Survival and Persistence of dried *Salmonella enterica* in Low Water Activity Conditions

by

Arisha Seeras

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Salmonella survives in low moisture products over an extended time. *Salmonella* also resists dry heat, which questions the assumption that *Salmonella* outbreaks from intermediate moisture foods result mostly from post-processing contamination. Some *Enterobacteriaceae* possess a genomic island called the locus of heat resistance (LHR), which confers heat resistance. This genomic island was also identified in *Salmonella* Senftenberg ATCC 43845, a strain with exceptional heat resistance. This study aimed to determine whether the locus of heat resistance improves survival of *Salmonella* during storage in low-moisture environments, and its resistance to dry heat. The LHR-positive *S. enterica* Senftenberg ATCC 43845 and the LHR-negative *S. enterica* Typhimurium ATCC 13311 were air dried and stored for up to 30 d at a relative humidity (RH) of 10%, 55%, or 75%. Saturated salt solutions were used to maintain a constant RH throughout storage. All experiments were conducted in triplicate and analyzed using one-way ANOVA. Cell counts of both strains of *Salmonella* were reduced by less than 1 log (CFU/g) after 30 d of storage at 10% RH. At 75% RH, the viability of the LHR-positive *S. enterica* ATCC 43845 was reduced by more than 8 log (CFU/g) after 3 d of storage, while viability of the LHR-negative *S. enterica* ATCC 13311 was reduced only by 2 log (CFU/g) after 3 d of storage. To further confirm the effect of the LHR, *S. enterica* ATCC 13311 was transformed with a plasmid pRK767 as a control or with pLHR, a plasmid obtained by cloning the LHR under control of the native promotor in pRK767. Cell counts of the LHR-complemented *S. enterica* ATCC 13311pLHR were reduced by 8 log (CFU/g) after 3 d of storage at 75% RH, which is comparable to results for the LHR-positive wild type strain, and cell counts of the LHR-negative *S. enterica* ATCC 13311pRK767 were reduced only by 3 log (CFU/g). Survival under low RH conditions of an isogenic strain without *kefB*

(*S. enterica* ATCC 13311pLHRΔ*kefB*) indicated that *kefB* might have a specific role during desiccation. Resistance to dry heat was determined by treatment at 110°C for 15 min. At a RH of 55% or lower, cell counts of all strains were reduced by less than 1 log (CFU/g). However, cell counts of the LHR-positive *S. enterica* ATCC 43845 and *S. enterica* ATCC 13311pLHR were reduced by 8 log (CFU/g) after heating to 110°C at 75% RH while the LHR-negative *S. enterica* ATCC 13311 and *S. enterica* ATCC 13311pRK767 were reduced only by 6 log (CFU/g). In conclusion, dried cells resist dry heat after equilibration in environments with a RH of less than 60% irrespective of the presence of the LHR. The LHR increases resistance to wet heat but reduces survival when cells are dried and reduces the heat resistance of dried cells.

Acknowledgements

Ten years ago, my father moved our family from Mauritius to Edmonton, with the aims of providing better opportunities and educational experiences for his children. If it wasn't for his and my mother's determination to offer their children a better life, I would not be at the University of Alberta today, finishing a Master's degree. Dad, thank you for encouraging me to follow in your footsteps, and for always reminding me that I have the power to achieve anything I put my mind to. Mom, thank you for your love and support throughout this degree, and for all the late-night cups of tea you made to help me study. Avishta and Ryan, thank you both for the much-needed comic relief after long days in the lab, and for the random stress-relief dance parties at home. Justin, thank you for being my rock throughout these last few years, and for always knowing what to say to make me feel better at the end of the day. For this, and so much more, I will always be grateful to you all.

I am lucky to have had the opportunity of working in a field that I am passionate about. I am especially thankful to my amazing supervisors, Dr. Lynn M. McMullen and Dr. Michael Gänzle, for believing that I could do this. Thank you, Dr. McMullen, for your guidance throughout, and especially for your support last year, I will never forget it. Thank you, Dr. Gänzle, for directing me to food microbiology during my undergraduate years; you helped me discover what I was passionate about back then, and have since then, helped me achieve my academic goal.

I also want to thank Patrick Ward, Ross Lowe and Rigoberto Garcia-Hernandez, for answering my countless questions about graduate school while working at AFDP, and for

helping make up my mind about going for a Master's degree. Thank you to everyone in 2-50 for making laboratory work fun throughout the day. I am especially thankful to Alma-Fernanda Sanchez Maldonado, and Januana Teixiera, for being exceptional mentors. To Yalu Yan, Paola Ares, Oanh Nguyen and Felicitas Pswarayi; thank you for the coffee breaks, the random chit-chats, and for being my guinea pigs whenever I attempted a new baking project. To Priya, Sandra, Divine, Saida, Ankush, Nitesh and Jimmy; thank you all for reminding me that I have a life outside of my lab, and helping me explore it in fun ways.

Lastly, I am very thankful to ALMA and NSERC for providing the funding for my project, and for being part of the MEaTnet program; without which I would not have had the opportunity of having hands-on training at Olds College, or earned valuable experience working in the meat industry.

Table of Contents

List of Tables

List of Figures

List of Symbols and Abbreviations

ANOVA	Analysis of variance
a_w	Water activity
min	Minutes
h	Hours
CFU	Colony Forming Units
E.	*Escherichia*
LHR	Locus of Heat Resistance
O/N	Overnight
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RH	Relative humidity
S.	*Salmonella*
SOC	Super Optimal broth with Catabolite Repression
spp.	Species
tet^R	Tetracycline resistance
TS	Tryptic Soy
TS^{tet}	Tryptic Soy with tetracycline
K^+/H^+	Potassium/Hydrogen antiporter

1. Introduction

Low moisture products are defined as foods that have a water activity (a_w) of 0.85 or lower and they appear to be dry or possess very low to no visible water (FAO/WHO, 2015). Foodborne illness outbreaks from low a_w foods ($a_w < 0.6$) dating as early as 1973, from powdered milk contaminated with *Salmonella enterica* serotype Derby, indicate that some species have the ability to overcome the low a_w hurdle for survival over an extended time (Finn et al., 2013a).

A reduction in the amount of water available in foods is one of many ways of achieving an environment in food that inhibits the growth of vegetative cells under non-refrigerated storage conditions. Water activity describes the amount of water that is not attached to food components and is available for various physico-chemical or biological reactions, such as enabling growth and survival of micro-organisms. Water activity of foods represents the ratio of the partial vapor pressure of water in a product at a certain temperature, compared to the partial vapor pressure of pure water at the same temperature (Brown AD., 1990). Lowering the amount of water available in a food matrix is a common food preservation technique as it inhibits microbial growth. While the minimal a_w needed for most bacterial growth is approximately 0.83 (ICMSF, 1996), bacterial physiological functions are impaired at a_w 0.6 or below; especially for *Salmonella*. Bacterial growth is dependent on the amount of water available in a product; if that water is bound, it impedes bacterial growth and survival during storage, resulting in a product with a low microbial load. Hence, the process of lowering the water activity of foods, where high a_w foods are deliberately dried (with the addition of solutes or by air/low heat drying), or foods are composed of low a_w ingredients, is effective in inhibiting bacterial growth. However, even though survival of microorganisms during storage in low a_w conditions occur, the degree of microbial load

reduction depends on factors such as storage humidity, the drying medium and temperature exposure pre- and post-storage. While *Salmonella* require a a_w of greater than 0.93 to grow and survive, several studies have reported their ability to survive in low a_w foods for an extended period of time (Andino & Hanning, 2015; Hiramatsu et al., 2005; Gruzdev et al., 2012b; Nummer & Smith, 2012). Depending on the food matrix, a reduction in the a_w of foods inhibits growth of *Salmonella*, indicating that the composition of the medium plays a major role in controlling growth of pathogenic micro-organisms post-processing (Goepfert et al., 1970; Deng et al., 2012).

The main source of contamination of low a_w foods is often believed to be associated with cross-contamination from environments and equipment where dormant *Salmonella* remain in a state of reduced metabolic activity, with approximately 5% or less transcribed genome, in the absence of moisture (Deng et al., 2012; Beuchat et al., 2013). However, the threat to food safety increases if the dormant micro-organisms are exposed to more favorable conditions through direct ingestion, which would stimulate growth in the intestinal tract of the host, as they remain infectious. While a number of studies have assessed tolerance to low a_w and subsequent resistance to other stresses in *Salmonella*, the bacterial mechanisms involved in survival under dry/desiccated conditions have not yet been thoroughly explored.

1.1 *Salmonella*.

Salmonella are Gram negative facultative rod-shaped bacteria, belonging to the *Enterobacteriaceae* family. They are found within the intestinal tract of humans or animals, or isolated from cross-contamination/shedding in soil, litter/manure, and food/feed products (Andino & Hanning, 2015). In Canada, non-typhoidal *Salmonella*-related illnesses make up approximately 5% of known foodborne illness cases; with poultry being the most common

source of *Salmonella* (Health Canada, 2016). *Salmonella, Campylobacter* spp. and *Clostridium perfringens* are considered to be the foodborne pathogens of the greatest concern in Canada (Health Canada, 2016). Non-typhoidal *Salmonella* are spread via the fecal-oral route, and induces symptoms such as diarrhea, nausea, fever, nausea and abdominal cramps within 72 hours of consuming infected food products (CDC, 2013; Health Canada, 2016). To date, *Salmonella* still pose a great food safety risk, due to their ability to adapt to environmental conditions beyond their normal growth conditions.

1.2 *Salmonella* outbreaks from low water activity products.

Salmonella, which are mesophilic bacteria with a temperature growth range of 2-54^{o}C, survive heat treatments greater than 60^{o}C (Andino & Hanning, 2015; Mercer et al., 2015), and increasing salmonellosis cases from low a_w foods confirm their ability to survive in low a_w foods (Table 1). Another micro-organism, well known for its ability to survive in low to very low a_w foods, is *Cronobacter sakazakii*, which have caused foodborne outbreaks associated with infant formula with a a_w of 0.2 (Iversen & Forsythe, 2004; Breeuwer et al., 2003). Low a_w food related salmonellosis outbreaks have been associated with several *Salmonella* serotypes including Tennessee, Agona, Montevideo, Typhimurium, Enteritidis, Infantis, and Senftenberg (Table 1).

While poultry remains the major source of *Salmonella*; at 70% in the US versus 29% in Canada, an increasing number of outbreaks originate from fruit, vegetables, nuts and other low a_w products (CDC, 2013; Health Canada, 2016). Over the last 10 years, several outbreaks were related to food products with a_w as low as 0.1 to 0.7 (Finn et al., 2013a; Table 1). While it is impossible to determine the exact infectious dose for individuals, investigation of past outbreaks from low a_w foods have resulted in the belief that *Salmonella* can have a much lower infectious dose if present in low a_w foods. While *Salmonella* have an infectious dose of 10^3 CFU/mL to

induce an illness, outbreaks associated with low a_w foods associated outbreaks have been traced back to much lower infectious doses (Blaser & Newman, 1982; Gill et al., 1983; PHAC, 2011). The increasing number of cases of salmonellosis from consumption of low a_w product is a troubling phenomenon, not only because of the high frequency of the outbreaks, but also the low infectious dose. This implies that *Salmonella* is the most likely pathogen to contaminate low a_w products and is best adapted to survive extended periods of storage time in a dormant stage. A salmonellosis infection is highly selective of the infective matrix; high infectious dose outbreaks require growth in a high moisture product with a known level prior to ingestion, to cause an infection in healthy adults (Mattick et al., 2001). The infective dose is lower for people with weakened immune systems, as children and the elderly tend to develop symptoms after consumption of contaminated high moisture products with counts as low as 10-1000 CFU/g (Archer et al., 1998, Doyle & Mazzotti, 2000). In low moisture products, however, the infectious dose seems to vary depending on the contaminated product.

Table 1. Outbreaks of salmonellosis from low moisture foods over a period of 10 years. *

Year	Product	Serovars	Water activity
2007	Dry pet food	Schwarzengrund	0.5-0.6
2008	Wheat cereal	Agona	0.4-0.5
2009	Peanut butter	Typhimurium	0.3-0.4
2010	Spices	Montevideo	0.5-0.6
2011	Pine nuts	Enteritidis	0.6-0.7
2012	Peanut butter	Bredeney	0.3-0.4
2013	Tahini paste	Montevideo, Mbandaka	0.1-0.2
2014	Chia powder	Newport, Hartford	0.5-0.6
2015	Nut butters	Paratyphi	0.3-0.4
2016	Pistachios	Montevideo, Senftenberg	0.6-0.7
2017	Hazelnuts	Typhimurium	0.5-0.6

* Adapted from the CDC website (http://www.cdc.gov/*salmonella*/outbreaks.html)

1.3 Factors responsible for survival of *Salmonella*.

Adaptation to osmotic stress consistently leads to accumulation of compatible solutes in bacteria, which triggers stress responses and subsequent survival to any other physical stresses. Sleator and Hill, (2010) assessed the compatible solute uptake in *Listeria monocytogenes* during stress, and observed that solute uptake induces a higher resistance to almost any preservation method currently used by food industries (Sleator & Hill, 2010). However, pre-adaptation to one stress does not automatically induce resistance to every other stress. Desiccated *S.* Typhimurium had exceptional survival when exposed to 2% H_2O_2 for 30 min, up to 100 ppm sodium hypochlorite and 0.25% didecyldimethylammonium chloride (DDAC), but their non-desiccated counterparts were undetectable, and exposure to organic acids with a pH as low as 3 proved to be more bactericidal to desiccation-adapted cells than to the non-adapted cells (Gruzdev et al., 2011). While air-dried *Salmonella* induced resistance to temperatures greater than 70^{o}C, a lower temperature heat treatment had a bacteriostatic effect (Mattick et al., 2001). Cross-protection between dry adaptation and heat resistance is strain dependent. Two different *Salmonella* serotypes, previously adapted to acidic cantaloupe and watermelon juice had different stress responses when pasteurized at 57^{o}C; *S.* Saphra had a greater D_{57} value when compared to its non-adapted counterpart, while acid adaptation made no difference to heat resistance of *S.* Poona (Sharma et al., 2005). Out of three strains of *Escherichia coli* O157:H7 pre-adapted to starvation, only two exhibited increased heat resistance (Rowe & Kirk, 2000).

1.4 Mechanism of survival of *Salmonella* in low water activity conditions.

A cell, in a state of extreme dryness, lacks free water and nutrients required for normal growth. Cellular integrity can be maintained as long as the pressure caused by the osmotic shift does not go beyond what the cytoplasmic membrane and cell wall can sustain; but in extreme

cases, the stress can lead to irreparable damage to the cell. Osmotic imbalance is described as a sudden shift in solute concentration externally, causing water to shift in or out of the cell to balance the osmolality of the cell and the environment (Wesche et al., 2008).

Osmotic stress is often induced by exposure to high osmolality media, where the amount of unbound water available to cells is restricted with the presence of ions and sugars. Osmotic stress in the presence of sodium chloride not only induces osmotic stress, but also ionic stress due to the presence of charged ions. In order to overcome the loss of water, cells respond by activating a set of regulatory systems to restore intracellular osmotic balance for survival (Bremer & Kramer, 2000). In *Enterobacteriaceae*, such as *Salmonella* and *E. coli*, these systems primarily include active uptake of K^+ ions and compatible solutes such as proline, ectoine, glycine betaine and dimethylsulphoniopropionate, trehalose and glutamate biosynthesis, modifications to cellular morphology, and fatty acid catabolism (Finn et al., 2013a; Kempf & Bremer, 1998; Canovas et al., 2001). These solutes are said to be compatible, as their uptake does not interfere with intracellular mechanisms required for growth and survival of bacterial cells. In low a_w environments, accumulation of these solutes has a protective effect on cellular membranes by enabling molecular stability of the membranes for prolonged survival of cells (Canovas et al., 2001, Purvis et al., 2005). While *Salmonella* can use compatible solutes to maintain osmotic balance, solute selection affects the effectiveness of the solutes as *Salmonella* favors uptake of betaine over proline-betaine, which is favored over proline (Csonka & Epstein, 1996, Abee & Wouters, 1999). Similarly, trehalose uptake plays an important role in desiccation tolerance of air-dried *E. coli*, hydroxyectoine uptake was more beneficial than trehalose biosynthesis during osmotic stress (Welsh & Herber, 1999; Manzanera et al., 2004).

Salmonella survives in low moisture conditions for an extended period of time and the presence and survival of *Salmonella* in farm environments, chicken litter, and food processing areas is well documented (Chen et al., 2013; Russo et al., 2013). By adapting several S*almonella* strains to low moisture in thermally treated compost with minimum moisture content of 20%, after exposure to 70°C, desiccation-adapted *Salmonella* in aged chicken litter, had higher survival rates after 6 h than their non-adapted counterparts which only survived up to 2 h (Chen et al., 2013). A salmonellosis outbreak in 2008 identified *Salmonella* Agona as the pathogen of concern and was the same strain that had caused an outbreak from the same toasted wheat cereal processing plant in 1998 (Russo et al., 2013). This highly resilient strain was believed to have entered a dormant state after the initial outbreak 10 years prior, but was re-introduced into clean processing areas during partial renovations in 2008 that unearthed the strain from areas that were condemned after the 1998 outbreak. These examples confirm how resilient some strains of *Salmonella* are when exposed to highly desiccated environments. Despite numerous studies on desiccation in *Salmonella*, cellular adaptation and survival mechanisms involved during desiccation stress are still far from being fully understood.

1.5 Genetic regulatory networks engaged during desiccation/osmotic stress.

Desiccation induces a variety of cellular responses in *Salmonella* but the fine-tuning of gene regulation depends on the strain selected, the method of desiccation, the drying matrix, and the drying surface. Multiple transcriptomic studies convincingly show that, irrespective of the *Salmonella* serovar and drying matrices selected, desiccation induces an upregulation of a multitude of genes involved in trehalose and glutamate biosynthesis, as well as fatty acid catabolism (Li et al., 2012; Gruzdev et al., 2011, Finn et al., 2013a). However, differences with

strain-specific and drying matrix-specific gene regulation were also observed, but expression of gene regulation were the most common.

In order to understand the mechanisms involved during desiccation of *Salmonella*, it is important to identify the genes responsible for inducing the desiccation stress responses. The most common genes identified during stress are *invA, dnaK, rpoE*, *otsB*, *kdpA* and *fadA* (Fong & Wang, 2016a; Deng et al., 2012; Li et al., 2012; Table 2). In an attempt to maintain basic cellular functions during extreme stress, cells redistribute resources to remain metabolically active, and as such, gene activation is selected based on which will ensure survival. For example, flagella assembly is a high-energy process, and more often during extreme stress, cells divert energy from flagella assembly to lower energy processes to promote survival (Li et al., 2012; Finn et al., 2013b). Dead cells can also be a nutrient source for the surviving cells during stress; some bacteria are known to produce extracellular polymorphic substances (EPS) during cell death induced by desiccation (Gruzdev et al., 2011). Desiccation killed 50% of *Salmonella* cells, and the EPS residues from the dead cells provided a source of extracellular solutes, which contributed to the overall nutrient content of the environment surrounding the surviving desiccated *Salmonella* cells (Gruzdev et al., 2011).

Table 2. Up and downregulation of genes in *Salmonella* exposed to desiccation stress.

Gene	Regulated	Description	Function	References	Drying matrix
rpoE	✓	Alternative sigma factor σ^E	Stress response in low a_w	33, 51	Deionized water, LB broth, peanut oil (a_w 0.3)
rpoS	✓	RNA polymerase sigma factor (σ^S)	General stress response	1, 36, 110, 105	*
otsA	✓	Trehalose-6-phosphate synthase	Trehalose biosynthesis	23, 74, 98, 111	LB broth, PBS, M9 (2% glucose)
otsB	✓	Trehalose-6-phosphate phosphatase	Trehalose biosynthesis	23, 44, 74, 98, 111	LB broth, peanut oil, PBS, M9 (2% glucose)
Pgm	✓	Phosphoglucose mutase	Trehalose biosynthesis	23	LB broth
proP	✓	Proline/betaine transporter	Uptake of osmo-protectants	43, 63	LB broth, TYG(NaCl)
kdpA	✓	K^+ translocating ATPase (A)	Potassium ion transport	51, 71	Deionized water
gltB	✓	Glutamate synthase large subunit	Glutamate biosynthesis	51, 74	Deionized water, PBS
gltD	✓	Glutamate synthase small subunit	Glutamate biosynthesis	53, 74	Deionized water, PBS
iscA	✓	*nifU*-like protein	Fe donor for Fe-S assembly	51	Deionized water
iscS	✓	Cysteine desulphurase	Fe-S cluster repair	72	*
sodA	✓	Manganese superoxide dismutase	Fe-S assembly in low [Fe]	114	*
sufD	✓	Cysteine desulphurase modulator	Fe-S assembly in low [Fe]	51	Deionized water
nifU	✓	Scaffolding protein	Fe-S cluster formation	10, 51, 74	Deionized water, PBS
yhgI	✓	Putative thioredoxin-like protein	Fe-S cluster formation	53	Deionized water
cyaY	✓	Bacterial frataxin ortholog	Fe donor for cluster repair	3, 115	*
yggX	✓	Oxidative stress related protein	Fe donor for cluster assembly	115	*

Table 2 continued

fadA	✓	3-ketoacyl-CoA thiolase	Degradation of LCFA	42, 44, 45, 74	PBS, peanut oil (a_w 0.52)
fadB	✓	FA oxidation complex (α subunit)	Degradation of LCFA	42	LB broth
rpoE	x	Alternative sigma factor σE	Stress response in low a_w	44	peanut oil (a_w 0.52)
dnaK	x	Heat shock chaperone protein	Encodes HSP70 protein	44	peanut oil (a_w 0.52)
invA	x	Invasion protein	Invasion and pathogenicity	44	peanut oil (a_w 0.52)

*No mention of drying matrix
✓: upregulated
x: downregulated

1.5.1 Downregulation of invasion genes during desiccation stress.

The *invA* gene is involved in invasion and pathogenicity of *Salmonella* and is upregulated in high a_w conditions in *S*. Typhimurium. However, during desiccation stress, downregulation of the *invA* gene is observed (Fong & Wang, 2016a), which suggests that energy redirection occurs during stress by inducing dormancy in pathogenicity-related genes, in favor for genes involved in survival during desiccation stress.

1.5.2 Regulation of RpoE/RpoS during stress.

The *rpoE* gene encodes for an alternate sigma factor σ^E, involved in the activation of a series of genes in response to various stressors, and the *rpoS* gene encodes for the general stress response alternative sigma factor, σ^S. RpoE regulation in *Salmonella* is a_w-dependent; it is downregulated when cells are dried in peanut oil with a a_w of 0.52 (Fong & Wang, 2016a), but air-drying or drying in peanut oil with a a_w of 0.3 results in upregulation of the *rpoE* gene (Gruzdev et al., 2012a; Deng et al., 2012). In *E. coli*, RpoE was responsible for survival under high temperature stress where regular σ factors could not function properly by managing mis-

folded proteins within the cytoplasm (Hiratsu et al., 1995). RpoS triggers a stress response against osmotic shock, heat, starvation, and oxidative stress (Dodd & Aldsworth, 2002). The *rpoS* gene is induced in *Salmonella* when exposed to low moisture conditions (Pratt et al., 2016), and *ΔrpoS* mutants are known to become sensitive to heat, starvation and acid stress (Abee & Wouters, 1999; Dodd & Aldsworth, 2002; Rees et al., 1995). While an upregulation of *rpoE* is observed after thermal stress in *S.* Typhimurium and *S.* Tennessee, desiccation induces a downregulation of *rpoE* (Fong & Wang, 2016a). The σ^E heat shock factor, encoded by the *rpoE* gene (Raina et al., 1995), improved survival of *S.* Typhimurium at 37ºC exposed to 0.85% NaCl solution while the σ^S factor was upregulated to a greater extent than σ^E at a NaCl-mediated a_w of 0.96 (McMeechan et al., 2007). It is likely that both the amount of unbound water available intracellularly, as well as the method of induction of desiccation could affect expression of *rpoE* in *Salmonella*.

1.5.3 Regulation of the *dnaK* gene during stress.

The gene *dnaK* encodes for the heat shock chaperone protein HSP70, which is required for binding and stabilization of other proteins during heat stress. Heat shock proteins (HSPs) generation is mediated by the heat shock transcription factor σ^H (RpoH) in response to heat stress, and once the temperature is lowered to regular growth temperatures, HSP generation slows down to normal levels (Rudolph et al., 2010). Exposure to mild heat induces subsequent heat resistance through the upregulation of *dnaK* which induces resistance by increasing the production of protein-stabilizing chaperones. When faced with low a_w conditions, *dnaK* is downregulated during storage for 6 h in peanut oil (Fong & Wang, 2016a), but upregulated after air-drying (Gruzdev et al., 2012a). This indicates that the matrix used for drying induces

different cellular regulatory networks in *Salmonella,* which can either induce cross-protection to subsequent stresses, or be detrimental to their survival.

1.5.4 Compatible solute uptake during osmotic/desiccation stress.

Even though bacterial cell walls can withstand considerable osmotic pressure, an increasing osmolality can negatively impact bacterial growth rate. When faced with conditions such as osmotic shock, desiccation and extreme heat, uptake of extracellular compatible osmo-protectants is activated, and the expression of trehalose biosynthetic operon (*otsBA*) is upregulated. Osmo-protectants are low molecular weight compounds that can easily enter or leave a cell to maintain proper intracellular osmotic balance (Fong & Wang, 2016a). The genes *proP*, *proU* and *osmU*, encoding for the transporters responsible for osmo-protectant uptake, show high up-regulation during desiccation. Mutations in the ProP system affects survival in *Salmonella.* Finn et al., (2013b) observed that mutations in the ProU and OsmU systems still provided surviving cells only if the ProP system was fully functioning whereas Δ*proP* mutants did not survive irrespective of functioning ProU and OsmU systems.

Genes *otsB* and *otsA* encode for the enzymes trehalose-6-phosphate phosphatase and trehalose-6-phosphate synthase, respectively, and are induced by osmotic shock, desiccation, extreme cold and elevated heat exposure for trehalose synthesis *in vivo* (Canovas et al., 2001; Purvis et al., 2005). The *pgm* gene encodes for the enzyme phospho-glucose mutase, which is required for the conversion of glucose-6-phosphate to glucose-1-phosphate, a precursor to numerous sugars produced *in vivo* (Canovas et al., 2001). Trehalose is a non-reducing disaccharide which, during desiccation or dehydration stress, functions as a water molecule replacement. It forms a viscous layer surrounding macromolecules to inhibit protein movement and membrane damage, until the required level of intracellular water is replenished (Purvis et al.,

2005). High trehalose concentrations within cells help them maintain the necessary turgor pressure to withstand exposure to media with minimal unbound water.

Trehalose production not only enables survival in extreme dehydration conditions, it also induces thermotolerance. Trehalose production improved the growth of *S.* Typhimurium at higher temperatures and improved growth of *E. coli* under osmotic stress (Canovas et al., 2001; Hengge-Aronis et al., 1993). Higher levels of trehalose biosynthesis have also improved growth of *E. coli* in high osmolality media at 41^{o}C (Purvis et al., 2005).

Mutations of either *ots* genes or the *pgm* gene decreased survival of either species during osmotic stress and prolonged storage. At temperatures greater than 45^{o}C, growth and survival of bacterial cells with the impaired *pgm* gene can be maintained with supplementation of galactose and glycine betaine in the growth media. Even with an impaired *otsA* gene, addition of glycine betaine replaced the trehalose requirement for growth under stress; however, addition of galactose did not. Galactose goes through the same pathway as trehalose production, and an *otsA* mutant would inhibit trehalose production for cell survival (Canovas et al., 2001). While *otsBA* mutations do not impair increased thermotolerance induced by high solute concentration, a combination of high osmolality and elevated temperature exposure significantly reduces thermotolerance of a *pgm* or *otsBA* mutant of *S.* Typhimurium. Media containing hexose sugars or inorganic salts tend to improve trehalose production and subsequent survival of strains during stress; however, concentrated pentose sugars-containing media, are less beneficial (Purvis et al., 2005).

1.5.5 Activation of the potassium ion active transport channel during stress.

Dehydration of *S.* Typhimurium SL 1344 induces upregulation of 90 genes involved in ribosome structure, amino acid transport and metabolism, stress response, transcription, energy

production and conversion, inorganic ion transport, and lipid/carbohydrate transport and metabolism, while 7 genes, 4 of which are involved in maintaining the virulence plasmid in *Salmonella*, were downregulated (Gruzdev et al., 2012a). The highest induction was in the *kdpABC* genes encoding for potassium ion (K^+) active transport channels. K^+ channels maintain osmotic balance through the flow of K^+ ions in and out of the cell, and are activated under osmotic stress or low K^+ growth conditions (Record et al., 1998).

Bacteria have the ability to maintain normal internal levels of K^+ through the induction of several K^+ uptake systems. Both *E. coli* and *Salmonella* have three major K^+ transporters: Kup, Trk and Kdp. As Kup and Trk systems both have low affinity to K^+ ions, they are activated to induce K^+ uptake in high [K^+] growth conditions. However, as the extracellular K^+ concentration decreases and the normal K^+ uptake level cannot be maintained, the Kdp system is activated and takes over as the main K^+ uptake system (Ballal et al., 2007). The Kdp-ATPase system is present in a variety of Gram negative (*Salmonella* and *E. coli*) and Gram positive (*Bacillus* spp. and *Clostridia* spp.) species and are required to maintain osmotic balance, regulate intracellular pH, genetic expressions and activate cellular enzymes (Laimins et al., 1981). Under low [K^+] conditions, *E. coli* induces the expression of the K^+-dependent ATPase (Kdp-ATPase) for K^+ uptake from the surrounding environment in an effort to maintain the normal intracellular [K^+]. The *E. coli* Kdp-ATPase system is made up of four protein subunits KdpF, KdpA, KdpB, and KdpC, that are encoded by genes located on the KdpFABC operon (Ballal et al., 2007). While KdpA binds and transports K^+ ions, KdpB acts as a phosphorylation site, KdpC is required for the Kdp-ATPase assembly and KdpF for complex stability. With a high affinity for K^+ ions, the Kdp-ATPase system is more effective in maintaining osmotic balance intracellularly than the other potassium uptake systems. The Kdp-ATPase system is independent of compatible solute

uptake and is only activated when a low K^+ ion concentration is detected. In fact, exposure to a NaCl-mediated osmotic shock in *Cyanobacteria* spp. suppressed expression of the Kdp system, thus confirming that Kdp induction is not mediated by osmotic shock (Ballal et al., 2005; Alahari et al., 2001). A Δ*kdpA* mutant does not enable long term *Salmonella* survival at 4^{o}C, indicating that a modification of the K^+ regulatory channels affect *Salmonella* survival post-desiccation and during long-term cold storage (Gruzdev et al., 2012a).

1.5.6 Glutamate synthesis.

Glutamate synthesis is initiated intracellularly to maintain osmotic balance during stress, and glutamate makes up almost 90% of the amino acids present in gram-negative bacteria in high osmolality media (Csonka & Epstein, 1996). There are two glutamate synthesis pathways which either require only glutamate dehydrogenase, or both glutamate synthetase and glutamate synthase. A high intracellular K^+ concentration induced higher glutamate dehydrogenase activity, which led to the assumption that a high concentration of intracellular K^+ ions act as a stimulus for glutamate synthesis (Csonka & Epstein, 1996). Glutamate synthesis is believed to facilitate the accumulation of osmo-protectants during desiccation stress (Finn et al., 2013a).

1.5.7 Function of Fe-S clusters during desiccation/osmotic stress.

Fe-S clusters are iron co-factors produced from the combination of bio-available iron with elemental sulphur, and are responsible for cellular processes such as electron-transfer, activation of genetic expression, enzyme activity and also act as Fe-S storage compounds (Gruzdev et al., 2011; Kiley & Beinert, 2003). Cluster formation helps to prevent oxidation when cells are exposed to desiccation stress, as oxidation is a major cause of cell death during desiccation. Fe-S clusters are present in [2Fe-2S], but the majority are assembled as [4Fe-4S] forms onto proteins in the presence of cis-residues, which are responsible for iron coordination

(Ding et al., 2004). It is estimated that the concentration of iron present in bacteria for iron co-factor biosynthesis is approximately 10 μM, as the majority of the iron present within a system is not bioavailable or stored in storage proteins such as ferritin (Ayala-Castro et al., 2008).

The *Isc* (Fe-S), *Suf* (S) and *Nif* (nitrogen fixation) system pathways are responsible for inducing Fe-S cluster formation. It is believed that the *Isc* pathway is required for housekeeping cluster formation, while the *Nif* pathway is for enzyme-related cluster formation. The *Suf* pathway is induced under stressful conditions (Ayala-Castro et al., 2008). The *iscA* gene has a high affinity to iron, which makes it a great iron donor. It also has the ability to coordinate Fe-S cluster formation and maturation, and is believed to be responsible for free iron collection during cluster formation (Ding et al., 2004). While bacteria such as *E. coli* have both *Isc* and *Suf* pathways for Fe-S cluster formation, other bacteria such as *Mycobacterium tuberculosis* utilize only the *Suf* pathway (Huet et al., 2005). Previous studies have indicated that the *Suf* pathway is better equipped for Fe-S cluster formation under stressful conditions such as oxidative or starvation stress in *E. coli*. The *Suf* pathway likely assembles the Fe-S clusters in low intracellular concentrations of bioavailable iron (Outten et al., 2004; Takahashi & Tokumoto, 2002). Furthermore, deletion of the *SufD* gene resulted in growth defects during stress, confirming the importance of the *Suf* gene for Fe-S assembly during stress (Outten et al., 2004).

During dehydration, cells of *Salmonella* undergo some level of oxidation and dehydration induces an upregulation of a number of oxidation stress-response genes, including *iscA* (*nifU*-like protein gene), *sufD* (cysteine desulphurase modulator gene) and *yhgI* (putative thioredoxin-like protein gene) (Gruzdev et al., 2012a). Upregulation of the *nifU* and *nifS* genes was also observed; these genes encode scaffolding proteins involved in Fe-S cluster formation (Gruzdev et al., 2012a).

In *S*. Typhimurium, the CyaY protein enables cluster biosynthesis and repair of clusters affected by oxidation, while the YggX protein enables Fe-S cluster formation and repair during extreme oxidative stress (Velayudhan et al., 2014; Gralnick & Downs, 2001). The CyaY protein, a bacterial homologue to the human protein frataxin, acts as an iron donor during Fe-S assembly, while YggX, another iron donor, is more involved in cluster repair than assembly (Adinolfi et al., 2009; Velayudhan et al., 2014). However, this is in contradiction with Adinolfi et. al. (2009), who claimed that the protein CyaY binds to cysteine desulphurase, encoded by the gene *iscS*, to act as an Fe-dependent inhibitor of the Fe-S cluster formation (Adinolfi et al., 2009).

1.5.8 Fatty acid catabolism induction during desiccation/osmotic stress.

Detection of an increased level of cyclopropane fatty acids (CFAs) is an indication of starvation or desiccation in bacterial cells, as CFAs protect cells against a range of stresses. Lipoproteins and phospholipids make up the fatty acid components present in bacterial cell membranes. To protect from stress, *Salmonella* cells induce modification of their fatty acid profiles to lower membrane fluidity during the stress. Since with desiccation more energy is needed by cells to survive, an increase in fatty acid degradation is observed as the *fadA* gene is upregulated to produce and have more ATP available for cells to increase production of stress-response proteins. The lower the lipid content of the cell membrane, the more rigid the cell membrane becomes, which prevents further loss of intracellular water during desiccation.

The *fad* locus is known to induce an upregulation of a variety of genes upon exposure to desiccation and starvation stress (Fong & Wang, 2016a). The most significantly induced expression in desiccation resistant *S*. Tennessee and desiccation sensitive *S*. Typhimurium LT2 was involved in the fatty acid metabolism pathway (51% in *S*. Tennessee v/s 35% in *S*. Typhimurium); with the *fadA* gene having the highest level of upregulation after 2 h of air drying

at 11% relative humidity (Li et al., 2012). The *fadA* gene is a fatty acid catabolic gene that encodes for FadA (3-ketoacyl-CoA thiolase), an enzyme involved in the degradation of long chain fatty acid into acetyl-CoA that, in turn, generates more ATP for cellular usage through the TCA cycle. The higher the intracellular energy production, the better the survival under stress conditions, which would explain the upregulation of the *fadA* gene during desiccation stress (Li et al., 2012; Kim et al., 2001)

1.6. Heat resistance of *Enterobacteriaceae*.

Survival of *Salmonella* during long-term starvation, desiccation and/or osmotic stress allows them to be resistant to food preservation processes, especially thermal treatments (Kieboom et al., 2006; Russo et al., 2013; Goepfert & Biggie, 1968; Mercer et al., 2015). Formation of Fe-S clusters, uptake of compatible solutes and a higher level of CFAs during desiccation stress, protects cells against a wide range of stresses such as heat, oxidative, and acid stresses, which implies that exposure to desiccation generates cells that are more resistant to other stresses. It is well established that the heat resistance of *Salmonella* is greatly impacted by the food matrix; the greater the amount of solids, fat, carbohydrates and lower unbound water all contribute to thermal resistance in *Salmonella*. Figure 1 shows the heat resistance of *Salmonella* in various matrices at a a_w of 0.5 or lower. The type of solute present in the drying matrix also has an effect on heat resistance. The presence of glycerol does not improve heat resistance, whereas the presence of sucrose increased the D-value (Mattick et al., 2001). Microbial species, growth conditions (stationary-phase grown versus exponential-phase grown), and previous exposure to stress, all contribute to heat resistance. Using a dry-adapted versus a non-adapted inoculum affects the heat resistance of *Salmonella* on treated almonds (ABC, 2007; Du et al., 2010; Danyluk et al., 2005). Similarly, stationary-phase cells have a greater ability to survive

stress, such as desiccation, in comparison to exponential-phase cells (He et al., 2011; Breeuwer et al., 2003; Danyluk et al., 2005; Humpfrey et al., 1995; Ng et al., 1969).

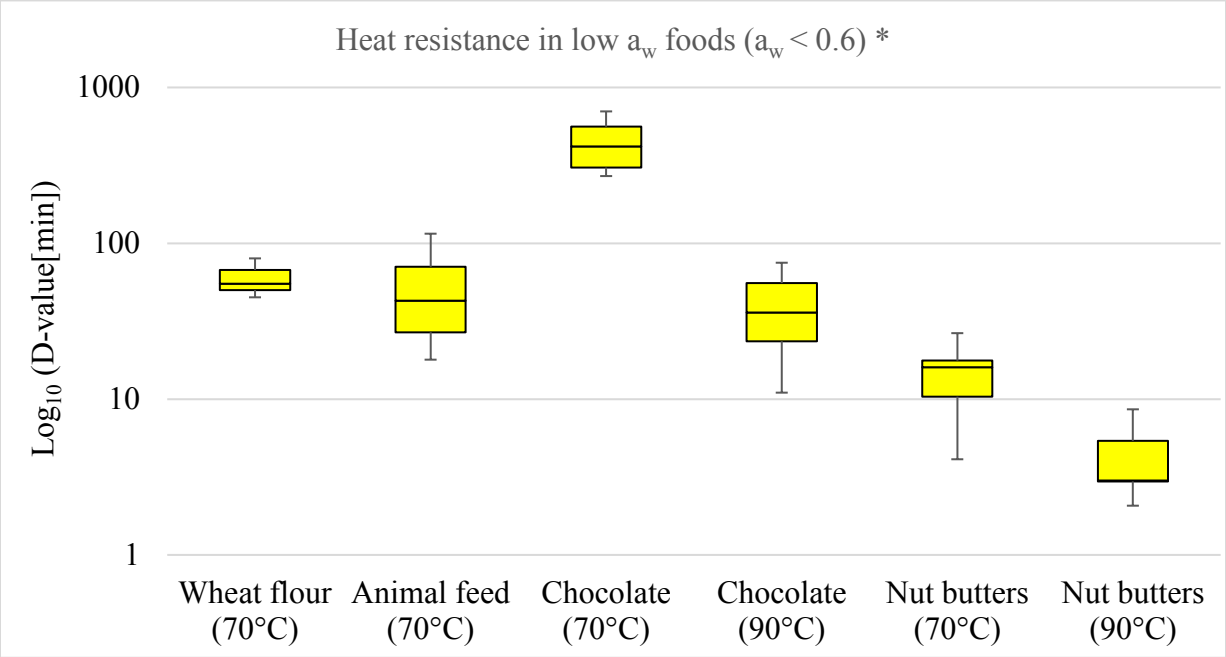

Figure 1. Heat resistance of multiple *Salmonella* strains in various matrices with a_w of 0.6 or less. The box plot shows log_{10} D-value (min) of *Salmonella* strains from previous studies treated at 70°C or 90°C, treated in 5 different matrices; 1 strain was tested in wheat flour (a_w 0.2-0.6; Archer et al., 1998), and 1 strain was tested in animal feed (a_w 0.2; Liu et al., 1969), 6 of the strains were tested in chocolate (a_w 0.5; Goepfert & Biggie, 1968; Lee et al., 1989; Krapf & Gatenbein-Demarchi, 2010; Barrile & Cone, 1970; da Silva do Nascimento et al., 2012), and 8 strains were tested in nut butters (a_w 0.3-0.5; Villa-Rojas et al., 2013; He et al., 2011; He et al., 2013; Ma et al., 2009).
*Adapted from Chen et al., 2009.

Most low a_w foods undergo some type of thermal treatment as one of the processing steps prior to packaging and storage. Validation studies have confirmed that specific heat treatments are effective in producing a 5-log reduction of bacterial counts, which meets the US federal requirements of at least having a 4-7 log reduction of vegetative foodborne pathogenic bacteria both in a laboratory setting and within various food matrices (Little et al., 2009; AIOE, 2012).

However, *Salmonella* control in low a_w food products and processing environments is challenging, as the D-values for thermal inactivation of *Salmonella* in food products increases astronomically when treating low a_w food products as compared to high a_w food products (Calhoun & Frazier, 1966). Thermal treatments performed on a 5-strain cocktail of *S. enterica* and *E. coli* O157:H7, air-dried and stored in peanut butter (a_w of 0.4) showed that 1 h exposure to 72°C only resulted in less than 2-log reduction of both strain sets, but when the temperature was increased to 90°C, a log reduction of 5.5-log and 7.1-log was observed in the *S. enterica* and *E. coli* cocktails, respectively (He et al., 2011).

Extreme heat resistance of *Salmonella* stimulated by low moisture environments in various food matrices is a phenomenon that was, and is still being studied (McDonough & Hargrove, 1968; Mattick et al., 2001; Krapf & Gatenbein-Demarchi, 2010; Santillana-Farakos et al., 2014). Adapting *Salmonella* strains to low moisture conditions using a high-solute broth (a_w of 0.65) then exposure to 55 to 60°C was detrimental to their survival, while strains exposed to greater than 70°C showed exceptional heat resistance (Mattick et al., 2001). Thermal treatments on *Salmonella* during chocolate conching results in a D_{60} of 306 min in cocoa butter, and D_{60} of 1008 min in dark chocolate (Krapf & Gatenbein-Demarchi, 2010).

1.6.1 The locus of heat resistance (LHR) mediates heat resistance of *Enterobacteriaceae.*

Thermal resistance is also well studied in *Escherichia coli* and studies have shown that heat stress is detected by the outer membrane porin (Omp) precursors, which induces the activation of σ^E and σ^H, which are bacterial transcription factors, required for RNA synthesis to induce the transcription of heat shock proteins (HSPs) and other genes needed for cellular recovery (Noor, 2015). While most strains of *E. coli* are heat sensitive, studies have confirmed

the presence of heat resistant strains in the food chain with D_{60} values from 5 to 60 min (Mercer et al., 2015; Dlusskaya et al., 2011).

Using comparative genomic analysis, Mercer et al. (2015) identified 6 genes arranged on an approximately 14kb genomic island that were unique to strains phenotypically classified as highly heat resistant. This island was termed the locus of heat resistance (LHR), and exhibited a D_{60} value of greater than 5 min in highly heat resistant strains of *E. coli* AW1.7 (Mercer et al., 2015). The LHR is comprised of 16 open reading frames (orfs; Figure 2) flanked by mobile elements. A BLAST (basic local alignment search tool) search located orfs 2, 9, 11, 13, 15 and 16 only in highly heat resistant strains such as *E. coli* AW1.7 (Mercer et al., 2015; Dlusskaya et al., 2011). The remaining ten orfs have convincing orthologs in other strains, but they were not identified in the same specific cluster as within the LHR. Additionally, the frequency of the LHR present within *E. coli* genomes is approximately 2% (Mercer et al 2015).

To further understand and confirm the role of the LHR in heat resistance, Mercer et al. (2015) cloned the full LHR and fragments of the LHR onto the low-copy vector pRK767 prior to transformation into the heat-sensitive *Escherichia coli* AW1.7 derivative, *E. coli* AW1.7ΔpHR1. The authors noted that *E. coli* AW1.7ΔpHR1pLHR had a very similar survival pattern as did the wild-type heat resistant *E. coli* AW1.7; confirming that presence of the LHR confers high heat resistance in *E. coli* AW1.7.

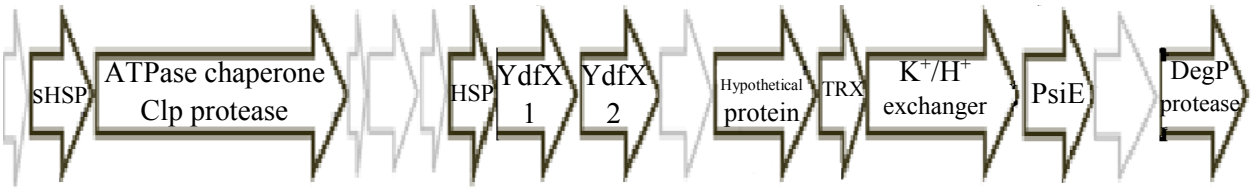

Figure 2. The locus of heat resistance (LHR), characterized from *Escherichia coli* AW 1.7. The LHR, known to induce highly heat resistance in some *Enterobacteriaceae*, is a 14.469 kb sized operon, made up of 16 open reading frames (orfs). Orfs 2, 9, 11, 13, 15 and 16 are orthologs that were found to be unique to highly heat resistant strains only. The black-lined orfs (2, 3, 7, 8, 9, 11, 12, 13, 14, 16) encode for predicted proteins such as heat shock proteins (small HSP C2; *orf2*, HSP 20; *orf7*), proteases (Clp protease; *orf3*, Zn-dependent protease; *orf15*, DegP-like; *orf16*), hypothetical proteins belonging to the YfdX family (*orf8*, *orf9*), other hypothetical proteins (*orf10*, *orf11*) a thioredoxin (TRX) (*orf12*), a potassium/hydrogen (K^+/H^+) exchanger (*orf13*), and a phosphate starvation gene (PsiE) (*orf14*) (Li & Gänzle, 2016; Mercer et al. 2017). The grey-lined orfs (1, 4, 5, 6, 10, 15) are less likely to be expressed, in comparison to the black-lined genes.

Sequence similarities indicate that some proteases found within the LHR cluster are similar to proteases from other strains, which further confirmed that moderately heat resistant and heat sensitive strains possess orthologs to some of the genes found on the LHR cluster. Therefore, a strain possessing the entire LHR sequence will express high heat resistance, and strains that have partial fragments of the operon would exhibit partial to no heat resistance. This was confirmed by Mercer et al., (2015) who separated the operon into three fragments: fragment one with *orf* 1-7; fragment two with *orf* 8-10; and fragment three with *orf* 11-16. Each fragment was transformed individually, as well as in groups, and strains with fragments 1 and 2 exhibited moderate heat resistance (D_{60} < 5min), confirming that fragment 3 is required to induce prolonged resistance to heat stress. All 16 orfs, as shown in Figure 2, are required to confer high heat resistance.

Studies conducted on the thermal resistance of *Salmonella* indicate that, in comparison with other *Salmonella* strains in pasteurized liquid egg, *S.* Senftenberg is one of the most heat resistant strains (Winter et al., 1946; Davidson et al., 1966). Genetic analyses in other

Enterobacteriaceae strains revealed the presence of the LHR, in other genera such as *Salmonella enterica* and *Enterobacter* (Mercer et al., 2017). Phylogenetic analysis of *S.* Senftenberg identified two thermotolerant loci (TLPQC1 and TLPQC2) on a 341.3 kb plasmid (pSSE-ATCC 43845); a plasmid that has properties similar to the IncHI2 R478 plasmid, but lack the antibiotic genes (Nguyen et al. 2017). Furthermore, a BLAST analysis confirmed that the identity of the genes on the second locus (TLPQC2) closely resembles the LHR in *E. coli* AW1.7 (Nguyen et al. 2017). Analysis of PCR products in this study confirmed that *Salmonella enterica* serovar Senftenberg ATCC 43845 possessed all three fragments that make up the LHR; which was responsible for the heat resistance exhibited by *S.* Senftenberg. This was further confirmed through the transformation of the wild-type, heat sensitive, and LHR-negative *Salmonella enterica* serovar Typhimurium ATCC 13311 with the LHR constructed on an empty plasmid (pRK767). The transformed strain, *S.* Typhimurium ATCC 13311pLHR, had a D_{60} > 5 min, proving that the LHR is also responsible for thermal resistance in LHR-positive *Salmonella* (Mercer et al., 2017). As such, it is possible that presence of both thermotolerant loci are not required to induce high heat resistance in *Salmonella*.

Resistance to heat in *Salmonella* differs in a high a_w environment, in comparison to low a_w conditions. While a study on over 300 strains confirmed that *S.* Senftenberg 775W was the most resistant strain in an aqueous medium (Ng et al., 1969), in a low a_w environment, heat resistance of *S.* Typhimurium surpasses that of *S.* Senftenberg 775W (Goepfert & Biggie, 1968; Liu et al., 1969).

In order to properly validate a processing step, a number of factors need to be monitored, including the strain selected for the validation as using a known heat sensitive strain for a thermal inactivation validation step would not provide the data necessary to design a plan that

would reduce the microbial load, especially if heat resistant strains are present. Bacterial stress responses include changes to cellular morphology, membrane modification, gene regulation and altering their cellular metabolism for survival. These responses trigger chaperone proteins and other components that, often than not, confer cross-protection against other stresses to ensure their survival. How different stresses trigger responses and how those responses induce cross-protection is a matter that requires more in-depth research to ensure that current microbial reduction strategies employed by the food industry are properly targeted. It is still not clear how many genes are involved in inducing resistance to desiccation and subsequent heat stress in *Salmonella*, and how the drying matrix, experimental methods, or strain selection affect survival. Much of the data currently available only offer a general idea of the interaction between desiccation and extreme heat resistance of *Salmonella*, but more work is needed in this area. Understanding the molecular mechanisms involved in *Salmonella* survival in low a_w stress is highly significant as it provides more detailed understanding of how *Salmonella* regulatory networks engage when faced with stressful conditions to further improve current interventions for *Salmonella* control. The data presented in this study will help bridge some of the knowledge gaps in the area of extreme heat resistance demonstrated by *Salmonella* adapted to low a_w conditions.

1.7. Research objectives

This study aimed to analyse and understand the survival and heat resistance of desiccation-adapted *Salmonella* stored under low water activity conditions. It is hypothesized that the LHR contributes to the survival of LHR-positive *Salmonella enterica,* adapted to low moisture conditions.

The specific objectives are:

1. To understand the regulation of the LHR in dried *Salmonella enterica*
2. To compare and contrast the effectiveness of the LHR in survival of LHR positive and LHR negative strains of *Salmonella* and *Escherichia coli* in low moisture conditions
3. To assess the impact of the LHR on the survival of dried *Salmonella* and *E. coli* after a short term, high heat exposure

2. Materials and Methods

2.1 Strains and Growth Conditions.

Cultures of *Salmonella enterica* serovar Senftenberg ATCC 43845 (LHR positive; Mercer et al., 2017), *Salmonella enterica* serovar Typhimurium ATCC 13311 (LHR negative), *Escherichia coli* AW1.7 (LHR positive; Mercer et al., 2015) and *Escherichia coli* AW1.7ΔpHR1, a heat sensitive and LHR negative, derivative of AW1.7 (Pleitner et al., 2012), were sub-cultured in Tryptic Soy (TS) (Difco, Becton Dickinson, US) broth, and maintained at -80°C in 70% glycerol. Prior to each experiment, the frozen cultures were streaked on TS agar plates, and incubated aerobically at 37°C overnight (O/N). Isogenic strains of *S. enterica* serovar Typhimurium ATCC 13311 (*S.* typhimurium pLHR, and *S.* Typhimurium pRK767) were previously generated by inserting the LHR, constructed on an empty plasmid pRK767 containing a tetracycline resistance (tet^R) gene, into the wildtype LHR negative strain (Mercer et al., 2017). The isogenic stock cultures were also maintained at -80°C in 70% glycerol, prior to streaking onto TS agar plates containing 15 $mgmL^{-1}$ tetracycline-HCL and incubated aerobically at 37°C.

2.2 Experimental design

2.2.1 Heat treatment to confirm presence of the LHR.

To confirm that the strains possessed or lacked the LHR, a heat test was performed. Both *Salmonella* and *E. coli* strains were inoculated from culture stocks stored at -80°C onto TS agar plates, and incubated O/N at 37°C. Several colonies were picked from each plate, and incubated O/N in fresh TS broth. A 100 µL aliquot of each O/N culture was transferred into sterile PCR tubes and heated at 60°C for 5 min, using a PCR thermocycler (Eppendorf Mastercycler^{TM} ep Gradient; Eppendorf, Hamburg, Germany). Enumeration of the surviving colonies was done by plating the serially diluted treated samples onto TS agar plates and incubating the plates O/N at 37°C.

2.2.2 Preparation of the dry inoculum for use in experiments

Several colonies were picked from TS agar plates containing colonies of *Salmonella* Senftenberg ATCC 43845, *Salmonella* Typhimurium ATCC 13311, *E coli* AW1.7 and *E. coli* AW1.7ΔpHR1, and incubated aerobically in 5 mL TS broth O/N at 37°C for the preparation of the dry prepared inoculum. An aliquot of each O/N inoculum was sub-cultured (1%) into fresh TS broth and incubated at 37°C between 18 to 20 h. A 100 µL portion of the stationary phase inoculum was spread onto TS agar plates (made with 20 mL TS agar) and incubated aerobically for 24 h at 37°C to produce a bacterial lawn (Danyluk et al., 2005).

The bacterial lawns were harvested from the surface of the plates with 1 mL of sterile TSB broth, and 1 mL of 0.1% peptone per strain, per plate, and into sterile 1.5 mL micro-centrifuge tubes. The harvested cells were washed three times by centrifugation (10,000 *g* for 3 min) at room temperature and re-suspended in each sterile media respectively to a final cell

count of approximately 2.0 x 10^{11} $CFUmL^{-1}$. The cell count per mL was determined with serial dilutions and plating onto TS agar. Sterile 0.1% peptone was used as the dilution medium throughout the remainder of the experiments.

2.2.3 Adaptation to dry conditions and culture storage at specific water activities (a_w).

A 25 µL aliquot of each re-suspended inoculum was transferred into individual sterile Co-star 24-well micro-titer plates (Corningware; Sigma-Aldrich, Darmstadt, Germany), and air-dried in a biosafety cabinet for 5 h. Dehydrated silica gel beads, and saturated solutions of sodium bromide and sodium chloride were used to create an a_w of 0.1, 0.55 and 0.75 respectively (Table 3), measured using a water activity meter (AquaLab PRE; Decagon Devices, WA, USA). A glass-bottomed air-tight container was used as a desiccator, containing different the desiccants mentioned to create the desired a_w of 0.1, 0.55 and 0.75. The plates were stored in the desiccators for up to 30 days at 37°C. Sterile 0.1% peptone (100 µL) was added to each well, and the dried pellets were scraped the bottom of the wells and re-suspended using a pipet tip, and then serially diluted and plated onto TS agar plates, which were incubated aerobically O/N at 37°C prior to enumeration to determine cell counts.

Table 3: Desiccants used to modulate the storage humidity at 37ºC.

Desiccants	Water activity (a_w)	Weight (g)	Temperature (°C)
Silica gel beads	0.1	100	37
Sodium bromide	0.55	104.9	37
Sodium chloride	0.75	33	37

2.2.4 Deletion of the LHR from *S*. Senftenberg ATCC 43845.

An attempt to delete the plasmid containing the LHR from *S*. Senftenberg ATCC 43845 was done using the plasmid curing protocol described by Pleitner et al. (2012), with 0.1%, 0.5% and 10% sodium dodecyl sulphate (SDS). This method was selected as it proved successful in creating a heat-sensitive derivative (*E. coli* AW1.7ΔpHR1) of the heat resistant *E. coli* AW1.7. Several single colonies were inoculated into 5 mL TS broth, and incubated O/N at 37 °C. The cultures were sub-cultured (10 %) into fresh broth with the various SDS concentrations, and incubated successively as the temperature increased from 37 °C to 42 °C, to plateau at 46 °C for a total of 10 subcultures. After each subculture, 100 µL was plated onto TS agar plates and 12 colonies from each plate was inoculated into fresh TS broth and grown O/N at 37 °C. A 100 µL aliquot of each O/N culture was transferred into PCR tubes, and heated at 60 °C for 30 min in the PCR thermocycler. The heated samples were transferred into fresh broth and incubated O/N at 37°C. Tubes with no growth were assumed to be strains that had lost the LHR.

2.2.5 Generating the *S*. Typhimurium ATCC 13311pLHRΔ*kefB* isogenic strain.

S. Typhimurium pLHRΔ*kefB* was generated by the insertion of the modified LHR (LHR Δ*kefB*) construct into the wildtype LHR negative *Salmonella* Typhimurium ATCC 13311. A pRF1-2-3C clone was generated from the LHR through the deletion of ORF13, and constructed onto the tet^R plasmid pRK767, prior to transformation into *E. coli* DH5α (Nguyen, unpublished report). The modified LHR plasmid (pLHRΔ*kefB*), was purified from *E. coli* DH5α and transformed into the wild-type LHR negative *S*. Typhimurium through electroporation, and grown on 15 $mgmL^{-1}$ tetracycline-HCL TS (TS^{tet}) agar plates, to generate the isogenic *S*. Typhimurium ATCC 13311pLHRΔ*kefB*.

Several *S*. Typhimurium ATCC 13311 colonies were inoculated into 5 mL fresh TS broth for 24 h, and incubated for 2 h in fresh TS broth. Cells were kept on ice all throughout the experiment. Using a plasmid preparation kit (GeneJet plasmid mini-prep kit; Fisher Scientific, Toronto Canada), the modified LHR plasmid, pLHRΔ*kefB*, was purified from *E. coli* DH5α (Nguyen, unpublished report) and kept on ice. The transformation was done as per a modified version of the High Efficiency Transformation protocol (New England BioLabs). A 500 µL aliquot was transferred into a sterile micro-centrifuge tube, centrifuged (5000 *g* for 4 min) and washed three times using 400 µL of 10% glycerol, maintained at 4 °C. The pellet was re-suspended in 80 µL of 10% glycerol, and 5 µL of the extracted plasmid was added. The tube was incubated for 5 min on ice, then poured into a transformation cuvette (80 µL) for the electroporation (200 Ohms, 2.5 kv, 25 capitance), using a transformation apparatus (BIO-RAD *E. coli* Pulser^{TM}; Bio-Rad Laboratories, California, USA). After the transformation, 900 µL of super optimal broth with catabolite repression (SOC) media (Nguyen, unpublished report) was poured into the cuvettes, transferred into a sterile micro-centrifuge tube, and incubated at 37 °C for 1 h to encourage growth in the nutrient-rich medium. Transformants were selected by plating 100 µL of the inoculum, and the remaining 900 µL on TS^{tet} agar plates and incubated O/N at 37 °C.

Plasmid insertion was confirmed with a colony PCR, using forward (F) and reverse (R) primer pairs (Table 4, 5) designed on Geneious (Biomatters, Auckland, New Zealand) to amplify three separate primer target regions of the LHR (Mercer et al., 2015). Colonies from each plate were transferred into 24.75 µL of a PCR master-mix solution (Table 6) for each primer pair, and amplified using a PCR thermocycler (Eppendorf Mastercycler^{TM} ep Gradient; Hamburg, Germany). Fragment amplification was done at an annealing temperature of 64 °C for 30 s, using

recombinant Taq DNA polymerase (Invitrogen, Ontario, Canada). Agarose gel electrophoresis (120V for 1 h on 1% agarose 0.5X TBE gel) was used to analyze the amplified products of the PCR, and visualized using SYBRsafe staining on the AlphaImager HP System.

Table 4: Primers used to identify the three fragments, with fragment sizes and respective orfs, which make up the LHR.

Fragments	**Fragment size** (kb)	**ORFs**	**Primers**	**Primer target region size** (kb)
F1	6	1 to 7	HR-F1(F), HS-R1(R)	1.7
F2	3.3	8 to 10	HR-F2.2(F), HR-R2(R)	2.8
F3	7	11 to 16	HS-F1(F), HR-R3(R)	2.8

Table 5: Primer sequences used in this study for each primer pair.*

Primers	Direction	Primer sequence (5' -> 3')
HR-F1	Forward (F)	GCTGTCCATTGCCTGA
HS-R1	Reverse (R)	AGACCAATCAGGAAATGCTCTGGACC
HR-F2.2	Forward	*TGTCTTGCCTGACAACGTTG*
HR-R2	Reverse	ATGTCATTTCTATGGAGGCATGAATCG
HS-F1	Forward	GCAATCCTTTGCCGCAGCTATT
HR-R3	Reverse	CTAGGGCTCGTAGTTCG

*Mercer et al., 2015

Table 6: Colony PCR reagents and volumes.

Master-mix reagents	**Volume** (µL)
Nuclease free water	18.5
10X buffer	2.5
Forward primer (F)	1.25
Reverse primer (R)	1.25
$MgCl_2$	0.75
dNTPs	0.5

2.2.6 Survival of isogenic strains at different water activities.

An inoculum of wild-type and isogenic strains of *Salmonella* spp. were prepared as described in paragraph 2.2.2, and stored at an a_w of 0.75 for up to 3 d at 37 °C. Samples were serially diluted and plated onto TS agar plates O/N at 37 °C for enumeration. Statistical analysis was performed using a two-way ANOVA.

2.2.7 Survival at 110°C of air-dried strains.

A 20 µL aliquot of prepared wild-type and isogenic *Salmonella* and *E. coli* inoculum were air-dried in 12x35 mm borosilicate glass vials (Screw-capped glass vials, Fisher Scientific, Toronto, Canada) for 7 h in a biosafety hood. The vials were loosely closed, and equilibrated for 24 h at a a_w of 0.75. The vials were placed into Pyrex round media storage bottles (Corning Inc., NY, USA), which was immersed into an oil bath containing pre-heated canola oil (Crisco, JM Smucker, USA) at 110 °C for 5, 15 and 30 min (see Appendix). The temperature was checked using a thermometer to ensure that it was at 110°C prior to every run. After heating, sterile broth (1 mL) was added to the dried pellets, cells were re-suspended, then serially diluted and plated on TS agar plates. Plates were incubated aerobically at 37°C O/N prior to enumeration. All experiments were done in triplicate. Statistical analysis was performed using an unpaired T-Test.

2.2.8 Survival of air-dried strains exposed to dry heat at 110°C.

The same steps were taken as described above for preparation of the inoculum. Using a heat-resistant floating micro-centrifuge (1.5-2.0 mL) tube rack (Fisher Scientific, Toronto, Canada), the vials were exposed to direct heat at 110°C for 5 min. A 1 mL aliquot of sterile broth was added to the dried pellet to re-suspended cells, serially diluted and plated on TS agar plates.

Plates were incubated aerobically at 37°C O/N prior to enumeration. All experiments were done in triplicate. Statistical analysis was performed using an unpaired T-test.

3. Results

3.1 Heat resistance of *Salmonella* and *E. coli.*

To confirm the heat resistance of LHR positive strains, *S.* Senftenberg ATCC 43845, *S.* Typhimurium ATCC 13311, *E. coli* AW 1.7 and *E. coli* AW1.7ΔpHR1 were heated to 60°C for 5 min. The samples were plated onto TS agar plates, and incubated for 24 h prior to cell counts. *S.* Senftenberg and *E. coli* AW 1.7 were exceptionally resistant to heat (Figure 3), whereas *S.* Typhimurium and *E. coli* AW 1.7ΔpHR1 were not heat resistant.

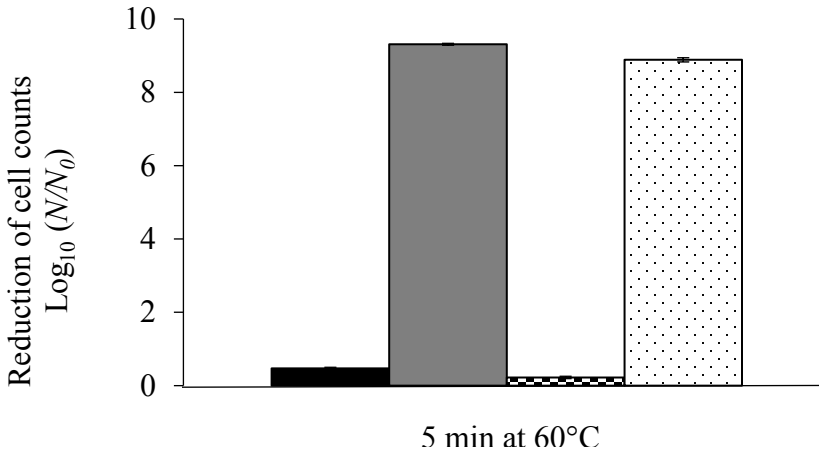

Figure 3: Reduction of cell counts after heating for 5 min at 60°C for *S.* Senftenberg ATCC 43845(LHR positive) (■) and *S.* Typhimurium ATCC 13311(LHR negative) (■), *E. coli* AW 1.7(LHR positive) (▩) and *E. coli* AW 1.7ΔpHR1(LHR negative) (□). Data are means ± standard deviation of the average of three replicates for each strain.

3.2 Survival of *Salmonella* spp. at various water activities.

Survival of *Salmonella* spp. in TS broth was measured after storage for up to 30 days. Bacterial lawns of wild-type *S.* Senftenberg and *S.* Typhimurium were washed and air-dried in micro-titer plates, and stored in a desiccator at varying humidity levels prior to sampling on TS agar plates. Figure 4 shows that storing LHR positive and LHR negative *Salmonella* at a a_w of less than 0.55 survival did not show a difference with presence or absence of the LHR in *Salmonella*. However, during storage in intermediate humidity conditions (a_w 0.75), cell counts of the wild-type LHR positive *S.* Senftenberg ATCC 43845 decreased faster than cell counts of the LHR negative *S.* Typhimurium ATCC 13311. Sampling of the *Salmonella* strains stored at a a_w of 0.75 was halted after day 10, as *S.* Senftenberg ATCC 43845 counts were below detection limit after 10 d of storage.

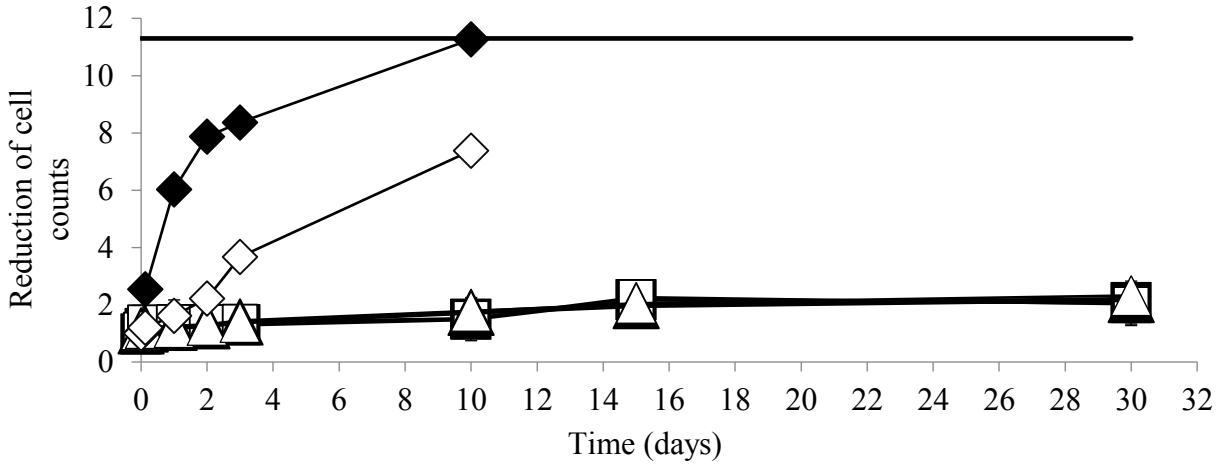

Figure 4: Survival of *Salmonella* Senftenberg ATCC 43845 and *Salmonella* Typhimurium ATCC 13311, dried in TS broth, after exposure to three a_w and storage for up to 30 d. *S.* Senftenberg ATCC 43845 and *S.* Typhimurium ATCC 13311 were air-dried for 5 h, then stored in an air-tight container using desiccants with varying water activities: ( ) ATCC 43845 at a_w 0.1; ( ) ATCC 13311 at a_w 0.1; ( ) ATCC 43845at a_w 0.55; ( ) ATCC 13311 at a_w 0.55; ( ) ATCC 43845at a_w 0.75; ( ) ATCC 13311at a_w 0.75. The ( ) represents the maximum limit for cell count reduction. Data are means ± standard deviation of the average of three replicates.

Survival of wild-type *Salmonella* spp. after drying in sterile 0.1% peptone and stored for up to 10 days was also assessed. Figure 5 shows the survival of *Salmonella* spp. that prepared with 0.1% sterile peptone as the drying medium. At a a_w of 0.75, the LHR positive *S.* Senftenberg ATCC 43845 and *S.* Typhimurium ATCC13311pLHR were less resistant to drying than the LHR negative *S.* Typhimurium ATCC 13311 and ATCC 13311pRK767. However, cell counts were reduced faster when compared to the same strains dried in a TS broth medium (Figure 4).

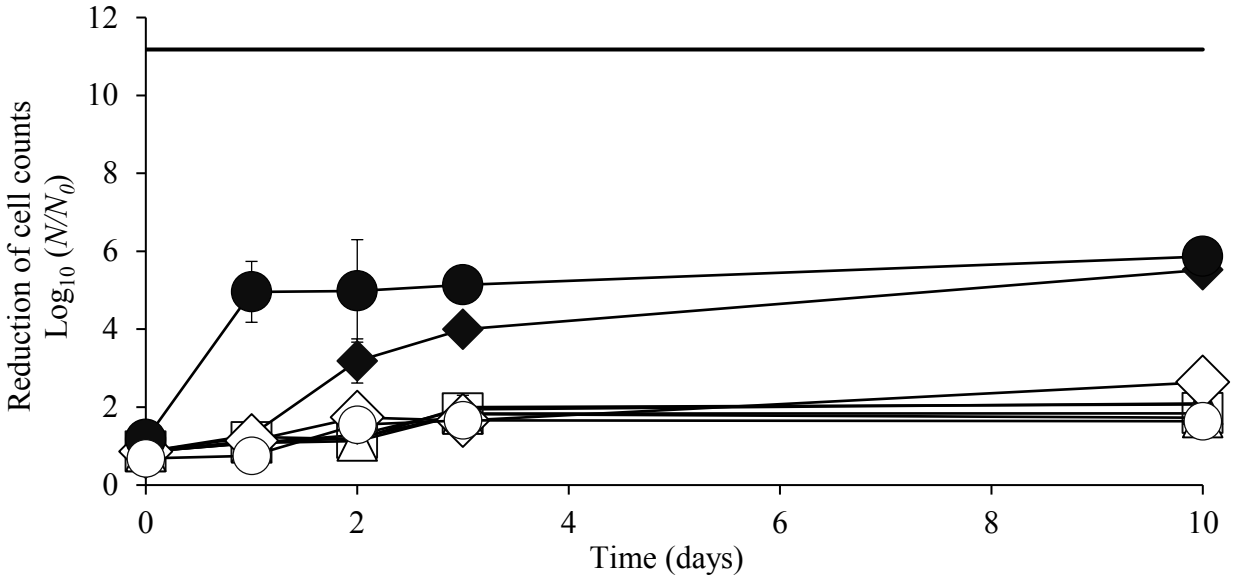

Figure 5: Survival of wild-type and isogenic *Salmonella* Senftenberg ATCC 43845 and *Salmonella* Typhimurium ATCC 13311, after exposure to three a_w and storage for up to 10 d. *Salmonella* spp. were air-dried in sterile 0.1% peptone for 5 h, then stored in an air-tight container using desiccants with varying water activities: (■) ATCC 43845 at a_w 0.1; (□) ATCC 13311 at a_w 0.1; (▲) ATCC 43845at a_w 0.55; (△) ATCC 13311 at a_w 0.55; (◆) ATCC 43845at a_w 0.75; (◇) ATCC 13311at a_w 0.75; (●) ATCC 13311pLHR; (○) ATCC 13311pRK767. The (—) represents the maximum limit for cell count reduction. Data are means ± standard deviation of the average of three replicates.

3.3 Survival of *Escherichia coli* spp. at different water activities.

Survival of wild-type *Escherichia coli* spp. was assessed after storage for up to 30 days. Concentrated inoculum of wild-type *E. coli* AW1.7 and *E. coli* AW1.7ΔpHR1 suspended in TS broth, were air-dried in micro-titer plates, and stored in a desiccator under varying humidity levels prior to sampling on TS agar. At a a_w of 0.75, cell counts of *E. coli* decreased irrespective of presence of the LHR (Figure 6).

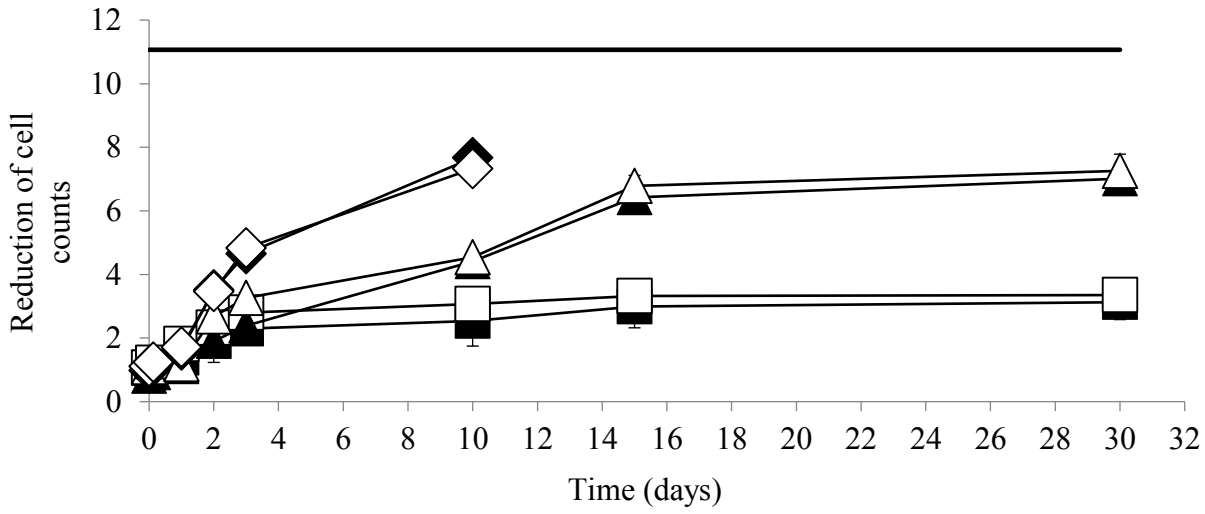

Figure 6: Survival of *E. coli* AW1.7 and *E. coli* AW1.7ΔpHR1, dried in TS broth, after exposure to three a_w and storage for up to 30 d. *E. coli* spp. were air-dried for 5 h, then stored in an air-tight container using desiccants with varying water activities: (—■—) AW1.7 at a_w 0.1, (—□—) AW1.7ΔpHR1 at a_w 0.1, (—▲—) AW1.7 at a_w 0.55, (—△—) AW1.7ΔpHR1 at a_w 0.55, (—◆—) AW1.7 at a_w 0.75, (—◇—) AW1.7ΔpHR1 at a_w 0.75. The (—) represents the maximum limit for cell count reduction. Data are means ± standard deviation of the average of three replicates.

LHR positive and negative *E. coli* strains dried in sterile 0.1% peptone medium was also assessed. Figure 7 shows that cell counts of both LHR positive and negative strains had no greater than 3-log reduction at a a_w of 0.55 or greater for up to 10 d. However, strains stored at a

a_w of 0.1 died faster between 2 and 3 d of storage. Further sampling of the *E. coli* strains stored at the varying water activities was stopped after 10 d of storage.

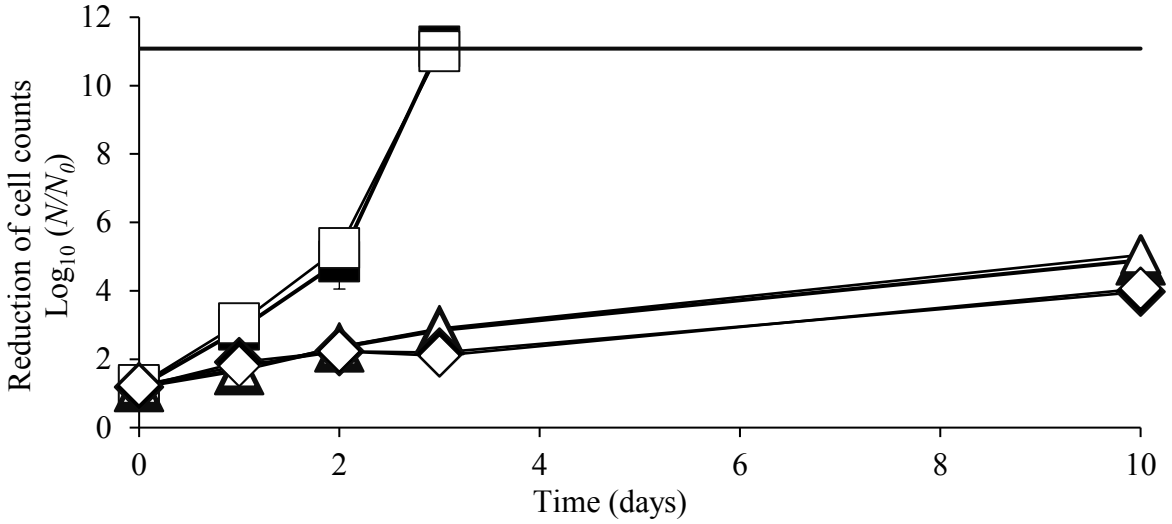

Figure 7: Survival of *E. coli* AW1.7 and *E. coli* AW1.7ΔpHR1, dried in sterile 0.1% peptone, after exposure to three a_w and storage for up to 10 d. *E. coli* spp. were air-dried for 5 h, then stored in an air-tight container using desiccants with varying water activities: ( ) AW1.7 at a_w 0.1, ( ) AW1.7ΔpHR1 at a_w 0.1, ( ) AW1.7 at a_w 0.55, ( ) AW1.7ΔpHR1 at a_w 0.55, ( ) AW1.7 at a_w0.75, ( ) AW1.7ΔpHR1 at a_w0.75. The ( ) represents the maximum limit for cell count reduction. Data are means ± standard deviation of the average of three replicates.

3.4 Deletion of the LHR from wild-type LHR positive, *S*. Senftenberg ATCC 43845.

To confirm the deleterious effect of the LHR, deletion of the wild-type LHR positive *S.* Senftenberg ATCC 43845 was attempted using the same curing protocol which was effective in deletion of the LHR from *E. coli* AW1.7 (Pleitner et al., 2012; Mercer et al., 2015). The protocol failed to generate LHR-negative derivatives of *S.* Senftenberg as the PCR results indicated presence of the LHR. Several other attempts also proved to be ineffective; the sub-culturing in increased temperatures and SDS concentrations did not induce a deletion of the LHR from *S.*

Senftenberg ATCC 43845. Therefore, an isogenic strain of *S.* Typhimurium ATCC 13311, generated from another study (Mercer et al., 2017) was used instead, to assess the effect of the LHR on the survival of dried *Salmonella* spp.

3.5 Survival of wild-type and isogenic *Salmonella* Typhimurium after exposure to a_w of 0.75.

Comparison of the wild-type and isogenic *Salmonella* strains was performed to confirm the deleterious effect of the LHR during storage under dry conditions. The inoculum was prepared as previously described, and air-dried in micro-titer plates. Sampling was performed for 3 d, as counts for the LHR positive wild-type and isogenic strains were below detection limit after 3 d of incubation at 37°C. The wild-type LHR positive *S.* Senftenberg had a very similar survival pattern compared to the isogenic *S.* Typhimurium ATCC 13311pLHR (Figure 8). Similarly, the wild-type *S.* Typhimurium ATCC 13311 survives the drying treatment just as well as the isogenic strain with the empty plasmid *S.* Typhimurium ATCC 13311pRK767. This confirms that the LHR is responsible for the poor survival of LHR positive *Salmonella* spp. after drying and storage in intermediate humidity conditions.

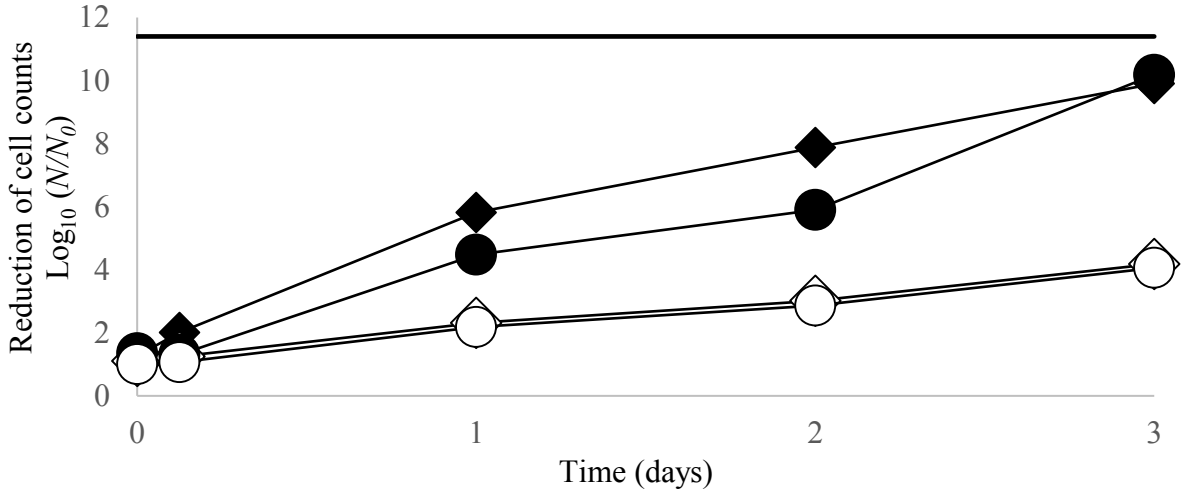

Figure 8: Reduction of cell counts of *S*. Senftenberg ATCC 43845 and *S*. Typhimurium ATCC 13311, *S*. Typhimurium ATCC 13311pLHR, and *S*. Typhimurium ATCC 13311pRK767, after drying and storage for up to 3 d. *Salmonella* strains were air-dried for 5 h in TS broth, then stored in a desiccator at a water activity of 0.75, for up to 3 days: (—◆—) ATCC 43845, (—◇—) ATCC 13311, (—●—) ATCC 13311pLHR, (—○—) ATCC 13311pRK767. The (——) represents the maximum limit for cell count reduction. Data are means ± standard deviation of the average of three replicates.

3.6 Transformation of pLHRΔ*kefB* into *S*. Typhimurium ATCC 13311.

Colony PCR was performed on all strains to confirm the presence of the LHR; results shown in Figure 9 indicate the presence, and lack of, the LHR in *S*. Typhimurium ATCC 13311pRK767 and *S*. Typhimurium ATCC 13311pLHR, respectively. Successful transformation of the modified LHR (pLHRΔ*kefB*) into the wild-type LHR negative *S*. Typhimurium is shown in Figures 10 and 11. Amplification of the LHR fragments of the wild-type *S*. Senftenberg ATCC 43845 and isogenic strains *S*. Typhimurium ATCC 13311pLHR and *S*. Typhimurium ATCC 13311pLHRΔ*kefB* are visible (Figure 11).

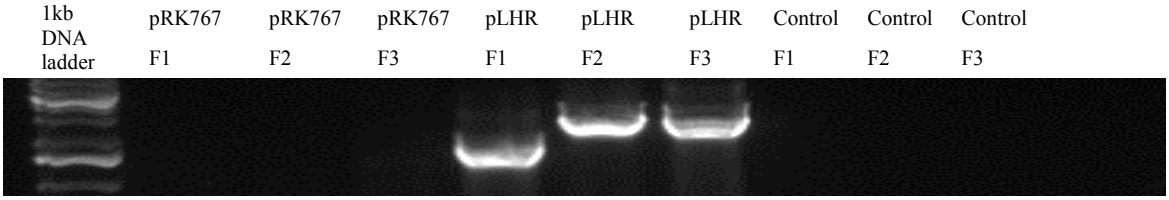

Figure 9: Gel electrophoretic analysis of PCR products recovered from a colony PCR of the isogenic strains, *S*. Typhimurium ATCC 13311pRK767 and *S*. Typhimurium ATCC 13311pLHR on a 1% agarose 0.5X TBE gel (120V for 1 h) with SYBRsafe staining. A 1kb+ ladder was used to identify the approximate size of the amplified bands. Lack of bands on the first three columns indicate absence of the LHR in ATCC 13311pRK767, followed by the amplified fragments F1, F2 and F3 of the LHR positive ATCC 13311pLHR.

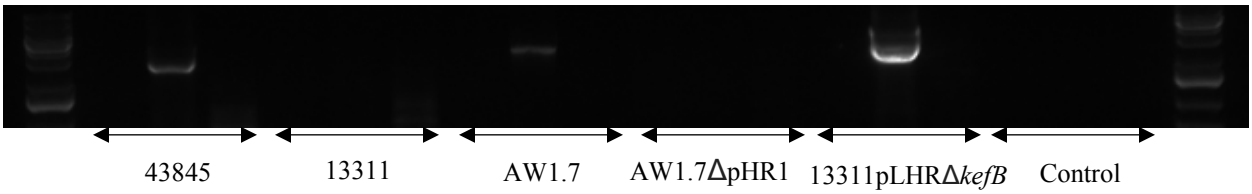

Figure 10: Gel electrophoretic analysis of PCR products obtained from amplifying fragments 1, 2 and 3 of the LHR in wild-type and isogenic *Salmonella* and *E. coli* strains. The image was obtained after loading the PCR products onto 1% agarose 0.5X TBE gel, and ran at 120V for 1 h with SYBRsafe staining. A 1kb+ ladder was used to identify the approximate size of the amplified bands. The first six columns on the gel indicate the wild-type LHR positive and LHR negative *S*. Senftenberg (43845) and *S*. Typhimurium (13311), respectively, where only the F2 band of *S*. Senftenberg showed the amplification. The next six sets are the wild-type *E. coli* AW 1.7 and its derivative, *E. coli* AW 1.7ΔpHR1 respectively, where only the F2 band of *E. coli* AW 1.7 showed the amplification. The last six columns represent *S*. Typhimurium ATCC13311pLHRΔ*kefB*, showing an over amplification of the F2 band, and the control containing the master-mix reagents only.

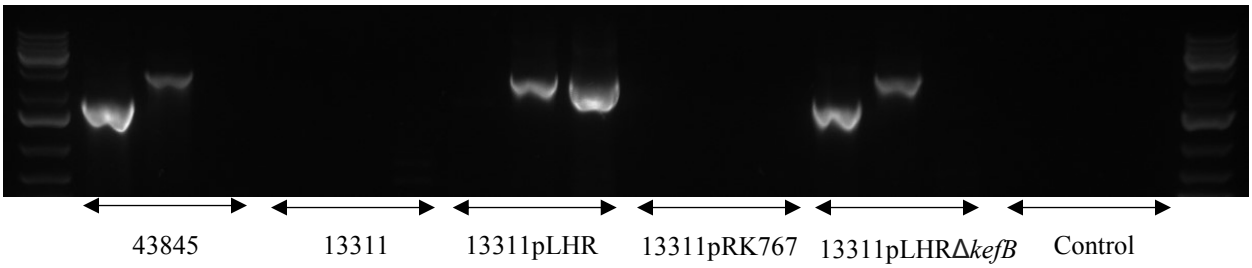

Figure 11: Gel electrophoretic analysis of PCR products obtained from amplifying fragments 1, 2 and 3 of the LHR in wild-type and isogenic *Salmonella* strains. The image was obtained from loading the PCR products onto 1% agarose 0.5X TBE gel, and ran at 120V for 1 h with SYBRsafe staining. A 1kb+ ladder was used to identify the approximate size of the amplified bands. The first six columns on the gel indicate the wild-type LHR positive ATCC 43845 (the F3 band was not properly amplified) and LHR negative ATCC 13311 respectively. The next six sets are the isogenic strains, *S.* Typhimurium ATCC13311pLHR (the F1 band was not properly amplified) and *S.* Typhimurium ATCC 13311pRK767 respectively. The last six columns represent the isogenic *S.* Typhimurium ATCC13311pLHRΔ*kefB* and the control containing the master-mix reagents only. A previous gel electrophoretic analysis of PCR products (data not shown) only identified fragments 2 and 3 in *S.* Typhimurium ATCC13311pLHRΔ*kefB*.

3.7 Survival of *S.* Typhimurium ATCC 13311pLHRΔ*kefB* after storage at aw of 0.75.

The survival of *S.* Typhimurium ATCC 13311pLHRΔ*kefB* under dry conditions was assessed after storage for 3 d. The inoculum for the new isogenic strain was prepared in 0.1% peptone, together with the other two wild-type and isogenic *Salmonella* strains. As shown in Figure 12, *S.* Typhimurium ATCC13311pLHRΔ*kefB* survives better than the LHR positive *S.* Typhimurium ATCC13311pLHR, but dies faster than the LHR negative *S. enterica* ATCC13311pRK767. This indicates that the absence of *kefB* on the LHR enables better survival than the full operon during storage at a_w of 0.75 for at least three days. Assessing the survival of the *S.* Typhimurium ATCC13311pLHRΔ*kefB* in 0.1% peptone salt was also attempted but, due to time constraints, was not completed. The preliminary data (data not shown) indicated that presence of NaCl in the drying medium decreased the survival in comparison to the strain dried

in 0.1% peptone. The potassium/hydrogen antiporter therefore, might have an important role in survival and storage of LHR positive dried *Salmonella* spp.

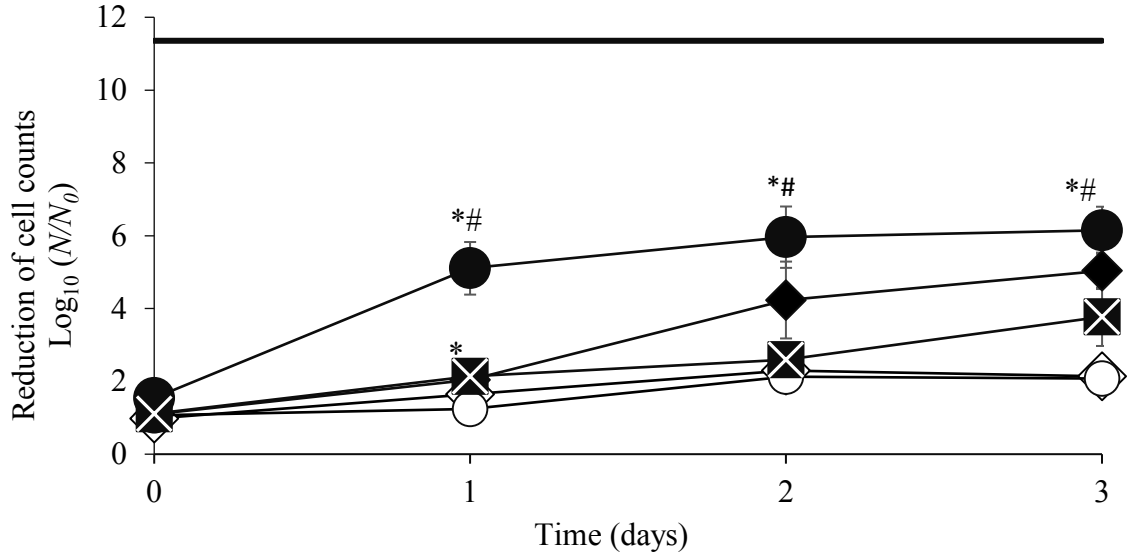

Figure 12: Reduction of cell counts of wild-type *Salmonella* spp. (*S*. Senftenberg ATCC 43845 and *S*. Typhimurium ATCC 13311) and LHR-inserted strains (*S.* Typhimurium ATCC 13311pLHR, *S*. Typhimurium ATCC 13311pLHRΔ*kefB* and *S.* Typhimurium ATCC 13311pRK767) after drying and storage for up to 3 d. *Salmonella* strains were air-dried for 5 h in 0.1% peptone, then stored in a desiccator at a water activity of 0.75, for up to 3 days: (—◆—) ATCC 43845, (—◇—) ATCC 13311, (—●—) ATCC 13311pLHR, (—⊠—) ATCC 13311pLHRΔ*kefB*, (—○—) ATCC 13311pRK767. The (—) represents the maximum limit for cell count reduction. Error bars represent the ± standard deviation of the average of three replicates for each strain at the specified a_w. Statistically significant ($P<0.001$) differences between isogenic LHR-positive and LHR-negative strains of the same species are indicated by an asterisk (*). Statistically significant ($P<0.001$) differences between isogenic strains carrying pLHR or pLHRΔ*kefB* are indicated by a number sign (#).

3.8 Preliminary assessment of heat resistance of dried *Salmonella* and *E. coli* strains.

Air-dried inocula were heated to determine how drying affected the heat resistance of the LHR positive strains. The air-dried inocula were stored at a a_w of 0.1, 0.55 and 0.75 prior to thermal treatment. Initial experiments were performed with strains of *Salmonella* and *E. coli* that were air-dried in glass vials and placed in Pyrex bottles. At a a_w of less than 0.75, no change in survival of both *Salmonella* and *E. coli* was observed after 5 min of heating, irrespective of the presence of the LHR (Figure 13). However, after storage at a a_w of 0.75, an increase in the cell count reduction for LHR positive for both wild-type and isogenic *Salmonella* strains was observed. Figure 13 also illustrated a reduction in cell counts after storage and heating for 15 min, which indicates that *E. coli* spp. can survive storage at a a_w of <0.55, irrespective of the LHR. Both wild-type and isogenic *Salmonella* strains had noticeable differences in reduction in cell counts when comparing the presence or absence of the LHR as the a_w increased from 0.1 to 0.55 after heating for 15 min. Exposure to 110°C greatly reduced the survival of LHR positive wild-type and isogenic strains of *Salmonella* and *E. coli* equilibrated at a a_w of 0.75. Based on the results, comparison of LHR positive to LHR negative *Salmonella* strains was more pronounced for the samples equilibrated at a_w 0.55 prior to heating, which confirmed that equilibration at an intermediate a_w inhibits the protective effects of the LHR during heat stress in *S.* Senftenberg ATCC 43845. No visible difference was observed from the *E. coli* strains, which indicated that air-drying and equilibration at varying water activities increases strain sensitivity irrespective of the LHR, in comparison to *Salmonella* spp.

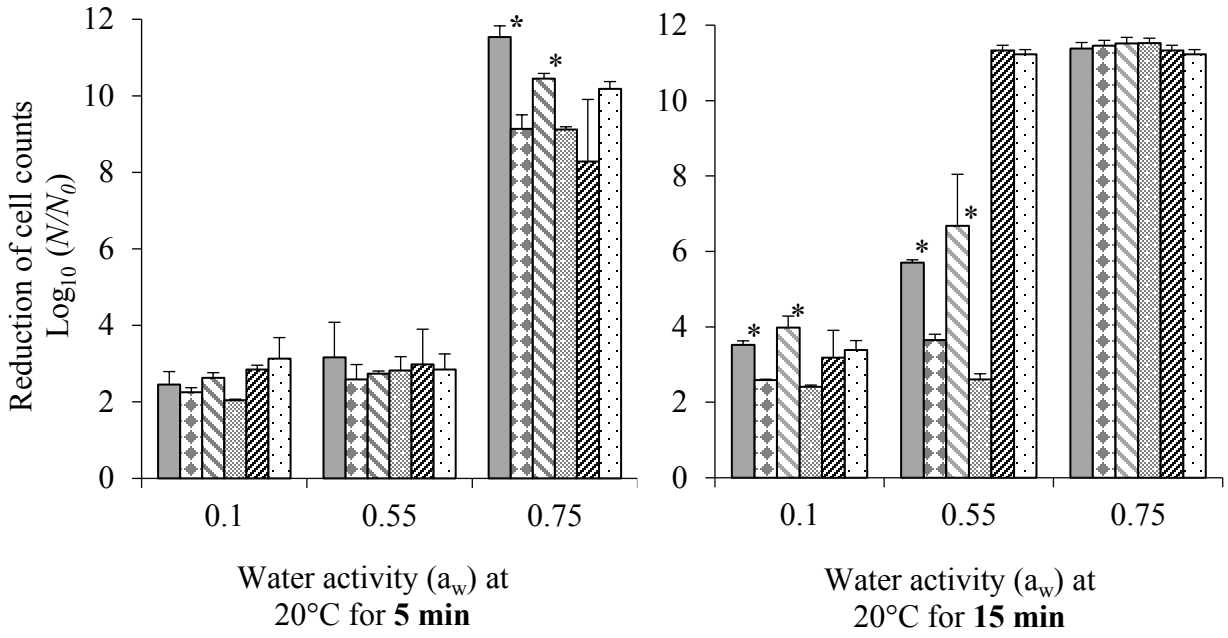

Figure 13: Combined graphs of the reduction in cell counts of LHR positive and LHR negative wild-type (*S.* Senftenberg ATCC 43845 and *S.* Typhimurium ATCC 13311) and isogenic (*S.* Typhimurium ATCC 13311pLHR, *S.* Typhimurium ATCC13311pLHRΔ*kefB* and *S.* Typhimurium ATCC 13311pRK767) *Salmonella* and *E. coli* strains after storage at various a_w and heat treated for 5 and 15 min. Strains were air dried for 5 h in glass vials prior to storage in modified environments simulating an a_w of 0.1, 0.55 and 0.75, for 24 h: (■) ATCC 43845, (▦) ATCC 13311, (▧) ATCC13311pLHR, (▩) ATCC 13311pRK767, (▨) *E. coli* AW1.7, (▤) *E. coli* AW1.7ΔpHR1. The log reduction of the air-dried, non-heat treated cells was approximately 1.8 log(CFU/g), and no significant difference was observed among strains. Vials were transferred into pyrex bottles, then exposed to 110°C for 5 and 15 min in an oil bath containing pre-heated canola oil. Data are means ± standard deviation of the average of three replicates for each strain at the specified a_w. **S**tatistically significant ($P<0.05$) differences between LHR-positive and LHR-negative strains of the same species are indicated by an asterisk (*).

3.9 Heat resistance of dried *Salmonella* and *E. coli* at 110°C

As the preliminary assessment confirmed that equilibration at a a_w of 0.55 results in the greatest difference between LHR positive and LHR negative *Salmonella* strains, a_w of 0.55 was selected to assess the effect of exposing *Salmonella* strains to 110°C for 5 min. The data shown in Figure 14 confirmed that while heating indirectly using a Pyrex bottle required 15 min of heating to observe a difference between the LHR positive and LHR negative *Salmonella*;

however, only 5 min was required if the glass vials were in direct contact with the pre-heated canola oil. This set of experiments also confirms that air-drying and equilibration at low a_w inhibits the heat resistance properties that the LHR exhibits in LHR positive *Salmonella enterica*.

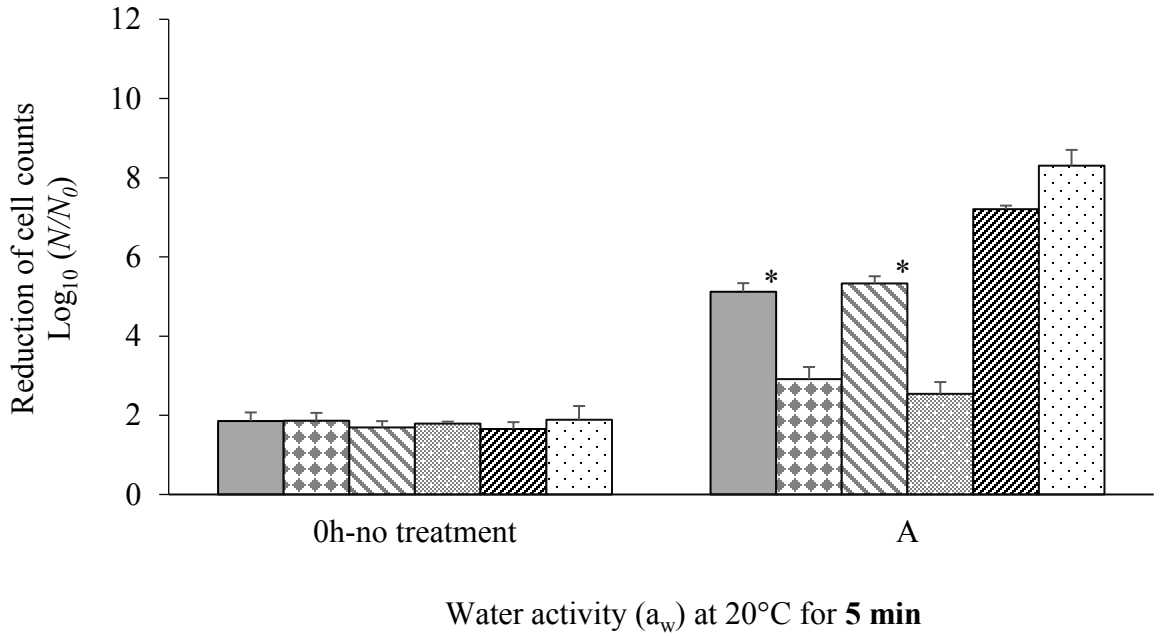

Figure 14: Heating of air-dried wild-type and isogenic *Salmonella* and *E. coli* spp., equilibrated at a a_w of 0.55 for 24 h in glass vials at 110ºC for 5 min: (■) ATCC 43845, (▦) ATCC 13311, (▨) ATCC13311pLHR, (▩) ATCC 13311pRK767, (▨) *E. coli* AW1.7, (▩) *E. coli* AW1.7ΔpHR1. Data are means ± standard deviation of the average of three replicates for each strain at the specified a_w. **S**tatistically significant ($P<0.05$) differences between LHR-positive and LHR-negative strains of the same species are indicated by an asterisk (*).

Discussion

The aim of this study was to identify the role of the LHR in desiccation survival of *Salmonella enterica* (*S.* Senftenberg and *S.* Typhimurium), in comparison to *E. coli* (*E. coli* AW1.7 and *E. coli* AW1.7ΔpHR1) in a controlled a_w environment. Additionally, the effect of desiccation on LHR-mediated high heat resistance was evaluated. The protocol that eliminated

the LHR from *E. coli* AW1.7 did not eliminate it from *S*. Senftenberg ATCC 43845. The LHR is situated on plasmids in *Cronobacter sakazakii* (Gajdosova et al., 2011) and *Klebsiella pneumoniae* (Bojer et al., 2010); however, it is most likely chromosomally included in *Salmonella*. Therefore, to confirm the role of the LHR during dry storage and subsequent heat stress, and to assess the role of *kefB* on the LHR, isogenic strains of *S*. Typhimurium, i.e. the LHR positive; *S*. Typhimurium ATCC 13311pLHR, *S*. Typhimurium ATCC 13311pLHRΔ*kefB* and the LHR negative *S*. Typhimurium ATCC 13311pRK767 were generated and included in the experiments.

4.1 Influence of the drying matrix during desiccation

Salmonellosis outbreaks from low a_w products confirm that the low water content of a product decreases the effectiveness of thermal treatments, and that an infectious dose much lower than 10^6 CFU/g (Todd et al., 2008) from different *Salmonella* serovars can provoke an infection (Archer et al., 1998, Doyle & Mazzotti, 2000). It is speculated that the nature of the food, such as a low a_w and high fat content, can protect the cells from the harsh environment in the stomach. *Salmonella* inoculated in high fat peanut butter survived better when exposed to a simulated gastro-intestinal system, which might contribute to the low infective doses observed in low a_w foods (Aviles et al., 2013). This improved survival in the peanut butter matrix compared to control cells supports the theory that the low infectious doses from contaminated foods such as chocolate and peanut butter relates to improved survival in the gastrointestinal environment enhanced by food components. Furthermore, using a contaminated non-fat dry milk powder, increased the survival of *Salmonella* in a food model composed of 20% milk powder and 80% peanut butter, in comparison to using contaminated peanut butter instead, in the same model formulation (Li et al., 2014).

Research reported in this thesis indicated that presence of NaCl in the drying medium impedes survival of LHR positive *Salmonella enterica* stored at intermediate a_w conditions (a_w 0.75); which was also observed by Hiramatsu et al. (2005), who demonstrated that the presence of NaCl in the drying matrix lowered the survival of *Salmonella* on paper discs. While presence of salt in a growth medium is known to induce ionic and osmotic stress, sucrose induces only osmotic stress. Osmotic stress response mechanisms in *Salmonella* are dependent on the nature of the solute; a higher upregulation of the *kdpFABC* genes was observed for K^+ ion transport when cells were exposed to 0.3 M NaCl, in comparison to 0.6 M sucrose (Balaji et al., 2005). A higher survival of *Salmonella* was observed in a low a_w food and in a simulated desiccation model only if sucrose was present in the drying matrix (Hiramatsu et al., 2005). However, current knowledge about the role of carbohydrate in heat resistance of *Salmonella* is contradictory. Strains grown in a low carbohydrate medium have higher heat resistance (Ng et al., 1969), a combination of high carbohydrate and lower fat peanut butter (33% fat, 42% carbohydrates) induces heat resistance of *Salmonella* adapted to a_w of 0.8 (He et al., 2011) whereas low carbohydrate and high fat peanut butter (49% fat, 24% carbohydrates) lowered the heat resistance of *Salmonella* (He et al., 2013) exposed to 90^{o}C for 20 min.

In E. coli, while sucrose uptake was more beneficial for survival of dried *E. coli* K-12, *E. coli* NISSLE 1917 was more effective in using trehalose as an osmo-protectant during desiccation stress (Louis et al., 1994). Furthermore, the decrease in survival of dried LHR positive and negative *E. coli* in TS broth (a_w of 0.75) in comparison to dried *E. coli* in 0.1% peptone (a_w of 0.75) in this study, indicated that presence of NaCl and other solutes in TS broth enabled survival in extreme dry conditions, but absence of the solutes induced resistance as the storage a_w increased to 0.75. This is in accordance with the study conducted by He et al. (2011),

who reported a 5-log reduction of an air-dried five-strain cocktail of *E. coli* 0157:H7 stored in peanut butter (a_w of 0.4) with 0.25% NaCl, in comparison to a 3.2-log reduction in salt-free peanut butter. However, they used a strain cocktail, which unfortunately does not afford differentiation among strains or serotype specificity when exposed to both stresses.

4.2 Effect of a controlled a_w environment on stressed cells

Desiccation induces a loss of intracellular water, which triggers regulation of genes for the activation of a multitude of stress responses to inhibit further water loss. *Salmonella* can survive in low a_w foods for weeks, months, or years (Russo et al., 2013; Archer et al., 1998; Podolak et al., 2010). In *Salmonella* exposed to stress in low a_w foods, the upregulation of the *fadA* gene occurs which confirm that CFAs are involved during desiccation stress (Fong & Wang, 2016b; Li et al., 2012). CFA upregulation is known to induce resistance to heat, pressure and acid, but lowers the fluidity of the membrane making it more stable to environmental stresses (Chen & Gänzle, 2016). Lowering the fluidity of the membrane restricts the intracellular water from leaving the cell, enabling cell survival when exposed to low water conditions.

Strains in this study were dried in TS broth and in 0.1% peptone. Several studies have confirmed the survival of *Salmonella* on dry surfaces for extended times (Humphrey et al., 1995; Gruzdev et al., 2012b; Finn et al., 2013a), which is in agreement with the results of this study where survival over 30 days for both LHR positive and negative *Salmonella* was observed after storage at a a_w of less than 0.6 in either drying media. Similar to *Salmonella*, *Staphylococcus aureus* and *Cronobacter* spp. also survive low a_w conditions for extended times (Edelson-Mammel et al., 2005; Beuchat et al., 2013). The protective effect of TS broth on survival of dried *E. coli* at a_w of 0.1 observed in this study is in accordance with the protective effect of glucose observed by Louis et al. (1994) after air-drying *E. coli* K-12 in minimal media. They also

observed that air-drying (a_w of 0.1) induced better survival of *E. coli* K-12 in comparison to freeze-drying. It is possible that the presence of dextrose (D-glucose) in TS broth promotes biosynthesis of trehalose in *E. coli*, which increased the resistance to extreme desiccation observed in this study.

Data from this study confirmed that after equilibration at a_w of 0.75, heat inactivation of *Salmonella* required temperature that exceeds the temperatures used for pasteurization of liquid or moist foods irrespective of the LHR. This is in accordance with He et al. (2013), who observed an approximately 5-log reduction of *S.* Typhimurium, equilibrated to a_w of 0.8, after exposure to 90^{o}C for 20 min. However, poor survival of the wild-type and isogenic strains equilibrated at a_w of 0.55 confirmed that the LHR does have a more pronounced negative effect on survival of *Salmonella*. A potential explanation for this observation is that equilibration to lower a_w conditions trigger cellular responses that take away the ability of the cell to overcome subsequent high heat stress in favor of survival during desiccation stress.

4.3 Species variation in survival during desiccation

Thermal treatments such as pasteurization provide safe food products for consumption and extend storage life by lowering the microbial load of the products (Corantin et al., 2005; Ma et al., 2009). However, previous low a_w-related salmonellosis outbreaks have confirmed that thermal treatments are less effective when applied in low a_w food products (Table 1). While the majority of *Salmonella* serovars are rapidly killed at pasteurization temperatures, some, such as *Salmonella enterica* serovar Senftenberg ATCC 43845 express remarkable resistance to 60°C or higher in high a_w food products (Ng et al., 1969; Davidson et al., 1966; Kornacki & Marth, 1993); a resistance that was recently attributed to presence of the LHR (Mercer et al., 2017).

Stationary-phase grown *Cronobacter sakazakii* exhibit remarkable survival to heat stress (Gajdosova et al., 2011; Breeuwer et al., 2003), and this resistance is related to the presence of the LHR (Mercer et al., 2015). Furthermore, LHR negative *Cronobacter sakazakii* mutants exhibited a lowered tolerance to osmotic stress (Orieskova et al., 2013), which confirmed that not only does the LHR enable heat resistance, but in presence of the required solutes, the LHR can also improve survival during osmotic stress. As such, it was expected that LHR positive *Salmonella* and *E. coli* would display similar resistance to desiccation and subsequent dry heat exposure. NaCl-mediated heat resistance was demonstrated by Mattick et al., (2001), Pleitner et al., (2012), and Peña-Meléndez et al., (2014) in *Salmonella* and *E. coli*. An upregulation of the LHR genes was observed after osmotic stress induction and growth in a medium containing 1-4% NaCl induced higher heat resistance in LHR positive *E. coli* AW1.7 (Mercer et al., 2015; Pleitner et al., 2012). A significant increase in survival (D_{60} > 20min) of LHR positive *Salmonella* and *E. coli* is observed after exposure to a growth medium containing 4% NaCl (Mercer et al., 2017). However, data from this study indicates that presence of NaCl in the drying medium impedes survival of LHR positive *Salmonella enterica* stored at intermediate a_w conditions (a_w 0.75).

Although the heat resistance data in this study demonstrate a pronounced difference when comparing *Salmonella* to *E. coli*, Miller et al., (1972) observed no significant difference when comparing the heat resistance of strains of *Salmonella* to *E. coli* during spray-drying of milk. They observed a greater survival of *Salmonella* during spray-drying of milk powder with a final a_w of 0.6, compared to drying to a a_w of 0.3 (Miller et al., 1972), which differs from the results of this study that did not show any significant difference in survival of strains equilibrated at a_w 0.55 or at 0.1, irrespective of the LHR. However, milk powder is a much more nutrient-dense

drying matrix than TS broth, and the variations in solute uptake could be responsible for the contradictory results.

4.4 Species and strain variations in survival after desiccation and high heat stress

High heat treatments (110-140^{o}C) are used for applications such as oil roasting of tree nuts, or dry roasting of cocoa beans for chocolate making and it is important to note that dry heat exposure results in a more pronounced heat resistance in comparison to wet heat (Krapf & Gatenbein-Demarchi, 2010; Abd et al., 2012). Exposure to 127^{o}C in hot oil for 1.5 min effectively reduced cell counts of *S*. Enteritidis and *S*. Senftenberg 775W inoculated on almonds by 5-log while retaining the required sensory aspect of roasted almonds (Du et al., 2010). Treating pecans with hot air (120^{o}C) for 20 min only gave a 2-log reduction (Beuchat & Mann, 2011), thus indicating that the contact method used on nuts defined the effectiveness of the thermal treatment. The current study confirms that, after exposure to wet heat, LHR positive *Salmonella* and *E. coli* strains exhibit exceptional heat resistance in comparison to their LHR negative counterparts. Additional protein expression work identified *orf2* (small HSP C2) and *orf7* (HSP 20) as heat shock proteins and *orf3* (Clp protease), *orf15* (Zn-dependent protease) and *orf16* (DegP-like protease) as heat-activated proteases on the LHR (Li & Gänzle, 2016). While heat shock proteins enable folding of newly synthesized proteins and prevent protein unfolding and aggregation, proteases excise and remove mis-folded or damaged proteins to induce greater protein turnover during heat-induced stress.

However, exposure to dry heat significantly lowers the protective effect of the LHR in *Salmonella*. Air-drying induces a downregulation of *dnaK* (Fong & Wang, 2016a), and the fact that the LHR does not induce heat resistance under dry conditions, indicates that the genes

encoding heat chaperone proteins and proteases of the LHR may not be useful for survival of heat stressed LHR positive *Salmonella* exposed to and stored in dry conditions.

Exposure to 57^{o}C in wet conditions induces a higher heat resistance in *S.* Senftenberg than in *S.* Typhimurium (Ng et al., 1969), which is expected due to the presence of the LHR. Genes identified on the LHR prevent protein mis-folding and denaturation, membrane damage and prevents oxidative stress during wet heat exposure. The increased survival of dried *S.* Typhimurium over the LHR positive *S.* Senftenberg after exposure to 110^{o}C in this study, while unexpected, is not unique as others have reported survival of S. Typhimurium at low a_w. Kirby & Davies, (1990) demonstrated that storage of *S.* Typhimurium LT2 at a a_w of 0.57 in TS broth, greatly increased the heat resistance of the strain. Mattick et al. (2001) observed greater survival of air-dried *S.* Typhimurium (a_w of 0.65 or less) after exposure to > 70^{o}C, whereas exposure to 65^{o}C or lower induced cell death. However, it seems that presence of the LHR is more detrimental, than beneficial for dried *Salmonella* exposed to high heat stress, an observation that was further confirmed by exposing the LHR positive *S.* Typhimurium ATCC 13311pLHR to dry heat. This variation among organisms can also be explained by the uptake of osmo-protectants, which are known to play a crucial role during desiccation survival in *Salmonella* and *E. coli*. This might explain why *S.* Typhimurium survived better during storage and subsequent heat stress, than did *S.* Senftenberg. Presence of the LHR, while beneficial during wet heat exposure, might actually inhibit osmo-protectant uptake during adaptation to drying, which is beneficial for LHR negative strains such as *S.* Typhimurium. Geopfert & Biggie, (1968) also observed *S.* Typhimurium survived dry heating better than *S.* Senftenberg in melted chocolate (low a_w and high sucrose), irrespective of the fat content of the chocolate. It is possible that in their case, the sucrose content enabled higher survival during heat and desiccation stress. *Salmonella* desiccated

on paper discs also responded better to desiccation stress if sucrose was present in the drying matrix (Hiramatsu et al., 2005).

While *Salmonella* and *E. coli* show comparable heat resistance, they behave very differently when exposed to desiccation stress. The lack of difference between LHR positive and negative *E. coli* survival, was unexpected. However, since *E. coli* AW1.7ΔpHR1 is a heat sensitive derivative of *E. coli* AW1.7, it is possible that the mechanisms engaged for desiccation survival are separate from heat resistance mechanisms that the heat sensitive derivative "lost" during plasmid curing of *E. coli* AW1.7.

4.5 Effect of *kefB* on desiccation survival of *S.* Typhimurium ATCC 13311pLHRΔ*kefB*

Comparative genomic analysis identified *kefB* as a gene on the LHR, which encodes for a potassium/hydrogen antiporter (Mercer et al., 2015). The purpose of using the isogenic strain without *kefB* (*S.* Typhimurium ATCC 13311pLHRΔ*kefB*) was to determine the role of the K^+/H^+ antiporter during desiccation. The KefB gene encodes for a K^+ efflux system, which is activated in the presence of glutathione and an electrophile such as methylglyoxal or N-ethylmaleimide (Ferguson et al., 1997). While previous literature mention several predicted mechanisms utilized by *Salmonella* for desiccation survival (Finn et al., 2013a), none have assessed the possible role of the glutathione-gated K^+ efflux system. This system enables cell survival by switching intracellular K^+ for extracellular H^+ ions to induce acidification of the cytoplasm. Cytoplasmic acidification, in turn, neutralize electrophiles that are generated through the breakdown of sugars and other compounds and prevent cellular toxicity (MacLean et al., 1998). It is possible that the expulsion of intracellular K^+ ions increases extracellular $[K^+]$, which impedes the activation of

the Kdp-ATPase system during desiccation. Data collected with the isogenic *Salmonella* indicated that the antiporter contributes to survival during dry storage.

While lack of NaCl improves survival of LHR positive dried *Salmonella*, it is possible that the other LHR genes have an impact on survival during storage at intermediate water activity. Considering how the strains were dried in TS broth, there is also a possibility that other solutes present in TS broth might have contributed to that deleterious effect. However, had this experiment been repeated in 0.1% peptone salt instead of TS broth, a better comparison would have shed more light on the effect of the K^+/H^+ antiporter during desiccation stress without the presence of other solutes.

4.6 Conclusion

There are still a lot of unresolved questions about the mechanisms involved in survival of *Salmonella* in low a_w foods. Based on all the data from previous studies that have analyzed current pathogen-control processing steps in the industry, it would be worthwhile to reassess those steps in an effort to further reduce outbreak risks. As was presented in this study, *S.* Senftenberg, a highly heat resistant strain, is resistant to extreme desiccation but not to intermediate water activity storage conditions, while *S.* Typhimurium is more desiccation resistant than *S.* Senftenberg at intermediate water activity. Furthermore, equilibration at an intermediate water activity has a bactericidal effect on LHR-positive dried *Salmonella enterica* prior to dry heat exposure in the presence of sodium chloride and dextrose (D-glucose) in TS broth. This bactericidal effect however, seems to be species specific as no change was observed in *E. coli*. As such, the appropriate strain selection is crucial when planning validation studies.

It is also important to understand the mechanisms behind heat resistance in *Salmonella* from low a_w food products, and how low a_w interacts with resistance to heat in *Salmonella*.

Current pasteurization temperatures are ineffective when treating highly contaminated low a_w foods, and exposure to a longer or higher temperature does not increase the log reduction of the contaminated product. Once HSPs and proteases are generated, the heat resistant strains can withstand high heat exposure for an extended time, thereby rendering the thermal processes futile. As such, a reassessment of the infectious dose of *Salmonella* as well as current thermal processes, is required when dealing with contaminated low a_w food products. Moreover, strain adapted to drying and subsequent storage was performed in broth and peptone only; had this experiment also been repeated in a food matrix, it would have provided additional information on whether the LHR behaves any differently if strains were treated in a food matrix. Understanding the molecular mechanisms involved in *Salmonella* survival in low a_w stress is highly significant as it provides more in-depth understanding of how regulatory networks for gene expression in *Salmonella* engage when faced with stressful conditions. This knowledge can, in turn, be used to further improve current interventions for control of foodborne pathogens in low water activity products.

Bibliography

1. Abee T., Wouters JA. 1999. Microbial stress response in minimal processing. Int. J. Food Microbiol. 50:65-91.
2. Abd SJ., McCarthy KL., Harris LJ. 2012. Impact of storage time and temperature on thermal inactivation of *Salmonella* Enteritidis PT 30 on oil-roasted almonds. J. Food Sci. 77(1): 42-47.
3. Adinolfi S., Iannuzzi C., Prischi F., Pastore C., Iametti S., Martin SR., Bonomi F., Pastore A. 2009. Bacterial frataxin CyaY is the gatekeeper of iron-sulfur cluster formation catalyzed by IscS. Nat Struct Mol Biol. 16(4):390-396.
4. AIOE. 2012. Validating the reduction of *Salmonella* and other pathogens in heat processed low moisture foods. Retrieved 02/28 2017, from: http://ucfoodsafety.ucdavis.edu/files/224455.pdf
5. Alahari A., Ballal A., Apte SK. 2001. Regulation of the potassium-dependent Kdp-ATPase expression in the nitrogen- fixing cyanobacterium *Anabaena torulosa*. J. Bacteriol. 183: 5578–5581.
6. Almond Board of California (ABC). 2007. Guidelines for Validation of Oil Roasting Processes. Retrieved 02/28 2017 from: http://www.almonds.com/sites/default/files/content/attachments/oil-roast-validation-guidelines.pdf
7. Andino A., Hanning I. 2015. *Salmonella enterica*: Survival, colonization, and virulence differences among serovars. Sci. World J Vol. 520179.
8. Archer J., Jervis ET., Bird J., Gaze JE. 1998. Heat resistance of *Salmonella* Weltevreden in low-moisture environments. J. Food Prot. 61:969–973.

9. Aviles B., Klotz C., Smith T., Williams R., Ponder M. 2013. Survival of *Salmonella enterica* serotype Tennessee during simulated gastric passage is improved by low water activity and high fat content. J. Food. Prot. 76: 333-337.
10. Ayala-Castro C., Saini A., Outten FW. 2008. Fe-S cluster assembly pathways in bacteria. Microbiol. Mol. Bio. Rev. 72: 110–125.
11. Balaji B., O'Connor K., Lucas JR., AndersonJM., Csnka LN. 2005. Timing of induction of osmotically controlled genes in *Salmonella enterica* serovar Typhimurium, determined with quantitative real-time reverse transcription-PCR. Appl. Environ. Microbiol. 71: 8273-8283.
12. Ballal A., Bramkamp M., Rajaram H., Zimmann P., Apte SK., Altendorf K. 2005. An atypical KdpD homologue from the cyanobacterium *Anabaena* sp. strain L-31: cloning, in vivo expression and interaction with *Escherichia coli* KdpD-CTD. J. Bacteriol. 187: 4921–4927.
13. Ballal A., Basu B., Apte SK. 2007. The Kdp-ATPase system and its regulation. J. Biosci. 32: 559–568.
14. Barrile JC., Cone FJ. 1970. Effect of added moisture on the heat resistance of *Salmonella* Anatum in milk chocolate. Appl. Microbiol. 19: 177-178.
15. Beuchat LR., Komitopoulou E., Beckers H., Betts RP., Bourdichon F., Fanning S., Joosten HM., Ter Kuile BH J. 2013. Low-water activity foods: increased concern as vehicles of foodborne pathogens. Food Prot. 76: 150-172.
16. Beuchat LR., Mann DA. 2011. Inactivation of *Salmonella* on pecan nutmeats by hot air treatment and oil roasting. J. Food Prot. 74: 1441-1450.

17. Blaser MJ., Newman LS. 1982. A review of human salmonellosis. I. Infective Dose. Rev. Infect. Dis. 4: 1096-1106.

18. Bojer MS., Struve C., Ingmer H., Hansen DS., Krogfelt KA. 2010. Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of *Klebsiella pneumoniae*. PLoS ONE. 5: e15467.

19. Breeuwer P., Lardeau A., Peterz M., Joosten HM. 2003. Desiccation and heat tolerance of *Enterobacter sakazakii*. J. Appl. Microbiol. 95: 967–973.

20. Bremer E., Krämer R. 2000. Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes, in Bacterial Stress Responses. Storz G., and Hengge-Aronis R. Washington, DC: ASM Press. Eds: 79–97.

21. Brown AD. 1990. Microbial water stress physiology: principles and perspectives. John Wiley & Sons, Ltd., Chichester, United Kingdom.

22. Calhoun CL., Frazier WC. 1966. Effect of available water on thermal resistance of three non-sporeforming species of bacteria. Appl. Microbiol. 14: 416-420.

23. Canovas D., Fletcher SA., Hayashi M., Csonka LN. 2001. Role of trehalose in growth at high temperature of *Salmonella enterica* serovar Typhimurium. Am. Soc. Microbiol. 183: 3365-3371.

24. Center for Disease Control and Prevention (CDC). 2013. Foodborne outbreak online database (FOOD). Retrieved 02/28 2017, from: http://www.cdc.gov/foodborneoutbreaks/Default.aspx

25. Center for Disease Control and Prevention (CDC). 2016. Reports of *Salmonella* outbreaks investigations. Retrieved 02/28 2017, from: http://www.cdc.gov/*salmonella*/outbreaks.html

26. Chen Y., Freier T., Kuehm., Moorman M., Scott J., Meyer J., Morille-Hinds T., Post L., Smoot., Hood S., Shebuski J., Banks J., GMA *Salmonella* control task force. 2009. Control of *Salmonella* in Low-moisture foods. Retrieved 02/28 2017, from http://www.gmaonline.org/downloads/technical-guidance-and-tools/SalmonellaControlGuidance.pdf

27. Chen YY., Gänzle MG. 2016. Influence of cyclopropane fatty acids on heat, high pressure, acid and oxidative resistance in *Escherichia Coli*. Int. J. Food Microbio. 222: 16–22.

28. Chen Z., Diao J., Dharmasena M., Ionita C., Jiang X., Rieck J. 2013. Thermal inactivation of desiccation-adapted *Salmonella* spp. in aged chicken litter. Appl Environ Microbiol. 79: 7013-7020.

29. Corantin H., Quessy S., Gaucher ML., Lessard L., Leblanc D., Houde A. 2005. Effectiveness of steam pasteurization in controlling microbiological hazards of cull cow carcasses in a commercial plant. Can. J. Vet. Res. 69:200-207.

30. Csonka LN., Epstein W. Osmoregulation. 1996. Neidhardt FC. (Ed.), *Escherichia coli* and *Salmonella* cellular and molecular biology. ASM Press, Washington DC.

31. Danyluk MD., Uesugi AR., Harris LJ. 2005. Survival of *Salmonella* Enteritidis PT 30 on inoculated almonds after commercial fumigation with propylene oxide. J. Food Prot. 68: 1613–1622.

32. Davidson CM., Boothroyd M., Georgala DL. 1966. Thermal resistance of *Salmonella* Senftenberg. Nature. 212: 1060-1061.

33. Deng X., Li Z., Zhang W. 2012. Transcriptome sequencing of *Salmonella* serovar Enteritidis under desiccation and starvation stress in peanut oil. Food Microbiol. 30: 311-315.

34. Ding H., Clark RJ, Ding B. 2004. IscA mediates iron delivery for assembly of iron-sulfur clusters in IscU under the limited accessible free iron conditions, in Stress and environmental regulation of gene expression and adaptation in bacteria. 2016. De Bruijn FJ. John Wiley & Sons, Ltd., New Jersey, USA.

35. Dlusskaya EA., McMullen LM., Gänzle MG. 2011. Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. J. Appl. Microbiol. 110: 840-849.

36. Dodd CE., Aldsworth TG. 2002. The importance of RpoS in the survival of bacterial through food processing. Int. J. Food. Microbiol. 74: 189-194.

37. Doyle E., Mazzotti AS. 2000. Review of studies on the thermal resistance of salmonellae. J. Food Prot. 63: 779-795.

38. Du W., Abd SJ., McCarthy LJ., Harris LJ. 2010. Reduction of *Salmonella* on inoculated almonds exposed to hot oil. J. Food Prot. 73: 1238-1246.

39. Edelson-Mammel SG., Porteous MK., Buchanan RL. 2005. Survival of *Enterobacter sakazakii* in a dehydrated powdered infant formula. J. Food Prot. 68: 1900–1902.

40. FAO/WHO. 2015. Code of hygienic practice for low moisture foods. Retrieved 02/28 2017, from www.fao.org/input/download/standards/13921/CXP_075e_2015.pdf

41. Ferguson GP., Nikolaev Y., McLaggen D., Maclean M., Booth IR. 1997. Survival during Exposure to the Electrophilic Reagent N-Ethylmaleimide in *Escherichia coli*: Role of KefB and KefC Potassium Channels. J. Bacteiol. 179: 1007-1012.

42. Finn S., Orla Condell O., McClure P., Amézquita A., Fanning S. 2013a. Mechanisms of survival, responses, and sources of *Salmonella* in low-moisture environments. Front. Microbiol. 4:331.

43. Finn S., Händler K., Condell B, Colgan A., Cooney S., McClure P., Amézquita A., Jay C., Hinton B., Fanning S. 2013b. ProP Is required for the survival of desiccated *Salmonella enterica* serovar Typhimurium cells on a stainless-steel surface. Appl. Environ. Microbiol. 79: 4376–4384.

44. Fong K., & Wang S. 2016a. Heat resistance of *Salmonella enterica* is increased by pre-adaptation to peanut oil or sub-lethal heat exposure. Food Microbiol. 58: 139-147.

45. Fong K., Wang S. 2016b. Strain-specific survival of *Salmonella* enterica in peanut oil, peanut shell and chia seeds. J. Food. Prot. 79: 361-368.

46. Gajdosova J, Benedikovicova K, Kamodyova N, Tothova L, Kaclikova E, Stuchlik S, Turna J, Drahovska H. 2011. Analysis of the DNA region mediating increased thermotolerance at 58^{o}C in *Cronobacter* spp. and other enterobacterial strains. Antonie Van Leeuwenhoek. 100: 279-289.

47. Gill ON., Sockett PN., Bartlett CL., Vaile MS., Rowe MS., Rowe B., Gilbert RJ., Dulake C., Murell HC., Salmaso S. 1983. Outbreak of *Salmonella* Napoli infection caused by contaminated chocolate bars. Lancet. 321: 574-577.

48. Goepfert JM., Biggie RA. 1968. Heat resistance of *Salmonella* Typhimurium and *Salmonella* Senftenberg 775W in milk chocolate. Appl. Microbiol. 16: 1939–1940.

49. Goepfert JM., Iskander IK., Amundson CH. 1970. Relation of the heat resistance of salmonellae to the water activity of the environment. Appl. Microbiol. 19: 429–433.

50. Gralnick J., Downs D. 2001. Protection from superoxide damage associated with an increased level of the YggX protein in *Salmonella enterica*. Proc. Natl. Acad. Sci. 98**:** 8030-8035.

51. Gruzdev N., McClelland M., Porwollik S., Ofaim S., Pinto R., Saldinger-Sela S. 2012a. Global transcriptional analysis of dehydrated *Salmonella enterica* serovar Typhimurium. Appl. Environ. Microbiol. 72: 7866-7875.

52. Gruzdev N., Pinto R., Saldinger-Sela S. 2012b. Persistence of *Salmonella enterica* during dehydration and subsequent cold storage. Food Microbiol. 32: 415–422.

53. Gruzdev N., Pinto R., Sela S. 2011. Effect of desiccation on tolerance of *Salmonella enterica* to multiple stresses. Appl. Environ. Microbiol. 77: 1667–1673.

54. He YS., Guo DJ., Yang JY., Tortorello ML., Zhang W. 2011. Survival and heat resistance of *Salmonella enterica* and *Escherichia coli* O157:H7 in peanut butter. Appl. Environ. Microbiol. 77: 8434–8438.

55. He YS., Li Y., Salazar JK., Yang JY., Tortorello ML., Zhang W. 2013. Increased water activity reduces the thermal resistance of *Salmonella enterica* in peanut butter. Appl. Environ. Microbiol. 79: 4763-4767.

56. Hengge-Aronis R., Lange R., Henneberg N., Fischer D. 1993. Osmotic regulation of *rpoS*-dependent genes in *Escherichia coli*. J. Bacteriol. 175: 259– 265.

57. Hiramatsu R., Matsumoto M., Sakae K., Miyazaki Y. 2005. Ability of shiga toxin producing *Escherichia coli* and *Salmonella* spp. to survive in a desiccation model system and in dry foods. Appl. Environ. Microbiol. 71: 6657-6663.

58. Hiratsu K, Amemura M, Nashimoto H, Shinagawa H, Makino K. 1995. The rpoE gene of *Escherichia coli*, which encodes sigma E, is essential for bacterial growth at high temperature. J. Bacteriol. 177: 2918–2922.

59. Health Canada. 2016. Yearly foodborne illness estimates from Canada. Retrieved 02/28 2017, from: http://healthycanadians.gc.ca/eating-nutrition/risks-recalls-rappels-risques/surveillance/illness-estimates-estimations-maladies/yearly-annuel-eng.php

60. Huet G., Daffe M., Saves I. 2005. Identification of the *Mycobacterium tuberculosis* SUF machinery as the exclusive mycobacterial system of [Fe-S] cluster assembly: evidence for its implication in the pathogen's survival. J. Bacteriol. 187: 6137–6146.

61. Humphrey TJ., Slater E., McAlpine K., Rowbury RJ., Gilbert RJ. 1995. *Salmonella* Enteritidis phage type 4 isolates more tolerant of heat, acid, or hydrogen peroxide also survive longer on surfaces. Appl. Environ. Microbiol. 61: 3161–3164.

62. International Commission on Microbiological Specification for Foods (ICMSF). 1996. Microorganisms in foods. Roberts TA, Baird-Parker AC, Tompkin RB, eds. Characteristics of microbial pathogens. London: Blackie Academic & Professional. 5: 513.

63. Iversen C., Forysthe S. 2004. Isolation of *Enterobacter sakazakii*, and other *Enterobacteriaceae* from powdered infant formula milk and related products. Food Microbiol. 21: 771–776.

64. Jovanovich SB., Martinelli M, Record Jr. MT., Burgess RB. 1988. Rapid response to osmotic upshift by osmo-regulated genes in *Escherichia coli* and *Salmonella* Typhimurium. J. Bacteriol. 170: 534-539.

65. Kempf B., Bremer E. 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch. Microbiol. 170: 319–330.

66. Kieboom J., Kusumaningrum HD., Tempelaars MH., Hazeleger WC., Abee T., Beumer RR. 2006. Survival, elongation, and elevated tolerance of *Salmonella enterica* serovar Enteritidis at reduced water activity. J. Food Prot. 69: 2681-2686.

67. Kiley PJ., Beinert H. 2003. The role of Fe-S proteins in sensing and regulation in bacteria. Curr. Opin. Microbiol. 6: 181–185.

68. Kim BH., Bang IS., Lee SY., Hong SK., Bang SH., Lee IS., Park YK. 2001. Expression of cspH, encoding the cold shock protein in *Salmonella enterica* serovar Typhimurium UK-1. J. Bacteriol. 183: 5580–5588.

69. Kirby RM., Davies R. 1990. Survival of dehydrated cells of *Salmonella* Typhimurium LT2 at high temperatures. J. Appl. Bacteriol. 68: 241–246.

70. Kornacki JL., Marth EM. 1993. Thermal inactivation of *Salmonella* Senftenberg and *Micrococcus freudenreichii* in retentates from ultra-filtered milks. LWT Food Sci. Tech. 26 : 21–27.

71. Krapf T., Gantenbein-Demarchi C. 2010. Thermal inactivation of *Salmonella* spp. during conching. LWT Food Sci. Tech. 43: 720-723.

72. Laimins LA., Rhoads DB., Epstein W. 1981. Genetics Osmotic control of Kdp operon expression in *Escherichia coli*. Proc. Natl. Acad. Sci. 78:464-468.

73. Lauhon C., Kambampati R. 2000. The iscS gene in *Escherichia coli* is required for the biosynthesis of 4-thiouridine, thiamin, and NAD. J. Biol. Chem. 275: 20096–20103.

74. Lee BH., Kermashal S., Baker BE. 1989. Thermal, ultrasonic and ultraviolet inactivation of *Salmonella* in thin films of aqueous media and chocolate. Food Microbiol. 6: 143-152.

75. Li H., Bhaskara A., Megalis C., Tortorello ML. 2012. Transcriptomic analysis of *Salmonella* desiccation resistance. Foodborne Patho. Dis. 9: 1143-1151.

76. Li H., Fu X., Bima Y., Koontz J., Megalis C., Yang F., Fleischman G, Tortorello ML. 2014. Effect of the local microenvironment on survival and thermal inactivation of *Salmonella* in low- and intermediate-moisture multi-ingredient foods. J. Food Prot. 77:67-74.

77. Li H. & Gänzle MG. 2016. Some like it hot: Heat resistance of *Escherichia coli* in Food. Front. Microbiol. 7: 1733.

78. Little CL., Jemmot W., Surnam-Lee SM., Hucklesby L., De Pinna E. 2009. Assessment of the microbiological safety of edible roasted nut kernels on retail sale in England, with a focus on *Salmonella*. J. Food. Prot. 72: 853-855.

79. Liu TS., Snoeyenbos GH., Carlson VL. 1969. Thermal resistance of *Salmonella* Senftenberg 775W in dry animal feeds. Avian Diseases. 13: 611-631.

80. Louis P., Triper HG., Galinski EA. 1994. Survival of *Escherichia coli* during drying and storage in the presence of compatible solutes. Appl Microbiol Biotechnol. 41: 684-688.

81. Ma L., Zhang G., Gerner-Smidt P., Mantripragada V., Ezeoke I., Doyle MP. 2009. Thermal inactivation of *Salmonella* in peanut butter. J. Food Prot. 72: 1596-601.

82. MacLean MJ., Ness LS., Ferguson GP., Booth IR. 1998. The role of glyoxalase I in the detoxification of methylglyoxal and in the activation of the KefB K+ efflux system in *Escherichia coli*. Mol Microbiol. 27: 563-571.

83. Manzanera M., Vilchez S., Tunnacliffe A. 2004. High survival and stability rates of *Escherichia coli* dried in hydroxyectoine. FEMS Microbiol. Let. 233: 347-352.

84. Mattick KL., Jørgensen F., Wang P., Pound J., Vandeven MH., Ward LR., Legan JD., Lappin-Scott HM., Humphrey TJ. 2001. Effect of challenge temperature and solute type on heat tolerance of *Salmonella* serovars at low water activity. App. Environ. Microbiol. 67: 4128–4136.

85. McDonough FE., Hargrove RE. 1968. Heat resistance of *Salmonella* in dried milk. J. Dairy Sci. 51: 1587-1591.

86. McMeechan A., Roberts M., Cogan TA., Jorgensen F., Stevenson A., Lewis C., Rowley G., Humphrey TJ. 2007. Microbiology. 153: 263-269.

87. Mercer RG., Walker B., Yang X., McMullen LM., Gänzle M. 2017. The locus of heat resistance (LHR) mediates heat resistance in *Salmonella enterica*, *Escherichia coli* and related genera of the *Enterobacteriaceae*. Food Microbiol. 64: 96-103.

88. Mercer RG., Zheng J., Garcia-Hernandez R., Ruan L., Gänzle MG., McMullen LM. 2015. Genetic determinants of heat resistance in *Escherichia coli*. Front Microbiol. 6: 932.

89. Miller DL., Goepfert JM., Amundson CH. 1972. Survival of *Salmonella* and *Escherichia coli* during the spray drying of various food products. J. Food Sci. 37: 828–831.

90. Ng H., Bayne HG., Garibaldi JA. 1969. Heat resistance of *Salmonella*: the uniqueness of *Salmonella* Senftenberg 775W. J. Appl Microbiol. 17: 78–82.

91. Nguyen SV., Harhay GP., Bono JL., Smith TPL., Harhay DM. 2017. Genome Sequence of the Thermotolerant Foodborne Pathogen *Salmonella enterica* Serovar Senftenberg ATCC 43845 and Phylogenetic Analysis of Loci Encoding Increased Protein Quality Control Mechanisms. Appl. Environ. Sci. 2: e00190-e00116.

92. Noor R. 2015. Mechanism to control the cell lysis and the cell survival strategy in stationary phase under heat stress. SpringerPlus. 4: 599.

93. Nummer B., Smith J. 2012. Survival of *Salmonella* in a high sugar, low water-activity, peanut butter flavored candy fondant. Food Control. 27: 184–187.

94. Orieskova M, Gajdosova J, Oslanecova L, Ondreickova K, Kaclikova E, Stanislav S., Turna J., Drahovska H. 2013. Function of thermotolerance genomic island in increased stress resistance of *Cronobacter sakazakii*. J. Food Nutr. Res. 52: 37-44.

95. Outten FW., Djaman O, Storz G. 2004. A Suf operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. Mol. Microbiol. 52: 861–872.

96. Peña-Meléndez M., Perry JJ., Yousef AE. 2014. Changes in thermal resistance of three *Salmonella* serovars in response to osmotic shock and adaptation at water activities reduced by different humectants. J. Food Prot. 77: 914-918.

97. Pleitner A, Zhai Y, Winter R, Ruan L, McMullen LM, Gänzle MG. 2012. Compatible solutes contribute to heat resistance and ribosome stability in *Escherichia coli* AW1.7. Biochim. Biophys. Acta. 1824: 1351-1357.

98. Podolak R., Enache E., Stone W., Black DG., Elliott PH. 2010. Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. J. Food Prot. 73: 1919-1936.

99. Pratt Z., Shiroda M., Static A., Lensmire J., Kaspar CW. 2016. "Osmotic and Desiccation Tolerance in *Escherichia Coli* O157:H7 and *Salmonella Enterica* Requires *rpoS* (σ^{38})", in Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria. 2016. De Bruijn FJ. John Wiley & Sons, Ltd., New Jersey, USA.

100. PHAC. 2011. *Salmonella enterica*: pathogen safety data sheet. Retrieved 02/28, 2017 from: http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/*salmonella*-ent-eng.php

101. Purvis JE., Yomano LP., Ingram LO. 2005. Enhanced trehalose production improves growth of *Escherichia coli* under osmotic stress. Appl. Environ. Microbiol. 71: 3761–3769.

102. Raina S., Missiakas D., Georgopoulos C. 1995. The *rpoE* gene encoding the sigma-E heat shock sigma factor of *Escherichia coli*. EMBO Journal. 14: 1043-1055.

103. Record TM. Jr., Courtenay ES., Cayley DS., Guttman HJ. 1998. Biophysical compensation mechanisms buffering *E. coli* protein-nucleic acid interactions against changing environments. Trends Biochem. Sci. 23: 190– 194.

104. Rees CED., Dodd CER., Gibson PT., Booth LR., Steward GSAB. 1995. The significance of bacteria in stationary phase to food microbiology. Int. J. Food. Microbiol. 28: 263-275.

105. Rowe MT., Kirk RB. 2000. Effect of nutrient starvation on the resistance of *Escherichia coli* O157:H7 to subsequent heat stress. J. Food Prot. 63: 1745–1748.

106. Rudolph B., Gebendorfer KM., Buchner J., Winter J. 2010. Evolution of *Escherichia coli* for growth at high temperatures. J. Biol. Chem. 285: 19029-19034.

107. Russo ET., Biggerstaff G., Hoekstra RM., Meyer S., Patel N., Miller B., Quick R. *Salmonella* Agona outbreak investigation team. 2013. A recurrent, multistate outbreak of *Salmonella* serotype Agona infections associated with dry, unsweetened cereal consumption, United States, 2008. J. Food Prot. 76: 227-230.

108. Rychlik I., Gregorova D., Hradecka H. 2006. Distribution and function of plasmids in *Salmonella enterica*. Vet. Microbiol. 112: 1–10.

109. Santillana-Farakos SM., Hicks JW., Frank JF. 2014. Temperature resistance of *Salmonella* in low-water activity whey protein powder as influenced by salt content. J. Food Prot. 77: 631-634.

110. da Silva do Nascimento M., Brum DM., Pena PO., Berto MI., Efraim P. 2012. Inactivation of *Salmonella* during cocoa roasting and chocolate conching. Int. J. Food. Microbiol. 159: 225-229.

111. Sharma M., Adler BB., Harrison MD., Beuchat LR. 2005. Thermal tolerance of acid-adapted and un-adapted *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in cantaloupe juice and watermelon juice. Lett. Appl. Microbiol. 41: 448–453.

112. Sleator RD., Hill C. 2010. Compatible solutes; a listerial passe-partout? Gut Microbes. 1: 77-79.

113. Spector M. P., Kenyon W. J. 2012. Resistance and survival strategies of *Salmonella enterica* to environmental stresses. Food Res. Int. 45: 455-481.

114. Spector MP., DiRusso CC., Pallen MJ., Garcia del Portillo F., Dougan G., Finlay BB. 1999. The medium-/long-chain fatty acyl-CoA dehydrogenase (fadF) gene of *Salmonella* Typhimurium is a phase 1 starvation-stress response (SSR) locus. Microbiology. 145: 15-31.

115. Takahashi Y., Tokumoto U. 2002. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J. Biol. Chem. 277: 28380–28383.

116. Todd EC., Greig JD., Bartleson CA., Michaels BS. 2008. Outbreaks where food workers have been implicated in the spread of foodborne disease. Part 4. Infective doses and pathogen carriage. J. Food. Prot. 71: 2339-2373.

117. Tsolis RM., Bäumler AJ., Heffron F. 1995. Role of *Salmonella* Typhimurium Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages. Infect Immun. 63: 1739-44.

118. Velayudhan J., Karlinsey JE., Frawley ER., Becker LA., Nartea M., Fang FC. 2014. Distinct roles of the *Salmonella enterica* serovar Typhimurium CyaY and YggX proteins in the biosynthesis and repair of iron-sulfur clusters. Infect Immun. 82: 1390-1401.

119. Villa-Rojas R., Tang J., Wang S., Gao M., Kang D., Mah J., Gray P., Sosa-Morales ME., Lopez-Malo A. 2013. Thermal Inactivation of *Salmonella* Enteritidis PT 30 in Almond Kernels as Influenced by Water Activity. J. Food Prot. 76: 26–30.

120. Welsh DT., Herber RA. 1999. Osmotically induced intracellular trehalose, but not glycine betaine accumulation promotes desiccation tolerance in *Escherichia coli*. FEMS Microbiol. Let. 174: 57-63.

121. Wesche A., Gurtler JB., Marks BP., Ryser ET. 2008. Stress, sub-lethal injury, resuscitation and virulence of bacterial foodborne pathogens. J. Food. Prot. 72: 1121-1138.

122. Winter AR., Stewart GF. McFarlane VH., Soloway M. 1946. Pasteurization of Liquid Egg Products III. Destruction of *Salmonella* in Liquid Whole Egg. Am. J. Public Health Nations Health. 36: 451-460.

Appendix

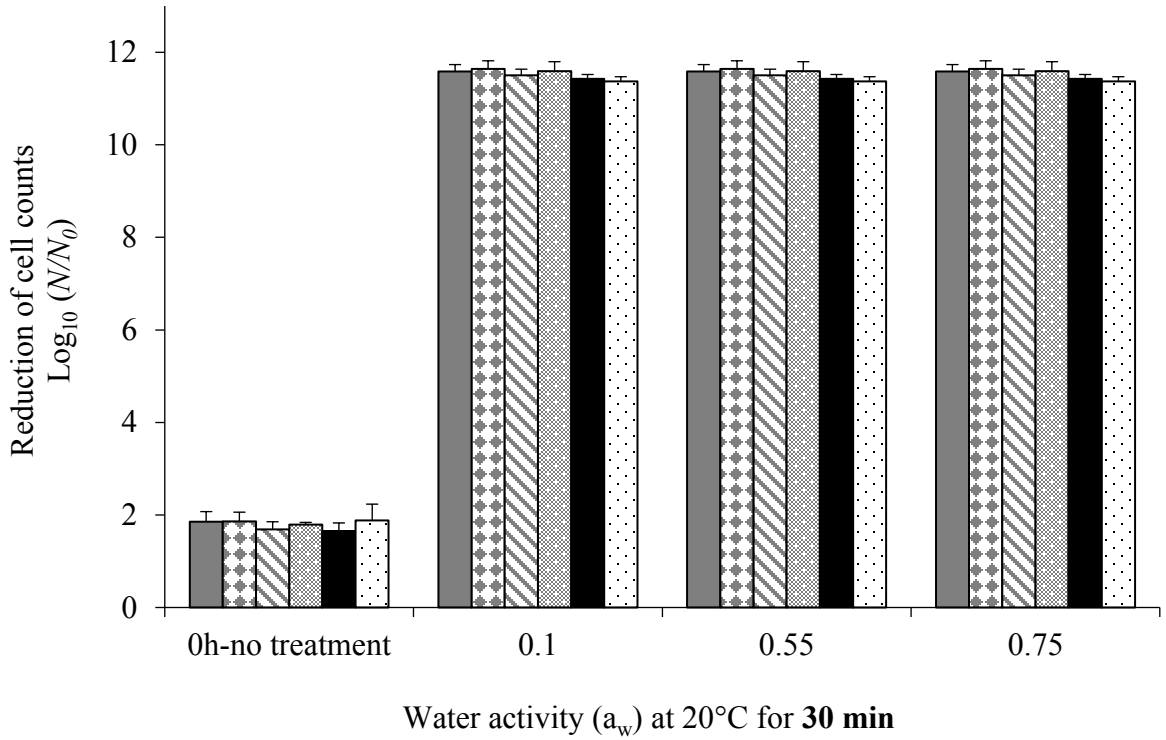

Figure 15: Reduction in cell counts of LHR positive and LHR negative wild-type and isogenic *Salmonella* and *E. coli* spp. after storage at various a_w prior to heat exposure for 30 minutes. Strains were air dried for 5 h in glass vials prior to storage in modified environments simulating an a_w of 0.1, 0.55 and 0.75, for 24 h: (■) ATCC 43845, (▦) ATCC 13311, (▨) ATCC13311pLHR, (▩) ATCC 13311pRK767, (▨) *E. coli* AW1.7, (▩) *E. coli* AW1.7ΔpHR1. Vials were transferred into a pyrex bottle, then treated at 110°C for 30 min in an oil bath. Data are means ± standard deviation of the average of three replicates.