INFLUENCE OF SUGAR ON THE SURVIVAL OF *SALMONELLA* IN A LOW-WATER ACTIVITY WHEY PROTEIN-BASED MODEL FOOD SYSTEM

by

STEPHANIE RENEE BARNES

(Under the Direction of JOSEPH F. FRANK)

ABSTRACT

Salmonella has been more prevalent in dried and low-water activity foods over the past few years. The purpose of this study was to determine the effects of sucrose, sorbitol, and fructose on *Salmonella* spp. survival, independent of a_w, in a low- a_w whey protein powder system. The type and presence of sugar supplemented into whey protein powders significantly influenced the survival of *Salmonella* at 0.42 and 0.54 water activity levels when samples were heat treated at 70°C. This was not observed at water activity level of 0.20. The addition of sugar had no influence on the survival of *Salmonella* spp. independent of water activity for whey protein powders stored at 37°C for 168 days. This suggests that sugar's impact on *Salmonella* survival is dependent upon the temperature and water activity that the food is subjected to during processing and storage.

INDEX WORDS: *Salmonella*, sugar, water activity, dried foods, whey protein isolate, sucrose, fructose, sorbitol, Weibull model

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STEPHANIE RENEE BARNES

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STEPHANIE RENEE BARNES

Major Professor:	Joseph F. Frank
Committee:	Mark A. Harrison
	Walid Q. Alali

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION

The genus *Salmonella*, a member of the *Enterobacteriaceae* family, contains two species and over 2,500 serotypes (Brenner & Farmer III, 2005; Popoff & Le Minor, 2005). *Salmonella* can be in nature in soils, water sources, foods, or any other products that are capable of coming into direct contact with animal feces (Brenner & Farmer III, 2005; Centers for Disease Control and Prevention (a), 2015; Ohl & Miller, 2001; Popoff & Le Minor, 2005). *Salmonella* not only grows within these niches, but it also can survive, even if subjected to sub-lethal stresses. If ingested, *Salmonella* can cause salmonellosis, which can generate either gastrointestinal or typhoid-like symptoms (Brenner & Farmer III, 2005; Centers for Disease Control and Prevention (a), 2015; Ohl and Miller, 2001; Popoff & Le Minor, 2005). This explains why *Salmonella* is the leading foodborne bacteria to cause both hospitalizations (35%) and deaths (28%) in the United States (Scallan et al., 2011).

Salmonella is unable to grow in dry environments; however, it can survive for prolonged periods of time. Its ability to thrive in undesirable conditions, coupled with a lowered infectious dose, explains why there have been numerous recalls and outbreaks associated with low- a_w foods (Beuchat et al., 2013; Hiramatsu et al., 2005; Janning et al., 1994). Temperature is a commonly used method to reduce pathogen presence in foods. During heating, as the a_w of a food decreases, the likelihood of survival of *Salmonella*

increases (Archer et al., 1998; Goepfert et al., 1970; Kirby & Davies, 1990; Mattick et al., 2001; Santillana Farakos et al., 2013).

The water activity of a food can be reduced by adding solutes, such as sugar or salt. It has been shown in literature that the type of solute used to lower water activity levels in a food or model system is influential in the survival of pathogenic bacteria. More specifically, multiple studies have been conducted in broth or sugar solutions to determine the impact of various sugars on the heat resistance of *Salmonella* (Baird-Parker et al., 1970; Corry, 1974; Gibson, 1973; Goepfert et al., 1970; Hiramatsu et al., 2005; Mattick et al., 2001; Peña- Meléndez et al., 2014; Sumner et al., 1991). However, this type of research has only been conducted on high and intermediate moisture foods/ model systems, not in low-water activity products ($a_w < 0.60$).

The purpose of this study was to determine the effects of sucrose, sorbitol, and fructose on *Salmonella* spp. survival, independent of a_w, in a low- a_w whey protein powder system. The objectives for this study were to determine if temperature of heat treatment, type of sugar supplemented into a whey protein powder, or concentration of sugar was influential in *Salmonella* inactivation.

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CHAPTER 2

LITERATURE REVIEW

General Characteristics of *Salmonella*:

Salmonella is classified into two species, *S. enterica* and *S. bongori* (Popoff & Le Minor, 2005). The *Salmonella* species *enterica* is further classified into 6 subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (Brenner & Farmer III, 2005; Popoff & Le Minor, 2005). Currently, there are currently more than 2,500 *Salmonella* serotypes (Popoff & Le Minor, 2005). *Salmonella* is a member of the *Enterobacteriaceae* family and γ–proteobacteria phylum (Brenner & Farmer III, 2005; Popoff & Le Minor, 2005). Members of the *Enterobacteriaceae* family are gram-negative, rod shaped, non-sporeforming, lack cytochrome oxidase, and are facultatively anaerobic (Brenner & Farmer III, 2005). *Salmonella* spp. have all of these characteristics as well as most being flagellated (Brenner & Farmer III, 2005; Giannella, 1996; Popoff & Le Minor, 2005).

Ingesting *Salmonella* can cause salmonellosis, which can produce two different sets of symptoms in humans (Centers for Disease Control and Prevention (a), 2015). Either typhoid or gastrointestinal symptoms can occur, depending upon which serotype of *Salmonella* is consumed (Ohl and Miller, 2001; Popoff & Le Minor, 2005). Although symptoms can take only a few to 48 hours to develop, most salmonellosis cases last four days to one week (Centers for Disease Control and Prevention (f), 2015). Symptoms include diarrhea, fever, nausea, vomiting, chills, headache, muscle pain, bloody stool, and

abdominal cramping (Brenner & Farmer III, 2005; Mayo Clinic, 2014). In rare cases, chronic arthritis or death can occur (Centers for Disease Control and Prevention (f), 2015; Ohl & Miller, 2001). *Salmonella* becomes problematic when it exits the intestines of the host and travels into the bloodstream and other parts of the body (Centers for Disease Control and Prevention (f), 2015). Once the bacteria become systemic, death can occur if the patient is not treated with the appropriate antibiotics (Centers for Disease Control and Prevention (f), 2015; Ohl & Miller, 2001).

Humans usually contract *Salmonella* through eating foods or drinking fluids contaminated with *Salmonella* (Brenner & Farmer III, 2005; Centers for Disease Control and Prevention (a), 2015; Ohl & Miller, 2001; Popoff & Le Minor, 2005). Often, these foods are of animal origin, but any food can become contaminated from a lack of good sanitation practices(Centers for Disease Control and Prevention (e), 2015; Giannella, 1996; Ohl & Miller, 2001). *Salmonella* can also be contracted from pets (especially reptile and bird) feces (Centers for Disease Control and Prevention (a), 2015). In these cases, humans become infected when they do not wash their hands after coming into contact with their pet's feces (Centers for Disease Control and Prevention (a), 2015).

Salmonella Pathogenesis

Once ingested, *Salmonella* passes through the stomach and enters into the mucous membrane of the small and large intestines (Giannella, 1996; Popoff & Le Minor, 2005). Before the infiltration process into the intestinal epithelium cells begins, *Salmonella* use fimbriae that aid in mucosa membrane attachment (Baumler et al., 1996; Ohl & Miller, 2001). Infiltration causes an inflammatory response to occur within the epithelial cells of the intestine (Giannella, 1996; Popoff & Le Minor, 2005). This response causes the

gastrointestinal problems commonly associated with salmonellosis (Giannella, 1996). *Salmonella* is able to enter these membranes by helping induce pinocytosis (Giannella, 1996). During pinocytosis, after the pathogen attaches to the epithelial cells, the cytoskeleton of the host cell forms ruffles that will engulf the pathogen within a large vesicle (Ohl & Miller, 2001; Popoff & Le Minor, 2005). *Salmonella* serovars that are similar to Enteritis will also induce the intestinal epithelium cells to recruit and bring in neutrophils into the intestinal lumen space (Galyov et al., 1997; Ohl & Miller, 2001). This process causes cytokine production (McCormick et al., 1993; Ohl & Miller, 2001).

The secondary hurdle for *Salmonella* survival within a host cell is the macrophages present within the mucosal membrane (Ohl & Miller, 2001; Popoff & Le Minor, 2005). Macropinocytosis is the process of bacterial entry into the host cell's phagocytes (Ohl & Miller, 2001). In this pathway, *Salmonella* enter into the host cell's macrophages and by macropinocytosis (described above in the ruffling steps), initiates other virulence mechanisms that will eventually bypass the phagocyte's protective properties, and allow for *Salmonella* survival within the host cell (Alpuche Aranda et al., 1992; Ohl & Miller, 2001; Popoff & Le Minor, 2005). After entry, the pathogen replicates and accumulates in the lymph nodes (Giannella, 1996; Popoff & Le Minor, 2005). From this point, the cells disperse throughout the body by the bloodstream (Giannella, 1996; Popoff & Le Minor, 2005). The ability to spread within a host is dependent on the strain, since most serovars are not able to survive in locations other than the intestines (Giannella, 1996; Popoff & Le Minor, 2005).

Salmonella Growth

Like most pathogens of concern, *Salmonella* prefers to grow in high moisture environments at neutral pH within a moderate temperature range (Chen et al., 2013). For products initially formulated with a lowered moisture content, high moisture environments can be created from a lack of packaging integrity that allows moisture migration into the food due to fluctuating temperatures or relative humidity conditions (Kotzekidou, 1998; Torres, 1987). Growth of *Salmonella* cannot occur below a water activity level of 0.95 (Troller & Christian, 1978). However, *Salmonella* grown in glycerol or sucrose supplemented tryptic soy broth, which lowers the water activity level, increases its ability to proliferate over several life cycles (Peña-Meléndez et al., 2014). Many foods, like meat, contain optimal conditions and nutrient levels for *Salmonella* growth and survival (Chen et al., 2013). *Salmonella* can grow and survive in a number of environments. This is probably why *Salmonella* is the leading foodborne bacteria to cause both hospitalizations (35%) and deaths (28%) in the U.S. (Scallan et al., 2011).

Heat Inactivation

Some foods are delicate and cannot tolerate high heat without loss of flavor, textural, nutritional, or other important quality factors (Doyle & Mazzotta, 2000). In an industrial setting, "hurdles" are applied to different processing stages to reduce bacteria that are potentially present in a food (Davidson & Harrison, 2003; Doyle & Mazzotta, 2000). Hurdles are processing steps performed to reduce pathogenic bacteria presence within a food. By using a hurdle approach, these perishable foods can be made safer for consumption by use of multiple hurdles such as preservatives, lowered time and temperature heat treatment, irradiation, or inclusion of food antimicrobials (Doyle & Mazzotta, 2000; Yousef & Courtney, 2003). Multiple hurdles are applied to foods that

contain one or more critical control points in order to preserve the safety of the product. To determine what hurdles are necessary, the composition of the food matrix needs to be considered. Lowering the pH and dehydration will increase the heat tolerance of bacterial cells (Craig et al., 1994; Doyle & Mazzotta, 2000). Conversely, supplementing a food with certain additives, like bacteriocins, can make these pathogens more sensitive to heat.

In the food industry, there are several different ways to heat treat a food to reduce the presence of pathogenic bacteria. Blanching, roasting, boiling, microwaving, baking, and canning/retort processing are just some of the ways to thermally treat foods. One of the most popular heating devices, the microwave oven, is reported to be sufficient for eliminating small bacterial populations (Doyle & Mazzotta, 2000). This is useful to prevent cross contamination that might occur from food preparation in the home. Microwave foods should be stirred (if possible) or moved off the center of the spinning plate, since microwaving causes an uneven heat distribution. This ensures that the geothermic center of the product is reached, and all surfaces have obtained adequate heating time/temperature to eliminate harmful bacteria. However, foods with large microbial loads would not benefit from this process due to its uneven heat distribution and longer time needed to kill pathogens (Doyle & Mazzotta, 2000).

To compensate for added heat stress, gram negative bacteria like *Salmonella* undergo a heat-shock response (Archer et al., 1998; Craig et al., 1994; Juneja & Novak, 2003; Lindquist & Craig, 1988; Nielsen et al., 2013; Yousef & Courtney, 2003) that alters cellular proteins and other genetic material in the cell. This response includes the expression of various heat-shock proteins (HSPs) that correct misfolded or denatured proteins and help the cell return to a normal state (Craig et al., 1994; Juneja & Novak,

2003; Nielsen et al., 2013; Schumann, 2007) These two tasks make heat shock proteins similar to protein chaperones and ATP-dependent proteases (Arsene et al., 2000; Hecker et al., 1996; Juneja & Novak, 2003). Once exposed to heat, the growth conditions of *Salmonella* do not significantly change whether the cells are planktonic or immobile (Nielsen et al., 2013).

Temperature abused foods, especially ready-to-eat foods, are of concern to the food industry because bacterial cells that have survived sublethal heat treatments have the potential to be more resistant to future stresses (Gruzdev et al., 2011; Juneja & Novak, 2003; Shachar & Yaron, 2006; Sirsat et al., 2011; Yousef & Courtney, 2003). Some of these potential stresses include a secondary heating step, exposure to sanitizers, or other non-thermal lethality processes (Gruzdev et al., 2011).

Pathogenic bacteria can be introduced to stress conditions through any part of the production process. *Salmonella* heated for ten minutes at 42ºC caused gene expression to become vastly differed from non-stressed cells (Sirsat et al., 2011). When comparing two different *Salmonella* strains, cells grown at 44ºC had higher survival rates than cells grown at lower temperatures (Ng et al., 1969). Desiccated *Salmonella* were more tolerant than nondesiccated cells to 1-5% bile salts, 0.5M NaCl, UV irradiation, multiple disinfectants, and 1 hour of heat treatment at 60ºC, 80ºC, and 100ºC (Gruzdev et al., 2011). When dehydrating *Salmonella*, using sucrose, trehalose, or a basic pH sterile double deionized water solution will increase survival rates (Gruzdev et al., 2012).

However, heat shock responses are temporary and can invoke varying degrees of stress tolerance dependent upon the serotype used (Gruzdev et al., 2011; Juneja & Novak, 2003). Ng et al. (1969), found that re-culturing *Salmonella* cells that survived an

initial growth temperature of 44ºC did not confer heat resistance when cells were grown at a lower temperature. *Salmonella* isolated from outbreak related low-water activity foods, were not more heat resistance than other *Salmonella* strains (Mattick et al., 2001). Dried *Salmonella* were more sensitive to citric and acetic acid exposure than non-desiccated cells (Gruzdev et al., 2011).

Improper heating of a food or other moderate to high temperature abuses during processing can result in survival of *Salmonella* and in some cases cause it to become more heat tolerant (Nielsen et al., 2013). The growth temperature, cell's stage of growth, and inherent differences between strains are just some of the determinant factors in the heat resistance of *Salmonella* (Ng et al., 1969). *Salmonella* Enteritidis cells that were previously stressed at higher temperatures had a greater heat tolerance than those grown at lower temperatures under the same secondary heat treatment (Yang et al., 2014).

Other stress factors encountered by *Salmonella*, such as desiccation, are also instrumental for its survival in moderate and high temperature environments. Desiccated *Salmonella* Typhimurium cells heat treated at 100°C and 120°C for one hour obtained log reductions of 0.37 CFU/ml and 2.14 CFU/ml respectively (Kirby & Davies, 1990). Kirby and Davies (1990), also showed that longer drying times did not significantly influence the survival curve when *Salmonella* were subjected to heat treatment at 135°C.

Water Activity

Water activity (a_w) is equal to the vapor pressure of a sample (p) over the vapor pressure of pure water (p_o) at a constant temperature (Chirife & Fontana, 2007; Reid, 2007). This definition is usually expressed by the following formula, $a_w = \frac{p}{p_o}$. Water

activity has also been used to describe the amount of energy that water has to undergo reactions or act as a solvent (Chirife & Fontana, 2007).

The amount of water available within a food determines many key properties, such as shelf stability and microbial safety (Schmidt, 2007). To lower the water activity of a food, solutes like sugar or salt can be added to the product (Beuchat et al., 2013; Tapia et al., 2007). Water activity can also be lowered by using heating techniques or other processing procedures such as freeze drying (Tapia et al., 2007). The definition for low water activity differs dependent upon the commodity item that is being examined. The a_w level in which microorganisms cannot proliferate is 0.60 (Beuchat et al., 2013). Low-a_w foods have been defined as those below $a_w < 0.85$ (Beuchat et al., 2013). For the scope of this project, low-a_w is defined as $a_w < 0.60$.

Pathogenic bacteria are not able to grow below a a_w level of 0.86 (Gurtler et al., 2014; Troller & Christian, 1978). Products below the a_w level for pathogenic bacterial growth still have the potential to cause health issues due to microbial survival. Even if a few cells are present on dried products, equipment surfaces, or other growth niches in a processing plant, there is the potential for cross contamination with a product containing a a_w level. This creates a health concern since pathogenic growth can occur (Gibson, 1973).

Salmonella in Low -Water Activity Foods

There have been numerous foodborne pathogens associated with low a_w food outbreaks. In 1998, 209 people contracted *Salmonella* Agona from consuming a toasted oat cereal (Centers for Disease Control and Prevention (b), 1998). Six hundred and twenty-eight people were infected with *Salmonella* Tennessee after consuming peanut

butter in 2006-2007 (Centers for Disease Control and Prevention (c), 2007). Peanut butter was implicated for *Salmonella* contamination in 2008-2009 when over 700 people contracted *Salmonella* Typhimurium (Medus et al., 2009). Over 1000 people, mostly children, were affected by *Salmonella* spp. contamination of potato chips containing adulterated paprika powder (Lehmacher et al., 1995). In Finland and Norway, *Salmonella* Typhimurium in chocolate caused over 350 reported illnesses (Kapperud et al., 1990). In 2014, chia powder was implicated as the source of salmonellosis in 31 cases (Centers for Disease Control and Prevention (d), 2014).

Many low a_w foods have long shelf lives. Even though *Salmonella* is not able to grow in low-water activity foods (not enough water is freely available), they can survive for long periods of time and continue to cause gastrointestinal symptoms if consumed by a host (Beuchat et al., 2013; Hiramatsu et al., 2005; Janning et al., 1994). In dried products, survival of low bacteria levels can cause infection (Beuchat et al., 2013). Tested chocolates from a contaminated chocolate outbreak in Norway and Finland revealed that ≤ 10 cells per 100 g of product were needed to cause illness (Kapperud et al., 1990). In several low-water activity food outbreaks, *Salmonella* was present at ≤ 1 cell/g in the contaminated food product (Buchanan et al., 2011). The low infectious dose of *Salmonella* in low a_w foods has been suggested to derive from the composition of the food matrix, which aids in cell survival in the gastrointestinal tract (Aviles et al., 2013; D'Aoust, 1977).

Due to multiple bacterial species being previously implicated in low-a_w food outbreaks; there have been numerous experiments into this field of study using other pathogens of concern. In nonfat dried milk, large microbial loads ($\geq 10^5$ CFU/g) of

Listeria can survive for 12 or more weeks at 25°C (Doyle et al., 1985). Silica gels at a water activity level of 0.2 that were stored at 22°C showed varying levels of microbial survival over a three month period (Janning et al., 1994). *Salmonella, Enterococcus faecium,* and *Lactobacillus plantarum* had high resistance to desiccated storage conditions, *Enterobacter cloacae* and *Escherichia coli* had intermediate resistance to storage over time, and the most sensitive group of microorganisms included *Pseudomonas aeruginosa*, *Aeromonas hydrophila,* and *Aeromonas sobria* (Janning et al., 1994). In infant cereal, *Bacillus cereus* had little to no loss in spore survival over 48 weeks of storage at temperatures ranging from 5-45°C and a_w levels of 0.27-0.28 and 0.53-0.55 (Jaquette & Beuchat, 1998).

Salmonella has become a big concern in dried and low-water activity (a_w) foods over the past few years. *Salmonella* in dried foods is very hardy and heat tolerant. Its resilience has been compared to having thermal resistance similar to bacterial spores (Doyle & Mazzotta, 2000). As the a_w of a food product or solution decreases, the likelihood of survival of *Salmonella* increases when exposed to various heat treatments (Archer et al., 1998; Goepfert et al., 1970; Kirby & Davies, 1990; Mattick et al., 2001; Santillana Farakos et al., 2013). More specifically, the starting water activity level of the food affects the heat resistance of *Salmonella* as opposed to the changed water activity level experienced during a heat treatment (Archer et al., 1998). The degree of *Salmonella* survival from heat treatment is dependent upon which strain is used and the additives used to lower the a_w level of the tested product at $a_w > 0.75$ (Corry, 1974; Goepfert et al., 1970). At 70°C and 80°C, Santillana Farakos et al. (2014) showed that *Salmonella* spp. survival in low-water activity whey protein powders was not significantly affected by salt

concentration. When using a paper disk model, *Salmonella* and STEC were able to survive for 70 days at 25°C and 35 days at 35°C (Hiramatsu et al., 2005). In this same experiment, most of the tested strains were detectable after 5 hours in both 70°C and 80°C heat treatments (Hiramatsu et al., 2005). *Salmonella* was only reduced by 3.2 logs after a heat treatment of 90°C for 50 minutes (Shachar and Yaron, 2006).

Sugar and its Influence on Pathogen Survival

Sugars are carbohydrates that can be found as monosaccharides or multiple unit structures (Wrolstad, 2012). The hydrophilic nature of sugars will bind to water within a food matrix to create a lower-a_w level within a food. Sugars are diverse in chemistry and other physical properties, which makes them well equipped for usage in a wide array of foods (Wrolstad, 2012).

Sucrose, more commonly known as table sugar, from cane or beet sugar, is the most commonly found disaccharide in the world due to its taste, cost, and stability (Linden & Lorient, 1999). Glucose and fructose are the products of sucrose hydrolysis (Linden & Lorient, 1999). Sucrose has a melting temperature of 160°C, and is very water soluble, 67g per 100g water, at room temperature (Linden & Lorient, 1999). Some of the most common uses of sucrose in foods include, binding water, flavor enhancer, texture modifier, and altering the lubricating or bulking properties of a product (Linden & Lorient, 1999).

One of the sweetest natural sugars is fructose, also known as fruit sugar and levulose (Osberger, 1991). Fructose can be found in honeys as well as an additive in desserts, dietetic foods and beverages, gums, and other dessert-like products or beverages (Osberger, 1991). Crystalline/pure fructose and high-fructose corn syrup (HFCS) are not

classified as the same sweeteners (Osberger, 1991). Crystalline fructose is over 99% fructose and has a higher sweetness perception than HFCS (Osberger, 1991). HFCS has a much lower percentage of fructose than pure fructose and contains a number of other sweeteners to complete this syrup (Osberger, 1991). Fructose has a greater solubility in water than both sucrose and dextrose, approximately 80% at room temperature (Osberger, 1991).

Sorbitol, a polyalcohol, is found in a variety of foods, such as fruits, sugar-free gums and candies, baking mixes, and dietary products (Dwivedi, 1991; Linden & Lorient, 1999). Some of the desirable properties of sorbitol include a high-water binding capacity, low sweetening effect, reduces sugar crystallization when added to sucrose or glucose, resistance to heating, and ability to increase shelf stability of fat products by complexing heavy metals (Dwivedi, 1991; Linden & Lorient, 1999). Glucose hydrogenation is the process by which sorbitol is generated for further usage in foods (Dwivedi, 1991; Linden & Lorient, 1999). Sorbitol is more soluble at room temperature than sucrose, with a maximum syrup concentration reaching 70% at 20°C (Linden &Lorient, 1999). Unlike most sugars, sorbitol cannot undergo Maillard browning reactions due to a lack of free carbonyl groups (Dwivedi, 1991). This characteristic makes sorbitol more heat tolerant than many other comparable sweeteners (Dwivedi, 1991).

Sorbitol dehydrogenase breaks down sorbitol into fructose for usage in human metabolism (Linden & Lorient, 1999; Osberger, 1991). Fructose is approximately three times sweeter than sorbitol, so less can be added to foods to create the same level of sweetness (Osberger, 1991).

Sugar and its Specific Influence on *Salmonella* Survival

The type of solute used to lower water activity levels in a food or model system influences survival of pathogenic bacteria. Hiramatsu et al. (2005) investigated the effects of desiccants on *Salmonella* and STEC survival by using a paper disk model. They found that sucrose had a protective effect on the tested pathogens, with higher sugar levels resulting in greater survival rates (Hiramatsu et al., 2005). Sucrose provided greater heat protection than NaCl and glucose-fructose solutions at water activity levels below 0.9 and heat below 74°C (Mattick et al., 2001). Sumner et al. (1991) showed that the heat tolerance of *Salmonella* Typhimurium ($a_w \leq 0.83$) and *Listeria monocytogenes* ($a_w \leq 0.90$) increases in sucrose based solutions with decreasing water activity levels. When adding sugar to test media to reduce the a_w, sucrose was found to be more protective than sorbitol, fructose, glucose, and glycerol when exposed to higher temperature stress (Baird-Parker et al., 1970; Corry, 1974; Goepfert et al., 1970). Goepfert et al. (1970) showed that at $a_w = 0.96$, the type of sugar examined is a factor on survival, independent of water activity. Baird-Parker et al. (1970) found similar results when examining sucrose, glycerol, and sodium chloride supplemented heart infusion broths in a range of reduced a_w levels ($a_w \leq 0.90$, except glycerol in which $a_w \leq 0.85$). *Salmonella* is more heat sensitive in glucose supplemented solutions ($a_w = 0.94$) from 48-50°C, but at higher temperatures (up to 60°C) glucose added broths provided more protection for *Salmonella* than NaCl added solutions (Aljarallah & Adams, 2007).

Most foods contain more than one sugar. Therefore, testing multiple sugar solutions or foods is important. Sucrose-glucose solutions ranging from $a_w = 0.706$-0.995, showed increased thermal resistance of *Salmonella* when water activity was reduced (Gibson, 1973). When using glucose-fructose solutions to lower the a_w of TSB broths,

lower a_w solutions protected *Salmonella* exposed to temperature treatments at or above 70°C (Mattick et al., 2001). The converse was found when cells were exposed to temperatures at 60°C or lower; low-a_w and temperature had a synergistic effect in increasing death rates than cells exposed to higher a_w sugar solutions (Mattick et al., 2001).

Previous research has shown that an equilibration to osmotic conditions is not influential in the heat tolerance of *Salmonella.* Storage of culture for up to one month in sugar solutions did not change the heat resistance of *Salmonella* when compared to cells heat treated in the same solution on day of inoculation (Corry, 1974). Osmotically shocked *Salmonella* and those adapted to sucrose added TSB had the highest survival rates compared to cells either adapted or shocked with glycerol or NaCl (Peña-Meléndez et al., 2014).

The influence of the amount of sugar (w/w) used to lower water activity levels of a broth or food to determine microbial heat resistance has not been thoroughly examined. Corry (1974) showed that dependent upon the strain tested, glycerol had some correlation between heat tolerance and the amount of solute added to a test solution. In mixed sugar solutions (a_w = 0.85-0.95), such as sucrose and either glycerol or glucose, the amount of sugars (w/w) present in solution was directly related to the D-value at 65°C (Corry, 1974).

Powdered infant formula has been connected to several outbreaks of pathogenic bacteria, especially *Salmonella* and *Cronobacter* (formerly known as *Enterobacter*) (Beuchat et al., 2013). The ability of microorganisms to survive within this food matrix is dependent upon the strain tested. *Salmonella* Enteritidis, *E. coli*, and *K. pneumonia* were

able to survive up to 15 months, *Pantoea* spp., *K. oxytoca* 5 strains of *E. sakazakii*, and *E. vulneris* survived over 2 years, and some *E. sakazakii* strains were present after 2.5 years of storage in milk-based infant formula (Barron & Forsythe, 2007). Forsythe (2014) and Gurtler & Beuchat (2007) found that *E. sakazakii*, just like many *Salmonella* spp. has a higher survival rate with a decrease in a_w levels, although it can survive in many different temperature (4-30°C) and a_w ranges (a_w 0.25-0.86).

Salmonella survives for extended periods of time on dried fruits, which can have lower- pH values and high-osmotic potential due to natural sugars present in the fruit before desiccation (Beuchat & Mann, 2014). At 25°C, *Salmonella* were recovered from date paste after 84 days, freeze-dried strawberries after 42 days, and dried cranberries and raisins after 21 days in storage (Beuchat & Mann, 2014). Out of 15 examined *Salmonella* (5) and STEC (10) strains, there was a ten times higher survival rate in sucrose supplemented squid chips than squid chips without sucrose (Hiramatsu et al., 2005). In commercial halva, $a_w = 0.176$, *Salmonella* Enteritidis was viable after 8 months of storage in refrigerated and room temperature storage conditions (Kotzekidou,1998). *Salmonella* was detected in peanut butter flavored candy fondant (containing high sugar levels) after six and twelve months in storage at 21°C (Nummer et al., 2012).

These studies highlight a lack of knowledge in determining whether the sugar content of a food, independently of water activity, affects *Salmonella* spp. survival. There is also a limited amount of information available on pathogen survival in model systems or foods at $a_w \leq 0.60$.

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CHAPTER 3

MATERIALS AND METHODS

Methodology for the water activity equilibration, inoculation of samples, packaging of samples, and sample recovery was adapted from those developed by Santillana Farakos et al. (2013).

Whey Protein-Sugar Preparation

*Bi*PRO© Non-Denatured Whey Protein Isolate Powder (>95% protein) (Davisco Foods International, Inc., Eden Prairie, MN) was added to sterile DI water to make a 4.5% solution of protein powder. This was stored in a refrigerator overnight. On the second day, sucrose (D (+)-Sucrose, 99+%, for biochemistry, RNAse free and DNAse free, Acros Organics, New Jersey), fructose (D-Fructose, Fisher Scientific, Pittsburgh, PA), or sorbitol (D-Sorbitol, Fisher Scientific, Pittsburgh, PA) was mixed with sterile DI water in a 50:50 (wt/wt) solution and steamed for 30 min to dissolve the sugar. Sugar solutions were then cooled to room temperature. The whey protein solution was divided into separate sterile containers and sufficient sugar solution was added to produce samples that would have 20 or 40% (wt/wt) sugar by dry weight. The protein/sugar samples were placed in sterile aluminum pans and placed in a -40°C freezer for at least 3 h. After freezing, pans were place in a freeze dryer (Freezemobile 25SL Unitop 600L, SP Scientific, VirTis, Gardiner, NY and Millrock Revo™ Series Freeze- Dryer, Millrock Technology, Kingston, NY) and dried for a minimum of 20 h. After drying, powders

were stored in bottles containing pouches of anhydrous calcium sulfate until placing in vacuum desiccators for equilibration.

Water Activity Equilibration

Sugar-protein powders were manually crushed by using a pestle into a homogenous size (checked by visualization) and placed in weigh boats within vacuum desiccators containing saturated salt solutions to equilibrate to a targeted water activity (a_w) level of either 0.11 (lithium chloride, 99+% anhydrous, Acros Organics, NJ), 0.43 (potassium carbonate, anhydrous, Amresco LLC, Solon, OH), or 0.58 (sodium bromide, 99+% anhydrous, Acros Organics). Water activity values were determined by using an Aqualab 4TEV a_w meter (Decagon Devices Inc., Pullman, WA) following manufacturer's instructions.

Preparation of *Salmonella*

Four *Salmonella* serovars previously associated with low a_w food outbreaks were used for this experiment: *Salmonella* Typhimurium (peanut), *Salmonella* Tennessee (peanut), *Salmonella* Agona (dry cereal), and *Salmonella* Montevideo (pistachios). Cultures were stored on cryobeads (CryoBank, Copan Diagnostics Inc., Murrieta, CA) in a -80°C freezer. Cultures were prepared by 2 transfers into 9 ml of TSB (tryptic soy broth, Becton, Dickinson and Company, Franklin Lakes, NJ) at 37°C for 24 h. A third transfer of 3 ml inoculum into 225 ml flasks of TSB were incubated at 37°C for 24 h. Cultures were then centrifuged at approximately 2,500 g for 30 min, the supernatant fluid was removed, and the pellet re-suspended with 2 ml of sterile 1% Bacto® peptone (Becton, Dickinson and Company, Franklin Lakes, NJ). The suspended pellets were placed in weigh boats and dried in vacuum desiccators containing anhydrous calcium

sulfate for a minimum of 2 days. The dried *Salmonella* were scraped into a centrifuge tube and the dried mass was crushed within the tube by using a sterile spatula to make one homogenous cocktail of the four serotypes. The initial population of the cocktail was approximately 10^9 CFU/g.

Sample Preparation

Equilibrated sugar powder (0.95 g) and 0.05 g of dried *Salmonella* cocktail were added to metal retort pouches (STOCK America Inc., Grafton, WI) to make 1 g samples. Equilibrated sugar powder (0.95 g) was also added to the pouches for uninoculated controls. Sample pouches were placed in individual FoodSaver® quart sized bags (Jarden Consumer Solutions, Sunbeam Products, Inc., Boca Raton, FL) and sealed using a FoodSaver® vacuum sealer (Jarden Consumer Solutions) as a means of primary containment. Metal retort pouches were then sealed using an impulse heat sealer. Oxygen transmission rate to samples were minimal due to the structure of the retort pouches. Pouches were composed of a laminate film containing polyethylene terephthalate, nylon, aluminum foil, and polyester (FLAIR Flexible Packaging Corporation, 2011; Lambert, 2015). Metal retort pouches were previously demonstrated to provide sufficient moisture barrier during water bath incubation (Santillana Farakos et al., 2013).

Experimental Design

Samples were subjected to heat treatment at either 37°C or 70°C. Samples treated at 37°C were placed in a conventional microbiological incubator and analyzed at 0, 7, 14, 21, 28, 42, 56, 84, 112, 140, and 168 days. Uninoculated control samples for monitoring water activity and background microflora (APC) were taken at 0, 84, and 168 days. Samples to be treated at 70°C were placed in a Precision™ circulating water bath (Thermo

Scientific, Thermo Fisher Scientific Inc., Waltham, MA) using custom designed racks that allowed water to circulate between the pouches. Samples at 70°C were analyzed at 0, 0.02, 0.08, 0.25, 0.5, 1, 4, 8, 16, 24, or 48 h. A 30 s come up time, for samples to initially reach 70°C, was added to each treatment time. Treated samples were immediately cooled in cold water for at least 30 s. Uninoculated controls were analyzed for a_w and aerobic plate counts at 0, 240, and 2880 min.

***Salmonella* Analysis**

To determine the level of *Salmonella*, pouches were cut open, contents scraped into stomacher bags using a sterile spatula, and diluted with 1% Bacto® peptone (Becton, Dickinson and Company). Stomacher bags containing diluted samples were pummeled (Seward, Lab Blender Stomacher 400, Davie, FL) for 1 min. Serial dilutions were made and samples were plated on tryptic soy agar (TSA, Becton, Dickinson and Company) (40.0 g/L) supplemented with 6.8 g/L sodium thiosulfate (5-hydrate, crystal) (Avantor Performance Materials, J.T. Baker, Center Valley, PA), 0.8 g/L ferric ammonium citrate (Sigma-Aldrich Corp., St. Louis, MO), and 3.0 g/L yeast extract (Becton, Dickinson and Company). This supplementation allowed easy identification and enumeration of *Salmonella* colonies. Plates were incubated at 37°C for 48 h. The limit of detection was determined to be 5 colonies per plate (represented by 3.00 CFU/g).

Controls were analyzed for water activity then plated to determine aerobic plate counts. Pouches were cut open, and sugar-protein samples were analyzed using an Aqualab 4TEV a_w meter (Decagon Devices Inc.) to determine the after treatment a_w levels. After recording these values, samples were diluted with 1 ml of 1% Bacto®

peptone (Becton, Dickinson and Company) and hand plated on supplemented TSA (Becton, Dickinson and Company). Plates were incubated for 48 h at 37°C.

Data Analysis

All treatments were repeated in triplicate. Log values of survivors were analyzed using two-way ANOVA with interaction terms on SAS 9.4 software (SAS Institute Inc., Cary, NC). Uninoculated a_w controls, after heat treatment, were compared using Tukey-Kramer's test on SAS 9.4 software (SAS Institute Inc., Cary, NC).

Validation of Method for Primary Modeling

Data from this study was compared to predictive models developed by Santillana Farakos et al. (2013) to validate data against that equivalent to the published model. Unsupplemented protein powder controls were compared to the pH 7 results found by Santillana Farakos et al. (2013) by using one-way ANOVA (SAS 9.4, SAS Institute Inc., Cary, NC). Validation of equivalent methodology used in this study against methods used by Santillana Farakos et al. (2013) was required for model comparisons.

Modeling *Salmonella* Inactivation Data

Survival curves were fit to the Weibull model (Mafart et al., 2002) using GInaFiT software (version 1.6, Katholieke Universiteit Leuven, Leuveen Belgium) (Geeraerd et al., 2005). The shape factor (β) and time to first log reduction (δ) values were compared to results reported by Santillana Farakos et al. (2013) to determine sugar's influence in a pre-existing whey protein powder model. Differences in shape factor (β) and first log reduction times (δ) were analyzed using one-way ANOVA on SAS 9.4 software (SAS Institute Inc., Cary, NC).

CHAPTER 4

RESULTS

Validation of Methodology

Figures 1 and 2 present data comparing the survival of *Salmonella* in inoculated unsupplemented whey protein powder collected in this study to values reported by Santillana Farakos et al. (2013). There were no significant differences between the two sets of data at 37°C and 70°C based on one-way ANOVA analysis. This indicates that the methodology used in this study is equivalent to that employed by Santillana Farakos et al. (2013).

Background Microbial Controls

Background microflora was analyzed at the beginning, middle, and end of each treatment. The background microflora at three log units lower than the inoculation level was considered acceptable. None of the samples examined exceeded this level of background microflora.

Salmonella Survival in a_w 0.20 Sugar Supplemented Powder at 70°C

Figure 3 presents data showing the survival of *Salmonella* in sugar supplemented protein powders equilibrated at $a_w = 0.20$ and held at 70°C for 48 h. The baseline data for all survival curves was from Santillana Farakos et al. (2013) which is the basis of an existing predictive model that does not include sugar as a predictive factor. The presence of sugar had no significant effect of survival at this a_w level. The type of sugar also did not influence survival. Average log reductions at 24 h for sugar supplemented and

unsupplemented protein powders ranged from 1.08 CFU/g to 1.85 CFU/g. After 48 h, log reductions for supplemented protein powders were between 0.95 CFU/g and 2.58 CFU/g. Unsupplemented protein powder had an average log reduction of 2.91 CFU/g after 48 h.

Table 1 presents data showing changes in water activity of the equilibrated protein powders during treatment at 70°C for 48 h. Water activity values taken after heating were significantly higher in fructose ($p= 0.0005$) and sucrose ($p= 0.0216$) supplemented protein powders than a_w values reported before heat treatment. Fructose supplemented protein powders increased from 0.22± 0.04 to 0.33± 0.04 and sucrose supplemented protein powders changed from 0.18± 0.02 to 0.28± 0.03 after heating. The heat treatment did not alter the water activity in the sorbitol supplemented powder.

Salmonella Survival in a_w 0.42 Sugar Supplemented Powder at 70°C

Figure 4 presents data on the survival of *Salmonella* for $a_w = 0.42$ in sugar supplemented protein powders at 70°C over 48 h. At this water activity, survival of *Salmonella* was significantly different in whey protein powders supplemented with sugar as compared to powders lacking sugars ($p<0.0001$). The type of sugar used within the protein powder was also shown to be significant ($p= 0.0123$). Unsupplemented protein had the lowest protective capability with an average log reduction of 5.82 CFU/g in 48 h. Sucrose had similar protective qualities as unsupplemented protein powder, with average log reductions of 5.60 CFU/g and 4.21 CFU/g for 20 and 40% (w/w) powders, respectively. Fructose and sorbitol provided the best protection for *Salmonella* over the 48 h of heat treatment. Average log reductions for each sugar ranged from 1.55 CFU/g to 2.50 CFU/g over the 48 h storage. *Salmonella* was not detected in unsupplemented

protein powders after 24 h of heat treatment, but *Salmonella* was detected in sugar supplemented powders after 48 h.

The presence of fructose in whey protein powder contributed to significant increases in a_w values from 0.39± 0.02 to 0.50± 0.02 after heating ($p< 0.0001$) (Table 1). The presence or absence of sucrose and sorbitol showed no notable changes in a_w after heat treatment.

Salmonella Survival in a_w 0.54 Sugar Supplemented Powder at 70°C

Adding sugar to the protein significantly ($p<0.0001$) enhanced *Salmonella* survival over 48 h in samples equilibrated to a_w of 0.54 and held at 70° C. (Figure 5). The type of sugar used in the protein powders also exhibited a significant effect ($p= 0.0140$) when compared to data on unsupplemented protein from Santillana Farakos et al. (2013). Plain protein powder and sucrose supplemented protein powder were associated with the highest log reductions of 5.72 CFU/g, 5.75 CFU/g, and 5.40 CFU/g in 0, 20, and 40% (w/w) sugar supplemented powders. Fructose supplemented powders provided more protection for *Salmonella* than sucrose supplemented and unsupplemented powders, but not as much as sorbitol supplemented powders at this a_w level. Average log reductions for fructose supplemented protein powders were (by increasing concentrations) 2.95 CFU/g and 2.42 CFU/g. Sorbitol supplemented powders achieved average log reductions of 2.03 CFU/g for 20% (w/w) powders and 2.11 CFU/g for 40% (w/w) powders. Most protein powders supplemented with sugar had protective effects after 48 h of heat treatment. Unsupplemented protein powder and 20% (w/w) sucrose supplemented protein powders were below the limit of detection after 24 h.

There were no differences in a_w values after heat treatment in unsupplemented and all three sugar supplemented protein powders (Table 1).

Salmonella Survival in a_w 0.20 Sugar Supplemented Powder at 37°C

Neither the amount of sugar added to the protein matrix nor the type of sugar examined significantly affected *Salmonella* survival at $a_w = 0.20$ held at 37 °C (Figure 6). When compared to the data of Santillana Farakos et al. (2013) the greatest survival of the pathogen occurred at this storage temperature and a_w range, with reductions ranging from only 0.39 to 2.24 log units after 168 days.

Table 2 presents the changes in a_w during storage at 37°C for 168 days. Water activity values after heat treatment for 168 days were higher in all 3 sugars examined. Sucrose supplemented protein powders increased from 0.18±0.05 to 0.23±0.03 after heat treatment for 168 days ($p= 0.0103$). There were significant increases in water activity, from 0.16±0.03 to 0.31±0.05, after heating fructose supplemented protein powder ($p< 0.0001$). Sorbitol supplemented protein powders had significant increases in a_w values (0.14±0.02 to 0.25±0.02) after 168 days ($p< 0.0001$).

Salmonella Survival in a_w 0.44 Sugar Supplemented Powder at 37°C

The addition of sugar as well as the type of sugar had no significant effect on survival of *Salmonella* at $a_w = 0.44$ (Figure 7). Inactivation over the 168 day period ranged from 1.97 CFU/g to 2.93 CFU/g log unit reduction.

Water activity values were significantly higher after heating ($p\leq 0.0452$) in fructose and sorbitol supplemented protein powders (Table 2). The a_w level of fructose supplemented powders rose from 0.41±0.03 to 0.50±0.03 after 168 days of heating, but sorbitol supplemented protein powders only increased from 0.40±0.04 to 0.44±0.02.

Sucrose supplemented protein powders did not have notable changes in a_w levels after heat treatment.

Salmonella Survival in a_w 0.54 Sugar Supplemented Powder at 37°C

The presence of sugar as well as the type of sugar used had no significant effect on survival of *Salmonella* in the protein powders equilibrated to a_w 0.54 and held at 37°C (Figure 8). Reductions achieved during 168 days of storage ranged from 2.16 CFU/g to 4.57 CFU/g log units.

Sucrose and sorbitol supplemented powders did not have noteworthy differences in a_w levels before and after heat treatment (Table 2). Fructose based protein powders had significant increases in water activity values from 0.49± 0.08 to 0.59± 0.03 after storage ($p= 0.0025$).

Primary Modeling of Survival

Survival data was fit to the Weibull model, which Santillana Farakos et al. (2013) determined was the best model for fitting low-water activity inactivation. Tables 3-8 show the shape factor (β) and time to first decimal reduction (δ) values for inactivation curves. Sucrose supplemented protein powders had higher β values than plain protein powder at 70°C. Also at 70°C, sorbitol supplemented protein powders had lower β values than unsupplemented protein powder. Fructose supplemented protein powders had higher β values than unsupplemented protein powder at 37°C.

Values for δ were log transformed to normalize the data. Sugar supplemented δ values were higher than those reported by Santillana Farakos et al. (2013) for all a_w values at 70°C. Therefore, it took longer for *Salmonella* to reduce by one log in powders containing sugar over unsupplemented powders. The converse was observed at the lower

heat treatment. At 37°C, δ values were higher for unsupplemented protein powders compared to powders supplemented with sugar.

CHAPTER 5

DISCUSSION

Salmonella inactivation varied in samples held at 70°C and was dependent upon the water activity and presence of sugar. In a_w = 0.42 and 0.54 powders, the presence of sugar within the protein matrix had a significant ($p<0.0001$) effect on the surviving *Salmonella* population. However, significant interaction effects between water activity and the presence of fructose ($p=0.0011$) or sorbitol ($p=0.0077$) in protein powders was observed. Table 10 shows the comparison of means for the log reductions of *Salmonella* at 70°C in these two sugar supplemented protein powders. From this analysis, it can be concluded that as the water activity increases, the presence of sugar has a greater magnitude of effect on the surviving population of *Salmonella*. Higher survival in the two monosaccharide supplemented powders could derive from these sugars having lower molecular weights than sucrose. Sugar solutions were made on a weight/weight (w/w) basis, not a molarity basis. Since sorbitol and fructose are half the weight of sucrose, it took twice as many molecules of fructose and sorbitol to make a 50:50 sugar/DI water suspension. This could account for the protective effects seen in the higher a_w fructose and sorbitol supplemented protein powders. Water activity had significant influence on *Salmonella* inactivation for all powders at a_w = 0.20 powders that were treated at 70°C.

Salmonella decreased to undetectable levels in unsupplemented powders at a_w = 0.42 and 0.54 held at 70°C for 24 hours. *Salmonella* continued to be detected in sugar

supplemented powders held under these conditions, with the exception of 20% sucrose supplemented protein powders at a_w = 0.54. Therefore, it can be concluded that at higher a_w values, sugars in dry protein-based foods can provide protection for *Salmonella* against heat inactivation. Similar results are reported in the literature. Reduced fat peanut butter spread (containing >40% total carbohydrates and corn syrup as a source of sugar), had the greatest *Salmonella* survival compared to 3 other reduced sugar peanut butter formulas heated to temperatures ranging from 70-90°C (Li et al., 2014). The reduced fat peanut butter spread used by Li et al. (2014) had a_w = 0.474; this was within the range of a_w levels examined in this study where sugar had a significant impact on *Salmonella* survival. At $a_w \geq 0.75$, the protective effects of sugar solutions have been reported as sucrose > glucose> sorbitol> fructose > glycerol, when heat treated to 57°C or 65°C (Goepfert et al. 1970 and Corry, 1974).This was not observed in the supplemented protein powders heat treated to either temperature in this study in which samples were stored at lower water activity values.

Sugar had no significant effect on the survival of *Salmonella* when samples were held at 37°C. Therefore, water activity remains a good predictor for the survival of *Salmonella* in high sugar dry foods not subjected to high heat. Santillana Farakos et al. (2013) found that at lower temperatures, 21°C and 36°C, *Salmonella* survival was higher with reduced a_w levels. Although sugar supplemented protein powders were not examined at 21°C, Santillana Farakos et al., (2013) observed stability of the *Salmonella* population in nonsupplemented protein powder after 6 months of storage of protein powder held at 21 and 37 °C which suggests that the mechanisms of survival are similar at the two temperatures.

Survival of *Salmonella* observed at 37°C differed from that observed by Aljarallah and Adams (2007). When using glucose or NaCl to reduce the a_w of test media to $a_w = 0.94$, they found that *Salmonella* was more sensitive to lower a_w solutions at temperatures ≤53°C. Similar results were reported by Mattick et al. (2001) with temperatures ≤ 55°C being sensitive for *Salmonella* and ≥ 70°C being protective for this microorganism at $a_w = 0.75\text{-}0.90$. Differences in cellular inactivation at different temperature could be caused by differences in cellular pre-stress conditions or injury to different genetic components of the cell (Aljarallah and Adams, 2007). In a dried paper disk model ($a_w = 0.56 \pm 0.02$), *Salmonella* was not detected after 35-70 days when stored at 25°C and after 15-35 days when stored at 35°C (Hiramatsu et al., 2005). However, survival rates increased at 25°C when sucrose was added to the dried disks or squid chips (Hiramatsu et al., 2005). The lack of significance in the presence of sugar at low temperatures differs from the findings of Goepfert et al. (1970) at higher water activity. They report that the solute used to modify the a_w level in a product determines the heat resistance of *Salmonella* at a_w= 0.96 (Goepfert et al., 1970). Similar results regarding solutes used to lower a_w (a_w= 0.85-0.99) were found by Baird-Parker et al. (1970).

Much previous research indicates that heat inactivation of *Salmonella* in low a_w, foods does not follow a log-linear pattern. Corry (1974) found that there is not a direct linear relationship between cellular heat resistance and lower a_w values. Various mathematical models have been developed or modified to describe nonlinear inactivation rates in microorganisms. The Weibull model is commonly used across multiple disciplines to describe failure within a system (Peleg, 2006). The formula for the Weibull model is provided below:

$$n = \left(\frac{t}{\delta}\right)^{p}$$

where n is the decimal reduction ratio, δ is the time to first log reduction, *t* is the time duration, and *p* (also represented as β in literature) is the shape parameter (Mafart et al., 2002).

Shape factor defines the shape of the curve's concavity (Couvert et al. 2005). With a β >1, the curve takes on a downward concave shape, which means microbial reduction occurred at an initial rapid rate (Peleg, 2006). At β <1, the curve is upwardly concave; therefore, after an initial rapid decline, microbial reduction decreases over time to create a tailing effect (Mafart et al. 2002 and Peleg, 2006). At 70°C**,** β values were less than one for all survival curves, denoting an upward concave shape. Shape factor values were higher in sucrose supplemented protein powders as compared to unsupplemented powder. This shows that these survival curves were closer to linearity than unsupplemented powder. β values for sucrose supplemented protein powder at $a_w = 0.20$ were similar to salt added whey protein powder β values at the same a_w level (Santillana Farakos et al., 2014).

Sorbitol supplemented protein powders generally had lower β values than plain protein powder β values. This denotes more upward concavity to the curve's shape. Upwardly concave survival curves were obtained in *Salmonella* inoculated peanut butter that was heat treated at 70°C, 80°C, and 90°C (Shachar and Yaron, 2006). Archer et al. (1998) found similar curve shapes when testing flour at 70°C, although data was not fit to the Weibull model. They reported an initial decline of *Salmonella* within the first few minutes, followed by a slow decline (tail) towards the end of the heating (Archer et al., 1998). In sucrose solutions heat treated at 58-64°C, *Listeria* survival curves increased in

upward concavity as a_w decreased (Fernández et al., 2007). This was observed with *Salmonella* β values in fructose supplemented protein powder at 70°C and sucrose supplemented protein powders at 37°C. When a_w was held constant, temperature did not appear to have an impact on the shape parameter. This is similar to results found by Fernández et al. (2007). Mattick et al. (2001) also reported increasing β values with rising temperatures, a result of significant tailing in the *Salmonella* inactivation curves.

Fructose supplemented protein powders had higher β values than unsupplemented powders at 37°C. These powders also exhibited Maillard browning reactions during the 6 month storage period as noted by subjective visual observation.

As mentioned previously, δ values were higher in all sugar supplemented protein powders than values reported for unsupplemented protein powders at 70°C. This shows that the presence of sugar has an initial protective effect in low-a_w foods at this higher temperature. Other researchers have used salt to reduce a_w levels in foods. Adding NaCl to whey protein powder with storage at 70°C and 80°C, did not affect *Salmonella* survival after 48 h beyond that predicted by a_w alone. (Santillana Farakos et al., 2014). Salt supplemented protein powders fit to the Weibull model had lower δ values than both unsupplemented and sugar supplemented protein powders at $a_w = 0.54$ (Santillana Farakos et al., 2014). The degree of protection for *Salmonella* at the higher a_w level ranged from NaCl < no supplement< sugar. This was not observed at lower water activity levels ($a_w < 0.54$).

Lower δ values observed for sugar supplemented powders at 37°C could be attributed to osmotic shock. Aljarallah and Adams (2007) state that cytoplasmic membrane injury at lower temperatures combined with additional osmotic shock

conditions created by the reduced a_w solutions could explain the lack of protection at the combined lower a_w and temperature levels. However, not all *Salmonella* cells are susceptible to this stress. *Salmonella* was detected after 182-242 days (6-8 months) on various dried fruits stored at 25°C (Beuchat and Mann, 2014). When adding desiccants to TSB to examine whether adapted or osmotically shocked *Salmonella* cells behave differently, sucrose was found to have the most protection over glycerol and NaCl at 55°C (Peña- Meléndez et al., 2014). *Salmonella* was detected in halva, a low-a_w confection containing high amounts of sugar, after 8 months of storage at room temperature (Kotzekidou, 1998).

The significance of the observed effects of sugar on Weibull model parameters cannot be determined until the data reported in this study is validated against predictions made by the model of Santillana Farakos et al. (2013).

In agreement with most of previous studies, increasing the a_w of a product decreases *Salmonella* survival. Sugar has an effect on survival of *Salmonella* at $a_w = 0.42$ and 0.54 values at 70°C. Since there are no effects of sugar at 37°C, a combination of a_w and temperature are important factors in the influence of sugar on *Salmonella* survival. To gain a more comprehensive understanding of the effects of sugar, independent of a_w, more sugar(s) or combinations of sugars, temperatures, and a_w levels should be examined. Validation of data collected in real foods is needed to determine if similar survival patterns are observed in a real-world context.

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Table 1: Changes in water activity of equilibrated protein powder with added sugars during heat treatment at 70°C for 48 hours[a]

Sugar	Targeted a_w of 0.11		Targeted a_w of 0.43		Targeted a_w of 0.58	
	Before	After	Before	After	Before	After
No sugar	0.13 ± 0.03^{A}	0.17^{A}	N/A	N/A	0.53 ± 0.01^{A}	0.54^{A}
Sucrose	0.18 ± 0.02^{A}	0.28 ± 0.03^{B}	0.43 ± 0.03^{A}	0.42 ± 0.01^{A}	0.53 ± 0.03^{A}	0.55 ± 0.04^{A}
Fructose	0.22 ± 0.04^{A}	0.33 ± 0.04^{B}	0.39 ± 0.02^{A}	0.50 ± 0.02^{B}	0.54 ± 0.03^{A}	0.58 ± 0.04^{A}
Sorbitol	0.22 ± 0.08^{A}	0.22 ± 0.07^{A}	0.40 ± 0.04^{A}	0.40 ± 0.03^{A}	0.54 ± 0.02^{A}	0.54 ± 0.03^{A}

[a]Different letters in rows indicate statistical differences before and after heat treatment ($p<0.05$). Sugar supplemented protein powder data based on 3 or more replications. Changes in a_w for protein powders supplemented with sugar are compared to data of Santillana Farakos et al. (2013) for unsupplemented powders.

Table 2: Changes in water activity of equilibrated protein powder with added sugars during heat treatment at 37°C for 168 days[a]

Sugar	Targeted a_w of 0.11		Targeted a_w of 0.43		Targeted a_w of 0.58	
	Before	After	Before	After	Before	After
Sucrose	0.18 ± 0.05^{A}	0.23 ± 0.03^{B}	0.44 ± 0.02^{A}	0.43 ± 0.01^{A}	0.54 ± 0.06^{A}	0.52 ± 0.02^{A}
Fructose	0.16 ± 0.03^{A}	0.31 ± 0.05^{B}	0.41 ± 0.03^{A}	0.50 ± 0.03^{B}	0.49 ± 0.08^{A}	0.59 ± 0.03^{B}
Sorbitol	0.14 ± 0.02^{A}	0.25 ± 0.02^{B}	0.40 ± 0.04^{A}	0.44 ± 0.02^{B}	0.52 ± 0.06^{A}	0.54 ± 0.03^{A}

[a]Different letters in rows indicate statistical differences before and after heat treatment ($p<0.05$). Sugar supplemented protein powder data based on 3 or more replications. Changes in a_w for protein powders supplemented with sugar are compared to data of Santillana Farakos et al. (2013) for unsupplemented powders.

Table 3: Weibull model parameters for *Salmonella* survival in protein powder supplemented with various sugars, equilibrated to a_w = 0.20±0.03, and held at 70°C[a]

Water activity	Sugar concentration	β	se β	log δ	se δ
0.20±0.03	No sugar	0.36	0.09	2.02	1.95
	Sucrose 20%	0.52	0.15	2.55	2.37
	Fructose 20%	0.15	0.05	2.41	2.54
	Sorbitol 20%	0.34	0.10	2.78	2.57
	Sucrose 40%	0.78	0.19	2.93	2.42
	Fructose 40%	0.21	0.07	3.06	3.05
	Sorbitol 40%	0.29	0.09	2.75	2.60

[a]Unsupplemented protein powder data is from Santillana Farakos et al. (2013). β (or p) represents the shape parameter. δ is defined as the time to first decimal reduction.

Table 4: Weibull model parameters for *Salmonella* survival in protein powder supplemented with various sugars, equilibrated to a_w = 0.42±0.01, and held at 70°C[a]

Water activity	Sugar concentration	β	se β	log δ	se δ
0.42±0.01	No sugar	0.22	0.02	0.31	0.23
	Sucrose 20%	0.26	0.03	0.73	0.67
	Fructose 20%	0.18	0.06	1.51	1.77
	Sorbitol 20%	0.21	0.06	1.88	2.00
	Sucrose 40%	0.37	0.07	1.91	1.76
	Fructose 40%	0.52	0.17	3.18	2.68
	Sorbitol 40%	0.22	0.08	2.51	2.62

[a]Unsupplemented protein powder data is from Santillana Farakos et al. (2013). β (or p) represents the shape parameter. δ is defined as the time to first decimal reduction.

Table 5: Weibull model parameters for *Salmonella* survival in protein powder supplemented with various sugars, equilibrated to a_w = 0.54±0.01, and held at 70°C[a]

Water activity	Sugar concentration	β	se β	log δ	se δ
0.54±0.01	No sugar	0.30	0.04	0.79	0.62
	Sucrose 20%	0.52	0.07	1.97	1.65
	Fructose 20%	0.58	0.13	2.71	2.29
	Sorbitol 20%	0.18	0.05	1.65	1.85
	Sucrose 40%	0.49	0.06	1.95	1.57
	Fructose 40%	0.69	0.16	2.95	2.39
	Sorbitol 40%	0.29	0.07	2.31	2.20

[a]Unsupplemented protein powder data is from Santillana Farakos et al. (2013). β (or p) represents the shape parameter. δ is defined as the time to first decimal reduction.

Table 6: Weibull model parameters for *Salmonella* survival in protein powder supplemented with various sugars, equilibrated to a_w = 0.20±0.02, and held at 37°C[a]

Water activity	Sugar concentration	β	se β	log δ	se δ
0.20±0.02	No sugar	0.28	0.15	5.94	5.95
	Sucrose 20%	0.14	0.24	4.11	5.03
	Fructose 20%	0.34	0.16	1.70	1.72
	Sorbitol 20%	0.30	0.07	1.18	1.04
	Sucrose 40%	0.07	0.20	5.76	7.20
	Fructose 40%	1.44	1.06	2.26	1.59
	Sorbitol 40%	1.02	0.40	2.09	1.40

[a]Unsupplemented protein powder data is from Santillana Farakos et al. (2013). β (or p) represents the shape parameter. δ is defined as the time to first decimal reduction.

Table 7: Weibull model parameters for *Salmonella* survival in protein powder supplemented with various sugars, equilibrated to a_w = 0.44±0.01, and held at 37°C[a]

Water activity	Sugar concentration	β	se β	log δ	se δ
0.44±0.01	No sugar	0.53	0.10	4.72	4.32
	Sucrose 20%	0.50	0.12	1.30	1.09
	Fructose 20%	0.76	0.20	1.58	1.24
	Sorbitol 20%	0.49	0.11	1.30	1.08
	Sucrose 40%	0.32	0.06	0.93	0.76
	Fructose 40%	0.66	0.13	1.55	1.13
	Sorbitol 40%	0.57	0.11	1.49	1.11

[a]Unsupplemented protein powder data is from Santillana Farakos et al. (2013). β (or p) represents the shape parameter. δ is defined as the time to first decimal reduction.

Table 8: Weibull model parameters for *Salmonella* survival in protein powder supplemented with various sugars, equilibrated to a_w = 0.54±0.01, and held at 37°C[a]

Water activity	Sugar concentration	β	se β	log δ	se δ
0.54±0.01	No sugar	0.45	0.08	4.29	4.01
	Sucrose 20%	0.49	0.12	1.07	0.95
	Fructose 20%	0.68	0.21	1.35	1.20
	Sorbitol 20%	0.33	0.07	0.90	0.80
	Sucrose 40%	0.40	0.09	1.01	0.88
	Fructose 40%	0.85	0.29	1.56	1.33
	Sorbitol 40%	0.27	0.08	0.46	0.59

[a]Unsupplemented protein powder data is from Santillana Farakos et al. (2013). β (or p) represents the shape parameter. δ is defined as the time to first decimal reduction.

Table 9: Time to reach limit of detection for *Salmonella* survival data in protein powder supplemented with various sugars at 3 a_w levels at 70°C[a]

Water activity	Sugar concentration	Time to DL
0.20±0.02	No sugar Sucrose 20% Fructose 20% Sorbitol 20% Sucrose 40% Fructose 40% Sorbitol 40%	**>** 48 h
0.42±0.01	No sugar	24-48 h
	Sucrose 20% Fructose 20% Sorbitol 20% Sucrose 40% Fructose 40% Sorbitol 40%	>48 h
0.54±0.01	No sugar	24-48 h
	Sucrose 20%	24-48 h
	Fructose 20% Sorbitol 20% Sucrose 40% Fructose 40% Sorbitol 40%	**>** 48 h

[a]Detection limit (DL) for this data was determined to be ≤ 5 colonies per plate. Unsupplemented protein powder data was reported by Santillana Farakos et al. (2013).

Table 10: Supplemented and unsupplemented protein powder comparisons of least square means values for 3 a_w levels at 70°C[a]

Sugar concentration	Water activity					
	0.20 ± 0.03		0.42 ± 0.01		0.54± 0.01	
	Fructose	Sorbitol	Fructose	Sorbitol	Fructose	Sorbitol
0*	8.29	8.29	6.26	6.26	5.62	5.62
20	8.00	8.41	8.10	8.22	7.89	8.12
40	8.15	8.52	8.23	8.22	8.08	7.81

[a]Sugar supplemented protein powder data based on 3 replications. *Unsupplemented protein powder (0%) data was reported by Santillana Farakos et al. (2013). Standard error for fructose and sorbitol supplemented protein powders was 0.35.

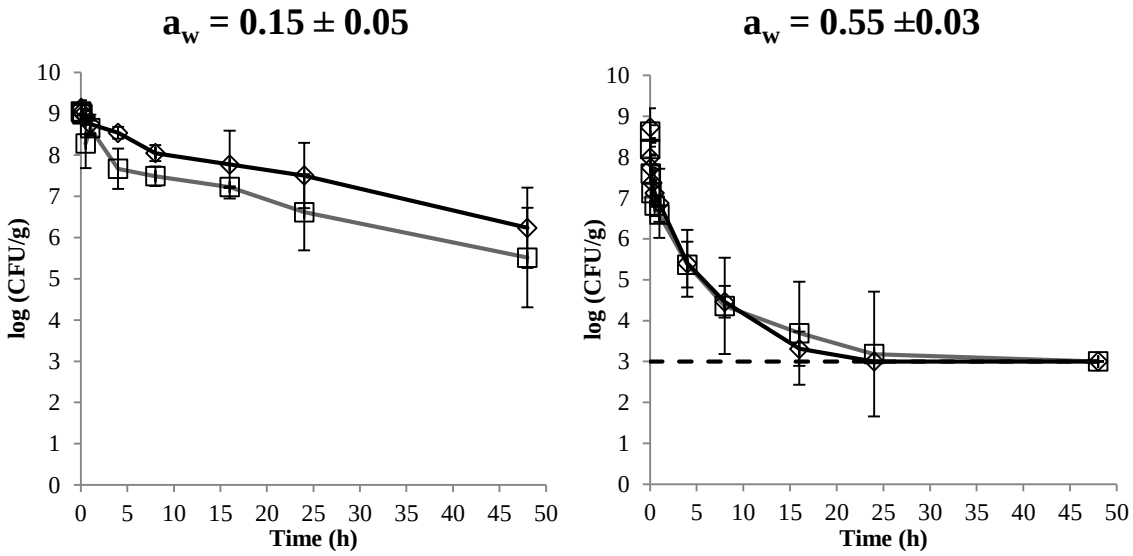

Figure 1: Validation of method to determine survival of *Salmonella* in whey protein powder (◇) against reported values from Santillana Farakos et al. (2013) (□). Data is for unsupplemented whey protein powder stored at 70°C. Error bars represent the standard deviation calculated from three replications. An x indicates that the count fell below the limit of detection, which is shown as the dashed line.

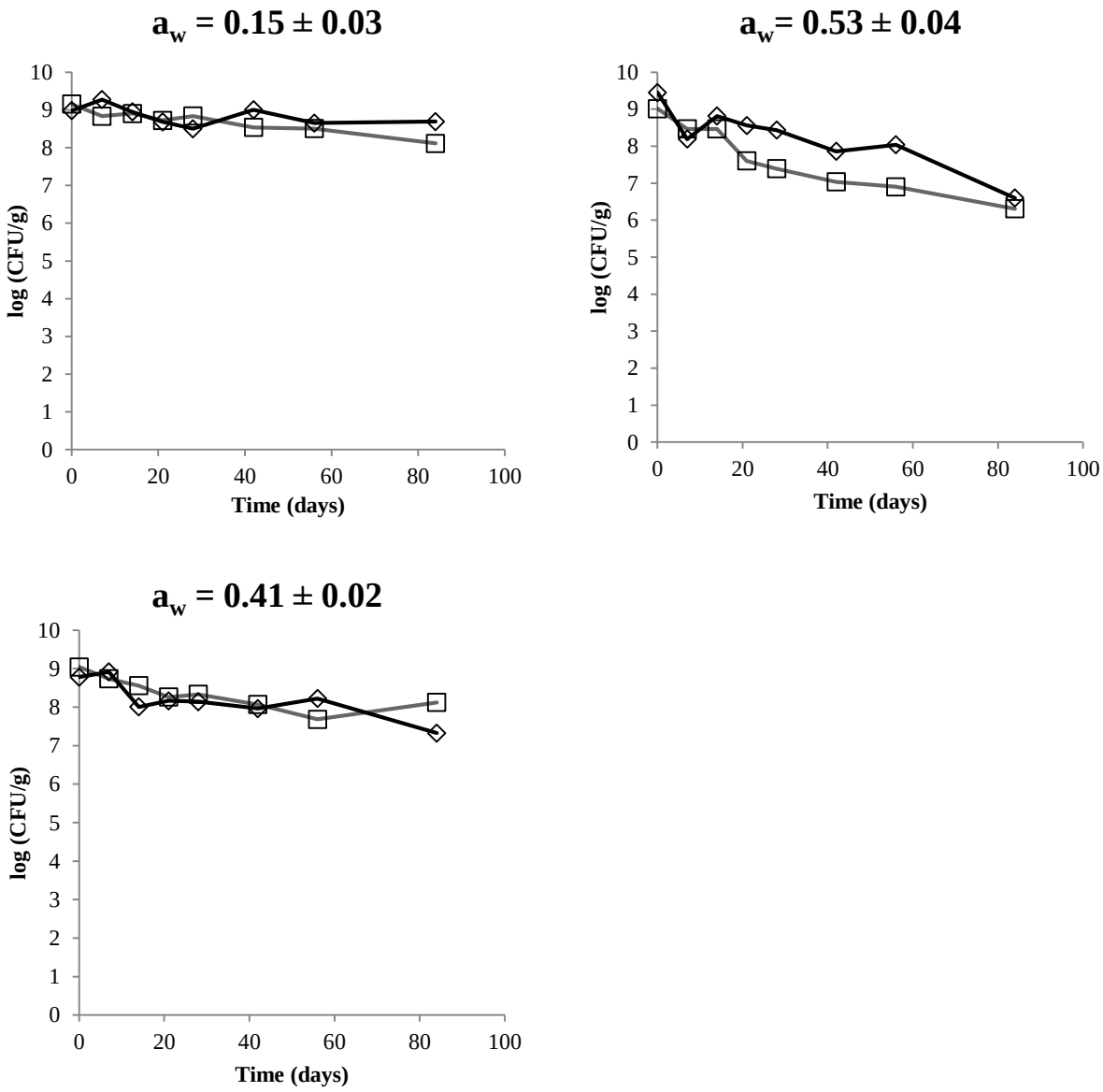

Figure 2: Validation of method to determine survival of *Salmonella* in whey protein powder (◇) against reported values from Santillana Farakos et al. (2013) (□). Data is for unsupplemented whey protein powder stored at 37°C.

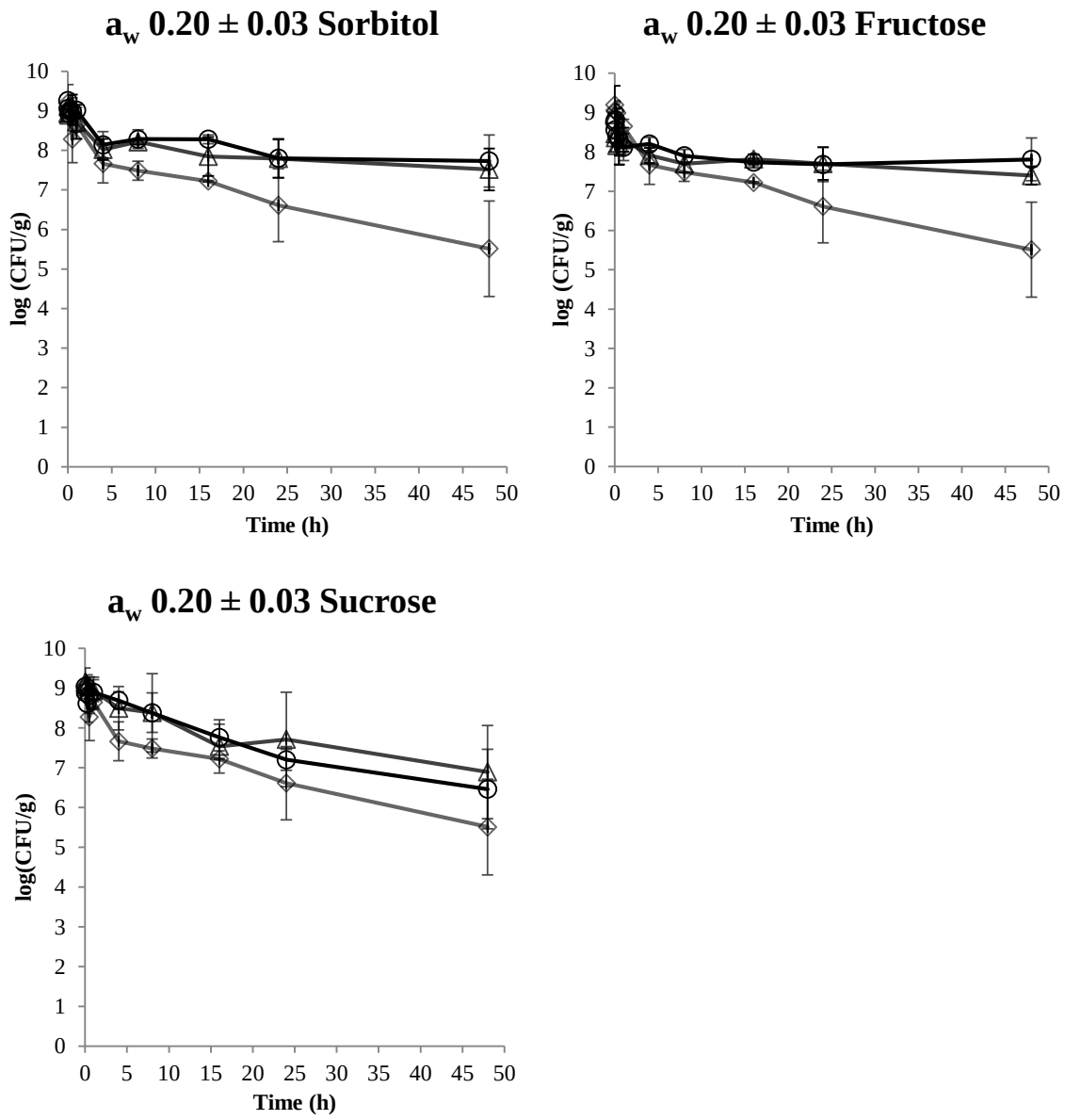

Figure 3: Survival of *Salmonella* at 70°C for 48 h in whey protein powder containing sorbitol, fructose, and sucrose equilibrated at a_w 0.20± 0.03. Sugars were added at concentrations of 0 (◇), 20 (△), and 40% (○). Error bars represent the standard deviation calculated from three replications. Unsupplemented protein powder data (0%) was reported by Santillana Farakos et al. (2013).

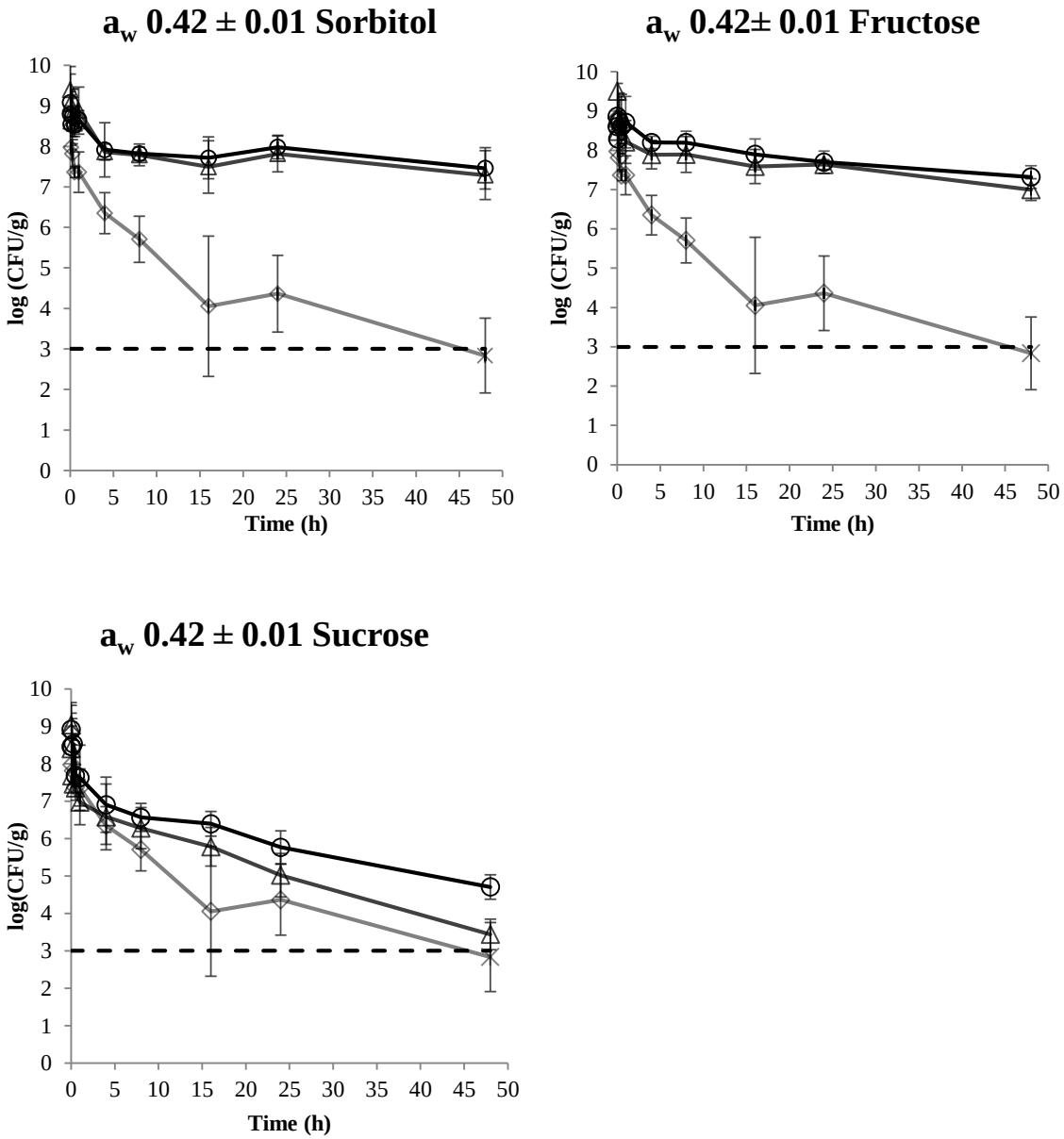

Figure 4: Survival of *Salmonella* at 70°C for 48 h in whey protein powder containing sorbitol, fructose, and sucrose equilibrated at a_w 0.42± 0.01. Sugars were added at concentrations of 0 (◇), 20 (△), and 40% (○). Error bars represent the standard deviation calculated from three replications. An x indicates that the count fell below the limit of detection, which is shown as the dashed line. Unsupplemented protein powder data (0%) was reported by Santillana Farakos et al. (2013).

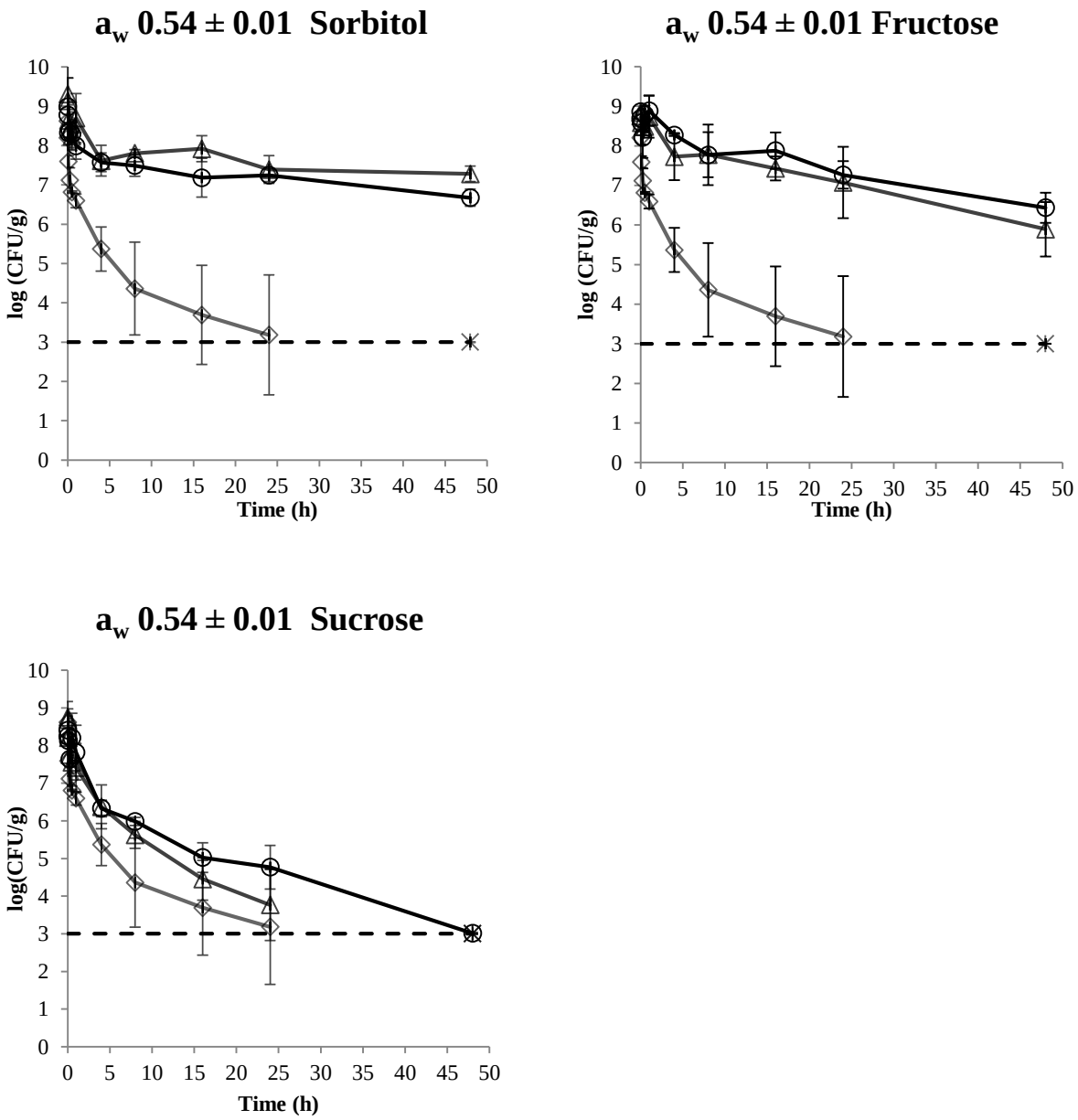

Figure 5: Survival of *Salmonella* at 70°C for 48 h in whey protein powder containing sorbitol, fructose, and sucrose equilibrated at a_w 0.54± 0.01. Sugars were added at concentrations of 0 (◇), 20 (△), and 40% (○). Error bars represent the standard deviation calculated from three replications. An x indicates that the count fell below the limit of detection, which is shown as the dashed line. Unsupplemented protein powder data (0%) was reported by Santillana Farakos et al. (2013).

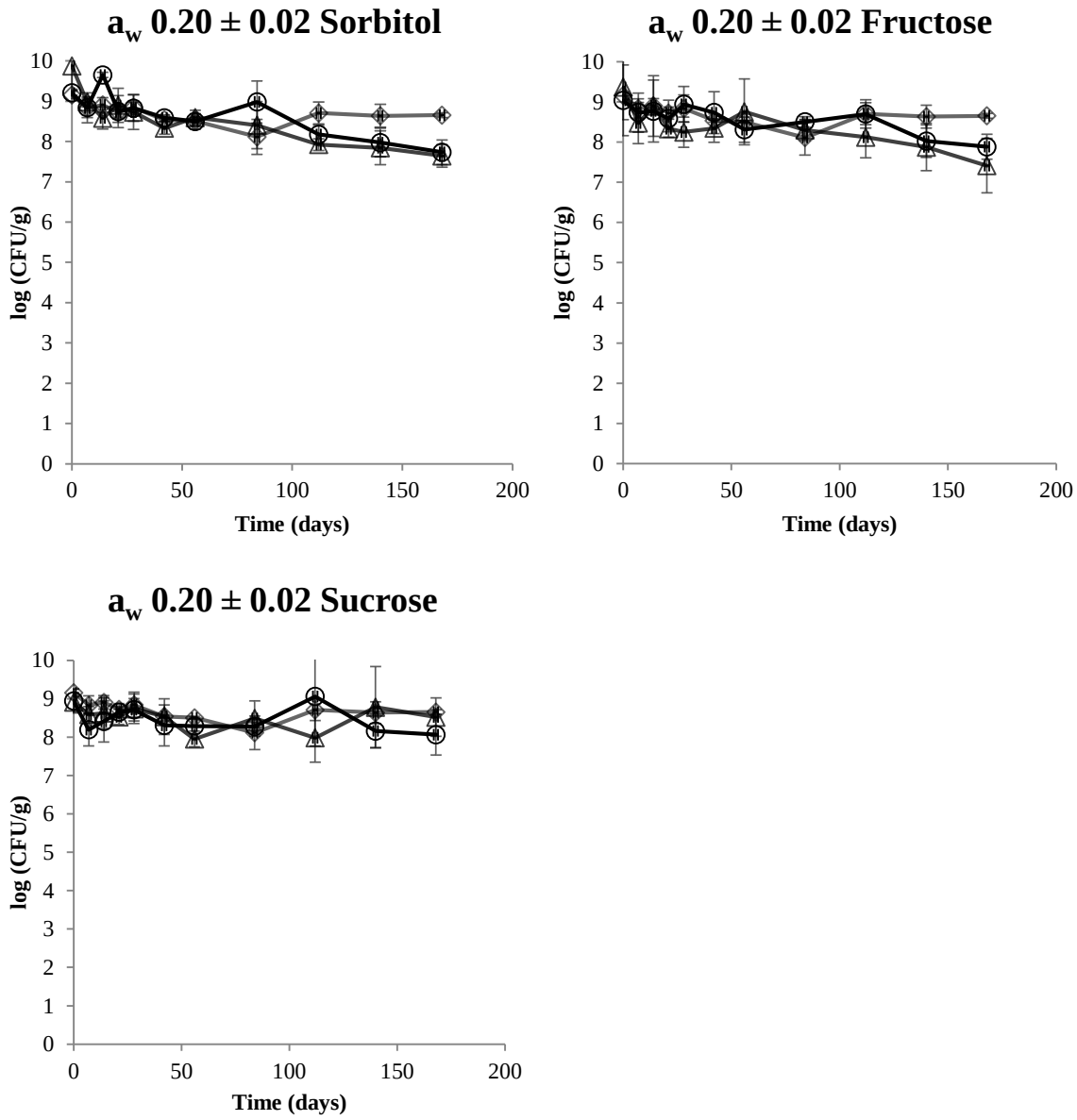

Figure 6: Survival of *Salmonella* at 37°C for 168 days in whey protein powder containing sorbitol, fructose, and sucrose equilibrated at a_w 0.20± 0.02. Sugars were added at concentrations of 0 (◇), 20 (△), and 40% (○). Error bars represent the standard deviation calculated from three replications. Unsupplemented protein powder data (0%) was reported by Santillana Farakos et al. (2013).

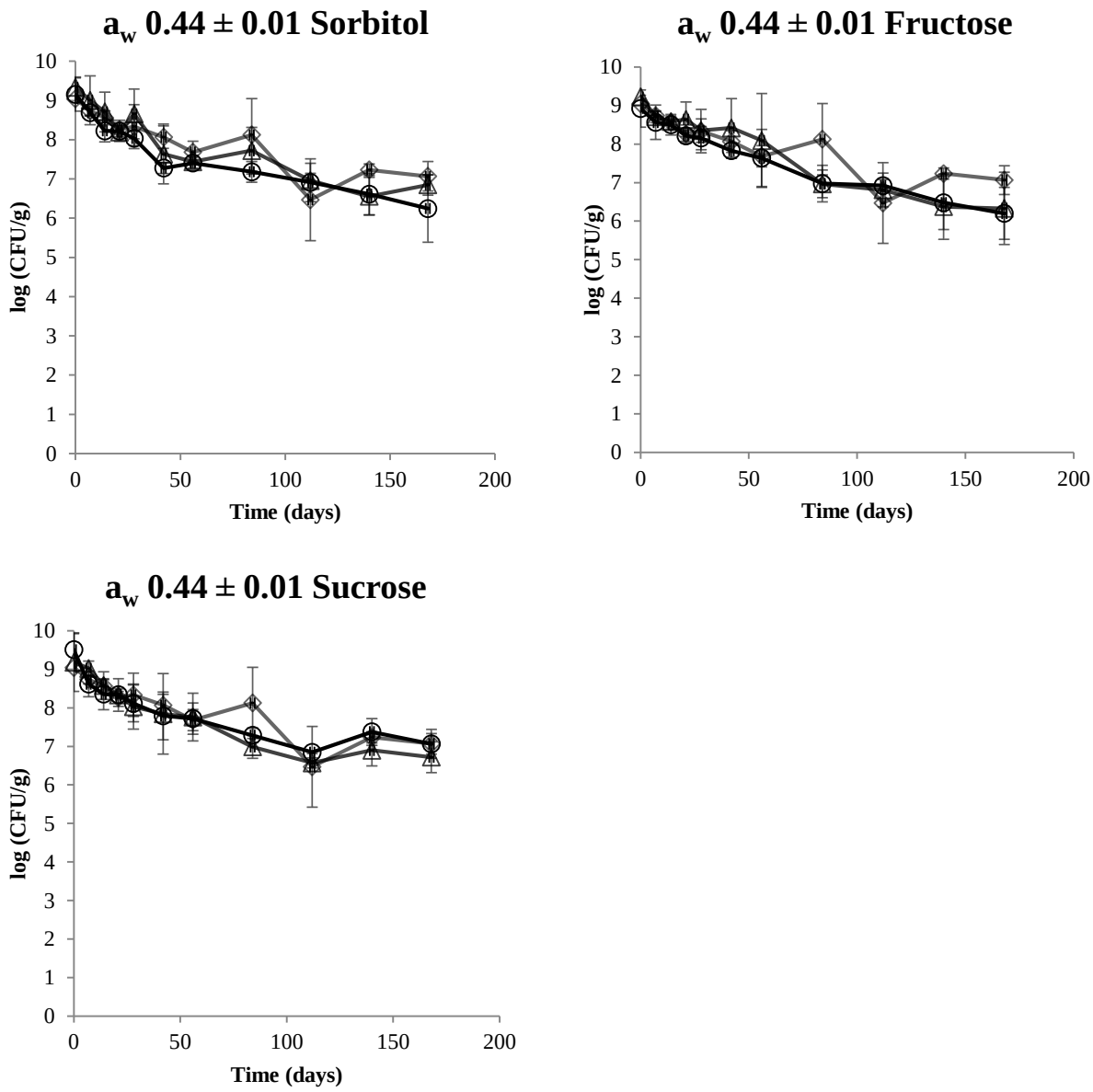

Figure 7: Survival of *Salmonella* at 37°C for 168 days in whey protein powder containing sorbitol, fructose, and sucrose equilibrated at a_w 0.44± 0.01. Sugars were added at concentrations of 0 (◇), 20 (△), and 40% (○). Error bars represent the standard deviation calculated from three replications. Unsupplemented protein powder data (0%) was reported by Santillana Farakos et al. (2013).

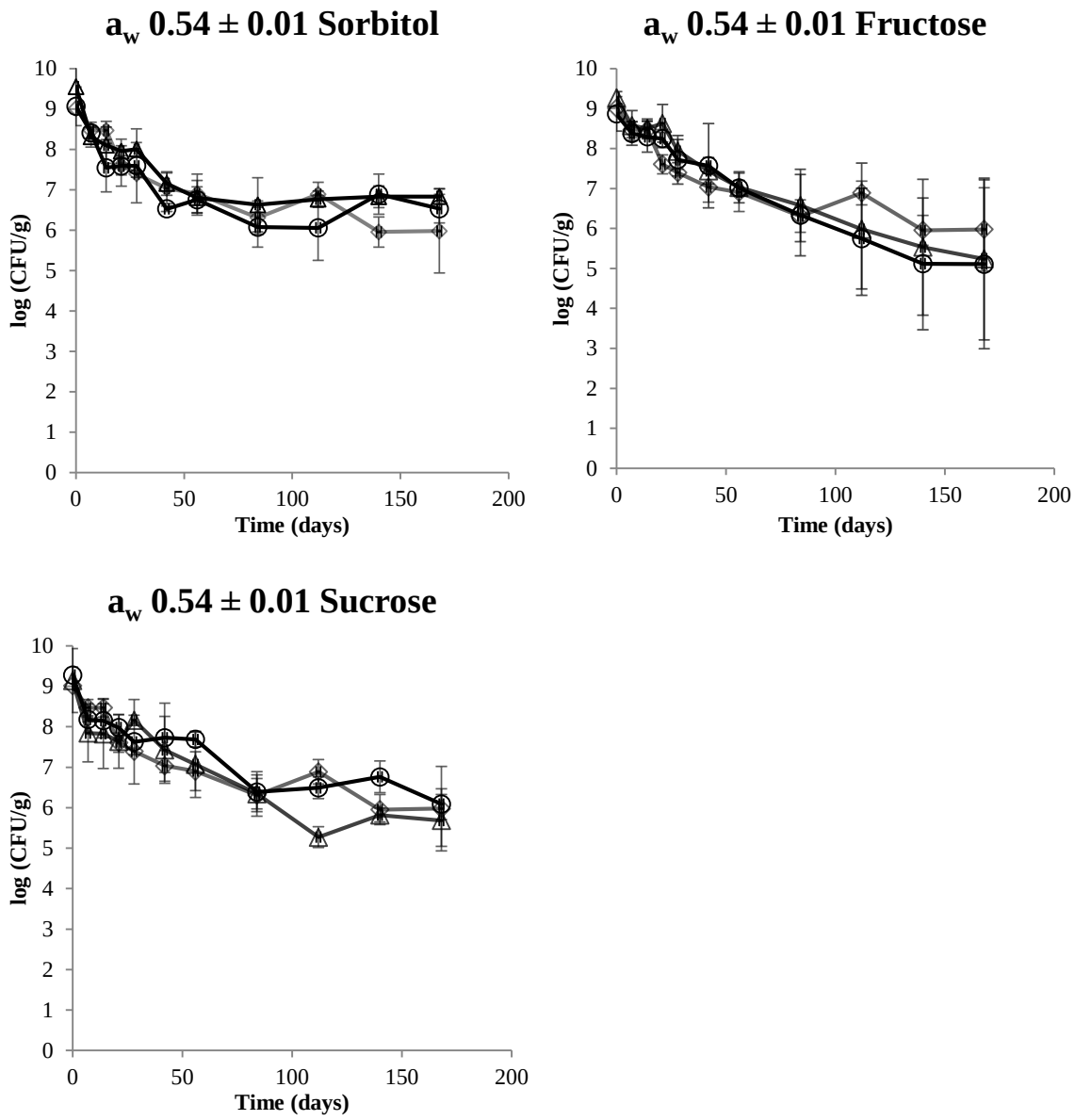

Figure 8: Survival of *Salmonella* at 37°C for 168 days in whey protein powder containing sorbitol, fructose, and sucrose equilibrated at a_w 0.54± 0.01. Sugars were added at concentrations of 0 (◇), 20 (△), and 40% (○). Error bars represent the standard deviation calculated from three replications. Unsupplemented protein powder data (0%) was reported by Santillana Farakos et al. (2013).

CHAPTER 6

CONCLUSIONS

The objectives of this study were to determine the effects of various sugars on the survival of *Salmonella,* independent of a_w, in a low-a_w whey protein powder model. The presence and type of sugar supplemented into whey protein powders significantly influenced the survival of *Salmonella* at water activity levels of 0.42 and 0.54 when samples were held at 70°C, but not at water activity levels of 0.20. The addition of various sugars had no influence on the survival of *Salmonella* spp. independent of water activity for whey protein powder stored at 37°C for 168 days. This suggests that sugar's impact on *Salmonella* survival is dependent upon the temperature that the food is subjected to during processing. Water activity was a significant factor affecting the survival of *Salmonella* for all temperatures examined, but combined effects of water activity and sugar were observed for fructose and sorbitol supplemented powders at 70°C.

Survival data was fit to the Weibull model to establish primary modeling parameters and to compare these values against previously reported data (Santillana Farakos et al., 2013). This data will eventually be incorporated into the published model (Santillana Farakos et al., 2013) to determine under what conditions the presence of sugar has a significant on survival of *Salmonella* within a low-a_w whey protein powder model.

Based on the data collected in this study, a useful predictive model may not be achievable unless more data is obtained. Future studies should be directed towards examining additional temperatures and a_w levels (such as a_w = 0.23-0.33) to improve upon the primary model developed in this study. Other future studies should validate the predictions of *Salmonella* survival with high sugar foods to determine the model's predicative capabilities. Finally, future studies should focus on using data collected in this study to modify the published low- a_w model developed by Santillana Farakos et al. (2013).