THE FATE OF *SALMONELLA* IN READY TO EAT CEREALS

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Abstract

This study was intended to provide insight into the ability of *Salmonella* to survive in ready-to-eat (RTE) cereal during storage, contaminated post processing. Sweetened toasted oat cereal (STOC) and toasted oat cereal (TOC) were used to elucidate the ability of *Salmonella* to remain viable for 3 months and the effect of sucrose on its survival. To date, this is the first study to report survival of *Salmonella* in ready to eat cereal during storage. Commercial cereal samples were inoculated with approx. 10^6 CFU/g of five different *Salmonella* strains belonging to four serovars (Agona, Typhimurium, Tennessee and Senftenberg) and re-dried within 24 h. Inoculated cereal was periodically sampled after drying on 1, 3, 5, 7, 14, 30, 60, and 90 days of storage at room temperature. The viable *Salmonella* count was determined using complex differential media and standard microbiological techniques. The count of most serovars increased during the cereal re-drying step in TOC, but not in STOC. During storage the *Salmonella* count remained greater than 10^7 CFU/g in TOC for the entire experimental period with the exception of serovar Senftenberg. The level of *Salmonella* in STOC declined during the first week of storage, but their final counts were more than 10^3 CFU/g. These results indicated that *Salmonella* was able to survive for at least ninety days in either type of cereal. The relevance of this research to the cereal industry is that it confirms the unique ability of this microorganism to survive conditions of very low water activity and stresses the importance of further processing to minimize the risk of transmission of this pathogen by cereal foods.

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1. Introduction

Salmonella was one of the first contagious bacterial agents recognized to cause disease and was first cultured by Georg Gaffky, a German scientist, in 1884 (62) . American scientists Salmon and Smith isolated *Salmonella enterica* serovar Typhi from infected pigs during this same period. This bacterial genus was later named *Salmonella* in honor of Salmon's great discovery. Since this time, *Salmonella* has earned itself the title of one of the leading causative agents for foodborne disease.

The widespread occurrence of *Salmonella* in nature is a result of its ability to adapt to a variety of less than ideal conditions. *Salmonella* has a unique capability to grow or survive in many extreme environments. For example, *Salmonella* spp. are known to grow at extreme high and low temperatures, modified atmospheric environments, and high and low pH environments (1, 33).. *Salmonellae* can survive in many hostile environments, but their ability to persist under dry conditions is of particular relevance to this study.

Salmonella infections are a major worldwide public health threat. Each year, there are an estimated 1.3 billion cases of non-typhoid salmonellosis that contribute towards more than three million deaths worldwide (70) . Of these approximate billion cases, approximately 2 million infections and 500 deaths occur in the U. S. each year (65)

Of greatest interest is the breadth of foods implicated in outbreaks during the last few decades. Although eggs and meat are typically the most common food matrices for *Salmonella* contamination, outbreaks linked to the consumption of dry foods are also of major concern. For example, a few products implicated in recent outbreaks include: peanuts, pecans, ready to eat cereal, infant formula, infant cereal and dry milk powders (33).

Due to the many recent large and highly publicized outbreaks in dry, ready-to-eat foods, there is heightened interest in determining how long and at what level *Salmonella* are capable of surviving in affected products (17) In particular, in the years since 2007, a series of food recalls have caused many experts, including Patricia Guthrie of the Canadian Medical Association, to call for increased enforcement and inspection in U.S. food production plants (42). The FDA in recent years has been burdened with increasing responsibilities in the face of a flat budget, producing an organization that is critically underfunded and understaffed, resulting in fewer and less comprehensive plant inspections (42, 68). Food and health safety advocates like Guthrie suggest three key steps to improve current regulatory conditions, including: better and more timely reporting of food poisoning cases among local, state and federal public health agencies, tougher and more accountable inspections of food processing and distribution plants, and better training for restaurant food handlers and food plant inspectors (42, 66, 68). Improvement in these key categories will help to prevent outbreaks before they occur, clean up unsanitary plant conditions, and ultimately decrease the number of overall outbreaks each year (including those caused by *Salmonella*).

The recent recalls and outbreaks have stressed the importance of understanding how *Salmonella* is capable of surviving under very low water activity. Toasted oats cereal represents one of the ideal models to study survival in a dry food because of its low water activity and harsh processing conditions. The research reported in this document was the first study aimed to determine the survival of *Salmonella* in ready-to-eat cereal.

2. *Enterobacteriaceae*

Salmonella is taxonomically classified within the *Enterobacteriaceae* family. More than 100 species and 31 bacterial genera belong to the *Enterobacteriaceae* family. Some genera most closely related to *Salmonella* include *Escherichia*, *Enterobacter*, *Erwinia, Klebsiella*, *Shigella*, and *Yersinia*. Although it is difficult to generalize about this family since it contains numerous species, the *Enterobacteriaceae* family does have distinct characteristics. For instance, this family tends to inhabit the intestinal tract of animals and humans (however, some genera can also be found in other environments) (73). Another characteristic feature is that these bacteria have a tendency to be glucose fermenters (although, as in the previous case, they often ferment other carbohydrates as well). Although not all *Enterobacteriaceae* affect humans or animals, this family nevertheless causes a sizable percentage of food outbreaks yearly (65).

3. *Salmonella*

Salmonella is a facultative anaerobic, Gram-negative bacteria belonging to the *Enterobacteriaceae* family. Bacterial taxonomists have identified two major *Salmonella* species, *S. enterica* and *S. bongori* and a total of six subspecies belonging to the first species. The most common subspecies, *Salmonella enterica* subsp. *enterica*, includes as many as 1,400 pathogenic serovars typically associated with foods, such as Typhimurium, Agona, Enteritidis, Montevideo, and Tennessee, among others. In addition to those serovars almost 1,000 more antigenically distinct *Salmonella* belong to the other five subspecies (33, 75).

Salmonella is a distinctive genus because of its complicated naming scheme, using sub-species, or serovar classification. This taxonomy, commonly referred to as the Kauffman-White scheme, is based upon differences in bacterial antigens (7, 69). Essentially, under this naming method, each *Salmonella* serovar is identified by a unique combination of antigens (O, H and Vi). Unlike serotypes of other bacteria, *Salmonella* serovars are given subspecies names (such as Typhimurium) rather than serological identifiers (such as *E. coli* O157:H7). Names of newly identified serovars are typically given after the place of their first isolation (i.e. *Salmonella* Tennessee). Although the complicated and unique naming scheme of *Salmonella* often causes misinterpretation of subspecies for species (*Salmonella* Tennessee rather than *Salmonella enterica* subsp. Tennessee, for instance), the current method is nevertheless the most accepted worldwide.

Another distinctive characteristic of *Salmonella* is their resilience to extreme environmental conditions, as compared to other organisms, such as non-O157 *Escherichia coli.* For example, some serovars are able to grow at temperatures as high as 54ºC, or as low as 2ºC (31). Additionally, they are capable of growth at pH values ranging from 4.5 to 9.5 (67). *Salmonella* are capable of survival in other extreme conditions, such as under modified atmospheres, or highly osmotic conditions, as examples. In essence, this organism quickly adapts to environmental conditions and thus is extremely difficult to control once it has contaminated a production plant.

The pervasive nature of *Salmonella* in the environment combined with intense animal husbandry practices, such as concentrated animal feedlot operations for cattle, have favored the proliferation of this human pathogen in the global supply chain. Of all the different sectors in the food industry, eggs, poultry and meat remain the most predominant reservoir for *Salmonella* (27, 33, 45, 67). By focusing efforts on these reservoirs, other, high-risk foods are overlooked, such as pork, lamb and mutton. The USDA instituted the "Final Rule on Pathogen Reduction and Critical Control Point Systems" in 1996 to apply efforts to all meats. The goal of the program is to require meat and poultry industries to implement HACCP in all production plants and randomly sample final product for *Escherichia coli*, an indicator organism to assess product quality (101). Although programs by the USDA have effectively reduced contamination in foods within scope, foods such as plant-based dry products that are out of scope have not shown the same results.

3.1. Characteristics of the disease: Salmonellosis

Foodborne *Salmonella* infections can lead to several clinical conditions: enteric (typhoid) fever, enterocolitis, and systemic infections (28, 33). Enteric (Typhoid) fever is a serious condition that is propagated by the Typhi or Paratyphi serovars of *Salmonella.* Symptoms include diarrhea, prolonged fever, abdominal pain, headaches, and prostration (28). Treatment is usually via antibiotics, however, increased resistance by Typhi and Paratyphi has greatly undermined current drug efficiency. This has caused unusually high fatality rates, especially in developing countries

Groups at highest risk for salmonellosis include immunocompromised individuals (elderly, newborns, infants), those with gastric hypoacidity, and frequent or recent users of antibiotics (25, 67). Due to these groups delayed or weak immune response, *Salmonella* is able colonize the intestine, causing serious illnesses like septicemia, aseptic reactive arthritis, Reiter's syndrome, and ankylosing spondylitis (67). Typically, healthy adults will experience only mild symptoms such as abdominal pain and non-bloody diarrheal stools. Treatment is usually limited to supportive therapy, such as fluid and electrolyte replacement.

Glynn and Bradley completed a study in 1992 on the dose-severity relationship of *Salmonella*. They discovered that when subjects previously ill with foodborne *Salmonella* were re-exposed, they experienced gastroenteritis with reduced severity, despite a higher dose (41). That study suggested that increased sanitation (which would

be expected to lower an average dose) would function to decrease the overall number of severe *Salmonella* infections.

4. *Salmonella* outbreaks related to dry and low a_w foods

Low water activity completely stops the growth of all microorganisms including vegetative pathogens such as *Salmonella*. Processed products such as peanut butter, chocolate, infant formula, ready to eat cereal and dried milk are characteristically low a_w foods. While these products do not support the growth of *Salmonella*, all have been the subject of food outbreaks of salmonellosis (13, 15, 16, 18, 83). Epidemiological and environmental investigations of these outbreaks have suggested that cross contamination plays a major part in the contamination of *Salmonella* in food products. Cross contamination could possibly explain the recent cluster of several serious and widespread *Salmonella* outbreaks in low water activity foods (Table 1). This has caused industry, government and consumers to express great concern, prompting action from the U.S. government (66, 68). The tipping point was the peanut butter outbreaks of 2007 and 2009, which signaled a pressing need for sweeping change of U.S. food regulations and enforcement.

The *Salmonella* Typhimurium outbreak of 2009, caused by contamination in peanut butter was the defining event that prompted a series of recently proposed changes in U.S. food regulations (96). This outbreak caused 714 documented cases of gastroenteritis resulting in nine deaths (18). The Center for Disease Control (CDC)

estimates that the 2009 outbreak was one of the largest food recalls ever issued in the United States, involving approximately 2,100 food products from 200 food companies with products containing peanuts (18). The outbreak was caused by the lack of good manufacturing practices, unsanitary processing conditions and criminal negligence, as company officials knowingly chose to ship tainted food product rather than halt production and fix the problem (42).

In contrast, the earlier peanut butter outbreak in 2007 was not caused by criminal negligence, but primarily plant maintenance,, as the outbreak was attributed to a leaky roof and faulty sprinklers (39). FDA inspectors examined raw materials, finished products, the plant environment, processing data and general inspection of the facility for potential sources of contamination (15, 103). The FDA collected 122 environmental samples, two of which tested positive (floor and drain sample from roaster room). According to Zink, the leaky roof and sprinklers in the facility may have allowed for growth of *Salmonella* in the plant and ultimately contamination in the finished product. The finished product eventually caused 628 cases from 47 U.S. states with no reported deaths.

Several dry foods have been implicated in salmonellosis outbreaks lately, with ready-to-eat cereal products having the greatest relevance to this study. Two branded toasted oat cereal and puffed rice products were contaminated with *Salmonella* Agona 10 years apart. The strains isolated in each of the outbreaks had identical pulsed field gel electrophoresis (PFGE) patterns, suggesting that the same organism was responsible for

both outbreaks. The first outbreak in 1998 had a total of 209 cases with 47 hospitalizations from a wide variety of states (13) . Based on this number, and because, in general, only a fraction of cases are reported, the CDC estimated that over 16,000 persons became ill as a result of this outbreak (8). In response, outbreak investigators examined potential cross contamination sources in or on the production lines, air handling systems, plant floor, ingredient delivery systems, and processing flow. They found widespread low-level contaminations of the organism in the plant environment, including contaminants on the floor, production equipment, and exhaust systems. The second and much smaller outbreak, was attributable to the same *Salmonella* Agona serovar, and sickened 28 individuals (13). The common link between the two outbreaks was they both were traced to the same plant exactly ten years apart. Therefore, low-level microbial contamination appeared to have remained in the plant for 10 years beyond the first outbreak and likely contributed toward the second.

Almonds (raw) have also been linked to foodborne infections caused by *Salmonella*. A series of outbreaks during 2001, 2003 and 2004 were reported to be associated with contamination due to *Salmonella* Enteritidis from orchard soils. Almonds are typically harvested (after they have reached maturity) by shaking the nuts off the tree and allowing them to sit on the ground to dry for 1 to 2 weeks (76, 86). Contamination is thought to occur during harvest and subsequent mixing of soil, hulls, shells and kernels during dehulling (86). In other words, one contaminated nut could cause an entire lot of finished product to be contaminated and ultimately be the source for a foodborne outbreak. In the 2003 outbreak, approximately 30 cases, including 7 hospitalizations,

were reported from 12 states (16). *Salmonella* was never found in the product; it is assumed that contamination was from the soil at the farm, where several environmental tests were positive for *Salmonella* Enteritidis.

A similar result was found from an earlier almond outbreak in 2001, which was also traced back to the soil. In the earlier outbreak, the California Department of Public Health declared that since three farms over a large geographic area were contaminated, the contamination source must also be widely distributed and possibly naturalized in the soil (11). Environmental survival may also contribute toward transmission and infection of animal hosts, which thus commences a cyclic contamination of the surrounding environment (95). Therefore, food manufacturers should be aware of their suppliers and conditions on associated farms. This information would help to determine if a final raw product is safe for consumption, and also establish a history for determining if contaminated conditions persist over time. Nevertheless, the consumption of raw almonds is highly risky as they lack a kill step during production, and also have low water activity.

A significant number of outbreaks due to dried seafood snacks have been reported in Japan. The rapid depletion of wild fish and shellfish stocks in recent years has greatly increased dependence on the international aquaculture industry as an alternative source for these popular food items (29). High-density farming conditions necessary to maximize output commonly concentrate marine animals into small areas that easily introduce widespread infection from multiple sources, such as from the use of

contaminated raw meats, offals, fish feed and animal feces. The use of contaminated feed in aquaculture underscores the great challenge this industry faces. It is clear that marine husbandry practices such as this are unsustainable and favor widespread bacterial contamination during rearing.

Problems in the aquaculture industry are especially relevant to the Japanese people, whose diet is heavily based in marine foods. Dried seafood snacks (such as dried squid chips) are both popular and unique to Japan and surrounding regions. Therefore it is not surprising that outbreaks of this product type have mostly occurred there. Predictably, Japan had 2 outbreaks from dried seafood snacks contaminated with *Salmonella* in 1999 that combined to sicken almost 2,000 individuals. The first outbreak, with 1,500 cases and 3 deaths, was caused by the ingestion of contaminated cuttlefish chips (43)**.** Contaminated well water that was used during processing of dried squid chips was the root cause for the second outbreak of 400 cases (2)**.** The massive scale of these two outbreaks underscored the importance of high quality feed and clean water as crucial to prevent repeat outbreaks of dried seafood snacks.

Chocolate, a seemingly safe product due to its low water activity, has been associated with several outbreaks since 1973. In that year, more than 200 cases were attributed to *Salmonella* Eastbourne in milk chocolate (91). The outbreak investigation found that the environmental contamination from storage rooms contaminated cocoa beans during dry roasting. Additional outbreaks involving salmonellosis from consumption of chocolate products are numerous. In 1982, 245 cases of serovar Napoli

contaminated chocolate imported from Italy. Five years later, in 1987, serovar Typhimurium contaminated Norwegian chocolates, causing 349 cases. Twelve additional cases in Finland were also attributed to this outbreak. Most recently, chocolates from a large firm in Germany were identified as the vehicle for a 2000 outbreak of *Salmonella* Oranienburg, which caused 439 cases throughout Europe (22, 37, 50).

Another *Salmonella* outbreak in Europe occurred as a result of paprika potato chip consumption in Germany. Contaminated paprika sickened over 1000 people in 1993 with no known deaths (33, 54). The outbreak occurred after as a company in Germany imported paprika from a South American supplier tainted with *Salmonella*. The high risk of *Salmonella* transmission via dried spices has been confirmed by a couple of recent outbreaks linked to white and black pepper. In 2009, more than 42 people got infected by consuming white pepper containing products in several Western states of the U.S. In addition, at the time of writing this document the Centers of Disease Control are still investigating a multi-state outbreak of *S.* Montevideo that has affected more than 230 people by consuming salami covered with contaminated black pepper (19).

Recent outbreaks involving *Salmonella* in dry foods demonstrate that this organism has a unique ability to resist extremely dry conditions. Table 1 presents an assortment of some of the most relevant *Salmonella* outbreaks linked to low water activity foods. Due to the complex global food supply chain; it is likely that many new foods will be linked to *Salmonella*. Thus, food manufacturers must respond by not only integrating a sufficient

processing "kill step," but also take steps to ensure hygienic plant and transport conditions.

Table 1: Foodborne outbreaks caused by *Salmonella* in selected dry products

Year	Country	Vehicle	Serovar(s)	Cases[a]	Deaths	Reference
1973	Trinidad	Milk Powder	Derby	3000[b]	NS[c]	33
1973	Canada and U.S.	Chocolate	Eastbourne	217	0	22
1982	U.K.	Chocolate	Napoli	245	0	33
1987	Norway	Chocolate	Typhimurium	361	0	50
1989	U.K.	Yeast Flavored Snack	Manchester	47	0	33
1993	Germany	Paprika Chips	Saintpaul, Javiana and Rubislaw	>670	0	54
1998	U.S.	Toasted Oat Cereal	Agona	209	0	13
1999	Japan	Squid Chips	*Salmonella* spp.	>453	0	49
1999	Japan	Cuttlefish Chips	Chester and Oranienberg	>1,500	3	43
2001	Canada and U.S.	Almonds	Enteritidis	168	0	33
2004	U.S.	Almonds	Enteritidis	47	0	86
2006	U.S.	Peanut Butter	Tennessee	628	0	15
2008	U.S.	Puffed Rice Cereal	Agona	28	0	16
2009	U.S.	Peanut Butter	Typhimurium	714	9	18

[a] Confirmed cases, unless stated otherwise

[b] NS, not specified

5. Overall microbiological load in dry or low a_w foods

In order to understand the route of *Salmonella* contamination, it is essential to know whether it is naturally present in the farm environment before it is harvested. If not, then the likely culprit is post-process contamination. Understanding the inherent

microbial load in dry foods will help manufacturers predict the greatest points of risk in their processing flow.

5.1 Microbial load in flours

Flours are not typically considered to be a common source of *Salmonella* transmission. This is primarily because cereal malts and flours are chiefly used as a raw material for more complex products that include a kill step, such as ready-to-eat cereal. The microbial load of this ingredient is highly variable and depends on the growing, dry milling, harvesting and storage conditions. Total plate count (TPC) for this product ranges between 10^3 and 10^6 CFU/g (59). Typically, the TPC for flours is comprised by yeasts (22% of total) and a variety of different bacteria. Molds counts are usually at concentrations of less than 10 CFU/g. Abnormal contaminants such as generic *E. coli*, and *Salmonella* do occasionally occur and should not be ruled out of concern (59) .

Two studies examined the *Salmonella* prevalence in wheat flour and found that of two different tests conducted on consecutive harvests, the percent of positive *Salmonella* tests ranged from 0.32 to 1.32% (77, 87). A similar study by Bulling et al. examined prevalence in dry milled wheat flour. Over four thousand samples were tested with an incidence rate of approximately 1%, indicating that the previous studies range of *Salmonella* contamination is representative for this food matrix (9). Typically, the microbiota of raw wheat is reduced by more than 1 log CFU by processing (aspiration and bran removal), but further reduction cannot be achieved unless a special kill step is

added before or after the milling process (46, 79). Even so, the addition of kill steps to the milling process is not typical due to the detrimental effects on flavor and texture. Therefore, microorganisms originally present on cereal grains, including the relatively rare occurrence of pathogens, can be expected to be present, albeit in substantially reduced numbers, in the milled cereal grain. William Sperber, a food safety and quality expert suggested in 2006 that the presence of contaminants, such as insect parts or fecal matter in the raw cereal grain explains the source of the occasional occurrence of pathogens, such as *Salmonella,* in milled cereal grains(88).

It is not surprising to have some contamination in flour, since cereal grains are prone to microbial proliferation during growth and harvesting while in contact with the soil. Postharvest, microbial growth is usually limited by good storage and milling practices. Under optimal storage conditions, condensation and moisture build up will be minimal, reducing potential microbial growth. Additionally, the dry milling process of flours generally removes 90-99% of microflora that are present in the kernel's bran, as this botanical layer is most prone to microbial contamination (33, 46, 79). The recent movement toward whole grain foods consequently introduces greater risk to the food supply since the bran, or the most contaminated part of the grain, is essentially added back to the endosperm-rich processed flour.

Overall, the prevalence of *Salmonella* in wheat flours is low. Therefore, public heath concerns for flour are typically also minimal since they are usually incorporated into products with a kill step (baking, frying). Flours cannot be ruled out as a possible

source of contamination, especially for whole grain products or those without a kill step. Even products with a kill step could potentially be at risk if ingredient-receiving areas are not sufficiently separated from the final product flow.

5.2 Microbial load in nuts

The culinary definition of nuts is extremely broad and includes botanically defined nuts (i.e. acorns), legumes (peanuts), drupes (almonds) and seeds (sesame) (51). . Similar to flour, microbial load in nuts depends on growing, processing, and storage conditions. During processing, the de-shelling process of nuts has the greatest potential to introduce contamination from the outer seed or hull into the kernel, otherwise known as the edible portion of the nut. If the outer seed coat (or hull) is not broken, the seed will be virtually sterile (59). Therefore, the goal of a nut manufacturer is to minimize contact between the hull and the inner meat of the nut, or to develop a kill step, such as roasting, to minimize contamination to the final product.

There is little published information on the microbial population of *Salmonella* in edible nuts. A recent study addressed this deficiency and found that of 727 samples (including almonds, cashews, hazelnuts, macadamia nuts, peanuts, pecans, pine nuts and walnuts), *Salmonella* was detected in 0.2 to 0.4 percent of all nuts tested (58). Based on that study, it is clear that *Salmonella* is present in nuts on rare occasions. Given that *Salmonella* is inherently present, Marcus et al. studied whether *Salmonella* and *E. coli* could survive in pecans after the conditioning process to determine if they would be

found in the finished product (61). Pecans require a process called wet conditioning to remove the outer hull. This process involves steaming or washing nuts in water (sometimes chlorinated) to make the kernel more pliable and less likely to break, a cost saving measure during processing. The same study observed survival of *Salmonella* but destruction of *E. coli* after conditioning in heavily contaminated nuts. This suggests that to be deemed safe, further kill steps are needed for pecans and other edible nut kernels.

Almonds were the vehicle of two recent salmonellosis outbreaks in 2001 and 2003 that prompted research to elucidate their susceptibility for contamination. In a 2007 study by Danyluk et al., over 9,000 almonds of different varieties were sampled over the course of five years and tested for microbial load (Table 2) (26). The *Salmonella* isolation frequency was reported to be less than 1%. The risk seems to be primarily related to raw almonds since roasting provides an effective kill step for roasted almonds. Raw almonds caused both the 2001 and 2003 outbreaks and thus should be avoided by consumers. Macadamia and walnuts were also tested similarly to almonds, with isolation frequencies in these products of 2.1% and 2%, respectively (78, 89). Based on these considerations, nuts may not be a prominent public health concern when processed by roasting; however, when minimally processed, they have a moderate risk of causing foodborne disease.

Table 2. Prevalence of *Salmonella* in different varieties of almonds surveyed over a five-year period. Taken from Danyluk, et al. (2006).

Year	Tested Samples	Number of Positive Samples
2001	2,003	12
2002	2,012	24
2003	1,764	15
2004	1,643	12
2005	1,852	18
Total	9,274	81

5.3 Microbial load in spices

Spices are notorious for harboring microorganisms due to the farm practices where they are typically grown. Many spices are grown, harvested and dried outdoors and subjected to soil, bird and insect contamination (64). The typical geographic location of spice production is in warm and humid locations that lay the groundwork for potential microbial contamination. Although many microorganisms are able to survive the drying process, the spores have the greatest advantage, as they offer the opportunity for later growth under certain conditions (temperature abused food). Several studies have found numerous aerobic mesophilic sporeformers dominating in the overall microbial load of spices (59) . When spices were tested for total plate count, spores comprised more than 50% of the overall microbial load. In addition to spores, coliforms, lactic acid bacteria, *Streptococcus* and *Salmonella* were also found. Another study investigated microbial prevalence in twenty different Asian spices. Of all spices, only red and black pepper

contained *Salmonella* spp., in percentages of 1.7 and 2.4%, respectively (44). Baxter and Holzapfel studied the microbial loads of 36 spices in South Africa (6). They found the highest level of contamination in black pepper, pimento and coriander. *Salmonella* was detected only in paprika, which is not a surprise, since this spice has been the causative agent of several recent outbreaks. The results of the above studies emphasize the need for safer methods of spice production, cleaning and sanitation.

Based on the microbial load in the aforementioned dry products, low water activity does not render food safe from *Salmonella* contamination. In addition to this information, it is also important to know how long this organism can persist in foods and what temperatures are sufficient to kill it. This information is important so food manufacturers can design appropriate kill steps to minimize potential contamination.

6. Survival of *Salmonella* in dry and low a_w foods

Outbreaks involving *salmonella*e have been linked to a variety of low a_w food products (Table 3). For this section the question yet to be answered: if *Salmonella* is found in a dry product, how long could it possibly survive?

Table 3. Selected *Salmonella* survival results in different foods with low water activity

Food	*Salmonella* serovar(s)	Inoculum (log CFU/g)	a_w or % moisture	Survival time (months)	Reference
Dried Milk Powder	Senftenberg, Typhimurium, New Brunswick		4%	5	63
Pasta	Infantis, Typhimurium		0.12%	12	75
Milk Chocolate	Eastbourne	8.0 5.0	0.41 0.38	9 at 21°C	92
Bitter Chocolate	Eastbourne	7.0 5.0	0.51 0.44	9 at 21°C 2.5 at 21°C	92
Peanut Butter	Agona, Enteritidis, Michigan, Montevideo, Typhimurium	5.7	0.20-0.33	5.5 at 21°C	10
		1.5	0.20-0.33	5.5 at 5°C 1.5 at 21°C	
Paprika Powder	Multiple Serovars			8	54

6.1 Survival of *Salmonella* in chocolate

Chocolate is the most popular confectionary product in the world and is one product with a relatively long shelf life due to its low a_w and water content. Despite these protective characteristics, several foodborne outbreaks due to chocolate products contaminated with *Salmonella* have occurred (7). Prevalence of *Salmonella* in chocolate is very low, with one study finding contamination as low as 3 CFU/g on average (50). Despite the relatively rare occurrence, when product is contaminated, survival of these few organisms is high. In 1976, Tamminga et al. found that *Salmonella* cells were able to persist in chocolate for as long as nine months (Table 3) (92).

Long-term survival in raw cocoa beans, the core product from which chocolate is derived, has also been documented. Craven et al. found that raw cocoa bean dust can lead to contamination of in-line and finished products due to inadequate standard time and temperature heating conditions (22). Shachar and Yaron reported that the combination of high fat and low a_w might be the key to the survival of *Salmonella* in this product (85). This synergistic combination makes a kill step difficult to justify, as the required time and temperature combination (Craven et al. reported at least 71ºC for 10 hours) has been reported to cause objectionable sensory characteristics that will ultimately make the product undesirable to the consumer.

Despite its scant prevalence, *Salmonella* in chocolate is a significant risk to human health. High fat content in this product protects *Salmonella* against gastric acid in the stomach, allowing colonization inside the gastrointestinal tract, leading to sickness (30). Thus, manufacturers producing chocolate find it difficult to eliminate *Salmonella* due to three reasons: (1) raw materials such as cocoa beans may carry *Salmonella*; (2) high fat content and low a_w increases thermal resistance, so even high-temperature heating processing may not kill *Salmonella* (and cause undesirable sensory characteristics); and (3) a few cells of *Salmonella* are capable of causing illness. More research should be conducted in this area to develop a kill step that will maintain a taste acceptable to consumers.

6.2 Survival of *Salmonella* in almonds and peanut butter

It is widely known that *Salmonella* can survive in peanut butter as a result of the two high profile outbreaks in 2007 and 2009. A study published in 2000 suggested that survival is highly dependent on storage temperature. In that study, peanut butter samples containing 6 log CFU/g of a 5 strain *Salmonella* cocktail were stored at 21°C and 5°C for 24 weeks (10) . After this time period, from 4.1 to 4.5 and from 2.9 to 4.3 log CFU/g reductions were observed, respectively. The authors concluded that the lower storage temperatures resulted in a greater number of *Salmonella* that were able to survive. At a lower initial inoculation (1.5 CFU/g), the results were similar as the 21°C trials were all negative for *Salmonella* while the lower storage temperature still had detectable levels Although the result of that study seems counterintuitive, it nevertheless suggests that allowing peanut butter to remain at room temperature (sealed) is safer than under refrigerated conditions.

In 2006, Uesugi et al. conducted one of the first studies on the survival of *Salmonella* in almonds (94). They measured survival over 18 month at four temperatures (-20, 4, 23 and 35°C) and found that at the first three temperatures (excluding 35ºC), survival decreased over time and calculated average change in counts of 0, 0, and -0.25 CFU/almond per month, respectively. Almond kernels stored at 35ºC had a reduction in viable cells, but after that no additional killing was observed , evidenced by a 1.1 log CFU/almond reduction from day 0 to 59 and 0 log CFU/almond from day 59 to 170. These studies are relevant because their observations show the ability of *Salmonella* to survive on nut and nut products throughout their typical shelf life of 1 to 2 years.

6.3 Survival of *Salmonella* in dry products (flours, milk powder, spices)

Few data are available on the survival of bacterial pathogens in flours. One study conducted by VanCauwenberge et al. found evidence that typical storage conditions may cause a reduction of approximately 3 log CFU/g after 24 hours (100). After this storage period, all samples remained positive for contamination of *Salmonella*. It is unlikely that flour would contain a *Salmonella* count as high as 3 log CFU/g, since the milling process removes most contaminants from the end product. Therefore, given that flour usually uses a kill step during cooking, combined with low prevalence and survivability of *Salmonella*, it is not of a major public health concern.

Dried milk powder has an extremely low water activity (approx. 0.03), undergoes a kill step (spray drying), and yet still carries some risk of *Salmonella* contamination (20). *Salmonella* has been the subject of several outbreaks linked to dried milk powder, with the largest (3,000 cases) occurring in Trinidad in 1973 (33). Given the high risk of *Salmonella* contamination of milk powder, McDonough et al. tested three serovars, Typhimurium, New Brunswick and Senftenberg, for survival under variable heat and moisture conditions in milk powder at different temperatures (4,16, 27 and 38°C) and moisture conditions typical of milk powders (~4%). In that experiment, *Salmonella* was able to survive for 15 weeks (63). Overall, the McDonough et al. study has limited relevance due to the short research period. Barron and Forsythe tested sub-lethally injured *Enterobacteriaceae* (including *Salmonella* Enteritidis) over a longer period of 2.5 years to determine their persistence in powdered infant formula (5). The researchers in that study divided species of *Enterobacteriaceae* into three groups based on their long-

term survival in a desiccated state. *Salmonella* was in the second most resistant group, as they survived as long as 15 months in the infant formula. In contrast to flours, once milk or infant formula has been dried to a powder, the reconstitution process does not usually include a kill step. This makes these products especially dangerous to infants (infant formula) and elderly adults (milk powder).

Lastly, spices such as dried paprika and pepper are infrequently contaminated with *Salmonella*. An outbreak of powdered paprika potato chips in Germany heightened concerns with regards to spices and *Salmonella*. A 1995 study found *Salmonella* capable of surviving in dry paprika for as long as 8 months (54) . Other than this outbreak, the only other incident involving *Salmonella* was in pepper (12). Urabe et al. further investigated survival in spices (red and black pepper), and also whether surviving *Salmonella* could grow in cooked food (added after cooking) (97). They found that of three serovars inoculated (Welteverden, Senftenberg, and Enteritidis), none were able to survive beyond 28 days. In cooked food such as egg and potato salads, each serovar was able to colonize and grow at 30ºC, but not at 10ºC. Given that spices are occasionally contaminated with *Salmonella*, it is imperative that proper food storage techniques are employed to mitigate the ability of *Salmonella* to survive and grow in foods.

Clearly, *Salmonella* is able to survive in a variety of products with low water activity (Table 3). From a previous section, we also know *Salmonella* is naturally present in many of these products and can also survive long periods of time. There is a follow up

question to these findings: do any of these products have a common link that makes them particularly susceptible to *Salmonella* contamination?

7. Commonality of dry/low a_w foods associated with *Salmonella* outbreaks

Typically, *Salmonella* has been associated with animal products such as meat, eggs or poultry (85) . However, as previously mentioned, many dry products have been implicated in recent outbreaks. Prior to several recent peanut butter outbreaks, for example, this product was thought to be generally safe from *Salmonella* contamination due to a low water activity, small water droplet size in the fat emulsion, and an inability of microbes to move between droplets (32). However, studies have shown several serovars of *Salmonella* can survive in peanut butter under room temperature or refrigerated conditions longer than 24 weeks, with little reduction in CFU/g (10, 71). Why is this? What makes peanut butter and other low water activity foods resistant? Is there a commonality between them? Plausible answers to these questions will be presented in the discussion section.

8. Characteristics of *Salmonella* Typhimurium, Tennessee, Senftenberg and Agona

Four serovars were tested in this study: *Salmonella* Typhimurium, Tennessee, Senftenberg, and Agona. Each was chosen based on three characteristics: desiccation resistance, ubiquity in low a_w foods, and notoriety. In 2006, *Salmonella* Typhimurium was the most common human serovar, representing 16.9% of

all cases (14) . In that same year, *Salmonella* Tennessee, Agona and Senftenberg made up about 2.4% of all cases combined (14) .

Despite the rarity of *Salmonella* Tennessee, Agona, and Senftenberg, they were chosen due to recent high profile outbreaks with which they are indentified. For instance, *S.* Tennessee was linked with peanut butter, Agona to cereal, Senftenberg to infant formula, and other dry products. Of particular interest is *Salmonella* Agona, which was reported as the causative agent in three outbreaks, although all of these were dry products (8). Although these serovars may not surface with regularity, they are nevertheless a very high risk for the dry food manufacturer merely based on previously documented cases.

Surprisingly, there are significant genetic differences between serovars within the species *Salmonella enterica* (the species for all four serovars). As an example, 2 to 8% of the Typhimurium genome is absent in many other serovars (33). This variability is the result of virulence genes that make their home on mobile genetic elements, such as plasmids. This gives one possible explanation for the different methods of attachment and invasion inside the human host that ultimately leads to foodborne illness.

9. Heat tolerance of *Salmonella*

Heat tolerance of *Salmonella* is believed to be closely related to the water content within the cell. Hiramatsu and collaborators hypothesized that in a viable organism, heat induces intense vibration of water molecules, causing breakage of disulfide and hydrogen

bonds of intracellular proteins (49). However, in a desiccated bacterium, this phenomenon may be limited substantially due to water availability. Those authors speculated that the lack of water prevents vibration, which in turn protects cellular proteins from denaturation at elevated temperatures (49). The combination of low water activity and heat in cereal, and many other dry products, may provide an optimal combination of conditions to produce thermally resistant *Salmonella*.

9.1 Heat tolerance in flours

Several experiments in wheat and corn flours have shown that death of *Salmonella* slows dramatically after the first few minutes of drying (34). At lower initial water activity, the D-values, or time required to kill 90% of the population were longer. A similar death curve was observed in another study that tested flour at various water activities, temperatures, and relative humidity conditions. The results of that experiment indicated that as the water activity decreases, heat tolerance increases, regardless of the relative humidity (3). The relevance of these studies to the food industry stems from the need that flour based products must employ a kill step in-line with the D-value of the most resistant organism, which may or may not be *Salmonella.*

9.2 Heat tolerance in nuts and nut products

Peanut butter was tested for thermal inactivation of various *Salmonella* serovars, a likely response to several recent outbreaks. Four temperatures (three typical roasting temperatures and one extreme temperature) were tested with three cocktails of

Salmonella: *S.* Tennessee outbreak isolates, other serovars, and unrelated *S.* Tennessee clinical isolates (60). The previous study found that current roasting temperatures are not sufficient for a 5-log kill of any *Salmonella* tested. However, the extreme temperature was able to achieve a 5-log reduction if subjected to a lengthy thermal treatment (90°C) for 30 minutes. A treatment such as this would have decreased shelf life and sensory qualities compared to typical peanut butter. Therefore, more research must be done to determine an appropriate inactivation procedure that will not negatively affect the sensory qualities of peanut butter. The general take away message from these studies is that as water content in dry foods decreases, heat tolerance increases. Therefore, although *Salmonella* is less likely to be found in dry products, it is more difficult to destroy than in moist products, and presents a great challenge to dry food manufacturers.

9.3 Heat tolerance in dried egg powders

Dried egg whites are noted to have 600-700 times the heat resistance as compared to liquid egg whites (40). Eggs provide another example of increased heat resistance as water activity and moisture decrease. A 1967 study proved this hypothesis by thermally heating dried egg white powder. In this experiment, one week of heating at 49-50ºC was required to eliminate *Salmonella* (74). Another experiment by Banwart and collaborators tested inoculated dried egg white powders at different moisture contents (10% vs. 4%) for 12 days at 50ºC. They found that 60% of *Salmonella* cells survived at the lower water content, while they were eliminated at 10% moisture (4). These studies provide yet another instance of the hardy nature of *Salmonella* when present in dry products.

10. Desiccation tolerance of *Salmonella*

Based on what is already known, *Salmonella* is heat tolerant, pervasive in nature, and survives long periods of time in dry products. Yet to be determined is how *Salmonella* tolerates dry conditions from a genetic perspective. The key to its survival has been linked to an operon (*proU*) and three genes within the locus: *proV, proW*, and *proX* (38) . Of particular interest is the gene *proX*, which has been reported to be responsible for expression of periplasmic (space between inner cytoplasmic membrane and external outer membranes) glycine betaine, which protects the cell against osmotic stress (90). Together with *proV* (responsible for protein binding and regulation of osmotic flow), these three genes regulate osmotic flow of glycine betaine and other osmolytes (such as sucrose) from the external environment and overall prevent the cell from desiccation (38, 90). Therefore, when *Salmonella* are subjected to dry conditions, these genes regulate and maintain turgor (minimum outward pressure in order to allow microbes to survive). Turgor is accomplished by the absorption of compatible solutes to water, such as trehalose, proline, or glutamine, as examples (38). Beyond these solutes, sucrose has also been found to extend and maintain cell life by a proposed mechanism, known as the "water replacement hypothesis" (49, 56). The water replacement hypothesis is particularly important to desiccation and heat tolerance due to the ubiquity of sucrose in food products.

Heat tolerance of *Salmonella* is highly related to desiccation resistance. Organisms that are sensitive to dry conditions undergo a protein conformational shift in the bacterial membrane that will eventually kill the cell when subjected to dry conditions

(49). A resistant organism like *Salmonella* that is able to uptake compatible solutes like sucrose has a competitive advantage to non-resistant organisms. Essentially, the uptake of sucrose reduces overall water content in the cell, which may result in fewer vibrations induced by heat. The overall advantage is that the bacterial membrane may retain cellular integrity, prolonging the life of the cell.

Hiramatsu, et al. reported that the survival of dried *Salmonella* cells substantially increased (up to 79 times) when sucrose was present in a desiccation model system using paper disks (49). The same study also found that dried squid chips containing sucrose maintained survival at 23 to 89 times greater than without sucrose. The relevance of that study was that it provided evidence that supported the water replacement hypothesis in both a simple (dried paper disks) and complex (dried squid chips) medium.

11. Impact of food additives on *Salmonella*

Several studies have shown that certain food additives have an effect on *Salmonella* survival under dry conditions. The aforementioned sucrose is one ingredient that has shown to enhance the survival of *Salmonella* in a variety of mediums. Salt is another, and it has been shown to contribute toward cell death (49).

11.1 Impact of sucrose to *Salmonella* survival

The mechanism of survival in presence of sucrose and trehalose is well studied. The aforementioned water replacement hypothesis, is one theory that attempts to explain

the ability of *Salmonella* and other organisms to survive under dry conditions. To better understand the mechanism by which the sugar replacement hypothesis occurs, it is important to understand how typical microorganisms would react to dry conditions. Essentially, as water is removed from the lipid bilayers, the headgroups are brought closer together, resulting in an increase in van der Walls interactions between acyl chains. The increase in affinity between acyl chains forces the dry bilayer into a gel phase at room temperature, which has been proposed to lead to phase separation (56). When rehydration occurs and the lipid bilayer undergoes another phase transition back to liquid crystalline stage (initial condition), cell leakage occurs, destroying the cell. Thus, by the water replacement hypothesis, *Salmonella* would be able to avoid this cellular leakage, thereby evading death.

The mechanism of this theory, therefore, is that when the cell dries in presence of sucrose (or trehalose), the effects of drying and rehydration (typically causing cell leakage, destroying the cell) are minimized because the lipid bilayer is maintained in the liquid crystalline phase (24, 49, 56). It is theorized that the sugar depresses the phase transition by hydrogen bonding to the polar headgroup. Variations in resistance to dryness could be related to a bacterial species ability to accumulate intracellular sucrose and/or trehalose.

Not only does sucrose enhance survival, it also increases heat resistance when present in solutions. A study conducted in 1974 established that of sucrose, glucose, glycerol and sorbitol, sucrose allowed for the greatest resistance (21). The reason for this

mirrors the already mentioned water replacement hypothesis, where cytoplasmic water is replaced by sucrose during dehydration. However, there is a lack of information in regards to resistance when exposed to ready-to-eat cereal, or many other ready-to-eat products. In other words, although a trend is observed with sucrose when present in a solution, there are few, if any studies examining this trend in ready-to-eat cereal products. More studies to support this theory would help establish specific heat treatment and storage guidelines to mitigate growth of *Salmonella* in food products. The main objective in this project is to determine the relationship between *Salmonella* spp. in ready-to-eat cereal by comparing survival in high and low sucrose containing cereal.

11.2 Impact of sodium chloride to *Salmonella* survival

Another ingredient commonly present in cereal is NaCl, or table salt. High salt concentrations in food have long been acknowledged for enhancing shelf life by the inhibition of microorganisms (72). The bacteriostatic effect of salt is believed to be caused by a decrease in water activity, compounded with osmotic effects caused by the concentration in solution.

Salmonella is typically inhibited at NaCl concentrations as low as 3%. An experimental example determined to test survival in presence of salt found that up to 1.5 log CFU/g was eliminated at a 5% NaCl concentration while under desiccant conditions (49). However, evidence suggests that the extent of the response of *Salmonella* to NaCl is largely adaptive, or food and serovar specific. (27). It is possible that salt could have

some effect on survival of *Salmonella* in cereal. However, given that most cereals (including the two featured in this study) contain less than 1% salt, the effect is both untested and unknown at the present time. Given that cereal typically contains less than 1%. With the addition of sugar, it is not clear what if any effect salt and sugar may have when both are present. This study will address this response activity.

12. Conclusion

The food industry is in the midst of a quandary in relation to dry foods and *Salmonella* contamination. Serial outbreaks in dry food products demonstrate the need for more research in this area. Specifically, research is needed to understand the length of time that *Salmonella* are able to survive, an appropriate inactivation temperature, and finally how to enact an effective kill step without seriously affecting product quality. The results of this project provide crucial data to understand the ability of *Salmonella* to survive in ready-to-eat cereal. The primary goal of this series of experiments was to determine whether time, type of cereal (sweetened or unsweetened), experimental design, or serovar are responsible for changes in microbial count. A secondary goal of this study was to determine whether sweetened cereals carry greater risk than unsweetened cereals by enhancing survival of *Salmonella,* according to the water replacement hypothesis.

The structure of this research was based on two primary null hypotheses:

Hypothesis I: The survival of *Salmonella* in low-water activity ready-to-eat toasted oat cereal is the same for different serovars.

Hypothesis II: The presence of sucrose does not influence the survival of *Salmonella* in low-water activity ready-to-eat toasted oat cereal.

Materials and Methods

A. Drying experiments

To establish an appropriate inoculation procedure, water activity and moisture were measured over 22 h periods. Boxes of sweetened toasted oat cereal (STOC) and unsweetened toasted oat cereal (TOC) were each purchased from a local supermarket, opened, and placed into a 5 quart colander (Good Grips, New York, NY). Volumes of 2 liters of deionized, sterilized water were poured evenly over each cereal and spread onto metal baking pans. Pans were placed inside a conventional laboratory oven (Precision, Woodstock, IL) at 40°C for 22 h and water activity (Decagon, CX-2, Pullman, WA) and moisture content were measured at 1, 3, 4, 5, 6, 7, 21, and 22 h. Moisture testing was conducted by a standard AOAC method (AOAC 925.10).

B. Assessment of background flora in toasted oat (TOC) and sweetened toasted oat cereals (STOC)

Before a test procedure could be developed, background flora was tested in both types of cereals. Five cereal boxes each, produced at various locations, dates and times, were purchased from a three local supermarkets. Cereal samples of 11 g of and 99 mL of peptone water were placed into sterile plastic stomacher bags and stomached for 2 min. Aliquots of 0.1 mL were transferred onto standard plate count agar (PCA, Neogen, Inc., Lansing, MI) in duplicate and incubated for 48 h at 37°C.

C. Bacterial strains and culture media

A total of 5 *Salmonella* serovars were used in this study (Table 4). Stock cultures were stored at -50°C in 20% glycerol. Stock cultures were cultivated in tryptic soy broth (TSB; Neogen, Inc.) for 24 h at 37°C before use in experiments.

Table 4: *Salmonella* serovars used in this study

Serovar	Source	Source	Associated Outbreak
Salmonella Senftenberg	Paradigm Labs	Shrimp	None
Salmonella Agona	FDA	Cereal	1998
Salmonella Typhimurium ATCC 700408	ATCC	N/A	None
Salmonella Typhimurium E2009005811	Minnesota Department of Health	Peanut Butter	2009
Salmonella Tennessee E2007000502	Minnesota Department of Health	Peanut Butter	2007

a. Inoculation Culture Preparation and Drying

Tubes containing 20 mL of tryptic soy broth (TSB) were inoculated with 100 μL of working cultures of each serovar and incubated for 24 hours at 37°C, in duplicate. After incubation, tubes were diluted in 2.0 L of sterile deionized water. These mixtures were poured over 340 g of STOC or TOC contained in a metal colander. After draining, the cereal samples were spread onto a metal baking pan. One blank portion (only sterile water poured over cereal) of STOC and TOC was prepared for water activity measurements. Pans were placed in an incubator at 40°C for 24 h for drying and subsequently each portion of cereal was packaged individually in eight 11-g quantities (representing each testing period 1, 3, 5, 7, 14, 30, 60 and 90 days) and stored at room

temperature, which was monitored to be between 22-23°C. All above inoculation and drying steps were done in duplicate in two separate, yet identical experiments.

b. Microbiological analysis

Cereals (TOC and STOC) were tested for *Salmonella* CFU/g and water activity (Model CX-2, Decagon Devices, Pullman, WA) in duplicate at each time period for each inoculation (twice each for two separate inoculations). Samples (11 g) (Samples were weighed in duplicate per test, and each test was duplicated) and 99 mL peptone were transferred into plastic stomacher bags (VWR, Westchester, PA) and stomached (Stomacher Lab Blender, Tekmar Company, Cincinnati, OH) for 2 min. From each stomacher bag, 1 mL aliquots were serially diluted (varied depending on elapsed test time) in 9-mL peptone water tubes. Volumes of 0.1 mL of serial dilutions were transferred onto a differential TSA agar (TSA media containing 0.8/L g ferric ammonium citrate and 6.8 g/L sodium thiosulfate) specifically formulated to identify *Salmonella* colonies producing hydrogen sulfide. Plates were incubated for 24 h at 37°C and subsequently counted to obtain the CFU/g values (Figure 1).

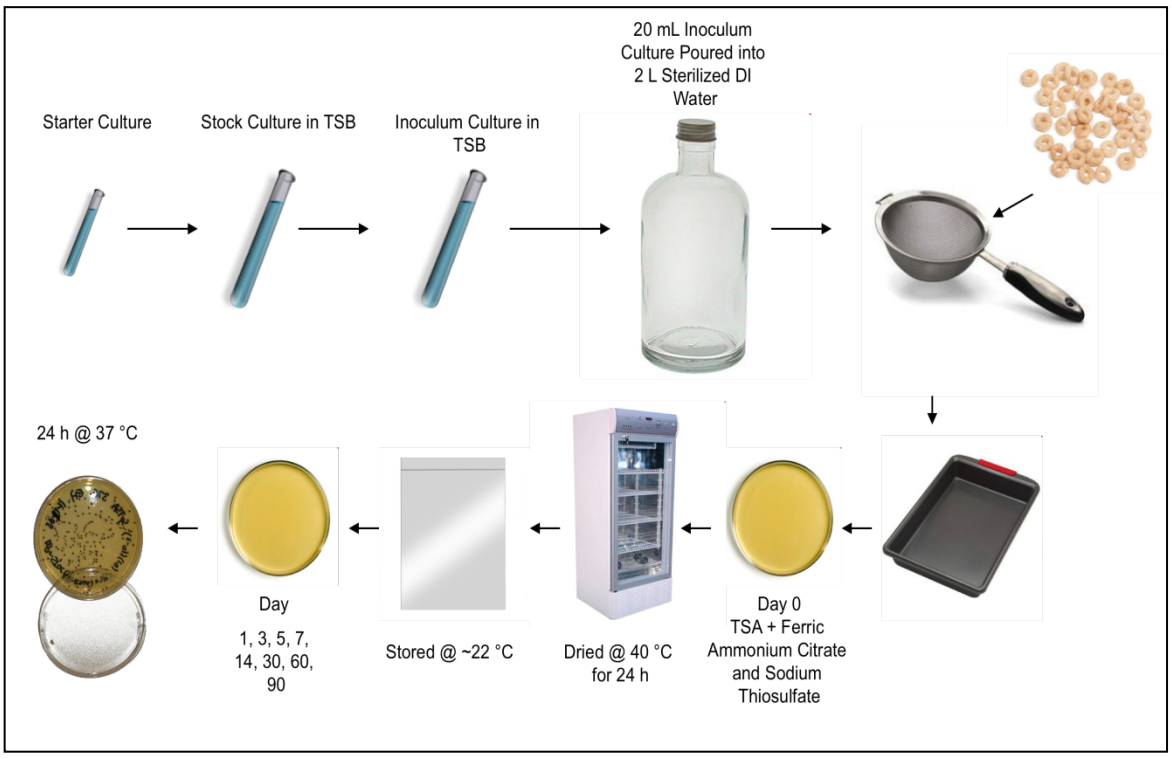

Figure 1: Graphical representation of cereal testing procedure for the detection of *Salmonella.*

D. Statistical analysis

The goal of this experiment is to determine whether time, type of cereal (sweetened or unsweetened), experimental design, or serovar were responsible for microbial count. To determine this, response feature analysis of variance was used to analyze the data from this experiment. This approach reduces multiple responses (or time periods) into a single response that can then be analyzed simply by one-way analysis of variance (standard ANOVA and t-tests) (23). Data was separated into two categories: overall mean of all repeated measures and slope (minus t=0). The mean value will identify the average count per serovar and type of cereal, while the slope will identify whether there is an upward or downward trend over time. A regression line was

calculated using a general linear mixed model based on repeated measures (inoculations) for each serovar and cereal type. The slope was caculated using two variables: the explanatory variable (time (months)) and the response variable (count (log CFU/g)). The regression line was calculated by using the standard equation $y = b_o + b_1 x$, where b_o is the intercept at time 0 (CFU/g value at the beginning of RTE cereal storage) and b_1 is the calculated slope (amount that y (count) changes when x (time) increases by 1 unit (time)). The slope of this line was calculated to show changes in bacterial counts over time. The slope included only data points produced during storage, or after the drying step. One-way analysis of variances, namely ANOVA, was used to determine statistically significant differences between the three previously named characteristics.

Results

1. Drying experiments

Moisture and water activity were tested during the preliminary drying experiment for both TOC and STOC. After inoculation in water and 22 hours of drying at 40 °C, both STOC and TOC achieved and average (three replicate measurements) a_w of 0.133 and 0.193, respectively. This compares to a starting a_w of 0.160 for TOC and 0.170 for STOC (Fig. 1).

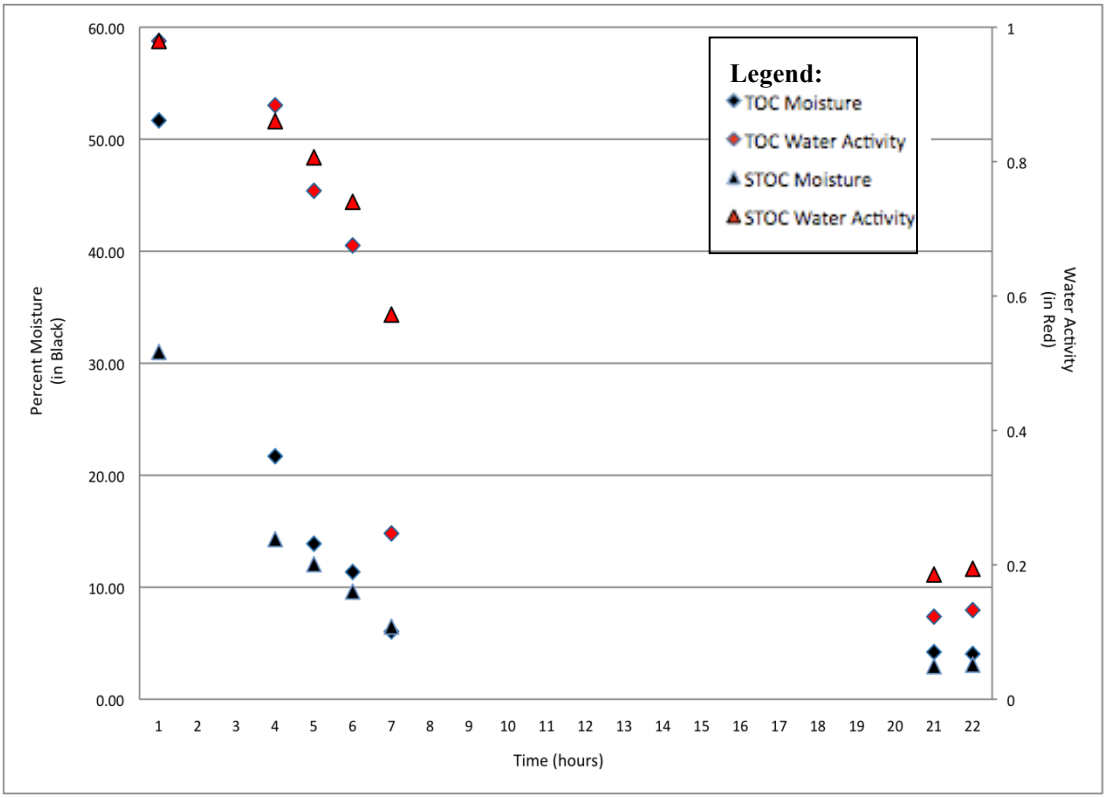

Figure 2. Reduction in moisture and water activity of TOC and STOC previously mixed with deionized water during incubation at 40°C.

For convenience, drying was conducted for a 24-hour period. After the two extra hours, the water activity of TOC and STOC were 0.110 and 0.142, respectively.

A control was run side by side with the inoculated TOC and STOC to monitor a_w as the experiment was stored in high density polyethylene packages for 90 days (Fig. 2) Overall, the water activity in experimental period was approximately 0.1-0.2 higher than during the initial testing phase (Fig. 2).

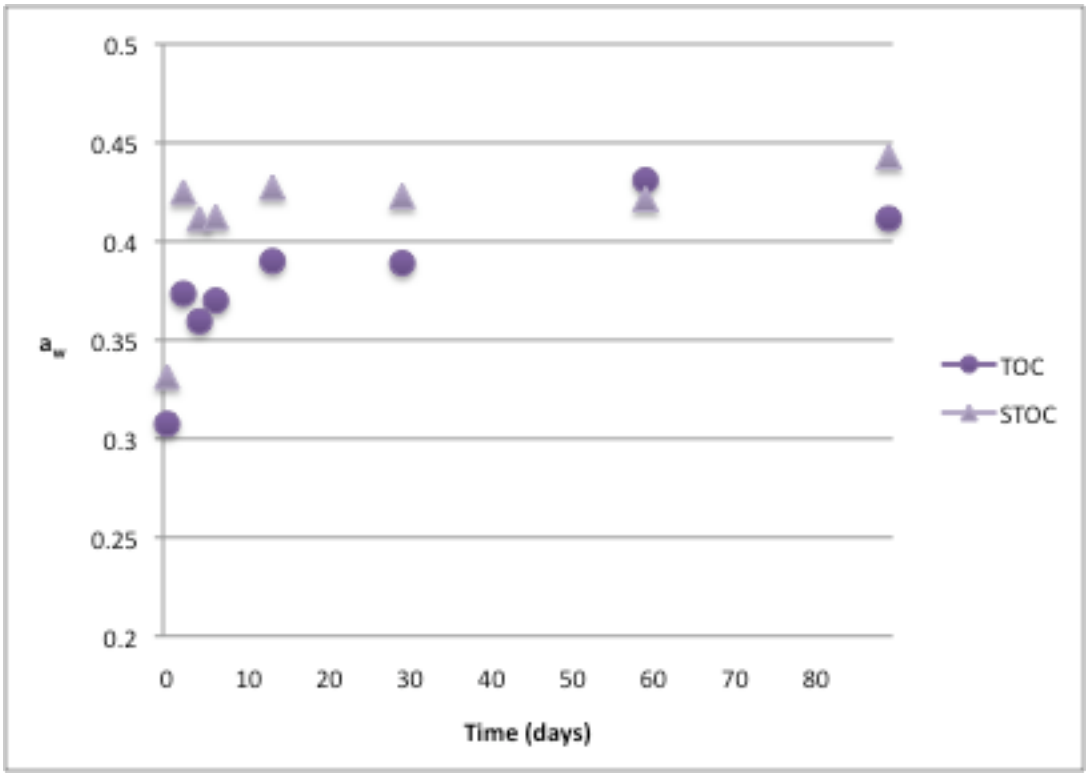

Figure 3. Water activity of toasted oats cereal during experimental phase stored at 22 °C for 90 days in thermally sealed high-density polyethylene bags.

2. Assessment of Background Flora

The background flora of TOC and STOC was tested on plate count agar during the exploratory testing phase. This test revealed that less than 100 CFU/g was observed in all background flora tests, with 7 out of 10 replicates having no microbial colonies. During

the experimental phase, background flora was tested again after each cereal box was opened. Out of twenty tests (plated in duplicate on plate count agar), only one had growth, a singular bacterial colony.

3. Survival Experiments

From 10 duplicated tests (or a total of 20 independent experiments) the average log count of *Salmonella* immediately after inoculation (before drying) was 6.58 ± 0.6 CFU/g (Table 5).

Table 5: Initial inoculation count of *Salmonella* onto STOC and TOC determined before drying

Type	Serovar	Replicate experiment	Initial count (log CFU/g)
TOC	*S.* Agona	1	6.18
TOC	*S.* Agona	2	6.58
STOC	*S.* Agona	1	5.98
STOC	*S.* Agona	2	6.32
TOC	*S.* Senftenberg	1	6.53
TOC	*S.* Senftenberg	2	6.41
STOC	*S.* Senftenberg	1	6.15
STOC	*S.* Senftenberg	2	6.45
TOC	*S.* Typhimurium (ATCC)	1	6.67
TOC	*S.* Typhimurium (ATCC)	2	6.63
STOC	*S.* Typhimurium (ATCC)	1	6.14
STOC	*S.* Typhimurium (ATCC)	2	6.38
TOC	*S.* Typhimurium (MDH)	1	7.15
TOC	*S.* Typhimurium (MDH)	2	6.77
STOC	*S.* Typhimurium (MDH)	1	6.66
STOC	*S.* Typhimurium (MDH)	2	6.74
TOC	*S.* Tennessee	1	6.97
TOC	*S.* Tennessee	2	7.18
STOC	*S.* Tennessee	1	7.05
STOC	*S.* Tennessee	2	6.63
	AVERAGE		**6.58**

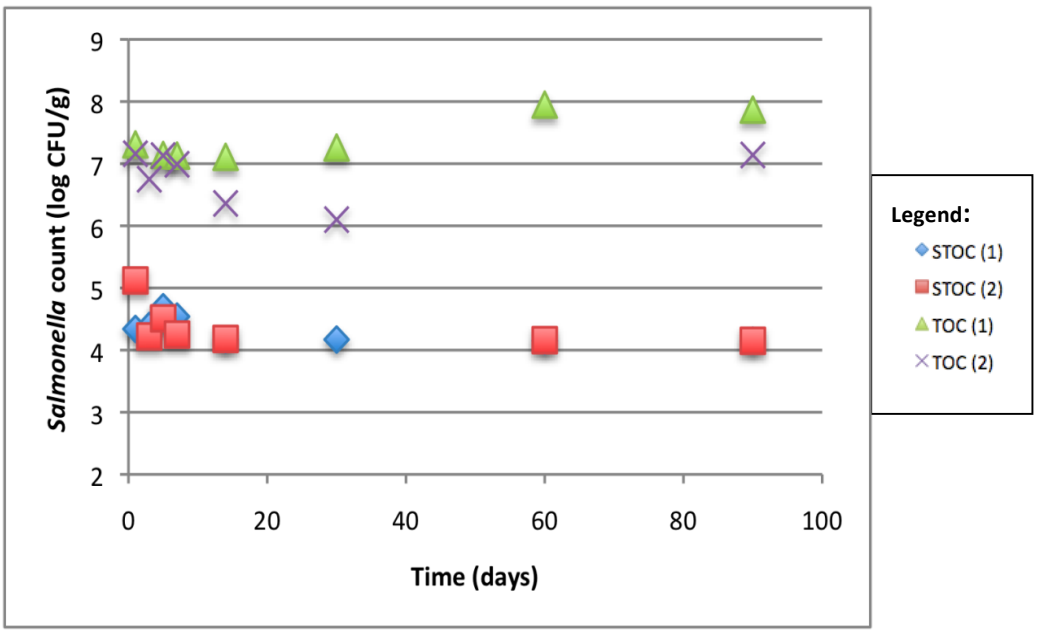

Figure 4: Count of *Salmonella* Agona on dried toasted oat cereals (STOC and TOC) during storage at room temperature.

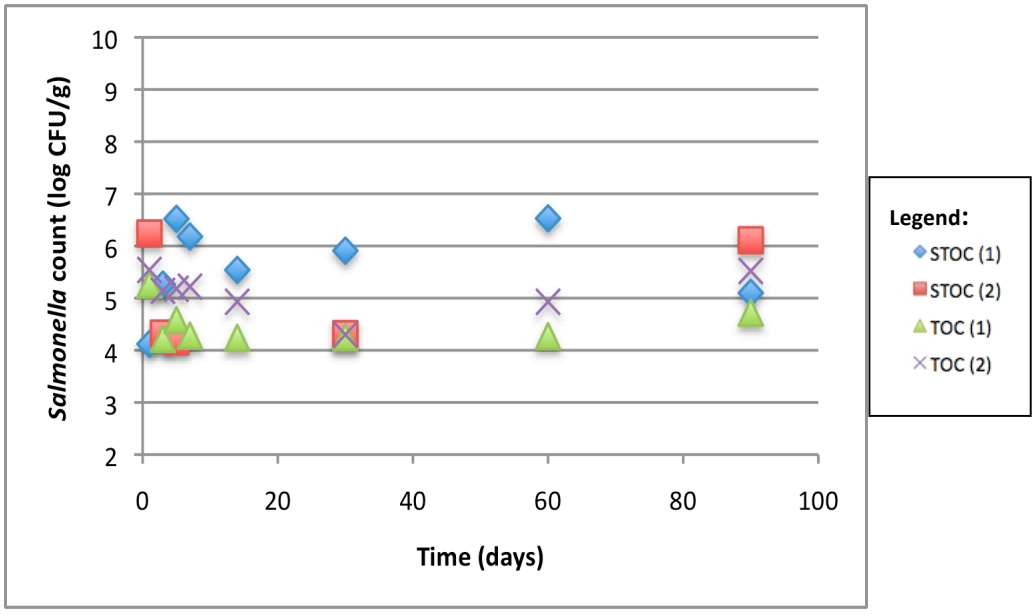

Figure 5: Count of *Salmonella* Senftenberg on dried toasted oat cereals (STOC and TOC) during storage at room temperature.

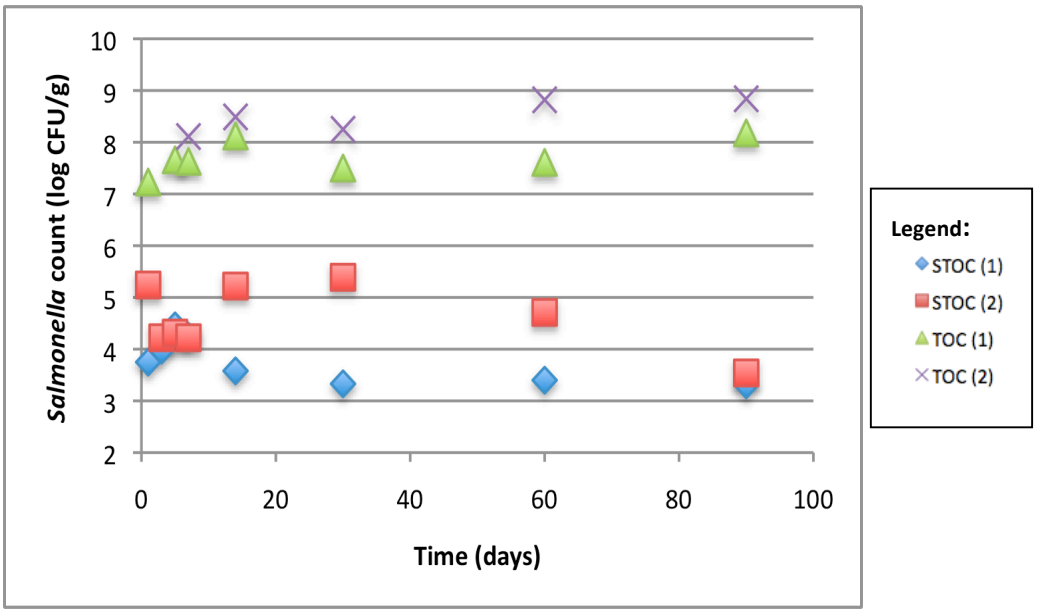

Figure 6: Count of *Salmonella* Typhimurium (ATCC strain) on toasted oat cereals (STOC and TOC) during storage at room temperature.

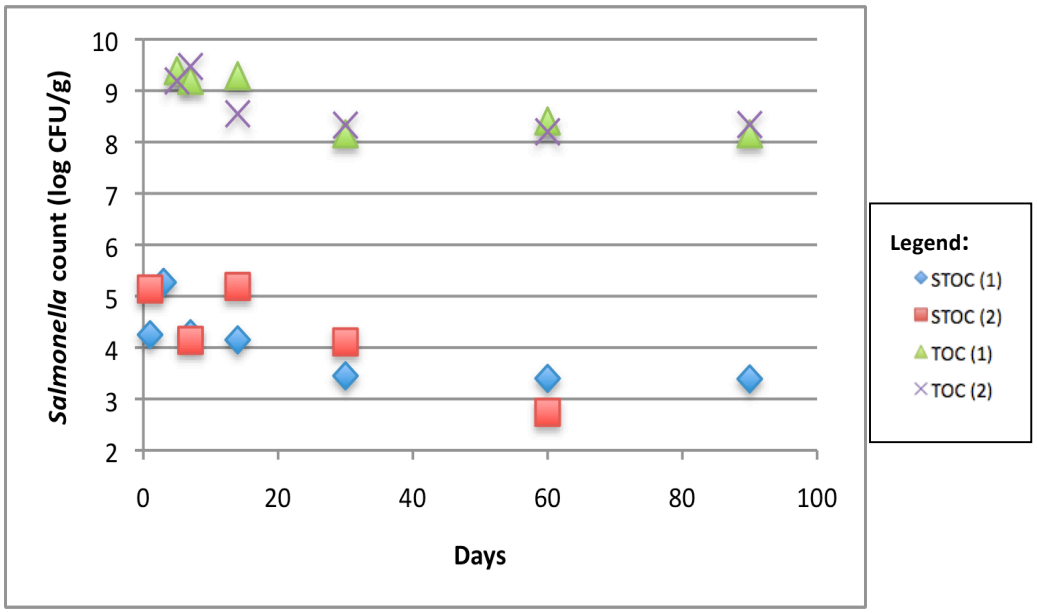

Figure 7: Count of *Salmonella* Typhimurium (MDH strain) on toasted oat cereals (STOC and TOC) during storage at room temperature.

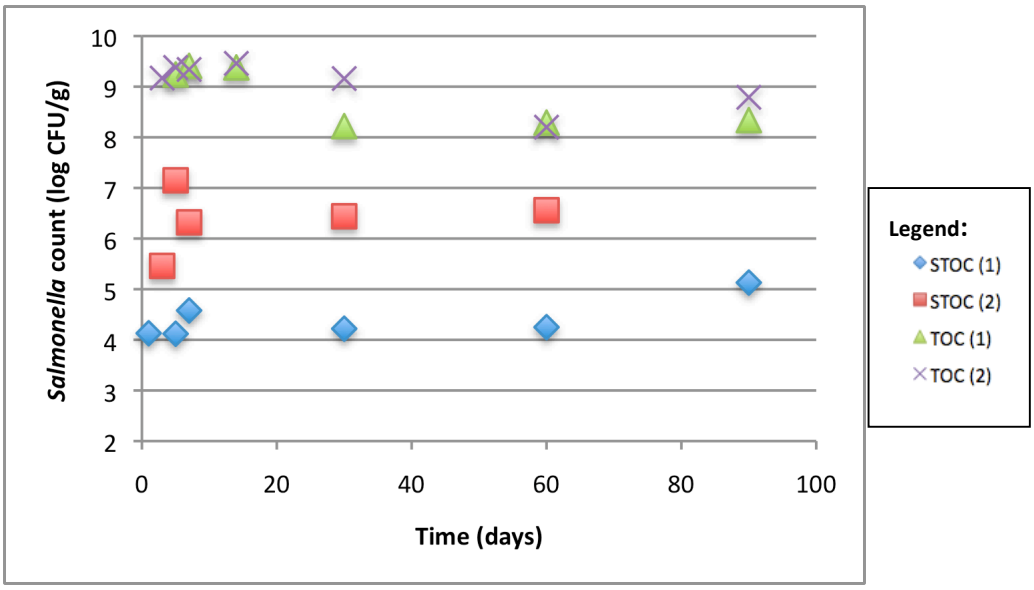

Figure 8: Count of *Salmonella* Tennessee on toasted oat cereals (STOC and TOC) during storage at room temperature.

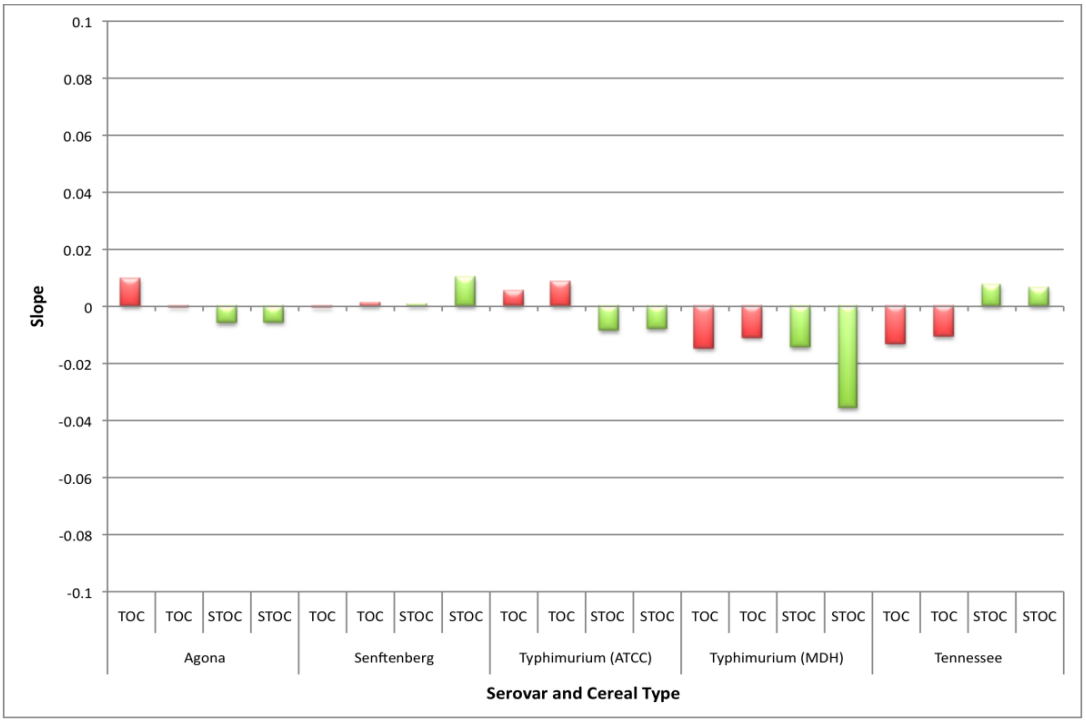

Figure 9: Average slopes for 90 day time trial (excluding inoculation and drying periods) of TOC and STOC inoculated with 5 serovars of *Salmonella*.

In TOC samples, the count of *Salmonella* Agona increased from approximately 6 log CFU/g to more than 7 log CFU/g during the drying period and before the cereals were packaged. However, the count on the first day after drying declined slightly to approx. 5 log CFU/g (Fig. 4). During the 90-day experimental period, the *S.* Agona count ranged from 10^6 to 10^8 log CFU/g in TOC, and by the end of the experiment, populations of 10^4 CFU/g were detected in STOC. In contrast *S.* Senftenberg count did not increase during the drying period in TOC and its numbers reached 10^5 CFU/g or less within the first 10 days (Fig. 5). In STOC, the *S.* Senftenberg counts were quite variable between trials, but the final levels were 5 to 6 log CFU/g.

Similar to *S.* Agona, the count of both *S.* Typhimurium strains and *S.* Tennessee markedly increased to 8 log CFU/g in TOC before the product reached final water activity (Figs. 6, 7, 8). During TOC storage, their numbers remained at more than 10^8 CFU/g for most of the trial period. The populations of both *S.* Typhimurium strains in STOC were gradually reduced during storage to reach levels of less than 10^4 CFU/g (Fig. 6, 7). The pattern of counts during storage for *S.* Tennessee in STOC resulted very similar to *S.* Senftenberg, also quite variable.

In figure 9, the response over time appears to be serovar dependent. Of all inoculations and serovars, 14 inoculations had a slightly decreasing slope, where 6 were increasing (Fig. 9). Of all inoculations, 19 of 20 had a slope less than 0.02 (both positive and negative direction).

4. Statistical Analysis

Statistical analysis clarifies the visual data from the average slope and count graphs. The analysis was conducted on cereal type, difference between experiment replicates, serovar, and serovar when dependent on cereal type. Cereal type and serovar were the only variables significant in the average count as well as the interaction of both of them (Table 6). Cereal type was the variable that had the greatest impact in explaining the differences observed within serovar. The slope was most dependent on the serovar, but cereal type was also significant (dependent on serovar) (Table 7). All analyses were significant at 95% confidence.

Table 6: Statistical analysis of aggregated *Salmonella* serovar means using ANOVA

	Degrees of Freedom	F Value	P-Value
Serovar	4	2.50	0.116
Cereal Type	1	66.80	<0.001
Repetitions	1	0.75	0.410
Serovar: Cereal	4	7.59	0.005
Residuals	9		

Table 7: Statistical analysis of aggregated *Salmonella* serovar slopes using ANOVA

	Degrees of Freedom	F Value	P-Value
Serovar	4	8.12	0.004
Cereal Type	1	1.06	0.329
Repetitions	1	0.14	0.713
Serovar: Cereal	4	5.71	0.014
Residuals	9		

Discussion

Each year, more than 2 billion packages of over 200 varieties of cereal are purchased in the United States, averaging approximately $75.00 per household (55). With household penetration in the United States at nearly 100%, ready to eat cereal is one of the most pervasive processed food products in the market today. As with other familiar food products such as eggs, the risk for widespread *Salmonella* contamination affecting many people is great, and therefore risk must be minimized at all levels of production and distribution. Therefore, more research is necessary to understand the ability of this organism to survive storage and heat treatment procedures to better develop an effective manufacturing procedure.

This study was intended to provide insight into the ability of *Salmonella* to survive in ready-to-eat (RTE) cereal during storage, post processing. Sweetened toasted oat cereal (STOC) was used in tandem with toasted oat cereal (TOC) to elucidate the ability of *Salmonella* to survive when in presence of sucrose. Although studies have shown that this organism survives in other dry foods, cereal is a unique food matrix due to its very low water activity and intense processing conditions.

What exactly makes ready to eat cereal unique from other low water activity food matrices? In terms of nutritional characteristics, the myriad types of low water activity products such as nuts, cereals and dried dairy powders are actually very different from one another. Nuts have a water activity of about 0.5, whereas cereals, when processed

into ready to eat cereal, often have water activities lower than 0.3 (98, 99). Dairy powders have water activities even lower than cereal. Fat content is also different, as nuts range from 30-50% fat by weight, whereas cereals are usually lower than 7%. Dairy powders will range in fat and vitamin content depending on the intended use. Like cereal, many vitamins and minerals are added during processing, whereas nuts are not usually fortified.

Could there be another link, other than nutritional characteristics, between these types of foods that may predispose them to *Salmonella* contamination? Perhaps it could be processing conditions, which will vary for each product. For example, almonds and peanuts are roasted; a fairly simple process, whereas ready to eat cereal is produced via a multistep and complicated manufacturing procedure, Depending on roasting conditions, this may not be sufficient to protect nuts from *Salmonella* contamination, whereas the cereal processing conditions will surely kill any microbes initially present. Likewise, some chocolate products do not undergo a sufficient kill step, and thus contamination could be at any point during processing. Dried dairy powders, similarly, do not get a sufficient heat treatment from spray drying alone, and require a pre-pasteurization step to ensure the final product is safe. LiCari and Potter demonstrated this to be true in a 1970 study (57). That study suggested that the most common point of contamination with dairy powders, similar to cereal, is likely after processing. Given the myriad varieties of processing conditions for different low water activity products, ready to eat cereal stands out on its own as a unique matrix due to a very low water activity, intense processing conditions and shelf stability.

These products really do not have many characteristics in common that would explain their predisposition toward *Salmonella* contamination. As previously mentioned, this organism is a very resilient microorganism; therefore, it is not likely the type of food matrix that renders this organism to survive, but the organism itself. *Salmonella* spp. is hardy and adaptable to many environments and thus will be the subject of many new contaminations in dry and low a_w food products in the years to come.

Recent *Salmonella* outbreaks have caused public outrage and intense media scrutiny. Most articles raise one most pertinent question: how is this microorganism able to contaminate the food supply? Many blame the Food and Drug Administration for inadequate surveillance; however, it is the food manufacturer who ultimately needs to understand risks inherent to the foods and production atmosphere (including equipment and design of plant) used to produce the final product. A good example of a food company failing to understand risks in their production process and/or ingredients is the cereal company with recurring *Salmonella* Agona contaminations. In this case, cereals produced from the same production facility in Northfield, Minnesota were contaminated with the same microorganism in both 1998 and 2008 (84). If risks were properly understood in the first instance, perhaps the second outbreak could have been averted. Effective corrective action steps can only be executed once risk is understood, and it is ultimately these action steps that will minimize risk and reduce reoccurrence of food outbreaks.

A. <u>Why is this study unique?</u>

Despite the food outbreaks in ready to eat cereal, to date, there are no documented studies on the survival of *Salmonella* in this food matrix. In this study, it is hypothesized that the water replacement hypothesis is a mechanism that allows *Salmonella* to survive less than ideal conditions. This hypothesis has not been tested in RTE cereal, despite the common use of sucrose in this food matrix.

In addition, previous studies lack realistic inoculation procedures, by either injuring (freeze drying) or not uniformly distributing cells (hand-mixing). This study employs a novel inoculation procedure that is uniform in nature and requires the cereal to be re-dried in order to achieve a similar a_w between initial and post inoculation.

B. Drying experiments

Water activity and moisture were tested at various points to determine the length of time necessary to dry the cereal back to the original water activity (Figure 3). In general, the temperature of 40ºC was chosen because injury to cells is minimal. A 24-hour drying period was chosen instead of 22 hours for convenience, but this additional time was still sufficient to achieve the desired water activity and moisture of the original sealed product.

In practice, the water activity values measured during the experiments experimental results were approximately 0.1-0.2 greater than in the exploratory drying experiments (Figure 3). During the experimental phase, from 10 to 15 pans of wet cereal were added to the drying apparatus, whereas only 1-2 pans were dried at once in the exploratory

experiments. Therefore, the disparity between experimental and exploratory phases can be explained by a difference in residual humidity of air inside the dryer between experiments. Basically, the residual humidity inside the experimental dryer was higher than the exploratory because a greater quantity of cereal effectively increased the quantity of water evaporated into the air per unit time. Considering the physical principles of water activity and thermodynamics, a food and its surrounding environment are at their lowest energy when they are equivalent. A greater the difference between the two will cause a faster rate of water loss in the item with the higher RH (53). Conversely, if the RH between food and air are closer together (such as in the experimental phase), the water loss rate will be slower. Therefore, because a greater quantity of cereal was dried in the experimental phase, water was lost at a slower rate than during the exploratory phase because of a higher RH inside the drying apparatus, explaining the discrepancy between results. However, the slight disparity in this case may be negligible, since bacteria are not capable of growth below a water activity of 0.8.

C. Background flora

Cereal grains commonly used to produce ready-to-eat cereal, such as wheat, do not typically harbor *Salmonella*. For example, *Salmonella* in wheat has been detected in less than 1% of samples reported (33, 88). This study did not find any natural contamination in the finished product. Out of 30 plates, four were inadvertently contaminated with a single colony that was attributed to the spread plating procedure. If counted, each contaminated plate contained 100 CFU/g or less. Therefore, given that the finished

product was virtually sterile, a unique non-selective agar was employed that contained only differential ingredients.

The unique agar used in this study was a modification of tryptic soy agar (TSA) that included two differential ingredients: ferric ammonium citrate and sodium thiosulfate. These two ingredients differentiated *Salmonella* by turning colonies black. The change in color is due to the production of the desulfhydrase enzyme that converts thiosulfate ($S_2O_3^{2-}$) to hydrogen sulfide (H_2S) (102). The hydrogen sulfide then reacts with ferric ions (Fe^{3+}) to create a black precipitate. Essentially, since there are few if any contaminants in the cereal, all black colonies on this unique agar can be confidently counted as *Salmonella*.

D. <u>Production practices and their significance on contamination of RTE cereal</u>

Salmonella cross contamination due to poor sanitation practices are enhanced by its ability to survive on clean surfaces for long periods of time. For example, Kusumaningrum et al. reported that *Salmonella* Enteritidis was capable of survival on dry stainless steel surfaces for extended periods of time (52). At an initial 10^5 CFU/cm^2 inoculation, between 20 to 100% of *Salmonella* was successfully transferred to food when in contact with the contaminated stainless steel surface. At least four days passed before the contaminated stainless steel surface was again free from food contamination. Under less contaminated conditions (10^3 CFU/cm^2), *Salmonella* was recovered 24 hours after inoculation. The relevance of this information is that long after an initial event (sneezes, poor ingredient quality, cross contamination, poor hygiene of an employee,

etc.), *Salmonella* continues to be a threat to food safety. Therefore, not only is it important to determine how long an organism is capable of survival in a food or on a surface, but also how processes may be continually improved to mitigate the chance of a reoccurrence of past mistakes.

Several outbreak investigations related to RTE cereal, chocolate and infant cereal have found poor equipment sanitation, inadequate cleaning and/or separation from clean zones, and poor employee practices, among other subpar practices (8, 22, 36, 52). In some cases, foodborne disease from *Salmonella* is due to poor facility/equipment design or inadequate maintenance. For example, a 200-person outbreak of *Salmonella* Eastbourne in chocolate was caused by poor design of the manufacturing facility (22). In the outbreak investigation report, it was discovered that poorly designed valves were capable of pumping insufficiently heated chocolate into storage tanks. Conversely, infant milk was contaminated with *Salmonella* Ealing due to poor equipment maintenance. In this outbreak, the contamination was traced to a hole in a spray dryer drum, which allowed the milk powder to pass semi-freely between the exterior environment and the drum (80).

Another case of faulty processing design occurred with the aforementioned peanut butter outbreak of 2007. Although the CDC officially deemed the investigation to be inconclusive, media outlets and a company spokesperson indicated otherwise (13, 39). In 2007, the company spokesperson indicated that the outbreak was traced to a leaky roof

and faulty fire sprinklers. Therefore, the introduction of water into the processing plant proved to be ideal conditions for cell proliferation and contamination of the product.

According to Howard Zink of the FDA, *Salmonella* is a problem in the food plant because: it survives long periods of dehydration, can be spread about a food plant in areas that look clean, lives and grows inside floor cracks, will not be eliminated by cleaning and sanitizing, and is nearly impossible to find by product testing alone (due to low prevalence) (104). Therefore, a production plant must be incredibly diligent to keep surfaces clean and routine maintenance to the highest standard. In the case of *Salmonella* Agona contamination in a cereal production facility, proper mitigation procedures were not followed, and the organism returned to re-contaminate their product.

E. Experimental culture results

This study is the first to demonstrate the ability of *Salmonella* to survive in dry, ready-to-eat cereal for at least 90 days, or three months. Given that the slope of each serovar over time (after drying) was essentially zero, *Salmonella* will likely survive in cereal for a period longer than 90 days (Figure 9). This study was not extended for a longer period because an average person will have consumed an entire box of cereal within a 90 day period, given that average U.S. consumption of cereal is about 7 boxes of cereal per person per year (assuming each box is 396 g and each serving size is 1 cup or 28 g) (55). In other words, an average U.S. consumer will eat approximately 0.6 boxes of cereal per month, or more depending on the size of the household. Based on those estimates, an average sized cereal box will be consumed within two months. Therefore,

despite the prediction that *Salmonella* will survive in RTE cereal longer than 90 days, consumers typically would have already eaten an entire box of cereal by then according to the aforementioned statistic. Therefore, 90 days was a sufficient period of time to mimic the average route of cereal, assuming that the consumer purchases the cereal within two months after production.

From the inoculation numbers prior to drying, it is clear that the method used was uniform as expected, although average survival over time was not. Unlike in a previous study testing the water replacement hypothesis in squid chips (49), this study did not find a statistical correlation between sucrose and increased survival in cereal, as the slope over time was statistically related to serovar, and not cereal type (Table 7). The addition of sucrose in STOC, had a marked effect in preventing growth during drying and it appeared to cause a slight reduction in counts during storage. However, other factors beyond sucrose may have impacted survival.

Average count of *Salmonella* in cereal was statistically dependant on cereal type, and to a certain extent serovar (although serovar was still dependant on cereal type) (Table 6). This is easily observed from the time dependant graphs (Figure 4-8) where STOC had much lower CFU/g than TOC, which surprisingly increased in count during the drying period. Given that bacteria are not known to grow below a water activity of 0.8, *Salmonella* were likely able to grow during the first few hours of drying while the water activity was still sufficient to support growth. However, even in this case, the sweetened cereal did not show similar results, contradicting this theory.

Although this study attempted to test the water replacement hypothesis in cereal, the experimental approach did not purposely address this theory, and was tailored more toward evaluating survival of *Salmonella.* Therefore, other factors could be at play that may have influenced the results, such as added salt in the cereal formulation.

One possible explanation for the increase in TOC count is based on the textural differences between the cereal types. The porous texture of TOC is achieved by quickly flashing water off of the dough by exposure to extremely high temperature and pressure conditions. The result is a crispy, porous textured cereal grain. STOC differs from TOC because it has a sucrose layer enrobing the cereal piece. One way to think about the difference is that TOC is like a chocolate covered donut. If one drop of water were transferred to the donut, the drop would likely stay immobilized, forming a droplet on top of the chocolate. However, if a drop were added to a cake donut instead, without frosting, the water would adsorb into the donut. This is a similar situation to what most likely occurred in the cereal inoculation. In other words, the TOC is like a sponge, whereas the STOC has a protective sugar layer, thereby protecting the cereal piece. The theory is that *Salmonella* was able to internalize in the TOC and colonize throughout the cereal piece. On the contrary, the inoculation solution on STOC primarily stayed on the surface of the cereal piece and did not internalize as in the TOC. This will not be evident from the a_w measurement as it is an average measure and not representative of microenvironments within a given food matrix (explained in detail below). Therefore, because of the potential more complete creation of a microenvironment within the TOC

cereal piece (at a higher a_w than the surface), the interior likely took longer to fully dehydrate, providing more opportunity for growth of *Salmonella*. The difference in physical structure of these two types of food likely contributed toward the discrepancy seen between the two types of cereal. The statistical analysis also supports this theory, as cereal type was most responsible, based on 95% confidence, for average *Salmonella* count.

Several studies support this hypothesis. Two related studies by Hills, et al. examined survival response of *Salmonella* in porous environments. Both studies found that microstructure, or porous environments, can have a marked effect on survival and growth of microorganisms (47, 48). The 1997 study found that the air-water distribution measured by NMR is a more effective means to predict survival than water activity in microporous food matrices. The reason for this is because water activity is an average measurement of the energy state of water, whereas nuclear magnetic resonance (NMR) will detect the mobility of water within a food system, and thus detect microenvironments within a porous material. In other words, in a porous food matrix such as cereal, water activity may have not been the best means to predict microbial survival and growth for the inoculation procedure.

Maillard reaction products generated during the drying process could be another possible explanation of the lower survival of STOC as compared to TOC. The Maillard reaction, or the nonenzymatic browning reaction, is a reaction between amino groups and reducing compounds. STOC contains several ingredients that increase the percent of

reducing sugars as compared to TOC. The most prevalent ingredient containing reducing sugars is honey, which has high concentrations of fructose and glucose (approximately 70%) (99). Although the Maillard reaction products could have some influence, it should be noted that the low drying temperature might have decreased the variety and quantity of Maillard products, thus limiting their effectiveness in promoting survival of *Salmonella.* The effects of Maillard reaction products, most notably melanoidin, on pathogenic bacteria have been investigated.

Einarsson et al. reported that histidine-glucose mixtures had the greatest inhibitory effect on bacterial growth of *Salmonella* and other microorganisms (35). The mechanism by which Maillard products inhibit growth of bacteria depend on the different substances formed during the reaction. For instance, the Maillard reaction product hydroxymethylfurfural (HMF) is known to have an inhibitory effect on the growth of pathogens. Two studies reported that melandoins (MEL) and aminoreductones (AR), both Maillard reaction byproducts, might also have inhibitory effects against pathogenic bacteria (81, 93). Rufian-Henares and Morales recently suggested that melandoins antimicrobial activity may be related to their ability to chelate magnesium and iron, both essential nutrients for growth of pathogenic bacteria (82). Given the robust research on Maillard reaction products inhibitory effect on pathogenic bacteria, it is possible that this had an effect on the STOC in this study. In specific, STOC contains all of the elements necessary for Maillard reaction products to form during the drying step: oat cereal contains histidine (among other amino acids), and STOC contains added glucose (in

quantities exceeding that of TOC). Therefore, it is possible that HMF, AR, and/or MEL were produced during drying that functioned to inhibit growth, especially in STOC.

Regardless of the differences in survival after drying, it is clear that *Salmonella* is capable of survival for periods up to 90 days after inoculation based on the results of this study. In future experiments, an alternative method could be employed to measure moisture by using NMR (in tandem with water activity and moisture testing) to measure microenvironments in the cereal to determine a proper inoculation procedure and heat treatment in order to minimize opportunity for growth. Additionally, it would be useful to detect the different Maillard products after drying and determine whether these have an inhibitory effect on microorganisms. Additionally, more research should be conducted to determine the thermal inactivation of *Salmonella* in cereal to encourage cereal manufacturers to employ a secondary kill step after completion of processing (additional steps are usually taken after the kill steps of cooking and extrusion, such as vitamin enrichment, for example) to mitigate the risk of *Salmonella* contamination in cereal.

Conclusion

This study scrutinized the survival of five *Salmonella* serovars in sweetened and unsweetened ready to eat toasted oat cereals. To date, this is the first study to report survival of *Salmonella* in ready to eat cereal. The results of the microbial analysis reveal that *Salmonella* is capable of survival in cereal for at least 90 days. Based on the slope data in this study, it is predicted that this organism is capable of surviving for a longer time period than 90 days.

The results in this study appeared to contradict the water replacement hypothesis. Potential explanations for this discrepancy could be porosity differences between cereals or Maillard reaction effects. The relevance of this to the cereal industry is that an additional kill step should be added to the processing flow after the cooking/extrusion step. Without a secondary kill step, manufacturers allow significant risk of contamination to remain in their product process.

More extensive testing is warranted, involving more *Salmonella* serovars and different inoculation procedures. Although conditions similar to the inoculation seen in this procedure are possible in a production plant, other procedures that do not require wetting the cereal should also be tested to verify the data seen in this study. Additionally, experiments should specifically be designed to test the water replacement hypothesis in these cereal matrices to determine the role of sucrose in extending survival in cereal, as it is a very common ingredient in the RTE cereal industry.

Further Studies

An epidemic of *Salmonella* outbreaks in low a_w products during the last ten years begs the question: why? Understanding this, as well as the most at risk ingredients and products will help to mitigate potential incidents. In this study, the ability of *Salmonella* to survive in ready to eat cereal was verified for at least 90 days.

The data in this study may serve as a springboard for other studies to follow to elucidate the ability of *Salmonella* to survive in cereal. Specifically, thermal inactivation of *Salmonella* in ready to eat cereal should be studied to determine the time and temperature conditions necessary to inactivate *Salmonella.* This data will encourage manufacturers to add a secondary kill step in their processing flow. Additionally, knowing the minimum conditions necessary for inactivation will minimize sensory changes to the product and prospectively yield an acceptable, yet safer, product.

In order to substantiate this research, future research should attempt a variety of inoculation procedures to determine efficacy. The best scenario would be to limit the wetting of the cereal in the inoculation so the drying step could be conducted more quickly. This may mitigate the time that *Salmonella* is potentially able to grow. Additionally, a longer testing period may elucidate more about the true survival time of *Salmonella* in RTE cereal, although most would be consumed by the three-month time period established in this study.

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Appendix

Data Used for Figures

Figure 2:

Sample	Time (hrs)	% Moisture	Avg % Moisture	a_w	Avg a_w
TOC	0	51.4	51.7	0.98	**0.98**
		52.1		0.98	
		51.6		0.98	
TOC	4	21.9	21.7	0.89	**0.88**
		21.6		0.88	
		21.6		0.88	
TOC	5	13.8	13.9	0.76	**0.76**
		14.0		0.75	
		13.9		0.76	
TOC	6	11.3	11.4	0.67	**0.68**
		11.4		0.68	
		11.4		0.68	
TOC	7	6.0	6.1	0.24	**0.25**
		6.1		0.25	
		6.1		0.25	
TOC	21	4.4	4.2	0.12	**0.12**
		4.3		0.13	
		4.0		0.12	
TOC	22	4.0	4.0	0.13	**0.13**
		4.0		0.13	
		4.1		0.14	

Sample	Time (hrs)	% Moisture	Avg. % Moisture	a_w	Avg a_w
STOC	0	51.4	51.7	0.98	**0.98**
		52.1		0.98	
		51.6		0.98	
STOC	4	23.9	23.8	0.86	**0.86**
		23.8		0.86	
		23.8		0.86	
STOC	5	20.7	20.1	0.81	**0.81**
		19.8		0.81	
		19.8		0.81	
STOC	6	16.6	16.0	0.74	**0.74**

		15.4		0.74	
		16.1		0.74	
STOC	7	10.9	10.8	0.54	**0.57**
		10.8		0.59	
		10.7		0.59	
STOC	21	4.8	4.9	0.18	**0.19**
		5.0		0.18	
		4.8		0.19	
STOC	22	5.0	5.1	0.19	**0.19**
		5.0		0.20	
		5.4		0.20	

Figure 3:

Time (day)	TOC a_a	Avg. TOC a_w	STOC a_a	Avg. STOC a_w
1	0.317	0.3075	0.347	0.3315
	0.298		0.316	
3	0.353	0.3735	0.416	0.425
	0.394		0.434	
5	0.361	0.3595	0.414	0.4115
	0.358		0.409	
7	0.372	0.37	0.409	0.4125
	0.368		0.416	
14	0.394	0.39	0.434	0.4275
	0.386		0.421	
30	0.403	0.389	0.412	0.423
	0.375		0.434	
60	0.418	0.431	0.417	0.4215
	0.444		0.426	
90	0.415	0.4115	0.445	0.443
	0.408		0.441	

Figures 4-8:

***S.* Agona**				
Time (days)	**STOC (1) (CFU/g)**	**STOC (2) (CFU/g)**	**TOC (1) (CFU/g)**	**TOC (2) (CFU/g)**
1	3.4×10^4	1.2×10^5	3.1×10^7	1.6×10^7
3	3.9×10^4	2.2×10^4		7.5×10^6
5	6.9×10^4	5.1×10^4	1.4×10^7	1.3×10^7
7	5.4×10^4	2.5×10^4	1.3×10^7	9.9×10^6
14	1.7×10^4	1.8×10^4	1.1×10^7	3.6×10^6
30	1.7×10^4		2.6×10^7	1.0×10^6
60	1.5×10^4	1.6×10^4	9.5×10^7	
90	1.5×10^4	1.5×10^4	8.7×10^7	1.4×10^7
***S.* Senftenberg**				
Time (days)	**STOC (1) (CFU/g)**	**STOC (2) (CFU/g)**	**TOC (1) (CFU/g)**	**TOC (2) (CFU/g)**
1	1.2×10^4	2.4×10^6	2.6×10^5	5.4×10^5
3	2.6×10^5	3.2×10^4	2.2×10^4	1.4×10^5
5	5.2×10^6	1.7×10^4	5.8×10^4	1.8×10^5
7	1.8×10^6		2.8×10^4	2.2×10^5
14	5.4×10^5		2.4×10^4	9.3×10^4
30	9.1×10^5	3.1×10^4	2.5×10^4	3.0×10^4
60	5.3×10^6		2.7×10^4	9.3×10^4
90	1.0×10^5	1.1×10^6	7.2×10^4	5.2×10^5
***S.* Typhimurium (ATCC)**				
Time (days)	**STOC (1) (CFU/g)**	**STOC (2) (CFU/g)**	**TOC (1) (CFU/g)**	**TOC (2) (CFU/g)**
1	7.5×10^3	2.4×10^5	2.3×10^7	
3	9.8×10^3	2.2×10^4		
5	4.5×10^4	3.1×10^4	6.6×10^7	
7	2.5×10^4	2.2×10^4	6.3×10^7	1.1×10^8
14	5.8×10^3	2.2×10^5	1.2×10^8	4.9×10^8
30	3.3×10^3	3.9×10^5	5.0×10^7	2.5×10^8
60	4.0×10^3	7.1×10^4	6.1×10^7	8.2×10^8
90	3.1×10^3	5.4×10^3	1.8×10^8	8.4×10^8
***S.*Typhimurium (MDH)**				
Time (days)	**STOC (1) (CFU/g)**	**STOC (2) (CFU/g)**	**TOC (1) (CFU/g)**	**TOC (2) (CFU/g)**
1	2.5×10^4	1.4×10^5		
3	2.7×10^5			
5			3.9×10^9	1.9×10^9
7	2.7×10^4	1.4×10^4	2.0×10^9	4.7×10^9
14	1.5×10^4	1.9×10^5	2.7×10^9	5.5×10^8
30	4.5×10^3	1.1×10^4	1.6×10^8	3.3×10^8
60	4.0×10^3	7.4×10^2	4.1×10^8	2.0×10^8
90	3.9×10^3		1.6×10^8	3.4×10^8
***S.* Tennessee**				
Time (days)	**STOC (1) (CFU/g)**	**STOC (2) (CFU/g)**	**TOC (1) (CFU/g)**	**TOC (2) (CFU/g)**
1	1.3×10^4			

3		4.6×10^5		1.7×10^9
5	1.2×10^4	1.5×10^7	2.4×10^9	3.8×10^9
7	5.8×10^4	3.2×10^6	4.1×10^9	3.4×10^9
14			3.8×10^9	4.6×10^9
30	2.2×10^4	4.4×10^6	2.2×10^8	1.6×10^9
60	2.5×10^4	5.8×10^6	2.9×10^8	2.0×10^8
90	1.3×10^5		3.4×10^8	7.9×10^8

Figure 9:

Serovar	Type	Slope
Agona	TOC	0.0098
	TOC	0.0002
	STOC	-0.0060
	STOC	-0.0056
Senftenberg	TOC	-0.0003
	TOC	0.0012
	STOC	0.0008
	STOC	0.0101
Typhimurium (ATCC)	TOC	0.0052
	TOC	0.0085
	STOC	-0.0085
	STOC	-0.0080
Typhimurium (MDH)	TOC	-0.0148
	TOC	-0.0109
	STOC	-0.0144
	STOC	-0.0357
Tennessee	TOC	-0.0133
	TOC	-0.0106
	STOC	0.0077
	STOC	0.0065