Thermal Inactivation of *Escherichia coli* O157:H7 and *Salmonella* Agona in Wheat Flour

Sarah Elisabeth Lillie Greene

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
in
Life Sciences
in
Food Science and Technology

Robert C. Williams, Chair.

Joseph E. Marcy

Sean F. O'Keefe

April 26, 2012

Blacksburg, VA

Keywords: thermal inactivation, *Salmonella* Agona, *E. coli* O157:H7, wheat flour, Sarah Elisabeth Greene

Thermal Inactivation of *Escherichia coli* O157:H7 and *Salmonella* Agona in Wheat Flour

Sarah Elisabeth Lillie Greene

ABSTRACT

Contaminated wheat flour has been identified as the probable vehicle of a multi-state outbreak of *Escherichia coli* O157:H7 associated with ready-to-bake cookie dough. Several cookie dough manufacturers are currently using heat-treated flour for ready-to-bake products, although data on thermal inactivation of foodborne pathogens in wheat flour remains scarce. The objective of this research was to first determine appropriate methods and parameters for bacterial inoculation and thermal treatment of wheat flour, and to subsequently determine the population reductions of *E. coli* O157:H7 and *Salmonella* Agona in artificially contaminated wheat flour following thermal treatment for 1, 5, 15 or 30 minutes at 55, 60, 65 or 70°C in a shaking water bath. Flour samples (a_w = 0.55) in sterile plastic bags were individually inoculated (~10^9 CFU/g), pulsified to distribute cultures, and pressed to a uniform thickness (1mm) prior to heat treatment. Following treatment, samples were rapidly cooled and diluted with peptone water; then plated onto Tryptic Soy Agar (TSA) and incubated at 37°C for 24 h prior to enumeration. The minimum heat treatments required for a 5-log reduction in microbial populations (~ 10^9CFU/g to ~ 10^4CFU/g) were 5 minutes at 70°C and 30 minutes at 70°C for *E. coli* O157:H7 and *S.* Agona, respectively. This research supports the hypothesis that the microbiological safety of ready-to-bake products may be improved by the use of heat-treated flour.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Rob Williams, and committee members, Dr. Joe Marcy and Dr. Sean O'Keefe, for their knowledge and support. Without their advice, insight, and generosity, this project would not have been successful. In addition, I would like to express my gratitude to the Virginia Agricultural Experiment Station for funding this project.

I would also like to recognize the students and staff who helped me so greatly throughout this research. A special thanks goes to (the now Dr.) Govind Kumar, who was instrumental in the development of methods used in this research, and Brett Driver, who was always willing to help out. I would also like to express my thanks to Dr. Hengjian Wang for his great kindness and willingness to help with all of the statistical analyses used in this research.

For all of the laughter and friendship that made my time in graduate school so special, I would like to think Kerri Martin, Bryan Aviles, Jeff Hamilton, Matt Schroeder, Mona Kumar, Jessica Maitland and Aníbal Concha Meyer. Our trips to Chicago, New Orleans and Delaware, picnics in the horticulture garden, and parties for every occasion will forever be among my fondest memories.

Lastly, I would like to thank my parents, David and Bobbie Greene, for their unwavering support and enthusiasm for all of my life's endeavors. Their own perseverance, intelligence, creativity and optimism will continue to serve as an example for me as I enter the next chapter in my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION AND JUSTIFICATION

The water activity (a_w) of a food is known to be one of the parameters affecting microbial growth and survival *(44)*. Water activity is defined as the ratio of the water vapor pressure of a food to the vapor pressure of pure water at the same temperature. The lower the water activity of a food, the less water is free to take part in the chemical and biological reactions required for microbial activity *(64)*. Foods with a water activity below 0.60 and moisture content below twenty five percent are generally considered to be low-moisture foods *(64)*.

Low-moisture foods have long been considered to be low risk products from a food safety standpoint because limited water availability within the product acts to hinder microbial growth *(38)*. However, following a number of recent foodborne illness outbreaks associated with low-moisture foods such as peanut butter *(16)*, black and red pepper *(5)*, raw almonds *(42)*, powdered infant formula *(1)*, and wheat flour *(59)*, it has become apparent that some pathogenic bacteria are capable of survival in such environments. Even without the capability of growth, the ability of pathogenic microorganisms to subsist in low-moisture products creates potential vehicles for foodborne illness.

Foodborne pathogens including *Salmonella*, Shiga toxin-producing *Escherichia coli* (STEC) and *Enterobacter sakazakii* have been associated with outbreaks in low-moisture foods *(1, 16)*. Outbreaks associated with low-moisture foods have resulted in illnesses and an economic burden in the United States. In 2010, the U.S. Department of Agriculture estimated the average case of

Salmonella or Shiga toxin-producing *E. coli* O157:H7 infection to incur over $1,900 and $6,600 in lost wages and medical costs, respectively *(97).*

Infections caused by *Salmonella* and Shiga toxin-producing *E. coli* O157:H7 have the potential to cause both serious illness and death. Low-moisture peanut products which were contaminated with *Salmonella* Typhimurium were linked to the deaths of nine consumers in 2008-2009*(3).* For *E. coli* O157:H7 infections, it has been estimated that five to ten percent of cases lead to the development of Hemolytic-Uremic Syndrome (HUS). HUS is a condition characterized by hemolytic anemia and acute renal damage. Approximately fifty percent of HUS cases suffer irreversible renal damage and ten percent of HUS cases will lead to death *(94).* Due to the potentially serious nature of the illnesses caused by both *Salmonella* and E. *coli* O157:H7, their presence in low-moisture foods is of concern to consumers, producers, and those who use said products as ingredients.

A number of the recent low-moisture associated outbreaks of foodborne illness have been linked to ingredients such as spices, nuts, pastes and flours. Such was the case for peanut butter and peanut paste during the 2008-2009 outbreak of Salmonellosis in the United States *(3).* The potentially contaminated peanut products were used in over 2,000 finished products across the country, resulting in a vast number of foodborne illnesses and product recalls. Other low-moisture ingredients such as paprika powder *(74)* and black and red pepper *(5)* have led to outbreaks of *Salmonella* in potato chips and salami products, respectively. In 1998, a puffed wheat and oat cereal was identified as the source of a multistate outbreak of *Salmonella* Agona in the United States *(2).*

Wheat flour is a commonly used ingredient in the U.S. food supply. In 2008, the USDA Economic Research Service (USDA- ERS) estimated per capita consumption of wheat flour in the U.S. to be over 135 pounds *(4).* Although the majority of wheat flour-containing products are cooked prior to reaching the consumer, a number of products such as pot pies, take and bake breads, and cookie dough come to the consumer uncooked. Products which have not been cooked have a greater potential to contain foodborne pathogens than their cooked counterparts *(57).*

The microbial safety of wheat flour came to into question following the 2009 outbreak of *E. coli* O157:H7 associated with raw refrigerated cookie dough. While a 2009 press release by the FDA states that "conclusions could not be made with regard to the root cause of the contamination," *(47)*, flour was identified as the most probable source of the outbreak in a 2011 publication in *Clinical Infectious Diseases (90)*. As of August 7, 2009, eighty consumers had been sickened across thirty-one states. Thirty five patients were hospitalized as a result, ten of which developed Hemolytic-Uremic Syndrome *(58).* As a precautionary measure, the manufacturer of the cookie dough began using "heat-treated flour," in its refrigerated cookie dough effective January 13, 2010 *(60).* A spokesperson for the company responded to public concerns stating "Although we do not know for certain that flour was the source of *E. coli* O157:H7, it is a raw agricultural commodity and as such can carry some risk…to further ensure the safety of our product, we've made a prudent decision to switch to heat-treated flour" *(59).*

Heat treatment of flour is carried out in efforts to reduce the number of foodborne pathogens present in the product. In addition to heat treatment, a number of methods have been developed for the reduction of foodborne pathogens in food powders such as flour. Alternative methods for

the decontamination of food powders include the use of gamma irradiation, microwave *(50),* and infrared *(92).* Due to high consumer acceptability and lower initial costs when compared to alternative treatments, thermal processing of flour remains an appealing method of decontamination for the food industry *(73).*

A number of experimental heat treatment methods have been developed specifically for wheat flour. These methods include high-temperature/short-time processes, lower-temperature/longer-time processes *(73),* and direct exposure to atmospheric steam. Certain food companies have recently introduced their own proprietary methods of flour heat treatment in efforts to increase food safety *(93).* However, published data concerning the efficacy of heat-treatment of flour remains scarce.

Industrial decontamination methods commonly utilize direct contact with steam or hot air, while experimental procedures commonly use hot air as the heating medium, with inoculated flour in some sort of container *(6, 73).*

In summation, low-moisture products such as wheat flour have recently been established as potential vehicles of foodborne illness such as salmonellosis and STEC infections. Previous studies have shown that the heat treatment of flour leads to a reduction in the number of non-pathogenic microorganisms such as *Saccharomyces cerevisiae, Lactobacillus plantarum* and *Bacillus subtilis* spores *(50, 73),* as well as *Salmonella* Weltevreden, a pathogenic bacteria *(6).*

The goal of this project is to use an experimental heat-treatment method to determine the thermal inactivation patterns of two outbreak-associated foodborne pathogens, *Salmonella* Agona and *E. coli* O157:H7, in wheat flour. Specifically, to identify the thermal treatment times required to

achieve a five-log reduction in bacterial populations at various experimental temperatures. An understanding of how strains of *E. coli* O175:H7 and *S.* Agona respond to heat treatment within wheat flour may be of use to future studies concerning pathogen reduction in this commodity.

Objectives

- To determine appropriate methods and parameters for bacterial inoculation and thermal treatment of wheat flour
- To determine thermal inactivation patterns of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Agona in wheat flour at 55, 60, 65 and 70°C, and treatments required to yield a 5-log reduction in microbial populations
- To identify an appropriate mathematical model to characterize the thermal inactivation of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Agona in wheat flour in response to experimental treatments

CHAPTER 2. LITERATURE REVIEW

Salmonella enterica: an overview

Salmonellae are Gram-negative rods which were first discovered by an American veterinary pathologist, Dr. Daniel Salmon, in 1885 *(30).* These non-spore forming members of the *Enterobacteriaceae* family are composed of two species: *Salmonella enterica* and *Salmonella bongori (14). S. enterica* are motile and zoonotic in nature; commonly residing in the intestinal tracts of birds, reptiles and farm animals *(33).* For the purpose of this paper, salmonellosis will refer to infections of non-Typhoidal *S. enterica.* Human salmonellosis is often contracted through the consumption of contaminated water, undercooked meat, raw eggs, and fresh agricultural products *(49).*

According to a study published by the CDC in 2010, approximately 40,000 cases of salmonellosis are reported in the U.S. annually *(33).* Acute symptoms of salmonellosis present themselves within 12-72 hours of consuming a contaminated food and include nausea, vomiting, abdominal cramps, mild diarrhea, and fever *(33).* Symptoms generally subside after one to two days and are self-limiting for healthy adults *(49).* However, very young, old or immunocompromised individuals may suffer more severe symptoms for extended time periods *(33).* As is the case in hospitals and nursing homes, where the mortality rate of *S. enterica* infections is estimated at 3.6% *(49).* An estimated 29 or 3 percent of patients will develop reactive arthritis (inflammation of the joints) or Reiter's syndrome (inflammation of the urethra and or cervix in females), respectively, following a *S. enterica* infection *(45).* These inflammatory sequelae are associated with the immunogenic marker HLA-B27*(54)* and may occur three to four weeks after the onset of acute symptoms *(49).*

Studies of foodborne outbreaks involving *S. enterica* suggest that ingestion of as few as 10-45 cells have the potential to cause disease *(41, 74)*. Infectious doses are known to be lower when the organism is incorporated into a high fat product *(40)* such as cookie dough, peanut butter, or chocolate. For example, the infectious dose of *S. enterica* serovar Napoli was found to be as low as 1.6 cell/g in chocolate *(52)*.

The pathogenicity of *S. enterica* is due to complex host-pathogen interactions and lie in the bacteria's ability to adhere and secrete proteins into gut epithelial cells of the host, using a type III secretion system (T3SS) *(56)*. Adherence is made possible by a variety of adhesions (Type 1 fimbriae) which mediate binding of the bacteria to the host cell *(100)*. For *S. enterica*, the major component of these fimbriae are encoded on a single gene known as *fimA(61)*. *S. enterica* commonly infect through attachment to the mucosa of the small intestine at the ileum and Peyer's patches, through invasion of M cells *(100)*. Peyer's patches refer to follicles within the small intestine which are intimately related to mucosa-mediated immune response *(100)*. Mediators used by *S. enterica* during the process of host cell invasion results in inflammation and fluid accumulation within the lumen, resulting in diarrhea *(82)*.

<u>*Salmonella*: nomenclature and taxonomy</u>

Salmonella nomenclature has been surrounded by controversy and confusion for many years. The earliest systems of *Salmonellae* identification were based on their nature as species-specific pathogens, i.e. *Salmonella cholerae-suis* or *Salmonella abortus-ovis (95)*. As new, non-host-specific species were discovered, this method of nomenclature became antiquated. In 1946, serotyping emerged as an important immunological method used in the classification of both *Salmonella* and *Escherichia* species *(64)*. The Kauffmann-White "one-species, one-serotype"

model used the O (somatic) and H (flaggelar) antigens to identify non-homologous serotypes as separate species of *Salmonella (14)*. With the use of DNA-DNA hybridization in the mid 1970's it was determined that all of the over 2,400 serovars *(64)* within the *Salmonella* genus belong to one of two species: *S. bongori* or *S. enterica (14)*. Furthermore, *S. enterica* was to be divided into six separate subspecies (I, II, IIIa, IIIb, IV, and VI) based on similarities in genomic make-up *(61)* and biochemical reactions *(14)*. Lastly, specific serovars were determined using serological classification based on the presence of multiple antigens *(64)*. With so many possible alphabetical and numerical combinations used for serovar identification, a common name, based on the geographic location of the original isolation, was given to each serovar *(95)*. For example, *Salmonella enterica* subspecies *enterica* serotype Agona is commonly referred to as simply "*Salmonella* Agona".

Bacteria within the *Salmonella* genus are proteobacteria *(61)* belonging to the *Enterobacteriaceae* family, and are therefore closely related other genus' of the family including *Escherichia*, *Shigella* and *Citrobacter* based on genetic sequence, G-C content, 16s rRNA, invasion genes and housekeeping genes *(77, 95)*. *S.* Agona belongs to subspecies I (*enterica*), which is the only subspecies known to cause illness in mammals *(86)*.

Outbreaks of foodborne illnesses associated with *Salmonella* Agona

Salmonella Agona was first isolated from cattle in Ghana in the early 1960's *(53)*. Nearly a decade later, the *S.* Agona had become the eighth most commonly identified serovar to be isolated from patients in the U.S. *(39)*. Since then, the serovar has been identified in a number of foodborne outbreaks involving toasted wheat and oat cereal *(2)*, fish meal used as animal feed *(39)*, fresh papayas *(85)*, anise seed-containing tea *(69)*, ready-to-eat meat *(62)*, powdered infant

formula *(15)*, an unidentified food at a Minnesota restaurant *(55, 85)*, and an unidentified peanut-flavored snack in the U.K. *(87)*.

Table 2.1. Outbreaks of foodborne illnesses associated with *Salmonella* Agona

Food	Year	Locations Effected	Reported Cases
Chicken, fed contained fish meal	1972	Paragould, Arkansas	17
Unidentified Snack	1994-1995	England and Wales	27
Toasted Cereal	1998	11 States	209
Aniseed-containing Tea	2002-2003	Germany	42
Powdered Infant Formula	2005	France	141
Unidentified, Chinese Buffet	2007	Hennepin County, Minnesota	3
RTE meat	2008	10 European Countries	163
Fresh Papayas	2011	25 States	106

*Compiled from *(2, 15, 39, 55, 62, 69, 85, 87)*

The presence of *S.* Agona in the food system is of concern not only due to its prevalence, but also due to its capacity for genetic adaptation. Recently, a number of multidrug resistant (MDR) strains of *S.* Agona have been identified in hospitals across Brazil *(79)*. The presence of mobile genetic information in the form of plasmids containing drug-resistant cassettes, genomic islands and transposons allow various strains of *S.* Agona to adapt and develop resistance *(37)* to common antibiotics such as including fluoroquinolones, ampicillin, chloramphenicol, and tetracyclines *(79)*.

Outbreaks of foodborne illnesses associated with non-Agona serovars of *S. enterica* in low-moisture foods:

S. Agona is one of the many serovars of *S. enterica* that have been associated with foodborne outbreaks. Foods that fall under the low moisture classification are of particular concern to food processors and public health officials due to the ability of *Salmonella* to survive in low moisture

products for extended periods of time *(38)*. Contaminated peanut butter *(3, 18)*, paprika powder *(74)*, puffed rice/corn/vegetable snacks *(29)*, dry pet food *(28)*, raw almonds *(42)*, and red and black pepper *(5)* have all been identified as sources of outbreaks of salmonellosis in recent years.

Table 2.2. Outbreaks of foodborne illnesses associated with non-Agona serovars of *S. enterica* in low-moisture foods

Food	Serovar	Year	States Effected	Reported Cases
Paprika Powder	Saintpaul, Javiana, Rubislaw	1993	NA	~1000
Powdered Milk	Tennessee	1992-1993	NA	48
Peanut Butter	Tennessee	2006-2007	47	628
Raw Almonds	Enteriditis	2003	12	29
Dry Pet Food	Schwarzengrund	2006-2007	19	70
Veggie Booty®	Wandsworth	2007	20	65
Dry Pet Food	Schwarzengrund	2007	18	62
Peanut Butter	Typhimurium	2008-2009	46	714
Pepper	Montevideo	2010	48	272

*Outbreaks in U.S., Canada or Germany. Compiled from *(1, 18, 27-29, 42)*

Monitoring of *Salmonella*-associated outbreaks

In the late 1990's the CDC began a surveillance system specifically designed to monitor cases of human salmonellosis across the U.S. *(100)*. As of 2004, there were six separate surveillance systems used to obtain data relating to occurrences of salmonellosis throughout the nation including the Public Health Laboratory Information System (PHLIS), the National Electronic Telecommunications System for Surveillance (NETSS), FoodNet, the National Antimicrobial Resistance Monitoring System (NARMS), and the Foodborne Outbreak Detection Unit *(24)*. In 1996, the National Molecular Subtyping Network for Foodborne Diseases Surveillance (PulseNet), was developed as a subtyping method using pulsed-field gel electrophoresis (PFGE)

to identify specific strains of *Salmonella* (and other foodborne pathogens) based on their unique genetic "fingerprint." PFGE is a molecular identification method in which purified microbial DNA is cut into fragments, then separated by size using a pulsed electrical field. Smaller fragments will move further down the gel than larger fragments, creating a unique series of bands.

Table 2.3. *Salmonella* surveillance in the U.S.

Surveillance System	Surveillance Type	Monitoring	Comprised Of	Methods
PHLIS	Passive	Confirmed cases	Individual states	Laboratory-based
NETSS	Passive	Non-confirmed cases	NA	Physician-based
FoodNet	Active	Confirmed cases that may be foodborne	Multiple States/Epidemiologists	Case-control studies, questionnaires
NARMS	Passive	Antimicrobial resistance	CDC, FDA, USDA, 16 state and local health departments	Molecular Techniques
Foodborne Outbreak Detection Unit	Passive	Outbreak related cases	Epidemiologists	Voluntary reporting to CDC

*Compiled from *(24)*

<u>*Escherichia coli*: an overview</u>

Escherichia coli are Gram-negative rods belonging to the family *Enterobacteriaceae;* first discovered in 1885 by Dr. Theodor Escherich *(64).* Although the species is often mentioned in reference to human illness, most strains of the bacteria are nonpathogenic *(17).* Nonpathogenic species are beneficial to humans in a variety of ways including synthesis of Vitamin K, protection against pathogenic bacteria, and serving as indicator organisms of fecal contamination in food or water *(48).*

When referring to pathogenic *E. coli*, the species is comprised of five major pathotypes: enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC),

enterohemorrhagic or Shiga toxin-producing *E. coli* (EHEC or STEC), and enteroaggregative *E. coli* (EAEC)*(77).* Various pathotypes of *E. coli* cause a diverse array of diseases ranging from diarrhea and hemolytic uremic syndrome (HUS), to urinary tract infections, pneumonia, meningitis and septicemia *(100).*

This paper will focus primarily on STEC strains due to their potential disease severity and implications to the food industry. Cases of STEC infection are often associated with the consumption of contaminated undercooked ground beef, unpasteurized juices, and fresh produce *(48).* According to the CDC, and estimated 265,000 cases of STEC infection occur annually in the U.S., with roughly 36% of those cases being caused by the strain O157:H7*(17).*

The symptoms of an STEC infection commonly occur within three to four days of consumption of a contaminated food and vary greatly in severity; often including abdominal cramps, bloody diarrhea and vomiting *(17).* For most healthy adults, the disease is self-limiting with symptoms subsiding after five to seven days after onset. Children, the elderly and immunocompromised individuals are at greatest risk of morality or the development of HUS, a disease characterized by kidney failure and hemolytic anemia, following an STEC infection *(48).*

Escherichia coli: nomenclature

Like *Salmonella*, there are a variety of ways in which *E. coli* may be classified. As mentioned previously, pathotyping (also called virotyping) is a method of broad categorization based upon similar pathogenicity of a group of *E. coli (77).* This method of typing is based upon the presence common virulence factors such as invasiveness, attachment, and production of toxins

(100). One pathotype may contain multiple serogroups and serotypes, which points to the fact that many *E. coli* strains may share common virulence factors.

More specifically, serotyping may be used for classification based on the highly variable reactivity of *E. coli* surface antigens (O/LPS and H/flagellar) to antibodies *(100)*. For example, the designation of *E. coli* O157:H7 indicates that the strain reacts with the 157th O-antibody (indicating serogroup) and the 7th H-antibody (indicating serotype). Strains of the same serogroup will react with a common O-antibody, while strains of the same serotype will react with a common H-antibody. In some cases, an *E. coli* strain will have a third serological classification based upon the presence of a capsule (K-antigen) interaction *(100)*.

<u>*Escherichia coli* O157:H7</u>

E. coli O157:H7 was first identified as human pathogen in 1982 following an outbreak of hemorrhagic colitis *(78)*. However, it was not until roughly a decade later that the strain was widely recognized as a major foodborne hazard. In 1993, a multistate outbreak of *E. coli* O157:H7 infections associated with undercooked Jack in the Box hamburgers resulted in the deaths of four children and the hospitalization of hundreds, many of whom suffered complications from HUS *(19)*. In response to the outbreak, President Clinton called a Congressional Hearing regarding the safety of the U.S. food supply. The FDA issued interim guidelines to its 1976 Model Food Code, and elevated its recommended internal cook temperatures for ground beef to 160°F for consumers, and 155°F for burgers prepared in food service establishments. In October of 1004, the USDA-FSIS declared *E. coli* 0157:H7 as an adulterant in raw ground beef *(98)*. Since coming into the public consciousness, foodborne

outbreaks of *E. coli* O157:H7 have been associated with a variety of meat and agricultural products.

Many of the virulence factors of *E. coli* O157:H7 are suspected to be the result of horizontal gene transfer *(78)*. These bacteria are able to attach to gut epithelial cells using adhesins prior to induction of actin reorganization or "pedestal formation" of the host cell. Actin rearrangement results in tight binding between bacteria and host cell *(100)* and causes "attaching and effacing lesions" of the brush border *(82)*. The major cell damage associated with *E. coli* O157:H7 is due to the production of Shiga-like toxins (also called verotoxins), *Stx*1 and *Stx*2, which interact with receptors in both intestinal and kidney cells *(100)*. Damage to kidney cells through interaction with Shiga-like toxins is responsible for the development of HUS, and may lead to death. Unfortunately, the use of antibiotic therapy such as fluoroquinolones and trimethoprim for treatment of *E. coli* O157:H7 infections seems to exacerbate, rather than alleviate, symptoms *(100)*.

The ability of *E. coli* O157:H7 to cause disease even in very low numbers is of public health concern *(38)*. Enumeration of *E. coli* O157:H7 present in meat patties consumed by those who developed *E. coli* O157:H7 infections revealed the infectious dose to be fewer than seven hundred organisms *(96)*. In low-moisture foods, the infectious dose of *E. coli* O157:H7 is suspected to be much lower, with between ten to one hundred ingested organisms capable of causing illness *(78)*.

Outbreaks of foodborne illnesses associated with *E. coli* O157:H7

Since the first major U.S. outbreak of *E. coli* O157:H7 in 1993*(19)*, there have been a number of foodborne outbreaks related to foods and beverages including unpasteurized apple juice *(20)*, contaminated drinking water *(22)*, romaine lettuce *(34)*, hazelnuts *(32)*, fresh spinach *(25)*, ready-to-bake cookie dough *(30)*, fresh strawberries *(8)*, and a number of raw ground beef products *(21, 23, 26, 31, 34).*

Table 2.4. Outbreaks of foodborne illnesses associated with *Escherichia coli* O157:H7

Associated Food/Beverage	Year	Number of States Effected	Confirmed Cases	Patients Hospitalized	Reported Deaths
Ground Beef from Fast Food Chain (Jack-in-the-Box)	1993	4	Over 500	NA	4
Unpasteurized Apple Juice	1996	NA	28 (70 suspected)	12	1
Ground Beef (Hudson Meat Co.)	1997	1	15	5	0
Contaminated Water at County Fair	1999	4	~1000 (unconfirmed)	NA	2
Ground Beef (ConAgra)	2002	3	28	7	0
Unidentified Food at Taco Bell	2006	5	52 (71 suspected)	53	0
Fresh Spinach	2006	199	26	102	3
Ground Beef (Topp's Brand)	2007	8	40	21	0
Ready-to-Bake Cookie Dough	2009	31	80	35	0
Romaine Lettuce	2011	10	60	30	0
Fresh Strawberries	2011	1	10	4	1
Lebanon Bologna	2011	5	14	3	0
In-Shell Hazelnuts	2011	4	8	4	0

*Compiled from *(19) (8, 20-23, 25, 26, 30-32, 34)*

Monitoring and prevention of *Escherichia coli* O157:H7-associated outbreaks

Surveillance, through both active and passive monitoring, allows the CDC to monitor trends and outbreaks associated with *E. coli* O157:H7. The information obtained regarding isolated cases and outbreak situations may be used to implement both prevention and control strategies for *E. coli* O157:H7 in the U.S. food system. In June of 2001, *E. coli* O157:H7 was added to the list of National Notifiable Diseases in the U.S. *(35).* A National Notifiable Disease is one of public health significance, and is mandated to be reported by physicians and private labs to the appropriate State Health Department. Reporting of a National Notifiable Disease to the CDC is highly encouraged, although voluntary, for State Health Departments. Surveillance of *E. coli* O157:H7-related illnesses currently takes place through both FoodNet and the Nationally Notifiable Diseases Surveillance System (NNDSS)*(35).* NNDSS collects its data from a variety of sources including its own laboratory, the Public Health Laboratory Information System (PHYLIS) and the CDC's National Escherichia coli reference laboratory. An annual summary of illnesses caused by *E. coli* O157:H7 and other EHEC strains can be found in the *Bacterial and Diarrheal National Case Surveillance: Annual Report.*

Since 1994, the U.S. meat industry has been working closely with the USDA-FSIS (Food Safety Inspection Service) in order to reduce the number of EHEC-related diseases, product recalls, and lost resources. Product sampling and verification of HAACP (Hazard Analysis and Critical Control Points) programs are implemented by the USDA-FSIS to encourage testing within the industry and minimize the occurrence of *E. coli* O157:H7 in the U.S. *(99).*

Wheat flour: an overview

Wheat flour is one of the most commonly utilized ingredients in the United States, with per capita consumption of commodity estimated at over 135 pounds per year *(4)*. With the exception of gluten free varieties, wheat flour is commonly found in most cereal, gravy, and baked goods in the U.S. Although, it must be noted that there are a variety of cereal grains that may be used for flour production including rice, corn, and rye.

Wheat flour is the product of particle reduction or "milling" of whole wheat kernels, or "wheat berries," which are composted of 3 parts *(68)*: The fiber- and essential fatty-acid-rich outer layer of the wheat berry, known as the bran, makes up approximately 10-15% of the kernel on a weight by weight basis. Due to its high fat content, the bran is susceptible to rancidity during transportation and storage, and therefore is often removed from refined wheat flours. The starchy middle portion, or endosperm, accounts for approximately 80 – 85% of the wheat berry by weight, and makes up the vast majority of refined white flour. Lastly, the germ is the nutrient rich innermost portion of the wheat kernel, composed of the highest amounts of protein, fat and B vitamins per gram. The germ only makes up approximately 3% of the whole kernel by weight, but is often discarded due to its tendency for rancidity after milling. "Whole-grain" flours, unlike refined flours, incorporate all 3 portions of the wheat kernel, and are therefore higher in nutrition but more susceptible to lipid oxidation *(68)*.

The thermodynamic properties of wheat flour, including thermal conductivity and specific heat, are important characteristics from a processing standpoint. *Thermal conductivity* (κ) is defined as "the amount of heat that flows per unit time through a food of unit thickness and unit area having unit temperature difference between faces," with units of W $m^{-1}K^{-1}$*(9)*. *Specific heat* (C_p)

refers to the amount of heat necessary to raise the temperature of a unit mass by a degree at a specified temperature, with units of kJ $kg^{-1}K^{-1}$. The specific heat of flour is approximately 1.371 kJ $kg^{-1}K^{-1}$ at 25°C, while the specific heat of water is 4.178 kJ $kg^{-1}K^{-1}$ at the same temperature *(66)*. The thermal conductivity of wheat flour is approximately 0.689 W $m^{-1}K^{-1}$ at 65.5°C *(88)*.

Occurrences of human pathogens in wheat flour

The microbial profile of wheat flour and other cereal grains have been examined both in the United States and abroad since the mid-1940s *(80)*. Between 2003 and 2005, the North American Miller's Association (NAMA), which represents a large portion of total dry-milled grains in North America, routinely collected samples of wheat flour in order to determine aerobic plate count (APC) as well as the presence of yeasts, molds, coliform bacteria, *Salmonella* and *E.coli (90)*. All microbial testing performed was in accordance with the *Compendium of Methods for the Microbial Examination of Foods* or the *Bacteriological Analytical Manual (43, 58)*. Coliform and generic *E. coli* counts were determined using both the Most Probable Number (MPN) method and 3M™ Petrifilm. The presence *E. coli* O157:H7 was not examined in the NAMA study because wheat flour had not been linked to any STEC outbreaks prior to the time of the survey *(91)*.

Results of the NAMA study showed coliform counts to be slightly higher than those reported in previous studies. However, the incidence of *Salmonella* in wheat flour was found to be significantly lower (0.14%) than previously reported occurrence rates (1.05%) *(90)*.

In a 2010 study carried out by the Institute of Environmental Science and Research Limited (ESR) for the New Zealand Food Safety Authority (NZFSA), both the occurrence of *Salmonella*

in wheat flour, and potential sources of contamination were examined *(51)*. This study highlights the fact that while *Salmonella* cannot grow in wheat flour, it may remain viable if present. Contamination was found to occur most commonly during pre-harvest production, transport, and milling or by human handlers in the production chain.

> "Overall, the risk of human salmonellosis due to contaminated cereal grains must be classified as low. However, the outbreak linked to flour indicates that when cereal contamination occurs it has the potential to affect large numbers of people, even if potential exposures occur via specialized behaviors (e.g. ingestion of uncooked home baking materials) or less common foods (e.g. uncooked muesli ingredients)" *(51)*.

Effect of water activity on the survival of microorganisms

Water activity is one of the most important factors for both microbial growth and thermal destruction. It is well documented that the thermal resistance of dehydrated microorganisms is much higher than that of the same organism in a hydrated state *(72)*. As water activity decreases, thermal tolerance of microorganisms increases *(67)*, with highest resistance in foods with water activities between 0.20 and 0.50 *(72)*. In a study of heat resistance of *Salmonella* Weltevreden in inoculated wheat flour (a_w 0.20 – 0.60), *z*-values were found to be 2.6 – 9.4 times greater than those expected for the same organism in environments with water activities greater than 0.60 *(6)*. It has been hypothesized that the presence of water molecules facilitates heat transfer, acting to denature proteins within the bacterial cell. Low-moisture products have water activities below 0.60 *(64)*, which is associated with increased thermal tolerance of microorganisms within these products.

Thermal inactivation studies: an overview

Before the advent of "alternative" methods such as infrared, high-pressure-processing (HPP), chlorination and ozone, thermal treatment was the primary means of microbial reduction for the food industry. The pasteurization of milk, canning of soup, cooking of meat and baking of bread all relay on thermal treatment for the safety of the product.

In the 1920s, Bigelow et al. sought to characterize a model of thermal inactivation of *Clostridium botulinum* spores *(10).* Through these studies, and those of Ball and Stumbo *(13)*, it was concluded that the thermal inactivation of both spores and vegetative cells follows mechanistic or linear pattern of inactivation when populations were plotted on a log scale. Since then, Bigelow's first order log-linear model has been the "primary modeling tool"*(11)* for the characterization of thermal inactivation of microorganisms within a food system, and is the basis for the calculation of D and *z* values as described below *(76).*

$$\text{Log}(N) = \log(N_0) - kt, \text{ or } \text{Log}(N) = \text{Log}(N_0) - t/D$$

Where N_0 denotes the initial microbial population within a food, N represents the microbial population present at a given time (t). D is equivalent to (1/k), which signifies the decimal reduction time, or time required for a reduction in the microbial population of 1-log cycle at a given temperature. A *z*-value is the temperature increase necessary for a reduction of the decimal reduction time (D-value) by 1 log cycle, and is related to D through following equation *(76)*:

$$\text{Log}(D) = \text{Log}(D_{ref}) - (1/z)(T-T_{ref})$$

Since D is a constant under isothermal conditions, D represents the D-value at a heating temperature (T), and D_{ref} represents the D-value at a reference temperature (T_{ref}).

A "thermal death time" is a multiple of the decimal reduction time (*f*D) which results in the pasteurization of the product based on recognized performance standards *(70)*. The *f* - value is variable, and dependent on the food product in question. For example, in the case of canning low acid foods, *f* = 12, because a 12 log reduction is required for commercial sterility.

Non-linear patterns of thermal inactivation

Not all thermal inactivation data seems to follow the traditional first order kinetics model proposed by Bigelow et al. *(13)*. Death curves that do not conform to the log-linear function are often biphasic in nature and display a "shoulder," a "tail," or a concavity upward or downward *(101)*. In 1963, Licciardello and Nickerson observed that even the data obtained by Bigelow displayed shouldering at lower temperature treatments (100-105°C) and a tailing at higher treatments (125-140°C), which Bigelow attributed to experimental error *(83)*.

A variety of microbial inactivation models which offer more flexibility in shape have been proposed and analyzed for goodness of fit in recent years *(11)*. These models include the Weibull function *(13)*, the two-fraction Cerf model *(36)*, the Whiting-Buchanan model *(101)*, and the R-fa_t model *(71)*, which view thermal and non-thermal inactivation on a basis of probabilities *(13)*. The approach to thermal inactivation as a distribution of probabilities rather than as a mechanistic effect is often referred to as "vitalistic"*(71)*. Vitalistic models are supported by the hypothesis that even cells within a population will have some level of variation in resistance to stress *(13)*.

The Weibull model of microbial inactivation is of particular interest to researchers because of its flexibility to fit both linear and biphasic death curves *(11)*. Two parameters, scale (α, also commonly referred to as k) and shape (β), are taken into consideration with this model *(13)*. The

dimensionless β is variable with temperature even within the same inactivation treatment (thermal, chemical, etc.) of the same organism within the same matrix. The equation for the Weibull model is a simple is a variation on the log-linear model and may be expressed by *(76)*:

$$Log(N) = log(N_0) - kt^{\beta}$$

A β greater than or less than one indicates concavity up or down, respectively, while a β equal to one satisfies the classical log-linear model of microbial inactivation *(11)* . In one review of fifty-five separate thermal inactivation studies carried out between 1954 and 2000, it was found that only seven contained data that fit a log-linear model, with only two having a log-linear fit over the entire temperature range studied *(13)*.

Considerations for choosing an appropriate thermal inactivation method:

A variety of methods have been developed in order to study thermal inactivation of microorganisms in food systems. When choosing an appropriate thermal inactivation method, one must consider the physical state of the inoculated product as well as the heat tolerance range of microorganism in question. The dimensions of the containers used during heat treatment should be taken into account, as heat transfer will differ greatly depending on the physical state and chemical makeup of the inoculated food product. Convenience in loading and unloading of inoculated product is also a practical consideration when choosing a container to be used during heat treatment. The heating medium (hot water or oil bath, hot plate, atmospheric steam, dry hot air, etc.) chosen should be appropriately selected to simulate the conditions of interest for the study. For example, hot water baths are only appropriate if the challenge temperatures do not exceed 90°C *(12)*.

Continuous temperature monitoring of both the heating medium and the inoculated product ensure that that the microorganisms are receiving the correct level of thermal treatment. Also, lag times for heating and cooling must be taken into account when analyzing thermal inactivation data. This lag time is often referred to as a "come-up time" when referencing the time necessary for every particle of the food product to reach treatment temperature *(75)*, and is not included in thermal treatment time.

The environment in which inactivation experiments are conducted should also be taken into consideration. If thermal inactivation trials mare performed in an open system, secondary environmental factors such as ambient temperature and relative humidity play an important factor in the thermal treatment. Monitoring of secondary factors is imperative for a successful thermal inactivation study *(12)*.

Methods of thermal inactivation in low-moisture foods

Previously used experimental methods for the thermal decontamination of food powders include hot air ovens in conjunction with small covered pans *(6)*, direct contact with streaming hot air *(50)*, and indirect contact with a streaming hot air source *(73)*. Thermal inactivation studies in other low-moisture foods have recently been carried out using small plastic or metal bags in circulating hot water or oil baths *(75)*.

A recent study by Ma et al. examined the heat resistance of various *Salmonella* servoars using small quantities of inoculated peanut butter contained in sterile plastic bags which were submerged in a circulating hot water bath for predetermined increments of time *(75)*. There are many benefits to using such a method with a powdered product. In a closed system, secondary

factors such as ambient temperature and relative humidity of the laboratory space can be better controlled. In addition, transfer of samples in and out of containers will be less prone to human error than with the use of narrow tubes or sealed disks. The use of a circulating hot water bath instead of a dry air oven may also lead to more continuous and uniform heating of inoculated samples. Finally, the ability for volume expansion of plastic bag also allows for cold diluent to be added directly into the sample after heating, thereby immediately halting heat treatment.

Previous thermal inactivation studies in powdered low-moisture foods

A series of studies were carried out between 2000 and 2005 in association with Unilever Bestfoods France-Amora-Maille which explored the impacts of various environmental factors on the decontamination of food powders *((46, 50, 72, 73).* All food powders used in the studies satisfy the definition of low-moisture foods set forth in James and Golden's *Modern Food Microbiology (64).* Powder granulometry, water activity, time and temperature of thermal treatment were all considered in the evaluation of microbial destruction of food powders including wheat flour and dried milk powder.

A study published in 2005 by Fine and Gervais *(50)* focused on an HTST process for decontamination of wheat flour in order to minimize organoleptic changes to the product such as Maillard browning. Ultra high heat (200-600°C) over short periods of time (0.1-30s) was used to investigate the thermal inactivation of *Bacillus subtilis* spores and *Saccharomyces cerevisiae* cells in wheat flour and on dried glass beads. Heat treatment was followed immediately by cooling with CO_2 gas (-70°C). Experiments were carried out using a closed system which involved a feeding hopper, direct exposure to a hot gas source, whirlwind, and direct exposure to a cold gas source. This process has been patented and developed by the Biotechnology and Food

Process Engineering laboratory (University of Burgundy, France). The process achieved a 2-8 log reduction depending on initial water activity. The most heat resistance for both spores and vegetative cells was observed when initial water activity was between 0.30 and 0.50. A reduction as low as 2 logs was seen at water activities between 0.30 and 0.40 *(50)*.

A study carried out by Ferret et al. examined the effect of particle size on the decontamination of food powders*(46)*. When food powders come in contact with liquids, agglomeration often takes place. Agglomeration of powders must be taken into account when discussing microbial inactivation, as heat penetration throughout the product may vary due to particle size. Particles with radii between 0.8 and 3.2 mm were shown to have similar microbial destruction rates, while food powders with particles over 5 mm were shown to have significantly less microbial destruction when treated with the same HTST process *(46)*.

In another study associated with the Unilever Bestfoods – France group *(73)*, *Saccharomyces cerevisiae* and *Lactobacillus plantarum* were separately inoculated into 30 g samples of wheat flour with a mortar to produce granules between 0.8 and 3.2 mm in size. Both a climactic chamber and a dryer were used to dry the inoculated sample to the desired water activity. A temperature of 5°C was maintained in the chamber during drying *(72)*. Dried samples were contained in a spherical metallic grid while being treated with hot air (150-200°C) for time periods of 5, 10, 20 or 30 seconds, then immediately cooled with CO_2 gas (-70°C). A thermocouple was placed in the center of the sphere to monitor temperature. Heating and cooling rates were found to be 50 and 75°C/s using this method. An 8 log reduction was achieved for the 30s/150°C/a_w=0.10, while organoleptic qualities of the flour were maintained. Optimum heat resistance was observed for samples with water activities between 0.30 and 0.50.

In a 1998 study by Archer et al., the effect of heating inoculated wheat flour samples in a hot air oven was examined *(6)*. Autoclaved flour was inoculated with *Salmonella* Weltevreden and dried in a drying chamber using silica gel with a saturated solution of lithium chloride to desired water activities. A sterile mini-food processor was then used to homogenize inoculated flour. Very small (0.02g) samples were placed into sterile sample pans and capped with lids before placing them into a preheated hot air oven. Time and temperature combinations ranged from 55°C/131°F – 77°C/170.6 °F for a duration of 0 – 150 minutes. Death curves were found to be biphasic, with rapid microbial destruction within the first 5-10 min, followed by a linear pattern of destruction. *D* values ranged from a $D_{60\text{-}62}$ of 875 minutes at an initial a_w of 0.4 to a $D_{63\text{-}65}$ of 29 minutes at an initial a_w of 0.5. Findings of this study supported the hypothesis that as initial water activity of a product decreased, survival of microorganisms within the product increased, irrespective of the time/temperature combination used during treatment. The *z*- values obtained from this study ranged from 15.2 to 53.9°C *(6)*.

CHAPTER 3. MATERIALS AND METHODS

Purification and preparation of cultures for *Escherichia coli* O157:H7 isolates

E. coli O157:H7 isolate EC1734 (clinical isolate from 2009 outbreak associated with cookie dough) and *E. coli* O157:H7 isolate EC1738 (food isolate obtained from refrigerated cookie dough) were obtained from the U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition, Laurel, MD, and stored at -70°C.

The following protocol was used for both *E. coli* O157:H7 isolates:

One loopful of stock culture was streaked onto Sorbitol MacConkey agar (SMAC) (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37°C for 48 hours. A well isolated colony was chosen and transferred to 9 ml Tryptic Soy Broth (TSB) (Fisher Scientific Co. LLC., Suwanee, Georgia, U.S.A.) and incubated at 37°C for 24 hours before confirmation using an API 20E test strip (Biomérieux, Marcy I'Etoile, France) and a latex agglutination assay for the O157 antigen (Remel, Lenexa, Kansas, U.S.A.). The confirmed *E. coli* O157:H7 isolates was used to make frozen stock cultures (in solution of 80% sterilized glycerol).

One loopful of each *E. coli* O157:H7 stock culture was inoculated individually into 9 ml TSB, incubated at 37°C, and transferred every 24 hours for 2 days. A loopful of each culture was then individually transferred into 40 ml TSB and incubated at 37°C for 24 hours. After incubation, 20 ml of each *E. coli* O157:H7 isolate were combined in a sterile centrifuge tube prior to centrifugation at 5,000 X g for 10 minutes. Cells were washed in sterile reverse-osmosis (RO) water, and the pellet was re-suspended in 4 ml of RO water to obtain an approximate 10^{11}CFU/ml concentration

Purification and preparation of cultures for *Salmonella* Agona

Freeze dried *Salmonella enterica* serovar Agona (ATCC BAA-707) was rehydrated according to ATCC instructions and added to 5 ml TSB. The solution was thoroughly mixed and one loopful of the suspension was streaked onto Tryptic Soy Agar (TSA) (Fisher Scientific Co. LLC., Suwanee, Georgia, U.S.A.) before incubation at 37°C for 48 hours. A well isolated colony was chosen and transferred into 9 ml TSB and incubated at 37°C for 24 hours in order to make frozen stock cultures (in solution of 80% sterilized glycerol).

One loopful of fluid stock culture was streaked onto XLT- 4 Agar (XLT4) (Fisher Scientific Co. LLC., Suwanee, Georgia, U.S.A) and incubated at 37°C for 48 hours. A well isolated colony was chosen and transferred to TSB and incubated at 37°C for 24 hours before confirmation using an API 20E test strip.

The following procedure was used for *S.* Agona: One loopful of stock culture was inoculated into 9 ml TSB, incubated at 37°C, and transferred every 24 hours for 2 days. A loopful of culture was then transferred into 40 ml TSB and incubated at 37°C for 24 hours prior to centrifugation at 5,000 X g for 10 minutes. Cells were washed in sterile reverse-osmosis (RO) water, and the pellet was re-suspended in 4 ml of RO water to obtain an approximate 10^{11}CFU/ml concentration.

Proximate analysis of wheat flour

Wheat flour was evaluated for moisture, ash, protein and lipid content prior to thermal inactivation studies. Methods used are indicated in Table 3.1.

Table 3.1. Methods used in proximate analysis of wheat flour

Characteristic Analyzed	Method	Source
Protein	Kjeldahl method	AOAC 920.87
Moisture	Oven drying at 95-100 °C	AOAC 934.01
Fat	Petroleum ether extraction on Soxtec units	AOAC 920.39C
Ash	Burned at 585 °C. "Wheat and flour testing method"	North American Export Grain Association, Inc. (NAEGA), 2004

Preparation of wheat flour

Three, five lb. bags of Pillsbury™ brand unbleached enriched wheat flour were purchased from a grocery store in Blacksburg VA, blended and stored in an air-tight container. Levels of background microflora were determined through random sampling of 1g portions of flour, followed by dilution with 1% peptone water (PW) (Fisher Scientific Co. LLC., Suwanee, Georgia, U.S.A) and plating onto TSA. A well-blended sample of flour was also tested for water activity (a_w) using a water activity meter (Decagon Devices AquaLab 4TE, Pullman, Washington, U.S.A.) prior to each day of experiments.

Inoculation of flour samples

One gram portions of flour were individually transferred into sterile 2oz plastic bags (Nasco Whirl-pak®, Fort Atkinson, Wisconsin, U.S.A.) and then inoculated drop-wise with 10µl of 10^{11}CFU/ml bacterial suspensions for each culture individually to achieve an approximately 10^9 CFU/g concentration. Samples were then manually massaged, followed by pulsification for 15 s using a Pulsifier (Microbiology International Pulsifier®, Microgen Bioproducts, Ltd., Camberley Surrey, U.K.) to distribute cultures. Pulsified samples were pressed between gauges to a uniform

thickness of 1mm within a pre-marked height of 30 mm within each bag. Any visible air pockets were manually removed from bags prior to sealing.

Positive control samples were prepared as described above but received no thermal treatment. Negative controls were inoculated with sterile RO water and thermally treated as described below.

Thermal treatment of inoculated flour

Heat treatment of inoculated flour was conducted in a shaking water bath (Thermo Scientific® Precision Reciprocating Shaker Bath, model 2870, Marietta, Ohio, U.S.A.) which was preset to 0.5°C above the to the desired treatment temperature (55, 60, 65 or 70°C) for at least 1 hour prior to each experiment. Sample bags were then clipped to one of four wire racks, with one rack assigned to each treatment duration (1, 5, 15 or 30 min) and weighted so that bags did come into contact with each other or sides of the water bath during treatment. The portion of the bag containing the flour sample was completely submerged in the bath at all times during heat treatment. Temperature was monitored throughout the heat treatment by inserting a thermocouple (FLUKE® 289 True RMS Multi-Meter, Everett, Washington, U.S.A.) into an RO water-inoculated flour sample. Additionally, the waterbath temperature was monitored using 3 thermocouples placed at separate locations in the waterbath (ECD series 5000 data-logger®, Model No. 5100, Portland, Oregon, U.S.A.).

Treatment times of 1, 5, 15 or 30 min at 55, 60, 65 or 70°C began after samples reached desired temperature. For each pathogen, six samples were tested per time/temperature combination.

Positive controls receiving no thermal treatment (time = 0) were represented in triplicate. Each sample was plated in duplicate, and each experiment was performed twice.

Microbiological analysis

Samples were removed from the water bath at the appropriate time intervals, and immediately submerged in ice water for 30 s before the addition of 9 ml PW per sample. Samples were shaken by hand for 15 s each, and allowed to rest in a sterile hood for approximately 15 min. Samples were then serially diluted in PW and surface plated (0.1 ml) onto TSA followed by incubation at 35°C for 24-48 hours. Colony counts were performed, and surviving population density was determined. For heat treatment durations of 15 and 30 min at temperatures of 65 and 70°C, enrichment was necessary as microbial populations dropped below the limit of detection (10^3 CFU/g). Tetrathionate (concentrated 2X, 9 ml) (Remel, Lenexa, Kansas, U.S.A.) was added to each sample inoculated with *Salmonella* Agona, and incubated at 37°C for 48 hours prior to plating on XLT-4 for detection. *E. coli* O157:H7-inoculated samples requiring enrichment were supplemented with *E. coli* broth (concentrated 2X, 9 ml) (Remel, Lenexa, Kansas, U.S.A.) and incubated for 48 hours prior to plating on SMAC and verified using a latex agglutination assay for the O157 antigen.

Statistical analysis

Statistical analysis of microbial population reduction as an effect of thermal treatment was analyzed using the GLM Procedure (SAS, Version 9.1.3) for each pathogen in a split-plot design. Using a complete randomized block model, treatment temperature was the major experimental unit, experimental replication was the block, and treatment durations were the sub-plot units.

To test the different responses to thermal treatment between *S*. Agona and *E*. coli O157:H7, the data was analyzed with the pathogen and treatment temperature as the main experimental units and the treatment duration as the sub-plot unit. The complete randomized block design was also used to test the effect of treatment temperature on population reduction at each time period ($P < 0.05$). The LSD (least significant difference) t–test was used to separate the main effect.

Weibull modeling was used to determine the line-of-best fit for death curves of both *S*. Agona and *E. coli* O157:H7 at every treatment temperature and duration. To analyze the data using Weibull model, the NLIN Procedure in SAS (nonlinear regression) was used (Marquardt computational method) on the following equation *(76)*:

$$\text{Log}(N) = \log(N_0) - kt^{\beta}$$

Where N represents the microbial population at a given time (t), N_0 represents the initial population, k represents the scale parameter and β represents the shape parameter in the Weibull function.

CHAPTER 4. RESULTS AND DISCUSSION

Results of proximate analysis of wheat flour

The results of a proximate analysis carried out on wheat flour used in all experiments are summarized in Table 4.1. Composition of flour with respect to moisture, protein and lipid, fell within a normal range for wheat flour according to the North American Miller's Association (NAMA) *(7)*.

Experimental parameters

Water activity was monitored prior to each experiment and remained at 0.46 +/- 0.02 pre-inoculation and 0.55 +/- 0.02 post-inoculation. Maintaining a stable water activity throughout all experiments was critical, as many studies have shown a direct effect between water activity and thermal inactivation kinetics of vegetative cells *(6, 72)*. Archer, et al. found thermal tolerance of *S.* Weltevreden in wheat flour to be greatest in samples with a_w between 0.30 and 0.40 *(6)*.

Approximate come-up times (time to reach treatment temperature) for 1g samples of wheat flour at treatment temperatures of 55, 60, 65 and 70°C were determined to be 40, 55, 75 and 95 s, respectively. The limit of detection for this study (10^3 CFU/g) was higher than initially intended; however, at a limit of 10^2 CFU/g, flour samples formed a thick "gravy" on the surface of the agar, making it very difficult to distinguish between colonies on plates that displayed greater than ~ 40 CFU. In order to increase the reliability of reported plate counts, the limit of detection was increased by a factor of 10. Background microflora was determined to be at approximately 10^2 CFU/g, which was below the limit of detection for heat treated samples. Occasionally, atypical colonies were present after enumeration of heat treated samples. However, the color and morphology of these colonies differed from that of either *S.* Agona or *E. coli* O157:H7, allowing for differentiation. Atypical colonies could have been a result of either background microflora

within the flour, or contamination pre- or post-treatment. No *Salmonella* or *E. coli* O157:H7 was detected during four analyses of background microflora performed over the four months of the study.

Previous studies involving thermal inactivation of vegetative cells in wheat flour utilized autoclaved flour *(6, 50, 73)* in order to minimize background microflora. However, as the background microflora used in this study fell below the limit of detection, it presented minimal issue when compared to the potentially undesirable effects associated with autoclaving. Undesirable effects of autoclaving may include moisture absorption which can lead to caking and uneven a_w throughout flour samples.

In this study, one gram samples of flour were individually inoculated in order to ensure that each sample received the same initial inoculation. Previous studies involving thermal inactivation of microorganisms in wheat flour inoculated a larger samples, blending with either a glass rod *(6)* or mortar and pestle *(50, 73),* and then subdivided the inoculated sample prior to heat treatment. Individual inoculation of samples may be more time consuming, but also may yield more consistent initial microbial populations.

The range of treatment temperatures and durations used for this study were based upon previous studies *(6)* and a desire to incur minimal organoleptic changes within the wheat flour that may be caused by Maillard browning *(73).*

Preliminary data was used to determine appropriate thermal treatments to yield a 1 through 5-log reduction of *S.* Agona in wheat flour. A 5-log reduction is a common performance standard for the decontamination of low-moisture foods *(12).* Although the target 5-log reduction may be

reached more rapidly with treatment at temperatures above 70°C, the aim of this study was to observe thermal inactivation patterns of pathogens in wheat flour. Four treatment temperatures between 55 and 70°C with durations of 1, 5, 15 and 30 minutes each were chosen to model the thermal destruction of *S.* Agona in order to observe patterns of thermal destruction leading to a 5-log reduction. Microbial reduction in response to heat treatment at 50°C was determined to be too mild to yield meaningful data at the chosen time periods. Hence, 55°C was chosen as the lowest treatment temperature. Heat treatment for durations exceeding 30 minutes resulted in populations of less than 10^3 CFU/g (limit of detection).

Thermal inactivation of *Salmonella* Agona

Heat treatment of wheat flour inoculated with *S.* Agona (approximately 10^9 CFU/g) resulted in a 5-log reduction following treatments for 30 minutes at 65 and 70°C. One replication of the experiment suggested that a 5-log reduction could be reached with a minimum treatment of 15 minutes at 70°C, however, a second replication failed to reach a reduction of 5-log at the same temperature and duration. A rapid decrease in microbial populations was observed during the first 5 minutes of heat treatment at all temperatures, which is not uncommon for thermal inactivation studies in low moisture foods *(6, 13, 65, 70, 75)*. Population reductions of 1.24, 2.81, 3.41 and 3.61 logs occurred as a result of the first minute of treatment as 55, 60, 65 and 70°C, respectively. This type of biphasic response to thermal treatment is commonly referred to as "tailing". Tailing was exaggerated as treatment temperatures were increased from 55 to 70°C, leading to an increasingly non-linear pattern of thermal inactivation.

Total log reductions of 3.89, 4.31, 5.11 and 5.22 were observed after treatment for 30 min at 55, 60, 65 and 70°C, respectively. The limit of detection (10^3 CFU/g) was reached following

treatment at 70°C for 30 minutes; however, *S.* Agona was detected in each sample after enrichment. The effects of heat treatment on populations of *Salmonella* Agona in wheat flour are summarized in Figure 4.1.1

A previous thermal inactivation study of *S.* Weltevreden in wheat flour at 55-77°C suggests that *S.* Weltevreden is more resistant to heat treatment *(6)* than the *S.* Agona used in this study. Treatment of *Salmonella*-inoculated flour samples at 70°C for 30 minutes resulted in a 3-log decrease in microbial populations the 1998 study *(6)*, while the same treatment resulted in a >5-log reduction in population of *S.* Agona in this study. Possible explanations for this discrepancy in population reduction in response to the same treatment temperature include variations in water activity and treatment methods. Flour used in the Archer study was equilibrated at a_w = 0.40, which is approximately 0.15 less than the flour used here. An increase in thermal tolerance of microorganisms at an initial a_w of 0.40 when compared to 0.55 is supported in the literature *(6, 73).* Differences in *Salmonella* serovars, treatment methods (oven vs. water bath) and sample size (0.02 g vs. 1.0 g) may also contribute to inconsistency in microbial reductions between this and the Archer study.

A 2009 study of thermal inactivation of *S.* Tennessee in peanut butter suggested similarly concave, biphasic patterns of microbial death *(75).* However, thermal inactivation data of *S.* Agona in wheat flour obtained from this study also showed a greater decrease in microbial populations than that of *S.* Tennessee in peanut butter at a_w = 0.45 *(75).* Thermal treatment at approximately 70°C for 30 minutes resulted in a 2-log reduction for *S.* Tennessee in peanut butter; an approximate 3-log difference in microbial reduction than what was found using *S.* Agona in wheat flour. Differences in *Salmonella* serovars or food matrices (pH, a_w, fat content,

thermal properties and sample size) may contribute to the inconsistency in thermal tolerance between the two studies. It has been hypothesized that the presence of fat in a food matrix may result in a protective effect to microorganisms during thermal processing *(52)*. Being that the fat content of peanut butter is much higher than that of wheat flour, this could be another possible reason for the variation in thermal tolerance between the two *Salmonella* serovars studied.

Statistical analysis of *Salmonella* Agona inactivation using various heat treatments

Populations of *S.* Agona at time zero for all samples were found to be statistically similar at approximately 10^9CFU/g. The effect of treatment duration on mean populations of *S.* Agona was found to be statically significant at all levels (0, 1, 5, 15, 30 min), while the effect of treatment temperature on mean populations of *S.* Agona at a given treatment duration were found to be statistically different for all levels, with the exception of 65 and 70°C. Statistically significant differences in mean populations of *S.* Agona following thermal treatment are listed in Table 4.2.

Modeling of Thermal Inactivation of *Salmonella* Agona

Due to the non-linear nature of thermal inactivation of *S.* Agona, a first-order (linear regression) model of thermal inactivation is inappropriate for the data. Based on the shape of the data, recent literature regarding non-linear models of thermal inactivation *(13, 70)*, and methods used in thermal inactivation studies which generated similar results *(63, 75, 76)*, a Weibull model was chosen. To analyze the data using Weibull model, the NLIN Procedure in SAS (nonlinear regression) was used (Marquardt computational method) on the following equation *(76)*:

$$\text{Log}(N) = \log(N_0) - kt^{\beta}$$

Where N represents the population of *S.* Agona at any time (t), N_0 signifies the initial population, and k and β represent two distinct variables at any given temperature. The use of Weibull modeling allowed for flexibility in the shape and gradient of thermal inactivation curves that result from various thermal treatments. The variable k signifies the rate of inactivation (gradient of curve), while β reflects the shape of the inactivation curve under isothermal conditions *(76).* The β variable was less than one in all of the Weibull functions used to model thermal inactivation of *S.* Agona in the current study. A β of less than one indicates that the thermal inactivation of *S.* Agona in wheat flour was concave (curving downward) in shape, which is the hallmark of tailing. Figures 4.1.2 – 4.1.5 illustrates the fit of Weibull functions to thermal inactivation data of *S.* Agona at treatment temperatures of 55 - 70°C.

Experimental population reductions of *S.* Agona closely matched those predicated through Weibull modeling, as they fell within the standard deviations of the experimental data. Due to the non-linear nature of the data, a correlation coefficient (R^2) could not be used to assess the goodness of fit of the Weibull models to the data sets. As an alternative, pseudo-R^2 was calculated to determine the acceptability of the models using the following equation *(76)*:

$$R^2 = 1 - \frac{\sum_1^n (Y_i - \hat{Y}_i)^2}{\sum_1^n (Y_i - \bar{Y})^2} ,$$

Where Y_i is the $\log_{10}$ population of *S.* Agona, $\hat{Y}_i$ is the $\log_{10}$ population as estimated by the Weibull model, $\bar{Y}$ is the average of the $\log_{10}$ population of *S.* Agona, and n is the number of data points of the inactivation curve. In SAS variance analysis output:

$$\text{Sum of Squares for "error"} = \sum_1^n (Y_i - \hat{Y}_i)^2$$

$$\text{Sum of Square for "corrected total"} = \sum_1^n (Y_i - \bar{Y})^2$$

The pseudo R^2 could be used to compare the goodness of fit of the Weibull model with that of a first-order linear regression model.

As treatment temperature increased, k (rate of microbial inactivation) increased, while β (concavity) decreased, with the exception of treatment at 70°C (Table 4.3). One possible explanation for this deviation from trend is the error incurred when populations of *S.* Agona neared the limit of detection at ~ 15 minutes of treatment, leading to an underestimate in the rate of thermal inactivation and an overestimate in concavity for the inactivation curve. It must be noted that although the puesdo-R^2 values are very close to one, this does not indicate a lack of error within the data. The presence of multiple variables (k and β) allows for a better fit than a model such as linear regression that contains only one variable.

Thermal inactivation of *E. coli* O157:H7

Treatment temperatures and durations used for thermal inactivation of *E. coli* O157:H7 were based on those selected for *S.* Agona for the purpose of comparison. Populations of *E. coli* O157:H7 (initial population ~ 10^9 CFU/g) decreased with increasing treatment temperatures and durations, as was expected and supported in previous thermal inactivation *(81, 84)* studies involving the pathogen. A 5-log reduction in populations of *E. coli* O157:H7 was reached with minimum treatments of 5 minutes at 70°C or 15 minutes at 65°C. Although a 5-log reduction is a common performance standard within the food industry, this may not be sufficient to ensure the safety of a product, given the USDA's zero-tolerance policy on *E. coli* O157:H7 in raw ground beef *(99)*. However, there is currently no such policy with regard to the pathogen in wheat flour.

Because the vast majority of thermal inactivation studies involving *E. coli* O157:H7 take place in food matrices with high water activities (i.e. juice, ground beef, poultry products) it is difficult

to draw comparisons between the inactivation patterns seen in this study with those of other studies. Microbial tolerance to thermal stress has been hypothesized to increase with decreasing water activity and increasing fat content *(67, 72).* The wheat flour used in this study is a unique food product due to its relatively low water activity, low fat content, and heterogeneous particle size distribution; making it difficult to draw comparisons of thermal inactivation data with studies in dissimilar food matrices.

As with *S.* Agona, a rapid decrease in microbial populations was observed within the first 5 minutes of heat treatment. Populations of *E. coli* O157:H7 were decreased by 0.62, 0.94, 1.92 and 3.94 logs following the first minute of treatment at 55, 60, 65 and 70°C, respectively. Tailing was exaggerated as treatment temperatures were increased from 55 to 70°C, leading to an increasingly non-linear pattern of thermal inactivation for *E. coli* O157:H7. This type of concavity in the death curve has been reported in many studies involving other foodborne pathogens *(63, 75, 76),* but less frequently in studies involving *E. coli* O157:H7. The majority of published thermal inactivation studies involving *E. coli* O157:H7 produced death curves that are log-linear in nature *(81, 84, 89).* As previously mentioned, these studies used high water activity meat products as test matrices, which could explain the discrepancy in thermal inactivation patterns of the organism. The limit of detection (10^3 CFU/g) was reached or exceeded following treatment at 70°C for 15 minutes and 65°C for 30 minutes. Exceeding the limit of detection prior to the terminal duration of the study (30 min) led to an underestimate of thermal destruction and overestimate of concavity at treatment temperatures of 65 and 70°C. Thermal destruction patterns at 70°C were skewed due to the limit of detection being exceeded at minute 15 of the possible 30 minutes of heat treatment. The overall shape of the data (0-30 min) obtained from a treatment at 70°C was concave, however, from treatment times 5 – 30 min, the pattern was a

roughly straight line at the limit of detection. The presence of *E. coli* O157:H7 was detected in each sample after enrichment. The effect of heat treatment on populations of *E. coli* O157:H7 in wheat flour is summarized in Figure 4.1.6.

Statistical analysis of *E. coli* O157:H7 inactivation at various heat treatments

Populations of *E. coli* O157:H7 at time zero for all samples were found to be statistically similar at approximately 10^9CFU/g. The effect of treatment duration on mean populations of *E. coli* O157:H7 was found to be statically significant at all levels (0, 1, 5, 15, and 30 min), as was the effect of treatment temperature on mean populations at all levels (55, 60, 65, and 70°C). Statistical analyses were carried out using the least significant difference (LSD) t-tests ($P < 0.05$). Statistically significant differences in mean populations of *E. coli* O157:H7 following thermal treatment are listed in Table 4.5.

Modeling of thermal inactivation of *E. coli* O157:H7

A Weibull model was chosen based on its capability of fitting non-linear data *(11, 13, 63)*, its use in prior thermal inactivation studies involving *E. coli* spp. *(11)*, and for ease of comparison with the thermal inactivation data of *S*. Agona. The Weibull model offered greater flexibility due to the presence of multiple parameters (k and β), when compared to a linear regression model. Predicted death curves fell within standard deviations of experimental values for the inactivation of *E. coli* O157:H7. Figures 4.1.7 – 4.2.1 illustrate the fit of Weibull functions to thermal inactivation data of *E. coli* O157:H7 in wheat flour at treatment temperatures of 55 - 70°C.

As denoted in Table 4.6, the rate of microbial inactivation of *E. coli* O157:H7, k, increased with increasing heat treatment, which was expected. However, as populations met or exceeded the limit of detection, the shape of the data became increasingly linear with increasing treatment

temperatures. This phenomenon is most apparent with treatment at 70°C, following heating for time periods at or above 5 minutes in duration. The very small β variable (0.0430) reflects the mostly linear nature of the data. When populations of *E. coli* met or exceeded the limit of detection at time periods of greater than 5 minutes, a great deal of error was introduced to the death curve at treatments of 70°C. The high pseudo-R^2 value for treatment at 70°C is another example of the flexibility that is introduced when using a model with multiple parameters. As previously mentioned, this goodness-of-fit does not necessarily indicate a lack of error within then data, but rather the ability of the Weibull model to apply to the experimental data.

Comparisons in thermal tolerance between *Salmonella* Agona and *Escherichia coli* O157:H7

In general, *E. coli* O157:H7 displayed greater tolerance to thermal treatments at 55 and 60°C when compared to *S.* Agona. At treatment temperatures of 65 and 70°C, *E. coli* O157:H7 experienced a greater decrease in populations than *S.* Agona after treatment durations of greater than 15 minutes (Figure 4.2.2). Similar findings were reported in a 2001 study involving thermal inactivation of *E. coli* O157:H7 and multiple *Salmonella* servoars in high fat (>18%) ground beef *(89)*. Overall, *E. coli* was found to be more resistant at temperature treatments under 58°C, and less resistant at temperatures between 58 and 70°C, when compared to *S.* Typhimurium at the same treatment temperature and duration. Thermal death studies carried out in liquid egg also indicated greater thermal tolerance of *E. coli* K12, as compared with *S. enteriditis* strain 13076, following heat treatment at 54, 56, 58 and 60°C *(51)*. Consistent with results of the current study, variation in thermal tolerance of *E. coli* spp. and *Salmonella* spp. in liquid egg were found to be greatest around 55°C, and lessened following heat treatment at 60°C. A 2012 comparison study of thermal inactivation of *E. coli* O157:H7 and *Salmonella* spp. in fresh fish found a

similar relationship between microbial death rates, in which differences in D-values between the two pathogens were greatest a 55°C and smallest at 65°C, over the 10 degree temperature range; with *E. coli* exhibiting greater thermal resistance than *Salmonella* at all treatment temperatures *(46)*. Thermal inactivation of *E. coli* O157:H7 was found to be more temperature dependent in high fat ground beef than *Salmonella* spp. over a range of 58 - 70°C, which was also apparent in our study *(89)*.

CHAPTER 5. CONCLUSIONS AND FUTURE RESEARCH

Although some take-and-bake dough processors have switched to using heat-treated wheat flour, published data thermal inactivation of foodborne pathogens in the commodity remains scarce. Using an experimental thermal treatment method, the minimum heat treatments required for a 5-log reduction (~10^9 CFU/g to ~10^4CFU/g) in microbial populations were 5 minutes at 70°C and 30 minutes at 70 °C for *E. coli* O157:H7 and *S.* Agona, respectively. This research supports the hypothesis that heat treatment of wheat flour may decrease potentially harmful microorganisms within the flour, and ultimately increase the microbiological safety of take-and-bake dough products.

The methods of inoculation and heat treatment described in this study could be used in future studies to yield basic information regarding thermal inactivation patterns of other foodborne pathogens or spoilage organisms in wheat flour or other low moisture food powders.

Although information obtained through this study may be informative regarding general thermal inactivation patterns of *E. coli* O157:H7 and *S.* Agona in wheat flour during an experimental process, a scaled-up model utilizing steam injection may yield data that is more applicable to the food industry. The experimental process used in our study uses indirect, dry-heating. A direct heating method with steam may lead to more effective heat transfer, and therefore, more rapid thermal inactivation of foodborne pathogens in wheat flour.

Future research may also benefit from examining the effect of heat treatment during wheat conditioning on microbial populations. Wheat conditioning involves soaking of whole wheat berries for a number of hours in warm water. If the conditioning water was to be heated to a

high enough temperature to reduce microbial populations, this could reduce overall processing time while removing bacterial contaminants from the surface of the kernels.

Regardless of when heat treatment takes place in the milling process, it would be beneficial to understand the effect of thermal treatment on the functional properties of wheat flour. Organoleptic properties and consumer acceptability of products made from heat treated flour would also be of interest to researchers and flour processors.

TABLES AND FIGURES

Table 4.1. Results of proximate analysis of wheat flour

	Moisture	**Ash**	**Protein**	**Lipid**	**Carbohydrate**
Average Percentage	13.15	0.56	12.09	0.87	73.33*
Standard Deviation	0.17	0.08	0.03	0.18	NA

*All analyses were carried out on a wet basis

* As calculated by 100% – (Moisture + Ash + Protein + Lipid)

Table 4.2. Differences in mean populations of *Salmonella* Agona in wheat flour following thermal treatments

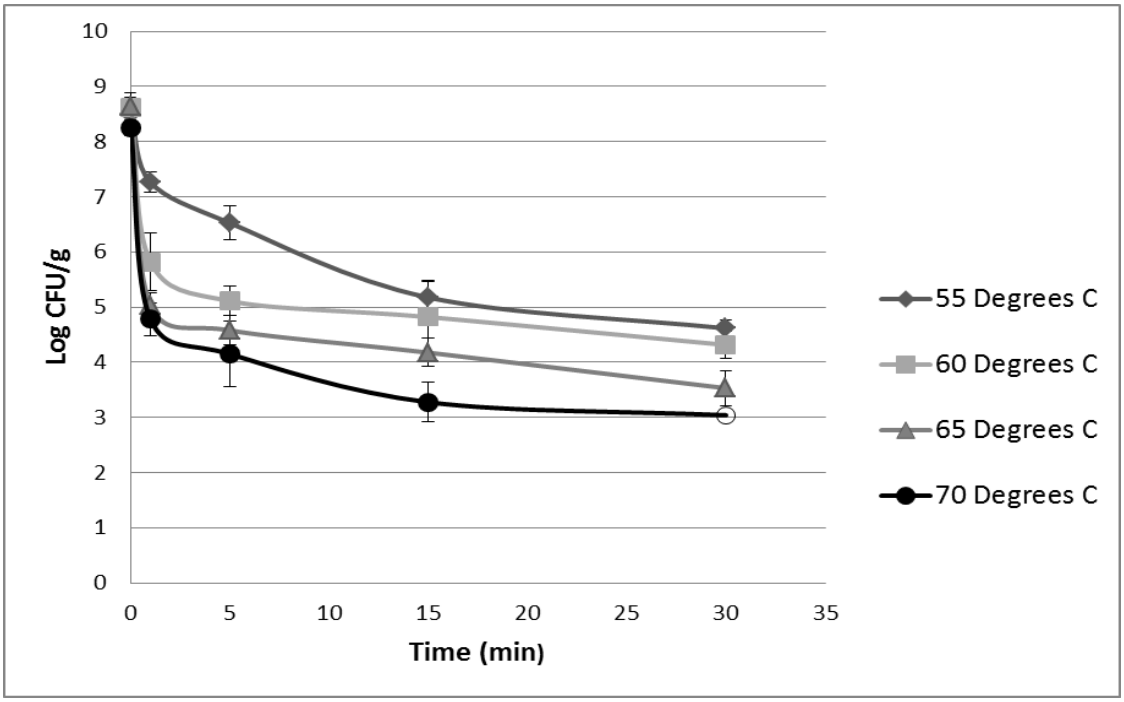
Treatment	**Temperature (°C)**			
Time (min)	**55**	**60**	**65**	**70**
0	8.50_{A}	8.26_{A}	8.64_{A}	8.26_{A}
1	7.27_{A}	5.82_{B}	4.93_{C}	4.78_{C}
5	6.53_{A}	5.11_{B}	4.53_{B}	4.14_{B}
15	5.17_{A}	4.82_{A}	3.68_{B}	3.28_{B}
30	4.61_{A}	$4.32_{A,B}$	$3.41_{B,C}$	3.03_{C}

* Mean populations (Log CFU/g) of *S.* Agona (n= 12) in response to thermal treatments

* Statistical analyses were carried out using the least significant difference (LSD) t-tests ($P < 0.05$)

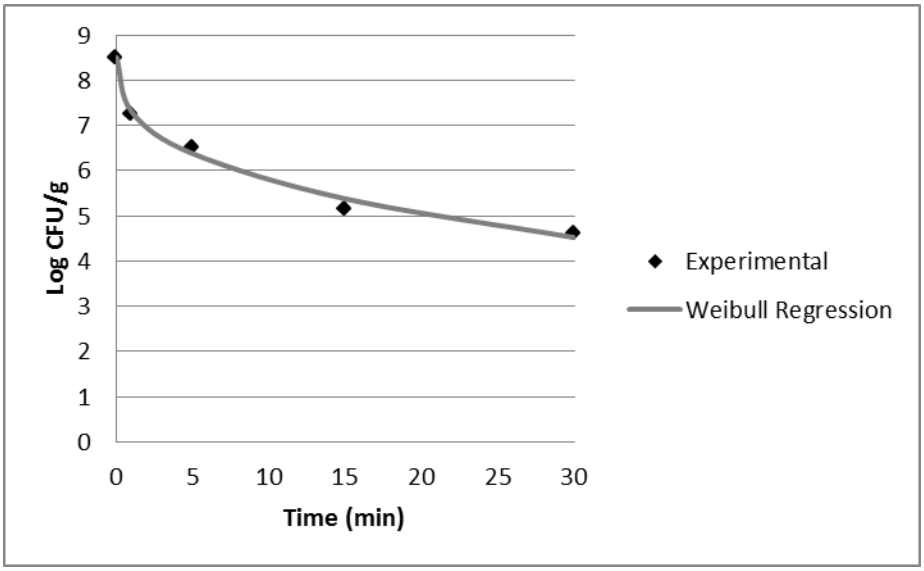
* Treatments within the same row with the same letter are not statistically different.

Figure 4.1.1. Effect of thermal treatment on populations of *Salmonella* Agona in wheat flour



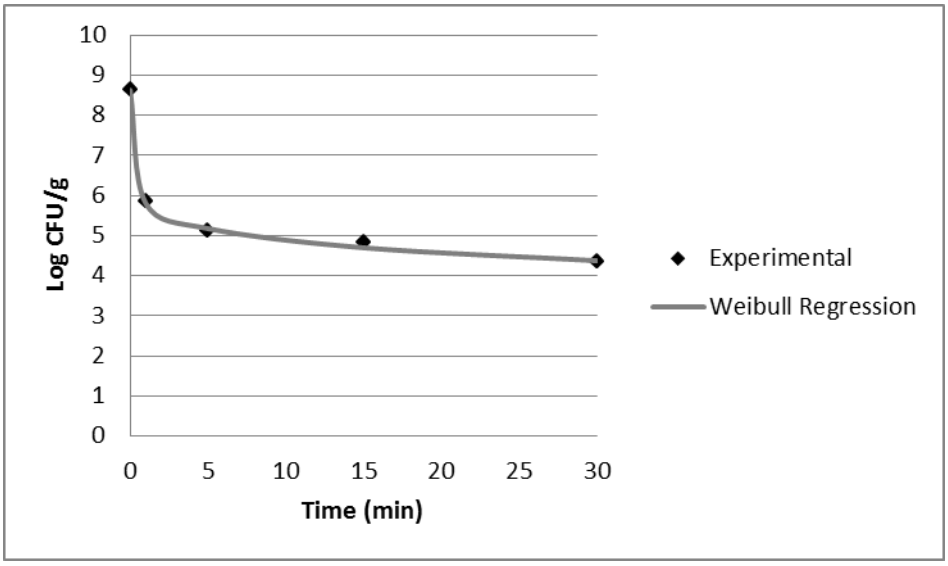
* Error bars indicate standard deviation (n=12) per treatment

Figure 4.1.2. Weibull regression as compared to experimental values of populations *Salmonella* Agona in wheat flour following thermal treatment for durations of 0, 1, 5, 15, and 30 minutes at of 55°C



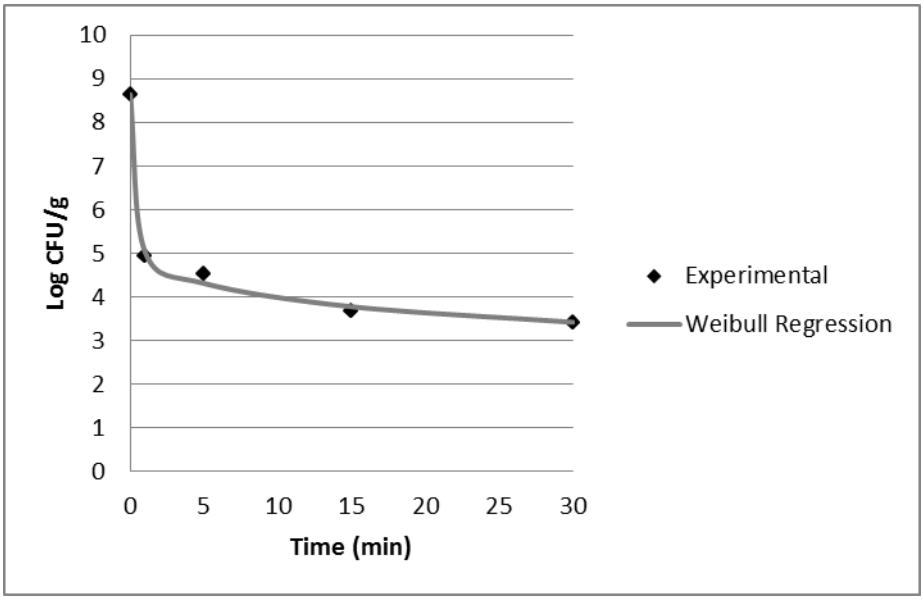
*Experimental data points represent mean population values (n = 12)

Figure 4.1.3. Weibull regression as compared to experimental values of populations *Salmonella* Agona in wheat flour following thermal treatment for durations of 0, 1, 5, 15, and 30 minutes at of 60°C



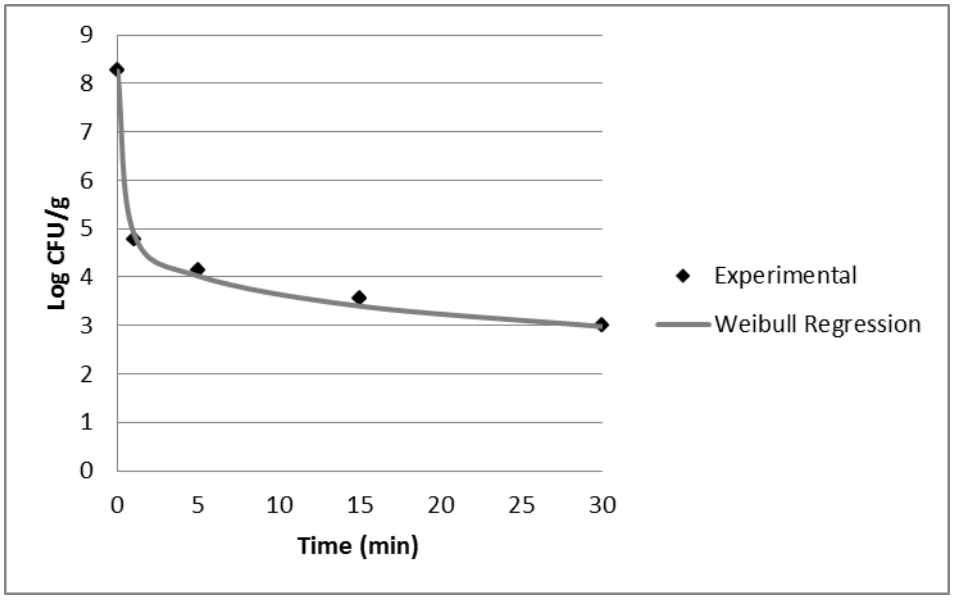
*Experimental data points represent mean population values (n = 12)

Figure 4.1.4. Weibull regression as compared to experimental values of populations *Salmonella* Agona in wheat flour following thermal treatment for durations of 0, 1, 5, 15, and 30 minutes at of 65°C



*Experimental data points represent mean population values (n = 12)

Figure 4.1.5. Weibull regression as compared to experimental values of populations *Salmonella* Agona in wheat flour following thermal treatment for durations of 0, 1, 5, 15, and 30 minutes at of 70°C

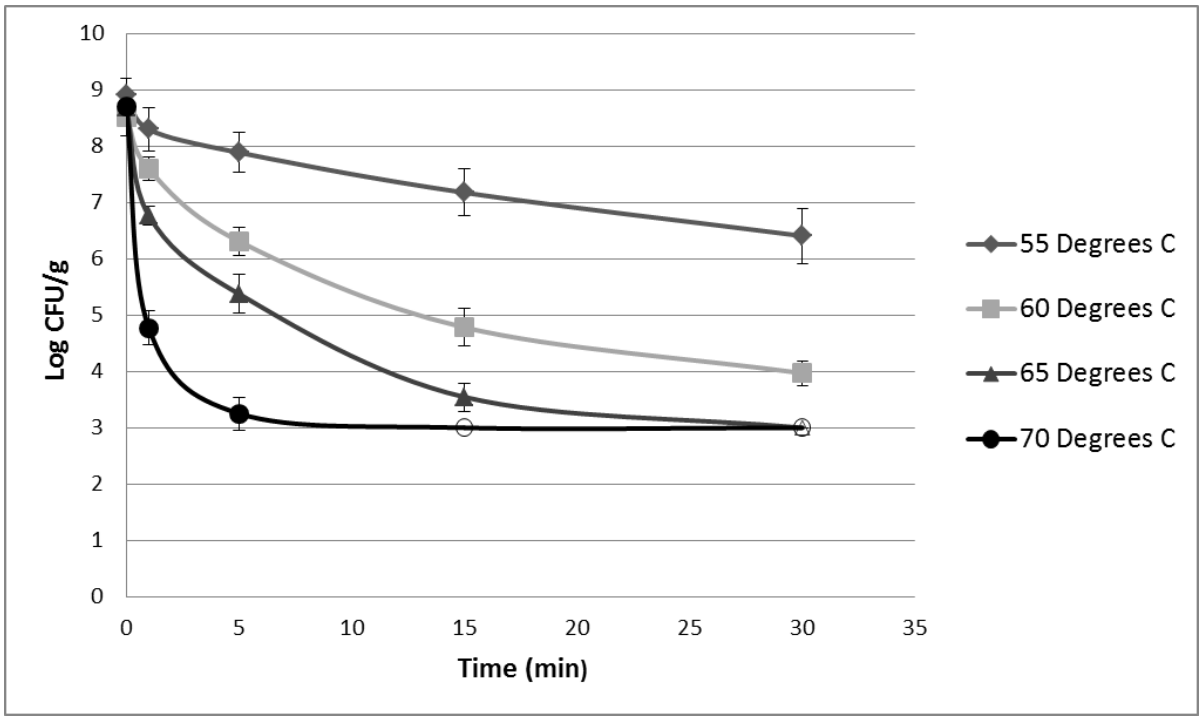


*Experimental data points represent mean population values (n = 12)

Table 4.3. Numerical values for k and β variables in Weibull function and Puesdo-R^2 for populations of *Salmonella* Agona at various treatment temperatures.

Temperature (°C)	**K**	**β**	**Pseudo-R^2**
55	1.2318	0.3469	0.9858
60	4.7905	0.0770	0.9846
65	7.3621	0.0583	0.9852
70	5.4778	0.0855	0.9862

Figure 4.1.6. Effect of thermal treatment on populations of *Escherichia coli* O157:H7 in wheat flour



* Error bars indicate standard deviation of *E. coli* O157:H7 populations following heat treatment performed in duplicate (n = 12)

* Open symbols indicate that the limit of detection was exceeded

Table 4.4. Differences in mean populations of *Escherichia coli* O157:H7 in wheat flour following thermal treatment

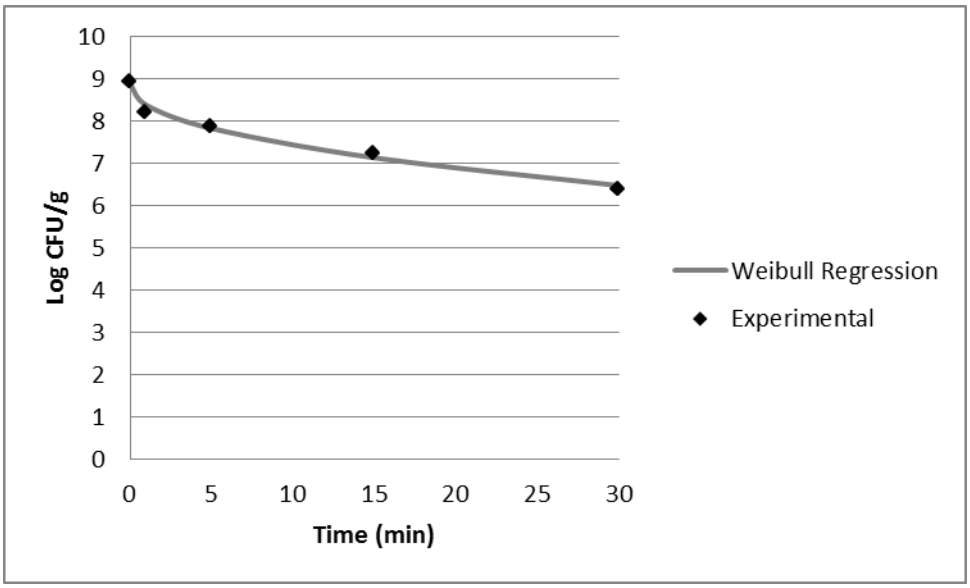
Treatment	**Temperature (°C)**			
Time (min)	**55**	**60**	**65**	**70**
0	8.92_{A}	8.68_{A}	8.66_{A}	8.55_{A}
1	8.20_{A}	7.61_{B}	6.80_{C}	4.77_{D}
5	7.89_{A}	6.32_{B}	5.37_{C}	3.20_{D}
15	7.24_{A}	4.91_{B}	3.56_{C}	3.03_{D}
30	6.39_{A}	4.05_{B}	$<3.00_{C}$	$<3.00_{C}$

*Mean populations (Log CFU/g) of *E. coli* O157:H7 (n= 12) in response to thermal treatments.

* Treatments within the same row with the same letter are not statistically different.

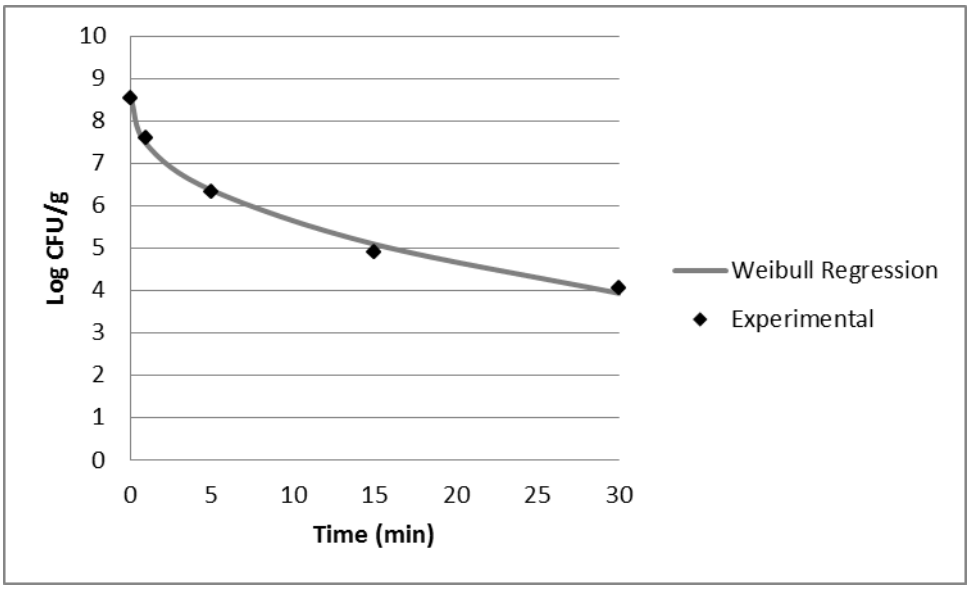
* Mean populations of < 3.000 indicate limit of detection was exceeded.

Figure 4.1.7. Weibull regression as compared to experimental values of populations *Escherichia coli* O157:H7 in wheat flour following thermal treatment for durations of 0, 1, 5, 15, and 30 minutes at of 55°C



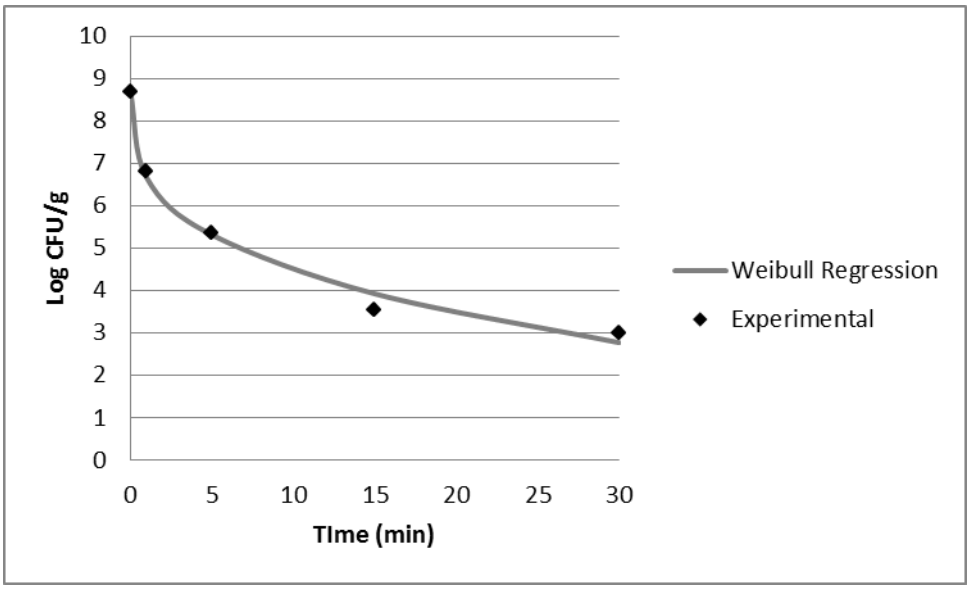
*Experimental data points represent mean population values (n = 12)

Figure 4.1.8. Weibull regression as compared to experimental values of populations *Escherichia coli* O157:H7 in wheat flour following thermal treatment for durations of 0, 1, 5, 15, and 30 minutes at of 60°C



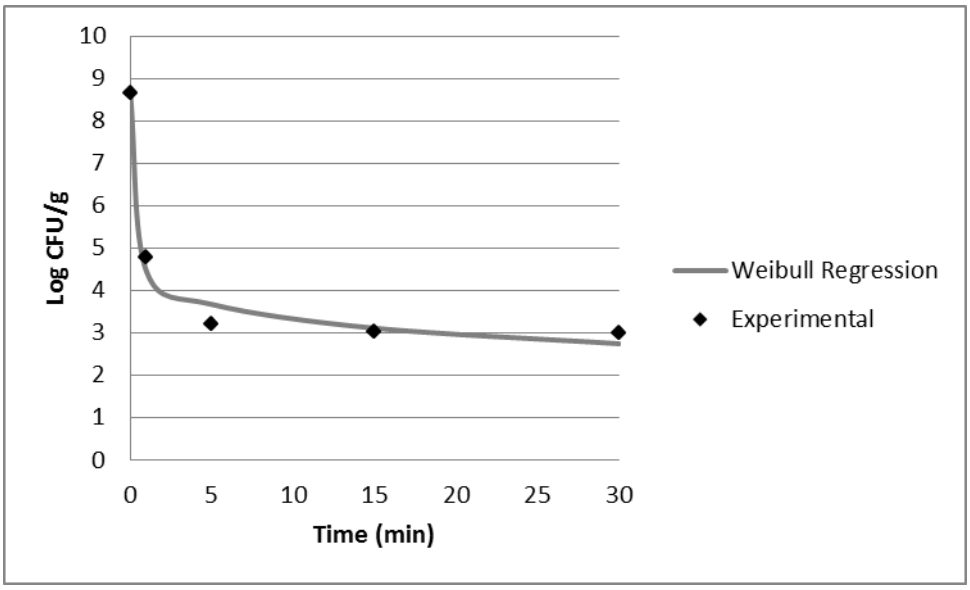
*Experimental data points represent mean population values (n = 12)

Figure 4.1.9. Weibull regression as compared to experimental values of populations *Escherichia coli* O157:H7 in wheat flour following thermal treatment for durations of 0, 1, 5, 15, and 30 minutes at of 65°C



*Experimental data points represent mean population values (n = 12)

Figure 4.2.1. Weibull regression as compared to experimental values of populations *Escherichia coli* O157:H7 in wheat flour following thermal treatment for durations of 0, 1, 5, 15, and 30 minutes at of 70°C

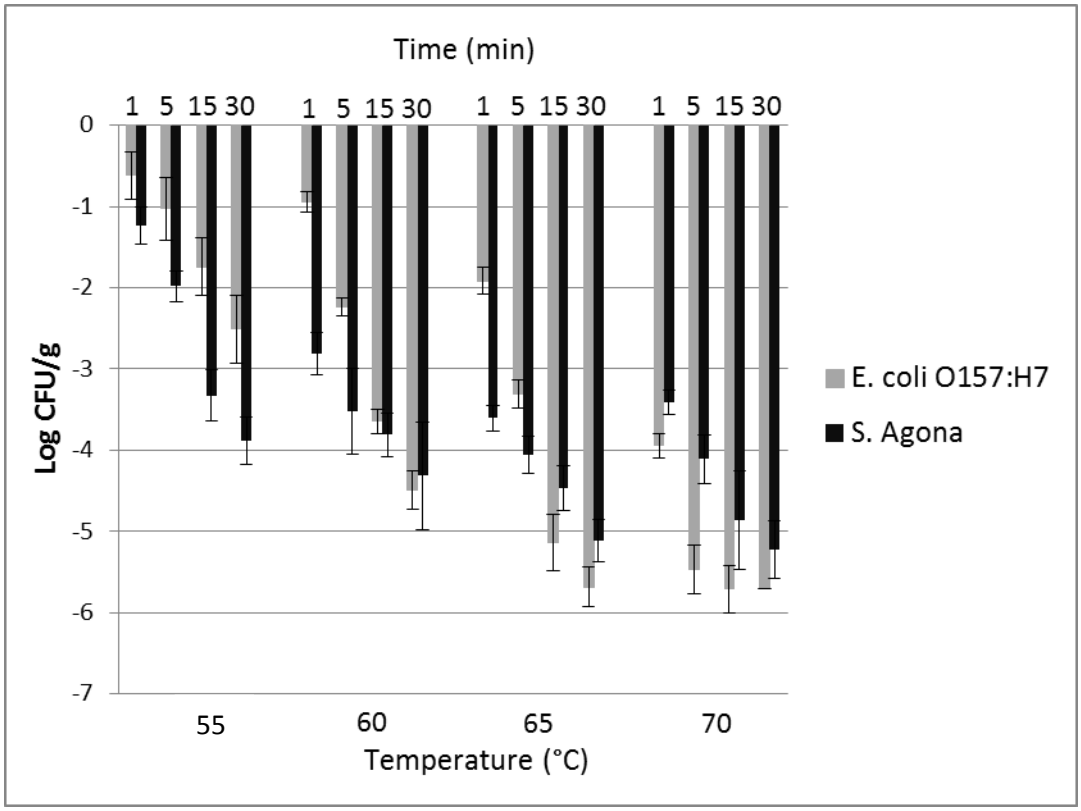


*Experimental data points represent mean population values (n = 12)

Table 4.5. Numerical values for k and β variables in Weibull function and Puesdo-R^2 for populations of *Escherichia coli* O157:H7 at various treatment temperatures

Treatment Temperature (°C)	**k**	**β**	**Pseudo-R^2**
55	0.4973	0.4602	0.9529
60	1.1629	0.4091	0.9925
65	2.1105	0.3075	0.9896
70	10.8196	0.0430	0.9817

Figure 4.2.2. Change in populations of *Escherichia coli* O157:H7 and *Salmonella* Agona in wheat flour as a result of thermal treatments at 55, 60, 65 or 70°C for 1, 5, 15 or 30 min



*Error bars indicate standard deviation of each treatment of duplicate treatments (n = 12)

Works Cited

1. Annonymous. 1993. *Salmonella* serotype Tennessee in powdered milk products and infant formula --- Canada and United States. p. 516-517. *In*, vol. 42. Morbility and Mortality Weekly Report.

2. Anonymous. 1998. Multistate outbreak of *Salmonella* serotype Agona infections linked to toasted oats cereal --- United States, April-May. p. 462-464. *In*, vol. 47. Morbitity and Mortality Weekly Report.

3. Anonymous. 2008-2009. Multistate outbreak of *Salmonella* Infections associated with peanut butter and peanut butter-containing products --- United States. p. 85-90. *In*, vol. 58.

4. Anonymous. 2010. Per capita annual wheat consumption - U.S. Available at: http://www.ers.usda.gov/briefing/wheat/consumption/html. Accessed June 6, 2011.

5. Anonymous. July 2009-April 2010. *Salmonella* Montevideo infections associated with salami products made with contaminated imported black and red pepper --- United States. p. 1647-1650. *In*, vol. 50. Morbidity and Mortality Weekly Report.

6. Archer, J., E. T. Jervis, J. Bird, and J. E. Gaze. 1998. Heat resistance of *Salmonella* Weltevreden in low-moisture environments. *Journal of food protection*. 61:969-973.

7. Association, N. A. M. 2010. Nutritional benefits of grain products. Available at: http://www.namamillers.org/nutrition_BenefitsofGrains.html. Accessed May 2, 2012.

8. Authority, O. H. 2011. Fresh strawberries from Washington County farm implicated in *E. coli* O157 outbreak in NW Oregon. Available at: http://www.oregon.gov/OHA/news/2011/2011-0808.pdf?ga=t. Accessed February 4, 2012.

9. Barbosa-Cánovas G.V. Food engineering - engineering properties of foods -. *In*, Encyclopedia of Life Support Systems (EOLSS) EOLSS Publishers, Oxford, UK.

10. Bigelow, W. D. 1921. The logarithmic nature of thermal death time curves. *The Journal of Infectious Diseases*. 29:528-536.

11. Black, D. G., F. Harte, and P. M. Davidson. 2009. *Escherichia coli* thermal inactivation relative to physiological state. *Journal of food protection*. 72:399.

12. Block, S. S. 2001. Disinfection, sterilization and preservation, 5th ed. Lippincott, Williams and Wilkins.

13. Boekel, M. A. 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International journal of food microbiology*. 74:139.

14. Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan. 2000. *Salmonella* nomenclature. *Journal of Clinical Microbiology*. 38:2465-7.

15. Brouard, C., E. Espié, F.-X. Weill, A. Kérouanton, A. Brisabois, A.-M. Forgue, V. Vaillant, and H. de Valk. 2007. Two consecutive large outbreaks of *Salmonella enterica* Serotype Agona infections in infants linked to the consumption of powdered infant formula. *The Pediatric Infectious Disease Journal*. 26:148-152 10.1097/01.inf.0000253219.06258.23.

16. Burnett, S. L., E. R. Gehm, W. R. Weissinger, and L. R. Beuchat. 2000. Survival of *Salmonella* in peanut butter and peanut butter spread. *Journal of Applied Microbiology*. 89:472-477.

17. CDC. *Escherichia coli* O157:H7 and other Shiga toxin-producing *Escherichia coli* (STEC). Available at: http://www.cdc.gov/nczved/divisions/dfbmd/diseases/ecoli_o157h7/. Accessed March 2, 2012.

18. CDC. Investigation information for outbreak of *Salmonella* Typhimurium infections, 2008–2009. Available at: http://www.cdc.gov/salmonella/typhimurium/. Accessed December 10, 2011.

19. CDC. 1993. Update: multistate outbreak of *Escherichia coli* O157:H7 infections from hamburgers -- western United States, 1992-1993. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/00020219.htm. Accessed May 4, 2011.

20. CDC. 1996. Outbreak of *Escherichia coli* O157:H7 infections associated with drinking unpasteurized commercial apple Jjuice -- British Columbia, California, Colorado, and Washington, October 1996. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/00044358.htm. Accessed March 1, 2012.

21. CDC. 1997. *Escherichia coli* O157:H7 infections associated with aating a nationally distributed commercial brand of frozen ground beef patties and burgers -- Colorado, 1997. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/00049079.htm. Accessed January 26, 2012.

22. CDC. 1999. Public health dispatch: outbreak of *Escherichia coli* O157:H7 and *Campylobacter* among attendees of the Washington County Fair -- New York, 1999. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4836a4.htm. Accessed Janurary 16, 2012.

23. CDC. 2002. Multistate outbreak of *Escherichia coli* O157:H7 infections associated with eating ground beef --- United States, June--July 2002. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5129a1.htm. Accessed January 16, 2012.

24. CDC. 2004. Salmonellosis - additional information : Surveillance. *In*.

25. CDC. 2006. Update on multi-state outbreak of *E. coli* O157:H7 infections from fresh spinach, October 6, 2006. Available at: http://www.cdc.gov/foodborne/ecolispinach/100606.htm. Accessed January 6, 2012.

26. CDC. 2007. Multistate outbreak of *E. coli* O157 infections linked to Topp's brand ground beef patties. Available at: http://www.cdc.gov/ecoli/2007/october/100207.html. Accessed Februsary 27, 20212. CDC. 2007.

27. Multistate outbreak of *Salmonella* serotype Tennessee infections associated with peanut butter --- United States, 2006--2007. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5621a1.htm. Accessed December 12, 2011.

28. CDC. 2007. *Salmonella* Schwarzengrund outbreak investigation, August 2007. Available at: http://www.cdc.gov/salmonella/schwarzengrund.html. Accessed February 28, 2012.

29. CDC. 2007. *Salmonella* Wandsworth outbreak investigation, June-July 2007. Available at: http://www.cdc.gov/salmonella/wandsworth.htm. Accessed February 28, 2012.

30. CDC. 2009. Multistate outbreak of *E. coli* O157:H7 infections linked to eating raw refrigerated, prepackaged cookie dough. Accessed June 6, 2011.

31. CDC. 2011. Investigation announcement: multistate outbreak of *E. coli* O157:H7 infections associated with Lebanon bologna. Available at: http://www.cdc.gov/ecoli/2011/O157_0311/index.html. Accessed March 6, 2012.

32. CDC. 2011. Investigation update: multistate outbreak of *E. coli* O157:H7 infections associtated with hazelnuts. Available at: http://www.cdc.gov/ecoli/2011/hazelnuts0157/index.html. Accessed February 28, 2012.

33. CDC. 2011. *Salmonella*. Available at: http://www.cdc.gov/salmonella/general/index.html. Accessed February 20, 2012.

34. CDC. 2012. *E. coli* Outbreak Investigations. Available at: http://www.cdc.gov/ecoli/outbreaks.html. Accessed May 1, 2012.

35. CDC. 2012. Shiga Toxin-Producing *Escherichia coli* (STEC) case surveillance. Available at: http://www.cdc.gov/nationalsurveillance/ecoli_surveillance.html. Accessed May 5, 2012.

36. Cerf, O. 1977. A review: tailing of survival curves of bacterial spores. *Journal of Applied Microbiology*. 42:1-19.

37. Chen, F. 2009. A genome map of *Salmonella* serovar Agona: numerous insertions and deletions reflecting the evolutionary history of a human pathogen. *FEMS Microbiology Letters*. 293:188-195.

38. Chen, Y., V.N. Scott, T.A. Freier, M. Moorman, J. Meyer, T. Morille-Hinds, Post, L. Smoot, L.A. Hood, S. Shebuski, and J. Banks, 2009. Control of *Salmonella* in low-moisture foods III:process validation and monitoring. p. 493-508. *In*, vol. 28(8). *Food Protection Trends*.

39. Clark, G., A., F. K. J. Gangarosa, and M. Thompson. 1973. Epidemiology of an international outbreak of *Salmonella* Agona. *The Lancet*. 302:490-493.

40. D'Aoust, J. Y. 1977. *Salmonella* and the chocolate industry. A review. p. 718-727. *In*, vol. 40. *Health Protection Branch, Health & Welfare Canada*.

41. D'Aoust, J. Y. 1985. Methods for the detection of foodborne *Salmonella* spp.: a review. *Southeast Asian Journal of Tropical Medicine & Public Health*. 26:195-208.

42. Danyluk, M. D., T. M. Jones, S. J. Abd, F. Schlitt-Dittrich, M. Jacobs, and A. J. Harris. 2007. Prevalence and amounts of *Salmonella* found on raw California almonds. *Journal of food protection*. 70:820-827.

43. Downes, F. P., and K. Ito, 2001. Compendium of methods for the microbiological examination of foods, 4th ed. American Public Health Association, Washington, D.C.

44. Doyle, M. P., and L.R.Beuchat, 2007. Food Microbiology: Fundamentals and Frountiers, 3rd. ed. ASM Press, Washington, D.C.

45. Dworkin, M. S. 2001. Reactive arthritis and Reiter's syndrome following an outbreak of gastroenteritis caused by *Salmonella enteritidis. Clinical Infectious Diseases*. 33:1010-1014.

46. Farret, E., F. Fine, and P. Gervais, 2005. Modeling of thermal microbial destruction of dried powder related to properties evolution and granulometry. *In*, COMSOL Multiphysics User's Conference, Paris, France.

47. FDA. 2009. Available at: http://www.fda.gov/NewsEvents/PublicHealthFocus/ucm169858.htm. Accessed May 16, 2011.

48. FDA. 2009. BBB- *Escherichia coli* O157:H7. Available at: http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/ucm071284.htm. Accessed March 3, 2012.

49. FDA. 2011. Available at: http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/ucm069966.htm. Accessed February 23, 2012.

50. Fine, F., and P. Gervais. 2005. A new high temperature short time process for microbial decontamination of seeds and food powders. *Powder Technology*. 157:108-113.

51. Glibert, S., R. Lake, P. Cressey, and N. King, 2010. Risky profile: *Salmonella* (non-typhoidal) in cereal grains. *In* Institute of Environmental Science and Research Limited, Christchurch Science Center.

52. Greenwood, M. H., and W. L. Hooper. 1983. Chocolate bars contaminated with *Salmonella* Napoli: an infectivity study. *BMJ*. 286:1394-1394.

53. Guinee, P. A., E. H. Kampelmacher, and H. M. Willems. 1961. Six new *Salmonella* types, isolated in Ghana (*S.* Volta, *S.* Agona, *S.* Wadsworth, *S.* Techimani, *S.* Mampong and *S.* tTafo). *Antonie Van Leeuwenhoek*. 27:469-72.

54. Hakansson, U. 1976. HLA-antigen B27 in cases with joint affections in an outbreak of salmonellosis. *Scandinavian Journal of Infectious Diseases*. 8:245-248.

55. Health, M. D. 2007. Salmonellosis, 2007. Available at: http://www.health.state.mn.us/divs/idepc/newsletters/dcn/sum07/salmonellosis.html. Accessed May 3, 2012.

56. Hensel, M. 2009. Bacterial secreted proteins: secretory mechanisms and role in athogenesis. Caister Academic Press.

57. http://digestive.niddk.nih.gov/ddiseases/pubs/bacteria/#4. Accessed June 3, 2011.

58. http://www.cjsan.gov/~ebam/bam-mm.html.. Accessed May 16, 2011.

59.http://www.foodbusinessnews.net/en/Opinion/Joshua%20Sosland/Understanding%20heat%20treated%20flour.aspx?p=1. Accessed May 3, 2011.

60. http://www.nestleusa.com/PubNews/PressReleaseLibraryDetails.aspx?id=DD7FA5AF-A9D5-484A-AB92-C08ADCFF52B0. Accessed May 3, 2011.

61. Huguette, J., S.M.M. Cohen, and W. Lin. 1996. PCR amplification of the fimA gene sequence of *Salmonella* typhimurium, a specific method for detection of *Salmonella* spp. *Applied and environmental microbiology*. 62:4303-4308.

62. Ireland, F. S. A. 2008. Report of an international outbreak Iinvestigation into a multi–country outbreak of *Salmonella* Agona, Europe, 2008.

63. Jagannath, A., T. Tsuchido, and J. M. Membre. 2005. Comparison of the thermal inactivation of *Bacillus subtilis* spores in foods using the modified Weibull and Bigelow equations. *Food Microbiology*. 22:233.

64. James, J. M., J.L. Martine, and D.A. Golden (ed.). 2005. Modern Food Microbiology, 7th ed. Springer, New York, N.Y.

65. Juneja, V. K., B. S. Eblen, and H. M. Marks. 2001. Modeling non-linear survival curves to calculate thermal inactivation of *Salmonella* in poultry of different fat levels. *International journal of food microbiology*. 70:37.

66. Kaletunc, G. 2007. Prediction of specific heat of cereal flours: a quantitative empirical correlation. *Journal of Food Engineering*. 82:589.

67. Kaur, Ledward, Park, and Robson. 1998. Factors affecting the heat resistance of *Escherichia coli* O157 : H7. *Letters in Applied Microbiology*. 26:325-330.

68. Kim, Y., R. Flores, and O. Chung, 2003. Physical and chemical characterization of wheat flour miling co-products. *Journal of Food Process Engineering*. 26:489-488.

69. Koch, J. 2005. *Salmonella* Agona outbreak from contaminated aniseed, Germany. *Emerging Infectious Diseases*. 11:1124-1127.

70. Kormendy, I., and L. Kormendy. 1997. Considerations for calculating heat inactivation processes when semilogarithmic thermal inactivation models are non-linear. *Journal of Food Engineering*. 34:33.

71. Lambert, R. J. W. 2003. A model for the thermal inactivation of micro-organisms. *Journal of Applied Microbiology*. 95:500-507.

72. Laroche, C. 2003. Unexpected thermal destruction of dried, glass bead-immobilized icroorganisms as a function of water activity. *Applied and Environmental Microbiology*. 69:3015-3019.

73. Laroche, C., F. Fine, and P. Gervais. 2005. Water activity affects heat resistance of microorganisms in food powders. *International journal of food microbiology*. 97:307-315.

74. Lehmacher, A., J. Bockemühl, and S. Aleksic. 1995. Nationwide outbreak of human salmonellosis in Germany due to contaminated paprika and paprika-powdered potato chips. *Epidemiology and infection*. 115:501-511.

75. Li, M., Z. Guodong, P. Gerner-Smidt, M. Vijaya, I. Ezeoke, and M. P. Doyle. 2009. Thermal inactivation of *Salmonella* in peanut butter. *Journal of food protection*. 72:1596-1601.

76. Lihan, H. 2009. Thermal inactivation of *Listeria monocytogenes* in ground beef under isothermal and dynamic temperature conditions. *Journal of Food Engineering*. 90:380.

77. Masao F. 2002. Phylogenetic analysis of *Salmonella, Shigella,* and *Escherichia coli* strains on the basis of the gyrB gene sequence. *Journal of Clinical Microbiology*. 40:2779-2785.

78. Mead, P. S., and P. M. Griffin. 1998. *Escherichia coli* O157:H7. *The Lancet*. 352:1207-1212.

79. Michael, R., D.A. B. Mulvey, L. Baker, O. Mykytczuk, E. M. F. Reis, and M. D. Asensi. 2004. Characterization of a *Salmonella enterica* serovar Agona strain harbouring a class 1 integron containing novel OXA-type b-lactamase (blaOXA-53) and 60-N-aminoglycoside acetyltransferase genes. *Journal of Antimicrobial Chemotherapy*. 54:354-359.

80. Milner, M., C. M. Christensen, and W. F. Geddes. 1947. Grain storage studies VI. wheat respiration in relation to moisture content, mold growth, chemical deterioration, and heating. *Cereal Chemistry*. 24:182-199 pp.

81. Murphy, R. Y., B. L. Beard, E. M. Martin, L. K. Duncan, and J. A. Marcy. 2004. Comparative study of thermal inactivation of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in ground pork. *Journal of Food Science*. 69:FMS97-FMS101.

82. N. Cary, V.D. Engleberg, S. Terence and S. Dermody. 2007. Schaechter's Mechanisms of Microbial Disease. Lippincott Williams and Wilkins, Philadelphia, PA.

83. Nickerson, J. J. 1963. Some observations on bacterial thermal death time curves. *Journal of Applied Microbiology*. 11:476-480.

84. Osaili, T. M., C. L. Griffis, E. M. Martin, B. L. Beard, A. E. Keener, and J. A. Marcy. 2007. Thermal inactivation of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in breaded pork patties. *Journal of Food Science*. 72:M56-M61.

85. Prevention, C. D. 2011, Investigation announcement: multistate outbreak of human *Salmonella* Agona infections linked to whole, fresh imported papayas. Available at: http://www.cdc.gov/salmonella/agona-papayas/072511/index.html. Accessed February 28, 2012.

86. S. Porwollik, C. Choy, P. Cheng, L. Florea, E. Proctor, and M. McClelland. 2004. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. *Journal of Bacteriology*. 186:5883-5898.

87. Shohat, T., M. S. Green, D. Merom, O. N. Gill, A. Reisfeld, A. Matas, D. Blau, N. Gal, and P. E. Slater. 1996. International epidemiological and microbiological study of outbreak of *Salmonella Agona* infection from a ready to eat savoury snack. *British Medical Journal*. 313:1107-1109.

88. Singh, P., R., D. R. Heldman, 2001. Introduction to Food Engineering, 3rd. Edition. Academic Press, San Diego, California.

89. Smith, S. E., J. L. Maurer, A. Orta-Ramirez, E. T. Ryser, and D. M. Smith. 2001. Thermal inactivation of *Salmonella* spp., *Salmonella* Typhimurium DT104, and *Escherichia coli* O157:H7 in ground beef. *Journal of Food Science*. 66:1164.

90. Sperber, W. H. 2003. Microbiology of milled cereal grains: issues in customer specifications. p. 7929-7931. *In*, vol. 3. International Association of Operating Millers' Technical Bulletin.

91. Sperber, W. H., and G. North American Millers' Association Microbiology Working. 2007. Role of microbiological guidelines in the production and commercial use of milled cereal grains: a practical approach for the 21st century. *Journal of food protection*. 70:1041.

92. Staack, N., L. Ahrné, E. Borch, and D. Knorr. 2008. Effect of infrared heating on quality and microbial decontamination in paprika powder. *Journal of Food Engineering*. 86:17-24.

93. Thomas, C. S. Date, 2011, New ConAgra System Tackles Raw Flour Risks. Available at: http://www.foodnavigator-usa.com/Market/New-ConAgra-system-tackles-raw-flour-risks. Accessed June 14, 2011.

94. Thorpe, C. M. 2004. Shiga Toxin—Producing *Escherichia coli* Infection. *Clinical Infectious Diseases*. 38:1298-1303.

95. Todar, K. 2008. *Salmonella.* Available at: http://textbookofbacteriology.net/salmonella.html. Accessed February 28, 2012.

96. Tuttle, J., T. Gomez, M. P. Doyle, J. G. Wells, T. Zhao, R. V. Tauxe, and P. M. Griffin. 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiology and infection*. 122:185-192.

97. USDA-ERS. 2010. Available at: http://www.ers.usda.gov/data/foodborneillness/html. Accessed June 6, 2011.

98. USDA-FSIS. 2002. New measures to address *E. coli* O157:H7 contamination. Accessed March 3, 2012.

99. USDA-FSIS. 2011. Microbiological testing program for *Escherichia coli* O157:H7. Available at: http://www.fsis.usda.gov/science/ground_beef_e.coli_testing_results/index.asp#1. Accessed March 3, 2012

100. Whitt, A. A. 2002. Bacterial Pathogenesis: A Molecular Approach. ASM Press, Washington, DC.

101. Xiong, R., G. Xie, A. E. Edmondson, and M. A. Sheard. 1999. A mathematical model for bacterial inactivation. *International journal of food microbiology*. 46:45.