

A PREDICTIVE MODEL FOR THE INACTIVATION OF *SALMONELLA* IN DRY FOODS

By

LISA MICHELLE TRIMBLE

(Under the Direction of JOSEPH F. FRANK)

ABSTRACT

Low water activity (a_w) foods and ingredients such as milk powder, almond butter, spices, and cake mix are naturally dry or have been dried through processing to $a_w \leq 0.85$. These products typically have a long shelf life, do not require a lethality step and do not require heating before consumption. *Salmonella* will not grow in such foods, but can survive for extended periods of time if introduced into the food or the process. Little is known about the influence of fat content on *Salmonella* survival in low a_w foods. The aim of this study was to determine the influence of fat content on the survival of *Salmonella* in dry foods and use this information to modify an existing predictive model so that it is valid for low and high fat foods. Survival data was obtained in whey protein powder supplemented with peanut oil which was equilibrated to various water activities below 0.60 and held at 21-80°C for 0-168 days. The Weibull model was selected to describe the data and a previously developed secondary model was modified to include foods with a fat content $\leq 50\%$ (w/w) based on the influence of temperature, a_w and fat content on survival. Predictions were validated in 4 foods within the range of the modeled data. The model was useful in predicting survival in low and high fat dry foods with a prediction performance of 66% and improved % bias and % accuracy compared to the unmodified model. Fat content (20% and 50% w/w) provided a protective effect toward survival at temperatures

$\geq 50^{\circ}\text{C}$ and additional fat content did not increase this effect. The presence of an emulsifier did not influence survival. Fat and sugar content did not influence the relative survival of 3 out of 4 serovars during storage. *S. Tennessee* was the most prevalent serovar and *S. Typhimurium* was not detected after 6 months of storage at 37°C . The revised model can be used as a quantitative support tool in risk mitigation strategies for low a_w foods containing fat.

INDEX WORDS: inactivation; fat; dry food; serotype; Weibull model; peanut flour;
Salmonella; predictive model; monoglyceride; water activity

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By

LISA MICHELLE TRIMBLE

B.S., Biology, University of West Georgia, 2003

M.S., Food Science, University of Georgia, 2012

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LISA MICHELLE TRIMBLE

Major Professor:	JOSEPH F. FRANK
Committee:	DONALD W. SCHAFFNER
	MARY ALICE SMITH
	XIANG-YU DENG

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2015

DEDICATION

To my parents and my grandparents

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CHAPTER 1

INTRODUCTION

Salmonella spp. are gram-negative, non-spore-forming, facultative anaerobes which belong to the family Enterobacteriaceae. They originate in the intestinal tracts of birds and mammals and may also colonize reptiles and insects. *Salmonella* spp. are widely distributed in the natural environment and are often reintroduced into the food chain through intensive husbandry practices (D'Aoust and Maurer, 2007).

Salmonellosis is one of the most common foodborne illnesses worldwide, with millions of cases occurring each year. The CDC Foodborne Diseases Active Surveillance Network (FoodNet) was established in 1996 for surveillance of foodborne illness cases and includes monitoring the incidence of salmonellosis (CDC, 2015). An estimated one million cases of foodborne salmonellosis occur in the United States annually (Scallan et al., 2011). Outbreak investigations have reported that ingestion of only a few cells can result in an infection (D'Aoust et al., 1975; D'Aoust and Maurer, 2007) and outcomes can range from gastroenteritis to death. Since 2007, 18 outbreaks of *Salmonella* infection have occurred in low water activity (a_w) foods such as peanut butter, sesame paste tahini, peanut paste, dry pet food and cereal (CDC, 2015). These outbreaks resulted in 1,691 illnesses, 498 hospitalizations and 10 deaths in the United States. One of the most widespread and devastating outbreaks occurred in peanut butter and peanut paste in 2009 and resulted in 714 illnesses and 9 deaths (CDC, 2015)

Although *Salmonella* cannot grow in dry foods, survival data demonstrate that certain serovars can persist in dry foods for long periods of time, even when subject to elevated temperatures

(Santillana Farakos, et al., 2013). The ability of certain serotypes to withstand uninhabitable conditions and subsequently cause foodborne illness can be attributed to their resilience and their ability to adapt to various stresses. The persistence of *Salmonella* depends on a variety of factors such as the components of the food matrix, the serotype, and environmental conditions such as temperature and a_w (Santillana Farakos et al., 2014). While it is well known that the resistance of *Salmonella* increases with decreasing a_w (Aljarallah, et al., 2007; Beuchat et al., 2013), the influence of certain food components and their interactions with a_w remain unclear (Goepfert et al., 1970; Mattick et al., 2001). Fat content has demonstrated a protective effect on *Salmonella* survival in peanut butter (Shachar and Yaron, 2006) and in an in-vitro digestive system (Aviles, et al., 2013).

In a previous investigation by our research group (Santillana Farakos et al., 2013), the influence of temperature, a_w and water mobility on *Salmonella* survival in dry foods was quantified by survival studies in a low a_w whey protein isolate (WPI) model food system. The Weibull model was the best of several models studied for describing the inactivation data and secondary model equations were developed and validated for survival in low a_w foods ($a_w < 0.60$) held at 21-80°C. While the performance of the secondary model was useful in predicting survival, the predictions were less reliable in peanut flour containing a low (12%) amount of fat.

This dissertation expanded upon the previous investigation (Santillana Farakos, et al., 2013) by examining *Salmonella* survival in a WPI model food system with added fat content. In light of research which suggests a protective effect of fat on survival, the usefulness of fat content as a predictor of *Salmonella* survival in dry foods has not been established. The objectives of this research were to 1) Determine the influence of temperature, a_w , and fat content on *Salmonella* inactivation in a dry WPI model food system held at processing temperatures applicable to dry

foods. 2) Use the survival data to expand the existing secondary predictive model so that predictions are valid for low fat and high fat foods. 3) Evaluate the performance of the Santillana Farakos et al., 2013 model in a low a_w , high fat peanut flour model food system containing an emulsifier. 4) Determine the influence of fat and sugar content on the relative survival of outbreak-associated serovars of *Salmonella* in low a_w WPI held at 36°C for 6 months. The results from these experiments will improve understanding of *Salmonella* inactivation kinetics in complex low a_w food matrices containing high amounts of fat and will aid in risk mitigation strategies for these foods.

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CHAPTER 2

LITERATURE REVIEW

General characteristics of *Salmonella* spp.

Enterobacteriaceae is a family of enteric bacteria which includes *Escherichia coli*, *Shigella* spp. and *Salmonella* spp. The genus *Salmonella* is divided into 2 species; *S. enterica* and *S. bongori* which together contain approximately 2500 serotypes. *S. enterica* is comprised of 6 subspecies which are characterized by genomic and biochemical traits. The majority of the serotypes (1454) are classified as *S. enterica* subspecies enterica (I) which typically colonize warm blooded animals (Brenner et al., 2000). Although *Salmonella* serotypes are genetically similar, only a fraction of the 2500 serotypes are capable of causing infection in humans. The severity of disease also differs among serotypes (Jones et al., 2008).

Salmonella spp. are gram-negative, rod-shaped, non-spore forming, facultative anaerobic bacteria (FDA, 2012). Part of their versatility is that they are chemoorganotrophs and are capable of metabolizing nutrients through fermentation and respiration. Salmonellae are able to grow over a wide range of temperatures from 5°C to 45°C (Marth, 1969). Optimal growth conditions for *Salmonella* spp. are at 37°C, water activity (a_w) > 0.93 and pH between 6.5 and 7.5 (DAoust and Maurer 2007).

Vehicles of infection include ingestion of contaminated foods of animal or plant origin, contact with sewage-contaminated water, and person to person transmission via the fecal-oral route (FDA, 2012) Cases of airborne transmission (Datta and Pridie, 1960) and potential secondary surface-to-surface transmission within the toilet and bathroom have also been documented

(Barker and Bloomfield, 2000; Johnson et al., 2013). Common food reservoirs of *Salmonella* spp. include poultry meat, eggs, aquaculture fish or shellfish, fresh fruits and vegetables, milk, and cheese (D'Aoust and Maurer 2007).

Diarrhea, abdominal cramps and fever typically occur between 12 to 72 hours after ingestion. In most cases salmonellosis is self-limiting and lasts 4 to 7 days (CDC, 2015). If dehydration occurs from severe diarrhea, hospitalization may be required. The spread of the infection from the intestines to the bloodstream and the major organs requires immediate treatment with antibiotics. Clinical manifestations of infection depend on the *Salmonella* serotype ingested (FDA, 2012). Non-typhoidal Salmonellosis is caused by serotypes other than *S. Typhi* and *S. Paratyphi A* and results in acute gastroenteritis. Complications of this type of infection may include septicemia, dehydration and reactive arthritis. Typhoidal salmonellosis is caused by *S. Typhi* and *S. Paratyphi A* and results in Typhoid Fever. Complications include septicemia, septic arthritis and chronic gallbladder infection.

Pathogenesis of *Salmonella*

After ingestion, *Salmonella* must clear several hurdles in the form of nonspecific host defenses before colonizing the intestinal mucosa. The natural antibacterial compound in saliva (lactoperoxidase), gastric acidity (pH=1.5-3.5), and intestinal peristalsis may prevent invasion (D'Aoust and Maurer, 2007). Once *Salmonella* colonizes the ileum and large intestine it binds to receptors on the epithelial cell surface. Pinocytosis is initiated when the organism induces ruffling of the enterocyte membrane. Proliferation occurs within the epithelial columnar cells of the intestine followed by spread to the lymph nodes and systemic circulation. Depending on the serotype and the host defenses, the infection is confined to the intestines or it may spread to the bloodstream and other organs. When the intestinal mucosa is invaded, an acute inflammatory

response is induced which causes the release of proinflammatory cytokines. This inflammatory reaction is the cause of symptoms such as chills, abdominal pain, fever and diarrhea. Activation of mucosal adenylate cyclase results in an increase in cyclic AMP, which induces the secretion of fluid and electrolytes into the intestinal lumen. Diarrhea is the result of this fluid production (Gianella, 1996).

Public Health burden

The global public health burden of gastroenteritis caused by *Salmonella* spp. has remained steady with an estimated 80.3 million foodborne cases occurring annually (Majowicz, et al. 2010).

National surveillance of *Salmonella* infections in the United States was established by the Centers for Disease Control and Prevention (CDC) in 1962. In 1996, the Foodborne Diseases Active Surveillance Network (FoodNet) began active, population-based surveillance of laboratory-confirmed infections of 9 foodborne pathogens (including *Salmonella*) in 10 state health departments. According to the 2012 FoodNet Surveillance Report, 457 *Salmonella* outbreak-associated cases were reported with 337 cases attributed to a food sources (CDC, 2014). An investigation based on data from 403 single source foodborne illness outbreaks reported distinct associations between food commodities and *Salmonella* serotypes. For example, *S. Enteritidis* or *S. Heidelberg* caused 91% of egg-associated outbreaks and 50% of leafy vegetable-associated outbreaks were caused by *S. Newport* or *S. Javiana* (Jackson, et al., 2013).

The possibility of a low infective dose makes *Salmonella* contamination in dry foods even more of a public health concern, particularly for high risk populations. A review of volunteer studies from 1951 suggested that ingestion of approximately 10^5 - 10^{10} cells of *Salmonella* are required for infection (Blaser & Newman, 1982). Conversely, an estimated 2-9 salmonellae per Christmas-wrapped chocolate ball were said to have caused an outbreak of *Salmonella* Eastborne

in the United States and Canada in 1974 (D'Aoust et al., 1975). Furthermore, the infective dose in contaminated paprika used in paprika- powdered potato chips was estimated as 4-45 organisms per 100 g of potato chips (Lemacher, et al., 1995). This discrepancy is likely attributed to differences in host susceptibility, the virulence of the infecting strain, and the composition of the food vehicle of infection (D'Aoust and Maurer, 2007). Children, the elderly, and immunocompromised individuals are the most susceptible to severe infections. In 2012, the highest incidence of FoodNet *Salmonella* infections (rate per 100,000 population) were in children aged <5 years (64.88) followed by age 5-9 years (19.29), age 70-79 years (18.38) and 80+ years (16.08) (CDC, 2014).

Adaptive capacity and resilience of *Salmonella*

Salmonella has demonstrated the ability to adapt to various stresses and survive for extended periods of time. *S. Typhimurium* survived in minced chicken held for 48 h at 2°C (Baker et al., 1986) and selected heat stress adapted mutants of *S. Typhimurium* grew at 54°C (Droffner and Yamamoto, 1992). Acid-adapted *Salmonella* demonstrated increased survival in fermented milk and cheese held at 5°C (Leyer and Johnson, 1993). Outbreak strains of *S. Tennessee* were shown to be more thermotolerant than *Salmonella* strains of other serotypes and non-outbreak clinical isolates of *S. Tennessee* in peanut butter heated to 71-90°C (Ma, et al, 2009). Similarly, outbreak strains of *S. Oranienburg* and *S. Enteritidis* were consistently the most resistant compared to 5 additional strains inoculated on to crushed cocoa beans held at 100°C or 102°C (Izurieta and Komitopoulou 2012).

Many strategies used to control *Salmonella* in food processing operations center on the sensitivity of the organisms to heat and acidic environments (FDA, 2009b). High salt concentrations are often used to hinder the growth of microorganisms by reduction of water

activity and therefore extending the shelf life of the food. However, low- a_w ($a_w \leq 0.60$) whey protein powder supplemented with 17% (w/w) NaCl showed no significant difference in the survival of *Salmonella* compared to unsupplemented powders held at 70°C and 80°C (Santillana Farakos, et al., 2014).

Mechanisms of survival may include filamentation (Mattick et al., 2000; Kieboom et al., 2006) biofilm formation, attachment to cellular or non-cellular surfaces, entry into a Viable but Nonculturable (VNBC) state (GMA, 2009; Podolak et al., 2010) and the activation of stress response genes. Transcriptome analysis of *S. Enteritidis* cells subjected to desiccation and starvation stress in a peanut oil matrix showed that the cells were in a physiologically dormant state and the only genes actively transcribed were those involved in regulatory, protective and heat/cold shock responses (Deng et al., 2012). Sublethal heat stress is known to induce the synthesis of heat shock proteins, which render the microbial cell resistant to a second challenge of potentially-lethal heat stress. The role of heat shock proteins is thought to be to assist cells in returning to their normal physiological state after the stress has subsided. As a result of the adaption to heat stress, the cells are cross-protected and their resistance to other treatments is also increased (Juneja and Novak, 2003).

***Salmonella* survival in dry foods**

A further illustration of the adaptive capacity of *Salmonella* spp. is the capability of certain serotypes to survive for long periods of time in low water activity (a_w) foods and food production environments. For example, *S. Agona* was documented as the cause of 2 outbreaks in toasted oat cereal (1998) and puffed rice cereal (2008) produced in the same facility (Russo, et al., 2013). After the 1988 outbreak, extensive efforts to decontaminate and remodel the production facility were performed, and production in the contaminated area was discontinued. In 2008, 21 isolates

of an identical strain of the 1988 outbreak strain of *S. Agona* were isolated from the puffed rice cereal and ill patients. In the months prior to the 2008 outbreak, facility maintenance required destruction of a wall adjacent to the production area where the contaminated toasted oat cereal was produced a decade earlier. It is hypothesized that disruption of the wall combined with wet cleaning reintroduced the desiccated 1998 outbreak strain of *S. Agona* into the cereal processing area (Russo, et al. 2013).

Water activity is defined as the partial vapor pressure of water in the food divided by the partial vapor pressure of pure water under the same temperature and pressure conditions (Labuza, 1968). The amount of available water in a food is a major factor in food stability, limiting and controlling microbial growth, determining the type of microorganisms present in foods, and product quality (Tapia et al., 2007). The water activity of a food can be decreased by freezing, heating and the addition of humectants and solutes like salt and sugar. Low water activity conditions create an osmotic pressure difference between the food and the hydrated microbial cell that disturbs the microorganism's internal equilibrium. Water migrates from the cytoplasm, membrane turgor pressure decreases, and the organism remains in the lag phase until homeostasis is restored (Ravishankar and Juneja, 2003). This osmotic pressure difference will inhibit the growth of toxigenic bacteria such as *Staphylococcus aureus* which requires $a_w > 0.85$ to grow, but it will not eliminate the risk of pathogenic bacteria such as *Cronobacter* and *Salmonella* (Tapia et al. 2007).

Low a_w ($a_w \leq 0.85$) foods and ingredients are those that are naturally dry or are dried through processing steps (Beuchat et al., 2013). Such foods include protein powder, nut butters, animal feed, spices, powdered infant formula, and chocolate. In many low a_w foods, a lethality process

is not required or the food has a low a_w ingredient that is introduced after the lethality step. Dry foods typically have an extended shelf-life and are not heated before consumption.

The risk of *Salmonella* contamination in dry foods and the public health consequences associated with this risk have become more widely recognized in recent years. The implementation of active surveillance of foodborne illness by FoodNet combined with advanced methods of detection have resulted in increased recalls of these products. Due to the extended shelf life of dry foods, contamination with *Salmonella* is likely to result in epidemic curves which depict dispersed illnesses and a range of dates that span for months or years. Epidemic curves illustrate the progression of an outbreak over time by plotting the date when a person became ill against the number of persons who became ill on that date (CDC, 2015). During an active outbreak, interpretation of the curves may be difficult because the curves are continuously updated as new data becomes available.

Pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* have been associated with or implicated in outbreaks in low a_w foods (Beuchat et al., 2013).

Cronobacter spp. are of particular concern in because they are widespread in dry processing environments and are more resistant to drying than other members of the Enterobacteriaceae family (Breeuwer, 2014). Outbreaks of *Cronobacter* infection are rare but result in severe illnesses in infants and some immuno-compromised adults (Gurtler et al., 2014).

On the other hand, outbreaks of *Salmonella* infection attributed to low a_w foods or ingredients have been recorded for decades. In 1953, desiccated coconut was implicated in a small outbreak in Australia (Wilson et al., 1955). Cocoa was the vehicle of infection for *S. Durham* in confectionary items sold in Sweden in 1970 (Gästrin et al., 1972). Thirty-three people in the United States and Canada became ill from consuming imported Belgian chocolate contaminated

with *S. Nima* in 1985 (Beuchat et al., 2013). A 1993 outbreak of *S. Saintpaul*, *S. Rubislaw*, and *S. Javiana* in paprika-powdered potato chips resulted in an estimated 1000 illnesses in Germany (Lehmacher et al., 1995). *S. Wandsworth* was implicated as the cause of 65 illnesses from consumption of a puffed rice snack with a vegetable coating in 2007 (CDC, 2011). More recently, *Salmonella* contamination was the cause of 28 FDA recalls of low a_w foods or ingredients in 2013 (FDA, 2013) and 44 recalls in 2014 (FDA, 2014).

Factors affecting survival in dry foods

The extent of *Salmonella* survival in low a_w foods depends on several factors including the thermal resistance of the cells, the history and physiological state of the cells, serotype, temperature, water activity and interactions with the components of the food matrix (Doyle and Mazzotta, 2000; Podolak, et al., 2010; Santillana Farakos, et al., 2013, 2014)

Sublethal temperatures used in processing of low a_w foods may contribute the survival and virulence of *Salmonella* in these foods. Resistance is highly influenced by cell recovery conditions, the strain tested, the culture conditions and history of the cells before the experiment and the surrounding solutes during heating (Doyle and Mazzotta, 2000).

The varying thermotolerance of *Salmonella* serotypes was demonstrated in the thermal reduction times of 8 *Salmonella* serotypes in dry corn flour (15% moisture) held at 49°C. *S. Tennessee* required 6.7 h for a 99% reduction, whereas *S. Newington* required 0.3 h (VanCauwenberge, et al., 1981). Furthermore, increased thermal resistance was observed for *S. Tennessee* when compared to *S. Agona*, *S. Montevideo* and *S. Typhimurium* in low a_w whey protein powder when the holding temperature was increased from 36°C to 70°C (Santillana Farakos et al., 2014). Greater thermal resistance was observed for *S. Typhimurium* in milk chocolate held in dry heat conditions at 70°C, 80°C and 90°C when compared to samples containing *S. Seftenberg*.

(Goepfert and Biggie, 1968). An analysis of literature data concerning *Salmonella* inactivation in dry foods showed a significant influence of serotype on the treatment time required for the first decimal log reduction (Weibull model parameter δ) (Santillana Farakos et al., 2014). By contrast, enhanced thermal resistance was not found to be serotype specific in peanut butter held at 71-90°C (Ma et al., 2009; Shachar and Yaron, 2006).

The history of the cells had a strong influence on survival in *Salmonella*-contaminated peanut butter treated at 80°C for 30 min. A second treatment at 70, 80 or 90°C resulted in greater thermotolerance of the cells, with less than a 2 log reduction at 90°C (Shachar and Yaron, 2006). Previously dried *Salmonella* inoculum resulted in decreased inactivation in chocolate and peanut butter-based crème filled crackers compared to wet-inoculated crackers (Beuchat and Mann, 2015). The thermal resistance of *S. Typhimurium* in concentrated milk was greater for cells grown at 43°C compared to cells grown at 22°C (Degas et al., 1972). The thermotolerance of exponential phase *Bacillus cereus* strains held at 50°C were enhanced after pre-exposure to non-lethal salt conditions. However, the adaptive salt stress response was less pronounced transition- and stationary phase cells (den Besten, et al., 2006).

Salmonella survival is influenced by the local microenvironments created by specific ingredients in dry, multi-ingredient foods. In other words, the properties of a specific ingredient may cause the formed environment in close proximity to the bacterial cell to demonstrate water activities and nutrient availability that differ from the bulk properties of the food. This heterogeneous nature of the microenvironment may impact pathogen survival in low- a_w foods (Li, et al., 2014). Along the same line, the influence of food components on bacterial inactivation in low a_w foods have been investigated. *Salmonella* survival in low a_w chicken base powder ($a_w < 0.30$) with adjusted salt contents (34%, 18.5%, 3.1% w/w) held at 21°C for 42 weeks was compared to

survival in intermediate moisture ($a_w \geq 0.68$) chicken base pastes with similar salt concentrations. Survival was unaffected by salt content independent of a_w , but was enhanced by $a_w < 0.30$ relative to the intermediate moisture paste (Shrestha and Nummer, 2016). Similarly, survival increased with decreasing a_w in whey protein powder held at 70°C and 80°C, but was unaffected by added 8% and 17% NaCl (w/w) (Santillana Farakos et al., 2014). Sucrose has demonstrated various levels of heat protection for *Salmonella* inactivation in reduced a_w test media (Gibson, 1973; Goepfert et al., 1970; Mattick et al., 2001) and a paper disk model (Hiramatsu et al., 2005). Early investigations on the influence of fat on bacterial survival show conflicting results in peanut oil and cod liver oil (Mitscherlich and Marth, 1984). As summarized by Mitscherlich and Marth, 1984, (Myers, 1969) reported extended survival times when previously dried or lyophilized bacteria were incorporated into oil, but shorter survival times were observed when moist bacteria was incorporated into oil. Further, Bindseil, 1917 reported that a 24 hour-old culture of *S. Typhi* survived for 150-230 days in flax oil, sesame seed oil and turnip oil, while survival was observed for 4 days in olive oil. *S. Typhi* survived in melted goose fat and lard stored at room temperature for 70 and 90 days, respectively. By contrast, a review by Lichtenstein, 1939 discussed evidence of a bactericidal effect of oil when used as a wound dressing. Cod liver oil and linseed oil were found to have a bactericidal effect, but olive oil was devoid of such effects. The quality of the oil was also found to affect survival, citing rancidity as an influencing factor.

The heat resistance of microbial cells varies according to their water content. Heating a microorganism in the presence of fat could be equivalent to dry heat conditions due to the localized absence of water around the bacterial cell. Dry heat conditions transfer heat by conduction, and therefore require high temperatures for extended periods of time. Heat is

absorbed by the outside surface of the item, and passes through to the center layer by layer which results in slow heat penetration (Pflug, et al., 2001). The limited heat conductivity by fats and oils was previously thought to be the cause of this protective effect (Pflug et al., 2001).

Decreased water activity has also been proposed to be the cause of the protective effect of oil. As summarized by Pflug, et al., 2001, studies by Senhaji, 1977 and Senhaji et al., 1976 examined the effect of added water on the survival of *B. subtilis* spores in soy oil. The authors concluded that increased resistance occurs as the result of decreased a_w of the oil during heating. An additional series of studies (Senhaji and Locin, 1975) found that spore survival in 33% oil-in-buffer emulsions resembled survival in phosphate buffer, while survival in a 2 phase 33% oil-in-buffer system resembled survival in oil (Pflug et al., 2001).

More recently, the influence of fat content on bacterial survival has been investigated in a variety of foods. An in-vitro digestion of high-fat and low-fat frankfurters showed a protective effect of fat against the gastric inactivation of *L. monocytogenes* (Barmpalia-Davis, et al., 2009). The D_{60} values for *L. monocytogenes* in chocolate-peanut spread ($a_w=0.46$, 39% fat, $D_{60}=37.5$ min) and peanut butter ($a_w=0.32$, 53% fat, $D_{60}=26$ min) were significantly higher than those in 1-4% fat liquid products (Kenney and Beuchat, 2004). A five-strain cocktail of *Salmonella* survived longer in yellow fat spreads with $\geq 61\%$ fat compared to those with $\leq 41\%$ fat when held at 21°C and 10°C (Holliday and Beuchat, 2003). *Salmonella*-contaminated high fat (24%) ground beef required 208 min at 58°C to achieve a 7 log reduction, whereas low fat (7%) ground beef required 53.5 min at the same temperature (Juneja and Eblen, 2000). Thermal inactivation of *L. monocytogenes* in whole-fat chocolate milk (4.0 % fat), in contrast, was not significantly different from inactivation in reduced-fat chocolate milk (1.0%), whole fat milk (3.5%) or a peanut beverage (3.1%fat) (Kenney and Beuchat, 2004). Furthermore, the level of natural lipids

(high, medium, low) in sardines and anchovies did not significantly affect the radiation resistance of *Salmonella* Typhimurium (Kamat and Thomas, 1998).

A review on the thermal resistance of Salmonellae by Doyle and Mazzotta, 2000 concluded that the heat resistance of *Salmonella* in dried foods is comparable to that of spores. A series of studies by Ababouch and Busta, 1987 and Ababouch et al., 1995 reported increased heat resistance of *B.cereus*, *B.subtilis*, *B. sporogenes* and *C. botulinum* spores suspended in edible oil when compared to spores suspended in buffer. The observed resistance increased with increasing temperature. Estimated inactivation rates based on literature D-values of 12 pathogens revealed a protective effect from inactivation for *Salmonella* cells in chocolate and *Bacillus cereus* spores in oil (van Asselt and Zwietering, 2006).

Along the same line, researchers have reported that the combination of high fat and low- a_w in foods have a synergistic effect on *Salmonella* survival. The heterogeneous nature of high fat, low a_w peanut butter held at 70°C and 90°C resulted in extrapolated Weibull model predictions of 260 min and 60 min for a 7-log reduction, respectively. The authors hypothesized that survival times may be even longer because of the tailing effect observed in the survival studies (Shachar and Yaron, 2006). *Salmonella* populations decreased by 1.7- $\log_{10}$ CFU/ml in tahini (sesame paste, $a_w=0.31$, 58% fat) after 28 d storage at 10°C. The D-values of previously stressed *Salmonella* cells in regular (33% fat), organic (50% fat) and no-salt organic(50% fat) peanut butter held at 72°C were significantly higher than reduced fat peanut butter (6.25% fat) held at the same temperature (He et al., 2011). In a similar study, inactivation of 3 *Salmonella enterica* serotypes in low-fat peanut butter (33% fat, 42% carbohydrate) decreased compared to regular peanut butter (49% fat and 24% carbohydrate) held at 25°C for 4 weeks (He et al., 2013). Aviles et al., 2013 reported cross-protection toward acid stress and enzyme activity from high fat, low

a_w peanut butters in an in-vitro digestive system. Foods associated with low infective doses of *Salmonella* (chocolate, cheese and meat) have a high fat content, which is hypothesized to protect the cells against gastric acidity in the digestive system so they are readily able to colonize the small intestine (D'Aoust and Maurer, 2007).

***Salmonella* and Peanut products**

Peanuts are used as ingredients in a wide variety of foods such as peanut butter, confections, pet treats and snack foods. The estimated prevalence of *Salmonella* on raw shelled peanuts was 0.67% with an average of 1.05 (range 0.74 to 5.25) MPN *Salmonellae* per 350 grams of peanuts (Miksch, et al., 2013). In previous years, the low prevalence of *Salmonella* on raw nuts was considered to pose a minimal public health risk due to low a_w conditions that do not support the growth of pathogens. However, the presence of *Salmonella* on raw peanuts can pose a significant public health risk in peanut-derived products if the pathogen survives the roasting process or post-process contamination occurs. The potential low infective dose of *Salmonella* associated with high fat, low a_w foods intensifies this risk. Few *Salmonella* outbreaks have occurred in peanuts or peanut products (Chang et al., 2013, Kirk et al., 2004; Schaffner et al., 2013) but several documented outbreaks in peanut butter and peanut paste have had widespread and devastating consequences (CDC, 2015). Research has demonstrated that once contamination occurs, *Salmonella* is likely to survive the duration of the shelf life of peanut butter (Burnett et al. 2000; Shachar and Yaron, 2006).

The water activity of peanut butter ranges between $a_w=0.20-0.33$ (Burnett et al., 2000) and the fat content of finished peanut butter must be less than 55% (FDA, 2013). Processing steps include dry roasting at 160°C for 40-60 min, followed by cooling, blanching and sorting of the peanuts. Grinding and milling occurs in two stages; the first stage reduces the nuts to a medium grind and

ingredients such as emulsifiers, salt, dextrose and sugar are added at temperatures between 60-74°C. Emulsifiers such as hydrogenated vegetable oils, monoglycerides, diglycerides or combinations of these are used to prevent oil separation (Woodroof, 1973). Selection of an emulsifier depends on the processing protocol, desired flavor release, fill temperature, and storage temperature (Lusas et al., 1989). Levels of emulsifier in peanut butter range from 1-5.5%, with 3.25% as the most common amount (Woodroof, 1973). The second grinding stage occurs between 71-77°C (up to 89°C) and reduces the nuts to a smooth, fine texture. The product is immediately cooled to 49°C or below to ensure proper fat crystallization. Jars are filled at temperatures between 30-43°C and are stored around 10°C (Woodroof, 1973). Storage studies by Burnett et al. 2000 attributed the differences of *Salmonella* survival at 21°C and 5°C to different peanut butter and peanut butter spread compositions. Natural peanut butter containing peanuts and salt had the highest inactivation rates of *Salmonella* after 24 weeks compared to commercial products containing hydrogenated vegetable oils, monoglycerides, peanut oil and other ingredients. The influence of these additional ingredients on the survival of *Salmonella* in peanut butter remains unclear.

The cause of the *Salmonella* outbreaks in peanut butter have been attributed to failure to validate roasting process, poor sanitation and facility maintenance, and post-process contamination (ConAgra Foods, Inc., 2007; FDA, 2009). The processing of a low a_w , high fat product with potentially sublethal processing temperatures for thermally-adapted *Salmonella* in combination with substandard operational practices, and poor sanitation and hygiene suggest that further research and control technologies are needed.

Control of *Salmonella* in dry processing environments

Salmonella contamination of low a_w foods and ingredients has been attributed to cross contamination, substandard operational practices, poor sanitation and hygiene, improper facility and equipment design, poor facility maintenance and inadequate ingredient control. (GMA, 2009). Failure to comply with standard safety practices such as implementation of an approved HACCP plan, validation and verification of antimicrobial intervention treatments, addressing pathogen-positive samples, and the presence of infected food production employees have also contributed to contamination in dry processing operations (Gurtler, et al., 2014).

Industry guidance documents outline several elements to prevent or minimize the ingress, spread, and growth of *Salmonella* dry food processing operations (GMA, 2009; ABC, 2007a; FDA, 2009). Minimization of contamination risk requires each process and each production facility to follow basic requirements such as good manufacturing practices (GMPs), strict application of zoning and dry cleaning, traffic control, and strict adherence to validated process limits (Anderson and Lucore, 2012).

The U.S. Food Safety Modernization Act (FSMA) requires processing establishments to have a Food safety plan, which documents an analysis of potential hazards for each process step, followed by activities performed in order to prevent, reduce or eliminate the hazard. The plan must include a written description of how safety records are maintained. The main principles included in a food safety plan are validation, monitoring and verification (Anderson and Lucore, 2012). Validation refers to a systematic order of activities aimed at controlling hazards. This broad set of activities includes a hazard analysis, determination of the most resistant pathogen and the level of inactivation required, assessment of the influence of food components on the pathogen survival, validation of the efficacy of the kill step, definition of the critical limits

needed to meet the regulatory standard, and the definition of specific operating and equipment parameters to achieve the desired inactivation level. Processors of low a_w foods can use scientific literature, government guidance documents, scientific validation experiments, predictive modeling to assist in development of a food safety plan for low a_w foods (Anderson and Lucore, 2012).

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CHAPTER 3

A REVISED MODEL FOR THE INFLUENCE OF TEMPERATURE, FAT CONTENT AND WATER ACTIVITY ON THE PERSISTENCE OF *SALMONELLA* IN LOW-WATER ACTIVITY FOODS¹

¹Trimble, L.M., Schaffner, D.W., and J.F. Frank. To be submitted to the *International Journal of Food Microbiology*

Abstract

Low water activity (a_w) foods containing fat are continually implicated as the vehicle of infection in outbreaks of *Salmonella* spp. The influence of fat content on survival in foods such as peanut butter remains unclear. Certain *Salmonella* serovars are capable of surviving for long periods of time in harsh temperatures and low moisture conditions. The objective of this study was to determine the influence of fat content on the survival of *Salmonella* in low a_w foods and use this information to modify an existing secondary inactivation model previously validated for low fat foods. Whey protein powder supplemented with peanut oil was equilibrated to 5 a_w ranges ($a_w < 0.60$), inoculated with a dried 4 strain cocktail of *Salmonella*, vacuum sealed and stored at 21, 37, 50, 60, 70 and 80°C for 48h, 28d or 168d. Survival data was fitted to the log-linear, Baranyi, Weibull, Biphasic-linear, Double Weibull and Geeraerd-Tail models. The Weibull model was chosen for secondary modeling due to its ability to describe the data ($F_{\text{test}} < F_{\text{table}}$) over all conditions. The influence of temperature, fat content and a_w on the Weibull model parameters was evaluated using Multiple Linear Regression and revised secondary models were developed based on parameter significance. Natural peanut butter, chia seed powder, toasted oat cereal and animal crackers within the temperature and a_w range of the modeled data were used to validate the revised model. Fat content influenced survival at temperatures $\geq 50^\circ\text{C}$, while a_w influenced survival at 37°C and 70°C independent of fat content. The revised model provided improved % bias and % discrepancy results compared to the previous model in addition providing fail safe predictions in all of the food products tested. Predictions were less reliable with decreasing a_w and increasing temperature. The results of this study provide information which will aid in the development of risk mitigation strategies for low a_w foods containing fat.

Introduction

Low water activity (a_w) foods and ingredients are naturally dry or have been dried through processing to $a_w \leq 0.85$ (Beuchat et al., 2013). These products typically have a long shelf life and do not require heating before consumption. Often, a lethality step is not required or these products have ingredients that are added after a lethality step. Such foods include cereals, milk powders, peanut butter, dry pet food, flour, spices, chocolate, and crackers. Low a_w is a barrier to growth for pathogenic bacteria since a minimum a_w of 0.87 is required for most bacteria to grow (Beuchat et al., 2013). Historically, low a_w foods were commonly thought to be microbiologically safe due to the unsuitable growth conditions in these foods. However, low a_w foods are frequently recalled for contamination with pathogenic bacteria. In 2014, 44 FDA recalls involved low a_w foods, spices and ingredients, with *Salmonella* contamination being the most frequent cause (FDA, 2014). Survival data demonstrate the highly adaptive capacity of *Salmonella* to survive in low a_w foods for extended periods of time, even when subject to high heat (Santillana Farakos, et al., 2013). However, the influence of food components on this adaptive capacity of *Salmonella* in low a_w foods remains unclear. Previous research suggests that fat has a protective effect on the heat inactivation of *Salmonella* (Juneja & Eblen, 2000) and the combination of high fat and low a_w in a food matrix has a synergistic effect on *Salmonella* survival (Aviles et al., 2013; Ma et al., 2009; Shachar and Yaron, 2006; Hiramatsu et al, 2005). Recent outbreaks of salmonellosis have occurred in high fat, low a_w foods such as sprouted chia powder, peanut butter, almond butter, and tahini sesame paste. These have resulted in 52 reported illnesses across 30 states, 7 hospitalizations and 1 death (CDC, 2015). *Salmonella* contamination of low a_w foods and ingredients has been attributed to cross contamination due to substandard operational practices, inadequate ingredient control, poor

sanitation and hygiene practices, and improper equipment design (GMA, 2009). Guidance documents for food industry preventative control measures include elements designed to prevent the ingress and spread of *Salmonella* spp. in processing facilities through the use of risk assessments, validation of control measures and establishment of corrective actions.

Validated secondary predictive models are a useful tool to assist in these risk mitigation strategies by providing quantitative support for microbiological risk assessments and Hazard Analysis and Critical Control Point systems. One such secondary model was developed and validated for *Salmonella* survival in low-fat, low a_w foods ($a_w \leq 0.60$) held at 21-80°C (Santillana Farakos, et al., 2013). The model was useful for predicting *Salmonella* in various low a_w foods with an acceptable prediction performance of 81%. However, model predictions were less accurate in foods containing low levels of fat (12%) when compared to non-fat foods. To the author's knowledge, there is no published model which quantifies the influence of fat content on *Salmonella* inactivation in low a_w foods. The aim of this study was determine the influence of fat content on the survival of *Salmonella* at low a_w ($a_w < 0.60$), and to use this information to develop a predictive model valid for low and high fat foods. Our approach was to modify the secondary model developed by Santillana Farakos et al., 2013 to also apply to high fat foods and then revalidate the new model. Survival of *Salmonella* was determined using a whey protein model system with peanut oil adjusted to various $a_w < 0.60$ with samples held at temperatures between 21-80°C.

Materials and Methods

Preparation of modified whey protein isolate powder

A 4% solution of BiPro whey protein isolate powder (WPI; 95% protein) (Davisco Foods International: LaSueur, MN) was homogenized with 20 or 50% peanut oil (w/w) (Planters, Kraft

Foods Group, Inc., Northfield, IL) using a hand-held homogenizer (Polytron PT 1200C, Kimatica: Bohmeia, NY) at 9000 rpms for 1 min. Homogenization was standardized during pilot studies using a particle sizer (Malvern masterciser 2000, Worcestershire, United Kingdom) with a small sample polydispersion unit and a 300 lens detector. The average particle size diameter was $< 100 \mu\text{m}$ for each emulsion (results not shown). Emulsions were poured into frozen sterile pans located in a -31°C freezer within 1 min of homogenization to maintain emulsion stability. Peanut oil emulsions were allowed to freeze overnight followed by freeze-drying from -40°C to 0°C for 24 h (Revo Opti-Dry Pro, Millrock Technology: Kingston, NY). Freeze-dried powders (approximate $a_w < 0.15$) were stored at 21°C in glass jars with calcium sulfate sachets to prevent moisture absorption. Prepared powders were used in experiments within 7d.

Equilibration of powders to target water activities

Freeze-dried powders were equilibrated to the target a_w levels ($a_w = < 0.60$) using saturated salt solutions in vacuum-sealed desiccators at 21°C . The target water activities were 0.11 (lithium chloride, Acros Organics, Trenton, NJ), 0.23 (potassium acetate, Fisher Scientific, Pittsburg, PA), 0.33 (magnesium chloride hexahydrate, Fisher Scientific), 0.43 (potassium carbonate, Amresco, Solon, OH), and 0.58 (sodium bromide, Acros Organics) (Fontana, 2007). Water activity was measured at 21°C using a_w meter (AquaLab Series 4TEV, Decagon Devices Inc., Pullman, WA) with ± 0.0003 precision.

Preparation of inoculum

A cocktail of *Salmonella* serovars associated with outbreaks in low a_w foods was used in this study. These include *S. Typhimurium* (peanut products), *S. Tennessee* (peanut butter), *S. Agona* (dry cereal) and *S. Montevideo* (spices, tahini paste). Serovars were cultured independently in tryptic soy broth (Difco: Franklin Lakes, NJ) using 3 consecutive transfers (9 ml, 9 ml, 225 ml)

for 24 h at 37°C. Each culture (225 ml) was then centrifuged at 3500 g for 30 min and the pellet was resuspended in 2 ml 1% Bacto Peptone (BD, Sparks, MD). Cell suspensions (2 ml) of each serovar were dried separately in weigh boats placed over a calcium sulfate desiccant (W.A. Hammond Drierite: Xenia, OH) in vacuum-sealed desiccators. Suspensions were dried to $a_w < 0.10$ for 2-5 d at 22°C.

Sample inoculation and packaging

Prior to inoculation, equal amounts of the 4 dried serovar cultures were combined, crushed and mixed into a homogenous powder using a metal spatula in a 25 ml tube. Equilibrated WPI samples were weighed (0.95 g) and combined with inoculum (0.05 g) in standard retort pouches (Stock America Inc., Grafton, WI) to provide a 1 g sample. This dry inoculation method resulted in initial concentrations of 10^9 CFU/g of *Salmonella*. Pouches were shaken to ensure an even distribution of powder and inoculum and were placed in food saver bags. Food saver bags were vacuum sealed (FoodSaver: United States) then heat sealed using an impulse sealer (PacWorld: Nazareth, PA).

Short-term Inactivation experiments

The vacuum-sealed retort pouches were stored in a circulating water bath (Precision, ThermoScientific, Waltham, MA) for 48h at 70°C and 80°C and for 28d at 50°C and 60°C. Water baths were equipped with custom-designed horizontal wire racks which ensured complete submersion and water circulation between pouches.

Long-term inactivation experiments

Controlled temperature incubators ($\pm 0.5^\circ\text{C}$) were used to store vacuum-sealed retort pouches for 6 months (168 days) at 37°C and 21°C. Pouches were stored in desiccators containing a calcium sulfate desiccant (W.A. Hammond drierite: Xenia, OH).

Experimental Plan

Samples in long-term experiments were analyzed at 0, 7, 14, 21, 28, 42, 56, 84, 112, 140 and 168 days. During short-term 70°C and 80°C experiments, survivors were recovered at 0, 1, 5, 15, 30, 60, 240, 480, 960, 1440, and 2880 min. At 50°C and 60°C, samples were analyzed after 0, 2, 6, 12, 24, 48, 96, 168, 336, 504, 672 h. Time 0 corresponds to the time required for the sample to reach the target temperature.

Each inactivation experiment was replicated three times. The a_w and background microflora of uninoculated controls were analyzed at time points from the beginning, middle and end of each experiment. *Salmonella* survivors were recovered on non-selective differential media containing tryptic soy agar (TSA, Becton Dickson and Company, Sparks MD) (40 g/L), ferric ammonium citrate (Sigma-Aldrich Co. St. Louis, MO) (0.8 g/l), yeast extract (Becton Dickson and Company, Sparks MD) (3.0g/l) and sodium thiosulfate (6.8 g/l) as described by Santillana Farakos, et al. 2013. Plates were incubated at 37°C for 48h. Statistically significant differences in survival between a_w , temperature and fat content combinations were analyzed by ANOVA using the Generalized Linear Model procedure with temperature and a_w interaction effects at the $\alpha = 0.05$ level of significance (SAS version 9.4, Cary, NC).

Development of predictive models

Model fitting and selection

Previous research indicates that survival curves of *Salmonella* spp. held in low water activity conditions show increased tailing with increased inactivation temperature (Taromina, 2014; Santillana Farakos et al., 2013), pronounced downward concavity with increased a_w levels (Mattick et al., 2001; Santillana Farakos et al., 2013) and the presence 2 subpopulations with

different resistances (Margas et al., 2014). Based on this criteria, the linear and non-linear inactivation models below were selected to be fit to the survival data from this experiment.

Baranyi growth model (Baranyi and Roberts, 1994) (1)

$$\log N_t = \log N_0 + \frac{\mu}{\ln(10)} * t - \frac{1}{\ln(10)} * \ln \left(1 + \frac{\exp \mu * t - 1}{10^{\log N_f - \log N_0}} \right)$$

Where N_t is the population at time t (CFU/g), N_0 is the population at time zero (CFU/g), μ is the maximum specific growth rate (min^{-1}) N_f is the final population ($\log_{10}$ CFU/g)

Weibull model (Mafart et al., 2002) (2)

$$\log N_t = \log N_0 - \left(\frac{t}{\delta} \right)^\beta$$

Where δ is the treatment time required for the first decimal log reduction and β is the shape factor.

Biphasic-linear model (Cerf, 1977) (3)

$$\log N_t = \log N_0 + \log(f * \exp(-k_{max1} * t) + (1 - f) * \exp -k_{max2} * t))$$

Where k_{max1} and k_{max2} represent the maximum specific inactivation rates for the heat resistant and heat sensitive populations, respectively. The heat sensitive and heat resistant fractions of the population are defined as $(1 - f)$ and f , respectively.

Geeraerd-tail model (Geeraerd et al., 2000) (4)

$$N_t = (N_0 - N_{resistant}) * \exp(-k_{gmax}) * t + N_{resistant}$$

Where $N_{resistant}$ represents the heat resistant population and k_{gmax} is the maximum specific inactivation rate (min^{-1}).

Log-linear model (Bigelow and Etsy, 1920) (5)

$$\log_{10} N_t = \log_{10} N_0 - \frac{k_{max} * t}{\ln 10}$$

Where t is the time (min), N is the population at time t (CFU/g), N_0 is the population at time zero (CFU/g), and $k_{\max}$ is the maximum specific inactivation rate (min^{-1}).

Double Weibull model (Coroller et al., 2006) (6)

$$N_t = \frac{N_0}{1+10^\alpha} \left[10^{-\left(\frac{t}{\delta_1}\right)^{p+\alpha}} + 10^{-\left(\frac{t}{\delta_2}\right)^p} \right] \quad \alpha = \log_{10} \left(\frac{f}{1-f} \right) \text{ where } f = \frac{10^\alpha}{1+10^\alpha}$$

Where N_0 , N_t and t are described previously and δ_1 and δ_2 represent the more sensitive subpopulation and the less sensitive subpopulation, respectively. The fraction of subpopulation 1 in the total population is represented by f , which varies from 0 to 1. The logit transformation of f yields the parameter α , which varies from negative infinity to positive infinity.

Data was fit to the models in equations 1-6 using GInaFit Version 1.6 (Geeraerd et al 2005; University Leuven, Belgium) and the Baranyi model using DMFit Version 3.5 (Institute of Food Research, Norwich, United Kingdom). To determine which model most appropriately described the data, the F value (F-test), the adjusted coefficient of determination (R^2_{adj}) and the root mean square error (RMSE) were calculated using Excel 2010 (Microsoft Corporation, Redmond, WA) according to equations (7)-(13). A global F-test ($F_{\text{test}} < F_{\text{table}}$), 95% confidence level) was used as the primary criterion to determine which model best described the data under all tested temperature, a_w and fat content conditions. If the model F_{test} value was $<$ than F_{table} , the model was judged to provide an acceptable fit to the data (Den Besten et al., 2006). Statistical parameters (highest R^2_{adj} , lowest RMSE) were used as the second criterion among datasets with more than one acceptable model fit. The model with the smallest number of parameters was chosen to maintain parsimony when the first two criteria were equally met.

$$f = \frac{MSE_{\text{model}}}{MSE_{\text{data}}} \quad (7)$$

$$MSE_{\text{data}} = \frac{RSS_{\text{data}}}{df_{\text{data}}} \quad (8)$$

$$MSE_{model} = \frac{RSS_{model}}{df_{model}} \quad (9)$$

$$RSS_{model} = \sum (\log N_{model} - \log N_{data})^2 \quad (10)$$

$$RSS_{data} = \sum (\text{average } \log N_{data} - \log N_{data})^2 \quad (11)$$

$$RMSE = \sqrt{\frac{RSS_{model}}{df_{model}}} \quad (12)$$

$$R_{adj}^2 = 1 - \frac{(n-1)(1-R^2)}{df_{model}} \quad (13)$$

$$\text{Where } R^2 = 1 - \frac{RSS}{TSS}$$

Where RSS_{data} is the residual sum of squares of the data, RSS_{model} is the residual sum of squares of the model, and df is the degrees of freedom where $df_{model} = n-p$ and $df_{data} = n-m$. (m is the number of time points in the curve, n is the total number of observations at time points in the included replications and p is the number of parameters in the model). TSS is the total sum of squares, which is the total amount of variation when model variation values are compared to the grand mean of the model variation values.

Evaluation of low-fat model fit parameters in high fat whey protein powder

Salmonella inactivation data obtained from the current experiment (20% and 50% fat (w/w)) at temperatures 21-80°C and 5 a_w ranges <0.60) were fit to the Weibull model secondary inactivation equations developed in unsupplemented whey protein powder and validated for low- a_w (a_w <0.60), low-fat (<12%) foods held at 21-80°C (Santillana Farakos, et al., 2013). Model performance evaluation indices (equations 14-17) by temperature and a_w were calculated for comparison of the reliability of the model for predictions in high fat and low fat foods.

Modification of existing secondary model

Previously published survival data for *Salmonella* in non-fat whey protein powder equilibrated to a_w <0.60 and held at 21-80°C (Santillana Farakos, et al., 2013) was added to the dataset to

increase the range of fat contents (non-fat, 20%, 50% (w/w)) included in the modified model. For each temperature and water activity combination, non-fat survival data was added from the 2nd and 3rd water mobility configurations as described by Santillana Farakos et al. 2013.

The influence of temperature, a_w and fat content on the survival of *Salmonella* was evaluated using the data analysis add-in for Multiple Linear Regression in Excel 2010 (Microsoft Corporation, Redmond, WA). The primary model parameters represent the dependent variables and temperature, a_w , and fat content represent the independent variables. Secondary model equations were developed based on the significance of the independent variables in a t_{test} . For each primary model parameter, an independent variable was included in the secondary model equation when the significance of the test was less than the level of confidence ($p < 0.20$).

Normality and uniform variance of residuals were verified using normal probability plots and residual plots.

Validation of modified secondary inactivation model

Inoculated and crushed Barnum's animal crackers (13 % fat, Mondelez International, East Hanover, NJ), toasted oats cereal (0.05 % fat, Kroger, Cincinnati, OH), micro-fine white chia powder (32% fat, Living Intentions, San Francisco, CA), and natural peanut butter (50 % fat, Kroger, Cincinnati, OH) were used to obtain *Salmonella* survival data (in duplicate) within the a_w , time and temperature range of the data used to develop the secondary model ($a_w \leq 0.60$ at 21-80°C for 0- 168 days). Plots of model predictions against observed experimental data and the corresponding correlation coefficient values (R , equation (14)) were used to estimate the strength of the linear relationship. Model reliability was evaluated using the accuracy factor and bias factor, expressed as the % discrepancy and % bias, respectively (eq.15-16) (Baranyi, et al., 1999). Model residuals (r ,) were calculated according to equation (17) and the percentage of

model residuals in the acceptable zone was used as a measure of model performance. A model is judged to be validated and acceptable if $\geq 70\%$ of the residuals are between -1 log CFU/g (fail safe) to 0.5 log CFU/g (fail dangerous) (Oscar, 2009).

$$R = \text{Correlation}(x, y) = \frac{SS_{xy}}{\sqrt{SS_{xx}SS_{yy}}} \quad (14)$$

where $SS_{xy} = \sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})$ and $SS_{xx} = \sum_{i=1}^n (x_i - \bar{x})^2$, $SS_{yy} = \sum_{i=1}^n (y_i - \bar{y})^2$

$$\%B_f = \text{sgn}(\ln B_f) \times (\exp^{|\ln B_f|} - 1) \times 100\% \quad (15)$$

$$\text{Where } B_f = 10^{\left[\frac{\sum_{i=1}^n \log(\log N_{\text{model}} / \log N_{\text{data}})}{n} \right]}$$

$$\text{sgn}(\ln B_f) = \begin{cases} +1 & \text{if } B_f > 0 \\ 0 & \text{if } B_f = 0 \\ -1 & \text{if } B_f < 0 \end{cases}$$

$$\% D_f = (A_f - 1) \times 100\% \quad (16)$$

$$\text{Where } A_f = 10^{\left[\frac{\sum_{i=1}^n |\log(\log N_{\text{model}} / \log N_{\text{observed}})|}{n} \right]}$$

$$r = \log N_{\text{observed}} - \log N_{\text{predicted}} \quad (17)$$

Results and Discussion

Inactivation of *Salmonella* in samples held at 21 to 80°C

Survival curves for *Salmonella* in a whey protein isolate model food system with varying amounts of fat (non-fat, 20%, 50% added fat by weight) at temperatures 21 to 80°C are shown in Figures 3.1-3.6. Table 3.1 presents the model fit statistics used as the criteria to determine which model (equations 1-6) most appropriately represents the data containing 20% and 50% fat. Populations of *Salmonella* at 22°C remained constant after 168 days of storage at all water activity ranges ($a_w < 0.60$) under study (Figure 3.1). The average log reduction per day was 0.005,

0.003, 0.005, 0.004 and 0.006 log CFU/day at $a_w=0.18\pm0.04$, 0.28 ± 0.04 , 0.37 ± 0.02 , 0.43 ± 0.03 and 0.54 ± 0.04 , respectively. The protective effect of added fat increased with increasing a_w when compared to non-fat powders during long-term storage ($p<0.0001$). At the lowest a_w range ($a_w=0.18$), the mean effect of fat was -0.67 when compared to non-fat powder, whereas the mean effect increased to 0.20 at the highest a_w range ($a_w=0.54$). The average log reduction for non-fat, 20% and 50% peanut oil whey protein powder across all a_w was 0.003, 0.005 and 0.005 log CFU/day, respectively.

Since the 168 day survival curves are essentially linear at 22°C, the log-linear model was appropriate in describing the data for all a_w and fat combinations ($F_{\text{test}} < F_{\text{table}}$). However, the fit was poor for the water activity ranges below 0.36 as shown in Table 3.1. The Baranyi model provided an acceptable description of the data with the exception of the 50% powder at $a_w=0.28\pm0.04$ for which the standard error was >200%. Half of the a_w and fat conditions were appropriately described ($F_{\text{test}} < F_{\text{table}}$) by the biphasic-linear model and Geeraerd-tail model. The Weibull and the Coroller model appropriately described the data for most of the conditions, with the exception of (50% fat, $a_w=0.27$ and 20% fat, $a_w=0.36$). Although the statistical fits are poor ($R^2_{\text{adj}} < 0.51$, RMSE > 0.27) for all conditions, the Weibull model provides the highest R^2_{adj} and RMSE values at 22°C.

At 37°C, survival significantly decreased ($p<0.001$) with increasing a_w for both non-fat and added-fat powders after 168 days of storage (Figure 3.2). Survival did not differ by fat content ($p=0.417$) and the influence of fat on survival was not dependent on a_w ($p=0.955$). The average log reduction for non-fat, 20% and 50% peanut oil whey protein powder across all a_w was 0.009, 0.012 and 0.011 log CFU/day, respectively. The average log reduction per day was 0.004, 0.008, 0.010, 0.012, and 0.018 log CFU/day for $a_w = 0.18\pm0.04$, 0.28 ± 0.03 , 0.37 ± 0.02 , 0.45 ± 0.04 and

0.54±0.03, respectively. The biphasic-linear model fit the data with the exception of the 20% fat at $a_w=0.36$. The Weibull, Coroller, Log-linear, and Geeraerd-Tail model appropriately described the data under all conditions ($F_{\text{test}} < F_{\text{table}}$). The Weibull model provided the highest R^2_{adj} values and the lowest RMSE values.

At 50°C, the presence of fat had a protective effect on survival ($p < 0.001$) when compared to non-fat powders after 28 days (Figure 3.3). However, additional fat content (from 20% to 50%) did not increase this protective effect ($p = 0.4025$). The average log reduction for non-fat, 20% and 50% peanut oil whey protein powder across all a_w ranges was 0.319, 0.044 and 0.059 log CFU/day, respectively. Survival did not differ by a_w ($p = 0.624$) and the influence of fat on survival was not dependent on a_w ($p = 0.991$). The average log reduction per day was 0.157, 0.145, 0.128, 0.136, and 0.137 log CFU/day for $a_w = 0.17 \pm 0.04$, 0.28 ± 0.05 , 0.35 ± 0.03 , 0.45 ± 0.05 and 0.56 ± 0.03 , respectively. The biphasic-linear, Geeraerd-tail and log-linear models fit the data with the exception of one fat- a_w combination (20% $a_w=0.17$, 50% $a_w=0.43$) (Table 3.1). The Coroller model did not fit the data at the two lowest a_w ranges ($F_{\text{test}} > F_{\text{table}}$). The Weibull and Baranyi models appropriately described the data at all conditions and provided similar statistical values (Table 3.1).

The presence of fat provided a protective effect ($p < 0.001$) on survival during 28 days of storage at 60°C when compared survival in non-fat powder (Figure 3.4). Additional fat content (from 20% to 50%) did not influence the protective effect ($p = 0.798$). The average log reduction for non-fat, 20% and 50% peanut oil whey protein powder across all a_w was 0.238, 0.051 and 0.059 log CFU/day, respectively. Survival was not influenced by a_w ($p = 0.085$) and the protective effect of fat was independent of a_w ($p = 0.5150$). The average log reduction per day was 0.106, 0.108, 0.114, 0.116, and 0.137 log CFU/day for $a_w = 0.18 \pm 0.04$, 0.27 ± 0.03 , 0.36 ± 0.02 ,

0.46±0.03 and 0.57±0.03, respectively. The Geeraerd-tail model fit the data with the exception of one condition (50% fat, $a_w=0.54$). The Weibull, Biphasic-linear, Log-linear, Coroller and Baranyi models appropriately described the data for all conditions ($F_{\text{test}} < F_{\text{table}}$). The Weibull and Coroller models provided the highest R^2_{adj} values and the lowest RMSE values (Table 3.1), followed by those of the biphasic-linear model.

The presence of fat (20%, 50%) in powders provided a protective effect for *Salmonella* survival ($p=0.022$) with 48h of treatment at 70°C. However, additional fat content (from 20% to 50%) did not increase survival ($p=0.930$). The average log reduction for non-fat, 20% and 50% peanut oil whey protein powder across all a_w was 2.60, 1.68 and 1.70 log CFU/day, respectively. As the a_w decreased, the heat resistance of *Salmonella* significantly increased ($p<0.001$). The average log reduction per day was 0.106, 0.108, 0.114, 0.116, and 0.137 log CFU/day for $a_w = 0.18\pm0.04$, 0.27 ± 0.03 , 0.36 ± 0.02 , 0.46 ± 0.03 and 0.57 ± 0.03 . The protective effect of fat was independent of a_w ($p=0.834$). The Geeraerd-tail and Biphasic-linear models provided acceptable fits for most conditions with the exception of one (50% fat, $a_w=0.44$). The Coroller and Log-Linear models did not fit the data well at the intermediate and high a_w ranges ($a_w>0.35$) under study (Table 3.1). The Weibull and Baranyi models provided an acceptable fit for all of the conditions ($F_{\text{test}} < F_{\text{table}}$). The Weibull model provided the highest R^2_{adj} values and the lowest RMSE values. Survival was enhanced by the presence of fat (20%, 50%) ($p<0.001$) when compared to non-fat powder during 48h of storage at 80°C. Additional fat content (from 20% to 50%) did not influence survival ($p=0.494$). The average log reduction per day for non-fat, 20% and 50% peanut oil whey protein powder across all a_w was 2.95, 1.06 and 1.01 log CFU/day, respectively. Survival did not differ by a_w ($p=0.060$) and the influence of fat and a_w were found to be independently related ($p=0.995$) across all levels of fat (non-fat, 20%, 50%). The average log

reduction per day was 1.45, 1.52, 1.74, 1.18, and 1.90 log CFU/day for $a_w = 0.18 \pm 0.03$, 0.27 ± 0.01 , 0.33 ± 0.02 , 0.43 ± 0.05 and 0.53 ± 0.04 , respectively. The Weibull, Biphasic-linear, Geeraerd-tail, log-linear and Baranyi models provided an acceptable fit for all conditions under study ($F_{\text{test}} < F_{\text{table}}$). The Coroller model was not appropriate for the 20% fat data at ($a_w = 0.18, 0.43$ and 0.55). The Weibull and Biphasic-linear models provided the highest R^2_{adj} and the lowest RMSE for all conditions (Table 3.1).

The Weibull model was chosen for secondary modeling due to its ability to describe the data under the greatest number of temperature, a_w and fat content (20%, 50%) conditions ($F_{\text{test}} < F_{\text{table}}$). Additionally, the Weibull model provided the highest R^2_{adj} and the lowest RMSE compared to other models. In cases where these values were similar to those of other models, the Weibull model was chosen to maintain the principle of parsimony. The two conditions in which the Weibull model did not provide a satisfactory fit were removed from the model dataset. Table 3.2 presents the corresponding Weibull model (equation 2) parameters (δ, β) for all conditions under study. Since many of the δ values differed by several orders of magnitude, the values were log transformed to achieve normality.

Modified secondary inactivation model

Based on equations (14-17), evaluation of the reliability of the predictions for the low-fat model developed by Santillana Farakos et al., 2013 in the whey protein powders with 20% and 50% (w/w) showed an overall performance of 56% of the residuals in the acceptable zone, a 5.36% bias and 23.12% discrepancy. Analysis by temperature (21-80°C) showed that the predictions by the low-fat model are the least reliable in high fat (20%, 50%) whey protein powder at 80°C (71 % bias, 133.79% discrepancy) 50°C (0.31% bias, 9.90% discrepancy), and 22°C (-2.09 % bias, 10.81% discrepancy). When analyzed according to a_w , the highest and lowest a_w ranges

($a_w=0.54$, $a_w=0.17$) were the least reliable; with 16.19 % bias and 51.79% discrepancy and 1.34 % bias and 9.96% discrepancy, respectively. The consistent instability of the published model at the a_w extremes ($a_w=0.54$, $a_w=0.17$) and range of temperatures (22°C, 50°C 80°C) in whey protein powders containing fat suggest that revised equations for the Weibull model parameters δ and β which account for fat content may provide improved accuracy in predictions.

The influence of temperature, a_w and fat content on the Weibull model parameters (δ , β) were evaluated using multiple linear regression. Linear equations for $\log \delta$ and β were developed based on the significance of the independent variables at $\alpha=0.20$. The analysis indicated that temperature ($p<0.001$), a_w ($p=0.017$) and fat content ($p<0.001$) had a significant influence on the treatment time required for the first decimal log reduction (δ). The temperature and a_w were found to have independent effects on δ ($p=0.300$). Fat content significantly influenced the shape of the inactivation curve (β) ($p=0.036$). The temperature and a_w interaction effect also significantly influenced β ($p=0.166$). The shape of the curve was not significantly influenced by temperature ($p=0.994$) and a_w ($p=0.239$). The modified secondary models developed for *Salmonella* spp. survival in low a_w foods ($a_w<0.60$) held at 21 through 80°C containing up to 50% fat (w/w) and are presented in Equations (18) and (19).

$$\log \delta = 7.45 - 0.0653 * T - 2.9285 * a_w - 0.03422 * \text{fat \%} \quad (18)$$

$$\beta = 0.537982 - 0.01583 * (T * a_w) - 0.0031 * \text{fat \%} \quad (19)$$

The standard errors (SE) for equation (17) $\log \delta$, the constant, temperature, a_w and fat content are 1.00, 0.39, 0.004, 0.71, and 0.004, respectively. For equation (18), the standard errors for β , the constant, $T*a_w$ interaction, and fat content are 0.32, 0.24, 0.01, 0.001, respectively.

Validation of the modified secondary inactivation model

Food products equilibrated to $a_w < 0.60$ and held at temperatures between 21°C and 80°C were used to obtain 280 CFU data points of *Salmonella* inactivation. Products included toasted oat cereal ($a_w=0.13$, $a_w=0.27$, $a_w=0.45$), animal crackers ($a_w=0.16$ and $a_w=0.25$), micro-fine chia seed powder ($a_w=0.52$) and natural peanut butter ($a_w=0.38$). All product and water activity combinations were stored at 22°C for 168 days, 37°C for 84 days and 168 days, 50°C for 4 days, 60°C for 28 days, 70°C for 24 h and 80°C for 1h. Thirty-five δ and β values were calculated using the GInaFit Excel Add-in in Excel 2010. Since many δ values within the dataset differed by several orders of magnitude, the values were log transformed to achieve normality. A plot of the predicted versus observed log δ and β values are presented in Figure 3.7. Table 3.3 shows the correlation (R) % bias, and the % discrepancy values for the predicted versus observed values of δ and β in low-fat and high fat food products for the revised models.

The prediction performance of the revised model fell short of the established criteria ($\geq 70\%$ of the residuals (-1 log CFU (fail safe) to 0.50 log CFU (fail dangerous)) (Oscar, 2009) with 55% of the residuals inherent in the model occurring in the acceptable zone. The degrees of discrepancy and bias found between the modified secondary model predictions and the data used to develop the modified model were 4 % bias and 24% discrepancy ($A_f=1.24$). A % bias of 1.00 indicates perfect agreement between the observed and predictive values, while the accuracy factor is the average difference between the predictions and the observations. The positive bias (4%) indicates that on average, the model overestimates the log transformed values by a factor of 1.24 ($A_f=1.24$) or 24% discrepancy.

When applied to survival data in the 4 food products under study, the revised model showed 66% of residuals in the acceptable zone (Table 3.3). Low-fat (0.05% toasted oats, 13% animal crackers) and high-fat (32% chia seed powder, 50% natural peanut butter) foods showed 62% and 64% of residuals in the acceptable zone, respectively. The positive % bias (1.17%) for the revised model predictions indicates a tendency to overestimate survival in all of the foods tested. Survival in high-fat foods (% bias 0.86) and low-fat foods (1.38 % bias) were both overestimated by the revised model. The predictions differ from observed values by an accuracy factor of 1.08 (8% discrepancy) in low-fat foods and 1.10 (10% discrepancy) in high fat foods. On the other hand, the model by Santillana Farakos et al., 2013 underestimated survival in a low-fat (12% fat) peanut meal (-9 % bias) by an average factor of 1.50 (50% discrepancy). The tendency of the revised model to overestimate rather than underestimate survival in dry foods containing low fat and high fat contents indicates that it can be applied to more complex food systems than the Santillana Farakos et al. 2013 model.

Furthermore, the current model can be used to estimate the expected log reduction for a low a_w food at temperatures 21-80°C. Figure 3.8 shows the revised model predictions versus the observed log reductions in 4 low a_w foods after 24 hours of treatment at 22-80°C. Data points above the line of equivalence indicate that the observed log reduction was greater than the predicted log reduction (fail safe). As shown in Figure 3.8, the least reliable predictions occurred at the lowest a_w ranges ($a_w < 0.26$) in low-fat foods (toasted oat cereal, animal crackers). The predicted log reductions at 22°C were the most accurate for both low-fat and high-fat foods. This corresponds with the previously discussed result (Figure 3.1) that at 22°C, the protective effect of fat increased with increasing a_w . The predicted log reductions in high-fat foods (chia seed powder $a_w=0.52$, natural peanut butter $a_w=0.38$) shift from fail safe to fail dangerous as

temperature increases from 22°C to 80°C. The most accurate predictions occurred at 37°C and 50°C where fat content begins to influence survival of *Salmonella* at 50°C (Figures 3.2 and 3.3). Along the same line, predicted log reductions at 70°C were below the line of equivalence (fail danger) which reflects the significant influence of a_w and fat content discussed previously (Figure 3.5).

The tendency of the model to underestimate survival in low-fat foods more frequently than in high-fat foods is represented in the Weibull model predicted versus observed log CFU/g plots for low and high fat foods (Figure 3.9). Points below the line of equivalence indicate inactivation predictions (CFU/g) which were greater than observed values (fail safe). The model underestimated survival in toasted oat cereal and animal crackers to the same degree (Figure 3.9(a)). Survival was underestimated in peanut butter more frequently than chia powder (Figure 3.9(b)), which is most likely due to the differences in water activity and fat content.

The validation results demonstrate the ability of the modified model to estimate the survival of *Salmonella* in low a_w foods ($a_w < 0.60$) containing fat (<50% w/w) held at temperatures 22-80°C. Like the Santillana Farakos et al., 2013 model, predictions by the modified model are less reliable in foods with decreasing a_w and increasing fat content. However, the accuracy of the predictions improved with the modified model, particularly in foods held at 80°C. To the authors' knowledge, the equations developed and validated in this study represent the first non-linear thermal inactivation model to incorporate fat content as a predictor for *Salmonella* survival in a low a_w food system. The collection of the survival data used to derive the modified model was designed to simulate the conditions (temperature, a_w , fat content, storage time) under which a low a_w food might be stored or contaminated with dried *Salmonella* spp. Therefore, the validated model is a credible tool for use under conditions within the range of the modeled data

(CAST, 2006). The model output can be used as a data source for exposure assessments as part of quantitative microbial risk assessments in low a_w foods (CAST, 2006). A useful decision-support tool in Hazard Analysis and Critical Control Point (HACCP) systems for low a_w foods is another possible application for the revised model. Low a_w food product research and development projects involving the effect of temperature on *Salmonella* inactivation based on model predictions could also be informative for the food industry. However, a predictive model is a simplified version of reality and its application should be supplemented with previous experience and knowledge of microbial principles (Ross et al., 2008). The authors acknowledge that the accuracy and precision of the current model may be inflated since the bias and discrepancy values are based on log-transformed mean calculations and are influenced by outliers (Oscar, 2005). Nevertheless, the revised model developed in this study will aid in the risk mitigation strategies for the control of *Salmonella* in low a_w foods containing low and high amounts of fat.

Conclusions

In the current study, a systematic approach was used to revise the Santillana Farakos et al., 2013 model based on the hypothesis that fat content influences the survival of *Salmonella* in low a_w foods. In powders held at 22°C, the protective effect of fat increased with increasing a_w . At temperatures $\geq 50^\circ\text{C}$, fat content provided a protective effect on survival in equilibrated whey protein powders ($a_w \leq 0.60$) when compared to unsupplemented whey protein powders under the same conditions. Additional fat content (20% versus 50%) did not increase this protective effect. Reduced a_w significantly increased survival in powders held at 37°C and 70°C. The Weibull model provided the best description of *Salmonella* inactivation in fat-supplemented protein powders equilibrated to $a_w \leq 0.60$ and held at temperatures from 21 to 80°C. A secondary model was developed for the treatment time required for the first decimal log reduction (δ) and the

shape factor (β) as influenced by temperature, a_w , and fat content. Although the revised model fell short of the established acceptable residual zone, it was useful in predicting survival in low a_w ($a_w \leq 0.60$) foods containing 0-50% fat and resulted in improved accuracy of predictions compared to the previously developed model. Future research could include expansion of the model to include additional fat percentages or types of fat. Temperatures which are applicable to the processing and storage of low a_w foods could also be included in the expansion of the model.

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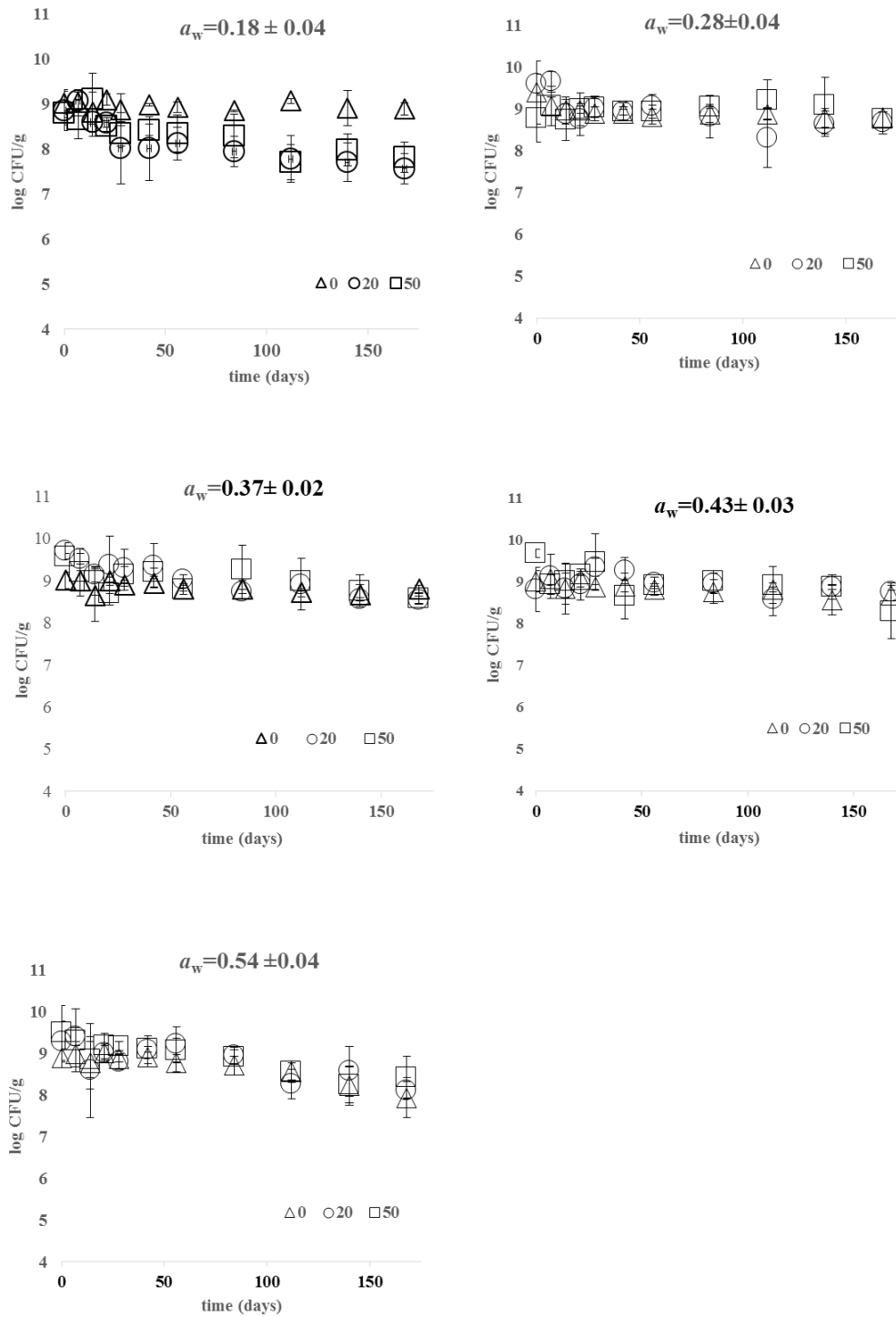


Figure 3.1. *Salmonella* inactivation at 22°C during 6 months (168 days) of storage in whey protein isolate powders with various fat contents (non-fat, 20%, 50% (w/w)) equilibrated to 5 water activity (a_w) ranges <0.60 . Error bars indicate the $\pm$ standard deviation of the average of three replicate survival curves for each a_w -fat content combination

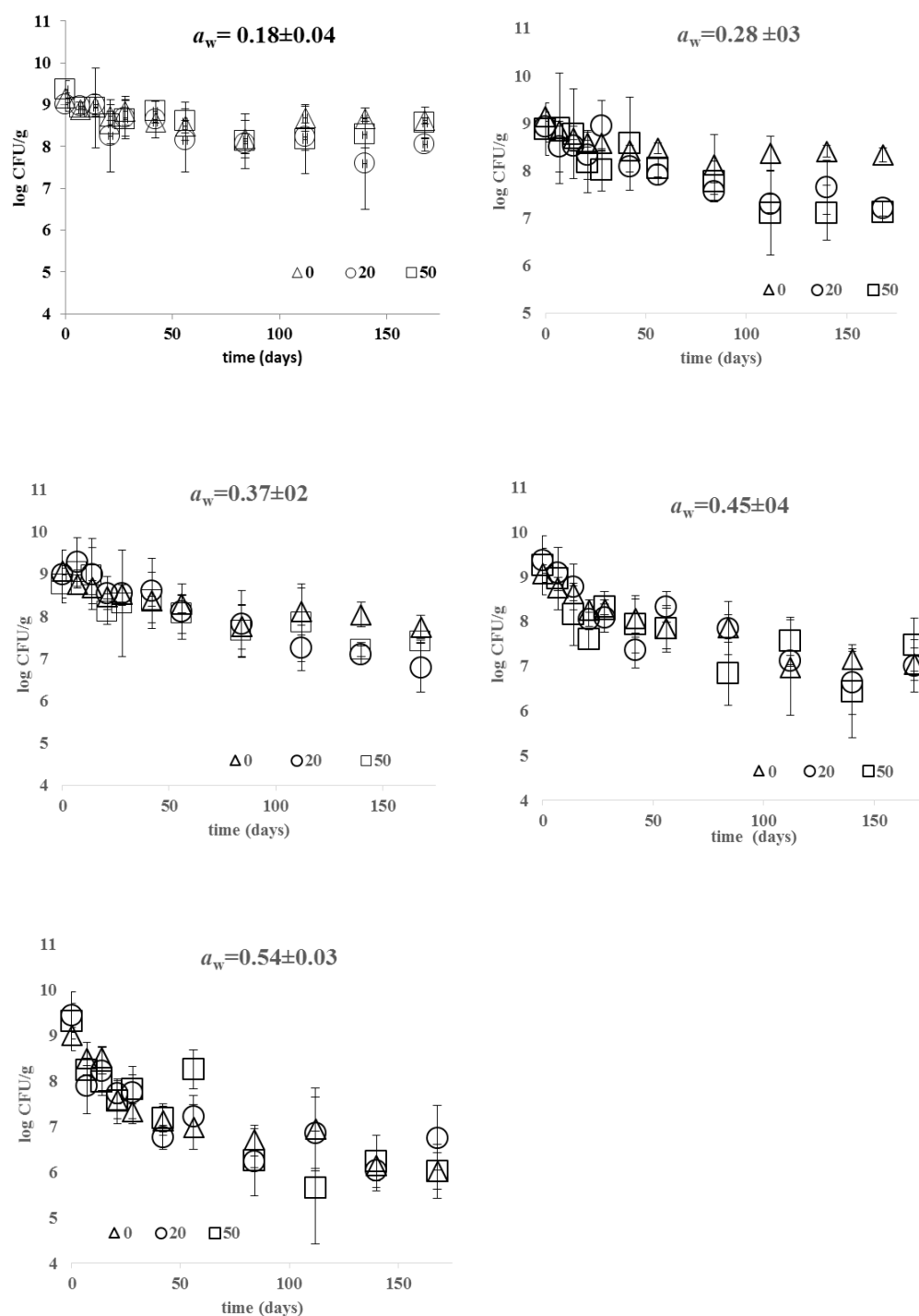


Figure 3.2. *Salmonella* inactivation at 37°C during 6 months (168 days) of storage in whey protein isolate powders with various fat contents (non-fat, 20%, 50% (w/w)) equilibrated to 5 water activity (a_w) ranges <0.60 . Error bars indicate the $\pm$ standard deviation of the average of three replicate survival curves for each a_w -fat content combination.

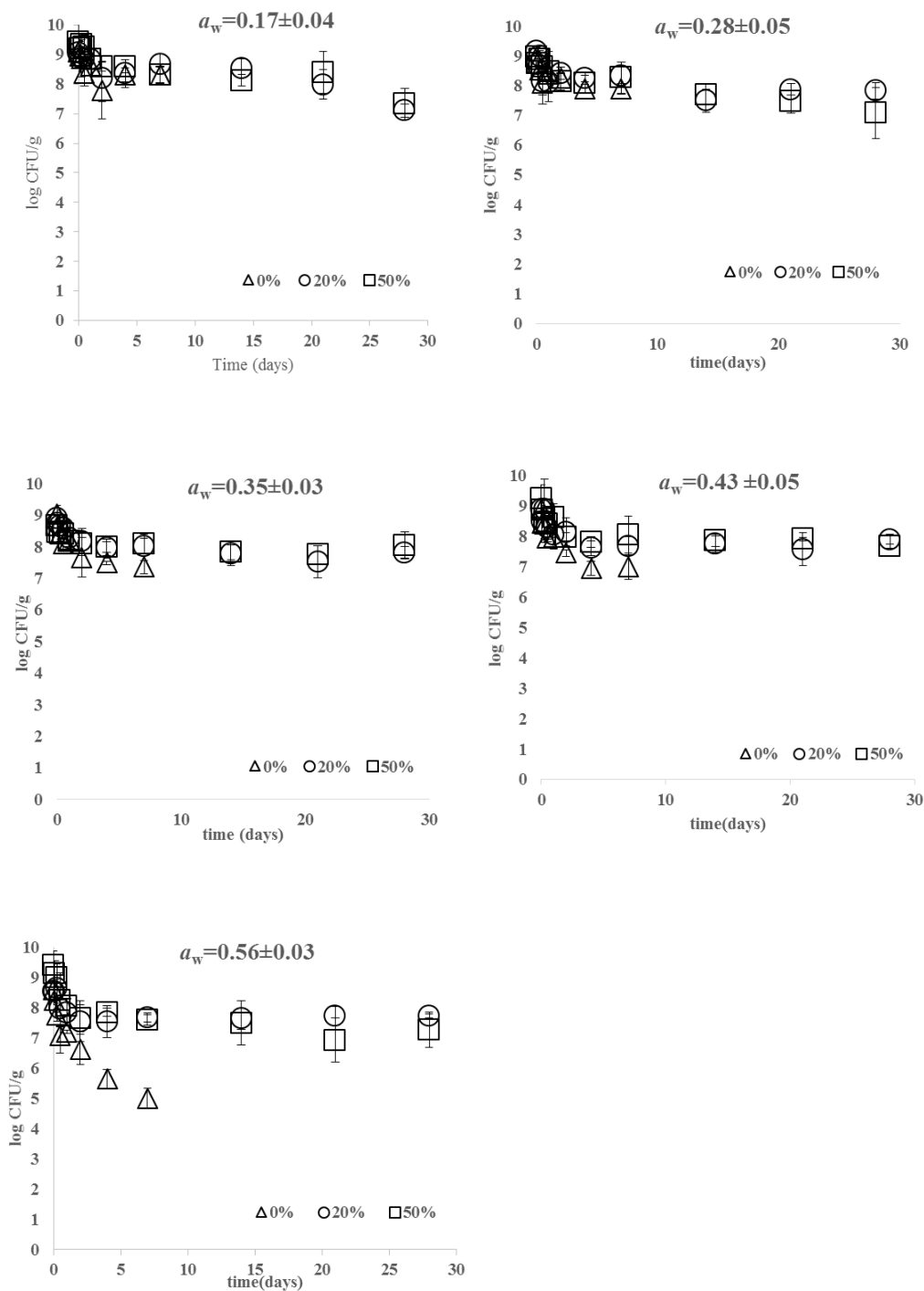


Figure 3.3. *Salmonella* inactivation at 50°C during 1 month (28 days) of storage in whey protein isolate powders with various fat contents (non-fat, 20%, 50% (w/w)) equilibrated to 5 water activity (a_w) ranges <0.60 . Error bars indicate the $\pm$ standard deviation of the average of three replicate survival curves for each a_w -fat content combination

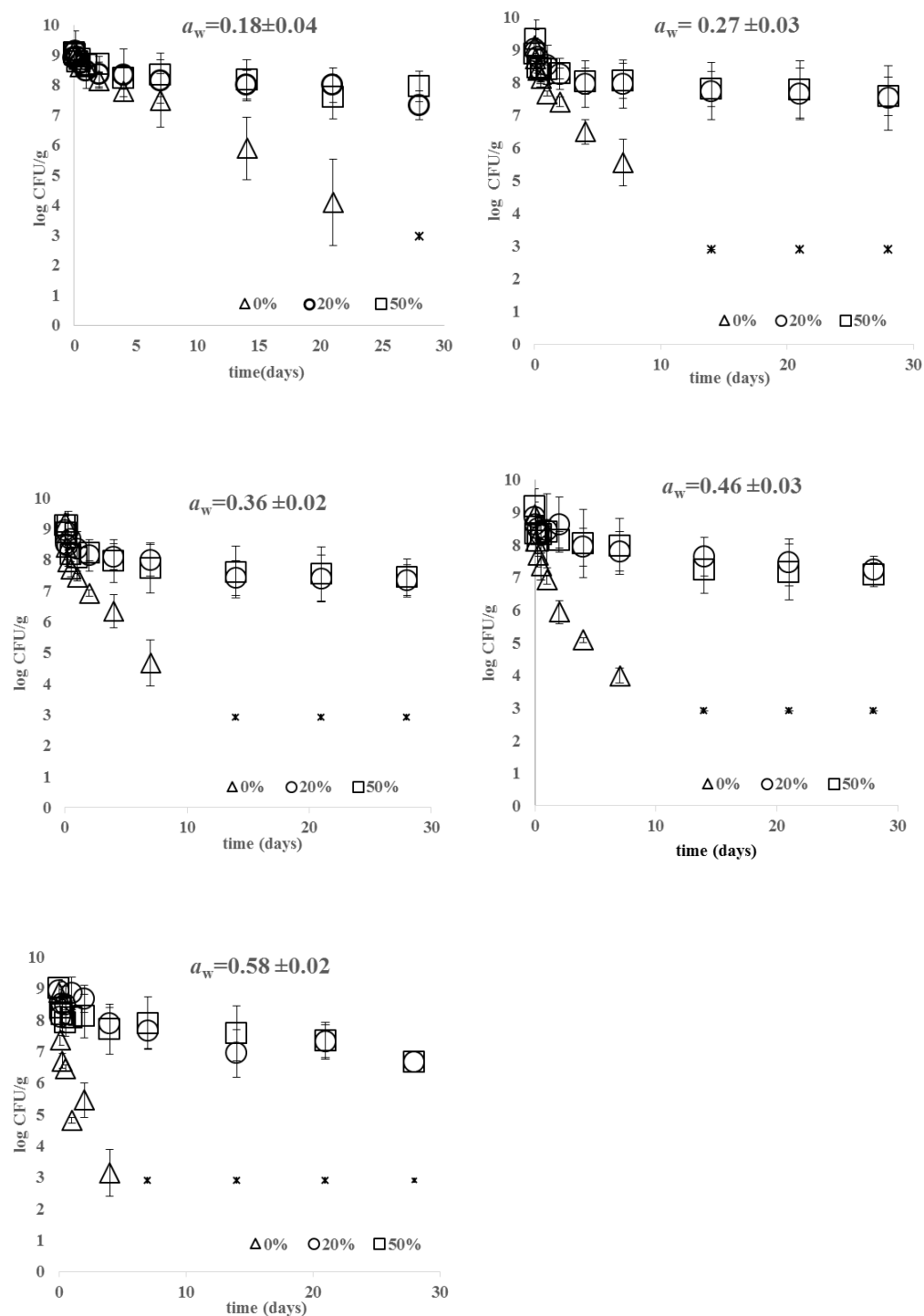


Figure 3.4. *Salmonella* inactivation at 60°C during 1 month (28 days) of storage in whey protein isolate powders with various fat contents (non-fat, 20%, 50% (w/w)) equilibrated to 5 water activity (a_w) ranges <0.60. Error bars indicate the $\pm$ standard deviation of the average of three replicate survival curves for each a_w -fat content combination. $\times$ indicates counts that were below the limit of detection (2.9 log CFU/g)

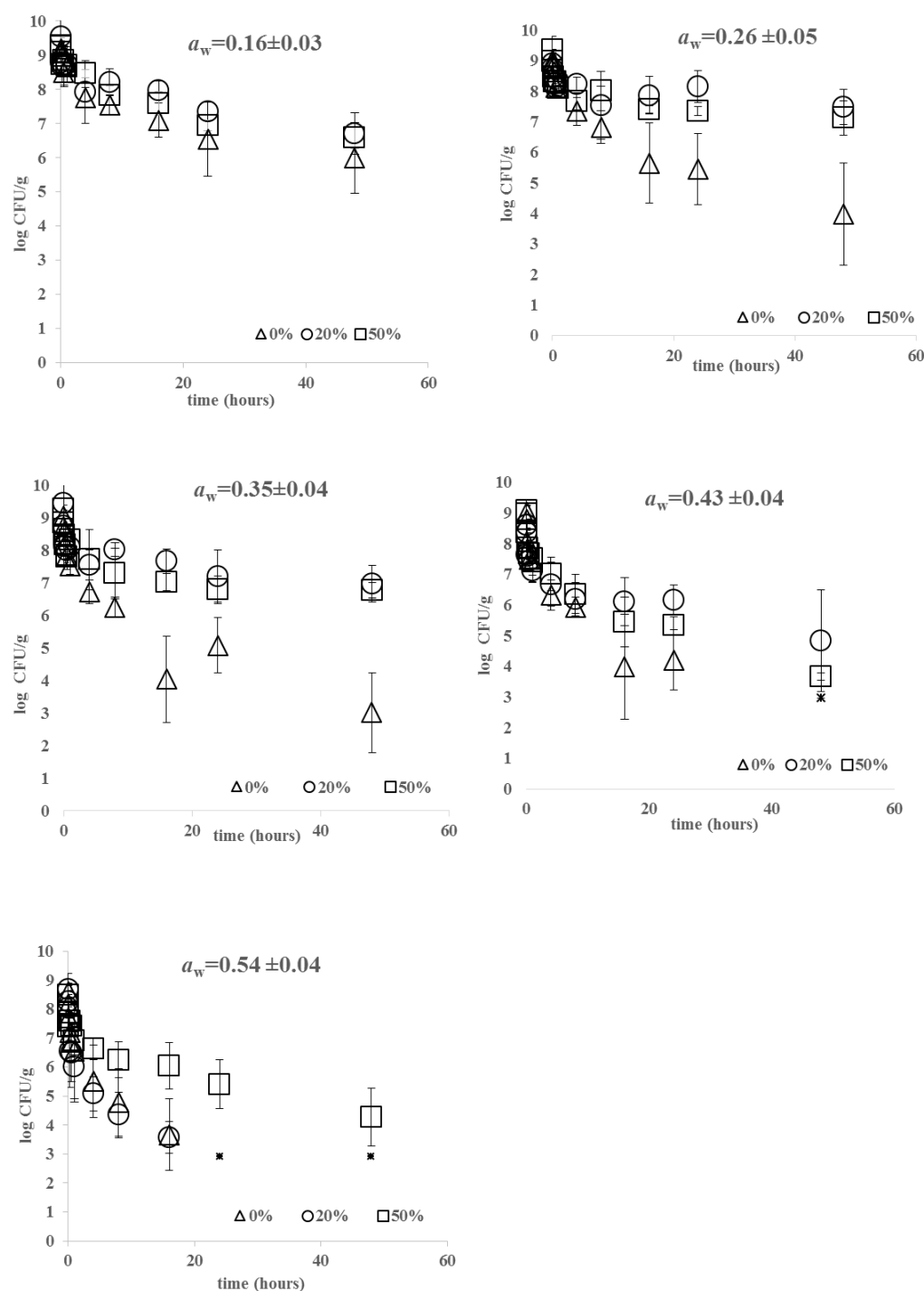


Figure 3.5. *Salmonella* inactivation at 70°C during 48 h (2 days) of storage in whey protein isolate powders with various fat contents (non-fat, 20%, 50% (w/w)) equilibrated to 5 water activity (a_w) ranges <0.60 . Error bars indicate the $\pm$ standard deviation of the average of three replicate survival curves for each a_w -fat content combination. * indicates counts that were below the limit of detection (2.9 log CFU/g)

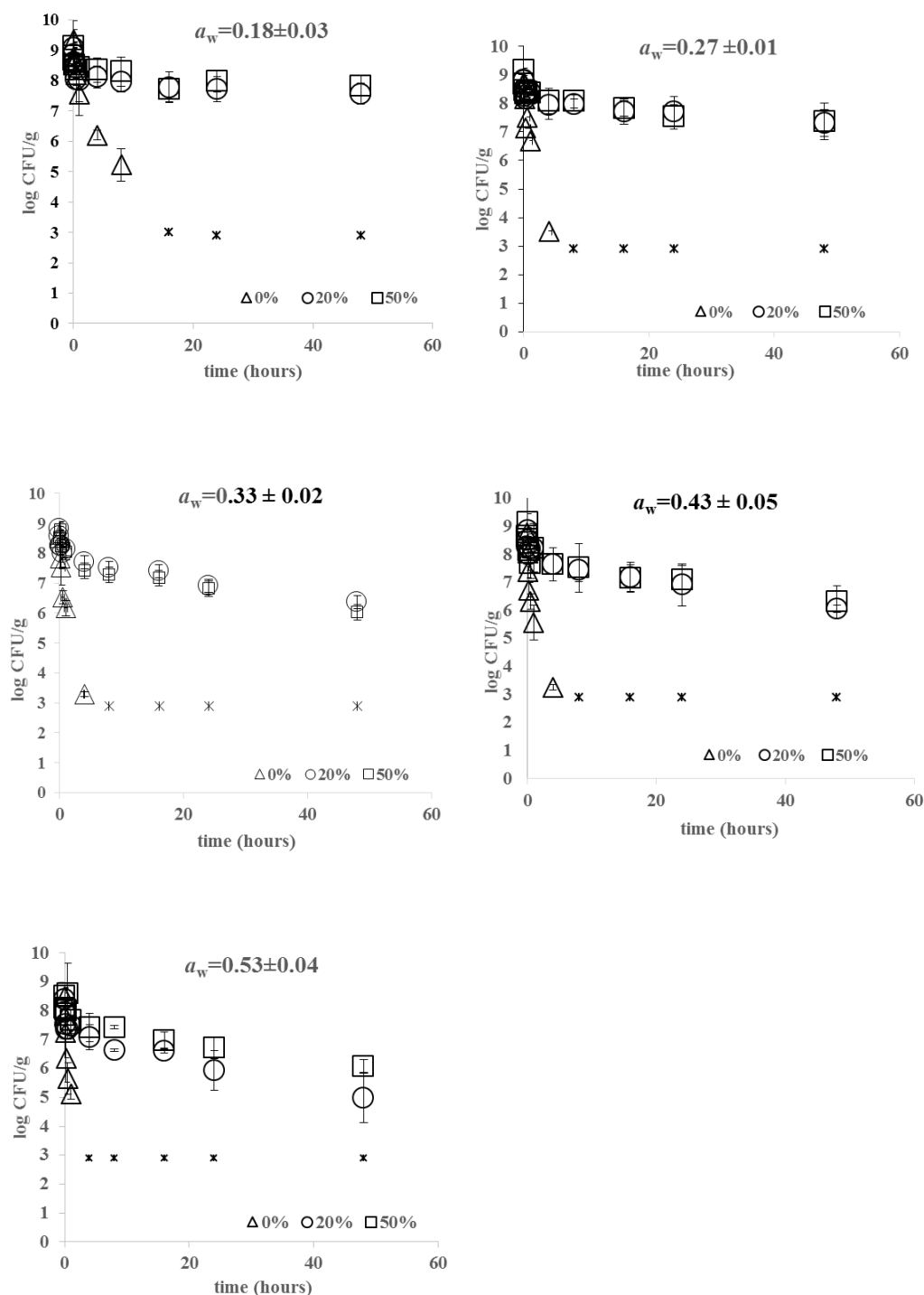


Figure 3.6. *Salmonella* inactivation at 80°C during 48 h (2 days) of storage in whey protein isolate powders with various fat contents (non-fat, 20%, 50% (w/w)) equilibrated to 5 water activity (a_w) ranges <0.60. Error bars indicate the $\pm$ standard deviation of the average of three replicate survival curves for each a_w -fat content combination. $\times$ indicates counts that were below the limit of detection (2.9 log CFU/g)

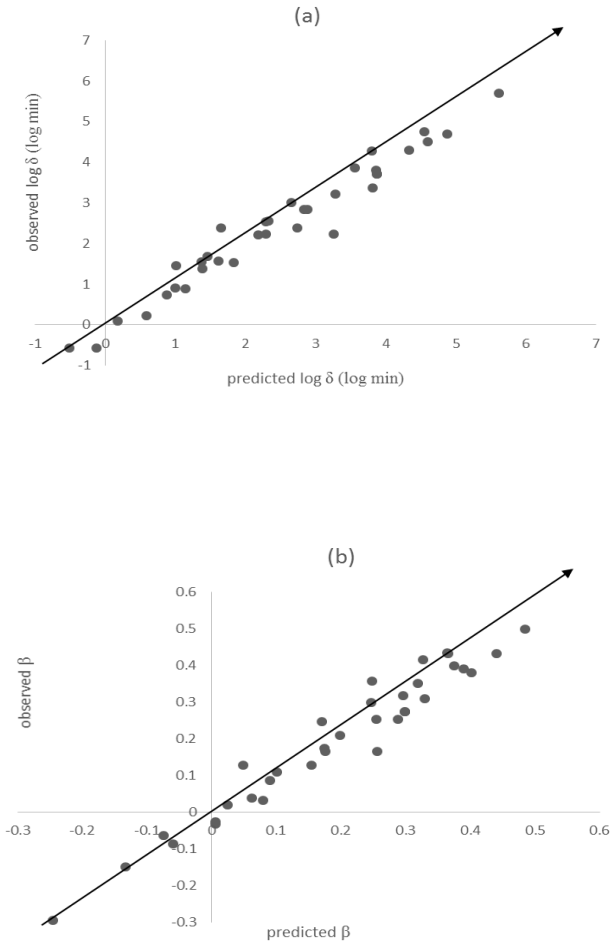


Figure 3.7. Model validation data for *Salmonella* inactivation in toasted oat cereal, chia seed powder, natural peanut butter and animal crackers equilibrated to $a_w = <0.60$ and held at 21-80°C for 24 h (a) Observed versus predicted values for Weibull model parameter (δ) the treatment time required for the first decimal log reduction (b) Observed versus predicted values for the Weibull model parameter (β) shape factor of the inactivation curve.

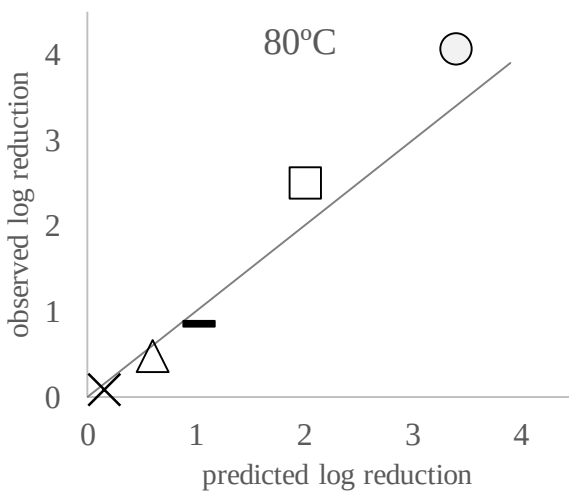
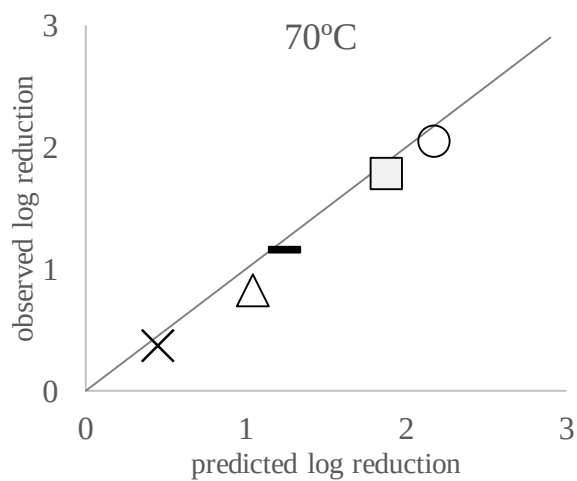
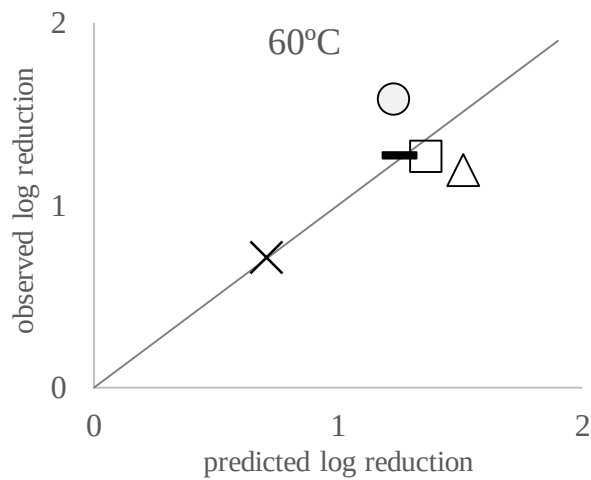
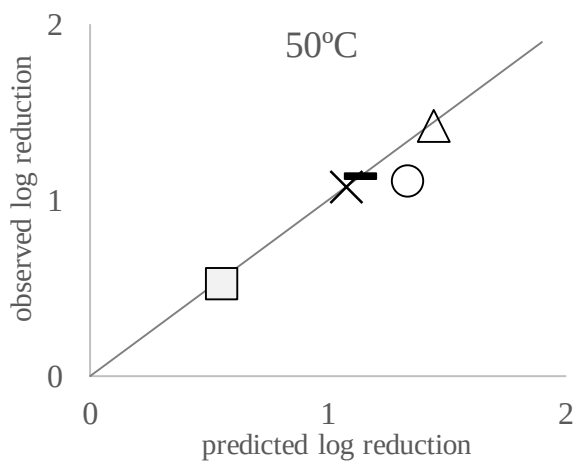
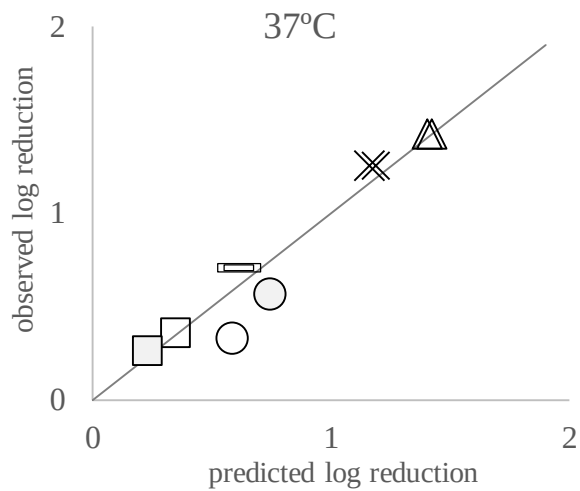
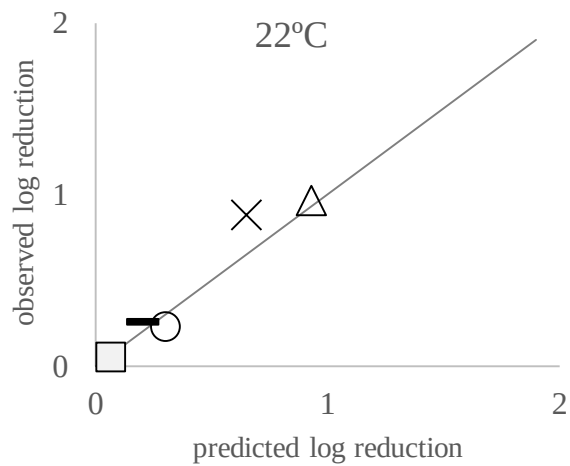


Figure 3.8. Predicted log reduction versus observed log reductions in 4 foods after 24 h of treatment at 21-80°C Points below the line of equivalence indicate predicted log reductions that were greater than observed reductions (fail danger). X= chia powder ($a_w=0.52$), Δ = natural peanut butter ($a_w=0.38$), $\square$ = toasted oat cereal ($a_w=0.45$), $\blacksquare$ = toasted oat cereal ($a_w=0.23$), — = toasted oat cereal ($a_w=0.13$), $\bigcirc$ = animal crackers ($a_w=0.19$), $\bigcirc$ = animal crackers ($a_w=0.25$).

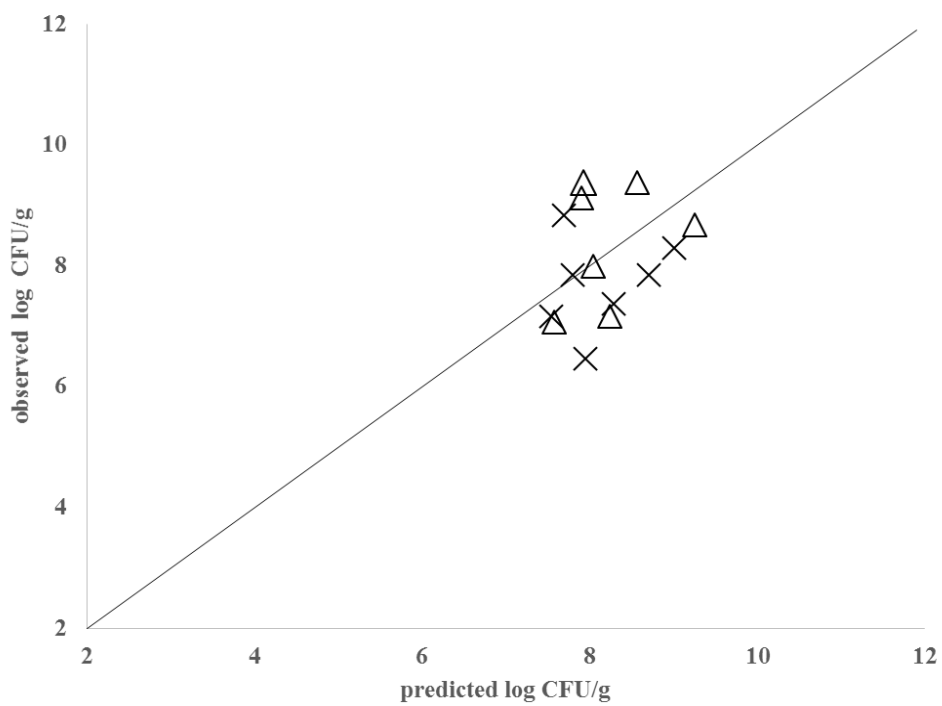
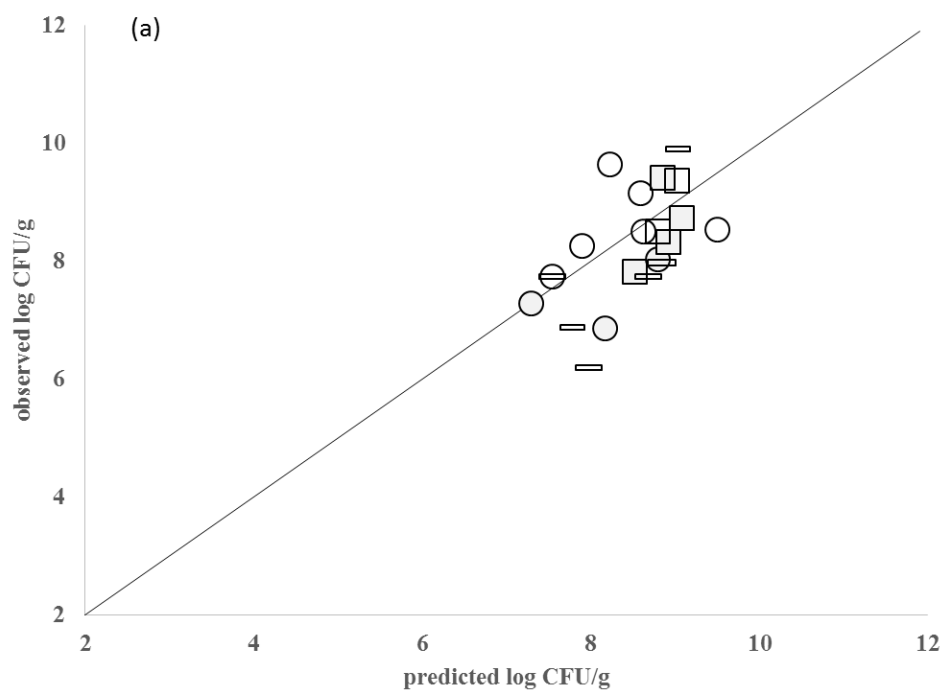


Figure 3.9. Predicted versus observed *Salmonella* counts (log CFU/g) in 4 low a_w ($a_w < 0.60$) food products after 24 h of treatment at temperatures 21-80°C (a) low-fat foods (toasted oat cereal $\blacksquare$ $a_w = 0.45$, $\blacksquare$ $a_w = 0.23$, $\bigcirc$ $a_w = 0.18$, 0.05% fat), (animal crackers $\bullet$ $a_w = 0.16$, $a_w = 0.25$, 13% fat) (b) high-fat foods (chia seed powder, X $a_w = 0.52$, 32% fat) (natural peanut butter, $\blacktriangle$ $a_w = 0.38$, 50% fat). Data points below the line of equivalence indicate predicted log CFU/g values that were greater than the observed log CFU/g values (fail safe).

Table 3.1. Statistical parameter fit data used to determine the best equation (Weibull, Biphasic, Geeraerd-tail, Log-linear, Corolller and Baranyi) to describe the data for *Salmonella* inactivation experiments in whey protein isolate powders with varying fat contents (20-50% w/w) and 5 water activity ranges ($a_w < 0.60$) at 6 T (°C). The highest adjusted R^2 (R^2_{adj}) and lowest root mean square error (RMSE) for each T, a_w and fat combination are shown in bold.

a_w^a	Fat %	T	Weibull ^b	Biph ^c	G-tail ^d	Log-lin ^e	Cor ^f	Baran _g	Weibull	Biph	G-tail	Log-lin	Cor	Baran
			R^2_{adj}					RMSE						
0.18	20	21	0.18	0.24	0.27	0.07	0.15	0.07	0.47	0.46	0.45	0.51	0.49	0.51
±0.02		37	0.33	0.32	0.35	0.32	0.32	0.32	0.52	0.52	0.51	0.52	0.51	0.52
		50	0.53	-	-	0.50	-	0.50	0.46	-	-	0.47	-	0.46
		60	0.46	0.47	0.41	0.42	0.48	0.42	0.50	0.49	0.52	0.51	0.49	0.51
		70	0.69	0.70	0.54	0.52	0.68	0.51	0.58	0.58	0.71	0.72	0.59	0.74
		80	0.48	0.54	0.51	0.28	-	0.37	0.44	0.41	0.43	0.52	-	0.48
0.17	50	21	0.01	-	-	-0.01	-	-	0.27	-	-	0.27	0.27	-
±0.01		37	0.25	0.21	0.24	0.16	0.23	0.16	0.49	0.50	0.48	0.51	0.49	0.51
		50	0.67	0.67	0.60	0.57	-	0.57	0.41	0.41	0.46	0.47	-	0.47
		60	0.52	0.51	0.49	0.42	0.51	0.42	0.41	0.42	0.43	0.45	0.42	0.45
		70	0.94	0.93	0.92	-	0.94	0.92	0.24	0.27	0.28	-	0.23	0.28
		80	0.53	0.52	0.49	0.37	0.53	0.48	0.34	0.35	0.36	0.40	0.32	0.36
0.27	20	21	0.37	0.40	0.37	0.28	0.43	0.28	0.38	0.38	0.38	0.41	0.37	0.41
±0.02		37	0.63	0.64	0.65	0.61	0.66	0.66	0.40	0.40	0.39	0.42	0.39	0.39
		50	0.49	0.50	0.44	0.31	-	0.39	0.41	0.41	0.43	0.48	-	0.45
		60	0.41	0.39	0.40	0.31	0.40	0.38	0.57	0.58	0.58	0.42	0.58	0.59
		70	0.32	0.93	0.26	0.09	0.30	0.19	0.51	0.52	0.50	0.55	0.52	0.56
		80	0.54	0.56	0.43	0.40	0.52	0.40	0.37	0.37	0.60	0.42	0.38	0.42
0.27	50	21	-	-	-	0.02	-	-	-	-	-	0.36	-	-
		37	0.44	0.43	0.45	0.47	0.43	0.47	0.66	0.67	0.66	0.65	0.66	0.65
		50	0.65	0.66	0.60	0.61	-	0.61	0.41	0.41	0.44	0.44	-	0.44

±0.02		60	0.46	0.48	0.43	0.34	0.48	0.42	0.47	0.46	0.48	0.52	0.46	0.49
		70	0.81	0.31	0.68	0.47	0.82	0.60	0.31	0.34	0.41	0.52	0.31	0.46
		80	0.62	0.60	0.51	0.45	0.61	0.50	0.37	0.38	0.42	0.45	0.38	0.43
0.36	20	21	-	-	0.05	0.04	-	0.04	-	-	0.34	0.37	-	0.37
±0.01		37	0.66	-	0.66	0.67	0.65	0.67	0.55	-		0.55	0.56	0.55
		50	0.66	0.67	0.66	0.45	0.68	0.65	0.30	0.29	0.30	0.38	0.29	0.30
		60	0.56	0.52	0.53	0.48	0.55	0.53	0.44	0.47	0.46	0.48	0.45	0.46
		70	0.65	0.66	0.48	0.37	0.63	0.37	0.46	0.45	0.55	0.61	0.47	0.61
		80	0.64	0.63	0.58	0.56	0.64	0.56	0.51	0.50	0.54	0.56	0.52	0.56
0.35	50	21	0.30	0.33	-	0.26	0.28	0.26	0.38	0.37	-	0.39	0.33	0.39
±0.03		37	0.44	0.54	0.55	0.54	0.43	0.54	0.49	0.49	0.48	0.49	0.48	0.49
		50	0.47	0.46	0.47	0.25	0.46	0.46	0.46	0.26	0.26	0.31	0.26	0.26
		60	0.48	0.50	0.46	0.32	0.50	0.43	0.55	0.54	0.56	0.63	0.54	0.58
		70	0.74	0.68	0.64	0.44	-	0.64	0.46	0.51	0.54	0.61	-	0.54
		80	0.71	0.71	0.64	0.61	0.71	0.61	0.50	0.50	0.56	0.58	0.51	0.58
0.43	20	21	0.51	0.50	-	0.50	0.49	0.50	0.33	0.34	0.34	0.33	0.34	0.34
±0.02		37	0.71	0.73	0.65	0.62	0.75	0.62	0.48	0.46	0.52	0.54	0.45	0.54
		50	0.39	0.44	0.46	0.17	0.45	0.45	0.47	0.45	0.45	0.55	0.45	0.45
		60	0.42	0.40	0.40	0.38	0.40	0.39	0.57	0.58	0.58	0.59	0.58	0.59
		70	0.71	0.73	0.62	0.57	0.74	0.57	0.65	0.63	0.75	0.67	0.62	0.80
		80	0.74	0.75	0.71	0.69	-	0.69	0.44	0.43	0.46	0.48	-	0.48
0.44	50	21	0.22	-	-	0.16	0.19	0.16	0.34	0.34	0.36	0.48	0.35	0.36
±0.01		37	0.49	0.48	0.48	0.38	0.48	0.48	0.68	0.69	0.69	0.75	0.68	0.69
		50	0.65	0.62	0.63	0.33	0.67	0.62	0.32	0.34	0.34	0.45	0.32	0.34
		60	0.49	0.47	0.45	0.42	0.47	0.43	0.57	0.60	0.59	0.60	0.58	0.60
		70	0.91	-	-	-	0.91	0.83	0.49	-	-	-	0.46	0.70
		80	0.59	0.62	0.50	0.48	0.57	0.48	0.61	0.59	0.67	0.52	0.62	0.68
0.55	20	21	0.26	0.24	-	0.28	0.30	0.28	0.49	0.49	-	0.48	0.47	0.48
±0.01		37	0.69	0.66	0.66	0.45	0.71	0.67	0.56	0.58	0.58	0.75	0.50	0.58
		50	0.27	0.43	0.45	0.08	0.45	0.45	0.48	0.42	0.42	0.54	0.42	0.42
		60	0.55	0.57	0.57	0.53	0.57	0.53	0.61	0.60	0.59	0.62	0.58	0.62
		70	0.81	0.85	0.72	-	-	0.72	0.84	0.75	1.02	-	-	1.02

		80	0.78	0.81	0.73	0.72	-	0.72	0.46	0.43	0.51	0.53	-	0.53
0.54	50	21	0.40	0.40	0.41	0.42	0.39	0.42	0.39	0.39	0.39	0.38	0.40	0.38
		37	0.72	0.70	0.70	0.70	0.71	0.71	0.63	0.64	0.65	0.65	0.64	0.65
±0.02		50	0.66	0.71	0.67	0.40	0.71	0.66	0.53	0.49	0.52	0.42	0.49	0.52
		60	0.55	0.59	-	0.49	0.53	0.49	0.48	0.45	-	0.51	0.49	0.51
		70	0.80	0.80	0.72	0.70	0.80	0.70	0.57	0.57	0.66	0.69	0.57	0.69
		80	0.73	0.72	0.67	0.65	0.72	0.65	0.41	0.52	0.45	0.47	0.42	0.47

^a average measured water activity of 3 survival curve replications at temperatures 21-80°C

^b (Mafart et al. 2002)

^c (Cerf, 1977)

^d (Geeraerd et al. 2005)

^e (Bigelow and Etsy, 1920)

^f (Coroller et al., 2006)

^g (Baranyi and Roberts, 1994)

^h $f_{\text{test}} > f_{\text{table}}$

Table 3.2. Fitted Weibull model parameters (δ , β) for *Salmonella* inactivation in whey protein isolate powders containing added fat (20% and 50% w/w)) equilibrated to 5 a_w ranges <0.60 at 21-80°C

Water activity ^a	Fat %	T °C	Log δ^b	Log se δ^c	β^d	Se β^e
0.18±0.02	20	21	2.37	2.70	0.16	0.13
		37	2.02	1.81	0.53	0.28
		50	2.75	1.68	2.70	1.11
		60	2.41	2.30	0.39	0.19
		70	-0.77	-0.51	0.19	0.05
		80	0.82	0.97	0.20	0.08
0.17±0.01	50	21	2.34	2.02	5.13	8.26
		37	2.05	2.13	0.26	0.16
		50	1.96	1.88	0.32	0.09
		60	2.33	2.26	0.31	0.12
		70	0.31	-0.08	0.35	0.04
		80	1.34	1.26	0.25	0.10
0.27±0.02	20	21	2.08	1.97	0.31	0.14
		37	1.82	1.46	0.60	0.19
		50	1.96	2.10	0.19	0.07
		60	2.21	2.29	0.31	0.15
		70	0.94	1.26	0.15	0.08
		80	1.01	1.06	0.21	0.08
0.27±0.02	50	21	- ^f	-	-	-
		37	1.78	1.58	0.67	0.32
		50	2.25	2.04	0.42	0.14
		60	2.24	2.27	0.28	0.12
		70	-0.51	-0.43	0.16	0.03
		80	0.44	0.56	0.18	0.05
0.36±0.01	20	21	-	-	-	-
		37	1.75	1.37	0.81	0.26
		50	2.27	2.14	0.25	0.07
		60	2.15	2.10	0.32	0.12
		70	-1.36	-0.97	0.12	0.03
		80	0.77	0.70	0.36	0.12
0.35±0.03	50	21	2.28	2.31	0.24	0.13
		37	1.88	1.54	0.68	0.27
		50	3.13	3.11	0.20	0.08
		60	1.77	1.95	0.24	0.10
		70	-0.27	-0.14	0.21	0.05
		80	0.60	0.51	0.36	0.10
0.43±0.02	20	21	2.12	1.68	0.56	0.22
		37	1.26	1.10	0.42	0.10
		50	2.06	2.27	0.19	0.08
		60	2.34	2.29	0.39	0.20
		70	0.24	0.36	0.30	0.10
		80	1.10	0.68	0.59	0.16
0.44±0.01	50	21	2.96	3.29	0.17	0.12
		37	1.11	1.20	0.32	0.13
		50	1.59	1.69	0.16	0.04
		60	1.66	1.83	0.24	0.09
		70	0.15	-0.14	0.44	0.06
		80	0.42	0.52	0.30	0.11

0.55±0.01	20	21	2.24	1.44	1.75	1.21
		37	0.29	0.39	0.25	0.06
		50	2.56	2.79	0.18	0.10
		60	2.28	2.08	0.56	0.24
		70	-0.46	-0.43	0.40	0.10
		80	0.48	0.34	0.37	0.09
0.54±0.02	50	21	2.20	1.65	0.78	0.41
		37	0.93	0.85	0.42	0.10
		50	0.92	1.10	0.21	0.06
		60	1.66	1.83	0.24	0.09
		70	0.34	0.21	0.41	0.09
		80	0.78	0.61	0.37	0.10

^a Average measured $a_w \pm$ sd of 3 replications at 21-80°C

^b Weibull model parameter for time required for first decimal log reduction (minutes)

^c Standard error of Weibull model parameter δ

^d Weibull model fit parameter that describes the shape of the curve

^e Standard error of Weibull model parameter β

^f Regression resulted in a positive slope

Table 3.3. Model performance indices for a revised model predicting *Salmonella* inactivation in low a_w ($a_w < 0.60$) foods with fat levels (0-50% w/w) held at 21-80°C.

Food product		R ^a	P ^b	% resid ^c	%B _f ^d	%D _f ^e
All	Pred vs obs ^f	0.48	0.004	66	1.17	9.34
Toasted oats, animal crackers,	δ ^g	0.98	<0.001	- ⁱ	-	-
chia seed powder, natural peanut butter	β ^h	0.98	<0.001	-	-	-
Low-fat (< 13%)	Pred vs obs	0.58	0.006	62	1.38	8.61
Toasted oats, animal crackers	δ	0.99	<0.001	-	-	-
	β	1.00	<0.001	-	-	-
High-fat (32-50%)	Pred vs obs	0.27	0.342	64	0.86	10.45
Chia seed powder, natural peanut butter	δ	0.96	<0.001	-	-	-
	β	0.97	<0.001	-	-	-

^a calculated correlation coefficient

^b significance of correlation test (p-value)

^c % of residuals in the acceptable zone (-1 log to 0.5 log)

^d calculated % bias

^e calculated % discrepancy

^f predicted versus observed CFU/g values in model validation experiments

^g Weibull model parameter, treatment time required for the first decimal log reduction

^h Weibull model parameter, shape factor

ⁱ not applicable

CHAPTER 4

VALIDATION OF SECONDARY MODEL PREDICTIONS OF *SALMONELLA* SURVIVAL IN A LOW-WATER ACTIVITY, HIGH FAT PEANUT FLOUR MODEL FOOD SYSTEM CONTAINING AN EMULSIFIER HELD AT 37°C OR 70°C ²

²Trimble, L.M., Patel, S.S., Barnes, S.R. and J.F. Frank. To be submitted to *The Journal of Food Protection*

Abstract

Certain *Salmonella* serovars are able to survive for extended periods of time in low water activity (a_w) peanut products such as peanut paste and peanut butter. These foods may contain ingredients such as salt, sugar or emulsifiers like monoglycerides. Predictive models are useful tools to estimate the inactivation of pathogens under specific environmental conditions. The objectives of this study were to a) determine the influence of temperature and 1.625% (w/w) of monostearin on the Weibull model parameters (δ and β) in a high fat (50% w/w), low a_w peanut flour model food system and b) evaluate the performance of secondary Weibull model parameters (δ and β) previously validated for *Salmonella* inactivation in low a_w foods ($a_w < 0.60$) held at 21-80°C. Supplemented peanut flours were equilibrated to $a_w = 0.46 \pm 0.04$ and were inoculated with a dried cocktail of *Salmonella* serovars (Tennessee, Agona, Montevideo, Typhimurium). Flours were stored for 48 hours at 70°C or 28 days at 37°C. The Weibull model parameters for the survival data were determined using GInaFit software. Temperature significantly influenced the δ values ($p=0.023$), while monostearin did not ($p=0.375$). Temperature ($p=0.70$) and monostearin ($p=0.344$) did not influence β values. At 37°C, the secondary model performance was acceptable with 100% of the residuals in the established acceptable zone and fail safe predictions in flours held for 28 days. At 70°C, the model performance fell short of the established criteria (70% of residuals in acceptable zone) with fail danger predictions after 1 h (unsupplemented) and 4 h (monostearin). Model predictions at both temperatures were more accurate in unsupplemented peanut flour. This study illustrates the temperature dependence of the Weibull model parameter δ and suggests that an alternative model may provide more accurate predictions of *Salmonella* survival in low a_w , high fat food matrices held at elevated temperatures.

Introduction

Salmonella infection remains one of the leading causes of foodborne illness in the United States (CDC, 2015). Researchers have estimated the prevalence of *Salmonella* on raw shelled peanuts in the United States to be 2.33% (Calhoun et al., 2013). Over the last 20 years, at least seven documented outbreaks of salmonellosis have occurred in peanuts or peanut products worldwide (Schaffner et al., 2013). Products such as peanut flour, peanut meal, peanut paste, and peanut protein have a low water activity ($a_w < 0.70$) and are used as ingredients in a variety of foods. In 2009, one of the most notable outbreaks of *Salmonella* in peanut products occurred in peanut paste and peanut butter, which resulted in 714 illnesses and 9 deaths across 46 US states (CDC, 2009).

Certain *Salmonella* serotypes have demonstrated the ability to persist for prolonged periods of time in low a_w conditions (Santillana Farakos et al. 2013). Studies based on food components such as fat, sugar, and salt have shown varying degrees of influence on the persistence of *Salmonella* in dry foods (Goepfert, et al. 1970, Kataoka, et al., 2014, Shrestha and Nummer, 2016). Emulsifiers such as monoglycerides, diglycerides or combinations of these are used to prevent oil separation in peanut butter. The amount of emulsifier in peanut butter ranges from 1-5.5%, with 3.25% as the most common amount (Woodroof, 1973). Monoglycerides have demonstrated growth inhibition of gram-negative foodborne pathogens at high concentrations (Bunkova et al. 2011).

Control of *Salmonella* in foods which have a history of rare, high concentration contamination events (peanut butter) requires the implementation of good agricultural practices and a HACCP program (Schaffner et al. 2013). Predictive models are a useful quantitative support tool in such risk mitigation strategies. A model for *Salmonella* inactivation has been developed and validated

for low a_w ($a_w < 0.60$) foods held at 21-80°C (Santillana Farakos et al., 2013). A recent analysis of literature data on *Salmonella* survival in low a_w foods showed that the food product type and composition influences the reliability of predictions by the model (Santillana Farakos, et al., 2014). The aim of this study is to determine the influence of a monoglyceride on the survival of *Salmonella* and evaluate the performance of this model in a low a_w , high fat peanut flour model food system containing a monoglyceride.

Materials and Methods

Preparation of peanut flour

Partially defatted, light-roast peanut flour (50% protein, 12% fat) (Golden Peanut Company, Alpharetta, GA), peanut oil (Planters, Kraft Foods Group, Inc., Northfield, IL) and monostearin (Tokyo Chemical Industry Company, LTD., Tokyo, Japan) were chosen as a model food system to simulate the characteristics of peanut butter. A 4% solution of peanut flour, 50% (w/w) peanut oil and 1.625% (w/w) monostearin was homogenized at 9000 rpm for 1 min (Polytron PT 120, Brinkmann, Instruments Services, Inc., Switzerland). Emulsions were poured into previously frozen pans located in a -30°C freezer within 1 min of homogenization to maintain emulsion stability. Pans were frozen overnight at -30°C and were freeze dried from -40°C to 0°C for 20 h (Revo Series Development Freeze-Dryer, Millrock Technology, Kingston, NY). Freeze-dried flours were stored in calcium sulfate jars until use within 7 days.

Equilibration of peanut flour

Freeze-dried peanut flours were equilibrated over saturated potassium carbonate salt (Amresco, Solon, OH) to $a_w = 0.46 \pm 0.04$ in a vacuum desiccator at 22°C. Water activity was measured using a_w meter (AquaLab Series 4TEV, Decagon Devices Inc., Pullman, WA) with ± 0.003 precision.

Sample inoculation and packaging

A four-strain cocktail of previously dried *Salmonella* serotypes associated with outbreaks in low a_w foods (*S.*Agona, *S.*Montevideo, *S.*Tennessee, and *S.*Typhimurium) was used in this study. Cultures were obtained from Dr. Larry Beuchat (Department of Food Science and Technology, The University of Georgia, Griffin, GA). The dried inoculum was prepared according to the method described by Santillana Farakos, et al, 2013. Equilibrated peanut flour (0.95 g) was combined with the dried inoculum (0.05 g) to make a 1 g sample. Initial concentrations of *Salmonella* were 10^9 CFU/g. Samples were vacuum-sealed, (FoodSaver, Sunbeam Products, Inc., Boca Raton, FL) and heat sealed (Impulse Heat Sealer, PackworldUSA, Nazareth, PA) in metal retort pouches (Stock America Inc., Grafton, WI) prior to treatment as described by Santillana Farakos, et al., 2013.

***Salmonella* inactivation experiments**

For short-term experiments, vacuum-sealed retort pouches were held at 70°C in a circulating water bath (Precision, ThermoScientific, Waltham, MA) equipped with horizontal wire racks to ensure complete submersion and water circulation between pouches. Samples were analyzed at 0, 1, 5, 15, 30, 60, 240, 480, 960, 1440, and 2880 min. For long-term experiments, pouches were held for 0, 2, 6, 12, 24, 48, 96, 168, 336, 504, and 672 h in controlled temperature incubators 37°C ($\pm 0.5^\circ\text{C}$). Each treatment was replicated three times. Uninoculated controls were analyzed for a_w and background microflora at the beginning, middle, and end of each replicated experiment. Survivors were recovered by dilution of treated samples in 1% Bacto Peptone and plating on non-selective differential media containing tryptic soy agar (TSA, Becton Dickson, Sparks MD) (40 g/L), ferric ammonium citrate (Sigma-Aldrich, St. Louis, MO) (0.8 g/l), yeast

extract (BD, Sparks MD) (3.0g/l) and sodium thiosulfate (6.8 g/l) as (BD, Sparks MD), as described in Santillana Farakos et al., 2013. Plates were incubated at 37°C for 48 h.

Inactivation model

Inactivation data were fit to the Weibull model (equation 1) using GInaFit Version 1.6 (Geeraerd et al 2005; University Leuven, Belgium)

$$\log N_t = \log N_0 - \left(\frac{t}{\delta}\right)^\beta \quad (1)$$

Values for the Weibull model parameters ((δ), treatment time (min) required for the first decimal log reduction and (β) defines the shape of the curve), the root mean square error (*RMSE*) and the adjusted coefficient of determination (R^2_{adj}) of the fit were provided by GInaFit. The influence of temperature and monostearin on the values of the Weibull model parameters δ and β were evaluated using multiple linear regression in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA) at $\alpha=0.05$ confidence level.

Validation of published model

Log δ values and β values were predicted using the secondary model equations developed by Santillana Farakos et al., 2013 (equations 2 and 3)

$$\log \delta = -0.10 \times T - 4.34 \times a_w + 9.91 \quad (2)$$

$$\beta = -0.006 \times T + 0.76 \quad (3)$$

where a_w represents water activity, T represents temperature and δ , β are defined above.

The quality of the secondary model predictions were measured by calculation of the bias factor, and accuracy factor expressed as % bias and % discrepancy (equations 4 and 5). The acceptable zone method (Oscar, 2005) was used to calculate the percentage of residuals (equation 6) within the acceptable zone (-1 log to 0.5 log). The model performance was deemed acceptable when $\geq 70\%$ of residuals were within the acceptable zone.

$$\%B_f = \text{sgn}(\ln B_f) \times (\exp^{|\ln B_f|} - 1) \times 100\% \quad (4)$$

$$\text{Where } B_f = 10^{\left[\frac{\sum_1^n \log(\log N_{\text{model}} / \log N_{\text{data}})}{n} \right]}$$

$$\text{sgn}(\ln B_f) = \begin{pmatrix} +1 \text{ if } B_f > 0 \\ 0 \text{ if } B_f = 0 \\ -1 \text{ if } B_f < 0 \end{pmatrix}$$

$$\% D_f = (A_f - 1) \times 100\% \quad (5)$$

$$\text{Where } A_f = 10^{\left[\frac{\sum_1^n |\log(\log N_{\text{model}} / \log N_{\text{observed}})|}{n} \right]}$$

$$r = \log N_{\text{observed}} - \log N_{\text{predicted}} \quad (6)$$

Where n represents the number of observations, $\log N_{\text{model}}$ and $\log N_{\text{data}}$ represent the log fitted and observed values at a given time point, respectively.

Results and Discussion

Salmonella inactivation data in a low a_w ($a_w=0.46\pm0.06$), high fat (50% w/w) peanut flour model food system with and without an emulsifier (1.625% monostearin w/w) held at 37°C or 70°C are presented in Figure 4.1. *Salmonella* exhibited non-linear inactivation kinetics with significant tailing after 14 days at 37°C and after 4 h at 70°C. Table 4.1 shows the Weibull model fit statistics and the model parameters (δ and β) for inactivation curves at both temperatures. A lower RMSE and a higher R^2_{adj} indicate a better fitting model. The values presented in Table 4.1 show that the Weibull describes the data well, with better fits occurring in the peanut flours without monostearin at 37°C and 70°C.

A total of 8 fitted (GInaFit) and predicted values of δ and β were obtained for the survival data in peanut flour. The $\log \delta$ values for flours held at 37°C were significantly higher ($p=0.023$) than

values for flours held at 70°C. The presence of monostearin did not influence ($p=0.375$) $\log \delta$ at either temperature. Likewise, the shape of the curves (β) were not influenced by temperature ($p=0.070$) or monostearin ($p=0.344$). Previous investigations have reported the influence of temperature on δ and the lack of influence of temperature (van Boekel, 2002) and food composition (Santillana Farakos et al., 2014) on the value of β .

Table 4.2 presents the secondary model performance indices for predictions by the model developed by Santillana Farakos et al., 2013, which has -2% bias and 16% discrepancy inherent in the model. A % bias of 1.00 indicates perfect agreement between the observed and predictive values, while the accuracy factor is the average difference between the predictions and the observations. For flours held at 37°C, the positive % bias indicates over prediction of survival (fail safe) by the model. The model performance was judged to be acceptable since 100% of the residuals were within the established acceptable zone. Predictions tended to be more accurate in the flours without monostearin (% bias closer to 1, lower % discrepancy) compared to flours containing monostearin.

On the other hand, predictions at 70°C were below the established criteria of $\geq 70\%$ of residuals in the acceptable zone (Table 4.2) and the % bias and % discrepancy are outside the error margins inherent in the Santillana Farakos et al., 2013 model equations. The negative bias indicates the model underpredicted survival to a greater extent in flours containing monostearin (-11% bias) compared to flours without monostearin (-9% bias). The % discrepancy indicates that predictions differ from observed values by a factor of 1.27 (27%) and 1.24 (24%). The deviation of predictions from fail safe to fail danger after 1 h (unsupplemented) and 4h (with monostearin) of treatment at 70°C is depicted in Figure 4.2b, which shows plots of the predicted versus observed log reduction in flours at each of the 11 time points previously discussed. Points

below the line of equivalence indicate predicted log reduction values that are larger than observed values (fail danger). The residual survival (tailing) at 70°C suggests the presence of 2 subpopulations with different resistances. The double Weibull model accounts for the adaption of cells in the more resistant and less resistant subpopulations with corresponding δ values and the same β value (Coroller et al., 2006) and therefore may describe the current survival data more appropriately.

The Weibull model secondary equations developed by Santillana Farakos, et al. 2013 were useful in predicting survival of *Salmonella* in equilibrated high fat peanut flour ($a_w = 0.48$) held at 37°C for 28 d. However, predicted log reductions in peanut flours held at 70° were underestimated after 1 h of treatment. Although the presence of 1.625% (w/w) monostearin did not significantly influence the Weibull model parameters, predictions in unsupplemented flour tended to be more accurate than those in flours containing monostearin. Along the same line, peanut butter containing hydrogenated vegetable oils, monoglycerides, and other ingredients demonstrated increased survival of *Salmonella* when held at 21°C and 5°C for 24 weeks compared to natural peanut butter containing peanut and salt (Burnett et al., 2000). This study illustrates the temperature dependence of δ and suggests that an alternative model may provide more accurate predictions of *Salmonella* survival at elevated temperatures.

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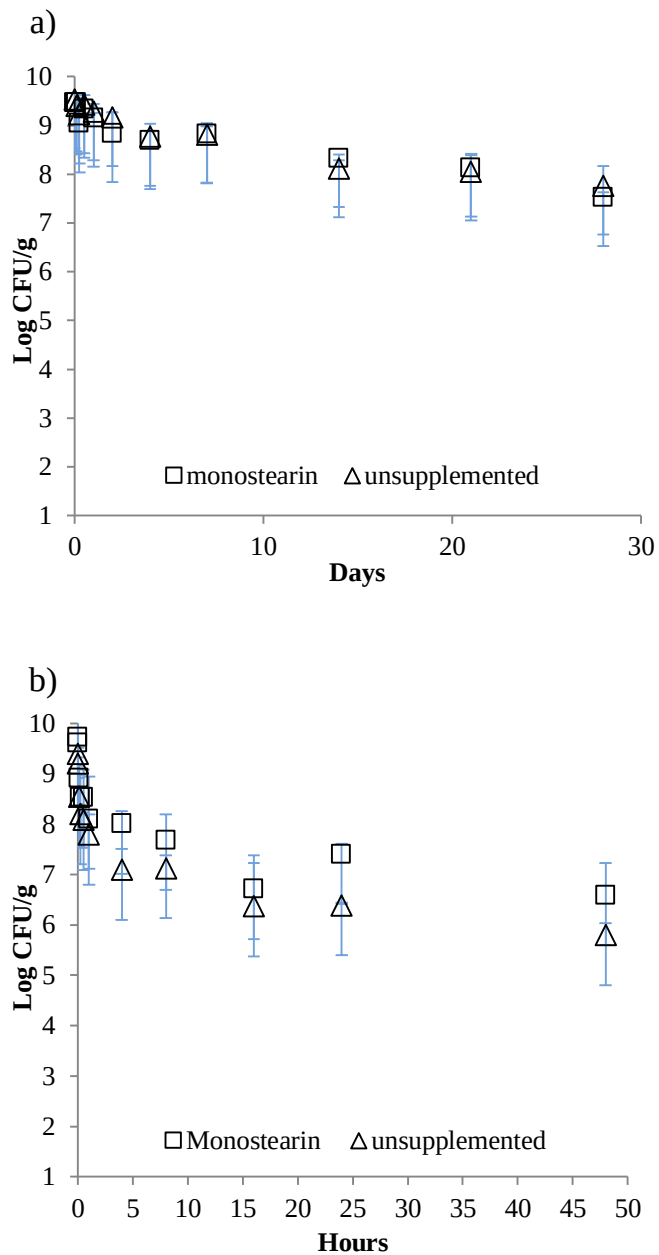


Figure 4.1 Inactivation of *Salmonella* at a) 37°C for 28 days ($a_w = 0.48 \pm 0.02$) and b) 70°C for 48 hours ($a_w = 0.51 \pm 0.01$) in a peanut flour model food system (50% peanut oil and 0.1625% monostearin or 50% peanut oil unsupplemented). Log CFU/g are the average of 3 replications. Error bars indicate $\pm$ sd

Table 4.1 Statistical parameter fits and fitted Weibull model parameters for *Salmonella* inactivation in peanut flours ($a_w=0.46\pm0.04$, 50% fat) held for 28d (37°C) or 48h (70°C)

T(°C)	Peanut flour	Goodness of fit ^a		Weibull model parameters			
		R^2_{adj}	RMSE	$\log \delta$ ^b	se δ ^c	β ^d	se β ^e
37	monostearin	0.80	0.28	2.42	75.60	0.57	0.13
	unsupplemented	0.89	0.21	2.36	53.42	0.54	0.09
70	monostearin	0.71	0.61	-0.70	0.34	0.21	0.06
	unsupplemented	0.91	0.35	-1.00	0.09	0.22	0.03

^a Weibull model goodness of fit statistical parameters adjusted R^2 , root mean squared error, best values in bold

^b time (min) required for the first decimal log reduction

^c standard error of δ

^d defined shape of curve where $\beta < 1$ represents upward concavity

^e standard error of β

Table 4.2. Secondary model^a performance indices for *Salmonella* survival in peanut flours ($a_w=0.46\pm0.04$, 50% fat) held for 28d at 37°C or 48h at 70°C

T (°C)	Peanut flour	% Residuals ^b	%B _f ^c	%D _f ^d
37	monostearin	100	0.47	1.99
	unsupplemented	100	0.65	1.97
70	monostearin	54	-11^e	27
	unsupplemented	64	-9	24

^a equations for Weibull model parameters developed by Santillana Farakos et al., 2013

^b % of residuals in the established acceptable zone (-1 log to 0.5 log)

^c % bias

^d % discrepancy

^e prediction performances that deviate from -2% bias and 16% discrepancy inherent in the model are shown in bold

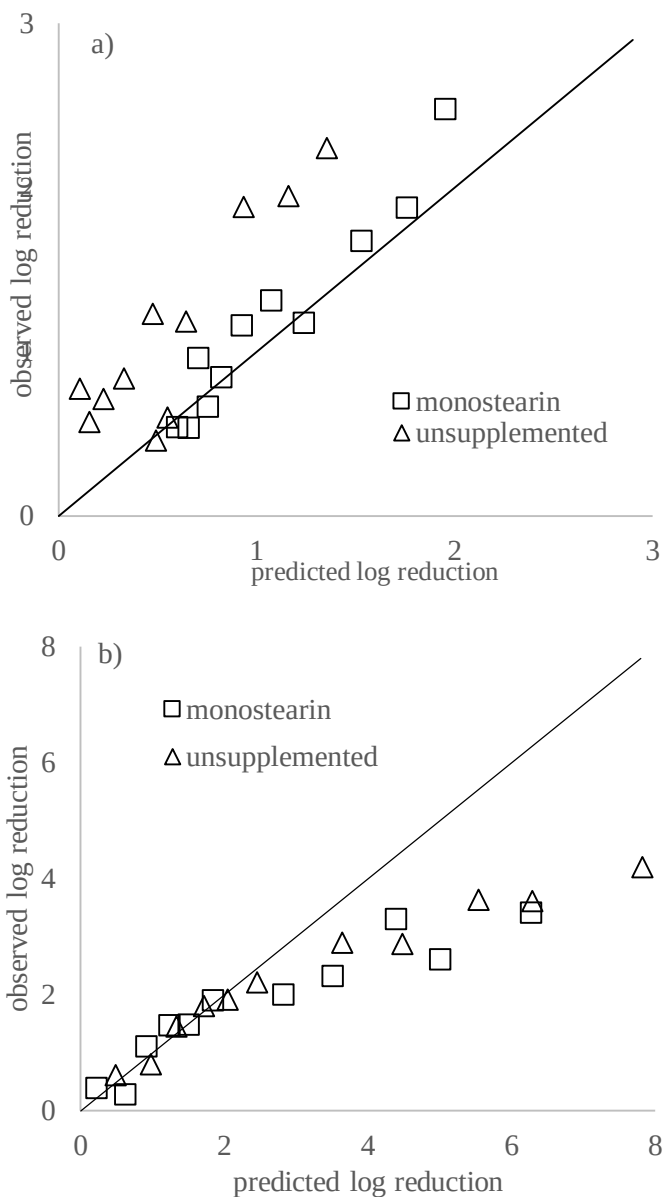


Figure 4.2. Predicted versus observed log reductions for a peanut flour model food system (50% peanut oil and 0.1625% monostearin or 50% peanut oil, unsupplemented) held for a) 28 d at 37°C ($a_w=0.48\pm0.02$) and b) 48h at 70°C ($a_w=0.51\pm0.01$). Predictions and observations are at timepoints (left to right) time zero, 2h, 8h, 12h, 1d, 2d, 4d, 7d, 14d, 21d and 28d (37°C) and time zero, 1 min, 5 min, 15 min, 30 min, 1h, 4h, 8h, 16h, 24h, and 48h (70°C).

CHAPTER 5

THE INFLUENCE OF SUGAR OR FAT CONTENT ON THE RELATIVE PERSISTENCE OF FOUR *SALMONELLA ENTERICA* SEROVARS IN A LOW-WATER ACTIVITY MODEL FOOD SYSTEM HELD AT 36°C ³

³Trimble, L.M., Barnes, S.R., Frye, J.G. and J.F. Frank. To be submitted to the *Journal of Food Protection*

Abstract

Salmonella has demonstrated the ability to survive in dry foods for several months and even years. Components of the food matrix, temperature, water activity (a_w), history of the cells and serotype have been shown to influence its persistence. Low a_w foods do not support microbial growth; yet they continue to be implicated in outbreaks of *Salmonella* infection. Many of these foods contain fat and/or sugar, which have been shown to provide a protective effect for *Salmonella* survival. The aim of this study was to evaluate the influence of a_w and high amounts of sugar (sucrose) or fat (peanut oil) on the relative survival of outbreak-associated *Salmonella* serovars in equilibrated ($a_w = 0.18 \pm 0.05$, $a_w = 0.54 \pm 0.06$) whey protein powder. Powders were inoculated with a dried 4 strain cocktail (Tennessee, Typhimurium, Agona, Montevideo), were vacuum sealed and stored at 36°C for 168 days. Survivors were recovered and presumptive colonies were randomly selected for serotyping at time zero and 168 days. A PCR multiplex assay was used to differentiate serotypes based on genetic profiles. A multinomial mixed logistic model tested for significant differences ($\alpha=0.05$) in the frequency of the surviving serotypes. *S. Typhimurium* was not detected after 168 days and its overall prevalence (0.03%) was too rare to analyze using this method. *S. Tennessee* was the most prevalent serotype, followed by *S. Agona*, *S. Montevideo* and *S. Typhimurium*. Fat and sugar content did not influence the persistence of the survivors ($p=0.144$). This study illustrates the importance of using a cocktail for thermal inactivation studies and should be considered when selecting strains for studies in low a_w foods containing fat and sugar.

Introduction

Preliminary reports from the Centers for Disease Control Foodborne Diseases Active Surveillance Network (FoodNet) identified 7,452 cases of foodborne salmonellosis in the United States in 2014. The incidence and overall public health burden due to *Salmonella enterica* have remained largely unchanged since 2006 (CDC, 2015). Over the past 50 years, approximately 35 outbreaks were caused by *Salmonella* contamination of low-water activity (a_w) foods, affecting at least 10,000 people worldwide (Beuchat et al., 2013). Many of these outbreaks occurred in dry foods containing fat and/or sugar. Such foods include peanut butter (*S. Tennessee*) (Sheth, et al., 2011), sesame paste tahini (*S. Montevideo*) (Unicomb, et al., 2005), halva (*S. Typhimurium*) (Beuchat et al., 2013), cake mix (*S. Typhimurium*) (Zhang, et al., 2007), a peanut butter-covered savory snack (*S. Agona*) (Shohat, et al., 1996), and chocolate (*S. Typhimurium*) (Kapperud, et al., 1990). Other serotypes implicated in *Salmonella* outbreaks in low a_w foods containing sugar or fat include *S. Braenderup* (almond butter) (CDC, 2014) and cocoa powder (*S. Durham*) (Beuchat et al., 2013).

Low a_w foods are not suitable for bacterial growth, which can be an asset in terms of controlling foodborne pathogens. However, some pathogens are capable of surviving in these conditions upon ingress into the processing facility or the food itself. *Salmonella* has demonstrated the ability to survive for prolonged periods of time at decreased a_w and elevated temperatures (Santillana Farakos, et al, 2013). Previous research indicates that survival depends on factors such as the environment (temperature, a_w , pH), the history of the bacterial cells, the serotype, and the components of the food matrix (Aljarallah & Adams, 2007; Komitopoulou & Penaloza, 2009; Li et al., 2014; Santillana Farakos, et al., 2014a & b; Shachar & Yaron, 2006). Studies

have demonstrated that fat content has a protective effect on survival (Hiramatsu et al., 2005; Shachar & Yaron, 2006; Santillana Farakos, et al., 2013) and certain sugars have a protective effect due to their influence on a_w (Hiramatsu et al., 2005). To the author's knowledge, the influence of fat and sugar content on the relative survival of specific serotypes in a low a_w food system has not been investigated. The aim of this study was to evaluate the influence of a_w ($a_w \leq 0.60$) and high amounts of fat or sugar ($\geq 40\%$ (w/w)) on the relative survival of 4 *Salmonella* serotypes in supplemented whey protein isolate powder held at 36°C for 168 days.

Materials and Methods

***Salmonella* inactivation experiments**

Previous experiments by our research group (Barnes, 2015) investigated the heat resistance of *Salmonella* in low a_w whey protein powders supplemented with peanut oil or sucrose. Briefly, a 4.0% solution of BiPro whey protein isolate (WPI, 95% protein, Davisco Foods International: LaSueur, MN) was homogenized with 50% peanut oil (w/w) (Planters, Kraft Foods Group, Inc., Northfield, IL) using a homogenizer (Polytron PT 1200C, Kimatica: Bohmeia, NY) for 1 min at 9000 rpm. Emulsions were poured into frozen metal pans located in a -31°C freezer within 1 min of homogenization. For sugar-supplemented powders, sucrose (D (+) 99+% Acros Organics, Trenton, NJ) was steamed with 50:50 (wt/wt) deionized water for 30 min and cooled to room temperature. Sugar solutions were combined with a 4.5% solution of BiPro WPI to achieve a 40% sucrose (wt/wt) solution and were frozen overnight at -40°C. Peanut oil emulsions and sugar solutions were freeze-dried from -40°C to 0°C for 24 h (Revo Opti-Dry Pro, Millrock Technology: Kingston, NY). Freeze-dried powders were equilibrated to $a_w = 0.18 \pm 0.05$ and $a_w = 0.54 \pm 0.06$. Three replications of one gram samples were inoculated with a previously dried cocktail of 4 *Salmonella* serotypes, were vacuum sealed and stored at 36°C for 168 days in

controlled temperature incubators as described in Santillana Farakos et al., 2013. The cocktail consisted of serotypes implicated in outbreaks in low a_w foods (*S. Typhimurium* (peanut products), *S. Tennessee* (peanut butter), *S. Agona* (ready-to-eat dry snack) and *S. Montevideo* (tahini). Cultures were obtained from Dr. Larry Beuchat, The University of Georgia, Griffin, GA. In previous validation experiments by our research group, the dried inoculum preparation method used in the current study resulted in equivalent prevalence percentages among the 4 serovars ($p=0.127$) (Santillana Farakos, et al., 2014a). Survivors were plated in duplicate on non-selective differential media consisting of tryptic soy agar (TSA, Becton Dickson and Company, Sparks MD) (40 g/L), ferric ammonium citrate (Sigma-Aldrich Co. St. Louis, MO) (0.8 g/l), yeast extract (Becton Dickson and Company, Sparks MD) (3.0g/l) and sodium thiosulfate (6.8 g/l) (Santillana Farakos et al., 2013).

Preparation of whole cell templates for serotyping

Presumptive *Salmonella* colonies were randomly selected from plated samples at the beginning (time zero) and end (168 days) of each replicated experiment. A total of 190 colonies were prepared for serotyping. Ten colonies per set of duplicate plates were selected and grown separately on TSA slants for 24h at 37°C. Growth was streaked for isolation on TSA and XLT4 plates and incubated for 24 h at 37°C. Ten isolated colonies were randomly chosen for each a_w and fat or a_w and sugar combination and were inoculated into 200 µl tubes of sterile deionized water. Tubes were stored at -80°C until use.

Determination of *Salmonella enterica* serotypes

The *Salmonella* multiplex assay for rapid typing (SMART) developed by Leader et al., 2009 was used to differentiate between serotypes based on their genetic profiles. Briefly, primers for 15 gene targets common in 50 of the most prevalent *Salmonella* serotypes were used in a single

multiplex PCR. Fluorescently-labeled PCR products were separated by capillary electrophoresis in an ABI 3130XL gene analyzer (Applied Biosystems, Foster City, CA). GeneMapper software Version 3.5 (Applied Biosystems, Foster City, CA) analyzed the size of the PCR products and the sizes were matched to the SMART typing codes generated by Leader et al., 2009.

Statistical analysis

A multinomial mixed logistic model (SAS version 9.4, Cary, NC) was used to test for significant differences ($\alpha=0.05$) between the frequency distributions of time zero powders and stored powders (168 days at 37°C). The treatment (a_w and fat or sugar) was the fixed effect and the replication was the random effect. The treatments for whey protein powders were as follows: 1) $a_w=0.18\pm0.05$, 40% sucrose 2) $a_w=0.15\pm0.03$, 50% peanut oil 3) $a_w=0.53\pm0.03$, 40% sucrose 4) $a_w=0.54\pm0.02$, 50% peanut oil. 5) The untreated controls were the time zero samples consisting of the inoculum and equilibrated ($a_w=0.18\pm0.01$, $a_w=0.55\pm0.04$) whey protein powder. A Laplace likelihood approximation was used to approximate the generalized linear mixed model. *S. Tennessee* was used as the reference. The prevalence of *S. Typhimurium* was too rare (0.03% of all colonies serotyped) to analyze using this method and was removed from the data analysis. The model compared the relative prevalence of *S. Agona* and *S. Montevideo* to the prevalence of *S. Tennessee* within the 5 treatments.

Results and Discussion

The prevalence of *Salmonella* colonies (serovars Typhimurium, Tennessee, Agona and Montevideo) before and after the 168 day sample storage at 36°C are presented in Table 5.1. Statistical analysis showed that a_w and sugar or fat content (treatments 1-4) did not influence the relative prevalence of the 2 serovars (Montevideo and Agona) included in the analysis with reference to *S. Tennessee* ($F=1.76$, $p=0.144$). *S. Typhimurium* occurred in the time zero

reference population, but was not detected after 168 days of storage (Table 5.1). The most prevalent serovars in decreasing order were Tennessee and Agona, followed by Montevideo and Typhimurium. This trend occurred in all of the treatments held at 37°C for 168 days. A comparable trend in the resistance of the same 4 serovars was documented in unsupplemented low a_w ($a_w \leq 0.60$) whey protein powder held for 168 days at 36°C, where the most prevalent *Salmonella* serotype was Agona followed by Tennessee, Montevideo and Typhimurium (Santillana Farakos, et al., 2014a). Similarly, a cocktail inactivation study by He et al., 2013 in high fat (49%) and reduced fat (33%) peanut butter ($a_w=0.20-0.80$, 24% and 42% carbohydrate) reported that *S. Typhimurium* and *S. Tennessee* were the least and most resistant serovars, respectively. Unlike the current study where *S. Typhimurium* was not detected after 6 months, it was detected in low numbers by Santillana Farakos et al., 2014a. Low numbers of a 2 strain cocktail of *S. Typhimurium* were also recovered after 6 and 12 months from heated (65°C) peanut butter-flavored candy fondant (10.5% fat, $a_w=0.65$) held at 22°C (Nummer et al., 2012).

The current study highlights the importance of using a cocktail in thermal inactivation studies to illustrate the worst-case scenario of survival, particularly in complex food matrices with a low a_w and a high fat or sugar content. *S. Typhimurium* was not detected after 6 months of storage in low a_w whey protein powders supplemented with fat or sugar, which provides direct evidence of the importance of the use of scientific reasoning when selecting strains for a cocktail. Further characterization of the behavior of *Salmonella enterica* in low a_w foods will require investigation of additional outbreak-associated serovars held for time and temperature combinations applicable to processing and storage of such foods.

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Table 5.1 Prevalence of *Salmonella* serovars in fat-supplemented and sugar-supplemented low a_w whey protein powders held at 36°C for 168 days ^a

T°C ^b	Treatment ^c	Number of colonies per <i>Salmonella</i> serotype			
		Tennessee	Montevideo	Agona	Typhimurium
36±0.5	0.23±0.03, 40% sucrose	20 (67) ^d	4 (13)	6 (20)	0(0)
	0.26±0.03, 50% oil	24 (80)	3 (10)	3 (10)	0(0)
	0.55±0.04, 40% sucrose	20 (69)	1 (3)	8 (28)	0(0)
	0.59±0.02, 50% oil	17 (57)	0 (0)	13(43)	0(0)
n/a	Untreated controls ^e	27 (40)	16 (24)	19 (28)	6 (9)

^a colonies were randomly selected from plated samples ^b average temperature ±sd

^c average a_w values of supplemented whey protein powder ±sd after 168 days of storage, percentages are by weight

^d percentage of total number of colonies in 3 replications per set of conditions ^e Untreated time zero (treatment 5) equilibrated powders

CHAPTER 6

CONCLUSIONS

Survival data was obtained for *Salmonella* survival in peanut oil supplemented low a_w ($a_w < 0.60$) whey protein isolate powder held at 22°C or 37°C for 168 days, 50°C or 60°C for 28 days and 70°C or 80°C for 48 hours. At temperatures $\geq 50^\circ\text{C}$, the presence of fat showed a protective effect on survival when compared to non-fat powders, but additional fat content (from 20% to 50%) offer additional protection. Increased a_w significantly decreased survival in powders held at 37°C and 70°C. *Salmonella* Tennessee was the most resistant serotype after 6 months of storage at 37°C and *Salmonella* Typhimurium was unable to survive the low a_w and high fat or sugar conditions of this study.

The revised secondary model represents the first model to include fat content as a predictor of *Salmonella* inactivation in low a_w ($a_w < 0.60$) foods held at temperatures 21-80°C. Overall the revised model predictions are fail safe for foods containing 0-50% fat (w/w) and are held for 1 min -168 days. While the previous low-fat model was reliable in predicting survival in high fat, low- a_w peanut flour held at 37°C, predictions for flour held at 70°C were consistently fail dangerous after 1 h of heat treatment. When compared to the low-fat model, the revised model provides improved accuracy and precision in more complex dry food matrices over the entire range of temperatures and water activities under study. The revised secondary model can be used as quantitative support for the development of exposure assessments, HACCP plans and FSMA food safety plans for processors of low a_w foods. Further validation studies should investigate the applicability of the revised model to foods with different types of fats. Since low a_w foods

have a long shelf life, expansion of the model to include storage times > 168 days could be beneficial to processors of low a_w foods.