Food safety interventions in the bakery industry: microbial safety from wheat milling to finished baked products

by

Daniel Vega

B.S., Universidad de Costa Rica, 2012
M.S., Kansas State University, 2016

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science Institute
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Abstract

Validation studies are a critical tool to control pathogens in food products for human consumption. Data from these studies lay the scientific foundation for strong food safety programs in processing plants to better ensure public health. Even though flour has been considered for years as microbiologically safe, there have been recent foodborne outbreaks associated with flour. Therefore, the first objective of this work was to validate the safety of flour during wheat tempering by the use of water or lactic acid (LA) at five different tempering temperatures (ambient, 120°, 130°, 140°, and 150°F) at two inoculation levels. Ambient water treatment resulted in a 0.4 log CFU/g reduction over 24 h of tempering at both inoculation levels, whereas ambient LA tempering reduced the *Salmonella* population by 1.2 and 1.4 log cycles at T0 (immediately after application) for low and high inoculation levels, respectively. By 12 h of ambient tempering of low-level inoculated wheat, only 1 of 3 replications indicated residual *Salmonella* viability, while two were negative by enrichment. For the high-inoculation level wheat, a 2.4 log CFU/g reduction was observed after 12 h and no further reductions were noted up to 24 h of tempering. For high inoculation level wheat, increasing tempering temperature for LA application to 130°F resulted in >5 log CFU/g reductions by 4 h, and no *Salmonella* was detected by enrichment at 140 and 150°F at any tempering time. The second to fourth objectives of this research were to validate a representative commercial oven baking process for the manufacture of cheesecake, fruit-filled pastries, and peanut butter bars against mixed inocula of pathogens. For cheesecake, it was found that the mean internal temperature increased from 17°C to ~97°C at the end of 50 min baking. The *Salmonella* population in cheesecake decreased by >5 log CFU/g by 37.5 min of baking and was completely eliminated after 50 min of baking (as determined by enrichment). The pH and water activity of cheesecake after baking and cooling

were 4.86 and 0.943, respectively. D-values of the *Salmonella* cocktail at 55, 58 and 61°C were 27.4, 13.8 and 4.9 min, respectively, whereas the *z*-value of the *Salmonella* cocktail was 8.2°C. For fruit-filled pastries, the mean internal temperature increased from 36.7°C to ~101°C at the end of 15 min baking. The *Salmonella* population in pastries decreased by >5 log CFU/g by 9 min of baking and was completely eliminated after baking and cooling (as determined by enrichment). The pH and water activity of pastries after baking and cooling were 5.33 and 0.677, respectively. D-values of the *Salmonella* cocktail at 55, 58 and 61°C were 32.8, 15.5 and 5.3 min, respectively, with a *z*-value of 7.7°C for the *Salmonella* cocktail determined. For peanut butter bars, internal temperature of the bars increased from ~25°C to ~91°C during 13 min of baking. *Salmonella,* Shiga toxin-producing *E. coli*, and *L. monocytogenes* population reductions ($P \leq 0.05$) were 2.4, 3.0, and 3.9 logs CFU/g, respectively, compared to raw dough levels. Water activity of bars decreased from 0.81 to 0.70, while pH increased from 7.0 to 8.7 during baking. Respective D-values (min) for *Salmonella* and STEC in a_w-adjusted dough were 9.4 and 10.2 at 85°C; 3.9 and 3.3 at 90°C; and 1.7 and 1.7, at 92.5°C, respectively. The calculated *z*-values for *Salmonella* and STEC were 10.1 and 10.1 respectively. The fifth study of this work involves the comparison of a hamburger inoculation study versus a surface inoculation study. During this research we obtained a 5.7 log CFU/g reductions of the hamburger surface inoculated bun versus a full lethality in a previous study, demonstrating differences between crust and crumb. The a_w of the crust decreased significantly ($p \leq 0.05$) during baking, while the crumb a_w had a similar value at the end of baking. This study demonstrates the importance of evaluating lethality differences between surface and internal parts of a bakery product.

Food safety interventions in the bakery industry: microbial safety from wheat milling to finished bakery products

by

Daniel Vega

B.S., Universidad de Costa Rica, 2012
M.S., Kansas State University, 2016

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science Institute
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Approved by:

Major Professor
Randall K. Phebus

Copyright

© Daniel Vega 2021

Abstract

Validation studies are a critical tool to control pathogens in food products for human consumption. Data from these studies lay the scientific foundation for strong food safety programs in processing plants to better ensure public health. Even though flour has been considered for years as microbiologically safe, there have been recent foodborne outbreaks associated with flour. Therefore, the first objective of this work was to validate the safety of flour during wheat tempering by the use of water or lactic acid (LA) at five different tempering temperatures (ambient, 120°, 130°, 140°, and 150°F) at two inoculation levels. Ambient water treatment resulted in a 0.4 log CFU/g reduction over 24 h of tempering at both inoculation levels, whereas ambient LA tempering reduced the *Salmonella* population by 1.2 and 1.4 log cycles at T0 (immediately after application) for low and high inoculation levels, respectively. By 12 h of ambient tempering of low-level inoculated wheat, only 1 of 3 replications indicated residual *Salmonella* viability, while two were negative by enrichment. For the high-inoculation level wheat, a 2.4 log CFU/g reduction was observed after 12 h and no further reductions were noted up to 24 h of tempering. For high inoculation level wheat, increasing tempering temperature for LA application to 130°F resulted in >5 log CFU/g reductions by 4 h, and no *Salmonella* was detected by enrichment at 140 and 150°F at any tempering time. The second to fourth objectives of this research were to validate a representative commercial oven baking process for the manufacture of cheesecake, fruit-filled pastries, and peanut butter bars against mixed inocula of pathogens. For cheesecake, it was found that the mean internal temperature increased from 17°C to ~97°C at the end of 50 min baking. The *Salmonella* population in cheesecake decreased by >5 log CFU/g by 37.5 min of baking and was completely eliminated after 50 min of baking (as determined by enrichment). The pH and water activity of cheesecake after baking and cooling

were 4.86 and 0.943, respectively. D-values of the *Salmonella* cocktail at 55, 58 and 61°C were 27.4, 13.8 and 4.9 min, respectively, whereas the *z*-value of the *Salmonella* cocktail was 8.2°C. For fruit-filled pastries, the mean internal temperature increased from 36.7°C to ~101°C at the end of 15 min baking. The *Salmonella* population in pastries decreased by >5 log CFU/g by 9 min of baking and was completely eliminated after baking and cooling (as determined by enrichment). The pH and water activity of pastries after baking and cooling were 5.33 and 0.677, respectively. D-values of the *Salmonella* cocktail at 55, 58 and 61°C were 32.8, 15.5 and 5.3 min, respectively, with a *z*-value of 7.7°C for the *Salmonella* cocktail determined. For peanut butter bars, internal temperature of the bars increased from ~25°C to ~91°C during 13 min of baking. *Salmonella,* Shiga toxin-producing *E. coli*, and *L. monocytogenes* population reductions ($P \leq 0.05$) were 2.4, 3.0, and 3.9 logs CFU/g, respectively, compared to raw dough levels. Water activity of bars decreased from 0.81 to 0.70, while pH increased from 7.0 to 8.7 during baking. Respective D-values (min) for *Salmonella* and STEC in a_w-adjusted dough were 9.4 and 10.2 at 85°C; 3.9 and 3.3 at 90°C; and 1.7 and 1.7, at 92.5°C, respectively. The calculated *z*-values for *Salmonella* and STEC were 10.1 and 10.1 respectively. The fifth study of this work involves the comparison of a hamburger inoculation study versus a surface inoculation study. During this research we obtained a 5.7 log CFU/g reductions of the hamburger surface inoculated bun versus a full lethality in a previous study, demonstrating differences between crust and crumb. The a_w of the crust decreased significantly ($p \leq 0.05$) during baking, while the crumb a_w had a similar value at the end of baking. This study demonstrates the importance of evaluating lethality differences between surface and internal parts of a bakery product.

Table of Contents

List of Figures ... xiii
List of Tables ... xv
Acknowledgements ... xvi
Dedication ... xvii
Chapter 1 - Introduction ... 1
Chapter 2 - Literature Review ... 4
Foodborne Illnesses ... 4
Listeria monocytogenes ... 8
Salmonella spp. ... 10
Escherichia coli ... 12
EPEC ... 13
EIEC ... 13
ETEC ... 14
EAEC ... 14
DAEC ... 14
EHEC ... 15
Flour Production ... 17
Bakery Industry ... 20
FSMA Validation in the Bakery Industry ... 21
Hazard Analysis ... 22
Preventive Controls ... 22
Oversight and Management of Preventive Controls ... 22
Supply Chain Program ... 23
Recall Plan ... 23
Impact of FSMA in Commercial Bakery Operations ... 24
Validation Studies and Research Importance ... 24
Chapter 3 - Research Questions ... 27
Experiment 1: Development of a Commercially Applied Antimicrobial Intervention Process During Wheat Milling ... 27

Experiment 2: Validation of a Simulated Commercial Baking Process to Control *Salmonella* spp. in Fruit-Filled Pastries ... 27
Experiment 3: Validation of a Simulated Commercial Baking Process to Control *Salmonella spp*. in Cheesecake ... 27
Experiment 4: Validation of a Simulated Commercial Baking Process to Control *Salmonella spp., STEC*, and *L. monocytogenes* in Peanut Butter Bars ... 28
Experiment 5: A Comparative Study of a Published Baking Validation Study versus a Surface Inoculation Study of Hamburger Bun Baking ... 28
Chapter 4 - Development of a Commercially Applied Antimicrobial Intervention Process During Wheat Milling ... 29
Introduction ... 29
Objectives ... 32
Materials and Methods ... 32
Experimental Design ... 32
Bacterial Cultures ... 32
Kernel Inoculation ... 34
Chemical Preparation ... 34
Tempering Process ... 35
pH Monitoring of Treated Kernels ... 36
Microbial Sampling and *Salmonella* Enumeration ... 37
Statistical Analysis ... 38
Results and Discussion ... 39
Heating Profile of the Kernels ... 40
pH of the Treated Kernels ... 41
Salmonella spp. Reductions by Tempering Treatments ... 43
10^4 CFU/g Inoculation Level Results ... 43
10^6 CFU/g Inoculation Level Results ... 45
Overall Results ... 48
Conclusions ... 49
Chapter 5 - Validation of a Simulated Commercial Baking Process to Control *Salmonella* spp. in Fruit-Filled Pastries ... 50

Introduction 50
Materials and Methods 51
Experimental Design 51
Bacterial Cultures 52
Flour Inoculation 53
Dough Preparation 54
Baking of Fruit-Filled Pastries 55
Thermal Destruction Parameters 56
Salmonella Enumeration and Enrichments 56
Statistical Analyses 57
Results and Discussion 57
Conclusion 63
Chapter 6 - Validation of a Simulated Commercial Baking Process to Control *Salmonella* spp. in Cheesecake 64
Introduction 64
Materials and Methods 65
Experimental Design 65
Bacterial Cultures 66
Flour Inoculation 67
Batter Preparation 67
Cheesecake Baking 68
Thermal Destruction Parameters 70
Bacterial Enumeration and Enrichment 70
Statistical Analyses 71
Results and Discussion 71
Conclusion 77
Chapter 7 - Validation of a Simulated Commercial Baking Process to Control *Salmonella* spp., Shiga Toxin-producing *E. coli,* and *L. monocytogenes* in Peanut Butter Bars 79
Introduction 79
Materials and Methods 81
Experimental Design 81

Bacterial Cultures 82
Flour Inoculation 83
Dough Preparation 84
Peanut Butter Bar Baking 84
Thermal Destruction Parameters 86
Bacterial Enumeration 87
Statistical Analyses 87
Results and Discussion 88
Conclusion 96
Chapter 8 - A Comparative Study of a Published Validation Study versus a Surface Inoculation Study of Hamburger Buns to better understand *Salmonella* lethality under dynamic matrix changes during baking 98
Introduction 98
Material and Methods 98
Bacterial Cultures 98
Hamburger Bun Preparation 99
Hamburger Bun Inoculation 100
Hamburger Buns Baking 101
Bacterial Enumeration and Enrichment 102
Results and Discussion 102
Conclusions 107
Chapter 9 - Conclusion And Future Research 109
References 112
Appendix A - 7- serovar *Salmonella* cocktail counts in pastry dough *vs.* heating time as plated on injury-recovery media (BHI agar overlaid with XLD agar). 125
Appendix B - Log D-values of 7- serovar *Salmonella* cocktail in pastry dough *vs.* dough temperature (°C) as calculated from counts on injury-recovery media (BHI agar overlaid with XLD agar). 126
Appendix C - 7- serovar *Salmonella* cocktail counts in cheesecake batter *vs.* heating time as plated on injury-recovery media (BHI agar overlaid with XLD agar). 127

Appendix D - Log D-values of 7-serovar *Salmonella* cocktail in cheesecake batter *vs.* batter temperature (°C) as calculated from counts on injury-recovery media (BHI agar overlaid with XLD agar). 128
Appendix E - 7- serovar *Salmonella* cocktail counts in peanut butter bars *vs.* heating time as plated on injury-recovery media (BHI agar overlaid with XLD agar). 129
Appendix F - Log D-values of 7- serovar *Salmonella* cocktail in peanut butter bars *vs.* batter temperature (°C) as calculated from counts on injury-recovery media (BHI agar overlaid with XLD agar). 130
Appendix G - 7-serotype *E. coli* cocktail counts in peanut butter bars vs. heating time as plated on injury-recovery media (BHI agar overlaid with MacConkey agar). 131
Appendix H - Log D-values of 7-serotype *E. coli* cocktail in peanut butter bars *vs.* batter temperature (°C) as calculated from counts on injury-recovery media (BHI agar overlaid with XLD agar). 132

List of Figures

Figure 4-1 Wheat temperature at different mixing time points in a Thermomix set at 100°C and a speed setting of 2. 40

Figure 4-2 *Salmonella* populations at 10^4 inoculation level before and after treatment combinations of water (W) or lactic acid (LA) at different temperatures at each sampling point. 45

Figure 4-3 *Salmonella* populations at 10^6 inoculation level before and after treatments combinations of water (W) or lactic Acid (LA) and different temperatures at each sampling point. 48

Figure 5-1 Mean temperature profile of pastries during oven baking (±SE) at 375°F (190.6°C) for 15 min of baking + 30 min ambient air cooling. 59

Figure 5-2 Relative humidity within oven during baking of fruit-filled pastries with average (± SE) baked at 375°F (190.6°C) for 15 minutes. 60

Figure 5-3 Average *Salmonella spp.* log counts and internal temperature (°C) of fruit-filled pastries during 15 min of baking at 375°F (190.6°C) oven temperature followed by 30 min of ambient air cooling (B+ C). 61

Figure 6-1. Mean temperature profile of cheesecake in oven during baking (±SE) at 300°F (148.9°C) for 50 min followed by 30 min ambient air cooling. 73

Figure 6-2. Relative humidity of the oven during cheesecake baking (with average ± SE) at 300°F (148.9°C) for 50 minutes. 74

Figure 6-3. Average *Salmonella spp.* log counts and internal temperature (°C) of cheesecake during 50 min of baking at 300°F (148.9°C) oven temperature followed by 30 min of ambient air cooling (B+ C). 75

Figure 7-1. Mean temperature profile of peanut butter bars during oven-baking (±SE) at 350°F (176°C) 13 min + 15 min of ambient air cooling. 90

Figure 7-2. Relative oven humidity during baking of peanut butter bars (± SE) baked at 350°F (176°C) for 13 minutes. 91

Figure 7-3. Average log counts and internal product temperature (°C) of peanut butter bars during 13 min of baking at 350°F (176°C) oven temperature followed by 15 min of ambient air cooling (B+ C). 92

Figure 8-1 Relative humidity of oven at 425°F (218.3°C) (± SE) during baking of hamburger buns for 13 minutes. 104

Figure 8-2 Average recovered *Salmonella* populations from surface region (upper 2 mm) and internal temperature (°C) of hamburger buns during 10 min of baking at 425°F (218.3°C). 105

List of Tables

Table 4-1 pH values for treated kernels at different sampling points. 42
Table 5-1 Ingredient list and weight of fruit-filled pastries sweet dough recipe.* 54
Table 5-2 pH and a_w of fruit-filled pastry dough during 15 minutes of baking at 375°F (190.6°C) oven temperature followed by 30 min of ambient air cooling (B+ C). 57
Table 5-3 D- and z- values for *Salmonella* in pastry dough at three different temperatures. 62
Table 6-1 Ingredient list and weights of the cheesecake recipe. 68
Table 6-2 pH and a_w of cheesecake dough during 50 minutes of baking at 300°F (148.9°C) oven temperature followed by 30 min of ambient air cooling (B+ C). 72
Table 6-3 D- and z- values for a 7-serovar *Salmonella* cocktail in cheesecake batter at three different temperatures. 76
Table 7-1 Ingredient list and weights for the peanut butter bars recipe.* 84
Table 7-2 pH and a_w of peanut butter bars during 13 minutes of baking at 350°F (176°C) oven temperature followed by 15 min of ambient air cooling (B+ C). 88
Table 7-3 D- and z- values for *Salmonella* and STEC for peanut butter bars at three different temperatures. 95
Table 8-1 Ingredient list and weight of the hamburger buns recipe.* 100
Table 8-2 a_w of crust and crumb, internal bun temperature, and oven temperature during baking of hamburger buns at 425°F (218.3°C). 103
Table 8-3 *Salmonella* log CFU/g of surface inoculated hamburger buns during baking at 425°F (218.3°C) compared to the previous study by Channaiah et al., (27) 106
Table 9-1 pH, a_w, target bacterial population, and bacterial population reductions of the different bakery research products investigated by our research group. 110

Acknowledgements

My dissertation and graduate student years would have not been possible without my family, friends, and mentors that have helped during these years. I want to thank Dr. Randall Phebus for supporting and helping me in every possible way to reach to this point. I would have not reached this point without your constant support and mentorship. Thank you for believing in me and giving me an opportunity to start this journey. I want to thank to Dr. Sara Gragg for your constant support and aid every time I needed it. Thanks Dr. Harshavardhan Thippareddi for your support and always willing to help. Thanks Dr. Paola Paez for being part of this journey since my bachelor's degree, thank you for believing in me during all these years.

I want to give thanks for my family that have always believed and supported me even when I thought I could not do it anymore. There have been some good and not so good moments along the way, but you were always ready to support me in whatever way I could possibly needed. I have learned so much from both of my parents but probably the most important lesson was to never give up and follow my dreams.

There has been many persons who helped me a lot during my graduate studies that taught me something, so I want to thank every person in the Food Science Institute that aided me in this journey from graduate students, undergraduate students, and interns and I can assure you that I tried to learn something from each of you.

Dedication

I dedicate my dissertation to my parents, brothers, nephew, and my friends that have supported and helped me along this path. Thank you all.

Chapter 1 - Introduction

The American Bakers Association (ABA) estimates that the bakery industry has a total economic impact of $154 billion dollars per year with total sales in the United States of $480 billion dollars (*3*). These numbers equal 2.46% of the U.S. GDP in 2020 (*3*). In 2020 it was estimated that the bakery industry generated 764,777 direct jobs and 1,528,016 indirect jobs (*3*). Therefore, the bakery industry has a great impact in the American economy and hires over 2 million persons between direct and indirect jobs. These jobs and the future of bakeries rely upon the food safety of their products.

When the Food Safety Modernization Act (FSMA) was signed into law, a new set of regulations were required for food producers across the United States, and bakeries were not exempted. Bakers and bakeries must comply with the new set of regulations to be in accordance with the new rule. The bakery industry is a very fragmented industry where a big percentage of the bakeries are considered small. Most of these small companies do not possess the resources to validate each of the bakery products being made in their bakeries. Therefore, it is highly important to develop tools that can help small to large bakeries and bakers to help comply with the new set of regulations established in the FSMA. One of the most important aspects included in FSMA is the utilization of validation studies, defined as studies that evaluate the ability of a specific food processing process or food formulation that will lead to the inactivation of bacterial pathogens (*85*). Currently, there is a lack of validation studies that ensure the safety of flour and different flour-containing bakery products.

The main ingredients required to produce bakery products come from cereals like wheat, rye, corn, among others. Cereals like wheat have previously been linked to different pathogenic

bacteria including *Salmonella, Escherichia coli,* and *Bacillus cereus* (*74*). Potential microbiological contamination of different cereal grains is a major concern for the industry, due to the impact on quality and properties of the grains (*74*). Microorganisms present in cereals are expected to be the same as the soil, storage containers, and those microorganisms present during harvesting. Contamination may come from sources like dust, water, insects, birds, pests, and others (*63*, *74*). Therefore, it is important to ensure the safety of the grains and the process of grain milling to produce flour that has lower risks of being contaminated.

Flour has been considered for years as microbiologically safe; however, there have been recent foodborne outbreaks associated to flour; therefore, there is a need to validate the safety of flour during its production and of flour as a main ingredient for bakery products (*2*, *35*, *51*, *83*, *116*). Several authors have performed screening assays to identify possible contaminants in wheat grain and flour, and found different composition of bacterial microbiota like *Serratia, Pantoea, Pseudomonas, Aeromonas, Escherichia, Enterobacter, Raoutella, Bacillus*, and some unidentified bacteria (*51*). Flour reported outbreaks have occurred in Canada, New Zealand, and the U.S.A. (*21*, *51*, *77*, *116*). Some of the recalled flour was found to be positive for *stx* genes and positive for *E. coli* O121 (*7*, *35*, *51*). Even though there is a reported low incidence of pathogens present in flour, this ingredient has still been linked to recent outbreaks of *Salmonella* Typhimurium phage type 42, *E. coli* O121, and *E. coli* O26 (*21*, *51*, *77*, *116*). The *Salmonella* Typhimurium phage type 42 outbreak in New Zealand in 2008 is considered to be the first outbreak to be confirmed by laboratory and epidemiologic investigation linked to the consumption of contaminated flour (*77*). Due to the recent outbreaks and the widespread use of flour by households, bakeries, and the food service industry, it is important to develop methods to ensure flour safety.

Bakery products have also been considered to be safe, but there have been multiple outbreaks related to baked goods including pastries, zeppoles, cream filled baked goods, among others (*124*, *127*, *128*). According to foodborne outbreak reports between 2004 and 2013, bread and bakery products caused 142 outbreaks and a total of 2,822 foodborne illnesses (*12*).

The bakery industry has stated that bakery products are not considered as high risk products and that most of the recalls are due to allergen mislabeling; however, there have been previous reports of bacterial contamination (*54*). Validated food safety plans to control bacterial contamination are required for food safety. Leaders inside the bakery industry have mentioned that it is common knowledge among the industry that the standard commercial oven or frying steps destroy pathogenic microorganisms, but scientifically validating it is important for the development of the food safety plan (*54*). These validation studies will vary depending on the risk associated with each product and must be assessed through the preventive control steps. According to different authors, there are no current scientific validation studies that assess the lethality of baking and frying of baking products (*28*, *100*, *105*, *113*). The development of baking validation studies that assess pathogen lethality specific for bakery products is important to give the bakeries the tools required to abide with FSMA regulations.

Hence, there is a need to fill the gaps in validation studies to ensure the safety of the products being produced in bakeries and by home bakers across the United States. There is a current need to develop alliances between bakers and research institutions to develop validation studies to assess the lethality of pathogens during the baking process in a variety of different products (*4*). During the development of the present work, we will be discussing validation studies for flour decontamination during wheat tempering and different bakery products that will provide the tools required by the industry to ensure the safety of their baked goods.

Chapter 2 - Literature Review

Foodborne Illnesses

According to the World Health Organization (WHO) "foodborne diseases are caused by contamination of food and occur at any stage of the food production, delivery, and consumption chain" (*119*). Foodborne diseases can result from different forms of contamination like polluted water, soil, air, and unsafe practices of production and storage (*119*). Most of these diseases will cause gastrointestinal problems, but some of them also cause gynecological, immunological, and neurological symptoms (*119*). The economic burden of foodborne illnesses is estimated to be of $95.2 billion in productivity loss and $15 billion for treating these diseases annually (*118*).

In the United States the Centers for Disease Control and Prevention (CDC) classifies foodborne illnesses into two major groups known as foodborne pathogens and unspecified agents (*17*). The known foodborne pathogens consist of 31 pathogens that includes bacteria, parasites, and viruses, many of which are tracked by public health systems. The unspecified agents are agents with lack of data to estimate their burden, unidentified agents like microbes, chemicals or other substances whose ability to cause a disease has not been proven (*17*). It is estimated that the known agents cause approximately 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths each year (*14*). It is also estimated that the unspecified agents in food in the US cause an additional 38.4 million gastroenteritis illnesses, 78,878 hospitalizations, and 1,686 deaths each year. Combined, these estimates total of 47.8 million illnesses, 127,839 hospitalizations and 3,037 deaths each year related to foodborne illnesses (*14*, *42*).

The top five pathogens that contribute to domestically acquired foodborne illnesses are norovirus, nontyphoidal *Salmonella*, *Clostridium perfringens, Campylobacter spp.* and *Staphylococcus aureus* (*17*). Nontyphoidal *Salmonella, norovirus, Campylobacter,* and *Toxoplasma* are the agents the cause the highest number of hospitalizations (*14*). Nontyphoidal *Salmonella*, *Toxoplasma*, *Listeria* and norovirus are the leading causes of deaths caused by foodborne illnesses(*17*).

Foodborne illnesses occur when people ingest a drink or food that is contaminated with a pathogen, a chemical or a toxin (*42*). Although most of these infections are typically caused by pathogens transmitted from human to human, others occur because of transmission from animals to humans. This is what we call a zoonotic pathogen or disease. Some of the most common and studied zoonotic pathogens are *Escherichia coli, Salmonella spp. Campylobacter spp.* and *Listeria monocytogenes* (*71*).

These same four pathogens are constantly surveyed by the CDC, where they are tracked to estimate the percentage of foodborne outbreaks attributed to these pathogens (*61*). This surveillance identified 3,981 outbreaks from 1998 through 2018 that either were confirmed or suspected to be caused by *Escherichia coli, Salmonella spp. Campylobacter spp.* or *Listeria monocytogenes* (*61*). Of those outbreaks, only 1,459 were confirmed or suspected to be linked to a specific food category, with 905 caused by *Salmonella spp.* 225 by *E. coli* O157*,* 44 by *Listeria,* and 255 by *Campylobacter* (*61*). This report demonstrated that *Salmonella spp*. was attributed to multiple food categories, *E. coli* O157 was mainly attributed to vegetable row crops and beef; *Listeria* was mainly attributed to dairy and fruits; and *Campylobacter* was linked to chicken but was broadly attributed to multiple food categories (*61*). Many outbreaks could not be linked to a specific agent and some illnesses could not be traced back to a specific etiological

agent. Some persons could be asymptomatic which further complicates the traceability efforts (*64*). If we look at the illness numbers by food category, 5.0% of the bacterial foodborne illnesses can be attributed to the grains-beans category (*88*). All of these pathogens are leading causes of diseases and can lead to severe complications and in some cases to death. Therefore, we need to aim for efforts to control and reduce pathogen levels of products that are produced for human consumption.

Efforts have been made to mitigate the incidence of foodborne pathogens. Some of these programs have been successful in decreasing the incidence of illnesses in the United States. Some authors claim that *E. coli* O157 illnesses have decreased compared to previous years (*31*). CDC stated that in 2009 *E. coli* O157 caused the lowest incidence of disease since 2004, and they attributed the success to efforts to control this pathogen since the 1990's (*13*). In a more recent report, CDC mentioned O157 infections decreased by 20% in 2019 compared to the previous three years; while the non-O157 Shiga toxin-producing *E.coli* (STEC) infections increased by 35% in the same period (*108*). These trends could be attributed to changes in regulatory policy where STEC are considered adulterants, and inspections are routinely looking for the presence of these pathogens in the food system. Others claim that making *E. coli* O157 an adulterant contributed to a decrease in the contamination of the food supply; therefore, a reduction in incidence of illness caused by these pathogens (*31*). However, according to a 2020 report from CDC the overall incidence of STEC increased in 2019 compared to the previous three years, which can be attributed to an increase in the non-O157 infections (*108*).

According to CDC reports, the incidence of *Salmonella spp*. remained unchanged in 2019 compared to 2016-2018 (*108*). Changes in *Salmonella spp*. incidence rates were serovar dependent; where Typhimurium and I 4,[5],12:i:- decreased, while Infantis was significantly

higher (69% increase) (*108*). One specific serovar that is important to highlight is *S*. Enteritidis, which is the most common cause of *Salmonella* infection since 2007. Foodborne illness from this serotype has not decreased (*108*). Enteritidis is commonly found in eggs and chicken. *Listeria* was one of the other pathogens where the number of infections in 2019 compared to the last three previous years did not change (*108*). The overall data shows that the target of Healthy People 2020 for reducing the number of foodborne illnesses was not met (*108*).

In the next section of this chapter, we will further discuss the pathogens studied in this research.

Listeria monocytogenes

Listeria monocytogenes belongs to the family of *Listeriaceae* which is divided into 6 species: *L. monocytogenes, L. innocua, L. welshnieri, L. seegleri, L. ivanovii,* and *L. grayi. Listeria monocytogenes* is the main pathogen of the genus (*8*). *Listeria* is a gram positive facultative pathogen that is motile by means of a flagellum. *Listeria* was initially isolated from rabbits and it has been found in ruminants, pigs, cats, and dogs (*56*, *71*). *Listeria* is considered to be ubiquitous and can occur in almost all raw food materials (*8*). One of the traits that has caused *Listeria* to cause so many foodborne outbreaks is its capability to survive and grow at temperatures as low as -0.4°C. Currently, there is a zero-tolerance policy for *Listeria* present in ready-to-eat foods due to its high mortality rate compared to other foodborne pathogens (*8*, *56*, *71*).

L. monocytogenes is one of the leading causes of death from foodborne pathogens (*56*). The severe infection can have a mortality rate of up to 30%, and if *Listeria*l meningitis occurs, the lethality can go up to 70% (*56*). It is estimated that the infectious dose to produce disease is less than 1,000 cells; however, the infectious dose varies across strains and susceptibility of individuals (*56*). The median illness incubation period for this pathogen is 3 weeks, but it can range from 3 to 70 days (*71*). This pathogen causes up to 2,500 illnesses and up to 500 deaths per year in the United States alone (*56*).

Listeriosis, which is the disease caused by *Listeria,* can cause serious complications in pregnant women, elderly, newborns, and individuals who are immunocompromised (*8*, *71*). In the case of pregnant women, the symptoms might be similar to a mild flu which can be followed in the upcoming days to week in abortion, stillbirths, septicemia of the newborn, or premature

birth (*71*). In the case of the newborns, they can be infected through the uterus or with surrounding bacteria in the vagina during the delivery of the newborn (*71*). Infants can develop symptoms at birth or a few weeks after. Some of the medical conditions associated with listeriosis are granulomatosis, septicemia, meningitis, or respiratory diseases (*71*). In the case of immunocompromised or elderly persons, *Listeria* can cause meningitis, meningoencephalitis, and less commonly septicemia (*71*).

Listeria spp. is salt tolerant and it has the ability to colonize food processing environments in moist and chilled conditions (*8*, *56*). This ability allows *Listeria* to contaminate a wide variety of different food products. *Listeria* has caused multiple outbreaks in humans coming from different food sources like milk, hot dogs, turkey meat, and others (*56*).

Listeria can survive in chilled environments and has been associated with salads, soft cheeses and shellfish stored under refrigerated conditions (*71*). It has also been found in fresh and frozen meat products, deli style cuts, cabbage, bean sprouts, cucumbers, potatoes, tomatoes, dairy products, raw milk, among others (*8*). Due to its widespread prevalence and ability to survive in colder environments, producers need to pay attention to cleaning and hygienic practices throughout the food supply chain (*8*). Certain factors increase the risk of food being contaminated with *Listeria spp.* like product exposed to contamination, product with no killing step, product with no preservation factors, post-processing contamination, long shelf-life in chilled conditions, and ready-to-eat products (*8*).

L. monocytogenes has been linked to multiple foodborne outbreaks, including Mexican-style soft cheese (1985), processed meat (2000), hot dogs (1999), turkey deli meat (2002), deli meat (2008), among many others (*56*). *Listeria spp.* has been found in raw wheat; however, it

was only found in 1 of 1,285 samples in the United States and in 2 samples (0.6%) in Canada (*84*, *123*). The occurrence of *Listeria* spp. in raw wheat could lead to contamination of bakery products and therefore flour must be considered as a potential source of transmission. In 2014 in Ottawa, two brands of bakery products were recalled due to possible contamination by *L. monocytogenes.* These food products were garlic bread and garlic cheese bread (*125*). *Listeria monocytogenes* has also been associated with products containing peanut butter (*129*), a common ingredient in bakery products.

Salmonella spp.

Salmonella is a motile, non-spore-forming, gram negative, rod-shaped bacterium that can be commonly found in the intestinal tracts of reptiles, wildlife, birds, farm animals, domestic pets, and humans (*56*, *63*, *71*). The genus *Salmonella* is divided into two species, both of which can cause disease in humans, *S. enterica* and *S. bongori* (*56*). *Salmonella enterica* is divided into subspecies, *S. enterica* subsp. *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* (*56*). *Salmonella* can be subdivided more into serotypes that differentiate the strains by their flagellar and surface antigenic properties (*56*). By 2007, there had been 2,579 different serotypes of *Salmonella* identified (*56*).

Depending on the serotype, *Salmonella* can cause two different illnesses, nontyphoidal disease and typhoid fever (*56*). Nontyphoidal disease is generally self-limiting; while, typhoidal tends to be more serious and has a higher mortality rate (*56*). Typhoid fever is caused by *S.* Typhi and *S*. Paratyphi. Both of these serovars have only been found in humans and the mortality rate can be up to 10% (*56*). Non-typhoidal salmonellosis is caused by serotypes that are not *S.* Typhi or *S*. Paratyphi, with a lower mortality rate of 1%. It is estimated that the infective dose for salmonellosis is as low as one cell, depending on serotype, age, and health of the susceptible

individual (*56*). The route of entry of this disease is oral, through the consumption of contaminated food or water, and/or feces (*56*). *Salmonella spp.* that cause the nontyphoidal infections have been linked to over one million cases each year in the U.S. (*56*).
CDC estimates that *Salmonella* causes 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States each year, with contaminated foods being the main source of infection (*25*). *Salmonella* is the second most common cause of foodborne illness in the United States, the number one cause of hospitalizations, and the number one cause of death from acquired foodborne illness (*17*). Even though a majority of the persons are vulnerable to salmonellosis, certain groups tend to be more susceptible to infection like young children, elderly, and immunocompromised individuals.

It is also well known that *Salmonella* is present in our meat and poultry products. Cattle have been found to be a reservoir of *Salmonella* and it can be found on their hides and shed through their feces (*6*, *38*, *78*). *Salmonella* is also found in swine, broilers, and poultry products (*40*, *104*). Since it can be found in the intestinal tract, it is shed through feces and can contaminate water sources and insects can work as a vector to a large number of different places (*63*). Therefore, *Salmonella spp.* is considered to be ubiquitous, and have been linked to outbreaks from a variety of food including tomatoes, cantaloupes, alfalfa sprouts, ground beef, among others (*63*). Of particular importance is *S.* Enteritidis*,* which is often implicated in outbreaks related to poultry and poultry products (*71*). This serovar has been associated with colonizing the ovary of laying hens and then can be transmitted through the eggs (*71*). *Salmonella* has been associated with multiple outbreaks with a wide variety of foods like cucumbers, pasta salads, cereals, flour, eggs, melon, sprouts, tuna, chicken, turkey, mushrooms, peaches, onions, and others (*24*). *Salmonella* has been found in a study performed in Australia in

unscreened wheat and has also been found in raw wheat in the United States (*32*, *80*). In a study that spanned three years (2012-2014), it was found that 1.23% of the total raw wheat samples were positive for *Salmonella spp.* (*84*). It was also determined that *Salmonella* spp. had high diversity indicating it came from different sources. A *Salmonella* Agbeni outbreak occurred in 2018-2019 that was linked to Duncan Hines cake mix (*15*). At least seven people from five different states were hospitalized during this outbreak (*15*). There is a risk of presence of *Salmonella* in the ingredients required to make bakery products, particularly flour and eggs, and outbreaks have been linked to a variety of bakery products and cake mixes.

Previous studies have found that *Salmonella* can be easily destroyed in heated products that have a high water activity (a_w) of >0.98, but at lower a_w higher temperatures are required to kill the pathogen (*8*). A recent recall (August, 2021) of Hostess hamburger and hot dog buns was due to possible *Listeria* and *Salmonella* contamination (*126*).

Escherichia coli

Most strains of *E. coli* are non-pathogenic and make up the predominance of the intestinal flora of humans and animals (*71*). *Escherichia* are gram-negative, non-sporing rods, often motile with peritrichous flagella (*8*). All of the species ferment glucose and produce acid or acid and gas, and this can be done aerobically and anaerobically (*8*). They are also catalase positive, oxidase negative, and reduce nitrates to nitrites (*8*). This species can provide benefits to the humans, like preventing colonization of the gastrointestinal (GI) tract by pathogens (*71*). However, there are groups of *E. coli* that can cause diseases in humans, pathogenic *E. coli*. Pathogenic *E. coli* might be differentiated by their serotype based on antigenic differences in the O antigen of the lipopolysaccharides, in the flagellar or H antigens, and lastly on the fimbrial

antigens (*71*). The pathogenic *E. coli* can be subdivided into six groups: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (*56*). Of these six groups, EHEC are widely studied due to the severity of the illness and the complications associated with this infection.

EPEC

EPEC strains are characterized by the presence of the Locus for Enterocyte Effacement (LEE) pathogenicity island (*56*). The LEE carries multiple virulence factors like the *eae* gene, which permits the adherence of EPEC to epithelial cells in the intestinal tract (*56*). The illness is associated with infantile diarrhea, and in developing countries mortality rates have been as high as 50% (*56*). It has been estimated than higher than 10 million bacteria are required to produce disease in healthy adults. Some of the reported food contaminated with EPEC that has caused outbreaks are raw beef and chicken, but other food contaminated with fecal material could be linked to EPEC infections (*56*).

EIEC

This group produces enterotoxin that is very similar to *Shigella* and can invade the colonic epithelial cells (*56*). The invasion phenotype is encoded on a virulence plasmid that ranges in size from 180kb to 220kb; however, these plasmids possess a high level of homology (*56*). The disease is typically a mild dysentery with very low mortality (*56*). The infective dose is estimated to be between 200 and 5,000 cells, depending on the virulence plasmids carried and susceptibility of the individual (*56*). Outbreaks associated with EIEC are scarce and there are no foods implicated with these outbreaks; since infected humans are the only known reservoir, any food contaminated with human feces could lead to possible infection from these pathogens (*56*).

ETEC

This group is highly motile and is characterized by the production of different virulence factors that include a heat-labile toxin and a heat-stable toxin (*56*). ETEC are known to cause a "travelers diarrhea" and is the cause of infant diarrhea in developing countries (*56*). The World Health Organization (WHO) has estimated that 380,000 persons die from ETEC every year, with most of these deaths occurring in children (*56*). The infectious dose for healthy adults is between 10 million and 10 billion cells. The illness produced is mainly self-limiting and mild and is characterized by watery diarrhea without blood or mucus (*56*). Infections are common in the United States but are commonly found in travelers to foreign countries (*56*). ETEC outbreaks are associated with the consumption of contaminated water and food, and humans seem to be the main source of infection. Some foods implicated in ETEC infections are mayonnaise, deli meats, brie cheese, and salads (*56*).

EAEC

EAEC is associated with persistent diarrhea in children around the world (*81*).These strains are known to cause an aggregative adherence in Hep-2 cell lines, which differentiates them from the other types of pathogenic *E. coli* (*81*). The adherence of these serotypes resemble stacked bricks against the cell line. This group can be identified by a gene associated with a plasmid; however, there is need to further investigate the role of this group as a cause of disease (*81*).

DAEC

DAEC has mainly been linked to diarrhea in children that are older than infants, with an increasing risk between the ages of 1 and 5 (*81*). DAEC does not usually produce heat-labile or

heat-stable enterotoxins, or Shiga toxins (Stx). DAEC adheres randomly to HeLa or Hep-2 cell lines and its symptoms are mild diarrhea without blood (*81*).

EHEC

EHEC are characterized by the production of Stx (*81*), which obtain their name due to their similarity with the toxin produced by *Shigella dysenteriae*. EHEC that produce these toxins are known as Shiga toxin-producing *Eschericiha coli* (STEC) (*56*). There are between 200 to 400 serotypes of STEC and a subgroup of STEC are called enterohemorrhagic *Escherichia coli* because they cause a more severe illnesses characterized by bloody diarrhea (*56*, *81*). Stx toxins are also associated with thrombotic thrombocytopenic purpura (TTP), hemorrhagic colitis and hemolytic uremic syndrome (HUS). These can be fatal in humans if left untreated(*22*, *56*, *71*). STEC have been isolated from cattle, pigs, goats, chicken, dogs, and cats (*71*).

STEC are estimated to cause more than 265,000 illnesses each year in the United States leading to more than 3600 hospitalizations, and 30 deaths (*22*). O157: H7 is the predominant strain of human infections and it is estimated that worldwide it accounts for 75% of STEC infections (*56*). A group of STEC referred as the "big 6" accounts for the majority of the non-O157: H7 infections; these are serogroups O26, O45, O103, O111, O121, and O145 (*22*, *56*). Infections by STEC have been associated with the consumption of contaminated ground beef in Europe, Canada, and the USA (*71*). However, outbreaks have also been linked to flour, lettuce, spinach, raw milk, mayonnaise, fruits, salami, and apple cider. It is generally recognized that STEC originated from infected animal feces (*71*).

In several studies, raw wheat was shown to be positive for the presence of pathogenic *E. coli* (*9*, *68*, *84*, *91*, *123*). STEC has been found in wheat, rye, buckwheat, spelt, and combinations of these grains (*68*). Researchers have characterized *E. coli* isolates and have found a high

diversity of the different strains indicating that raw wheat has multiple sources of contamination (*9*, *53*, *68*, *91*). Twenty different STEC serogroups from 123 positive samples were found in German flour (*91*). Some of the strains identified have been positive for *stx* and *eae* genes, which indicates a likelihood of producing human diseases. In recalled flour associated with foodborne outbreaks, all samples were positive for *stx* (*9*, *10*, *50*, *91*).

Regarding flour and bakery products, there have been multiple outbreaks and recalls due to *E. coli* prevalence. *E. coli* O157 has been related with outbreaks due to contaminated cookie dough and frozen pizza (*18*, *83*, *94*). In the frozen pizza outbreak there were 21 people from 10 states that were part of this outbreak, where four individuals developed hemolytic uremic syndrome (*18*). An outbreak in 2009 traced to prepackaged cookie dough sickened 77 people in 30 different states of which 35 had to be hospitalized (*94*). In this outbreak, the company had to recall 3.6 million packages of chocolate chip dough and they claimed that flour was the only ingredient that had not cleared the supplier level testing (*94*). STEC O26 and O121 have also been linked to flour outbreaks including flour produced in the USA and Canada. In the USA outbreak, 63 people from 24 states were reported as infected and both *E. coli* O26 and O121 were detected from the implicated flour by Whole Genome Sequencing (WGS) (*32*, *41*). In Canada, there was another outbreak related to flour where *E. coli* O121 was identified as the etiological agent (*82*). In this outbreak, 29 people were infected across six different provinces, with eight people hospitalized and one that developed HUS (*83*). A recent recall of flour and flour related products due to contamination with *E. coli* O26 caused 21 people from nine different states to become ill and three required hospitalization (*23*).

Products made with the STEC-contaminated flour were also recalled. Some of these products include biscuit mix, jalapeno breader, cake mix, pancake mix, bread mix, muffin mix, and brownie mixes (*41*).

Flour Production

Kernels (or berries) are the part of the wheat plant from which the flour is produced (*130*). Flour can then be defined as the product of finely ground cereal grains or portions of plants that contain starch, which are mainly used as part of bakery goods. Wheat kernels are composed of three main parts, the endosperm, bran, and germ, which are separated during flour production and have different uses (*130*). The bran is the outer shell of the kernel and is only used for whole wheat flour, and it mainly consists of soluble fibers and some trace minerals (*130*). The endosperm, which represents around 83 percent of the kernel weight, is milled to produce the white flour. It has a high portion of protein and carbohydrates. The germ only represents around 2.5% of the kernel and is usually removed from the flour because it tends to cause rancidity of the baked products (*130*).

Even though there are over 30,000 different varieties of wheat, the most common ones are hard and soft red wheat (*130*). Hard red winter (HRW) accounts for about 40% of the United States wheat production and is primarily grown in the great plains (*89*). HRW is mainly used for bread flour but is also utilized to improve blending (*89*). HRW has a medium to high protein content which makes it good for all-purpose flour and chewy breads, when compared to other wheat varieties (*89*). Even though wheat or flour with lower moisture content tends to be more shelf stable, moisture content is often standardized between 12 -14% (*89*).

Cereals like wheat have been linked to different pathogenic bacteria such as *Salmonella, Escherichia coli,* and *Bacillus cereus* (*10*, *74*). Cereal grains that might possess microbiological contamination is a major worry for the grains industry, this because of the repercussions the contaminants might have on the quality and properties of the grains (*74*). Microorganisms present in cereals like wheat, rye, corn, and others are generally the same as the soil, storage containers, and those microorganisms present during harvesting. These microorganisms come from sources like dust, water, insects, birds, pests, and others (*63*, *74*). The bacterial profile of the milled grains is very similar to the ones of the whole grain, but usually in smaller numbers due to the different steps of processing of the grains (*81*). The low a_w of these products restrict the growth of microorganisms if stored correctly (*63*). Therefore, it is important to minimize the presence of pathogens in grains and during milling to produce flour that has lower risks of being contaminated.

The wheat kernels are cleaned and tempered prior to milling. After tempering, the kernels are broken by rollers and separated depending on the type of flour being produced. During milling, the endosperm is gradually reduced in size and separated from the bran and germ and are sifted through fine screens (sieves) several times until the right flour particle is achieved (*1*, *130*). The resulting flour fractions are blended to produce various grades of products (*1*).Tempering is a process that is used to help soften the husk using a small amount of water, then the moisture penetrates to the heart of the wheat kernel (*112*). To temper the wheat, the moisture content is adjusted to 15 – 16 % (*109*, *112*). After water is added, the wheat kernels are usually held for 12 to 18 hours depending on the hardness of the wheat (*109*, *112*). The introduction of water has been shown to increase the number of bacteria after the conditioning

process, when samples had 10^4 log CFU/g or greater (*7*). In case of *E. coli,* it was detected in previously uncontaminated wheat after the conditioning step of wheat milling (*7*).

Previous studies have demonstrated that EHEC and *Salmonella* spp. can survive for extended periods of time for up to two years in wheat flour (*47*, *52*). Even though flour is a low water activity ingredient that has been historically regarded as microbiologically safe, flour has been associated with foodborne outbreaks (*2*, *35*, *50*, *51*, *83*, *116*). Several authors have performed screening assays to identify possible contaminants in wheat and flour, finding different compositions of bacterial microbiota including *Serratia, Pantoea, Pseudomonas, Aeromonas, Escherichia, Enterobacter, Raoutella, Bacillus*, and some unidentified bacteria (*51*). Outbreaks traced to flour have occurred in different countries like Canada, New Zealand, and the U.S.A. (*21*, *51*, *77*, *116*). Other studies reported *Salmonella* prevalence to be less than 2%; and levels of 0.15 to 0.44 MPN/100g was determined for *E. coli* O121 (*7*, *35*, *51*). In recalled flour, it was found that all the samples taken were positive for *stx* genes and positive for *E coli* O121 (*7*, *35*, *51*). Even though there is a low incidence of pathogens present in flour, this ingredient has been linked to recent outbreaks of *Salmonella* Typhimurium phage type 42, *E. coli* O121, and *E. coli* O26 (*21*, *51*, *77*, *116*). The *Salmonella* Typhimurium phage type 42 outbreak in New Zealand in 2008 is considered to be the first outbreak to be confirmed by laboratory and epidemiologic investigation linked to the consumption of contaminated flour (*77*). There has been multiple flour and flour related products that have been recalled or have caused an outbreak. Some of these products include flour, bread flour, all-purpose flour, unbleached flour, biscuit mix, jalapeno breader, cake mix, pancake mix, bread mix, muffin mix, and brownie mixes (*23*, *41*). Due to the recent outbreaks and the widespread use if flour by households, bakeries, and the food service industry, it is important to develop methods to ensure flour safety.

Bakery Industry

According to the American Bakers Association (ABA), the bakery industry has a total economic impact of $154 billion per year with total sales in the United States of $480 billion (*3*). This represents 2.46% of the U.S. GDP in 2020 (*3*). It is estimated that there are over 3,000 independent bakeries of which 65% of them have less than 10 employees. There are another 6,000 bakeries that generate $3 billion and there are three commercial bakeries that account for 55% of the total commercial bakery revenues in the United States (*60*). In 2020, it was estimated that the bakery industry generated 764,777 direct jobs and 1,528,016 indirect jobs (*3*). According to ABA, when FSMA was introduced into the bakery industry, the new regulations required bakers across the United States to fall within compliance of the new law. Being such a fragmented industry and having a big percentage of bakeries being considered small, the development of tools that can help the industry comply with the new set of regulations included in FSMA became paramount.

Even though bakery products have historically been considered to be safe, there have been multiple outbreaks related to bakery goods including pastries, zeppoles, cream filled baked goods, among others (*124*, *127*, *128*). According to reports between 2004 and 2013, bread and other bakery products caused 142 outbreaks and 2,822 total of foodborne illnesses (*12*). Therefore, there is a need to fill the gaps in validation studies to ensure the safety of the products being produced in bakeries across the United States. A collaboration was made by the AIB International, ABA, Kansas State University, and the University of Georgia to develop validation studies to assess the lethality of pathogens during the baking process in a variety of different products (*4*). During the development of the present research, we will be discussing the

validation studies for different bakery products that will help develop those tools required by the industry to ensure the safety of their bakery goods.

FSMA Validation in the Bakery Industry

The Food Safety Modernization Act (FSMA) was signed into law on January 4, 2011 by President Barack Obama. This act established regulations for the food industry to prevent illnesses caused by foods and it regulates possible food safety issues in the whole supply chain. This law applies to everyone involved in the food chain from growers to food processors, and suppliers. The Food and Drug Administration (FDA) is in charge of overseeing the implementation of FSMA, and when the law came into effect, they set different implementation dates based on company size as following,

- September 19, 2016: Large food businesses
- September 18, 2017: Small food business with fewer than 500 full time employees
- September 17, 2018: Very small business that earn less than $1 million

The implementation of the law applies to all domestic and foreign food facilities that are registered in the section 415 of the Food, Drug, & Cosmetic Act (*44*). FSMA placed the responsibility of preventing foodborne illnesses onto the food manufacturers. That is why food facilities are required to implement written preventive control plans. The foundation of the plan is prevention and response in case of deviations.

The most important aspect of the FSMA for food processing facilities is the establishment of the food safety plan which can be divided into five components: hazard analysis, preventive controls (PC), oversight and management of preventive controls, supply chain program, and recall plan. We will briefly discuss each of these components.

Hazard Analysis

This is the first step in the food safety plan. Food producers need to consider what biological, chemical, and physical hazards are reasonably likely to occur. These hazards can be naturally present in the ingredients or might be intentionally introduced. Each hazard identified requires a preventive control that must be written and implemented to control the hazards (*44*)

Preventive Controls

Here the food processors have the flexibility to address the PC in the food products they are producing. PC must be written and implemented to ensure that the hazards will be reduced or eliminated to ensure the food product is not adulterated. There are different types of PC (*44*):

- <u>Process controls</u> are the procedures implemented to make sure that control parameters are satisfied. These can include chilling, cooking, acidification, and others. The process controls must have critical limits that are appropriate to the applicable control in the food safety system.
- <u>Food allergen controls</u> are designed to prevent allergen cross-contact and to ensure that any allergens present in the food product are listed on the labels of the packaged foods.
- <u>Sanitation controls</u> are procedures and processes to maintain the food processing facility in sanitary conditions to minimize or avoid hazards from environmental sources, employees, or allergen hazards.
- <u>Other controls</u> are any control that is not previously mentioned that can aid in the control of the hazards identified.

Oversight and Management of Preventive Controls

Once food processing facilities have identified the PC for a hazard, the facility must ensure that the controls are being met through the following actions (*44*):

- Monitoring procedures are utilized to ensure that the PC are being performed appropriately. This includes thermal treatment values, which must be documented.
- Corrections are the steps that are taken in case an issue arises during food production.
- Corrective actions are necessary when a problem is identified, and the corrections are taken during the PC implementation. The idea is to reduce the likelihood that an issue will happen again, to assess the affected food, and prevent the product from entering commerce if it is adulterated. They must be documented.
- Verification of the actions required to make sure that the PC are being implemented consistently and that they are effective in reducing the hazard risk. This part of the plan includes scientifically validated processes to ensure control measures are effective in controlling the hazard. Verification includes checking of the records to ensure that monitoring and corrective actions are being conducted. They must be documented.

Supply Chain Program

Food producers must have a risk-based supply chain program if their hazard analysis identifies a hazard that requires a PC or if a control will be implemented in the food processing facility. This requires manufacturers to make sure they are receiving their ingredients from approved suppliers. If the hazard is being controlled in the food processing plant, then the supply chain program might not be required (*44*).

Recall Plan

In case that the PC plan has identified a hazard that requires a PC, then a recall plan must be established. The recall plan must include all steps required to remove the product from markets and how to notify the suppliers and the general public. Identifying the product and appropriate disposal of the product must be addressed (*44*).

Impact of FSMA in Commercial Bakery Operations

The bakery industry points out that bakery products are not considered to be high risk products and that most recalls are due to allergen mislabeling; however, there have been reports of pathogen contamination (*54*). Validation of processing steps to control bacterial contamination is required to ensure food safety. These validations studies will be vary depending on the risk of each product and must be assessed through the preventive control steps.

Different authors have pointed out the void of scientific validation studies that assess the lethality of baking and frying of bakery products (*28*, *100*, *105*, *113*). Such studies for specific bakery products are important to give the bakeries the tools required to be in compliance with FSMA regulations.

Validation Studies and Research Importance

A pathogen inactivation study can be defined as a study that evaluates the ability of a specific food manufacturing process or food formulation to inactivate bacterial pathogens (*85*). To determine if a pathogen inactivation challenge study is required, food processors must perform a hazard analysis to assess the biological hazards and their potential growth and inactivation (*85*). Some factors to include in the decision making are routes of contamination, intrinsic food factors, use of processing technologies, and the historical safe use of the specific food product. Some of the most important intrinsic factor are a_w and pH; both of these factors combined or by themselves can inhibit or support the growth of pathogen in the food matrix (*85*). If these two parameters do not ensure pathogen control in the product, a challenge study might be required. Specific food product data is then required to ensure food safety. The evaluation of

the food product safety should be performed by expert microbiologists and food technologists who understand the characteristics of the microbial inhibition in the food product (*85*). Inactivation studies help determine if thermal or antimicrobial steps provide an adequate log reduction of the target pathogen to render the product as safe (*85*).

Ideally, these studies should be performed at a commercial processing facility or a laboratory that has pilot food processing facilities. The food produced should be processed in a way that will mimic the conditions during commercial applications and the product produced should be representative of normal food production (*85*). However, small changes are allowed to select for the "worst-case scenario", this based on the knowledge of the food being tested. It has been recommended that if performing a thermal inactivation study, the lower a_w should be used due to the fact that pathogens show increased resistance under low moisture conditions (*85*).

Another important factor to consider is the strains selected to perform the challenge study, where it has been recommended to use several strains in combination. This will help to account for variability among organisms (*85*). The strains selected should also be appropriate for the product that is being tested, which can include microbial strains that have been previously linked to the food product or process. Ideally high inoculation levels should be used to be able to effectively enumerate the target magnitude of reduction (usually a 5-log reduction is desired) (*85*).

Therefore, the series of validation studies reported here will greatly help the bakery industry to increase their food safety assurance, to ensure their processes are in compliance with the FSMA regulations, and to avoid paying for individualized studies that can cost thousands of dollars. Therefore, the development of these present studies is of vital importance to the bakery industry. There have been already multiple products that have been scientifically validated by our research team to ensure that bakery products have a validated kill step that will effectively reduce the risk

of microbial contamination. Some of these products include brownies, pies, hamburger buns, bread, donuts, muffins, and cream filled pastries (*27*, *28*, *30*, *57*, *102*, *113*, *121*). However, there are many other products that have not been validated; therefore, in the present work we will be discussing the results of peanut butter bars, cheesecake, and fruit-filled pastries baking validations.

Chapter 3 - Research Questions

Experiment 1: Development of a Commercially Applied Antimicrobial Intervention Process During Wheat Milling

Can a 5% lactic acid solution reduce *Salmonella* spp. population levels when applied during wheat tempering at increasing wheat/lactic acid mixtures temperatures (ambient, 120, 130, 140 and 150 ^{0}F) and using tempering durations of 1 to 24 hours?

Experiment 2: Validation of a Simulated Commercial Baking Process to Control *Salmonella* spp. in Fruit-Filled Pastries

Is baking fruit-filled pastries at 375°F oven temperature following industry parameters effective at reducing/eliminating *Salmonella* spp. populations from inoculated raw flour?

What are the D- and *z*-values of the *Salmonella* cocktail in pastry dough?

Experiment 3: Validation of a Simulated Commercial Baking Process to Control *Salmonella spp*. in Cheesecake

Is baking of cheesecake at 300°F following industry parameters effective to reducing/eliminating *Salmonella* spp. populations from inoculated flour?

What are the D- and *z*-values of the *Salmonella* cocktail in cheesecake dough?

Experiment 4: Validation of a Simulated Commercial Baking Process to Control *Salmonella spp.*, *STEC*, and *L. monocytogenes* in Peanut Butter Bars

Is baking of peanut butter bars at 375°F following industry parameters effective to reducing/eliminating *Salmonella* spp., *STEC,* and *L monocytogenes* populations from inoculated flour?

What are the D- and *z*-values of the three independent pathogen cocktails during heating of peanut butter bars dough with modified (lowered) water activity?

Experiment 5: A Comparative Study of a Published Baking Validation Study versus a Surface Inoculation Study of Hamburger Bun Baking

Are there differences in *Salmonella* spp. survival in the crumb and crust during oven baking of hamburger buns due to differences in the intrinsic parameters associated with each bun fraction?

Chapter 4 - Development of a Commercially Applied Antimicrobial Intervention Process During Wheat Milling

Introduction

Salmonella spp. is the leading cause of foodborne outbreaks, illnesses, and number of hospitalizations (*26*). Every year one out of six Americans will become sick from foodborne disease, resulting in approximately 128,000 hospitalizations and 3,000 deaths (*17*). Low moisture ingredients and foods are usually not considered as foods that cause foodborne disease (*2*). The most probable reason for not being considered as potentially dangerous is because they commonly do not provide the proper conditions to support bacterial growth (*2*, *74*). *Salmonella* spp., however, have been linked to multiple outbreaks in low moisture foods (*2*).

Cereals have been shown to be sporadically contaminated by different pathogenic bacteria like *Salmonella, Escherichia coli,* and *Bacillus cereus* (*74*). Potential microbiological contamination of different cereal grains is a major concern for the industry due to the impact on quality and properties of the grains (*74*). Microbial contamination present in different cereals like corn , wheat, rye, and others is expected to be the same as those microorganisms present during harvesting and can come from multiple sources like pests, water sources, birds, and others (*63*, *74*). The bacterial profile of the milled grains is very similar to that of the whole grain, but usually in smaller numbers due to the different steps of processing of the grains (*81*). The low a_w of these products restrict the growth of microorganisms if stored correctly (*63*).

Hard red winter (HRW) wheat accounts for about of 40% of the United States wheat production and is primarily grown in the great plains (*89*). HRW is mainly used for bread flour but is also utilized as an improver for blending (*89*). HRW has a medium to high protein content which makes it good for all-purpose flour and chewy breads, compared to other wheat varieties (*89*).

Even though wheat or flour with lower moisture content tends to be more shelf stable, moisture content is often standardized between 12 -14% (*89*).

Tempering is a process that is used to help soften the husk with water, then the moisture penetrates to the heart of the wheat (*112*). This facilitates a more complete separation of the husk from the endosperm during milling. To temper the wheat, first the moisture content is determined and water is added to adjust the kernels to 15 – 16 % moisture content (*109*, *112*). After water is added wheat kernels are usually held for 12 to 18 hours depending on the hardness of the wheat (*109*, *112*). During milling, the endosperm is gradually reduced in size and separated from the bran and germ by the use of sieves (*1*). The flour fractions are blended to produce various grades of products (*1*).

In wheat milling, water is introduced as part of the conditioning process. The introduction of water during the conditioning process has led to an increase in number of mesophilic bacteria after the conditioning process (*7*). It was found that conditioning changed the range and maximum counts of microbial populations in wheat products and the *E. coli* counts increased (*7*). *E. coli* was detected in previously uncontaminated wheat after the conditioning step of wheat milling (*7*).

Flour is a low moisture ingredient that has been associated with recent foodborne outbreaks (*2*). Several authors have performed screening assays to identify possible contaminants in wheat and flour, and found that the number of positive samples for *Bacillus cereus*, *Escherichia coli*, and *Salmonella* are very low (*7*, *35*). Even though there is a low incidence of pathogens present in flour, this ingredient has still been linked to recent outbreaks of *Salmonella* Typhimurium phage type 42 and *E. coli* O121 (*21*, *77*). The *Salmonella* Typhimurium phage type 42 outbreak in New Zealand in 2008 is considered to be the first outbreak to be confirmed

by laboratory and epidemiologic investigation linked to the consumption of contaminated flour (*77*). Due to the recent outbreaks and the widespread use of flour by households, bakeries, and the food service industry, it is important to develop methods to ensure flour safety.

Previous researchers have tried to find antimicrobial interventions to reduce the pathogen load during wheat tempering by using organic acid and saline solutions (*95*, *96*). The previous papers have looked at seasonality and survivability of enteric bacteria in wheat kernels and looked at the effect of pathogen reductions after tempering with different antimicrobials combinations. Some authors highlight the need to develop new techniques to reduce the microbial load of grains without impacting the quality of the grains (*74*). It has been stated before that after a step to reduce pathogens is added to the process the microbial integrity must be maintained throughout the rest of the process (*1*). This research was conducted to understand different treatment combinations (water or lactic acid at 5% at five different tempering temperatures) during simulated wheat milling tempering conditions to reduce the pathogen load to safe levels.

Objectives

This study was conducted to validate the antimicrobial effectiveness of heated 5% lactic acid when applied to cleaned, inoculated and pre-heated wheat kernels during the tempering phase of milling. Studies evaluated 5% lactic acid applied at increasing wheat/lactic acid solution temperatures (ambient, 120, 130, 140 and 150 ^{0}F) and using tempering durations of 0 to 24 hours to eliminate *Salmonella* spp. immediately prior to flour milling.

Materials and Methods

Experimental Design

This study utilized a randomized complete block design with repeated measurements and subsampling for 10^4 and 10^6 CFU/g inoculation levels. At each inoculation level, tempered wheat kernels were subjected to 10 different treatment combinations corresponding to a factorial combination of two solutions [water or 5% lactic acid (LA)] and five temperature levels (ambient, 120°F, 130°F, 140°F, 150°F). Three independent replications served as the blocking factor. Two samples of kernels ranging from 16 to 35 g were collected from each treatment in each replication at 0, 4-, 12-, 18- and 24-hours post-treatment. Residual *Salmonella* populations were determined in order to calculate log CFU/g reductions resulting from each treatment.

Bacterial Cultures

Six *Salmonella* serovars were used in this research and were selected based on previous research performed by this group and their relationship with low water activity environments. Three of the serovars of *Salmonella* were obtained from the American Type Culture Collection (ATCC; Newport 6962, Senftenberg 775W 43845 and Typhimurium 14028). *Salmonella* Tennessee and one non-typed, dried pet food isolate were donated by Richter International, Inc.

(Columbus, OH). *Salmonella* 4,[5],12: i: (mono-phasic) was obtained from Dr. Brian Lubbers (Kansas State University Veterinary Diagnostic Laboratory). Cultures were stored at -80°C on cryoprotect beads (Key Scientific, Stamford, TX). To activate pure cultures for the study, a single bead was transferred to 10 mL of tryptic soy broth (Difco™, Sparks, MD; TSB) and incubated 24 h at 37°C to create parent strains, which were stored at 4°C until needed.

To achieve a 10^4 CFU/g inoculation level, a loopful (10 µL) of each of the six parent strains were transferred into separate 15 mL centrifuge tubes with 10 mL of TSB (Difco™, Sparks, MD) and incubated for 24 h at 37°C. After incubation tubes were centrifuged at 2900xg for 15 minutes at 25°C using a Allegra X-14R centrifuge (Beckman Coulter, Brea, CA). Pellets were rehydrated with 10 mL of phosphate buffered saline (VWR, Radnor, PA; PBS). Once pellets were rehydrated, equal amounts of all the harvested *Salmonella* strains were mixed to create the bacterial cocktail used as master inoculum.

To achieve a 10^6 CFU/g inoculation level, a 10 µl inoculating loop was used to transfer to a 15 mL centrifuge tube containing 10 mL of TSB (Difco™, Sparks, MD) and was grown at 37°C for 24 h. Then a sterile swab was used to spread the working culture on tryptic soy agar (Difco™, Sparks, MD; TSA) plates. Plates were then incubated at 37°C for 24 h. After 24 h, plates were harvested using 5 mL of 0.1% peptone (Becton, Dickinson and Company) solution four times, to collect an approximate volume of 20 mL. After collection of the lawns, tubes were centrifuged at 2900xg for 15 minutes at 25°C using an Allegra X-14R centrifuge (Beckman Coulter, Brea, CA). Pellets were then rehydrated with 20 mL of PBS (VWR, Radnor, PA; PBS). Once pellets were rehydrated, equal amounts of all of the harvested *Salmonella* cultures were mixed to create the bacterial cocktail used as the master inoculum.

Kernel Inoculation

Hard red winter (HRW) wheat kernels pre-dried to approximately 8.1% moisture, were weighed (6 kg) and equally divided into three sanitized sealable plastic tubs (9.4 L, Rubbermaid, Atlanta, GA). Kernels were spread evenly in each tub and mist-inoculated inside a biosafety cabinet by spraying 20 mL of the *Salmonella* cocktail culture (1 mL per 100 g of kernels) uniformly across the kernel layer. After placing the lid to seal the tub, inoculated kernels were mixed manually by shaking the tub inside of the biosafety cabinet for ~30 sec. Inoculated kernels were then dried to the original pre-inoculation weight by placing the tub with open lid inside an incubator (Lab-Line®, Imperial III Incubator, Melrose Park, IL) at 45°C for 10 h. During this drying, the moisture content of the kernels was monitored every hour and once it was close to the target moisture content every 30 min, using a Dickey-John grain moisture tester (Grainger, Lake Forest, IL). Once the moisture content of 8.1% was reached, the inoculated kernels were mixed again by shaking the sealed tubs, and then stored in a Ziploc bag inside a sealed tub at ambient temperature (~25°C) for 7-days. On the day of the experiment all three tubs were combined into one single container, mixed and then used as the inoculated kernels for the tempering*antimicrobial intervention evaluation.

Chemical Preparation

A 5% lactic acid solution was prepared from 88% L-lactic acid (Corbion, Purac®, Lenexa, KS; LA). To confirm the LA concentration, an aliquot from the mixed solution was titrated using the following method:

1. Transfer 5 mL of sample from the solution to a clean beaker.
2. Add 50 mL of deionized water to the beaker.
3. Add 5 drops of phenolphthalein indicator solution (ChemWorld, Kennesaw, GA)

while swirling the beaker continuously.

4. Titrate using 0.25 N NaOH (BDH, VWR International, LLC, Radnor, PA) until the solution turns pink for more than 10 seconds.

5. Confirm concentration of lactic acid by using the following calculation:

$[(0.25 \times 0.09008 \times \text{mL NaOH}) / 5] \times 100 = \%$ LA in application solution.

Both treatment solutions, water (W) and 5% LA, were placed into 100 mL glass bottles and set inside incubators set to target temperatures of 120, 130, 140 and 150°F to pre-warm. One bottle was left at room temperature the night before each experiment (~74°F).

Tempering Process

The dried kernels at 8.1% MC were tempered to a target moisture content of 15.50%. To determine the amount of each solution to add, we used the AACC International Method 26-95.01 formula (*110*):

Equation 1

$$\text{weight of water to add} = \left(\frac{\text{100-original moisture (\%)}}{\text{100-desired moisture (\%)}} - 1\right) \text{x weight of sample}$$

Water (W) or 5% LA were used for tempering the *Salmonella* spp. inoculated kernels. To simulate a commercial wheat tempering process, a Vorwerk Thermomix TM 31 with a butterfly mixing attachment (Vorwerk, Thousand Oaks, CA) was utilized. 500 g of the inoculated kernels were placed inside the Thermomix along with the pre-heated treatment solution. The Thermomix blender was run at speed 2 with a temperature setting of 100°C for 4, 8, 10 or 12 minutes to achieve target wheat temperatures of 120, 130, 140, and 150°F, respectively. Preliminary studies were completed to establish Thermomix heating times at these settings required to reach each target wheat temperature. For the ambient temperature treatment, 500 g of the inoculated kernels were mixed with the solutions (water or lactic acid) in a sealable bag and were hand mixed for 2

minutes. Upon completion of the mixing period, treated kernels were placed into Ziploc bags, sealed and set inside incubators set to the different target tempering temperatures of 120, 130, 140, or 150°F. The ambient temperature treatments were placed in the Ziploc bags and left at ambient temperature (~74°F). All treated wheat kernel samples were held for 24 hours, with subsamples drawn at time 0 (after reaching treatment temperature), 4, 12, 18 and 24 hours to determine residual *Salmonella* population levels.

pH Monitoring of Treated Kernels

Non-inoculated kernels were treated following the same protocol (treatment*temperature) of the inoculated kernels. The pH of kernels was measured at 6 sampling points after reaching target tempering temperature (0, 0.5, 1, 4, 12, and 24 h). The pH of the kernels was measured on duplicate samples, and the average of the duplicate samples is reported.

pH monitoring was only done for one study replication; thus, statistical analysis of the data was not performed. For measuring the pH of the samples, AACC International Method 02-52.01 Hydrogen-Ion Activity (pH)—Electrometric Method was used with some minor changes (*111*). In brief, 10 g of the treated kernels were placed into 7-oz Whirl-Pak (Madison, WI) bags and 100 mL of distilled water was added. Bags were shaken in a shaker incubator (Excella E24 Incubator Shaker Series; New Brunswick Scientific, Edison, NJ) for 15 minutes at 70 rpm and 25°C. After shaking the bags, samples were rested for 10 minutes at which point the pH was determined on the supernatant of the bag using a Corning Pinnacle 530 pH meter (Nova Analytics Corp., Woburn, MA).

Microbial Sampling and *Salmonella* Enumeration

Samples were collected using a sterile plastic spoon at each of the different sampling points. To prevent temperature loss of the tempered kernels, two full spoons were collected while the samples where at the different temperatures targeting 25 g of sample. Duplicate samples of each treatment combination were taken at each sampling point to account for possible variances within the kernels inoculation, due to the heterogenous shape of the kernels. These analytical samples were then placed into sterile stomacher bags containing 75 mL of chilled (~4°C) buffered peptone water (Becton, Dickinson and Company; BPW). Samples were then rested for 30 minutes in the chilled BPW (Becton, Dickinson and Company) before being hand mixed for 30 seconds. Each sample was serially diluted in 0.1% peptone water and plated in duplicate on xylose lysine deoxycholate agar (Thermo Scientific™ Remel, Waltham, MA, XDL agar) that had been previously overlaid with 14 mL of TSA on top. This plating method was used to account for sublethally injured *Salmonella* cells arising from the different treatment combinations (*65*). All plates were incubated at 37°C for 18-24 h and black colonies were counted. *Salmonella* count was calculated by dilution factor and sample weight according to the following formula.

Equation 2

$$\textit{Salmonella}\text{ level in analytical sample} = \frac{\text{Sample Weight}(g)+75(mL)}{\text{Sample Weight}(g)} \times \text{Dilution Factor} \times \text{Colony Count (CFU)}$$

In accordance, a count of one from a sample of 25 g gives rise to the adjusted count of 4, which is app. the lower detection limit (LDL) of the study. Adjusted count was subjected to $\log_{10}$ transformation for statistical analysis purposes. After analyzing all treated wheat samples quantitatively by direct plating protocol, the homogenized samples in BPW were stored

overnight at 4°C. For any samples in which no *Salmonella* CFUs were detected on plates, 25 mL of stored homogenate were added to 225 mL of BHI broth followed by incubation at 37°C for 24 h. These enriched samples were then streaked onto XLD agar plates that were subsequently incubated for 24 h at 37°C to detect any presumptive *Salmonella* presence in samples at very low (undetectable by direct plating) levels.

Statistical Analysis

Log_{10} transformed count data were analyzed under the linear mixed model. Interactions between model fixed effects were examined using type III tests. Pair-wise comparisons between two levels of a fixed effect were performed based on the two-sided test for non-zero difference in means. Disregarding the significance of treatment-by-time interaction, the effect of treatment was examined at each time point. Fixed effects were reported in the form of least squares (LS) means (on the log_{10} scale), standard errors, and mean difference (on the log10 scale). No multiplicity adjustment was applied. SAS statistical analysis was executed via Statistical Analysis Software (SAS version 9.4; Cary, NC) PROC MIXED with option DDFM=KR.

Counts at 10^4 CFU/g Inoculation Level

Only the two treatment groups at the ambient temperature level were statistically analyzed since counts at other temperature levels were mostly non-detectable (ND). Fixed effects of the model included replication, solution, time, and solution-by-time interaction. Random effects of the model include replication-by-solution (the error term vector corresponding to repeated measurement over time) and replication-by-solution-by-time (i.e., the error term vector corresponding to subsampling). The variance-covariance structure of replication-by-solution was

taken as first-order autoregressive according to the model fitting criteria. The variance-covariance structure of replication-by-solution-by-time was taken as variance components.

Counts at 10^6 CFU/g Inoculation Level

Only the four treatment groups at the ambient and 120°F temperature level were statistically analyzed since counts at other temperature levels were mostly non-detectable (ND). Fixed effects of the model included replication, solution, temperature, time, and all the two-way and three-way interactions. Random effects of the model include replication-by-solution-by-temperature (the error term vector corresponding to repeated measurement over time) and replication-by-solution-by-temperature-by-time (i.e., the error term vector corresponding to subsampling). The variance-covariance structure of replication-by-solution-by-temperature was taken as first-order autoregressive according to the model fitting criteria. The variance-covariance structure of replication-by-solution-by-temperature-by-time was taken as variance components.

Results and Discussion

Two wheat kernel inoculation levels (10^4 and 10^6 CFU/g) were used, as determined after one week of storage at room temperature in aerobic conditions to mimic what might be a natural contamination scenario. After one week of storage the inoculation levels were 4.0 and 6.2 log CFU/g. Previous authors have studied the effect of storage conditions of wheat kernels on bacterial populations (*95*). The paper from Sabillón et al., (90) found *Salmonella* spp. reductions in hard red winter wheat of 0.79 ± 0.01 log CFU/g after seven days of storage at 24.2°C; which is a very similar temperature of storage utilized in our studies; however, the moisture content of the hard wheat kernels was 12.1%, which is 3.4% lower than our target moisture content during

tempering in our study (*95*). After the week of storage at 24.2°C, they had a *Salmonella* spp. population of 6.01 ± 0.01 log CFU/g, very similar to the current study`s high target inoculation level of 6.2 log CFU/g. Sabillón et al., (90) reported that increasing temperatures and time during storage conditions decreased the microbial populations present in inoculated kernels where *Salmonella* and *E. coli* O157:H7 survival populations decreased in HRW kernels after 7 days of storage by 0.31 and 0.14 log CFU/g if stored at 2°C; decreased by 0.33 and 0.02 log CFU/g if stored at 10.8°C; decreased 0.79 and 0.38 log CFU/g if stored at 24.2°C; and, decreased by 1.14 and 1.44 log CFU/g if stored at 32°C, respectively (*95*).

Heating Profile of the Kernels

Results of the heating profile of the kernels can be observed in Figure 1. The Vorwerk Thermomix TM 31 with a butterfly mixing attachment (Vorwerk, Thousand Oaks, CA) was utilized with 500 g of kernels and ran at speed 2 with a temperature setting of 100°C for 4, 6, 8, 10, and 12 minutes to observe the heating curve of the kernels.

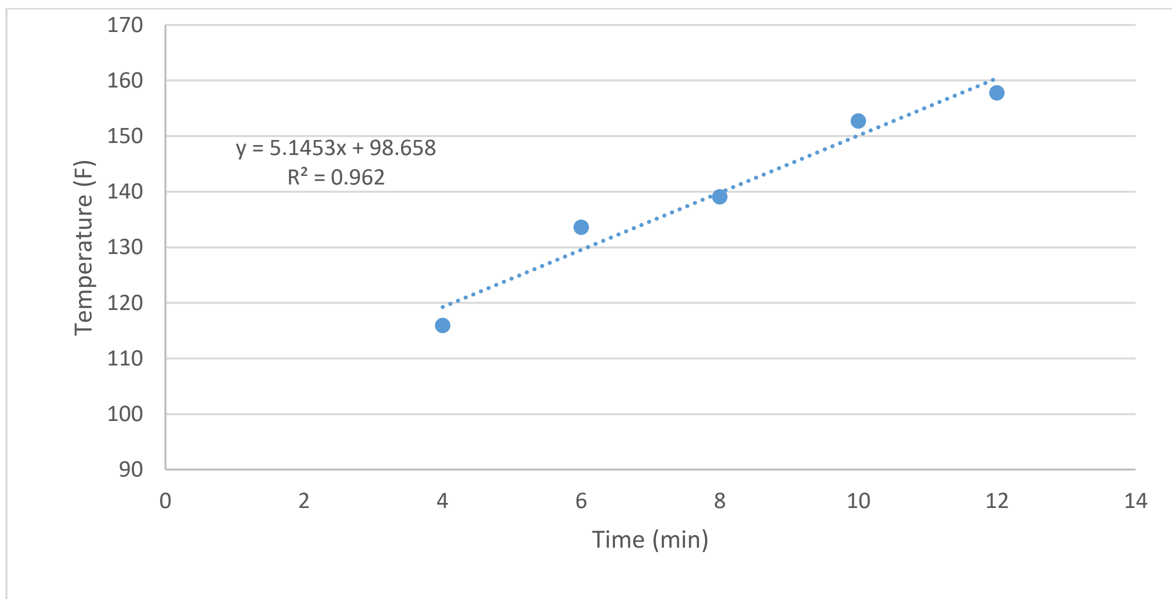


Figure 4-1 Wheat temperature at different mixing time points in a Thermomix set at 100°C and a speed setting of 2.

The heating equations and R^2 values are depicted in the graph. As shown the heating profile time and temperatures match very closely the target temperatures of the tempering process and were deemed appropriate for the inoculated study. Is important to mention that setting of the Thermomix was set at 100°C and therefore the temperature of the kernels might had a fast rise in the temperature of the kernels which could have aided in the lethality of the pathogens tested.

pH of the Treated Kernels

We hypothesized that the wheat pH of the LA treated kernels would be buffered out after treatment of the kernels. Buffering capacity is defined as the resistance of a solution to change in pH with the addition of acid or alkali (*114*). The buffering capacity of foods is determined by protein content, amounts of dipeptides, and the contents of base or acid groups (*97*). It has been previously reported that cereals including wheat have a lower buffering capacity than protein based foods (*79*). In a study where the buffering capacity of feedstuff ingredients was determined, it was found that wheat had one of the lowest acid buffering capacities; however, the type nor the wheat variety were reported (*49*). Our results indicate a kernel pH value (6.70 for our ambient water control group at time 0) to be very similar to the ones found in the literature in feedstuffs, where it was determined that initial pH of wheat is 6.33 (*49*).

Table 4-1 pH values for treated kernels at different sampling points.

Treatment*	Treatment Time**					
	0H	0.5H	1H	4H	12H	24H
AW	6.70	6.53	6.44	6.39	6.49	6.40
120W	6.79	6.40	6.36	6.55	6.64	6.50
130W	6.75	6.48	6.25	6.65	6.58	6.59
140W	6.70	6.58	6.50	6.60	6.54	6.66
150W	6.51	6.53	6.61	6.75	6.58	6.60
AL	4.16	3.89	3.78	3.97	3.95	4.03
120L	3.48	3.75	3.92	4.04	4.30	4.46
130L	3.65	4.17	4.01	4.31	4.69	4.55
140L	3.83	4.22	4.56	4.65	4.95	4.89
150L	3.70	3.86	4.34	4.59	4.77	5.00

*AW: ambient water; AL: ambient 5% lactic acid; numbers: kernel and solution treatment temperatures (°F)
** After reaching target temperature

The pH of the samples treated with water ranged at different temperatures from 6.25 to 6.79. The pH value of the water-treated samples did not vary greatly across time*temperature treatments (6.25-6.79). With regards to the LA treatment, a trend was observed where the kernel pH values increased from immediately after treatment (0 hours) to the final pH value at the end of the tempering process (24 hours). At all of the treatment temperatures, the pH increased with increasing storage time as the LA was buffered. The initial lower values of pH were reported for the 120°F and 130°F LA treatments. The 130°F temperature application of LA has been reported in previous literature to have a higher lethality compared to LA applied at ambient temperature, in different food matrices (*86*). At the end of the 24 hours, the AL-treated sample had the lowest pH (4.03). It is interesting to note that at increasing temperature we can see a higher pH value of the samples treated with LA; this trend was observed with the water-treated samples.

Salmonella spp. Reductions by Tempering Treatments

Water and LA tempering treatments were highly effective in reducing and/or eliminating *Salmonella* spp. populations at both the 10^4 and 10^6 CFU/g inoculation levels. Due to very low to no quantitative recovery of *Salmonella* in samples receiving most of the water and lactic acid tempering treatments, statistical analysis of the data could not be pursued for all of the treatment*temperature interactions. Results will show the statistical analysis where the data allowed to perform analysis.

10^4 CFU/g Inoculation Level Results

In Figure 4-2, the survival rate of *Salmonella* spp. can be visualized after treating the kernels inoculated at a target level 10^4 CFU/g. The initial inoculation level was confirmed to be 4.0 log CFU/g of kernels. The AW treatment resulted in little to no *Salmonella* reductions (<0.5 log CFU/g) compared to the non-treated kernels. Similar results were found by previous researchers, where they found that tempering with water did not reduce pathogen population levels in soft wheat and hard wheat, when tempering with water at a temperature application of 23 ± 1°C (*95*, *96*). Ambient lactic acid (AL) provided an immediate reduction of 1.2 log CFU/g after treatment and the bacterial counts decreased over 24 h of tempering to have total reductions of up to 3.6 log CFU/g. When we compared AW to ambient 5% LA (AL), the AL reductions were significantly ($p < 0.05$) higher than AW at each of the five sampling points. The bacterial reductions for AL are very similar to the ones found by Sabillón et al., (90), where they used lactic acid at 5% at 23 ± 1°C and found reductions of 1.8 log CFU/g for soft wheat and 2.6 log CFU/g for hard wheat immediately after treatment. Sabillón et al., (90) report very similar reductions for *E. coli* O157 and non-O157 Shiga toxigenic *Escherichia coli* with the addition of the 5% LA.

In the current research, it can also be observed that increasing tempering temperature provided additional *Salmonella* population reductions; however, we were not able to find similar research to be able to compare our results. The increase in wheat tempering temperature using water at 120°F (48.89°C) resulted in a 1.1 log CFU/g reduction at time 0. As water tempering temperature increased at time 0 to 130°F, the *Salmonella* population was substantially reduced by 3.8 log CFU/g, and at higher water temperatures no recovery was detected. No *Salmonella* was recovered in water-tempered wheat at ≥ 120°F by the 4 h sampling using direct plating. At time 0, AL provided an immediate 1.2 log CFU/g *Salmonella* reduction in tempered kernels. Reductions at time 0 increased to 2.9 and 3.3 log CFU/g with LA tempering at 120 and 130°F, respectively. Although very low levels (right at the limit of detection by direct plating) of *Salmonella* were detected throughout for 24 h tempering period using ambient LA, by the 4 h sample collection point no *Salmonella* was detected at 120°F or greater tempering temperture, equating to ≥ 4.0 log CFU/g reductions. *Salmonella* was not detected by enrichment with LA treatment at 120, 130, 140 and 150°F after 24 hours of tempering.

Comparing the heat treatments using water or LA, it would appear that moderate to higher tempering temperture (120-150°F) is the greatest contribution to *Salmonella* reductions, whether using water or 5% LA, when tempering times of at least 4 h are utilized. Tempering with 5% LA solution, however, provides a more immediate *Salmonella* reduction at ≥ 120°F compared to water.

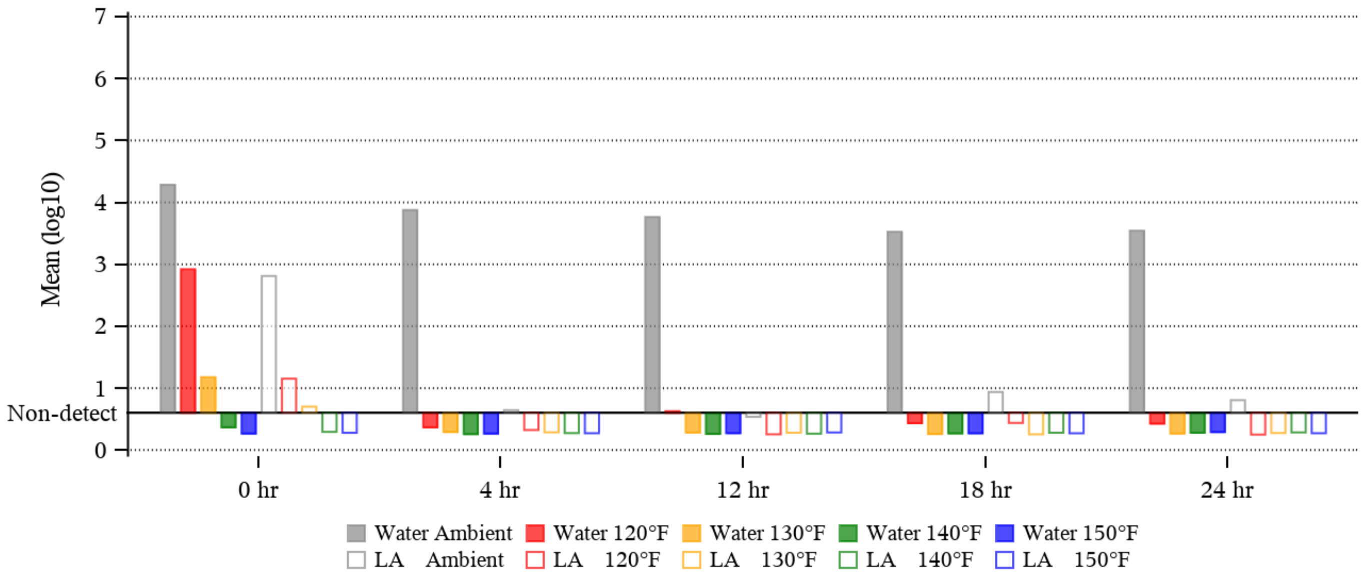


Figure 4-2 *Salmonella* populations at 10^4 inoculation level before and after treatment combinations of water (W) or lactic acid (LA) at different temperatures at each sampling point.

10^6 CFU/g Inoculation Level Results

In Figure 4-3, the survival rate of *Salmonella* spp. can be observed after treating the kernels inoculated at a target level 10^6 log CFU/g. The average initial inoculation level was confirmed to be 6.2 log CFU/g of kernels. The AW treatment resulted in 0.4 log CFU/g reductions over 24 h of tempering, demonstrating a stable *Salmonella* population level in the tempered wheat. These results are very similar to the ones observed at the lower inoculation level. The reduction for AL was 1.4 log CFU/g at time 0 and by 4 h, 2.2 log CFU/g where it remained stable 2.4 log CFU/g through 24 h. The treatment, time, and temperature main effects were significant ($p < 0.001$). The temperature*time interaction was also significant ($p = 0.010$). The difference in *Salmonella* reductions between AW and AL tempering treatments was significant ($p=0.017$). These results are also similar to the ones found with the lower inoculation level. As depicted in Figure 4-3, it appears that AL resulted in an initial reduction and then acid

was buffered out since the reduction was not increased after holding for 24 hours at ambient temperature. These results can be confirmed by the results of the pH values of the kernels after treatment, where we can observe an increasing pH of the kernels at the end of the tempering process (Table 4- 1).

As mentioned before in the research by Sabillón et al., (90), they found very similar reductions to the ones found on this paper with 5% LA at ambient temperature in HRW (2.6 log CFU/g). Another study found a reduction of *Enterobactericeae* populations of 2.5 log CFU/g after treating kernels with 5% LA at 23°C (*96*). These researchers also found that 5% LA was effective in reducing yeast and mold counts. Other authors evaluted the effect of adding Sodium Bisulfate during tempering and found reduction of *E. coli* O121 and O26 of up to 4 log CFU/g, withouth changes in the wheat flour quality (*93*).

The 120°F water tempering treatment showed reductions of 1.5 log CFU/g at time 0, while 5% LA at 120°F showed reductions of 4.3 log CFU/g immediately after treatment. This shows a difference in the reductions between 120°F water and LA of 2.8 log CFU/g which is significantly higher ($p<0.001$). After 24 hours of holding at the 120°F temperature, there was still recoverable populations for water and LA of 2.2 and 0.8 log CFU/g, respectively, where this difference between water and LA is significant ($p=0.032$). At 130°F, water tempering showed at time 0 reduction of 4.7 log CFU/g, while LA showed a near complete reduction of < 6 log cycles (only one sample had colonies by direct plating out of six samples). After 24 hours of holding at 130°F, only one of the water treated samples had colonies, which demonstrates a reduction of > 6 log CFU/g. *Salmonella* was not detected by enrichment with the LA treatment at 130°F at any tempering time. At 140°F, water showed an immediate reduction of 5.5 log CFU/g, while LA had no recoverable colonies. After 4 hours of holding at 140°F, both treatments showed no

recoverable colonies. At 150°F we found no recoverable colonies at any of the sampling points for both treatments. *Salmonella* was not detected by enrichment with LA treatment at 140°F and 150°F, at any tempering time, and no *Salmonella* was not detected by enrichment with water treatment at 140 and 150°F after 24 hours of holding time.

Research performed on dry corn flour inoculated with 8 serovars of *Salmonella* at a level of 10^5 CFU/g at 15% moisture content found that 99.9% of all *Salmonella* were killed after 24 hours of dry heat application at 120°F (*115*), results similar to our findings. These authors claim that natural contamination of dry foods might be reduced with the application of heat treatments in this temperature range (*115*).

As seen in the lower inoculation level study, increasing tempering temperature provided additional *Salmonella* population reductions. The 140 and 150°F tempering treatments were the only two temperatures that had no recoverable populations after tempering for 24 hours for both of the treatments. The figures show a trend that 5% LA provides higher reductions than water at every temperature tested, and much shorter tempering time can be used to eliminate high levels of *Salmonella* in the contaminated wheat.

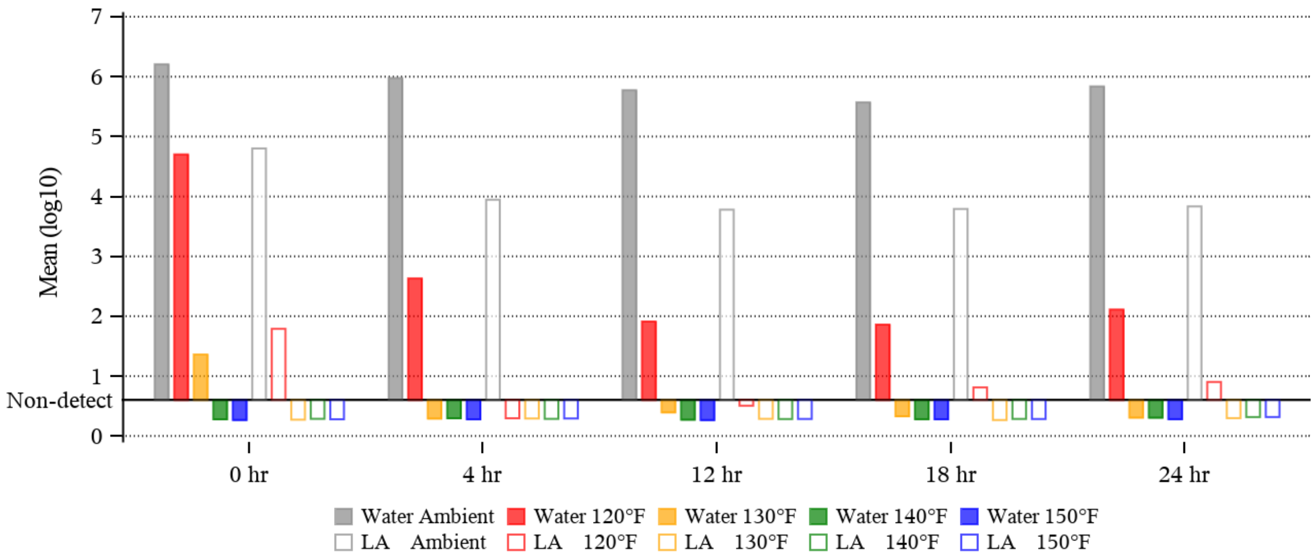


Figure 4-3 *Salmonella* populations at 10^6 inoculation level before and after treatments combinations of water (W) or lactic Acid (LA) and different temperatures at each sampling point.

Overall Results

HRW kernels treated with a 5% LA solution effectively reduced the load of *Salmonella* contaminated dry wheat kernels. Ambient water treatment resulted in a 0.4 log CFU/g reduction over 24 h of tempering at both inoculation levels, whereas ambient LA tempering reduced the *Salmonella* population by 1.2 and 1.4 log cycles at T0 (immediately after application) for low and high inoculation levels, respectively. By 12 h of ambient tempering of low-level inoculated wheat, only 1 of 3 replications indicated residual *Salmonella* viability, while two were negative by enrichment. For the high-inoculation level wheat, a 2.4 log CFU/g reduction was observed after 12 h and no further reductions were noted up to 24 h of tempering. For high inoculation level wheat treated with ambient LA, increasing tempering temperature for LA application to 130°F resulted in >5 log CFU/g reductions by 4 h. At 140°F we found reductions of up to 5.5 log CFU/g immediately after treatment, and no population survival at any other of the sampling points. *Salmonella* was detected by enrichment at 150°F at any tempering time.

Reports from flour milling companies (personal communications) have found an increased thermal resistance of *E. faecalis* if dry heat was applied to the tempering process, this before the addition of tempering liquid. However, with the addition of tempering liquid, results from these experiments are very similar to the ones found by industry, where they found 2- 3 log CFU/g reductions of *E. faecalis* immediately after treatment and non-detectable colonies after 30 minutes of holding temperature at ~150°F. With the addition of the ambient water, this report

found small reductions on the bacterial populations even after holding at ambient temperature for up to 24 hours.

Conclusions

This study demonstrates an effective antimicrobial application for wheat kernels taking advantage of the pre-milling tempering stage of processing that would provide millers with a preventive control step to increase the safety of raw flour. The results of this study showed that tempering HRW kernels with a 5% LA solution effectively reduces the load of *Salmonella,* even at very high contamination levels. With the addition of 5% LA solution, particularly warmed to 120-130°F, very high levels of *Salmonella* can be inactivated almost immediately during the tempering step. Furthermore, increasing the temperature of the tempering during wheat milling showed an enhanced antimicrobial activity to undetectable levels. Therefore, these treatments may have the capability to increase the safety of the wheat milling process.

Chapter 5 - Validation of a Simulated Commercial Baking Process to Control *Salmonella* spp. in Fruit-Filled Pastries

Introduction

Salmonella is a common causative agent that has caused foodborne illnesses linked to flour and baked products (*92*). *Salmonella* spp. causes approximately one million foodborne illnesses, 19,000 hospitalizations, and 380 deaths annually, being credited with causing 11% of the total acquired foodborne illnesses in the U.S. (*17*). *Salmonella* spp. have been linked to multiple bakery products like hamburger buns, sandwiches, cheese and spinach pie, desserts with dairy cream, oven baked pastries, frozen pastries, cream cake, and Turkish delight, among others (*37*, *67*, *70*, *102*).

According to Center for Science in the Public Interest (2015), bakery products caused 142 foodborne outbreaks and 2,822 illnesses between 2004-2013 (*12*). In a study performed in Turkey, it was found that up to 11.8% of bakery products contained *Enterobacteriaceae* and at least one sample had *Salmonella* spp. present (*102*). In Illinois, a pastry company was linked to approximately 100 persons becoming sick after eating one of their products; in this case the suspected cause of disease was *Staphylococcus aureus* (*127*). Another foodborne disease outbreak in Rhode Island was traced to a cream-filled pastries that sickened at least 79 persons, where *Salmonella* was the causative agent (*128*). This outbreak was linked to two deaths and the bakery was ordered to remain closed (*124*). These outbreaks linked to pastries did not only sickened hundreds of persons, but it led to product recalls in at least three different states. It has been stated previously that food recalls can cost companies millions of dollars to ensure

consumer safety and regain trust in customers perceptions (*59*). These outbreaks highlight the importance of preventive steps to control foodborne diseases associated with bakery products.

To ensure safety of their products commercial bakeries should address the following: identify hazards, build preventive controls (PC), create a supply chain approval program, and establish a recall plan (*44*). The Food Safety Modernization Act (FSMA) required that by 2018, very small business (less than $1 million per year) must be in accordance with the new compliances guidelines (*43*), which include many bakeries. FSMA mandates that processors scientifically validate their food manufacturing processes that are considered important to prevent/control food safety hazards (*43*).

Currently there is a need of scientifically validated processes that mimic industry parameters to ensure the safety of bakery products. To the best of our knowledge, there has been no scientifically validated study that ensures the safety of fruit-filled pastries. The objective of this study was to validate a simulated commercial baking process for fruit-filled pastries to control *Salmonella*. *Salmonella* was utilized in this study because it has been linked to multiple bakery product outbreaks primarily through contaminated raw flour and has been found to survive well in dried environments.

Materials and Methods

Experimental Design

This research project was divided into two independent studies: 1) fruit-filled pastries baking validation against a 7-serovar *Salmonella* cocktail, and 2) determination of the thermal D- and *z*-values of the *Salmonella* cocktail in the pastry dough. Pastry dough was prepared from inoculated bread flour. In the baking validation study, pastry dough was rolled into a 3 mm

thickness and cut into squares of 4” x 4”. Dough squares were arranged on a baking sheet and approximately 15 g of pre-made fruit filling was placed in the center of each dough square. With a water moistened brush, two sides of the square were wetted and folded to form triangles, that were then pinched with a fork to crimp the edges. Pastries were then proofed for 30 minutes at 37°C (98.6°F) with a relative humidity of 72%. After proofing, pastries were baked at 190.6°C (375°F) for 15 minutes, followed by 30 minutes of ambient air cooling (B+C). Survival of the *Salmonella* population was determined at 3 minutes during baking and then every 3 minutes and at the end of a cooling period for a total of six sampling points. The *Salmonella* populations were determined in the flour, dough, and at each of the six sampling points. Non-inoculated bread flour was used to prepare pastry dough to determine the pH, water activity (a_w), internal temperature of the product, and proximate analyses of the dough. Relative humidity and temperature of the oven during baking of the bars were also measured. The D- and z- value part of the study of the *Salmonella* cocktail was determined by the use of Thermal Death Time disks (TDT) (6.0 cm diameter and 0.5 cm height; University of Nebraska, Lincoln, NE) and temperature controlled water baths (Model 2864, Thermo Fisher Scientific™, Marietta, OH). Both the baking validation and thermal destruction parameter studies were conducted as randomized complete block designs with three replications as blocks.

Bacterial Cultures

Seven *Salmonella* serovars were used in this research and were selected based on previous research performed by this group and their relationship with low water activity environments. Three serovars of *Salmonella* were obtained from the American Type Culture Collection (ATCC; Newport 6962, Senftenberg 775W 43845 and Typhimurium 14028), and

Salmonella Tennessee and three dry pet food isolates were donated by Richter International, Inc. (Columbus, OH). All cultures were individually grown using a similar method described by Channaiah et al., (29). In brief, all the bacterial cultures were activated in brain heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD) broth from frozen beads kept at -80°C. Then a 10 µl inoculating loop of culture was transferred to a 15 mL centrifuge tube containing 10 mL of BHI broth and grown at 37°C for 24 hours. The working culture was then grown as lawns on BHI agar plates using a sterile swab and harvested using 1 mL of 0.1% peptone (Becton, Dickinson and Company) solution twice. Equal amounts of all the harvested bacteria were mixed to create the bacterial cocktail to be used to inoculate flour.

Flour Inoculation

Bread flour (200 g) was weighed into a sanitized sealable plastic tub (9.4 L, Rubbermaid, Atlanta, GA), spread evenly, and then mist-inoculated inside a biosafety cabinet by spraying ~2 mL of the respective bacterial culture (~1 mL per 100 g of flour mix) uniformly across the flour layer. After sealing the tub, inoculated flour was mixed manually by shaking the tub inside of the biosafety cabinet for 30 sec. Inoculated flour was then dried back to the original pre-inoculation weight by placing tub with open lid inside an incubator (Lab-Line®, Imperial III Incubator, Melrose Park, IL) at 37°C for 3 h. Dried, inoculated flour was mixed again using a spatula and manually shaking the sealed tubs, and then stored in the sealed tub at ambient temperature (~25°C) overnight. The inoculated flour was then used the next morning to make the pastry dough.

Dough Preparation

The recipe and ingredients to prepare the pastries were supplied by AIB International, Inc. (Manhattan, KS) (Table 5-1). The dry ingredients were weighed in a plastic container and stirred together. Then shortening and water was added to a mixing bowl (Artisan®, KitchenAid®, St. Joseph, MI) and then the dry ingredients were added on top of the water-shortening mixture and mixed at speed-1 for 1 minute using a sanitized hook attachment. After the initial minute of mixing, the dough was mixed for 12 minutes at speed 2. After mixing, the dough was rolled out into a 3 mm sheet thickness, followed by cutting squares of 4” by 4”. The 15 g of pre-made fruit filling was added and folded into triangles and pressed with a fork along the pastry edge. Pastries were then proofed for 30 minutes at 37°C (98.6°F) with a relative humidity of 72%. After proofing, pastries were baked at 190.6°C (375°F) for 15 minutes, followed by 30 minutes of ambient air cooling (B+C) on a wire rack.

Table 5-1 Ingredient list and weight of fruit-filled pastries sweet dough recipe.*

Ingredient	Weight (g)
Bread Flour	225.0
Sugar	40.5
Salt	2.3
Nonfat Dry Milk	18
Dried Whole Eggs	5.6
AP Shortening	22.5
Monoglyceride (GMS 520)	6.8
Compressed Yeast	18
Water	101.3
Vanilla Extract	2.3
Roll in Fat	133

*Recipe provided by AIB International and yields 8 pastries.

Baking of Fruit-Filled Pastries

Fine-gauge thermocouples [Type-T Thermocouples (Omega Engineering Inc., Stamford, CT)] connected to an eight-channel data logging system (USB-TC with MCC DAQ software, Measurement Computing, Norton, MA) were used to monitor the temperatures of dough and oven during the baking step. Thermocouples were placed in the geometric center of the pastries. For the measurement of the internal temperatures of the pastries, three pastries had thermocouples inserted per replication, and three replications were performed. To measure the internal temperature of the oven two fine-gauge thermocouples were placed inside the oven in different locations and an average was calculated per replication. The relative humidity of the oven was measured while the non-inoculated fruit-filled pastries were baked. To measure relative humidity, a SCORPION® 2 Profiling system (Markel Food Group., Sinking Springs, PA) was used.

For the oven baking study, flour was inoculated with the *Salmonella* cocktail (~7 log CFU/g), dried overnight to the original pre-inoculation weight of the flour, and used to create the fruit-filled pastries (50-g units). Pastries were baked at 375°F (190.6°C) for 15 minutes, followed by 30 min of ambient air cooling (B+C). Surviving *Salmonella* populations were determined at minute 3 of baking, and then every 3 min using an injury-recovery method by agar overlay plating protocol. Sampling points were randomized for each replication. At each sampling point, samples were placed into chilled 100 mL 0.1% peptone solution and stomached for 1 min in an AES Blue Line Smasher (Biomerieux, Marcy-l' Etoile, France), and placed in a cooler until plated (within 30 min).

To determine pH and a_w, pastry dough was prepared using non-inoculated flour following the recipe in Table 5-1. Later, the pre-made fruit filling were added to the pastries before

sampling at each of the sampling points mentioned before. For a_w measurements, samples were transferred into a_w cups (Decagon Devices, Inc., Pullman, WA), sealed, allowed to reach ~25°C, and a_w was measured using AquaLab Dewpoint 4TE a_w meter (Decagon Devices, Inc.). Sample pH were measured at 25°C using calibrated ExStik® Waterproof pH meter (Extech Instruments, Waltham, MA) by directly placing the pH electrode into a sample bag that contained approximately 25 g of product with 10 mL of distilled water (slurry method).

Thermal Destruction Parameters

D- and *z*- values of the bacterial cultures were determined by a method described by Channaiah et al., (29), with modification, using thermal-death-time (TDT) disks (6.0 cm diameter and 0.5 cm height; University of Nebraska, Lincoln, NE) and temperature-controlled water baths (Model 2864, Thermo Fisher Scientific™, Marietta, OH). Briefly, the D-values of the *Salmonella* cocktail were determined using ~4 g of dough placed into TDT disks, and heating at 55, 58, and 61°C with *Salmonella* population survival determined (using injury-recovery media) during the heat treatment sampling at intervals of 30 minutes, 15 minutes, and 5 minutes respectively.

Salmonella Enumeration and Enrichments

Salmonella enumeration and enrichment in the baking validation and D-values studies were conducted as described by (*30*) using injury-recovery media, BHI agar overlaid with xylose lysine deoxycholate (XLD; Becton, Dickinson and Company) agar. Approximately 15 ml of XLD were placed on top of the BHI as overlay media after 6 hours of incubation at 37°C. For any samples in which no *Salmonella* CFUs were detected on plates, 10 mL of stored homogenate

were added to 90 mL of BHI broth followed by incubation at 37°C for 24 h. These enriched samples were then streaked onto XLD agar plates that were subsequently incubated for 24 h at 37°C to detect any presumptive *Salmonella* presence in samples at very low (undetectable by direct plating) levels.

Statistical Analyses

Baking validation and D- value determinations were independent studies, each designed as randomized complete block with three replications as blocks. The statistical analyses of the *Salmonella* population, pH and a_w data were conducted by analysis of variance at $P \leq 0.05$ using SAS version 9.3 (SAS Institute, Cary, NC). The mean D- and *z*-values were calculated from the linear regression graphs plotted using Microsoft Excel 2011 (Microsoft Corp., Redmond, WA) for each replication individually.

Results and Discussion

Table 5-2 pH and a_w of fruit-filled pastry dough during 15 minutes of baking at 375°F (190.6°C) oven temperature followed by 30 min of ambient air cooling (B+ C).

Sampling point	pH	a_w
Dough pre- proof	5.25 ± 0.32	0.927 ± 0.0010 ^{A}
Dough post- proof	5.46 ± 0.02	0.920 ± 0.0013 ^{A}
3 minutes	5.17 ± 0.07	0.911 ± 0.0028 ^{A}
6 minutes	4.87 ± 0.14	0.885 ± 0.0142 ^{A}
9 minutes	4.98 ± 0.32	0.788 ± 0.0334 ^{B}
12 minutes	5.11 ± 0.26	0.767 ± 0.0030 ^{B}
15 minutes	4.80 ± 0.17	0.726 ± 0.0081 ^{BC}
Baking + Cooling (B + C)	5.33 ± 0.46	0.677 ± 0.0233 ^{C}

Columns with different letters superscripts are statistically different (P≤0.05).

Table 5-2 shows the mean and standard deviation of the water activity (a_w) of the pastries at each of the pull times. This data demonstrates a significant decrease ($P < 0.05$) in a_w from the pre-proof dough sample (0.927 ± 0.0010) until after B+C (0.677 ± 0.0233). The lowest recorded a_w was after the B+C (0.677 ± 0.0233). Previous articles have followed similar protocols to determine a_w of baked products during the baking steps (*69*, *72*). The change in a_w during the baking step could be due to moisture evaporation, protein coagulation, and starch gelatinization (*69*). The initial a_w of this product is comparable to other baked products that presented a_w values ranging from 0.94 to 0.97 before the thermal treatment. These products include hamburger buns, muffins, and donuts (*27*, *28*, *30*).

Table 5-2 also shows the average and standard deviation of the pH of the sweet dough pastries during the baking step at each of the pull times across three replications. No significant changes ($P > 0.05$) occurred from pre-proof (5.25 ± 0.32) to B+ C (5.33 ±0.46). These results are different from the changes observed in baking of peanut butter bars (Chapter 7) where pH significantly increased after B + C. In research performed on similar baking validation studies for brownies, pH values are very similar to the ones found in this research, where the starting pH value was of 5.74 and at the end of the baking validation the value was 5.81 for the crumb (*113*), and like in the present study, the pH values were not significantly different from each other. Other products like sesame-topped bread and hamburgers buns had very similar initial pH values compared to the ones found on this research, of 5.16 ± 0.06 and 5.46 ± 0.04, respectively (*28*, *100*).

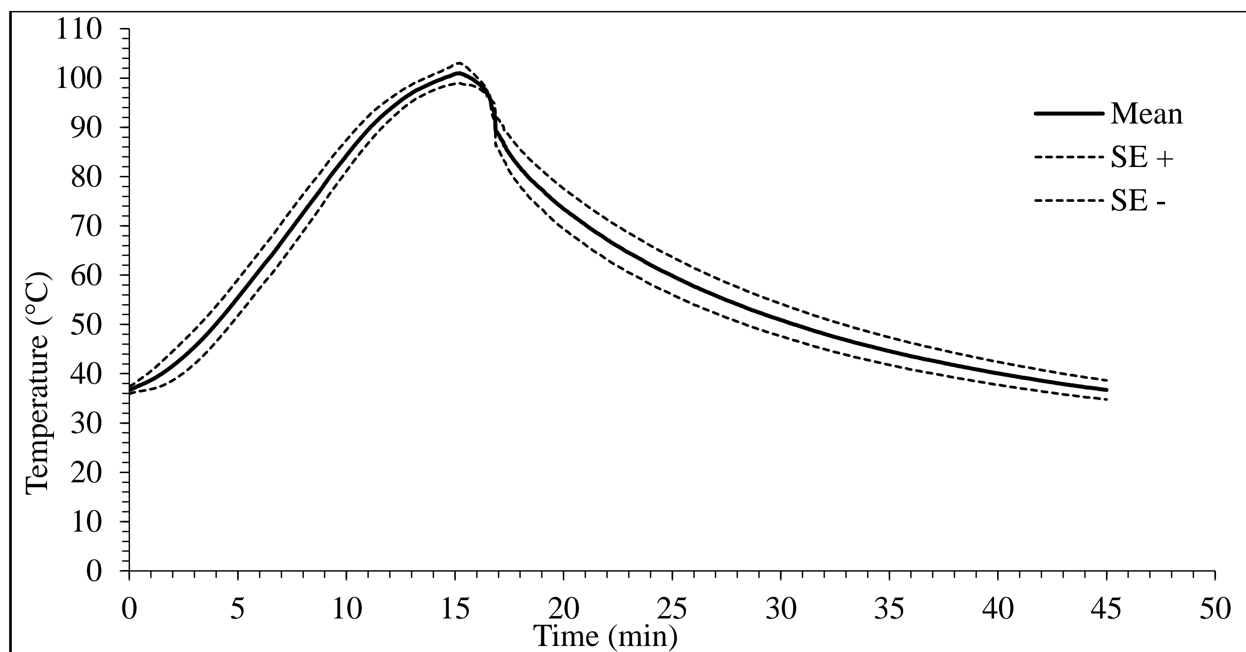


Figure 5-1 Mean temperature profile of pastries during oven baking (±SE) at 375°F (190.6°C) for 15 min of baking + 30 min ambient air cooling.

Figure 5-1 shows the average internal temperature of the center of the fruit-filled pastries during the baking process. Thermocouples were placed in random pastries in the middle of the product and the selection of the pastries during each replication was randomly assigned. The thermocouples collected the temperature data at one second intervals during the 15 min of baking + 30 min of ambient air cooling. The data within replication was averaged and then all three replications were averaged and used as the internal temperature of the pastries during baking. The highest mean internal temperature of the pastries was 100.96°C; this temperature was achieved seconds after removing the pastries from the oven after the 15 minutes of baking. Pastries were removed from the oven after 15 minutes of baking and then were cooled at ambient temperature for 30 minutes on a baking rack. At the end of the cooling period bars had an internal average temperature of 33.32°C. Other authors have found similar values of temperature achieved after 15 minutes of baking at 350° F, where the value of peanut butter cookies reached an internal temperature of 109°C at the end of baking (*72*). Wyatt and Guy (115) mentioned that an internal

baking temperature of 108°C for 1 minute was enough to destroy *Salmonella* and *S. aureus;* however, holding temperatures of 25 and 35°C can support the growth of these microorganisms, placing importance on the handling and distribution of baked products (*121*).

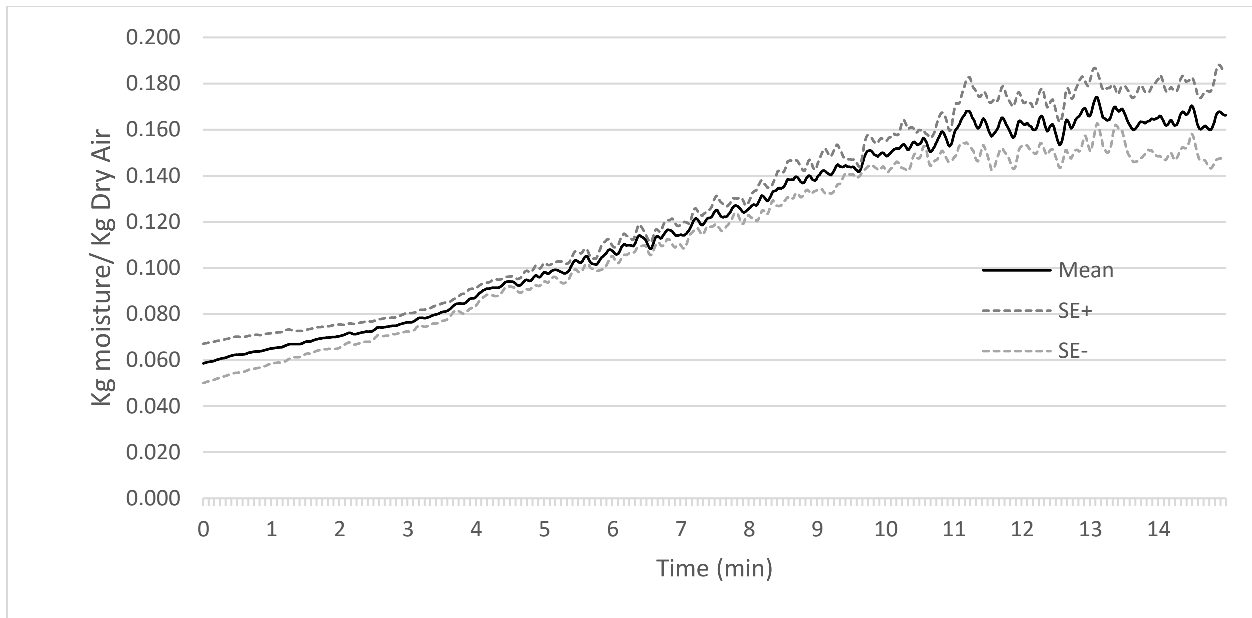


Figure 5-2 Relative humidity within oven during baking of fruit-filled pastries with average (± SE) baked at 375°F (190.6°C) for 15 minutes.

Figure 5-2 shows the average relative humidity ± standard error of the relative humidity (RH) inside the oven during the baking process; three replications were performed. To measure RH of the oven, a SCORPION® 2 Profiling system (Markel Food Group., Sinking Springs, PA) was used. This graph shows the how the humidity inside the oven increases from 0.060 to approximately 0.170 kg moisture/ kg dry air during the baking step of the fruit-filled pastries. Authors have stressed the importance of RH in baking, where it has been stated that relative humidity has a pronounced effect on the heat resistance of *Salmonella* during baking (*117*). The same author found that the surface of the product is the food portion with least lethality in comparison to the center of the product (*117*). Wang (*111*) found that at the same temperature

with increasing relative humidity less time was required to generate similar lethality values of *Salmonella* Agona in high fat matrix products (*117*). These findings highlight the importance of controlling and understanding the RH of the oven in baking of different food products. These findings of *Salmonella* thermal resistance might be explained by the dynamic surface a_w, especially in the surface of the products where changes in a_w are more noticeable (*117*).

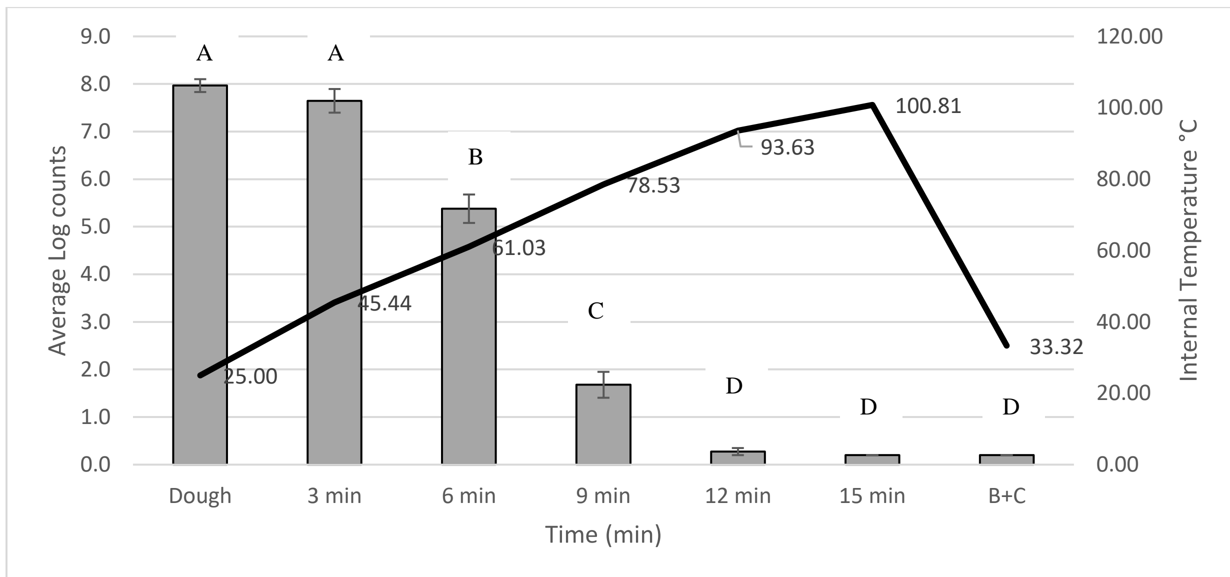


Figure 5-3 Average *Salmonella* spp. log counts and internal temperature (°C) of fruit-filled pastries during 15 min of baking at 375°F (190.6°C) oven temperature followed by 30 min of ambient air cooling (B+ C).

* Columns with different letter superscripts are statistically different (P≤0.05).

Figure 5-3 shows the average *Salmonella* spp. population of the pastries at each of the sampling points, over-imposed with the average internal temperature of the pastries at each point. All of the data points are average values from three replications. As can be noted at the end of the baking, a *Salmonella* reduction of > 5 log CFU/g was achieved, thereby validating the baking step as a highly effective control step in ensuring the microbiological safety of fruit-filled pastries. During baking *Salmonella* counts decreased to 1.7 log CFU/g (a 6.3 log cycle reduction) by 9

minutes of baking. After 12 min of baking, over a 7.7 log CFU/g reduction was achieved compared to the inoculated uncooked dough. After 15 minutes of baking, no viable *Salmonella* population was detected (> 8.0 log CFU/g reduction), as confirmed by enrichment. Previous research performed by different groups have validated different bakery products that achieved >5 log CFU/g. These products include muffins, donuts, hamburger buns, pita chips, and pretzels filled with peanut butter (*28*, *29*, *30*, *69*, *80*). In an inoculated study performed on pumpkin filled pies, it was determined that an internal baking temperature of 108°C for 1 minute was enough to destroy *Salmonella* and *S. aureus* (*121*). This study also mentioned that after baking of the pies, holding temperatures of 25 and 35°C supported the growth of both microorganisms; while, a holding temperature of 4°C was an effective inhibitor of growth (*121*).

Table 5-3 D- and z- values for *Salmonella* in pastry dough at three different temperatures.

Temperature	D-value (min)	z-value (°C)
55°C	32.8 ± 3.62	
58°C	15.5 ± 2.59	7.7 ± 0.46
61°C	5.3 ± 0.12	

Calculated D- and z- values for *Salmonella* spp. in pastry dough at 55, 58, and 61°C are provided in Table 5-3. Other authors have determined D- and z values in products such as hamburger buns at the same temperatures of 55, 58, and 61°C (*28*). The D- values obtained in this research (32.8, 15.5, and 5.3) are higher than the ones obtained in hamburger buns of 28.64, 7.61, and 3.14 min, respectively (*28*). The differences in D- and z- values between the hamburger bun dough and the pastry dough could be due to specific product characteristics like ingredients, pH, aw, product composition, and baking parameters. Other researchers have looked at differences in D- and z- values with different inoculated ingredients (flour or walnuts) and found no significant difference except at 70°C (*29*). It has been noted that increasing values of protein,

fat, and sugar contents of products will increase the thermal resistance of microorganisms (*113*). Studies performed on heat tolerance of *Salmonella* serovars in low water activity products and changes in temperature and solute type found that low a_w was detrimental for survival at temperatures of 55 and 60°C; while, low a_w at temperatures >70°C was a protective factor (*76*). This could be an important determinant to calculate D- and z- values for future studies of products that possess low a_w.

Conclusion

The current research shows a common commercial fruit-filled pastry baking process, that achieved over 100°C at 15 minutes of baking resulted in > 6 log CFU/g reduction of *Salmonella* spp. by 9 minutes of baking and > 8 log CFU/g by 15 minutes of baking. Therefore, this study validated the ability of baking process for fruit-filled pastries to eliminate high levels of *Salmonella* contamination potentially entering the raw dough mix via contaminated ingredients. The results of this research are specific to the baking parameters established in this paper, and any changes in the ingredients or baking parameter may affect pathogen lethality. The D – values calculated in this study could be used to provide information about the heat resistance of *Salmonella* in dough and help determine microbial predictive modeling to inactivate *Salmonella* in fruit-filled pastries. Determination of water activity and product composition are important characteristics of food products that must be considered when calculating D- and z- value studies.

Chapter 6 - Validation of a Simulated Commercial Baking Process to Control *Salmonella* spp. in Cheesecake

Introduction

Salmonella spp. is estimated to cause 1.35 million infections, 26,500 hospitalizations and 3,000 deaths each year (*25*). The main route of infection of *Salmonella* spp. is through food (*25*). *Salmonella* is also the second most common cause of foodborne illness in the United States, the number one cause of hospitalizations, and the number one cause of death from acquired foodborne illness (*17*). CDC has also reported that the incidence of *Salmonella* spp., has not changed in recent years (*108*). The incidence of reported illnesses from *Salmonella* spp. are serovar dependent, where some serovars like Typhimurium and I 4,[5],12:i:- decreased while others like *S.* Infantis was significantly higher (69% increase) (*108*). S. Enteritidis, which is the most common cause of *Salmonella* infection since 2007, has not decreased in the most recent reports available from 2019 (*108*).

Cereals like wheat have been linked to different pathogenic bacteria including *Salmonella* (*74*). Potential microbiological contamination of different cereal grains is a major concern for the industry due to the impact in quality and properties of the grains (*74*). Microorganisms present in cereals like wheat, rye, corn, and others are expected to be similar as those found in production associated with the soil, storage containers, and harvesting/transportation equipment. Microorganisms present during harvesting can come from sources like dust, water, insects, birds, pests, and others (*63*, *74*). Flour is a low water activity ingredient that has been historically regarded as microbiologically safe; however, it has been associated with recent foodborne disease outbreaks (*2*, *35*, *51*, *83*, *116*). The low a_w of these products restrict the growth of

microorganisms if stored correctly (*63*). Therefore, it is important to ensure the safety of the grains and the process of wheat milling to produce flour that has lower risks of being contaminated. However, pathogens such as *Salmonella* can contaminate and survive in raw flour for extended periods; therefore, thermal lethality processes in bakery operations are important in order to ensure the safety of finished food products. Published validation studies on baking to control *Salmonella* in specific bakery products are scarce.

Cheesecakes have been associated with previous foodborne outbreaks. One outbreak was in the United Kingdom in which 7 people become infected and 4 were hospitalized due to *S.* Enteritidis (*87*, *90*). Previous studies performed with cheesecake have demonstrated that *Salmonella* Enteritidis can survive when it was inoculated to 10^6 in a cheesecake batter (*55*). Therefore, there is a need to validate a simulated commercial baking process for cheesecake to control *Salmonella* and to determine D- and z-values of a 7-serovar *Salmonella* (Hartford, Newport, Senftenberg, Tennessee, Typhimurium, and two untyped pet food isolates) cocktail in cheesecake batter.

Materials and Methods

Experimental Design

This research was split into two independent studies: 1) cheesecake baking validation against a 7-serovar *Salmonella* cocktail, and 2) determination of the D- and *z*-values of the *Salmonella* cocktail in cheesecake batter. Cheesecake batter (2245 g) was made from inoculated bread flour. During the baking validation part of the study, the cheesecake batter was divided among five 6-inch aluminum pans (350 g batter in each pan). The 5 filled pans were placed onto

a baking sheet, which was placed into a preheated oven at 300°F (148.9°C). Cheesecakes were oven-baked for 50 minutes, followed by 30 minutes of ambient air cooling (B+C) on a wire rack. Surviving *Salmonella* populations were then determined at 12.5 minutes of baking and then every 12.5 minutes, and at the end of the cooling period for a total of five sampling points. The *Salmonella* populations were also determined in the raw flour and raw batter, for a total of seven sampling points. Another batch of cheesecake batter was prepared using flour that was non-inoculated to determine pH, water activity (a_w), internal temperature of cheesecake batter during baking, and proximate analyses. Relative humidity and temperature of the oven during baking of the cheesecakes were also measured. In D- and z-value part of the study, Thermal Death Time disks (TDT) (6.0 cm diameter and 0.5 cm height; University of Nebraska, Lincoln, NE) and temperature-controlled water baths (Model 2864, Thermo Fisher Scientific™, Marietta, OH) were used to characterize the heat tolerance of the *Salmonella* cocktail in cheesecake batter. Both baking validation and thermal destruction parameter studies were conducted as randomized complete block design with three replications as blocks.

Bacterial Cultures

Seven *Salmonella* serovars were used in this research and were selected based on previous research performed by this group and their relationship with low water activity environments. Three serovars of *Salmonella* were obtained from the American Type Culture Collection (ATCC; Newport 6962, Senftenberg 775W 43845 and Typhimurium 14028), and *Salmonella* Tennessee and three untyped dry pet food isolates were donated by Richter International, Inc. (Columbus, OH). All cultures were individually grown using a similar method described by Channaiah et al., (29). In brief, all the bacterial cultures were activated in brain

heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD) broth from frozen beads stored at -80°C. A 10 µl inoculating loop was used to transfer each culture to a 15 mL centrifuge tube containing 10 mL of BHI broth, and was incubated at 37°C for 24 h. The working cultures were then grown as 24-h lawns on BHI agar plates using a sterile swab, and were harvested using 1 mL of 0.1% peptone (Becton, Dickinson and Company) solution twice. Equal amounts of all the harvested *Salmonella* serotypes were mixed to create the bacterial cocktail used as master inoculum.

Flour Inoculation

Bread flour (200 g) was weighed into a sanitized sealable plastic tub (9.4 L, Rubbermaid, Atlanta, GA), spread evenly, and then mist-inoculated inside a biosafety cabinet by spraying ~2 mL of the master inoculum (~1 mL per 100 g of flour mix) uniformly across the flour layer. After sealing the tub, inoculated flour was mixed manually by shaking the tub inside of the biosafety cabinet for 30 sec. Inoculated flour was then dried back to the original pre-inoculation weight by placing the tub with open lid inside an incubator (Lab-Line®, Imperial III Incubator, Melrose Park, IL) at 37°C for 3 h. Dried, inoculated flour was mixed again using a spatula and manually shaking the sealed tubs, and then stored in the sealed tub at ambient temperature (~25°C) overnight. The inoculated flour was then used the next morning to prepare the cheesecake batter.

Batter Preparation

The recipe and ingredients to prepare the cheesecakes were supplied by AIB International, Inc. (Manhattan, KS) (Table 6-1). The cream cheese was softened by warming to

60°F (15.6°C) and was added to a mixing bowl and mixed for 1 minute at low speed (Artisan®, KitchenAid®, St. Joseph, MI). After softening of the cream cheese, the flour and sugar were added to the mixing bowl and mixed for 3.5 minutes at low speed. While the bowl contents were mixing, the sour cream along with the eggs and vanilla were tempered to an approximate temperature of 68°F. Once the wet ingredients were tempered, they were added to the mixing bowl and mixed for 2.5 minutes at low speed. To make the cheesecake crust, 50 g of pre-made graham cracker crust was placed into circular 6-inch aluminum pans and spread out while pressing to the bottom of the pan. Then 350 g of the batter was poured over the cracker crust for each of the five pans of cheesecake. Finally, the five cheesecakes were placed onto a baking sheet pan and were baked at 300°F (148.9°C) for 50 minutes, followed by 30 minutes of ambient air cooling (B+C) on a wire rack.

Table 6-1 Ingredient list and weights of the cheesecake recipe.

Ingredient	Weights (g)
Cream Cheese	1100.0
Sugar	423.9
Bread Flour	24.2
Sour Cream	187.0
Liquid Whole Eggs	253.8
Vanilla Extract	5.8
Graham Crust	250.0

Cheesecake Baking

Fine-gauge thermocouples [Type-T Thermocouples (Omega Engineering Inc., Stamford, CT)] connected to an eight-channel data logging system (USB-TC with MCC DAQ software, Measurement Computing, Norton, MA) were used to monitor and record the temperatures of batter during the baking step. Thermocouples were placed in the center of the cheesecakes. To

measure the internal temperatures of the cheesecakes, three individual cheesecakes had thermocouples inserted per replication, and three replications of the internal temperature were performed. To determine the internal air temperature of the oven two fine-gauge thermocouples were placed inside the oven in different locations and an average was calculated per replication; three replications were performed. The relative humidity of the oven was measured while non-inoculated cheesecakes were baked. To measure relative humidity a SCORPION® 2 Profiling system (Markel Food Group., Sinking Springs, PA) was used.

The bread flour was inoculated with the *Salmonella* cocktail (8 log CFU/g), dried overnight to the original pre-inoculation weight of the flour, and used to create the cheesecakes (total weight of each cheesecake was 400 g). Cheesecakes were baked at 300°F (148.9°C) for 50 minutes, followed by 30 min of ambient air cooling (B+C). Surviving *Salmonella* populations were determined at 12.5 minutes and every 12.5 minutes thereafter using an injury-recovery agar overlay plating protocol. Sampling points were randomized for each replication. At each sampling point, samples were rapidly placed into chilled 50 mL of 0.1% peptone water (Becton, Dickinson and Company) and stomached for 1 min in an AES Blue Line Smasher (Biomerieux, Marcy-l' Etoile, France). Homogenized samples were then placed in a cooler until plated (within 30 minutes).

To determine pH and a_w, cheesecakes were prepared using non-inoculated flour and were sampled at each of the six sample points mentioned before. For a_w measurements, samples were transferred into a_w cups (Decagon Devices, Inc., Pullman, WA), sealed, allowed to reach ~25°C, and a_w was measured using AquaLab Dewpoint 4TE a_w meter (Decagon Devices, Inc.). Sample pH was measured at 25°C using a calibrated ExStik® Waterproof pH meter (Extech

Instruments, Waltham, MA) by directly placing the pH electrode into a sample bag that contained approximately 25 g of product with 10 mL of distilled water (slurry method).

Thermal Destruction Parameters

D- and *z*- values of the *Salmonella* cocktail in cheesecake batter were determined by a method described by Channaiah et al., (29), with modifications, using Thermal Death Time (TDT) disks (6.0 cm diameter and 0.5 cm height; University of Nebraska, Lincoln, NE) and temperature-controlled water baths (Model 2864, Thermo Fisher Scientific™, Marietta, OH). Briefly, the D-values of *Salmonella* cocktail were determined using ~5 g of batter placed inside TDT disks, and heating at 55, 58, and 61°C with *Salmonella* population survival determined (using injury-recovery media) during the heat treatments, sampling at intervals of 20 minutes, 10 minutes, and 5 minutes, respectively for the three temperatures.

Bacterial Enumeration and Enrichment

Salmonella enumeration and enrichment in the baking validation and D-values studies were conducted as described by (*30*) using injury-recovery media, BHI agar (Becton, Dickinson and Company) overlaid with xylose lysine deoxycholate (XLD; Becton, Dickinson and Company) agar. Approximately 15 mL of XLD were placed on top of the BHI as overlay media after 6 hours of incubation at 37°C. For any samples in which no *Salmonella* CFUs were detected on plates, 10 mL of stored homogenate were added to 90 mL of BHI broth followed by incubation at 37°C for 24 h. These enriched samples were then streaked onto XLD agar plates that were subsequently incubated for 24 h at 37°C to detect any presumptive *Salmonella* presence in samples at very low (undetectable by direct plating) levels.

Statistical Analyses

Baking validation and D- value determinations were independent studies, each designed as randomized complete block with three replications as blocks. The statistical analyses of the *Salmonella* population, pH and a_w data were conducted by analysis of variance at $P \leq 0.05$ using SAS version 9.3 (SAS Institute, Cary, NC). The mean D- and *z*-values were calculated from the linear regression graphs plotted using Microsoft Excel 2011 (Microsoft Corp., Redmond, WA) for each replication individually.

Results and Discussion

The recipe used in this study is very similar to the one used by previous researchers, with the main difference being that in the current study margarine was not used (*55*). In the research by Hao et al., (51) they used whole eggs that were added one at a time and mixed until smooth, and after all the eggs were added 1 mL of two concentration of master inoculum was added to achieve an inoculation level of 10 or 10^6 (*55*); while, in our study we inoculated the flour used to make the batter. One of the main differences of Hao et al., (51) study is the heating profile of the baking step; where, they placed the cheesecake inside a preheated oven at 232°C for 10 minutes and then the temperature was dropped to 121°C for one hour (*55*). The temperature used for this study was of 148.9°C for 50 minutes of baking.

Table 6-2 pH and a_w of cheesecake dough during 50 minutes of baking at 300°F (148.9°C) oven temperature followed by 30 min of ambient air cooling (B+ C).

Sampling point	pH	a_w
Batter	4.80 ± 0.02	0.954 ± 0.0031
12.5 minutes	4.79 ± 0.03	0.961 ± 0.0047
25 minutes	4.78 ± 0.05	0.954 ± 0.0066
37.5 minutes	4.79 ± 0.01	0.961 ± 0.0032
50 minutes	4.82 ± 0.02	0.941 ± 0.0054
Baking + Cooling (B + C)	4.86 ± 0.03	0.943 ± 0.0069

Table 6-2 shows the mean and standard deviation of the water activity (a_w) of the cheesecakes at each of the sampling times. This data indicates that the a_w did not change significantly with any of the pull times ($P > 0.05$). The lowest recorded a_w was after 50 min of baking (0.941 ± 0.0054). Although there are differences in the baking parameters and recipe compared to other studies performed with cheesecake, the a_w are very similar where it was found by previous authors to be a_w 0.98 ± 0.001 in batter and after baking (*55*). Both studies did not find significant differences in the pre- and post-baking water activity values.

Table 6-2 also shows the average and standard deviation of the pH of the cheesecake during the baking step at each of the pull times. pH did not significantly change from cheesecake batter (4.80 ± 0.02) to the B+ C (4.86 ± 0.03) ($P > 0.05$). When compared to previous cheesecake studies, we found very similar pH values; where, pH of the batter was of 4.96 ± 0.02 and the pH of the cheesecake was of 4.93 ± 0.02. None of the studies found differences in the pH of cheesecake batter to fully cooked product (*55*).

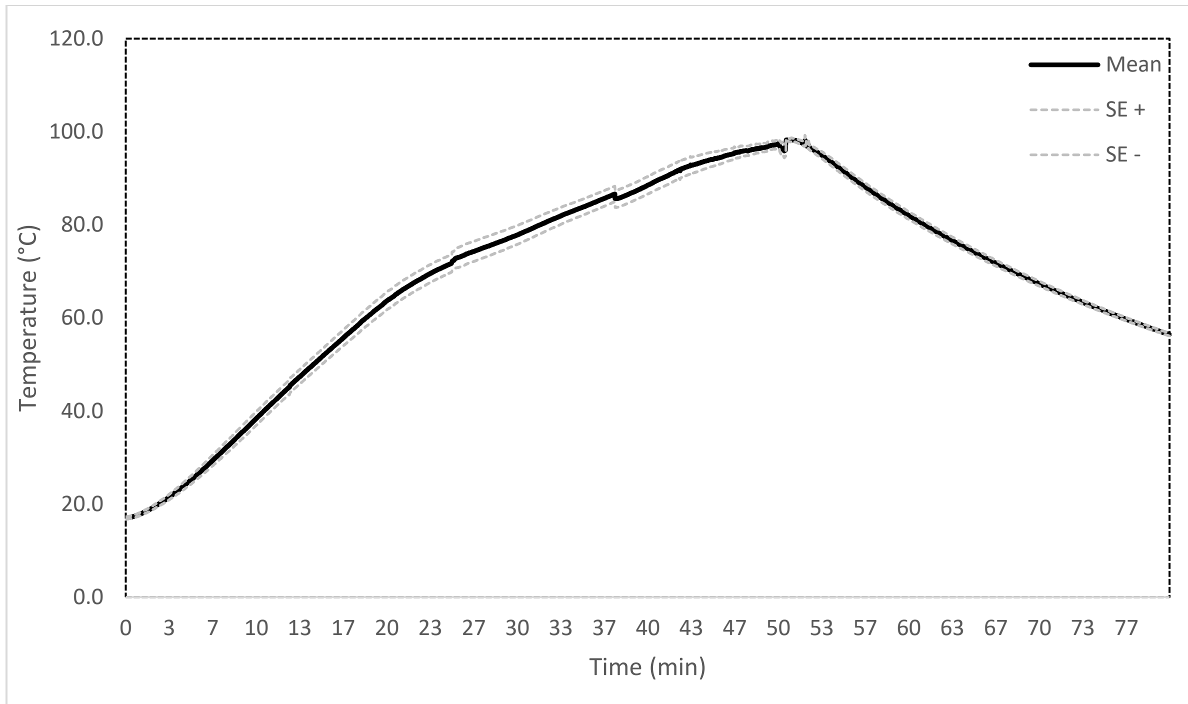


Figure 6-1. Mean temperature profile of cheesecake in oven during baking (±SE) at 300°F (148.9°C) for 50 min followed by 30 min ambient air cooling.

Figure 6-1 shows the average internal temperature of the cheesecake during the baking step. To measure the internal temperature, thermocouples were placed in different cheesecakes in the middle of the product and the thermocouples were placed in different cheesecake locations within the oven chamber in each of the replications. The thermocouples were programmed to record data in one second interval during the 50 min of baking + 30 min of ambient air cooling. The data within replication was averaged and then all three replications were averaged and used as the internal temperature of the cheesecake batter during baking. The average value was recorded as the internal temperature of the cheesecake during baking. The highest mean internal temperature of the cheesecakes was of 97.4°C; this temperature was achieved at 50 minutes of baking, and it was held for a couple of seconds before it started dropping after removing the cheesecakes from the oven for ambient air cooling. After the 50 minutes of baking, the baking pan with the five cheesecakes was removed from the oven, the individual cheesecakes were placed on a baking rack

and let cool down at room temperature 25°C for 30 minutes. When the thermocouples were first placed inside the cheesecakes the reported temperature was 17.0°C and at the end of the cooling period the cheesecakes had an internal average temperature of 56.4°C. In previous studies on cheesecake where they had a two-step baking process where the product was placed inside an oven at 232°C for 10 minutes to then the temperature decreased to 121°C for one hour, it was found that the highest internal temperature achieved was 68°C (*55*). The highest temperature achieved in Hao et al., (51) study was almost 30°C lower than in our study of 97.4°C; thus, *Salmonella* lethality of the two baking schedules were very different.

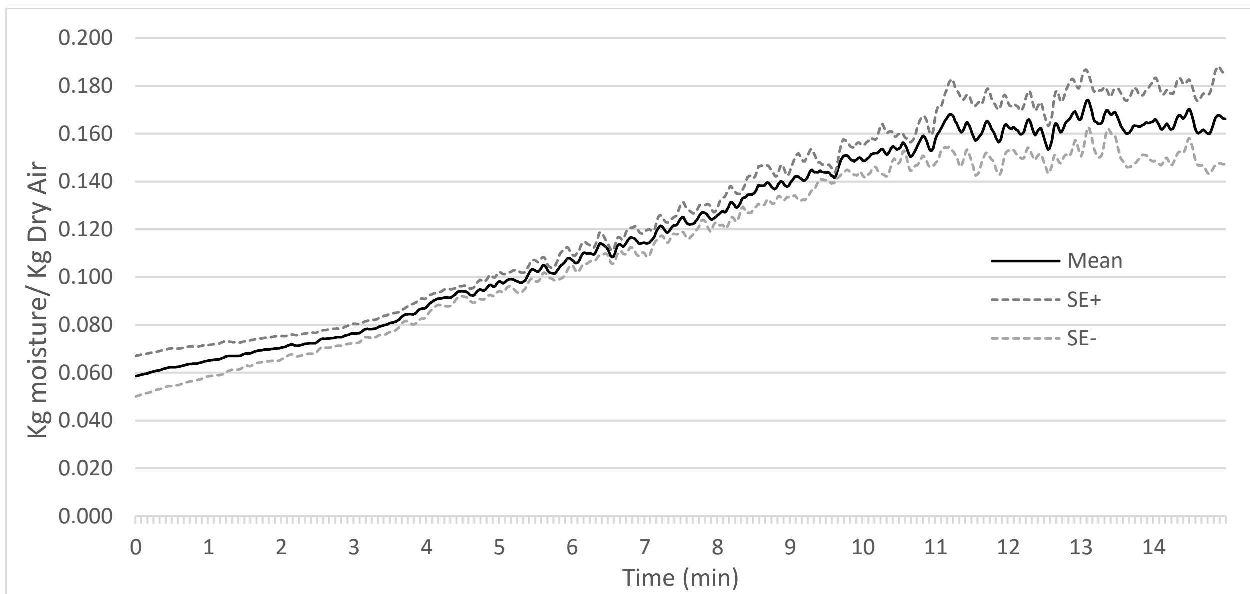


Figure 6-2. Relative humidity of the oven during cheesecake baking (with average ± SE) at 300°F (148.9°C) for 50 minutes.

Figure 6-2 shows the average relative humidity ± standard error of the relative humidity (RH) inside the oven during the baking process. To measure RH of the oven, a SCORPION® 2 Profiling system (Markel Food Group., Sinking Springs, PA) was used. This graph shows that the humidity inside the oven increased from 0.060 to 0.0170 kg moisture/kg dry air during the baking step of the cheesecake. Relative humidity plays an important role in the thermal

resistance of *Salmonella* spp. during baking steps (*117*). It has been demonstrated before that the surface of baked products has a lower bacterial lethality compared to the center of the products; and that at an increasing relative humidity, less time is required to reduce *S*. Agona in high fat food matrices (*117*). These findings highlight the importance of controlling and understanding the RH of the oven in baking of different food products.

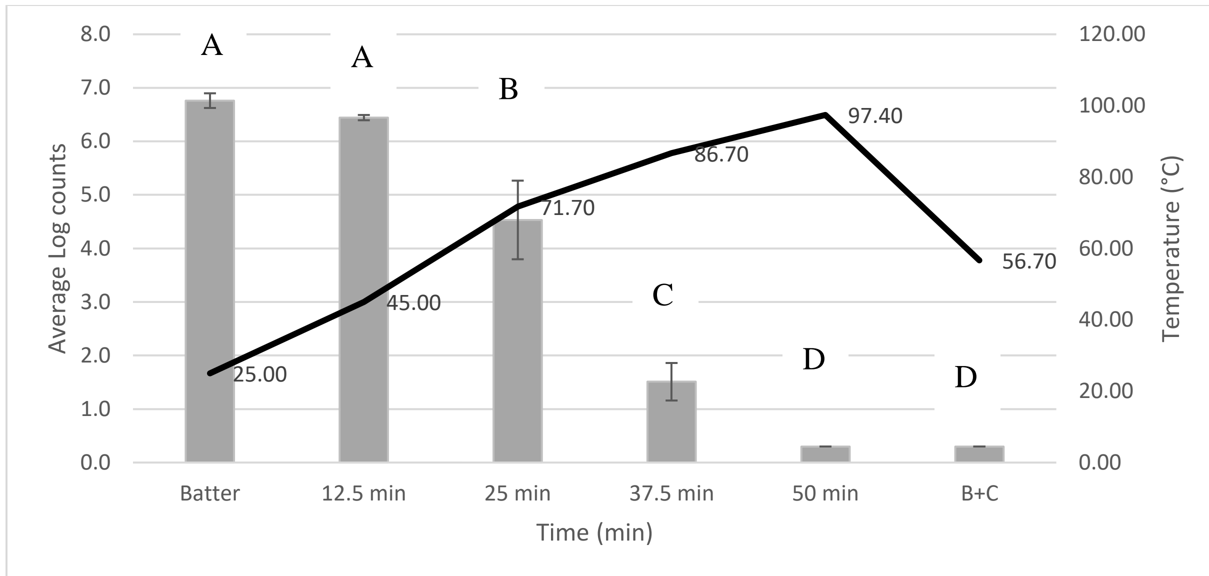


Figure 6-3. Average *Salmonella* spp. log counts and internal temperature (°C) of cheesecake during 50 min of baking at 300°F (148.9°C) oven temperature followed by 30 min of ambient air cooling (B+ C).

* Columns with different letter superscripts are statistically different (P≤0.05).

Figure 6-3 shows the average residual *Salmonella* spp. population of cheesecakes at each of the sampling points, over-imposed with the average internal temperature of cheesecakes at each of the sampling points. All of the data points are average values from three replications. At the end of 50 min of baking, a *Salmonella* reduction of > 6.5 log CFU/g was achieved; thus validating the baking step as an effective *Salmonella* control step. After 37.5 min of baking, over a 5-log CFU/g reduction was achieved compared to the inoculated batter. During baking *Salmonella* counts decreased to < 0.30 log CFU/g (detection limit) at 50 minutes of baking, resulting in >6.5 log

CFU/g reduction. After 50 minutes of baking, we found no bacterial population survival, confirmed by enrichment, which provides a full lethality of the inoculated samples. In similar research performed on cheesecake, it was found that *S.* Enteritidis survived (enrichment positive) in three of nine samples in products with similar compositions that were inoculated at 10^6 (*55*); however, the heating profile in this study was very different from the one used in the current research. In the previous study, where they had two formulations (regular and low fat), it was found that the lower fat content did not have an effect on the survival of *S.* Enteritidis (*55*). These authors highlight the importance of temperature achieved during baking to assess the lethality of *Salmonella* achieved, where it was mentioned that the center of the product had more survival compared to the sides of the product (*55*). Other products like muffins, donuts, hamburger buns, pita chips, and pretzels filled with peanut butter have demonstrated lethality >5 log CFU/g, similar to the results found in this study (*28*, *29*, *30*, *69*, *80*). In another study performed on pumpkin filled pies, an internal temperature of 108°C for 1 minute was enough to destroy *Salmonella* and *S. aureus* (*121*).

Table 6-3 D- and z- values for a 7-serovar *Salmonella* cocktail in cheesecake batter at three different temperatures.

Temperature	D-value (min)	z-value (°C)
55°C	27.4 ± 1.77	
58°C	13.8 ± 0.77	8.2 ± 1.01
61°C	4.9 ± 0.83	

The D- and z- values for *Salmonella* spp. in cheesecake batter at 55, 58, and 61°C are provided in Table 6-3. Other researchers that studied *Salmonella* survival population during cheesecake baking stressed that D- values for cheesecake have not been previously generated. Moreover, they did not perform a D- value study claiming that the temperatures required for D-

value studies are too high and would negatively impact the quality of the final product (*55*). Other researchers have determined D- values in different bakery products and found values in hamburger bun dough of 28.64, 7.61, and 3.14 minutes at 55°C, 58°C, and 61°C, respectively (*28*). Comparing these lethality values to the results found in the present study, the values in cheesecake batter are higher compared to the hamburger buns. The differences in D- and z-values between the hamburger buns and the cheesecake could be due to specific product characteristics like ingredients, pH, aw, product composition, and baking parameters. One of the main ingredients in the cheesecake is cream cheese, and the cheesecake has a higher fat content than the hamburger buns (18.12% and 3.79%, respectively) (*28*). Previous researchers have demonstrated that increasing values of protein, fat, and sugar contents of products increased the thermal resistance of microorganisms (*55*, *113*). FDA in its draft guidance for industry have mentioned that generally as the moisture of a product decreases the general heat resistance of microbes will increase; for fat content is mentioned that at higher fat content higher thermal resistance; and, the presence of sugar can increase the thermal resistance of microorganisms mainly due to the decrease in water activity of the products (*46*). Other researchers studied the heat tolerance of *Salmonella* serovars in low water activity products and found that low a_w was detrimental for *Salmonella* survival at temperatures of 55 and 60°C; while, low a_w at temperatures >70°C always acted as a protective factor (*76*).

Conclusion

The current work presents data to validate a commercial cheesecake preparation and baking process, mimicking industry parameters. The mean internal temperature of cheesecake increased from 17 to ~97°C at the end of 50-min baking time. The *Salmonella* population in

cheesecake decreased by >5 log CFU/g by 37.5 min of baking and was completely eliminated after 50 min of baking (as determined by enrichment). This study validated the effectiveness of baking process for cheesecake by demonstrating a >6.5 log CFU/g reduction in *Salmonella* population after 50 minutes of baking at 300°F. The pH and water activity of cheesecake at B+C were 4.86 and 0.943, respectively. D- values of the *Salmonella* cocktail at 55, 58, and 61°C were 27.4, 13.8, and 4.9 min, respectively, whereas the *z*-value of the *Salmonella* cocktail was 8.2°C. This study validated a baking process for cheesecake; however, additional research should be conducted if the recipe and/or baking parameters are modified. The D- and *z*-values determined in cheesecake batter can be used by the bakery industry to evaluate the expected lethality of their baking processes. The D- values calculated in this study could be used to provide information about the heat resistance of *Salmonella* in batter and help determine microbial predictive modeling to inactivate *Salmonella* in cheesecake. Determination of water activity and product composition are important characteristics of food products that must be considered when calculating D- and z- value studies.

Chapter 7 - Validation of a Simulated Commercial Baking Process to Control *Salmonella* spp., Shiga Toxin-producing *E. coli,* and *L. monocytogenes* in Peanut Butter Bars

Introduction

Peanut butter bars belong to a segment of the bakery industry known as snack and granola bars (*39*). This category of product sold over $6.14 billion during 2018 (*39*). These products have seen an increase in their sales because they can be perceived as healthy, natural, and a good source of protein (*39*). The ingredients present in peanut butter bars makes this product a high fat and sugar content product with a relatively low water activity (a_w). Peanut butter and flour are the two main ingredients of peanut butter bars and these ingredients have been recognized as potential vehicles for different pathogens (*120*).

Flour is usually regarded as a safe product; however, some studies found that it can be contaminated during milling, packaging, transportation, and storage (*7*, *120*). Flour can be traced back as the source of contamination of different outbreaks (*21*, *45*). Flour has a low a_w that does not allow the growth of pathogens; however, pathogens can survive for long periods during storage and reproduce once the flour is rehydrated by mixing it with the product ingredients (*7*). Some studies have found that coliforms can be found at all of the stages of the flour milling process (*7*). *E. coli* has been detected sporadically in flour end products derived from the outer grains, and it is believed that contamination can come during the conditioning process (*7*). *Salmonella* has also been found in flour at low levels in different studies (*7*, *35*).

Peanut butter is a high fat (55% fat content) and low a_w (0.20 to 033) product that has been found contaminated with *Salmonella* spp. (*11*). *Salmonella* Mbandaka, Tennessee and

Typhimurium have been found at low levels in opened jar of peanut butter (*11*, *19*, *20*, *98*). Different authors have found that *Salmonella* spp. can survive for 24 in both refrigerated and room temperature in different types of peanut butters and spreads (*11*, *66*). Other researchers have found that *L. monocytogenes* can survive in peanut butter formulations after small contaminations during processing and that it can survive in the product for up to 420 days (*33*). In a similar study it was found that *Salmonella* and *E. coli* O157:H7 can survive at room temperature or refrigerated temperatures in peanut butter for up to 9 weeks (*66*). Several researchers have tried to reduce the levels of pathogens present in peanut butter; however, it is believed that due to the physical characteristics of this product *Salmonella* and *E. coli* have a higher thermal resistance when compared to other products (*58*, *75*, *99*).

Salmonella spp. was the second most common cause of single etiology confirmed outbreaks with a total of for 113 outbreaks (29%) for the year 2017 (*26*). *Salmonella* spp. is a risk in ready to eat or ready to bake products with 37 positive samples out of 207 (17.9%) (*70*). Peanut butter has been linked to several outbreaks in the US where *Salmonella* spp. has been the responsible pathogen (*5*).

Shiga toxin-producing *Escherichia coli* (STEC) was the third most common cause of foodborne outbreaks from a single etiology, causing five percent of outbreaks and six percent of the total hospitalizations (*26*). *E. coli* O157 was found in eight out of 133 samples in a study on ready to eat and ready to bake products (*70*). STEC has been recently linked to outbreaks in soynut butter (2017) and flour (2016 and 2019) (*16*, *45*).

Listeria monocytogenes is a ubiquitous bacterium that is commonly associated with post-processing contamination. For the year 2017, *L. monocytogenes* was linked to eight foodborne outbreaks in the US (*26*). A study of the incidence of different bacteria in ready to bake products

found that *L. monocytogenes* was present in 6.8% of the samples analyzed (*70*). There are few papers that report the survival rate of *L. monocytogenes* in baked products (*69*).

While almost all bakery products go through a cooking step, process validation studies are required to ensure consumer safety (*28*). Therefore, validated bakery processes that ensure reduction of pathogens to safe levels are necessary. Bakers must incorporate preventive control steps during manufacturing to eliminate these pathogens. This study was conducted to validate a representative commercial oven baking process for the manufacture of cereal-based peanut butter bars against mixed inocula of *Salmonella* (serotypes Hartford, Newport, Senftenberg, Tennessee, Typhimurium, and two untyped pet food isolates), STEC (O26, O45, O103, O111, O121, O145, and O157), and *Listeria monocytogenes* (5 strains). This study also was conducted to determine D- and z-values against a mixed cocktail of *Salmonella,* STEC and *Listeria monocytogenes* during heating of peanut butter cereal bar dough adjusted to an a_w of 0.65 representative of surface drying during oven baking.

Materials and Methods

Experimental Design

This research project was divided in two independent studies: 1) peanut butter bars baking validation against a 7-serovar *Salmonella* cocktail, a 7-strain *E. coli* cocktail, and a 5-strain *Listeria monocytogenes* cocktail, and 2) determination of the D- and *z*-values of the three pathogen cocktails in peanut butter bars dough. Peanut butter bar dough was prepared from inoculated oat flour. In the baking validation study, peanut butter bar dough was rolled into a sheet of ½" thickness and cut into bars of 1.5" x 4". Bars were baked at 177°C (350°F) for 13 minutes, followed by 15 minutes of ambient air cooling (B+C). Survival populations of the three pathogens were determined after one minute of baking, every two minutes thereafter during

baking, and at the end of the cooling period for a total of 8 sampling points. The pathogen populations were determined in the flour, dough, and at each of the eight sampling points. Non-inoculated flour was used to prepare peanut butter bar dough to determine the pH, water activity (a_w), internal temperature of the product, and proximate analyses of the dough at each of the sampling points. Relative humidity and temperature of the oven during baking of the bars were also measured. The D- and z- values of the three pathogen cocktails were determined by the use of Thermal Death Time disks (TDT) (6.0 cm diameter and 0.5 cm height; University of Nebraska, Lincoln, NE), Clarity vacuum pouches (3-mil 6" X 8") (BUNZL, Riverside, MO), and temperature-controlled water baths (Model 2864, Thermo Fisher Scientific™, Marietta, OH). Both baking validation and thermal destruction parameter studies were conducted as randomized complete block designs with three replications as blocks.

Bacterial Cultures

Seven *Salmonella* serovars were used in this research and were selected based on previous research performed by this group and their relationship with low water activity environments. Three serovars of *Salmonella* were obtained from the American Type Culture Collection (ATCC; Newport 6962, Senftenberg 775W 43845 and Typhimurium 14028), and *Salmonella* Tennessee and three untyped dry pet food isolates that were donated by Richter International, Inc. (Columbus, OH). Six of the *E. coli* strain (O157, O145, O111, O103, O45, and O26) were obtained from Dr. John Luchansky (USDA ARS Eastern Regional Research Center, Wyndmoor, PA), while O121 (which was isolated from flour associated with a recent STEC O121 outbreak and obtained from flour milled in the state of Missouri) was provided by the U.S. Food and Drug Administration (FDA, Silver Spring, MD). Four strains of *Listeria*

monocytogenes (B-33043, B-33260, B33054, and B-33245) were obtained from the ARS culture collection (NRRL) (Peoria, IL) and one strain was obtained from the American Type Culture Collection (ATCC; *Listeria monocytogenes* 7644). All cultures were individually grown using a similar method described by Channaiah et al., (29). In brief, all the bacterial cultures were activated in brain heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD) broth from frozen beads stored at -80°C. Then a 10 µl inoculating loop was used to transfer an aliquot of culture to a 15 mL centrifuge tube containing 10 mL of BHI broth and grown at 37°C for 24 hours. The working culture was then grown as lawns on BHI agar plates using a sterile swab, and harvested using 1 mL of 0.1% peptone (Becton, Dickinson and Company) solution twice. Equal amounts of all the harvested strains of each pathogen were mixed to create the mixed strain cocktail of each of the respective pathogens.

Flour Inoculation

Oat flour (250 g) was weighed into a sanitized sealable plastic tub (9.4 L, Rubbermaid, Atlanta, GA), spread evenly, and then mist-inoculated inside a biosafety cabinet by spraying ~2.5 mL of the respective pathogen cocktail (~1 mL per 100 g of flour mix) uniformly across the flour layer. After sealing the tub, inoculated flour was mixed manually by shaking the tub inside of the biosafety cabinet for 30 sec. Inoculated flour was then dried back to the original pre-inoculation weight by placing the tub with open lid inside an incubator (Lab-Line®, Imperial III Incubator, Melrose Park, IL) at 37°C for 3 h. Dried, inoculated flour was mixed again using a spatula and manually shaking the sealed tubs, and then stored in the sealed tub at ambient temperature (~25°C) overnight. The inoculated flour was used the next morning to make the peanut butter bar dough.

Dough Preparation

The recipe and ingredients to prepare the peanut butter bar were supplied by AIB International, Inc., Manhattan, KS (Table 7-1). The wet ingredients were weighed in a sanitized mixing bowl (Artisan®, KitchenAid®, St. Joseph, MI) and mixed for one minute at speed-1 using a sanitized paddle attachment. All dry ingredients were then added to the bowl, placed in the mixer, and mixed for 1 min at speed 1. After mixing, the dough was rolled out into a ½" thick sheet, followed by cutting bars to a size of 1.5" by 4".

Table 7-1 Ingredient list and weights for the peanut butter bars recipe.*

Ingredient	Weight (g)
Oat Flour	200.0
Cinnamon	3.6
Soda	6.4
Salt	1.6
Baby Oats	60.6
Brown Sugar	114.4
Peanut Butter	284.0
Water	82.0
HFCS 42	44.2
Vanilla	8.8

*Each prepared batch yields eight 55-g bars.

Peanut Butter Bar Baking

Fine-gauge thermocouples [Type-T Thermocouples (Omega Engineering Inc., Stamford, CT)] connected to an eight-channel data logging system (USB-TC with MCC DAQ software, Measurement Computing, Norton, MA) were used to monitor the temperatures of dough and the oven chamber during the baking step. Thermocouples were placed in the geometric center of the

bars. For the measurement of the internal temperatures of the bars, three bars had thermocouples inserted and were randomly selected per replication, and three replications were performed. To measure the internal temperature of the oven, two fine-gauge thermocouples were placed inside the oven in different locations and an average was calculated per replication. The relative humidity of the oven was measured while the peanut butter bars were baking. To measure relative humidity, a SCORPION® 2 Profiling system (Markel Food Group., Sinking Springs, PA) was used.

In preparation for baking, flour was inoculated with the target pathogen cocktail (~7 log CFU/g), dried overnight to the original pre-inoculation weight of the flour, and used to create peanut bar dough (50-g bars). Bars were baked at 177°C for 13 minutes, followed by 15 min of ambient air cooling (B+C) on a wire rack. Surviving pathogen populations were determined at minute 1 of baking and then every 2 min subsequently using an agar overlay plating protocol to improve detection of injured cells. Sampling points were randomized for each replication. At each sampling time, samples were quickly placed into 100 mL of chilled 0.1% peptone solution and stomached for 1 min in an AES Blue Line Smasher (Biomerieux, Marcy-l' Etoile, France). These homogenized samples were then held in a cooler until plated (within 30 min).

To determine pH, moisture content, and a_w, peanut butter bars were prepared using non-inoculated flour and were sampled at each of the 8 sampling points mentioned before. For a_w measurements, samples were transferred into a_w cups (Decagon Devices, Inc., Pullman, WA), sealed, allowed to reach ~25°C, and a_w was measured using an AquaLab Dewpoint 4TE a_w meter (Decagon Devices, Inc.). Sample pH was measured at 25°C using calibrated ExStik® Waterproof pH meter (Extech Instruments, Waltham, MA) by directly placing the pH electrode into a sample bag that contained approximately 25 g of product with 10 mL of distilled water

(slurry method). To determine moisture content of the samples, a dry oven method was used using an oven of 70°C for 24 hours. One baked and cooled bar per replication was sent to the analytical laboratory in the Animal Sciences and Industry Department at Kansas State University for analyses of moisture, fat, protein, and carbohydrates.

Thermal Destruction Parameters

D- and *z*- values of the three pathogen cocktails in raw peanut butter bar dough were determined by a method described by Channaiah et al., (29) with modifications, using Thermal Death Time (TDT) disks (6.0 cm diameter and 0.5 cm height; University of Nebraska, Lincoln, NE), Clarity vacuum pouches (3-mil 6" X 8") (BUNZL, Riverside, MO), and temperature-controlled water baths (Model 2864, Thermo Fisher Scientific™, Marietta, OH). Since full lethality of the target pathogens was not achieved, it was decided to perform the D- and z- value studies using the lowest a_w achieved during baking (0.65). the purpose of the modified a_w was to calculate the D- and z- values studies under the premise of the worst case scenario. To modify the a_w of the peanut butter bars, the dough was mixed the day before of the experiment and placed and extended over a cafeteria tray lined with aluminum foiled and placed inside an incubator at 40°C until the water activity of the dough reached our target content. With the modified a_w dough we proceeded to perform the D- and z- value studies. The D-values of the *Salmonella* cocktail were determined using ~8 g of dough in TDT disks, and heating at 85, 90, and 92.5°C with the surviving *Salmonella* population enumerated (using injury-recovery media) during the heat treatments for 7 minutes, 3 minutes, and 30 seconds respectively. The D-values of the STEC cocktail were determined using ~10 g of dough in Clarity vacuum pouches and pressed down to ~10 mm and vacuum packaged, and placed inside controlled water bath at 85,

90, and 92.5°C. Surviving STEC population was enumerated (using injury-recovery media) during the heat treatments for 7 minutes, 3 minutes, and 1 minute, respectively.

Bacterial Enumeration

Salmonella enumeration and enrichment in the baking validation and D-values studies were conducted as described by (*30*) using injury-recovery media, BHI agar overlaid with xylose lysine deoxycholate (XLD; Becton, Dickinson and Company) agar. For *E. coli* enumeration and enrichment in the baking and D-values studies were performed using injury-recovery media, BHI agar overlaid with MacConkey Agar (Becton, Dickinson and Company) agar. For *Listeria monocytogenes* enumeration and enrichment in the baking were performed using injury-recovery media following a procedure by Yan, Gurtler, & Kornacki (116), with minor modifications utilizing TSA (Becton, Dickinson and Company) agar overlaid with modified oxford agar base supplemented with Moxolactam supplement (MOX; Remel).

Statistical Analyses

The statistical analyses of all three pathogen populations, pH and a_w data were conducted by analysis of variance at $P \leq 0.05$ using SAS version 9.3 (SAS Institute, Cary, NC). The mean D- and *z*-values were calculated from the linear regression graphs plotted using Microsoft Excel 2011 (Microsoft Corp., Redmond, WA) for each replication individually.

Results and Discussion

Proximate analysis of the peanut butter bars after B+ C showed a fat value of 21.60% ± 0.77; a moisture content of 10.84% ± 0.87; and a protein content of 11.39% ± 2.68.

Table 7-2 pH and a_w of peanut butter bars during 13 minutes of baking at 350°F (176°C) oven temperature followed by 15 min of ambient air cooling (B+ C).

Sampling point	pH	a_w
Dough	7.06 ± 0.07 [F]	0.811 ± 0.02 [a]
1 minute	7.19 ± 0.07 [F]	0.804 ± 0.02 [a,b]
3 minutes	7.34 ± 0.07 [D]	0.793 ± 0.02 [a,b,c]
5 minutes	7.43 ± 0.07 [D]	0.757 ± 0.02 [b,d,c]
7 minutes	7.79 ± 0.07 [C]	0.749 ± 0.02 [e,d,c]
9 minutes	8.06 ± 0.07 [B]	0.733 ± 0.02 [e,d]
11 minutes	8.25 ± 0.07 [B]	0.654 ± 0.02 [g]
13 minutes	8.58 ± 0.07 [A]	0.671 ± 0.02 [g,f]
Baking + Cooling (B + C)	8.65 ± 0.07 [A]	0.706 ± 0.02 [e,f]

* Columns with different letter superscripts are statistically different ($P \leq 0.05$).

Table 7-2 shows the mean and standard deviation of the water activity (a_w) of the peanut butter bars at each of the pull times. The a_w significantly decreased ($P \leq 0.05$) from the dough sample (0.811 ± 0.02) until after B+C (0.706 ± 0.02). Previous articles have followed similar protocols to determine a_w of baked products during baking steps (*69*, *72*). The decrease in a_w could be due to starch gelatinization, moisture evaporation, and protein coagulation (*69*). Studies performed in pita chips and peanut butter pretzels demonstrated a decrease in a_w during baking process (*69*). A similar study was performed with peanut butter cookies where the a_w of the product was very similar to the initial a_w of the product in this research (*72*). Other researchers have stated that a_w decreases with baking times as it can be noted in the research by Kottapalli et al., (65). Other baked products performed by the same research group have found higher initial a_w compared

to this product, with values ranging from 0.94 to 0.97 before the thermal treatment. These products include hamburger buns, muffins, and donuts (*27*, *28*, *30*). The lowest a_w observed during the current study was 0.65 determined at 11 min of baking. This value was further utilized to perform the D- and z- values study of the peanut butter bar product, as this a_w would likely be the point in baking where the least thermal lethality against pathogens would occur.

Table 7-2 also shows the average and standard deviation of the pH of the peanut butter bars during the baking step at each of the pull times. pH values significantly increased ($P \leq 0.05$) during baking from a dough value of 7.06 ± 0.07 to the end of baking + cooling (B+C) time with a value of 8.65 ± 0.07. We hypothesize that the changes in the pH are due to the presence of baking soda in the recipe of the peanut butter bars. Baking soda when its baked goes from from sodium bicarbonate into sodium carbonate, which is a stronger alkaline salt; therefore increasing the pH of the bars during the baking step. The results are mean values from three replications. Lathrop et al., (68) found very similar pH values for cookies formulated with peanut butter with pH values of 6.85 ± 0.05 for the inoculated product; while, a study with peanut butter filled pretzels found pH values of 6.46 ± 0.047 (*69*, *72*). In another study performed on muffin batter and nut muffin batter, the initial pH was of 6.61 ± 0.12 and 6.50 ± 0.2, respectively, which then significantly increased to 7.52 ± 0.01 and 7.30 ± 0.02 at the end of the cooling period (*29*, *30*). These results are very similar to the ones found in the current research.

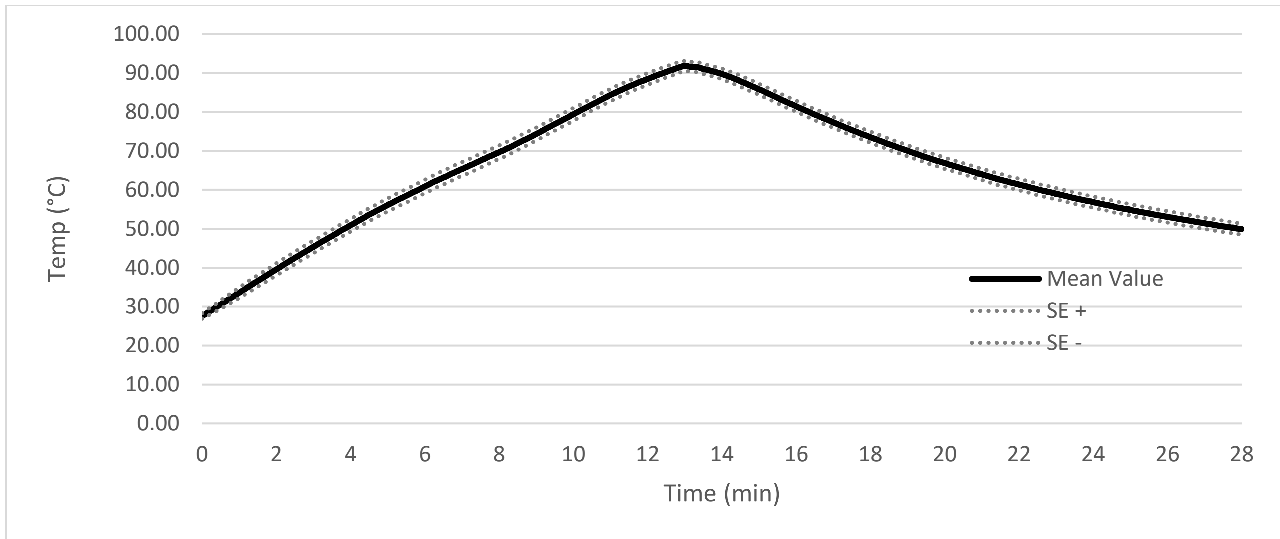


Figure 7-1. Mean temperature profile of peanut butter bars during oven-baking (±SE) at 350°F (176°C) 13 min + 15 min of ambient air cooling.

Figure 7-1 shows the internal average temperature of the peanut butter bars during the baking step. Thermocouples were placed in the geometric center of the product and then bars were randomly selected to place the thermocouples in different positions inside the oven for each of the replications. The thermocouples collected the temperature data at one second intervals during the 13 min of baking + 15 min of ambient air cooling. This data was averaged from three replications and with this information we can estimate the internal temperature of the bars during the baking step. The highest mean internal temperature of the bars was 91.88°C. This temperature was achieved at 13 minutes of baking, immediately after the bars were taken out of the oven and placed on a wire rack for 15 minutes to cool. In a study of peanut butter cookies, the average highest internal temperature of the cookies was 92°C after baking for 15 minutes at 350°F (*72*). These results are very similar to the ones found in this study.

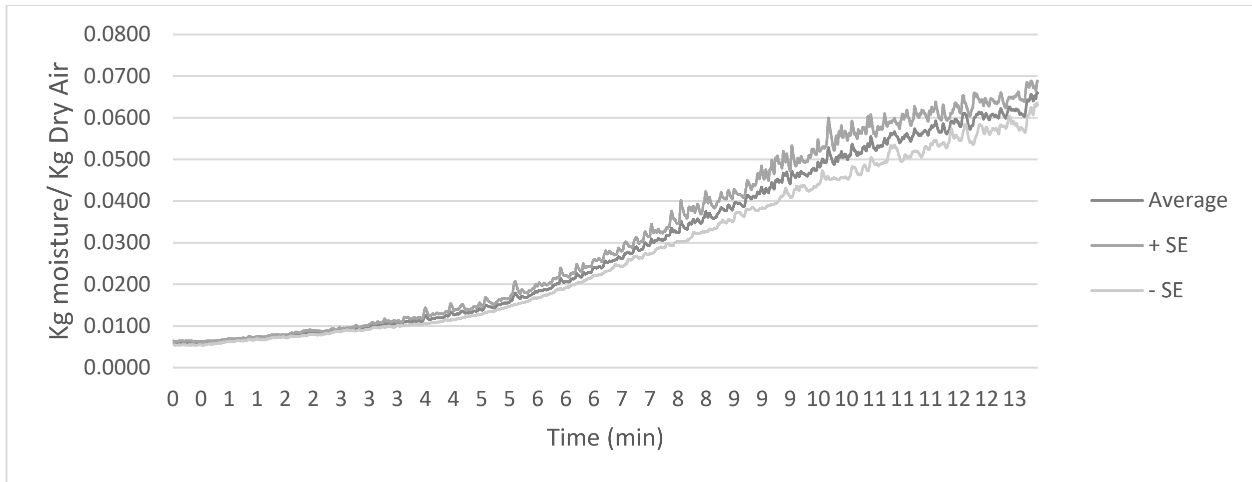


Figure 7-2. Relative oven humidity during baking of peanut butter bars (± SE) baked at 350°F (176°C) for 13 minutes.

Figure 7-2 shows the average relative humidity ± standard error of the relative humidity (RH) inside the oven during the baking process; three replications were performed. To measure RH of the oven, a SCORPION® 2 Profiling system (Markel Food Group., Sinking Springs, PA) was used. The relative humidity inside the oven increased during the baking process from 0.007 to 0.0065 kg moisture/kg dry air. Several researchers have stressed the importance of moisture; where, increasing moisture before or during heating can substantially lower the heat resistance of *Salmonella* spp. (*62*).

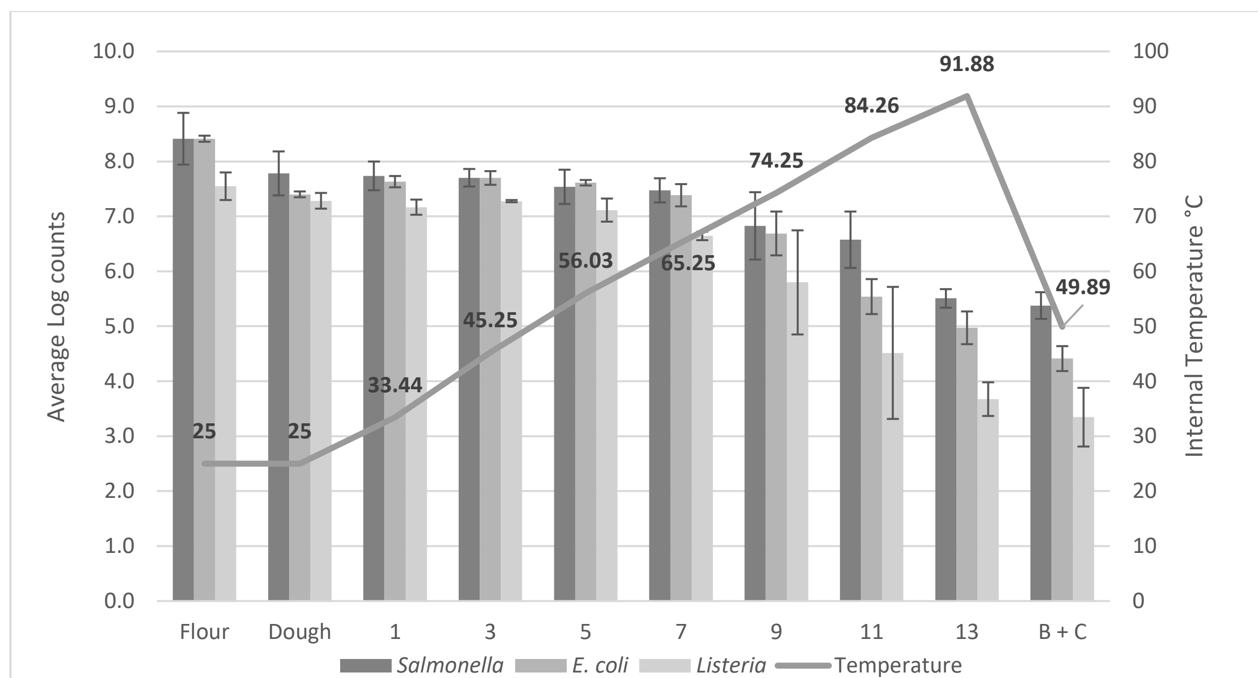


Figure 7-3. Average log counts and internal product temperature (°C) of peanut butter bars during 13 min of baking at 350°F (176°C) oven temperature followed by 15 min of ambient air cooling (B+ C).

Figure 7-3 shows the average microbial population survival in the peanut butter bars at each of the sampling points (for all three pathogens), this is over-imposed with the average internal temperature of the bars at each point. All of the samples are average values from three replications. Raw peanut butter bar dough contained 7.3-7.7 log CFU/g of the three pathogen cocktails. Little to no pathogen population reductions (≤ 0.5 log CFU/g) were observed during the initial 7 minutes of baking. Slightly higher thermal lethality was noticed as the internal product temperature reached 74.25°C (9 min of baking). At this point in baking, pathogen population reductions approached 1 log cycle. At the end of 13 min of baking, only a 2-log cycle reduction had been achieved for all of the pathogens present in the bars. Reviewing the scientific literature and compared to several bakery product validations our research group has conducted, this is the first cereal-based product to demonstrate notable *Salmonella* survival at the end of the baking and cooling process, and this is first bakery product performed by this group that does not achieve a 5-log kill by the end of the

baking step. Previous products investigated include cookies, tortillas, donuts, muffins, hamburger buns, bread, and cheesecake (*27*, *28*, *29*). The average total kill was of 2.4, 3.0 and 4.0 log CFU/g at the end of the baking validation for *Salmonella* spp*.,* STEC, and *Listeria monocytogenes*, respectively. Several authors have studied pathogen survival in different bakery products and have found higher lethality rates after the baking step in products including muffins, donuts, hamburger buns, pita chips, and pretzels filled with peanut butter (*28*, *29*, *30*, *69*, *80*). All of the previous products had a much higher a_w (hamburger buns 0.97; muffins 0.92; pita chips 0.98; and peanut butter filled pretzels 0.95) compared to the a_w of the peanut butter bars (0.811) before the baking step, which might have greatly impacted the lethality of the bacteria in the bars (*28*, *30*, *69*). Authors have stressed that at lower a_w bacteria tend to be more heat resistant; therefore, supporting the lower lethality observed in this study (*34*, *62*, *101*, *107*). It has been established that one of the principal causes of thermal inactivation of bacteria under high moisture conditions is the destabilization of the ribosomes (*107*). Therefore, some authors have hypothesized that at lower a_w will reduce the molecular water mobility of the cell which will help stabilize the ribosomal units against damage that may irreversible when compared to systems with higher moisture (*107*).

A study with cookies that had peanut butter as a key ingredient had very similar internal temperature values, where at 13 minutes of baking reported a temperature of 97°C, while in this study at the end of baking the internal temperature was 92°C (*72*). In the study by Lathrop et al., (68), they found positive *Salmonella* samples at 13 minutes of baking at 350°F (176°C) oven temperature which is the same temperature utilized in the current study to bake the peanut butter bars (*72*). In their study, they found that after baking for 13 minutes there was still *Salmonella* spp. present in 44% of the samples (*72*). A study by Michael et al., (76) comparing the thermal resistance of *Salmonella* and STEC and found that *Salmonella* survived for longer periods of time

in the baked product, which are similar results found in this research (*80*). In another study of peanut butter filled pretzels it was found that *Salmonella* spp. had a higher thermal resistance than *L. monocytogenes* (*69*). These other studies have similar results to the ones found in this study where *Salmonella* had the highest survival rate of the three pathogens analyzed.

Different intrinsic properties such as a_w, pH, and the food product matrix can affect the thermal resistance of pathogens (*72*). The properties of peanut butter like low a_w and high fat content can affect the heat tolerance of microorganisms (*99*). Several authors have stated that generally *Salmonella* has a higher heat resistance with a decrease product a_w, whereas peanut butter dries during heating there is a decrease in the inactivation rates (*72*, *99*). One study evaluated the thermal resistance of *E. coli* O121 and found that this strain had more survival upon desiccation than O157; however, it is still less thermally resistant than previous studies performed in *Salmonella* spp (*106*). In another study, it was found that a_w affected the inactivation kinetics of the same three pathogens *E. coli* O157*, Salmonella,* and *L. monocytogenes;* where, *L. monocytogenes* had the highest thermal resistance, at a a_w of 0.3 -0.5 (*103*). In the present study, the characteristics of the peanut butter bars [e.g. low water activity (0.65), higher fat content, non-uniform dough (presence of oat particulates)] likely contributed to the lower lethality rate compared to other bakery products previously analyzed.

Since full lethality against the inoculated pathogens was not achieved by the end of the baking procedure, the studies to determine the D- and z- values were performed giving consideration to matrix effects and dynamic changes in a_w during baking. As noted in the previous results, the peanut butter bar not only has a low a_w but also has a higher fat content than the previous products researched. It is believed that the combination of these two factors provides the bacteria a protective effect and therefore an increased thermal resistance.

To ensure that the modeling tools are accurate and provide conservative lethality estimates, and reflect actual baking parameters, it was decided to perform the D- and z- value studies at the lowest a_w found during the baking parameters. It is well known that a lower a_w will have a lower lethality on the bacteria while baking. It has been stated that there is a greater thermal resistance with decreasing % moisture content and a_w; where, product moisture content impacted lethality more than a_w (*48*). Therefore, the selection of the lowest a_w to perform D- and z- values will ensure that the lethality will accurately mimic baking parameters and product safety can be ensured, once the correct temperature, time, and a_w were selected.

Based on the previous information, D- and z- values were calculated for the three pathogens mentioned before. For this part of the study, the calculations of these values were based on the lowest a_w found during the baking validation part of the study (0.65). To lower the water activity to this value, the mixed dough was placed inside an incubator at 37°C between 10 to 12 hours, until the water activity value reached the targeted value.

Table 7-3 D- and z- values for *Salmonella* and STEC for peanut butter bars at three different temperatures.

	D- values		z- values	
Temperature (°C)	*Salmonella*	STEC	*Salmonella*	STEC
85	9.4 ± 1.11	10.2 ± 0.47		
90	3.9 ± 0.34	3.3 ± 0.39	10.05 ± 2.18	10.12 ± 0.55
92.5	1.7 ± 0.40	1.7 ± 0.20		

In Table 7-3, it can be noted the D- and z- values for *Salmonella* and *E.coli*, where it can be noted that both of these pathogens have similar values. Previous authors have found higher thermal resistance from *Salmonella* compared to *E. coli* (*80*). It has also been found that *E. coli* demonstrates higher levels of thermal inactivation than *Salmonella* in peanut butter (*57*). However, as far as we know this is one of the first papers that has attempted to modify the a_w to perform the thermal inactivation studies, which might have impacted the thermal resistance of

the bacteria. This research included the D- and z- value calculations for *L. monocytogenes;* however, the lethality achieved with the same temperatures used for the other pathogens did not allow us to accomplish a good lethality study (data not shown). Even though a higher *L. monocytogenes* population reduction was observed compared to *Salmonella* and STEC in the baking study (Figure 7-3), *L. monocytogenes* survival in the reduced a_w dough was higher, suggesting the need for additional research on the pathogen's mechanism for greater survival.

Previous researchers have observed higher thermal resistance of *Salmonella* compared to *Listeria* but D- and z- values studies were not performed (*69*). Other papers have found a higher resistance of *L. monocytogenes* compared to *Salmonella* in peanut butter after microwave heating at a_w of 0.3 to 0.5 (*103*). In a recent paper that determined D- and z- values for brownie dough *L. monocytogenes* had lower thermal resistance than *Salmonella* spp. at 64, 68, and 72°C; however, presented a higher thermal resistance at 76°C (*113*). One important factor that might have affected the increased thermal resistance of *L. monocytogenes* is that during the drying period the inoculated dough was kept at 37°C which could have led to the bacterial cells having an increased heat resistance compared to cells held at normal physiological temperatures. Previous researchers have found up to a two-fold increase in thermal resistance of *L. monocytogenes* after being sublethally heat shocked (*73*).

Conclusion

Reduced a_w bakery products may present an elevated pathogen survival risk. Previous studies on bakery products have achieved over a 5-log reduction by the end of the baking step. This is one of the first products to have a notable level of survival of microbial populations at the end of the baking process. Intrinsic characteristics of peanut butter cereal bars (e.g., lower a_w and

higher fat content) are likely contributors to the decreased process lethality observed in the current study compared to other bakery products and should be considered in food safety plans for similar products providing a lower lethality rate compared to previous analyzed bakery products. During a previous baking validation of these bars, *L. monocytogenes* demonstrated a lower survival rate compared to STEC and *Salmonella.* The current study found similar heat resistance for STEC and *Salmonella*. However, *L. monocytogenes* survival in the reduced a_w dough was higher, suggesting the need for additional research on the mechanism for greater survival. This higher resistance acquired by these pathogens make peanut butter a risky ingredient, and when utilized in bakery product formulations, industry must ensure that the finished products are free of pathogen contaminants. Similar studies in our laboratory on a wide range of bakery products have achieved >5-log reductions in *Salmonella* and STEC populations after the commercial-simulated baking step. Intrinsic characteristics of peanut butter bars (e.g., lower a_w and higher fat content) are likely contributors to the decreased process lethality observed in the current study and should be considered in food safety plans for similar products.

Chapter 8 - A Comparative Study of a Published Validation Study versus a Surface Inoculation Study of Hamburger Buns to better understand *Salmonella* lethality under dynamic matrix changes during baking

Introduction

This chapter will discuss the results of a *Salmonella* spp. inoculated study on the surface of hamburger buns during baking. Hamburger buns were made using a common industry recipe and was evaluated during baking for a_w of crumb and crust, oven temperature, internal temperature, relative humidity of the oven, and survival of *Salmonella* spp. populations during baking. The results of this study will be compared with the results of a previous study titled "Validation of Baking To Control *Salmonella* Serovars in Hamburger Bun Manufacturing, and Evaluation of *Enterococcus faecium* ATCC 8459 and *Saccharomyces cerevisiae* as Nonpathogenic Surrogate Indicators" (*28*). The comparison will allow us to understand more precisely differences in *Salmonella* lethality between the crust and crumb of bakery products during baking due to dynamic physical changes that occur.

Material and Methods

Bacterial Cultures

Three *Salmonella* serovars were used in this research and were selected based on the previous published research article "Validation of Baking To Control *Salmonella* serovars in

Hamburger Bun Manufacturing, and Evaluation of Enterococcus faecium ATCC 8459 and Saccharomyces cerevisiae as Nonpathogenic Surrogate Indicators" (*28*). The three serovars of *Salmonella* were obtained from the American Type Culture Collection (ATCC; Newport 6962, Senftenberg 775W 43845 and Typhimurium 14028). All cultures were individually grown using the method described by Channaiah et al., (29). In brief, all the bacterial cultures were activated in brain heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD) broth from frozen beads stored at -80°C. A 10 µl inoculating loop was used to transfer each culture to a 15 mL centrifuge tube containing 10 mL of BHI broth, and was incubated at 37°C for 24 hours. The working cultures were then grown as 24-h lawns on BHI agar plates using a sterile swab, and were harvested using 1 mL of 0.1% peptone (Becton, Dickinson and Company) solution twice. Equal amounts of all the harvested *Salmonella* serotypes were mixed to create a 3-strain *Salmonella* cocktail.

Hamburger Bun Preparation

The recipe and ingredients to prepare the hamburger buns were supplied by AIB International, Inc. (Manhattan, KS) (Table 8-1). Dough ingredients were added to a 20-qt (18.9-liter) McDuffee bowl, which was placed into a Hobart A-20 stand mixer (Hobart, Troy, OH) with fork agitator attachment. Ingredients were mixed for 1 min on low and then for 12 min on medium speed. After dough mixing, the dough was divided, panned and proofed as described in Channaiah et al., (29).

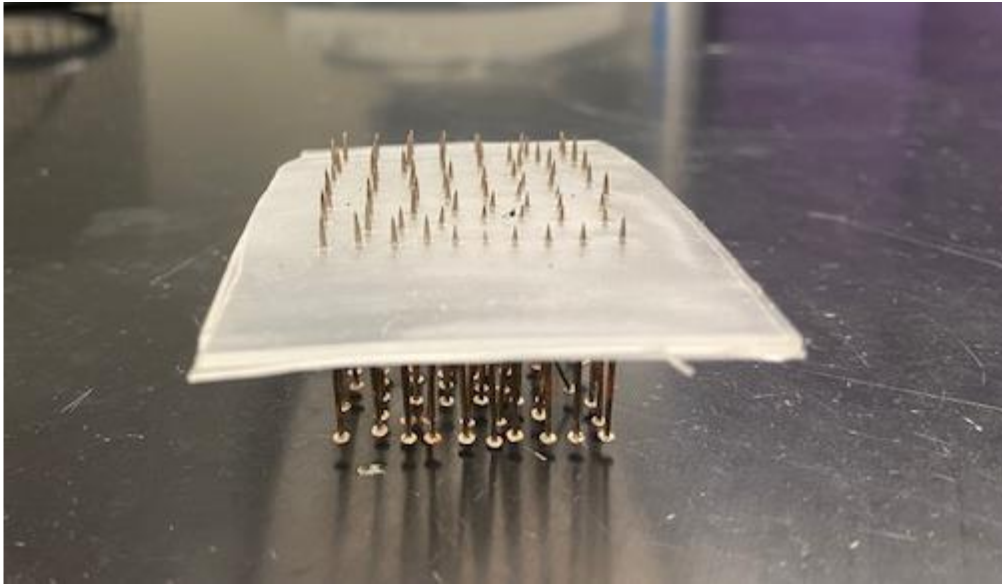
Table 8-1 Ingredient list and weight of the hamburger buns recipe.*

Ingredient	Weight (g)
Bread Flour	700
Sugar	84
Salt	14
Sodium Stearoyl Lactylate	3.5
Calcium Propionate	1.8
Yeast Food	0.7
Shortening	42
Yeast Compressed	17
Ascorbic Acid Solution (1.6%)	1.5 ml
Water	478.5 ml

*Recipe yields six buns.

Hamburger Bun Inoculation

For the development of this comparison study, the flour was not inoculated like in the previous research chapters. Instead, hamburger buns were made following the recipe provided by AIB and before placing in the oven they were inoculated by placing 0.8 mL of inoculum cocktail (11 log CFU/g) on the surface of the hamburger buns and spread across the surface using an L-shape spreader. After the inoculum was placed on the bun dough surface, a small inoculation device that contained uniformly spaced pins secured in plastic (Picture 8-1) was used to penetrate the top surface of the proofed bun dough inoculated surface to a depth of ~2 mm, thereby translocating the pathogen contamination slightly subsurface within a zone that become the bun crust during baking. After the inoculation, we allowed 30 minutes of attachment time before placing the hamburger buns in the oven. This inoculation protocol provided the opportunity to assess the baking lethality against *Salmonella* in the crust as dynamic a_w and moisture changes occurred in the oven.



Picture 8-1 Inoculating needles with a penetration depth of 2 mm to perform surface inoculation of hamburger buns.

Hamburger Buns Baking

Fine-gauge thermocouples [Type-T Thermocouples (Omega Engineering Inc., Stamford, CT)] connected to an eight-channel data logging system (USB-TC with MCC DAQ software, Measurement Computing, Norton, MA) were used to monitor the temperatures of dough during the baking step. Thermocouples were placed towards the geometric center of the buns. For the measurement of the internal temperatures of the buns, three buns had thermocouples inserted per replication, and three replications were performed. To measure the internal temperature of the oven two fine-gauge thermocouples were placed inside the oven in different locations and an average was calculated per replication; three replications were performed. The relative humidity of the oven was measured while the buns were baking. To measure relative humidity, a SCORPION® 2 Profiling system (Markel Food Group., Sinking Springs, PA) was used.

During baking, the inoculated buns (proofed dough) inoculated with the *Salmonella* cocktail (~9 log CFU/g) were placed towards the center of the oven. Buns were baked at 425°F (218.3°C) for 10 minutes. Surviving *Salmonella* populations were determined at minute 0 and

every 2 min thereafter using an agar overlay plating protocol to improve detection of injured cells. Sampling points were randomized for each replication. At each sampling point, samples were taken with a scalpel and tweezers and only the top part of the buns was collected (top 2 – 3 mm), rapidly placed into 30 mL of chilled 0.1% peptone solution, and stomached for 1 min in an AES Blue Line Smasher (Biomerieux, Marcy-l' Etoile, France). Sample homogenates (bags) were then placed in a cooler until plated (within 30 min).

To determine a_w, hamburgers buns were sampled at each of the 5 samples points mentioned before. For a_w measurements, samples were transferred into a_w cups (Decagon Devices, Inc., Pullman, WA), sealed, allowed to reach ~25°C, and a_w was measured using an AquaLab Dewpoint 4TE a_w meter (Decagon Devices, Inc.).

Bacterial Enumeration and Enrichment

Salmonella enumeration in this baking validation study was conducted as described by Channaiah et al.(*30*) using injury-recovery media; BHI agar overlaid with xylose lysine deoxycholate (XLD; Becton, Dickinson and Company) agar. For samples demonstrating no *Salmonella* recovery by direct plating, an enrichment protocol was used as previously described Channaiah et al., (29) as a qualitative determination of complete pathogen elimination.

Results and Discussion

In this section we will discuss the results obtained from the surface inoculation study and we will mainly compare our results to the ones found in "Validation of Baking To Control *Salmonella* Serovars in Hamburger Bun Manufacturing, and Evaluation of *Enterococcus faecium* ATCC 8459 and *Saccharomyces cerevisiae* as Nonpathogenic Surrogate Indicators" (*28*), which was the main objective of this research.

Table 8-2 a_w of crust and crumb, internal bun temperature, and oven temperature during baking of hamburger buns at 425°F (218.3°C).

Sampling Time	Water Activity Crust	Water Activity Crumb	Bun internal temperature (°C)	Oven Temperature (°C)
0 minutes	0.9521 ^{A}	0.9672 ^{b}	25.00	-
2 minutes	0.9495 ^{A}	0.9717 ^{ab}	44.47	177.58
4 minutes	0.8865 ^{B}	0.9758 ^{a}	57.88	185.86
6 minutes	0.8843 ^{B}	0.9718 ^{ab}	80.57	185.86
8 minutes	0.8798 ^{B}	0.9750 ^{a}	94.70	191.52
10 minutes	0.7967 ^{C}	0.9749 ^{a}	100.46	190.67

* Columns with different letter superscripts are statistically different ($P \leq 0.05$).

Table 8-2 shows the mean water activity (a_w) of the hamburger buns at each of the sampling times for both crumb and crust components of the product. The a_w significantly decreased for the crust of the bun ($P \leq 0.05$) from the pre-baked dough sample (0.9672) until the end of 10 minutes of baking (0.7967). The lowest recorded a_w was at 10 minutes of baking (0.7967) for the crust of the bun. In the case of the crumb, a significant difference was found between time 0 and 10 minutes of baking ($P \leq 0.05$). The change in a_w during the baking step could be due to multiple reasons like moisture evaporation, protein coagulation, and starch gelatinization (*69*). The initial a_w of this product is comparable to other baked products that presented a_w values ranging from 0.94 to 0.97 before the thermal treatment; the other products include our comparison study of hamburger buns but also products like muffins and donuts (*27*, *28*, *30*). In the current study, crumb a_w remained mostly constant at 0.97 throughout the entire 10 min of baking. Conversely, a_w of the crust steadily declined with increasing baking time and increasing bun temperature. The reduction in crust a_w occurred rapidly and ultimately reached a value of 0.7967 at the end of baking. The difference in the a_w between the crust and crumb of the hamburger buns could be due to the difference in temperature and moisture evaporation rate in the two different areas of the buns. The internal temperature of the hamburger bun (crumb) reached 100.46°C while the temperature of the crust

was likely closer to the temperature of the oven (190.67°C). These temperature differences and a_w values between the crust and crumb are likely impactful not only on the a_w of the sample but also on the lethality of the *Salmonella* survival population.

Different properties of food matrices such as a_w and pH can alter the thermal resistance of pathogens (*72*). Low a_w can affect the heat tolerance of microorganisms (*99*). Several authors have stated that generally *Salmonella* has a higher heat resistance in lower a_w environments and with the results of this study and we see a significant change in the a_w of the surface of the bun during baking that could lead to a decrease in the inactivation rates of the bacterial population (*72*, *99*). Some authors have stressed that the product surface is the food portion that achieves the least lethality compared to the center of the product (*117*).

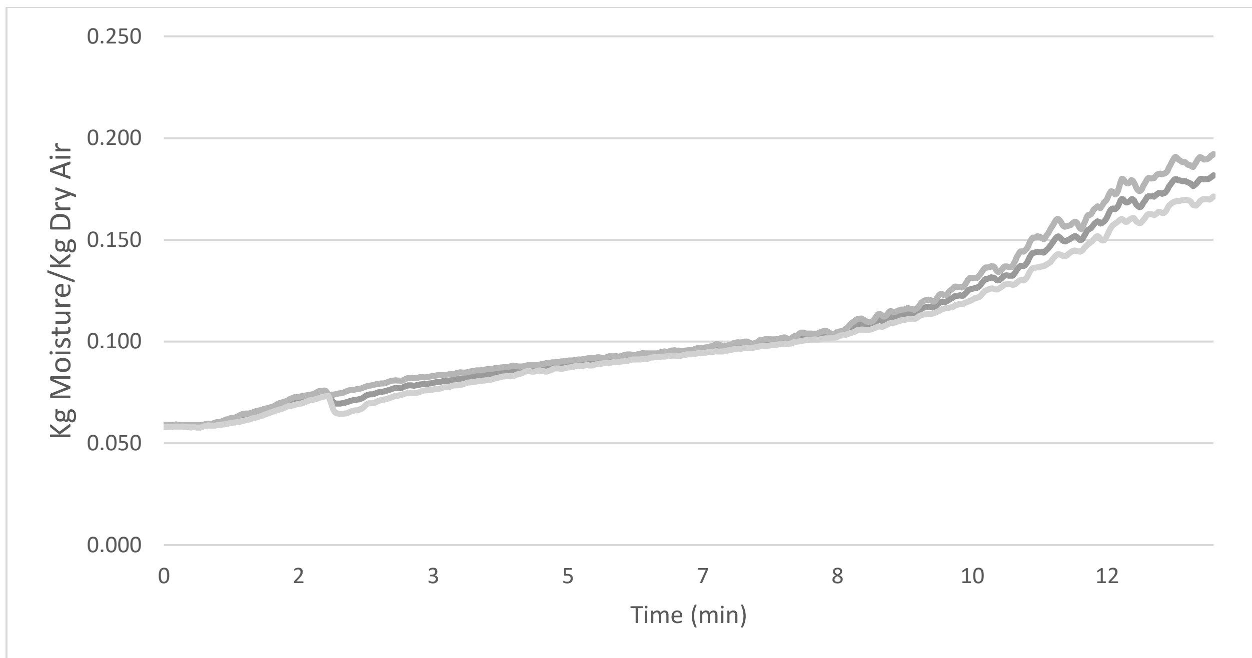


Figure 8-1 Relative humidity of oven at 425°F (218.3°C) (± SE) during baking of hamburger buns for 13 minutes.

Figure 8-1 shows the average relative humidity ± standard error (RH) inside the oven during the baking process; three replications were performed. To measure RH of the oven, a

SCORPION® 2 Profiling system (Markel Food Group., Sinking Springs, PA) was used. The humidity inside the oven increased during the baking step from 0.055 to 0.175 kg moisture/kg dry air. Authors have stressed the importance of RH in baking, where relative humidity has a pronounced effect in the heat resistance of *Salmonella* spp. during baking (*117*). Wang (111) has found that at the same temperature with an increasing relative humidity less time was required to obtain similar lethality values of *Salmonella* Agona in products that were high in fat content. These findings highlight the importance of understanding and controlling the relative humidity of the oven during baking of different food products. These findings of *Salmonella* spp. thermal resistance might be explained by the dynamic surface a_w, especially on the surface of the products where changes in a_w are more noticeable (*117*).

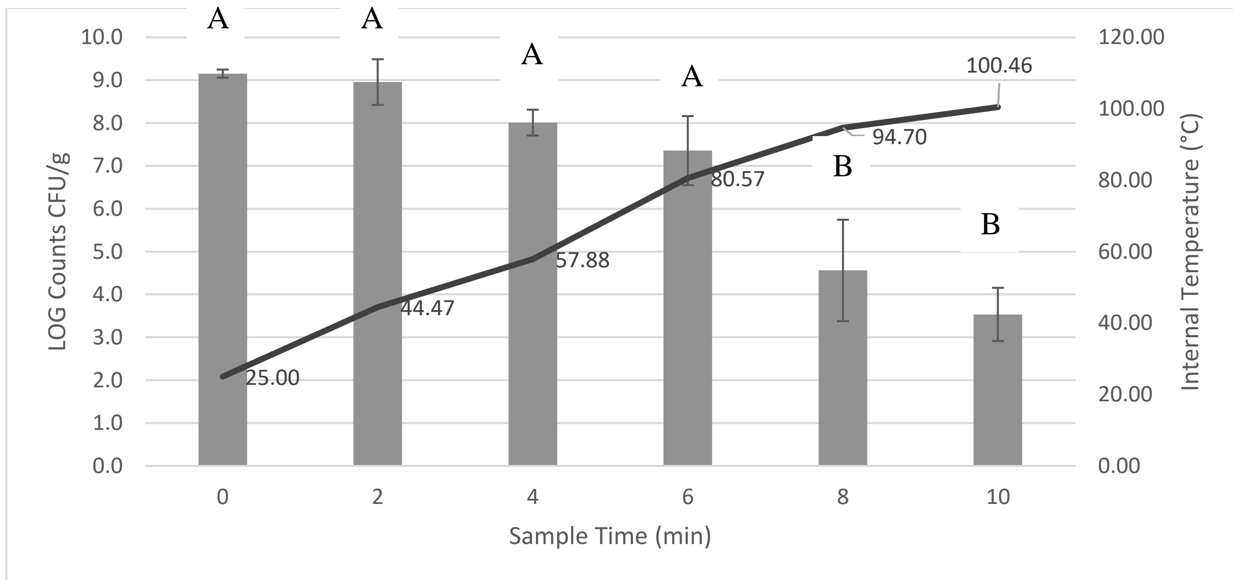


Figure 8-2 Average recovered *Salmonella* populations from surface region (upper 2 mm) and internal temperature (°C) of hamburger buns during 10 min of baking at 425°F (218.3°C).

* Columns with different letter superscripts are statistically different ($P \leq 0.05$).

Figure 8-2 shows the average residual *Salmonella* spp. population of the surface inoculated hamburger buns at each of the six sampling points, over-imposed with the average internal (crumb) temperature of the hamburger buns at each point. All of the sample data points are average values

from three replications. After 10 min of baking, there is a 5.7 log CFU/g reduction compared to the surface inoculated raw dough. In the previous research performed by Channaiah et al., (27), whereby flour was inoculated with the same *Salmonella* serovars resulting in uniform distribution across the crumb and crust parts of buns, >5 log cycle reductions were recorded. In an inoculated study performed on pumpkin filled pies it was determined that an internal baking temperature of 108°C for 1 minute was enough to destroy *Salmonella* and *S. aureus* (*121*). However, as noted in Figure 8-2, a temperature of 108°C was not able to be achieved by the ten minutes of baking at 425 °F.

Table 8-3 *Salmonella* log CFU/g of surface inoculated hamburger buns during baking at 425°F (218.3°C) compared to the previous study by Channaiah et al., (27)

Sampling Time	Present Study	Previous Research		
	***Salmonella* Cocktail**	***S.* Seftenberg**	***S.* Newport**	***S.* Typhimurium**
	Log Counts CFU/g			
0 minutes	9.2	5.7	7.3	7.6
2 minutes	9.0	*	*	*
4 minutes	8.0	*	5.7	*
6 minutes	7.4	1.0	0.6	1.1
8 minutes	4.6	0.5	*	0.5
10 minutes	3.5	0.5	*	0.5

* Denotes a sample where the data point is not available.

Table 8-3 shows the average of three replications of the inoculation level and *Salmonella* spp. survival at different sampling times during baking of hamburger buns at 425 °F. It can be observed that the surface inoculation level of the buns is between 1.6 to 3.5 log CFU/g higher than the study by Channaiah et al., (27). This can be explained by the fact that in the previous study the flour was inoculated and mixed with the rest of the ingredients to create the dough; therefore, the mixing had a dilution effect on the inoculum. While, in our present study the inoculum was placed on top of the buns and therefore the same dilution factor does not take place. One of the other important differences between the two studies is the thermal lethality in

the present study was evaluated by the use of a cocktail of the three serovars; while, in the previous study lethality was evaluated on each serovar individually.

In the present study, after 10 minutes of baking at 425°F, a 5.7 log CFU/g reduction of the *Salmonella* cocktail was achieved; however, we still had a surviving *Salmonella* population of 3.5 log CFU/g. Channaiah et al., (27) found that after 10 minutes of baking at 425°F the *Salmonella* populations were under the limit of detection. Depending on the serovar analyzed, Channaiah et al., (27) observed reductions between 5.2 to 7.1 log CFU/g at the 10 minutes point of baking.

Conclusions

Due to the differences in the methodology of both studies, it is challenging to compare the results of both research studies. However, we found that after 10 minutes of baking at 425 °F we could not achieve a full *Salmonella* spp. lethality when high *Salmonella* levels were confined to the top 2 mm in the zone of crust formation. This contrasts with the results of the Channaiah et al., (27) where they found that after 10 minutes of baking all three serovar populations were under detection limit of the detection protocol. It is important to note that in the present study the dough inoculation level was between 2 and 4 log CFU/g higher, which likely has an impact on the results. At the end of baking in the present study both the internal temperature of the buns and the oven temperature were at its highest point; therefore, having pull times at the end of baking (13 minutes) instead of our 10 minutes sampling point, could have aided in achieving higher bacterial population reductions. Another factor that complicates the comparison of the *Salmonella* spp. is the large variations in the inoculation level for the three serovars in the study by Channaiah et al., (27).

An important finding of the present study is the significant differences between the a_w of the crust and crumb of the sample. The lowest a_w was achieved by the end of the 10 minutes of baking and as discussed can have an impact on the lethality of the *Salmonella* spp. population. The difference in a_w could possibly be explained by the temperature difference achieved in the crumb compared to the crust of the product.

Chapter 9 - Conclusion And Future Research

As it was discussed during the present work, foodborne pathogens can be present in different stages of food production from wheat milling to finished bakery products. Due to the inherent risk these pathogens present, control steps must be followed to protect the safety of human foods. Even though a zero-risk is no possible, the introduction of critical control points during the different steps of food processing could reduce the risk significantly. These risk mitigation strategies can help reduce likelihood of acquiring a foodborne transmitted illness.

Some of the risk mitigation strategies include evaluation of cleaning and disinfection in processing plants, validation studies for critical control points, prevent cross contamination and consumer education and outreach. Companies can include these strategies through the implementation of GMPs and PCs at processing facilities. The combination of all of these strategies can help protect our food system. One of the most important aspects of these strategies are the validation studies that have been thoroughly discussed in the present research work. The addition of control steps to reduce pathogen levels during food processing of both ingredients and ready to eat products is going to play a key role in the safety of the bakery industry.

Due to risks mentioned before, this group has performed extensive research in developing scientific studies that validate the safety of bakery ingredients and products. In table 9-1 the different bakery products that have been researched by this group can be observed. The pH and the a_w of the products displayed in this table correspond to the values at the end of baking and cooling of each product. This table shows that the peanut butter bars is the bakery product that achieved the lowest lethality of all the products analyzed.

Table 9-1 pH, a_w, target bacterial population, and bacterial population reductions of the different bakery research products investigated by our research group.

Product	pH	a_w	Organism	Reductions
Hamburger buns	5.46	0.73	*Salmonella* spp.	≥7.6
Hamburger buns surface	*	0.767	*Salmonella* spp.	5.7
Soft cookies	8	0.474	*Salmonella* spp.	≥6.6
Hard cookies	7.73	0.138	*Salmonella* spp.	≥7.4
Muffins	7.52	0.928	*Salmonella* spp.	6.5
Nut muffins	7.52	0.701	*Salmonella* spp.	≥7.0
Bread rolls	5.23	0.81	*Salmonella* spp.	≥7.5
Donuts	5.51	0.944	*Salmonella* spp.	≥6.7
Tortillas	6.62	0.934	*Salmonella* spp.	≥7.3
Fruit-filled pastries	5.33	0.677	*Salmonella* spp.	≥7.7
Cheesecakes	4.86	0.943	*Salmonella* spp.	≥6.5
Peanut butter bars	8.65	0.706	*Salmonella* spp.	2.3
Peanut butter bars	8.65	0.706	STEC	3.0
Peanut butter bars	8.65	0.706	*L. monocytogenes*	4.0

Bolded products are the products that are discussed in the present work.

Peanut butters bars are a product that have a lower a_w before baking of the product, but also possess a higher fat content compared to the other products researched by this group. This finding is important and could guide future research regarding the safety of bakery products. Research might need to be shifted to products that tend to be drier and with higher fat contents. It is also hypothesized that the heterogenous mixture of the peanut butter bars, mainly to the oats present, could aid in a protection effect for the different pathogens tested. Therefore, it becomes of a highly pressing subject to better understand what might be the underlying mechanisms that

provide the pathogens this survival or protection effect, that might come from changes in the properties of the food or due to physical characteristics of the product.

Even though extensive research has been done to validate the safety of different bakery products, there are still some research questions that need to be answered. We need to understand how a_w changes during baking and the impact these changes have on the thermal resistance of pathogens of concern like *Salmonella* and STEC. There is also a need to better understand how different hygroscopic ingredients might also affect the thermal resistance of pathogens in different food matrices. It has also been studied and researched before that fat provides bacteria protection from thermal treatments; however, mechanisms of action and the impact of high fat content ingredients might have in this mechanism have not been fully elucidated.

The conclusion of this document highlights the importance of developing tools that the industry requires to validate their FSMA and HACCP plans to reduce the likelihood of causing a foodborne outbreak. The research present in this document can aid different actors involved in developing a safer food system and can be beneficial for bakers, government agencies and industry.

References

1. Akins-Lewenthal, D. 2012. Supply Chain Management to Maintain Microbiological Integrity of Processed Grain Ingredients. *Cereal Foods World* 57:115–117.

2. Akins, D. 2014. Flour food safety. Denver, CO.

3. American Bakers Association. 2021. The Economic Impact of Baking in the United States.

4. American Institute of Baking. Baking Process Kill Step Calculators.

5. Andrews, J. 2012. 2009 Peanut Butter Outbreak: Three Years On, Still No Resolution for Some. *Food Saf. News*.

6. Beach, J. C., E. A. Murano, and G. R. Acuff. 2002. Prevalence of *Salmonella* and Campylobacter in Beef Cattle from Transport to Slaughter. *J. Food Prot.* 65:1687–1693.

7. Berghofer, L. K., A. D. Hocking, D. Miskelly, and E. Jansson. 2003. Microbiology of wheat and flour milling in Australia. *Int. J. Food Microbiol.* 85:137–149.

8. Blackburn, C. D. W., and P. J. McClure. 2009. Foodborne pathogens: Hazards, risk analysis and control: Second EditionFoodborne Pathogens: Hazards, Risk Analysis and Control: Second Edition.

9. Boss, R., and J. Hummerjohann. 2019. Whole Genome Sequencing Characterization of Shiga Toxin – Producing *Escherichia coli* Isolated from Flour from Swiss Retail Markets. *J. Food Prot.* 82:1398–1404.

10. Bundesinstitut für Risikobewertung. 2020. *Escherichia coli* in flour - sources, risks and prevention 1–28.

11. Burnett, S. L., E. R. Gehm, W. R. Weissinger, and L. R. Beuchat. 2000. Survival of *Salmonella* in peanut butter and peanut butter spread. *J. Appl. Microbiol.* 89:472–477.

12. Center for Science in the Public Interest. 2015. Outbreak Alert! 2015.

13. Centers for Disease Control and Prevention. 2010. CDC Report Shows Success in Fighting *E. coli* O157:H7.

14. Centers for Disease Control and Prevention (CDC). 2018. Burden of Foodborne Illness: Questions and Answers.

15. Centers for Disease Control and Prevention (CDC). 2019. CDC Investigation Notice: Final Update on the Outbreak of *Salmonella* Agbeni Infections.

16. Centers for Disease Control and Prevention (CDC). 2020. *E. coli* (*Escherichia coli*).

17. Centers for Disease Control and Prevention (CDC). 2018. Estimates of Foodborne Illness in the United States.

18. Centers for Disease Control and Prevention (CDC). 2007. Multistate Outbreak of *E. coli* O157:H7 Infections Linked to Totino's and Jeno's Frozen Pizza (FINAL UPDATE).

19. Centers for Disease Control and Prevention (CDC). 2007. Multistate Outbreak of *Salmonella* Serotype Tennessee Infections Associated with Peanut Butter --- United States, 2006--2007.

20. Centers for Disease Control and Prevention (CDC). 2009. Multistate Outbreak of *Salmonella* Typhimurium Infections Linked to Peanut Butter, 2008-2009 (FINAL UPDATE).

21. Centers for Disease Control and Prevention (CDC). 2016. Multistate Outbreak of Shiga toxin-producing *Escherichia coli* Infections Linked to Flour (Final Update).

22. Centers for Disease Control and Prevention (CDC). 2012. National Enteric Disease Surveillance: STEC Surveillance Overview Surveillance System Overview: National Shiga toxin-producing *Escherichia coli* (STEC) Surveillance 0–3.

23. Centers for Disease Control and Prevention (CDC). 2019. Outbreak of *E. coli* Infections Linked to Flour.

24. Centers for Disease Control and Prevention (CDC). 2021. *Salmonella*.

25. Centers for Disease Control and Prevention (CDC). 2020. *Salmonella* Homepgage.

26. Centers for Disease Control and Prevention (CDC). 2017. Surveillance for Foodborne Disease Outbreaks United States , 2017 : Annual Report.

27. Channaiah, L. H., M. Michael, J. Acuff, K. Lopez, D. Vega, G. Milliken, H. Thippareddi, and R. Phebus. 2018. Validation of a Simulated Commercial Frying Process to Control *Salmonella* in Donuts . *Foodborne Pathog. Dis.* 15:763–769.

28. Channaiah, L. H., E. S. Holmgren, M. Michael, N. J. Sevart, D. Milke, C. L. Schwan, M. Krug, A. Wilder, R. K. Phebus, H. Thippareddi, and G. Milliken. 2016. Validation of Baking To Control *Salmonella* Serovars in Hamburger Bun Manufacturing , and Evaluation of *Enterococcus faecium* ATCC 8459 and *Saccharomyces cerevisiae* as Nonpathogenic Surrogate Indicators 79:544–552.

29. Channaiah, L. H., M. Michael, J. C. Acuff, K. Lopez, R. K. Phebus, H. Thippareddi, and G. Milliken. 2019. Validation of a nut muffin baking process and thermal resistance characterization of a 7-serovar *Salmonella* inoculum in batter when introduced via flour or walnuts. *Int. J. Food Microbiol.* 294:27–30.

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

31. Crim, S. M., P. M. Griffin, R. Tauxe, E. P. Marder, D. Gilliss, A. B. Cronquist, M. Cartter, M. T. Angelo, D. Blythe, K. Smith, S. Lathrop, S. Zansky, and R. Paul. 2015. Preliminary Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food — Foodborne Diseases Active Surveillance Network , 64:495–499.

32. Crowe, S. J., L. Bottichio, L. N. Shade, B. M. Whitney, N. Corral, B. Melius, K. D. Arends, D. Donovan, J. Stone, K. Allen, J. Rosner, J. Beal, L. Whitlock, A. Blackstock, J. Wetherington, L. A. Newberry, M. N. Schroeder, D. Wagner, E. Trees, S. Viazis, M. E. Wise, and K. P. Neil. 2017. Shiga Toxin–Producing *E. coli* Infections Associated with

Flour . *N. Engl. J. Med.* 377:2036–2043.

33. Cunningham, A., B. Flemmer, N. Dobmeier, B. Montgomery, B. Kottapalli, and D. Akins-Lewenthal. 2018. Survival of *Listeria monocytogenes* in Peanut Butter under Shelf-stable Conditions.International Association of Food Protection. Salt Lake City.

34. Doyle, M. E., and A. S. Mazzotta. 2000. Review of Studies on the Thermal Resistance of *Salmonellae*. *J. Food Prot.* 63:779–795.

35. Eglezos, S. 2010. Microbiological Quality of Wheat Grain and Flour from Two Mills in Queensland , Australia. *J. Food Prot.* 73:1533–1536.

37. Eriotou, E. 2015. Prevalence of *Listeria monocytogenes* and *Salmonella* spp. in Ready-to-Eat Foods in Kefalonia, Greece. *J. Bacteriol. Parasitol.* 06.

38. Fegan, N., P. Vanderlinde, G. Higgs, and P. Desmarchelier. 2004. Quantification and prevalence of *Salmonella* in beef cattle presenting at slaughter 892–898.

39. Finkel, E. 2018. State of the Industry 2018: Snack bars focus on nutrition. *Snack Food Wholes. Bak.*

40. Foley, S. L., A. M. Lynne, and R. Nayak. 1997. *Salmonella* challenges : Prevalence in swine and poultry and potential pathogenicity of such isolates 1 , 2. *Am. Soc. Anim. Sci.*

41. Food and Drug Administration (FDA). 2017. FDA Investigated Multistate Outbreak of Shiga toxin-producing *E. coli* Infections Linked to Flour.

42. Food and Drug Administration (FDA). 2020. Foodborne Pathogens.

43. Food and Drug Administration (FDA). 2020. FSMA Final Rule for Preventive Controls for Animal Food.

44. Food and Drug Administration (FDA). 2020. FSMA Final Rule for Preventive Controls for Human Food. Elsevier.

45. Food and Drug Administration (FDA). 2019. Outbreak Investigation of *E. coli*: Flour

(May 2019).

46. Food and Drug Administrration. 2016. Hazard Analysis and Risk-Based Preventive Controls for Human Food: Draft Guidance for Industry 3:1–23.

47. Forghani, F., M. Den Bakker, J. Liao, A. S. Payton, A. N. Futral, and F. Diez-gonzalez. 2019. *Salmonella* and Enterohemorrhagic *Escherichia coli* Serogroups O45 , O121 , O145 in Wheat Flour : Effects of Long-Term Storage and Thermal Treatments. *Front. Microbiol.* 10:1–12.

48. Garces-vega, F. J., E. T. Ryser, and B. P. Marks. 2019. Relationships of Water Activity and Moisture Content to the Thermal Inactivation Kinetics of *Salmonella* in Low-Moisture Foods. *J. ofFood Prot.* 82:963–970.

49. Giger-Reverdin, S., C. Duvaux-ponter, D. Sauvant, O. Martin, I. Nunes do Prado, and R. Muller. 2002. Intrinsic buffering capacity of feedstuffs. *Anim. Feed Sci. Technol.* 96:83–102.

50. Gill, A., C. Carrillo, M. Hadley, R. Kenwell, and L. Chui. 2019. Bacteriological analysis of wheat fl our associated with an outbreak of Shiga toxin-producing *Escherichia coli* O121. *Food Microbiol.* Elsevier Ltd 82:474–481.

51. Gill, A., C. Carrillo, M. Hadley, R. Kenwell, and L. Chui. 2019. Bacteriological analysis of wheat flour associated with an outbreak of Shiga toxin-producing *Escherichia coli* O121. *Food Microbiol.* Elsevier Ltd 82:474–481.

52. Gill, A., T. McMahon, F. Dussault, and N. Petronella. 2020. Shiga toxin-producing *Escherichia coli* survives storage in wheat flour for two years. *Food Microbiol.* Elsevier Ltd 87:103380.

53. Gonzalez-Escalona, N., and J. A. Kase. 2019. Virulence gene profiles and phylogeny of Shiga toxin-positive *Escherichia coli* strains isolated from FDA regulated foods during 2010-2017. *PLoS One* 1–26.

54. Gorton, L. 2016. FSMA will change life for bakers. *Bak. Bus.*

55. Hao, Y. Y., A. J. Scouten, and R. E. Brackett. 1999. Cheesecake: A potential vehicle for Salmonellosis? *J. Food Prot.* 62:26–29.

56. Hartman, G. 2012. Bad Bug Book Handbook of Foodborne Pathogenic Microorganisms and Natural Toxins Introduction. *Book* 1:259.

57. He, Y., D. Guo, J. Yang, M. Lou Tortorello, and W. Zhang. 2011. Survival and Heat Resistance of *Salmonella* enterica and *Escherichia coli* O157 : H7 in Peanut Butter. *Appl. Environ. Microbiol. p.* 77:8434–8438.

58. He, Y., Y. Li, J. K. Salazar, J. Yang, and L. Tortorello. 2013. Increased Water Activity Reduces the Thermal Resistance of *Salmonella* enterica in Peanut Butter. *Appl. Environ. Microbiol. p.* 79:4763–4767.

59. Henegan, C. 2016. More than money: What a recall truly costs. *Food Dive*.

60. Hyslop, G. 2018. Indsutry insight into US bakery market. *Bak. Snacks*.

61. Interagency Food Safety Analytics Collaboration. 2020. Foodborne illness source attribution estimates for 2018 for *Salmonella*, *Escherichia coli* O157 , *Listeria monocytogenes* , and *Campylobacter* using multi-year outbreak surveillance data , United States. GA and DC.

62. Izurieta, W. P., and E. Komitopoulou. 2012. Effect of Moisture on *Salmonella* spp. Heat Resistance in Cocoa and Hazelnut Shells. *Food Res. Int.* Elsevier B.V. 45:1087–1092.

63. Jay, J. M., M. J. Loessner, and D. A. Golden. 2005. Modern Food Microbiology Seventh. Springer, New York, NY.

64. Jones, J. L., L. Wang, O. Ceric, S. M. Nemser, D. S. Rotstein, D. A. Jurkovic, Y. Rosa, B. Byrum, J. Cui, Y. Zhang, C. A. Brown, A. L. Burnum, S. Sanchez, R. Reimschuessel, and S. Bernard. 2019. Whole Genome Sequencing Confirms Source of Pathogens Associated with Bacterial Foodborne Illness in Pets Fed Raw Pet Food. *J. Vet. Diagnostic Investig.*

65. Kang, D.-H., and D. Y. . Fung. 2000. Application of thin agar layer method for recovery

of injured *Salmonella* typhimurium. *Int. J. Food Microbiol.* 54:127–132.

66. Kilonzo-Nthenge, A., E. Rotich, S. Godwin, and T. Huang. 2009. Consumer Storage Period and Temperature for Peanut Butter and Their Effects on Survival of *Salmonella* and *Escherichia coli* O157 : H7. *Food Prot. Trends,* 29:787–792.

67. Kimura, A. C., M. S. Palumbo, H. Meyers, S. Abbott, R. Rodriguez, and S. B. Werner. 2005. A multi-state outbreak of *Salmonella* serotype Thompson infection from commercially distributed bread contaminated by an ill food handler. *Epidemiol. Infect.* 133:823–828.

68. Kindle, P., M. Nüesch-inderbinen, N. Cernela, and R. Stephan. 2019. Detection , Isolation , and Characterization of Shiga Toxin – Producing *Escherichia coli* in Flour. *J. Food Prot.* 82:164–167.

69. Kottapalli, B., S. P. V Nguyen, T. I. M. Perez, and A. Cunningham. 2019. Thermal Inactivation of *Salmonella* and *Listeria monocytogenes* in Peanut Butter – Filled Pretzels and Whole Wheat Pita Chips. *J. Food Prot.* 82:238–246.

70. Kotzekidou, P. 2013. Microbiological examination of ready-to-eat foods and ready-to-bake frozen pastries from university canteens. *Food Microbiol.* 34:337–443.

71. Krause, D. O., and S. Hendrick. 2010. Zoonotic Pathognes in the Food Chain BMC Veterinary Research.

72. Lathrop, A. A., T. Taylor, and J. Schnepf. 2014. Survival of *Salmonella* during Baking of Peanut Butter Cookies 77:635–639.

73. Linton, R. H., M. D. Pierson, and J. R. Bishop. 1990. Increase in Heat Resistance of *Listeria monocytogenes* Scott A by Sublethal Heat Shock 53:924–927.

74. Los, A., D. Ziuzina, and P. Bourke. 2018. Current and Future Technologies for Microbiological Decontamination of Cereal Grains. *J. Food Sci.* 83:1484–1493.

75. Ma, L. I., G. Zhang, P. Gerner-smidt, V. Mantripragada, I. Ezeoke, and M. P. Doyle.

2009. Thermal Inactivation of *Salmonella* in Peanut Butter. *J. Food Prot.* 72:1596–1601.

76. Mattick, K. L., F. Jørgensen, P. Wang, J. Pound, M. H. Vandeven, L. R. Ward, J. D. Legan, and T. J. Humphrey. 2001. Effect of Challenge Temperature and Solute Type on Heat Tolerance of *Salmonella* Serovars at Low Water Activity. *Am. Soc. Microbiol.* 67:4128–4136.

77. Mccallum, L., S. Paine, K. Sexton, M. Dufour, K. Dyet, M. Wilson, D. Campbell, D. Bandaranayake, and V. Hope. 2013. An Outbreak of *Salmonella* Typhimurium Phage Type 42 Associated with the Consumption of Raw Flour. *Foodborne Pathog. Dis.* 10.

78. Mcevoy, J. M., A. M. Doherty, J. J. Sheridan, I. S. Blair, and D. A. Mcdowell. 2003. The Prevalence of *Salmonella* spp . in Bovine Faecal , Rumen and Carcass Samples at a Commercial Abattoir 693–700.

79. Mennah-Govela, Yamile A. Bornhorst, G. M. 2021. Food Buffering Capacity: Quantification Methods and its Importance in Digestion and Health. *R. Soc. Chem.* 12:543–563.

80. Michael, M., J. Acuff, K. Lopez, D. Vega, R. Phebus, H. Thippareddi, and L. H. Channaiah. 2020. Comparison of Survival and Heat Resistance of *Escherichia coli* O121 and *Salmonella*in Muffins. *Int. J. Food Microbiol.* Elsevier 317:108422.

81. Montville, T. J., K. R. Matthews, and K. E. Kniel. 2012. Food Microbiology: An IntroductionThird. ASM Press, Washington.

82. Morton, V., J. Cheng, D. Sharma, and A. Kearney. 2017. An outbreak of Shiga toxin-producing *Escherichia coli* O121 Infections Associated with Flour—Canada, 2016–2017. *Canada Commun. Dis. Rep.* 43:154–155.

83. Morton, V., J. M. Cheng, D. Sharma, and A. Kearney. 2017. An Outbreak of Shiga Toxin–Producing *Escherichia coli* O121 Infections Associated with Flour — Canada, 2016–2017Morbidity and Mortality Weekly Report.

84. Myoda, S. P., S. Gilbreth, D. Akins-lewenthal, S. K. Davidson, and M. Samadpour. 2019.

Occurrence and Levels of *Salmonella*, Enterohemorrhagic *Escherichia coli* , and *Listeria* in Raw Wheat. *J. Food Prot.* 82:1022–1027.

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

86. Niebuhr, S. E., A. Laury, G. R. Acuff, and J. S. Dickson. 2008. Evaluation of Nonpathogenic Surrogate Bacteria as Process Validation Indicators for *Salmonella* enterica for Selected Antimicrobial Treatments , Cold Storage , and Fermentation in Meat. *J. Food Prot.* 71:714–718.

87. Osimani, A., L. Aquilanti, and F. Clementi. 2016. Salmonellosis Associated with Mass Catering: A survey of European Union Cases Over a 15-Year Period. *Epidemiol. Infect.* 144:3000–3012.

88. Painter, J. A., R. M. Hoekstra, T. Ayers, R. V Tauxe, C. R. Braden, F. J. Angulo, and P. M. Griffin. 2013. Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbreak Data, United States, 1998–2008. *Emerg. Infect. Dis.* 19:407–415.

89. Plain Grain Inc. 2020. Hard Red Winter Wheat 2020 Regional Quality Survey.

90. Poetter, C., G. Kunstmann, D. Peter, and F. Mattner. 2014. Containment of a Cheesecake-Associated Outbreak of Salmonellosis in 3 Different Hospitals, Detected by Continuous Microbiologic Surveillance. *Am. J. Infect. Control*. Association for Professionals in Infection Control and Epidemiology, Inc. 42:816–817.

91. Projahn, M., M. C. Lamparter, P. Ganas, S. C. Lorenz-wright, D. Maede, A. Fruth, C. Lang, and E. Schuh. 2021. Genetic Diversity and Pathogenic Potential of Shiga toxin-producing *Escherichia coli* (STEC) Derived From German Flour. *Int. J. Food Microbiol.* 347.

92. Richter, K. S., E. Dorneanu, K. M. Eskridge, and C. S. Rao. 1993. Microbiological Quality of Flours. *Am. Assoc. Cereal Chem.* 38:367–369.

93. Rivera, J., A. Deliephan, J. Dhakal, C. G. Aldrich, and K. Siliveru. 2021. Significance of Sodium Bisulfate (SBS) Tempering in Reducing the *Escherichia coli* O121 and O26 Load of Wheat and Its Effects on Wheat Flour Quality. *Foods* 10:1479.

94. Rothschild, M. 2011. Flour Prime Suspect in 2009 Cookie Dough Outbreak. *Food Saf. News*.

95. Sabillón, L., J. Stratton, D. Rose, and A. Bianchini. 2020. Reduction in Pathogenic Load of Wheat by Tempering with Saline Organic Acid Solutions at Different Seasonal Temperatures. *Int. J. Food Microbiol.* Elsevier 313.

96. Sabillón, L., J. Stratton, D. Rose, R. Flores, and A. Bianchini. 2016. Reduction in Microbial Load of Wheat by Tempering with Organic Acid and Saline Solutions. *Cereal Chem.* 93:638–646.

97. Salaun, F., B. Mietton, and F. Gaucheron. 2005. Buffering Capacity of Dairy Prodcuts. *Int. Dairy J.* 15:95–109.

98. Scheil, W., S. Cameron, C. Dalton, C. Murray, and D. Wilson. 1998. A South Australian *Salmonella* Mbandaka outbreak investigation using a database to select controls. *Aust. N. Z. J. Public Health* 22:536–539.

99. Shachar, D., and S. Yaron. 2006. Heat Tolerance of *Salmonella* enterica Serovars Agona , Enteritidis , and Typhimurium in Peanut Butter. *J. Food Microbiol.* 69:2687–2691.

100. Shrestha, S., W. Birbari, T. Sheehan, and K. A. Glass. 2016. Thermal Inactivation of *Salmonella* on Sesame-topped Bread during baking using High and Low oven humidity. *Food Prot. Trends,* 36:116–124.

101. Silva, F. V. M., and P. A. Gibbs. 2012. Thermal Pasteurization Requirements for the Inactivation of *Salmonella*in Foods. *Food Res. Int.* Elsevier Ltd 45:695–699.

102. Siriken, B., O. Cadirci, G. Inat, and S. Pamuk. 2009. Microbiological examination of meatball, cream cake and Turkish delight (lokum). *J. Anim. Vet. Adv.* 8:2049–2054.

103. Song, W., and D. Kang. 2016. Influence of Water Activity on Inactivation of *Escherichia coli* O157 : H7 , *Salmonella* Typhimurium and *Listeria monocytogenes* in Peanut Butter by Microwave Heating. *Food Microbiol.* Elsevier Ltd 60:104–111.

104. Altekruse, N. Bauer, A. Chanlongbutra, R. Desagun, A. Naugle, W. Schlosser, R. Umholtz, and P. White. 2006. *Salmonella* Enteritidis in Broiler 12.

105. Stewart, C. M., M. B. Cole, and D. W. Schaffner. 2003. Managing the Risk of Staphylococcal Food Poisoning from Cream-Filled Baked Goods To Meet a Food Safety Objective. *J. Food Prot.* 66:1310–1325.

106. Suehr, Q. J., N. M. Anderson, and S. E. Keller. 2019. Desiccation and Thermal Resistance of *Escherichia coli* O121 in Wheat Flour. *J. Food Prot.* 82:1308–1313.

107. Syamaladevi, R. M., J. Tang, R. Villa-Rojas, S. Sablani, B. Carter, and G. Campbell. 2016. Influence of Water Activity on Thermal Resistance of Microorganisms in Low-Moisture Foods: A Review. *Compr. Rev. Food Sci. Food Saf.* 15:353–370.

108. Tack, D. M., L. Ray, P. M. Griffin, P. R. Cieslak, J. Dunn, and T. Rissman. 2020. Preliminary Incidence and Trends of Infections with Pathogens Transmitted Commonly Through Food — Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2016–2019.

109. Technical, A. 2009. Experimental Milling: Introduction, Equipment, Sample Preparation, and TemperingAACC International Approved Methods. AACC International.

110. Technical, A. 2009. Experimental Milling: Temper TableAACC International Approved Methods. AACC International.

111. Technical, A. 2009. Hydrogen-Ion Activity (pH)—Electrometric MethodAACC International Approved Methods11th Ed.

112. Toprak, D., and C. Degirmencilik. Cleaning and Tempering of Wheat. *World Milling and Tempering of Wheat*.

113. Unger, P., L. H. Channaiah, A. Singh, A. Singh, M. Babb, Y. Yang, and M. Michael. 2021. Validation of brownie baking step for controlling *Salmonella* and *Listeria monocytogenes*. *Food Sci. Nutr.*

114. Van Slyke, D. D. 1922. On the measurement of buffer values andon the relationship of buffer value to the dissociation constant of the buffer and the concentration and reaction of the buffer solution. *J. Biol. Chem.* Â© 1922 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology. 52:525–570.

115. VanCauwenberge, J., R. Bothast, and W. Kwolek. 1981. Thermal Inactivation of Eight *Salmonella* Serotypes on Dry Corn Flour. *Appl. Environ. Microbiol.* 42:688–691.

116. Vasser, M., J. Barkley, A. Miller, E. Gee, K. Purcell, M. N. Schroeder, C. Basler, and K. P. Neil. 2021. Multistate Outbreak of *Escherichia coli* O26 Infections Linked to Raw Flour — United States, 2019.

117. Wang, W. 2017. Validation of baking to inactivate *Salmonella*in high-protein and high fat model foods. Illinois Institute of Technology.

118. World Health Organization. 2020. Food Safety.

119. World Health Organization. 2021. Foodborne diseases.

120. Wu, S., S. C. Ricke, K. R. Schneider, and S. Ahn. 2017. Food safety hazards associated with ready-to-bake cookie dough and its ingredients 73:986–993.

121. Wyatt, C. J., and V. H. Guy. 1981. Growth of *Salmonella* typhimurium and *Staphylococcus aureus* in Retail Pumpkin Pies. *J. Food Prot.* 44:418–421.

122. Yan, Z., J. B. Gurtler, and J. L. Kornacki. 2006. A Solid Agar Overlay Method for Recovery of Heat-Injured *Listeria monocytogenes*. *J. Food Prot.* 69:428–431.

123. Zhang, H., E. Yamamoto, J. Murphy, C. Carrillo, K. Hardie, and A. Locas. 2021. Microbiological Survey of Wheat Flour Sold at Retail in Canada , 2018 to 2019 84:647–

654.

124. Anonymous 2011. Bakery Linked To *Salmonella* Deaths Ordered Closed. *Manufacturing.net*.

125. Anonymous 2014. Bakery products recalled due to Listeria. *Bak. J.*

126. Anonymous 2021. Hostess recalls hamburger and hot dog buns for possible *Listeria* and *Salmonella* risks. *Food Saf. News*.

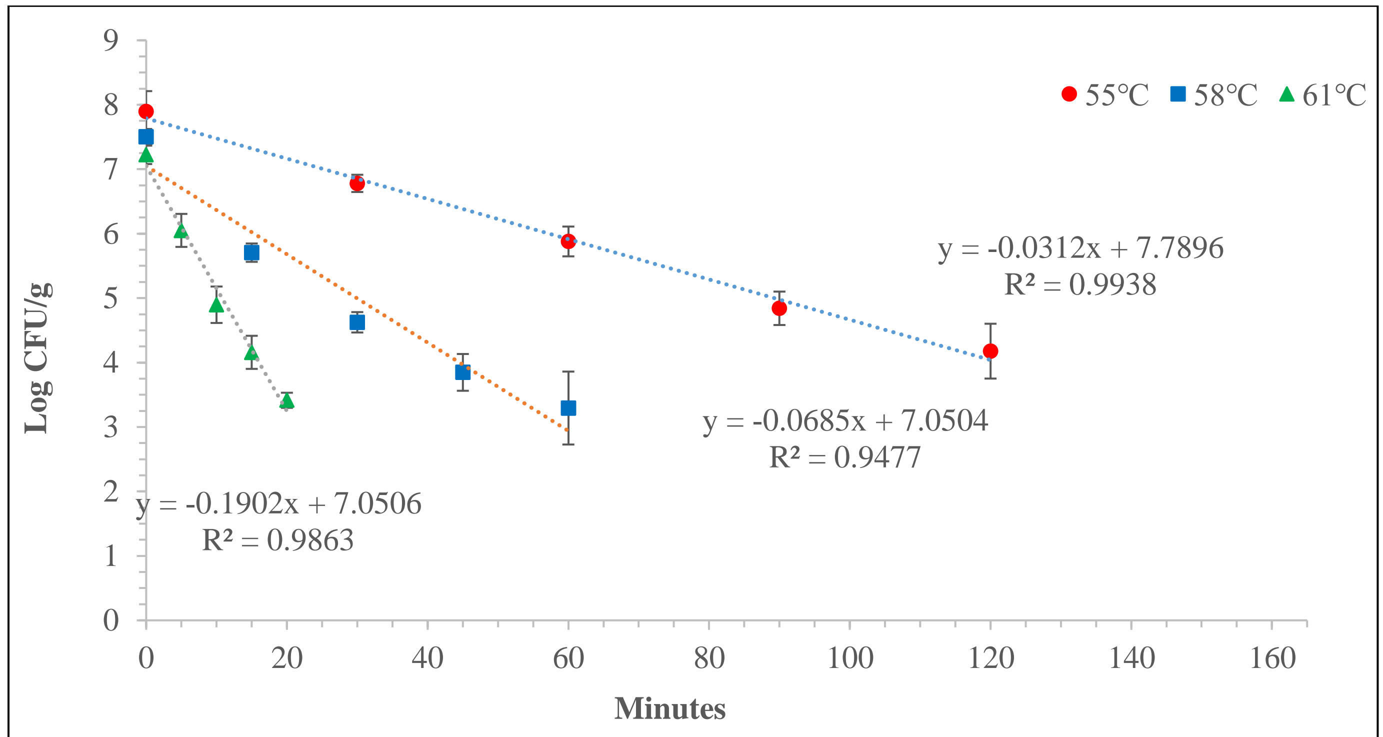
127. Anonymous 2010. Illinois Pastries Linked to 100 Illnesses. *Food Saf. News*.

128. Anonymous 2011. Outbreak in Rhode Island Linked to Pastries. *Food Saf. News*.

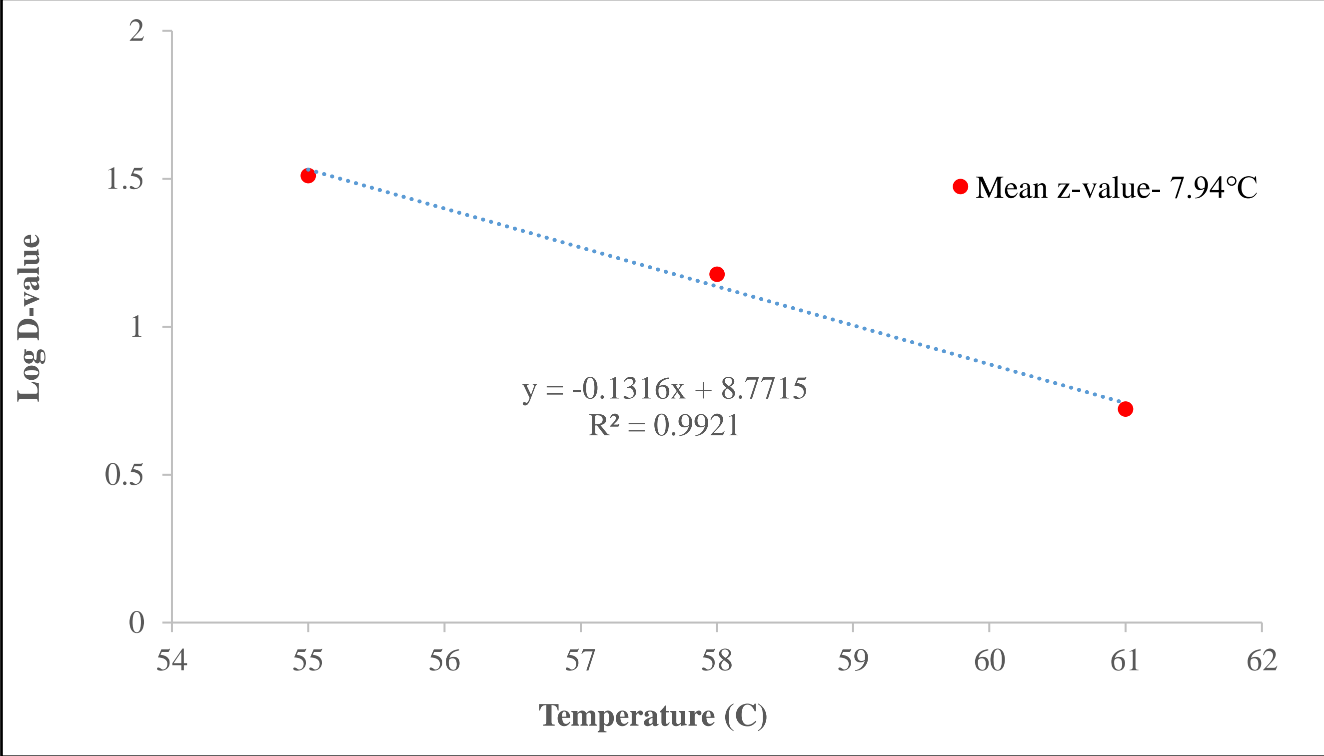
129. Anonymous 2020. Positive *Listeria* test results spur international recall of peanut spread. *Food Saf. News*.

130. Anonymous 2021. Wheat Kernel. *BAKERpedia*.

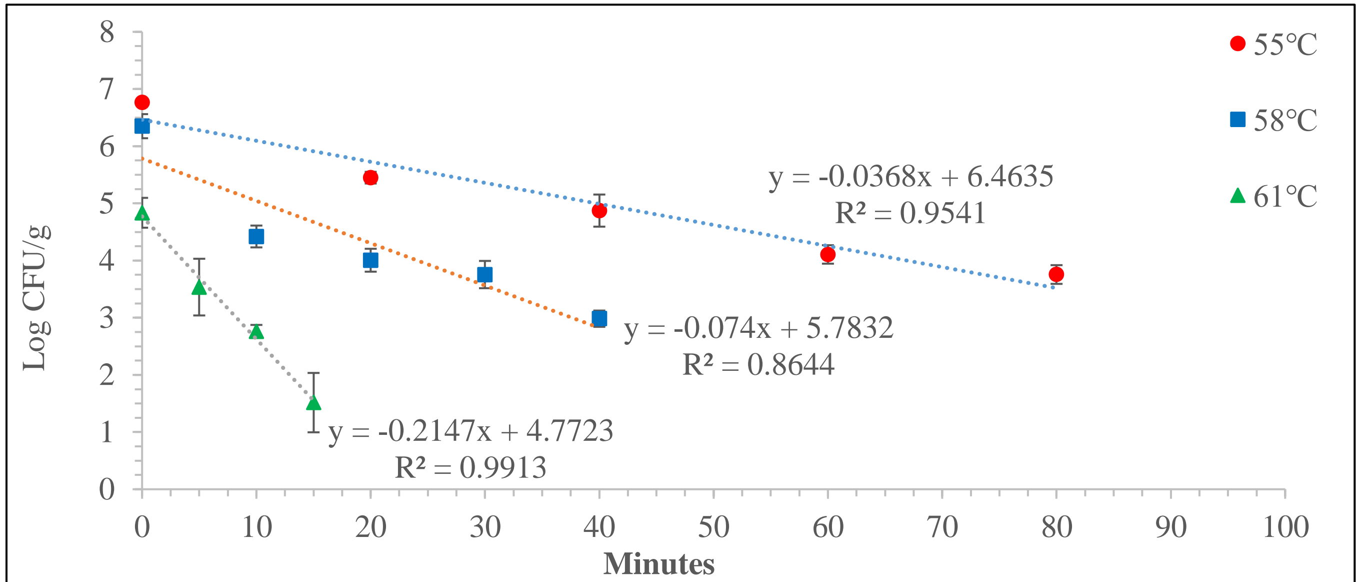
Appendix A - 7- serovar *Salmonella* cocktail counts in pastry dough *vs.* heating time as plated on injury-recovery media (BHI agar overlaid with XLD agar).



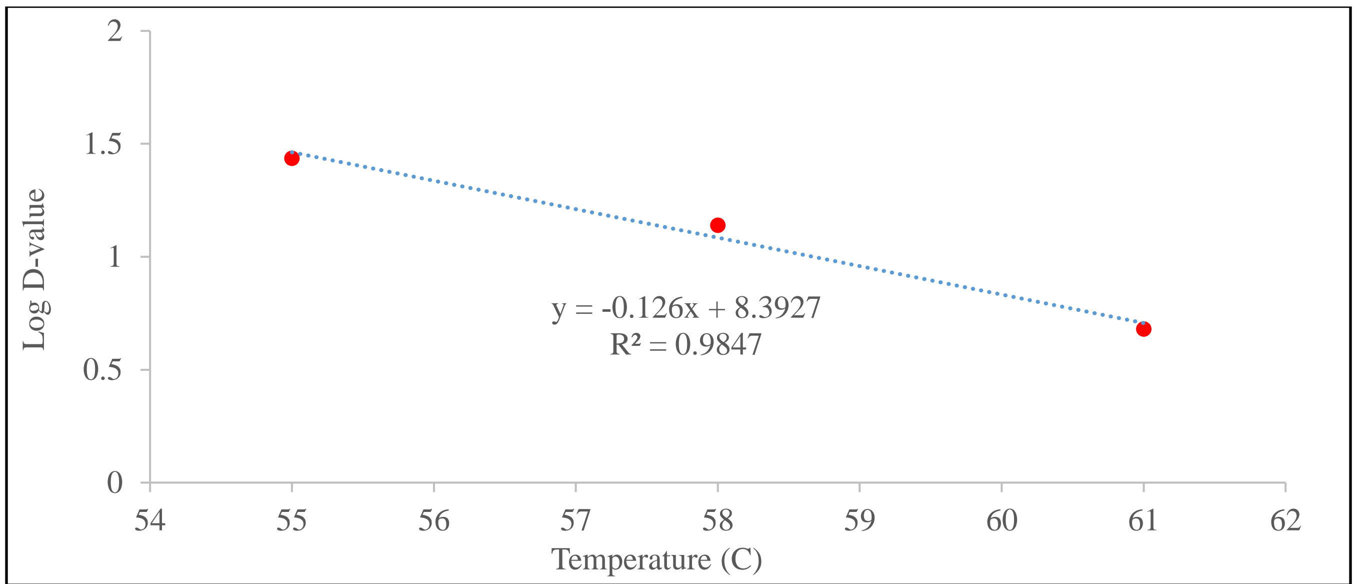
Appendix B - Log D-values of 7- serovar *Salmonella* cocktail in pastry dough *vs.* dough temperature (°C) as calculated from counts on injury-recovery media (BHI agar overlaid with XLD agar).



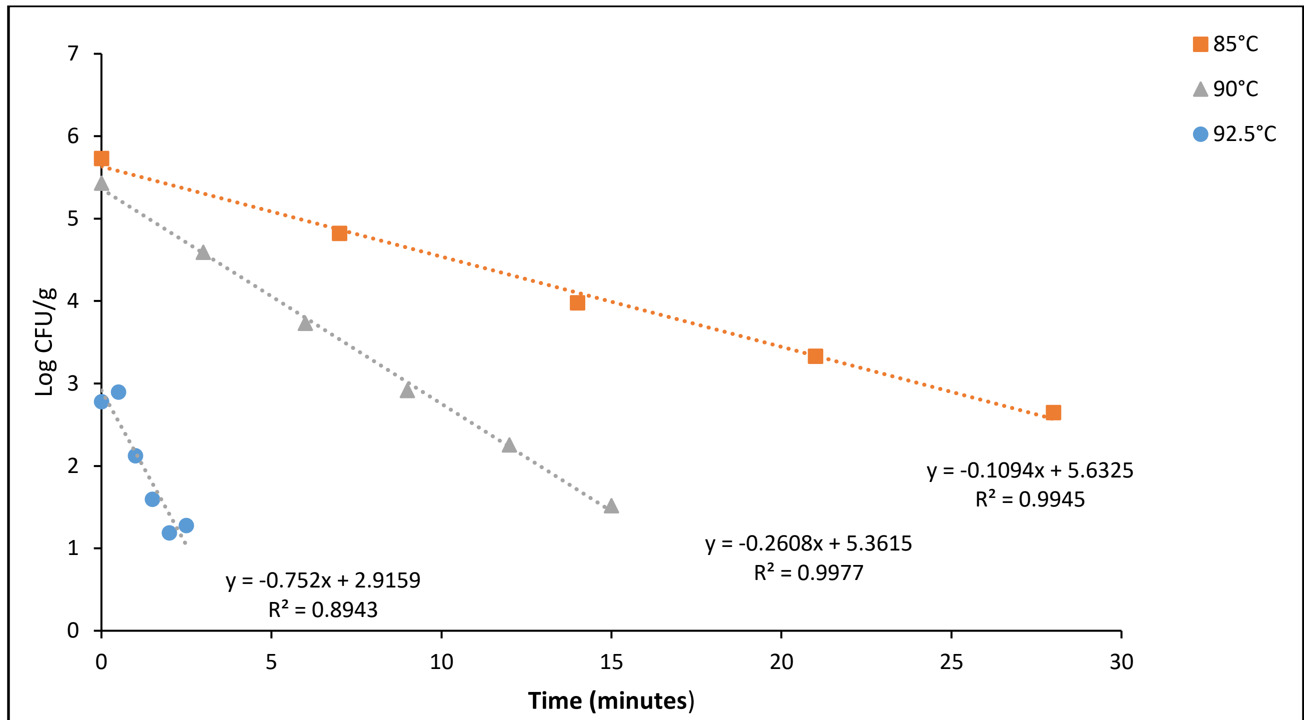
Appendix C - 7- serovar *Salmonella* cocktail counts in cheesecake batter *vs.* heating time as plated on injury-recovery media (BHI agar overlaid with XLD agar).



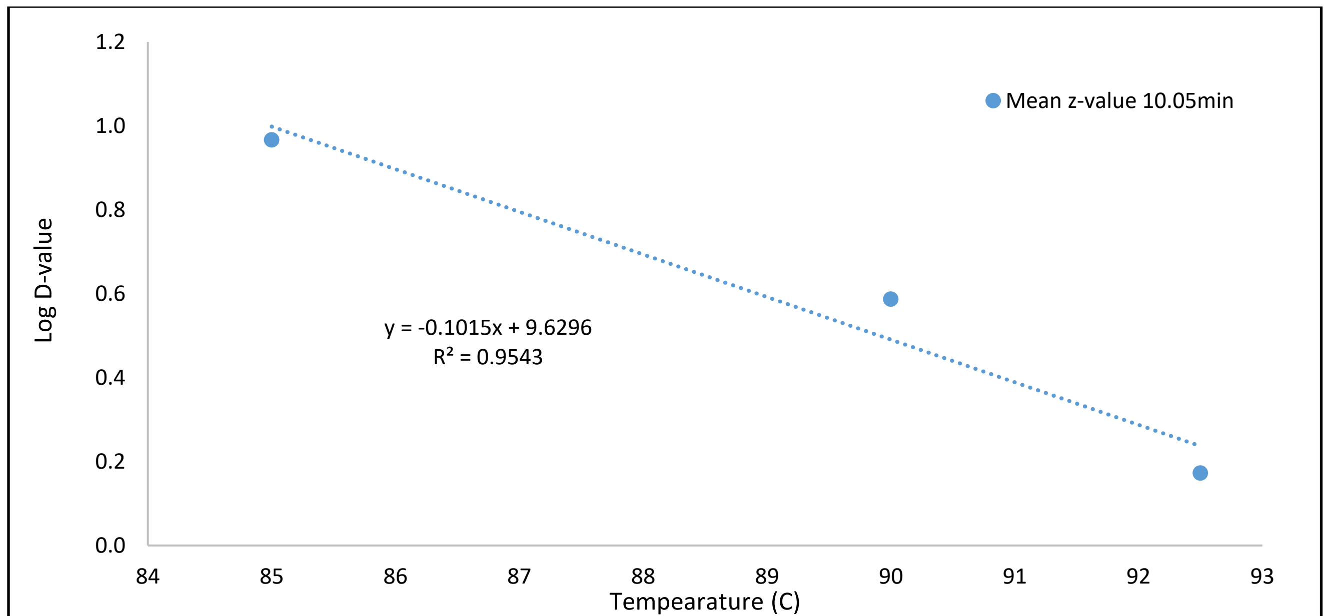
Appendix D - Log D-values of 7-serovar *Salmonella* cocktail in cheesecake batter *vs.* batter temperature (°C) as calculated from counts on injury-recovery media (BHI agar overlaid with XLD agar).



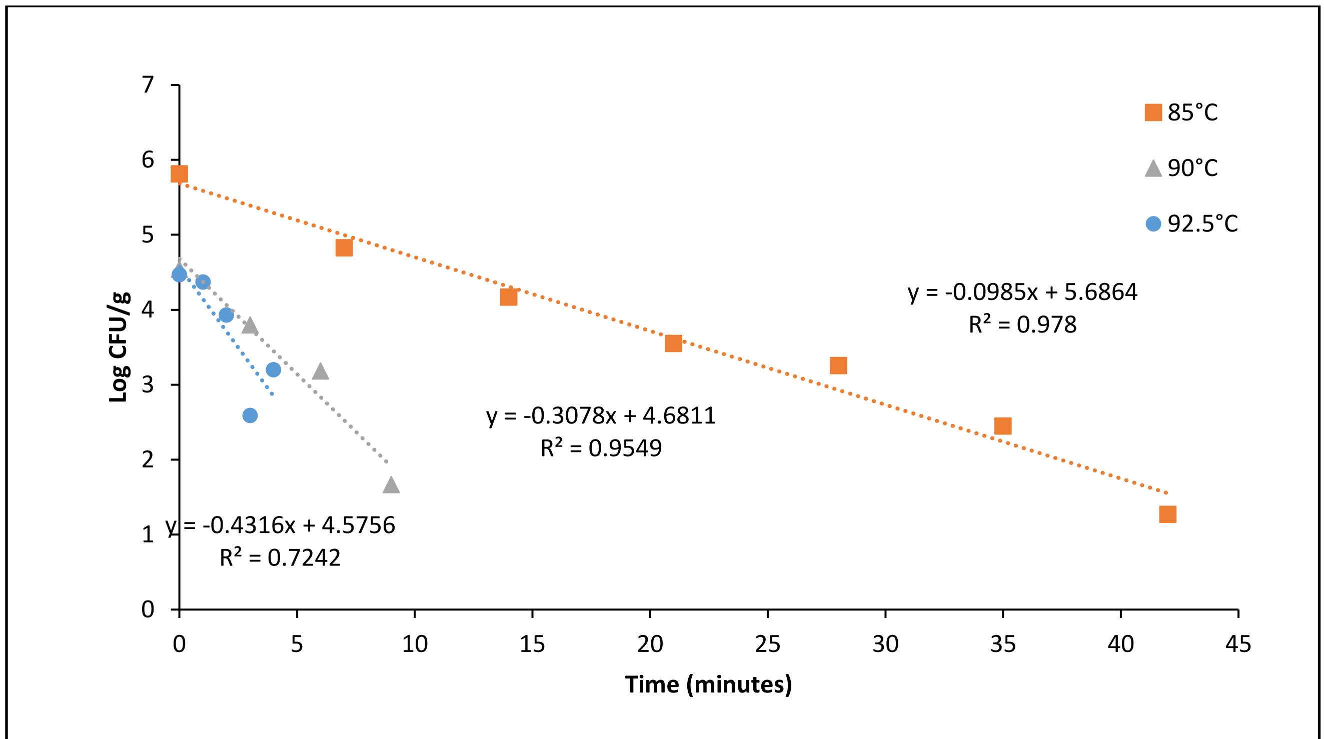
Appendix E - 7- serovar *Salmonella* cocktail counts in peanut butter bars *vs.* heating time as plated on injury-recovery media (BHI agar overlaid with XLD agar).



Appendix F - Log D-values of 7- serovar *Salmonella* cocktail in peanut butter bars *vs.* batter temperature (°C) as calculated from counts on injury-recovery media (BHI agar overlaid with XLD agar).



Appendix G - 7-serotype *E. coli* cocktail counts in peanut butter bars vs. heating time as plated on injury-recovery media (BHI agar overlaid with MacConkey agar).



Appendix H - Log D-values of 7-serotype *E. coli* cocktail in peanut butter bars *vs.* batter temperature (°C) as calculated from counts on injury-recovery media (BHI agar overlaid with XLD agar).