatively anaerobic, motile, non-spore-forming bacilli classified as a member of the family *Enterobacteriaceae,* able to grow on a large variety of culture media (Wray and Wray, 2000). In addition, *Salmonella* isolates are characterized by their ability to ferment glucose into gas and acid on triple sugar iron (TSI) agar medium, but not utilize sucrose or lactose in differential media (D'Aoust et al., 1989). Nevertheless, in certain cases *Salmonella* has demonstrated fermentation of sucrose and lactose through plasmids (Le Minor et al., 1974). In a study of breeder/multiplier and broiler production houses, 60% of meat and bone meal contained *Salmonella*, and feed was considered to be the source of *Salmonella* due to the nature of the pathogen in poultry breeder/multiplier houses (Jones et al., 1991). It was noted that *Salmonella* contamination in U.S. broiler production changed little between 1969 and 1989 (Jones et al., 1991).

Salmonella contamination of rendered products is most likely during post-rendering handling failures in sanitation (Kinley, 2009). Recontamination with *Salmonella* also may occur via aerosols that flow through processing areas (Davies et al., 1997; Magwood et al., 1965; Orthoefer et al., 1968). Samples taken using swabs from the raw materials area in a rendering processing plant had a higher *Salmonella* contamination rate, up to 95%, compared with 15.2% in the finished product area (Davies et al., 1997).

The primary serovars found in animal feeds are *S*. Senftenberg, *S*. Montevideo, and *S*. Cerro (Jay et al., 2005). Li et al. (2012) presented surveillance data from the Feed Contaminants Program (2002-2009) and *Salmonella* Assignment (2007-2009) of the U.S. FDA, which monitors trends of *Salmonella* contamination in animal feeds. A total of 2,058 samples from animal feeds, feed ingredients, pet foods, pet treats, and supplements for pets between 2002-2009 were collected and sampled for *Salmonella* presence and identity. From these samples, 257 were positive for *Salmonella* (12.5%). Of 45 *Salmonella* serotypes identified, *Salmonella* Senftenberg and Montevideo were the most frequently recovered serotypes. These findings provided the animal feed industries with *Salmonella* prevalence information that can be used to address *Salmonella* contamination problems. Several studies have determined the contamination rates and most prevalent serovars of *Salmonella* in a variety of animal feeds and feed ingredient samples collected from animal feed facilities and rendering plants as well as through retrospective analysis of gathered surveillance data (Davies et al., 1997; Ge et al., 2013; Li et al., 2012; Papadopoulos et al., 2009). Gong and Jiang (2017) reported that overall *Salmonella* prevalence in animal feeds ranged from 12.5 to 22.9% at a low contamination level (<10 MPN/g) in the United States, with higher contamination rates, up to 34.4%, observed in some feed ingredients such as animal bone meals and blood meals. Gong and Jiang (2017) stated *Salmonella* serotypes Typhimurium,

Infantis, and Senftenberg were found in both the raw materials receiving area and the finished meal loading-out area, indicating a potential of cross-contamination between these areas in a rendering processing environment.

According to the U.S. FDA under section 402(a)(1) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 342(a)(1), non-direct human contact animal feeds or feed ingredients are adulterated by *Salmonella* if one or more of the following serotypes pathogenic to the animals intended to be consumed is detected, such as *S*. Pullorum, Gallinarum, or Enteritidis in poultry feed, *S*. Cholerasuis in swine feed, *S*. Abortuseque in horse feed, *S*. Abortusovis in sheep feed, and *S*. Newport and Dublin in dairy and beef feeds (FDA, 2010). Nevertheless, detection of any *Salmonella* serotype in pet food or pet treats identifies the product as adulterated, as they are direct-human-contact animal feed and will not undergo a commercial heat step or other commercial process to kill the pathogen (FDA, 2010). The FDA FSMA mandated new rules on current GMPs (CGMPs), with sanitation preventive controls against *Salmonella* contamination in foods for animals. Implementing these rules will help the rendering industry that produces/uses pet food, animal feed, and raw materials (FDA, 2013).

Salmonella contamination has not been a concern only for the U.S. but also to the world. A Joint Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO) Expert Meeting on Animal Feed Impact on Food Safety concluded that ensuring safe feed is an important component of efforts to reduce and prevent microbiological hazards (FAO-WHO, 2008). Consequently, the European Food Safety Authority (EFSA) conducted a microbiological risk assessment in feedstuffs for food-producing animals and recognized the possibility of introducing *Salmonella* in the animal production system via feed consumption (Fink-Gremmels, 2012).

Although the level of pathogen contamination in feed that represents a public health risk is undefined, studies have attempted to link contaminated feed to human infections. A study by Clark et al. (1973) started with an epidemiologic investigation of an international outbreak of *S.* Agona reported by scientists associated with the U.S. CDC. *S.* Agona was identified as a public health problem in the United States, the United Kingdom, the Netherlands, and Israel during 1969 and 1970 (Clark et al., 1973). The cause was an initial isolation from Peruvian fish meal that was followed by recovery of *S.* Agona from domestic animals and subsequently from humans via restaurant food. This foodborne disease outbreak in the United States was traced back to Paragould, Arkansas, to a local restaurant, and then back to a Mississippi poultry farm that fed Peruvian fish meal. This led to the FDA's incorporation of animal feed into the definition of food, inducing the rendering industry to focus more on the importance of biological safety of the foods they produced. That is why, in 1984, the rendering industry founded the Animal Protein Producers Industry (APPI) to continually educate and ensure safety of industry products as a means to regulate biosecurity within the industry, such as *Salmonella* screening or developing protocols such as Hazard Analysis and Critical Control (HACCP) plans (Franco, 2006; Kinley, 2009).

1.4. *E. faecium* NRRL B-2354 as a surrogate for *Salmonella*

E. faecium NRRL B-2354 is a Gram-positive, spherical cell, and facultatively anaerobic organism (Byappanahalli et al., 2012). The organism was originally isolated from dairy utensils in 1927 by G.J. Hucker (Kornacki, 2012) and in 1960 was deposited in the U.S. Department of Agriculture Agricultural Research Service (USDA-ARS) NRRL culture collection as NRRL B-2354. (Kopit et al., 2014). A 16S rRNA gene sequencing and biochemical assay concluded that strain NRRL B-2354 was most similar to members of *E. faecium* (Ma et al., 2007), a discovery that changed the strain assignment at NRRL and ATCC. The Almond Board of California first

identified *E. faecium* NRRL B-2354 as a suitable surrogate for *Salmonella* during almond thermal processing. Jeong et al. (2011) used *E. faecium* NRRL B-2354 as a surrogate for *Salmonella enterica* serovar Enteritidis phage type 30 (SE PT30) on the surface of almonds subjected to moist-air heating; based on study findings it was determined to be a conservative surrogate for SE PT30 during moist-air heating. Bianchini et al. (2014) determined if *E. faecium* NRRL B-2354 was an adequate surrogate organism for a *Salmonella* cocktail of *S.* Braenderup NVSL 96-12528, *S.* Oranienburg NVSL 96-12608, *S.* Typhimurium ATCC 14028, *S.* Enteritidis IV/NVSL 94-13062, and *S.* Heidelberg/Sheldon 3347-1 during extrusion. Results indicated that the minimum temperature needed to achieve a 5.0-$\log_{10}$ reduction of *E. faecium* was 73.7°C using a model 2003 GR-8 single screw extruder (C.W. Brabender Instruments, South Hackensack, NJ) with a feeder with screw size of 18 mm and pitch of 19 mm set to run at 10 rpm was used to supply meal into the extruder. Above 80.3°C, enumeration of *E. faecium* yielded counts below the detectable levels (<10 CFU/g). *Salmonella* was reduced by 5.0 $\log_{10}$-cycles at 60.6°C, and above 68.0°C at extrusion of 21.6 g/ min and 24.4 g/ min, respectively. The counts of this organism in the product were below the detection limit. The data showed *E. faecium* was reduced to a higher extent indicating its use as a surrogate would provide an appropriate margin of error in extrusion processes designed to eliminate this pathogen.

Rachon et al. (2016) studied survival and the heat resistance of cocktails of *Salmonella*, *L. monocytogenes* and the pathogen surrogate *E. faecium* NRRL B-2354 in four low moisture foods (confectionary formulation, chicken meat powder, pet food and savory seasoning). The inactivation kinetics of the pathogens and surrogate at temperatures between 70 and 140°C were different between each organism and product. *E. faecium* NRRL B-2354 was a suitable *Salmonella* surrogate for three of the low moisture foods studied, but not for the confectionary formulation.

Therefore, Rachon et al. (2016) concluded that heating low moisture food in moisture-tight environments (thermal cells) to 111.2, 105.3 or 111.8°C using Weibull model could accurately predict 5.0 log_{10}-cycles reductions of *Salmonella, L. monocytogenes* and *E. faecium* NRRL B-2354, respectively.

Verma et al. (2018) evaluated oat flour inoculated with *E. faecium* NRRL B-2354 and a *Salmonella* cocktail of *S.* Agona, *S.* Mbandaka, *S.* Enteritidis, *S.* Tennessee, and *S.* Montevideo. Inoculated material was extruded in a lab-scale single-screw extruder running at different screw speeds (75 to 225 rpm) and different temperatures (75, 85, and 95°C). On comparing *Salmonella* and *E. faecium* NRRL B-2354, the results indicated that the two microorganisms showed a different response to processing depending upon fat content, moisture content, and screw speed. Verma et al. (2018) suggested *E. faecium* NRRL B-2354 was an acceptable surrogate for *Salmonella* due to its higher thermal resistance.

Recent studies are focused on low-moisture food products, with thermal processing or radio-frequency pasteurization. Tsai et al. (2019) aimed to evaluate impacts of water activity (a_w) on the survival of *Salmonella* and *E. faecium* NRRL B-2354 in cocoa powder at three different process temperatures (70, 75, and 80°C) and two a_w levels (0.30 and 0.45, at 22°C). *E. faecium* demonstrated less heat resistance than *Salmonella* when a_w was increased to 0.45. D-values for *Salmonella* at a_w 0.45 were 31.6-7.0 min at 70-80 °C compared to 25.8-4.7 min for *E. faecium.* Liu et al. (2018) investigated the influence of a_w on thermal resistances of *E. faecium* NRRL B-2354 and *S.* Enteritidis PT 30 in wheat flour. Under all tested conditions, *E. faecium* NRRL B-2354 exhibited equal or higher (1.0-3.1 times) D- and *z*-values than those of *Salmonella*. Overall, *E. faecium* NRRL B-2354 could be used as a conservative surrogate for *Salmonella* in thermal processing of wheat flour and treatment temperatures between 75 and 85°C. Wei et al. (2019)

aimed to develop a practical radio-frequency (RF) pasteurization of ground black pepper. RF heating of ground black pepper was conducted for 120 s and 130 s, which resulted in a final average surface temperature of 78.1 and 80.1 °C. It was shown to provide more than 5.9 $\log_{10}$ CFU/g reduction for *Salmonella* spp. and a reduction of 3.9 $\log_{10}$ CFU/g for *E. faecium* NRRL B-2354 with 130 s of treatment time.

The FDA FSMA, signed into law January 2011, gave mandate for the need for validation of food safety intervention technologies and hazards control. The hazard analysis and risk-based preventive controls requirements described in the law for food/feed manufacturers, including those of animal foods/feeds producing industry, "shall identify and implement controls, including critical control points, to prevent or significantly minimize potential hazards and shall verify that the preventive controls are adequate and effective," (FSMA, 2011). Food manufacturers cannot directly introduce foodborne pathogens like *Salmonella* into their facilities for process validation, as it can become very hard to eliminate them from the facilities. Therefore, it is necessary to find a surrogate which can behave the same as or has a higher resistance than *Salmonella* for validation studies within food facilities (Wei et al., 2019). Surrogate microorganisms are organisms with characteristics and behaviors similar to a comparable specific pathogen and are extremely useful in validating the effectiveness of lethality and/or decontamination steps during food manufacture (Bianchini et al., 2014).

1.5. **Thermal death time**

Thermal inactivation of a microorganism is always dependent on time/temperature control. The thermal death time (TDT) is defined as the time needed to reduce a given number of organisms at a specific temperature in a specific food product, or medium (Teixeira, 2006). The decimal reduction time (D-value) is defined as the time needed to kill 90% of a particular organism at a specific temperature. A high D-value at a given temperature indicates an increased thermal resistance of a microbial population in a product (Heldman and Hartel, 1998). D-value is determined from bacterial death rate; D_0 refers to the D-value of an organism at 250°F (121.1°C). The *z*-value reflects the temperature change needed to traverse one $\log_{10}$ on a thermal destruction curve. The F_0 is a useful reference in designing thermal processing, and it equals the time needed for a specific reduction of microorganisms at 250°F (121.1°C). F_0 is a universal standard value to show the capacity of a heat process (Zhang, 2011).

In the food industry there have been numerous research studies regarding different factors such as cooking methods, food composition, packaging type and product type and their impact on the thermal resistance of pathogens. *Salmonella* is readily destroyed at milk pasteurization temperatures (Jay, 2005). In a study on the heat resistance of *S*. Senftenberg 775W, Ng et al. (1969) found this strain to be more heat sensitive in the log phase than in the stationary phase of growth. These authors also found that cells grown at 44°C were more heat resistant than those grown at either 15°C or 35°C. Although *S*. Senftenberg 775W has been reported to be 30 times more heat resistant than *S*. Typhimurium (Ng et al., 1969), the latter organism was found to be more resistant to dry heat than the former (Goepfert, 1968). These authors tested dry heat resistance in milk chocolate. Murphy et al. (2000, 2004) demonstrated that six *Salmonella serovars* (*S*. Senftenberg, *S*. Typhimurium, *S*. Heidelberg, *S*. Mission, *S*. Montevideo, and *S*. California) and *L.*

monocytogenes had significantly different 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 with kinetic rate constants (approximately 2.303/D) of 0.076 to 9.68 min^{-1} obtained for *Salmonella* at a temperature range of 55 to 70°C.

Ramirez-Lopez (2006) studied the thermal resistance of spore forming bacteria using ground beef as a model medium for raw rendering materials using temperatures of 91, 95, 96 °C and concluded that ≥96°C was necessary to inactivate sporeforming organisms isolated from rendering materials. Results from laboratory experiments and pathogen growth models were able to provide estimates of the times and temperatures required to inactivate vegetative cells and spores, and recommended a validation of the processing method as a means to ensure a realistic condition to determine sterilization of raw rendered products. Glenn (2006) reported problems with the enumeration of the bacteria by traditional aqueous buffer dilution methods due to the high fat content of raw poultry rendering material. His research objective was to measure microbial loads in raw poultry rendering materials, but 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.

Kinley et al. (2010) determined the status of bacterial contamination in rendered animal products and analyzed *Salmonella* and enterococci isolates from the samples. The total bacterial counts ranged from 1.7 to 6.7 log_{10} CFU/g, with the highest counts reported in blood meal and the lowest in meat meal. Both blood meal and feather meal were more frequently contaminated ($P<0.05$) with enterococci than any other meal types. The D-values for the *Salmonella* isolates at 55, 60 and 65°C were in the ranges of 9.27-9.99, 2.07-2.28, and 0.35-0.40 min, respectively. Rachon et al. (2016) studied suitable storage times of inoculated foods that could be applied in

heat resistance studies or process validations with similar cell viability and heat resistance characteristics. The Weibull model and the first order kinetic (D-value) methods were used to express inactivation data and calculate the heating time to achieve 5.0 $\log_{10}$ reductions at temperatures ranging from 70°C to 140°C. At higher temperatures (>100°C), calculated heating times based on D-values to achieve 5.0-$\log_{10}$ reductions were significantly lower than the times calculated using the Weibull model, because the initial heat shoulder until microbial inactivation was observed to begin was not taken into account, and the product had not yet reached the target temperature. This finding shows the inadequacy of forcing the application of first order kinetics when product temperature is increasing, and when holding times at target temperatures cannot be reliably be controlled, as in food processes like extrusion and continuous heat treatments without moisture evaporation. Channaiah et al. (2016) was able to prove thermal lethality on a 3-strain cocktail of *S. enterica* serovars of *S*. Typhimurium (ATCC 14028), *S*. Newport (ATCC 6962) and *S*. Senftenberg 775W inoculating a commercial muffin baking process utilizing an oven temperature at 190.6°C for 21 min. A ≥5.0 $\log_{10}$ CFU/g reduction in *Salmonella* populations was demonstrated by 17 min of baking, and a 6.1 $\log_{10}$ CFU/g reduction in *Salmonella* population after 21 min of baking. A D-value experiment included in the study of the *Salmonella* cocktail in muffin batter produced D-values of 62.2 ± 3.0, 40.1 ± 0.9 and 16.5 ± 1.7 min at 55, 58 and 61°C, respectively; the z-value was 10.4 ± 0.6°C. Although this study is focused on the baking industry it gives an example of a validation standard in a baking process.

Jones-Ibarra et al. (2017) studied raw poultry offal inoculated with a mixture of *Salmonella* serovars Senftenberg, Enteritidis, and Gallinarum subjected to heating at 150, 155, and 160 °F (65.5, 68.3, and 71.1°C) for up to 15 min. Mean D-values for the *Salmonella* cocktail at 150, 155, and 160°F were 0.254±0.045, 0.172±0.012, and 0.086±0.004 min, respectively. The *z*-value was

21.95±3.87°C. Their results indicated that a 7.0-log-cycle inactivation of *Salmonella* may be obtained from the cumulative lethality encountered during the heating come-up period. Hayes (2013) conducted research on the thermal death of four pathogenic strains of *Salmonella* recognized by the FDA as hazardous in animal feeds (*S*. Cholerasuis, *S*. Enteritidis, *S*. Newport, and *S*. Dublin, tested in beef rendered material (bone, tissue) and poultry offal materials. In thermal treatments up to 420 s at 240°F (115.6°C), *S*. Cholerasuis was last detected at 120 s, *S*. Enteritidis at 120 s, *S*. Newport at 300 s and *S*. Dublin at 360 s in inoculated beef materials. In thermal treatments up to 420 s at 240°F (115.6°C), *S*. Cholerasuis, *S*. Enteritidis, *S*. Newport, and *S*. Dublin were last detected at 360 s respectively. Hayes (2013) concluded that further research was needed at 240°F (115.6°C) for longer time intervals to ensure that the *Salmonella* serovars are destroyed and to identify the impact of particles on thermal conductivity through the rendering matrices. This is due because after periods of appearing to be destroyed, some unidentified microorganisms reappeared at later treatment times.

Zhang (2011) reported on *Geobacillus stearothermophilus* as a surrogate bacterium to validate thermal treatments for testing inoculated and uninoculated rendered poultry materials from three different plants. Processing at 290°F (143.3°C) at 0, 1 and 2 minutes of heating indicated the organism to 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 was the first known study on the use of surrogate bacteria as an indicator organism to validate the thermal treatment for the rendering industry.

The rendering industry provides a utility for the one-third to one-half of each animal produced for meat, milk, eggs, and fiber that is not consumed by humans. These raw materials provide for a sustainable management of animal carcasses and produce many useful products such

as meat and bone meal, meat meal, poultry meal, hydrolyzed feather meal, blood meal, fish meal, and animal fats. The most important and valuable use for these animal by-products is as feed ingredients for livestock, poultry, aquaculture, and companion animals. Food safety must be based on sound verified science, and continued progress is dependent on the commitment of every level of production to prevent, eliminate or reduce a significant hazard. That is why the FDA FSMA and its implemented rules require the development and application of process preventive controls and a Food Safety Plan, applied in particular with the *Final Rule: Current Good Manufacturing Practice, Hazards Analysis, and Risk-Based Preventive Controls for Food for Animals* (21CFR§507). The objectives of the following study were designed to provide both short- and long-term benefits to the poultry rendering industry by providing data to describe and validate the lethality of high heat processing to *S.* enterica on poultry carcass offal (blood and feathers), generate scientific data allowing the comparison of thermal lethality of *Salmonella* to the surrogate *E. faecium* NRRL B-2354 to determine degree of difference in lethality by application of heat, verify the utility of *E. faecium* NRRL B-2354 for thermal inactivation, demonstrating its usefulness for in-plant validation of high heat processing during the rendering of chicken by-products, and compare the D-value results of the Baranyi and Roberts Model with the Linear Model for best fit.

2. MATERIALS AND METHODS

2.1. **Microorganisms and inoculum preparation**

Isolates belonging to *Salmonella enterica* serovars Senftenberg, Heidelberg, and Typhimurium, recovered from poultry products or chicken harvesting environments, were chosen from the Food Microbiology Laboratory culture collection (Department of Animal Science, Texas A&M University, College Station, TX) and revived from -80°C by incubating in 10 mL sterile brain heart infusion (BHI; Becton, Dickinson and Co.) broth for 24 h at 35°C. Following the initial revival passage in BHI and incubated for 24 h at 35°C, a second revival passage in BHI and incubated for 24 h at 35°C was completed to activate isolates for subsequent inoculum preparation. Following revival of isolates, a cocktail of *S*. Senftenberg, Heidelberg, and Typhimurium isolates was prepared by blending equivalent volumes into a sterile 50.0-mL conical tube and centrifuged (2191 x g in a Jouan B4i centrifuge, 25±2°C, 15 min) to pelletize the cells, pouring off the supernatant and then adding 30.0 mL 0.1% peptone water (Becton, Dickinson and Co., Sparks, MD, USA) to wash cells. The resulting suspension of cells was centrifuged again under identical settings; the resulting supernatant was poured off and remaining pellet hydrated with 3.0 mL of 0.1% peptone water to obtain a 9.0-10.0 $\log_{10}$ CFU/ml inoculum cocktail. A preliminary experiment was conducted to verify that counts of overnight cultures of individual *Salmonella* isolates utilized for the cocktail do not differ from one another (Appendix A).

Isolate B-2354 of *E. faecium* (Orla-Jensen 1919) Schleifer and Kilpper-Balz 1984 was received from the USDA Agricultural Research Service Culture Collection (NRRL) and revived in 10 mL sterile brain heart infusion (BHI) broth for 24 h at 27°C. Following the initial revival passage, a second revival passage was completed in like fashion to activate and prepare isolate for

subsequent inoculum use. Following revival of isolates, the *E. faecium* NRRL B-2354 was prepared by blending equivalent volumes of *E. faecium* NRRL B-2354 isolates into a 50.0-mL conical tube and centrifuged (2191 x g in a Jouan B4i centrifuge, 25+2°C, 15 min) to wash the cells, pouring off the supernatant and adding 30.0 mL 0.1% peptone water. This procedure was repeated twice; in the last step, the supernatant was poured off and the remaining pellet was hydrated with 3.0 mL of 0.1% peptone water to obtain a 9.0-10.0 log_{10} CFU/ml inoculum. A preliminary experiment was conducted to verify that counts of overnight cultures of *E. faecium* NRRL B-2354 isolates utilized for the cocktail do not differ from one another (Appendix B).

2.1.1. **Sample inoculation with *Salmonella* cocktail or *E. faecium* NRRL B-2354**

Chicken blood and chicken feathers were selected for inoculation and subsequent thermal lethality analysis of *Salmonella* cocktail. Raw samples were obtained on-site a commercial rendering establishment located in the southern United States. For blood, inoculation was achieved by pipetting 0.1 mL of the prepared *Salmonella* cocktail for the purpose to inoculate approximately 8.0-9.0 log_{10} CFU/ml inoculum into 50.0-mL conical tube containing 25 mL chicken blood as a means to simulate a contaminated sample size to a commercial process and vortexed for 1 min for inoculum mixture. A total of seven inoculated samples were prepared. For inoculation of feathers, 10 g of feathers were weighed to simulate a contaminated sample size to a commercial process and into a 50.0-mL conical tube, followed by adding 0.1 mL of prepared inoculum and vortexed for 1 min for inoculum mixture. A total of seven inoculated samples were prepared. For the two sample matrices, non-inoculated samples were aseptically collected and analyzed for presence and numbers of background *Salmonella* (Appendix C). Chicken feathers and blood identical in composition and from the same rendering establishment were also collected and inoculated in

identical fashion with *E. faecium* NRRL B-2354 as the method used for *Salmonella* cocktail inoculation (Appendix D).

2.2. **Sample thermality processing**

Metal vessels (1” x 6” galvanized steel and 0.2 millimeters thickness by 1” iron screwcap Southland® Memphis, TN) (Figure 1) were used to simulate the thermal processing conditions of the commercial rendering establishment with respect to material contacting rendered material during commercial processing. A VWR™ Enviro-Safe® K 50531 thermometer was set inside of an open metallic vessel control filled with 50 mL of distilled water to obtain an approximate reading of the vessel and be able to monitor temperature of process throughout all of the heating process.

Figure 1. Metal vessels used to hold sample materials during thermal rendering experiments of chicken feathers or chicken blood.

2.2.1. ***Salmonella* cocktail and *E. faecium* NRRL B-2354 D-value in chicken blood**

Metal vessels were partially submerged in distilled water in a stainless steel cookpot (11.4 L, TRAMONTINA Inc., Sugar Land, TX, USA) on a Precision™ Induction Cooktop (Figure 2), programmed to ensure a heat application inside of either 82, 87, or 93°C (180, 190, or 200°F, respectively).

Figure 2. Metal vessels immersed in distilled water in a stainless-steel cooktop on a Precision™ induction cooktop.

Vessels were allowed to heat before loading in inoculated sample material for the purpose of heating the metal vessel and avoiding an extended period of “come up” of temperature. Once the metal vessel reached 82°C (180°F), the inoculated chicken blood sample was poured immediately into a metal vessel and placed in distilled water heated to 82°C (180°F) in a Precision™ induction cooktop for 0, 0.5, 1, 2, 3, 4 or 5 min and spaced sufficiently in order to not allow any samples to touch one another (Figure 3).

Figure 3. Metal vessels spaced sufficiently in order for sample separation.

The metal vessels were removed at their respective time points and immediately placed in ice-laden cold water (0°C) to halt further microbial destruction due to heat; vessels cooled immediately once placed in ice-laden container. (Figure 4).

Figure 4. Metal vessels in ice-laden cold water (0°C) to halt heat transfer.

Chicken blood samples were then subjected to serial dilution by diluting sample into 9 mL of 0.1% peptone water with 1 mL of sample (1:10 dilution) and enumeration of surviving *Salmonella* on bismuth sulfite (Hi-Media™ L.B.S. Marg, Mumbai, India) agar (BSA) with 1 g/L sodium pyruvate (Sigma-Aldrich, Co., St. Louis, MO, USA) (Gurtler and Kornacki, 2009) to allow for repair and detection of sub-lethally injured salmonellae. Surviving *E. faecium* NRRL B-2354 were enumerated on Kenner Fecal (KF) *Streptococcus* agar (KFSA; Becton, Dickinson and Co.) with 2, 3, 5-Triphenyltetrazolium Chloride (TTC, 1%) (Sigma-Aldrich, Co., St Louis, MO, USA) and 1 g/L sodium pyruvate (Gurtler and Kornacki, 2009) (Appendix E) to allow for repair and detection of sub-lethally injured cells on Petri plates, and incubated at 35-37°C for 24-48 h before inspection of typical colonies and counting.

2.2.2. ***Salmonella* cocktail *and E. faecium* NRRL B-2354 D-values in chicken feathers**

Metal vessels were partially submerged in distilled water in a stainless steel cookpot (11.4 L, TRAMONTINA Inc.) on a Precision™ induction cooktop (Figure 2) and programmed to ensure a heat application inside of 87, 93, or 98°C (190, 200, or 210°F, respectively) for the purpose of heating the metal vessels and avoiding having the product experience prolonged "come up" of temperature. Once they reached the target temperature, inoculated chicken feather samples were aseptically placed immediately in the metal vessels using tweezers (Sigma-Aldrich, Co., St. Louis, MO, USA) and immersed in distilled water in a Precision™ induction cooktop for 0, 3, 6, 9, 12, 15, or 18 min, this time points where chosen consulting with a commercial rendering establishment process and to obtain accurate D-values, vessels were spaced sufficiently in order to not allow any samples to touch one another (Figure 3). The metal vessels were removed at their respective time points and immediately placed in ice-laden cold water (0°C) to halt further microbial destruction due to heat (Figure 4), and then placed aseptically in stomacher bags using tweezers with 90 mL 0.1% peptone water and placed in a Stomacher 400 blender (Seward Laboratory Systems Inc., Bohemia, NY, USA) for 1 min. Samples were then subjected to dilution. Surviving *Salmonella* were enumerated on BSA with 1 g/L of sodium pyruvate and surviving *E. faecium* NRRL B-2354 on KFSA containing 1% TTC and 1 g/L sodium pyruvate on Petri plates. Plates were then incubated at 35-37°C for 24-48 h before inspection of typical colonies and counting.

2.3. **Cumulative thermal lethality for *Salmonella* and *E. faecium* NRRL B-2354 in chicken blood and feathers**

Consulting with the commercial rendering establishment to simulate the commercial rendering process in a laboratory setting, pathogen- or surrogate-inoculated chicken blood and feathers samples were heated to 93°C (200°F) for 5 min or 149°C (300°F) for 18 min, respectively.

Chicken blood and feathers samples were obtained from a commercial rendering establishment located in the southern United States. For chicken blood, inoculation was achieved by pipetting 0.5 mL of the prepared *Salmonella* cocktail or *E. faecium* NRRL B-2354 into a 50.0-mL conical tube containing 50 mL chicken blood this is due to obtain a larger volume for a simulated contaminated sample and vortexing for 1 min for inoculum mixture. For inoculation of feathers, 10 g of feathers were weighed (the 10 g were kept the same as previous experiment this due to properly mix the inoculum and sample when vortexing) into a 50.0-mL conical tube, followed by adding 0.5 mL of prepared inoculum to increase cell counts and vortexing for 1 min.

Chicken blood samples were placed in metal vessels that were partially submerged in peanut oil due to its high boiling point of 441-445°F in a stainless-steel cookpot (11.4 L, TRAMONTINA Inc.) in a Precision™ Induction Cooktop tuned to 243°C (470°F) (Figure 5).

Figure 5. Metal vessels immersed in peanut oil in a stainless-steel cooktop on a Precision™ induction cooktop.

The metallic vessels were removed after come-up temperature of 93°C (200°F) and inoculated samples were placed in the vessel and subjected to 5 min of heating at 93°C (200°F). Vessels were then removed and immediately placed in ice-laden cold water (0°C) to halt further microbial destruction due to heat, and then subjected to dilution and enumeration of surviving *Salmonella* on BSA with 1 g/L of sodium pyruvate and *E. faecium* on KFSA with 1% TTC and 1 g/L of sodium pyruvate. Inoculated Petri plates were then incubated at 35-37°C for 24-48 h for before inspection of typical colonies and counting.

Chicken feathers samples were placed in a metallic vessel and vessels were immersed in peanut oil in a Precision™ induction cooktop pre-heated to 243°C (470°F) (Figure 5) to ensure a heat application inside of the vessel of 149°C (300°F), and spaced sufficiently in order to not allow

any samples to touch one another and thus insulate heat transfer. The metallic vessels were removed at "come-up" temperature of 149°C (300°F) and inoculated samples were placed in the vessels and immersed again in the heated peanut oil in the cooktop and cooked for 18 min of heating at 149°C (300°F). Following heating, vessels were immediately placed in ice-laden cold water (0°C) to halt further microbial destruction due to heat, and sample material was then transferred to a filter stomacher bag containing 90 mL peptone water, and placed in a Stomacher 400 blender for 1 min. Following mixing, samples were subjected to serial dilution and plating of surviving *E. faecium* on KFSA with 1% TTC and 1 g/L of sodium pyruvate. Surviving *Salmonella* were plated on BSA supplemented with 1 g/L of sodium pyruvate as a repair agent for sublethally injured cells on Petri plates. Inoculated plates were incubated at 35-37°C for 24-48 h for before inspection of typical colonies and counting.

2.4. **Statistical analysis**

Experimental design to obtain accurate D-values in chicken blood were seven time points (0, 0.5, 1, 2, 3, 4, 5 min) by three heating temperatures (180, 190, or 200°F). The organisms, temperature, replications, model, and D-value were used to obtain an analysis of variance (*N*=36). Chicken feathers experimental design to obtain accurate D-values were seven time points (0, 3, 6, 9, 12, 15, 18 min) by three heating temperatures (190, 200, or 210°F). The organisms, temperature, replications, model, and D-value were used to obtain an analysis of variance (*N*=36) For cumulative lethality trials, two identically prepared independent samples were completed per sample matrix combinations for both *Salmonella* and *E. faecium* NRRL B-2354 (*N*=6). D-value experimental design did not had duplicates. All experiments were replicated three times (*N*=3). Plate count data were utilized to generate a D-value of *Salmonella* and *E. faecium* NRRL B-2354 as a function of cooking temperature, sample matrix, and statistical modeling and were $\log_{10}$-

transformed prior to subsequent data analysis. ComBase (University of Tasmania/USDA-ARS) was used to determine D-value using both the Baranyi and Roberts and the linear model functions of DM Fit. Statistical analyses of D-values were performed using JMP Pro v12 (SAS Institute Inc., Cary, NC). To determine whether replication exerted a significant effect on statistical outcomes a Restricted Maximum Likelihood (REML) ($p<0.05$) was completed. Once replication effects were determined to be non-significantly affecting analysis outcomes, an analysis of variance (ANOVA) was used to determine differences among D-values as affected by main effects and/or interactions of main effects. Statistically significant differences amongst main effects and their interactions ($p<0.05$) were compared using Tukey's Honest Significant Differences (HSD) test. The *z*-values were determined as the negative inverse of the slope of the best-fit linear regression line ($\log_{10}$ D-values). A mean *z*-value was generated for both *Salmonella* and *E. faecium* NRRL B-2354 in each sample type allowing processors to alter time-temperature cooking schedules yielding predicted *Salmonella* or surrogate lethality.

3. RESULTS AND DISCUSION

3.1. *Salmonella* and *E. faecium* NRRL B-2354 D-values on chicken blood

To determine if replication was significant on pathogen and surrogate D-values, a Restricted Maximum Likelihood (REML) analysis ($p<0.05$) was completed. Analysis output indicated replications did not exert a significant effect on resulting data, and produced only 0.48% of total data variation ($P=0.958$) (Table 1). Data were re-analyzed by analysis of variance (ANOVA) where replication was not included as a main or random effect (Table 2). The ANOVA for both organisms in chicken blood was statistically significant ($P=0.0001$) (Table 2). The mean thermal process constant (*z*-value) for *Salmonella* cocktail and *E. faecium* NRRL B-2354 in blood was 25.22±3.69 and 125.56±89.02°F, respectively. *E. faecium* NRRL B-2354 demonstrated a higher temperature difference to traverse one $\log_{10}$ on the thermal destruction curve compared to *Salmonella*, resulting in an increased resistance to heating. The *z*-value in chicken blood for *Salmonella* is in agreement with the report of Jones-Ibarra et al. (2017) in poultry offal (various chicken carcass components): 21.95±3.87°F.

In Table 3, the *Salmonella* 180°F D-value for chicken blood differed from that of the pathogen surrogate (0.99 min versus 0.55 min for *Salmonella* versus *E. faecium*) ($P=0.0006$). In this case, the surrogate did not have similar heat-treatment response, it had a lower heat resistance. Only microbes having similar or greater thermal resistance than pathogen(s) of concern are acceptable surrogates. In addition, modeling systems can be included to analyze similar characteristics of the pathogen of concern. Nevertheless, the D-values for the pathogen versus the surrogate organism did not differ at temperatures of 190 and 200°F. This agrees with the study of Bianchini et al. (2014), as *E. faecium* demonstrated similar thermal resistance characteristics as

Salmonella in a balanced carbohydrate-protein meal. Jones-Ibarra (2017), whose study D-values in raw poultry offal for the *Salmonella* cocktail (*S*. Senftenberg, *S*. Enteritidis, and *S*. Gallinarum) at temperatures of 150, 155, or 160°F were 0.254±0.045, 0.172±0.012, and 0.086±0.004 min, respectively. As temperature was increased the D-value also incremented, indicating an increased thermal resistance of the microorganisms (190°F versus 200°F for *Salmonella* and *E. faecium*). In conclusion, these values are in agreement in previous studies making it a suitable surrogate for thermal processing treatment on chicken blood (Goepfert, et al. 1968, Ng. et al., 1969).

Table 1. REML variance component estimates for replications in blood D-value

Random Effect	Variance Ratio	Variance Component	Standard Error	95% Lower CL [a]	95% Upper CL	Wald p-value	Total Variance (%)
Rep #	0.004	8.4358e-5	0.001	-0.003	0.003	0.957	0.482
Residual		0.017	0.005	0.010	0.034		99.518
Total		0.017	0.005	0.010	0.033		100.000

[a] CL: Confidence limit

Table 2. Analysis of variance of *Salmonella* and *E. faecium* for chicken blood D-values

Source	DF	Sums of Square	Means Squares	F Ratio	*P*>F
Model	11	4.577	0.416	23.791	<0.0001*
Error	24	0.420	0.017		
C. Total	35	4.997			

P>F determined statistically significant at p<0.05.

Table 3. Least squares means for D-values in chicken blood for the interaction of microorganism x heating temperature

Organism, Cook Temperature (°F)	D-value[a]	R^2	Pooled SE[b]	*P*>F
Salmonella, 180	0.99A	0.81±0.22	0.053	0.0006
E. faecium, 180	0.55B	0.89±0.08		
Salmonella, 190	0.48B	0.93±0.05		
E. faecium, 190	0.49B	0.86±0.06		
Salmonella, 200	0.58B	0.66±0.37		
E. faecium, 200	0.53B	0.77±0.21		

[a] Means not sharing a capitalized letter (A, B) differ by Tukey's Honestly Significant Differences (HSD) test at p=0.05.
[b] Pooled SE = Pooled Standard Error.

Table 4 indicates the interaction of microorganisms x D-value determination model. The statistical analysis indicates a non-statistically significant difference (P=0.7340). Tukey's HSD determined that microorganism x model differ, the Baranyi and Roberts model D-values of *Salmonella* and *E. faecium* were 0.37 and 0.23 min, respectively (Baranyi et al., 1993, Baranyi and Roberts 1994, 1995). The Baranyi and Roberts model has been reported to be strongly correlated compared to the Linear Model meaning that Baranyi and Roberts better models the data, and may give a more accurate predicted D-value. The Baranyi and Roberts model offers good predictive capabilities (Grijspeerdt and Vanrolleghem, 1999). It is also truly dynamic model in the sense that it can deal with time varying environmental conditions and in the view of the growing attention given to quantitative risk analysis of food production (Mcmeekin and Ross, 1996; Foegeding, 1997), this is an indispensable asset. However, the literature shows a limited number of studies on the thermal validation studies comparing the Baranyi and Roberts and linear regression models for determining D-values of microbes. The Baranyi and Roberts model should be a model used for future predictive microbiological studies.

Table 4. Least squares means for D-values in chicken blood for the interaction of microorganism x D-value determination model

Organism, Baranyi/Roberts & Linear Model	D-value	R^2	Pooled SE[a]	*P*>F
Salmonella, Linear	0.98	0.69±0.28	0.044	0.7340
E. faecium, Linear	0.81	0.74±0.13		
Salmonella, Baranyi and Roberts	0.37	0.91±0.19		
E. faecium, Baranyi and Roberts	0.23	0.94±0.05		

[a] Pooled SE = Pooled Standard Error.

Table 5 describes the interaction between microorganism x temperature x model main effects. There was not a statistically significant difference between D-values as a result of this interaction (*P*=0.3535).

The Baranyi and Roberts model for determination of D-values demonstrated a better fit/prediction for D-values analysis (Grijspeerdt and Vanrolleghem, 1999; Fakruddin et al., 2011). As demonstrated in Baranyi et al. (1996), this dynamic inactivation model can describe shoulders and/or tails as well as the possible log linear decrease of a microbial population in a suitable way. In conclusion, the only statistically significant was the interaction of microorganism x heating temperature (*P*=0.0006) which supports the utility of *E. faecium* being a surrogate organism for the pathogen *Salmonella*. It also agrees with Liu and Schaffner (2007) definition that an ideal surrogate for thermal processing validation would be a non-pathogenic organism that provides similar response to the target pathogenic organism when it's subjected to the same thermal treatments or microbial reduction intervention.

Table 5. Least squares means for D-values in chicken blood for the interaction of microorganism x temperature x model

Organism, Cooking Target Temperature (°F), Model	D-value	R^2	Pooled SE[a]	*P*>F
Salmonella, 180°F, Linear	1.25	0.85±0.11	0.076	0.353
E. faecium, 180°F, Linear	0.80	0.83±0.06		
Salmonella, 180°F, Baranyi and Roberts	0.71	0.77±0.32		
E. faecium, 180°F, Baranyi and Roberts	0.29	0.97±0.02		
Salmonella, 190°F, Linear	0.65	0.90±0.05		
E. faecium, 190°F, Linear	0.74	0.82±0.03		
Salmonella, 190°F, Baranyi and Roberts	0.30	0.97±0.01		
E. faecium, 190°F, Baranyi and Roberts	0.25	0.91±0.06		
Salmonella, 200°F, Linear	1.04	0.33±0.09		
E. faecium, 200°F, Linear	0.90	0.59±0.09		
Salmonella, 200°F, Baranyi and Roberts	0.11	0.99±0.00		
E. faecium, 200°F, Baranyi and Roberts	0.16	0.96±0.04		

[a] Pooled SE = Pooled Standard Error.

3.2. *Salmonella* and *E. faecium* NRRL B-2354 D-values on chicken feathers

To determine if replication was a significant main effect impacting D-values, a Restricted Maximum Likelihood (REML) (p<0.05) was completed. Analysis output indicated that replications did exert a significant effect on resulting data, but produced 0.0% of total data variation (*P*=0.0011) (Table 6). Hence, data were re-analyzed by ANOVA where replication was not identified as a main or random effect (Table 7). The ANOVA for both organisms in chicken feathers was statistically significant (*P*=0.0001) (Table 7). The mean thermal process constant (*z*-value) for *Salmonella* and *E. faecium* NRRL B-2354 in feathers was 291.64±367.2 and 230.74±213.1°F, respectively. This was because of the feather samples low water content (Paşayev et al., 2017; Reddy and Yang 2007; Hernandez and Santos, 2012). Chicken feathers moisture content was reported as 16.18% on a dry weight basis at 20°C and a relative humidity of 80% (Paşayev et al., 2017). An explanation for this deviation of *z*-value is that feathers will take more time heating in a closed system vessel to eliminate the organisms.

According to Fisher and Phillips (2009), the heat resistance of *E. faecium* is associated with its membrane structure and has been related to the lipid and fatty acid content. *E. faecium* has also been shown to be an acceptable surrogate for the study of thermal inactivation of bacteria in different products (Annous and Kozempel, 1998; Li et al., 1993; Piyasena et al., 2003). Research has also indicated that *E. faecium* is an adequate surrogate for *Salmonella* for validation of thermal processes in almonds and in beef jerky, which are low-moisture, low a_w products similar to the rendered products described here (Almond Board California, 2007; Borowski et al., 2009; Jeong et al., 2011).

Table 6. REML variance component estimates for replications in feathers D-values

Random Effect	Variance Ratio	Variance Component	Std Error	95% Lower CL[a]	95% Upper CL	Wald *p*-Value	Total Variance (%)
Rep #	-0.0818	-0.0256	0.0078	-0.0410	-0.0101	0.0011	0.00
Residual		0.3130	0.0943	0.1872	0.6270		100.00
Total		0.3130	0.0943	0.1872	0.6270		100.00

[a] CL: Confidence limit.

Table 7. Analysis of variance of *Salmonella* and *E. faecium* D-values for chicken feathers

Source	DF	Sums of Squares	Mean Squares	F Ratio	*P*>F
Model	11	40.935	3.7214	12.948	0.0001
Error	24	6.897	0.2874		
C. Total	35	47.833			

P>F determined statistically significant at p<0.05.

Table 8. Least squares means for D-values in chicken feathers for the interaction of microorganism x heating temperature

Organism, Cooking Target Temperature (°F)	D-value	R^2	Pooled SE[a]	*P*>F
Salmonella, 190°F	1.72	0.84±0.14	0.218	0.4609
E. faecium, 190°F	2.26	0.88±0.14		
Salmonella, 200°F	2.04	0.75±0.23		
E. faecium, 200°F	2.02	0.79±0.17		
Salmonella, 210°F	1.96	0.73±0.30		
E. faecium, 210°F	2.24	0.78±0.27		

[a] Pooled SE = Pooled Standard Error.

In Table 8, for the interaction of microorganism x heating temperature, the D-value for chicken feathers was not statistically different (*P*=0.4609). Studying the thermal destruction of *S.* Enteritidis in feeds, Himathongkham et al. (1996) observed a linear relationship between *Salmonella* reduction and temperature. Although in our results it was not statistically significant, there appears to be a correlation of organism x heating temperature by *E. faecium* having greater or equal D-values of *Salmonella* in all three temperatures (*Salmonella*, 1.72, 2.04, 1.96 min versus *E. faecium,* 2.26, 2.02, 2.24 min) (Table 8). Himathongkham et al. (1996), though not working specifically with *E. faecium* and thermal lethality, suggested that heating temperature could be the most important factor on the inactivation of bacterial contaminants in food and feeds (Bianchini et al., 2012). Table 9 describes the interaction of microorganism x model; statistical analysis indicated the output was not statistically significantly differently (*P*=0.537). It confirms that by analyzing D-values by the Baranyi/Roberts and Linear model, they will differ since the Baranyi and Roberts model will describe shoulders and/or tails in the data output compared to the log linear decrease of the microbial population. Nonetheless, the R^2 indicates a better fit in the Baranyi and Roberts model with *Salmonella* and *E. faecium* as explained before it takes the whole data points into account.

Table 9. Least squares means for D-values in chicken feathers for the interaction of microorganism x D-value determination model

Organism, Baranyi/Roberts & Linear Model	**D-value**	**R^2**	**Pooled SE[a]**	***P*>F**
Salmonella, Linear	2.98	0.58±0.16	0.178	0.5371
E. faecium, Linear	3.15	0.68±0.20		
Salmonella, Baranyi and Roberts	0.83	0.96±0.05		
E. faecium, Baranyi and Roberts	1.22	0.95±0.03		

[a] Pooled SE = Pooled Standard Error.

Table 10 reports the outcome of statistical analysis of the interaction of microorganism x temperature x model, showing no statistical significance with respect to the interaction impacting resulting D-values (*P*=0.8527). Although the interactions are not statistically significant, there is an opportunity to observe that D-values interactions in the Linear Model are similar to the pathogen and surrogate. The Baranyi and Roberts model demonstrated *E. faecium* as having a higher thermal resistance than *Salmonella*. Tsai et al. (2019) reported on the correlation between *Salmonella* and the *E. faecium* in low a_w products by correlating it with thermal inactivation treatments in both organisms. Kinley (2009) had *S.* Senftenberg in its cocktail as the highest heat resistant salmonellae and its $D_{149°F}$=0.36±0.18 relates to this study is $D_{210°F}$ 0.620±0.9 demonstrating that this organism is an adequate organism to include in a cocktail for thermal validation studies.

Table 10. Least squares means for D-values in chicken feathers for the interaction of microorganism x temperature x model

Organism, Cooking Target Temperature (°F), Model	D-value	R^2	Pooled SE[a]	*P*>F
Salmonella, 190°F, Linear	2.48	0.74±0.14	0.309	0.8527
E. faecium, 190°F, Linear	2.92	0.80±0.18		
Salmonella, 190°F, Baranyi and Roberts	0.97	0.94±0.01		
E. faecium, 190°F, Baranyi and Roberts	1.60	0.95±0.01		
Salmonella, 200°F, Linear	3.17	0.55±0.15		
E. faecium, 200°F, Linear	3.17	0.63±0.06		
Salmonella, 200°F, Baranyi and Roberts	0.89	0.94±0.08		
E. faecium, 200°F, Baranyi and Roberts	0.88	0.94±0.05		
Salmonella, 210°F, Linear	3.30	0.45±0.06		
E. faecium, 210°F, Linear	3.38	0.60±0.30		
Salmonella, 210°F, Baranyi and Roberts	0.62	0.99±0.0		
E. faecium, 210°F, Baranyi and Roberts	1.18	0.96±0.04		

[a] Pooled SE = Pooled Standard Error.

3.3. **Cumulative thermal lethality for *Salmonella* and *E. faecium* NRRL B-2354 in chicken blood and feathers**

Consulting with the commercial rendering establishment to simulate the commercial rendering process in a laboratory setting, the inoculated chicken blood and feathers were heated to 93°C (200°F) and 149°C (300°F) for 5 and 18 min, respectively. Data generated indicate significant lethality to the *Salmonella* cocktail of 7.4±0.03 $\log_{10}$ CFU/mL in blood and 8.6±0.02 $\log_{10}$ CFU/g in feathers, respectively (Table 11). *E. faecium* NRRL B-2354 yielded lethality of 7.8±0.04 $\log_{10}$ CFU/mL in blood and 8.6±0.07 $\log_{10}$ CFU/g in feathers (Table 12).

Table 11. Inactivation of *Salmonella* in blood and feathers during cooking under conditions used in commercial rendering

Salmonella (log_{10} CFU/g) Pre-Heating	*Salmonella* (log_{10} CFU/mL) Post-Heating
Blood[a]	
7.4±0.03	ND[c]
Feathers[b]	
8.6±0.02	ND

[a] Values depict means of three replications containing two samples each (*N*=6) ± one sample standard deviation from mean. Limit of detection for blood = 1 CFU/mL

[b] Values depict means of three identical replications containing two samples each (*N*=6) ± one sample standard deviation from mean. Limit of detection for feathers = 10 CFU/g.

[c] ND=Non-detectable

Table 12. Inactivation of *E. faecium* NRRL B-2354 in blood and feathers during cooking under conditions used in commercial rendering

E. faecium (log_{10} CFU/g) Pre-Heating	*E. faecium* (log_{10} CFU/g) Post-Heating
Blood[a]	
7.8±0.04	ND[c]
Feathers[b]	
8.6±0.07	ND

[a] Values depict means of three replications containing two samples each (*N*=6) ± one sample standard deviation from mean. Limit of detection for blood = 1 CFU/mL

[b] Values depict means of three identical replications containing two samples each (*N*=6) ± one sample standard deviation from mean. Limit of detection for feathers = 10 CFU/g.

[c] ND=Non-detectable

4. CONCLUSION

Overall, the findings from this research demonstrate lethality to *Salmonella* and *E. faecium* NRRL B-2354, as a surrogate for validation studies, in rendered products. There was only one statistically significant difference in the chicken blood interaction of microorganism x heating temperature. Baranyi and Roberts model-produced D-values were better fits as compared to linear regression-derived D-values for inoculated chicken blood. A replication effect was not detected in chicken blood D-value work.

Chicken feathers had a replication effect in its analysis, but indicated no variance contribution by the replication effect. Statistical differences in the interactions of microorganism x temperature, microorganism x model, microorganism x temperature x model was likewise not significant at p=0.05. The interaction of microorganism x temperature did not impact resulting D-values in feathers by Tukey's HSD test. In the microorganism x temperature x model interaction, a higher thermal resistance of *E. faecium* was observed with the Baranyi and Roberts model through the three increasing heating temperatures, though the statistical model did not indicate a significant effect of the interaction. Nonetheless, reviewing R^2 values indicate that the Baranyi and Roberts model has a good D-value correlation

Even though the analysis produced non-statistical difference in the interactions by heating chicken blood and feathers at 200 or 300°F at 5 or 18 min, respectively, both *Salmonella* and *E. faecium* NRRL B-2354 were inactivated to non-detectable levels, validating the commercial rendering establishment process and compliance with FDA regulations. The Baranyi and Roberts model proved to be a better model than the Linear Model by providing with time varying environmental conditions, as well its good predictive capabilities. In combination with the application of other food safety preventive controls, these new scientific data describing the

inactivation of the pathogen *Salmonella* via high heat rendering are expected to assist commercial rendering establishments in providing safe raw materials for animal and human food manufacture. As well, verifying the utility of *E. faecium* NRRL B-2354 as a useful non-pathogenic surrogate for in-plant validation of *Salmonella* inactivation during the rendering of chicken feathers and blood. Future research should address evaluation of raw material composition, particularly fat content, and the resulting impacts of chemical composition on foodborne pathogen inactivation during high-heat rendering.

REFERENCES

Almond Board of California, 2007. Guidelines for validation of dry roasting processes. Available at http://www.almondboard.com/Handlers/ Documents/Dry-Roast-Validation-Guidelines.pdf. Accessed 9 September 2019.

Anderson, D. 2006. Rendering operations, In: Essential rendering all about the animal by-Products industry. Edited by D.L. Meeker. National Renderers Association, Arlington, V.A. 31-52.

Annous, B. A., Kozempel, M. F. 1998. Influence of growth medium on thermal resistance of *Pediococcus* spp. NRRL B-2354 (formerly *Micrococcus freudenreichii*) in liquid foods. J. Food Prot. 61, 578-58.

Baranyi, J., Pin, C., Ross, T., 1999. Validating and comparing predictive models. Int. J. Food Microbiol. 48, 159-166.

Baranyi, J., Roberts, T., McClure, P., 1993. A non-autonomous differential equation to model bacterial growth. Food Microbiol. 10, 43-59.

Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. 23, 277-294.

Baranyi, J., Roberts, T.A., 1995. Mathematics of predictive food microbiology. Int. J. Food Microbiol. 26, 199-218.

Bianchini, A., Stratton, J., Weier, S., Hartter, T., Plattner, B., Rokey, G., Hertzel, G., Gompa, L., Martinez, B., Eskridge, K.M., 2014. Use of *Enterococcus faecium* as a surrogate for *Salmonella enterica* during extrusion of a balanced carbohydrate-protein meal. J. Food Prot. 77, 75-82.

Bianchini, A., Stratton, J., Weier, S., Hartter, T., Plattner, B., Rokey, G., Hertzel, G., Gompa, L., Martinez, B., Eskridge, A.M., 2012. Validation of extrusion as a killing step for *Enterococcus faecium* in a balanced carbohydrate-protein meal by using a response surface design. J. Food Prot. 75, 1646-1653.

Borowski, A. G., Ingham, S. C., Ingham, B. H. 2009. Lethality of home-style dehydrator processes against *Escherichia coli* O157:H7 and *Salmonella* serovars in the manufacture of ground-and-formed beef jerky and the potential for using a pathogen surrogate in process validation J. Food Microbiol. 72, 2056-2064.

Boyer, C.I., Bruner D.W., Brown J.A., 1958. *Salmonella* Organisms Isolated from Poultry Feed. J. Avian. Dis. 2, 396.

Buchanan, R., Whiting, R., Damert, W., 1997. When is simple good enough: A comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. Food Microbiol. 14, 313-326.

Bucher, O., D'Aoust, J.Y., Holley, R.A., 2008. Thermal resistance of *Salmonella* serovars isolated from raw, frozen chicken nuggets/strips, nugget meat and pelleted broiler feed. Int. J. Food Microbiol. 124, 195-198.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J., 2012. Enterococci in the environment. Microbiol. Mol. Biol. Rev 76, 685-706.

Ceylan, E., Bautista, D.A., 2015. Evaluating *Pediococcus acidilactici* and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for *Salmonella* for in-plant validation studies of low-moisture pet food products. J. Food Prot. 78, 934-939.

Channaiah, L.H., Michael, M., Acuff, J.C., Phebus, R.K., Thippareddi, H., Olewnik, M., Milliken, G., 2017. Validation of the baking process as a kill-step for controlling *Salmonella* in muffins. Int. J. Food Microbiol. 250, 1-6.

Clark, G.M., Kaufman A.F., Gangarosa, E.J., Thompson, M.A. 1973. Epidemiology of an International Outbreak of *Salmonella* Agona. Lancet 2, 490-493.

Cox, N.A., Bailey, J.S., Thomson, J.E., Juven, B.J. 1983. *Salmonella* and other *Enterobacteriaceae* found in commercial poultry feed. Poultry Sci. 62, 2169-2175

D'Aoust, J.-Y. 1989. *Salmonella*. In M.P. Doyle (ed.), Foodborne Bacterial Pathogens. Marcel Dekker, New York, NY., 327-445

Davies, R. H., Nicholas R. A. J, McLaren I. M., Corkish J. D., Lanning D. G., Wray C. 1997. Bacteriological and serological investigation of persistent *Salmonella* Enteritidis infection in an integrated poultry organization. Vet. Microbiol. 58:277-293.

Davies, R.H., Wray, C. 1997. Distribution of *Salmonella* contamination in ten animal feed mills. Vet. Microbiol. 57, 159-169.

Davis, M., 2003. Feedstuffs as a vehicle of cattle exposure to *Escherichia coli* O157:H7 and *Salmonella enterica*. Vet. Microbiol. 95, 199-210.

European Food Safety Authority (EFSA). 2008. Scientific opinion of the panel on biological hazards on a request from the Health and Consumer Protection, Directorate General, European Commission on microbiological risk assessment in feedingstuffs for food-producing animals. EFFSA J. 720:1-84.

Fakruddin, M., Mazumder, R.M., Mannan, K.S.B., 2011. Predictive microbiology: Modeling microbial responses in food. Ceylon Journal of Science (Bio. Sci.) 40, 121-131.

FDA. 2010. Compliance Policy Guide Sec. 690.800 *Salmonella* in Animal Feed. Draft Guidance. U.S. Food and Drug Administration. Rockville, MD.

Fink-Gremmels, J., 2012. Animal feed contamination: Effects on livestock and food safety, ed. Philadelphia, PA pp 33-49.

Fisher, K., Phillips, C., 2009. In vitro inhibition of vancomycin-susceptible and vancomycin-resistant *Enterococcus faecium* and *E. faecalis* in the presence of citrus essential oils. Brit. J. Biome. Sci. 66, 180-185.

Foegeding, P.M., 1997. Driving predictive modelling on a risk assessment path for enhanced food safety. Int. J. Food Microbiol. 36, 87-95.

Food and Agriculture Organization of the United Nations, World Health Organization (FAO-WHO). 2008. Animal feed impact on food safety. Report of the FAO/WHO expert meeting, 8-12 October 2007. FAO, Rome.

Franco, D.A. 2005. A Survey of *Salmonella* serovars and most probable numbers in rendered-animal-protein meals: inferences for animal and human health. J. Environ. Health 67, 18-23.

Franco, D.A., Swanson W.1996. The Original Recyclers. The Animal Protein Producers Industry, The Fats & Proteins Research Foundation, and The National Renderers Association. First Ed. Alexandria, VA.

Gabis, D.A. 1991. Environmental factors affecting enteropathogens in feed and feed mills. In: Colonization Control of Human Bacterial Enteropathogens in Poultry, Blankenship, L.C. (ed). Academic Press, San Diego, CA, pp. 23-28.

Ge, B., LaFon, P.C., Carter, P.J., McDermott, S.D., Abbott, J., Glenn, A., Ayers, S.L., Friedman, S.L., Paige, J.C., Wagner, D.D., Zhao, S., McDermott, P.F., Rasmussen, M.A., 2013.

Retrospective analysis of *Salmonella*, *Campylobacter*, *Escherichia coli*, and *Enterococcus* in animal feed ingredients. Foodborne Pathog. Dis. 10, 684-691.

Geeraerd, A., Herremans, C., Van Impe, J., 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. Int. J. Food Microbiol. 59, 185-209.

Glenn., L.M. 2006. Isolation and identification of thermally resistant bacteria in raw poultry. MS Thesis, Clemson Univ., Clemson. SC

Goepfert, J.M., Biggie R. A. 1968. Heat resistance of *Salmonella* Typhimirium and *Salmonella* Senftenberg 775W in milk chocolate. Appl. Microbiol. 16, 1939-1940.

Gong, C., Jiang, X. 2017. Characterizing *Salmonella* contamination in two rendering processing plants. J. Food Prot. 80, 265-270.

Grau, F.H., Brownlie L.E. 1968. Effect of some pre-slaughter treatments on the *Salmonella* population in the bovine rumen and feces. J. Appl. Bacteriol. 31, 157-163.

Grijspeerdt, K., Vanrolleghem, P., 1999. Estimating the parameters of the Baranyi model for bacterial growth. Food Microbiol. 16, 593-605.

Gurtler, J.B., Kornacki, J.L., 2009. Comparison of supplements to enhance recovery of heat-injured S*almonella* from egg albumen. Lett. Appl. Microbiol. 49, 503-509.

Hacking, W.C., Mitchell, W.R., Carlson, H.C. 1978. *Salmonella* investigation in an Ontario feed mill. Can. J. Com. Med. 42, 400-406.

Hayes, M., 2013. Validation of thermal destruction of pathogenic bacteria in rendered animal products. PhD Dissertation. Clemson University. Clemson, S.C.

Heldman, D., Hartel R. W. (ed.). 1998. Principles of Food Processing. Aspen Publishers, Gaithersburg, MD.

Hernandez, A.L.M., Santos, C.V., 2012. Keratin fibers from chicken feathers: Structure and advances in polymer composites, Keratin: Structure, properties and applications. Nova Science Publishers, Inc., pp. 149-212.

Himathongkham, S., das Graças Pereira, M., Riemann, H., 1996. Heat destruction of *Salmonella* in poultry feed: Effect of time, temperature, and moisture. Avian Dis. 72-77.

Hofacre, C.L., White D.G., Maurer J.J., Morales C, Lobsinger C., Hudson C. 2001. Characterization of antibiotic-resistant bacteria in rendered animal products, Avian Dis. 45, 953-961.

Isa, J., Boycott, B., Brouchton, E. 1963. A survey of *Salmonella* contamination in animal feeds and feed constituents. Can. Vet. J. 4, 41-43.

Jay, J.M. 2005. Modern Food Microbiology, 7th Edition. Aspen Publishers Inc., Gaithersburg, MD.

Jeong, S., Marks, B.P., Ryser, E.T., 2011. Quantifying the performance of *Pediococcus* sp. (NRRL B-2354: *Enterococcus faecium)* as a nonpathogenic surrogate for *Salmonella* Enteritidis PT30 during moist-air convection heating of almonds. J. Food Prot. 74, 603-609.

Jiang, X., 2016. Prevalence and characterization of *Salmonella* in animal meals collected from rendering operations. J. Food Prot. 79, 1026-1031.

Jin, Y., Tang, J., 2019. Improved design of aluminum test cell to study the thermal resistance of *Salmonella enterica* and *Enterococcus faecium* in low-water activity foods. Food Control 104, 343-348.

Jones, F.T., 2011. A review of practical *Salmonella* control measures in animal feed. J. App. Poult. Research 20, 102-113.

Jones, F.T., Axtell R. C., Rives D.V., Scheideler, S. E., Tarver Jr. F. R., Walker R.L, Wineland M.J. 1991. A survey of *Salmonella* contamination in modern broiler production. J. Food Prot. 54, 502-507.

Jones-Ibarra, A.M., Acuff, G.R., Alvarado, C.Z., Taylor, T.M., 2017. Validation of thermal lethality against *Salmonella enterica* in poultry offal during rendering. J. Food Prot. 80, 1422-1428.

Kaufmann, A. F., Feeley J.C., 1968. Culture survey of *Salmonella* at a broiler-raising plant. Public Health Rep. 83:417-422.

Kinley, B., 2009. Prevalence and biological control of *Salmonella* contamination in rendering plant environments and the finished rendered meals. PhD Dissertation. Clemson University. Clemson, S.C.

Kinley, B., J. Rieck, P. Dawson, X. Jiang. 2010. Analysis of *Salmonella* and enterococci isolated from rendered animal products. Can. J. Microbiol. 56, 65-73.

Kopit, L.M., Kim, E.B., Siezen, R.J., Harris, L.J., Marco, M.L., 2014. Safety of the surrogate microorganism *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. Appl. Environ. Microbiol. 80, 1899–1909.

Kornacki JL.,2012. *Enterococcus faecium* NRRL B-2354: tempest in a teapot or serious foodborne pathogen. Food Safety Magazine. http://www.foodsafetymagazine.com/magazine-archive1/april-may-2 012/enterococcus-faecium- nrrl-b-2354-tempest-in-a-teapot-or-seriousfoodborne-pathogen.

Le Minor, L., Coynault C., Pessoa G. 1974. Determinisme plasmidique du caractere atypique “lactose postif” de souches de *S*. Typhimurium et de *S*. oranienburg isolates au Bresil lors d’epidemies de 1971 a 1973. Ann. Microbiol. 125A, 261-285.

Le Minot, L., Coynault C., Rhode R., Rowe B., Alcksic S. 1973. Localisation plasmidique de determinant genetique du caractere atypique “saccharose +” des *Salmonella*. Ann. Microbiol. 124B, 295-306.

Li, Y., Hsieh, F, Fields, M. L., Huff, H. E., Badding, S. L. 1993. Thermal inactivation and injury of *Clostridium sporogenes* spores during extrusion of mechanically deboned turkey mixed with white corn flour. J. Food Process. Preserve. 17, 391-403.

Liu, B., Schaffner, D.W., 2007. Quantitative analysis of the growth of *Salmonella* Stanley during alfalfa sprouting and evaluation of *Enterobacter aerogenes* as its surrogate. J. Food Prot. 70, 316-322.

Liu, S., Ozturk, S., Xu, J., Kong, F., Gray, P., Zhu, M.-J., Sablani, S.S., Tang, J., 2018. Microbial validation of radio frequency pasteurization of wheat flour by inoculated pack studies. J. Food Eng. 217, 68-74.

Liu, S., Rojas, R.V., Gray, P., Zhu, M.J., Tang, J., 2018. *Enterococcus faecium* as a *Salmonella* surrogate in the thermal processing of wheat flour: Influence of water activity at high temperatures. Food Microbiol. 74, 92-99.

Magwood, S. E., Fung J., Byrne J. L. 1965. Studies on *Salmonella* contamination of environment and product of rendering plants. Avian Dis. 9:302-308.

McKellar, R., Knight, K., 2000. A combined discrete–continuous model describing the lag phase of listeria monocytogenes. Int. J. Food Microbiol. 54, 171-180.

McMeekin, T.A., Ross, T., 1996. Shelf life prediction: Status and future possibilities. Int. J. Food Microbiol. 33, 65-83.

Meeker, D.L., Hamilton C.R. 2006. An overview of the rendering industry. Edited by D.L. Meeker. National Renderers Association, Arlington, V.A.

Murphy, R.Y., Beard, B.L., Martin, E.M., Keener, A.E., Osaili, T., 2004. Predicting process lethality of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in ground,

formulated, and formed beef/turkey links cooked in an air impingement oven. Food Microbiol. 21, 493-499.

Murphy, R.Y., Marks B.P., Johnson E.R., Johnson M.G. 2000. Thermal inactivation kinetics of *Salmonella* and *Listeria* in ground chicken breast meat and liquid medium. J. Food Sci. 65(4), 706-710.

Murphy. R.Y., Osaili T., Duncan L.K., Marcy J.A. 2004. Thermal inactivation of *Salmonella* and *Listeria monocytogenes* in ground chicken thigh/leg meat and skin. Poult. Sci. 83, 1218-1225.

Nesse, L.L., Nordby, K., Heir, E., Bergsjoe, B., Vardund, T., Nygaard, H., Holstad, G., 2003. Molecular analyses of *Salmonella enterica* isolates from fish feed factories and fish feed ingredients. Appl. Environ. Microbiol. 69, 1075-1081.

Ng, H., Bayne H. G., Garibaldi J.A. 1969. Heat resistance of *Salmonella*: The uniqueness of *Salmonella* Senftenberg 775W. Appl. Microbiol. 17, 78-82.

Ockerman, H.W., Hansen C.L. 1988. Animal By-Product Processing. Ellis Horwood Ltd., Chichester, England.

Ockerman, H.W., Hansen, C.L. 2000. Animal by-product processing and utilization. CRC Press, Taylor & Francis Group, Boca Raton, F.L.

Orthoefer, J. G., Schrieber, J. B. Nichols, Schneider N. 1968. *Salmonella* contamination in a rendering plant. Avian Dis. 12:303-311

Paşayev, N., Kocatepe, S., Maraş, N., Soylak, M., Erol, M., 2017. Investigation some characteristics of chicken feather's rachis. IOP Conference Series: Materials Science and Engineering 254.

Piyasena, P., McKellar, R. C., Bartlett, F. M. 2003. Thermal inactivation of *Pediococcus* spp. in simulated apple cider during high-temperature short-time pasteurization. Int. J. of Food Microbiol. 82, 25-31.

Pomeroy, B.S., M.K. Grady. 1961. *Salmonella* Organisms Isolated from Feed Ingredients. Proc. U.S. Livestock Sanit. Ass. 65, 449.

Rachon, G., Penaloza, W., Gibbs, P.A., 2016. Inactivation of *Salmonella*, *Listeria Monocytogenes* and *Enterococcus faecium* NRRL B-2354 in a selection of low moisture foods. Int. J. Food Microbiol. 231, 16-25.

Ramirez-Hernandez, A., Inestroza, B., Parks, A., Brashears, M.M., Sanchez-Plata, M.X., Echeverry, A., 2018. Thermal inactivation of *Salmonella* in high-fat rendering meat products. J. Food Prot. 81, 54-58.

Ramirez-Lopez, L.M. 2006. Heat inactivation of thermo-resistant bacteria isolated from poultry offal. MS Thesis, Clemson University. Clemson. SC.

Reddy, N., Yang, Y., 2007. Structure and properties of chicken feather barbs as natural protein fibers. J. Polym. Environ. 15, 81-87.

Shrimpton, D.H., Monsey J.B., Hobbs B.C., Smith M.E. 1962. A laboratory determination of the destruction of alpha amylase and *Salmonella*e in whole eggs by heat pasteurization. J. Hyg. 60:153-162.

Teixeira. A.A. 2006. Simulating thermal food processes using deterministic models. 73-106 in Thermal Food Processing. D. Sun, ed. CRC Press, Taylor & Francis Group, Boca Raton, FL.

Tsai, H.C., Ballom, K.F., Xia, S., Tang, J., Marks, B.P., Zhu, M.J., 2019. Evaluation of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during cocoa powder thermal processing. Food Microbiol. 82, 135-141.

U.S. Food and Drug Administration. 2013. CGMPs and Preventive controls for food for animals. FDA Food Safety Modernization Act. Available at: https://www.fda.gov/animal-veterinary/animal-food-feeds/food-safety-modernization-act-and-animal-food. Accessed September 11 2019.

U.S. Food and Druga Administration (FDA). 2010. Guidance for FDA staff: compliance policy guide Sec. 690.800 *Salmonella* in animal feed (draft guidance). Available at:http://www.fda.gov/lCECI/ComplianceManuals/CompliancePolicyGuidanceManual/default.htm. Accessed September 9 2019.

Venkitasamy, C., Zhu, C., Brandl, M.T., Niederholzer, F.J.A., Zhang, R., McHugh, T.H., Pan, Z., 2018. Feasibility of using sequential infrared and hot air for almond drying and inactivation of *Enterococcus faecium* NRRL B-2354. LWT Food Sci. Technol. 95, 123-128.

Verma, T., Wei, X., Lau, S.K., Bianchini, A., Eskridge, K.M., Subbiah, J., 2018. Evaluation of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during extrusion of low-moisture food. J. Food Sci. 83, 1063-1072.

Watkins, J.R., Flowers A.I., Grumbles L.C. 1959. *Salmonella* organisms in animal products used in poultry feeds. Avian Dis. 3, 290-301.

Wei, X., Lau, S.K., Stratton, J., Irmak, S., Subbiah, J., 2019. Radiofrequency pasteurization process for inactivation of *Salmonella* spp. and *Enterococcus faecium* NRRL B-2354 on ground black pepper. Food Microbiol. 82, 388-397.

Wray C., Wray, A. 2000. *Salmonella* in Domestic Animals. CABI Publishing, Wallingford, Oxon, United Kingdom.

Xu, J., Yang, R., Jin, Y., Barnett, G., Tang, J., 2020. Modeling the temperature-dependent microbial reduction of *Enterococcus faecium* NRRL B-2354 in radio-frequency pasteurized wheat flour. Food Control.

Zhang, Y. 2011. Thermal destruction of *Geobacillus stearothermophilus* in rendered animal co-products. M.S. thesis. Clemson University, Clemson, S.C.

APPENDIX A

***Salmonella* serovars Senftenberg, Typhimurium, and Heidelberg**

A preliminary experiment was conducted to verify that counts of overnight cultures of individual *Salmonella* isolates utilized for the cocktail do not differ from one another. Isolates belonging to *S. enterica* serovars Senftenberg, Heidelberg, and Typhimurium, recovered from poultry products or chicken harvesting environments, were chosen from the Food Microbiology Laboratory culture collection (Department of Animal Science, Texas A&M University, College Station, TX) and revived from -80°C by incubating in 10 mL sterile brain heart infusion (BHI) broth for 24 h at 35°C. Following the initial passage, a second passage was completed in like fashion to activate isolates for subsequent preparation. Following revival of isolates, *Salmonella* isolates were serially diluted on peptone water and plated on tryptic soy agar (TSA) (Becton, Dickinson and Co., Sparks, MD, USA™) and incubated for 24 h at 35-37°C before inspection of colonies and counting. Plate counts were $\log_{10}$ transformed prior to data analysis. Microsoft® Excel® (Redmond, WA, USA, 2016) was used for data analysis.

Table A1. *Salmonella enterica* serovars Senftenberg, Typhimurium, and Heidelberg at 24 h growth ($\log_{10}$ CFU/mL).

Serovar	Replication 1	Replication 2	Replication 3	Mean±Std. Dev.
Senftenberg	8.7	8.9	8.8	8.8±0.07
Typhimurium	9.1	9.1	8.9	9.0±0.08
Heidelberg	9.0	9.1	8.9	9.0±0.1

APPENDIX B

***Enterococcus faecium* NRRL B-2354**

A preliminary experiment was conducted to verify that counts of overnights of *E. faecium* NRRL B-2354 isolate utilized do not differs from one another. Isolate B-2354 of *E. faecium* (Orla-Jensen 1919) Schleifer and Kilpper-Balz 1984 was ordered from the USDA Agricultural Research Service Culture Collection (NRRL) and revived in 10 mL sterile brain heart infusion (BHI) broth for 24 h at 27°C. Following the initial passage, a second passage was completed in like fashion to activate and prepare isolate for subsequent use. Following revival of isolate, *E. faecium* NRRL B-2354 isolates were serially diluted on peptone water and plated on tryptic soy agar (TSA) (Becton, Dickinson and Co., Sparks, MD, USA™) and incubated for 24 h at 35-37°C before inspection of colonies and counting. Plate counts were $\log_{10}$ transformed prior to data analysis. Microsoft® Excel® (Redmond, WA, USA, 2016) was used for data analysis.

Table B1. *E. faecium* NRRL B-2354 at 24 h growth ($\log_{10}$ CFU/mL).

***E. faecium* NRRL B-2354**	
Replication 1	10.0
Replication 2	10.0
Replication 3	9.7
Mean±Std. Dev.	9.9±0.1

APPENDIX C

Non-inoculated samples for numbers of background salmonellae on chicken blood and feathers.

Samples were obtained from a commercial rendering establishment located in the southern United States. 25 mL of blood were poured into a 50.0-mL conical tube and vortexed for 1 min for mixture. 10 g of feathers were weighed and then placed in stomacher bags and poured 90 mL 0.1% peptone water and placed in a Stomacher 400 blender for 1 min and plated on bismuth sulfite agar incubated for 24-48 h at 35-37°C before inspection of colonies and counting.

Table C1. Non inoculated blood for background salmonellae (log_{10} CFU/mL).

Uninoculated Blood	
Replication 1	5.6
Replication 2	4.9
Replication 3	4.3
Mean+Std. Dev.	4.9±0.6

Table C2. Non-inoculated feathers for background salmonellae (log_{10} CFU/g).

Uninoculated Feathers	
Replication 1	7.1
Replication 2	6.9
Replication 3	6.9
Mean+Std. Dev.	7.0±0.1

APPENDIX D

Non-inoculated samples for numbers of background enterococci on chicken blood and feathers.

Samples were obtained from a commercial rendering establishment located in the southern United States. 25 mL of blood were poured into a 50 mL conical tube and vortexed for 1 min for mixture. 10 g of feathers were weighed and then placed in stomacher bags and poured 90 mL 0.1% peptone water and placed in a Stomacher 400 blender for 1 min and plated on KF *Streptococcus* and incubated for 24-48 h at 35-37°C before inspection of colonies and counting.

Table D1. Non-inoculated blood for background enterococci (log_{10} CFU/mL)

Uninoculated Blood	
Replication 1	5.4
Replication 2	6.1
Replication 3	5.0
Mean+Std. Dev.	5.5±0.5

Table D2. Non-inoculated feathers for background enterococci (log_{10} CFU/g)

Uninoculated Feathers	
Replication 1	5.5
Replication 2	5.9
Replication 3	5.8
Mean+Std. Dev.	5.8±0.2

APPENDIX E

Recovery of *Salmonella* inoculated on following 5 min heating at 93°C (200°F) as a function of recovery.

The potential for sub-lethal injury to occur during thermal processing was identified and the medium had to be modified to the selective plating medium for *Salmonella* allowing injured cells to be detected at counts not statistically different (P=0.675) from that of a non-selective plating medium. *S.* serovars Senftenberg, Heidelberg, and Typhimurium were inoculated into blood and were subjected to 93°C (200°F) in pre-heated metal vessels for 5 min and immediately placed in ice-laden cold water (0°C) to halt further microbial destruction due to heat. *Salmonella* were counted by preparation of serial dilutions and plating on non-selective tryptic soy agar, bismuth sulfite overlay of tryptic soy agar and selective media supplemented with differing injured cell repair agents. 1 g/L sodium pyruvate, 1 g/L 3'3'-Thiodipropionic acid, (TDP) (Gurtler and Kornacki, 2009) to allow for repair and detection of sub-lethally injured[1] cells on Petri plates, and incubated at 35-37°C for 24-48 h before inspection of typical colonies and counting.

---

[1]Injury is the inability of a microbe to grow and form colonies on a selective plating medium; this is expected to produce an over-estimation of process lethality, potentially allowing inadequately rendered product to be deemed safe during routine testing.

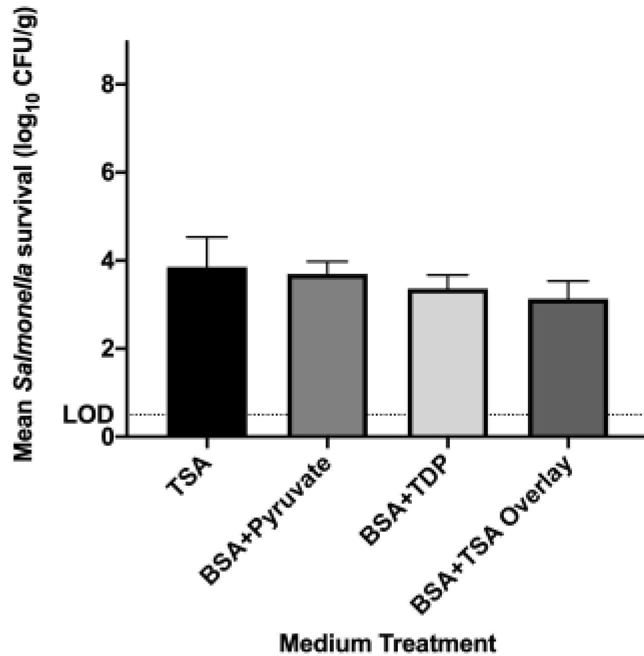

Figure E1. Inoculated *Salmonella* recovery on 5 min heating at 93°C (200°F) as a function of recovery medium (*P*=0.675).
Columns depict means of three identical replications; error bars depict the standard error about means (SEM). Means were compared by one-way analysis of variance (ANOVA). TSA (tryptic soy agar); BSA + pyruvate (bismuth sulfite agar + 1 g/liter sodium pyruvate); BSA+TDP (bismuth sulfite agar + thiodipropionic acid); BSA+TSA overlay (bismuth sulfite agar base layer covered with 10 mL sterilized TSA).

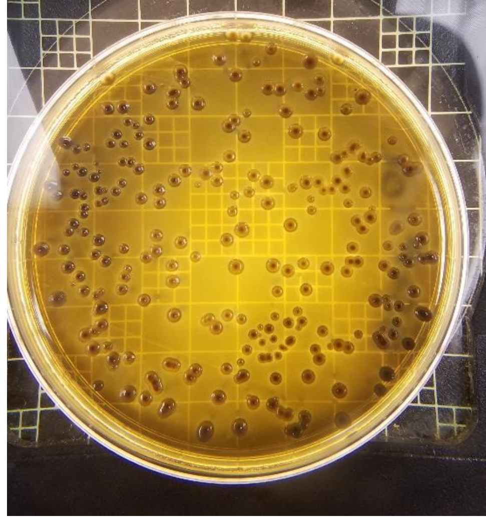

Figure E2. BSA + sodium pyruvate.

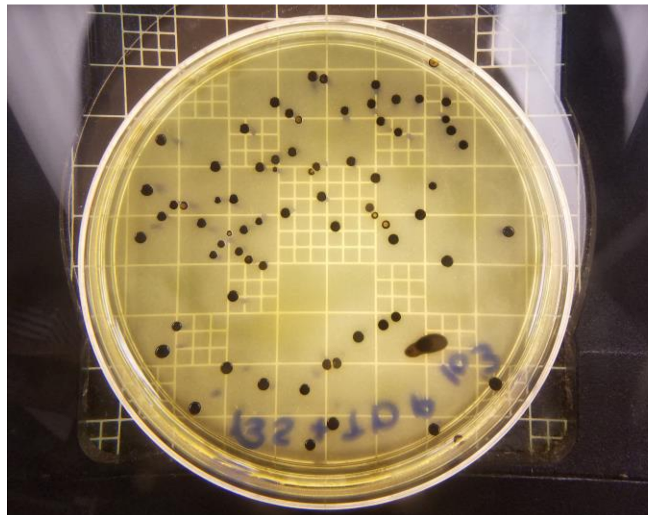

Figure E3. BSA + TDP.

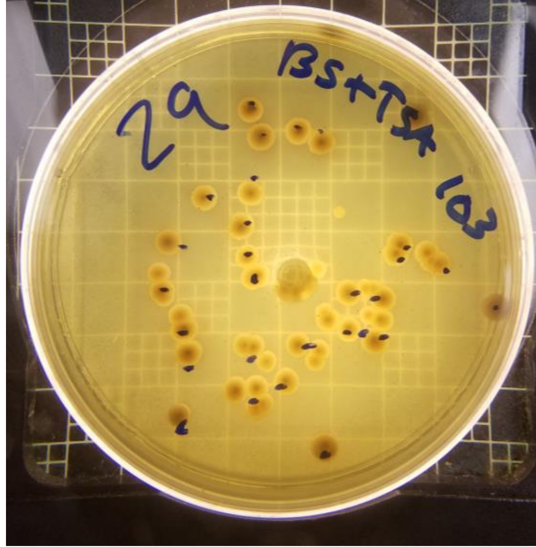

Figure E4. BSA + TSA Overlay.