Thermal Inactivation Kinetics of *Salmonella* Serovars on Dry Cereal

A Thesis

Submitted to the Faculty of the Graduate School of the University of Minnesota

By

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In Fulfillment of the Requirements for the Degree of Master of Science

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August 2011

Acknowledgements

I would like to thank Dr. Francisco Diez-Gonzalez for all his help, guidance, comments, and critiques throughout this project. Without any of the advice or guidance provided I would not have been able to finish this project.

For their support and helpful advice, I would also like to thank Dr. Ryan Fink and Dr. Stelios Viazis and the rest of the Food Safety Microbiology Laboratory: Dr. Stephen Lumor, Dr. Luna Akhtar, Dr. Hongshun Yang, Zhe Hou, Na Wang, Camila Gadotti, Dorey Gray , Ellen Hystead and Abena Opoku. These individuals made my time in the lab much more interesting, especially during the long nights and weekends in the lab. I would also like to thank Nicole Pearson and Kristina Zevenbergen and the rest of the undergraduates who helped throughout the project in media making, Grace Nelson, and Hannah Clarke. I could not thank them enough for all the help provided.

I would also like to thank my family, Cindy Kurowski, Lee Chick, and Will Chick for all the support given throughout the two years of this project. Thanks dad for assuring me that the very long nights in the lab builds character. I would like to thank all my friends for their support, for which I cannot name all of them except Hetvi Damodhar, for their support throughout this project.

Without any of these elements, this project would not have succeeded.

Abstract

Outbreaks of *Salmonella* linked to low water activity foods have been an increasing concern to the food industry, as *Salmonella* is the leading causative agent of bacterial foodborne illness in the United States. Some of the most recent foods linked to outbreaks include low water activity foods, such as peanut butter, dry cereal, and black pepper. Several studies have indicated that the thermal resistance of *Salmonella* at low water activity increases significantly, but very few kinetic studies have been published. The goal of this research was to determine the kinetic parameters of thermal resistance of *Salmonella* in a low water activity food. Cereal with water activity levels between 0.1 and 0.5 a_w was used as the model food, and the effect of sucrose addition was also assessed.

The model toasted oat cereal was inoculated with 10^8 CFU/mL of one of three *Salmonella* serovars, dried overnight at 40°C and equilibrated to 0.11, 0.33 or 0.53 water activity. The cereal was then ground, put into sealed capillary tubes and heated at temperatures between 65 and 105°C. The capillary tubes were removed at intervals, immediately cooled and then plated onto *Salmonella*-specific differential tryptic soy agar. The inactivation curves were then plotted and lines of best fit were used to calculate D and Z-values for each serovar at a given temperature and water activity. Sucrose (25%) was also added to the cereal to assess its effect on thermal resistance.

Using *Salmonella* serovars Typhimurium, Tennessee and Agona, at 0.53 water activity, the D-values ranged from 172 to 208 min at 65°C and from 4.3 to 6.5 min 80°C among the three serovars. D-values at 80°C increased 4-fold when the water activity

was reduced to 0.33. All serovars became more resistant to thermal treatments as the water activity decreased, and at 0.11 a_w D-values greater than 135 min were measured at 85°C.

At 0.53 a_w the D-values of each of the three serovars increased between 32 and 102% at 80^{o}C once sucrose was added to the cereal. At 0.33 a_w the serovars' D-values increased by 95 to 152% at 85^{o}C compared to the cereal without sucrose. Once the water activity was lowered to 0.11, each of the three serovars had a decrease in D-values of between 46 and 85% at every temperature tested. The Z-values for the three serovars at 0.33 and 0.53 a_w were approximately 10°C, but this value increased from 20 to 50% if the serovars were tested at 0.11 a_w. There was not a statistically significant change in the Z-values due to the addition of sucrose.

The thermal resistance of *Salmonella* increases dramatically at very low water activity. This increase may render some food processes, once thought to be able to control *Salmonella,* ineffective at controlling these pathogenic bacteria in dry foods. More research is required to further understand the mechanisms of the organism's ability to survive such a large increase in temperature within these low water activity environments.

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Chapter 1 - Introduction

1.1 General characteristics of *Salmonella*

Both species within the genus *Salmonella* are Gram negative, non-spore forming rods described as facultative anaerobes. *Salmonella* has both fermentative and respiratory pathways for metabolizing nutrients. *Salmonella* serovars are oxidase negative and catalase positive. Most serovars produce hydrogen sulfide, ornithine, and are capable of decarboxylating lysine. They are also able to use citrate as a primary carbon source but do not hydrolyze urea (2). While most serovars of *Salmonella* are peritrichious, there are non-motile serovars that have no flagella.

1.2 Environmental factors

Salmonella is natural inhabitant of the mammalian gastro-intestinal (GI) tract. It is excreted from the GI tract within the feces and can survive in soils for a long time, even when the feces have become dry (3). After excretion, *Salmonella* can persist in soils, which may serve as a vector for contamination of crops such as fruits and vegetables. Though *Salmonella* is very prevalent, its survival is dependent upon the physical and chemical characteristics of the environment. The environmental factors that may affect the growth of *Salmonella* include, but are not limited to, pH, temperature and water activity (4).

The optimum growth pH for most *Salmonella* serovars ranges between 6.5 and 7.5, but is able to grow slowly at pH values of 4.5 and 9.5. At pH values below 4.5, *Salmonella* is still capable of surviving thanks to its acid tolerance response (ATR), a

stress response mechanism that allows it to survive in acidic conditions typically of fermented and acid foods (5). When *Salmonellae* are grown in a mild acidic medium between pH 5.5 to 6.0 it can trigger the acid tolerance response that would allow it to survive when transferred into a more acidic medium of less than pH 4.5. The acid tolerance response is characterized by the expression of 43 proteins mostly in the membrane, which allows *Salmonella* to be grown in even lower conditions than pH 4.5. This acid tolerance response influences the growth rate of the cells, the pH balance within the cell, and has a cross-over protection effect to protect from low water activity and heat (6).

The growth range of *Salmonella* is typically between 5.5 and 45^{o}C, depending on the specific serovar and the environmental conditions (6). Under ideal conditions the optimum growth temperature for *Salmonella* is 37^{o}C, though some serovars are able to grow as high as 54^{o}C and as low as two degrees Celsius. These lower growth temperatures are usually achieved by having the cells conditioned to grow under low temperature conditions (7).

The “acid tolerance response” is not the only stress response that imparts resistance to *Salmonellae*. Enhancement of heat resistance can also increase survivability, if *Salmonella* is subjected to sub lethal temperatures of less than 50^{o}C for 15 to 30 minutes (6). Similar to the acid tolerance response, heat treatment also induces the expression of “heat shock” proteins that can increase the heat resistance of the cells. It is likely that this type of stress response system may have a cross protection effects under a variety of environmental conditions (8).

1.3 Nomenclature

While there have been many changes throughout the years on how to classify the different species, subspecies, and serovars of *Salmonella.* The first major nomenclature system was the Kauffman-White scheme, which organized all of the different "species" of *Salmonella* by the different antigens found within each "species". The antigens they were using were the somatic O antigen and the flagellar H antigen (1,9).

The somatic O antigen is a designation of the composition of the O antigen that is connected to the lipopolysaccharide layer in the cell membrane containing polysaccharides, lipids, and/or proteins (9). The differences between each designation for the flagellar H antigen are differences among the amino acid composition within the proteins of the flagella. This becomes more complex when many serovars have two sets of flagellar antigens, and each one of those can typically have two phases with different antigens (2). The Kauffmann-White system designated each serovar as species organized in five different subgenera. Each of these classifications were assigned based on biochemical phenotypes.

The Kaufmann-White system is no longer used due to the advancement of DNA-based species and subspecies taxonomy (2,10). The CDC's currently used system from the World Health Organizations recommendations includes two species, *Salmonella enterica* and *Salmonella bongori* (11,12). Within these two species there are over 2,500 serovars (13). All but twenty of these serovars are within the species *enterica*, which is

further broken down into six subspecies. The six subspecies are *enterica, salamae, arizonae, diarizonae, houtenae, and indica.*

Table 1.3.1 Distribution of *Salmonella* serovars per subspecies.

***Salmonella* species and subspecies**	**No of serovars**
S. enterica subsp. *enterica*(I)	1,504
S. enterica subsp. *salamae (II)*	502
S. enterica subsp. *arizonae* (IIIa)	95
S. enterica subsp. *diarizonae (IIIb)*	333
S. enterica subsp. *houtenae (IV)*	72
S. enterica subsp. *indica (VI)*	13
S. bongori (V)	22
Total	2,541

Data obtained from (2,13)

Each of these subspecies is differentiated from one another through biochemical and genetic typing schemes (1). The large number of serovars originates from a multi-antigen serological system that includes at least three different polyvalent sera targeting at least 7 different antigens that include O and H. The naming of specific serovars is relatively arbitrary, but is based on location of first isolation of a strain with a unique serological pattern. With the current system the correct method of nomenclature for the serovar Typhimurium is now *Salmonella enterica* subspecies *enterica* serovar Typhimurium.

1.4 Disease characteristics

The infections caused by *Salmonella* are generally called "salmonellosis" and can be broken down into three kinds of diseases: enteric fever, enterocolitis, and systemic infection. The enteric fever includes the well-known typhoid fever, while the systemic infections are caused by non-typhoid *Salmonella* serovars. Enteric fever starts with an incubation period of between 7 and 28 days, and is diagnosed when the infectious agent is isolated in the blood or feces. The symptoms of enteric fever typically include diarrhea, abdominal pain, spiking fever, and prostration (2). Occasionally, infected individuals become asymptomatic carriers of enteric fever after recovering from very acute symptoms.

Enterocolitis is caused by non-typhoid *Salmonella* serovars and usually requires between eight and 72 hours of incubation within the host (4). The enterocolitis infection caused by non-typhoid *Salmonella* is characterized by non-bloody diarrhea and abdominal pain. These symptoms can last as long as five days requiring only supportive therapy of fluid and electrolyte replacement, to balance the loss of liquids from diarrhea. While the enterocolitis infection is generally self-limiting, depending upon the infecting serovar and the immune system status of the host, it can become a serious systemic and possibly a septicemia infection. Other chronic *Salmonella* infections include the autoimmune diseases of reactive arthritis and ankylosing spondylitis (14,15).

While bouts of *Salmonella* induced enterocolitis may be self-limiting, those other conditions may require hospitalization. These diseases can become more complicated to treat due to the increased emergence of antibiotic resistances within the serovars.

Some of the strains were resistant to specific antibiotics used in husbandry, while others were resistant to the antibiotics commonly used against human infections (2). The appearance of serovars such as the multiple antibiotic resistant serovar *Salmonella* serovar Typhimurium DT104, have led some countries to ban the use of certain antibiotics from non-human uses, such as fluoroquinolones, in effort to keep them free from resistances (4)

Protection against *Salmonella* infection can be accomplished by vaccination, but to date there are very few vaccines available. The vaccines developed for *Salmonella* diseases are mainly for the protection against *Salmonella* serovar Typhi due to the severity of the disease. Live oral vaccines have been used for typhoid fever, though they have many side effects and are not effective in all populations (16).

Live attenuated vaccines are the focus of new research as new genetic modifications are being made. Avirulent strains, such as serovar Typhi strain Ty21a, which lacks the Vi capsular antigen and reduces the number of biosynthetic LPS enzymes, has been a new focus of attenuation (17). It also has a frameshift in the *rpoS* gene, causing unstable RNA polymerase, which along with the mutations mentioned above, reduces its virulence (2,17).

A second vaccine, called Typhim Vi, contains the antigens from the capsular Vi antigen of *Salmonella* serovar Typhi (18). This vaccine is a single dose containing the antigens preserved in phenol (2,19). In addition to human immunization, a number of vaccines have been approved for treatment of chickens to reduce their colonization. The FDA has approved avirulent serovars available for the food industry to inoculate

chickens. The live avirulent serovar Typhimurium for use in broiler chickens and breeder chickens for the poultry industry was approved in 1999 (2). Europeans also have a vaccine for *Salmonella* serovar Enteritidis as a protection from Enteritidis infections in the egg industry.

1.5 Infectious dose and mechanism of infection

Similar to other pathogens, the infectious dose for *Salmonella* depends upon the strength of the host's immune system. Many things can affect the infectious dose such as the age and relative health of the host, history of past contact with the pathogen, the amount and type of food in the stomach, the health and population of the native gut flora, the host's history of antibiotic use, etc (2,20). While most of what is listed above is directly related to the relative health of the host, the composition of the food should be of special mention. The food composition can determine the infective dose of the pathogen, mainly through the fat content of the food (Table 1.5.1). The higher the fat content of the food, the more opportunities there are for the *Salmonella* cells to be within the hydrophobic micelles of the fat, protecting it from the hydrolytic effect of the acids of the stomach.

Once a large enough population of *Salmonella* has been ingested, the cells have three steps to a successful infection: surviving the acid processes of the stomach, effacement, attachment, and invasion of the intestinal epithelial cells. *Salmonella* can survive the stomach if at least one of three conditions is present: a high initial infective dose, a lack of stomach contents, or a large amount of stomach contents (20). The

higher the infectious dose that is consumed, the higher the chance of enough cells would survive to cause the infection. If the cells have induced their acid tolerance response, than they are even more likely to survive the acidic conditions of the stomach. The second condition is dependent on a small amount of a contaminated food ingested on an empty stomach that would reduce its transit time in the stomach. This condition would increase the likelihood of *Salmonella* reaching in to the intestine. In the last case,

Table 1.5.1 Infective dose of *Salmonella* serovars estimated in select foods

Food	Serovar	Infectious dose (CFU)	Reference
Eggnog	Meleagridis	10^4-10^7	(21)
Eggnog	Anatum	10^5-10^7	(21)
Goat cheese	Zanzibar	10^5-10^{11}	(22)
Carmine dye	Cubana	10^4	(23)
Imitation ice cream	Typhimurium	10^4	(24)
Chocolate	Eastbourne	10^2	(25)
Hamburger	Newport	10^1-10^2	(26)
Cheddar cheese	Heidelberg	10^2	(27)
Cheddar cheese	Typhimurium	10^0-10^1	(28)
Chocolate	Napoli	10^1-10^2	(29)
Chocolate	Typhimurium	$\leq 10^1$	(30)
Paprika potato chips	Saintpaul	$\leq 4.5 \times 10^1$	(31)
Alfalfa sprouts	Newport	$\leq 4.6 \times 10^2$	(32)
Ice cream	Enteritidis	$\leq 2.8 \times 10^1$	(33)

Data from (2)

if the stomach is overfilled, the cells of *Salmonella* may not be exposed to the full effects of the gastric acid. Once through the stomach, the contents containing the cells will pass into the small intestine to continue the invasion process.

Once the cells reach the small intestine, attachment depends upon finding a suitable attachment site. The continued sloughing of the outer most intestinal epithelial walls, competition with the natural gut flora, and the constant movement of the bowels may hinder attachment to intestinal cells. Survival of *Salmonella* cells would also require avoiding the natural enzymatic activity of the proteases, lipases, and other natural enzymes, and the activity of bile acid.

The attachment sites that cells of *Salmonella* require are specialized cells of the submucosal layer of the intestinal wall called Peyer's patches, which are lymphatic follicles found in the small intestine (2,34). Once the pathogen has attached to the Peyer's patch several changes happen within the target host cell. The *Salmonella* cells will deliver its own extracellular proteins, while promoting the influx of calcium ions into the host cell. Some of the extracellular proteins will cause the actin to change into microfilaments around the *Salmonella* cells. Most of the other proteins will cause a cytoskeleton rearrangement of the epithelial host cell. This rearrangement with the formation of the microfilaments is sometimes deemed as "membrane ruffling", and helps *Salmonella* to be taken into the cell through pinocytosis of the host cell. Once *Salmonella* is internalized it can stay localized to the small intestine, or it can infect the blood stream to cause the systemic infections.

1.6 Prevalence in food

As *Salmonella* is able to tolerate many different environments, i.e. high salinity, high osmotic pressure, high acidity, etc., it is also able to colonize and survive in many

foods of different composition. *Salmonella* is a known contaminant of many high moisture protein sources, such as beef, poultry, and pork (35-37). Studies have isolated many *Salmonella* serovars in these foods and observed their prevalence in the retail products. Within these retail products these studies have sampled and measured a prevalence rate of 4.2%, 10.7%, and 60.8% for ground beef, broiler chickens, and liquid whole egg, respectively (37,38,39).

While *Salmonella* is a known contaminant of high moisture protein sources, there are many studies that have also investigated the prevalence of *Salmonella* in low moisture foods and feeds. Studies have measured the prevalence of *Salmonella* in animal feeds (40). One of these studies was able to isolate *Salmonella* from dry feed. Between 8% and 16% of the samples of individual feed components (meat, bone, fish and poultry meal supplements) taken were positive for the presence of *Salmonella*. The environmental areas of feed processing plants has also been reported to be contaminated with *Salmonella*. In one study, samples from different points of the production line in ten different grain mills and from a total 3,721 samples, 12.5% were positive for *Salmonella* in these mills (41).

Table 1.6.1 – Low moisture foods reported to harbor *Salmonella.*

Food	Number of Samples	Positive Samples
Chocolate	7	2
Black Pepper	7	6
Pasta	4	3
Coconut	2	1

Source - (42)

Animal feeds are not the only dry foods that have been known to be contaminated with *Salmonella.* Raw almonds implicated in outbreaks with *Salmonella* were tested positive. Follow up studies determined that 0.87% of all samples in a 5-year period were positive for *Salmonella* (43). Sesame seeds have also have been the source of contamination with a study isolating *Salmonella* in 12.5% of raw seed samples, and 11% in processed sesame paste and halvah (44). Several other low moisture foods have also been surveyed, and the results are summarized in Table 1.6.1. Though the sample sizes for some of these foods were small, the study illustrated the wide variety of low moisture foods that *Salmonella* can survive in.

The prevalence of *Salmonella* has also been determined in different flours that were meant for human consumption. Table 1.6.2 shows the data from (84) in which flours of 5 different sources were determined. Wheat was the largest sampled source of grain, for which in over 4,300 samples only six were positive for *Salmonella.* Each of the other four samples of grain flour did not have a sample that was positive for *Salmonella*. This is a reduction from previous studies measuring *Salmonella* prevalence in wheat flour of being 1.3 to 0.3% among at least 1100 samples (84).

Table 1.6.2 – Prevalence of *Salmonella* in different grain flours

Grain	Sample Size	Positive Samples
Wheat	4,358	6
Corn	1,772	0
Oat	714	0
Whole Wheat	286	0
Durum	180	0

Source – (84)

From the data in Table 1.6.2, the prevalence of *Salmonella* within grains was low among these samples. The researchers of that study concluded that *Salmonella* may not be a natural contaminant of dry grain flours, but may be a secondary contaminant due to the processing conditions. Due to the approved amounts of insect fragments and rodent filth in wheat flour, *Salmonella* could contaminate during processing of the grains (84,112). When these insects and rodents contaminate and thrive on the flour, the feces, or the fragments, of these animals could contaminate the flour with the gastrointestinal bacteria.

1.7 Outbreak history

According to the Centers for Disease Control (CDC), *Salmonella* is the foodborne pathogen that causes most of the bacterial outbreaks, since at least 1983 (45). Between 1983 and 2002, the CDC has reported more than 1,800 outbreaks confirmed for a wide variety of *Salmonella* serovars (45-48). Tables 1.7.1 and 1.7.2 include data compiled from the CDC's food borne outbreaks surveillance surveys conducted every five years.

The number of outbreaks attributed to *Salmonella* did not increase between 1982 and 2002 versus the total number of cases (Tables 1.7.1, 1.7.2). Of the twenty years of data tracked, 15 out of the 20 years have total reported and confirmed *Salmonella* cases between 1,700 and 5,000 a year. Two of those five years are extreme outliers, where two large outbreaks accounted for a significant portion of the 10,000+ cases for those years.

The first of the outlier years was in 1985, when a Chicago dairy was involved in an outbreak that, at the time, was the largest outbreak in CDC history, with more than 16,000 culture confirmed samples taken from patients for the outbreak strain of serovar Typhimurium (45,49). The second outlier year was in 1996, when a company was responsible for a very large outbreak of *Salmonella* serovar Enteritidis in ice cream. The number of cases attributed to this specific outbreak could not be confirmed by the CDC, but data on the entire year's cases was available. During 1994, 919 confirmed ice cream cases were reported while 1993 and 1995 had 32 and 60 respectively (48). While, this does not seem like a large number, at least two papers estimated that the actual number of cases based on epidemiological data and formulas were 224,000 (50,51).

Confirmation of a food as the vehicle of a foodborne outbreak or detection of *Salmonella* in a food product can trigger the recall of the contaminated product from the market. The FDA regularly publishes through its website recall notices to become available for the general public. This website has data available on the food product recalls issued in the last six years. These recalls are sometimes from an industry ingredient source that may have been sold to many other producers, which creates a large web of recalls, such as the case involving the Peanut Butter Corporation of America recall of 2009. Table 1.7.3 was compiled through this website and cross referencing other sources, such as the CDC and review articles, which contains some of the largest recalls of the last six years for *Salmonella* and earlier noteworthy outbreaks.

Table 1.7.1 Foodborne outbreaks linked to *Salmonella*

Year	Total outbreaks/ cases with a confirmed agent	Confirmed bacterial outbreaks/cases	Confirmed *Salmonella* outbreaks/cases	Confirmed bacterial outbreak deaths/*Salmo-nella* deaths	Percentage of outbreaks with a confirmed agent
1983	505/14,889	127/7,082	72/2,427	35/7	37
1984	543/16,420	128/7,307	78/4,479	12/3	34
1985	495/31,079	143/22,132	79/19,660	76/20	44
1986	467/12,781	119/4,855	61/2,833	11/7	39
1987	387/16,500	83/8,928	52/1,846	5/2	35
1988	451/15,732	139/7,156	94/2,987	19/14	41
1989	505/15,867	171/6,557	117/4,920	17/14	44
1990	532/19,885	196/9,002	136/6,290	15/1	45
1991	528/14,876	173/6,335	122/4,146	10/5	41
1992	407/11,015	117/4,156	80/2,834	8/4	36
1993	489/17477	135/10,402	68/7,122	9/1	34
1994	653/16,234	148/5,487	70/2,858	3/1	30
1995	628/17,800	155/10,017	90/8,449	11/9	31
1996	477/22,607	112/14,219	69/12,450	3/2	32
1997	504/11,940	105/3,696	60/1,731	2/0	33
1998	1,314/27,258	258/8,919	125/2,731	27/6	13
1999	1,343/24894	217/6,403	111/3,463	5/2	28
2000	1,417/26,122	247/6,905	127/2,850	15/2	24
2001	1,243/25,130	235/7,034	111/3,141	10/7	37
2002	1,330/24,966	227/8,626	111/4,636	13/3	37

Compiled from: (45-48)

Table 1.7.2 Percentage of foodborne disease attributed to *Salmonella*

Years	Percentage of confirmed bacterial outbreaks attributed to *Salmonella*
1983-1987	57
1988-1992	69
1993-1997	55
1998-2002	49

Compiled from: (45-48)

Another notable source of outbreaks has been associated with powdered infant formula (Table 1.7.4). The source of contamination in each of the infant formula outbreaks was spray dried milk. These outbreaks suggest that *Salmonella* has a unique heat resistance, considering that spray dried milk receives two heat treatments, once during the pasteurization process and again during the spray dry process.

Table 1.7.3 Recent *Salmonella* outbreaks and recalls linked to low water activity foods.

Company	Food	Year of Public Recall	Serovar	Human Cases
Not Available[4]	Peanut butter Coated snack[4]	1995[4]	Agona phage type 15	27[4]
Not Available[3]	Dried squid chips	1999	N/A	453[3]
Paramount Farms of California[1]	Raw almonds	2004	Enteritidis	29
Peter Pan and Great Value[1]	Peanut butter	2007	Tennessee	628[2]
Mars Petcare US[1]	Dry pet food	2007	Schwarzengrund	62[2]
Malt-O-Meal[2]	Puffed rice and wheat cereals	2008	Agona	28[2]
Peanut Corporation of America[1]	Peanut butter	2009	Typhimurium	714[2]
Mincing Overseas Spice Company and Wholesome Spice Company[1]	Black and red pepper	2009	Montevideo	272[2]
Basic Food Flavors[1]	Hydrolyzed vegetable protein	2010	Tennessee	0

Sources – [1]FDA Recall List 2004-2010 as of November 14, 2010
[2]CDC Salmonella Homepage and Outbreak Update Page as of November 20, 2010
[3]From (2)
[4]From (52)

Table 1.7.4 Outbreaks of *Salmonella* in infant formula

Salmonella Serotype	Number of infants affected	Location	Year	Reference
Earling	48	UK	1985	(53)
Tennessee*	≥3	USA, Canada	1993	(54)
Virchow*	48	Spain	1994	(55,56)
Anatum	17	UK, France	1996-1997	(56)
London	30	Republic of Korea	2000	(57)
Agona	44	France	2004	(58)
Agona	93	France	2005	(58)

Table adapted from (59) with information from (58)
*Laboratory confirmed for lactose fermentation (54,55)

1.8 Microbial death kinetics

Microbiologists have developed different methods to model the growth and death of microbes in different conditions (60). Growth models are generally based upon first order kinetics, due to the idea that during the log phase of cell growth each cell divides into two cells at a near constant rate that varies upon conditions. These doublings can be easily modeled in the first –order kinetic method which uses the instantaneous growth rate, "μ."

$$\frac{N}{N_o} = e^{\mu t}$$

(Equation 1)

N=Population at a given time
N_o= Initial Population
T=Time
μ=Growth rate
Source-(60)

Some death kinetics models use a logarithmic approach (first-order kinetics), while others use non-logarithmic approaches. The main difference between growth kinetics and death kinetics involves the negative rate constant "k". Many of the death models that are assumed linear use the equation:

$$\frac{N}{N_o} = e^{-kt}$$

(Equation 2)
Source-(60)

The decimal reduction value - one of the main values used in determining how effective a treatment is at inactivating a microbial population, can be determined

through equation 1. The decimal reduction value, hereby referred as the D-value, is the time taken at a specific temperature for a population to be reduced by 90%, which is a one logarithm$_{10}$ reduction. The relationship between the D-value and the rate constant "k":

$$D = \frac{\ln(10)}{k}$$

(Equation 3)
Source-(60)

While a D-value is useful in determining the heat resistance of an organism at a certain temperature, it is generally more useful knowing the response of an organism at multiple temperatures. This temperature change response is known as the "z-value" which is defined as the increase in temperature required for the D-value to be reduced by one logarithmic cycle. The z-value can be defined graphically (Equation 4) or through the Arrhenius equation (Equation 5 with the Arrhenius equation as Equation 6) (60,61).

$$z = \frac{T_1 - T_2}{LogD_1 - Log\ D_2}$$

(Equation 4)
Source-(60)

The other is a graphical method needing at least one logarithmic cycle of the D-values:

$$z = \frac{\ln(10)\ RT_1T_2}{E_a}$$

(Equation 5)
Source - (111)

$$k = Ae^{\frac{-E_a}{RT}}$$

(Equation 6)
Source - (111)

In either case the units of the Z-value is degrees Celsius, and the temperature in either equation 4 or 5 is also in Celsius (60,111). For the Arrehnius equation (Equation 6) the temperature is in Kelvin.

1.9 Water activity

The moisture content of a food is an important intrinsic characteristic that defines many aspects of its quality, deterioration reactions, and the growth of microorganisms. While moisture content is a defined parameter of a food, the effects of water concentrations differ by the composition of food. Different substances hold water in different ways, due to the different kinds of chemical bonds each molecule is involved in. Instead of moisture content, the "water activity" (a_w) or relative vapor pressure provides a parameter that could be better linked to changes in the food. The relative vapor pressure is defined as the ratio of the vapor pressure of water above a food to the vapor pressure of pure water at a constant temperature ($RVP = {}^{P}/_{P_o}$) (63).

The relative vapor pressure is an important parameter within a food, because it is an indicator of the chemical potential of the water that can be used for chemical and biological reactions. For example, although the moisture content of egg yolk with 10% sugar or 10% salt by weight is the same, the water activity is 0.978 and 0.865

respectively (64). Even though they have roughly the same amount of water shown by the moisture content, the difference shown by the water activity is the amount of "active" water that can be involved in reactions. Due to the different intermolecular forces of sugar and salt, they bind water differently causing the lowered water activity of the salted egg yolk.

Water activity has been shown to be a vital indicator to what reactions can occur in a medium, whether the reactions are microbial growth, lipid oxidation, Maillard reactions, etc. The following is the minimum required water activity for growth of different broad classifications of microorganisms.

Table 1.9.1 Minimum water activity growth requirement of microorganisms

Group of Microorganisms	Minimal growth a_w Required
Most Bacteria	0.91-0.88
Most Yeasts	0.88
"Regular" molds	0.80
Halophilic Bacteria	0.75
Xerotolerant Molds	0.71
"Xerophilic" Molds and "Osmophilic" Yeasts	0.62-0.60

Source - (2)

The minimum water activity of 0.88 for growth of "most" bacteria (Table 1.9.1) varies depending mainly upon the Gram reaction of the bacteria. Most Gram-negative bacteria have relatively higher water activity requirements for growth than Gram-positive organisms. For example most *Enterobacteriaceae* cannot grow below 0.93, while Gram-positive *Staphylococcus aureus* has been reported to grow at values as low as 0.83 a_w (2). The water activity can also regulate different biological processes such as toxin production and flagella synthesis, and can cause responses such as cell elongation (2,65). Many microorganisms increase their osmolytes in response to a

reduction of the water activity in the environment. Those increases of osmolytes can be an accumulation of potassium ions or synthesizing an abundance of different amino acids or simple carbohydrates, in an effort to balance the osmotic pressures (66,67).

1.10 Thermal resistance studies in intermediate to high moisture foods

Before 1950, there were multiple thermal resistance studies which set the foundations of the field of thermo-bacteriology. Studies during that era used a wide range of temperatures, media, agents, and organisms and compared them directly. After 1950 more studies were directed towards non-sporeforming bacteria, specifically towards *Salmonella* and *E. coli*. There was more research done on how resistances adjusted to a changing environment, whether it was changes in pH, moisture content, or specific composition changes of the growth medium. One of the earliest studies to show heat resistance as a dynamically changing attribute was a study using *Salmonella* serovars in eggs at different pH levels. The pH studied was 5.5 and 8.0, where the D-values were two to five times higher than at pH 8.0 (68).

Since the thermal resistance of *Salmonella* was shown to change depending on the environment, there have been numerous studies showing the heat resistance of *Salmonella* in individual foods. These foods have included chicken a la king, different concentrations of condensed soup, and many different meats (69-73). Most of these papers had the general idea that the heat resistance of *Salmonella* varied by not only the serovar, but by the composition of the food. For these studies the food components that researchers studied were mainly total salt and fat content which had a direct

relationship with the heat resistance. Investigators were also interested in comparing heat resistances of different organisms of other genera to the heat resistance of *Salmonella* serovars. *Staphylococcus* and *Bacillus* were two of the genera that were compared to *Salmonella* (72,74,75). In those studies, vegetative cells of *Bacillus* and *Staphylococcus* had similar heat resistances to *Salmonella* in the individual foods that they were studied in.

There have been few studies that studied the heat resistance of *Salmonella* in peanut butter, which is an intermediate moisture (0.45 a_w) and high fat (53% fat) food (76). While many of the foods previously mentioned had Z-values between five and nine degree Celsius, the Z-values reported in peanut butter were 55.9^{o}C (76). This is an unusually high Z-value for any organism, and the authors only attributed this result to the high fat and low water activity food.

1.11 Thermal resistance studies in low moisture foods

Due to outbreaks involving dried powders and animal feeds, the heat resistance of *Salmonella* in dry products has attracted significant interest for food microbiologists. The dry products studied first were bone meal and animal feed (77,78,79,80). These studies measured the effects of different water activity values on the heat resistance of *Salmonella* in dry powders. This heat resistance could be seen clearly in bone meal, where the efficacy of heat varied widely by the water activity. While they did not measure the D-values, they did measure the change in viable cells (Table 1.11.1).

Table 1.11.1 Reduction of *Salmonella* in bone meal at different water activity levels

Temperature (°C)	Water activity in bone meal	Heating time (Minutes)	Reduction in number of viable cells
55	0.99	40	10-fold
75	0.88	40	10^2-fold
75	0.77	40	10-fold
90	0.88	20	10^6-fold
90	0.77	40	10^4-fold
100	0.62	20	10^3-fold

Source - (79)

It was concluded decreasing the water activity had a significant effect on the heat resistance of *Salmonella*. Another study reported similar patterns of increasing heat resistance at higher moisture content, but they did not measure water activity (77). In meat and bone meal *Salmonella* serovar Seftenberg was measured with at temperatures between 52 and 85°C with three moisture content levels, 5, 10, and 15% (77). The D-values of the cells tested at 15% moisture content were the smallest, while at a 10% moisture level the D-values were at least two-fold greater. The heat resistant experiments of cells in the 5% moisture level measured D-values between two to three times as high as the 10% level.

In addition to the relationship with moisture content, changes in heat resistance as dependent on solutes, pH, and water activity have been observed recently. The first of two of these publications studied the differences between sucrose and glycerol at different water activities and concentrations (81). Since glycerol decreases the water activity more effectively than sucrose, the water activity for a glycerol solution is lower than its glucose counterpart at the same percent concentration. The study measuring

sucrose and glycerol at different concentrations measured the massive increase in heat resistance when the solute lowering the water activity is sucrose instead of glycerol.

Table 1.11.2 D-values of *Salmonella* serovars Typhimurium (STy) and Tennessee (STe) at 57.2^{o}C as affected by different water activities obtained with sucrose and glycerol.

	Sucrose			**Glycerol**		
Water activity	% Conc. (w/w)	STy D-value (min)	STe D-value (min)	% Conc. (w/w)	STy D-value (min)	STe D-value (min)
0.75	ND	ND	ND	57.7	8.8	6.0
0.87	63.7	61.5	35.9	ND	ND	ND
0.90	58.6	46.7	23.5	33.9	2.6	1.7
0.93	51.3	30.5	21.3	ND	ND	ND
0.96	39.6	14.3	9.5	ND	ND	ND
0.99	14.5	1.1	0.8	4.9	1.1	1.1

Adapted from - (81)

These researchers also compared the effects of sorbitol, fructose, glycerol, and sucrose at a 0.96 water activity (81). A D-value of 16 minutes at 57.2^{o}C was measured with sucrose, while heat treatments in the presence of sorbitol, fructose and glycerol resulted in D-values of 5.5, 1.3, and 1.2, respectively. This compared to another publication studying the same solutes of fructose, glycerol, and sorbitol, as well as glucose and sucrose. These studies cannot be compared directly though, because the researchers did not evaluate the solutes on basis of water activity, but instead at the 30 and 70% concentration levels (Table 1.11.3)(82).

Table 1.11.3 D_{65} values of *Salmonella* in various solutes at 70% and 30% (w/v) concentration in 0.1 M phosphate buffer pH 6.5

Solute	Concentration (% w/v)	D_{65} values of *S.* Typhimurium 7M 4987 (min)	D_{65} values of *S.* Senftenberg (min)
Sucrose	70	53	43
	30	0.7	1.4
Glucose	70	42	17
	30	0.9	2
Sorbitol	70	20	17
	30	0.7	2.1
Fructose	70	12	8.5
	30	0.5	1.1
Glycerol	70	0.9	0.7
	30	0.2	0.95

Source - (82)

The large increase in heat resistance due to the addition of sucrose compared to other compounds has been attributed to the particular effect of these substances on bacterial cells (82). Sucrose, glucose, fructose, and glycerol have been hypothesized to cause plasmolysis, which dehydrates the cells and stabilizes the proteins and enzymes. Of these sugars and polyols, sucrose cannot be internalized by *Salmonella,* while glucose, fructose and glycerol are taken up by the bacterial cells. This internalization mechanism of glycerol, glucose, and fructose replaces some of the intracellular water, stabilizing other components as well. While the internalization mechanism of glycerol does stabilize cell components, it does not protect as well as the dehydration and non-internalization plasmolysis mechanism of sucrose.

The heat resistance of *Salmonella* as affected by water activity has also been studied in cereal flours directly. Flour is a food with a particularly low water activity that

is often contaminated with *Salmonella* (83,84). Two reports specifically investigated the heat resistance of *Salmonella* in flour. One study compared eight different serovars in corn flour, and the second report studied one serovar in wheat flour (83,85). The study using corn flour only measured the D-values at 49°C which varied between 0.3 to 9.9 hours (83). The second study measured serovar Weltevreden at six different temperatures over six different water activities and the data is summarized in Table 1.11.4 (85).

Table 1.11.4 Z-values of *Salmonella* serovar Weltevreden in flour at different water activities

Range of water activity values	Z-value (°C)
0.60-0.56	30.3
0.50-0.46	53.9
0.45-0.41	19.6
0.36-0.40	15.2
0.31-0.35	29.2
0.25-0.30	34.7

Source - (85)

While that study reported unusually high Z-values, it seemed to corroborate the dependence of heat resistance on water activity. These results suggested that the heat resistance is very dynamic and did not just increase as the water activity decreased, but that had an apparent maximum between 0.46-0.50 a_w. The authors hypothesized an explanation for the dynamic values measured, and speculated that water activity may not have been equilibrated thoroughly, or that the heating method changed the water activity of the flour as it was heated. The heating method would have changed each of

the test parameters equally, but the methods used to set the water activity may have affected the data. The study used a super saturated solution of lithium chloride (a_w=0.11 @23°C) to lower the flour's water activity. Since they used only one desiccating salt, they were removing the flour after it had been in the same desiccator for different time periods. Those samples that were in the longest had the lowest water activity. However, they did not let the flour equilibrate, and were merely measuring the water activity of the surface of the flour, because desiccators remove water from the outside of the flour, and remove more as it equilibrates. If the samples were truly equilibrated, all of the flour samples would have been 0.11 a_w throughout the entire sample. If this error was the source of the changing Z-values, it would be assumed that the longer the flour was kept in the desiccator more of the flour would be closer to 0.11 a_w. Though the trends they found may still be valid because as each sample was removed and had the surface water activity measured, the samples had wide variations of their Z-values.

To try to understand the unique heat resistance of *Salmonella* under low water activities, two studies observed the potential role of ribosomes (86-88). These studies treated cells with the antibiotics chloramphenicol and rifampin to stop protein synthesis, and other reports used magnesium chloride to stabilize the ribosomes during heat treatment. The role of chloramphenicol and rifampin would theoretically stop any extra proteins from being synthesized, if the mechanism of low water activity heat resistance would involve the expression of stress response proteins (88,89). The cells were incubated in a solution of glucose-fructose (a_w=0.95) for 48 hours with the

antibiotics to rule out the possibility that the heat resistance was induced by a reduction in the osmotic pressure. The antibiotic treatment did not have any effect on heat resistance unless the antibiotics were added to cells after 48 hours of growth.

1.12 Linear versus non-linear inactivation models

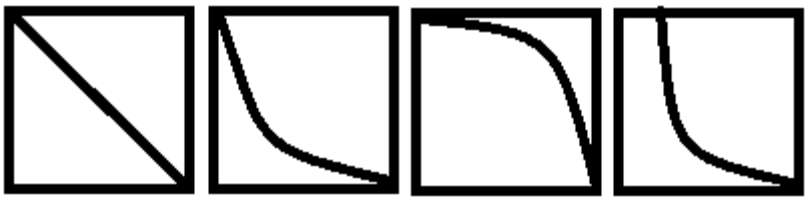
One of the issues that studies on thermal resistance often encounter is the choice of mathematical model to describe the inactivation data. While the data in most studies could satisfy a linear correlation in a logarithmic scale, several investigations reported non-linear inactivation curves (90,91). These non-linear graphs had different characteristics that may involve a large initial drop, a lagging period ("shouldering"), or a period at the end with no decline in the population ("tailing"). These characteristics were evident since the earliest studies on thermal resistance in 1929 that described the death curves of different bacteria (90). These studies reported an initial one or two log CFU decrease in the population at the beginning of the experiments, which the researchers had problems explaining why such a large percentage of the total population was inactivated in the first part of the experiment.

During this time period, the proposed mechanism of action of any stress on a cell was termed the monomolecular theory, where the stress reacts with one molecule or a single set of molecules within the cell. This hypothesis meant that whatever happened in one cell must happen in every other cell at the same time, because they all have exactly the same molecules, causing a constant reaction rate throughout the time measured. However, those initial and rapid cell viability reductions mentioned before

challenged this hypothesis, which was explained through different theories, such as, variation in resistance, inter- cellular clumping, or changes in states of dormancy as response to stress.

The variation in resistance has not been well understood, and researchers have speculated to be from the natural variation in size of the cells. The different sizes of the organism may cause the chemical or thermal diffusion rates to differ among the individual organisms. This hypothesis was tested by comparing their research to other thermal studies. The other studies used for comparison involved "Bacterium typhosum" and *Staphylococcus aureus*, fruit flies, anthrax spores and mustard seeds (91). While these comparisons involve subjects that are immensely different and were not truly equivalent studies, it showed an important relationship. Different organisms can have different death patterns which can also vary using the same organism versus different agents.

Through these early studies, the following graph shapes characterized different organism's death curves. Each kind of graph is attributed to different organisms seen in the chart below.



A B C D

(Redrawn from (91))

Figure 1.12.1 Different possible death curves

During the early 20th century, it was theorized only four types of heat inactivation curves were observed based upon the organism's characteristics (93). Under curve type A, small and medium spores such as *B. subtilis* and *B. anthracis* are typical. *Escherichia coli* responses were characteristic of curve type B. Based on this classification the inactivation of *E. coli* and some yeasts follow curve type C. Finally, curve type D was only observed with large spores. These different curves opposed the monomolecular theory of inactivation, and a multi-molecular theory of inactivation was proposed, instead. Other studies did not observe inactivation curves looking like graph "A" above, but rather the other three. These graphs had less linear reaction rates, where some would decrease as the experiment went on (graph "B" and "D"), or others would increase (graph "C"). This was explained by the inactivating element needing to react with multiple vital sites within the cell. The energy needed to inactivate these sites can have natural variation between individual cells, due to stages of growth and microenvironments.

These shoulders or tails were not reported in every study, and varied even with different trials of the same experiment. To account for the tailing or shouldering, researchers have either taken partial parts of the death curve to calculate D-values, or used mathematical models to account for it. These models range from computer calculated best-fit models to the Weibull method. When researchers have taken partial parts of the death curves only the most linear parts of the graph are typically used, omitting the tail or the shoulder if present (76-78,92,93).

One study focused on the “tailing” of the graph using chloramphenicol, an antibiotic which inhibits protein synthesis (88,89). The addition of chloramphenicol did not affect the initial viable count reduction, but it influenced the “tailing” of the biphasic death curves. The antibiotic reduced the D-values of the tail from 89 minutes at 59°C to 64 minutes using 100 µg/ml (86). Even with the addition of chloramphenicol the initial section of the graph had D-values of 22 to 25 minutes at 59°C. Though the D-values of the tailing were still larger than the D-values of the initial sections of the graph, they were lowered due to the antibiotic. The researchers explained that the evidence of tailing was due to a failure of chloramphenicol to completely stop protein synthesis (86).

The results of the study with chloramphenicol were in agreement with another study that speculated the mechanism behind causing the tailing was due to protein synthesis, although it was observed the tailing was only present when the test temperature was below 60°C. Above that temperature the inactivation curves followed a more linear model (94). Attempts from that study, and another report measuring *Salmonella* in chocolate milk, powder to subculture and retest the tailing for greater heat resistances have indicated no enhancement of resistance (78). This is in disagreement of a study which measured *Salmonella* in peanut butter, which measured a higher resistance of the cells when retesting the cells for a second heat treatment (76,95).

Chapter 2 - Materials and Methods

2.1 Strains and culture preparation:

The *Salmonella* strains used in this research were *S.* Typhimurium E2009005811, *S.* Tennessee E200700502 and *S.* Agona. The first two strains were provided by the Minnesota Department of Health and they were isolated from patients linked to the peanut butter outbreaks of 2009 and 2007, respectively (96,97). The *S.* Agona serovar was originally isolated from an outbreak related to toasted oats cereal from 1998. The stock cultures of the three serovars were stored in a 1:1 ratio of glycerol and tryptic soy broth (TSB; Neogen, Inc., East Lansing, MI) at -55^{o}C. The working cultures of each serovar were prepared from frozen stocks and inoculated into TSB, grown overnight at 37^{o}C and then stored at 4^{o}C. To test the working cultures, they were re-transferred once a week and streaked onto tryptic soy agar (TSA; Neogen, Inc.) containing 0.8 g/L ferric ammonium citrate and 6.8 g/L sodium thiosulfate. Periodically the serovars were also streaked onto bismuth sulfate agar (Neogen, Inc.) and xylose lysine deoxycholate agar (Neogen, Inc.) for confirmation.

2.2 Inoculation and drying procedure

From working cultures, serovar cultures were grown overnight in 40 mL TSB and added to bottles containing 360 mL sterile water and gently shaken. Twenty grams of a commercially available toasted oat cereal were added to the bottles and mixed by repeated inversion for one minute. The inoculated cereal samples were strained with sterile kitchen strainer and spread out on sterile perforated kitchen baking sheets. The

baking sheets with cereal were placed in an incubator at 40^{o}C for 12 to 18 h to allow drying. The cereal samples were then ground using sterilized mortar and pestle in a bio-safety cabinet, placed on aluminum foil trays and equilibrated to specific water activities in desiccators to control the water activity.

For the trials involving added sucrose, 7.5 g of commercially available powdered sucrose was added after the cereal was spread upon the baking sheet. Sucrose was mixed into the cereal by piling the cereal and adding the sucrose in small increments by folding and mixing the sucrose into the wet cereal with sterile spoons. Once trays were removed from the incubator at the end of the drying period, the trays were weighed to ensure that 6.5±0.5 grams, or 25% by weight, of sucrose were added. Sucrose containing cereal was also ground and equilibrated as described for the trials with no sucrose.

2.3 Preparation of individual samples

The 26.5 g, or 20 g without sucrose, ground cereal sample was separated and set in two separate foil trays. The foil trays were then stored into two separate desiccators of differing water activity for 7 to 12 days to fully equilibrate the samples. The foil trays containing the cereal were removed and the water activity was measured to ensure the samples were ±0.02 a_w of the desiccator the samples were put in. If the cereal was not within 0.02 a_w of the target water activity within 7 to 12 days, the sample was not used. These samples were placed into sterile 12 cm^3 syringes and cereal was transferred into capillary tubes (1.5-1.8 x 90 mm borosilicate glass) by inserting the tubes through the

luer-lock tip of the syringes. Ram rods (118 mm x 1 mm stainless steel) wiped with 70% ethanol were used to fill the capillary tubes when necessary. The cereal filled tubes were heat sealed using a propane hand torch and placed in a 10% commercially available chlorine bleach solution for at least one minute. If the bleach was observed whitening the cereal, the samples were not used. After all capillary tubes were filled the water activity was measured using the remaining cereal in the syringe. Water activity of the samples was measured using a water activity meter (Pawkit Model, Decagon Devices,Inc., Pullman, ID) calibrated every other day according to the manufacturer's procedure. Once all the needed capillary tubes were sealed, the water activity of the remaining cereal in the syringe was measured. If the remaining cereal's water activity varied by more than 0.02 a_w, none of the capillary tubes were used.

2.4 Thermal testing

All of the sealed capillary tubes were placed into either an oil bath (High Temp Bath 160 A, Fisher Scientific, Inc., Waltham, MA) or water bath (Isotemp 205, Fisher Scientific, Inc.) calibrated once a month. The water baths were set at the testing temperatures between 60 to 95^{o}C and the oil bath from 85 to 105^{o}C. At predetermined time intervals, two capillary tubes were removed and immediately placed in an ice bath for one minute. From the ice bath, the tubes were placed in a solution of 10% bleach and rinsed with sterile water. Each heat sealed capillary tubes were then placed in separate sterilized 24 x 150 mm screw cap test tubes each containing a magnetic stir bar (25 x 5 mm) and vortexed until the capillary tube was pulverized and the cereal exposed.

Ten milliliters of phosphate buffer (PB) were added to the test tubes and mixed for 10 seconds. These buffer suspensions were further diluted by transferring 1 mL serially into 9-mL PB tubes. Volumes of 0.1 mL of dilutions were spread plated in duplicate, on TSA containing with 0.8 g/L ferric ammonium citrate and 6.8 g/L sodium thiosulfate. The plates were incubated for 24 hours at 37^{o}C before counting colonies.

2.5 Calculations

The counts of surviving serovar cells were calculated using the aerobic plate count formula from the Food and Drug Administration's Bacteriological Analytical Manual (98). Each capillary tube sample was calculated individually and averaged with its replicate. The inactivation curves were plotted using Excel 2007 by either using a linear plot of the time versus the log of the plate count, or on an exponential semi-log plot using the time versus the plate count of each interval. Tailing, defined as three consecutive points that counts were not reduced more than 0.25 logarithms, was removed if it was prevalent.

2.6 Habituation studies

The habituation test was performed by inoculating 20 g samples of the toasted oat cereal as the procedure above. The cereal samples were ground and water activity values were measured. The 20 g samples were separated into five syringes and stored in a heat sealed stomacher bag at room temperature, and removed and tested in

intervals. Once a syringe was removed from the stomacher bag, the bag was resealed with the remaining syringes.

2.7 Rehydration studies

The water activity of 20 g samples of inoculated cereal was measured, and one gram samples added to sterile 24 x 150 mm test tube with a 25 x 5 mm magnetic stir bar. Volumes of 30 mL of PB were added and vortexed for 30 s. Using 5 mL disposable syringes and a 4" 22 gauge hypodermic needle (Air-Tite Product Co., Inc.) the fluid from the 24 x 150 mm test tube was used to fill (1.5 – 1.8 x 90 mm) capillary tubes. The capillary tubes were immediately flame sealed and tested in the same procedure as the other thermal inactivation studies.

2.8 Statistics

The total count for each interval within every trial was calculated by averaging the counts of the two duplicate capillary tubes. D-values were calculated using a semi-logarithmic method, where the inactivation curves was plotted using the total bacterial count at each sampling interval against time. The slope of this curve was found with an exponential trend line using Microsoft Excel 2010. If tailing was present, defined as three testing intervals at the end of a trial with less than a 0.25 log reduction, the tailing was removed. The inactivation curve had the D-value calculated if there were more than 5 intervals, after removal of the tail and a regression coefficient (R^2) above 0.80. The D-value was then calculated by the following equation: $D=\frac{ln(10)}{Slope}$. The reported D-

value for each condition and temperature was an average of the three replicate trials. Error for each D-value was found by calculating the 95% confidence interval with Microsoft Excel 2010.

Z-values were calculated by plotting, on a semi-logarithmic graph, each of the four D-values against the temperature they were tested at for each serovar. An exponential trend line was then calculated using Microsoft Excel 2010, and the Z-value was calculated using the following equation: $Z=\frac{ln(10)}{Slope}$. Statistical differences were measured using the average of the Z-value of all three serovars and using a two tailed student t-test with significance defined as $p<0.05$.

Chapter 3. Results

3.1 Overall results

All trials were measured with the attempt to graph at least seven data points over four 5^{o}C intervals at each water activity level tested, with each condition performed in triplicate. To increase accuracy and lower variance of the inactivation curves, each data point is an average of two samples for each trial performed. From a total of 216 experiments, 42 trials had a relatively high linear regression coefficients (R^2>0.95) observed between the reduction of the viable count and time (Fig. 1). While only 19% of all trials had correlations values above 0.95, 103 trials (48%) had correlation values above 0.90. The remaining 113 trials (52%) had correlation values between 0.80 and 0.90, where trials with correlation values lower than 0.80 were not included in determining average D-values. In many trials, the lack of linearity was due to a marked reduction of more than one log CFU/g right after the initial data point (Fig. 2B, D, F). In those cases, the linearity is often observed when the initial data point is ignored, though the D-values were calculated with the inclusion of the initial data point.

The shapes of the inactivation curves were occasionally characterized by a period of very slow inactivation at the end of the trials (Fig. 3). This period of very slow inactivation was defined as a period at the end of the trial with three or more intervals with less than a 0.25 log CFU/g reduction. These 'tailing' periods were not observed in every trial, but were seemingly random. There were 16 trials with observed tailing without the addition of sucrose compared to six with the addition. Cultures obtained from these periods of tailing did not have any significant differences in heat resistance

did not have any significant differences in heat resistance when grown and challenged through the same methods again.

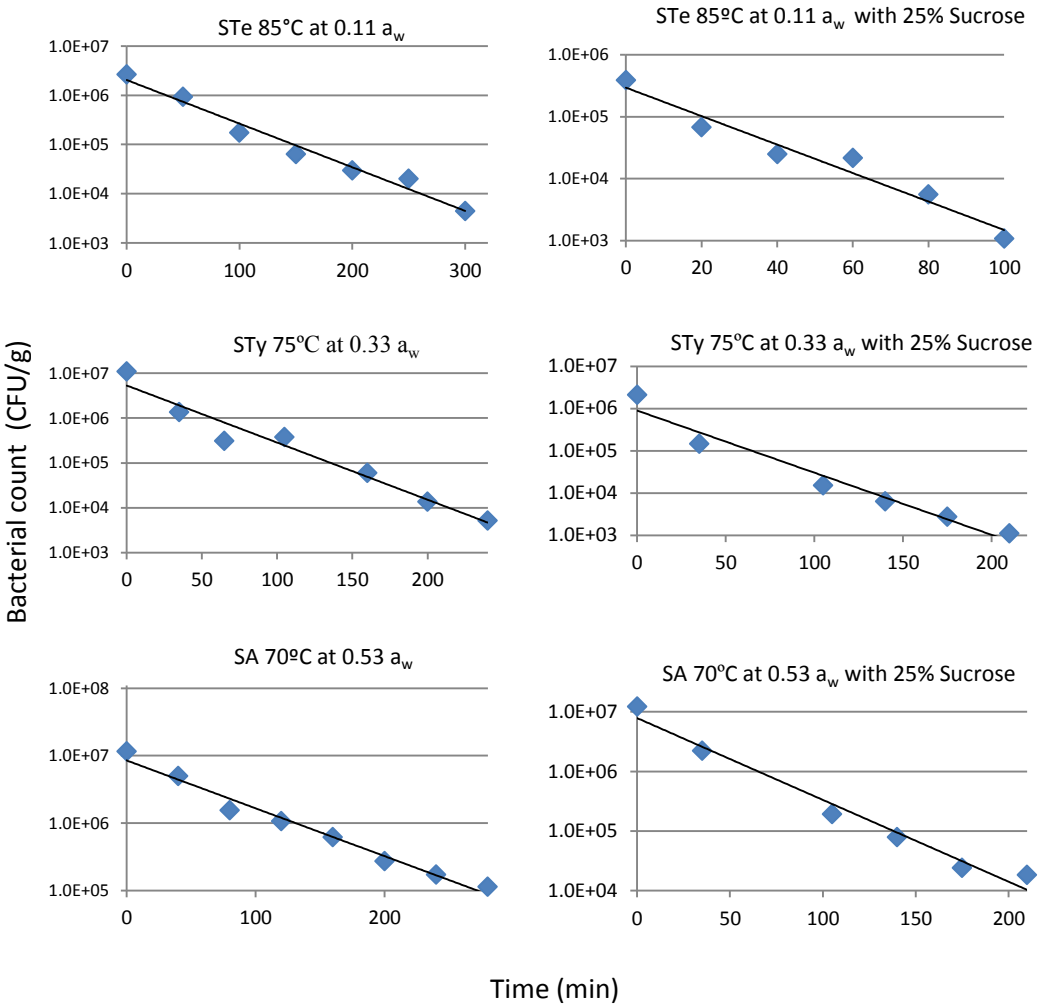


Figure 3.1.1 Representative plots of thermal inactivation experiments of *Salmonella* in dry cereal at different water activities (a_w) and temperatures that had linear regression coefficients (R^2) greater than 0.95. STy, STe, and SA are abbreviations for *Salmonella* serovars Typhimurium, Tennessee and Agona, respectively.

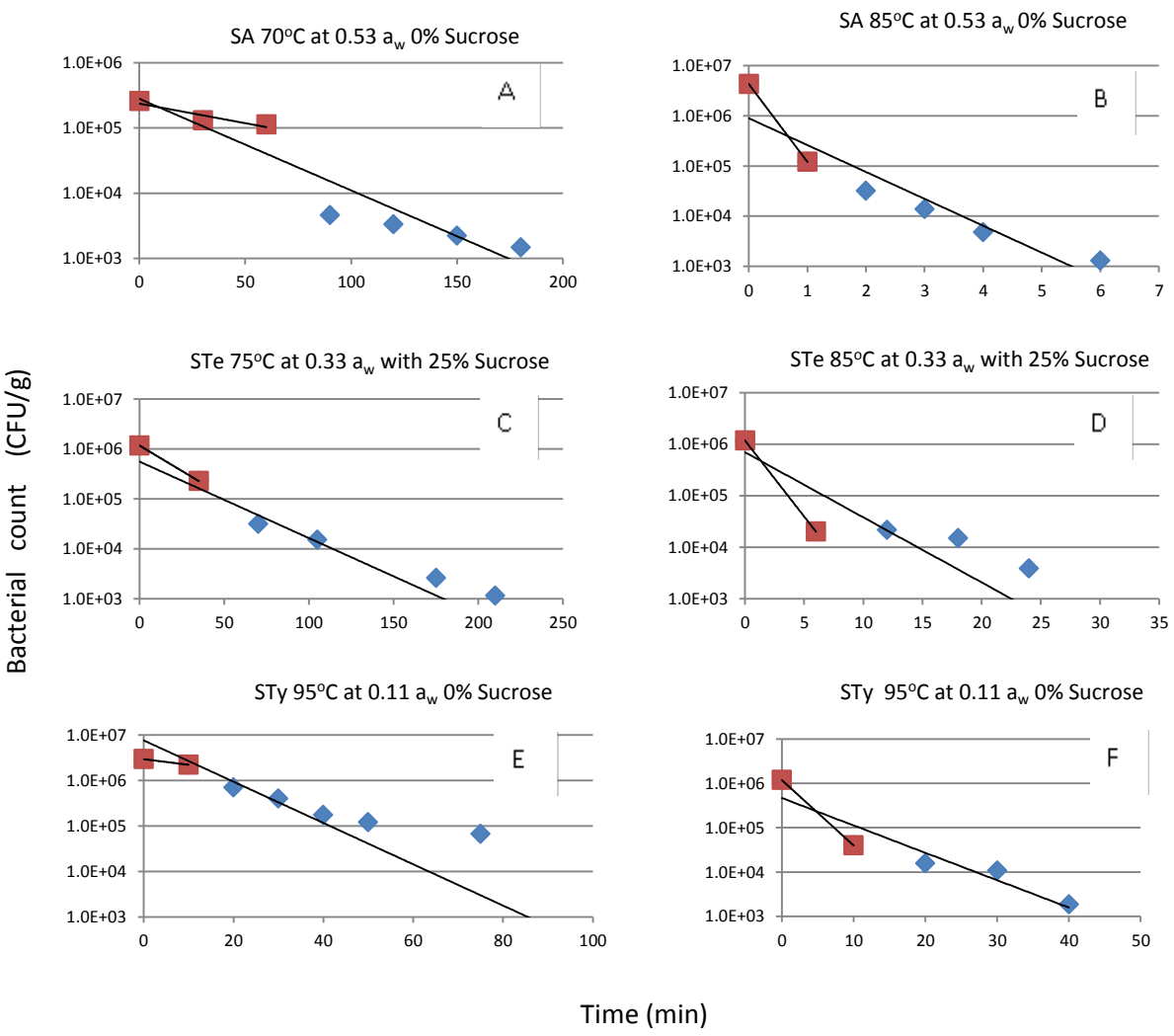


Figure 3.1.2 Representative plots of thermal inactivation experiments of *Salmonella* in dry cereal at different water activity (a_w) and temperatures that had different characteristic reductions of the initial population. STy, STe, and SA are abbreviations for *Salmonella* serovars Typhimurium, Tennessee and Agona, respectively.

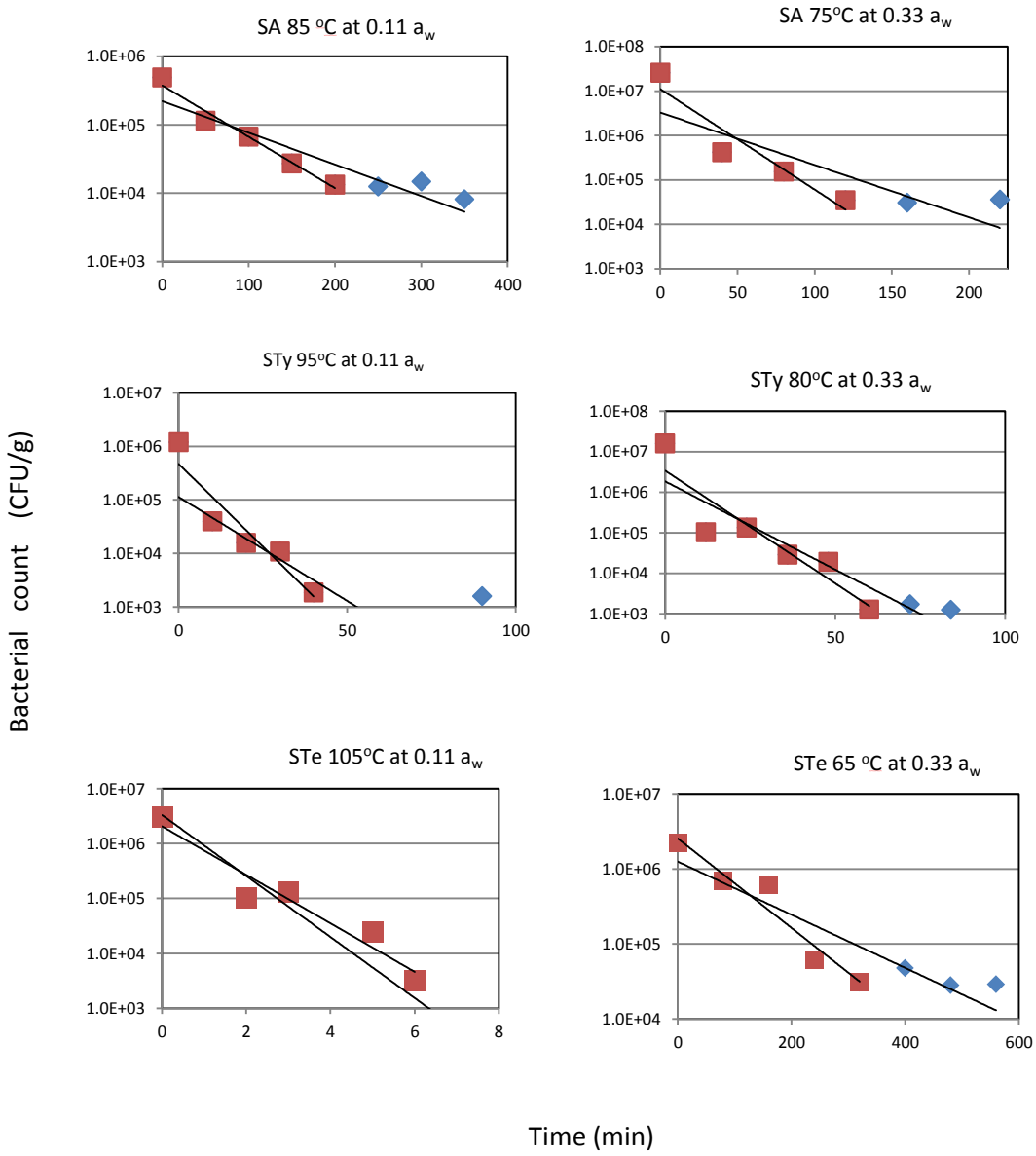


Figure 3.1.3 Representative plots of thermal inactivation experiments of *Salmonella* in dry cereal at different water activity (aw) and temperatures that had linear regression coefficients (R^2) greater than 0.80 if some data points at the end of the experiments (blue diamond) were eliminated from calculation. STy, STe, and SA are abbreviations for *Salmonella* serovars Typhimurium, Tennessee and Agona, respectively.

In cereal samples without sucrose, D-values of each of the three serovars decreased as the temperature increased, but the extent of decline was markedly affected by water activity (Table 3.1.1). The D-values of all strains increased significantly as the water activity declined. At 75°C, the D-values ranged from 27 to 29 min at 0.53 a_w but similar values increased almost 3-fold at 0.33 a_w. Comparing the individual D-values at each temperature and water activity level, significant differences were observed between the water activity levels. The average increase in D-values in trials without the addition of sucrose for each three serovars ranged between a two and six fold increases when the water activity was lowered from 0.53 a_w to 0.33 a_w, while a 17 to 22 fold increase at 85°C when it was further lowered from 0.33 a_w to 0.11 a_w. Among serovars, little differences in D-values were observed at 0.33 and 0.53 a_w but *S*. Agona had consistently larger values at temperatures above 95°C at 0.11 a_w in plain cereal.

Addition of sucrose to cereal resulted in similar effects of water activity and temperature to those observed with samples without sucrose. D-values declined as the treatment temperature increased, and water activity reductions caused an enhancement in thermal resistance at the same temperatures. The effect that the addition of sucrose had on the thermal resistance could be easily quantified through calculating percent change of the D-values (Table 3.1.2). At 0.11 a_w consistent reductions in D-values for all three serovars of at least 45% were observed at temperatures between 85°C and 100°C in sucrose-containing samples compared to plain cereal. In contrast, treatment with sucrose at 0.33 a_w resulted in larger D-values and the

percent change was close to 100% at 85°C (Table 3.1.2). Sucrose addition also increased the D-values at 0.53 a_w, but at low temperatures a few negative changes were recorded.

Table 3.1.1 Rate of thermal inactivation of *Salmonella* in cereal measured as decimal reduction time (D-values) as affected by water activity (a_w), temperature and sucrose content. STy, STe, and SA are abbreviations for serovars Typhimurium, Tennessee and Agona, respectively.

Water Activity	Temperature (°C)	D-values (min)					
		STy	STe	SA	STy	STe	SA
		No sucrose			With sucrose (25%)		
0.11	85	105.8	133.9[a]	117.0	49.5	49.4[a]	63.8
	90	NT	NT	NT	19.3	15.5	24.3
	95	16.4[b]	23.1	53.7[c]	5.7[b]	6.4	8.3[c]
	100	10.7	10.8[d]	15.8	3.2	3.5[d]	5.0
	105	3.5	2.4	5.2	NT	NT	NT
0.33	70	171.9	178.3	174.2	NT	NT	NT
	75	79.9	80.3	75.8	86.7	85.0	100.2
	80	23.3	20.5[e]	24.9[f]	41.1	27.6[e]	35.8[f]
	85	5.4	5.9[g]	6.7	13.6	11.5[g]	13.2
	90	NT	NT	NT	2.8	3.4	3.6
0.53	65	203.3	172.4	207.9	NT	NT	NT
	70	93.2	74.6	106.4	90.4	82.8	80.2
	75	26.9	28.1	29.0	28.5	25.4	30.9
	80	6.1[h]	6.5	4.3[i]	12.3[h]	8.6	8.3[i]
	85	NT	NT	NT	2.8	2.9	2.4

Each D-Value is an average of three replicates
NT, Not tested
Same superscript letters are significantly different between levels of sucrose ($P<0.05$)

Table 3.1.2. The percent change of D-values of *Salmonella* serovars Typhimurium (Sty), Tennessee (Ste), and Agona (SA) in dry cereal as a result of addition of 25% sucrose.

Temp. (°C)	Percent change of D-Values due to addition of 25% sucrose								
	0.11 a_w			**0.33** a_w			**0.53** a_w		
	STy	**STe**	**SA**	**STy**	**STe**	**SA**	**STy**	**STe**	**SA**
70	ND	ND	ND	ND	ND	ND	-3.0	11.0	-24.6
75	ND	ND	ND	8.5	5.9	32.2	5.9	-9.6	6.6
80	ND	ND	ND	76.4	34.6	43.8	101.6	32.3	93.0
85	-53.3	-63.1	-45.5	151.9	94.9	97.0	ND	ND	ND
95	-65.2	-72.3	-84.5	ND	ND	ND	ND	ND	ND
100	-70.1	-67.6	-68.4	ND	ND	ND	ND	ND	ND

Percent change = [(D-value with sucrose/D-value without sucrose) × 100] – 100.

The D-values of each of these conditions were plotted in a semi-logarithmic method against the temperature and an exponential curve fitting was obtained (Fig. 4). In almost all of the cases the correlation coefficient of a linear regression had values greater than 0.95 (Table 3.1.3). Based on those regressions, the change in temperature required to obtain a 90% reduction in D-values, also known as Z-value were calculated for each serovar and water activity (Table 3.1.4). The Z-value was also calculated as an average of all three serovars together to find the statistical significance of the effect of lowering the water activity on all the serovars in general. As the water activity is lowered from 0.53 to 0.33, the slopes remained similar which resulted in close, statistically insignificant changes to the Z-values. Although the heat resistance can be

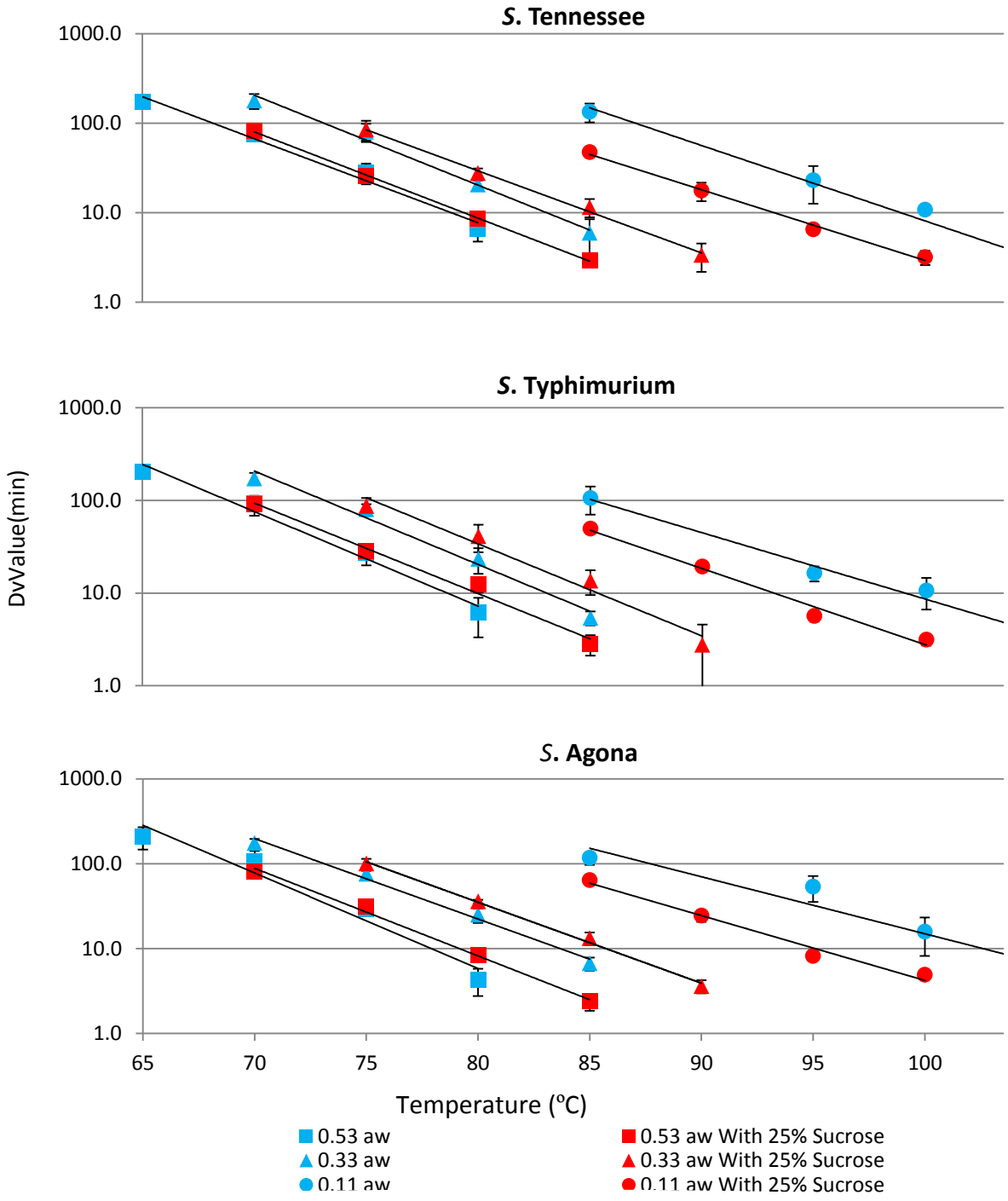


Figure 3.1.4. Effect of temperature on D-values of *Salmonella* serovars in dry cereal and the influence of water activity (a_w) and the addition of sucrose.

Table 3.1.3 Regression parameters of correlation of D-values versus the temperature of thermal inactivation determinations of *Salmonella* serovars Typhimurium (Sty), Tennessee (Ste) and Agona (SA) in dry cereal as affected by water activity (a_w)and sucrose addition.

	No Sucrose								
	0.11 a_w			**0.33 a_w**			**0.53 a_w**		
	Intercept	Slope	R^2	Intercept	Slope	R^2	Intercept	Slope	R^2
STy	1.3×10^8	-0.166	0.99	2.4×10^9	-0.232	0.98	1.1×10^9	-0.235	0.98
STe	2.2×10^9	-0.194	0.98	2.6×10^9	-0.232	0.99	2.5×10^8	-0.216	0.98
SA	7.8×10^7	-0.155	0.93	8.4×10^8	-0.218	0.99	5.9×10^9	-0.260	0.96
	25% Sucrose								
	0.11 a_w			**0.33 a_w**			**0.53 a_w**		
	Intercept	Slope	R^2	Intercept	Slope	R^2	Intercept	Slope	R^2
STy	4.9×10^8	-0.190	0.98	3.1×10^9	-0.229	0.97	6.5×10^8	-0.225	0.99
STe	2.4×10^8	-0.182	0.99	6.6×10^8	-0.212	1	4.5×10^8	-0.222	1
SA	1.8×10^8	-0.176	0.98	1.5×10^9	-0.220	1	1.5×10^9	-0.238	1

Table 3.1.4 Change in temperature needed to obtain a 90% reduction in D-values, or Z-value of *Salmonella* serovars Typhimurium (Sty), Tennessee (Ste), and Agona (SA) in dry cereal as affected by water activity (a_w) and sucrose addition.

Water Activity	Z-values (^oC)							
	STy	STe	SA	Average	STy	STe	SA	Average
	No sucrose				With sucrose (25%)			
0.11	13.91	11.86	14.90	13.6[a]	12.11	12.64	13.10	12.6[a]
0.33	9.92	9.95	10.56	10.1[b]	10.05	10.88	10.49	10.5[b]
0.53	9.79	10.65	8.88	9.8[b]	10.23	10.37	9.69	10.1[b]
Same superscript letters are not significantly different between water activity levels (P<0.05)								

seen to rise due to the Y-intercepts of each graph rising, shifting the nearly parallel lines upwards, signaling an increase in thermal resistance. These trends between 0.33 a_w and 0.53 a_w were present with and without the addition of sucrose, and sucrose did not have a statistically significant effect at these water activity levels.

As the water activity was further lowered to 0.11 a_w the slopes decreased significantly when all the D-values were plotted, while the Y-intercepts generally decreased, causing a large statistically significant increase compared to either water activity level. The Z-value of each serovar increased from 2 to 4^{o}C, compared to either 0.33 or 0.53 a_w. The same trends were observed with the addition of sucrose, but the samples with the added sucrose were significantly less based on the results of a t-test.

The heat resistance of each serovar had the same general trend of increasing D-values as the water activity of the environment was decreased, and 0.11 a_w the addition

of sucrose markedly decreased the D-values. When each serovar's heat resistance was compared to the other two serovars, differences were noticeable. Serovars Typhimurium and Tennessee did not have a single statistically significant difference at any temperature tested at any of the three water activity levels, even with the addition of sucrose.

Serovar Agona differed from both serovars at each water activity level at certain temperatures (Table 3.1.5). Without the addition of sucrose, the significant differences between Agona and the other two serovars were only observed as the water activity was lowered to 0.11 a_w. The most statistically significant differences were seen when additional sucrose was present, in which the D-values of Agona were different at every temperature tested. With and without the addition sucrose, the heat resistance of Agona did not have any observed statistical differences between the other two serovars at 0.33 a_w, though it did have significant differences at 0.53 a_w when additional sucrose was present.

As seen in Table 3.1.3, the slope of all the D-values had the same general trend of decreasing as the water activity was lowered from 0.33 a_w to 0.11 a_w. Sucrose had a similar impact on the slopes of each serovar, except Tennessee which was the only serovar that had an increase in Z-value, though small, with the addition of sucrose at 0.11 a_w. This is opposed to the trends observed in each of the other serovars.

Table 3.1.5 Statistical differences between serovars of the rate of thermal inactivation of *Salmonella* in cereal measured as decimal reduction time (D-values) as affected by water activity (a_w), temperature and sucrose content. STy, STe, and SA are abbreviations for serovars Typhimurium, Tennessee and Agona, respectively.

Water Activity	Temperature (°C)	D-values (min)					
		STy	STe	SA	STy	STe	SA
		No sucrose			With sucrose (25%)		
0.11	85	105.8	133.9	117.0	49.5[a]	49.4	63.8[a]
	90	NT	NT	NT	19.3[b]	15.5	24.3[b]
	95	16.4[c]	23.1	53.7[c]	5.7[d]	6.4[e]	8.3[d,e]
	100	10.7	10.8	15.8	3.2[f]	3.5[g]	5.0[f,g]
	105	3.5	2.4[h]	5.2[h]	NT	NT	NT
0.33	70	171.9	178.3	174.2	NT	NT	NT
	75	79.9	80.3	75.8	86.7	85.0	100.2
	80	23.3	20.5	24.9	41.1	27.6	35.8
	85	5.4	5.9	6.7	13.6	11.5	13.2
	90	NT	NT	NT	2.8	3.4	3.6
0.53	65	203.3	172.4	207.9	NT	NT	NT
	70	93.2	74.6	106.4	90.4	82.8	80.2
	75	26.9	28.1	29.0	28.5	25.4[i]	30.9[i]
	80	6.1	6.5	4.3	12.3[j]	8.6	8.3[j]
	85	NT	NT	NT	2.8	2.9	2.4

Each D-Value is an average of three replicates
NT, Not tested
Same superscript letters are significantly different between serovars ($P<0.05$)

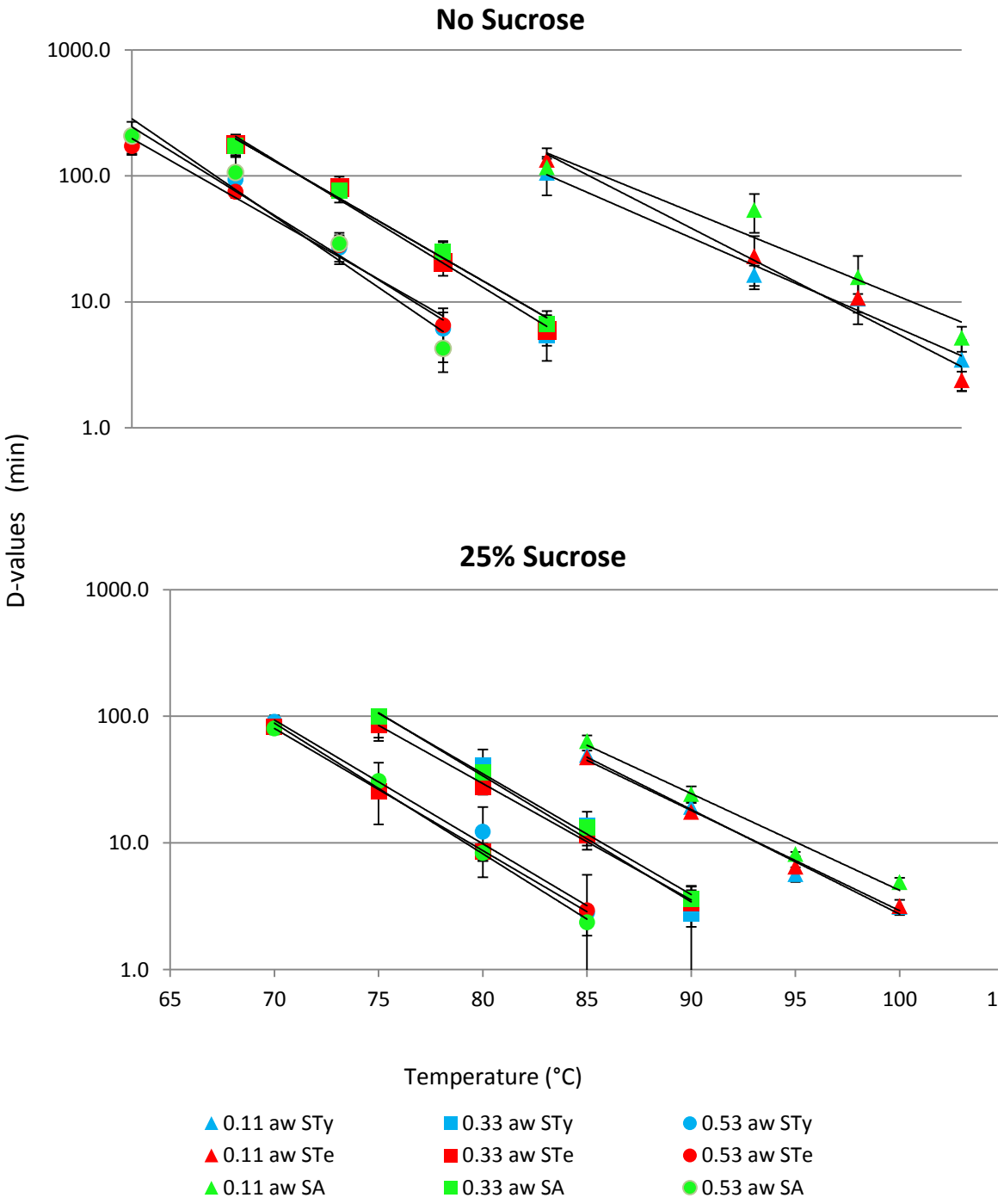


Figure 3.1.5. Graphical comparison of serovar correlation of temperature and D-values from Table 3.1.1.

3.2 Rehydration

The D-value of *Salmonella* serovar Typhimurium were drastically reduced when 30 mL of PBS were added to one gram samples of 0.05 a_w cereal. The D-value for serovar Typhimurium was only 3.0 minutes at 52°C, compared to the 54°C D-value of 3 minutes measured for serovar Typhimurium that had been grown in tryptic soy broth. The times taken from mixing the one gram sample with the 30 mL of PBS until the samples were in the water bath were less than two minutes. There was no observed tailing and the initial drop of at least one logarithm was present in each of the six trials.

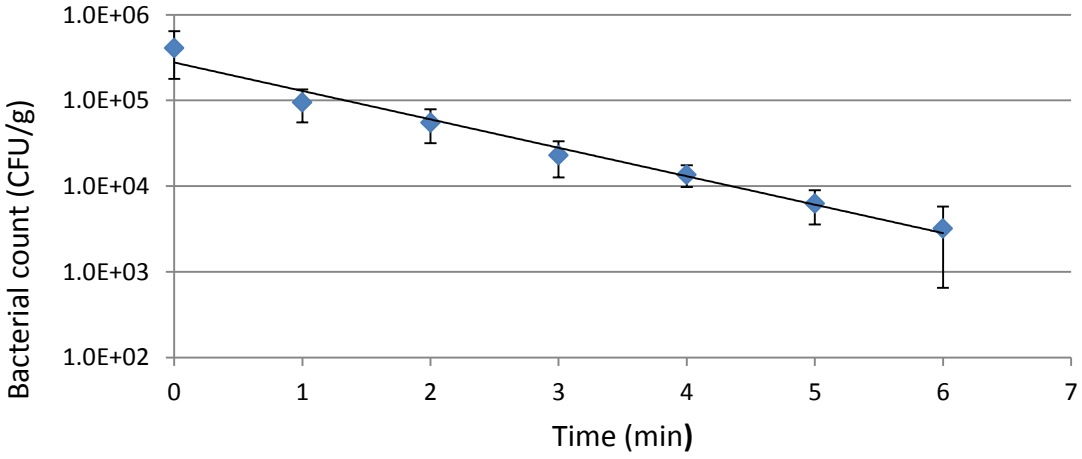


Figure 3.2.1. Thermal inactivation of *Salmonella* Typhimurium at 52°C that had been first dried in a 0.05 a_w cereal and re-suspended in phosphate buffered saline before heat treatment.

3.3 Habituation

A series of experiments were conducted to determine if the thermal resistance of *Salmonella* changed during storage of dry cereals. *Salmonella* serovar Typhimurium's D-values (Fig. 7) at 95^{o}C decreased over time when stored at room temperature. The overall variance in water activity was 0.04 a_w, while it was held at room temperature. Over the course of the 31 day period, the D-value decreased by 57.7%, while the initial inoculum did not decrease over the course of the 31 days. The individual trials which were performed in duplicate, had no observed tailing, and the initial drops were constant throughout the experiment.

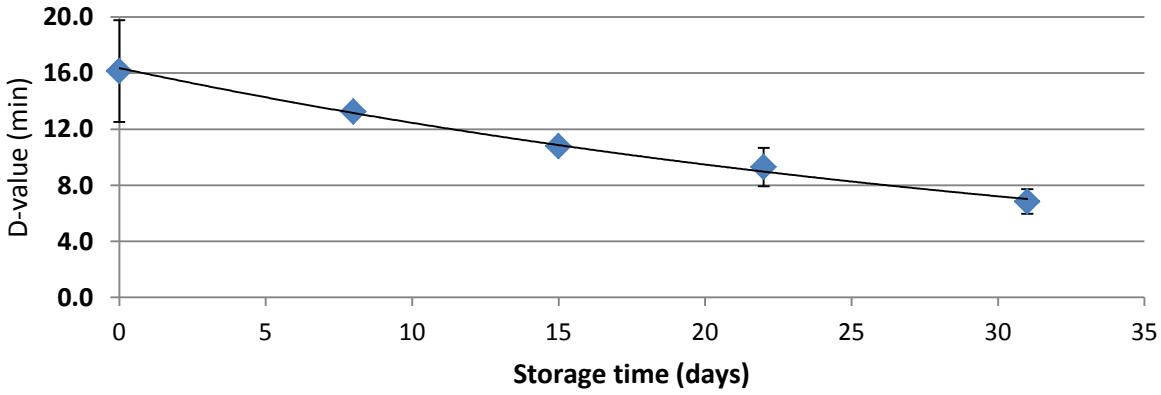


Figure 3.3.1 Effect of storage time on D-values of Salmonella Typhimurium in dried cereal at 0.10 ±0.04 a_w. The 95% confidence intervals are shown.

Chapter 4. Discussion

4.1 General increase of heat resistance

The current study assessed the heat resistance of three *Salmonella* serovars at different low water activity levels using a commercially available toasted oat breakfast cereal. Several studies that have reported an inverse relationship between heat resistance of *Salmonella* and lowering the water activity of the environment (77,81,82). The increase in heat resistance as the water activity is lowered is greater in *Salmonella* than in other organisms that have been compared in past studies (65,83,99). Very few studies, however, have measured the thermal resistance of *Salmonella* serovars below 0.5 a_w, and most of those used peanut butter (0.45 a_w) as the test matrix (76,95). The heat resistance of *Salmonella* has also been determined in dry carbohydrate based foods in two separate studies that used corn or wheat flour (83,85).

Those two studies that evaluated heat resistance in flours utilized different methods to this investigation. Those studies used dry air as a heat source while the inoculated flour was spread into thin layers on foil trays. Only one of these studies measured how the thermal resistance of *Salmonella* changed as the water activity differed (85). That study had varying results as the water activity was lowered, and no clear trend was apparent. In contrast, in our study D-values consistently increased as the water activity was lowered from 0.53 a_w to 0.11 a_w. The heat resistance did not only increase, but the sensitivity to temperature changes, defined by the Z-value, also increased significantly (Table 3.1.4) when each of the three serovar's Z-value was

averaged. The average Z-values were significantly larger at 0.11 a_w among all serovars, but they did not differ between 0.53 and 0.33 a_w.

The explanations of this increasing heat resistance are limited. The theories that have been postulated include membrane modifications or the action of removing the intracellular water molecules strengthens the proteins through forcing closer interactions. The membrane modification theory arose from the discovery of *Mycobacterium tuberculosis* being able to form spores after two weeks in low nutrient media (100). Due to this discovery, it was questioned by the researcher of this current study whether *Salmonella* may also be able to change its membrane physiology to becoming more heat resistant. While there are many more processes involved with the formation of a spore than a modification of a membrane, it was questioned whether *Salmonella* would be able to change its membrane during the drying process, not form a true spore. The current study tested this theory by rehydrating cells (0.05 a_w) with an excess of phosphate buffer solution and then immediately testing the heat resistance. Based on this hypothesis, we thought that if *Salmonella* is able to change its physiology to become more heat resistant, the cells at 0.05 a_w would have an initial heat resistance close to the D-values of cells at 0.11 a_w (Table 3.11) and heat resistance would gradually decline as the cells were rehydrated. Instead the heat resistance of the cells at 0.05 a_w was immediately reduced to being susceptible to only few minutes of heating at 52^{o}C (Figure 3.2.1), compared to the 0.11 a_w cells being resistant to temperatures of 105^{o}C (Table 3.1.1). While this test does not have any conclusive evidence against a change the

physiology of the cell membrane, it indicates that the unique heat resistance is rapidly lost.

The second theory is the stabilization of the cell components through desiccation, in which the loss of water immobilizes the proteins, creating closer interactions in both the intra and inter-molecular structures of the proteins. This loss of water would also leave less area for the native structure to react with and being denatured. If this theory was true, then once the cells were rehydrated the heat resistance would decrease rapidly, as present in the rehydration test. Though this simple test did not confirm this theory, it may support a rapid change in the cells. The rehydration test of this study seemed to favor this mechanism, but the habituation test did not, due to the loss of heat resistance over time. While the water activity did not change, and the initial population did not decrease over the course of 31 days, the heat resistance did. This would indicate a change in the proteins under this theory of stabilization by desiccation.

Another consideration for stabilization by desiccation that should be observed for this theory is the large gain of heat resistance seen for *Salmonella* serovar Tennessee that was not observed in *Escherichia coli* O157:H7 (Appendix A). If the theory was true and the force of desiccation was enough to make an organism more tolerant to temperatures, this should be seen in other organisms, especially *E. coli*. *E. coli* is a member of a genus relatively close to *Salmonella* and has 73% genetic relatedness (101). While this test did not disprove this theory, it does add yet another consideration.

4.2 Effect of sucrose

In past studies, sucrose seemed to enhance heat resistance, but the results of this investigation did not agree with this trend. Past studies have indicated that sucrose increases the denaturation temperature of individual proteins in solution, which could be the explanation of the increased heat resistance of bacteria, especially as the water activity is lowered (82,103,103). Microorganisms are known to uptake certain substances, 'osmolytes,' in high osmotic pressure environments (104-106). These osmolytes, such as trehalose, glycerol, betaines, counteract the effects of high osmotic pressure. Sucrose and polyols in general, have been theorized to possibly be used as osmolytes and cause a degree of plasmolysis which further dehydrates and stabilizes the proteins of the cell through desiccation (82,103).

The findings of this study seem to follow the trends observed in previous studies, but this effect appears to reach an apparent minimum water activity to where sucrose can cause an increase in heat resistance. The trends within this study had minor to significant increases of the heat resistance due to sucrose at 0.53 a_w. The addition of sucrose even caused some test conditions to have a decrease in heat resistance compared to the cereal with added sucrose. As the water activity was lowered to 0.33 a_w the increases became more consistent and significant with some conditions having two or three fold higher D-values (Table 3.1.2).

Once the water activity was lowered to 0.11 a_w the addition of sucrose had a negative effect on the D-values at every temperature tested and for all serovars. Though not all of these decreases had defined statistical significance each condition

tested had at least a 50% reduction of D-values at every temperature with the addition of sucrose. This decrease in heat resistance due to sucrose had not been observed, possibly because past studies used aqueous media to measure the heat resistance with sucrose.

Between the 0.33 and 0.53 water activity levels the effect of sucrose was greater as the temperature was increased. Each of the serovars had an increase in D-value as the testing temperature was raised to 80^{o}C or higher (Table 3.1.2). Except for Agona at 0.33 a_w, each serovar at 0.33 and 0.53 a_w had at least a two fold increase, when both values were positive. At 0.33 a_w this high temperature increase due to sucrose was even larger when the temperature was raised to 85^{o}C.

4.3 Linear deviations

While the trends of increasing the heat resistance by decreasing the water activity were true among every trial, there were certain characteristics that were not observed in all trials, specifically “tailing” and “initial drops.” The phenomenon of ‘tailing’ was a reduced inactivation rate of a small subset of the population for the entire duration of the heating trials. Ten percent of all of the trials tested were observed to have these very slow rates of inactivation which were observed at the ends of the trial. For this study, tailing was defined as three concurrent intervals with less than a 0.25 logarithmic reduction (Figure 3.1.3). This is not the first study to observe tailing, but past studies have accounted for tailing through the use of simple and complex kinetic equations to account for them, or removing them entirely for the calculations (76-

78,92). This study chose to remove the tailings when calculating the kinetic rates, due to the inconsistent occurrences of the tailing and the large effects these had on the kinetic values.

An explanation for the tailing is difficult since it could not be consistently reproduced. A few studies that have observed the tailings have tried to explain them through the concept of varied resistances within the test population. Previous studies (78,86,94) have tested this theory by retesting populations sub-cultured from trials that have exhibited these tailings with the same methods. These past studies, along with this current study, have not had any consistent significant increase in heat resistance when testing cells cultured from these tailings.

Other explanations, which this current study did not address, have used microenvironments, and differing rates of heat shock protein induction (HSPs). The microenvironment theory explains that the cells exhibiting the tailing phenomenon could be in a favorable microenvironment protecting the cells from thermal destruction. These favorable microenvironments could differ in water activity, different compositions or amounts of solutes. The different concentrations of the solutes within these microenvironments could cause varied extent of plasmolysis of the individual cells which dehydrates the cells to different degrees. These differences in desiccation could cause contrasting effects upon the heat resistance, where some could be dehydrated just enough to cause an increase in heat resistance, while others are dehydrated to cause negative effects on the heat resistance.

The theory involving the heat shock proteins is that the HSPs, which are usually synthesized during an adaptation procedure, are actually synthesized during the heating trials in a small subpopulation which forms the tailing. This theory is strengthened by a previous study that added chloramphenicol to the medium, to cease any protein synthesis during the heating trials. The addition of chloramphenicol reduced the D-values of tailing by 25%, though tailing was still twice as prevalent as the rest of the curves (86). The researchers of that study concluded that the remaining tailing cells were caused by a failure of chloramphenicol to completely stop all synthesis in each cell.

Much like the tailing, the initial test intervals during the trials were often characterized by an inactivation rate different from the rest of the curve (Figure 3.1.2). Each of the three serovars exhibited an immediate reduction of more than half a logarithmic cycle in 90% of the trials, and more than one logarithm in 71% of the trials, referred to as "initial drops". The initial drops of at least half of a logarithmic cycle were consistently observed between all serovars and conditions. These initial drops have been observed throughout other studies as well, though the shapes of the drops have differed. Studies measuring the resistance of *Salmonella* serovars in different media have reported inactivation rates that were lower at the beginning ('shoulders') (107), higher inactivation rates at the beginning ('initial drops') (77), or nearly linear inactivation curves without shoulders or initial drops (108). There have also been studies that have shown both shouldering and initial drops without changing the conditions (69,73). Theories to account for these changes involve the same

microenvironments that could account for the tailing, or different growth phases of the cells where some growth phases are affected by the heat treatment more severely.

4.4 Differences between serovars

Each of the three serovars had increases in their respective thermal resistances as the water activity was decreased. *Salmonella* serovars Typhimurium and Tennessee did not have any significant differences in heat resistance between each other (Table 3.1.5). The similar heat resistances of serovars Tennessee and Typhimurium may be because each serovar was isolated from peanut butter outbreaks. While serovar Agona was isolated from a cereal outbreak in 2008, was not significantly different at most conditions tested without the addition of sucrose. Though one of the significant differences was notable, as serovar Agona had D-values three times higher than serovar Typhimurium at 95^{o}C in a 0.11 a_w environment.

There were significant differences among the three serovars with to the addition of sucrose. Serovar Agona's heat resistance was significantly higher at nearly every temperature tested at the 0.11 a_w than both serovars when sucrose was added. The largest difference seen was between serovars Agona and Typhimurium at 100^{o}C at 0.11 a_w. The heat resistance of Serovar Agona also caused some differences between the serovars at 0.53 a_w, where it was 33% lower than Typhimurium at 80^{o}C and 18% higher at 75^{o}C.

Serovar Agona had the most significant changes in heat resistance due to the addition of sucrose compared to the trials without additional sucrose. Serovar Agona

had a significant increase at every temperature tested at 0.33 a_w and a significant decrease at 0.11 a_w. Serovars Typhimurium and Tennessee, however, were only significantly different due to a decrease in heat resistance at 0.11 a_w with the addition of sucrose.

Chapter 5. Conclusions and Future Research

5.1 Conclusion

As *Salmonella* continues to be among the top causative agents of food borne diseases, it becomes more imperative to fully understand the mechanisms by which this pathogen contaminates foods. It becomes not only important to understand how it can grow but also how it is able to survive some of the harsh environments it is found. As this study has observed, serovars of *Salmonella* in low water activity may not be inactivated by current heat processes found within commercial thermal treatments.

Within low water activity environments, the heat resistance of *Salmonella* increases by orders of magnitude compared to higher water activity environments. As the water activity of the cereal was lowered to 0.53 a_w, the D-values were as high as 90 minutes at 70^{o}C. In a 0.33 a_w environment, the D-values increase to 175 minutes at 70^{o}C. As water activity levels drop to 0.11, the cells of *Salmonella* are able to withstand boiling temperatures for as long as 10 to 15 minutes. With the addition of sucrose, the cells had a general increase in D-values at 0.33 and 0.53 a_w. As the water activity was lowered, the addition of sucrose decreased the D-values of each serovar between 40 to 80%.

The average Z-value of all three serovars was about 10^{o}C with and without the addition of sucrose at 0.53 and 0.33 water activities. As the water activity decreased to 0.11, the Z-value increased to 12.5 and 13.5^{o}C with and without the addition of sucrose, respectively. These values are a large increase compared to the reported 6^{o}C Z-values seen for *Salmonella* in select poultry meats (69).

To be able to control this pathogen knowledge of the mechanism behind this massive increase in heat resistance compared to high water activity foods must be elucidated. The current theories upon the mechanisms of this increase do not completely capture the events underlying this enhanced resistance. The control of these mechanisms may allow for more effective processing of dry foods to be able to inactivate the pathogen completely.

5.2 Future studies

The future studies of *Salmonella* in low water activity environments should focus upon the mechanisms *Salmonella* utilizes to increase its thermal resistance. To elucidate these mechanisms the genetic regulations that determine this increase in heat resistance should be examined as the water activity is lowered. By examining the genes involved the increase in heat resistance, one may be able to establish whether the increase is an actual physical modification, or a mechanical consequence of the desiccation process.

A method to investigate this process would be through the use of microarray experiments. By using the same dry cereal samples at different water activity levels, different gene expression levels could be qualified as up regulated or down regulated. These changes in the cell could be compared to the genetic expression of conserved housekeeping genes in a fully hydrated sample that does not change as the water activity is lowered to 0.11 a_w. As the water activity is lowered, certain genes would be up regulated or down regulated in response to the changing environment.

If the data from this current study is any indication of the genetic mechanisms, a large change of gene expressions as the water activity approaches 0.53 a_w and another large expression change between 0.33 and 0.11 a_w should be expected. If there is a genetically mediated mechanism, it may also be useful to compare it to mutant strains that do not have the genes to produce enzymes to create osmolytes, such as trehalose, betaines, and glycerol. Mutant strains of *Salmonella* serovar Typhimurium have been modified to be deficient in producing these osmolytes, such as the OtsA and OtsB negative mutants that have been used in previous studies (113). Furthermore, the direct effect sucrose has upon thermal resistance could also be examined using microarray experiments. With an addition of sucrose, the same large expression of genes at 0.53 a_w would be expected based on this study's data. The second large expression as the water activity approaches 0.11 a_w may not be observed.

With these microarray experiments, it would also be important to understand how rapid these changes take place. By placing the cells in different low water activity solutions and removing cells at certain times, the speed of the acclimation to the low water activity environment and the osmotic protection system could be found. This acclimation project would not be possible to measure with the methods of this current project due to the long drying time; it would be able to be performed in liquid solutions of low water activity.

To fully understand these mechanisms it must also be ascertained whether this low water activity protection mechanism is unique to *Salmonella*. A simple way to test this would be to measure strains of various bacteria and test them under the same

conditions of this current study. While strains of *E. coli* would seem promising to the large genetic relatedness to *Salmonella*, it may also be interesting to note other bacteria capable of thriving in harsh conditions, such as strains of *Pseudomonas* (106).

Any of these experiments would greatly improve the ability of commercial food processing to control this pathogen in dry environments. To fully be able to control this pathogen, the mechanisms behind the pathogen's large increase in heat resistance must be understood beyond that of our current understanding.

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Appendix A - Comparing the Heat Resistance of *Escherichia coli* O157:H7 to *Salmonella* serovar Tennessee in Flour

A.1 Culture preparation:

The stock *Salmonella* culture of *Salmonella* serovar Tennessee E2009005811 used for this project was provided by the Minnesota Department of Health and was originally isolated from a peanut butter outbreak in 2007. The stock culture of *E. coli* O157:H7 strain 43895 used was a CDC clinical isolate from an outbreak involving a fast food company in 1983 (ATCC). The stock cultures were stored in a 1:1 ratio of glycerol and tryptic soy broth (TSB; Neogen, Inc., East Lansing, MI) at -55^{o}C. The working cultures of each serovar was prepared from the frozen culture and inoculated into TSB, grown overnight at 37^{o}C and then stored at 4^{o}C. The working cultures were re-transferred once a week. The working culture for serovar Tennessee was streaked onto tryptic soy agar with added ferric ammonium citrate and sodium thiosulfate when it was retransferred. Periodically the serovar was also streaked onto bismuth sulfate agar and XLD agar for confirmation. The working culture of *E coli* O157:H7 was streaked onto sorbitol MacConkey agar (Neogen, Inc., East Lansing, MI)) with supplemental cefixime and tellurite for confirmation.

A.2 Inoculation:

From the working cultures *E. coli* O157:H7 or *Salmonella* serovar Tennessee were transferred to 20 mL of tryptic soy broth and grown at 37^{o}C overnight. The overnight cultures were centrifuged at 3000 G for 10 minutes and the supernatant was decanted

except for 0.5 mL. The remaining supernatant and pellet was vortexed again and added to 2.5 g of commercial brand corn starch and mixed with a sterile spoon. Fifty grams of commercial brand flour was added to the inoculated corn starch and mixed together with a spoon. The mixture was then sifted three times in a commercial brand autoclaved kitchen sifter to evenly distribute the cells. The water activity of the mixture was then measured with a portable water activity meter (Decagon – pa_wkit).

A.3 Preparation of individual samples

The inoculated flour mixture was placed into sterile 12 cm^3 syringes and flour was transferred into capillary tubes (1.5-1.8 x 90 mm borosilicate glass) by inserting the tubes through the luer-lock tip of the syringes. Ram rods **(**118 mm x 1 mm stainless steel) wiped with 70% ethanol were used to fill the capillary tubes when necessary. The flour filled tubes were heat sealed using a propane hand torch and placed in a 10% commercially available chlorine bleach solution for at least one minute. If the bleach was observed wetting the flour, the samples were not used. After all capillary tubes were filled the water activity was measured using the remaining cereal in the syringe. Water activity of the samples was measured using a water activity meter (Pawkit Model, Decagon Devices,Inc., Pullman, ID) calibrated every other day according to the manufacturer's procedure.

A.4 Thermal testing

All of the sealed capillary tubes were placed into either an oil bath (High Temp Bath 160 A, Fisher Scientific, Inc., Waltham, MA) or water bath (Isotemp 205, Fisher Scientific, Inc.) calibrated once a month. The water baths were set at the testing temperatures between 55 to 65^{o}C. At predetermined time intervals, two capillary tubes were removed and immediately placed in an ice bath for one minute. From the ice bath, the tubes were placed in a solution of 10% bleach and rinsed with sterile water. Each heat sealed capillary tubes were then placed in separate sterilized 24 x 150 mm screw cap test tubes each containing a magnetic stir bar (25 x 5 mm) and vortexed until the capillary tube was pulverized and the cereal exposed. Ten milliliters of phosphate buffer (PB) were added to the test tubes and mixed for Y min. These buffer suspensions were further diluted by transferring 1 mL serially into 9-mL PB tubes. Volumes of 0.1 mL of dilutions were spread plated in duplicate, on TSA containing with 0.8 g/L ferric ammonium citrate and 6.8 g/L sodium thiosulfate. The plates were incubated for 24 hours at 37^{o}C before counting colonies.

A.5 Plating

The content of the sealed capillary tubes was removed by the stir bar when the 24 x 150 mm test tube was vortexed. Ten milliliters of phosphate buffer were added to the ground capillary tubes within the 24 x 150 mm test tube, with further dilutions made using nine milliliters of phosphate buffer. The samples were spread plated, in duplicate, on tryptic soy agar (ACU media) with 0.8 gram ferric ammonium citrate and

6.8 grams sodium thiosulfate per liter for *Salmonella* samples or sorbitol MacConkey (ACU media) for *E. coli* O157:H7 samples.

A.6 Calculations

The total plate counts were calculated using the aerobic plate count formula from the Bacteriological Analytical Manual. Each of the tube was calculated individually and averaged with the other duplicate. The death curves were made using Excel 2007 by either using a linear plot of the time versus the log of the plate count, or on an exponential semi-log plot using the time versus the total plate count of each interval. If tailing was prevalent, it was removed from the plots if three consecutive points did not decrease by more than 0.25 logarithms.

A.7 Results

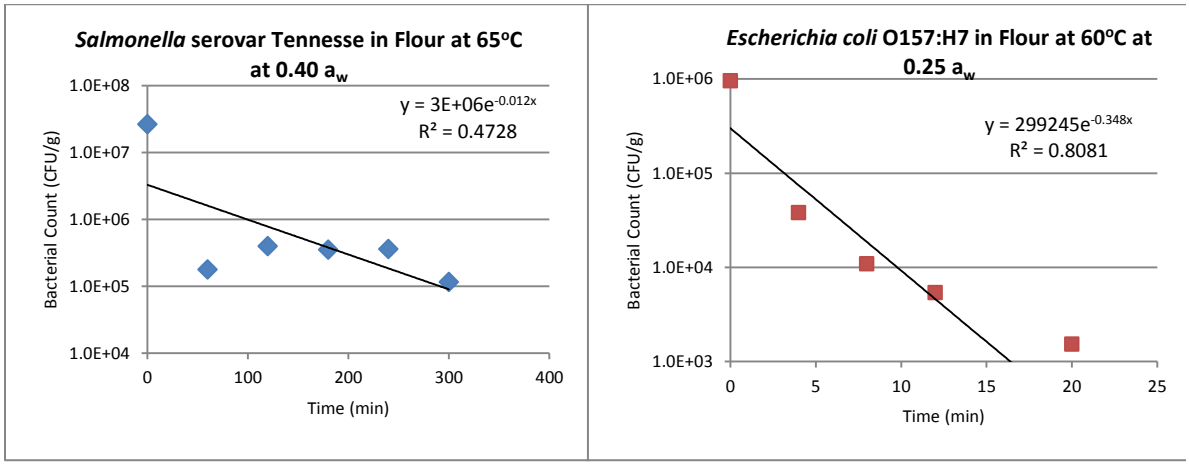

Figure A.7.1 – Examples of inactivation curves of *Salmonella* serovar Tennessee and *Escherichia coli* O157:H7 in flour.

Table A.7.1 – D-values of *Escherichia coli* O157:H7 and *Salmonella* serovar Tennessee in flour at different water activities and temperatures.

	0.25 a_w			0.40 a_w		
Organism	D_{55} (min)	D_{60} (min)	D_{65} (min)	D_{55} (min)	D_{60} (min)	D_{65} (min)
E. coli O157:H7	18.2[1]	8.1	1.8[1]	6.4	NT	0.8
S. Tennessee	NT	NT	NT	213.7	NT	154.4

[1] - Single trial
NT - Not tested

The heat resistance of *Salmonella* serovar Tennessee was compared to *Escherichia coli* O157:H7 in a flour matrix. The heat resistance was directly compared in flour of water activity of 0.40 between the two organisms at 55 and 65^{o}C. The D-values of *Salmonella* serovar Tennessee were 33 fold higher at 55^{o}C and 123 fold higher at 65^{o}C than that of *E. coli* O157:H7. When the water activity was lowered to 0.25, the D-values of *E. coli* O157:H7 increased between two and three fold at comparable temperatures.

A.8 Discussion:

A past study compared the heat resistance of a cocktail of eight *Salmonella* serovars and *E. coli* O157:H7 in ground beef (109). The D-values of the *Salmonella* cocktail were 16.34 and 0.15 minutes at 55 and 63^{o}C respectively. The values of *Salmonella* in ground beef were comparable to the D-values of *E. coli* O157:H7 with 20.08 and 0.16 minutes using 55 and 63^{o}C.

The heat resistance of these organisms is comparable in ground beef, but once the water activity was lowered, the heat resistance of *Salmonella* increased greatly, while *E. coli*'s did not.

The actual D-value for *Salmonella* was most likely larger than what is reported in Table A.7.1. The D-value is calculated from the large initial drop observed in all the trials as seen in Figure A.7.1. With this initial drop ignored, there is little decrease in the bacterial count beyond this large reduction, which would increase the D-value. Though this initial drop was observed in *E. coli* O157:H7 as well, there was a continual reduction in the intervals after the drop, unlike *Salmonella.*

The results of this test indicate that while there is an increase in heat resistance of *Salmonella* as the water activity is lowered, it is not observed in *E. coli* O157:H7. If the single results of *E. coli* O157:H7 did not deviate with more replicates, the increase observed as the water activity was lowered was two to three fold of the D-value. Though this rise is significant, it is not comparable to the increase of *Salmonella.*

A.9 Limitations of the testing

This test was performed as a method for direct comparison of the heat resistance of *E. coli* O157:H7 to that of *Salmonella*. The methods, while they still fulfilled the objective, did have flaws. The flour that was used was not equilibrated in a desiccator to be able to adequately control the water activity. The water activity that was measured for the flour was the natural water activity of the flour before it was used. It should also be studied whether the cells of *E. coli* O157:H7 has an increase in heat resistance if they are allowed to equilibrate to the water activity of the flour environment if given more time than this study allowed.

Appendix B – Experimental Data

Each of the heat challenges were conducted in triplicate, except for the habituation and rehydration. The individual parameters for the measurement of *Salmonella* serovars in different water activity levels is shown in the following tables.

Table B.1 - D-values of three *Salmonella* serovars at 0.11 a_w without added sucrose. Correlations are based on a line of best fit.

	D-Values (min)					
Temperature (°C)	Typhimurium	Correlation (R^2)	Tennessee	Correlation (R^2)	Agona	Correlation (R^2)
85	69.7	0.99	127.8	0.96	100.6	0.99
	127.8	0.94	109.5	0.96	115	0.84
	120	0.96	164.3	0.94	135.3	0.97
95	13.3	0.97	12.6	0.86	41.8	0.96
	17.7	0.80	29.9	0.90	47	0.91
	18.1	0.89	26.35	0.87	71.9	0.95
100	14	0.87	11.3	0.82	8.1	0.96
	10.8	0.92	11	0.89	18.85	0.88
	7	0.9	9.9	0.82	20.2	0.90
105	3.8	0.84	2.1	0.95	4.2	0.97
	4.6	0.82	2.8	0.96	5.1	0.85
	2	0.99	2.26	0.93	6.25	0.96

Table B.2 - D-values of three *Salmonella* serovars at 0.33 a_w without added sucrose.

Correlations are based on a line of best fit.

	D-Values (min)					
Temperature (°C)	Typhimurium	Correlation (R^2)	Tennessee	Correlation (R^2)	Agona	Correlation (R^2)
70	181.3	0.89	177.0	0.95	192.0	0.89
	188.5	0.87	209.4	0.86	177.2	0.93
	145.8	0.98	148.6	0.97	153.5	0.82
75	90.7	0.83	95.6	0.89	72.0	0.89
	71.9	0.86	82.3	0.85	82.5	0.91
	77.0	0.96	62.9	0.86	72.9	0.90
80	21.5	0.90	22.6	0.81	27.4	0.88
	18.0	0.85	20.5	0.91	20.0	0.89
	30.3	0.81	18.3	0.92	27.4	0.94
85	6.3	0.88	8.5	0.80	7.7	0.88
	4.7	0.86	4.8	0.84	5.6	0.89
	5.3	0.86	4.5	0.86	6.7	0.93

Table B.3 - D-values of three *Salmonella* serovars at 0.53 a_w without added sucrose.

Correlations are based on a line of best fit.

	D-Values (min)					
Temperature (^oC)	Typhimurium	Correlation (R^2)	Tennessee	Correlation (R^2)	Agona	Correlation (R^2)
65	209.1	0.98	153.3	1.00	176.9	0.86
	191.7	0.98	171.9	0.98	176.9	0.83
	209.1	0.98	191.9	0.91	270.0	0.91
70	95.8	0.95	82.1	0.79	74.1	0.94
	104.5	0.89	71.9	0.91	109.7	0.95
	79.3	0.85	69.8	0.98	135.5	0.98
75	33.8	0.84	25.3	0.81	28.0	0.98
	22.1	0.81	35.4	0.90	26.1	0.95
	24.7	0.82	23.5	0.98	32.8	0.98
80	8.24	0.91	5.7	0.85	5.8	0.93
	3.4	0.90	5.5	0.92	3.5	0.99
	6.7	0.93	8.27	0.96	3.5	0.86

Table B.4 - D-values of three *Salmonella* serovars at 0.11 a_w with 25% added sucrose.

Correlations are based on a line of best fit.

	D-Values (min)					
Temperature (°C)	Typhimurium	Correlation (R^2)	Tennessee	Correlation (R^2)	Agona	Correlation (R^2)
85	47.0	0.92	53.6	0.87	64.0	0.92
	53.6	0.82	45.2	0.92	69.8	0.85
	48.0	0.94	43.5	0.97	57.7	0.87
90	20.4	0.81	14.8	0.95	26.2	0.83
	20.2	0.80	16.2	0.91	26.2	0.87
	17.2	0.96	21.7	0.90	20.6	0.86
95	4.9	0.87	6.5	0.84	8.1	0.85
	6.0	0.82	6.1	0.81	7.9	0.87
	6.1	0.82	6.9	0.80	8.5	0.80
100	3.3	0.89	3.1	0.87	5.0	0.89
	3.4	0.87	3.7	0.83	5.2	0.85
	2.7	0.93	2.7	0.84	4.5	0.81

Table B.5 - D-values of three *Salmonella* serovars at 0.33 a_w with 25% added sucrose.

Correlations are based on a line of best fit.

	D-Values (min)					
Temperature (°C)	Typhimurium	Correlation (R^2)	Tennessee	Correlation (R^2)	Agona	Correlation (R^2)
75	90.3	0.87	106.1	0.9	98.8	0.92
	101.5	0.84	70.0	0.94	88.9	0.93
	68.4	0.93	78.9	0.85	112.9	0.82
80	54.8	0.92	29.3	0.91	37.3	0.86
	35.4	0.90	29.5	0.93	33.9	0.91
	33.0	0.82	24.0	0.94	36.1	0.82
85	11.3	0.81	13.0	0.86	13.0	0.87
	11.73	0.81	12.7	0.81	15.3	0.91
	17.7	0.87	8.8	0.9	11.3	0.90
90	4.4	0.80	2.34	0.83	4.1	0.83
	1.16	0.92	4.4	0.80	3.0	0.91
	2.7	0.85	3.3	0.80	3.7	0.82

Table B.6 - D-values of three *Salmonella* serovars at 0.53 a_w with 25% added sucrose. Correlations are based on a line of best fit.

	D-Values (min)					
Temperature (°C)	Typhimurium	Correlation (R^2)	Tennessee	Correlation (R^2)	Agona	Correlation (R^2)
70	73.8	0.96	91.8	0.95	80.2	0.94
	85.6	0.89	76.5	0.91	87.6	0.91
	111.8	0.93	80.0	0.92	72.9	0.98
75	32.4	0.80	27.7	0.95	33.3	0.84
	25.0	0.81	24.6	0.88	28.9	0.86
	28.1	0.91	23.8	0.96	30.4	0.91
80	13.6	0.81	8.1	0.90	8.4	0.86
	10.1	0.84	9.9	0.90	7.3	0.87
	13.2	0.89	7.7	0.95	9.3	0.90
85	2.6	0.80	3.0	0.85	2.4	0.88
	2.3	0.80	2.7	0.82	1.9	0.88
	3.5	0.92	3.1	0.80	2.8	0.87

Table B.7 – D-values of individual days tested as the cells of *Salmonella* serovar Typhimurium were stored at room temperature. Correlations are based on a line of best fit.

First Replicate				**Second Replicate**			
Day	D-Value (min)	Water Activity	Correlation (R^2)	Day	D-Value (min)	Correlation (R^2)	Water Activity
1	14.3	0.10	0.90	1	18.0	0.89	0.10
8	13.3	0.06	0.82	8	13.2	0.87	0.06
15	10.8	0.07	0.83	15	10.8	0.84	0.07
22	8.6	0.10	0.87	22	10.0	0.83	0.10
31	7.3	0.13	0.88	31	6.4	0.94	0.13

Table B.8 –Individual replicate D-values of *Salmonella* serovar Typhimurium when the cells were rehydrated in phosphate buffer from 0.05 a_w and tested at 52^{o}C. Correlations are based on a line of best fit.

Replicate	D-Value (min)	Correlation (R^2)
1	2.37	0.90
2	2.40	0.93
3	4.09	1.00
4	3.91	0.82
5	2.68	0.98
6	3.13	0.95