

VALIDATION OF BAKING TO CONTROL *SALMONELLA* SEROVARS IN HAMBURGER
BUN MANUFACTURING, AND EVALUATION OF *ENTEROCOCCUS FAECIUM* ATCC
8459 AND *SACCHAROMYCES CEREVISIAE* AS NONPATHOGENIC SURROGATES FOR
THERMAL PROCESS VALIDATION

by

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Abstract

With the implementation of the Food and Drug Administration's Food Safety Modernization Act, the food industry must scientifically verify that current production processes provide sufficient protection against pathogens. This study was conducted to validate a simulated commercial baking process for hamburger buns to control *Salmonella* spp. contamination and to determine the appropriateness of using non-pathogenic surrogates (*Enterococcus faecium* ATCC 8459 or *Saccharomyces cerevisiae*) for in-plant process validation studies. Wheat flour was separately inoculated (~6 log CFU/g) with three *Salmonella* serovars (Typhimurium, Newport or Senftenberg) or *E. faecium*. Dough was formed, proofed, and baked to mimic commercial manufacturing conditions. Non-inoculated dough was used to evaluate *S. cerevisiae* (Baker's yeast) survival during baking. Buns were baked for 9, 11 and 13 min in a conventional oven set at 218°C, with internal bun temperature profiles recorded. *Salmonella* serovars and *S. cerevisiae* were reduced by >6 log₁₀ CFU/g after 9 min of baking. *E. faecium* was detected by direct plating after 11 min of baking but not after 13 min. After 13 min of baking, all three target organisms were eliminated (>6 log CFU/g reduction) in the buns. D- and z-values of *Salmonella* spp. (3-serovar cocktail), *E. faecium*, and *S. cerevisiae* in bun dough were also determined. D-values of *Salmonella* spp. and *E. faecium* during heating of dough were 28.64 and 133.33, 7.61 and 55.67, and 3.14 and 14.72 min at 55, 58 and 61°C, respectively; whereas, D-values of *S. cerevisiae* were 18.73, 5.67 and 1.03 min at 52, 55 and 58°C, respectively. The z-values of *Salmonella* spp., *E. faecium* and *S. cerevisiae* were 6.58, 6.25 and 4.74°C, respectively. *E. faecium* demonstrated greater thermal resistance than *Salmonella* spp. and *S. cerevisiae*, making it an appropriate (and conservative) surrogate to establish thermal process lethality in the validation of commercial

baking operations. The low thermal tolerance of *S. cerevisiae* relative to *Salmonella* limits its usefulness as a potential surrogate for process validations.

Table of Contents

List of Figures	vii
List of Tables	ix
Acknowledgements	x
Dedication	xi
Chapter 1 - Introduction	1
Chapter 2 - Literature Review	5
Thermal Destruction of Microorganisms	5
Response to Heat Treatments	5
Measures of Thermal Destruction	6
D-value	6
z-value	7
F-value	8
Burden of Foodborne Illness	9
<i>Salmonella enterica</i>	13
Invasion	14
<i>Enterococcus faecium</i> ATCC 8459	16
<i>Saccharomyces cerevisiae</i>	17
<i>Salmonella</i> spp. Contamination of Flour Products	18
Chapter 3 - Objectives	22
Chapter 4 - Validation of Baking to Control <i>Salmonella</i> Serovars in Hamburger Bun Manufacturing, and Evaluation of <i>Enterococcus faecium</i> ATCC 8459 and <i>Saccharomyces cerevisiae</i> as Nonpathogenic Surrogates for Thermal Process Validation	23
Introduction	23
Materials and Methods	26
Experimental design	26
Inoculum preparation	27
Inoculation of flour	28
Dough preparation	28
Oven selection and optimization to mimic industry standard	30

Hamburger bun baking	30
Microbiological analyses	31
D- and z-values determinations	32
Physical/chemical analyses of hamburger buns.....	33
Results and Discussion	34
Water activity and pH of hamburger buns	34
Validation of the bun baking process.....	35
Determination of D- and z-values in bun dough.....	40
Chapter 5 - Conclusions.....	47
References.....	49
Appendix A - Images	61

List of Figures

Figure 1.1 Categorization of jobs dependent on the U.S. baking industry	1
Figure 1.2 Distribution of bakery products by percent of U.S. sales	2
Figure 2.1 Generalized D-value graph	7
Figure 2.2 Generalized z-value graph	8
Figure 2.3 Burden of illness pyramid	10
Figure 4.1 Water activity (a_w) of hamburger buns during the baking process.....	34
Figure 4.2 Mean internal temperature profile of hamburger buns during 13 min baking process (218°C) followed by 30 min of cooling	36
Figure 4.3 Survival of <i>Salmonella</i> serovars (<i>S. Typhimurium</i> , ST; <i>S. Newport</i> , SN; <i>S.</i> <i>Senftenberg</i> , SS), <i>Enterococcus faecium</i> (EF) and <i>Saccharomyces cerevisiae</i> (SC) in hamburger buns during baking at 218°C oven temperature; enumerated using selective media	37
Figure 4.4 Survival of <i>Salmonella</i> serovars (<i>S. Typhimurium</i> , ST; <i>S. Newport</i> , SN; <i>S.</i> <i>Senftenberg</i> , SS), <i>Enterococcus faecium</i> (EF) and <i>Saccharomyces cerevisiae</i> (SC) in hamburger buns during baking at 218°C oven temperature; enumerated using injury- recovery media	37
Figure 4.5 Thermal inactivation of <i>Salmonella</i> spp. and <i>E. faecium</i> in hamburger bun dough: viable cells enumerated on selective media	39
Figure 4.6 Thermal inactivation of <i>Salmonella</i> spp. and <i>E. faecium</i> in hamburger bun dough: viable cells enumerated on injury-recovery media	39
Figure 4.7 Thermal inactivation of <i>Salmonella</i> spp. at 55, 58 and 61°C in hamburger bun dough: viable cells enumerated on XLD agar	41
Figure 4.8 Thermal inactivation of <i>Salmonella</i> spp. at 55, 58 and 61°C in hamburger bun dough: viable cells enumerated on BHI agar with XLD agar overlay	41
Figure 4.9 Thermal inactivation of <i>E. faecium</i> at 55, 58 and 61°C in hamburger bun dough: viable cells enumerated on m-E agar	42
Figure 4.10 Thermal inactivation of <i>E. faecium</i> at 55, 58 and 61°C in hamburger bun dough: viable cells enumerated on BHI agar with m-E agar overlay	42

Figure 4.11 Thermal inactivation of *S. cerevisiae* in hamburger bun dough at 52, 55 and 58°C as enumerated on PDA supplemented with 100 ppm chloramphenicol..... 45

Figure 4.12 Log D-value versus temperature for *Salmonella* spp. (SS) enumerated on XLD agar and BHI agar overlayed with XLD; *E. faecium* (EF) enumerated on m-E agar and BHI overlayed with m-E agar; *S. cerevisiae* (SC) enumerated on PDA supplemented with 100 ppm chloramphenicol..... 46

List of Tables

Table 4.1 Dough recipe for hamburger buns	29
Table 4.2 D-values (min) and z-values (°C) of a 3-serovar <i>Salmonella</i> spp. cocktail, <i>Enterococcus faecium</i> ATCC 8459, and <i>Saccharomyces cerevisiae</i> during heating of hamburger bun dough	43

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Dedication

This work is dedicated to my friends and family who were always nearby offering support and encouragement. Thank you, Nick and Donka, for setting me on the path and helping me weather the late nights and early mornings. Thank you, Minto, for your help troubleshooting when I wasn't sure what questions to ask. Thank you, Sarah, for always being ready with whatever I needed. Thank you, Clinton, for your patience while gently pushing me through all the times I struggled. Thank you to all the other pairs of hands who contributed in meaningful ways. I couldn't have made it here without you all.

Chapter 1 - Introduction

While often taken for granted, baked goods are a staple of the American diet. The American Bakers Association (ABA) estimated the U.S. baking industry had a total economic output of about \$294.9 billion, contributing to about 1.84% of U.S. GDP, and employed nearly 1.87 million Americans (Fig. 1.1) in 2012 (ABA, 2013). Wages and taxes collected from these businesses and their employees were estimated at about \$91.1 mil and \$37.3 mil, respectively (ABA, 2013).

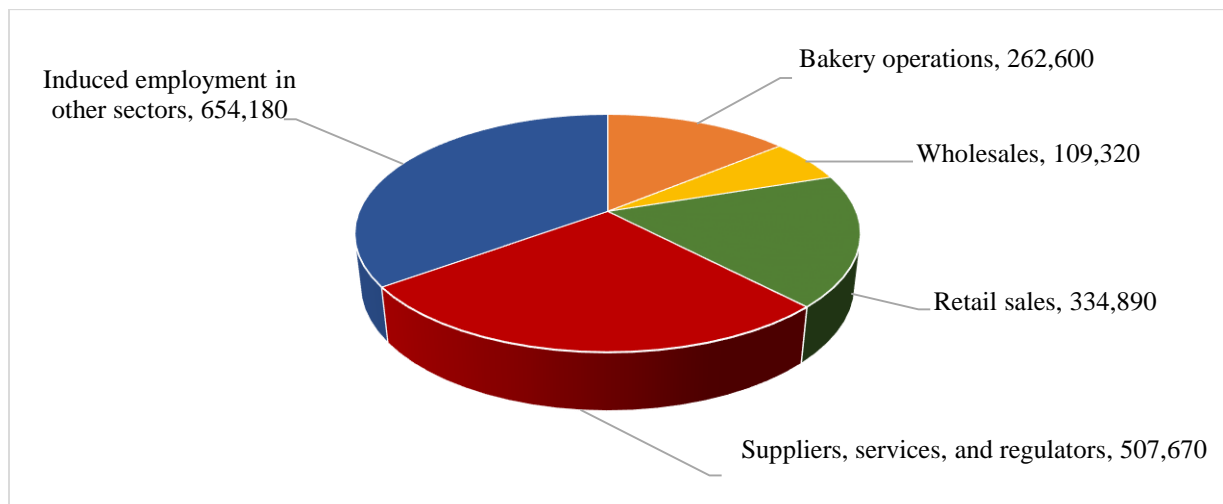


Figure 1.1 Categorization of jobs dependent on the U.S. baking industry

The diversity of baked goods on the market is also impressive. White, wheat, and rye breads predominate at about 40% of commercial bakery sales; “rolls, buns, muffins, bagels, and croissants” are the second largest category, making up 20% of sales, followed by soft cakes at 10% (Bakery and Snacks, 2012). The “remaining sales [come] from pies, pastries, donuts, and sweet goods” (Bakery and Snacks, 2012). This distribution of products is displayed in Fig. 1.2

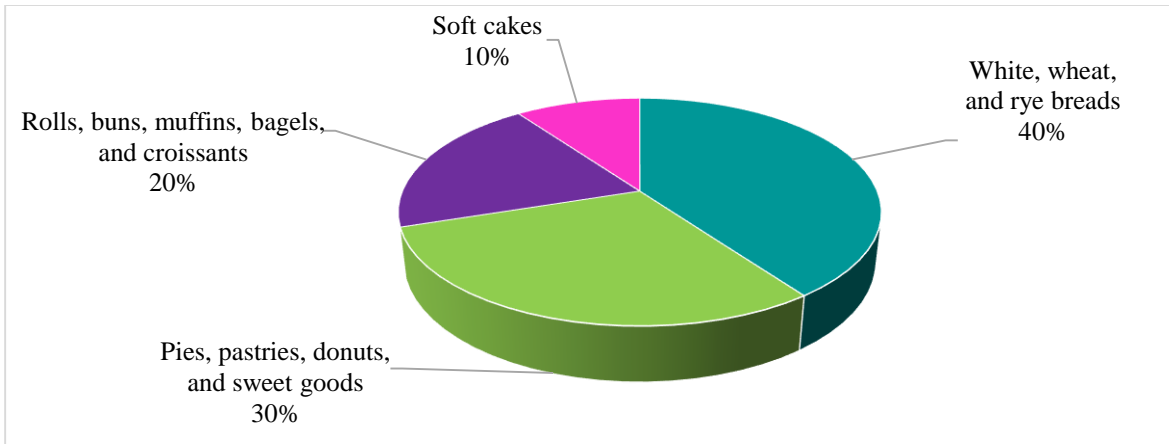


Figure 1.2 Distribution of bakery products by percent of U.S. sales

To ensure that business continues to boom for the baking industry, processing facilities must remain in compliance with recent updates to federal food safety standards. With the formal signing of the Food Safety Modernization Act (FSMA) on January 4, 2011, facilities will soon be required to register with the U.S. Food and Drug Administration (FDA) (FDA, 2011). In order to remain a registered facility with the authority to distribute food products, each facility must develop “science-based mitigation strategies” for Hazard Analysis and Risk-based Preventative Controls (HARPC) (FDA, 2011)(Wayne Labs, 2014). To qualify, the following steps must be taken: (1) evaluate any hazards (biological, chemical, physical, radiological, or intentionally introduced), (2) establish preventative controls for each identified hazard, (3) verify controls effectively reduce hazards to an appropriate level of risk, (4) utilized corrective actions should the risk of hazard exceed the defined minimal level, and (5) document all processes (FDA, 2011)(Wayne Labs, 2014).

Unlike facilities operating under Hazard Analysis Critical Control Points (HACCP) regulations, many FDA processors, including bakeries, do not presently have explicit, published

guidelines quantifying what are or are not acceptable processing parameters (Wayne Labs, 2014). This knowledge gap represents a vital hole which must be filled.

Conducting in-plant validation research is necessary to verify that the systems in place are operating with the anticipated result (Jeong *et al.*, 2011)(Kopit *et al.*, 2014). Ideally, all pertinent organisms would be evaluated in these conditions, however, this is generally infeasible for pathogenic organisms as containment and decontamination of industrial equipment is not practicable (Jeong *et al.*, 2011) (Kopit *et al.*, 2014). It thus falls to laboratories to identify non-pathogenic indicator organisms with similar or slightly elevated survival characteristics as surrogate species (Kopit *et al.*, 2014). It is equally acceptable to choose surrogate species which are inherent to the food matrix of interest or to add an organism for evaluation (Kornacki, 2012).

The most widely referenced surrogate for *Salmonella* spp. is *Enterococcus faecium* NRRL B-2354, which is a clonal relative of *E. faecium* ATCC 8459, sharing over 99% genome sequencing identity (Kopit *et al.*, 2014). Despite recent concerns regarding its BSL-1 classification, this strain has been used in the food industry for greater than 65 years as a non-pathogenic surrogate during thermal processing (Kornacki, 2012). Operations and food matrices evaluated with *E. faecium* surrogates include pasteurization of milk, ice cream mix, and juice products; microwave processing of liquids; and dry heating of wheat-based products, ground beef, and almonds (Kopit *et al.*, 2014)(Kornacki, 2012). Several strains of *E. faecium* are also commonly used as adjunct cultures added to fermented breads, cheeses, and other dairy products for flavor development, bacteriocin production, and competitive exclusion of pathogenic microflora (Giraffa, 2014)(Kopit *et al.*, 2014)(Kornacki, 2012)(Tan *et al.*, 2013).

Although its use as a surrogate is not reported in the literature, *Saccharomyces cerevisiae*, which is standard ingredient in many baked products, has been of interest to bakers as an

indicator of thermal process control as they seek non-pathogenic microbial options for in-plant process verification activities.

Chapter 2 - Literature Review

Thermal Destruction of Microorganisms

Thermal processing is the most widely used control method for elimination of pathogens in food products (Wu, 2014). Differences in the heat resistance of microorganisms have forced prolonged evaluation of the survival capabilities of individual microorganisms in diverse food matrices (Beney *et al.*, 2003). Factors such as fat content, water activity, pH, and pressure have a combined influence on the relative hostility of the microenvironment in food products (Beney *et al.*, 2003). Furthermore, microorganisms may display an array of responses to thermal stress.

Response to Heat Treatments

Cells express varying responses to lethal heat ranging from survival to damage to death (Wu, 2014). Damaged cells fall into two categories: irreversibly damaged or sublethally damaged (Wu, 2014). Sublethally damaged cells are of particular concern because they are difficult to detect but may demonstrate complete recovery and restoration of virulence over time (Wu, 2014). While most cellular structures and functions are impaired by thermal treatments, damage to the outer membranes is typical and may impede protein production (Wu, 2014). Exposure to heat commonly results in degradation of the lipid components of cellular membranes, resulting in the leakage of substrates such as Mg^{2+} , K^+ , amino acids, nucleic acids, or proteins (Wu, 2014). This leakage exacerbates the strain as Mg ions are needed for structural stability of ribosomes and is believed to inhibit the action of ribonuclease (Wu, 2014). These injured cells are much more fastidious than their healthy counterparts; additional nutrients and incubation time are often needed to detect and enumerate sublethally damaged microorganisms (Wu, 2014).

Measures of Thermal Destruction

With a constant exposure to heat, microorganisms are destroyed at a logarithmic rate which is proportionate to the quantity of organisms present (Goff, n.d.). By expressing the thermal resistance of a microorganism in terms of its rate of destruction, comparisons can be made between species (CDC, 2009). In addition to the aforementioned criteria, the rate of destruction is of course heavily influenced by the temperature to which the microorganism in a given food matrix is exposed.

D-value

An initial step to determine the thermal resistance of a microorganism is to calculate its D-value, or the time in minutes required to inactivate 1-log cycle, or 90%, of the population of the microorganism of interest (FDA, 2014)(Goff, n.d.)(Sperber, 2007). This is commonly done by inoculating the food matrix of concern with a known quantity of the microorganism, exposing the contaminated food product to a constant level of heat, and evaluating the surviving population at known time intervals. Graphing these data points such that time lies on the x-axis and the population of interest lies on the y-axis, the rate of thermal destruction can be calculated using the formula $D = \frac{t}{\log N_0 - \log N_t}$ where t is the time in minutes of the sampling interval, N_0 is the population present at the beginning of the interval, and N_t is the population surviving at the end of the interval (Fig. 2.1) (Goff, n.d.). Alternatively, the D-value can also be calculated as the absolute value of the inverse slope of the line of best fit through several data points taken at regular time intervals (Michael *et al.*, 2014). This process is then repeated at a series of temperatures which either mimic operational limits or deficiencies for a process. For each temperature, the temperature treatment is denoted as a subscript (e.g. $D_{58^\circ\text{C}} = 8$ minutes) (CDC, 2009)(FDA, 2014). Similarly, the term thermal death time is used to express the amount of time

required to destroy a specified quantity of the microorganism of interest in a particular food by thermal processing at a specified temperature (Goff, n.d.).

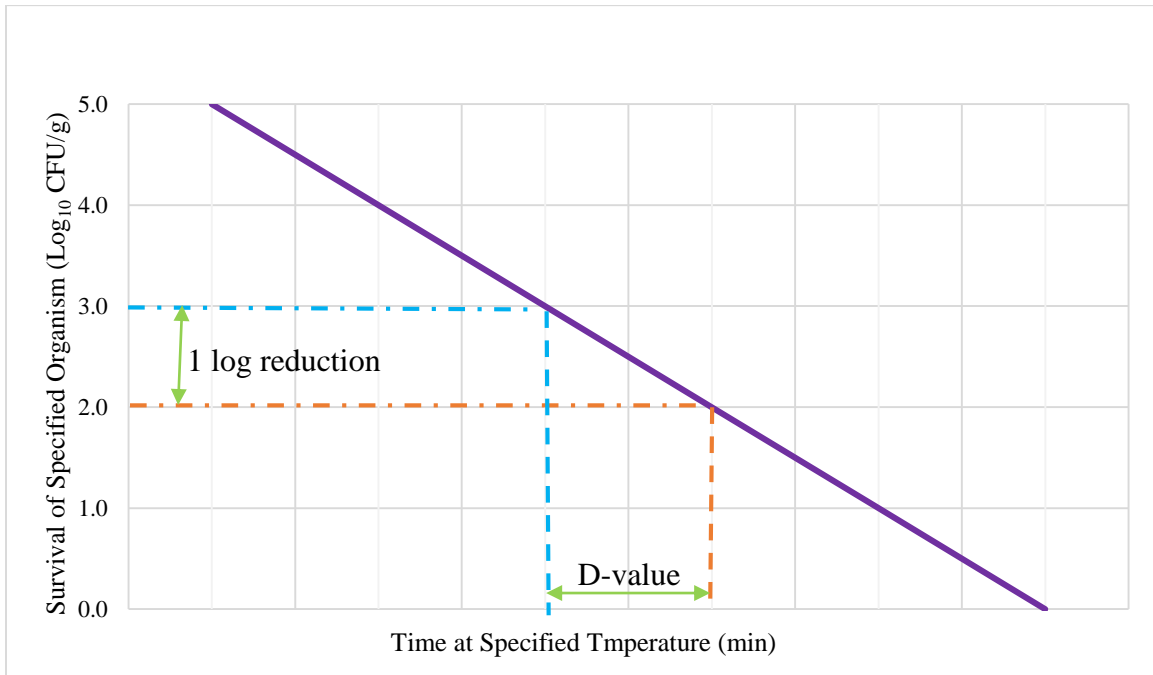


Figure 2.1 Generalized D-value graph

z-value

Following analysis of the rate of destruction at assorted temperatures for a given microorganism in a given food matrix, these data can be graphed with temperatures on the x-axis and D-values on the y-axis. The absolute value of the inverse slope of the line of best fit through these data points is referred to as the *z-value* (FDA, 2014)(Michael *et al.*, 2014). Alternatively, if only two D-values are known, the following equation can be used: $z = \frac{T_1 - T_2}{\log D_1 - \log D_2}$ where T_1 is the temperature at the first D-value (D_1) and T_2 is the temperature at the second D-value (D_2) (Fig. 2.2) (Goff, n.d.). The *z-value* quantifies how much the temperature must be adjusted to alter known D-value(s) by a factor of 10 (Goff, n.d.)(Sperber, 2007). An increasingly large *z-value* denotes a depressed lethality to an increase in temperature (Goff, n.d.). *z-values* are commonly used to predict D-values at temperatures which were not experimentally evaluated (FDA, 2014).

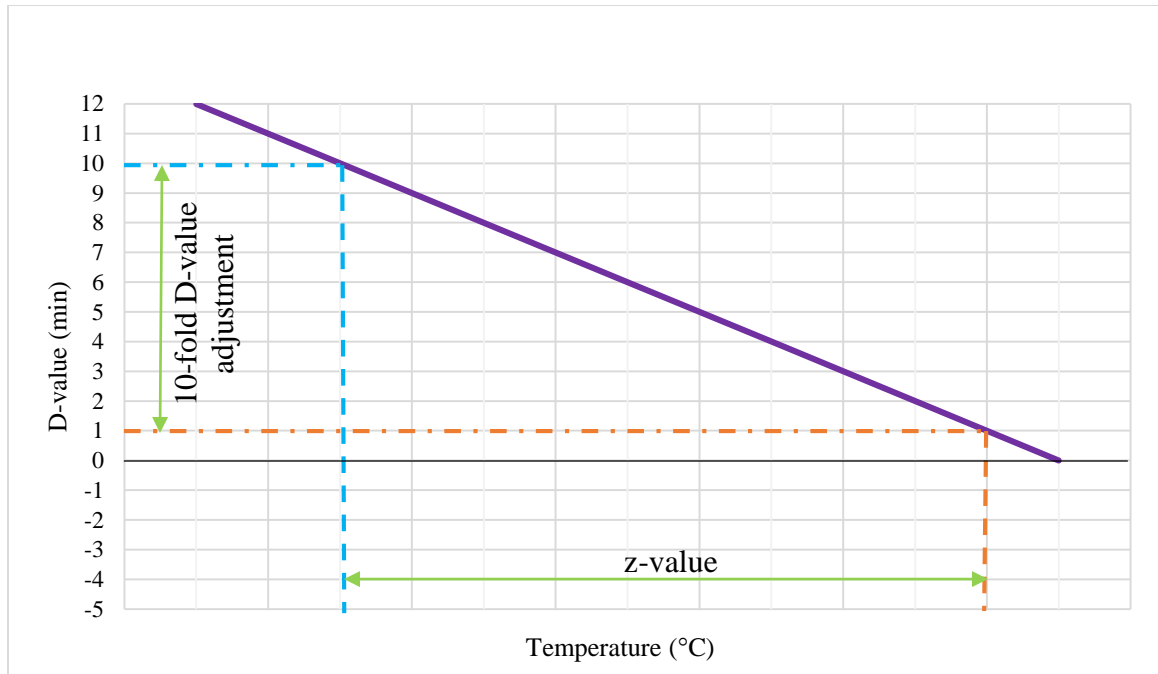


Figure 2.2 Generalized z-value graph

F-value

Knowledge of the D- and z-values of an organism in a particular food matrix allows for the determination of an F-value. F-values are used to describe the time required to destroy or inactivate a given quantity of a microorganism in a given food matrix under specified conditions; they are commonly used to derive the time required to yield a commercially sterile product (Goff, n.d.). As thermal death curves are a logarithmic function, food is considered commercially sterile when the probability of the survival of a microorganism is less than 10^{-6} (Goff, n.d.). As a result, most processing operations calculate F-values as a 12D process for the most thermally resilient pertinent microorganism (FDA, 2014). Convention also dictates that if parameters are not explicitly stated to assume the process describes a thermal exposure of 250°F and a z-value of 18°F (FDA, 2014).

Burden of Foodborne Illness

Food safety is a growing sector whose continued development stands to benefit millions of lives each year. Nearly half of the principle foodborne pathogens in the United States have been identified since the 1970s, substantiating concerns regarding the possibility of other species yet to be isolated and described (Tauxe, 2002). The prevalence of foodborne pathogens is a function of ecology and technology (Tauxe, 2002). While some species are likely relatively novel, other species are simply being attributed as the etiological agent for outbreaks in unprecedented food sources (Tauxe, 2002). Additionally, well-documented pathogens continue to evolve new mechanisms to bypass current intervention technologies (Tauxe, 2002). Globalization of the food trade aids the spread of these adaptations (Tauxe, 2002). In short, despite recent scientific breakthroughs, foodborne illness remains a concerning topic worthy of continued research.

In order to make sound food safety policy decisions, several attempts have been made to estimate the economic and social costs of these preventable conditions (Scallan *et al.*, 2011). In 1995, the U.S. Centers for Disease Control and Prevention (CDC) began partnering with state and local health agencies to create the Foodborne Diseases Active Surveillance Network (FoodNet), a program used to monitor trends in the incidence of foodborne illness (CDC, 2015). Although FoodNet covers 15% of the U.S. population, several barriers remain to collecting accurate nation-wide estimates of foodborne illness (CDC, 2015). Most instances of foodborne illness result in relatively minor, non-specific symptoms of gastrointestinal distress, including stomach cramps, nausea, and diarrhea or vomiting, which clear without medical intervention (Behravesh *et al.*, 2011)(Tauxe, 2002). As a result, many cases of foodborne illness likely go unreported or undiagnosed (Fig. 2.3) (Scallan *et al.*, 2011). Additionally, it is often difficult to ascertain the origin of the etiological agent (Scallan *et al.*, 2011). These challenges are

compounded in the case of pathogens, such as *Listeria monocytogenes*, which may display a long latent period before symptoms of infection become apparent. Nevertheless, these illnesses are not to be underestimated; cases of foodborne illness are relatively common, costing substantial losses due to missed workdays, and may result in lifelong complications or death, especially in persons with depressed immune systems (Behravesch *et al.*, 2011)(Hoffman, 2012).

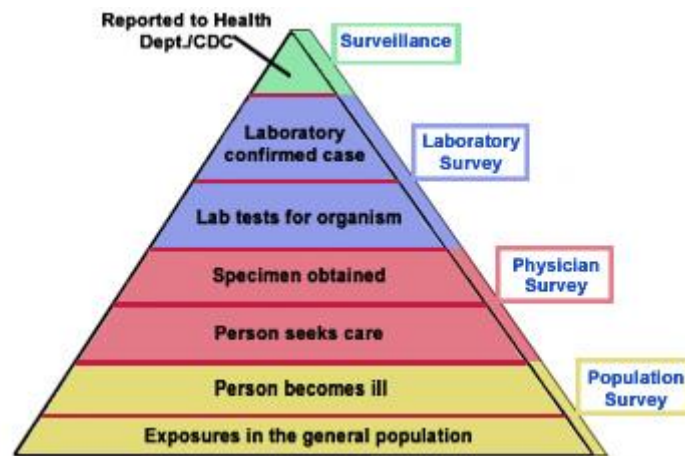


Figure 2.3 Burden of illness pyramid
(source: <http://www.cdc.gov/foodnet/surveillance.html>)

The two methods of data recovery for foodborne illness include passive surveillance, in which diagnostic laboratories voluntarily report positive test results to public health agencies, and active surveillance, in which public health agencies regularly contact diagnostic laboratories regarding the incidence of positive test results. Scallan *et al.* (2011) includes data from both surveillance methods to estimate the overall incidence of foodborne illness across the United States. By combining data collected in relation to foodborne outbreaks with data from five surveillance programs [FoodNet, the National Notifiable Diseases Surveillance System (NNDSS), the Cholera and Other *Vibrio* Illness Surveillance (COVIS) System, the National Tuberculosis Surveillance System (NTSS), and the Foodborne Disease Outbreak Surveillance

System (FDOSS)], a mathematical model could be used to estimate the national average incidence of foodborne illness with 90% confidence intervals (Scallan *et al.*, 2011). Thirty-one major pathogens were investigated (those with asterisks were identified as a foodborne pathogen since the early 1970s), including: Astrovirus*, *Bacillus cereus*, *Brucella* spp., *Campylobacter* spp.*, *Clostridium botulinum*, *C. perfringens*, *Cryptosporidium* spp.*, *Cyclospora cayetanesis**, enterotoxigenic *Escherichia coli* (ETEC)*, Shiga toxin-producing *E. coli* (STEC)*, *Giardia intestinalis*, Hepatitis A virus, *Listeria monocytogenes**, *Mycobacterium bovis*, Norovirus*, Rotavirus*, nontyphoidal *Salmonella* spp., *Salmonella enterica* serotype Typhi, Sapovirus, *Shigella* spp., *Staphylococcus aureus*, *Streptococcus* spp. group A, *Toxoplasma gondii*, *Trichinella* spp., *Vibrio cholera**, *V. vulnificus**, *V. parahemolyticus**, other *Vibrio* spp.*, and *Yersinia enterocolitica** (Scallan *et al.*, 2011)(Tauxe, 2002).

By comparing data for organisms which were reported to different networks which utilize different surveillance methods (passive or active), Scallan *et al.*, (2011) were able to better estimate the effects of underreporting and underdiagnosis leading to the creation of pathogen-specific multipliers and uncertainty distributions. Utilizing data collected from 2000 to 2008, Scallan *et al.* (2011) estimate an annual incidence of foodborne illness of 9.4 million cases with a 90% confidence interval spanning from 6.6 to 12.7 million cases. These illnesses are distributed by pathogen type: 5.5 million (59%) caused by viruses, 3.6 million (39%) attributed to bacteria, and 0.2 million (2%) parasitic infections (Scallan *et al.*,2011).

The severity of foodborne illness extends beyond mild malaise. Scallan *et al.* (2011) also investigated the frequency of hospitalizations and death due to consumption of contaminated food in the United States. Of the estimated 55,961 (90% CI 39,534-75,741) hospitalizations, 64% were associated with bacteria, 27% with viruses, and 9% with parasites (Scallan *et al.*, 2011).

Mortality was estimated at 1,351 (90% CI 712-2,268) with 64% caused by bacteria, 25% by parasites, and 12% by viruses (Scallan *et al.*, 2011). Nontyphoidal *Salmonella* spp. were the most common causative agents, resulting in 35% of hospitalizations and 28% of deaths (Scallan *et al.*, 2011). Recent FoodNet data suggest that the 2014 incidence of laboratory-confirmed nontyphoidal *Salmonella* spp. infections remains relatively unchanged at approximately 15.45 cases per 100,000 (Gao *et al.*, 2011)(MMWR, 2015). While these estimates may seem like a relatively small proportion of the national population, it is important to remember that these numbers represent people with families who suffered from potentially preventable illnesses.

The true cost of foodborne illness exceeds monetary expenses for medical care and lost productivity. After the initial onset of disease, some pathogens have the potential to induce chronic sequelae. Quality-adjusted life years (QALYs) are one method for evaluation of the burden of qualitative disease outcomes, such as ability to perform normal daily activities and live a comfortable life (Hoffmann *et al.*, 2012). By combining quantitative and qualitative measures of loss, a more complete picture is formed which can be used to more effectively compare the impacts of pathogens and direct food safety policy decisions (Hoffmann *et al.*, 2012). Hoffmann, Batz, and Morris (2012) evaluated annual cost of illness and QALYs lost for fourteen foodborne pathogens which account for 95% of illnesses and hospitalizations and 98% of deaths identified by Scallan *et al.* in 2011. Hoffman *et al.* (2012) found the cost of foodborne illnesses in terms of 2009 U.S. dollars to be about \$14 billion (with an uncertainty ranging from \$4.4 billion to \$33.0 billion) and a total loss of 61,000 QALY (with an uncertainty ranging from 19,000 to 145,000 QALYs). Roughly 90% of these losses were caused by five pathogens, including over \$3.3 billion and 17,000 QALYs lost due to non-typhoidal *Salmonella* spp. infections (Hoffman *et al.*, 2012).

Salmonella enterica

Salmonella spp. have long been identified as a major threat to food safety and public health. Despite years of intense study, *Salmonella* spp. remain a complicated subject. Underneath the family Enterobacteriaceae, the genus *Salmonella* is presently divided into two species: *S. enterica* and *S. bongori* (Cooke *et al.*, 2007). Of the seven subspecies, warm-blooded animals are susceptible to *S. enterica* subspecies *enterica* (Cooke *et al.*, 2007). In total, approximately 2,600 serovars have been identified thus far per the “presence or absence of capsular antigens, flagellar antigens, envelope antigens, or reactions to specific antisera” (Gray & Fedorka-Cray, 2002), 2002)(Cooke *et al.*, 2007). For simplicity, strains are usually referred to as genus *Salmonella* followed by the name of the serovar.

Salmonella spp. are not fastidious and give the appearance of peritrichiously flagellated, Gram-negative rods (Gray & Fedorka-Cray, 2002). Salmonellae have the ability to utilize either citrate or D-glucose as sole sources of carbon (Guthrie, 1992). Growth of *Salmonella* spp. is inhibited at a_w below 0.94, pH less than 3.8 or greater than 9.0, temperature less than 7°C or exceeding 45°C (Gray & Fedorka-Cray, 2002)(Guthrie, 1992). Although they do not form spores or microcysts, Salmonellae are known for their tolerance to salt and desiccation (Gray & Fedorka-Cray, 2002)(Guthrie, 1992). Due to their participation in bacterial conjugation, transfer of plasmids, which encode antibiotic resistance, between strains is of great concern (Guthrie, 1992). As a facultative anaerobes, they are well equipped to survive in the gut (Guthrie, 1992).

Transmission of *Salmonella* spp. commonly occurs through fecal contamination of food or water (Gurthrie, 1992). Consumption of *S. Typhi* or *S. Paratyphi* A, B, or C results in severe illness, termed enteric fever, which should be treated with appropriate antibiotics as it is a systematic infection (Cooke *et al.*, 2007)(Gray & Fedorka-Cray, 2002). Consumption of non-

typhoidal *Samonella* serovars typically results in self-limiting gastroenteritis, or salmonellosis, which should be treated with supportive therapy to avoid promotion of a prolonged carrier state (Gray & Fedorka-Cray, 2002)(Guthrie, 1992). Symptoms of salmonellosis develop within 12-72 h and include headache, stomach cramps, nausea, vomiting, and/or diarrhea (which may or may not be bloody); these symptoms usually dissipate after 1-4 days (Guthrie, 1992)(Raffatellu *et al.*, 2007). Behravesh *et al.* (2011) analyzed data collected by FoodNet spanning from 1996 to 2005; they analyzed 121,536 reports of laboratory-confirmed bacterial infections and found 215 (39%) of these cases were the result of salmonellosis. While most infections caused by non-typhoidal *Salmonella* spp. are relatively mild, some cases of salmonellosis may result in bacteremia or focal infections, which require medical interventions (Guthrie, 1992).

Invasion

Intracellular survival and proliferation is essential for infection and dissemination to other hosts (Boumart *et al.*, 2014)(Raffatellu *et al.*, 2007). Among *Salmonella* spp., the Trigger mechanism of invasion, utilizing type 3 secretion system 1 (T3SS-1) encoded by *Salmonella* pathogenicity island 1 (SPI-1), has long been associated with invasion of M-cells in the distal ileum (Boumart *et al.*, 2014)(Morgan, 2007)(Raffatellu *et al.*, 2007). However, Boumart, Velge and Wiedemann (2014) identify *Salmonella* spp. as the first to express the ability to utilize both the Trigger mechanism and the Zipper mechanism, which is moderated by Rck (an outer membrane invasin protein). Rck invasin is encoded by the *rck* gene which is found on the large virulence plasmid (Boumart *et al.*, 2014). The *rck* gene is most frequent among the host impartial *S. enterica* serovars Enteritidis and Typhimrium, and can also be identified in some strains of *S. Dublin* (Boumart *et al.*, 2014). Both of these mechanisms alter the structure of actin in the host

cell cytoskeleton and prompt the host cell to absorb bacterial cells (Boumart *et al.*, 2014)(Morgan, 2007)(Raffatellu *et al.*, 2007).

The majority of internalized *Salmonella* inhabit vacuoles (Boumart *et al.*, 2014)(Morgan, 2007)(Raffatellu *et al.*, 2007). After encapsulation in a *Salmonella*-containing vacuole (SCV), bacterial effector proteins encoded on T3SS-1 must be suppressed to prevent host cell recognition, inflammatory response, and lysosomal targeting (Boumart *et al.*, 2014)(Raffatellu *et al.*, 2007). The next stages of SCV maturation, including formation of *Salmonella*-induced filaments (SIFs) for nutrient uptake and promotion of bacterial replication, are controlled by upregulation of type 3 secretion system 2 (T3SS-2) effector proteins encoded on *Salmonella* pathogenicity island 2 (SPI-2) (Boumart *et al.*, 2014)(Morgan, 2007). Prior to release from the SCV, T3SS-1 dominance is reinstated to facilitate invasion into adjacent cells (Boumart *et al.*, 2014).

It is now recognized that *Salmonella* also replicate in the cytosol of epithelial cells (Boumart *et al.*, 2014). In approximately 20% of invasions, propagation of *Salmonella* in the intracellular fluid may exceed that of the SCV (Boumart *et al.*, 2014). Termed hyper-replication, this bimodal propagation state may yield production of greater than 100 bacteria per host cell (Boumart *et al.*, 2014). Death of these highly colonized cells results in release of virulent *Salmonellae* into the lumen of the gut and perpetuation of infection into deeper tissues (Boumart *et al.*, 2014)(Gray & Fedorka-Cray, 2002)(Raffatellu *et al.*, 2007). After gaining access to the lamina propria, *Salmonellae* may colonize macrophages, thereby avoiding immune response (Gray & Fedorka-Cray, 2002). Access to the lymph system aids in further dissemination throughout the host (Gray & Fedorka-Cray, 2002). Cells may travel to the liver and spleen where

proliferation continues, inducing systemic or focal infections, or they may be sequestered in the gall bladder, resulting in an asymptomatic carrier state (Gray & Fedorka-Cray, 2002).

***Enterococcus faecium* ATCC 8459**

Enterococcus faecium belongs to the lactic acid bacteria (LAB) group; they are ubiquitous, being commonly found in animal gastrointestinal tracts, food products of animal origin, such as fermented sausages and cheeses, and soil or plant material (Giraffa, 2014)(Tan *et al.*, 2013). They are identified as “Gram-positive, oxidase-negative, catalase-negative, non-spore-forming cocci that occur singly, in pairs, or in short chains” (Giraffa, 2014). As LAB, *E. faecium* are facultative anaerobes and convert carbohydrates to lactic acid (Giraffa, 2014). *Enterococci* are well known for their hardiness; cells will continue growth in the range of 5°C to 50°C, from a pH of 4.6-9.9, in up to 6.5% NaCl, or up to 40% bile salts (Giraffa, 2014).

Tolerance to inhospitable environments unfortunately extends to antimicrobial resistances among many enterococci (Giraffa, 2014)(Kopit *et al.*, 2014)(Kornacki, 2012)(Tan *et al.*, 2013). The possibility of the spread of antibiotic resistance from enterococci to other genera via bacterial conjugation serves as the foundation for debate concerning the continued use of *E. faecium* as a processing aid (Kornacki, 2012)(Kopit *et al.*, 2014). Incidence of nosocomial infections caused by *E. faecalis* (80%) and *E. faecium* (20%) has been increasing over the past 30 years (Kopit *et al.*, 2014)(Kornacki, 2012). To quantify the perceived threat, Kopit *et al.* (2014) sequenced the genome of *E. faecium* NRRL B-2354, *E. faecium* ATCC 8459, and clinical strains TX0082 and 1,231,502. Surrogate *E. faecium* strains lacked, or contained nonfunctional copies of, virulence genes, and displayed reduced ability to form biofilms (Kopit *et al.*, 2014). Surrogate strains were also more sensitive to antibiotics, displaying sensitivity to vancomycin, streptomycin, gentamicin, ampicillin, penicillin, cephalosporins (cefoxitin and cefazolin),

chloramphenicol, tetracycline, polymyxin b, and novobiocin (Kopit *et al.*, 2014)(Kornacki, 2012). To alleviate concerns regarding acute toxicity, Tan *et al.* (2013) orally administered 10^{11} CFU *E. faecium* YF5, a potential probiotic isolated from sourdough, to mice for 8 days. No changes were observed in the animals' activity, behavior, coat quality, or biopsied ceca from controls (Tan *et al.*, 2013). These data support the continued use of *E. faecium* ATCC 8459 and NRRL B-2354 as processing aids.

Saccharomyces cerevisiae

Saccharomyces cerevisiae is a unicellular yeast; its dimensions range from 1 to 16 μm long by 1 to 8 μm wide (Pyler and Gorton, 2008). Yeast utilize alcohol fermentation, converting carbohydrates to carbon dioxide and ethanol, given the presence of nitrogen, sulfur, minerals, and nutrients (Pyler and Gorton, 2008). *S. cerevisiae* cells usually exist in haploid or diploid state, but may enter polyploidy states as well (Pyler and Gorton, 2008). In the presence of sufficient nutrients, any of the ploidy states may undergo asexual vegetative growth, termed budding, and form strands of pseudohyphae (Dickinson, 2004)(Pyler and Gorton, 2008). This is desirable for the formation of pure cultures demonstrating useful fermentative characteristics for baking (Pyler and Gorton, 2008).

Yeast cells also have the ability to utilize sexual reproduction (Dickinson, 2004)(Pyler and Gorton, 2008). Meiosis yields four haploid spores, which are contained in a saclike structure called an ascus (Dickinson, 2004)(Pyler and Gorton, 2008). The cells contained in an ascus demonstrate the greatest level of stress tolerance over stationary phase yeast cells which in turn exceed log phase yeast cells (Dickinson, 2004)(Pyler and Gorton, 2008). Cellular damage following exposure to harsh environmental conditions may impede the ability of yeasts to reproduce regardless of the method of replication; exposure to high temperatures may disrupt

cellular membranes, degrade ribosomes, or deteriorate DNA resulting in loss of viability and cellular death (Dawes, 2004).

***Salmonella* spp. Contamination of Flour Products**

As pathogens become better understood, it is important to reevaluate standard food industry practices to ensure the threat is adequately controlled. In the event silo capacity is exceeded, unprocessed wheat may be stored at farm-level silos or on ground pads, which may become contaminated by birds, rodents, or other pest species (Eglezos, 2010)(Sperber *et al.*, 2007). Nonetheless, Berghofer *et al.* (2003) cite flour as “the cleanest end product of the milling process” as the majority of exterior grain kernel contaminants remain attached to the outer layers, such as the bran, germ, and pollard (Berghofer *et al.*, 2003). Despite the removal of the exterior of the kernels, flour should not be classified as a ready-to-eat (RTE) food product. The low water activity (a_w) of wheat during storage (a_w 0.40-0.65) and processing (a_w 0.68-0.70) inhibits the proliferation of pathogens, but does not guarantee their elimination, even after extended periods of storage for up to one year (Berghofer *et al.*, 2003)(Eglozos, 2010) (McCallum *et al.*, 2013). Additionally, moisture may condense in milling equipment along with accumulated flour residues (Berghofer *et al.*, 2003)(Eglozos, 2010). Improperly cleaned facilities and equipment or aerosols generated when processing incoming grain may contaminate outbound flour (Berghofer *et al.*, 2003).

Production of vast quantities of flour, being a non-homogenous solid product, leads to impracticality in obtaining a representative sample for laboratory analyses. Simply put, flour is prone to sporadic pockets of contaminants instead of uniformly detectable contamination, and it is not realistic to expect regular microbial sampling to ensure food safety (Speber *et al.*, 2007). Nevertheless, a few attempts have been made to quantify the incidence of flour contamination. A

survey of nine Australian flour mills across two harvest seasons found the incidence of *Salmonella* spp. contamination of wheat flour to be less than 0.5% in 412 samples (Berghofer *et al.*, 2003). Another Australian study, conducted during the 2006-2007 wheat season, estimates the prevalence of *Salmonella* spp. in wheat flour to be less than 0.7% and a mean aerobic plate count (APC) of 4.2 log₁₀ CFU/g (Eglezos, 2010). Similarly, a study of U.S. wheat published by Richter *et al.* in 1993 found the mean APC to vary from 10³ to 10⁴ CFU/g, depending on the variety of wheat, and the prevalence of *Salmonella* spp. contamination to be 1.32%. This work was followed by a review of North American milled grain samples taken from 2003-2005 which suggested that APC have remained relatively unchanged while incidence of *Salmonella* spp. contamination dropped to 0.14% (n= 4,358) (Sperber *et al.*, 2007).

Despite the low incidence of flour contamination, an increasing number of recalls and outbreaks have been attributed to *Salmonella* spp. in flour. The most recent recall of a flour product occurred on April 2, 2015. Navajo Pride (Farmington, NM) recalled 5-, 25-, and 50-pound bags of bleached, all-purpose flour after routine screening of a 5-pound sample that tested positive for *Salmonella* spp. (FDA, 2015). A few months prior, on November 14, 2014, another positive routine test lead Lundberg Family Farms to recall 25-pound bags of brown rice flour (FDA, 2014). The affected lots were marketed in retail store bulk bins (FDA, 2014). Nearly 400 tons of soybean flour, distributed over a 10-month period, were recalled on October 4, 2011 by Thumb Oilseed Producer's Cooperative for possible *Salmonella* spp. contamination (FDA, 2011). None of these recalls have been associated with human illness.

Looking into the more distant past, however, flour was identified as the most likely source of *Salmonella* Paratyphi B phage type 1 which caused an outbreak in Australia in 1952 (Berghofer *et al.*, 2003)(Eglezos, 2010). *S. Typhimurum* phage type 42 (STM42) was implicated

in a 2008 salmonellosis outbreak in New Zealand (Eglezos, 2010)(McCallum *et al.*, 2013). STM42 was successfully isolated from unopened bags of flour at estimated concentrations of one cell per 50 g to one cell per 300 g (McCallum *et al.*, 2013). The event resulted in at least 67 illnesses and 12 hospitalizations (McCallum *et al.*, 2013). In the resulting investigation, cases were 12.5 times more likely to have consumed raw cake or pancake batter than controls (McCallum *et al.*, 2013).

In 2000, a multistate outbreak of *S. Thompson* was traced to commercially distributed hamburger buns (Kimura *et al.*, 2005). At least 55 cases of salmonellosis were identified in relation to the event; nine people were hospitalized (Kimura *et al.*, 2005). In order to maintain virulence, a sufficient quantity of cells must survive passage through the stomach (Eglezos, 2010)(Kimura *et al.*, 2005). An increasing fat content seems to exert a sparing effect on bacterial cells, resulting in a diminished minimum infectious dose (Eglezos, 2010)(Kimura *et al.*, 2005). In the case of a hamburger bun saturated with grease, the infectious dose of *Salmonella* spp. may drop from 1,000 cells to 100 cells or less (Eglezos, 2010)(Kimura *et al.*, 2005). In this event, it was speculated that the buns were likely contaminated during slicing or packaging by an ill bakery worker, but, as the other symptomatic bakery worker predominantly mixed dough, it is important to verify that current industry standard baking parameters are providing adequate safety to consumers (Kimura *et al.*, 2005).

Variation in the thermal resistance among *Salmonella* serovars is well documented (Ng *et al.*, 1969). While *S. Senftenberg* is generally regarded as the most heat tolerant serovar, the ordering of thermal resistance of *Salmonella* strains has been shown to be dependent on the food matrix being evaluated (Ng *et al.*, 1969). Prior exposure to less hospitable environments,

including desiccation and reduced pH, may lead to increased resistance to thermal treatments (Beney *et al.*, 2003)(Gruzdev *et al.*, 2011).

Chapter 3 - Objectives

The objectives of this study were centered on providing the baking industry with base-line, quantifiable data for hamburger bun process validation and justification of baking parameters in regard to HARPC compliance.

The first phase of this research was to evaluate the potential survival of target microorganisms in the late phases of baking. In the event of a process control failure, it is important to have an understanding of the relative threat of pathogen survival. The primary source of biological hazards in hamburger bun manufacturing was determined to be contaminated raw ingredients. As such, goals for the baking study were: 1) Ensure a typical baking process for hamburger buns reduces the threat posed by *Salmonella* spp. contamination in raw ingredients to an acceptable level; 2) Evaluate the potential use of *Enterococcus faecium* ATCC 8459 as a surrogate for *Salmonella* spp. during in-plant process validations; and 3) Evaluate the potential use of *Saccharomyces cerevisiae* (Fleischmann's Compressed Yeast) as a potential indicator for *Salmonella* spp. survival during baking.

The second phase of this project was to quantify parameters necessary for thermal inactivation (D- and z-values) of *Salmonella* spp, *E. faecium* ATCC 8459, and *S. cerevisiae* (Fleischmann's Compressed Yeast) in hamburger bun dough. This allows direct comparison of the relative thermal tolerance between the three target species and provides definitive justification supporting the use of surrogate indicator organisms. Additionally, calculation of D- and z-values in hamburger bun dough allows processors to determine equivalent time-temperature lethality for similar hamburger bun processes.

Chapter 4 - Validation of Baking to Control *Salmonella* Serovars in Hamburger Bun Manufacturing, and Evaluation of *Enterococcus faecium* ATCC 8459 and *Saccharomyces cerevisiae* as Nonpathogenic Surrogates for Thermal Process Validation

Introduction

Approximately 2,800 commercial bakeries and 6,000 retail bakeries operate in the United States, with a market value of nearly \$30 billion per year. Baked breads account for ~40% of commercial bakery sales followed by rolls, buns, muffins and bagels (~20% of sales) (Bakery and Snacks, 2012). The Food Safety Modernization Act (FSMA) mandates that the food industry focus on establishing preventative controls to proactively reduce food safety hazards. Title 1 (Sec. 103) of FSMA calls for food processing facilities to conduct and document product or process-specific hazard analyses and institute risk-based preventative controls to ensure product safety (FSMA, 2011). In the baking industry, a wide array of items are manufactured, each with unique characteristics, processing parameters and compositional components. Scientific evaluation of associated pathogen risk factors and adequacy of processing steps to mitigate these risks is thus a necessity.

Although not directly linked to improper production practices, there were 4,200 illnesses associated with bakery products reported in the U.S. between 1998 and 2011, with bread products linked to 30 of these outbreaks and 706 illnesses (CSPI, 2009). *Salmonella* spp. are commonly associated with foodborne illnesses attributed to low-water activity foods and ingredients, such as milk powders, powdered infant formula, dry seasonings and flour (Akins, 2014). *Salmonella* spp. can be introduced into bakery products prior to thermal processing through ingredients such as eggs (Board, 1969)(FSIS, 2008), milk products (El-Gazzar & Marth,

1992)(Ahmed *et al.*, 2000), flour (Dack, 1961)(Richter *et al.*, 1993), milk chocolate (D'Aoust, 1977), coconut (Goepfert, 1980), peanut butter (Scheil *et al.*, 1998), fruits (Golden *et al.*, 1993), spices (Hara- Kudo *et al.*, 2006), and yeast flavorings (Joseph *et al.*, 1991). Moreover, *Salmonella* cells that have survived desiccation (i.e. in stored dry ingredients) have been shown to exhibit greater thermal resistance during processing (Gruzdev *et al.*, 2011). The *Salmonella* infective dose has been estimated to be less than a thousand cells for many strains (Blaser & Newman, 1982), and can be as low as one cell (FDA BBB, 2012), depending on age, health of the host, and serovar/strain differences among members of the same genus. *Salmonella* contamination levels as low as 0.04-0.05 CFU/g of food have been linked to outbreaks (Lehmacher *et al.*, 1995).

Historically, *S. Senftenberg* 775W has been reported to be a notable heat resistant serovar, particularly in high-moisture foods (Ng *et al.*, 1969). According to the U.S. Centers for Disease Control and Prevention, *S. Typhimurium* has been the most prevalent pathogenic serovar since 1997, and *S. Newport* has been reported to be the third most common pathogenic serovar associated with foodborne outbreaks (CDC, 2013). Although *Salmonella* spp. cannot grow in foods or ingredients with a water activity <0.93 (such as in flour), it can survive for months and grow when favorable conditions become available, such as rehydration of flour (Eglezos, 2010). Between October 2008 and January 2009, 67 *S. Typhimurium* Phage Type 42 cases, including 12 hospitalizations, were reported in New Zealand and were traced to consumption of a contaminated uncooked baking mixture containing flour (McCallum *et al.*, 2013).

In order to validate and/or verify in-plant food safety processes without risking facility contamination, surrogates for specific pathogens are often identified and characterized through laboratory studies. Surrogates should be non-pathogenic and demonstrate similar growth and

survival characteristics to the specific pathogen of interest (Kornacki, 2012). *Enterococcus faecium* NRRL B-2354 [deposited at the American Type Culture Collection (Manassas, VA) as the Biosafety Level-1 *Micrococcus freudenreichii* Guillebeau ATCC 8459] has been used by the food industry for over 60 years for a variety of purposes, including use as a surrogate for pathogenic *Salmonella* spp. in thermal processing (Kornacki, 2012). Kopit *et al.* (2014) reported that *E. faecium* NRRL B-2354 has relatively high acidic and thermal resistances, and does not possess virulence or antibiotic resistance genes, supporting its use for food process thermal validation studies. *E. faecium* NRRL B-2354 has been characterized for its thermal destruction parameters and survival characteristics during dry and moist roasting of almonds, and is recommended by the Almond Board of California for in-plant validation of thermal processes for almonds to control *Salmonella* spp. (ABC, 2014)(Jeong *et al.*, 2011).

Although most bakery products undergo a putative kill step at the point of production, such as baking or cooking, these control points generally lack published scientific validation. Hence, an attempt is made to develop a kill-step validation protocol that suits the needs of U.S. bakery industries. We used hamburger bun manufacturing as the model to develop a scientific validation protocol for bakery products in general. We chose hamburger buns, as this is one of the most popular bakery products consumed in the U.S. and in Canada. Thermal inactivation data for *Salmonella* spp. in dough and buns/bread is scarce in the literature. Therefore, the objectives of this study were to validate a simulated commercial baking process for hamburger buns to control *Salmonella* spp. contamination introduced via raw ingredients, determine the appropriateness of using non-pathogenic surrogates (*E. faecium* ATCC 8459 or *Saccharomyces cerevisiae*) for in-plant process validation studies, and determine thermal inactivation parameters (D- and z-values) of *Salmonella* spp. and potential surrogates in hamburger bun dough.

Materials and Methods

Experimental design

For the hamburger bun baking study, flour was inoculated individually with three *Salmonella enterica* serovars, or *E. faecium*, and used to make dough. The dough was divided into dough pieces of proper weight, formed into a flat disk and placed in a pan. *S. cerevisiae* (Baker's yeast added as part of the standard dough formula) was similarly enumerated in non-inoculated baking trials. After proofing, the pan was put into an oven and baked at 218.3°C for 13 minutes. The equipment (Hobart A-200 stand mixer, McDuffee bowl, fork agitator, baking pan) was washed with detergent and wiped with 70% ethyl alcohol between the dough inoculated with different organisms to avoid cross contamination. Bun sampling times [9, 11 and 13 min of baking, and 13 min of baking followed by 30 min post-bake room temperature cooling (B+C)] were evaluated to determine target organism survival during the simulated commercial hamburger bun baking process. The 9 and 11 minutes baking times were included to represent the minimum baking process which still produced an acceptable product from a quality standpoint. Since the time-temperature criteria are most important for effective pathogen destruction, thermocouples were used to measure the internal temperature. This experiment was designed as a randomized complete block (replications being blocks) with seven treatments: flour, pre-proof dough, post-proof dough, 9 min bake, 11 min bake, 13 min bake, and B+C. Analysis of variance for the surviving target microbial populations (log₁₀ CFU/g) was conducted using SAS version 9.3 (SAS Institute, Cary, NC). Three independent replications were conducted for each target organism, and all microbial enumerations were done in duplicate.

For the D- and z-value study, flour inoculated with a 3-serovar cocktail of *Salmonella enterica*, or single strains of *E. faecium* and *S. cerevisiae*, was used to prepare dough. Dough was

placed in Whirl-Pak filter bags, heat sealed and heated in water baths set at 55, 58 and 61°C for *Salmonella* spp. and *E. faecium*, and at 52, 55 and 58°C for *S. cerevisiae*. A lower temperature was used for *S. cerevisiae* as a complete destruction of the inoculated population was observed during the come-up time for 61°C sampling, and D-value calculations were not possible. The D- and z-value study was designed as a randomized complete block, with replications as blocks. Three independent replications (as represented by new inoculum preparation, different lots of inoculated flour, and separate days of preparation/baking) were conducted and all microbial enumerations were done in duplicate. Linear regression graphs were plotted using Microsoft EXCEL, 2011, and the D- and z-values were calculated.

Inoculum preparation

Salmonella enterica serovars [Typhimurium (ATCC 14028), Newport (ATCC 6962) and Senftenberg (ATCC 4385)] and *Enterococcus faecium* ATCC 8459 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All bacterial cultures were propagated in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) and stored at -80°C on protectant beads in glycerol (Pro-Lab Diagnostics Microbank Bacterial Preservation System, Fisher Scientific). Working cultures were activated from frozen state by transferring one bead into 10 mL brain heart infusion broth (BHI; Oxoid Ltd., Basingstoke, Hampshire, England) and incubated for 24 h at 37°C. Individual cultures from BHI broth were then propagated as lawns on BHI agar plates for 24 h at 37°C (eight plates per organism). Lawns were harvested by washing each plate twice with 1 mL 0.1% peptone water (PW), using a disposable L-spreader to dislodge cells from the agar surface, and pipetting the resultant fluid into a 50 mL conical vial (providing ~16 mL of concentrated inoculum). When *Salmonella* spp. was used as a cocktail in

the D- and z-value study, all three serovars were mixed in equal quantities before the inoculation procedure.

Inoculation of flour

Flour (400 g) was evenly spread shallowly in a plastic tub (35.6 x 21.6 x 14.0 cm), which was placed within a large biohazard bag, and was mist inoculated inside a Class II Type A2 biosafety cabinet with the respective cultures individually at 1 mL inoculum per 100 g flour (4 mL total) to achieve a target of ~6 log CFU/g. Open tubs of inoculated flour were placed into a 37°C incubator and allowed to dry until the original flour weight was achieved. Inoculated flour was transferred into 1 gallon Ziploc bags, sealed and hand-mixed, and then stored at ambient temperature for 48 h prior to use. Final target bacterial concentrations of the flour were determined by direct plating immediately prior to dough preparation.

Dough preparation

All activities involving inoculated dough preparation and baking were conducted in a Biosafety Level-2 pilot food processing laboratory at Kansas State University using approved personnel safety protocols. The study utilized a “no-time” dough recipe, defined as one that minimizes fermentation time, representative of commercial hamburger bun manufacturing. Ingredients and the dough recipe provided by AIB International (Manhattan, KS) are presented in Table 4.1. *S. cerevisiae* yeast cakes were purchased from Fleischmann’s AB Mauri (Fleischmann’s Compressed Yeast; Chesterfield, MO). Dough ingredients were added to a 20 qt. McDuffee bowl, and the bowl and mixer were covered with a large plastic bag to control biological aerosols. Ingredients were mixed with a Hobart A-20 stand mixer with fork agitator attachment for 1 min on low and then 12 min on medium speed. After mixing, the inoculated

dough was allowed to rest in the bowl for 5 min to allow aerosols to settle before the plastic cover was removed.

Table 4.1 Dough recipe for hamburger buns

Ingredient	Quantity
Dry:	
Bread Flour, Bread	700 g
Sugar, Granulated	84 g
Salt	14 g
Sodium Stearoyl Lactylate	3.5 g
Calcium Propionate	1.8 g
Yeast Food, no oxidants	0.7 g
Shortening, all-purpose	42 g
Yeast, compressed	17 g
Liquid:	
Ascorbic Acid Solution (1.6%)	1.5 mL
Water	478.5 mL

The dough was transferred onto a flour-dusted (non-inoculated) stainless steel table, and samples were obtained for the pre-proof target culture enumeration. The dough was hand rolled into balls ($71 \text{ g} \pm 0.5 \text{ g}$) and allowed to rest for 10 min at room temperature. Dough balls were then molded into compartments of a standard greased bun pan (eight buns per pan). Pans with dough were placed into a proofing cabinet at 81% relative humidity and 43.3°C for approximately 60 min, or until the dough rose to a height of 32 mm above the top of the pan.

Upon achieving this height, the post-proof dough was sampled for enumeration of target microbial populations and then remaining buns were baked.

Oven selection and optimization to mimic industry standard

The wholesale baking industry typically uses ovens that utilize a combination of heat transfer mechanisms (radiant, convection, and conduction) to bake hamburger buns. These ovens are well suited for large-scale production, as they accommodate up to several hundred full-sized pans at one time, with the large batch size leveling out the transfer of heat (232.2°C for 10 minutes being a common industry practice). In this investigation, an electric oven (Model: ACR3130BAW0, Amana, Whirlpool Corp., Benton Harbor, MI) was chosen to bake hamburger buns. Industry standard steel hamburger bun pans (inner dimensions 4” top diameter, 3½” bottom diameter, 5/8” depth) were cut to fit inside the electric oven. Running a single modified pan in this type of system would produce an over-baked bun in minimal processing time. A series of hamburger bun baking trials were carried out at different temperature and time periods to determine the optimum hamburger bun bake profile simulating the common baking industry practice. The buns baked at 218.3°C (425°F) for 13 minutes were found to be optimum; matching the baking industry’s hamburger bun end use quality parameters such as crust color, appearance, size, texture and internal temperature.

Hamburger bun baking

The temperature of the empty conventional oven was set at 218.3°C and confirmed using an 8-channel data logging system (Measurement Computing USB-TC with MCC DAQ Software, Norton, MA) and type T thermocouples (Fine Gauge Thermocouples, Omega Engineering Inc., Stamford, CT). One pan containing eight buns was used for each baking treatment replication for each target organism. Two of the eight numbered bun positions on the tray were randomly

assigned to each of the four sampling times (9, 11 and 13 min of baking, and B+C). Before placement of the pan into the oven, thermocouples were inserted into the geometric center of the two buns that were randomly designated to receive the B+C treatment, and one thermocouple was affixed to the side of the pan to monitor oven air temperature, with readings logged at 1s intervals over the defined baking periods. At each bake time, two buns were removed from the oven and analyzed as duplicate samples to determine surviving target organism population. One of the two buns allowed for B+C treatment was used to measure post-bake bun pH and water activity (a_w), and the other was analyzed for surviving target bacterial population. At each sampling time, the oven door was opened and the two buns were quickly removed using sanitized tongs, placed into stomacher bags containing pre-chilled (4°C) PW, and hand-massaged to minimize further thermal lethality of the treatment.

In a follow-up study to establish the time required at 218.3°C for pathogen lethality in hamburger buns, dough was inoculated, prepared, and baked as previously described except sampling times were shifted forward and more frequent. Baking treatments were evaluated at the following intervals: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 min for *S. Newport*; 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 min for *S. Typhimurium* and *S. Senftenberg*; 7.00, 7.75, 8.50, 9.25, 10.00, 10.75, 11.50, and 12.25 min for *E. faecium*.

Microbiological analyses

Duplicate 10-g samples of inoculated flour, pre-proof dough (10 g), post-proof dough (71 g), and baked whole buns (71 g) in PW were homogenized for 1 min in a lab blender (AES CHEMUNEX Smasher™, bioMérieux Inc., Hazelwood, MO). Serial dilutions of each sample were spread plated on selective and injury-recovery media. For selective plating, *Salmonella* serovars were enumerated on xylose lysine desoxycholate agar (XLD; Difco, Becton Dickinson)

incubated at 37°C for 24 h, *E. faecium* was enumerated on m-*Enterococcus* agar (m-EA; Difco, Becton Dickinson) incubated at 37°C for 48 h, and *S. cerevisiae* was enumerated on potato dextrose agar (PDA; Oxoid Ltd.) supplemented with 100 ppm of chloramphenicol (Oxoid Ltd.) incubated at 35°C for 48 h. To quantify sublethally injured *Salmonella* spp. and *E. faecium* populations, dilutions were also plated on non-selective BHI agar incubated for 6 h, overlaid with 10 mL of respective selective agar (at 50°C), and incubated for an additional 24 or 48 h for *Salmonella* spp. or *E. faecium*, respectively. Bags containing all homogenized samples were stored at 4°C until results were obtained from direct plating. In cases where no viable *Salmonella* or *E. faecium* were detected by direct plating, 25 mL of stored sample homogenate were transferred into 225 mL of BHI broth and incubated for 24 h at 37°C, followed by streaking the enriched broth sample onto an appropriate selective agar for qualitative detection of surviving populations below the detection limit (0.5 log CFU/g).

D- and z-values determinations

Flour was inoculated with the 3-serovar *Salmonella* spp. cocktail or *E. faecium* ATCC 8459 to achieve a target level of ~6 log CFU/g, and dough was prepared as described previously. Polyethylene Whirl-Pak filter bags (Catalog No.: 01-812-5, Fisher Scientific) were trimmed to 13 x 20.5 cm and edges were sealed using a FoodSaver® Vacuum Sealing System (Sunbeam Products, Boca Raton, FL). Small lead fishing line weights were attached to the bottom edge of each sample bag in order to submerge samples in the hot water bath and ensure adequate water circulation space around all bags. After proofing, 25 g of inoculated dough was transferred into these prepared bags, the dough inside the bag was pressed to a uniform thickness of ~0.5 cm, and the bags were vacuum-sealed using the FoodSaver® Vacuum Sealing System. The aforementioned data logging system was used to monitor temperatures of the hot water baths and

the dough (measured by inserting probes in the center of sealed dough samples dedicated to temperature monitoring only). Once target time-temperature parameters were achieved in the dough, a sample bag was quickly removed from the water bath and submerged in ice water for rapid cooling. Sampling time intervals at 55, 58 and 61°C were 25, 6, and 2 min, respectively, for *Salmonella* spp., and 85, 23, and 10 min, respectively, for *E. faecium*. Sampling time intervals for *S. cerevisiae* at 52, 55 and 58°C were 10, 2.5 and 0.5 min, respectively.

Heat-treated dough samples (25 g) were diluted with 75 mL of chilled PW, homogenized for 1 min using a lab blender, and serially diluted. For *Salmonella* spp. and *E. faecium* enumeration, dilutions were spread plated on selective agars (XLD or m-E, respectively) and injury-recovery agars (BHI overlaid with XLD or m-E, respectively, after 6 h incubation). For *S. cerevisiae* enumeration, dilutions were spread plated on PDA supplemented with 100 ppm of chloramphenicol. D- and z-values were calculated as described by Michael *et al.* (2014). D-values were calculated as absolute values of the inverse of the slopes of the regression lines of the log of viable bacterial cells versus time; whereas, z-values were calculated as absolute values of the inverse of slopes of the regression lines of the log of D-values versus temperatures.

Physical/chemical analyses of hamburger buns

For each replication, one bun from the B+C treatment was used to determine final product pH and a_w . For pH measurements, 15-g of the interior bun crumb was added to 100 mL deionized water, and the mixture was stirred continuously with a spatula until a stable pH reading (Oakton Instruments, Vernon Hills, IL) was obtained. The a_w of the crumb and crust of baked buns was determined separately using an AquaLab Series 4TEV water activity meter (Pullman, WA). Water activities were determined for pre- and post-proof dough, along with separate crumb and crust measurements during baking (2, 4, 6, 8, 10, 12, 14 min), and after

baking (14 min + 30 min cool time) buns. At each sampling point, within 10 sec of removal from the oven, the crumb and crust of buns were separated, placed in water activity cups and sealed with Parafilm M™ (American National Can, Chicago, IL) until analyzed (within 30 min).

Results and Discussion

Water activity and pH of hamburger buns

The mean pH of fully baked hamburger buns was 5.46 ± 0.04 . From 9 to 14 minutes of baking, the a_w of the crumb remained constant (0.971 ± 0.005) whereas the a_w of the crust of buns decreased from 0.965 to 0.728 over this baking period (Fig. 4.1). After buns were cooled for 30 min, however, the a_w of the crust increased to 0.861 ± 0.026 . These a_w measurements of breadcrumb and crust were similar to those reported by Czuchajowska *et al.* (1989) for finished bread loaves.

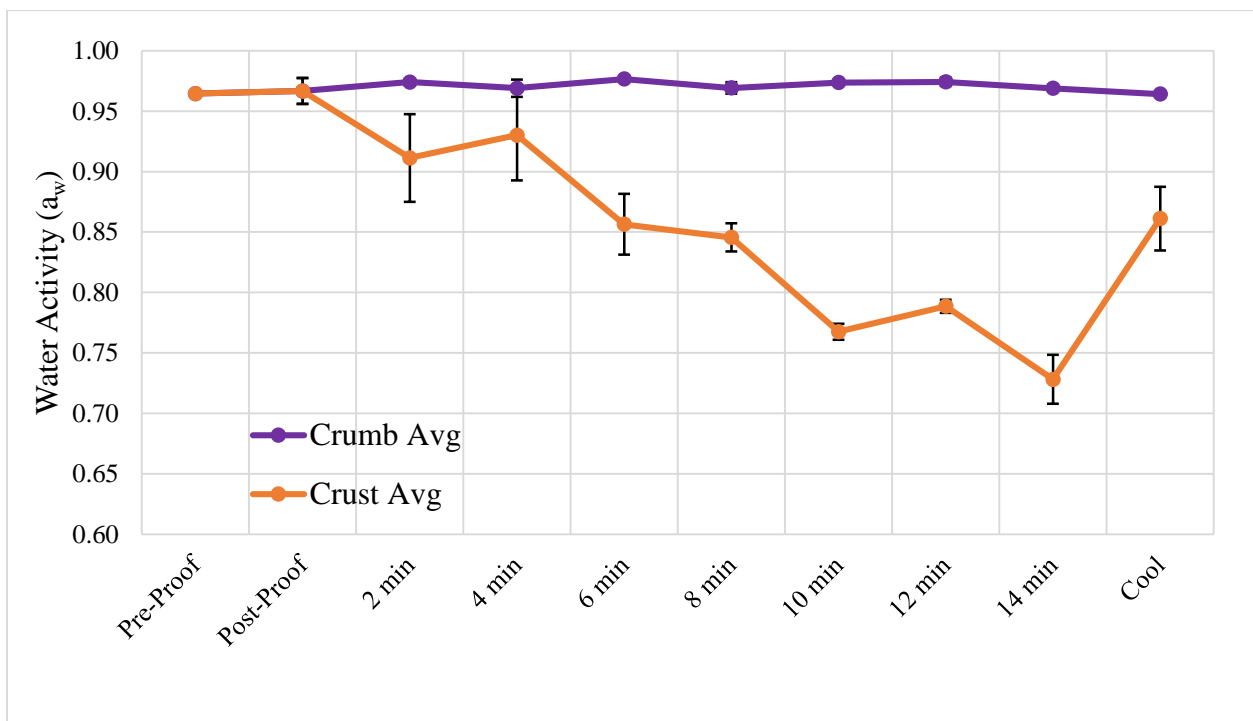


Figure 4.1 Water activity (a_w) of hamburger buns during the baking process

The 0.97 a_w of the crumb paired with the observed 5.46 pH suggests the opportunity for growth of *Salmonella* spp. inside the buns during storage at ambient temperature as *Salmonella* spp. may continue to grow at a_w as low as 0.94 (Smith *et al.*, 2004). This suggests that adequate thermal treatment during baking of buns is vital to eliminate the potential food safety risk of *Salmonella* spp. contamination in raw bun ingredients.

Validation of the bun baking process

This study sought to verify that a standard hamburger bun baking process is capable of delivering a desired lethal effect to ensure destruction of pathogenic microorganisms that may be introduced via raw ingredients. The internal heating profile (average of 15 baking runs) of hamburger buns during 13 min of baking in a 218.3°C conventional oven, followed by 30 min of ambient temperature cooling, is shown in Fig. 4.2. Crumb temperatures increased to ~100°C during the first 8 min of baking, and remained at this temperature for the next 5 min while buns were in the oven and for ~1 min after removal from the oven. Internal bun temperatures decreased to 50°C during the first 9 min of ambient cooling. Population levels of *Salmonella* serovars, *E. faecium*, and *S. cerevisiae* during proofing, baking and cooling as enumerated on selective and injury-recovery/non-selective agars are presented in Figs. 4.3 and 4.4, respectively. Surviving *Salmonella* serovar populations were similar ($P > 0.05$) throughout the process at various sampling times, with no viable cells enumerated by direct plating on selective or injury-recovery media (detection limit of 0.22 log CFU/g) after the minimum 9 min of baking. All samples that tested negative by direct plating were also negative for all three *Salmonella* serovars after enrichment (indicating >6 log CFU/g reductions) for all three serovars tested. In a similar study, Lathrop *et al.* (2014) inoculated peanut butter cookie dough with a 5-serovar cocktail of

Salmonella spp. (Tennessee FSL-R8-5221, Tornow FSL-R8-5222, Hartford FSL-R8-5223, Typhimurium FSL-WI-030 and Agona FSL-S5-517) and baked at 177°C for up to 15 min. They reported that *Salmonella* spp. was detectable in the peanut butter cookies (after the enrichment) when baked for 14 min; however, *Salmonella* spp. was not detected in the cookies after 15 min of baking. The longer *Salmonella* spp. inactivation time in Lathrop *et al.* (2014) study compared to this study (9 min) could be because of the differences in the food matrices used in the respective studies (peanut butter cookies vs. dough; and differences in fat content and water activity values) or the result of reduced baking temperatures.

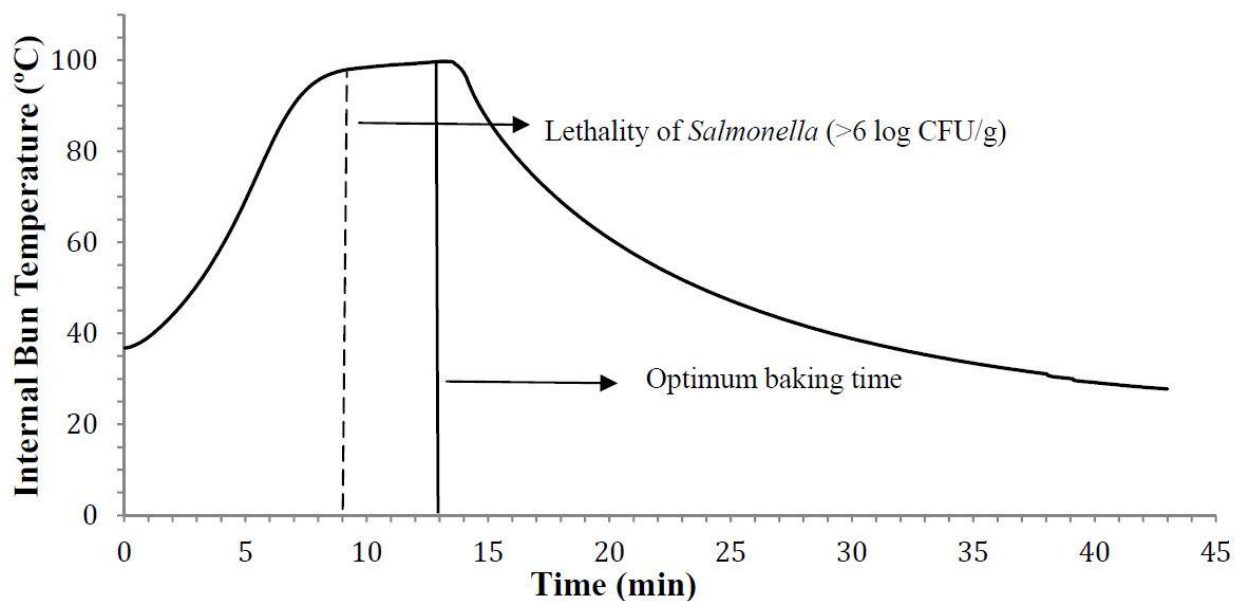


Figure 4.2 Mean internal temperature profile of hamburger buns during 13 min baking process (218°C) followed by 30 min of cooling

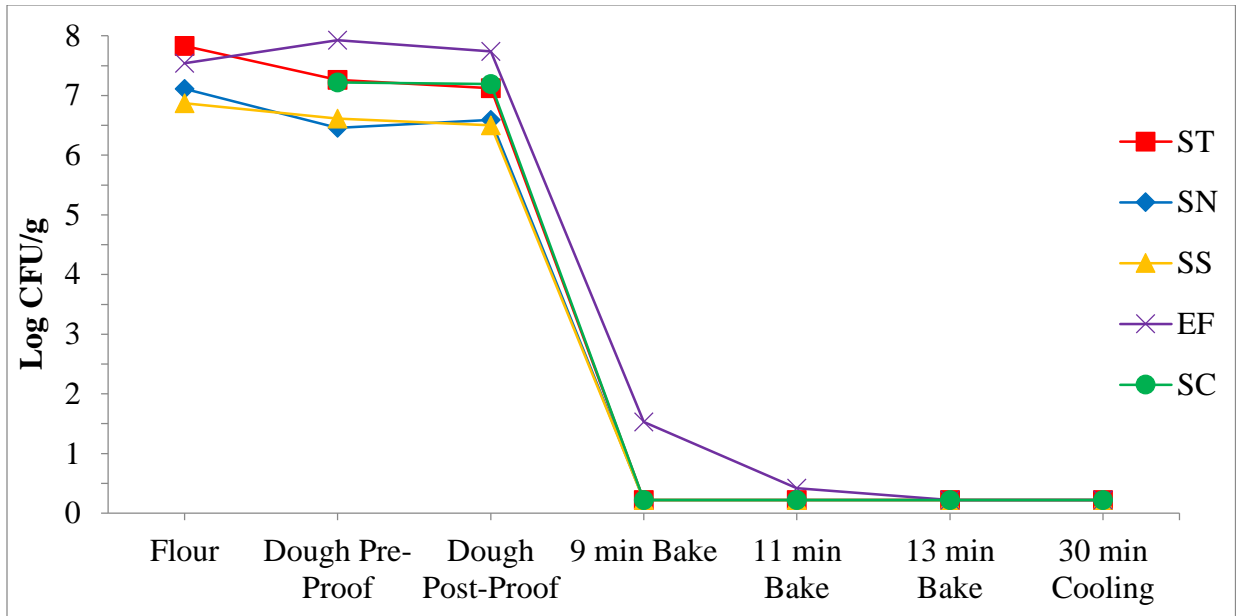


Figure 4.3 Survival of *Salmonella* serovars (*S. Typhimurium*, ST; *S. Newport*, SN; *S. Senftenberg*, SS), *Enterococcus faecium* (EF) and *Saccharomyces cerevisiae* (SC) in hamburger buns during baking at 218°C oven temperature; enumerated using selective media

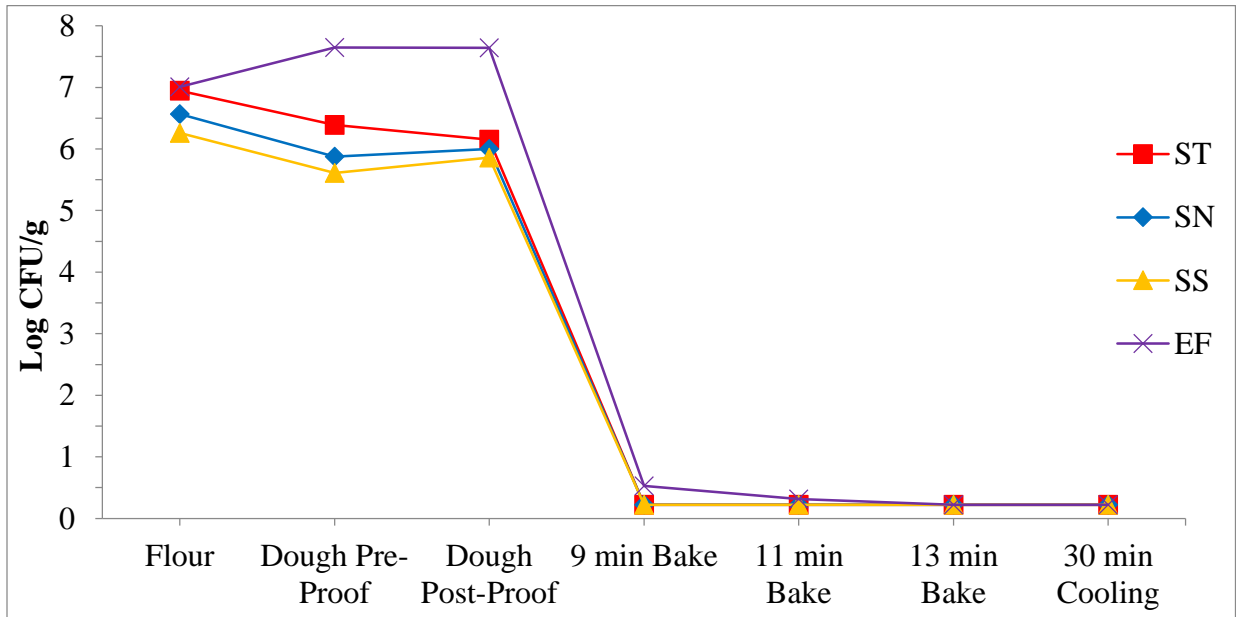


Figure 4.4 Survival of *Salmonella* serovars (*S. Typhimurium*, ST; *S. Newport*, SN; *S. Senftenberg*, SS), *Enterococcus faecium* (EF) and *Saccharomyces cerevisiae* (SC) in hamburger buns during baking at 218°C oven temperature; enumerated using injury-recovery media

Enterococcus faecium demonstrated greater thermal resistance compared to the *Salmonella* serovars. Although the survival of *Salmonella* serovars and *E. faecium* were similar ($P > 0.05$) until post-proofing, survival of *E. faecium* on both selective and injury-recovery media was observed up to 11 min of baking compared to no detectable *Salmonella* serovars at 9 min of baking. The *S. cerevisiae* population during the process was similar ($P > 0.05$) to that of *Salmonella* serovars (Fig. 4.3); however, this reflects similar population levels in pre-baked dough (~ 7 log CFU/g), and no viable organisms detected after 9 min of baking. It is likely that yeast were inactivated to populations below the detection level within a short baking time.

A follow-up study was conducted to determine the time required at these processing parameters to reduce selected microbial populations to the detection limit; mean data for three replications are summarized for selective and injury-recovery media in Figs. 4.5 and 4.6, respectively. The survival of *S. Newport* was no longer detected in samples collected after 7.0 min of baking on injury-recovery media. *S. Senftenberg* was reduced to a similar level by 8.0 min of baking as determined by direct plating on injury-recovery media. *S. Typhimurium* demonstrated intermediate recovery on injury-recovery media until 9.0 min of baking. In accordance with the initial data, *E. faecium* displayed the greatest thermal resistance and survived at detectable levels on injury-recovery media until 11.50 min of baking.

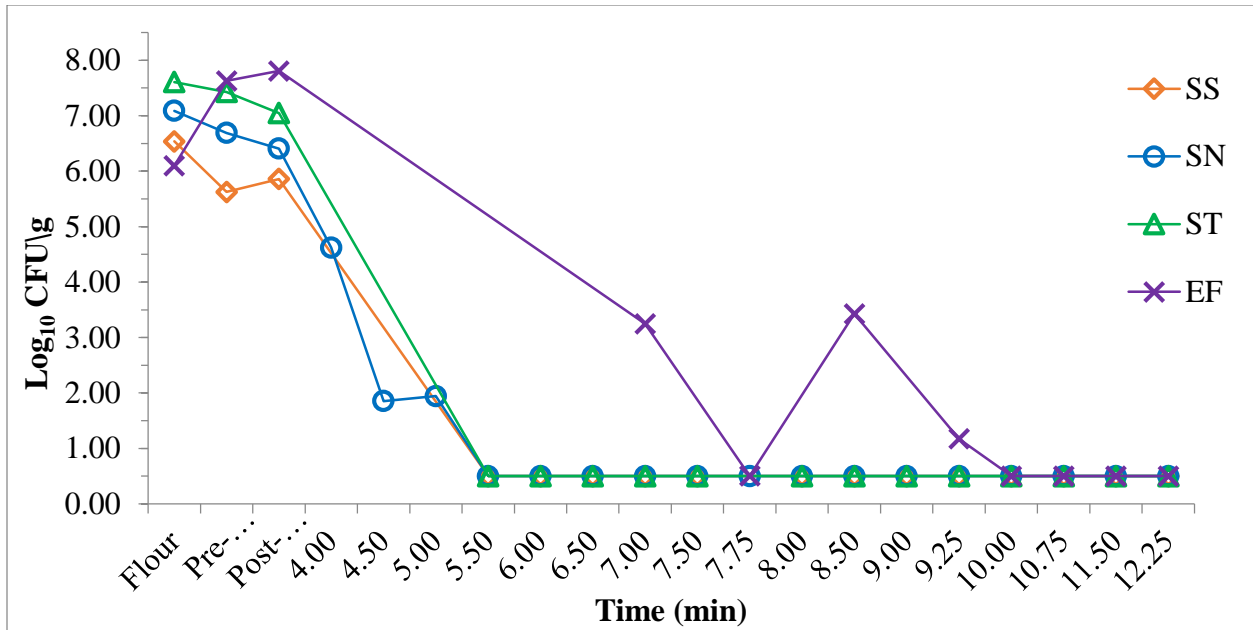


Figure 4.5 Thermal inactivation of *Salmonella* spp. and *E. faecium* in hamburger bun dough: viable cells enumerated on selective media

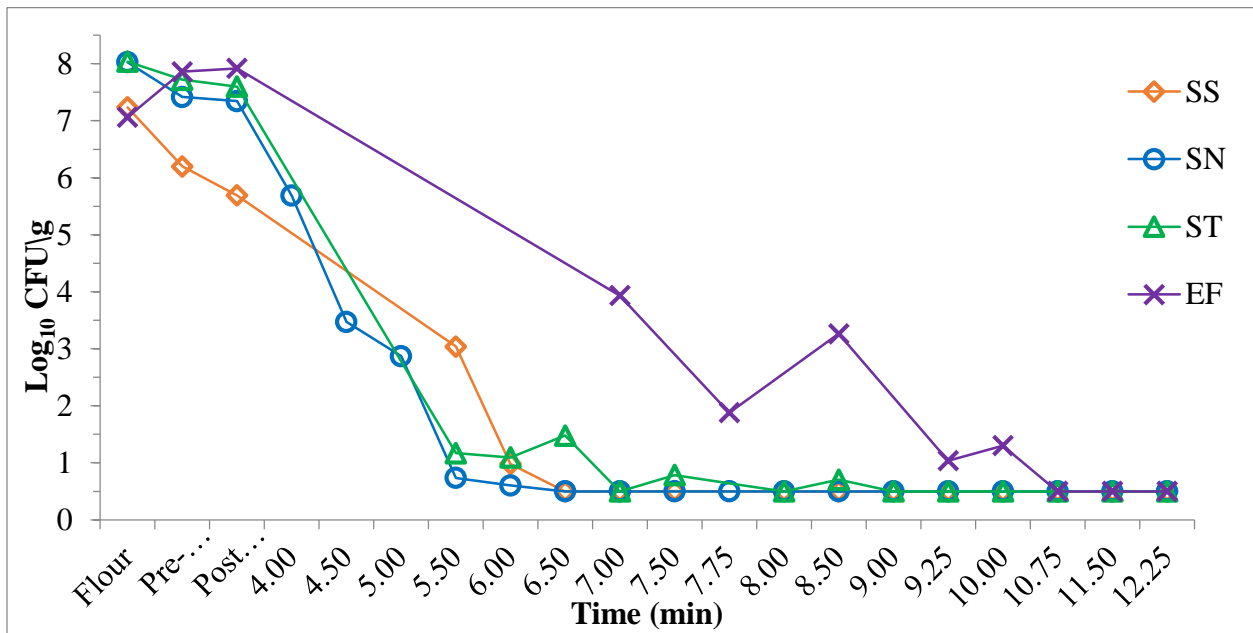


Figure 4.6 Thermal inactivation of *Salmonella* spp. and *E. faecium* in hamburger bun dough: viable cells enumerated on injury-recovery media

Determination of D- and z-values in bun dough

While *S. Senftenberg* is generally regarded as the most thermally resistant strain, a cocktail of relevant *Salmonella* spp. is often used to generate D- and z-values as it represents the range of contamination possibilities in the industry (Doyle & Mazzotta, 1999). Furthermore, some *Salmonella* spp. strains react to heat differently in different food matrices. For example, in most experiments with eggs, *S. Enteritidis* shows greater heat resistance than *S. Typhimurium*, while in molten milk chocolate *S. Typhimurium* had greater heat tolerance compared to *S. Senftenberg* (Doyle & Mazzotta, 1999).

Salmonella spp. populations during the thermal inactivation of 3-serovar cocktail on selective and injury-recovery media are presented in Figs. 4.7 and 4.8, respectively; whereas, *E. faecium* populations on selective and injury-recovery media are presented in Figs. 4.9 and 4.10, respectively. The *Salmonella* spp. $D_{55^{\circ}\text{C}}$, $D_{58^{\circ}\text{C}}$, and $D_{61^{\circ}\text{C}}$ values were 21.30 and 28.64, 7.53 and 7.61, and 2.29 and 3.14 min on selective and injury-recovery media, respectively; and $D_{55^{\circ}\text{C}}$, $D_{58^{\circ}\text{C}}$ and $D_{61^{\circ}\text{C}}$ were 87.21 and 133.33, 45.33 and 55.67, and 6.14 and 14.72 min for *E. faecium* on selective and injury-recovery media, respectively (Table 4.2). The calculated z-values of *Salmonella* spp. were 6.22 and 6.58°C, and that of *E. faecium* were 5.20 and 6.25°C on selective and injury recovery media, respectively.

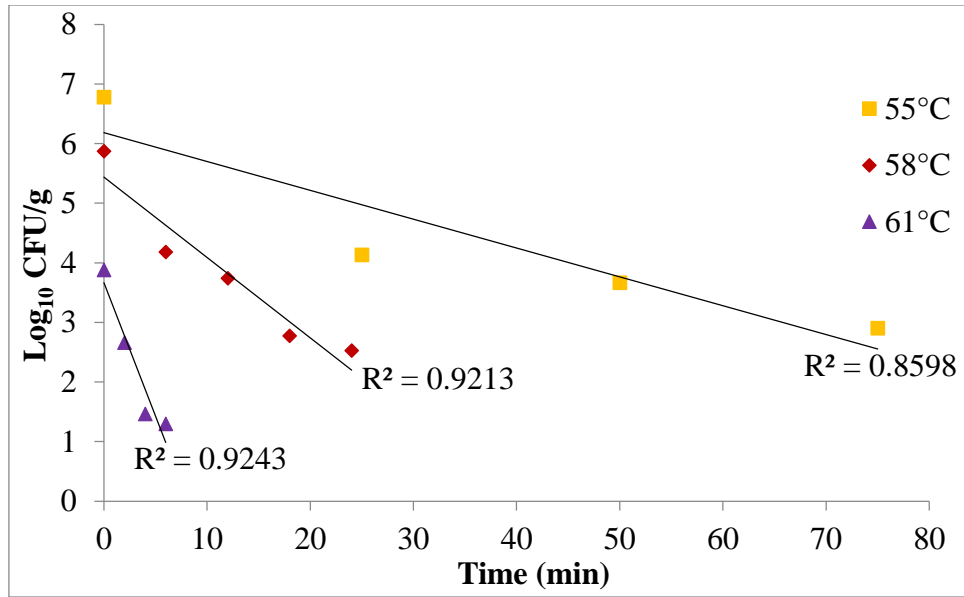


Figure 4.7 Thermal inactivation of *Salmonella* spp. at 55, 58 and 61°C in hamburger bun dough: viable cells enumerated on XLD agar

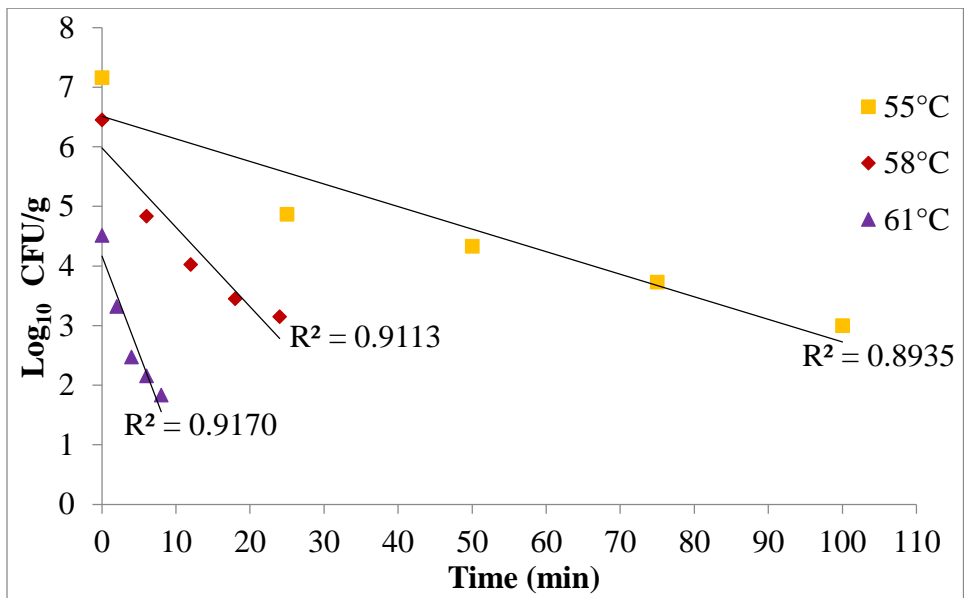


Figure 4.8 Thermal inactivation of *Salmonella* spp. at 55, 58 and 61°C in hamburger bun dough: viable cells enumerated on BHI agar with XLD agar overlay

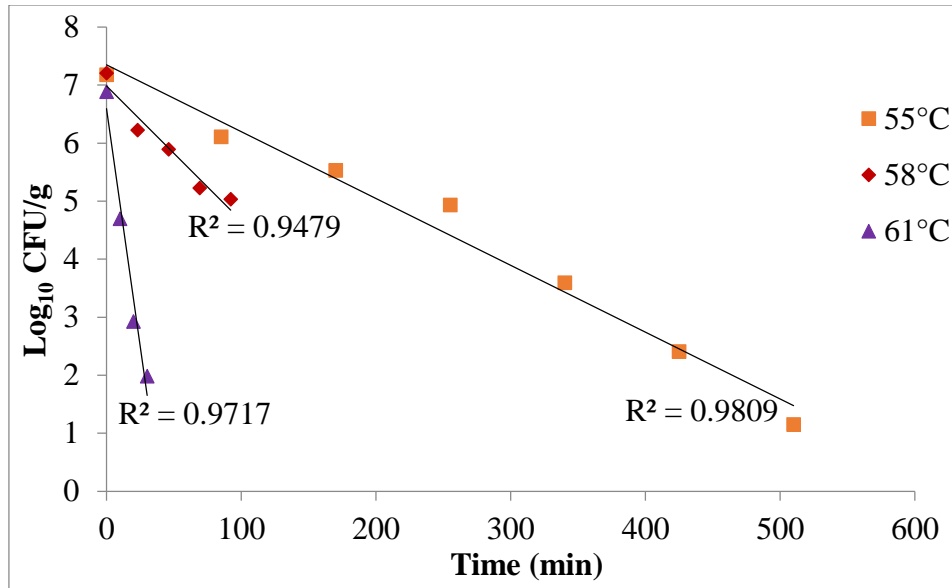


Figure 4.9 Thermal inactivation of *E. faecium* at 55, 58 and 61°C in hamburger bun dough: viable cells enumerated on m-E agar

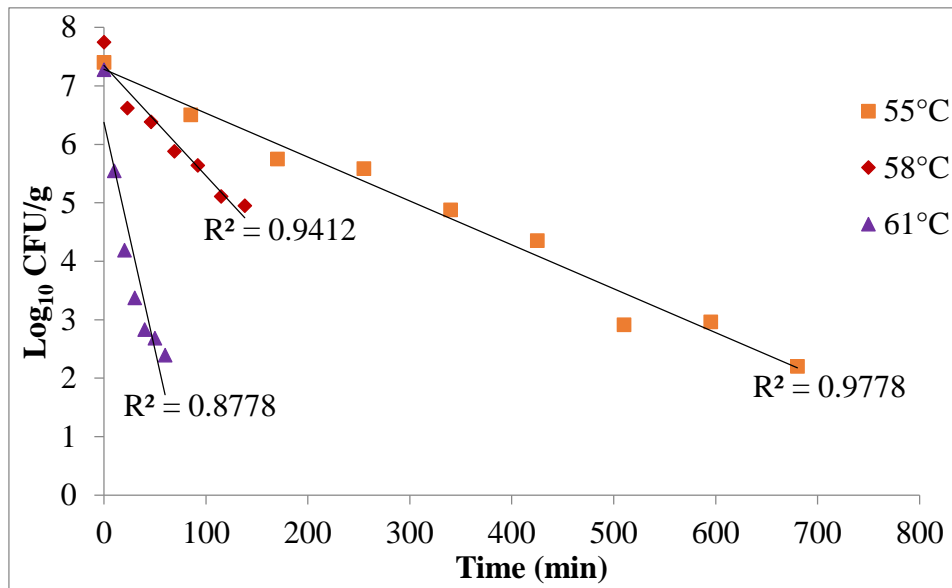


Figure 4.10 Thermal inactivation of *E. faecium* at 55, 58 and 61°C in hamburger bun dough: viable cells enumerated on BHI agar with m-E agar overlay

Table 4.2 D-values (min) and z-values (°C) of a 3-serovar *Salmonella* spp. cocktail, *Enterococcus faecium* ATCC 8459, and *Saccharomyces cerevisiae* during heating of hamburger bun dough

		<i>Salmonella</i> spp.		<i>E. faecium</i>		<i>S. cerevisiae</i>
		BHI/XLD ^a	XLD ^b	BHI/m-E ^c	m-E ^d	PDA ^e
Temperature (°C)	52	ND ^f	ND	ND	ND	18.73 ± 0.72
	55	28.64 ± 5.19	21.30 ± 2.61	133.33 ± 0.00	87.21 ± 4.74	5.67 ± 1.51
	58	7.61 ± 0.61	7.53 ± 0.61	55.67 ± 9.00	45.33 ± 6.79	1.03 ± 0.21
	61	3.14 ± 0.32	2.29 ± 0.21	14.72 ± 4.11	6.14 ± 0.47	ND
	z-value	6.58 ± 0.96	6.22 ± 0.32	6.25 ± 0.80	5.20 ± 0.05	4.74 ± 0.34

^a Injury-recovery media, Brain Heart Infusion (BHI) agar with Xylose Lysine Desoxycholate (XLD) agar overlay

^b Selective medium, XLD agar

^c Injury-recovery media, BHI agar with m-*Enterococcus* (m-E) agar overlay

^d Selective medium, m-*Enterococcus* agar

^e Potato Dextrose agar plus choramphenicol

^f ND: Not Determined

The greater D- and z-values of *Salmonella* spp. and *E. faecium* on the injury recovery media compared to the corresponding D-values on the selective media, confirms that a sub-population of injured bacterial cells is able to survive heating in dough at the three temperatures studied in the current study. Injured pathogenic cells can recover when favorable environmental conditions are available and may pose a foodborne illness risk.

McCormick *et al.* (2003) reported the D-value of *Salmonella* Typhimurium in low-fat ready-to-eat turkey bologna as 4.63 and 0.95 min at 57 and 60°C, respectively. They also reported the z-value of *S. Typhimurium* as 5.56°C. D- and z-values reported by McCormick *et al.* (2003) are different than values determined in this study because of differences in the composition of bologna and bun dough, along with differences in *Salmonella* strains used. Bianchini *et al.* (2014) reported that during extrusion of balanced carbohydrate-protein meal, minimum temperature required to achieve a 5-log reduction for a 5-strain cocktail of *Salmonella*

enterica (Branderup NVSL 96-12528, Oranienburg NVSL 96-12608, Typhimurium ATCC 14028, Enteritidis IV/NVSL 94-13062 and Hedelber/Sheldon 3347-1) was 60.6°C compared to 73.7°C for *Enterococcus faecium* NRRL B-2354. The authors stated that *E. faecium* can be used for the in-plant thermal inactivation validation studies for *Salmonella* spp. during extrusion, as the inactivation temperature for *E. faecium* was higher compared to *Salmonella* spp.

Populations of *S. cerevisiae* vs. time during thermal inactivation at 52, 55 and 58 °C are presented in Fig. 4.11. The D-values of *S. cerevisiae* in hamburger bun dough were 18.73, 5.67 and 1.03 min at 52, 55 and 58°C, respectively, and the z-value of *S. cerevisiae* was 4.74 (Table 4.2). These D and z values for *S. cerevisiae* were considerably lower than those for *Salmonella* spp. and *E. faecium*. In comparison, the $D_{58^{\circ}\text{C}}$ values of the three microorganisms (7.61 ± 0.61 for *Salmonella*, 55.67 ± 9.0 for *E. faecium*, and 1.03 ± 0.21 for *S. cerevisiae*) point out the challenges for using *E. faecium* or *S. cerevisiae* as surrogates for *Salmonella* spp. during baking validation studies. In such instances, the differences in D-values should be taken into consideration. López-Malo *et al.* (1999) reported that the D-values of *S. cerevisiae* in Sabouraud glucose 2% broth were 18.3, 4.8 and 2.7 at 50, 52.5 and 55°C, respectively. The D-values reported by López-Malo *et al.* (1999) were lower than those reported in this study because of the differences in the heating medium (liquid vs. solid matrix). However, López-Malo *et al.* (1999) reported similar z-values (4.2°C) as those reported in the current study, indicating that the thermal sensitivity of *S. cerevisiae* to the change in the temperature were similar in Sabouraud broth and hamburger buns.

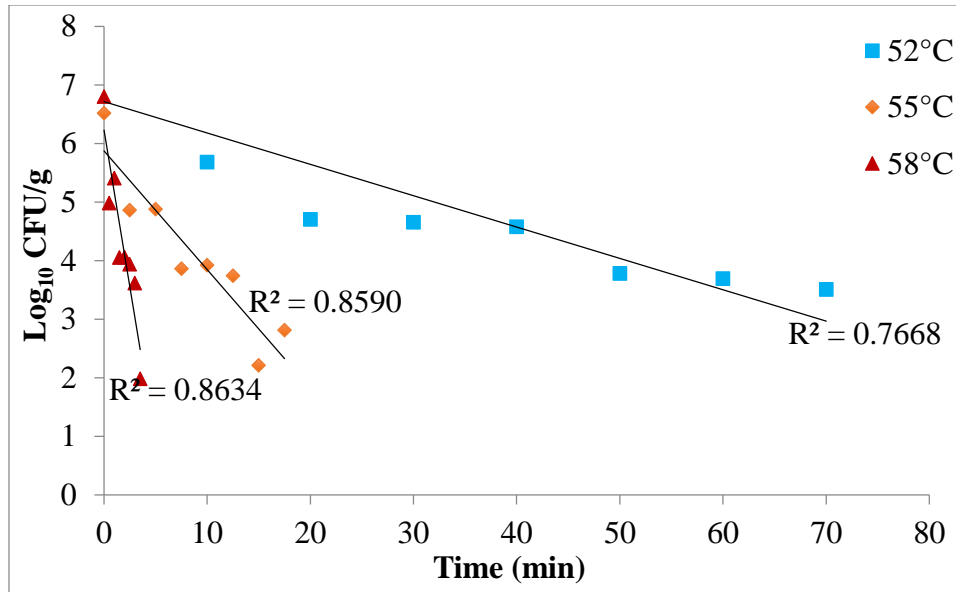


Figure 4.11 Thermal inactivation of *S. cerevisiae* in hamburger bun dough at 52, 55 and 58°C as enumerated on PDA supplemented with 100 ppm chloramphenicol

The present study demonstrated that the typical hamburger bun baking process will eliminate *Salmonella* spp. populations (>6 log CFU/g reductions) utilizing oven temperatures $\geq 218.3^{\circ}\text{C}$ (425°F) and baking for at least 9 min. Also, considering the internal temperature *vs.* time recorded in this study, it is clear that all the *Salmonella* cells were destroyed within 9 min, prior to the optimum bake time (as determined and utilized in these studies). The thermal resistance of *S. cerevisiae* in hamburger bun dough is lower than *Salmonella* spp., and therefore, *S. cerevisiae* is not recommended as a surrogate for *Salmonella* spp. in thermal inactivation studies (Fig. 4.12). Greater survival of *E. faecium* during bun baking and higher D-values of *E. faecium* compared to that of *Salmonella* spp. suggest *E. faecium* can be used as a surrogate for *Salmonella* spp. for baking studies in processing facilities if needed, although this would result in an overestimation of the lethality required (Fig. 4.12).

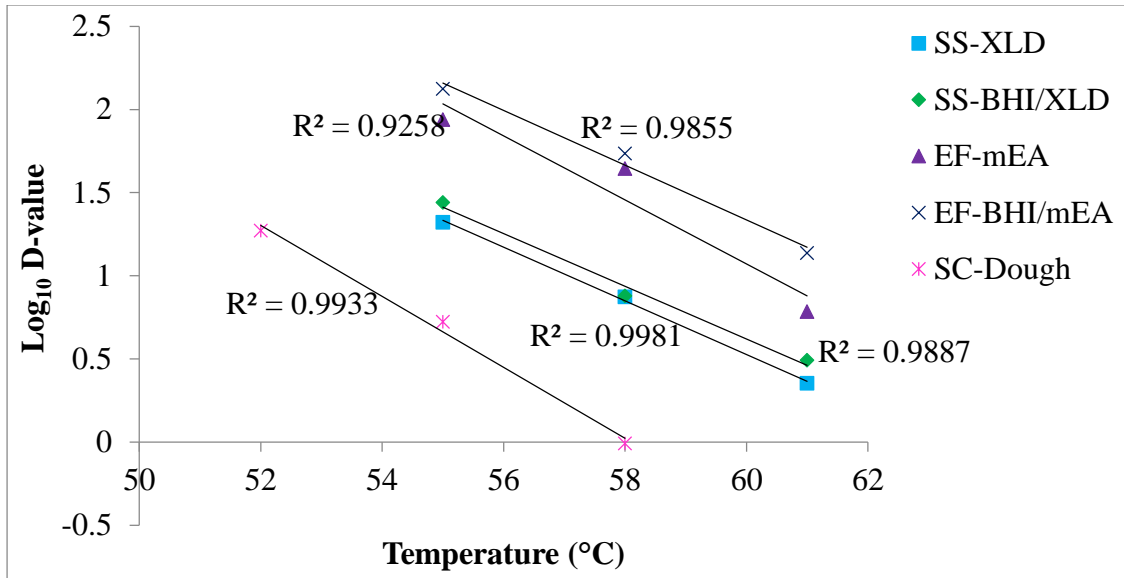


Figure 4.12 Log D-value versus temperature for *Salmonella* spp. (SS) enumerated on XLD agar and BHI agar overlayed with XLD; *E. faecium* (EF) enumerated on m-E agar and BHI overlayed with m-E agar; *S. cerevisiae* (SC) enumerated on PDA supplemented with 100 ppm chloramphenicol

Chapter 5 - Conclusions

Hamburger bun dough exposed to an oven temperature and baking time typical of a commercial baking process was highly lethal to all three microbial populations studied. The high humidity of the forming crumb combined with internal temperatures approaching that of steam is an extremely destructive combination. While the a_w of the crust decreases during baking, elevated peak temperature exposure ensures adequate lethality. The general hamburger bun baking process evaluated in these experiments is sufficient to reduce *Salmonella* spp. contamination in raw ingredients by > 6 log cycles. In the known incidents of *Salmonella* spp. contamination of flour, this level of lethality should be sufficient to yield a product which is safe for human consumption.

Due to dramatic shifts in temperature during the baking process, *Saccharomyces cerevisiae* in hamburger bun dough were unable to survive any of the baking treatments examined. While it may be appealing for bakers to avoid addition of an artificial surrogate, the thermal tolerance of *S. cerevisiae* was poor compared to *Salmonella* spp.; therefore, a lack of recovery of *S. cerevisiae* cannot approximate the survival of *Salmonella* spp. making it an ineffective surrogate.

Enterococcus faecium ATCC 8459, on the other hand, was recovered from hamburger buns that were under-baked while corresponding *Salmonella* spp. enrichments were negative. These data were further reinforced by calculation of the D-values in hamburger bun dough. *E. faecium* consistently outperformed *Salmonella* spp. in terms of survival at lethal temperatures. Work by other scientists confirms these findings in other food matrices and verifies the lack of virulence factors in *E. faecium*, lending further credibility to its use as a conservative surrogate for *Salmonella* spp. in hamburger bun baking validations.

These studies are meant to entice discussion regarding the survival characteristics of bacteria in bakery products. Only a small fraction of the baking industry is represented by this work. While it provides a starting point for justification of standard production guidelines, more research in this field is of critical importance. In order to comply with FSMA, these experiments need to be repeated across the broad range of diverse bakery products and also for other pathogenic bacteria which may contaminate raw ingredients. Furthermore, laboratory scale research can only serve to open the door for extensive commercial scale validations. What has been observed in a laboratory must be certified by in-plant analyses to continue to hold merit.

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Appendix A - Images



Figure A.1 Inoculation of flour was performed in a Class II Type A2 biosafety cabinet



Figure A.2 Ingredients were mixed with a Hobart A-20 stand mixer with fork agitator attachment in a Biosafety Level-2 pilot food processing laboratory



Figure A.3 Dough was hand rolled into balls prior to resting and proofing



Figure A.4 Dough balls were allowed to rest for 10 min at room temperature after rolling



Figure A.5 Dough balls were molded into compartments of a standard greased bun pan for proofing



Figure A.6 Thermocouples were inserted into the geometric center of buns and one thermocouple was affixed to the side of the pan to monitor oven air temperature

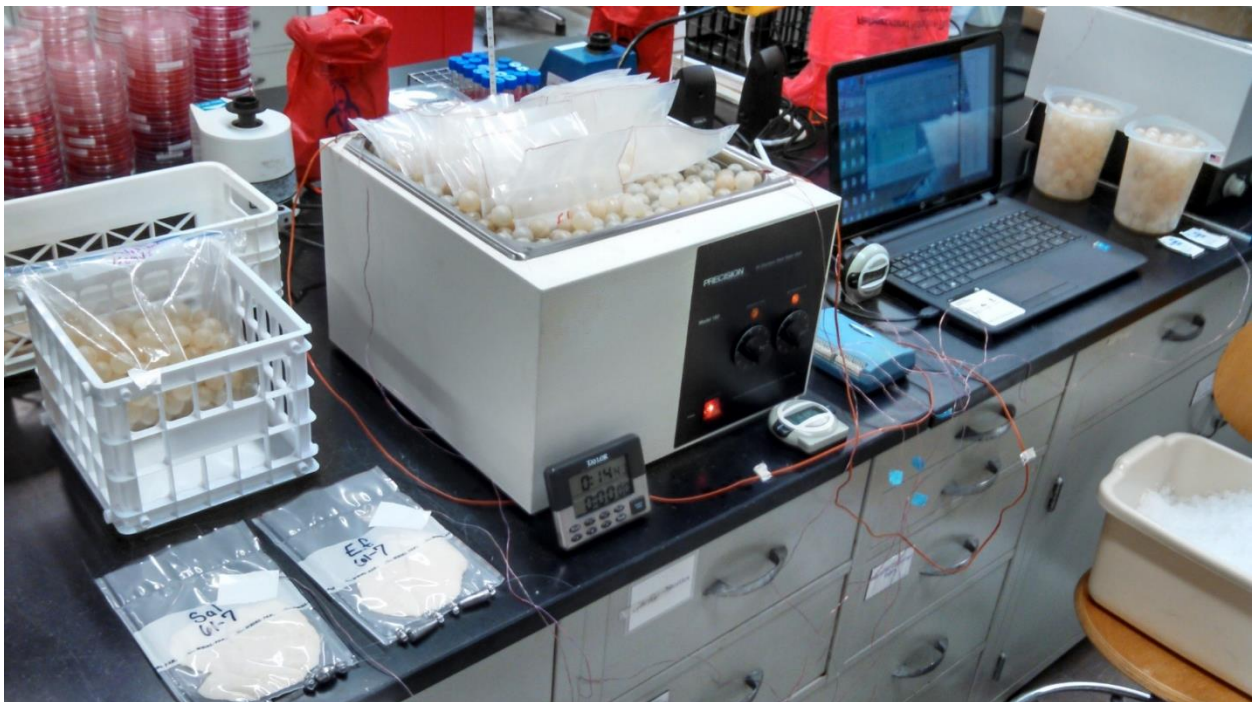


Figure A.7 D- and z-value study configuration