12-2016

Evaluation of thermal process lethality in meat for non-pathogenic Escherichia coli as a surrogate for Salmonella

Morgan Alyse Redemann
Purdue University

EVALUATION OF THERMAL PROCESS LETHALITY IN MEAT
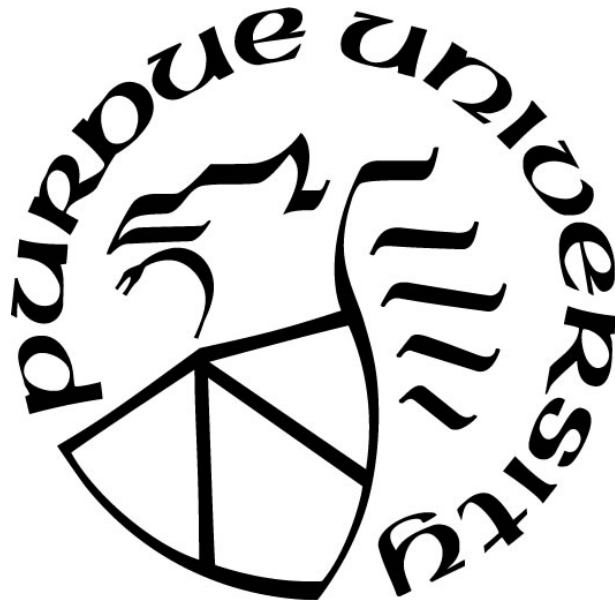# FOR NON-PATHOGENIC *ESCHERICHIA COLI* AS A SURROGATE
FOR *SALMONELLA*

by

Morgan Alyse Redemann

A Thesis

Submitted to the Faculty of Purdue University
In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Food Science

West Lafayette, Indiana

December 2016

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF THESIS APPROVAL

Dr. Manpreet Singh, Chair
> Department of Food Science

Dr. Fernanda San Martin
> Department of Food Science

Dr. Stacy Zuelly
> Department of Animal Sciences

Approved by:
> Dr. Carlos Corvalan
>> Head of the Departmental Graduate Program

For my parents and my sister Lauran, for their constant love and support in achieving my goals and dreams

For my guardian angel, Andrew, who never stopped believing in me

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CCP	Critical Control Point
CDC	Center for Disease Prevention and Control
CFR	Code of Federal Regulations
CFU	Colony Forming Units
FMIA	Federal Meat Inspection Act
GAP	Good Agricultural Practices
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Point
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
NARMS	National Antimicrobial Resistance Monitoring System
ODPHP	Office of Disease Prevention and Health Promotion
PW	Peptone Water
RTE	Ready-to-Eat
SSOP	Sanitation Standard Operating Procedures
STEC	Shiga-toxin *Escherichia coli*
TAL	Thin Agar Layer
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UHT	Ultra High Temperature
USDA-FSIS	United States Department of Agriculture – Food Safety Inspection Service
VRBG	Violet Red Bile Glucose Agar
VTEC	Verocytotoxin *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
XLD	Xylose Lysine Deoxycholate

ABSTRACT

Author: Redemann, Morgan, A. MS
Institution: Purdue University
Degree Received: December 2016
Title: Evaluation of Thermal Process Lethality in Meat for Non-Pathogenic *Escherichia Coli* as a Surrogate for *Salmonella*
Major Professor: Manpreet Singh

Non-typhoidal *Salmonella* is the leading cause of foodborne illness in the United States, resulting in about 20,000 hospitalizations and nearly 380 deaths annually. The meat processing industry has been especially plagued by *Salmonella*, from meat-inherent sources and more alarmingly, cross-contamination. For ready-to-eat (RTE) meat products specifically, this can cause significant problems in processing facilities ensuring safe product for consumption, resulting in foodborne illness.

The development of standard lethality compliance guidelines by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) within Appendix A assists processors in confirming that *Salmonella* is inactivated in RTE beef and poultry products, based on a "worst case" raw product contamination condition. While this is beneficial, means of thermal process validation are limited. However, the identification of five non-pathogenic *E. coli* strains isolated from cattle may provide a method of validation for processors. Previous studies have investigated the behavior of the isolates individually in response to a variety of microbial interventions, including cooking, fermentation, freezing, refrigerated storage, and antimicrobial treatments as compared to the behavior *E. coli* O157:H7 as well as *Salmonella*. Based on the results of these studies, it was sensible to study the behavior of the combined non-pathogenic *E. coli* isolates in ground beef at varying fat contents under thermal processing conditions

compared to *Salmonella* to determine its potential for use to validate thermal processing. Therefore, the objective of this study was to determine if the non-pathogenic *E. coli* isolates could be used as a surrogate for a mixed culture of *Salmonella* as means to validate thermal processing parameters in accordance with Appendix A.

For lower temperatures outlined in Appendix A (130, 135, 140, 145°F (54, 57, 60, and 63°C)), the non-pathogenic *E. coli* inoculum has significantly different ($P < 0.05$) decimal-reduction values (D-values), in that they are significantly greater than *Salmonella* D-values across all five fat content levels (5, 10, 20, 25, 30%). At temperatures greater than 145°F (63°C), no significant differences ($P > 0.05$) existed between the inoculums across fat content, indicating that the two inoculums were being inactivated at similar rates. These results suggest that the most appropriate use of the non-pathogenic *E. coli* surrogates would be for predicting, ensuring, and validating thermal processing for the inactivation of *Salmonella* at lower temperatures, specifically those that fall within the "danger zone" that support rapid bacterial growth (40 - 140°F (4 - 60°C)). Beyond temperature 145°F (63°C), the non-pathogenic *E. coli* inoculum offers no substantial advantage, as it is being inactivated as rapidly as *Salmonella.* Due to its prolific growth and high-density yield, the absence of the *E. coli* inoculum can ensure the inactivation *Salmonella* at higher thermal processing temperatures. However, investigation of the effects of meat product attributes (pH, water activity, moisture, fat and muscle distribution) as well as considerations of additional variables, risks, and parameters of facility-conducted thermal processing trials is recommended to gain further insight on thermal processing behavior of both non-pathogenic *E. coli* inoculum and *Salmonella.*

CHAPTER 1.   INTRODUCTION

Non-typhoidal *Salmonella* is the leading cause of bacterial foodborne illnesses in the

United States annually (Scallan and others 2011). Out of nearly 56,000 hospitalizations

and 1,351 deaths due to foodborne illness, *Salmonella* was responsible for 35% and 28%

of these cases, respectively. This has resulted in nearly $3.7 million for total cost of the

Salmonella infections annually, accounting for medical and productivity costs (USDA

2014). According to the Annual Foodborne Illness Surveillance Report, it has been

estimated that there are 15.3 incidents of *Salmonella*-related illness per 100,000

individuals, even surpassing the incidence rate of *Camplyobacter* of 13.3 incidents per

100,000 individuals (CDC 2016a). As a result of this, in collaboration with the Office of

Disease Prevention and Health Promotion (ODPHP), FoodNet constructed food safety

objectives to reduce the incidence rate of foodborne illness through the "Healthy People

2020" initiative in 2006. For *Salmonella*, the most recent 2015 food safety report card for

the initiative has cited no change in the overall incidence rate, while other pathogens,

such as *Escherichia coli* 0157:H7, have decreased. Many sources varying among many

serotypes of *Salmonella* has been cited as a major factor for the static incident rate (CDC

2016a). Thus, it is imperative to continue exploring means of ensuring food safety during

processing.

Salmonella is typically associated with poultry products, but in recent years, there

has been outbreaks in more atypical products such as cucumbers, cantaloupe, and peanut

butter, among others. Additionally, the infective dose of *Salmonella* can be as low as 15

cells or as high as 10^5 cells, depending on the serotype (Mead and others 1999; Foley and

Lynne 2008). *Salmonella* serovars belonging to *S. enterica* cause the most cases of

foodborne illness in humans, of which more than 2,500 serotypes have been identified (CDC 2015b). The National Antimicrobial Resistance Monitoring System (NARMS) has observed patterns of multi-drug resistance for serovars including but not limited to *S.* Enteritidis, *S.* Newport, and *S.* Typhimurium, stimulating a call to action to prevent microbial contamination in the food system.

The Food Safety Inspection Service under the United States Department of Agriculture (USDA-FSIS) is primarily responsible for setting standards to ensure the safety of processed foods. The formal establishment of the Hazard Analysis Critical Control Points system (HACCP) for food processing facilities in 1996 has significantly impacted the safety and controls within the food industry. Validation, verification, and monitoring of processing systems are three essential overarching principles that encompass the means of identifying, quantifying, and mitigating or eliminating risks and risk factors.

Surrogate and indicator organisms have been in use since the 1800s, originally developed as a measure of water quality and sanitation. Fecal contamination, identified by the presence of naturally occurring *Escherichia coli,* was specifically measured as an indicator organism. This concept evolved to its current form for processing and Good Manufacturing Practices (GMPs) integrity at food production facilities, and is now known as an "index organism" (Medema and Payment 2003). Surrogate organisms originated from this concept of the early indicator organism, but instead of being naturally occurring, they are introduced into a system undergoing evaluation as an inoculum (Busta and others 2003). Surrogate organisms are typically non-pathogenic, so that they can be used in processing facilities without risk to health and food safety.

However, easy preparation, enumeration and differentiation from other microflora as well as rapid, sensitive, and inexpensive detection is desired. Surrogate organisms should also exhibit similar behavior to the target organism demonstrated through thermal death time values, D-values, and z-values. Thus, their most appropriate application is for processing treatment evaluation (Busta and others 2003) The canning industry is a primary example of using surrogate organisms to validate the destruction of *Clostridium botulinum* spores.

While various studies have analyzed microbial interventions in meat to characterize the five non-pathogenic *E. coli* strains individually (see Table 1 - P1, P3, P8, P14, and P68 isolated from beef cattle) little research has been completed to investigate their use for thermal processing validation. The purpose of this research is to determine if the non-pathogenic *E. coli* isolates can be used as surrogates for thermal processing validation and compare their thermal behavior to *Salmonella*. This research utilizes a five strain cocktail of the non-pathogenic *E. coli* isolates (Table 1) to correlate performance characteristics with a mixed culture of *Salmonella* in ground beef with varying fat content and at different lethality temperatures in accordance with the standards in Appendix A. Based on the comparison of the thermal death time of the surrogates and *Salmonella*, the secondary objective of this research is to determine if fat content level impacts microbial performance characteristics under thermal processing conditions. This will provide insight as to whether the non-pathogenic *E. coli* isolates could be used as surrogates for *Salmonella* to validate thermal processing in meat according to Appendix A.

CHAPTER 2.   LITERATURE REVIEW

2.1   Introduction

Each year, about 9.4 million cases of foodborne illness in the United States are caused by 31 major pathogens (Scallan and others 2011). About 3.6 million of these cases, nearly 39% of all foodborne illness, is caused by bacteria annually, of which, non-typhoidal *Salmonella* composes 11% as the leading illness-causing pathogen (Scallan and others 2011). Thirty-five percent of hospitalizations and 28% of deaths were the result of non-typhoidal *Salmonella* (Scallan and others 2011). The meat industry specifically has been plagued with pathogen outbreaks, which is concerning for thermally processed, ready-to-eat (RTE) meat products. Acid/alkaline tolerance, low temperature tolerance, thermotolerance, and desiccated environment survival are some of the adaptations that *Salmonella* has been reported to develop through sublethal injury (Foster 2001; Phillips and others 1998; Wesche and Ryser 2013). These survival and adaptation mechanisms of *Salmonella* have compelled meat processing facilities to implement stringent controls and protocols to ensure thorough processing for complete inactivation. The implementation of the Hazard Analysis Critical Control Point (HACCP) System with good manufacturing practices (GMPs) and sanitation standard operating procedures (SSOPs) has provided more control for manufacturing facilities to improve food safety, and further developments in detection technologies continue to emerge. Of these technologies, real-time methods to confirm pathogen inactivation have been developed as means to evaluate thermal processing efficacy, specifically the use of non-pathogenic surrogate microorganisms in processing facilities. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) established guidelines for the use of

microbial surrogate microorganisms in processing studies and validations that require

further study. Appropriately selected surrogates have inactivation characteristics and

kinetics that are used to predict those of the target pathogen, and also behave similarly to

the target pathogen when exposed to processing parameters within a specific food

product. This is specifically used for analyzing thermal death time values (D- and z-

values). Studies have been conducted to analyze the use of non-pathogenic *Escherichia*

coli surrogates for specific treatments and processing of meats to control target pathogens

such as *E. coli* 0157:H7 as well as pathogenic *Salmonella* serotypes. However, there is a

lack of data that specifically addresses and complies with the mandates of Appendix A.

The Appendix A guidelines provide time/temperature requirements to reduce specified

microbial loads in RTE meats, and are recognized by the United States Department of

Agriculture's Food Safety Inspection Service (USDA-FSIS) as validated process

schedules. Evidence demonstrating that the non-pathogenic *E. coli* organisms can be used

as surrogates for *Salmonella* would continue to increase the confidence in food safety and

processing validations and promote more effective processing and controls.

2.2   Current Surveillance of *Salmonella*

FoodNet, a collaborative program established in 1995 under the Center for

Disease Control (CDC), conducts surveillance for major pathogens in the United States

diagnosed by laboratory testing of samples from patients (CDC 2015a). Among these

pathogens*, Salmonella* has continued to be the most frequent case of infection with 15.3

cases per 100,000 individuals in the United States, according to the FoodNet 2014

Annual Foodborne Illness Surveillance Report (CDC 2016a). Aligned with objectives

from the Healthy People 2020 initiative (ODPHP 2016), the 2015 Food Safety Report has

concluded that there has been no change in the incidence of culture-confirmed infections since the 2006-2008 baseline studies and objective to decrease the incidence rate to 11.4 cases per 100,000 individuals. *Salmonella* is the most frequent cause of infection, along with *Campylobacter*, due to its complexity of many sources varying by many serotypes.

2.3   *Salmonella*

Salmonella is a member of the family *Enterobacteriaceae,* which includes gram-negative, non-spore forming-bacilli that are facultative anaerobes. *Salmonella* is further divided into two species categories, *S. enterica* and *S. bongori* (CDC 2016b; CDC 2015c). *S. bongori* is most common to cold-blooded animals and can infect humans, but rarely, while over 99% of serotypes belong to the *S. enterica* species, which is associated with warm-blooded animals (CDC 2015c; Fookes and others 2011). The *S. enterica* category causes most foodborne illness in humans and more than 2,500 serotypes have been identified (CDC 2015c), which can further be divided into typhoidal and non-typhoidal categories. Typhoidal *Salmonella* causes systemic disease and the risk is relatively low in the United States, while non-typhoidal *Salmonella* is much more common. According to the CDC, non-typhoidal *Salmonella* is a leading cause of gastroenteritis worldwide and the infective dose is based on serotype, from as much as $10^3 - 10^5$ cells to as low as 15 - 20 cells (FDA 2015). This wide range is a cause of concern in RTE and thermally processed meat products, thus mandating guidelines such as Appendix A, providing time/temperature requirements to reduce specified microbial loads in RTE meats.

2.4   Salmonella Infection

Consumption of contaminated food products, including meat, poultry, eggs, milk, seafood, and fresh produce accounts for 95% of human salmonellosis cases (Mead and others 1999; Foley and Lynne 2008). Disease syndromes from non-typhoidal *Salmonella* include gastroenteritis, bacteremia, and focal infections (Darwin and Miller 1999; Foley and Lynne 2008). Symptoms of diarrhea, fever, and abdominal cramps typically manifest themselves between 12 and 72 hours after infection, and usually lasts 4 to 7 days. Most individuals recover without treatment (symptoms are self-limiting), but the elderly, infants, and immunocompromised populations are more likely to experience severe illness (CDC 2016d). A low percentage of cases may result in septicemia and invasive infections of organs and tissues, which lead to extra-intestinal diseases such as osteomyelitis, pneumonia, and meningitis. The cause of this small percentage of cases in the past has been reported to due to *S.* Choleraesuis, as well as *S.* Typhimurium (Cohen, Bartlett, and Corey 1987). As the leading cause for bacterial foodborne illness, *Salmonella* has detrimental impact amongst regulatory agencies and consumers to trust in food processing systems.

The fecal-oral transmission of *Salmonella* is the primary mode of infection in humans and animals, and the infective dose can range from $15 - 20$ cells to $10^3 - 10^5$ cells (Foley and Lynne 2008; FDA 2015). This range is partially influenced by serotype characteristics, as well as the nature of the contaminated food matrix (Giannella and others 1972; FDA 2015; Foley and Lynne 2008). The human stomach has inherent barriers to *Salmonella* colonization, including low pH and presence of organic acids (Foley and Lynne 2008). However, it has been reported that *Salmonella* has developed acid shock protein mechanisms over time to survive low pH environments and increase

acid tolerance (Foster 1991). *Salmonella* types that are able to adapt to the high acid

environment colonize in the small intestine, colon, and cecum using fimbriae or pili

(Darwin and Miller 1999). These mechanisms are constantly adapting through sublethal

injury, paralleling the use of antimicrobials in medicine and agriculture. Therefore, there

is a sense of urgency for study of serotypes developing resistance and adaptive

mechanisms.

2.5   Resistance and Adaptations

       Environmental stress and sublethal injury during processing induces development

of a variety of adaptations in *Salmonella*. Several studies have investigated acid and

alkaline resistance/tolerance, growth at sub-optimal temperatures, thermotolerance

adaptations, and survival under desiccation. *Salmonella* has been observed to develop an

Acid Tolerance Response through the production of Acid Shock Proteins (ASPs) during

growth in log phase (Foster 2001). The sigma factor (σ^s) and *rpoS* gene have been found

to be responsible for this adaptation (Foster 2001). The 1965 study conducted by Liston

found *Salmonella* to be able to grow near refrigeration temperatures, affecting its

metabolism (Liston 1965). Increased heat resistance has been found to be a result of a

combination of factors, including nutrient deprivation, and acid/alkaline shock (Wesche

and Ryser 2013). Wesche and Ryser also observed that sublethal injury encourages

morphological changes which contribute to biofilm formation in manufacturing facilities

(Wesche and Ryser 2013). Upregulation in nutrient-uptake transporter genes *proU* and

osmU genes allow *Salmonella* to maintain osmotic balance to survive in low water

activity environments (Deng and others 2012; Finn and others 2013a,b). These

adaptations increase the likelihood of growth, spoilage, and development of bacterial

virulence factors (Wesche and Ryser 2013). Thus, there are more opportunities for pathogenesis, particularly foodborne illness upon product consumption.

2.6   Sources of *Salmonella*

Salmonella is typically associated with consumption of contaminated poultry, beef, pork, eggs, milk, seafood, nut products, and fresh produce (Foley and Lynne 2008). Recent outbreaks in food products include Cucumbers (*S.* Newport, 2014), Foster Farms Chicken (*S.* Heidelberg, 2013), Cantaloupe (*S.* Typhimirium and Newport, 2012), Turkish pine nuts (*S.* Enteritidis, 2011), and peanut butter (*Salmonella* Typhimurium, 2009) (CDC 2016e). According to FoodNet/Foodborne Diseases Active Surveillance Network, the top 10 serotypes of culture-confirmed *Salmonella* infections include: Enteritidis, Typhimurium, Newport, Javiana, I 4, [5], 12:|:-, Heidelberg, Saintpaul, Infantis, Muenchen, and Oranienburg (CDC 2014). It has been reported that pathogens such as *Salmonella* and *Clostridium perfringens* are likely to be found in raw meat prior to processing, as well as in fermented sausages with high moisture (Jenson and others 2014). International trade records have demonstrated that *Salmonella* and Shiga toxigenic *Escherichia coli* (STEC, VTEC, EHEC) are of most importance (Jenson and others 2014). Primary sources of contamination from the animal include the gastrointestinal tract and hide, thus requiring careful separation from carcass meat (Jenson and others 2014). The hide has specifically been found to be the major source of carcass contamination, in which bacteria are introduced onto the carcass through initial cuts and hide removal(Arthur and others 2010). While animals are natural reservoirs for *Salmonella*, contamination can also occur through and by other means during processing. Due to demand for product, production pressures result in reducing cooking times,

extending production runs, and reducing cleaning frequency, which potentially compromises food safety (Bell and Kyriakides 2002). For RTE meat products, controls and processing validations are essential to mitigate or eliminate cross contamination.

2.7   Current Methods for Mitigation of *Salmonella* and Meat-Inherent pathogens

Supporting systems of Hazard Analysis Critical Control Point System (HACCP) include sanitation standard operating procedures (SSOPs), good manufacturing practices (GMPs), and a "zero-tolerance" policy for visible contamination of feces and ingesta (Jenson and others 2014). For the success of HACCP, pre-requisite programs such as SSOPs and GMPs must be practiced and followed by personnel in the manufacturing facility. A well-trained workforce is required for the success of all risk interventions and controls.

To address cross-contamination along with sources of *Salmonella* and other pathogens, a variety of interventions and controls are integrated into the HACCP plan. On the personnel side, gloves and better hand washing practices reduce contamination from the hands. Cross-contamination from personnel and equipment must be closely monitored, incorporating careful practices of separating carcass meat from the hide and gastrointestinal tract. Pre-slaughter interventions such as stress minimization during transport (specifically for cattle), sourcing clean cattle, and management of hide contamination can minimize this introduction of bacteria during initial incising cuts (Arthur and others 2010). De-hairing (pork and goat processing) and chemical decontamination are additional means to minimize microbial loads on hides (Carlson and others 2008). In-process unit interventions include a multitude of sanitizing systems for knives, methods for separation of carcass meat, and specific handling procedures for the

removal of the gastrointestinal tract and other internal organs (Jenson and others 2014). To minimize the growth of microorganisms transferred to the carcass, rapid temperature reduction and maintaining low temperatures during shelf-life are essential. Reduced temperatures and periodic cleaning in the cutting room can reduce growth during this step. Carcass chilling, especially initial chilling, further reduces the risk of pathogenic growth; the carcass surface temperature should be below 7°C (45°F), the minimum growth temperature for *Salmonella* and *E. coli* (Arthur and others 2010).

2.8   Regulatory Bodies in Meat Safety

The Food Safety and Inspection Service (FSIS) is an agency of the United States Department of Agriculture responsible for overseeing the safety and wholesome nature of meat, poultry, and egg products (USDA-FSIS 2016). One of the first federal consumer protection measures emerged in 1906 in response to Upton Sinclair's novel, *The Jungle*, exposing the unsanitary conditions in meat-packing establishments. The Federal Meat Inspection Act (FMIA) established sanitary standards and inspection protocol for animal processors, including the requirement of continuous presence of government inspectors at all meat-manufacturing establishments (USDA-FSIS 2016). This could be a very early form of validation of meat safety during this era. In the early 1900s, methods relied heavily on organoleptic means, using sight, touch, and smell to prevent meat from diseased animals to enter the food supply. As processing technologies advanced into the late 1950s, there was a growing demand to keep pace with the industrialized meat processing methods. A study in 1976 by consulting firm Booz, Allen, and Hamilton recommended a delegation of inspection responsibilities from the inspectors to the establishment. Inspectors were now responsible for verification of meat safety (USDA-

FSIS 2016). Microbiological criteria for finished product was also recommended. These recommendations were deemed radical at the time by consumer groups and FSIS, resulting in follow-through on only a few of the proposals (USDA-FSIS 2016). However, change in the meat industry was expedited in the 1990s after a large outbreak of *E. coli* O157:H7 that resulted in 4 children deaths and hundreds sickened. This led to structural changes within FSIS and the establishment of the HACCP System in 1996. The role of FSIS was to reduce the risk of foodborne illness in meat and poultry products by identifying potential hazard points and implementing standards at each processing step to mitigate or eliminate the hazard of interest (USDA-FSIS 2016).

2.9   Hazard Analysis and Critical Control Points (HACCP)

Development of HACCP led improvement of food safety in production facilities by providing structure for integration of measures to reduce the risk of not only biological, but also chemical and physical hazards in food. The system also invokes accountability for production facilities to maintain acceptable levels of food safety during processing (USDA-FSIS 2016). The seven principles that comprise the HACCP system are: 1) Conduct a hazard analysis, 2) Critical control point identification (CCP), 3) Critical limits established for preventative measures for each CCP, 4) Establish CCP monitoring requirements/procedures for using monitoring results to adjust processes/maintain control, 5) Establish corrective actions at times of deviation from a critical limit, 6) Maintain record procedures for the HACCP plan, and 7) Establish verification procedures for the HACCP plan (USDA-FSIS 2016). For the meat and poultry industries, FSIS mandated the utilization of HACCP in all meat and poultry production facilities.

At the core of the seven principles of HACCP lies the concept of validation, verification, and monitoring or reassessment (CFR 2016a). Validation confirms that the HACCP plan is ensuring that both plan and measures are purposeful and meeting intentions, while verification ensures the plan is adequate, i.e. "working as intended." Overall process validation to ensure safety and quality of foods must be incorporated in the HACCP plan to ensure that it works correctly and fulfills its purpose. According to the National Advisory Committee on Microbiological Criteria for Foods (NACMCF), verification is defined as any activity, other than monitoring, that determines the validity of the HACCP plan and ensures that the HACCP system is operating according to the plan (Dickson 2013). It is also referred to as a set of methods, procedures, and tests to evaluate the HACCP system and determine if it is compliant with the plan (USDA-FSIS 2016). Thus, validation is an activity that supports verification activities, utilizing data from studies and technical information to determine if the HACCP plan is able to control the food safety hazards (Dickson 2013). To ensure that CCPs, process validations, and the HACCP plan is under control, monitoring the system through observations and measurements is critical. In the case of misalignment and deviations, corrective actions must be taken to regain control of the deviated CCP in order to ensure the quality and safety of the product. As stated by 9 CFR 417 - HACCP Systems, the HACCP plan must be incorporated for numerous types of products and associated processes, from slaughter to thermally processed and fully cooked products (CFR 2016a). Initial validation is critical to the integrity of the HACCP plan, testing critical limits, monitoring results, and establishing corrective actions.

2.10 Ready-To-Eat (RTE) Meats

Processes such as curing, comminuting, cooking, and drying are used to create RTE meats, which are readily available at grocery stores. Deli meats are included in this category. The convenience of RTE meats and the expectation that the consumers will minimally heat (if needed) these products makes it a high-risk product category for public health. As with many food products, there are chemical, physical, and microbiological hazards associated with RTE foods. Nitrite, a compound used in deli meats, is used to enhance color and prevent growth of *Clostridium botulinum* spores; the addition of nitrite is a CCP (Jenson and others 2014). The legal limit in the United States is not more than 200 ppm for sodium nitrite, and not more than 500 ppm for sodium nitrate in finished meat products (CFR 2016b). Physical hazards can include contamination from processing equipment such as cutting blades, while microbiological hazards encompass an array of cross-contamination points, from raw materials to retail stores (Jenson and others 2014). One of the most prominent pathogens found in RTE meat, *Listeria*, could enter food manufacturing facilities and thrive in a variety of ways, including entrance via raw materials and ingredients, from the environment, and from food manufacturing staff. As a psychrotroph, it thrives in the most difficult areas to clean in refrigerated environments, in-process systems, as well as non-food contact areas (Jenson and others 2014). These include direct handling by operators, and inadequate processing and refrigeration (Jenson and others 2014). Pathogens more likely to be found in raw meat include *Salmonella* and *C. perfringens* (Jenson and others 2014).

2.11   Thermal processing

Thermal processing includes different types of heat treatment, which controls microbial loads in foods. Heat is transferred to food via conduction, convection, or radiation to destroy microorganisms, extend shelf life, and to impart changes that improve food quality. Processes to extend shelf life include pasteurization, sterilization, and ultra-high temperature (UHT), and processes to improve food quality include blanching, cooking, baking, roasting, and frying (Sanguansri 2016). Thermal processing is a critical step in pathogen control in food processing and is typically a CCP in the HACCP plan for food safety management systems. Due to the critical nature of this unit operation, validation of thermal processes is important to obtain sufficient level of pathogen control without compromising product quality. A target pathogen, food spoilage organism, enzyme inactivation, or changes in food characteristics (texture, color, flavor) are used to determine the time-temperature combination required to ensure safe food (Sanguansri 2016). The main components of thermal processing include decimal reduction time (D-value), z-value, and lethality of a thermal process (F-value).

2.12   Decimal Reduction Time (D-value)

Decimal reduction time, referred to as the D-value, is defined as the heating time required for the 90% reduction of the microbial concentration at a constant temperature (Sanguansri 2016). Where, N is the number of surviving cells at time t (Lewis 2006), the logarithmic reduction model is as follows:

$$\log \frac{N}{N_0} = -\frac{t}{D}$$

Factors that affect the D-value include the type of microorganism, temperature, and medium/food matrix, which encompasses pH, redox potential, and composition). It is widely accepted that microbial thermal destruction, in general, follows first-order kinetics (Lewis 2006).

2.13   z-value

The temperature increment specific to a microorganism required for a 10-fold change in the rate of thermal destruction is called the z-value (Lewis 2006). The z-values also refer to the temperature coefficient of different food components, such as Vitamin C, to describe the temperature increment required for change or destruction. Microbiological safety and quality z-values typically differ by $10 - 20°C$, thus it is important to consider these values when developing an optimal thermal process (Tucker and Featherstone 2011).

2.14 F-value: Lethality of Thermal Processes

The time required to achieve a given reduction ratio in the number of microorganisms at a given constant temperature is the F-value (Lewis 2006):

$$F = D \log\left(\frac{N_0}{N}\right)$$

In terms of thermal processing, if the F-value is calculated at 250°F or 121°C, it is known as the sterilization value for foods. This is used as an indicator to determine alternate processing temperatures without compromising safety of the products.

2.15 Guidelines and Validation for RTE meat

As part of HACCP, RTE meats must comply with guidelines set forth by the USDA-FSIS Appendix A (USDA-FSIS 1999). These guidelines provide time/temperature requirements to reduce specified microbial loads in RTE meats. The guidelines are recognized by FSIS as validated process schedules and are expected to have been conducted for each product, utilizing a time-temperature relationship to deliver a '*Listeria monocytogenes* 6D cook' or any other time-temperature combination which provides the same microbial destruction (USDA-FSIS 1999). This specific 6D cook encompasses other pathogens such as *Salmonella* and vegetative cells of *C. perfringens*, as these are more heat-labile than *Listeria* (Jenson and others 2014). For *Salmonella*, FSIS has implemented a minimum 6.5- $\log_{10}$ reduction in RTE beef products and a 7-$\log_{10}$ reduction for fully and partially cooked poultry products (USDA-FSIS 2006). In-plant validation is a key aspect of the HACCP plans to ensure processing plants are not compromising safety of processed foods. For validation of thermal processing of RTE

meat plants, probes and data loggers are used to record temperature data as secondary

proof for adequate processing of foods and maintaining documentation. Each batch cook

is verified to confirm that the specified time-temperature combination is being applied to

the product.

2.16   Thermal Process Validation: Surrogates, Indicators, and Markers

The concept of a surrogate organism – a non-pathogenic substitute marker for the

pathogen or organism of interest – stems from the concept of an indicator organism

(Sinclair and others 2012). The difference is that an indicator organism is naturally

occurring, while a surrogate is introduced into the system as an inoculum (Busta and

others 2003). The indicator concept was developed in the 1800s, when quality and

treatment of water was a large issue at hand (Medema and Payment 2003). The 1800s

brought about an era of discovery with the paralleling of recognition of the scientific field

of bacteriology and seeking ways to improve water quality and treatment (Medema and

Payment 2003). At this point in history, it was understood that the pathogens in water

were from fecal sources, and the levels of contamination led to the development of

practices to measure fecal contamination levels using an indicator concept (Medema and

Payment 2003). Early practices included the use of slow sand filtration to reduce the

bacteria in water by greater than 90% to below 100 bacteria/ml (Medema and Payment

2003). Additional studies of fecal microorganisms in conjunction with these findings led

to the concept of the indicator organism, specifically using the category of *Escherichia*

coli to indicate fecal pollution. *E. coli* was most appropriate for this application, as it is

found in the feces of warm-blooded animals, therefore including human vectors, and

demonstrates predominance over other thermotolerant coliforms in human and animal

excreta (Dufour 1977). The application and interpretation of *E. coli* levels in water were presence-driven, in that, when *E. coli* was absent, the pathogens were absent (Medema and Payment 2003). This specific application and interpretation is now defined by the term 'index organism.' The current interpretation of the presence of indicator organisms is more specifically related to the application of processing or treatment, in which its presence represents a failure of Good Manufacturing Practices (GMPs). Thus, the current and most appropriate use of the term 'indicator organism' is in conjunction with the process or treatment evaluated, including process indicator or disinfection indicator (Medema and Payment 2003). Used within the HACCP plan, index organisms, indicators, and surrogates provide valuable insight into the treatments and processing of products, when applied appropriately. However, the limitations of indicator organisms, such as their presence in very low numbers and uneven distribution in foods, makes it difficult to rely on indicator organisms to validate a process when trying to demonstrate high levels of log reductions following a process.

2.17 Surrogates vs. Indicator Organisms

Selection of surrogate microorganisms to validate a process requires consideration of many factors. The first step in surrogate selection is determining the pathogen of interest, which requires information about previous outbreaks, isolation of pathogens from the product, survival characteristics and environmental adaptations including effects of processing on survival (Busta and others 2003; National Advisory Committee on Microbiological Criteria for Foods 2010).

Specific criteria should be considered when selecting surrogates and have been outlined by Busta and others (2003) and the National Advisory Committee on Microbiological Criteria for Foods. These criteria are as follows:

- Nonpathogenic: No risk to safety nor health in processing facility

- Predict lethality of target pathogen using inactivation characteristics and kinetics

- Similar behavior to target microorganism: Susceptible to injury under processing parameters similar to target pathogen, varying by food product (thermal death time values, D- and z- values)

- Growth, populations: Genetically stable, stable and consistent growth for reproducibility; yield highly dense populations that remain constant until utilization

- Detection: Easy to differentiate and enumerate, rapid enumeration, cost effective detection methods

- Attachment to product: Similar to target

- Does not become a spoilage organism on processing equipment

Surrogates organisms differ from index and indicator organisms in that they are introduced into a system as an inoculum. In the context of industry processing validations, it is essential that the surrogates selected are not pathogenic, since introducing pathogens to processing facilities is not advised. The non-pathogenic nature of surrogate organisms is a primary benefit to using them for processing validations. An additional benefit of surrogate organisms is that they can be used in "worst case" processing conditions studies in both lab and processing facility environments (Anderson and Lucore 2012). It is typically advisable that when using surrogate organisms for in-

plant validation, the products in which the surrogate organisms are introduced are further processed and cooked to render them safe and prevent any public health issues.

Unlike surrogate organisms, indicator organisms are typically naturally occurring in the product undergoing processing. Typical applications for indicator organisms include validation and verification of Good Manufacturing Practices (GMPs), Good Agricultural Practices (GAPs), Sanitation Standard Operating Procedures (SSOPs), and other food safety and hygiene integrity systems (Busta and others 2003). Indicator organisms, as opposed to surrogate organisms, are best used in these programs, which are essential to mitigating the risk of pathogenic contamination. Additionally, inadequate processing for safety is marked by the presence of indicators, providing evidence of failure to comply with the aforementioned programs (Busta and others 2003). It is expected that impacts and effects on the concentration of the indicator organisms will directly correlate to the target organism. The absence or low concentration of an indicator organisms demonstrates that the product was not exposed to circumstances that would risk contamination by the target pathogens (Busta and others 2003). Utilizing indicator organisms mandates dependence on the presence of the indicator organism in the food when the target pathogen may be present, and absent when the target is absent or eliminated after processing (Busta and others 2003).

While indicators can be used for thermal processing validation, they provide more insight into process control attributes, such as GMPs and good product handling practice programs (Marshall and others 2005). Given the limitations of indicator organisms, surrogate organisms can provide a different type of control to processors and food safety overseers specifically for validation purposes, as it relies on a known inoculum level and

its relation to the target pathogen. Specifically, the most appropriate use for surrogates is to analyze effects and responses of processing conditions (Busta and others 2003).

2.18 Surrogate Use

Surrogate organisms are not a new concept, but their use has been more recently explored to address food safety needs as detection methods improve. Historically, surrogates have been used in the canning industry to validate the destruction of *Clostridium botulinum* spores in low acid foods, specifically *C. botulinum* include *C. sporogenes* and *Bacillus stearothermophilus* (Busta and others 2003). Produce processing, though it does not contain a kill step, utilizes surrogates to evaluate cleaning, sanitation, and disinfection of equipment, as they provide an added process control advantage (Busta and others 2003). Additional foods using surrogate organisms include but not limited to: animal feed, poultry feed, almonds, dry roasted almonds, ground and formed beef jerky, whole muscle turkey jerky, and fresh meat (Marshall and others 2005; Anderson and Lucore 2012). Indicators or surrogates used in validation studies include: mesophilic aerobic bacteria (total plate count), coliforms, *E. coli* Biotype I/II, *Enteroccocus faecium*, *Pediococcus* spp., and lactic acid bacteria (Dickson 2013). Many surrogates are process-specific, thus research into the applications of the surrogates is essential to conduct a representative study and validation (Dickson 2013). Surrogates are not limited to processing efficacy evaluation; they can also be used for fermentation, freezing, and refrigerated storage studies (Keeling and others 2009).

2.19 Non-Pathogenic *E. coli* Surrogates

Research conducted by Marshall and others (2005) identified 113 isolates of *E. coli* from cattle for use as indicator organisms for *E. coli* O157:H7. The organisms were

characterized through combinations of selected microbial intervention treatments to evaluate temperature sensitivity and thermal death times. Out of the 113 isolates, five *E. coli* indicator isolates were validated for integrity as verification of current microbial intervention practices used in the meat industry. Four of the five isolated were found to be more versatile indicators of *E. coli* O157:H7 reduction on beef carcass tissue across seven different treatment conditions (Marshall and others 2005). Only one isolate was found to be significantly different for more than one treatment. Thus, these findings demonstrate that no single isolate can thoroughly represent microbial intervention efficacy, and the use of a cocktail of surrogates for validation provides an additional margin of safety.

Many studies have been conducted using non-pathogenic *E. coli* surrogates in the meat industry to evaluate a variety of processing and storage conditions for validation purposes. These include antimicrobial treatments, cold storage (freezing and refrigeration), fermentation, cooking, and inoculated pack studies (Niebuhr and others 2008; Keeling and others 2009; Dickson 2013). The five strains (American Type Culture Collection (ATCC) 1427, 1428, 1429, 1430, 1431) identified by Marshall and others were found to be representative of *E. coli* 0157:H7 and *Salmonella*e in meat products (Dickson 2013). Additionally, FSIS permits them for in-plant studies (Dickson 2013). These surrogates are all non-pathogenic and isolated from cattle hides with the objective to determine similarity to *E. coli* 0157:H7 (Marshall and others 2005). In terms of acid and heat tolerance, these 5 surrogates were found to be most similar to *E. coli* 0157:H7 (Marshall and others 2005). A study conducted by Keeling and others (2009) found that these five surrogates provide a margin of safety due to greater survival demonstrated by

higher populations in cooking, fermentation, freezing, and refrigerated storage for meat (Keeling and others 2009). For refrigerated and frozen storage, the surrogates did not exemplify statistically lower populations compared to *E. coli* 0157:H7 (Keeling and others 2009). A study on fermentation showed that 4 of the 5 surrogates tested had less reduction than *E. coli* 0157:H7. For thermal processing across temperatures of 60, 65, and 71°C (140, 149, and 160°F), all surrogates had higher D-values than the pathogenic strains, demonstrating more thermal resistance as compared to *E. coli* 0157:H7, but were not statistically different from the D-values of the pathogen (Keeling and others 2009). Despite statistical differences among individual surrogates as compared to *E. coli* 0157:H7, overall, they were found to be equivalent, conservative, or marginal to population reductions of the pathogen. Definitions of the aforementioned evaluations include equivalent as no statistical difference, conservative as demonstrating a lower population reduction or greater D-value than *E. coli* 0157: H7, and marginal as demonstrating a higher population reduction or lesser D-value as compared to *E. coli* 0157:H7 (Keeling and others 2009). In the context of thermal inactivation, all surrogates across all temperatures in the study were found equivalent or conservative to *E. coli* 0157: H7. Thus, the strains in combination would be useful and representative of the target organism in thermal processing validations.

A study conducted by Niebuhr and others (2008) focused on these same organisms as surrogates for five strains of *Salmonella enterica* for antimicrobial treatments, fermentation, freezing, and refrigerated storage of meat. Antimicrobial treatments demonstrated population reduction specific to the surrogate as compared to the *S. enterica* cocktail based on the specific treatment (specific spray washes and tissue

types). Similar to Keeling (2009), fermentation resulted in 4 of the 5 surrogates having lower reductions than that of the target pathogen, in this case, *S. enterica*, therefore providing a margin of safety (Niebuhr and others 2008; Keeling and others 2009). It was found that these same surrogates could be used for specific process evaluations and validations for *Salmonella* (Niebuhr and others 2008). Freezing and refrigerated storage demonstrated greater and equivalent surrogate survival compared to *S. enterica* (Niebuhr and others 2008).

The results of previously conducted studies by Keeling, and Niebuhr have looked at specific treatments to meat products to evaluate characteristics of *E. coli* surrogates as compared to target pathogens. Of the criteria provided by NACMCF, these specific studies addressed criteria requiring in-depth research, specifically evaluating inactivation characteristics and kinetics, and comparing behavior and responses of the surrogates to the target microorganism when subjected to processing parameters; thermal death time values, D-values, and z-values (Busta and others 2003; Dickson 2013). There is limited information and studies on the potential use of these *E. coli* surrogates to validate thermal processing targeting *Salmonella* for RTE meat products. To develop and establish food safety guidelines in accordance with Appendix A, research needs to demonstrate similarities between the *E. coli* isolates and *Salmonella*e for thermal lethality processes.

CHAPTER 3. THERMAL PROCESS LETHALITY IN GROUND BEEF FOR NON-PATHOGENIC *ESCHERICHIA COLI* AS A SURROGATE FOR *SALMONELLA*

3.1 Abstract

Food processors are seeking more technologically advanced ways to ensure food safety. In 1999, the Food Safety Inspection Service under the United States Department of Agriculture (USDA-FSIS) developed Appendix A, compliance guidelines for thermal lethality, specifically 6.5 and 7.0-$\log_{10}$ reductions of *Salmonella* for meat and poultry products. As pathogen detection in the food industry continues to improve, processors have more information available to ensure integrity and food safety in processing parameters. Five non-pathogenic *E. coli* isolates were identified by Marshall and others (2005) as potential indicators or surrogate organisms for *E. coli* O157:H7 in beef, and Niebuhr and others (2007) found the five *E. coli* isolates to have similar behavior of *Salmonella* for non-thermal microbial interventions. To continue to improve food safety in the meat industry, especially in ready-to-eat (RTE) products, the five *E. coli* isolates were evaluated for use as a surrogate for *Salmonella* in ground beef under thermal processing conditions. Ground beef at five fat content levels across nine temperatures from Appendix A was inoculated with either non-pathogenic *E. coli* inoculum or a cocktail of *Salmonella* and heated in a water bath at specific temperatures. Each fat/temperature combination for each inoculum was enumerated to determine the decimal-reduction values (D-values). The D-values of *E. coli* and *Salmonella* were compared to determine if significant differences existed, indicating whether the non-pathogenic *E. coli* isolates could be used as a surrogate for *Salmonella* in compliance

with Appendix A. As expected, D-values for both inoculums decreased as temperature increased. Across fat content, significant differences ($P < 0.05$) existed at the lowest four temperatures (54, 57, 60, and 63°C (130, 135, 140, and 145°F)) but in general, temperatures above 63°C (145°F) demonstrated no significant differences ($P > 0.05$). At these temperatures, both *E.coli* and *Salmonella* were inactivated rapidly with similar D-values. At the lower temperatures, *E. coli* D-values were consistently higher than those of *Salmonella*, which provides a margin of safety if they were to be used as surrogates for *Salmonella.* The *E. coli* surrogates have potential to become a technology for thermal processing validations in combination with Hazard Analysis Critical Control Point (HACCP) plans to ensure the safety of meat products.

3.2   Introduction

About 11% out of 3.6 million cases of foodborne illness annually are caused by pathogenic, non-typhoidal *Salmonella* (Scallan and others 2011) About 35% of hospitalizations and 28% of deaths have been the result of *Salmonella*, making it the leading illness-causing pathogen. FoodNet, under the Center for Disease Prevention and Control (CDC), has estimated there are 15.3 cases per 100,000 individuals of *Salmonella*-related foodborne illness in the United States, according to the FoodNet 2014 Annual Foodborne Illness Surveillance Report (CDC 2016a). Aligned with objectives from the Healthy People 2020 initiative, the 2015 Food Safety Report has concluded that there has been no change in the incidence of culture-confirmed infections since the 2006-2008 baseline studies and objectives to decrease the incidence rate to 11.4 cases per 100,000 individuals (ODPHP 2016). Based on this recent report, *Salmonella* remains as the most

frequent cause of infection, along with *Campylobacter*, due to its complexity of many sources varying by many serotypes.

Salmonella has been found in meat, poultry, eggs, milk, seafood, and a contaminant of fresh produce and processed foods containing contaminated ingredients (CDC 2016c). Salmonellosis symptoms include primarily mild to severe diarrhea (acute gastroenteritis), abdominal cramps, fever, as well as nausea, vomiting, and headache (CDC 2015b). Invasive Salmonellosis infections can result in bacteremia, meningitis, osteomyelitis, and septic arthritis, and most commonly occur in people who are very young or old, or have compromised immune systems. This has resulted in nearly $3.7 million for total cost of the *Salmonella* infections annually, accounting for medical and productivity costs (USDA 2014).

Improved food safety and process controls has been the result of implementing Hazard Analysis Critical Control Point (HACCP) programs in food production facilities. The core principles of HACCP include routine validation, verification, and monitoring of processing systems to ensure and improve food safety. As foodborne pathogen detection technologies continue to improve, validation and verification methods have also improved, becoming more preventative in nature. Using non-pathogenic bacteria as surrogate microorganisms for pathogens has provided an opportunity to validate thermal processing parameters, specifically for meat and poultry products.

The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) established lethality standards for *Salmonella* in partial and fully cooked beef and poultry products in 1999 in Appendix A. The standards require a minimum 6.5 log_{10} reduction for beef and 7.0 log_{10} reduction for ready-to-eat (RTE) poultry (USDA-

FSIS 1999). Previous research has identified five non-pathogenic strains of *Escherichia coli* (Table 1) that has responded to meat processing antimicrobial interventions similar to *E. coli* 0157:H7 (Marshall and others 2005). An additional study investigated the use of the five strains individually as compared to *Salmonella enterica* for non-thermal interventions, including antimicrobial treatments, cold storage, and fermentation in meat with results suggesting potential for use in meat process validations for *Salmonella* reduction individually and collectively (Niebuhr and others 2007). Based on prior findings, it was reasonable to investigate the performance characteristics of the five strains under thermal processing as compared to *Salmonella*. Thus, to ensure compliance with Appendix A, non-pathogenic surrogate organisms present an opportunity to validate thermal processing without compromising food safety at a processing facility. The objective of this study was to compare the performance characteristics of the five non-pathogenic *E. coli* isolates to a mixed culture of *Salmonella* at varying fat contents of ground beef at different lethality temperatures to determine if the *E. coli* isolates could be used as surrogates to validate thermal processing parameters.

3.3   Materials and Methods

Salmonella isolates were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and non-pathogenic *E. coli* surrogates were obtained from Iowa State University. Isolates were stored at -80°C on sterile glass beads in cryotubes containing 20% glycerol. Tryptic Soy Agar (TSA; Neogen Corp., Acumedia, Lansing, MI, USA) slants were made for each of the five surrogates and five *Salmonella* strains.

3.3.1   Non-pathogenic *E. coli* Surrogates

Table 1 contains the reference and ATCC accession numbers according to Marshall and others (2005) for the non-pathogenic *E. coli* surrogates. The five strains were originally isolated from cattle hides to be used as indicator organisms for *E. coli* O157:H7 (Marshall and others 2005).

3.3.2   *Salmonella* Isolates

The *Salmonella* isolate reference information can be found in Table 2. The cocktail was composed of five different strains: *S.* Enteritidis, *S.* Typhimurium, *S.* Heidelberg, *S.* Newport, and *S.* Choleraesuis. Four of the five strains (*S.* Enteritidis, *S.* Typhimurium, *S.* Heidelberg, *S.* Newport) are the most common serovars responsible for cases in the United States. *S.* Choleraesuis is not commonly found in the United States, but it is predominant in Asia, and is typically associated with pork products (Foley and Lynne 2008; Morrow and Funk 2001).

3.3.3   Meat Preparation

Ground beef with 5, 10, 20, 25, and 30% fat content, typical fat contents that would be found in retail for ground beef, was used in the study. The ground beef was inoculated with non-pathogenic *E. coli* surrogates and *Salmonella* isolates separately to conduct the thermal tolerance experiments. Frozen ground beef in 1-pound chubs were adjusted to the correct fat contents (5, 10, 20, 25, and 30% fat) at and obtained from the Iowa State University Meats Laboratory. For three replicates per temperature per fat content, frozen chubs for each fat content was subdivided into 40g batches in sterile Whirl-Pak bags for inoculation. Excess meat was held in frozen storage at -20°C (-4°F).

3.3.4   Inoculum Preparation (Non-Pathogenic *E. coli* Surrogates and *Salmonella* Cocktail)

Starter cultures for both *E. coli* and *Salmonella* were sourced from the TSA slants. Individual tubes containing 10 ml of Tryptic Soy Broth (TSB; Neogen Corp., Acumedia, Lansing, MI, USA) were inoculated with one surrogate, and incubated at 37°C (98.6°F) for 18 – 24 hrs. From the 10 ml TSB test tubes, 100 µl of each surrogate was transferred into conical tubes (50 ml Nunc sterile propylene centrifuge tubes, Catalog No. 339653, ThermoFisher Scientific) containing 25 ml of TSB, then incubated at 37°C (98.6°F) for 18 – 24 hrs. After incubation, the five conical tubes were centrifuged at 4700 xG for 10 minutes at 4°C (40°F) to form a pellet (Sorvall Legend XTR, ThermoFisher Scientific, Asheville, NC). Excess TSB was removed, and the pellets were reconstituted with 10 ml of 0.1% peptone water (PW: Neogen Corp., Acumedia, Lansing, MI), then vortexed to create a homogenous mixture. Each conical tube containing one surrogate was dispensed into another 50 ml conical tube to combine the cultures to create the cocktail, and vortexed to mix. Target enumeration in the inoculum was 8 – 9 $\log_{10}$ CFU/ml.

3.3.5   Meat Inoculation and Preparation for Heating

Ground beef was inoculated with either *E. coli* surrogates or the *Salmonella* cocktail as separate bulk samples to achieve an inoculation level of 6 logs higher than background microflora levels. Target enumeration of the inoculum was 8 – 9 $\log_{10}$ CFU/ml. Prior to inoculation, a 2g sample of non-inoculated meat was placed in a sterile Whirl-Pak filter bag (Nasco Whirl-Pak, 18 oz./532ml flat wire, No. B01341) to serve as the negative control. The bag was sealed tightly to prevent cross contamination during experimentation. Additionally, a temperature reference bag for the thermocouple of the data logger (HH80AU, Thermocouple Dual Input Meter with USB and DC Power Jack,

Type K Thermocouple, Omega Engineering) was prepared using 2g of non-inoculated meat. Following this, 30g of remaining meat was weighed and inoculated with 5 ml of the non-pathogenic *E. coli* or *Salmonella* inoculum (as separate bulk samples) resulting in a final average concentration prior to heating of Ca. ~ 9 – 12 $\log_{10}$ CFU/g and ~ 8 – 11 $\log_{10}$ CFU/g, respectively. The inoculated bag was hand-massaged for 1 minute, and then subdivided into ten sterile bags (FisherBrand Sterile Sampling Bags 3 inch x 5 inch, No. 14955175, Fisher Scientific) containing 2g of inoculated meat. Each 2g sample bag was flattened to remove air to a thin layer (approximately 1 – 2 mm in thickness). The flattened bags were heat sealed (ULine Tabletop Poly Bag Sealer, 20", Model No. H-306), then placed in a cold refrigerator at 4°C (40°F) for 42 – 48 hrs to simulate potential industry storage conditions and ensure attachment to the meat.

3.3.6 Thermal Inactivation and Enumeration

For each fat content of the inoculated ground beef, the ten 2g sample bags were placed in a temperature controlled water bath (Thermoscientific AC-150 Haake, A25B – Haake Bath, Asheville, NC) for thermal inactivation. The water bath was stabilized at the following temperatures for the studies: 54°C (130°F), 57°C, (135°F), 60°C (140°F), 63°C (145°F), 66°C (150°F), 68°C (155°F), 71°C (160°F), 74°C (165°F), 77°C (170°F). The come-up time (the time required for the reference bag to come up to the stabilized water bath temperature) was recorded, and the first sample removed was assigned at time $t = 0$. Temperature data was measured using the HH80AU temperature data logger with the thermocouple secured inside the temperature reference bag. The data logger HH800SW software recorded the real-time temperature data. Bags were removed from the water

bath at predetermined time intervals (temperature dependent) and immediately placed in an ice-water bath.

After chilling the samples in the ice-water bath, samples were aseptically transferred into sterile Whirl-Pak filter bags with 10 ml of 0.1% PW and stomached (Seward, Stomacher 400 Circulator, Worthing, UK) for 1 minute at 260 rpm. The homogenized samples were then serially diluted in 0.1% PW, then enumerated by spread plating using the one-step thin agar layer (TAL) method (Kang and Fung 2000). Non-inoculated meat was plated as negative controls, while the inoculated and untreated meat samples for each inoculum were plated as positive controls. The inoculum was plated to ascertain the populations of the non-pathogenic *E. coli* or *Salmonella* cocktail that was used in the study. Non-pathogenic *E. coli* surrogate-inoculated samples were enumerated on Violet Red Bile Glucose Agar (VRBG; Neogen Corp., Acumedia, Lansing, MI) with a non-selective overlay of TSA. *Salmonella*-inoculated samples were enumerated on Xylose Lysine Deoxycholate (XLD; Neogen Corp., Acumedia, Lansing, MI) with a non-selective overlay layer of TSA. Plates were incubated at 37°C (98.6°F) for 18 – 24 hrs.

The TAL method for enumeration was used to resuscitate heat-injured cells, which may otherwise inactivate when applied directly onto selective media (Kang and Fung 2000). Thus, this method supports conservative estimates for bacterial enumeration, allowing for recovery of heat-injured bacteria that have the potential to cause disease upon human consumption.

3.3.7 Calculating D-values and z-values

Thermal death time curves with a correlation coefficient of (r^2) of 0.84 - 0.99 were used to determine D-values. Bacterial counts from two plates from each sample point were used to calculate the average bacterial concentration/g, and then converted to $\log_{10}$ CFU/g. The z-value was estimated by determining the linear regression of the $\log_{10}$ D-values against temperature (degrees Celsius), calculating the absolute value of the inverse of the slope. For the z-value calculation, temperatures 54 - 71°C (130 – 160°F) were used to determine the slope (in degrees Celsius), excluding tailing observed at temperatures greater than 71°C (160°F) to obtain the best fit line.

3.3.8 Statistical Analysis

Each temperature and fat content combination was independently replicated three times. Bacterial enumeration data was transformed to $\log_{10}$ CFU/g and analyzed via linear regression using Microsoft Excel 2016 software (Microsoft Corp., Redmond, Washington). The experiment was executed as a randomized complete block design with factor treatments of inoculum type (non-pathogenic *E.coli* or *Salmonella*), fat content, and temperature. Significance of the factors was set at ($P < 0.05$). Least-Squares Means was used for mean separation. The data was analyzed by analysis of variance (ANOVA) using SAS ® 9.3 (SAS Institute; Cary NC) to determine whether the non-pathogenic *E. coli* can be used as a surrogate for *Salmonella* across a range of temperatures and meat fat content to validate thermal processing parameters.

3.4   Results and Discussion

Appendix A mandates a 6.5-$\log_{10}$ reduction in RTE beef and a 7.0- $\log_{10}$

reduction in poultry for *Salmonella* for temperatures 54 - 74°C (130 - 165°F). The limit

of detection for this study was set at 30 CFU/g, which provides a margin of safety when

calculating D-values. As expected, increase in temperature resulted in decreasing D-

values for both the non-pathogenic *E. coli* surrogates and *Salmonella* disregarding fat

content (Figures 1 - 9). Based on the analysis of variance (ANOVA) and Least Squares

Means, significant differences between *E. coli* and *Salmonella* were determined within

each temperature and across each fat content. The D-values of *E. coli* surrogates were

observed to be higher than those for *Salmonella.* At temperatures 54, 57, 60, and 63°C

(130, 135, 140, and 145°F), *E. coli* and *Salmonella* were significantly different ($P < 0.05$)

across fat content of ground beef in this study (Figures 1 - 4). No significant differences

($P > 0.05$) existed between fat contents within each inoculum, apart from temperature

57°C (135°F). The difference of fat content at 30% at 57°C (135°F) could be attributed to

an enhanced protective effect that increases D-values, as observed in studies by Juneja

and Eblen (2001) and Ahmed (1995). This effect was only seen at 57°C (135°F) (Figure

2). Temperatures 66, 68, 71, 74, and 77°C (150, 155, 160, 165, and 170°F) in general

demonstrate no significant differences ($P > 0.05$) between the two inoculums across fat

content (Figures 5 – 9).

For either inoculum, no apparent pattern or relationship was observed between z-

value and fat content (Table 3). The z-values of *Salmonella* were larger than those of the

E. coli inoculum, meaning that a larger change in temperature is required to reduce the D-

value by 90% at all fat levels in comparison to the *E. coli* inoculum. The z-values paired

by fat content between *E. coli* and *Salmonella* differ significantly ($P < 0.05$), in which

Salmonella z-values were greater than *E. coli* z-values; a larger change in temperature is required to reduce the D-value by 90% at all fat levels in comparison to the *E. coli* inoculum.

In summary, significant differences ($P < 0.05$) in the D-value were observed between the two inoculums at lower temperatures (54, 57, 60, 63°C (130, 135, 140, and 145°F)) within each fat content. At temperatures greater than 63°C (145°F) for the D-value, no significant differences ($P > 0.05$) exist between the two inoculums. Paired by fat content, the z-values of *E. coli* and *Salmonella* differ significantly ($P < 0.05$); *Salmonella* z-values were greater than *E. coli* z-values. Thus, *Salmonella* demonstrated greater temperature stability than the non-pathogenic *E. coli*. Higher process lethality is achieved with a lower z-value as observed for the *E. coli* surrogates (less temperature stable than *Salmonella*); *Salmonella* D-values are consistently less than those observed for *E. coli*.

Larger D-values observed at lower temperatures 54 - 63°C (130 -145°F)) for both inoculums are consistent with the finding that bacteria grow most rapidly in the "danger zone," between temperatures 4 - 60°C (40 - 140°F) (USDA-FSIS 2013). For temperatures above the "danger zone," the only significant differences ($P < 0.05$) between inoculums were at 68 and 71°C (155 and 160°F) at 5% fat. The minimal amount fat content may have promoted more effective heating for the inoculums, since water and solid materials have a higher thermal conductivity than fat (Potter and Hotchkiss 1998). In general, the effect of fat level on D-values is not significant ($P > 0.05$) for both inoculums, with the exception of temperature 57°C (135°F) (Figure 2). Within temperature 57°C (135°F) the D-value for non-pathogenic *E. coli* at 30% fat is significantly different ($P < 0.05$). It is

unclear why this particular fat content and temperature combination demonstrated significant difference ($P < 0.05$), but it could be attributed to the summation of a few factors, including inevitable experimental variation and the composition of the 2g samples. Juneja and Eblen (2000) observed that increased fat resulted in poor heat penetration, which resulted in an increased D-value (Juneja and Eblen 2000). Ahmed (1995) and Juneja and others (2001b) cite the protective effect as a potential reason for increased D-values at higher fat content for their studies, since fat has a lower thermal conductivity than water (Ahmed and others 1995; Juneja and others 2001a; Ma and others 2007). Though no significant differences ($P > 0.05$) were detected in this study, *Salmonella* had the highest D-values at 30% fat, as compared to the mixed results of *E. coli.* While previous studies conducted by Ahmed (1995), Juneja and others (2001b), and Juneja and Eblen (2000) found that in general, higher fat levels had a protective effect on *Salmonella* spp. inoculum, other studies acknowledge deviations from this conclusion or did not observe this distinct pattern. The results in the present study are in agreement with Vasan and others (2014), in which no significant differences ($P > 0.05$) were observed across fat levels within each inoculum, potentially due to methodology or strain selection (Vasan and others 2014). For the present study, these same factors of strain selection, methodology, or meat composition could also have resulted in lack of significant differences. Orta-Ramirez and others (2005), and Mogollón and others (2005) offer explanations related to physical properties of the meat used as well as chemistry based on fat content that could influence D-values (Orta-Ramirez and others 2005; Mogollón and others 2009). Comparing ground beef to whole muscle, Orta-Ramirez found that the homogeneous mixture of fat and muscle in ground beef may have a diluting effect instead

of a protective effect, whereas whole muscle fat distribution is more defined and separated, allowing for more effective attachment (Orta-Ramirez and others 2005). Mogollón found that whole muscle beef had a greater thermal resistance than coarsely ground beef, and cites that the osmotic potential across whole muscle cells may influence thermal resistance to increase (Mogollón and others 2009). This is exemplified in ground beef or pureed beef, in which the mixture is more homogeneous, which may result in more free water for suspension of the inoculum (Mogollón and others 2009). Consequently, the thermal conductivity of water would increase the susceptibility of the inoculum to thermal inactivation, thus mitigating time differences to inactivate microorganisms between varying fat levels (Mogollón and others 2009).

There are no apparent patterns in the data between z-values and fat content (Table 3), and the z-values were found to be larger than those cited in other studies including Murphy and others (2004) and Juneja and Eblen (2000). Using ground beef as the heating medium, Murphy and others (2004) used a six-strain cocktail of *Salmonella* spp. resulting in a z-value of 5.74°C, while Juneja and others (2000) used an eight-strain cocktail of *S.* Typhimurium DT104 and found the highest z-value to be 8.08°C. Juneja and others (2001b) determined the z-value to be 9.11°C for an 8-strain cocktail in ground beef. A study of thermal inactivation of *Salmonella* in ground poultry at varying fat levels by Juneja and others (2001a) also found that there was no statistically significant effect of fat content on z-values (Juneja and others 2001b). Inconsistencies among published literature has been attributed to varying degrees of heat resistance due to serotype, product formulation/composition, and temperature range utilized to calculate the z-value (Juneja and Eblen 2000; Juneja and others 2001b; Murphy and others 2004). As cited in such

studies, it must be emphasized that it is inappropriate to directly compare z-values across studies, since z-values are dependent on a variety of factors, including meat composition, strain selection, cell physiology, and thermal lethality methodology (Juneja and others 2001b, Murphy and others 2004).

The aforementioned factors could also explain the lack of distinct patterns in this study for D-values. A sample size of 2g was used for each bag to achieve the thinnest layer possible, promoting equal heating of each individual sample in the water bath. Especially among higher fat contents, it is difficult to obtain a perfectly homogenous mixture of fat to tissue. Although air was removed by flattening the bag, it is inevitable that very small amounts of air could remain in the sample, permitting some variability. Fat has a lower thermal conductivity than water, and air has an even lower thermal conductivity than fat, thus the level of heat penetration efficacy may have led to variability (Potter and Hotchkiss 1998).

Additional experimental limitations include the rapidity of removal times for samples from the water. It was more difficult to remove each sample with very short time intervals before all bacteria were inactivated (1 – 5 seconds). For some temperatures above 63°C (145°F), the limit of detection (30 CFU/g) was used to determine the D-values to provide a margin of safety. For temperatures greater than 63°C(145°F), though no differences exist; the D-values for *E. coli* were consistently greater than the D-values for *Salmonella*. Beyond this temperature, the non-pathogenic *E. coli* inoculum offers no significant advantage, since it is inactivated as rapidly as *Salmonella*. However, inactivation of *Salmonella* can be ensured with the absence of the non-pathogenic *E. coli* at temperatures greater than 63°C (145°F). The 48-hr 4°C (40°F) incubation period could

have caused physiological changes to individual strains in both inoculum, including cold shock adaptations that modify growth dynamics in cold environments, which is well-known in *E.coli* spp. (Beales 2004). Another factor could be variation in individual thermal resistance, which was also observed by Juneja and others (2001), and could also impact thermal lethality in this study (Juneja and others 2001a).

Though direct comparison of the data to Appendix A is an oversight of several factors, the D-values of *E. coli* inoculum at 30% fat across temperatures were most similar to the 'worst case' product lethality compliance guidelines required for a 6.5 or 7.0-$\log_{10}$ reduction. Studies conducted by Juneja and Eblen (2000), and Juneja (2001) have supported the conclusion that higher fat content reduces thermal conductivity, therefore resulting in increased D-values to inactivate bacteria. In this study, 30% fat content has the most conservative (larger) D-values, which ensures a margin of safety if these values were to be used for beef products regardless of fat content in accordance to Appendix A. In 2005, an update of Appendix A provided a range of fat levels (1 – 12% fat) for poultry and their respective lethality time/temperature combinations for reducing *Salmonella*. Again, it would be inappropriate to utilize direct comparison of calculated log-reductions of the surrogates from this study to the lethality times outlined in Appendix A (for both beef and poultry with fat levels) due to factors such as meat composition and fat level that may affect D-values.

The guidelines in Appendix A are set for cooked beef, roast beef, and corned beef, disregarding fat content of each meat. These lethality time/temperature combinations are based on the most conservative estimates or "worst case" product to ensure inactivation of *Salmonella.* Based on this assumption, despite differences in the

meat type, the non-pathogenic *E. coli* inoculum could be used as a surrogate for *Salmonella*. D-values for *E. coli* are consistently higher and significantly different ($P <$ 0.05) than *Salmonella* up to temperature 63°C (145°F). As expected, D-values for both inoculums decrease with temperature increase. It was hypothesized that both *E. coli* and *Salmonella* would be almost immediately inactivated at temperatures greater than 63°C (145°F), thus mitigating differences in lethality times between the two inoculums. But because both inoculums are inactivated so rapidly at these higher temperatures, a higher lethality time is not necessarily required for the *E. coli* surrogates relative to *Salmonella*.

At lower temperatures across all fat contents (54, 57, 60, and 63°C (130, 135, 140, and 145°F)), *E. coli* D-values are at least two times greater than the *Salmonella* D-values, serving as an acceptable surrogate. At higher temperatures (> 63°C (145°F)), as long as the D-value of *E. coli* is equivalent or greater than that of *Salmonella*, *E. coli* can be used as a surrogate for *Salmonella*. Based on the criteria determined by Busta and others (2003) and the NACMCF, the non-pathogenic *E. coli* surrogates meet many of the requirements. In addition to being non-pathogenic, the surrogates demonstrated thermal inactivation behavior (D-values) similar to or greater than *Salmonella*, with larger D-values (more time required) for thermal processing, assuring that the inactivation of the *E. coli* surrogates ensures inactivation of *Salmonella*. The surrogates also demonstrate high density enumeration in comparison to *Salmonella* in the meat prior to t=0 (Ca. 9 – 12 $\log_{10}$ CFU/g and 8 – 11 $\log_{10}$ CFU/g, respectively), stability in growth upon preparation, and it is easy to differentiate from native flora in ground beef once plated (Busta and others 2003). Based on the criteria fulfilled, the *E. coli* surrogates provide a margin of safety to ensure the thermal inactivation of *Salmonella* in RTE ground beef.

However, further research is required to determine if the surrogates behave similarly in different cuts of meat and poultry, in addition to comparison of additional strains of pathogenic *Salmonella* to compare thermal lethality and resistance characteristics. Investigation of ingredient additions (seasonings, oil, etc.) would provide further insight regarding effects on thermal lethality for more complex RTE meat products. More research is needed to investigate the effects of additional, specific components of RTE meat products (oils, seasonings, additional ingredients, etc.) that may impact thermal lethality of *Salmonella* or other pathogens. Finally, a scaled-up process or pilot plant trial would be beneficial to identify additional factors, risks, and critical control points as realistic measures to consider for thermal process validation using the *E. coli* surrogates

Although the *E. coli* surrogates demonstrate the potential for thermal process validation of RTE beef products, its success is dependent on the HACCP plan in processing facilities. Appendix A compliance standards and any thermal processing validations can only be successful as long as the integrity of the HACCP plan is maintained.

3.5   Tables and Figures

Table 1. Non-pathogenic *E. coli* strains isolated from beef cattle.

E. coli Strains	ATCC Accession Number
P1	BAA-1427
P3	BAA-1428
P8	BAA-1429
P14	BAA-1430
P68	BAA-1431

Table 2. *Salmonella* isolates obtained from ATCC and Iowa State University.

Salmonella Strains	Source
S. Enteritidis	ATCC 4931
S. Typhimurium	ATCC 700720
S. Choleraesuis	ATCC 13312
S. Newport	ATCC 6962
S. Heidelberg	Iowa State University

Table 3. z-values for *Escherichia coli* surrogates and *Salmonella* for each fat content.

Fat Content (%)	*E. coli* surrogates z-value (°C)	*Salmonella* z-value (°C)	*E. coli* surrogates z-value (°F)	*Salmonella* z-value (°F)
5	6.74	10.3	15.2	25.4
10	7.07	9.90	15.1	25.1
20	6.9	10.5	17.3	28.9
25	7.36	12.4	17.7	35.8
30	6.9	10.4	16.8	29.2

Figure 1. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b**
Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1** or **2**
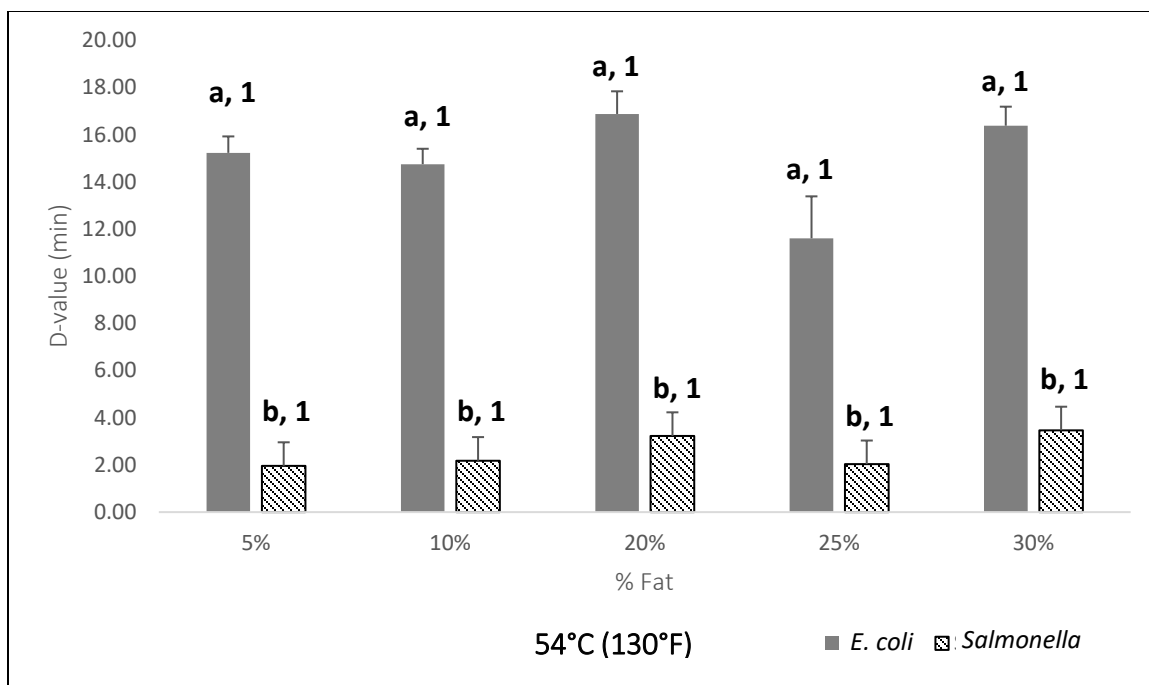
Figure 2. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b** Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1**, **2, or 3**

Figure 3. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b**
Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1** or **2**
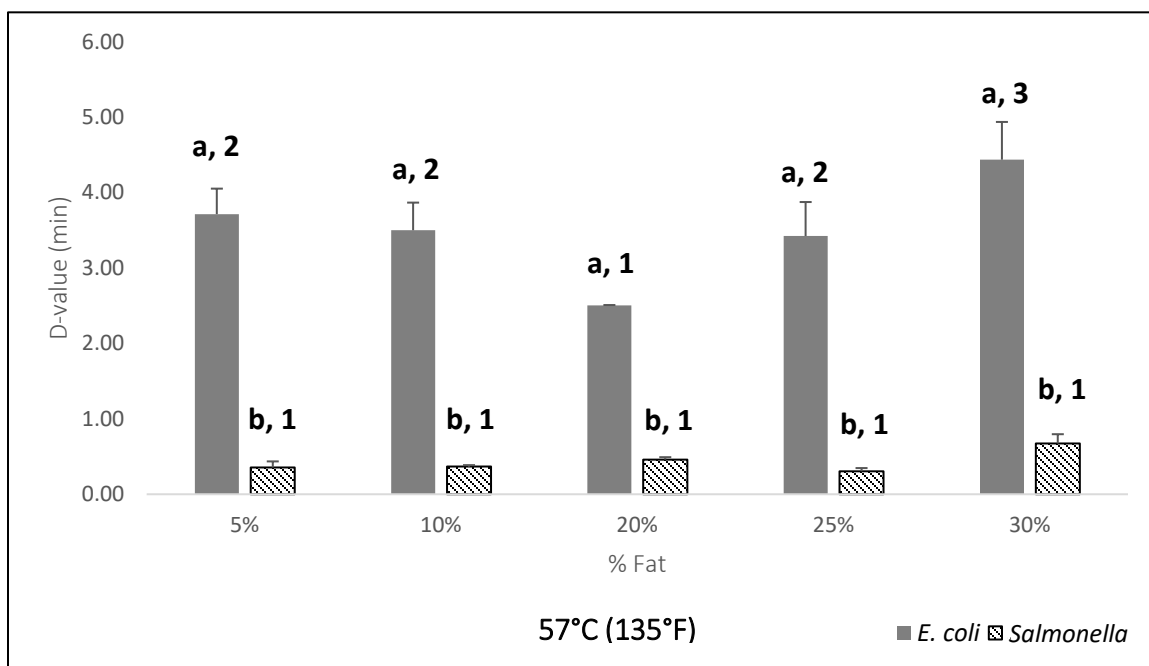
Figure 4. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b**
Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1** or **2**
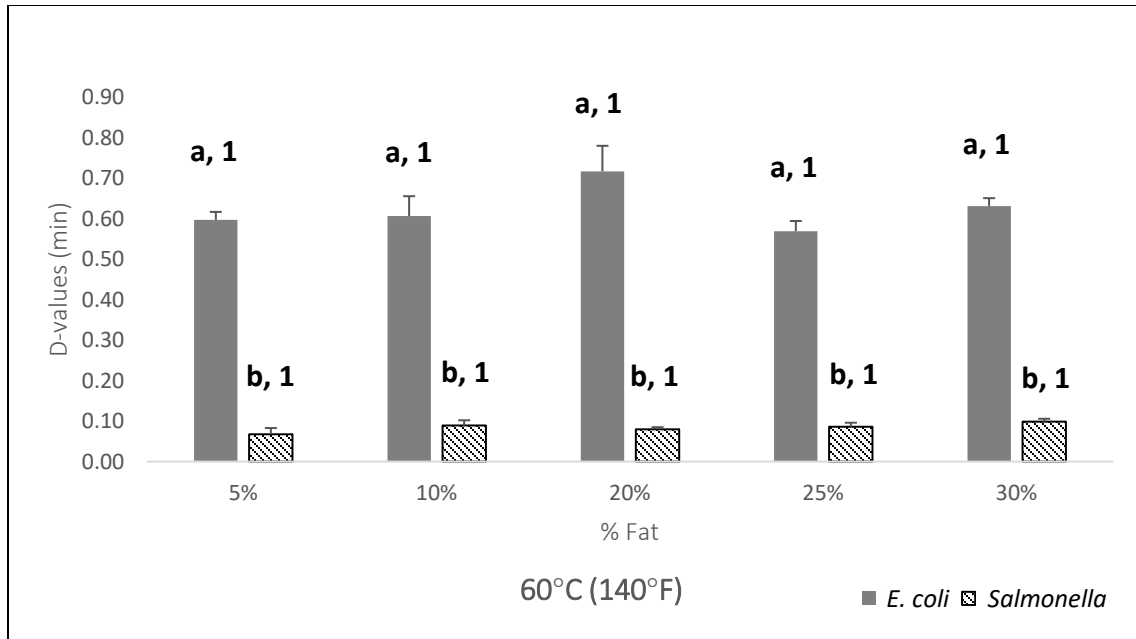
Figure 5. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b**
Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1** or **2**

Figure 6. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b**
Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1** or **2**
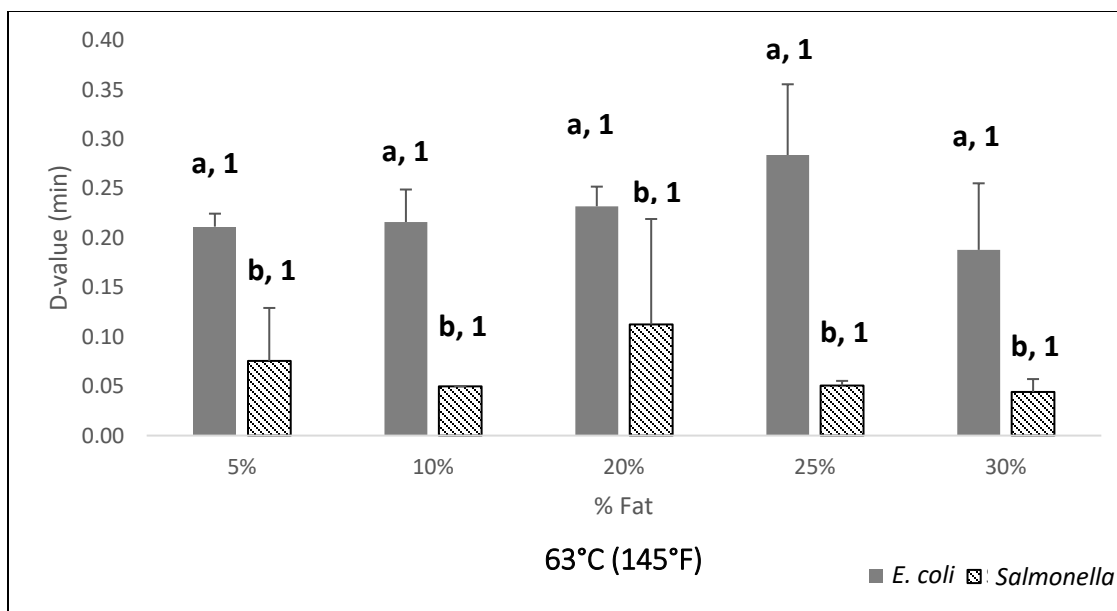
Figure 7. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b**
Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1** or **2**
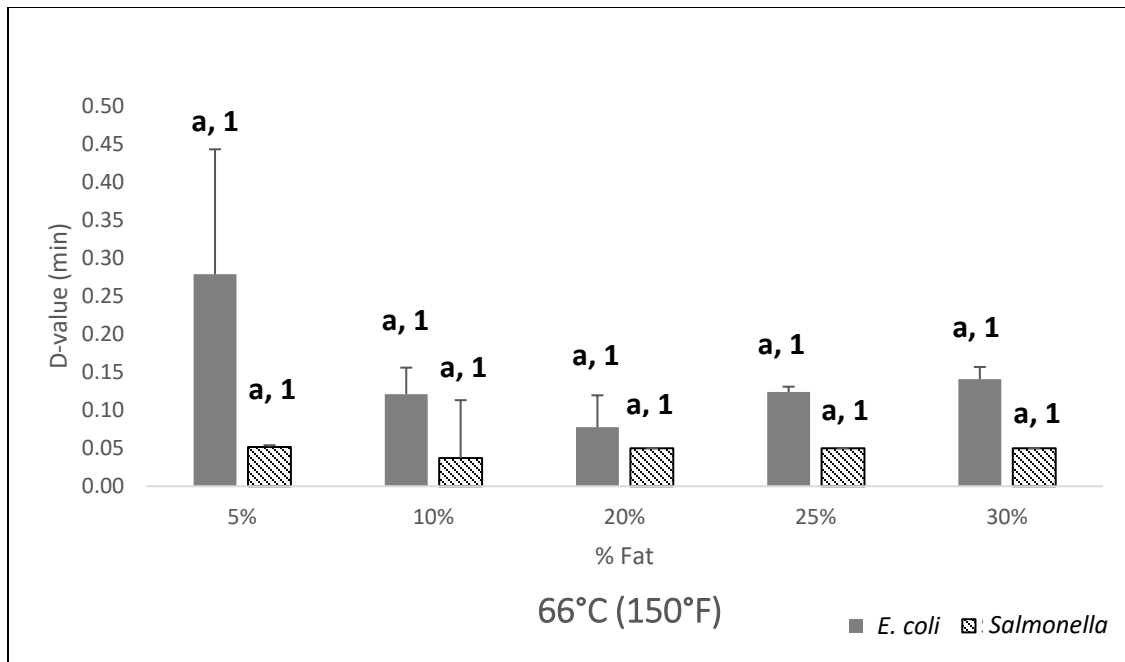
Figure 8. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b**
Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1** or **2**
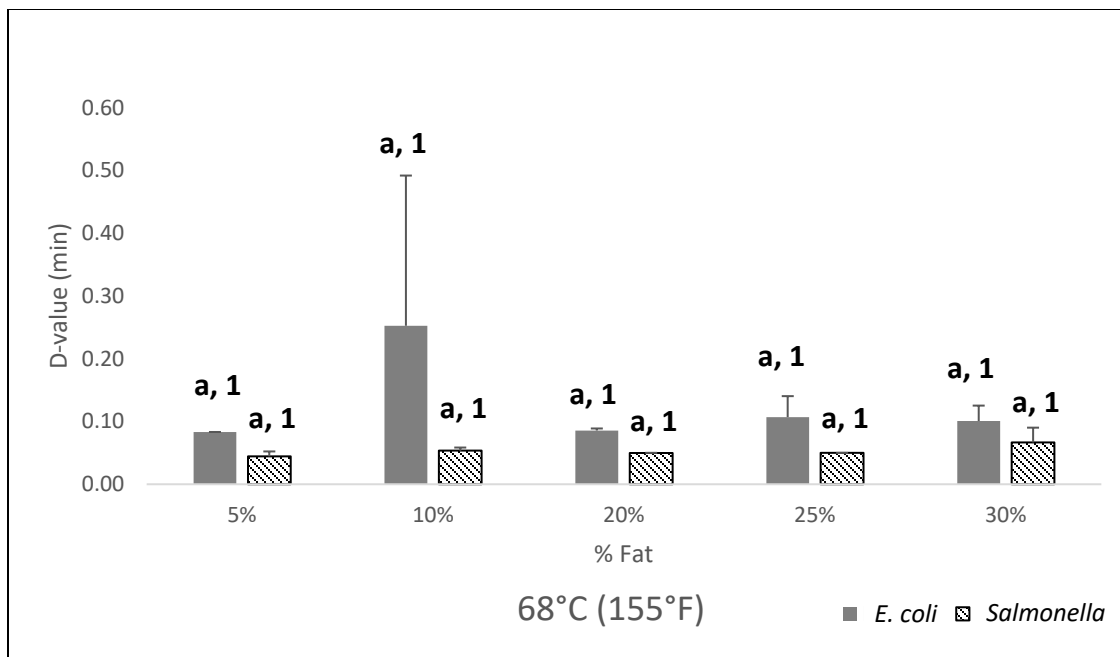
Figure 9. D-values (min±std. dev.) of *Escherichia coli* surrogates and *Salmonella* in ground beef with varying fat content.

Significant differences of *E. coli* or *Salmonella* at varying fat levels denoted by **a** or **b**
Significant differences of a given pathogen (*E. coli* or *Salmonella*) at different fat contents denoted by **1** or **2**
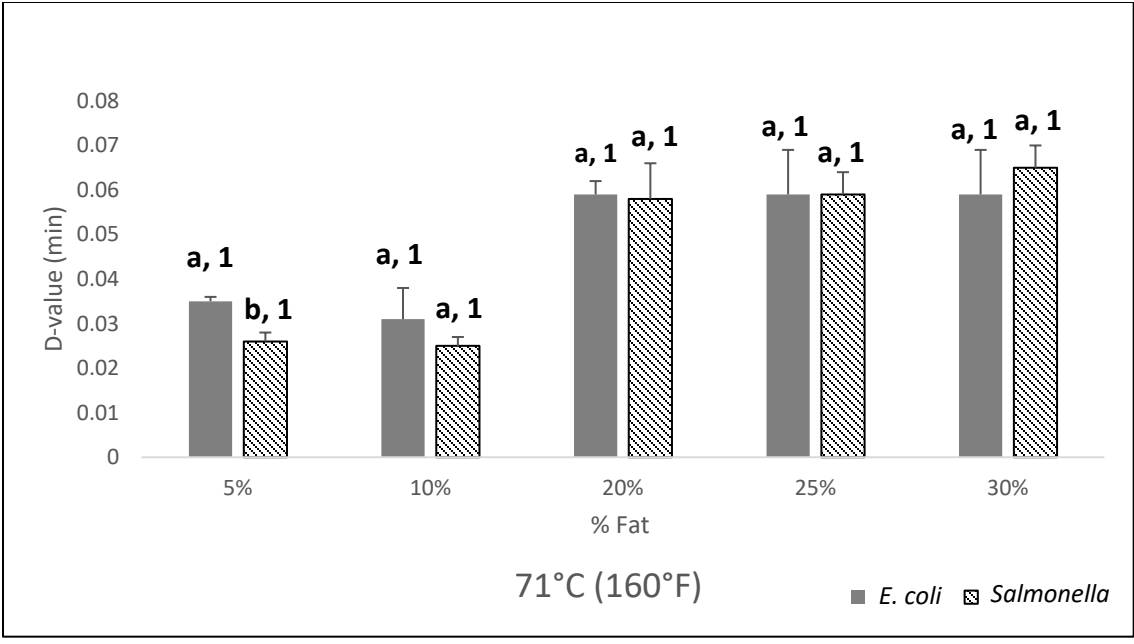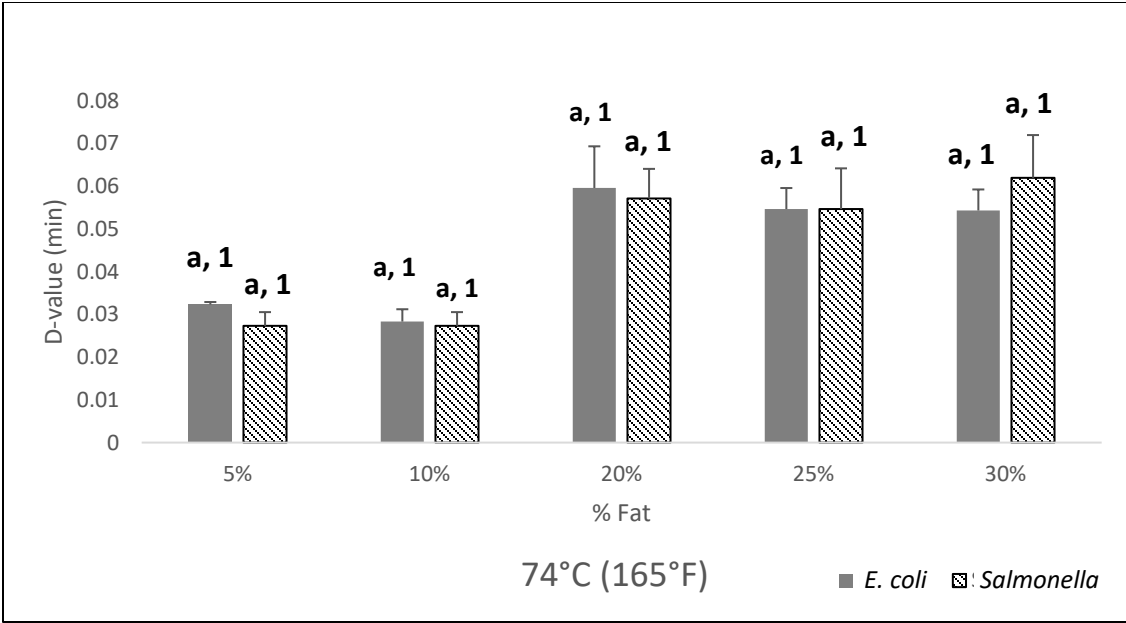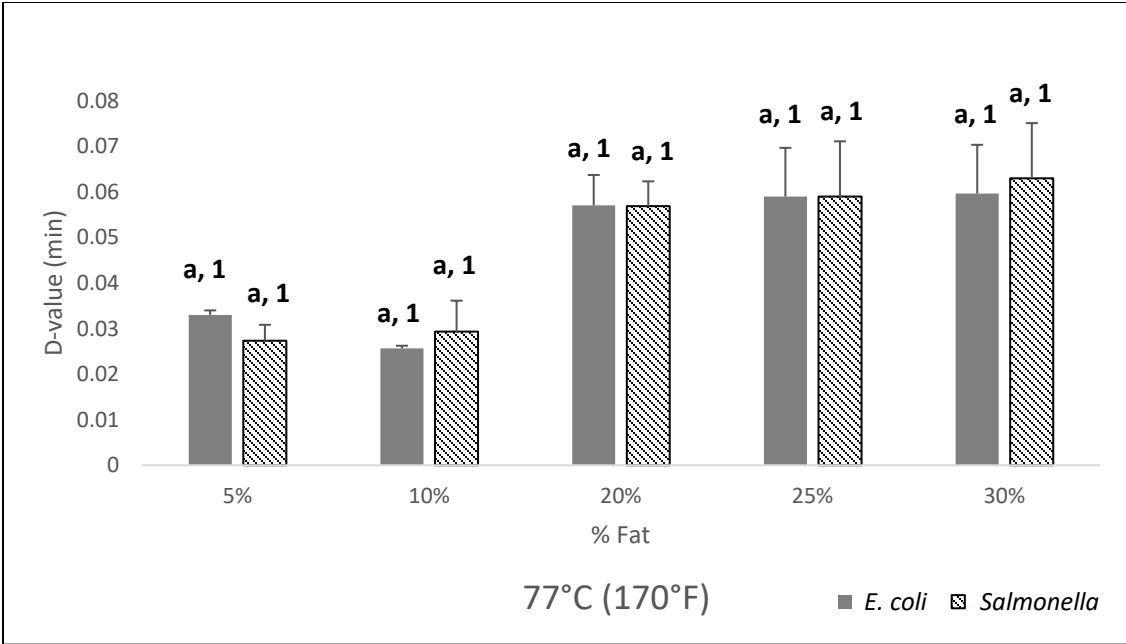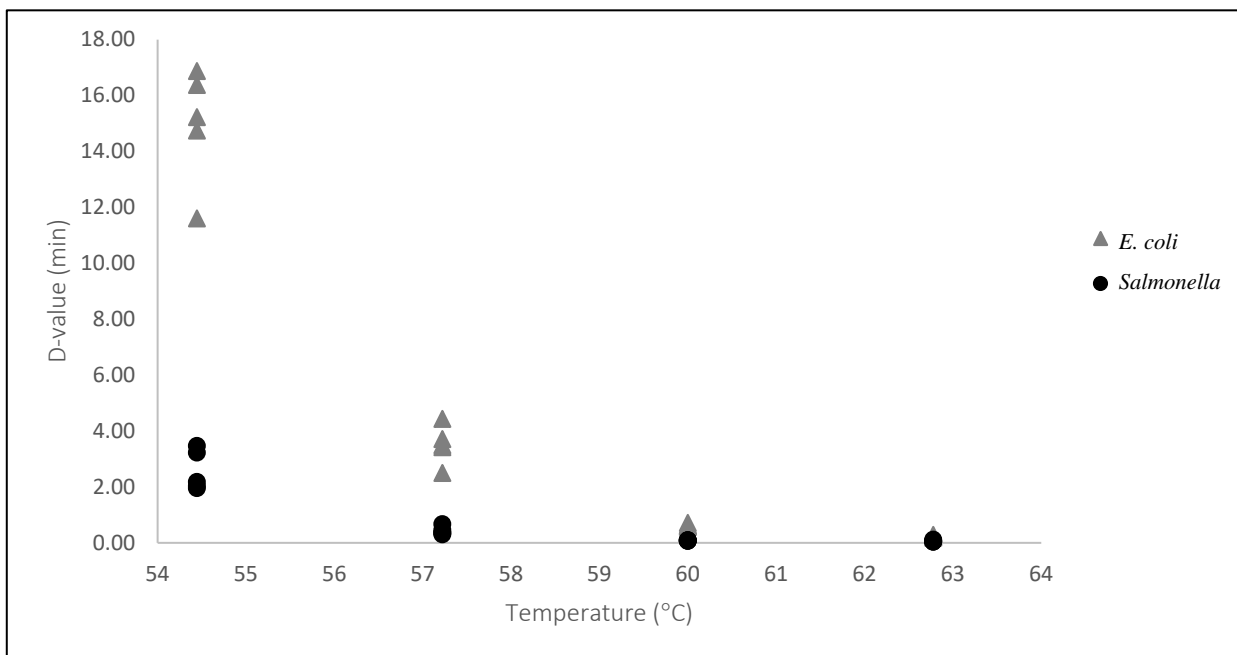
Figure 10. Average D-values of *Escherichia coli* surrogates and *Salmonella* for lower temperatures 54 - 63°C (130 – 145 °F).

CHAPTER 4.   OVERALL CONCLUSIONS

The work of this thesis provides an investigation of the thermal lethality of five non-pathogenic *E. coli* isolates to be used as surrogates for *Salmonella* based on the compliance guidelines outlined in Appendix A for RTE meat products (USDA-FSIS 1999). This thesis is among a few recent studies investigating the use of specific non-pathogenic isolates as surrogates for pathogen lethality to be used in thermal processing operations in industry. It specifically provides additional information about the five non-pathogenic *E. coli* isolates as related to Appendix A thermal lethality guidelines for meat and poultry to supplement other studies (Marshall and others 2005; Niebuhr and others 2008; Keeling and others 2009) investigating the characteristics and potential use of the isolates in the food industry.

Parameters used to develop guidelines for Appendix A were based on defined "worst case" raw product, using the highest levels of *Salmonella* in the data from their studies (USDA-FSIS 1998). No distinct patterns in the data were revealed in the present study in terms of fat within each temperature, apart from 30% fat, which generally required the most time for thermal inactivation. The study conducted by Vasan (2014) found no statistical difference in the effect of fat content on D-values, and though Juneja and Eblen (2000) determined significant difference of D-values with lag time based on fat content, some variation in D-values alone across fat was also observed (Juneja and Eblen 2000). Another factor that could have influenced the results is variation of thermal resistance of *Salmonella* strains selected for study, an aspect also observed also by Juneja and others (2001b). Comparing lethality D-values from this study directly to the time/temperature guidelines for 6.5 and 7.0-log$_{10}$ reductions in Appendix A, 30% fat had

the most similar time/temperature profile for lethality; the D-values in general were highest at 30% fat for both inoculums. Previous studies by Juneja (2001) and Ahmed (1995) also found that in general, higher levels of fat in meat had a protective effect at lower temperatures, therefore more time was required to inactivate pathogens (Ahmed and others 1995; Juneja and others 2001). The time/temperature data for 30% fat could be considered as the "worst case" raw product for thermal inactivation of the non-pathogenic *E. coli* inoculum.

The non-pathogenic *E. coli* was significantly different from *Salmonella* ($P <$ 0.05) and had consistently higher D-values than *Salmonella* for temperatures 54, 57, 60, and 63°C (130, 135, 140, and 145°F). Bacteria grow most rapidly in the temperature range of 4 - 60°C (40 - 140°F), which is considered the "danger zone," therefore, it is that more time is required to inactivate pathogens at cooking temperatures within this range (USDA-FSIS 2013). The data is consistent with the conclusions of bacterial growth behavior within the "danger zone" - lower temperatures required longer periods of time to inactivate both non-pathogenic *E. coli* and *Salmonella*, with the *E.coli* isolates requiring two to ten times more time varying with fat content. The significant difference ($P < 0.05$) of *E. coli* to *Salmonella,* in this case, provide a margin of safety for pathogen inactivation at these temperatures. In general and as expected, temperatures greater than 145°F (63°C) resulted in no significant differences ($P > 0.05$) between inoculums and fat content, as both *E. coli* and *Salmonella* were completely inactivated rapidly.

Based on the findings, the non-pathogenic *E. coli* inoculum would be best used as a surrogate for lower processing temperatures (54, 57, 60, and 63°C (130, 135, 140, and 145°F)). The time/temperature profile for the *E.coli* inoculum at 30% fat provides the

most conservative or "worst case" model to ensure thermal inactivation of *Salmonella.*
Beyond 63°C (145°F), since no significant differences ($P > 0.05$) exist due to rapid
inactivation of both inoculums, the non-pathogenic *E. coli* isolates offer minimal margin
of safety relative to *Salmonella.* This is not necessarily a cause for concern, since both
inoculum were inactivated rapidly at temperatures greater than 63°C (145°F). At
temperatures greater than 63°C (145°F), the non-pathogenic *E. coli* inoculum offers
minimal advantage as a surrogate, since it is inactivated as rapidly as *Salmonella.*
However, with a high density population and prolific growth, the absence of the *E. coli*
inoculum can be an indicator of the inactivation of *Salmonella* at higher temperatures.
More research is needed to investigate the effects of additional, specific components of
RTE meat products (oils, seasonings, additional ingredients, etc.) that may impact
thermal lethality of *Salmonella* or other pathogens. Thus, this study provides insight on
the potential of *E.coli* as a surrogate for *Salmonella*, but many factors must be considered
such as type and cut of meat, product composition, strain selection, cell physiology, and
methodology; a few examples of many factors that may be encountered in the food
industry.

Although the *E. coli* surrogates demonstrates the potential to validate thermal
processing parameters, the integrity of the HACCP plan ultimately determines validation
of processing parameters. This study offers specific means for thermal process validation,
but it must be supported by HACCP plans in processing facilities.

CHAPTER 5.   FUTURE RESEARCH

To supplement the present study, further research is needed in more tangential aspects to gain further insight on effects on thermal lethality behavior of the surrogates and its relationship to meat quality. Attributes such as water activity, moisture content, humidity, and pH have different effects on the lethality performance of pathogens. Distribution of fat and muscle tissue also has been found to effect thermal processing of meat. Thus, proximate analysis of attributes would provide further insight into the factors affecting behavior of both *E. coli* surrogates and *Salmonella* to thermal processing. The meat in this study also contained minimal levels of native microflora, which could impact growth and lethality of the surrogate and pathogen inoculum. Additionally, identifying the most thermally resistant bacteria from the surrogates or *Salmonella* may provide insight into shared characteristics that resulted in thermal resistance.

Different types and cuts of meat have a variety of effects on thermal inactivation of pathogens. Specifically for *Salmonella*, a study by Mogollón and others (2009) found that whole muscle thermal resistance was greater than thermal resistance of coarsely ground beef. This was in agreement with the conclusion from Orta-Ramirez and others (2005) that the physical arrangement of components within a food matrix may cause thermal resistance variation (Orta-Ramirez and others 2005). Since the composition is different than beef products, it is recommended that additional thermal lethality studies should be conducted with different cuts and types of meat to evaluate the effects of muscle and fat orientation and distribution on microbial inactivation.

Development of an equation to model the relationship of the surrogates to *Salmonella* is another area that should be explored. This model should be simple, in that

an input value for temperature can predict the time required to eliminate the surrogates, thus inactivating the pathogens. A simple model that could be easily used and interpreted by processing facilities to confirm thermal processing parameters would be well-received and beneficial to the industry.

A scaled-up, on-site trial of the study is recommended in the processing facility to compare thermal lethality data, and understand individual differences specific to the processing facility and equipment. The laboratory environment, while beneficial in controlling many parameters, lacks processing equipment that is used in manufacturing facilities to cook meat. Pilot plant trials or full-scale trials would be helpful to understanding additional risks that must be considered in the HACCP plan. Dickson (2013) cites that the log reductions and controlling variables and risks is more achievable in the smaller scale and highly controlled conditions of the lab environment, which may differ significantly from the full-scale operation (Dickson 2013). Additionally, processing equipment varies from facility to facility, therefore obtaining preliminary thermal inactivation data for the specific meat undergoing processing would be essential to create validation parameters.

It is important to understand the Appendix A compliance guidelines for lethality performance, and have a HACCP plan, but these parameters are only effective if they are implemented and utilized properly by the processing facility. Food safety can be ensured by the integrity of the HACCP plan in combination with compliance guidelines. Therefore, the efficacy of the HACCP plan is critical to the efficacy of Appendix A compliance guidelines as well as thermal processing validation.

APPENDIX

<u>Compliance Guidelines For Meeting Lethality Performance Standards For Certain Meat And Poultry Products (Temperatures 130 – 145°F): Appendix A (USDA-FSIS 1999)</u>

5.1.1.1 Introduction

Establishments producing ready-to-eat roast beef, cooked beef and corned beef products and certain ready-to-eat poultry products are required by FSIS to meet the lethality performance standards for the reduction of <u>Salmonella</u> contained in ℒℒ 318.17(a)(1) and 381.150(a)(1) of the meat and poultry inspection regulations. Further, FSIS requires meat and poultry establishments, if they are not operating under a HACCP plan, to demonstrate how their processes meet these lethality performance standards within a written process schedule validated for efficacy by a process authority (ℒℒ 318.17(2)(b)and (c) and 381.150 (2)(c) and (d)).

To assist establishments in meeting the lethality requirements, FSIS is issuing these compliance guidelines, which are based upon the time/temperature requirements contained in previous regulations. Establishments may choose to employ these guidelines as their process schedules. FSIS considers these guidelines, if followed precisely, to be validated process schedules, since they contain processing methods already accepted by the Agency as effective.

Also within these guidelines, FSIS has provided discussion regarding disposition of product following heating deviations and advice for the development of customized procedures for meeting the lethality performance standards.

5.1.1.2 Guidelines for Cooked Beef, Roast Beef, and Cooked Corned Beef

1. Cooked beef and roast beef, including sectioned and formed roasts, chunked and formed roasts, and cooked corned beef can be prepared using one of the following time and temperature combinations to meet either a 6.5-$\log_{10}$ or 7-$\log_{10}$ reduction of <u>Salmonella</u>. The stated temperature is the minimum that must be achieved and maintained in all parts of each piece of meat for a least the stated time:

Compliance Guidelines For Meeting Lethality Performance Standards For Certain Meat And Poultry Products (Temperatures 130 – 145°F): Appendix A (Continued)

Minimum Internal Temperature		Minimum processing time in minutes or seconds after minimum temperature is reached	
Degrees Fahrenheit	Degrees Centigrade	6.5-$\log_{10}$ Lethality	7-$\log_{10}$ Lethality
130	54.4	112 min.	121 min.
131	55.0	89 min.	97 min.
132	55.6	71 min.	77 min.
133	56.1	56 min.	62 min.
134	56.7	45 min.	47 min.
135	57.2	36 min.	37 min.
136	57.8	28 min.	32 min.
137	58.4	23 min.	24 min.
138	58.9	18 min.	19 min.
139	59.5	15 min.	15 min.
140	60.0	12 min.	12 min.
141	60.6	9 min.	10 min.
142	61.1	8 min.	8 min.
143	61.7	6 min.	6 min.
144	62.2	5 min.	5 min.
145	62.8	4 min.*	4 min.*

Compliance Guidelines For Meeting Lethality Performance Standards For Certain Meat And Poultry Products (Temperatures 146 – 160°F): Appendix A (Continued)

Minimum Internal Temperature		Minimum processing time in minutes or seconds after minimum temperature is reached	
Degrees Fahrenheit	Degrees Centigrade	$6.5\text{-}\log_{10}$ Lethality	$7\text{-}\log_{10}$ Lethality
146	63.3	169 sec.	182 sec.
147	63.9	134 sec.	144 sec.
148	64.4	107 sec.	115 sec.
149	65.0	85 sec.	91 sec.
150	65.6	67 sec.	72 sec.
151	66.1	54 sec.	58 sec.
152	66.7	43 sec.	46 sec.
153	67.2	34 sec.	37 sec.
154	67.8	27 sec.	29 sec.
155	68.3	22 sec.	23 sec.
156	68.9	17 sec.	19 sec.
157	69.4	14 sec.	15 sec.
158	70.0	0 sec.**	0 sec.**
159	70.6	0 sec.**	0 sec.**
160	71.1	0 sec **	0 sec.**

Compliance Guidelines For Meeting Lethality Performance Standards For Certain Meat
And Poultry Products: Appendix A (Continued)

* Past regulations have listed the minimum processing time for roast beef cooked to
145°F as "Instantly." However, due to their large size, most of these roasts dwell at
145°F, or even at higher temperatures, for at least 4 minutes after the minimum internal
temperature is reached. FSIS has revised this time/temperature table to reflect this and
emphasizes that, to better ensure compliance with the performance standard,
establishments should ensure a dwell time of at least 4 minutes if 145°F is the minimum
internal temperature employed.

**The required lethalities are achieved instantly when the internal temperature of a
cooked meat product reaches 158°F or above.

2. Cooked beef, including sectioned and formed roasts and chunked and formed
roasts, and cooked corned beef should be moist cooked throughout the process
or, in the case of roast beef or corned beef to be roasted, cooked as in paragraph
(3) of this compliance guide. The moist cooking may be accomplished by
placing the meat in a sealed, moisture impermeable bag, removing the excess
air, and cooking; by completely immersing the meat, unbagged in water
throughout the entire cooking process; or by using a sealed oven or steam
injection to raise the relative humidity above 90 percent throughout the cooking
process.

3. Roast beef or corned beef to be roasted can be cooked by one of the
following methods:

- Heating roasts of 10 pounds or more in an oven maintained at 250°F
  (121°C) or higher throughout a process achieving one of the
  time/temperature combinations in (1) above;

- Heating roasts of any size to a minimum internal temperature of 145°F
  (62.8 °C) in an oven maintained at any temperature if the relative
  humidity of the oven is maintained either by continuously introducing
  steam for 50 percent of the cooking time or by use of a sealed oven for
  over 50 percent of the cooking time, or if the relative humidity of the
  oven is maintained at 90 percent or above for at least 25 percent of the
  total cooking time, but in no case less than 1 hour; or

- Heating roasts of any size in an oven maintained at any temperature that
  will satisfy the internal temperature and time combinations of the above
  chart of this compliance guide if the relative humidity of the oven is

maintained at 90 percent or above for at least 25 percent of the total cooking time, but in no case less than 1 hour. The relative humidity may be achieved be use of steam injection or sealed ovens capable of producing and maintaining the required relative humidity.

4. Establishments producing cooked beef, roast beef, or cooked corned beef should have sufficient monitoring equipment, including recording devices, to assure that the time (accuracy assured within 1 minute), the temperature (accuracy assured within 1°F), and relative humidity (accuracy assured within 5 percent) limits of these processes are being met. Data from the recording devices should be made available to FSIS program employees upon request.

5.1.1.3 Guidelines for Cooked Poultry Rolls and Other Cooked Poultry
        Products

1. Cooked poultry rolls and other cooked poultry products should reach an internal temperature of at least 160 °F prior to being removed from the cooking medium, except that cured and smoked poultry rolls and other cured and smoked poultry should reach an internal temperature of at least 155°F prior to being removed from the cooking medium. Cooked ready-to-eat product to which heat will be applied incidental to a subsequent processing procedure may be removed from the media for such processing provided that it is immediately fully cooked to the 160 °F internal temperature.

2. Establishments producing cooked poultry rolls and other cooked poultry products should have sufficient monitoring equipment, including recording devices, to assure that the temperature (accuracy assured within 1°F) limits of these processes are being met. Data from the recording devices should be made available to FSIS program employees upon request.

REFERENCES

Ahmed NM, Conner DE, Huffman DL (1995) Heat-Resistance of Escherichia Coli O157:H7 in Meat and Poultry as Affected by Product Composition. J Food Sci 60:606–610. doi: 10.1111/j.1365-2621.1995.tb09838.x

Anderson D, Lucore LA (2012) Validating the Reduction of Salmonella and Other Validating the Reduction of Salmonella and Other Pathogens in Heat Processed Low-Moisture Foods.

Arthur TM, Brichta-Harhay DM, Bosilevac JM, and others (2010) Super shedding of Escherichia coli O157:H7 by cattle and the impact on beef carcass contamination. Meat Sci 86:32–37. doi: 10.1016/j.meatsci.2010.04.019

Beales N (2004) Adaptation of Microorganisms to Cold Temperatures, Weak Acid Preservatives, Low pH, and Osmotic Stress: A Review. Compr Rev Food Sci Food Saf 3:1–20. doi: 10.1111/j.1541-4337.2004.tb00057.x

Bell C, Kyriakides A (2002) Industry Focus : Control of Salmonella.

Busta FF, Suslow TV, Parish ME, and others (2003) The Use of Indicators and Surrogate Microorganisms for the Evaluation of Pathogens in Fresh and Fresh-Cut Produce. Compr Rev Food Sci Food Saf 2:179–185. doi: 10.1111/j.1541-4337.2003.tb00035.x

Carlson B, Ruby J, Smith G., Sofos J, Bellinger G, Warren-Serna W, Centrella B, Bowling R, Belk K. (2008) Comparison of antimicrobial efficacy to multiple beef hide decontamination strategies to reduce levels of Escherichia coli O157:H7 and Salmonella. Journal of Food Protection 71, 2223–2227

CDC. Center for Disease Prevention and Control (2014) FoodNet–Number and Incidence of *Salmonella* Infections by Serotype 2013. Available from: http://www.cdc.gov/foodnet/data/trends/tables/2013/table5.html. Accessed 2016 July 20.

CDC Center for Disease Prevention and Control (CDC) (2015a) Foodborne Diseases Active Surveillance Network (FoodNet). Available from: http://www.cdc.gov/foodnet/about.html. Accessed 2016 October 4.

CDC. Center for Disease Prevention and Control (2015b) Traveler's Health – Salmonellosis (Nontyphoidal). Available from: http://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/salmonellosis-nontyphoidal. Accessed 2016 October 4.

CDC. Foodborne Diseases Active Surveillance Network (CDC-FoodNet) (2016a) FoodNet 2014 Annual Illness Surveillance Report – Salmonella. Available from: http://www.cdc.gov/foodnet/reports/annual-reports-2014.html. Accessed 2016 October 4.

CDC. Center for Foodborne Diseases Active Surveillance Network (CDC-FoodNet). (2016b) FoodNet 2015 Preliminary Data. Available from: http://www.cdc.gov/foodnet/reports/prelim-data-intro.html. Accessed 2016 October 4.

CDC. Centers for Disease Prevention and Control (2015c) Salmonella – Technical Information. Available from: http://www.cdc.gov/salmonella/general/technical.html#one. Accessed 2016 October 4.

CDC (Centers for Disease Control and Prevention) Foodborne Diseases Active Surveillance Network (FoodNet, CDC) (2016a) FoodNet 2014 Annual Illness Surveillance Report – *Salmonella*. Available from: http://www.cdc.gov/foodnet/reports/annual-reports-2014.html. Accessed 2016 October 4.

CDC - Foodborne Diseases Active Surveillance Network (FoodNet, CDC). (2016b) FoodNet 2015 Preliminary Data. Available from: http://www.cdc.gov/foodnet/reports/prelim-data-intro.html. Accessed 2016 October 4.

CDC. Center for Disease Prevention and Control (2016d) What is Salmonellosis? Available from: https://www.cdc.gov/salmonella/general/. Accessed 2016 August 1.

CDC. Center for Disease Prevention and Control (2016e) Reports of Selected *Salmonella* Outbreak Investigations. Available from: https://www.cdc.gov/salmonella/outbreaks.html. Accessed 2016 August 20.

CFR. Code of Federal Regulations. Title 9, Ch. III, Part 417 (2016a) Hazard Analysis and Critical Control Point Systems. Available from: http://www.ecfr.gov/cgi-bin/text-idx?c=ecfr&tpl=/ecfrbrowse/Title09/9cfr417_main_02.tpl. Accessed 2016 October 22.

CFR. Code of Federal Regulations. Title 21, Ch. I, Part 172. (2016b) Hazard Analysis and Critical Control Point Systems. Available from: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.175. Accessed 2016 June 10.

Darwin KH, Miller VL (1999) Molecular Basis of the Interaction of Salmonella with the Intestinal Mucosa Molecular Basis of the Interaction of Salmonella with the Intestinal Mucosa. 12:405–428.

Deng X, Li Z, Zhang W (2012) Transcriptome sequencing of Salmonella enterica serovar Enteritidis under desiccation and starvation stress in peanut oil. Food Microbiol 30:311–315. doi: 10.1016/j.fm.2011.11.001

Dickson J (2013) for Small and Very Small Processors : Validation of Antimicrobial Interventions. 33:95–104.

Dufour AP. (1977) Escherichia coli: the fecal coliform. In: Bacterial indicators/health hazards associated with water. Hoadley, A.W. and Dutka, B.J. (Eds.) ASTM, Philadelphia. pp. 48-58.

Finn S, Condell O, McClure P, and others (2013a) Mechanisms of survival, responses, and sources of salmonella in low-moisture environments. Front Microbiol 4:1–15. doi: 10.3389/fmicb.2013.00331

Finn S, Hinton JCD, McClure P, and others (2013b) Phenotypic characterization of Salmonella isolated from food production environments associated with low-water activity foods. J Food Prot 76:1488–1499. doi: 10.4315/0362-028x.jfp-13-088

Foley SL, Lynne AM (2008) Food animal-associated Salmonella challenges: pathogenicity and antimicrobial resistance. J Anim Sci. doi: 10.2527/jas.2007-0447

Fookes M, Schroeder GN, Langridge GC, and others (2011) Salmonella bongori provides insights into the evolution of the salmonellae. PLoS Pathog. doi: 10.1371/journal.ppat.1002191

Foster JW (1991) Salmonella Acid Shock Proteins Are Required for the Adaptive Acid Tolerance Response. 173:6896–6902.

Foster JW (2001) Acid Stress Responses of Salmonella and E. coli : Survival Mechanisms , Regulation , and Implications for Pathogenesis. J Microbiol 39:89–94.

Giannella RA, Broitman SA, Zamcheck N (1972) Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro. Gut 13:251–6. doi: 10.1136/gut.13.4.251

Jenson I, Vanderlinde P, Langbridge J, Sumner J (2014) Safety of Meat and Meat Products in the Twenty-first Century.

Juneja VK, Eblen BS (2000) Heat inactivation of *Salmonella* Typhimurium DT104 in beef as affected by fat content. Lett Appl Microbiol 30:461–7. doi: 10.1046/j.1472-765x.2000.00755.x

Juneja VK, Eblen BS, Marks HM (2001a) Modeling non-linear survival curves to calculate thermal inactivation of Salmonella in poultry of different fat levels. Int J Food Microbiol 70:37–51. doi: 10.1016/S0168-1605(01)00518-9

Juneja VK, Eblen BS, Ransom GM (2001b) Thermal Inactivation of Salmonella spp . in Chicken Broth, Beef, Pork, Turkey, and Chicken: Determination of D- and Z-values. J Food Sci 66:146–152. doi: 10.1111/j.1365-2621.2001.tb15597.x

Kang DH, Fung DY (2000) Application of thin agar layer method for recovery of injured *Salmonella* Typhimurium. *International Journal of Food Microbiology.* 54:127-132.

Keeling C, Niebuhr SE, Acuff GR, Dickson JS (2009) Evaluation of Escherichia coli biotype 1 as a surrogate for Escherichia coli O157:H7 for cooking, fermentation, freezing, and refrigerated storage in meat processes. J Food Prot 72:728–32.

Lewis MJ (2006) Thermal Processing. Food Process Handb 33–70. doi: 10.1002/3527607579.ch2

Liston J (1965) G rowth of SaImoneIIa. Science (80-) 641–645.

Ma L, Kornacki JL, Zhang G, and others (2007) Development of thermal surrogate microorganisms in ground beef for in-plant critical control point validation studies. J Food Prot 70:952–957.

Marshall KM, Niebuhr SE, Acuff GR, and others (2005) Identification of Escherichia coli O157:H7 meat processing indicators for fresh meat through comparison of the effects of selected antimicrobial interventions. J Food Prot 68:2580–6.

Mead PS, Slutsker L, Dietz V, and others (1999) Food-related illness and death in the United States. Emerg Infect Dis 5:607–625. doi: 10.3201/eid0505.990502

Medema G, Payment P (2003) Safe drinking water: an ongoing challenge. … Drink Water 11–45.

Mogollón MA, Marks BP, Booren AM, and others (2009) Effect of beef product physical structure on salmonella thermal inactivation. J Food Sci. doi: 10.1111/j.1750-3841.2009.01253.x

Morrow W, Funk J. (2001) Salmonella as a Foodborne Pathogen in Pork. Department of Animal Science, North Carolina State University. Available from: https://projects.ncsu.edu/project/swine_extension/publications/factsheets/816s.pdf. Accessed 18 November 2016.

Murphy RY, Martin EM, Duncan LK, and others (2004) Thermal process validation for escherichia coli o157:h7,salmonella, and listeria monocytogence in ground turkey and beef products. J Food Prot 67:1394–1402.

National Advisory Committee on Microbiological Criteria for Foods (2010) Parameters for Determining Inoculated Pack / Challenge. J Food Prot 73:140–202.

Niebuhr SE, Laury a, Acuff GR, Dickson JS (2008a) Evaluation of nonpathogenic surrogate bacteria as process validation indicators for Salmonella enterica for selected antimicrobial treatments, cold storage, and fermentation in meat. J Food Prot 71:714.

Niebuhr SE, Laury a, Acuff GR, Dickson JS (2008b) Evaluation of nonpathogenic surrogate bacteria as process validation indicators for Salmonella enterica for selected antimicrobial treatments, cold storage, and fermentation in meat. J Food Prot 71:714.

Office of Disease Prevention and Health Promotion (ODPHP). 2016. Food Safety. Available from: https://www.healthypeople.gov/2020/topics-objectives/topic/food-safety/objectives. Accessed 2016 October 4.

Orta-Ramirez A, Marks BP, Warsow CR, and others (2005) Enhanced Thermal Resistance of Salmonella in Whole Muscle Compared to Ground Beef. J Food Sci 70:m359–m362. doi: 10.1111/j.1365-2621.2005.tb11480.x

Phillips LE, Humphrey TJ, Lappin-Scott HM (1998) Chilling invokes different morphologies in two *Salmonella enteritidis* PT4 strains. J Appl Microbiol 84:820–826. doi: 10.1046/j.1365-2672.1998.00417.x

Potter NN and Hotchkiss JH. (1998) Heat Preservation and Processing. *Food Science*. 5th Edition. Springer.

Sanguansri P (2016) Traditional Thermal Processing. Elsevier

Scallan E, Hoekstra RM, Angulo FJ, and others (2011) Foodborne illness acquired in the United States-Major pathogens. Emerg Infect Dis 17:7–15. doi: 10.3201/eid1701.P11101

Sinclair RG, Rose JB, Hashsham SA, and others (2012) Criteria for selection of surrogates used to study the sate and control of pathogens in the environment. Appl Environ Microbiol 78:1969–1977. doi: 10.1128/AEM.06582-11

Tucker G, Featherstone S (2011) 8 Cook Values and Optimisation of Thermal Processes.

United States Department of Agriculture - Food Safety and Inspection Service (USDA-FSIS) (1998) Lethality and Stabilization Performance Standards for Certain Meat and Poultry Products : Technical Paper.

USDA-FSIS (1999) Time-Temperature Tables for Cooking Ready-To-Eat Poultry Products. Available from: http://www.fsis.usda.gov/wps/wcm/connect/9ab2e062-7ac8-49b7-aea1-f070048a113a/RTE_Poultry_Tables.pdf?MOD=AJPERES. Accessed 2016 October 4.

USDA-FSIS (2013) "Danger Zone" (40°F - 140°F). Available from: http://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/safe-food-handling/danger-zone-40-f-140-f/CT_Index. Accessed 2016 November 1.

United States Department of Agriculture (USDA) (2014) Cost of foodborne illness estimates for *Salmonella* (non-typhoidal). Available from: http://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses.aspx#48498. Accessed 2016 October 4.

Vasan A, Geier R, Ingham SC, Ingham BH (2014) Thermal tolerance of O157 and non-O157 Shiga toxigenic strains of Escherichia coli, Salmonella, and potential pathogen surrogates, in frankfurter batter and ground beef of varying fat levels. J Food Prot 77:1501–11. doi: 10.4315/0362-028X.JFP-14-106

Wesche A, Ryser E (2013) Stress adaptation, survival and recovery of foodborne pathogens. Guid to Foodborne Pathog 422–436.