Influence of Physiological State, Prolonged Dry Storage, and Passage through Simulated Digestion on the Survival and Gene Expression of *Salmonella enterica* sv. Tennessee

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ABSTRACT

Salmonella enterica serotypes have been linked to outbreaks associated with low water activity foods. The ability of biofilm forming pathogens, such as *Salmonella,* to survive thermal and chemical processes is improved; it is unclear if biofilms will also improve survival to desiccation and gastric stresses. The purpose of this study was to quantify the effect of physiological state (planktonic versus biofilm) and prior exposure to desiccation on *Salmonella* survival and gene expression after passage through an *in-vitro* digestion model.

Cells of *Salmonella enterica* serotype Tennessee were deposited onto membranes for planktonic cells or on glass beads to create biofilms. The cells were subsequently dried at room temperature and stored in dried milk powder ($a_w = 0.3$) for up to 30 days. *Salmonella* survival was quantified by serial dilution onto brilliant green agar before desiccation, after desiccation, after 1-day storage and after 30-day storage. At each sampling both physiological states were tested for survival through a simulated gastrointestinal system. RNA was extracted at the identical time points and relative gene expression determined for genes associated with stress response (*rpoS, otsB*), virulence (*hilA, hilD, invA, sipC*) and a housekeeping gene 16S rRNA using quantitative real-time PCR.

The physiological state and length of storage effected the survival and gene expression of *Salmonella* within the desiccated milk powder environment and after passage through an

in-vitro digestion system ($p<0.05$). Larger numbers of *S*. Tennessee were recovered by plate counts for biofilm cells, compared to planktonic cells. However, the numbers of 16S rRNA gene copies were not significantly different suggesting entry of *S*. Tennessee into a viable but non-culturable state. Prolonged storage in dry milk powder was not associated with increased cross-protection to gastric stress. Increased expression of stress response genes *rpoS* and *otsB* correlated with survival, indicating cross protection of low water activity and acid stress. Increased expression of virulence-associated genes was seen in cells exposed to short periods of dry storage, suggesting an increased virulence potential.

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List of Abbreviations

a_w: Water activity

b: Biofilm

BGA: Brilliant Green Agar

BPW: 1% (wt/vol) peptone water

c: Control non-digested

CFU: Colony Forming Units

g: Gastric Phase of *in vitro* digestion

i: Intestinal Phase of *in vitro* digestion

LPS: Lipopolysaccharide

p: Planktonic

PIF: Powdered Infant Formula

PWC: 1% Peptone solution supplemented with 0.2% cellulase

qRT-PCR: Quantitative Real-Time PCR

T_d: Dew Point Temperature

T_s: Sample Temperature

TSB: Tryptic Soy Broth

Chapter 1: Introduction

Contamination of food products by pathogenic microorganisms is of great concern to both food manufacturers and to the health of the general public. Food-borne illnesses attributed to known pathogenic microorganisms were associated with an estimated to cause 9.4 million illnesses annually in the USA (Scallan et al., 2011). Approximately 1 million cases are attributed to non-typhoid serotypes of *Salmonella enterica*, resulting in 35% of hospitalizations and 28% of deaths due to known foodborne pathogens (Scallan et al., 2011). Increasing numbers of these outbreaks are attributed to foods with low water activity or moisture, such as dry milk, infant formula, chocolate and peanut butter (Podolak et al., 2010). Interestingly, a reduced infectious dose of some serotypes of *Salmonella* is reported in low moisture foods such as chocolate (Blaser and Newman, 1982; Kapperud et al., 1990). It is unclear if these estimated low infectious doses are due to reduced recoverability from low water activity foods. Alternatively, exposure to desiccation in the food may lead to improved survival in the stomach, improving the likelihood of reaching the small intestine to cause disease. This phenomenon, known as cross-protection has been reported to improve survival of acid stressed *Salmonella enterica* subjected to thermal processing (Sirsat et al., 2011). Additionally, expression of virulence genes could be increased leading to increased numbers of infection events.

Foods can become contaminated through direct contact with a contaminated surface or indirectly when dust particles or water is incorporated (Kusumaningrum et al., 2003). An increasing problem in the food industry is the persistence of bacteria in biofilms, which adhere to surfaces and are highly resistant to current sanitation practices (Carpentier and Cerf, 1993; Mittelman, 1998; Podolak et al., 2010;). Biofilms are community structures made by bacteria composed of an extra cellular matrix, which houses a single bacterial species or in many cases

are complex populations of multiple bacterial species (Prouty, Gunn, 2003; Rickard et al., 2003). Bacteria able to produce biofilms create microenvironments in which survival is promoted despite harsh environmental conditions including heat, chemicals and desiccation. *Salmonella enterica* is known to persist within biofilms, and within biofilms tolerance to disinfectants and antimicrobials is increased (Du et al., 2010; Giaouris et al., 2005). This suggests a possible strategy *Salmonella* may use to persist within the dry environment found in low moisture food processing plants (Carpentier et al., 1993; Giaouris et al., 2005; Giaouris et al., 2006; Kusumaningrum et al., 2003; Stepanovic et al., 2004).

The purpose of this study is examine the effect of physiological state (biofilm vs. planktonic cells) on the survival of *Salmonella* at low water activities, and identify potential cross-protection to the harsh conditions of the stomach (low pH, enzymes) that may promote human illness. An isolate of *Salmonella enterica* serotype Tennessee obtained from contaminated peanut butter associated with a multistate outbreak was used (Sheth et al., 2011). Cells were grown to stationary phase as planktonic cells or allowed to form biofilms on silica beads. Cells were subsequently washed to remove residual nutrients and dried at room temperature to simulate low nutrient conditions within a dry foods processing plant. Such a contamination event may have occurred from a liquid with suspended cells such as bird feces or condensation drip from a roof or pipe. The biofilm treatments would represent a contamination in which a biofilm was allowed to form such as a holding tank. The planktonic cells were pipetted onto nitrocellulose membranes, enabling the recovery of all the inoculated cells. The formation of biofilms onto silica beads, while not necessarily reflective of materials used for food processing enable the measurement of water activity of the cell environment using a dewdrop method. Cells were dried at room temperature in a bio-safety cabinet until water

activity measurements reflective of powdered milk were achieved (a_w 0.3) and subsequently stored in airtight containers to prevent introduction of moisture to the system. The membranes and beads were placed in dry milk powder for a period up to one month in airtight containers. This storage time reflects the time it would take from manufacturing until it is likely to be used by the consumer. After desiccation, *Salmonella* was passed through an *in vitro* digestion system utilizing enzymes and a pH representative of the gastric and intestinal environments. The survival of *Salmonella* was determined by plate counts and by quantification of the number of 16S rRNA gene copies, allowing for identification of viable but non-culturable bacteria. To better understand the effect of physiological state and stress on the survival and virulence of *S.* Tennessee quantitative real time PCR will be used to measure relative expression of key stress and virulence responsive genes. This study examines both physiological states of *Salmonella* exposed to desiccation, low nutrients, acid and bile stress, conditions not previously studied together. Results of this study will be important for the development of control strategies for *Salmonella* in processing environments and may also highlight the importance of more sensitive detection strategies for *Salmonella* and other in low moisture environments.

Chapter 2. Literature Review

2.1 Foodborne Illnesses and Epidemiology

It is estimated that one million cases of domestically acquired foodborne illnesses (11% of all cases) are due to non-typhoidal serotypes of *Salmonella enterica*, second behind norovirus. Non-typhoidal *Salmonella* infections are the leading cause of deaths and hospitalizations associated with foodborne pathogens in the USA (CDC, 2011). The vehicles for these staggering statistics are divided between the variety of foods and reservoirs for *Salmonella*. Common vehicles for *Salmonella* include poultry, eggs and recently bean sprouts and other fresh, minimally processed produce. *Salmonella enterica* serotype Typhimurium, for example, caused food related outbreaks in ice cream, cheddar cheese and chocolate, among others (D'Aoust, 1994). Recently, an outbreak occurred involving a low water activity, long-term storage food (peanut butter) contaminated with *Salmonella enterica* ser. Tennessee in all but four U.S. states (CDC, 2007).

Contamination of milk powder products has led to outbreaks involving *Salmonella* and other food borne pathogens. Outbreaks occurred all over the world including the United Kingdom, Bulgaria, France, Spain and the United States (Cahill et al., 2008). In Trinidad, 3,000 infants were infected with *Salmonella enterica* ser. Derby from a contaminated powdered milk plant (Cahill et al., 2008; Weissman et al., 1977). One of the biggest concerns when powdered milk is contaminated is the use of it in powdered infant formula (PIF). In 2005, an outbreak in France had 141 confirmed cases of children less than one year old with 36% of them having to be hospitalized and 99% reporting bloody diarrhea (Brouard et al., 2007). A larger outbreak in South Korea reported 2,000 infants infected with *Salmonella enterica* ser. London from powdered infant formula (Cahill et al., 2008). Incidences of contamination in individual

containers of powdered infant formula were infrequent within a production lot, making it difficult to determine if the contamination occurred before or after the container was opened (Cahill et al., 2008). However, the occurrence of so many cases, across multiple households suggests contamination occurred within the processing plant.

2.2 Salmonella

Salmonella is a genus of Gram-negative, facultative anaerobic, rod-shaped bacteria that have caused a great number of infections, hospitalizations and deaths due to foodborne illness (CDC, 2011). It is divided into two species: *Salmonella enterica* and *Salmonella bongori.* The *enterica* species is associated with the majority of human disease with rare incidences of *S. bongori.* *S. enterica* contains six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* (Tindall et al., 2005). *Salmonella bongori* does not contain subspecies. The majority of *Salmonella* isolates from humans belong to subspecies enterica (I), although subspecies arizonae (IIIa) and diarizonae (IIIb) infections do occur in humans and result not only in gastrointestinal distress but also extra-intestinal infections such as septicemia and chronic urinary tract infections (Mahajan et al., 2003). *Salmonella* serotypes vary not only on antibody reaction to flagella (H) and lipopolysaccharide (LPS) (O) groups but also in their survival and infectious dose, with some strains having adaptations of great concern to the food industry. The most common serovars involved in human illnesses in the USA in 2009 (the most recent report) include *Salmonella enterica* subsp. enterica serovars : Enteritidis, Typhimurium, Newport and Javiana (CDC, 2011).

2.3 Salmonellosis Symptoms and Pathogenesis

Salmonellosis is a manifestation of symptoms caused by infection of the small intestinal mucosal lining by select serotypes of *Salmonella*. Typhoid and paratyphoid strains produce symptoms of enteric fever and diarrhea after an incubation time of 7-28 days. Non-typhoid strains produce symptoms 8 to 72 hours after an infectious dose is consumed. It is characterized by non-bloody diarrhea and abdominal cramps. Chronic symptoms such as arthritis and Reiter's syndrome can also develop from the infection (Dworkin et al., 2001).

In order for the bacterium to infect the mucosal lining of the small intestine it must survive the many hurdles produced by the body. Saliva contains enzymes, such as lactoperoxidase as well as antibacterial compounds such as lactoferrin and lysozyme, which can kill bacterial cells (Tenovuo, 2002). *Salmonella* must also endure the high acidity and pepsin in the stomach. Additional antimicrobial enzymes (bile salts, peptidases, lipases) secreted from the gall bladder and pancreas along with presence of mucus, cell sloughing and competition with gut micro flora also act to prevent small intestinal colonization. In order to overcome these defenses a sufficient amount of cells be ingested to insure that enough cells survive to invade epithelial cells (Blaser and Newman, 1982). The necessary amount is variable based on many factors such as: the individual's age, the food consumed, and the virulence of the strain (Blaser et al., 1982). Determining the infectious dose can be done indirectly through epidemiological studies or directly through volunteer experiments (Kothary and Babu, 2001). Volunteer experimental feeding studies suggested that the infectious dose varied between serotypes but typically 10^5-10^{10} organisms were necessary (Kothary et al., 2001; McCullough and Eisele, 1951a,b,c,d. Outbreak studies suggested comparable doses to the volunteer studies, but also suggested doses as low as 100 cells for some foods (D'Aoust, 1994; Kothary et al., 2001). Outbreak investigations,

however, are indirect approximations based on recovered cell numbers from the food product and the suggested serving size of a food.

Once *Salmonella* adheres to the mucosal lining, proteinaceous appendages are produced and invasion of the epithelial cells begins by altering the host cell's ruffling and cytoskeleton (Ohl and Miller, 2001). Once inside the host cell, the *Salmonella* cells begin to divide within the vacuoles along with, in some cases, enterotoxin production and virulence plasmid replication (Ohl et al., 2001). All these processes result in severe epithelial cell destruction. The mass amounts of cells broken open cause the clinical signs of disease including diarrhea and abdominal pain.

2.4 *In vitro* Digestion

Simulated digestion has been used to investigate cells and food components without having to use human subjects. The basic premise is to simulate relevant stresses, which the sample would encounter if ingested. These stresses include pH, heat and enzymes. The procedure is divided into two phases, gastric and intestinal. The gastric phase contains a pH around 2.5 with the addition of pepsin. The intestinal phase consists of a pH around 6.8 with the addition of pancreatin and bile salts. The residence time in each phase is adjusted based on estimated residence time based on the food matrix. Studies on porcine diet have used test tubes in a shaking incubator tempered to the right pH of the digestive phase with the addition of enzymes to achieve this (Wilfart et al., 2008). Other methods to simulate mechanical forces during digestion include peristaltic pump usage. For this study the method utilizes porcine temperatures and enzymes, which are closely related to human conditions. This method also measures tempers the pH of the solution once during each phase. Other methods maintain the

pH over the time of the phase to also account for responses and pH equilibration by the body once a substance is introduced. These techniques allow for changes to be made to response times during digestion and manipulate them to study differences such as age (Wolters et al., 1993). Enzymes for in vitro digestion are specific to the food matrix to be used. Within an animal feed study pepsin was used to degrade protein and pancreatin to simulate ileal digestion. The main components of milk powder are protein, sugar and fat. To simulate the digestion of protein and fat, pepsin and pancreatin in conjunction with bile salts are used. To compare *in vivo* and *in vitro* digestability organic matter and nitrogen content was measured. The *in vitro* digestion model correlated well with *in vivo* data for intestinal digestion. Gastric digestability was increased in the *in vitro* model compared to *in vivo* (Hur et al, 2011; Wilfart, et al., 2008).

2.5 Water Activity and the Food Industry

Water activity (a_w) is the amount of water available for biological use. It is determined by comparing the vapor pressures of the water in the food (P) to pure water (P_0) at the same temperature. The equation is as follows: $a_w = P/P_0$ (Fellows, 2009). Water activity is a more important characteristic compared to moisture content because water activity directly relates to what is available for microorganisms to use for growth while moisture content is the total amount of water in the sample. A food product can have high moisture content but if the water is bound by solutes such as salt or sugar, the microorganism is not able to acquire the water. Examples of high moisture low a_w foods include salted butter and jellies, which utilize sugar to bind the water. The goal of water activity reduction in food processing is to either bind the water or remove it from the food product.

Water activity is measured more commonly using a hygrometer. A commonly used hygrometer is the chilled mirror dew point measurement. It is commonly used in food science research for its ease of use and low cost. Utilizing dew point, a property dictated by temperature and vapor pressure, the vapor pressure and therefore the water activity of a sample can be determined. A sample is placed in a container within a sealed chamber containing a temperature controlled mirror, optical sensor, fan and infrared thermometer (Barbosa-Canovas et al., 2008). The sample equilibrates the humidity in the chamber and as the mirror is cooled the air around it contracts until it reached its dew point. The optical sensor processes a reflectance change in the mirror at the dew point temperature (T_d) and the infrared thermometer measures the sample temperature (T_s). $A_w = (P_0(T_d))/(P_0(T_s))$ (Barbosa-Canovas et al., 2008) where the vapor pressure of water is at the same temperature as the sample or the mirror. Other methods exist such as: the electric hygrometer, measuring electrical conductance change based on vapor pressure, the polymer hygrometer, measuring fiber expansions due to vapor pressure, and the thermocouple psychrometer, utilizing a wet bulb to measure evaporation temperatures (Barbosa-Canovas et al., 2008).

There are different water activity requirements to prohibit growth for each microbe. Molds and yeasts such as *Saccharomyces* sp. can replicate in a minimum water activity of 0.61. *Salmonella* requires a water activity of above 0.91 to grow (Barbosa-Canovas et al., 2007). The lowest water activity associated with bacterial growth is 0.85, which permits growth of *Staphylococcus aureus.* For this reason only foods with a water activity below 0.85 are classified as low water activity foods and are subject to less regulation than higher a_w foods. By decreasing the water activity, bacterial growth can be controlled. Pathogens, however, can still survive at low water activities. *Salmonella,* for example, has been known to survive low water

activity environments for two years (Hiramatsu et al., 2005; Kieboom et al., 2006; Kim et al., 2007; Stepanovic et al., 2004; Wesche et al, 2009).

There are many techniques to lower a foods water activity and the use of them is dependent on the final food product needed. Sugar is added to jams to reduce the water activity while still maintaining the moisture levels of the product. For some cereals the process of heat extrusion causes an explosive decompression of the product and removes a large amount of the water.

2.6 Powdered Milk

Powdered milk is utilized in a variety of food products from powdered infant formula to chocolate. By drying milk components microbial growth can be controlled, nutritional milk components can be added to non-aqueous products, and milk can be reformulated to achieve desired standards (Chandan et al., 2008; Fellows, 2009). Powdered milk is usually kept at a water activity of 0.30 during storage (Barbosa-Canovas et al., 2008). In industry the majority of milk powder is skim or full-cream. Skim milk powder consists of 36% protein, less than 1% fat, 51% lactose, 8% ash water and 3-4% moisture. Full-cream milk powder consists of 26% protein, 27% fat, 38% lactose, 6% ash and 3% moisture (Chandan et al., 2008). Cream powder, consisting of the cream separated from the skim milk, and buttermilk powder are other milk powder products.

Milk powder is produced by first evaporating the milk and then spray drying it into a powder. The process begins with full-cream milk being either separated into skim milk and cream or standardized to contain the proportions for full-cream milk powder. The following steps for the skim and full-cream milks are identical. The milk is preheated to denature the whey

protein and to allow for successful moisture evaporation in later steps. The milk solids are condensed to reduce spray-drying time. The condensation is done at low temperatures and under a vacuum. For the spray drying water evaporation is achieved by atomizing the milk particles, increasing the surface area compared to the volume. The condensed milk is pumped through the atomizer or spray nozzle and is introduced to dry heated air causing the small droplets to form a powder. The moist air is separated from the powder in a cyclone chamber and the powder is collected (Chandan et al., 2008; Fellows, 2009; LiCari and Potter, 1970).

Although the low water activity of milk powder is efficient in preventing further microbial growth it does not eliminate contamination. The process of spray drying has been shown to have up to a 4-log reduction of some *Salmonella enterica* serotypes. Some serotypes experienced decreased reductions including *S.* Tennessee, which only had a 3-log reduction at normal spray drying temperatures (LiCari et al., 1970). Manipulating solid concentrations and moisture percentages have suggested higher log reductions but, again, each *Salmonella* serotype tested had varying results (Podolak et al., 2010). A 6-log reduction was achieved with *Salmonella* ser. Typhimurium in a 20% solids concentrated milk solution but increasing the solids (40%) the solution before spray drying further increased survival after spray-drying (Miller et al.,1972; Podolak et al., 2010). *Salmonella* persists in large numbers in the reduced water activity of powdered products such as milk and eggs for at least one year (Kafel and Radkowski, 1986; Podolak et al., 2010).

2.7 Stress Responses and Cross-protection

Stress responses induce changes within an organism in order to maintain necessary conditions and processes functioning. Stress responses increase survival through many

mechanisms from membrane protein changes to maintain intracellular homeostasis to inducing DNA and protein repair mechanisms to prevent further cell damage. Stress response can also be characterized by development of long, filamentous cells when grown in salt solutions (aw 0.93) (Kieboom et al., 2006). Virulence is maintained within this filamentous cell morphology as well as increased survival during digestion (Stackhouse et al, 2012). *Salmonella* stress responses vary depending on the type and intensity of stress, combination of stresses and the state of the cells. Cross protection is described as an increased survival to stress due to previous stress adaptations. Cross-protection has posed a problem for the food industry through increased pathogen survival because of the processing methods utilized to control microbial growth can also contribute to resistance to other methods.

Thermal stresses are commonly used to inactivate or kill cells. When a food product is improperly heated or temperature abused some organisms have shown an increase in virulence and resistance to other stresses (Datta et al., 1993; Podolak et al., 2010; Sirsat et al., 2011). Exposure of *Listeria monocytogenes* and *E. coli* to sub-lethal temperatures is associated with an increase in virulence gene expression (Datta et al., 1993; White-Ziegler et al., 2007). *E. coli* began expressing *papB* at 37°C, which encodes for fimbriae associated with host virulence (White-Ziegler et al., 2007). *Listeria monocytogenes* began producing more hemolysis listeriolysinn O at 37°C, increasing the virulence of the already dangerous bacteria after heat shock (Datta et al., 1993). *Salmonella enterica* serotype Typhimurium under thermal stress expressed *ibpA, uspA, uspB, htpG, dnaK and htrA*, common heat shock protein genes responsible for repairing protein damage and reducing stress from apoptosis signals, as well as genes responsible for increased host cell attachment (Sirsat et al., 2011). Descriptions of the genes are as follows: *ibpA* (protein folding and stabilization), *uspA* (adaptation to environmental change),

uspB (adaptation to environmental change)*, htpG* (encodes for chaperone Hsp90 involved in adaptation to environmental change)*, dnaK* (encodes for chaperone Hsp70 involved in adaptation to environmental change) *and htrA* (a serine protease involved in protein degradation) (Sirsat et al., 2011). Overall these genes maintain necessary component of the cell working to adapt the cell into the new environment.

During acid stress, membrane transport is regulated to maintain internal cellular homeostasis. Osmotic stress also changes membrane transport and works to maintain water content. Reducing the diffusivity of membranes as well as protecting components from changes such as protein denaturation, membrane disruption, DNA degradation, help the cell survive under both stresses. Low water activity stresses have increased the survival of microorganisms to both long-term nutrient deficient storage and heat stresses (4, 25, 37 and 70°C) (Hiramatsu et al., 2005). *Salmonella* was shown to retain initial cell numbers up to two years in a dry environment (Hiramatsu et al., 2005). Acid conditions cause a cross protection effect against heat, cold, osmotic, and oxidative stresses (Xu et al., 2010a). Acidic conditions on biofilms have been recently investigated to reveal a difference between virulence gene expressions between planktonic and biofilm cells (Xu et al., 2010a). This study looked gene expression of *stn*, involved in enterotoxin production, and *invA*, involved in cell invasion, at different pH including pH 5,6,7. This resulted in biofilm cells having increased *invA* expression at all pH's compared to planktonic cells. These pH's, however, are not indicative of stresses within digestion.

The genes *rpoS* and *rpoE* are involved in stress responses in a variety of microorganisms (Balaji et al., 2005; Du et al., 2011). Both encode for sigma factors regulating the expression of other genes. Both sigma factors respond to osmotic, pH, starvation and thermal stresses (Balaji et al., 2005; Du et al., 2011; Rychlik and Barrow, 2005; White, 2007). RpoE has been suggested to

have a role in flagella regulation due to the osmotic shock in the small intestine increasing the ability of *Salmonella* to invade the epithelial cells (Du et al., 2011).

Salmonella pathogenicity genes have been quantified relative to 16s rRNA qRT-PCR values. *InvA* is involved in *Salmonella*'s ability to invade epithelial cells (Galan et al., 1992). It is used to screen for presence of *Salmonella* because the gene is present only in members of the *Salmonella enterica* subspecies enterica and can be quantified using real-time PCR (qRT-PCR) to predict the virulence of a strain because it is important for invasion (D'Souza et al., 2009; Xu et al., 2010a). A regulator of invasion genes is *hilA* (Bajaj et al., 1995). *HilD* is a de-repressor of *hilA* and important to allow for high levels of invasion gene expression. When invading cells, *Salmonella* utilizes *sipC* to control actin nucleation and type III secretion involved in cytoskeleton rearrangement (Chang et al., 2005; Myeni, Zhou, 2010). *AvrA* codes for protein AvrA, en effector involved in the inflammatory and programmed cell death of host cells (Ben-Barak et al., 2006; Wang et al., 2009). The *stn* gene encodes for the enterotoxin produced by salmonella. It is partially responsible for the nausea, diarrhea, fever and abdominal cramps of salmonellosis (Xu et al., 2010a). All of the above genes have been used in qRT-PCR analyses for *Salmonella.* While analyzing virulence gene allows for some conclusions, it does not necessarily correlate with pathogenesis. Many factors influence pathogenesis of an organisms such as age, cell sloughing, post-transcriptional protein modifications as well as many other factors. In order to obtain conclusions on pathogenesis, live feeding trial must be conducted for the organism of interest.

Since the low water activity food outbreaks there has been an increased effort to study the effect of food processing procedures and stresses on pathogenic microorganisms. *Salmonella* has been studied under heat, low water activity, high salt, low nutrient availability, and chemical

stresses. Most of these stresses were introduced on vegetative planktonic cells grown originally in high nutrient broth. It is suggested that broth-grown bacteria are not indicative of real food industry conditions because of the differences in survival (Carpentier and Cerf, 1993; Giaouris et al., 2005; Podolak et al., 2010; Van Houdt and Michiels, 2010; Xu et al., 2010a; Xu et al., 2010b). Since biofilms are the common forms found in the environment (Rickard et al.,2003) and are known to be a problem in the food industry (Carpentier et al., 1993), a focus on its interactions is necessary for a more realistic view.

2.8 Biofilms

Biofilms are bacterial populations adhered to each other and/ or a surface within a matrix (Costerton et al., 1995). Biofilm formation begins with planktonic cells adhering to a surface, and then individual cells excrete exo-polysaccharides that serve to embed the cells in a matrix. Other planktonic cells can attach to the biofilm passively; however only members of the biofilm typically grow and divide (Rickard et al., 2003). Detachment of small numbers of cells from the biofilm or dislodgement of the entire biofilm is possible throughout the life of the biofilm (Xu et al., 2010b). The cells within the biofilm that are not attached are able to dislodge and become fully functioning planktonic cells again. Biofilms formation protects the microorganisms within from stresses such as heat, acid, antimicrobials, oxidation and desiccation (Xu et al., 2010b). The production of the extracellular matrix and other byproducts is very unique in each biofilm-former, as is the genomic profile. Biofilms become more complex when a multispecies micro-ecosystem forms, with symbiotic growth strategies, promoting the survival of bacteria in environments, which would otherwise be inhospitable (Rickard et al., 2003). Biofilms allow for co-aggregation of different cell species and therefore a close contact environment (Rickard et al.,

2003), which could increase the horizontal gene transfer events. The genes available have a greater chance of providing an advantage since only organisms capable of living in the specific environment are present.

Biofilms are very difficult to control, posing a problem within a food-processing environment where a stress resistant form of an organism can survive to contaminate food. Biofilms can adhere and accumulate on food contact surfaces, causing food safety concerns (Carpentier et al., 1993). The ease of adherence to plastic, stainless steel, glass and even Teflon® surfaces in the form of biofilms increase the need for solutions to biofilms (Carpentier et al., 1993; Giaouris and Nychas, 2006; Podolak et al., 2010; Prouty, Gunn, 2003; Stepanovic et al., 2004) Contamination can also be caused by infected water sources where biofilms make pathogens more resistant to disinfectants such as chlorine (Steed, Falkinham, 2006). Overall biofilms pose a great danger to the food industry because of its ability to persist, survive and spread. *Salmonella enterica* ser. Typhimurium and other serotypes of *Salmonella* are able to form strong biofilms on a variety of different surfaces (Kim et al., 2007; Kusumaningrum et al., 2003; Patel and Sharma, 2010; Stepanovic et al., 2004).

There are many ways in which biofilms have been grown and dislodged for analysis (Lindsay, vonHoly, 1997). To simulate a food-processing environment, biofilms were grown on stainless steel, glass, and plastics. The procedure most commonly used to grow biofilms involves the introduction of a broth culture of a biofilm-former into direct contact with the desired growth surface. The culture is allowed to grow in a constant log-growth phase with frequent growth media replacement. Surfaces are washed to remove non-attached cells between every media change (Giaouris et al., 2006; Kim et al., 2007; Lindsay et al., 1997; Prouty et al., 2003; Stepanovic et al., 2004; Xu et al., 2010a). In order to dislodge the cells methods such as

sonication, bead vortexing, surface vortexing and surface scraping have been used. None of these methods result in complete removal of biofilms for analysis, as biofilms are still visible by scanning electron microscopy (Lindsay et al., 1997). To analyze all of the cells within the biofilms for cell counts and genetic analysis as many cells as possible must be dislodged. There is a difference between late colonizing cells and early colonizing cells (Rickard et al., 2003) and both must therefore be dislodged. A technique that might prove to be effective is using an enzymatic degradation of critical matrix polysaccharides. *Salmonella* has been shown to require the production of cellulose in order to form biofilms (Prouty et al., 2003). The use of cellulose to remove *Salmonella* biofilms from gallstones has indicated a possible use for enzymatic disruption as a gentler method of removal (Prouty et al., 2003). In order to have a substantial amount of biofilm for water activity analysis, biofilms can be grown on the same silica beads used to dislodge the cells. Growth and retrieval of biofilms on beads were shown to be effective in flowing water environments (Steed et al., 2006). Analyzing *Salmonella* in biofilm under non-aqueous desiccation environments has never been done before. New techniques to allow for the analysis of biofilms are important in the pursuit of biofilm understanding.

2.9 Quantitative Real-time Polymerase Chain Reaction

Once the cells are available for analysis, gene expression is extremely critical characteristic to understand what goes on within an individual organism and a living system of organisms. qRT-PCR analyses are a very effective ways to analyze and quantify gene expression. qRT-PCR allows for quantification of gene expression based on specific primer integrations during PCR. When a primer is ligated into a specific DNA sequence it emits a measureable fluorescence with which quantities can be interpolated from a standard sample

curve. It is a quick way to analyze specific gene expression quantities, which would otherwise be done using more time consuming and less precise methods such as regular PCR with gel electrophoresis (D'Souza et al., 2009). qRT-PCR methods have already been used for other studies of *Salmonella* spp. (Chan, Kim, Falkow, 2005; D'Souza et al., 2009; Xu et al., 2010a).

16S rRNA gene is one of the more common genes utilized for qRT-PCR standards. It is always expressed in live cells because it encodes for the 16S unit of the 30S unit of prokaryotic ribosomes. Each organism has an average number of 16S rRNA gene copies which can be used to determine the living cell counts based on qRT-PCR fluorescence values for the gene (Mizusaki, Takaya, Yamamoto, Aizawa, 2008; Xu et al., 2010a). The relative gene expression can also be calculated from 16S rRNA values (Livak, Schmittgen, 2001; Xu et al., 2010a).

2.10 Conclusion

Understanding survival, persistence, growth and resistance of bacterial within an environment is a crucial area of study in order to provide solutions to bacterial contamination. Foodborne pathogens and spoilage microorganisms have a huge negative impact on public health, the food industry and the economy. The food processing industry is high-risk area for bacterial contamination, some of which can cause tremendous crises. Food processing environments have been shown to be incomparable to broth culture conditions and scientific analysis must therefore be tailored to mimic the appropriate conditions in order to draw powerful conclusions. The increased importance on the role of biofilms related to bacteria's effect within an environment has centered the focus of study further to biofilm analysis. *Salmonella* spp. are major players in foodborne outbreaks and must therefore be emphasized. Designing a method for growing and analyzing biofilms is of great importance for future research. A study of the genetic expression

profiles comparable to food processing environments will provide much needed information to conduct further experiments and provide solutions. Using up to date genetic analysis techniques like qRT-PCR and microarrays provide the most effective route to holistic biofilm stress responses. Scientists must evaluate, re-evaluate, invent and propose new methodologies and questions to pursue significant and beneficial answers to the world's problems.

Chapter 3: Materials and Methods

3.1 Bacterial Strain and Preparations of Inocula

Salmonella enterica serotype Tennessee (*S.* Tennessee) previously isolated from peanut butter during the peanut butter outbreak of 2006 (Sheth et al., 2011) was inoculated from a -80°C stock into tryptic soy broth (TSB, Difco, Sparks MD) and incubated shaking (125 RPM) at 37°C for 24 hours. This served as the initial inocula of planktonic cells or for formation of biofilms onto glass beads.

Biofilm formation on glass beads was adapted from the method of Crawford *et al*. 2008. Briefly, *Salmonella* Tennessee was incubated within TSB in a sterile Erlenmeyer flask containing sterile 2.3mm Zirconia/ Silica beads (Biospec Products, Inc., OK) arranged in a single layer. The *Salmonella*- bead mixture was incubated statically at 25°C for 48 hours. The liquid was carefully decanted, taking care to minimize movement of beads and the beads were subsequently washed by gently swirling the beads in 1% (wt/vol) buffered peptone (BPW, Sigma-Aldrich, Co., MO) to remove unattached cells. The growth and washing steps were repeated twice, then the beads were separated into samples (n=72) of 30 beads with a concentration of 8.5 log CFU/sample based on plate counts.

Planktonic *Salmonella* Tennessee cells were incubated in TSB for 48 hours, after which the uppermost 10 ml of the culture was transferred to a sterile 15ml polypropylene centrifuge tube and centrifuged at 4,000 x g for 10 minutes to collect cells. The supernatant was removed and the cells were washed with 10 ml 1% BPW three times and then re-suspended in 10 ml 1% BPW. The washed cells (100 µl) were pipetted onto 0.22µm nitrocellulose membrane filter discs

(25mm size, Millipore, MA) (n=72). Each membrane contained 8.9 log CFU/filter disc based on plate counts.

The samples were divided as follows: biofilm (B) and planktonic (P) each having the following time points: initial (0) (n=18), desiccated 2 hours (2) (n=18), desiccated 24 hours (24) (n=18), desiccated 720 hours (720) (n=18), desiccation treatments performed as described in 3.2. Survival and gene expression were determined for all samples before (n=6) and after passage through an *in vitro* digestion system after the gastric phase (n=6) and after passage through gastric phase and an intestinal phase (n=6). Half of each physiological state at each time point (n=3) were processed for microbial analysis and half were processed for RNA analysis as described in section 3.3.

Initial samples (0) of cells in biofilms on beads (n=18) and planktonic cells on membranes (n=18) were stored in 1ml 1% peptone supplemented with 0.2% cellulase (PWC, Sigma)(n=3) and 0.5ml RNAprotect Bacterial Reagent (Valencia, CA)(n=3), or transferred to a simulated digestion system as described in section 3.4 (n=12).

3.2 Drying of *Salmonella* Biofilms and Planktonic Cells and Storage in Dry Milk Powder

The remaining biofilm samples (n=54) and membranes (n=54) were arranged in a single layer in sterile uncovered petri dishes and allowed to dry for 2 hours within an ABSL2 biosafety cabinet (CCI) (Figure 1). After two hours both the beads and membranes reached 0.30 a_w determined by measuring dew point using a dew point water activity meter (Aqualab 4TE, Aqualab, WA). Immediately after drying, six samples (0) of each physiological state were processed as described in 3.3 to determine bacterial counts after drying (n=3) and RNA profile (n=3). The remaining 0 samples from each physiological state were transferred to a simulated digestion system as described in section 3.4 (n=12). The remaining dried beads (n=36) and

membranes samples (n=36) were placed in individual, airtight 2 ml glass vials. Each vial was filled with enough dry skim milk powder (a_w: 0.29-0.31) to completely cover the beads or membrane (approximately 3-5g). Samples were labeled and stored at 25°C for 24 hours (24) or 720 hours (720). At the specific time interval the beads or membranes were removed from the milk powder and processed as described in 3.3 and 3.4.

3.3 Microbiological analysis and RNA extraction

Cells were dissociated from the beads by incubation in 1 ml of PWC with constant mixing by gently rocking for 1 hour, followed by 5 minutes of low speed vortexing to maximize detachment but minimize physical lysis. Following biofilm detachment, bacterial counts were determined from 10-fold serial dilutions using 1% BPW as a diluent. Aliquots (100 µl) were plated onto Brilliant Green Agar (BGA) (Difco, NJ). Plates were incubated for 48 hours at 37°C.

Cells were dissociated from the membranes in 1ml of PWC with 0.75 g of 2.3mm sterile glass beads, and incubated at 25°C on a rocking platform for 1 hour, followed by 5 minutes of low speed vortexing to maximize detachment but minimize physical lysis. Following the dissociation from membranes, bacterial counts were determined as described for biofilm cells. Three technical replicates were performed for each sample. Three biological replicates were performed for the entire experiment.

RNA was extracted using the combined mechanical and chemical lysis per manufacturer's instructions using the RNeasy Mini Kit (Qiagen). Extracted RNA samples were frozen at -80°C until analyzed for expression as described in 3.5

3.4 Survival of *Salmonella enterica* serovtype Tennessee Through a Simulated Gastrointestinal System

The survival of *Salmonella* Tennessee from each physiological state (b, p) and treatment (0, 2, 24, 720) was evaluated using a simple *in vitro* digestion model involving a simulated gastric and small intestinal phase (Figure 1) which shows high correlation with *in vivo* digestibility coefficients for organic matter, starch, protein, and energy (Boisen and Fernández, 1997; Noblet and Jaguelin-Peyraud, 2007; Wilfart et al., 2008). Beads or membranes at each time point were transferred to 2.0ml micro centrifuge tubes containing 1ml 0.1M potassium phosphate buffer pH 6.0. Tubes were, rocked for 1 to 2 minutes at ambient temperature. Fresh porcine pepsin (806 U/mg protein, Sigma-Aldrich) was made by suspension in double deionized water (DDH2O) to a concentration of 25mg/ml and maintained on ice and used within 5 minutes of suspension. The gastric phase was simulated as follows: 0.2 M HCl was added to each tube to reduce pH between 2.0 and 3.0 using colorpHast pH-paper (EMD Chemicals Inc.,NJ) and 40 μL of 25 mg/mL of freshly prepared porcine pepsin in distilled water was added. Tubes were vortexed, and immersed in a water bath (Thermo-Fisher) set at 42°C for exactly 1 minute to bring the temperature of the slurry to 39°C (the average swine body temperature). Tubes were transferred to a shaking incubator (175 rpm) set at 39°C for 3 h. After the gastric phase, cells from three samples of each physiological state and treatment were collected by centrifugation at 10000 xg for 5 minutes and cells were resuspended in 1 ml of PWC. Cells were enumerated and RNA extracted as described in 3.3. The small intestinal phase was simulated as follows: upon completion of the gastric phase, 400 μl of 0.2 M phosphate buffer pH 6.8 was added to the remaining gastric tubes from each physiological state (n=3) and pH adjusted to 6.8 using 1 M NaOH. The pH of each tube will be verified using colorpHast pH-paper. Then, 40 μL of 100

mg/mL of freshly prepared porcine pancreatin (Sigma-Aldrich) and 40 μL of 25 mg/ml freshly prepared bile salts (Sigma-Aldrich) were added. Tubes were vortexed briefly and incubated shaking at 39°C for 5 h. Samples will be collected to culture *Salmonella* after the small intestinal digestion phase as described in 3.3.

3.5 Gene expression of *Salmonella* Tennessee as impacted by physiological state and desiccation

The extracted RNA from the three biological replicates for each of the physiological state, time points and digestion phases were analyzed using Quantitative Real-Time PCR (qRT-PCR). Total RNA was converted to cDNA and primer specific cDNA amplified using the One-Step quantitative reverse transcription PCR kit (USB, Cleveland, OH) per manufacturer's instructions. Each 15μl reaction contained 25 ng of total RNA, 0.16 μl of M-MLV RT (reverse transcriptase), 0.16 μl of RNase inhibitor, 10 μl of HotStart-IT^{TM}SYBR®Green qPCR Master Mix 2X (USB® 75770 Cleveland, OH, USA), 0.4μl of fluorescein as passive reference dye (USB® 75767 Cleveland, OH, USA), 0.5 mM of forward and 1mM of reverse primers. PCR conditions consisted of: 1 cycle 50°C for 10 minutes for reverse transcription of RNA, 1 cycle 95°C for 2 minutes for activation of HotStart-IT polymerase and reverse transcriptase inactivation, followed by, 40 cycles of denaturation at 95°C for 15 sec followed by a variable annealing temperatures and real time detection for 39 sec and 72°C for 60 sec. Primers sets and their anneal temperatures are as follows: 16S rDNA, *hilA* and *rpoS* (53°C), *invA* and *otsB* (53.75°C) and *hilD* and *sipC* (50°C). Dissociation curves were produced to insure that only a single peak, at the appropriate melting temperature of the amplicon was detected for each primer set. Primers used were collected from previously published papers summarized in Table 1 and

optimized for use in this experiment. cDNA synthesis and amplification was carried out with an iQ5^{TM} Optical system Real-Time PCR detection system (Bio-Rad). Ct values were normalized using *16S rRNA* as a housekeeping gene and gene expression was calculated by Pfaffl method for relative quantification (ratio=$(E_{target})^{\Delta Ct, target}$/ $(E_{ref})^{\Delta Ct, ref)}$. Six technical replicates were performed for each biological replicate.

3.6 Data analysis

Bacterial counts were log transformed to approximate normal distribution. Statistical analyses were performed using JMP (version, SAS, Cary, NC) statistical software. 2-way ANOVAs were performed to test for differences in bacterial counts and differences in relative gene expression. Models tested were: physiological state, desiccation time and digestive stress. P-values < 0.05 were considered significant.

3.7 Tables and Figures

Table 1: Genes and primers for virulence and stress response genes.

Gene Target	Function	Primers	Reference
Survival			
16S rRNA	Transcription	F: TGTAGCGGTGAAATGCGTAG R: CAAGGGCACAACCTCCAAG	Mizusaki *et al.,* 2008
Virulence			
hilA	Regulator for SPI-1 virulence	F: CATGGCTGGTCAGTTGGAG R: CGTAATTCATCGCCTAAACG	Mizusaki *et al.,* 2008
sipC	Cell invasion SPI-1	F: CTGTGGCTTTCAGTGGTCAG R: TGCGTTGTCCGGTAGTATTTC	Mizusaki *et al.,* 2008
invA	Cell invasion SPI-1	F: CACGCTCTTTCGTCTGGCA R: TACGGTTCCTTTGACGGTGCGA	D'Souza *et al.,* 2009
hilD	Regulator of *hilA*	F: AGCAGTTTCACTTTAGTTTGCTTTC R: CGTTTTCAGATGTTCAAATACCTCT	Mizusaki *et al.,* 2008
Stress Response			
rpoS	Transcription	F: GTTGGACGCGACTCAGCTTT R: TTTTACCACCAGACGCAGGTT	Balaji *et al.,* 2005
otsB	Trehalose Production	F: TTAACCGTATCCCCCGAACTC R: CCGCGAGACGGTCTAACAAC	Balaji *et al.,* 2005

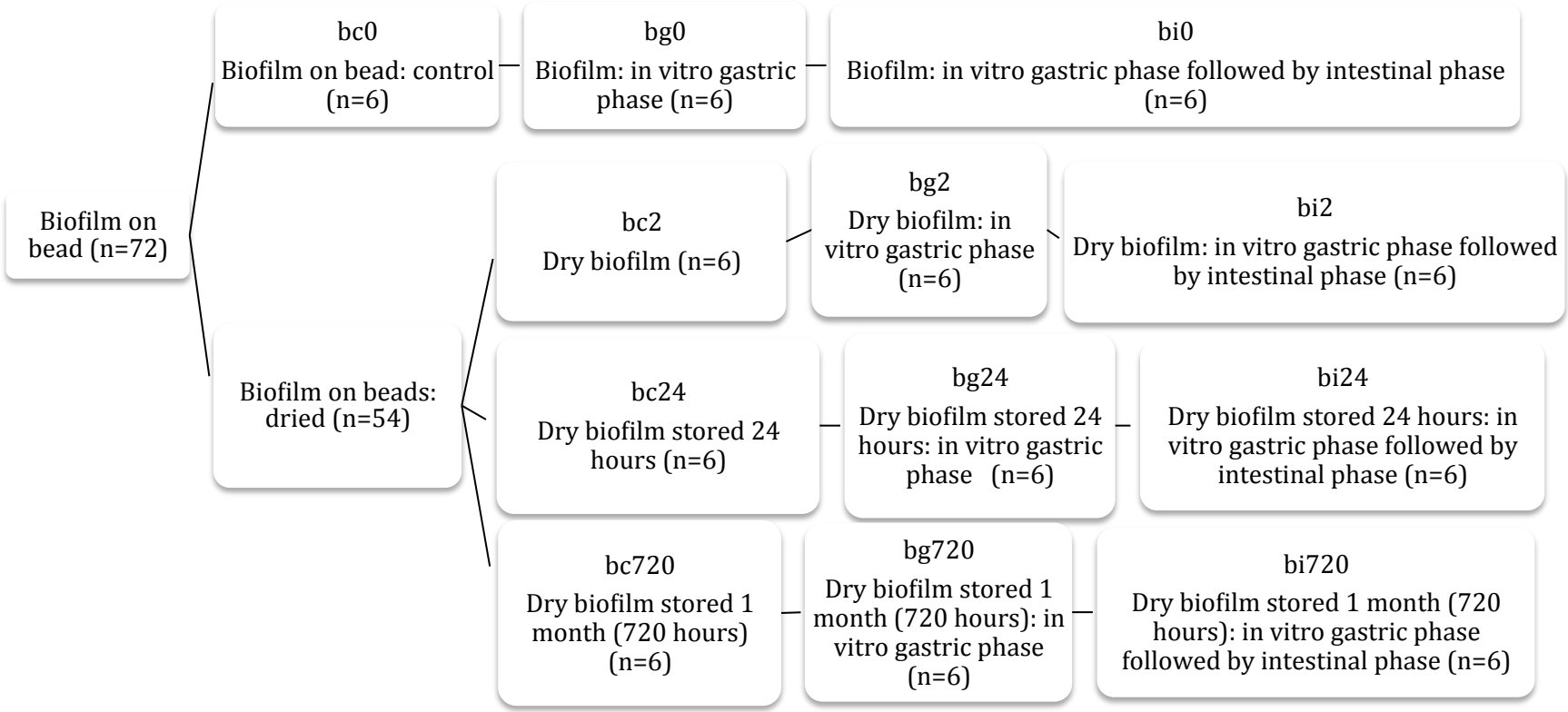

Figure 1a: Diagram depicting treatments of biofilm state cells including desiccation, storage at a_w 0.3 and simulated digestion. The sample number represents the combined sample number of each sample processed for RNA extraction and gene expression analysis or for enumeration by serial dilution and plating on BGA.

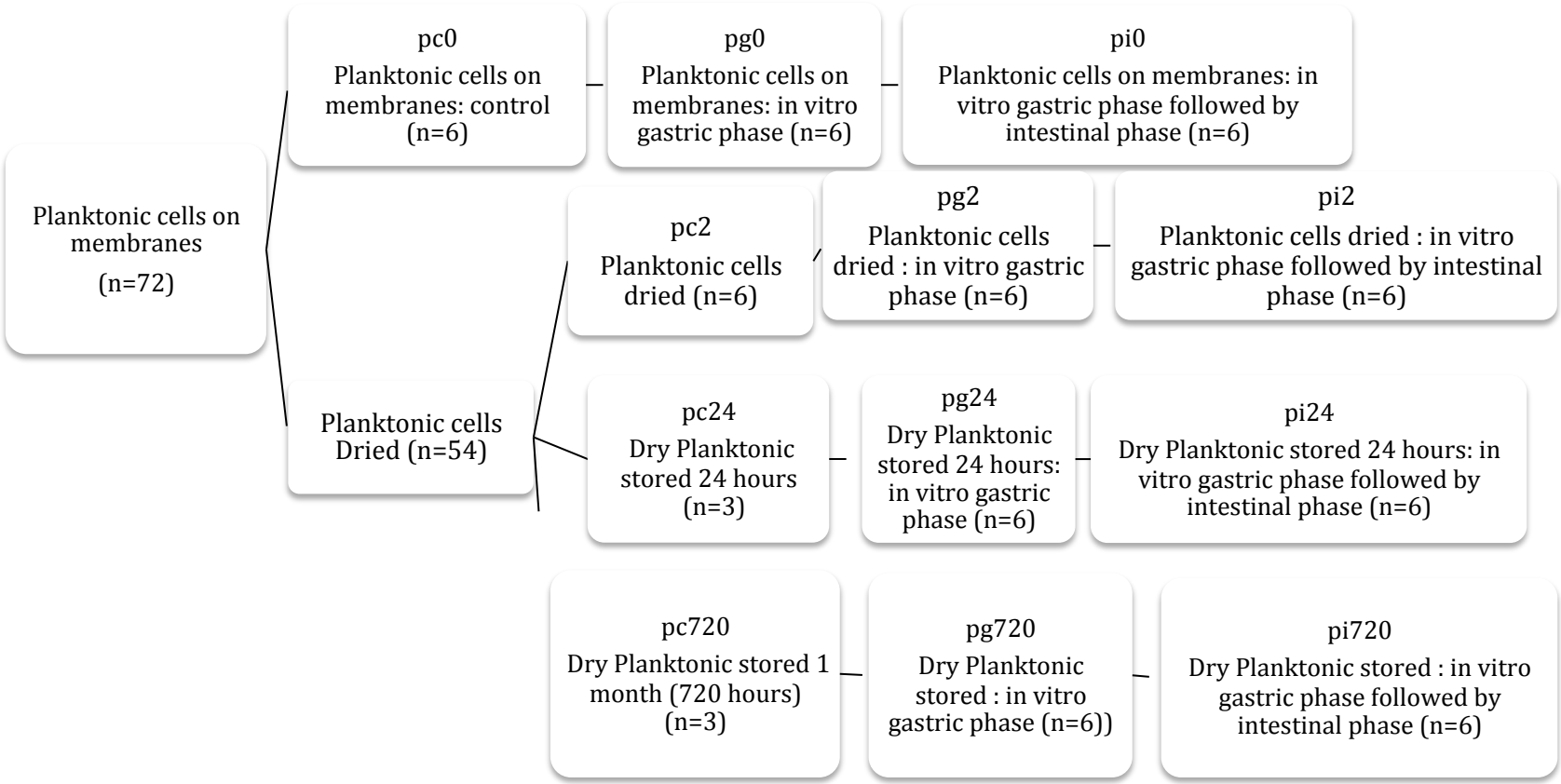

Figure 1b: Flow diagram of planktonic cell treatments including desiccation to a_w 0.3 , storage for a period up to 30 days and passage through simulated digestion. The sample number represents the combined sample number of replicates processed for RNA extraction and gene expression analysis or for enumeration by serial dilution and plating on BGA.

Chapter 4: Results and Discussion

4.1 Effect of Physiological State, Simulated Digestion and Dry Storage on Survival of *S.* Tennessee

Numbers of *Salmonella enterica* ser. Tennessee recovered on BGA from dried milk powder and after simulated digestion was influenced by the physiological state of the cells, and length of storage in dried milk powder (Figure 2). Utilizing relative 16S rRNA copy number changes to quantify survival of active *Salmonella* yielded less dramatic changes through the treatments over all but the trends did correlate with plate count data in some treatments (Figure 2 and Table 2). No 16S rRNA gene expression was detected in un-inoculated milk powder, indicating that the copies detected in inoculated samples are only the inoculated strain.

The physiological state of *S.* Tennessee cells prior to drying and subsequent storage significantly affected the recoverable numbers after prolonged storage in dry milk powder (a_w 0.3) (Table 2). Populations of recoverable *S.* Tennessee in the biofilm state were reduced by 2.8 log CFU/sample throughout the entire 720h study; in contrast a significantly larger reduction (5.4 log CFU/sample) was determined for planktonic cells ($p < 0.05$). Despite the decrease in *S.* Tennessee recovered on the BGA media, there was not a significant difference in log copies of 16S rRNA copies for either planktonic or biofilm cells. (Figure 2a, n720 samples).

Incubation in simulated gastric fluid was associated with a significant reduction in culturable *S.* Tennessee (Figure 2b, g samples) for both biofilm and planktonic cells, with larger reductions in culturable cells noted for planktonic cells. The exception was that cells in a biofilm for 720 hours were recovered in greater numbers after the gastric phase, compared to the non-treated cells (Figure 2b). The number of culturable cells recovered from samples stored for different periods of time were significantly different, however there was no generalizable trend associated with time (Table 2b). No significant changes in numbers of 16S rRNA gene

copies were observed for cells in the biofilm state (Table 2b, Figure 2b). In contrast a one log reduction in number of 16S rRNA gene copies was determined for planktonic cells held for 2 hours, after longer periods of storage the number of active cells was comparable to time 0 (Table 2).

Incubation in simulated intestinal phase conditions was associated with increased recovery of *S*. Tennessee on BGA compared to the gastric phase and non-treated cells (Table 2c). The length of storage in dry milk powder was correlated with an increase in number of recovered planktonic cells though this trend was not apparent with biofilm cells (Table 2c). The length of storage in the milk powder significantly reduced the number of active cells, indicated by numbers of log copies 16S rRNA genes compared to log CFU/sample, in the simulated intestinal phase intestinal stage (Table 2c). The changes obtained from plate count data suggest decreases in recoverability over time as well as large decreases within the gastric treatments and increases in many intestinal treatments. The changes in the non-digested and gastric treatments do not correlate to similar trends within 16S copy number data.

Despite the inclusion of cellulose in the media and chemical digestion, complete lysis did not occur, therefore the number of 16S rRNA gene copies in the biofilm state were underestimated. This is evident based on the increased number of cells recovered on BGA. This could be due to decreased efficiency during RNA extraction. Another possibility is a change in 16S rRNA expression within these treatments. Some studies suggest heterogeneity in copy number associated with environmental response (Dahllof, et al, 2000). This study utilized *rpoB* as an endogenous gene control for 16S rRNA and suggested variability 16S rRNA copy number. These differences were seen in denaturing gradient gel electrophoresis analysis but are still applicable to RNA studies.

The discrepancies between plate counts and number of 16S rRNA copy numbers are a product of viable but non-culturable (VBNC) cells than changes to survival. Similar results have been noted using *rpoS* as a marker for viability (Kusumoto, et al, 2012). The results obtained using *rpoS* indicated steady copy numbers while a decrease in CFU's was noted. This is in conjunction with what is seen within this study, and therefore supports VBNC as an explanation for the different results obtained from both techniques. An increase in VBNC cells is associated with prolonged dry storage. This suggests an increased likelihood of false negative results in low moisture foods that have been stored for long periods of time, especially when the contamination is through biofilm cells (Trevors, 2011). This increased number of VBNC cells may explain the lower infectious dose of *S. enterica* serotypes associated with low moisture outbreak vehicles. Since the dose is estimated based on number of recovered cells in the product, the presence of VBNC cells maybe associated with a decreased number of cells per serving. The lack of significant differences within many of the 16S rRNA gene copy numbers indicates long-term digestive stress survival with even some growth within the intestinal phase. Further research into longer dry-storage times is needed as well as comparison experiments with other organisms.

4.2 Effect of Physiological State, Simulated Digestion and Dry Storage on Gene Expression of *S.* Tennessee

Quantitative real-time PCR analyses of several genes associated with stress response and virulence were analyzed. Results indicated differences in normalized expression associated with the physiological state, length of exposure and simulated gastric digestion. Exposure to low water activity and gastric conditions were associated with increased expression of *otsB,* encoding for trehalose-6-phosphatase, which converts trehalose phosphate to trehalose. Trehalose is a compatible solute, which allows

microorganisms to protect themselves from osmotic stress. Trehalose binds water that would otherwise be lost from the cell during drying and dry environment exposure. In doing so it protects against cell collapse due to water loss. Trehalose also protects cell components from heat stress by covering cell components and absorbing the heat before the cell components (Purvis et al., 2005).

The magnitude of change of *ots*B for biofilm and planktonic cell was affected by the amount of time the cells were subjected to low water activity storage (Figure 3a). Within the non-digested control, expression increase was greatest for biofilm and planktonic cells previously exposed to 24 hours of dry storage. Gastric stressed biofilm cells desiccated for 2 hours (bg2) had a large increase in expression (21 fold), which was significantly greater than non-desiccated control and cells stored for 24 hours (10 fold) (Figure 3b). Planktonic cells stored for 24 hours had a similar peak at pg24 (21 fold) compared to the non-desiccated control. These increases suggest trehalose production is an important strategy used by *Salmonella* for both physiological cell states in dry storage. For the intestinal treatments, planktonic cell expression is significantly greater than biofilm cells at 2 hours and levels equalize after 24 hours of storage (Figure 3c). The low expression value for bi2 may reflect increased accumulation of trehalose from prior passage through the gastric phase (bg2), which was associated with elevated levels of *ots*B. Alternatively, the presence of the exopolysaccharide matrix may further protect the biofilm cells from bile salts and peptidases. While quantification of trehalose concentration in the cell was not measured in this experiment the increased expression of an important gene in the conversion of sugars to trehalose may be associated with enhanced recovery by plating onto BGA of biofilms at 24 hours (Figure 2a) . Previous studies report the expression on trehalose synthesis genes of *Salmonella* enterica ser. Typhimurium can increase osmotic stress tolerance 7 fold (Howells et al., 2002). This could be measured on these treatments in future studies.

In *Salmonella,* the alternative σ factor, RpoS is expressed in response to stress including extreme temperatures, low nutrients, osmotic stress and low pH (Rychlik, et al. 2005). Expression of *rpo*S occurred for all desiccated cells quickly and was detected at time 0 and greater increases detected for cells stored for 24 hours (Figure 3a). However, these increases were significantly affected with physiological state, with larger increases noted for planktonic cells, especially at 720 hours. As expected large increases in *rpoS* occurred for *S.* Tennessee during the simulated gastric phase when the cells were exposed to low acid for 2h (Figure 3b). The largest increase in relative *rpo*S expression was seen in biofilm cells stored for 2 hours and planktonic cells desiccated for 24 hours suggesting that the period of adaptation is influenced not only by the time but also the physiological state of the cells. This correlates with *otsB* levels previously discussed and demonstrates the influence of dry storage on stress response. Expression levels of *rpoS* during the intestinal phase were reduced compared to the gastric phase, however low levels of *rpoS* was expressed in the intestinal phase, likely in response to bile salts and digestive enzymes. This timed stress response is important because the emulsifying effect of bile salts within the intestines can disrupt membrane stability.

Previous studies performed on planktonic cells suggest a two-hour adaptation period for resistance to an acid environment to manifest (Rychlik and Barrow, 2005). Biofilms might have a shorter period. The effect of this acid tolerance and subsequent cross protection can be seen in the survival data presented previously. Interestingly, the biofilm samples stored for 720 h at low water activity had smaller magnitude of expression when exposed to gastric pH, suggesting adaptation to acid stress is conferred by prior exposure to low water activity.

Virulence genes, such as *invA, hilD, hilA* and *sipC* are partially regulated by *rpoS. hilA* and *hilD* regulate the expression of a variety of virulence genes such as *invA*. *invA* produces a part of a type III secretion system involved in cell penetration. *sipC* encodes for an actin disruption protein that is transported by the type III system as part of cell invasion.

Within the non-digested biofilm treatments, only the 24 hour stored treatment had a significant *hilA* fold change (Figure 4a) Gastric stressed *biofilm* hilA expression peaked after 2 hours of desiccation, while planktonic cells had their greatest increase after 720 hours of storage . For short-term dry storage, significant increases of *hilA* seem to correlate with lower increases within the intestinal phase. This is not true, however, for planktonic cells after a month of storage, since intestinal levels did not seem affected by high gastric levels.

For *hilD*, no significant difference is observed for non-digested treatments. Post gastric biofilm desiccated for 2 hours and stored for 24 hours, as well as planktonic cells stored for 720 hours had the greatest expression for gastric treatments (Figure 4b). Within the intestinal treatments expression of *hilD* was increased 2-fold in planktonic cells stored for 2 and 24 hour periods, while biofilm cells stored for the same period did not increase expression within the intestinal phase. The balance of *hilA* and *hilD* can increase or decrease other virulence gene expression. Throughout the time points it seems different amounts of *hilD* are required to affect *hilA* levels. This might be due to other repressors or quorum sensing repression of *hilA*, as previously studied (Rychlik et al., 2005). This would suggest planktonic cells are able to use *hilA* during intestinal digestion to increase its activation of pathways necessary to invade the epithelial cells (Bajaj, Hwang, Lee, 1995) while biofilm cells do not seem gain an advantage, within the intestinal phase.

InvA levels increase at similar time intervals as *hilA*. The only significant increase in *invA* expression (Figure 5a,). Planktonic cells, however, have a peak expression of i*nvA* after storage for 24 hours as well as significant increases in expression for 2 hours and 720 hours non-digested. Similarly, *hilA* levels are increased at these time points. Gastric stressed biofilms result in an 11- fold increase after 2 hours of dry storage. Subsequent time points do not follow the trend. Planktonic cells demonstrate a large significant increase when stored desiccated for 24 hours before exposure to gastric conditions (26 fold) but smaller increases for cells stored desiccated for 2 hours or 720 hours.. These trends between biofilm and planktonic cells correlate with *rpoS* levels previously discussed. Planktonic cells desiccated for 2 hours port intestinal phase had large fold gene expression differences compared to the other intestinal samples (9.5). This is supported by a decrease in levels within the intestinal phase while gastric levels increased for planktonic cells.

SipC has its significant increases within non digested biofilm samples stored for 2 and 24 hours and all the planktonic non-digested samples. Post gastric samples for biofilms stored for 2 hours and planktonic cells stored for 24 hours contain the greatest expression levels within the gastric treatments (Figure 5b). Planktonic cells are greatly different having a 17 fold increase compared to the 5.2 fold increase for biofilm cells for these treatments. Biofilm cells obtain the high expression level of *sipC* within post intestinal biofilm cells stored for 24 hours. This is not true for other biofilm desiccation times within intestinal stress. Planktonic cells, however do maintain significant increased levels during the all desiccation times within the intestinal phase.

For both i*nvA* and *sipC* short-term dry storage increases expression levels. There are also significant differences between the physiological states predominantly having planktonic cells maintain expression levels throughout the treatments. This might suggest that while biofilm cells seem to survive better, planktonic cells retain their virulence as well as their stress response levels.

There are dramatic differences between biofilm and planktonic cell gene responses. Biofilm cells seem to benefit more from cross protection brought on by prior stress responses. Planktonic cells seem to have an advantage over biofilm cells to invade within the small intestines, an advantage increased during short-term dry storage. Further research can expand on time points between 24 hours and one month to illuminate the changes within these time periods.

4.3: Tables and Figures

Table 2a: Comparison of culturable biofilm (b) and planktonic (p) *Salmonella* Tennessee recovered on brilliant green agar (n=6) and the log copy number of cells (n=6) detected by quantitative real time PCR amplification of 16S rDNA using SYBR green after no digestion treatment (c, control). Desiccation time points include 0 hours, 2 hours, 24 hours, and 720 hours.

Physiological State	Desiccation Time (h)	Log CFU/sample	Log 16S rDNA Copy Number	(Log Copy Number)/ (Log CFU/sample)
Biofilm	0	8.5± 0.1[a]	6.0± 0.6[h]	0.7
	2	7.8± 0.1[b]	6.3± 0.5[h]	0.8
	24	7.7± 0.4[b]	6.0± 0.9[h]	0.8
	720	5.7± 0.2[c]	6.3± 0.4[h]	1.1
Planktonic	0	8.9± 0.1[d]	8.3± 0.3[i]	0.9
	2	8.0± 0.1[e]	8.3± 0.3[i]	1.0
	24	7.3± 0.2[f]	8.2± 0.1[i]	1.1
	720	3.5± 0.6[g]	8.3± 0.3[i]	2.4

Samples not connected by same letter are significantly different.
[1] ND, not detected, below limit of detection and excluded from statistical analysis.
Log copy number/ log CFU/sample above 1 indicates VBNC cells.

Table 2b: Comparison of culturable biofilm (b) and planktonic (p) *Salmonella* Tennessee recovered on brilliant green agar (n=6) and the log copy number of cells (n=6) detected by quantitative real time PCR amplification of 16S rDNA using SYBR green post gastric phase (g). Desiccation time points include 0 hours, 2 hours, 24 hours, and 720 hours.

Physiological State	Desiccation Time (h)	Log CFU/sample	Log 16S rDNA Copy Number	(Log Copy Number)/ (Log CFU/sample)
Biofilm	0	4.5 ± 0.4^{a}	6.4 ± 0.4^{e}	1.4
	2	3.3 ± 0.5^{b}	6.1 ± 1.1^{e}	1.8
	24	4.7 ± 0.1^{a}	6.6 ± 0.7^{f}	1.4
	720	6.9 ± 0.1^{c}	6.2 ± 0.5^{e}	0.9
Planktonic	0	3.50 ± 0.2^{b}	8.5 ± 0.3^{g}	2.4
	2	< 3.0 [1] (ND)	7.4 ± 1.7^{h}	2.5
	24	5.7 ± 0.2^{d}	8.2 ± 0.3^{g}	1.4
	720	3.3 ± 0.4^{b}	8.3 ± 0.2^{g}	2.5

Samples not connected by same letter are significantly different.
[1] ND, not detected, below limit of detection and excluded from statistical analysis.
Log copy number/ log CFU/sample above 1 indicates VBNC cells.

Table 2c: Comparison of culturable biofilm (b) and planktonic (p) *Salmonella* Tennessee recovered on brilliant green agar (n=6) and the log copy number of cells (n=6) detected by quantitative real time PCR amplification of 16S rDNA using SYBR green post intestinal phase (i). Desiccation time points include 0 hours, 2 hours, 24 hours, and 720 hours.

Physiological State	Desiccation Time (h)	Log CFU/sample	Log 16S rDNA Copy Number	(Log Copy Number)/ (Log CFU/sample)
Biofilm	0	9.2± 0.2[a]	8.1± 0.4[g]	0.9
	2	8.7± 0.1[b]	7.9± 0.9[gi]	0.9
	24	9.7± 0.1[c]	6.6± 0.6[h]	0.7
	720	7.8± 0.2[d]	8.4± 0.2[gj]	1.1
Planktonic	0	8.4± 0.2[e]	8.0± 0.4[gi]	1.0
	2	9.2± 0.1[a]	7.5± 0.8[i]	0.8
	24	10.3± 0.1[f]	8.6± 0.4[j]	0.8
	720	8.3± 0.01[e]	6.8± 0.3[h]	0.8

Samples not connected by same letter are significantly different.
[1] ND, not detected, below limit of detection and excluded from statistical analysis.
Log copy number/ log CFU/sample above 1 indicates VBNC cells.

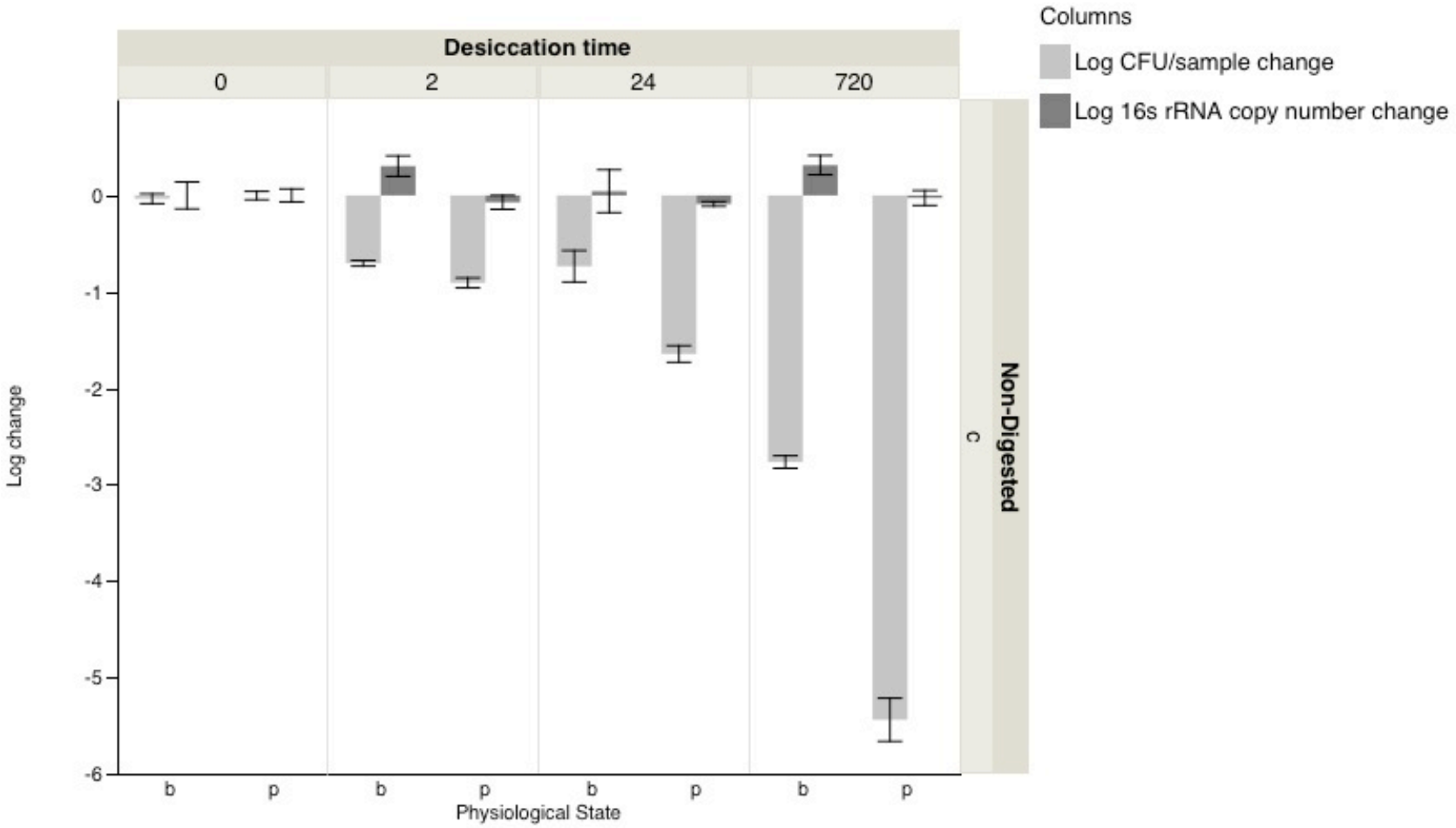

Figure 2a: Control, no digestion: Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) on changes in plate counts on BGA (n=6) and 16S rRNA gene copy numbers (n=18), using SYBR green qRT-PCR. All values reflect the log change compared to the time 0 for each physiological state (b: 8.5 log CFU/sample, 6.0 log copy number, p: 8.9 log CFU/sample, 8.3 log copy number. Each error bar is constructed using 1 standard deviation from the mean.

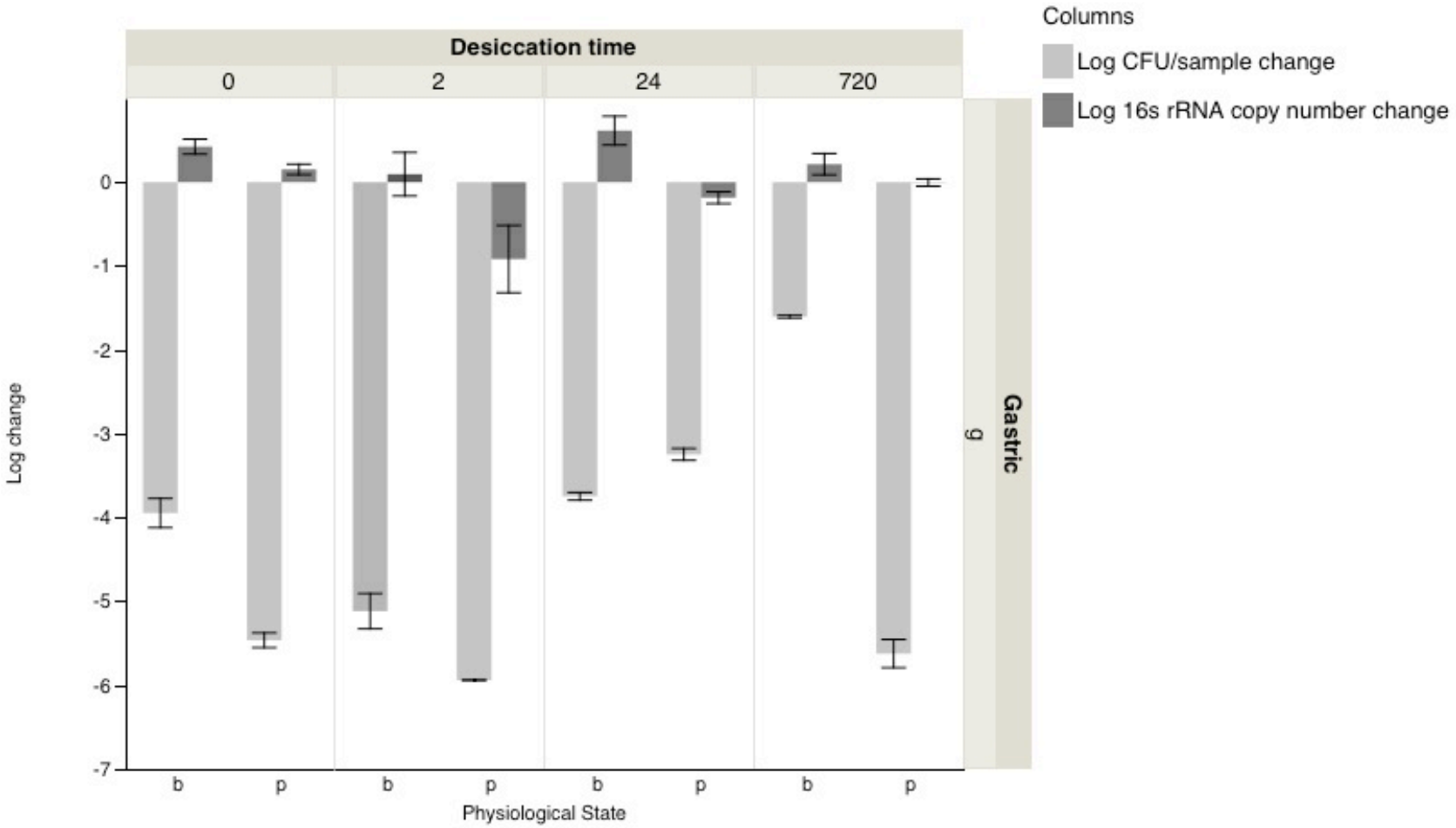

Figure 2b: Gastric phase (g): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) on changes in plate counts on BGA (n=6) and 16S rRNA copy numbers (n=18), using SYBR green qRT-PCR. All values reflect the log change compared to the time 0 for each physiological state (b: 8.5 log CFU/sample, 6.0 log copy number, p: 8.9 log CFU/sample, 8.3 log copy number. Each error bar is constructed using 1 standard deviation from the mean.

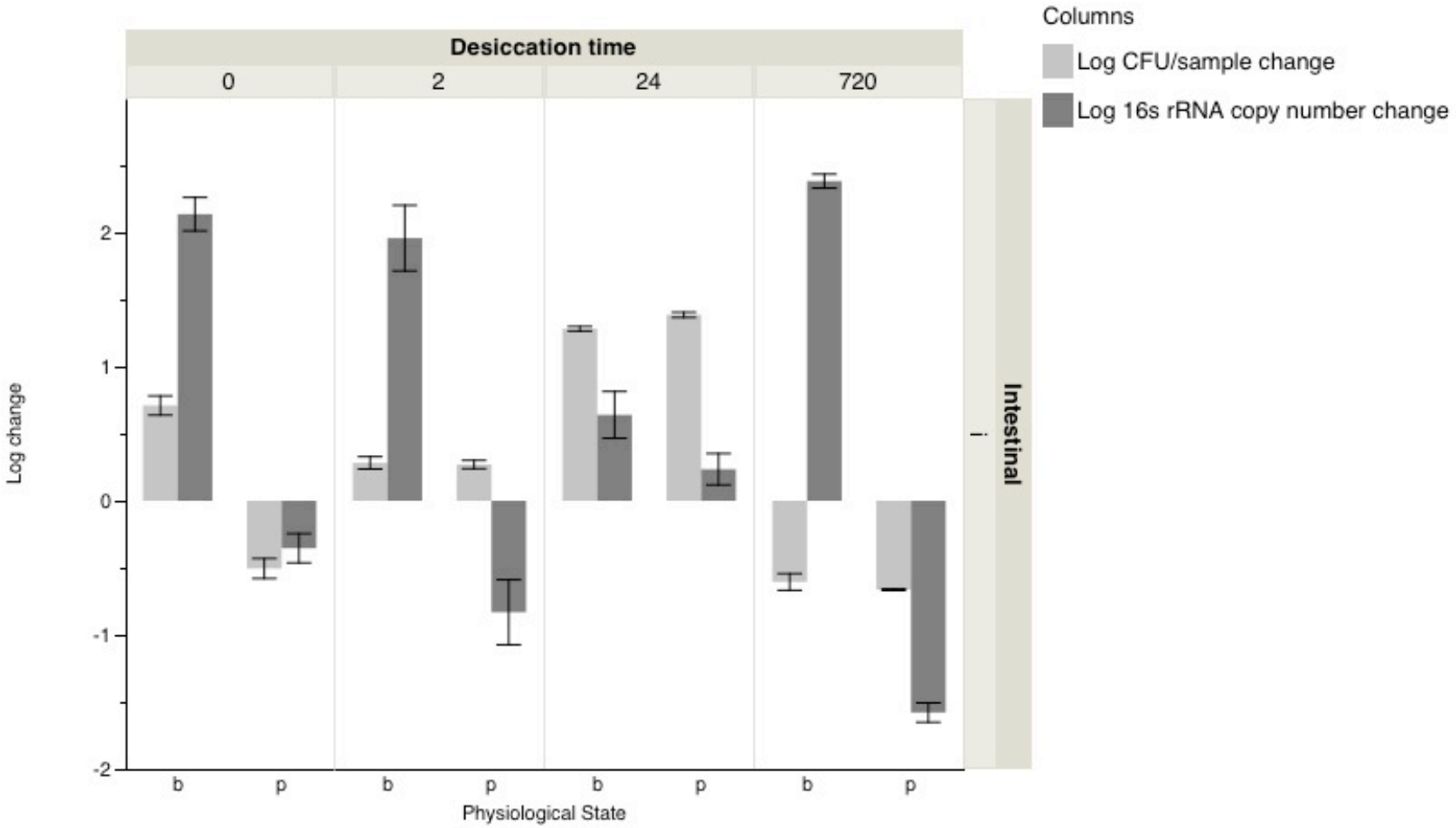

Figure 2c: Intestinal Phase (i): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) on changes in plate counts on BGA (n=6) and 16S rRNA copy numbers (n=18), using SYBR green qRT-PCR. All values reflect the log change compared to the time 0 for each physiological state (b: 8.5 log CFU/sample, 6.0 log copy number, p: 8.9 log CFU/sample, 8.3 log copy number. Each error bar is constructed using 1 standard deviation from the mean.

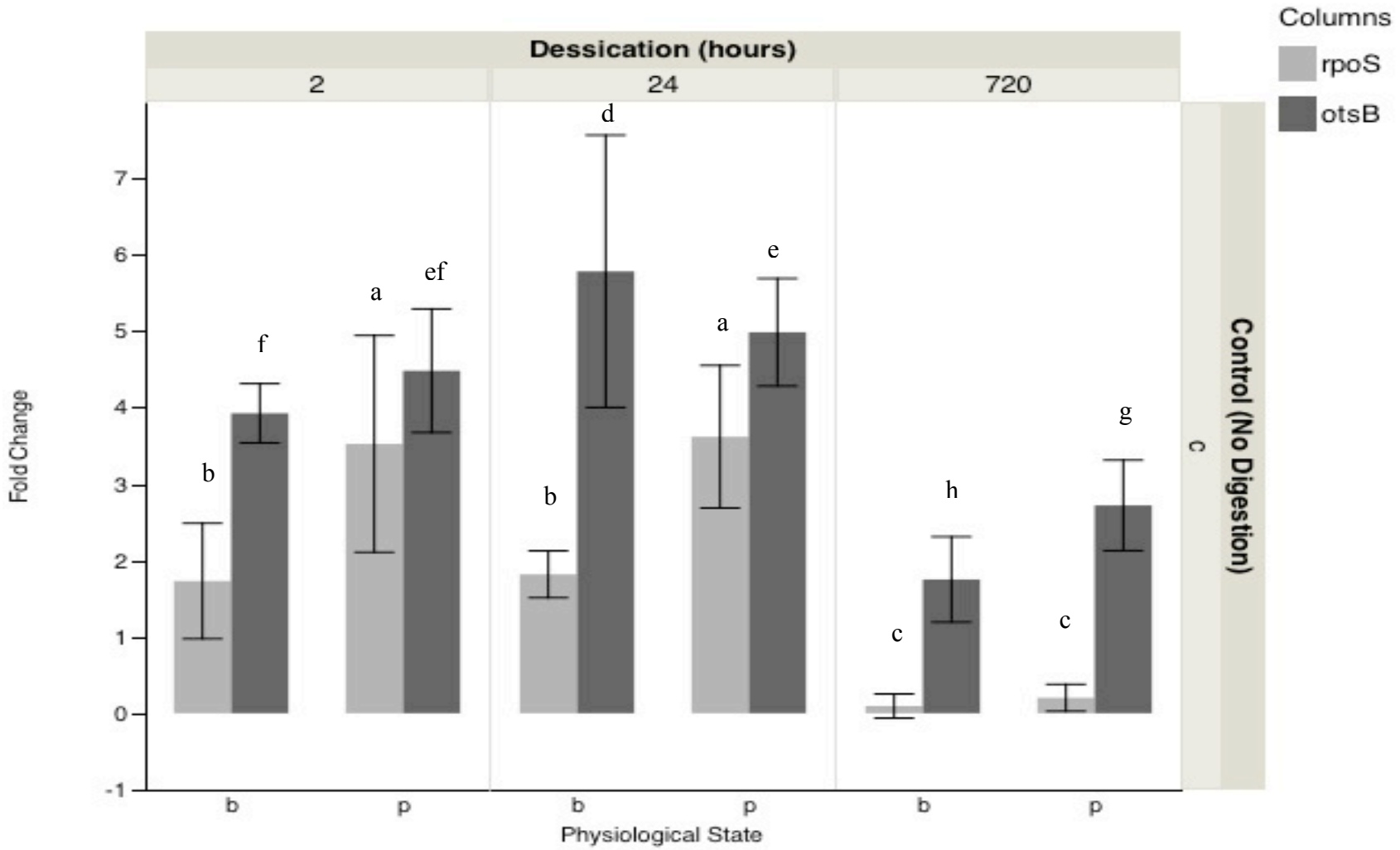

Figure 3a: Non-digested control (c): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) in expression of *otsB* and *rpoS* genes detected using SYBR green qRT-PCR. Copy numbers were normalized based on expression of 16S rDNA Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

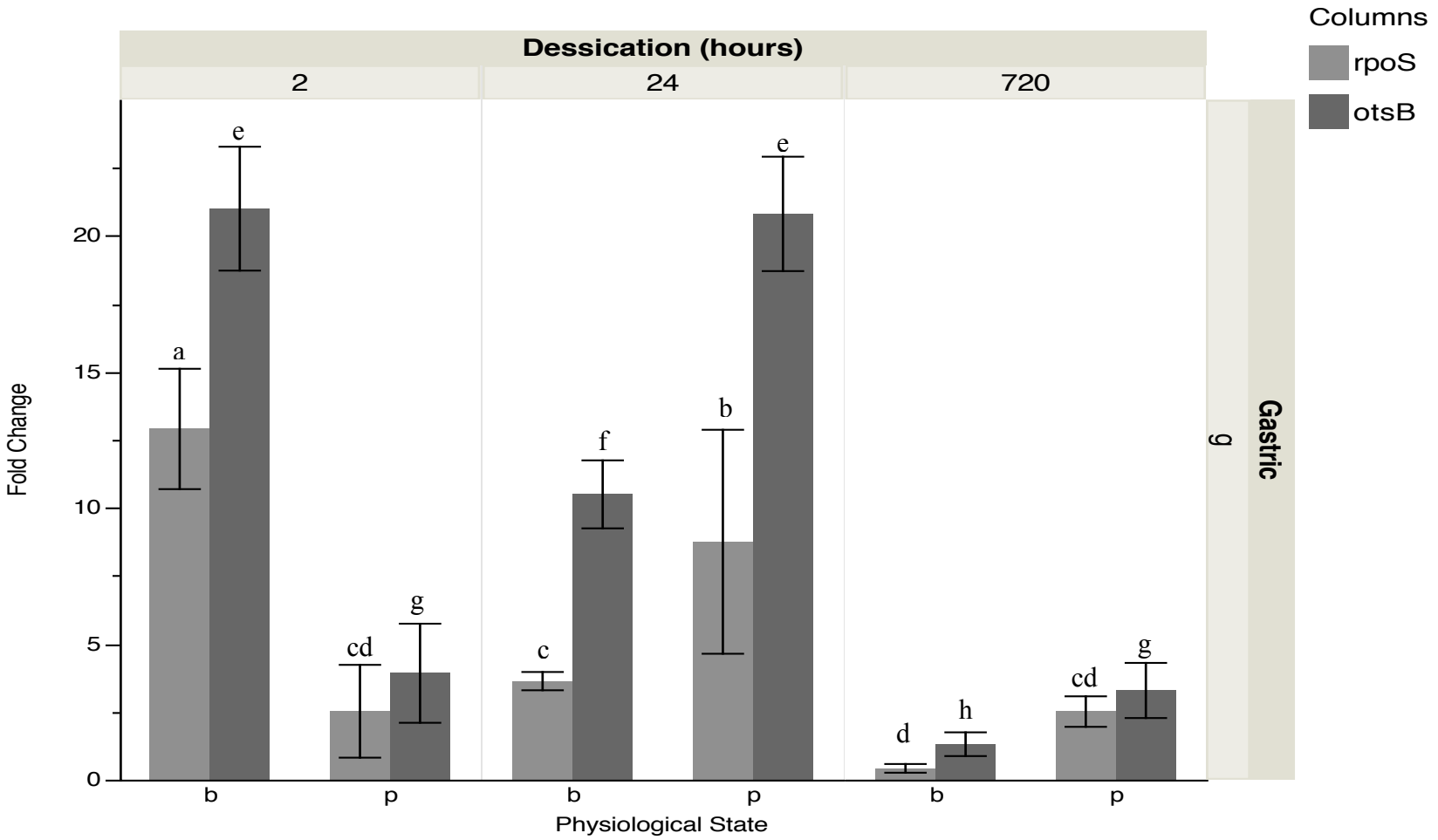

Figure 3b: Gastric Phase (g): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) in expression of *otsB* and *rpoS* genes detected using SYBR green qRT-PCR. Copy numbers were normalized based on expression of 16S rDNA. Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

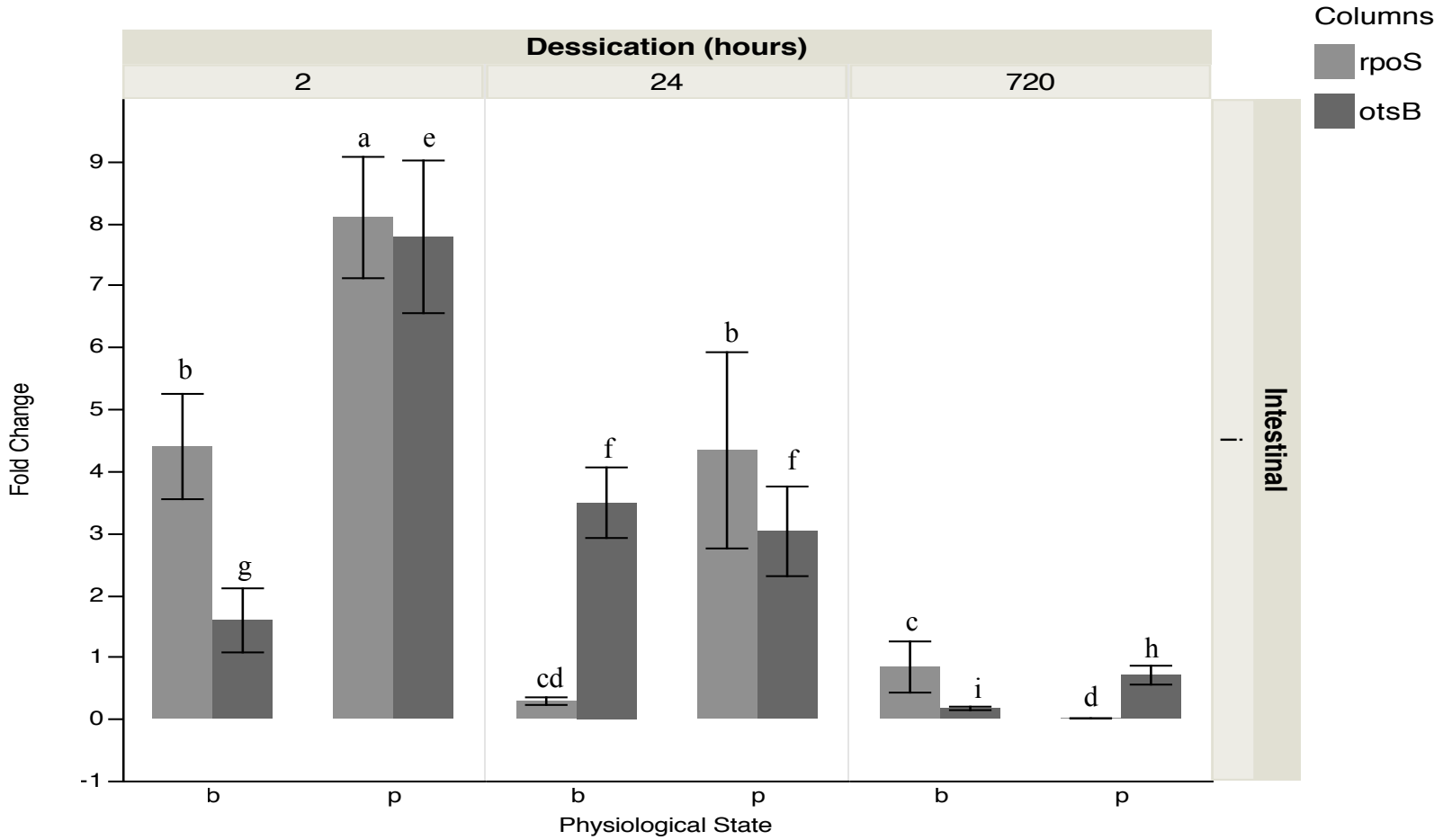

Figure 3c: Intestinal Phase (i): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) in expression of *otsB* and *rpoS* genes detected using SYBR green qRT-PCR. Copy numbers were normalized based on expression of 16S rDNA. Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

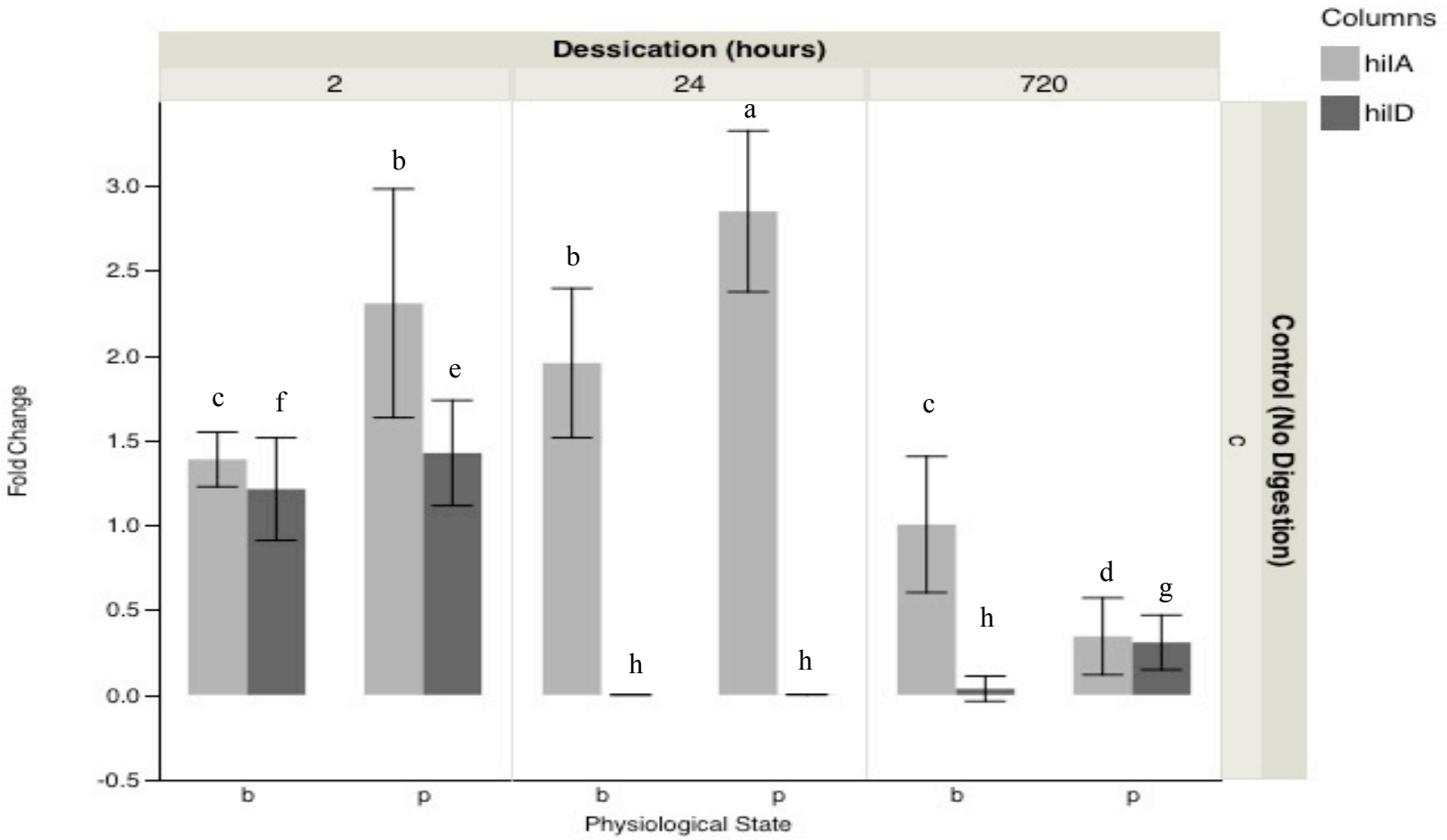

Figure 4a: Non-digested control (c): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) in expression of *hilA* and *hilD* genes detected using SYBR green qRT-PCR. Copy numbers were normalized based on expression of 16S rDNA. Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

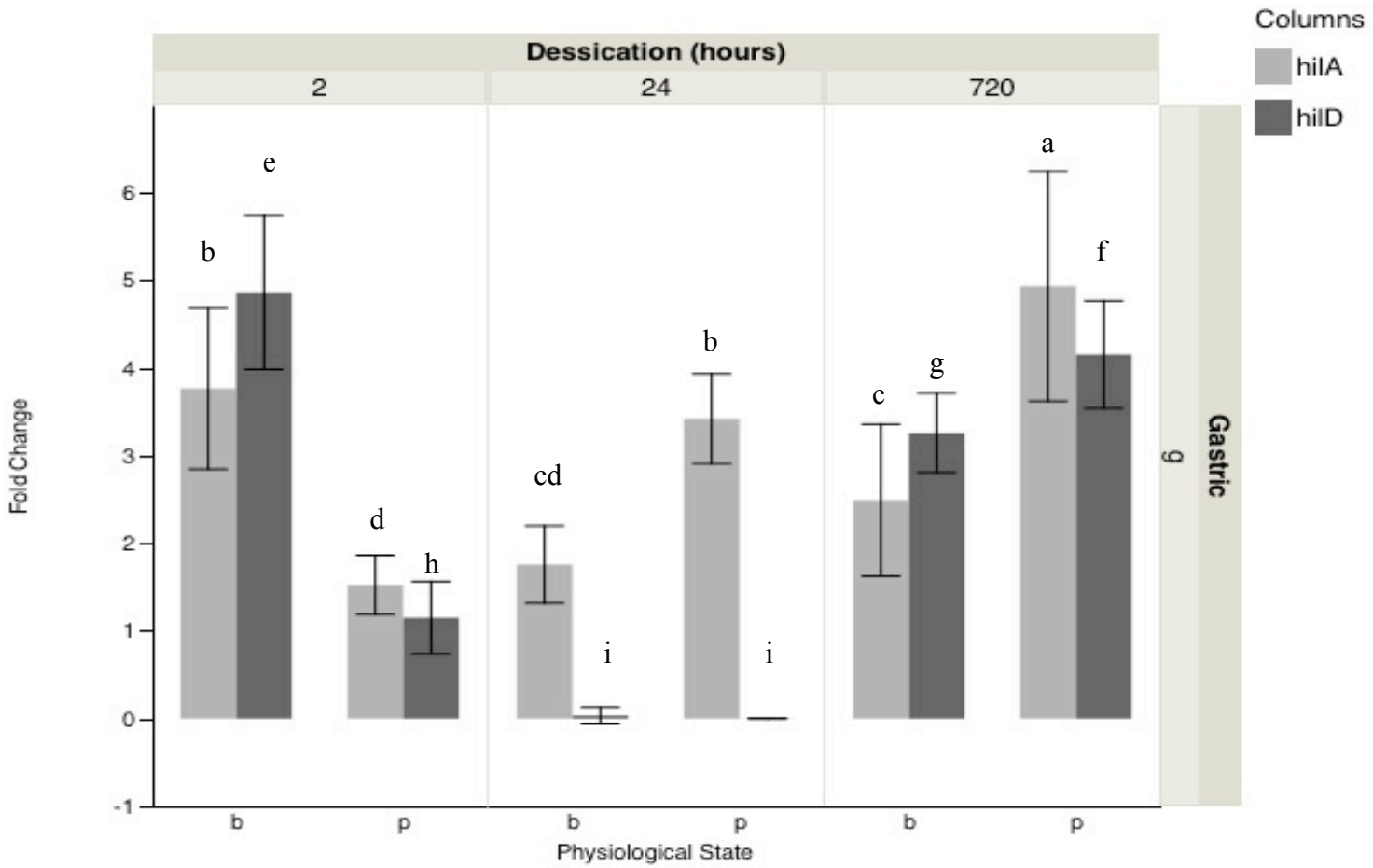

Figure 4b: Gastric Phase (g): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) in expression of *hilA* and *hilD* genes detected using SYBR green qRT-PCR. Copy numbers were normalized based on expression of 16S rRNA genes. Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

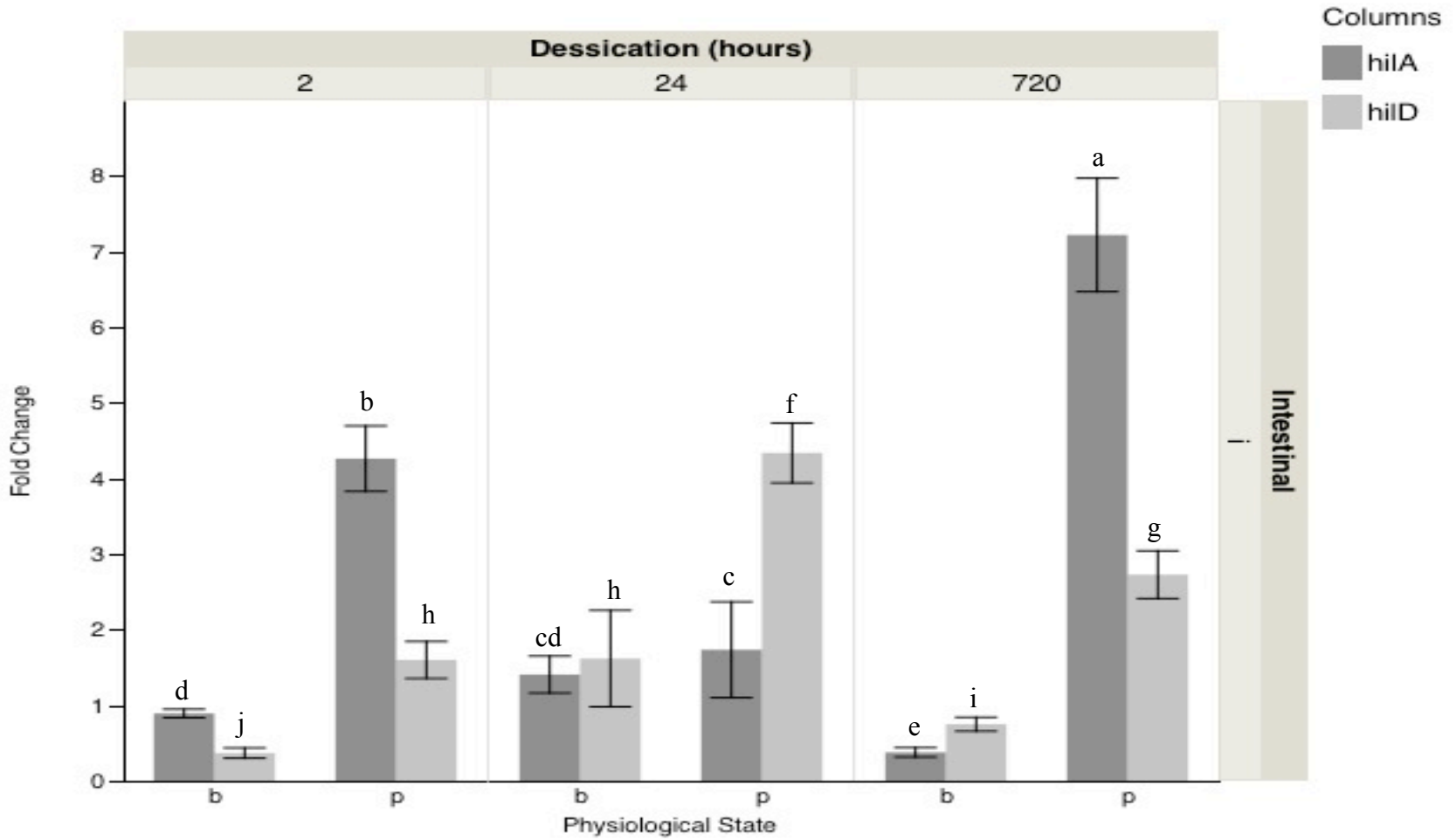

Figure 4c: Intestinal Phase (i): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) on expression of *hilA* and *hilD* genes detected using SYBR green qRT-PCR. Copy numbers were normalized based on expression of 16S rRNA genes. Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

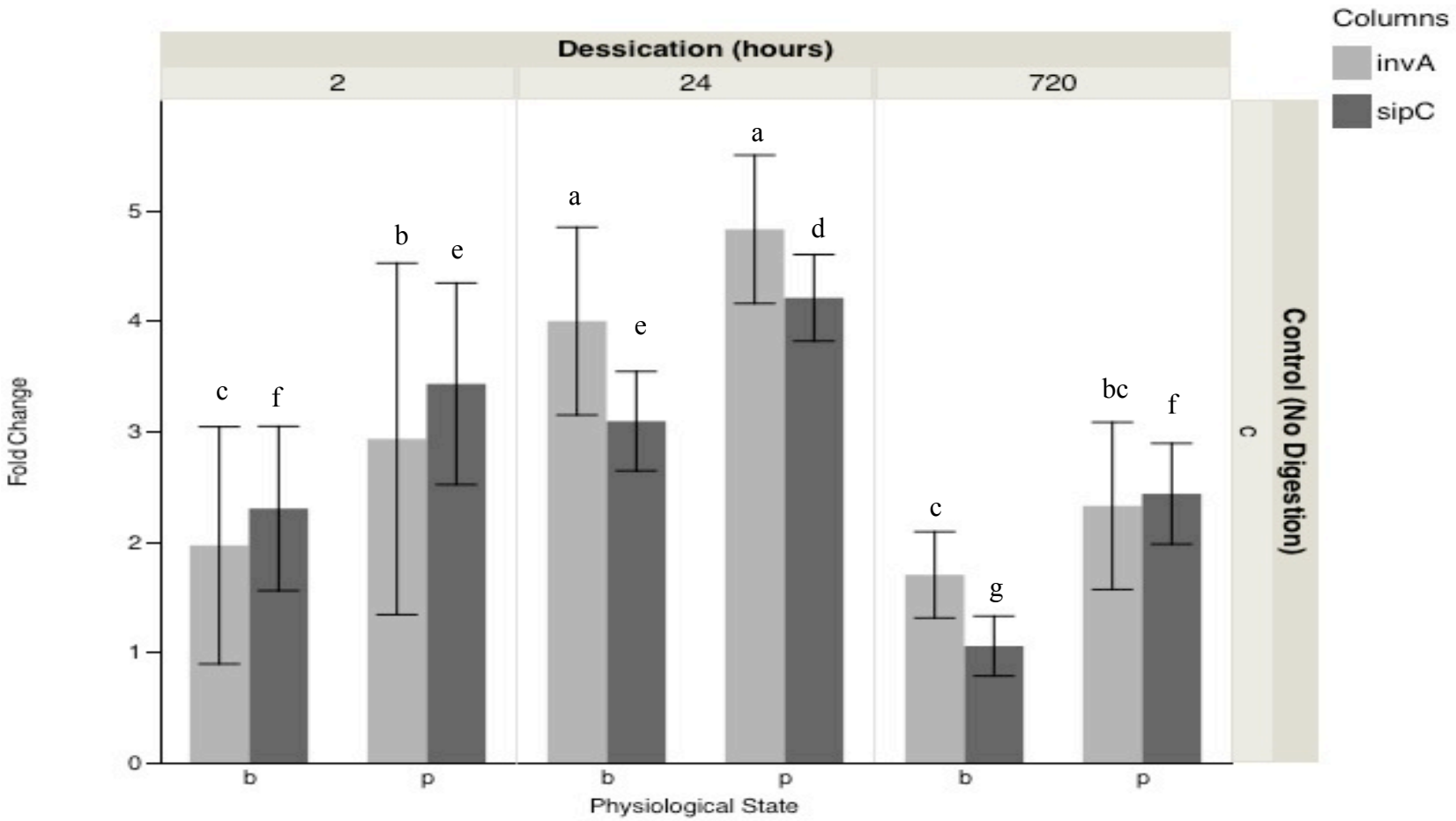

Figure 5a: Non-digested control (c): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) on expression of *invA* and *sipC* genes detected using SYBR green qRT-PCR. Copy numbers were normalized based on expression of 16S rRNA genes. Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

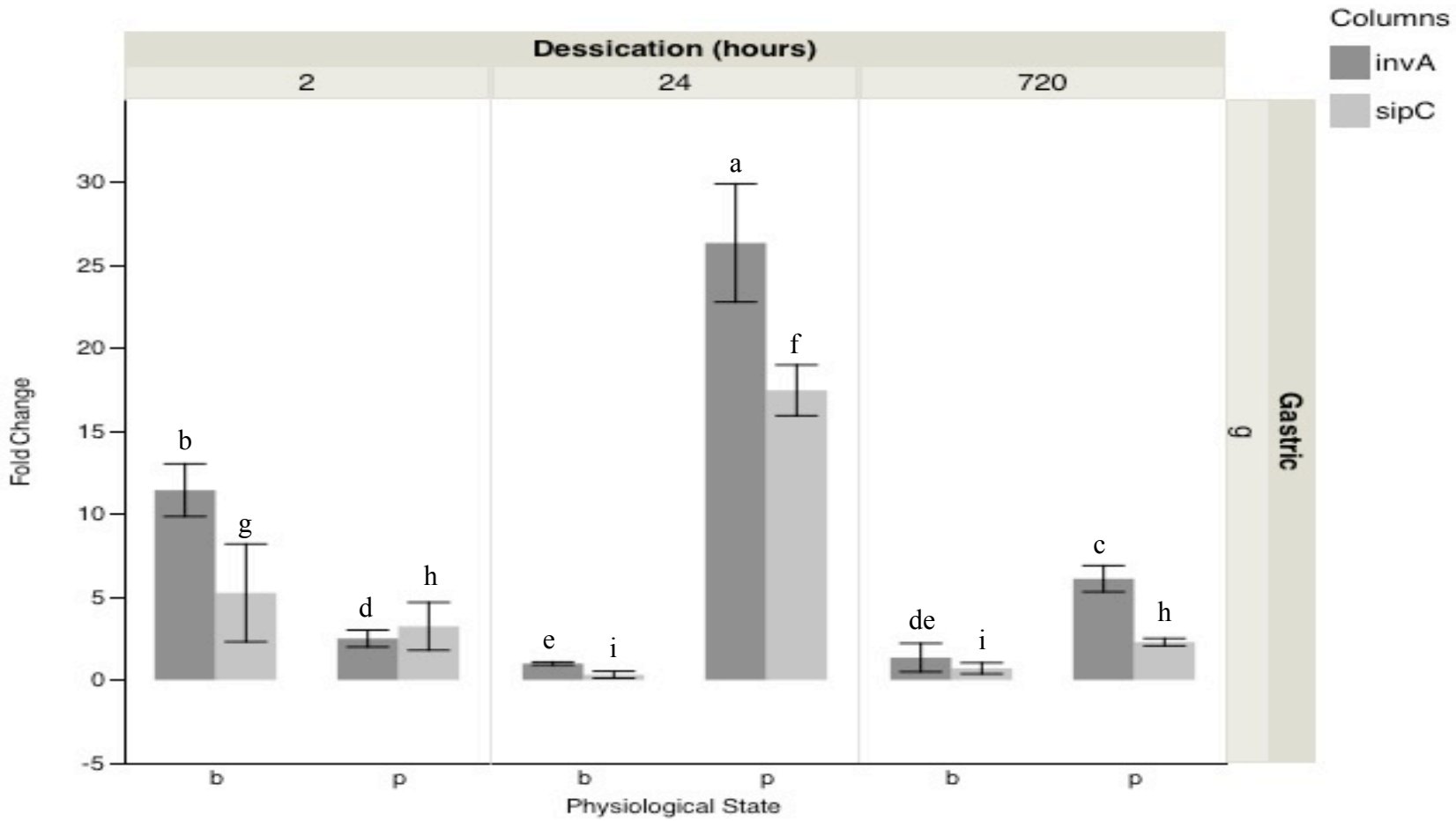

Figure 5b: Gastric Phase (g): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) on expression of *invA* and *sipC* genes detected using SYBR green qRT-PCR. Copy numbers are normalized based on expression of 16S rRNA genes. Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

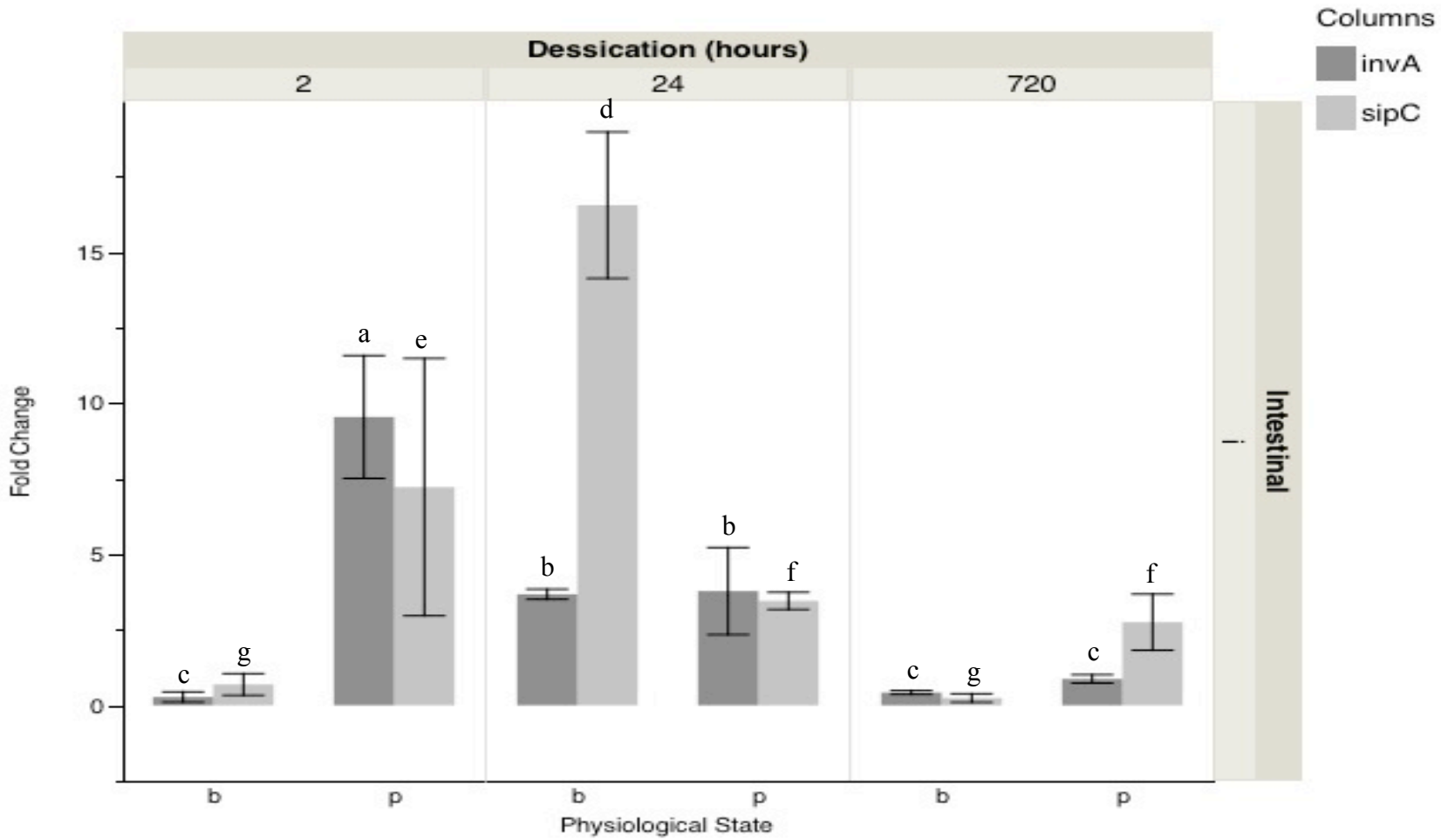

Figure 5c: Intestinal Phase (i): Effect of physiological state (biofilm (b), planktonic (p)) and desiccation time (in hours) on expression of *invA* and *sipC* genes detected using SYBR green qRT-PCR. Copy numbers are normalized based on expression of 16S rRNA genes. Values reflect the fold change of each treatment compared to the time 0 of each physiological state. Error bars represent the standard deviation of six replicates from the sample mean.

Chapter 5: Conclusions

5.1: Impact

This study further supports the need to consider the physiological state of a cell along with its prior stresses. The increases in magnitude of virulence gene expression in desiccation stressed cells, as well as the small reduction in cell numbers over time indicating *Salmonella* is a continuing threat to public health and a major hurdle for the food industry.

Although *Salmonella* Tennessee was the focus of this study, the questions and methods presented can and should be expanded to other food borne pathogens. Little work has been done comparing biofilm to planktonic cells and even less work incorporating multiple environmental stresses even though that is what is usually found in processing facilities. The effect multiple stresses have on not only survival but also gene expression and recoverability affects the food industry. This is important when evaluating food samples using plating verification. VBNC cell's ability to cause false negatives in instances where there is contamination can inhibit the prevention of outbreaks. Further research into alternative verification methods that discriminate between live and dead cells is needed to detect contamination with greater accuracy.

5.2: Method Application

With the development of new technology an methodologies more complex studies can be performed, coming closer to modeling the complicated web of interactions between microorganism and the food industry. In this study a dry processing environment such as a milk powder processing facility was modeled. Placing desiccation with starvation along with pH, heat and enzyme stresses resulted in information that can be

used in risk assessment and later on, intervention strategies. Similar studies can be conducted, for instance in fruit concentrate plants with *E. coli*.

Low infectious dose *Salmonella* outbreaks brought about new questions to answer and a reevaluation of processing techniques of certain products such as low a_w foods. This study further illuminates the need of reassessment, specifically including biofilms and different storage time points within microbiological testing. The reassessment should not only include recoverability but also gene expression related to infection and cross protection.

5.3: Future Directions

The focus of further study should first be on utilizing these methods on other *Salmonella* serotypes such as *S.* Typhimurium in order to compare the results to better-characterized organisms. Another improvement would be expanding the time points before and after 24 hours in order to see curves of gene expression change and determine if what was seen are the peaks or if only the sides of a curve were observed. Expanding the variety of genes would also allow for determination of the most important reactions if any differences were obtained. In order for the increased virulence hypothesis to be accepted, the cells would also have to be tested on live human subjects. Many projects can stem from the findings, hopefully leading to better pathogen control discoveries.

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