IDENTIFICATION OF A GRAS SURROGATE FOR *SALMONELLA* IN LOW WATER ACTIVITY FOODS

By

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(Under the Direction of

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ABSTRACT

Salmonella contamination of low water activity (a_w) foods is a food safety issue. This project was aimed to identify a surrogate for *Salmonella* in low a_w foods. *Pediococcus acidilactici* ATCC 8042, was identified as a potential generally-recognized as safe (GRAS) surrogate for *Salmonella* and was compared to a five-strain cocktail of *Salmonella* and to *Enterococcus faecium* NRRL-B2354, an already recognized surrogate. Thermal kinetics parameters were calculated in toasted oats cereal (TOC) and peanut butter, while single-time point inactivation measurements were determined in almonds. Both surrogates were inactivated in TOC at similar rates as *Salmonella* at temperatures between 85-95 °C and in peanut butter at 63-77 °C and there was no statistical difference between the two surrogates. In almonds, however, *Pediococcus* was more sensitive to heating at 138 °C than *Salmonella* and *Enterococcus*. This research recognized a viable GRAS surrogate for *Salmonella* for use in peanut butter and TOC.

INDEX WORDS: *Salmonella,* Low water activity foods, Bacterial surrogates

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CHAPTER 1

LITERATURE REVIEW

Salmonella enterica subs. *enterica* is the most common bacterial cause of foodborne disease in the United States. Current estimates approximate that *Salmonella* causes 1 million illnesses per year *(64).* These infections typically cause gastrointestinal stress, but can become more complex, resulting in hospitalizations and death. While it is commonly associated with poultry, eggs, and dairy products, *Salmonella* can contaminate foods previously thought to be safe.

Low water activity foods ($a_w \leq 0.6$) are one class of food products that have been associated with *Salmonella* outbreaks. Low water activity foods were thought to be safe since the low water activity prevents bacterial metabolic activity. However, there have been many outbreaks of *Salmonella* associated with low water activity foods. These have included toasted oats cereal, peanut butter, and raw almonds. Given the ready-to-eat status of all these food products, the impact of these outbreaks was large. Peanut butter, especially, caused large numbers of illnesses in three separate *Salmonella* outbreaks associated with peanut butter. One outbreak, in 2007, caused 628 illnesses *(6)*. Given the impact of the low water activity food associated outbreaks, controls of *Salmonella* in these foods should be addressed.

Not only is the control of *Salmonella* in low water activity foods a public health need; it is a regulatory necessity. The Food Safety Modernization Act (FSMA) requires a food safety plan for each food facility. This plan must include a risk analysis of all

bacterial, chemical, and physical hazards and appropriate controls to reduce the risk of the identified hazards *(52)*. These controls must be validated, using scientific evidence that proves the control produces the effect needed.

One method validating *Salmonella* control measures in foods is by using a bacterial surrogate in the food matrix. A bacterial surrogate is a non-pathogenic organism that has similar responses to a control in a food, compared to the target pathogen *(67)*. A good surrogate is non-pathogenic, easy to use, easy to store, genetically stable, and has similar growth and survival characteristics to the target pathogen. An additional key characteristic is GRAS status. Generally Recognized As Safe (GRAS) food additives have been either approved for use by a scientific committee or have been regularly used in foods *(15)*. GRAS microorganisms are typically approved through the second categorization. GRAS species include *Lactococci*, *Lactobaccilli*, and *Pediococci.* These microorganisms are fermentative and included in fermented foods such as cheese and yogurt.

Very few surrogates for *Salmonella* in low water activity foods have been identified. One, *Enterococcus faecium* NRRL-B2354, has been accepted by the California Board of Almonds for validating almond roasting processes *(11)*. *E. faecium* NRRL-B2354 is non-pathogenic but is not a GRAS microorganism. This may cause regulatory hurdles. Other surrogates for *Salmonella* in low water activity foods have been reported in the scientific literature; however, they have yet to be accepted for practical application in processing plants.

Given the requirement of validating controls for *Salmonella* in low water activity foods, and the lack of viable surrogates in those food matrices, a need for more surrogates

for *Salmonella* in low water activity foods is evident. Therefore, this project was undertaken to identify a GRAS surrogate for *Salmonella* in different low water activity foods. This project falls into two parts. First, the identification of potential surrogates in a low water activity food matrix. This included using bioinformatics tools to find surrogates and screening them. The second part was comparing the thermal kinetics parameters of the GRAS surrogate with a *Salmonella* cocktail and *E. faecium* NRRL-B2354 in toasted oats cereal, peanut butter, and almonds.

1.1 *Salmonella* Characteristics

1.1.1 General Traits of *Salmonella*

Bacteria belonging to the genus *Salmonella* are Gram-negative pathogenic microorganisms. They are classified within the Enterobacteriaceae family in the Proteobacteria phylum. The different *Salmonella* species are rod-shaped facultative anaerobes *(37)*. Most *Salmonella* serovars are motile, using peritrichous flagella to move, while some serovars (Pullorum and Gallinarum) do not have flagella *(49)*. One of the unique biochemical characteristics of this genus that distinguish *Salmonella* from other Enterobacteriaceae genera is its ability to reduce sulfate and produce hydrogen sulfide.

Salmonella is a bacterium that has a unique ability to adapt to changes in the environment. While it prefers to grow in warm, moist environments, it is particularly capable responding to stresses *(49)*. The optimal conditions for *Salmonella* are typically a temperature of 37°C, in a wet environment with simple carbohydrates as source of energy. However, *Salmonella* species can endure acid, desiccation, and temperature stresses *(49)*. This allows the bacteria to survive under adverse conditions for some time until the environment becomes more supportive of growth.

1.1.2 *Salmonella* Nomenclature

The genus *Salmonella* contains two species, *enterica* and *bongori,* which both cause disease in humans. *Salmonella enterica* is the more common species and the more diverse, with six subspecies. The subspecies are: *enterica, salamae, arizonae, diarizonae, houtenaie,* and *indica (63).* Both species of *Salmonella* are further divided into serovars, by the Kaufman-White scheme. This biochemical determination separates the species into serovars through identification of the differences between surface antigen properties (flagellar and other antigens) *(37).* Specifically, the determination examines the differences between the O antigens, a polysaccharide found on the surface of the cell's lipopolysaccharide and the H antigen, a portion of the bacterial flagella. The O antigen can change, depending on the composition of the sugar components, the bonds between the sugar molecules, and the bonds between the antigens *(63).* The other antigen used for serotyping, the H antigen, is a portion of the bacterial flagella. *Salmonella* can express either one or two types of H antigen. The antigens themselves are the filamentous portion of the flagella *(63).* The diversity within the combinations of antigens is immense, resulting in more than 2,500 serovars *(37).*

1.1.3 Disease Characteristics and Impact

Salmonella can cause two types of diseases in humans: enteric fever (typhoid and paratyphoild fever) and nontyphoidal. Enteric fever is caused by *Salmonella* serovars Typhi, Paratyphi A, and Sendai *(24).* Enteric fever is characterized by long lasting fever, headaches, stomach pains, diarrhea, and lethargy *(37).* Enteric fever is often life threatening as it frequently involves an invasive infection of multiple organs that leads to death in close to 2% of the cases. Typhoidal and paratyphoidal *Salmonella* is associated

with contaminated water, animal products, or contact with infected persons *(24)*. It is usually not directly associated with food.

Nontyphoidal *Salmonella* causes gastrointestinal illness in humans. Most serovars of *Salmonella* cause nontyphoidal illness, which is typified by diarrhea, vomiting, and abdominal pain *(24)*. Typical infections start between 12 to 72 hours after ingestion *(49)*. In most cases, the infection is limited to 5-7 days without treatment *(24)*. Complications can occur, including serious dehydration and systemic infections. These can result in hospitalizations and occasionally death.

Nontyphoidal *Salmonella* can be acquired through contact with infected persons, animals, and contaminated food. Foodborne *Salmonella* infections are widespread and have been associated with a wide variety of foods. The infectious dose can vary as well depending on the food type and the serovar. In most foods, the infectious dose is approximately 10^5 cells, however in high fat foods, the infectious dose has been reported to be much lower. In outbreaks related to foods such as chocolate and cheese, investigators found that infections developed with low doses of 10 to 100 cells *(18)*.

Salmonella is the most common bacterial cause of foodborne disease in the United States. Scallan et al. estimated 1 million illnesses, 19,000 hospitalizations, and 370 deaths per year *(64)*. Those public health figures have been calculated to cost more than $11 billion annually and $11,000 per case of *Salmonella (65)*. Decreasing the prevalence of *Salmonella* would have both a large health benefit and economic impact. Globally, the burden of nontyphoidal *Salmonella* is 93.8 million illnesses and 155,000 deaths per year *(51)*. Of these cases, 80% are foodborne illnesses, which results in 75 million illnesses per year. The global estimates also state that 22 million cases and

200,000 deaths are caused by typhoid fever every year. These estimates demonstrate that *Salmonella* has a large impact on both national and global health.

1.1.4 Reservoirs

Salmonella is a natural inhabitant of the gastrointestinal (GI) tract of multiple mammal, bird, reptile and amphibian animals including those species typically used as livestock. The GI tract appears to be a favorable environment with the preferred growth temperature and accessible nutrients *(49)*. The bacterium can be spread in the human food chain through the transfer of feces onto food products. Food can be contaminated directly, such as in poultry slaughter plants from the feces of infected chickens to other chickens or at the kitchen, to other foods. Food can also be contaminated indirectly, for example, via animal manure onto fresh produce crops.

Salmonella has been shown to be a frequent inhabitant of the GI tract of livestock. On farm surveys have shown a prevalence of 16.2% on poultry farms, 57.3% on swine farms, and 17.9% on dairy farms *(60)*. This is not the case in horticultural farms. Prevalence of *Salmonella* is markedly smaller, approximately 3.1%, in environmental samples of horticultural environments *(60)*. Higher *Salmonella* prevalence in livestock has also been determined indirectly by several finished product surveys. *Salmonella* was detected in 44.7% of chicken breasts in retail and the predominant serovars were Heidelberg and Kentucky *(36)*. In contrast, its prevalence in other commodities such as low water activity nuts was reported to be significantly lower. Macadamia nuts had the highest prevalence with 4.2% while Salmonella-positive samples was less than 1% in pecans, cashews and hazelnuts *(76)*.

However, the difference in environmental prevalence does not seem to be reflected in outbreak data. An attribution study of all 2013 outbreaks showed there was no significant difference between any of the food groups that produced *Salmonella* outbreaks in 2013 *(25)*. In fact, seeded vegetables were the largest attribution group, followed by eggs and poultry. This suggested that there is a difference between the overall prevalence of *Salmonella* on the farm and in food products and the actual foods associated with illness.

1.1.5 *Salmonella* and low water activity foods

While *Salmonella* is associated with poultry products, dairy foods and fresh produce, it has been increasingly associated with low water activity (a_w) foods. Low water activity foods are defined as those with a_w values of less than 0.6, because 0.6 is the minimum water activity needed for microbial growth *(70)*. Low water activity entirely inhibits bacterial growth in foods since bacteria require water for most metabolic activities. However, a reduced water activity in a food does not protect against contamination with pathogenic microorganisms (Table 1.1). In the last ten years, many *Salmonella* outbreaks have been associated with low water activity foods. Those reports were initially surprising, since low water activity foods were thought to pose lower risk than high water activity foods. The lack microbial growth lowers the risk of large populations of pathogenic bacteria in foods.

Table 1.1. Outbreaks of *Salmonella* in low water activity foods in the last 10 years

Year	Serotype	Source	No. of Cases
2016	Virchow	Shake and Meal Products	33
2016	Montevideo and Senftenberg	Pistachios	11
2015	Paratyphi B variant L(+) tartrate(+)	Nut Butter	10
2014	Newport, Hartford, and Oranienburg	Sprouted Chia powder	31
2014	Braenderup	Nut Butter	6
2013	Montevideo and Mbandaka	Tahini Sesame Paste	16
2012	Bredeney	Peanut Butter	42
2012	Infantis	Dry Dog food	49
2009	Typhimurium	Peanut Butter	529
2009	Montevideo, Newport, and Senftenberg	Pistachios	8
2008	Agona	Rice and Wheat Puff Cereal	28
2007	Tennessee	Peanut Butter	628

(17)

In 2008, Malt-O-Meal cereals had an outbreak of gastroenteritis caused by *Salmonella enterica* serovar Agona. This outbreak sickened 28 people in 17 states, and resulted in the hospitalization of 12 people *(62)*. Outbreak investigations identified the vehicle of infection to be rice and wheat puff breakfast cereals. What made this outbreak rather remarkable is that the pulse field gel electrophoresis pattern (PFGE), which is a type of molecular fingerprinting tool used in outbreak investigations, matched the PFGE pattern from a 1998 outbreak of *Salmonella* Agona from the same company *(62)*. The 1998 outbreak was larger, with 209 cases and 47 hospitalizations *(3)*. This earlier outbreak was traced back to toasted oats cereal manufactured in the same facility as the puffed rice and wheat in the 2008 outbreak. The linkage of the strain type within the facility suggests that the strain of *Salmonella* survived in the facility for 10 years. This

was particularly intriguing, since *Salmonella* is not a spore former, and would need to use different mechanisms of survival to persist in a harsh environment for years.

Among low water activity foods, peanut butter has also been a frequent vehicle of a series of *Salmonella* outbreaks. In the last ten years there have been three peanut butter outbreaks, with two of those outbreaks causing more than 500 illnesses each. The most recent *Salmonella* outbreak in peanut butter was in 2012 caused by a relatively rare serovar Bredeney. This 20-state, 42-case outbreak was traced back to New Mexico *(9).* The *Salmonella* Bredeney outbreak triggered a large recall of most of the company's products, since the source of contamination was traced back to the entire manufacturing facility. Those included not only peanut butter, but also almond butter and roasted nuts.

The 2009 peanut butter outbreak was a much larger outbreak spanning multiple products and multiple companies. This outbreak was identified as originating in a company called Peanut Corporation of America that used to be located in Georgia. This outbreak involved 529 cases reported in 43 states *(8).* This outbreak lead to 116 hospitalizations and unfortunately, 8 deaths and was caused by a *Salmonella* serovar Typhimurium. The magnitude of this outbreak was partially due to the extent of distribution of peanut butter and peanut products as ingredients in multiple products. The Peanut Corporation of America directly marketed only a fraction of its production for consumer, but they supplied the peanut butter and other products to be used in many other products, such as crackers, ice cream, pastries, etc. Because of this, 54 companies had to recall 431 products from the market *(8)*. The scope and complexity of this outbreak and its resulting massive recall evidenced the need for improved food safety protection in the food supply.

The first reported peanut butter outbreak in the United States was caused by serovar Tennessee and involved 628 patients *(6)*. This outbreak was also quite widespread spanning 47 states and was traced back to two brands of peanut butter, Great Value and Peter Pan, produced in the same processing plant. Given the scale of the outbreak, and the unexpected carrier, the first peanut butter outbreak indicated for the first time that a product that was considered as extremely safe because of its low water content, it could still be a vector for foodborne illness.

An earlier series of outbreaks associated with to low water activity foods, were the two raw almond outbreaks in 2001 and 2004. In 2001, an outbreak of *Salmonella* Enteritidis phage type 30 sickened 168 people *(40)*. This outbreak mostly occurred in Canada, with only 11 cases of United States residents. However, the epidemiological investigation traced the contamination back to Californian farms. The investigation revealed that *Salmonella* was detected in environmental samples of farms and processing equipment *(40)*. A second outbreak, in 2004, sickened 29 people in the US and Canada *(4)*. This was also a *Salmonella* Enteritidis outbreak however, the strains were different. As a result of these two outbreaks, the California Board of Almonds and the USDA require a process that produces a 4 log reduction in *Salmonella* before sale *(5)*.

All of these outbreaks illustrated three major issues. First, unexpected and low risk foods could still be contaminated with pathogens. Even though a food has not yet been implicated in an outbreak does not mean that it will not ever be. Secondly, *Salmonella* has a greater ability to survive in difficult environments than previously expected. The cereal outbreaks demonstrated that *Salmonella* may be able to survive in a plant environment for ten years. This unique trait may cause repeated outbreaks with the

same strain multiple times, which was unexpected. Lastly, there is an enhanced risk and difficulty in tracing a contaminated ingredient, instead of a single food. Contaminated ingredients can cause recalls of many products, instead of a single one, and complicate finding the source of an outbreak.

1.2 Regulations and Control Measures

1.2.1 Food Safety Modernization Act

Because of the issues raised by these outbreaks, and others as well, the US government passed the Food Safety Modernization Act (FSMA) in 2011. This law focused almost entirely on prevention and endeavored to change the way companies assess food safety, starting with including risk-based analyses of control processes. FSMA has six main rules: preventative controls for human food, produce safety, preventative controls for animal food, foreign supplier verification, intentional adulteration of food, and sanitary transport of food *(52)*. These rules have started to be implemented and will be fully implemented in 2024 *(16)*. They cover many parts of the food industry, from produce farms, to manufacturing facilities, to transport.

The Preventative Controls for Human Food Rule was included in the law to change the way food manufacturers analyze the food safety risks in each facility. This regulation also applies to low water activity food manufacturers. Before the passage of FSMA, the regulations for food manufacturers did not require any analysis of manufacturing processes, instead were rules that every facility should follow. The new rule requires risk-based analysis of the manufacturing process and controls of the identified hazards *(52).*

In order to address most of the requirements, the FDA requires all facilities to have a food safety plan. First, a hazard analysis must be developed and written. This hazard analysis is expected to identify all biological, chemical, and physical hazards in the process. The significant hazards must be addressed in the next part of the plan. When a hazard is identified through risk analysis, controls must be identified and applied. This must include: process controls, food allergen controls, sanitation controls, supply-chain controls, recall plan, and other necessary controls *(13)*. For all of these controls, monitoring procedures, corrective actions, and verification procedures must be in place.

Before they are in place all controls must be validated, meaning they must be proved scientifically that the control will be effective. Validation procedures must either be supported by published scientific evidence or technical evidence performed by the facility *(20)*. A properly validated control measure must show that the treatment will be an effective control of the hazard. For example, a process that uses heat treatment to control a pathogen must have been shown to achieve the needed reduction in pathogens. This reduction can be a certain log reduction or a complete kill. The validation must be done at conditions that most closely resemble the actual process controls, since variables can change the degree of pathogen kill, making in ineffective. Verification using technical data in the plant setting is the most effective, since it is the exact control measure used. However, introducing pathogens into a facility is not an acceptable method of verification. The challenge of verifying thermal inactivation of pathogens is that models are not always perfect estimates, but technical studies in a facility are impossible with a pathogen. This is an issue that needs to be addressed. The new requirements in the

Preventative Controls Rule are being enforced and there may not be an adequate validation method for pathogen inactivation in some foods.

1.2.2 Bacterial Surrogacy

Instead of modeling the process in a laboratory setting for validation of thermal treatment, a bacterial surrogate can be used. Surrogates have similar characteristics as the target pathogen in food, except that they are non-pathogenic. Harmless microorganisms would be then suitable for in-plant validation studies. To be considered a surrogate, a bacterial strain must be non-pathogenic, have similar survival and growth characteristics as the target pathogen, and easy to use *(67)*. Before use, scientific evidence should demonstrate that the surrogate organism behaves identically or very close to the target pathogen in the exact same environmental and testing conditions. In food, this means the same food matrix, same inoculation method, and same thermal treatment.

A widely accepted surrogate for *Salmonella* in almonds, a low water activity food, is *Enterococcus faecium* NRRL B-2354. This strain has been approved by the California Almond Board for nut roasting validation *(11)*. A 2003 *Salmonella* Enteritidis outbreak from almonds revealed that almonds were also at risk of Salmonella transmission. This outbreak sickened 29 people in 12 states and was traced back to raw almonds *(45)*. To address the potential for *Salmonella* contamination, it was reported that *E. faecium* NRRL B-2354 was a viable surrogate for validating dry roasting or steam heating processes in almond processing *(41, 42)*.

E. faecium NRRL B-2354 was adopted as a surrogate because it met most of the surrogate guidelines. This strain was proven to even slightly over predict the thermal inactivation of *Salmonella* Enteritidis PT30 in almond heating *(42)*. The slight over

performance is preferred in surrogacy, as it gives the manufacturer an additional safety factor to ensure that the process is capable of delivering adequate inactivation. *E. faecium* NRRL B-2354 is also non-pathogenic but belongs to a bacterial genus not previously considered as a generally recognized to be safe (GRAS) organisms. *Enterococcus* species are naturally inhabitants of the GI tract of animals and have been used as fecal indicators. While some *Enterococcus faecium* species are associated with nosocomial infections, *E. faecium* NRRL B-2354 lacks virulence genes *(47)*. Because of the lack of pathogenicity, the strain has been deemed to be safe to use.

Even if a strain is predicted to be safe through genetic characterization, it may not be allowed within a facility. There are strict regulations on what can be added to or adjacent to foods. This may pertain to surrogates. Regulators may not be certain that the food will not be contaminated. Instead of using a bacterial strain that may not be approved for use in a manufacturing facility, a GRAS strain could be used as a very viable alternative.

Generally Recognized as Safe (GRAS) food additives are allowed for use in food without lengthy regulatory approval. GRAS ingredients are proven to be safe through scientific evidence presented by experts or their common use in foods since January 1, 1958 *(15)*. GRAS microorganisms are usually approved under the second category. These are usually found in fermented foods, such as cheese and yogurt. The lactic acid bacteria used in foods are GRAS and allowed in food manufacturing facilities. Using bacterial strains already approved for use in foods as a surrogate would make the regulatory approval simpler.

1.2.3 Thermal Kinetics

An approach to predict and understand the thermal inactivation of bacteria is through the use of mathematical models. A model uses the decrease in cell numbers over time to predict the rate of cell death in foods as a function of temperature. The simplest mathematical method used is the log linear method, or the Bigelow and Etsy model. This model is based on first order reaction kinetics, which assumes a constant rate of enzymatic inactivation and results in a straight line of the regression of the logarithm of the concentration versus time points *(71)*. The linear regression is used to find the thermal parameters that describe the rate of microbial inactivation. The time needed for a logarithm reduction of a microbial count at a specific temperature in a food product is defined as D-value *(72)*.

The D-value is calculated from the direct linear regression of log10 CFU vs time, through the formula, D-value = - 1/(slope of the regression line) *(72)*. Once the D-value is determined for at least four different temperatures, then a z-value can be calculated as well. The z-value is defined as the change in temperature needed for a 10-fold increase in D-value *(72)*. Both values are built on two assumptions. The first is that bacterial inactivation follows first order reaction kinetics *(71)*. The second is that each bacterium has the same probability of dying *(71)*. Neither of these assumptions are met in all foods with all bacterial strains. Individual bacterial cells do not always have the same possibility of dying and enzymes do not always follow first order kinetics. This can result in “shoulders” or “tails” in inactivation curves that are not well described by the log-linear model. As a result, alternative models that better fit the data must be used.

One of the models that have proven to offer a suitable alternative to describe inactivation is the Weibull model. This is a non-linear model, which incorporates variable rates of cell death. The Weibull model was developed to predict the time to failure after a stress (such as heat) and was applied to microbiology *(71)*. The model uses two distribution parameters, α and β to describe the regression. The δ parameter is the scale or time parameter and the β parameter describes the shape of the curve *(71)*. When the β parameter is less than one the shape of the curve is concave down, meaning the remaining cells have a smaller probability of dying than the initial cell population. When the β parameter is greater than one, the curve is concave up which is interpreted as the cells have a greater chance of dying than the initial population. When β is equal to one, the probability of cell death equal and then the data can fit log-linear model.

When used in food, the Weibull model can be applied to develop process calculations. In foods, a model is needed to define processing parameters. The processing parameters determined are employed in lieu of the processing parameters from the log linear model. The Weibull model is based on the δ value instead of the D-value *(1)*. The δ value can be used to calculate a similar value to the z-value.

1.2.4 Processing Overview of Selected Low Water Activity Foods

1.2.4.1 Production of Toasted Oats Cereal

Both Malt-O-Meal outbreaks were identified as originating from a gun puffed line in the manufacturing plant *(3, 62)*. A gun puff is a high heat, high pressure method of drying cooked grains and extruded products *(30)*. A typical gun is run at temperatures from 200-260 °C and 200 psi *(30)*. When manufacturing puffed rice, pressurized steam is used to cook the rice in the gun puff chamber *(73)*. The pressure is released following

cooking, and the rice dries quickly, which changes the texture and “puffs” the rice. A similar method is used to process wheat berries for puffed wheat cereal, except there is a pre-treatment of the berries in a salt solution. After being puffed, the grains are further dried to a moisture content of 1-3% *(30)*.

A gun-puffed extruded product, like toasted oats cereal, is made through mixing raw ingredients and feeding the mix through a cooking extruder. This is formed into shapes at a temperature around 70 °C *(30)*. These are dried before being puffed. Similar to the whole grain cereals, the extruded product is dried further to a moisture of 1-3% *(30)*. This drying process can also include sifting out pieces that may have broken during the gun puffing process. The cereal is then packed into cereal boxes.

1.2.4.2 Production of Peanut Butter

Commercial creamy peanut butter is a mixture of roasted peanuts, sugar, salt, and stabilizers *(44)*. When manufacturing peanut butter, incoming raw peanuts are shelled, roasted, blanched, then ground into a paste. Peanut roasting is a lengthy process, approximately 40-60 min at 160 °C *(44)*. During the roasting process, the nut dries from 5% moisture to 0.5% moisture, then the nut browns, changing flavor, color, and texture *(74)*. The nuts are then cooled and blanched. Blanching removes the skin and cleans the nut.

To create peanut butter, the prepared peanuts are ground into paste in two steps. The first grinding reduces the peanuts to a medium grind and the second grind reduces the peanuts into a paste *(74)*. At this step, salt may be added depending on the formulation. Grinding may produce some heat, where temperatures can reach 80 °C *(74)*.

Stabilizers are added to prevent the separation of oil from the rest of the peanut paste. Stabilizers in peanut butter include: hydrogenated vegetable oils, monoglycerides, diglycerides, or any combinations of them *(74)*. With the addition of the stabilizers, sugar and salt may also be incorporated at this point. Since the stabilizers can be solid at room temperature, they are added warm to the second grinding process. This should be at a temperature of 60-74 °C *(44)*. The peanut butter is then cooled and packed into jars.

1.2.4.3 Pasteurization and Roasting of Almonds

The USDA and California Board of Almonds require all almonds to be processed using a method that delivers a 4-log CFU reduction of *Salmonella (5).* To achieve this reduction, almonds are chemically and heat treated. Propylene oxide is used to pasteurize almonds in a pressurized chamber for 4 h *(55)*. The almonds are then ventilated for 2 d to achieve a reduction of propylene oxide on the almond. This chemical treatment is capable of obtaining a 5-log CFU reduction of *Salmonella*.

Heat can be used to achieve a 4-log CFU reduction as well. Hot air roasting of almonds is one of the most common almond roasting techniques used. This process runs at temperatures between 130 and 155 °C, for times between 10 and 40 min, depending on the roasting temperature used *(75)*. However, *Salmonella* can be very resistant to heat, and these processes may not achieve a 4-log CFU reduction. Treatment with radio frequency, moist air, oil roasting, and steam heating methods have been investigated as possible alternatives for enhancing the reduction of *Salmonella.*

1.3 Bacterial Response to Desiccation Stress

1.3.1 Desiccation Response and Stress Cross Protection

The increased association of *Salmonella* to low water activity foods has generated extensive interest in elucidating its response to a desiccated environment. *Salmonella* can survive in low water activity foods for months. For example, the epidemiological investigation of the peanut butter outbreak in 2009 detected *Salmonella* in five month old peanut butter *(49)*. Such observations have confirmed that *Salmonella* can survive long term in harsh environments, which may trigger the cellular response to other stressors.

When bacteria are introduced into a stressful environment, such as dry conditions, the cells must respond and protect themselves. When exposed to a low moisture environment, bacteria activate mechanisms to retain cellular water to be able to maintain biochemical functions. A change in water flow across the membrane causes the cell to transport potassium and solutes into the cell *(61)*. The influx of solutes balances the osmotic pressure from within the cell to the environment around it. This ion flow keeps cell turgor initially. Long term changes to the cell membrane follow, which preserve cytoplasmic water *(49)*. The cell requires internal water to keep essential proteins hydrated and to move nutrients. Without cytoplasmic water, vegetative cell viability is gradually reduced.

The second step of cellular response to desiccation is long-term adaptation to the environment. This step involves cellular mechanisms that the cell activates to survive. Long term desiccation stress responses involve modifications of the membrane, re-naturing misfolded proteins, and conserving as much energy as possible.

Some studies have investigated *Salmonella's* cellular response to desiccation. Deng et al. conducted a transcriptomic study in peanut oil, a low water activity food *(27)*. This report evaluated the transcriptome during a period of nine days. Changes to the proteins transcribed were initially observed, and an overall decrease of metabolic activity were noted afterwards. To survive desiccation, the cell must synthesize new proteins to adapt to stress. The stress response of the cell involves alternate sigma factors to modify protein synthesis, chaperone proteins to promote re-folding of other proteins, and proteins that alter the cell membrane. Under desiccation conditions, cells transcribe the alternate sigma factors RpoH and RpoE, which are also involved in heat shock response *(27)*. Those sigma factors direct the transcription of other proteins involved in the heat shock response, such as chaperone proteins DnaK and DnaJ *(27)*. The desiccation response appears to use similar protein responses to respond to other stresses. A common stress pathway often leads to cross-protection to survive multiple stresses after being exposed to only one stress.

Another transcriptomic study reported similar results. Gruzdev et al desiccated *Salmonella* Typhimurium on filter paper for 22 hours and found 90 upregulated genes *(34)*. This observation suggested that there is a relatively significant initial stress response needed to survive desiccation. The same investigation generated mutants in some of those genes to confirm their role on desiccation resistance. When two stress regulators were deleted, RpoE and Fnr, survival after desiccation was decreased *(34)*. RpoE is involved in heat shock response and Fnr is a global stress regulator. The deletion of either of these resulted in cell death, during desiccation.

The transcriptomic studies are an effective approach to elucidate how the cell reacts to desiccation stress and to what extent the cellular response to desiccation is similar to the response to other heat stresses. *Salmonella* has been reported to have increased thermal tolerance when desiccated, which may be due to stress cross protection. Early theories of the increased stress resistance stated that the cell survived increased temperatures due to the lack of water in the cell. When cells are in a wet environment, there is plenty of water to hydrate essential proteins. During heating, the water molecules vibrate next to protein bonds and denatures those bonds *(56)*. In a desiccated state, the water by protein bonds do not exist, as a result, proteins retain their native structure even at higher temperatures. The lack of water hydrating proteins could also cause the proteins' dipoles to interact, which may also be involved in stabilizing them *(56)*.

However, increased evidence suggests that the enhanced thermal stability may also be due to stress response. The transcriptomic studies discussed above indicated that the cells synthesized proteins that were involved in heat stress response *(31, 33)*. The synthesized proteins allowed the cell to pre-adapt to the stress. There was no lag time for the cell to respond to the new stress. Guzdev et al. observed that in rehydrated cultures, cells that had been desiccated then re-introduced into a wet environment, survived heat better than cultures that had not been dehydrated at all *(35)*. This finding suggested that the cell's response was not just due to the water content in the environment, but it was a response due to new proteins in the cell.

Salmonella's increased thermal resistance after desiccation is not the only stress that leads to cross protection. Desiccated cells have been shown to be more resistant to salt, UV, sanitizers, and bile salts *(35)*. The stress response of *Salmonella* can allow the

cells to survive even more stressful environments. Cross protection would favor the survival in environments that were assumed to be safe for consumers. The presence of those cellular mechanisms may lead to foodborne disease outbreaks in foods previously assumed to be safe.

1.3.2 Thermal Resistance of *Salmonella* in Low Water Activity Foods

Drying foods has been an effective preservation technique to delay food spoilage used by humans for millennia. The lack of water in dry foods is known to stop microbial growth and preserve foods for long periods of time. This enhanced preservation can be explained by the concept of "low water activity" of the foods. Water activity is a parameter that describes the influence of water on the food system. It is mathematically defined as the ratio of equilibrium of partial vapor pressure of water in the system to the equilibrium partial vapor pressure of pure water at the same given temperature *(59)*. This ratio is a good predictor of whether microbes can grow in the food. High water activity foods have water available for microbial growth, while lower water activity foods do not have sufficient water to support growth. Based on multiple studies, the minimum water activity for growth of multiple organisms has been determined. Table 1.2 shows a few food related microorganisms.

Table 1.2. The minimum water activity needed for microbial growth

Minimum Water Activity	Microorganisms
0.95	*Escherichia, Shigella, Bacillus*
0.91	*Salmonella, Listeria monocytogenes*
0.87	*Staphylococcus aureus*
0.61	Molds, yeasts
<0.60	No microbial growth

Taken from *(70)*

Growth is completely inhibited at water activity conditions below 0.6, but the viability of surviving cells can still be observed for multiple species. As discussed before, *Salmonella* is more thermally resistant in low water activity foods. This has been reported in a variety of food matrixes. Studies have shown large differences among D-values of *Salmonella* in low water activity foods. Table 1.3 shows examples of D-values of *Salmonella* in various food matrices.

Table 1.3. D-values of *Salmonella* in low water activity food matrices

Food Matrix	Temperature (°C)	D-value (min)	Reference
Wheat flour	70	20.7	*(31)*
Peanut Butter	71	29.3	*(50)*
Pecans	120	20	*(18)*
Pet Food	71.1	5.45	*(21)*
Confectionary	80	40.07	*(57)*

As a comparison, the time needed for a 6.5 log CFU reduction of *Salmonella* in ground beef at 70°C is less than a minute *(43)*. This difference indicates that there is a significant increase in the thermal stability of *Salmonella* when the water activity is decreased. The table also illustrates the effect of different matrices on thermal stability. There is a large difference in the temperature needed between to achieve a 1-log CFU reduction for pecans and peanut butter. Similarly, at almost the same temperature, the D-value in peanut butter is almost 5-fold of similar value in pet food.

There has been some research into the specific effects of solutes on the thermal resistance of *Salmonella*. He et al. used four different formulations of peanut butter to investigate the differences in *Salmonella* inactivation kinetics *(38)*. That study observed that a peanut butter with higher carbohydrate content and lower fat level had greater D-

values than a peanut butter with higher fat and lower carbohydrates *(38)*. This finding may suggest that the carbohydrates in the food, including added sucrose, have a protective effect on the cells.

The effect of specific solutes on the thermal resistance of *Salmonella* has been studied in liquid media. Those reports indicated that solutes can both change the water activity of the matrix and the thermal resistance of *Salmonella*. Sucrose has the largest effect on the heat resistance of *Salmonella* in a broth media, compared to cultures containing glycerol, sorbitol, or fructose *(33)*. While that study was not performed in a low water activity food, its results may still be applicable. When compared to the peanut butter study above, it can be speculated that the sugar added to the peanut butter may have had a protective effect.

1.3.3 Lactic Acid Bacteria Stress Responses

As discussed above, a bacterial surrogate must have similar characteristics as the target pathogen. To identify a non-pathogenic organism that behaves similarly to *Salmonella* in low water activity foods, the stress responses of non-pathogenic organisms could be a viable approach. Lactic acid bacteria (LAB) are a very diverse bacterial category and many genera are considered GRAS organisms because they have been used in food fermentations. Their GRAS status makes particularly attractive for their application as surrogates. Because of their GRAS status and history of use in food products, they are proven to be non-pathogenic. However, to be a surrogate in low water activity foods, they must have similar properties to *Salmonella.*

The stress response of lactic acid bacteria to osmotic stress has similarities to *Salmonella's* response. Both quickly import osmoprotectants into the cell to stabilize

turgor. However, in Gram negative bacteria, the initial response to osmotic stress includes increasing the cellular concentration of potassium. This response does not occur in LAB cells *(48)*, instead they uptake more amino acids and other osmoprotectants to retain turgor when under osmotic stress.

Some LAB also synthesize stress response proteins when subjected to osmotic stresses. Some strains of *Lactococcus lactis* synthesize heat shock proteins after exposure to osmotic stress induced by salt *(46)*. These proteins include the chaperone proteins DnaK and DnaJ, which are also induced in *Salmonella* under osmotic stress. Other studies have found similar responses in other LAB species. The expression of chaperone proteins under osmotic shock is a common response across many species of lactobacilli and lactococci. The expression of small heat shock proteins and proteases is also a common general stress response in many LAB *(48)*.

The occurrence of stress cross protection is not limited to *Salmonella.* In fact, many species of bacteria use components of the same stress response system to react to multiple stresses. Having a similar response to the same stress across species may allow for comparisons. Cross protection is not a novel system in *Salmonella* it may be common.

CHAPTER 2

MATERIALS AND METHODS

2.1 Selection and Screening of Potential *Salmonella* Surrogates

2.1.1 Use of NCBI BLAST to Identify Surrogates

To identify potential surrogates, a comparison of genetic relatedness was conducted. Phylogenetic trees were built to determine the possible genetically related GRAS strains to *Enterococcus faecium.* To identify potential strains, the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) was utilized. BLAST is a search algorithm used to quickly search large databases for genetically similar sequences *(2).* Since *Salmonella* is Gram negative, and there are very few Gram-negative GRAS microorganisms, similar genes in *E. faecium* were used. Based on Grudzdev et al, the FNR/CRP family of transcriptional regulators was compared, since they are up regulated in *Salmonella* during desiccation stress. Unlike rpoS, which is also upregulated by *Salmonella* during desiccation stress, FNR/CRP transcriptional regulators are found in both *Salmonella* and *E. faecium (34).* While The two FNR/CRP transcriptional regulators were entered as search queries, using the BLAST server defaults, which are the non-redundant protein sequences as the database and the protein-protein BLAST algorithm. The scoring matrix used was BLOSUM62, with the expect threshold set at 10 and the word size as 6. The *E. faecium* proteins were excluded from the search. Proteins from GRAS microorganisms with a similarity score of more than 50% were downloaded and used for tree building.

2.1.2 Phylogenetic Tree Building

The similar GRAS proteins and query protein were compiled into a single file in FASTA format. Each file was aligned, a method used to find similarities between the proteins, using the program MUSCLE *(28)*. This was done on the Sapleo cluster at the University of Georgia's Advanced Computing Resource Center. The resulting file was converted into Phylip format using the Centre for Integrative Bioinformatics Alignment Format Converter *(19)*.

Before building a phylogenetic tree, the amino acid replacement model selection software ProtTest was utilized *(26)*. This program finds the best-fit model of evolution with the given data. The recommended model was applied to the tree building. To build the phylogenetic tree, the program RAxML was used *(68)*. This program is a determination of the maximum likelihood of an evolutionary relationship between the given proteins. The trees were produced through imputing the aligned sequences and analyzing them with the amino acid replacement model found with ProtTest and the GAMMA rate of heterogeneity. GAMMA was selected since it is the best model for small trees *(69)*. The phylogenetic trees were developed using a rapid bootstrap analysis and maximum likelihood search. The resulting bipartitions tree was viewed using FigTree *(58)*.

2.1.3 Bacterial Strains and Cultivation

The cell cultures chosen from the phylogenetic comparisons were either grown from frozen (-60°C) glycerol stocks available at the Center for Food Safety culture collection or purchased from ATCC as lyophilized pellets. All cultures were grown

through inoculating the either frozen or lyophilized bacteria into broth cultures of either tryptic soy broth (TSB, BBL Beckton Dickinson and Co., Franklin Lakes, NJ), De Mann, Rogosa, and Sharpe broth (MRS, BBL), or brain heart infusion broth (BHI, BBL) according to table 2.1. These were grown in shaker cultures overnight. Stocks were re-grown every two months. *Salmonella* was confirmed by streaking out the cultures on xylose lysine deoxycholate agar (XLD, BBL). The stocks were stored at 4°C.

Table 2.1 Bacterial strains used in this research and their growth media

Bacteria	**Strain**	**Media Used**
Salmonella Agona	F 5567	TSA/TSB
Salmonella Typhimurium	ATCC 14028	TSA/TSB
Salmonella Enteritidis	2415	TSA/TSB
Salmonella Heidelberg		TSA/TSB
Salmonella Tennessee	K4643	TSA/TSB
Salmonella Newport	MH57137	TSA/TSB
Lactococcus lactis	ATCC 7962	MRS
Pediococcus acidilactici	ATCC 8042	MRS
Pediococcus acidilactici	HPS	MRS
Pediococcus claussenni	ATCC BAA-344	MRS
Streptococcus thermophilus	ATCC 19257	BHI
Enterococcus faecium	NRRL-B2454	TSA/TSB

2.1.4 Inoculation onto Toasted Oats Cereal (TOC)

The procedure was adapted from Chick, 2011 *(22)*. Aliquots (100 µL) of stock culture was inoculated into 40 mL of broth media. Depending on the strain, either TSB, MRS, or BHI broth were used for cultivation. All cultures were grown overnight at 35°C in a shaker culture. Samples of 20 g of commercial toasted oats cereal (TOC) were weighed into sterile stomacher bags. In a biosafety cabinet, the TOC was mixed with the 40 mL broth culture and 360 mL of sterile water and bags was shaken by and for 1 min.

The TOC samples were strained through a sterile metal strainer and placed on a sterile metal tray to dry. Trays was placed in a 37°C incubator for 18-24 h to dry. Dried, inoculated TOC samples were ground using a sterile mortar and pestle. Ground TOC were stored in an open petri dishes in a desiccator. The desiccator contained saturated solutions of magnesium chloride (MgCl) to allow equilibration of TOC at a water activity of 0.33.

2.1.5 Initial screening at 75°C

Ground TOC was stored for 7 to 12 days in the desiccator before thermal inactivation experiments. After equilibration, ground cereal samples were packed into 0.2 mL sterile PCR tubes until half-full. The water activity of each sample was measured before thermal inactivation. For each time point three PCR tubes were combined into one sample. For screening, viable counts were determined at an initial time zero and after 30 min at 75°C. All microbiological testing was performed in triplicate. The samples were placed into a pre-heated heating block (Thermo-Fisher, Waltham, MA) and placed in an ice bath after 30 min. The PCR tubes were then immersed in a 10% bleach solution for 10 seconds. The opened tubes were placed into tubes of 10 mL of 0.1% buffered peptone water (PW, BBL). The tubes were then serially diluted in 0.1% PW and plated in duplicate onto either trypticase soy agar (TSA, BBL), MRS, or BHI. The plates were then incubated at 37°C for 18-24 hours.

2.2 Thermal Inactivation of *Salmonella* and Surrogates on TOC

For comparison of the inactivation kinetics, a *Salmonella* cocktail was prepared, as well as *P. acidilactici* and *E. faecium* NRRL-B2354. The *Salmonella* cocktail included the strains listed below (Table 2.2). The strains were inoculated onto TOC according to

section 2.1.4. Inactivation preparation. Inactivation kinetics were determined at 80, 85, 90, and 95°C. At 80°C, samples were collected every 30 min for 3 h. At 85°C, samples were collected every 15 minutes for 1.5 hours. At 90°C, samples were collected every 10 min for 1 h. At 95°C, samples were collected every 10 min for 1 h.

Table 2.2 *Salmonella* cocktail serovars, strain numbers, and sources

Serovar	Strain	Source
Enteritidis	2415	Almonds
Agona	F5567	Cereal
Typhimurium	ATCC 14028	
Tennessee	K4643	Peanut Butter
Newport	MH57137	

2.3 Thermal Inactivation Kinetics of *Salmonella* and Surrogates in Peanut Butter

2.3.1 Inoculation of Peanut Butter

To examine the surrogacy potential for *E. faecium* and *P. acidilactici* in other low moisture foods, a comparison between a *Salmonella* cocktail, *E. faecium*, and *P. acidilactici* in peanut butter was performed. For this experiment, the *Salmonella* serovar Newport was replaced with Heidelberg in the cocktail. The updated cocktail is below (Table 2.3).

Table 2.3 *Salmonella* cocktail serovars, strain numbers, and sources

Serovar	Strain	Source
Enteritidis	2415	Almonds
Agona	F5567	Cereal
Typhimurium	ATCC 14028	
Tennessee	K4643	Peanut Butter
Heidelberg	MH27651	

The inoculation and thermal inactivation methods were adapted from Ma et. al *(50)*. For the *Salmonella* cocktail, each serovar was inoculated into its own aliquot of 20 mL of TSB. *E. faecium* and *P. acidilactici* were inoculated into five separate aliquots. This produced a total of 100 mL of inoculum. The cultures were incubated overnight in a shaker culture at 35 °C.

To prepare the inoculum, the bacteria was centrifuged at 906 g for 10 minutes and washed with 20 mL phosphate buffered saline (PBS). The washed culture was centrifuged, re-suspended in 8 mL PBS, and consolidated into a single tube. The consolidated culture was centrifuged for 15 minutes and re-suspended in 600 µL of PBS. This was used as the inoculum. To inoculate into peanut butter, 60 g of creamy peanut butter was weighed into sterile stomacher bags and placed into a 50 °C water bath to melt. Prior to inoculation, the peanut butter was removed from the water bath and allowed to cool to 37 °C. The melted peanut butter was inoculated and mixed with a sterile spatula for 4 minutes. The bag was then stomached at 260 rpm for 2 minutes. The inoculated peanut butter was stored in a sealed bag for 3 to 7 days before use.

2.3.2 Thermal Inactivation in Peanut Butter

For each thermal inactivation, 1 (±0.05) gram samples of inoculated peanut butter were weighed into 7 oz sterile stomacher bags. The peanut butter was spread into a thin rectangle, approximately 3 × 6 cm. The bags were suspended into a hot water bath. Three samples were removed at pre-determined time points and held in an ice bath for 1 minute. Temperature was monitored by a thermocouple in a bag of uninocculated peanut butter. A sample of the inoculated peanut butter was used to determine water activity.

After being removed from the ice bath, the samples were stomached with 9 mL BPW with 0.1% Tween 80 for 2 minutes at 260 rpm. The samples were further diluted using BPW and plated onto the appropriate media. Plates were incubated for 18-24 hours at 38 °C.

Thermal inactivation curves were performed at four temperatures: 63, 68, 73, and 77 °C. At 63 °C, samples were collected at 15 and 30 minutes, then every 30 minutes for 3 hours. At 68 °C, samples were collected at 7 and 15 minutes, then every 15 minutes for 1.5 hours. At 73 and 77 °C, samples were collected at 5 minutes and 10 minutes, then every 10 minutes for 1 hour. Each inactivation curve was performed in duplicate. Data was analyzed using the Weibull model; the methods are found in section 2.6.

2.4 Validation of Heat Resistance on Almonds

In 2014, the California Board of Almonds approved the use of *E. faecium* NRRL-B2354 as a surrogate for *Salmonella* in almond processes *(11)*. This method includes a proof of heat resistance study, which uses one temperature and time to show heat resistance. This was adapted to investigate the heat resistance of *P. acidilactici.*

The *Salmonella* cocktail used in section 2.3, *E. faecium, P. acidilactici*, and *Salmonella* Enteritidis PT30 were inoculated onto almonds to compare the heat resistance. The California Board of Almonds used *Salmonella* Enteritidis PT 30, the causative agent in the 2001 outbreak, to validate *E. faecium* as a surrogate in almonds *(42)*. *Salmonella* Enteriditis PT30 was added to compare the heat resistance of a single serovar versus a cocktail. Almonds were inoculated by adding 20 µL of stock solutions into 10 mL of broth culture media. The broth cultures were incubated in a shaker culture at 35 °C for 18-24 h. For the single strain inoculations, 1 mL of broth was spread onto 5

plates of solid media. For the *Salmonella* cocktail, 1 mL of each serovar was spread onto 1 plate of solid media, for a total of 5 plates. These plates were incubated at 38 °C for 24 h. This procedure produced a bacterial lawn.

To prepare the inoculum, 6 mL of PW was pipetted onto the prepared plates. A sterile cell scraper gently suspended the cells in the PW. The inoculum was pooled, for a total inoculum of 25 mL, and mixed on a stir plate for 1 min. Nonpareil supreme 20/22 raw, chemically pasteurized almonds (Nuts.com, Cranford, NJ) were weighed (200 g) into sterile stomacher bags. After mixing, the inoculum was pipetted into the almonds and shaken for 1 min. To contain the drying almonds, a ¼ in.-wire mesh covered the top of a metal tray. Two layers of filter paper was placed on top of the wire mesh. Inoculated almonds were placed on the filter paper and loosely covered with tin foil. The trays were left to dry in a biosafety hood for 24 h. The dried almonds were stored in sterile stomacher bags for 24-48 h.

Heat resistance was determined by measuring the log CFU reduction after 15 min at 138 °C. Six 25 g samples of inoculated almonds were weighed into stomacher bags. Three samples were used to find initial counts and three were used to measure the log reduction. The samples were placed on trays made from wire mesh and placed into a hot air oven (Thermo-Fisher, Waltham, MA). The time was measured starting once the oven reached 138 °C. This took approximately 5 min. Following the 15 min, the almonds were placed into a stomacher bag, then immersed in an ice bath for 3 min.

To plate the samples, 25 mL PW was added to the almonds sample. This was shaken for 1 min. The bag then rested for 4 min, then was shaken for 15 s. The sample

was then serially diluted in 9 mL PW and plated on the appropriate media. The plates were incubated for 18-24 h at 38 °C. The log reduction after 15 min was measured.

2.5 Sequencing and Confirmation of the Identity of GRAS Surrogate

Whole genome sequencing was conducted to confirm the identity of the *Pediococcus acidilactici* strain. To extract DNA for the sequencer, the DNeasy Blood and Tissue extraction kit was used (Qiagen, Hilden, Germany). Since the surrogate was gram positive, the protocol required the use of an enzymatic lysis buffer before extraction. The DNA was sequenced using an Illumina HiSeq 200 (Illumina, San Diego, CA). Raw sequence reads (FASTQ) were uploaded to the server. Sequence quality was measured using FASTQC. Sequence assembly was performed using SPAdes *(53)*. The resulting contigs file was uploaded an run using BLAST to identify the surrogate *(39)*. The genome was annotated using the RAST server and SEED Genome Viewer *(7, 12, 54)*.

2.6 Statistical Analyses

To analyze the TOS and peanut butter data and compare the curves, Microsoft Excel with the add-in GInaFit was used to fit the data to a Weibull model *(32)*. The δ and β values were determined and to compared between the curves. The root mean square error (RMSE) and R^2 value were employed to analyze the fit of the curve. For comparison of δ values among strains, a one-way ANOVA analysis of δ values was performed, followed by a Tukey-Kramer HSD test, using JMP (SAS, Cary, NC). Following the completion of all time points, z-values were calculated through the equation $z=(T_1-T_2)/(\text{Log}(\delta_1)-\text{Log}(\delta_2))$ *(72)*. Regressions of the temperature versus the log of the δ-values was performed to determine z-values. The z-value was approximated

through plotting a linear regression curve and determining the change in temperature needed for a 1 log CFU reduction along the curve.

CHAPTER 3

RESULTS

3.1 Phylogenetic Trees Using *E. faecium* FNR/CRP Transcriptional Receptors

E. faecium NRRL-B2354 has two FNR/CRP family transcriptional receptors. Their accession numbers are WP_010738809.1 and WP_002289290.1 *(10, 14)*. These two transcriptional receptors were used as BLAST queries to identify potential similar proteins in GRAS microorganisms. Each transcriptional regulator had its own tree built to maximize the identification of potential surrogates. For the first transcriptional receptor, WP_01078809.1, the BLAST search found similar transcriptional receptors in the genera *Pediococcus* and *Lactobacillus*. ProtTest found the substitution model VT to be the best fit, and it was incorporated as the model of protein substitution. The resulting tree, with a maximum likelihood score of -2106, is in figure 3.1.

This tree shows that there were no close phylogenetic relationships between *E. faecium* FNR/CRP transcriptional regulators and other bacteria. *E. faecium* was clearly separated from other bacteria. This relationship was well supported, with a bootstrap value of 100. Phylogenetic proximity is measured through linear distance between branches, and there was a long distance between *E. faecium* and the nearest branch. There have been many mutations in both *E. faecium* and *Lactobacillus* since they diverged. What was interesting about the tree is that there is a distant relationship between *E. faecium* and *P. acidilactici*, though both are thermally resistant in low water activity

foods *(21)*. This suggests the method of thermal resistance may not be dependent on FNR/CRP transcriptional regulators.

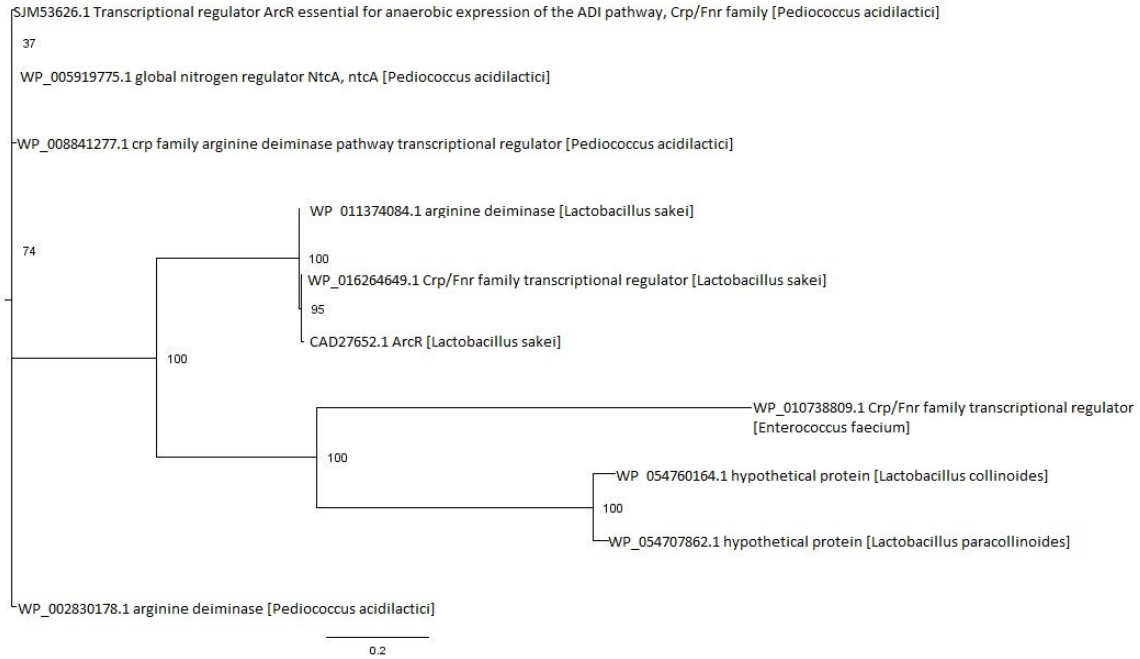

Fig. 3.1 Unrooted phylogenetic tree of WP_01078809.1 and related proteins

The second tree, with WP_002289290.1, compared *E. faecium* to *Lactobacillus* and *Lactococcus* species. ProtTest found the substitution model to be LG, and it was used in the subsequent tree building. The phylogenetic tree, with a maximum likelihood value of -1491, is shown in figure 3.2.

Figure 3.2 displays the relationship between *E. faecium* and *L. lactis.* A circular tree was used to better show the relationship between *E. faecium* and *L. lactis.* While they are not closely related, they share a well-supported branch (bootstrap value of 100). This relationship suggests a shared ancestor. While the linear distance between the individual

proteins and the shared ancestor is long, they still may have similar FNR/CFP transcriptional receptors. The large difference between the *Lactobacillus* species and both *E. faecium* transcriptional receptors shows there is no clear relationship between the two. Therefore, *Lactobacillus* species were removed from consideration of potential surrogates.

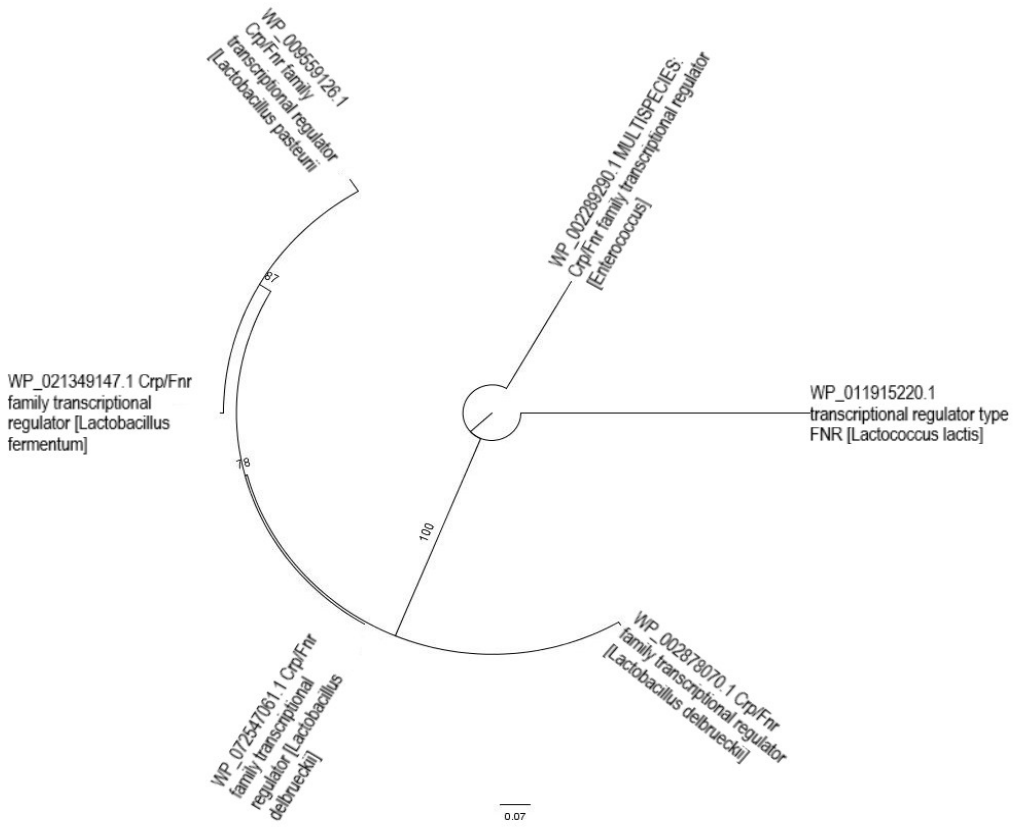

Fig. 3.2 Phylogenetic tree off WP_002289290.1 and related proteins

3.2 Screening of Potential *Salmonella* Surrogates

Each potential surrogate strain was screened once to get the log reduction after a half hour. The results are found in table 3.1.

Table 3.1 Extent of bacterial inactivation at 75°C in toasted oats cereal

Species	Strain	Log CFU Reduction
Salmonella Agona	F 5567	0.001
Salmonella Typhimurium	ATCC 14028	0.17
Lactococcus lactis	ATCC 7962	0.22
Pediococcus acidilactici	ATCC 8042	0.09
Pediococcus acidilactici	HPS	0.01
Pediococcus claussenni	ATCC BAA-344	>2.0
Streptococcus thermophilus	ATCC 19257	0.97
Enterococcus faecium	NRRL-B2454	0.20

The potential surrogates listed in Table 3.1, were compared to the two *Salmonella* serovars tested. To be considered for further testing, the potential surrogates had to have a similar log CFU reduction to the two *Salmonella* serotypes. *Streptococcus thermophilus* and *Pediococcus claussenni* were eliminated because their heat inactivation was 1.0 Log CFU or greater than *Salmonella*. *Lactococcus lactis* ATCC 7962, *Pediococcus acidilactici* ATCC 8042, and *Pediococcus acidilactici* HPS all had Log CFU reduction values comparable to *Salmonella*. However, *P. acidilactici* HPS was no longer considered since preference was given to commercially available strains. To choose between *L. lactis* ATCC 7962 and *P. acidilactici* ATCC 8042, a test at 95°C was performed.

A thermal kinetics trial at 95°C was performed to determine the best surrogate. The procedure is found in section 2.2. For this trial, *L. lactis* ATCC 7962 had very low counts and the thermal kinetics could not be determined. However, *P. acidilactici* ATCC 8042 had similar survival rate to *Salmonella* and the data is reported below.

3.3 Comparative Thermal Kinetics of *Salmonella* and Surrogates on TOC

3.3.1 Thermal Kinetics of *Salmonella* and Surrogates at 80, 85, 90, and 95 °C

For each temperature, at least two independent experiments were performed. Measurements during each of the individual trials were performed in triplicate. The average a_w for all samples was 0.34±0.05. At 80°C, the test was performed over 180 min, with seven total time points. The average microbial counts (Log CFU) versus time at 80°C is shown in Figure 3.3.

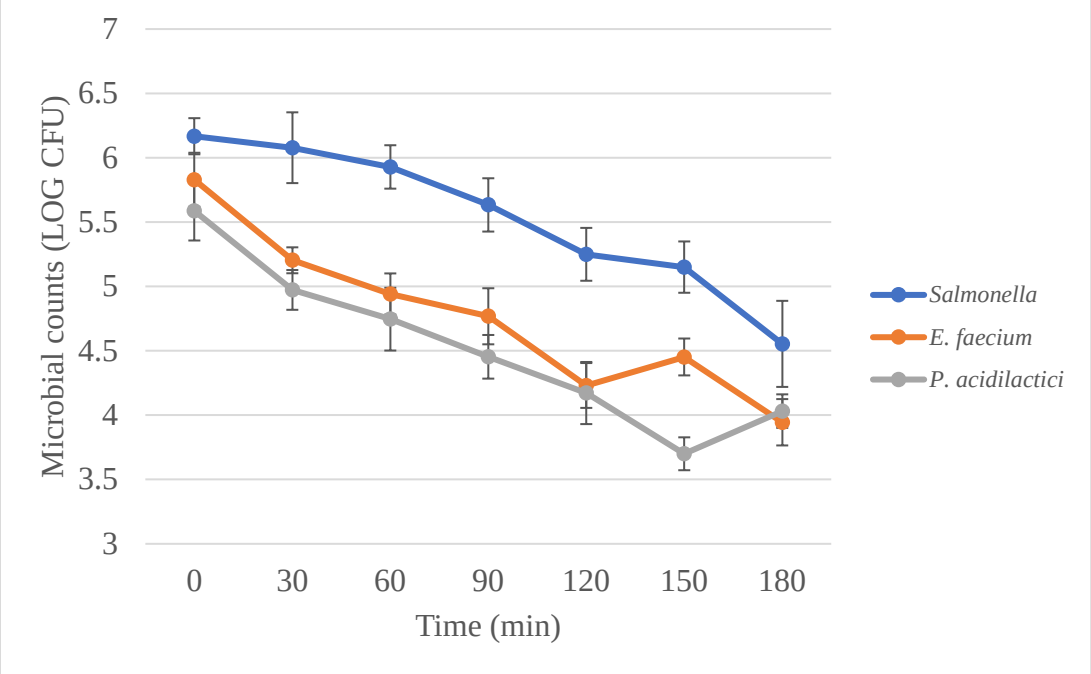

Figure 3.3 Inactivation of bacterial strains at 80°C in toasted oats cereal pre-equilibrated at a_w of 0.33. *Salmonella* included a cocktail of five strains.

Figure 3.3 shows that not only *E. faecium* and *P. acidilactici* were inactivated at a faster rate than *Salmonella*, the shape of the curves was different. After calculating the

inactivation rate parameters, the difference between *Salmonella* and the two LAB was confirmed (Table 3.2). *Salmonella* inactivation rate was almost twice the inactivation rate of *E. faecium* and *P. acidilactici.* A one-way ANOVA showed significant differences between ($p \leq 0.05$) δ values of the bacteria tested. A Tukey-Kramer HSD test for variances between means split the bacteria into two separate groups. *Salmonella* (group A) had significantly higher δ values than *E. faecium* and *P. acidilactici* (group B).

Table 3.2 Summary of thermal inactivation parameters at 80°C calculated with the Weibull model in toasted oats cereal.

Bacteria	δ value (min)	β value	R^2 value	RMSE
Salmonella	139.6 ± 17.2^{A}	1.36	0.96	0.10
E. faecium	69.5 ± 7.0^{B}	0.71	0.95	0.16
P. acidilactici	63.2 ± 3.2^{B}	0.56	0.87	0.23

Not only were the δ values different, but the β values were different as well. The β value in the Weibull model represents the shape of the curve. In the Weibull model, the shape can be concave ($\beta<1$) or convex ($\beta>1$). In concave curves, the probability of a cell dying decreases over time and the opposite is true in convex curves. At 80°C, the β value of *Salmonella* was 1.36, indicating that the curve is convex. The β values of *E. faecium* and *P. acidilactici* were 0.71 and 0.56, respectively indicating that the curves of the surrogates were convex.

At 85°C, the thermal inactivation curves become closer than at 80°C (Figure 3.4). The curves are all convex and have similar rates of inactivation. The average water activity of all samples was 0.33±0.04. Table 3.3 shows the thermal kinetics values for the

trials at 85°C. Despite that the δ values of *P. acidilactici* was almost three-fold smaller than the *Salmonella* δ value, a one-way ANOVA calculation resulted in no significant difference ($p \geq 0.05$) among the three bacteria. Both the R^2 value and the root mean square error value indicated that the Weibull model fit the data well. For all the bacteria, the R^2 value is above 0.9. The RMSE for all bacteria were low suggesting that the curve fit the data well.

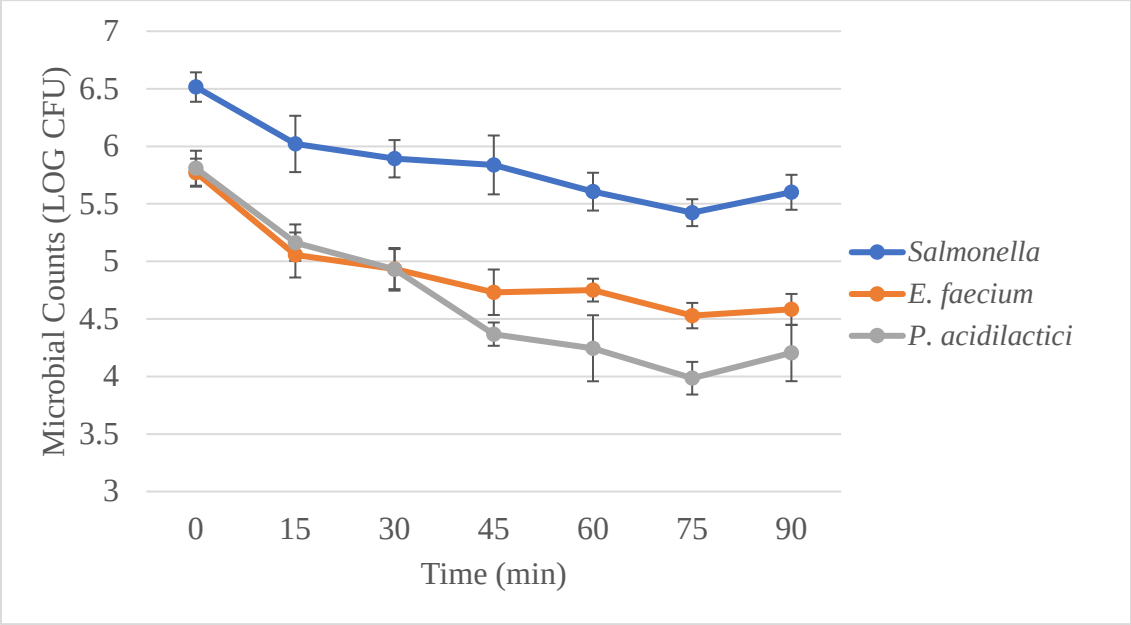

Figure 3.4 Inactivation of bacterial strains at 85°C in toasted oats cereal pre-equilibrated at a_w of 0.33. *Salmonella* included a cocktail of five strains.

Table 3.3 Summary of thermal kinetics parameters at 85°C calculated with the Weibull model in toasted oats cereal.

Bacteria	δ value	β value	R^2 value	RMSE
Salmonella	86.8 ± 47.2^A	0.50	0.92	0.11
E. faecium	45.5 ± 14.7^A	0.33	0.93	0.11
P. acidilactici	27.0 ± 4.0^A	0.52	0.91	0.28

At 90°C, the inactivation kinetics were closer than the previous temperatures. As can be seen in Figure 3.5, the shape of the curves were similar, as well as the rate of inactivation (Table 3.5). The average a_w for all samples was 0.33±0.04.

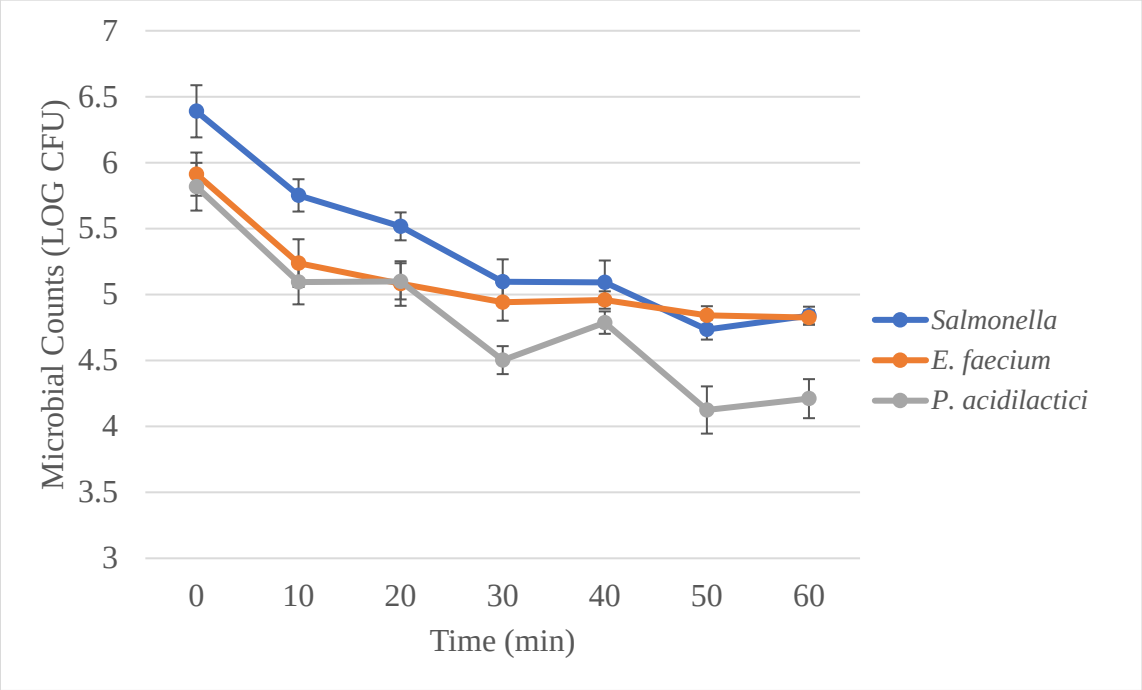

Fig. 3.5 Inactivation of bacterial strains at 90°C in toasted oats cereal pre-equilibrated at a_w of 0.33. *Salmonella* included a cocktail of five strains.

Table 3.4. Summary of thermal kinetics parameters at 90°C calculated with the Weibull model in toasted oats cereal.

Bacteria	δ value	β value	R^2 value	RMSE
Salmonella	23.4±7.4^A	0.51	0.93	0.15
E. faecium	41.1±8.1^A	0.26	0.97	0.06
P. acidilactici	24.7±2.0^A	0.56	0.84	0.41

At 90°C, the δ value of *Salmonella* was very similar to *P. acidilactici*'s and even smaller than the *E. faecium*'s, but it was not statistically significantly ($p \geq 0.05$).

At 95°C, the curves were more steep and faster than at lower temperatures, and the thermal inactivation time was short. This can be seen in figure 3.6.

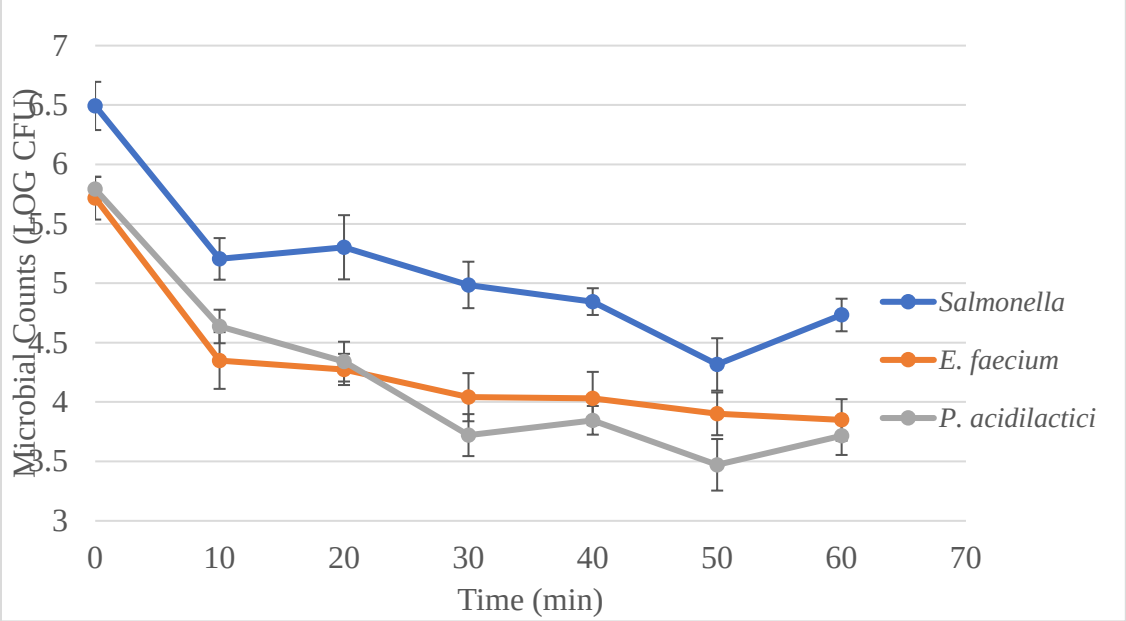

Fig. 3.6 Inactivation of bacterial strains at 95°C in toasted oats cereal pre-equilibrated at a_w of 0.33. *Salmonella* included a cocktail of five strains.

Table 3.5 Summary of thermal kinetics parameters at 95°C calculated with the Weibull model in toasted oats cereal.

Bacteria	δ value	β value	R^2 value	RMSE
Salmonella	7.8 ± 2.9^{A}	0.33	0.87	0.28
E. faecium	3.3 ± 2.5^{A}	0.19	0.94	0.07
P. acidilactici	7.0 ± 2.8^{A}	0.36	0.92	0.22

At 95°C, there was a marked reduction in δ values compared to the δ values at 90°C. This occurred across all bacteria. However, the bacteria still all had similar δ values, with a one-way ANOVA ($p \geq 0.05$) resulting in no significant difference among them. This data supported the idea there is a good potential for both *P. acidilactici* and *E. faecium* to be used as surrogates for *Salmonella* in commercial toasted oats cereal.

3.3.2 Comparison of z-values

Another parameter that characterizes the thermal stability of bacteria is the z-value. The z-value is the number of degrees (C) needed to change the δ value by one log *(72)*. This can be done through mathematical equation or estimation using a graph. The equation used is $z=(T_1-T2)/(\text{Log}(\delta_1)-\text{Log}(\delta_2))$ *(72)*. The z-value can be estimated through graphing the log of the δ values versus the temperature in °C (Figures 4.5, 4.6, 4.7). A regression line is plotted and the z-value is found through observing the change in degrees when the delta value is reduced by 1 log. The z-values calculated were 11.9, 9.5 and 15.7 °C, for *Salmonella*, *E. faecium* and *P. acidilactici*, respectively.

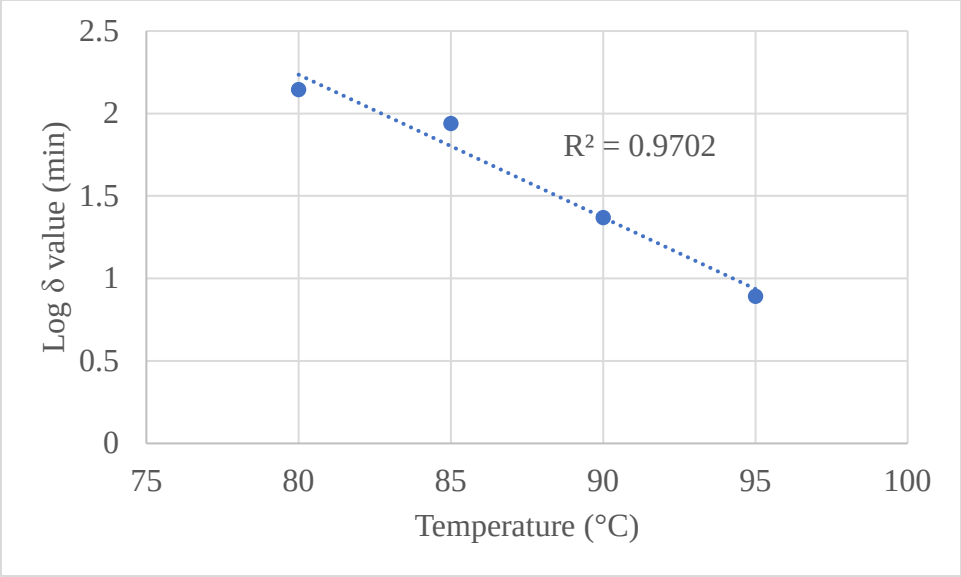

Fig. 3.7 Linear regression of temperature vs. Log δ value of heat inactivation of *Salmonella* in toasted oats cereal.

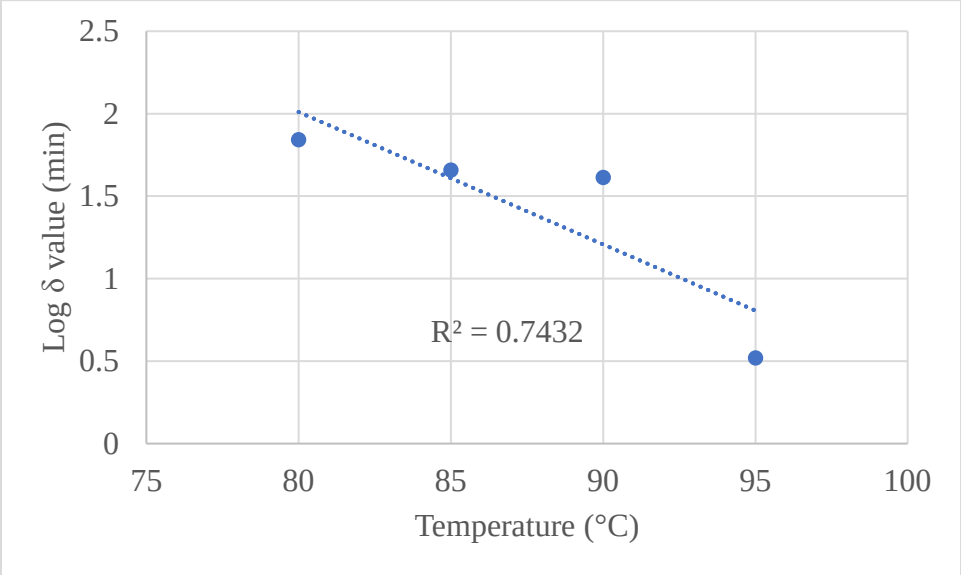

Fig. 3.8 Linear regression of temperature vs. Log δ value of heat inactivation of *E. faecium* in toasted oats cereal for calculation of z-value.

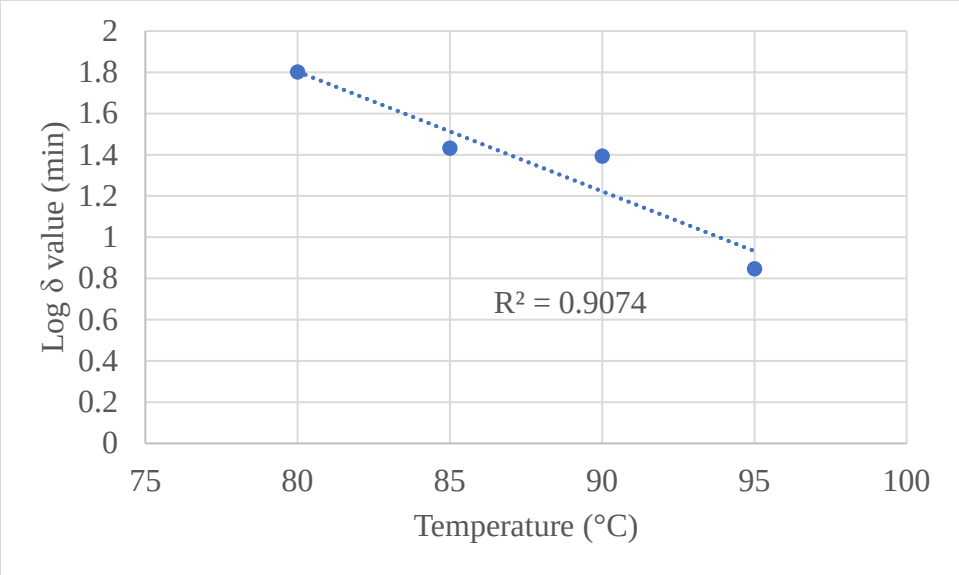

Fig. 3.9 Linear regression of temperature vs. Log δ value of heat inactivation of *P. acidilactici* in toasted oats cereal for calculation of z-value.

As can be observed in figures 3.7-3.9, the linear regression for *Salmonella* and *P. acidiliactici* was satisfactory ($R^2 \geq 0.9$). It follows that the graph estimations of both were close to the mathematical estimations, with the graph estimations being approximately 12 and 15 min, respectively. The graphical estimation for *E. faecium* is higher than the mathematically determined value, at 12.5 min. However, the regression line is not as well fit ($R^2=0.74$), and the data points used to determine the z-value were overestimated by the regression line. Therefore, the graphical version is higher.

3.4 Comparative Thermal Kinetics of *Salmonella* and Surrogates in Peanut Butter

3.4.1 Determination of δ values for *Salmonella, E. faecium* and *P. acidilactici*

At 63 °C, the average water activity of the samples was 0.39±0.07. The inactivation curves are in Figure 3.10. Figure 3.10 demonstrates that the inactivation of *Salmonella* was markedly faster than the other bacteria. Both *E. faecium* and *P. acidilatici* have an initial decline in population; however, it is less than *Salmonella.*

Salmonella has a more rapid initial inactivation and has a slight decline for the entire time series. A comparison of the kinetic parameters is reported in table 3.6.

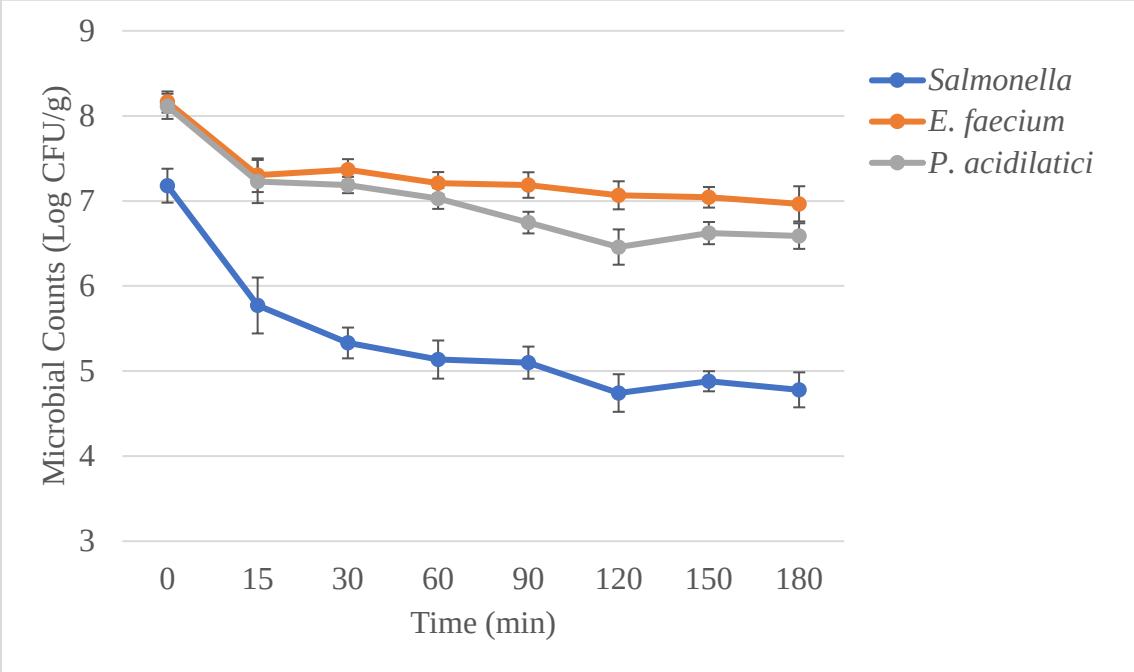

Fig. 3.10 Inactivation of bacterial strains at 63°C in peanut butter. *Salmonella* included a cocktail of five strains.

Table 3.6 Summary of thermal kinetics parameters at 63°C calculated with the Weibull model in peanut butter.

Bacteria	**δ value (min)**	**β value**	**R^2 value**	**RMSE**
Salmonella	2.0 ± 0.2^{A}	0.19	0.96	0.14
E. faecium	94.2 ± 26.0^{B}	0.16	0.93	0.09
P. acidilatici	$31.1\pm7.0^{A, B}$	0.27	0.93	0.13

Based on kinetics parameters *Salmonella* was inactivated more than 15-fold ($p \leq 0.05$) faster than *E. faecium.* Both surrogates were more heat resistant than *Salmonella* in peanut butter, which shows the differences in kinetic parameters between food matrixes.

At 68 °C, a similar pattern of differences between *Salmonella* and the two surrogates was observed (Figure 3.11).

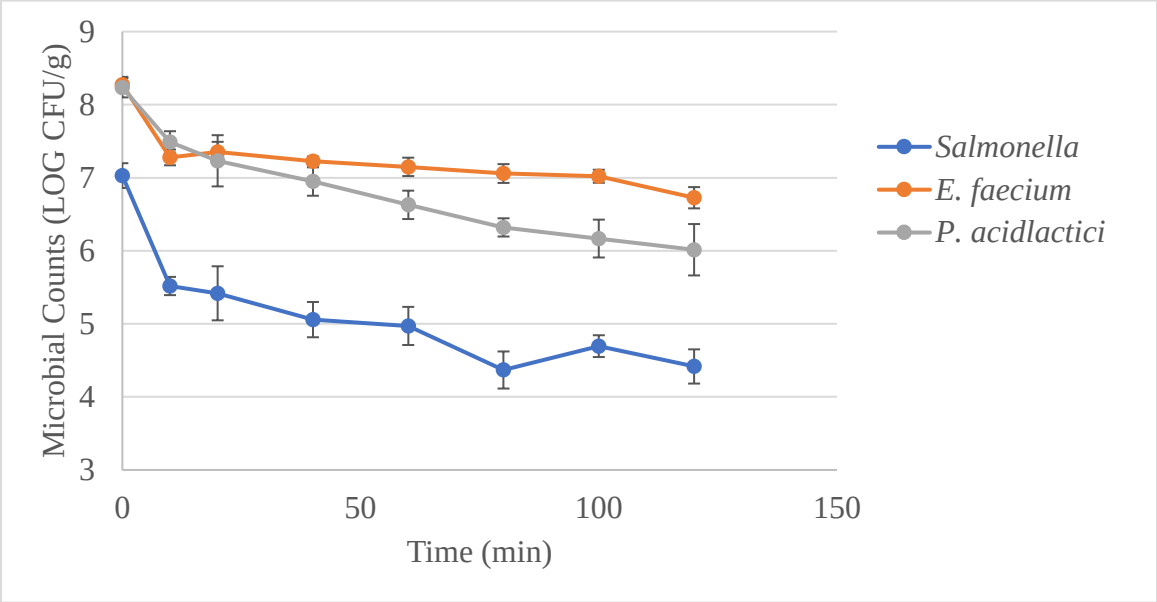

Fig. 3.11 Inactivation of bacterial strains at 68°C in peanut butter. *Salmonella* included a cocktail of five strains.

The average water activity of all samples was 0.44±0.03. Figure 3.11 displays similar inactivation curves. Both surrogates are inactivated slower than *Salmonella*, but the difference is not as large as at 63 °C.

Table 3.7 Summary of thermal kinetics parameters at 68°C calculated with the Weibull model in peanut butter.

Bacteria	δ value (min)	β value	R^2 value	RMSE
Salmonella	6.0±2.0[A]	0.27	0.90	0.27
E. faecium	27.0±7.3[A]	0.18	0.91	0.14
P. acidilactici	21.1±0.9[A]	0.46	0.99	0.05

Table 3.7 shows the kinetics parameters at 68 °C. Despite the graphical differences from Fig. 3.11 and differences of more than 3-fold, there were no significant differences ($p \geq 0.05$) between *Salmonella* and the two surrogates based on δ value. Compared to 63 °C, the δ value was greater for *Salmonella*. Typically, the δ value decreases as the temperature increases, as can be seen for both *E. faecium* and *P. acidilactici*.

At 73 °C, the thermal inactivation curves spread further than at 68 °C. Figure 3.12 displays the different inactivation curves. It follows the trend of the previous two temperatures, where *Salmonella* is inactivated faster than the two surrogate strains. The average water activity of all samples was 0.34±0.06, which is lower than previous time points. The thermal kinetics parameters can be seen in table 3.8.

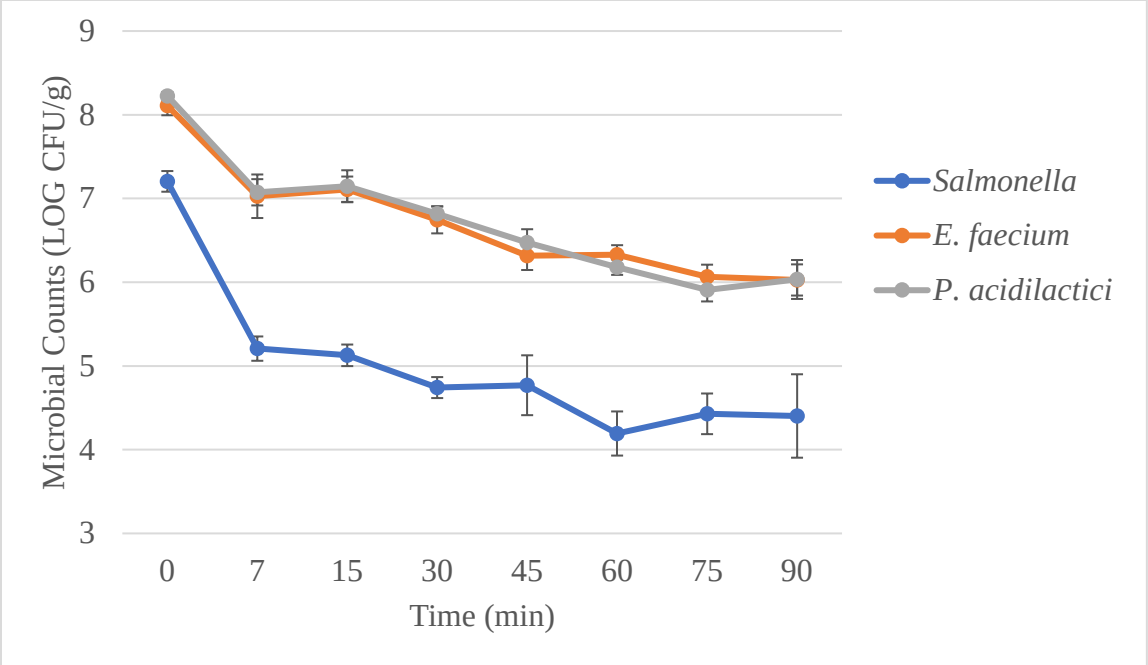

Fig. 3.12 Inactivation of bacterial strains at 73°C in peanut butter. *Salmonella* included a cocktail of five strains.

Table 3.8 Summary of thermal kinetics parameters at 73°C calculated with the Weibull model in peanut butter.

Bacteria	δ value (min)	β value	R^2 value	RMSE
Salmonella	1.5 ± 0.5^{A}	0.25	0.88	0.32
E. faecium	$13.0\pm3.4^{A, B}$	0.37	0.94	0.17
P. acidilactici	15.5 ± 5.9^{B}	0.45	0.94	0.19

Table 3.8 summarizes the kinetics parameters at 73 °C, which demonstrates there was a significant difference ($p\leq0.05$) between *P. acidilactici* and *Salmonella*. The δ value of *Salmonella* was almost 10 times smaller, but there were no significant differences between the two surrogates and *E. faecium* and *Salmonella.* All the δ values decreased as the temperature increased, which was expected. With the raise in the temperature, the

variability of the *Salmonella* data increased. The R^2 value was below 0.9 and the root mean square error was increased.

At 77 °C, the inactivation curves followed a similar trend to the previous three temperatures. The average water activity of all the samples was 0.38±0.06. The inactivation curves are in figure 3.13.

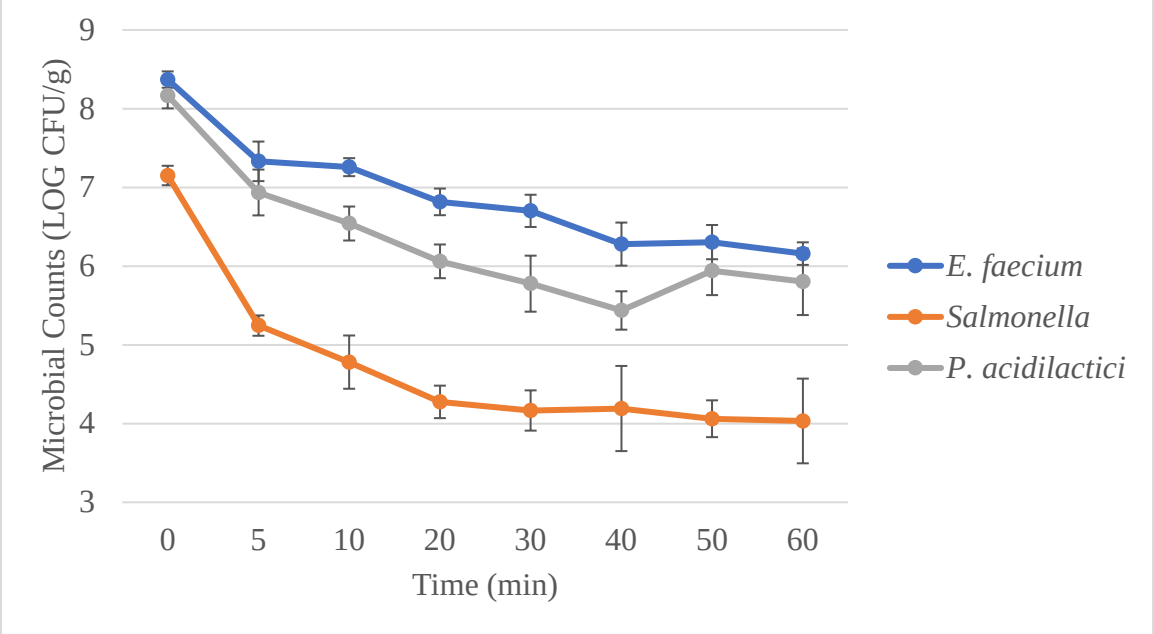

Fig. 3.13 Inactivation of bacterial strains at 77°C in peanut butter. *Salmonella* included a cocktail of five strains.

Figure 3.13 shows the inactivation curves at 77 °C. Like the previous three temperatures, *Salmonella* was inactivated faster than the two surrogates. There was more variation in the samples, with the standard deviation for some time points being larger than previous temperatures. The kinetic parameters are presented in table 3.9

Table 3.9 Summary of thermal kinetics parameters at 77°C calculated with the Weibull model in peanut butter.

Bacteria	δ value (min)	β value	R^2 value	RMSE
Salmonella	0.4 ± 0.1^{A}	0.22	0.89	0.28
E. faecium	8.9 ± 0.9^{B}	0.38	0.95	0.15
P. acidilactici	$2.6\pm1.9^{A, B}$	0.27	0.87	0.33

Table 3.9 displays the kinetics parameters at 77 °C, which shows that there was a significant difference ($p \leq 0.05$) between *Salmonella* and *E. faecium*, but there was no significant difference between *Salmonella* and *P. acidilactici.* At the previous temperature, this trend was reversed, where *P. acidilactici* was significantly different to *Salmonella.* There was a consistent decrease δ values for all bacteria for 68-77 °C, which was used to find z-values, found in the next section.

3.4.2 Z-values of *Salmonella, E. faecium,* and *P. acidilactici* in Peanut Butter

To compare the sensitivity of each bacteria or cocktail to increases in temperature, the z-values were calculated. The z-value calculations followed the methods in section 2.2. The z-values determined were 7.6, 13.6 and 13.0 °C, for *Salmonella*, *E. faecium* and *P. acidilactici*, respectively. For both surrogate bacteria, the z-values were calculated using the δ values from 63 and 77 °C. For *Salmonella,* the z-value was calculated using the δ value from 68 and 77 °C because the δ value from 63 °C did not follow the trend established by the other temperatures.

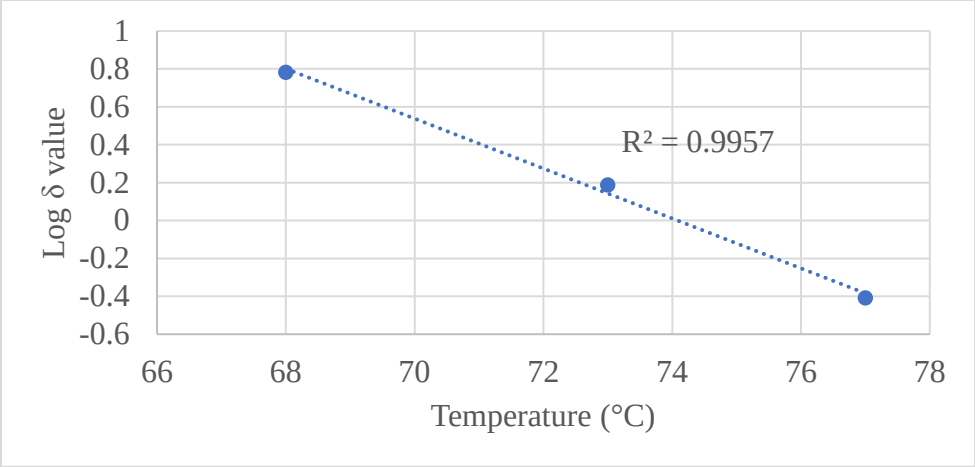

Fig. 3.14 Linear regression of temperature vs. Log δ value of heat inactivation of *Salmonella* in peanut butter for calculation of z-value.

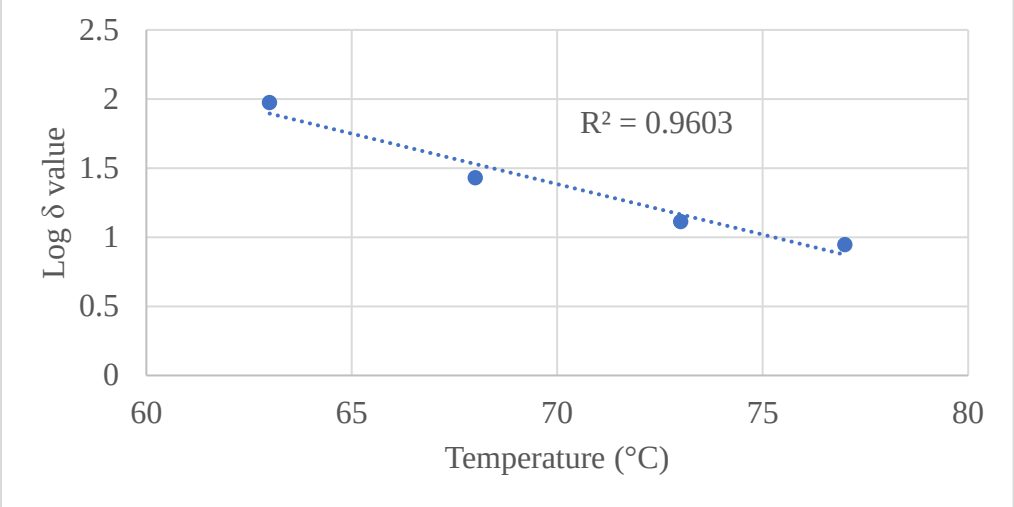

Fig. 3.15 Linear regression of temperature vs. Log δ value of heat inactivation of *E. faecium* in peanut butter for calculation of z-value.

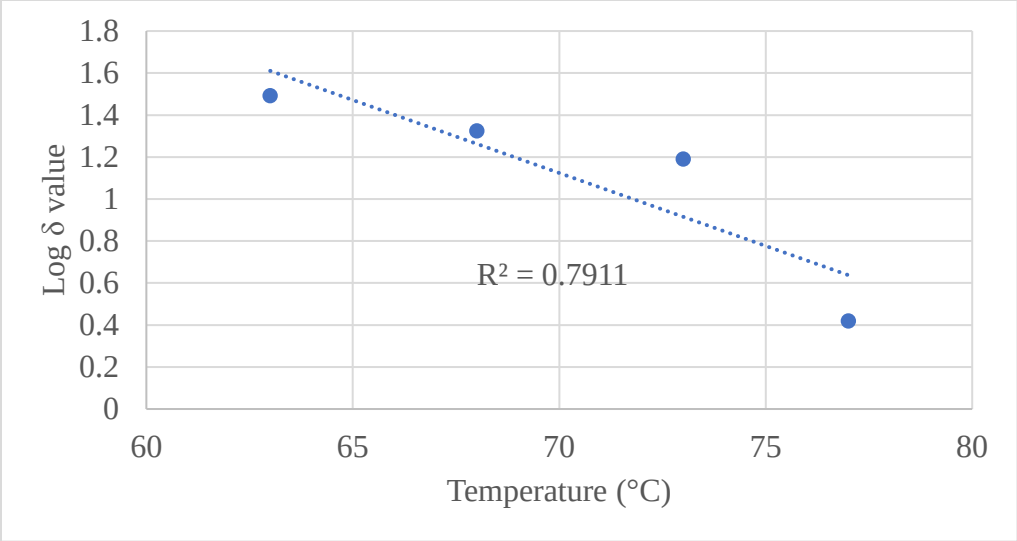

Fig. 3.16 Linear regression of temperature vs. Log δ value of heat inactivation of *P. acidilactici* in peanut butter for calculation of z-value.

Figure 3.14 was created using three data points, instead of four, to find a fit line. With the δ value from 63 °C, the linear regression was not well fit (R^2=0.47), instead of a well-fit line (R^2=0.99). Both regression lines for the surrogates are moderately well fit ($R^2 \geq 0.75$), which allows for estimation from graphs. Comparing the mathematically determined z-values, the graphs show the z-values were within normal ranges. The approximate z-values were 7.5, 14, and 14 for *Salmonella, E. faecium,* and *P. acidilactici,* respectively. These values are similar to the z-values determined mathematically.

3.5 Evaluation of Heat Resistance of *Salmonella* and Surrogates on Almonds

The California Board of Almonds has approved the use of *E. faecium* NRRL-B2354 for use in validating the dry air heating processes. To compare the heat resistance, a quick study was performed which examined the log reduction of all bacteria after 15 min at 138°C. The results are in table 3.10.

Table 3.10 Average log reduction after 15 min at 138 °C on almonds

Bacteria	Log Reduction
Salmonella	6.7 ± 1.3^{A}
Salmonella Enteritidis PT 30	5.5 ± 0.9^{A}
E. faecium	3.2 ± 0.9^{A}
*P. acidilactici**	6.8 ± 0.1^{A}

**P. acidilactici* was reduced to undetectable levels after the heat treatment

Table 3.10 summarizes the log reduction after the heat treatment. *E. faecium* is the most resistant; however, it is not significantly more resistant than the two *Salmonella* treatments. *P. acidilactici* was the least resistant, with the heat treatment reducing the bacteria to undetectable levels (10 CFU/g). Both *Salmonella* treatments had similar reductions, with the Enteritidis PT 30 treatment being slightly more resistant.

3.6 Whole Genome Sequence of *P. acidilactici* ATCC 8042

To confirm the identity of the surrogate used in the experiments, a whole genome sequence of the surrogate was obtained. The resulting sequence was confirmed as *Pediococcus acidilactici* using the BLAST suite. The combined contigs file had a 98% similarity to an uploaded genome in the BLAST database. This confirmed the identity of the surrogate. A further analysis of the genome was performed to identify possible genes of interest. A summary of the annotated genome is shown in figure 3.17.

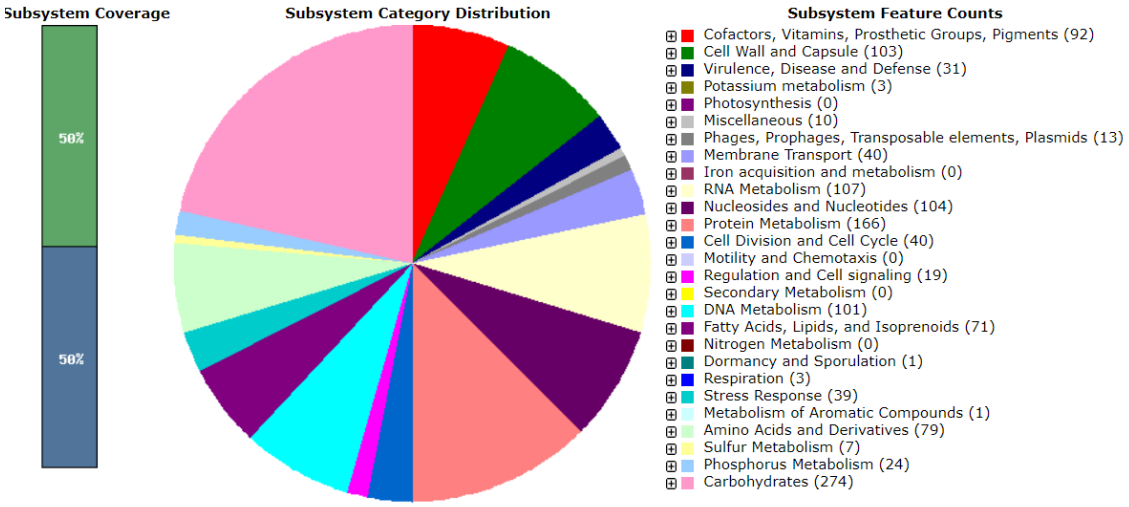

Fig. 3.17 Annotated genome of *P. acidilactici* ATCC 8042 as seen in the SEED Genome viewer *(54)*

Many of the genes identified are used for general maintenance and growth of the cell. These include genes for carbohydrate metabolism, cellular division, and fatty acid synthesis. However, genes related to stress response are identified as well. These findings are summarized in table 3.11.

Table 3.11 Summary of *P. acidilactici* subsystems related to cellular stress response

Subsystem Category	Subsystem Feature	Subsystem
Regulation and Cell Signaling	Regulation and Cell Signaling	Lsyr-family proteins in *Escherechia coli*
		HPr catabolite repression system
		DNA-binding regulatory proteins
		Stringent Response, (p)ppGpp metabolism
		Cell envelope-associated LytR-CpsA-Psr transcriptional attenuators
Stress Response	Osmotic Stress	Osmoregulation
		Choline and betaine uptake and biosynthesis
	Oxidative Stress	Protection from reactive oxygen species
		Oxidative stress
		NADPH:quinone oxidoreductase 2
		CoA disulfide thiol-disulfide redox system
		Glutathione: Redox cycle
		Glutaredoxins
	Cold Shock	CspA family of proteins
	Heat Shock	Heat shock dnaK gene cluster extended
	Detoxification	Uptake of selanate and selenite
		HFL operon

Table 3.11 displays the genetic susbsystems that are associated to cellular stress response, such as osmotic and heat stress, as well as the cellular regulation genes. These genes could be involved in the cellular response to desiccation, and the resulting increased heat resistance, as has been seen in the previous sections.

CHAPTER 4

DISCUSSION

4.1 Comparative Thermal Kinetics of *Salmonella* and Surrogates on TOC

The goal of this project was to find a GRAS surrogate for *Salmonella* with possible applications in different food matrices. The ultimate purpose was that such surrogate could be used in commercial food production facilities to validate heat inactivation processes. The surrogate would contribute to the safety of the process. The findings of these experiments suggested that both *P. acidilactici* and *E. faecium* can be used as a surrogate at temperatures between 85-95°C (Tables 3.3-3.5) in toasted oats cereal. At 80°C, however, *Salmonella* was twice more resistant than both potential surrogates, and they may not perform as ideal surrogate (Table 3.2).

The results are inconsistent with previously reported data. The thermal inactivation rates of *Salmonella* were greater in this research, compared to previous work in our lab where the thermal resistance of individual *Salmonella* serovars was measured in toasted oats cereal. Chick et. al reported δ values at 85°C of 0.55, 1.01, and 2.87 minutes at the same water activity (0.33) for individual *Salmonella* serovars Typhimurium, Tennessee, and Agona, respectively *(23)*. While all of these serovars were included in the cocktail, the δ value in the present research was more than 50-fold, at 87.8 minutes.

What was not investigated in this study and could explain the differences between this study and Chick et. al, was the potential differences between the thermal resistance of

Salmonella serovars or strains. Even though they were not statistically different, Chick et. al observed differences between the δ values of the three serovars used *(23)*. This study used a cocktail and did not perform any further differentiations to determine whether one specific serovar survived better than the others did. There could be one serovar determining the differences in δ values.

Other studies have found that the thermal resistance of *E. faecium* and *P. acidilactici* in different low water activity foods was greater than *Salmonella*. Jeong et. al reported that *E. faecium* was approximately 30% more resistant than *Salmonella* Enteritidis PT 30 on almonds when subjected to moist air heating *(42)*. This was also observed in dry pet food. *P. acidilactici* and *E. faecium* were both more thermally resistant than a *Salmonella* cocktail *(21)*. The similar trend was not observed in this study in toasted oats cereal. A possible explanation could be because the *Salmonella* cocktail was more resistant than the single *Salmonella* strains used by other studies, or that the effect of the food matrix changes the relative resistances.

At all temperatures, there was no significant difference between *E. faecium* and *P. acidilactici* in TOC. This lack of difference enhances the potential for the replacement of *E. faecium* by *P. acidilactici*. *P. acidilactici* is a GRAS status microorganism, which would allow for easier approval for this bacterium to enter a food facility. *E. faecium* is the *Salmonella* surrogate that has been adapted for almonds but has not been declared as GRAS ingredient. This lack of GRAS status may limit the approval for *E. faecium* for application in other commodities.

From the z-value graphs, two things can be found. One, that the mathematically determined z-values are approximately the estimated z-values found in the graph. A 1 log

CFU reduction in δ value is approximately 12°C for *Salmonella*, 13°C for *E. faecium*, and 15°C for *P. acidilactici*. These are very close for *Salmonella* and *P. acidilactici* but is two degrees off the estimation for *E. faecium.* This follows the fits of the curves. Both *Salmonella* and *P. acidilactici* have well-fit ($R^2 \geq 0.9$) linear regression lines. This is not the case for *E. faecium*, where the 90°C time point was much higher than what would be predicted by the linear regression curve and led to a curve that was not as well fit ($R^2=0.74$).

4.2 Comparative Thermal Kinetics of *Salmonella* and Surrogates in Peanut Butter

A good surrogate bacterium has similar or higher thermal resistance than the target. Both potential surrogates had consistently higher δ values than *Salmonella* at all temperatures. This supports the idea that both *E. faecium* and *P. acidilactici* could be good potential surrogates for *Salmonella* in peanut butter. This observation is supported by research that observed *E. faecium* to have similar characteristics be considered a surrogate for *Salmonella* in peanut paste at 85 °C *(29)*. Secondly, similar to cereal, at all temperatures there was no significant difference between *E. faecium* and *P. acidilactici.* This lack of difference confirmed there is potential for *P. acidilactici* to replace *E. faecium* as a surrogate.

There have been several studies examining the heat resistance in peanut butter. The results were similar when using the Weibull model. Previous investigations have reported comparable results. While this study involved lower temperatures than previous studies, there were similar temperatures in the 70-80 °C range. Shachar and Yaron examined the heat resistance of *Salmonella* Agona, Enteritidis, and Typhimurium at 70, 80, and 90 °C *(66)*. At 70 °C, the δ value found was 0.25 minutes. Ma et. al found δ

values ranging from 0.79 to 4.29 minutes at 71 °C for different *Salmonella* strains *(50)*. At 77 °C, the δ values ranged from 0.59 to 1.00 minutes. All these values were comparable to the values found in this study. At 73 and 77 °C, the δ values for *Salmonella* were 1.5 and 0.4 minutes, respectively. Other studies have found *Salmonella* to be more resistant; however, those studies used the log-linear model, and they cannot be directly compared to our findings.

4.3 Heat Resistance of *Salmonella* and Surrogates on Almonds

From the previous two sections, it was clear that there was a relationship between the heat resistance of *E. faecium* and *P. acidilactici* in both toasted oats cereal and peanut butter. This strongly suggested the potential for replacing *Enterococcus* since *P. acidilactici* is a GRAS microorganism. Because *E. faecium* is accepted as a surrogate for *Salmonella* in dry heating of almonds, a small heat resistance study was undertaken to investigate whether *P. acidilactici* was a good potential replacement for *E. faecium* on almonds.

The results of this study, however, indicated that *E. faecium* and *P. acidilactici* did not have similar resistance to heat on almonds. The results confirmed that the approved surrogate was more resistant than both a *Salmonella* cocktail and a *Salmonella* strain related to an almond outbreak. The average extent of inactivation was slightly larger than the expected value, at 3.2 instead of 2.5, but this study added a come up time to the 15 min heat resistance time *(11)*. However, the heat treatment of *P. acidilactici* reduced the population to undetectable levels. This lack of heat resistance is inconsistent with the thermal inactivation observed in TOC and peanut butter.

In contrast with the protocols used for TOC and peanut butter, the almond testing was conducted at a markedly higher temperature and the bacteria was inoculated on the surface of the almonds, instead of dispersed throughout the food matrix. The change of matrix has been known to influence thermal resistance. The findings confirm the impact of the food characteristics on the heat resistance of *P. acidilactici* and *Salmonella*. This investigation corroborates the impact of the food matrix on the heat resistance of bacterial strains.

Almonds are similar to peanut butter in being a low water activity and high fat food, but they do not have the added sugar, salt, and structure that peanut butter has. The addition of sucrose may be a factor in increased heat resistance for *Salmonella,* so it may follow that the same is true for *P. acidilactici (33, 38).* Similar to *P. acidilactici,* both *Salmonella* strains were more susceptible to heat treatment than expected. Other studies of using dry air ovens to inactivate *Salmonella* have observed smaller reductions *(42, 75).* *E. faecium* was twice as resistant as the *Salmonella* cocktail and the single serovar (Table 3.10). This is a larger difference between the pathogen and surrogate than previously reported. Jeong et al. observed that *E. faecium* was only 30% more resistant to heat in a moist air oven *(42)*. Our investigation determined differences between the pathogen and surrogate to be in the range of 200%. While this still confirms surrogacy, the difference between the two studies is notable. The *Salmonella* strains in this study appeared to be less thermally resistant than the Jeong paper. This could be due to strain differences in the cocktail and heating methods.

4.4 Whole Genome Sequence and Genomic Comparison

Most of the thermal inactivation data established a close relationship between the thermal inactivation parameters of *E. faecium* NRRL-B2354 and *P. acidilactici* ATCC 8042. At all temperatures and across food matrixes both bacteria had similar thermal inactivation kinetics parameters. They both share *Salmonella*'s ability to survive thermal treatment in a desiccated state. With the hypothesis that the resistance in *Salmonella* is due to a stress response, it was hypothesized that there would be similar genes in surrogates. Given a similar phenotype, there may be a similar genotype.

Due to the complex nature of desiccation resistance and heat tolerance, the role of transcriptional regulators was identified as a potential point of similarity. A common transcriptional regulator would activate similar responses in the cell. Gruzdev et. al identified two transcriptional regulators, *rpoE* and *fnr*, that are necessary for desiccation resistance *(34)*. *E. faecium* contains two transcriptional receptors in the same protein family, CRP-FNR. Given the established surrogacy, the CRP-FNR protein family was chosen as a potential similarity between the two.

However, as can be seen in figure 3.1, there was no close evolutionary relationship between the transcriptional receptors of *E. faecium* and *P. acidilactici.* Since the thermal kinetics parameters were similar, a close relationship between the required genes would be expected. This could mean that the FNR-CRP family of transcriptional regulators was not directly associated with the stress cross protection of desiccation resistance and increased thermal resistance.

The whole genome sequence of *P. acidilactici* did not provide clear answers either. There are only two genetic subgroups in the genome related to osmotic stress and

one related to heat shock. Both osmotic stress genetic subgroups related to the intake and synthesis of osmoprotectants and the heat shock stress response was the synthesis of chaperone proteins. Both of these systems are needed for the osmotic stress and heat shock, but they are not novel. Most LAB import osmoprotectants to survive osmotic stress and express heat shock proteins, but not all of them survive thermal treatment after desiccation *(48)*. The whole genome sequence did not yield a clear alternative mechanism for desiccation resistance and heat tolerance.

CHAPTER 5

CONCLUSION

5.1 Conclusion

Salmonella enterica is the most common bacterial source of foodborne illness and remains a major public health concern. One of the many types of food that have been implicated in *Salmonella* illnesses are low water activity foods. This type of food is not only a vector for *Salmonella but* can cause *Salmonella* to be more thermally resistant. Because of the Food Safety Modernization Act, these foods need to have validated controls for *Salmonella* to assist in reducing *Salmonella* illnesses.

The goal of this study was to identify a GRAS surrogate for *Salmonella* in low water activity foods. It has identified one, *Pediococcus acidilactici* ATCC 8042, to be used as a surrogate in toasted oats cereal and peanut butter. In toasted oats cereal, both *P. acidilactici* and *E. faecium* can be used as a surrogate in the temperature range of 85-95 °C. While both surrogates were less resistant to heat than *Salmonella,* they were not significantly different ($p \leq 0.05$) and can be used as a surrogate.

In peanut butter, both surrogates were more resistant than *Salmonella* at all temperatures from 63-77 °C. At some temperatures, either surrogate was significantly more resistant than *Salmonella.* This was not a consistent trend where one surrogate was significantly higher at all temperatures. At 63 °C, *E. faecium* was significantly more resistant, while at 73 °C *P. acidilactici* was significantly more resistant. This confirms that both surrogates can be used for validation in peanut butter.

At all temperatures in both toasted oats and peanut butter *P. acidilactici* and *E. faecium* were not significantly different from each other, which suggests the potential to use either bacteria as a surrogate in the foods. This was not true in almonds. There was a clear difference in the thermal resistance of *P. acidilactici.* The lack of heat resistance in almonds is an example of the effect of a food matrix on a bacteria's heat resistance.

The use of surrogates in thermal validation is an important tool and is needed to ensure the safety of the food system. This study identified a surrogate that can be used in the validation of cereal and peanut butter processing. This surrogate can be introduced into a food facility easily, given its GRAS status. This study also reports that if a surrogate works in some food matrixes, it does not guarantee the use in all food matrixes. An easier method of identification of potential surrogates should be elucidated, so that more processes can be validated.

5.2 Future Research

One hypothesis of this study was that through comparing CRP/FNR transcriptional receptors a GRAS surrogate could be identified. While this did not prove to be a factor in identifying a GRAS surrogate, the potential for using a surrogate to identify the similarities between heat resistant bacteria remains. The exact mechanism of desiccation resistance and heat tolerance of *Salmonella* has not been elucidated. There have been two studies showing the genes expressed under desiccation, but transcriptomic studies have not given a clear answer on the exact mechanism.

A comparison between the transcriptomes of *Salmonella* and both surrogates could give some more answers. All three bacteria were resistant to desiccation and became tolerant to heat after desiccation. They do not have many other similarities,

though. *Salmonella* is Gram negative; both surrogates are Gram positive. *Salmonella* is a pathogen; neither surrogate is pathogenic. A comparison of the transcriptomes during desiccation could show the similar genes used under the stress.

The results of this study could be used to identify the genes used in *Salmonella's* desiccation response. The similar genes used by the surrogates could be identified as well. These genes could be found in other nonpathogenic strains, leading to the identification of more surrogates. This study reported the differences in thermal tolerance between food matrixes, and the two surrogates identified may not be the best surrogates for *Salmonella* in each food matrix. An easier method of identifying surrogates could lead to more surrogate options and better modeling in each food. This would make the food supply safer.

CHAPTER 6

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APPENDIX A

THERMAL INACTIVATION IN TOC

Table A.1 Microbial counts (LOG CFU) in TOC at 80 °C

Time (min)	*E. faecium* 9/5		*E. faecium* 11/2		*Salmonella* 9/7		*Salmonella* 10/31		*P. acidilactici* 12/20		*P. acidilactici* 1/19	
	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD
0	5.82	0.21	5.83	0.21	6.21	0.12	6.13	0.16	5.88	0.36	5.3	0.10
30	5.22	0.07	5.19	0.13	6.02	0.28	6.14	0.27	5.27	0.10	4.68	0.20
60	4.97	0.14	4.92	0.18	5.88	0.15	5.9	0.19	5.02	0.15	4.47	0.33
90	4.91	0.13	4.63	0.31	5.60	0.22	5.67	0.19	4.45	0.17		
120	4.09	0.16	4.37	0.19	5.05	0.21	5.45	0.20	4.41	0.25	3.93	0.23
150	4.76	0.12	4.14	0.17	5.02	0.20	5.28	0.20	4.12	0.14	3.28	0.11
180	3.62	0.23	4.27	0.13	4.55	0.33			4.19	0.14	3.87	0.12

Table A.2 Thermal kinetics parameters in TOC at 80 °C

Bacteria	Date	δ Value	RMSE	R-square adjusted	β Value
E. faecium	5-Sep	76.54	0.21	0.93	0.91
Salmonella	7-Sep	122.45	0.12	0.96	1.29
Salmonella	31-Oct	156.92	0.07	0.96	1.44
E. faecium	2-Nov	62.43	0.11	0.96	0.51
P. acidilactici	20-Dec	59.97	0.15	0.95	0.56
P. acidilactici	19-Jan	66.37	0.32	0.79	0.57

Table A.3 Microbial counts (LOG CFU) in TOC at 85 °C

Time (min)	*Salmonella* 9/15		*E. faecium* 9/18		*Salmonella* 10/25		*E. faecium* 10/27		*P. acidilactici* 12/19		*P. acidilactici* 1/18	
	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD
0	6.50	0.13	5.59	0.12	6.53	0.12	5.95	0.12	5.82	0.17	5.80	0.13
15	5.74	0.30	4.79	0.19	6.30	0.19	5.32	0.20	5.06	0.16	5.27	0.16
30	5.58	0.15	4.65	0.17	6.20	0.17	5.21	0.18	4.83	0.22	5.03	0.15
45	5.59	0.30	4.35	0.21	6.09	0.21	5.12	0.19	4.37	0.06	4.37	0.14
60	5.29	0.17	4.45	0.15	5.92	0.15	5.05	0.04	4.18	0.33	4.31	0.24
75	5.04	0.13	4.23	0.11	5.80	0.11	4.83	0.11	4.13	0.14	3.84	0.15
90	5.38	0.19	4.50	0.12	5.82	0.12	4.67	0.15	4.25	0.17	4.16	0.32

Table A.4 Thermal kinetics parameters in TOC at 85 °C

Bacteria	Date	δ Value	RMSE	R-square adjusted	β Value
Salmonella	15-Sep	39.54	0.16	0.87	0.31
Salmonella	25-Oct	133.98	0.05	0.97	0.68
E. faecium	18-Sep	30.88	0.14	0.90	0.22
E. faecium	27-Oct	60.22	0.08	0.96	0.44
P. acidilactici	19-Dec	23.01	0.16	0.93	0.42
P. acidilactici	18-Jan	31.00	0.40	0.88	0.62

Table A.5 Microbial counts (LOG CFU) in TOC at 90 °C

Time (min)	*Salmonella* 9/22		*E. faecium* 9/25		*Salmonella* 10/19		*E. faecium* 11/9		*P. acidilactici* 12/14		*P. acidilactici* 1/16	
	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD
0	6.20	0.21	5.91	0.13	6.58	0.19	5.92	0.20	5.73	0.18	5.90	0.18
10	5.50	0.16	5.27	0.17	6.00	0.09	5.21	0.19	4.90	0.16	5.29	0.17
20	5.10	0.11	5.19	0.06	5.94	0.10	4.98	0.27	5.01	0.10	5.19	0.18
30	4.66	0.14	4.97	0.17	5.53	0.20	4.92	0.11	4.38	0.08	4.62	0.13
40	4.84	0.29	5.04	0.06	5.35	0.04	4.88	0.08	4.74	0.02	4.83	0.15
50	4.19	0.12	4.82	0.09	5.28	0.04	4.86	0.05	3.97	0.16	4.28	0.20
60	4.44	0.05	4.89	0.05	5.24	0.08	4.76	0.06	4.25	0.16	4.17	0.14

Table A.6 Thermal kinetics parameters in TOC at 90 °C

Bacteria	Date	δ Value	RMSE	R-square adjusted	β Value
Salmonella	22-Sep	16	0.21	0.91	0.5
E. faecium	25-Sep	49.28	0.07	0.96	0.29
Salmonella	19-Oct	30.86	0.10	0.96	0.53
E. faecium	9-Nov	33.01	0.04	0.99	0.23
P. acidilactici	14-Dec	22.71	0.28	0.76	0.46
P. acidilactici	16-Jan	26.74	0.53	0.91	0.66

Table A.7 Microbial counts (LOG CFU) in TOC at 95 °C

Time (min)	*Salmonella* 9/29		*E. faecium* 10/3		*E. faecium* 10/11		*Salmonella* 11/7		*P. acidilactici* 12/7		*P. acidilactici* 12/21	
	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD
0	6.44	0.17	5.90	0.16	5.53	0.20	6.55	0.24	5.88	0.10	5.71	0.11
10	5.04	0.12	4.79	0.18	3.91	0.30	5.37	0.23	4.58	0.12	4.69	0.16
20	5.27	0.30	4.64	0.11	3.91	0.15	5.33	0.24	4.05	0.21	4.63	0.12
30	5.03	0.16	4.47	0.17	3.62	0.23	4.95	0.23	3.36	0.16	4.09	0.19
40	4.80	0.11	4.37	0.17	3.69	0.27	4.89	0.12	3.66	0.09	4.04	0.15
50	4.34	0.20	4.36	0.10	3.44	0.26	4.29	0.24	3.12	0.19	3.82	0.24
60	4.75	0.22	4.30	0.28	3.40	0.07	4.72	0.06	3.41	0.11	4.02	0.21

Table A.8 Thermal kinetics parameters in TOC at 95 °C

Bacteria	Date	δ Value	RMSE	R-square adjusted	β Value
Salmonella	28-Sep	4.85	0.25	0.85	0.29
E. faecium	2-Oct	5.82	0.03	1.00	0.21
E. faecium	11-Oct	0.79	0.10	0.98	0.17
Salmonella	7-Nov	10.72	0.32	0.89	0.37
P. acidilactici	7-Dec	4.17	0.28	0.92	0.37
P. acidilactici	21-Dec	9.88	0.17	0.93	0.35

APPENDIX B

THERMAL INACTIVATION IN PEANUT BUTTER

Table B.1 Microbial Counts (LOG CFU/g) in peanut butter at 63 °C

Time (min)	*P. acidilactici* 3/1		*Salmonella* 2/28		*E. faecium* 2/27		*E. faecium* 3/5		*Salmonella* 3/6		*P. acidilactici* 3/8	
	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD
0	8.17	0.17	6.86	0.25	8.19	0.11	8.14	0.14	7.50	0.15	8.05	0.12
15	7.43	0.30	5.65	0.25	7.14	0.22	7.46	0.17	5.89	0.41	7.03	0.21
30	7.21	0.07	5.21	0.18	7.42	0.13	7.31	0.12	5.45	0.18	7.16	0.12
60	7.12	0.10	4.99	0.23	7.27	0.14	7.15	0.11	5.29	0.22	6.94	0.15
90	6.85	0.15	5.09	0.27	7.21	0.06	7.16	0.24	5.10	0.10	6.64	0.11
120	6.60	0.25	4.77	0.24	7.19	0.08	6.95	0.25	4.71	0.20	6.31	0.17
150	6.71	0.15	5.01	0.14	7.25	0.10	6.83	0.14	4.75	0.10	6.54	0.11
180	6.59	0.27	4.91	0.27	7.03	0.28	6.90	0.14	4.65	0.15	6.58	0.03

Table B.2 Thermal kinetics parameters in peanut butter at 63 °C

Bacteria	Date	δ Value	RMSE	R-square adjusted	β Value
P. acidilactici	1-Mar	38.1	0.08	0.97	0.31
Salmonella	28-Feb	1.78	0.16	0.95	0.15
E. faecium	27-Feb	120.18	0.12	0.89	0.05
E. faecium	5-Mar	68.31	0.06	0.98	0.26
Salmonella	6-Mar	2.16	0.12	0.99	0.23
P. acidilactici	7-Mar	24.01	0.18	0.89	0.23

Table B.3 Microbial Counts (LOG CFU/g) in peanut butter at 68 °C

Time (min)	*P. acidilactici* 2/12		*Salmonella* 2/14		*E. faecium* 2/15		*P. acidilactici* 2/22		*Salmonella* 2/23		*E. faecium* 2/26	
	Count	**SD**	**Count**	**SD**	**Count**	**SD**	**Count**	**SD**	**Count**	**SD**	**Count**	**SD**
0	8.45	0.08	7.09	0.11	8.29	0.13	8.01	0.18	6.97	0.23	8.26	0.08
10	7.71	0.10	5.55	0.05	7.26	0.08	7.27	0.19	5.49	0.20	7.30	0.13
20	7.41	0.26	5.46	0.53	7.36	0.13	7.06	0.44	5.37	0.21	7.34	0.15
40	7.16	0.21	4.93	0.26	7.23	0.04	6.74	0.18	5.18	0.22	7.22	0.08
60	6.78	0.24	5.09	0.15	6.95	0.17	6.47	0.14	4.85	0.38	7.34	0.09
80	6.41	0.07	4.51	0.27	6.86	0.11	6.23	0.18	4.23	0.23	7.26	0.15
100	6.27	0.25	4.94	0.25	6.94	0.13	6.07	0.27	4.45	0.05	7.10	0.05
120	6.12	0.29	4.27	0.16	6.51	0.18	5.90	0.41	4.56	0.31	6.94	0.11

Table B.4 Thermal kinetics parameters in peanut butter at 68 °C

Bacteria	**Date**	**δ Value**	**RMSE**	**R-square adjusted**	**β Value**
P. acidilactici	12-Feb	20.2	0.07	0.99	0.48
Salmonella	14-Feb	4.03	0.25	0.92	0.27
E. faecium	15-Feb	19.69	0.16	0.91	0.25
P. acidilactici	22-Feb	22.04	0.02	1.00	0.44
Salmonella	23-Feb	8.06	0.30	0.88	0.27
E. faecium	26-Feb	34.23	0.12	0.91	0.11

Table B.5 Microbial Counts (LOG CFU/g) in peanut butter at 73 °C

Time (min)	***Salmonella* 11/13**		***E. faecium* 11/16**		***Salmonella* 1/8**		***E. faecium* 1/9**		***P. acidilactici* 1/10**		***Salmonella* 1/23**		***P. acidilactici* 1/22**		***E. faecium* 1/24**	
	Count	**SD**	**Count**	**SD**	**Count**	**SD**	**Count**	**SD**	**Count**	**SD**	**Count**	**SD**	**Count**	**SD**	**Count**	**SD**
0	7.18	0.13	8.01	0.12	7.17	0.16	8.09	0.10	8.28	0.05	7.27	0.08	8.17	0.04	8.21	0.12
7											5.21	0.15	7.07	0.16	7.03	0.26
15			7.03	0.05			7.10	0.18	7.12	0.14	5.13	0.13	7.18	0.23	7.19	0.23
30	5.16	0.24	6.40	0.07	5.06	0.08	6.81	0.19	6.84	0.06	4.00	0.06	6.80	0.12	7.03	0.23
45	4.97	0.21	5.87	0.14	4.93	0.34	6.35	0.22	6.24	0.21	4.41	0.53	6.71	0.11	6.73	0.15
60	4.22	0.36	6.09	0.05	4.92	0.17	6.25	0.07	6.20	0.10	3.43	0.26	6.16	0.08	6.65	0.22
75	4.95	0.25	5.91	0.09	5.01	0.22	5.90	0.13	5.81	0.11	3.33	0.26	6.01	0.17	6.38	0.22
90	4.59	0.38	5.85	0.22	4.38	0.41	5.93	0.10	6.02	0.31	4.24	0.70	6.05	0.16	6.30	0.24

Table B.6 Thermal kinetics parameters in peanut butter at 73 °C

Bacteria	Date	δ Value	RMSE	R-square adjusted	β Value
Salmonella	13-Nov	1.82	0.37	0.87	0.24
E. faecium	16-Nov	8.21	0.23	0.92	0.35
Salmonella	8-Jan	0.83	0.05	0.94	0.2
E. faecium	9-Jan	15.1	0.10	0.98	0.46
P. acidilactici	10-Jan	9.62	0.18	0.96	0.4
Salmonella	23-Jan	1.97	0.54	0.82	0.32
P. acidilactici	22-Jan	21.4	0.21	0.91	0.49
E. faecium	24-Jan	15.54	0.17	0.92	0.29

Table B.7 Microbial Counts (LOG CFU/g) in peanut butter at 77 °C

Time (min)	*E. faecium* 1/29		*Salmonella* 2/1		*P. acidilactici* 2/6		*Salmonella* 2/7		*E. faecium* 2/5		*P. acidlactici* 2/9	
	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD	Count	SD
0	8.40	0.13	7.08	0.13	8.04	0.23	7.22	0.12	8.34	0.08	8.29	0.09
5	7.25	0.14	5.12	0.14	6.83	0.40	5.37	0.12	7.42	0.36	7.04	0.18
10	7.40	0.04	4.66	0.31	6.56	0.16	4.90	0.36	7.12	0.19	6.52	0.27
20	6.92	0.14	3.86	0.21	6.38	0.27	4.69	0.20	6.71	0.19	5.75	0.16
30	6.94	0.12	4.20	0.25	6.07	0.44	4.13	0.26	6.47	0.29	5.48	0.27
40	6.46	0.07	4.28	0.60	6.02	0.21	4.10	0.49	6.10	0.48	4.85	0.27
50	6.44	0.22	4.42	0.14	6.18	0.21	3.70	0.33	6.17	0.22	5.70	0.41
60	6.25	0.17	4.42	0.76	6.37	0.40	3.64	0.31	6.07	0.12	5.23	0.45

Table B.8 Thermal kinetics parameters in peanut butter at 77 °C

Bacteria	**Date**	**δ Value**	**RMSE**	**R-square adjusted**	**β Value**
E. faecium	29-Jan	9.77	0.17	0.94	0.36
Salmonella	1-Feb	0.29	0.46	0.80	0.16
P. acidilactici	6-Feb	0.67	0.19	0.92	0.15
E. faecium	5-Feb	7.93	0.13	0.97	0.4
Salmonella	7-Feb	0.49	0.11	0.99	0.27
P. acidilactici	8-Feb	4.58	0.47	0.82	0.39

APPENDIX C

HEAT RESISTANCE ON ALMONDS

Table C.1 Heat resistance at 138 °C after 15 min in almonds

Bacteria	**Initial Count (LOG CFU/g)**	**Final Count (LOG CFU/g)**	**Log Reduction**
Salmonella	8.04	Undetectable	8.04
PT 30	8.33	1.97	6.36
E. faecium	7.22	3.08	4.15
P. acidilactici	6.88	Undetectable	6.88
Salmonella	8.33	2.90	5.43
PT 30	8.54	3.94	4.61
E. faecium	7.18	4.93	2.25
P. acidilactici	6.74	Undetectable	6.74