ABSTRACT

JAYEOLA, VICTOR. Identification of Molecular Mechanisms Mediating Long-Term Survival of *Salmonella* on Low-Moisture Foods. (Under the direction of Dr. Sophia Kathariou).

Salmonella is a leading foodborne pathogen globally causing numerous cases of foodborne illnesses, hospitalizations and deaths annually. Low-moisture foods (LMFs) are important vehicles for *Salmonella* outbreaks due to the ability of this pathogen to survive on LMFs for long periods. However, the molecular mechanisms underlying the survival of *Salmonella* on LMFs are poorly characterized. The purpose of this dissertation was to identify molecular determinants required for the survival of *Salmonella* on LMFs.

In the first study, the survival of *Salmonella* on a panel of dried fruit stored at 4 and 25 ^{o}C was assessed. Enumerations on growth media revealed rapid decline in the population of *Salmonella* on the dried fruit, with longer survival on dried apple stored at 4 ^{o}C compared to 25 ^{o}C. However, fluorescence microscopy and laser scanning confocal microscopy after staining with the LIVE-DEAD *Bac*Light Bacterial Viability kit showed that a large proportion (56-85%) of *Salmonella* cells present on dried fruit were viable at the times when *Salmonella* could not be recovered on growth media. Findings from this study revealed that *Salmonella* can transition into the viable but non-culturable state on dried fruit. Therefore, microscopic and molecular methods should be used in addition to culture-based methods for efficient detection and enumeration of *Salmonella* contaminating dried fruit.

In the second study, the global transcriptome of *Salmonella* on selected LMFs was analyzed. The LMFs were inoculated, dried to original water activity (A_w), and stored 25 ^{o}C in the dark. The total RNA of *Salmonella* on the LMFs after 3 hours of drying and at 1, 5 and 12 weeks was isolated, ribo-depleted, sequenced and analyzed. The transcriptional response of *Salmonella* on the LMFs was rapid as several genes were differentially expressed as soon as after

3 h of drying compared to the inoculum suspension. For all products, the transcriptome of *Salmonella* after drying at 3 h was noticeably different from transcriptome at 1, 5 and 12 weeks of storage, with no major changes in transcriptome thereafter. Several differentially-expressed genes were identified in all tested LMFs at all tested times, while some were upregulated only in specific LMF-time combinations. Some of the differentially expressed genes included those involved in fatty acid biosynthesis, osmoprotectant biosynthesis and transport, DNA repair and RNA degradation, biosynthesis of aromatic compounds and stringent response factors.

Transposon sequencing analysis (Tn-Seq) was employed in the third study to determine the genetic determinants required for the survival of *Salmonella* on LMFs. Tn*5*-based mutant libraries of three different strains of *Salmonella* were negatively selected for survival on pistachios, followed by analysis of the distribution of distinct Tn*5* insertion sites immediately after inoculation (T_0), after drying (T_1), and at 120 days (T_{120}). Tn*5* insertion sites with reduced relative abundance at T_1 *vs* T_0 or T_{120} *vs* T_1 were localized in DNA repair, lipopolysaccharide biosynthesis and stringent response genes. Testing of 14 single-gene deletion mutants revealed that mutants in *sspA*, *barA*, *uvrB*, *damX*, *rfbD*, *uvrY*, *lrhA*, *yifE*, *rbsR* and *ompR* were impaired for survival on pistachios. The findings highlight the value of the combined mutagenesis and sequencing strategy to identify novel genes important for the survival of *Salmonella* in low-moisture foods.

In the fourth study, we used a nitrocellulose-based screening protocol to identify *Salmonella* mutants with impaired desiccation tolerance. Multi-gene and single-gene deletion mutants of *S.* Typhimurium strain 14028s were screened for desiccation tolerance on nitrocellulose membrane, and mutations that resulted in impaired survival were identified. Several genes, including those involved in the biosynthesis of thin aggregative fimbriae (*csgD*

and *csgA*), fimbriae genes (*fimY* and *fimA*), and *recA* were identified to be important for the desiccation tolerance of *Salmonella*. Survival assessment of mutants lacking these genes on in-shell pistachios revealed that while certain determinants such as thin aggregative fimbrae and *recA* were required for survival on model abiotic surfaces (NC) and LMFs (pistachios), others such as fimbriae that were important for survival on abiotic surfaces may not be required for survival on LMFs.

The fifth study was performed to assess the capacity of a surrogate construct, *S.* Typhimurium MMH112 (harboring deletions in the major *Salmonella* pathogenicity islands SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5) as an avirulent control in the *Galleria mellonella* infection model. Fourth-instar *G. mellonella* larvae were inoculated with bacterial suspensions of *S.* Typhimurium 14028s (WT) and *S.* Typhimurium MMH112 and monitored daily for survival. The mortality of inoculated *G. mellonella* larve was comparable between both strains, suggesting that *S.* Typhimurium MMH112 was virulent in *G. mellonella* and thus not suitable as an avirulent control in the infection model. Findings highlight the need for a *Salmonella* surrogate as an avirulent control in the *G. mellonella* infection model.

In the final study, an ampicillin-based selection protocol was used to selectively enrich for *Listeria monocytogenes* mutants unable to grow on produce in order to identify genetic factors required for growth on produce. The protocol was validated by assessing the relative recovery of *L. monocytogenes* strain 2011L-2858 and its cold-sensitive mutant L1E4 (incapable of growth at 4 ^{o}C) following inoculation of cantaloupe rind with a 1:1 mixture of the strains and incubation at 4^{o}C with or without ampicillin. Significant increase in the relative abundance of L1E4 compared to 2858 on cantaloupe rind in the presence of ampicillin proved the efficacy of this protocol to enrich for non-replicating cells. Application of this protocol to transposon mutant

libraries from three *L. monocytogenes* strains yielded several mutants unable to grow on cantaloupe. Thus, ampicillin selection can facilitate discovery of genes essential for growth of *L. monocytogenes* on fresh produce.

Findings from these studies showed diverse mechanisms employed by *Salmonella* to survive on LMFs. These mechanisms include the induction of the viable but non-culturable state, DNA recombination and repair, osmolarity regulation, lipopolysaccharide biogenesis, stringent response, stress-induced sigma factors, and fimbriae formation. Elucidation of the molecular basis of the survival of *Salmonella* on LMFs may aid the development of improved detection and inactivation strategies, thereby reducing the food safety burden associated with contamination of low-moisture foods.

Identification of Molecular Mechanisms Mediating Long-Term Survival of *Salmonella* on Low-Moisture Foods

by

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DEDICATION

I would like to dedicate this dissertation to organizations and individuals who give indigent and disadvantaged students access to education.

BIOGRAPHY

Victor Jayeola was born in the city of Ibadan, Nigeria. He completed his high school at Adifase High School in Ibadan and received his Bachelor of Science degree in Food Science and Technology from the University of Agriculture Abeokuta in Ogun State, Nigeria. After completing a mandatory one-year National Youth Service Program as a computer literacy instructor in Owerri, Nigeria, he received a MasterCard Foundation Scholarship to pursue a MS degree in Food Science at Michigan State University. His M.S research, under the guidance of Dr. Elliot Ryser, investigated the growth of *Listeria monocytogenes* and *Salmonella* in fresh-cut produce during simulated commercial transportation, retail storage, and display. After completing his M.S in 2015, Victor joined the research group in the laboratory of Dr. Sophia Kathariou at North Carolina State University, Raleigh, NC to pursue a doctorate degree in Microbiology with a minor in Biotechnology. His doctoral work has been focused on identification of molecular mechanisms mediating long-term survival of *Salmonella* on low-moisture foods.

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CHAPTER 1

LITERATURE REVIEW

1.1 *Salmonella*: nomenclature, physiology, and pathogenesis

Salmonella is a genus of Gram-negative, facultatively anaerobic bacteria that belongs to the *Enterobacteriaceae* family. They are rod-shaped, about 2-5 microns long and 0.5 -1.5 microns wide, and motile by peritrichous flagella (Andino and Hanning, 2015). The genus *Salmonella* comprises two species, *S. enterica* and *S. bongori*, of which only *S. enterica* is a human pathogen (Brenner et al, 2000). Based on biochemical characteristics and genome composition, *S. enterica* is further divided into six subspecies; *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* (Tindall et al, 2005). *S. enterica* subsp. *enterica* is responsible for most (> 90%) human *Salmonella* infections and is further classified into serovars based on three antigenic determinants; somatic (O), capsular (K), and flagellar (H) antigens, with more than 2500 serovars identified (Grimont et al, 2007). However, only a small proportion of these serovars have been involved in human infections, with 20 serovars responsible for 95% of all human salmonellosis in the United States from 2009 to 2015 (Dewey-Mattia et al, 2018). The other subspecies of *S. enterica* are less well characterized and they have been mostly isolated from reptiles while *S. bongori* is predominantly associated with cold-blooded animals (Fookes et al, 2011, Lamas et al, 2017).

In terms of clinical manifestations, S. *enterica* subsp. *enterica* is divided into typhoidal and non-typhoidal serovars. The typhoidal serovars are Typhi, Sendai and Paratyphi A, and are mostly transmitted via contaminated water and person-to-person contact and cause enteric fever in humans, while the non-typhoidal serovars cause primarily gastroenteritis, and are mostly

transmitted via contaminated food (Gal-mor et al, 2014). This review focus on non-typhoidal *S. enterica* subsp. *enterica* due its importance as a major foodborne pathogen.

Salmonella enterica subsp. *enterica* serovars are mesophilic, with optimum growth at 35-37^{o}C, pH of 6.5-7.5, and water activity (A_w) of 0.94-0.99 (Percival et al, 2014). They are catalase positive, oxidase negative, ferment mannitol, glucose, sorbitol to produce acid and gas, utilize citrate as a sole carbon source, decarboxylate lysine, and form hydrogen sulfide (H_2S) on triple sugar iron agar (Percival et al, 2014). The natural reservoirs of *Salmonella* include domestic and wild animals, such as poultry, swine, cattle, wild birds, turtles, cats and rodents, although different serovars may have unique ecological niche (Andino and Hanning, 2015; Percival et al, 2014). Although an enteric organism, *Salmonella* is capable of prolonged survival outside animal hosts and has been isolated from marine environments, vegetation, irrigation and surface water, soil, and other environments (Martinex-Urtaza et al 2004; Bell et al, 2015).

The genetic traits of *Salmonella* allow it to persist and proliferate in different conditions and infect a wide spectrum of hosts. The genome size of *Salmonella* is typically > 4600 kb with about 52-53% GC content and more than 4000 open reading frames, and some *Salmonella* strains harbor plasmids encoding various resistance and virulence genes (McClelland et al, 2001; Andino and Hanning, 2015). All *S. enterica* strains are considered pathogenic due to the presence of several virulence factors, including the *Salmonella* pathogenicity islands (SPI-1 to SPI-5) which contain major virulence determinants (Ibarra and Steele-Mortimer, 2009). The severity of *Salmonella* infections in humans varies depending on the health status of the host, strain, and dosage. Salmonellosis symptoms range from self-limiting, uncomplicated gastroenteritis in healthy individuals to life-threatening illness in infants, young children, elderly people and immunocompromised patients (Majowicz et al, 2010). *Salmonella* is also associated

with systemically invasive disease and bacteremia, especially in immunocompromised hosts (Feasey et al, 2012)

The mechanisms of infection of *Salmonella* in humans involve different stages, starting with survival in the acidic pH of the stomach by the activation of the acid tolerance response (ATR) in order to maintain pH homeostasis with the extracellular environment (Foster et al, 1991). *Salmonella* that survives the acidic pH environments can reach the bowel, traverse the intestinal mucus layer, and adhere to intestinal epithelial cells by expressing adhesins encoded in SPI-3 and SPI-4 (Fàbrega et al, 2013). Upon adherence to the epithelial cells, *Salmonella* expresses the type III secretion systems (T3SS) encoded by SPI-1 and induces phagocytosis into large vesicles called *Salmonella*-containing vacuoles (SCVs) inside the epithelial cells (Fàbrega and Vila, 2013). SCVs are the only intracellular compartment in the epithelial cells that provides a conducive environment for *Salmonella* survival and proliferation (Francis and Vila, 1993). Inside the SCVs, *Salmonella* expresses a second T3SS (encoded by SPI-2) which is important for its survival and proliferation inside the host epithelial cells and macrophages (Fàbrega and Vila, 2013). Mature SCVs migrate from the epithelium and are engulfed by phagocytes where *Salmonella* continues to survive and replicate, and the migration of the phagocytes facilitates dissemination of *Salmonella* in the host via the bloodstream leading to systemic infection (Fàbrega and Vila, 2013; Eng et al, 2015).

1.2 Low-moisture foods

Low-moisture foods (LMFs) are foods and food ingredients that are either naturally low in moisture or processed to achieve low moisture (Beuchat et al, 2012). Total moisture content of foods consists of chemically bound and unbound moisture (free moisture), with the latter available for microbial growth (Finn et al, 2013). Free moisture in foods is measured as the water

activity (Aw), which is the ratio of the vapor pressure of water in food compared to that of pure water at the same temperature (Beuchat et al, 2012). According to the Food and Drug Administration (FDA), low-moisture foods are foods with A_w below 0.6 (FDA, 2016). Examples of low-moisture foods include confectionery, chocolate, honey, crackers, spices, infant formula, dried egg, dry petfood, dried animal and vegetable products, and nuts. LMFs are considered shelf-stable and of low food safety risk because their A_w is below the level required for microbial growth (Lund et al., 2000). The minimum A_w required for the growth of most foodborne bacterial pathogens is 0.85 while xerophilic spoilage yeasts and mold can grow, although slowly, at A_w of 0.6 to 0.7 (Beuchat et al, 2012). However, association of diverse LMFs with foodborne outbreaks has raised serious food safety concerns.

Although LMFs cannot support microbial growth, some microorganisms, including pathogens are able to survive as vegetative cells or spores on LMFs for extended periods of time. The food safety risk associated with LMFs is exacerbated in LMFs that are consumed as ready-to-eat foods (RTE). The European Commission Regulation defines RTE as food intended by the producer for direct consumption without processing to eliminate or reduce to an acceptable level microorganism of concern (ECR, 2005). Furthermore, inactivation strategies such as thermal treatment that are effective against pathogens in high-moisture foods are much less effective against pathogens in LMFs, thus rendering decontamination of LMFs extremely difficult (Podolak et al, 2010). Additionally, most pathogens contaminating LMFs tend to develop higher tolerance to other stressors, a phenomenon termed ‘cross-protection’, although the mechanisms involved are poorly understood (Gruzdev et al, 2012).

1.3 Low-moisture foods as important vehicles for *Salmonella* outbreaks

Salmonella is a leading cause of foodborne illnesses globally (Hoffmann et al, 2017). In the United States alone, *Salmonella* is estimated to cause 1 million cases of foodborne illnesses annually, accounting for 28% of all hospitalizations and 35% of all deaths attributed to known foodborne bacterial pathogens (Mead et al, 1999; Scallan et al, 2011). The overwhelming majority (> 83%) of foodborne outbreaks associated with LMFs in the US between 2007 and 2018 involved *Salmonella* (CDC, 2019). The US Department of Agriculture Economic Research Service (USDA-ERS) estimated the cost of foodborne illness from *Salmonella* in 2013 to be $ 3.7 billion (USDA-ERS, 2014). LMFs are important vehicles of *Salmonella* outbreaks, accounting for 21% of investigated *Salmonella* outbreaks reported by the Centers for Disease Control and Prevention (CDC) from 2007 to 2018 (CDC, 2019). Worldwide, LMFs were involved in numerous outbreaks resulting in 7,315 cases of foodborne illnesses and 63 deaths between 2007 and 2012, with *Salmonella* responsible for 53% of these outbreaks (Santillana and Joseph, 2014).

Diverse LMFs have been involved in *Salmonella* outbreaks and selected LMF-associated Salmonella outbreaks are listed in Table 1. An example of a large-scale *Salmonella* Typhimurium outbreaks associated with LMFs was the 2008-2009 outbreak associated with peanut butter that caused 714 cases of human infections, 171 hospitalizations and 9 deaths, and resulted in one of the largest product recalls in the US with an estimated loss of $1 billion in sales (CDC, 2009; Cavallaro et al., 2011). The outbreak lasted for 8 months (September 6, 2008 to April 4, 2009) and involved a *S.* Typhimurium strain with a distinct pulsed-field gel electrophoresis (PFGE) pattern that was different from *Salmonella* from raw meat and poultry products (Cavallaro et al., 2011). A 2018 *Salmonella* outbreak associated with Kellog's Honey

Smack cereal caused 135 cases of illnesses in 36 states and resulted in 34 hospitalizations (CDC, 2019). PFGE and whole genome sequencing (WGS) analysis of isolates from 91 ill people and 8 food samples confirmed *S.* Mbandaka as the contaminant in this outbreak that lasted for about 7 months (CDC, 2019). Overall, various serovars of *Salmonella* have been involved in outbreaks involving LMFs, suggesting that the ability to persist and remain infectious on LMFs is conserved among diverse serovars (Podolak et al, 2010; Beuchat et al, 2013).

1.4 Routes of contamination and control measures against *Salmonella* on low-moisture foods

Salmonella is persistent in the environment, including food processing facilities, and can contaminate LMFs through diverse routes (Finn et al, 2013). Contamination of LMFs can occur on the farm (especially for seeds and nuts), but contamination of LMFs often involves cross-contamination of finished products due to ineffective cleaning and sanitation procedures, poor equipment and facility design, and poor manufacturing practices (Podolak et al, 2010; Finn et al, 2013). Below I will discuss the routes of contamination and the failures in the execution of control measures for some LMF-associated *Salmonella* outbreaks. Contaminated almonds were involved in a *Salmonella* outbreak in 2000/2001 that caused 168 cases in several states in the US and Canada (Isaacs et al, 2005). The source of contamination was traced to the orchards of one of the suppliers (Isaacs et al, 2005). The huller-sheller facility that was used to process the contaminated almonds became contaminated and then served as the source of contamination for batches from different suppliers (Isaacs et al, 2005). Although the supplier with the *Salmonella*-positive almonds denied using manure or biosolids on their farm and affirmed that the irrigation water used on the farm was solely from the California Aqueduct system, contaminated manure and irrigation water are important sources of contamination (Isaacs et al, 2005).

Table 1: Selected *Salmsonella* outbreaks associated with low-moisture foods

Year	Food	Serotype	No. of cases	Reference(s)
2000	Almonds	*S*. Enteritidis PT30	168	Isaacs et al, 2005
2001	Peanuts	*S*. Stanley, S. Newport	109	Kirk et al, 2004
2002	Tahini	*S*. Montevideo	68	Unicomb et al, 2005
2003	Aniseed (Tea)	*S*. Agona	42	Koch et al, 2005
2006	Peanut butter	*S*. Tennessee	715	Sheth et al, 2011
2007	Rice-corn snack	*S*. Wandsworth	75	CDC, 2007
		S. Typhimurium		
2008	Peanut butter	*S*. Typhimurium	628	Cavallaro et al., 2011
2008	Cereal	*S*. Agona	28	Russo et al, 2013
2011	Pine nuts	*S*. Enteritidis	43	Irvin et al, 2012
2014	Chia powder	*S*. Newport	31	CDC, 2014
		S. Hartford		
		S. Oranienburg		
2016	Pistachios	*S*. Montevideo	11	CDC, 2016
		S. Senftenberg		
2018	Cereal	S. Mbandaka	135	CDC, 2018

In another outbreak, investigators identified 42 cases of salmonellosis among infants (< 13 months old) who consumed aniseed-containing herbal tea in Germany in 2002/2003 (Koch et al, 2005). Although the cases were sporadic, *Salmonella* Agona strains isolated from several case-patients and the implicated herbal tea showed the same PFGE pattern, suggesting a common source of contamination (Koch et al, 2005). Further investigation revealed that one of the growers of aniseeds had used untreated, contaminated manure (Koch et al, 2005). The majority of the parents of the case-patients reported using boiling water to prepare the tea, and some parents reported quickly cooling tea by adding cold water (Koch et al, 2005).

In 1998, a multistate *Salmonella* outbreak associated with toasted oat cereal products manufactured in one plant caused more than 400 cases of illnesses in the United States (Breuer, 1999). PFGE analysis of clinical isolates, isolates from the contaminated products, and from the processing facilities confirmed that a single *Salmonella* Agona strain was involved in the outbreak (Breuer, 1999). Although the direct source of contamination was not identified, the processing facility implemented several correctional changes, including removal of contaminated equipment, stripping all surfaces to bare concrete followed by decontamination, and installation of new production lines (Russo et al, 2013). However, in 2008 there was another multistate outbreak associated with unsweetened puffed rice and puffed wheat cereals processed in the same facility and contaminated with the same *S.* Agona strain implicated in the 1998 outbreak (Russo et al, 2013). An investigation into the source of contaminations revealed that the products were likely contaminated in a destoner machine (for stone removal) used just before packaging (Russo et al, 2013). A wall that had been constructed to seal off a section of the facility after the 1998 outbreak was demolished a few weeks leading to the onset of the 2008 outbreak due to maintenance work, thereby allowing the destoner to draw in air from the

contaminated area (Russo et al, 2013). The destoner machine tested positive to the outbreak *S.* Agona strain (Russo et al, 2013). Similarly, the source of contamination of infant formula with *Salmonella* was traced to contaminated dust drawn from contaminated insulation material through a hole in the dryer (Rowe et al, 1987).

Investigation of the 2008–2009 peanut-associated *Salmonella* outbreak revealed blatant disregard of good manufacturing processes (Podolak, 2010). The investigators reported use of unclean equipment, containers and utensils, storage of raw materials and finished products in the same area, and overall improper facility and equipment design (Podolak, 2010). Before the outbreak, *Salmonella* had been isolated from finished products at the facility, but the manufacturer failed to clean the production lines and validate the critical control points before manufacturing products that were eventually implicated in the outbreak (Podolak, 2010). In a previous peanut-associated outbreak involving *Salmonella* Tennessee that caused 715 cases in 2006-2007, FDA investigators reported that the roasting temperature of peanuts was not measured during processing, and roasted peanuts were held in uncovered bins (Sheth et al, 2011). Investigators also discovered that the outbreak strain been previously been isolated from environmental samples from the plant in 2004, 2 years before the outbreak (Sheth et al, 2011).

Overall, contamination of LMFs with *Salmonella* could occur via diverse routes, including, but not limited to the few discussed above. Contamination of LMFs often result from failures in food safety systems on the farm (e.g., good agricultural practices) or in the processing facilities (e.g., hazard analysis critical control points, HACCP). Therefore, adherence to food safety guidelines will significantly reduce the food safety risk associated with LMFs.

1.5 Survival of *Salmonella* in low-moisture environment and low-moisture foods

Although the low A_w of LMFs is not conducive for growth, *Salmonella* survives on diverse LMFs for an extended period (Podolak et al, 2010). Moreover, *Salmonella* outbreaks involving LMFs often last for months, thus indicating long-term survival of *Salmonella* on the implicated LMFs (Beuchat et al, 2013). The survival of *Salmonella* on diverse LMFs, such as chocolate and confectionary products, peanut butter, tree nuts, flours, spices, etc. have been documented (Podolak et al, 2010; Beuchat et al, 2013). *Salmonella* can survive on abiotic surfaces, such as stainless steel and plastics that are commonly used in food processing (Margas et al, 2014). Several strains of different serovars survived on stainless steel surfaces, showing approx. 2 log reduction over 30-day storage under ambient conditions (Margas et al, 2014). *Salmonella* survived on stainless steel surfaces for more than 6 weeks (Finn et al, 2013). Another study reported survival of *Salmonella* on plastic surfaces for more than 250 days, although longer survival was observed on almonds, chocolates and radish sprout seeds (all having A_w < 0.6) than on plastic surfaces (Hokunan et al, 2016). These studies provide evidence that *Salmonella* can persist on food-contact surfaces, which can in turn serve as sources of contamination in consumers' homes.

Several factors, such as storage temperature, inoculation level, inoculum preparation and inoculation methods, and strain types affect the survival of *Salmonella* on LMFs (Finn et al, 2013). *Salmonella* survive noticeably longer in LMFs at freezing and refrigeration temperature, compared to ambient temperature (Kimber et al, 2012; Brar et al, 2015; Beuchat and Mann, 2014). Inoculated at approx. 4 log CFU/g, the population of *Salmonella* showed no reductions on almonds and pistachios stored at -19 and 4^{o}C for 12 months but was reduced by 2.5 log on the nuts stored at 25^{o}C for the same period (Kimber et al, 2012). Similar observations were made in

another study where the population of *Salmonella* was stable on walnut kernels stored at -20 and 4^{o}C but declined at the rate of 0.1 log CFU/g per month on kernels stored at 23^{o}C (Blessington et al, 2012). Furthermore, *Salmonella* survived more than 100 days longer on dried fruit (dates, raisins, strawberries, cranberries) stored at 4^{o}C than at 25^{o}C (Beuchat and Mann, 2014). Reductions in the population of *Salmonella* inoculated in peanut butter and peanut butter spread was two times higher in samples stored at 25^{o}C than 4^{o}C for 24 weeks (Burnett et al, 2000). Therefore, preservation of LMFs at refrigeration temperature can indeed enhance the survival of *Salmonella* on these products.

Product composition or formulation can also impact the survival of *Salmonella* on LMFs. The survival of *Salmonella* in various peanut products (in-shell peanuts, roasted peanuts, unblanched peanut kernel, peanut brittle, pacËoca, and peÂ-de-mocËa) was significantly influenced by the A_w, lipid, carbohydrate and protein content (Nascimento et al, 2018). *Salmonella* tended to survive longer on peanut product with high carbohydrate content at A_w of 0.3 while high-fat content at higher A_w of 0.68 reduced survival on the peanut confectionary products (Nascimento et al, 2018). Other studies have also reported higher tolerance of *Salmonella* to desiccation on LMFs with high carbohydrate content, and that high-fat content was inhibitory to survival when the A_w was increased from 0.3 to 0.6 (He et al, 2011; Kataoka et al, 2014). Reduced survival on LMFs with high-fat content and relatively high Aw may be due to generation and mobility of free radicals in LMFs with high A_w which could have bactericidal effects on *Salmonella*. Nonetheless, the hurdle effect of the chemical composition and A_w of LMFs on the survival of *Salmonella* needs further elucidation

The inoculation level of *Salmonella* impacts survival on LMFs, with higher inoculum levels resulting in longer survival (Podolak et al, 2010). The population of *Salmonella* inoculated

on walnut kernels at 9.9 and 6.3 log CFU/g reduced by 3.4 and 6 log CFU/g, respectively after storage for about 3 years (Blessington et al, 2012). However, other survival assessment studies did not observe variations in survival based on the levels of inoculum used (Komitopoulou and Pe aloza, 2009; Miksch et al, 2012; Kimber et al. 2012). The survival of *Salmonella* inoculated on almonds and pistachios at 10, 6 and 4 log CFU/g and stored at -19, 4 and 25 ^{o}C was independent on the inoculum level (Kimber et al, 2012). Similarly, reductions in the population of *Salmonella* inoculated on raw peanut and pecan kernels at 3 and 6 log CFU/g, and on cocoa butter oil, crushed cocoa and hazelnut shells, cocoa beans and almond kernels at 6 and 8 log CFU/g did not correlate with inoculum concentrations (Miksch et al, 2012; Komitopoulou and Pe aloza, 2009). Therefore, the impact of inoculum concentrations on survival may be dependent on other factors, such as the characteristics of LMFs (surface area, chemical composition, etc.).

The methods of preparation of the *Salmonella* inoculum used in survival assessment studies considerably impacted survival (Komitopoulou and Pe aloza, 2009; Beuchat and Mann, 2014). In general, *Salmonella* grown on agar media tended to survive longer than when cultured in broth medium (Komitopoulou and Pe aloza, 2009; Beuchat and Mann, 2014; Bowman et al, 2015). It has been hypothesized that possible lower A_w of agar compared to broth media may preadapt *Salmonella* to low-moisture conditions, thus enhancing its survival on LMFs (Beuchat and Mann, 2014). The survival of *Salmonella* in low-moisture environments varies considerably among different strains, although the variation does not correlate with serovars (Margas et al, 2014, Hokunan et al, 2016). Reductions in the population of four S. Typhimurium strains (DT104, HR, SD, and FH/St/165) on stainless steel surfaces varied by almost 4 log, and similar observations were made with different strains of other serovars (Margas et al, 2014). In addition, the survival of *Salmonella* on chocolate, almonds, and radish seeds varied among the four strains

of serovars Stanley, Typhimurium, Chester, and Oranienburg tested (Hokunan et al, 2016). Therefore, most survival assessments used cocktail mixtures of different *Salmonella* strains to account for variations in survival.

1.6 Molecular mechanisms mediating the survival of *Salmonella* in low-moisture environments

The ability of *Salmonella* to survive in LMFs and dry environments for an extended period is well characterized but the underlying mechanisms and determinants are not fully understood. Findings from previous studies suggest that desiccation tolerance of *Salmonella* is mediated by complex mechanisms involving diverse molecular pathways and biological processes (Finn et al, 2013; Podolak et al, 2010). The underlying mechanisms are dependent on other environmental factors, such as pH, temperature, solute concentration, in addition to water activity (Finn et al, 2013; Podolak et al, 2010). Mechanisms and strategies that are relevant to the desiccation tolerance of *Salmonella*, including the induction of viable but non-culturable state (VBNC), accumulation of compatible solutes, filamentation and thin fimbriae aggregation formation, cell envelope biogenesis, and others are discussed below.

1.6.1 Induction of the VBNC state in *Salmonella* on low-moisture foods

Bacteria in the VBNC state are not able to grow under laboratory conditions at which they would normally grow, although they remain viable (Oliver, 2010). Several metabolic markers, including the uptake of labeled amino acids, maintenance of cellular membrane and genomic DNA integrity, respiration, gene transcription and translation, have been measured to evaluate the viability of bacteria in the VBNC state (Rahman et al, 1994; Oliver 2010, Ramamurthy et al, 2014). Strong evidence suggests that the transition of bacteria into the VBNC state is a survival strategy in response to stressful conditions, and not an intermediate phase

towards cell death (Oliver, 2010; Ramamurthy et al, 2014; Zhao et al, 2017). Stressors, such as chlorination, oxidative stress, osmotic stress and exposure to commercial household detergents and inorganic salts have been shown to induce the VBNC state in diverse serovars of *Salmonella* (Xu et al, 2018; Highmore et al, 2018; Oliver et al, 2005, Robben et al, 2018).

The induction of the VBNC state in *Salmonella* in low-moisture conditions is largely uncharacterized despite extensive investigations of the capacity of *Salmonella* to survive desiccation. This knowledge gap may be because most survival assessment studies did not assay for VBNC, as culture-based detection and enumeration methods were employed in these studies (Kimber et al, 2012; Margas et al 2014; Hokunan et al, 2016; Beuchat et al, 2014; Nascimento et al, 2018). Therefore, it remains unclear if reductions in CFU reported in previous survival studies reflected loss of viability or transition of *Salmonella* into the VBNC state. Indeed, the few survival assessments studies that tested for VBNC reported that reductions in cell viability were significantly less than reductions in culturability (growth on media) due to formation of VBNC *Salmonella* under low-moisture conditions (Gruzdev et al, 2012; Morishige et al, 2017). Enumeration of *Salmonella* desiccated on polystyrene surfaces for 100 weeks at 4^{o}C on growth media revealed approx. 5 log reduction in population, although viability assessment revealed only a 50% reduction in viable cells (Gruzdev et al, 2012).

There are speculations that VBNC *Salmonella* may have been involved in foodborne outbreaks where none or low levels of *Salmonella* could be recovered from foods associated with the outbreaks (Makino et al, 2000; Gruzdev et al, 2012; Zhao et al, 2017). Although there has been no direct evidence linking VBNC *Salmonella* to outbreaks or infections in humans, stressors in LMFs, such as osmotic stress, desiccation, and antimicrobial agents have been experimentally shown to induce the VBNC state in *Salmonella* (Caro et al, 1999; Nicolo et al,

2011; Gruzdev et al, 2012). Therefore, the existence of VBNC *Salmonella* in LMFs is highly probable and could pose potential food safety and public health risks. In addition to being undetectable using the gold-standard culturing methods, VBNC bacteria tend to have higher tolerance to stressors compared to vegetative bacteria due to their reduced metabolic activity, alterations in membrane fatty acid composition, modulation of membrane-bound proteins and increase in peptidoglycan cross-linking (Signoretto et al, 2000; Muela et al, 2008; Chaiyanan et al, 2007).

Previous studies reported that VBNC *Salmonella* can become vegetative and proliferate under favorable conditions (Senoh et al, 2012; Morishige et al, 2017; Ramamurthy et al, 2014). Supplementation of growth media with pyruvate and catalase, and co-culturing with eukaryotic cells are some of the methods that have been employed to resuscitate VBNC *Salmonella* (Gupte et al, 2003; Senoh et al, 2012; Morishige et al, 2017). However, in these studies, only a fraction of the VBNC population were resuscitated while a significant proportion remained unculturable (Gruzdev et al, 2012; Gupte et al, 2003; Senoh et al, 2012; Morishige et al, 2017). So far there are no standard resuscitation protocols as resuscitation of VBNC bacteria seemed to depend on the factors that induced formation of VBNC, age of the VBNC cells, different strains, and other unknown factors (Oliver 2010; Zhao et al, 2017). Therefore, further investigations of the existence and the induction of VBNC *Salmonella* in LMFs, as well as the resuscitating factors, virulence, and other characteristics of VBNC *Salmonella* that may be relevant to food safety are needed.

1.6.2 Accumulation of compatible solutes

Exposure of bacteria to low-water activity environments causes an imbalance in the osmotic gradient between the environment and bacterial cytoplasm resulting in water efflux, cell

shrinkage and death (Csonka, 1989). Bacteria with high tolerance to osmotic stress, such as *Salmonella,* can balance osmolarity across the cell membrane and the environment by accumulating low molecular weight, highly soluble compatible solutes (Csonka and Hanson, 1991, Sleator and Hill, 2002). Compatible solutes (osmoprotectants), such as proline, glycine betaine, ectoine and trehalose are electrically neutral and can be accumulated at high concentrations without damaging cellular components and affecting enzyme function (Luzardo et al, 2000, Sleator and Hill, 2002). Some compatible solutes (e.g., trehalose) are synthesized *de novo* by bacteria upon exposure to osmotic stress, while others (e.g., proline and glycine betaine) are transported into the cell from the environment (Csonka, 1989, Finn et al, 2013).

Trehalose, a non-reducing disaccharide, can be synthesized from glucose, maltose, and maltodextrins through at least five distinct biosynthetic pathways (Avonce et al, 2012). The most widely distributed and first to be identified trehalose biosynthesis pathway in bacteria includes synthesis of trehalose-6-phosphate from an enzymatic condensation of UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (OtsA), followed by dephosphorylation of trehalose-6-phosphate by trehalose-phosphatases (OtsB) to form trehalose and inorganic phosphate (Elbein et al, 2003). Trehalose biosynthetic genes were upregulated in *Salmonella* under desiccation on paper disks and stainless-steel, and on powdered milk, black pepper, dry petfood and chocolate, suggesting the importance of trehalose biosynthesis to the desiccation tolerance of *Salmonella* on abiotic surfaces and LMFs (Li et al, 2012; Finn et al, 2013; Crucello et al, 2019).

Some osmoprotectants are transported into the cytoplasm from the environment rather than synthesized *de novo*, probably in order to reduce glucose and ATP expenditure during biosynthesis of trehalose (Sleator and Hill, 2002). The transportation of osmoprotectants (K^+-

glutamate, proline, glycine betaine, ectoine, etc.) from the environment into the cytoplasm of bacteria is mediated by several transporters and permeases (Csonka, 1989; Sleator and Hill, 2002). The most characterized of these transporters include the membrane-bound ATP-binding cassette transporters ProU and OsmU, and a member of major facilitator superfamily permeases, ProP (Finn et al, 2013, Frossard et al, 2012; Sleator and Hill, 2002). ProP and ProU mediate the uptake of most osmoprotectants in *E. coli* and *Salmonella* and they have similar affinities for betaine, proline and ectoine (Cairney et al, 1985; Jebbar et al, 1992; Sleator and Hill, 2002, Frossard et al, 2012). Moreover, *proU* and *osmU* were upregulated in *Salmonella* desiccated on stainless steel and paper disks (Li et al, 2012; Finn et al, 2013a), and the deletion of *proP* significantly impaired long-term survival of *Salmonella* on stainless steel (Finn et al, 2013a). Similarly, *osmV* encoding an osmoprotectant ABC transporter ATP-binding protein, was upregulated on *Salmonella* on powdered milk, black pepper, chocolate, and dry petfood (Crucello et al, 2019).

However, other transcriptional studies did not report differential expression of osmoprotectant biosynthesis genes and transporters in *Salmonella* desiccated on plastic and polycarbonate surfaces (Gruzdev et al, 2012; Maserati et al, 2018). Moreover, transposon mutants of *Salmonella* lacking known osmoprotectants genes were not impaired for short-term survival on plastic surfaces (Mandal et al, 2017). The discrepancy among different studies regarding the relevance of known osmoprotectants may be due to differences in the type of surfaces tested (stainless steel, filter paper or plastic), desiccation conditions (desiccation inside a desiccator or under ambient conditions) and sampling times (few hours to weeks), and the sensitivity of the analysis (microarray, RNA-sequencing or transposon-sequencing). Furthermore, most of the transcriptional studies tested desiccation tolerance of *Salmonella* only

on abiotic surfaces, and the study by Crucello et al (2019) analyzed transcriptome on LMFs in a single trial. Therefore, the transcriptional response and importance of osmoprotectant-related genes in *Salmonella* contaminating LMFs needs to be characterized.

1.6.3 Filamentation and thin fimbriae aggregation formation

Filamentation of bacterial cells occurs when bacteria elongate and replicate DNA without septation into daughter cells, thus forming long multi-nucleoid filamentous cells (Takeuchi et al, 2005). Filamentation has been proposed as a survival strategy of *Salmonella* under suboptimal A_w (Finn et al, 2013). *Salmonella* formed filaments when grown in broth supplemented with NaCl, sucrose, or glycerol solution to obtain suboptimal A_w of 0.95, 0.94 and 0.92, respectively, at 37^{o}C for 24h (Mattick et al, 2000). This finding was corroborated by another study that reported filamentation of *Salmonella* on agar media with A_w of 0.94 to 0.97 (Kieboom et al, 2006). It is important to note that these studies did not directly assay for desiccation tolerance of *Salmonella* but rather the growth of *Salmonella* under reduced A_w. Therefore, filamentation of *Salmonella* observed in these studies may have been as a result of an incomplete replication cycle rather than a survival strategy. Moreover, the A_w of media used in these studies were remarkably higher than what is obtained in of LMFs ($A_w < 0.6$), and it is highly unlikely that the low A_w of LMFs would be permissible for cell elongation and DNA replication. Therefore, investigations into the formation and roles of filamentation of *Salmonella* contaminating LMFs are needed.

Production of thin aggregative fimbriae and cellulose (Tafi or Curli) has been reported to enhance survival of *Salmonella* on dry surfaces (White et al, 2006). Tafi, hair-like protein appendages with adhesins at the tip ends, and cellulose are important for attachment, biofilm formation and stress tolerance of *Salmonella* (Austin et al, 1998; Gibson et al, 2006; Tan et al,

2016). *Salmonella* mutants lacking *csgD*, which encodes a positive regulator of Tafi and cellulose, showed about 30-fold higher reductions in population on plastic surfaces and were significantly less tolerant to lyophilization in comparison to the wild type (White et al, 2006; Gibson et al, 2006). Interestingly, none of the previous transcriptome studies of *Salmonella* on LMFs and abiotic surfaces reported differential expression of genes involved in Tafi or cellulose production (Li et al, 2012; Finn et al, 2013b; Gruzdev et al, 2012; Maserati et al, 2017, Crucello et al, 2019). It is possible the genes involved in Tafi and cellulose production are not induced under desiccation, although Tafi and cellulose production may be important for survival. Moreover, the importance of Tafi and cellulose production for survival of *Salmonella* on LMFs is yet to be determined.

1.6.4 Cell envelope biogenesis

The cell envelope plays important roles in bacterial homeostasis and stress response (Sohlenkamp and Geifer, 2016). The lipopolysaccharide (LPS), a major component of the outer cell membrane, have been shown to be important for desiccation tolerance of *Salmonella*, as mutations in genes encoding various component of the LPS impaired survival (Mandal and Kwon, 2017). Moreover, *Salmonella* mutants lacking the O-polysaccharide unit of the LPS were more sensitive to desiccation on Formica melamine resin laminate surfaces (Garmiri et al, 2008). The exact role of LPS in enhancing the desiccation tolerance of *Salmonella* remains to be elucidated, although there are speculations that polysaccharides may act as a water reservoir or impact the topology of membrane-bound proteins that may be relevant for survival (Garmiri et al, 2008). However, these hypotheses are yet to be experimentally investigated. The involvement of the LPS in the survival of *Salmonella* in low-moisture conditions raises food safety concerns as LPS is a known virulence determinant of *Salmonella* and plays important

roles in swarming motility, host invasion and oxidative stress tolerance (Toguchi et al, 2000; Thomsen et al, 2003; Choi; Groisman et al, 2013; Bogomolnaya et al, 2014).

Fatty acid metabolism may be important for the survival of *Salmonella* on LMFs. Genes involved in fatty acid degradation *fadA, fadE, fadH, fadI* as well as genes associated with short-chain fatty acid biosynthesis of propionic (*prpEBCD*) and butyric acid (*gabTBD*) were upregulated in *Salmonella* under desiccation (Li et al, 2012). Moreover, *fabA* and *cfa* which are involved in unsaturated and cyclopropane fatty acid synthesis, respectively, were significantly upregulated in *Salmonella* inoculated on granulated sugar (Chen et al, 2014). Since bacteria do not store lipids in the cytoplasm (Zhang and Rock, 2008), possible changes in the fatty acid composition of *Salmonella* under desiccation would likely occur in the cell membrane. Alteration of the fatty acid composition of lipid bilayer membranes is an important adaptation strategy for bacteria (Parsons and Rock, 2013; Zhang and Rock, 2008). Exposure of *Salmonella* to different growth temperatures, lactic acid, essential oils, heat shock and magnetic field significantly altered the fatty acid composition of the membrane (Balamurugan, 2010; Siroli *et al*, 2015; Mouhoub *et al*, 2017; Porta *et al*, 2010). Membrane fatty acid composition impacts membrane fluidity, permeability, phase transition temperature and possible the topology of membrane-bound proteins (Dubois-Brissonnet *et. al*, 2011; Beranova *et al*, 2008; Casadei *et al*, 2002; Halverson and Firestone, 2000; Mouhoub *et al*, 2017). However, the membrane fatty acid composition of *Salmonella* in low-moisture environments is yet to be characterized.

1.6.5 Membrane porins and transporters

Proteins that are involved in signal transduction, inorganic ion and compatible solute transport have been identified as important determinants for survival of *Salmonella* in low-moisture environments (Finn et al, 2013; Podolak et al, 2010). The two-component EnvZ/OmpR

phosphorelay signal transduction system is important for the detection of osmotic changes in the environment and the regulation of the expression of membrane-bound porins (Feng et al, 2003; Wang et al, 2012). The EnvZ, an inner cytoplasmic membrane-bound histidine kinase, autophosphorylates in high-osmolarity conditions and then phosphorylates the response regulator OmpR which in turn regulates the expression of several stress-induced proteins, including outer membrane porins OmpF and OmpC (Lee and Kim, 2017, 2000; Oropeza and Calva, 2009; Cameron and Dorman, 2012). These porins are involved in the passive diffusion of osmoprotectants in both *Salmonella* and *E. coli* (Kempf and Bremer, 1998). Upregulation of *ompC* has been reported in *Salmonella* in high-osmolarity NaCl solutions (Balaji et al., 2005). Upregulation of *ompC* and *ompL* was reported in *Salmonella* on chocolate, black pepper, powdered milk and dog foods (Crucello et al, 2019). However, none of the membrane porin genes were differentially expressed in *Salmonella* desiccated on abiotic surfaces (Deng et al., 2012; Gruzdev et al., 2012; Li et al., 2012; Finn et al., 2013a).

The *Salmonella* genes involved in inorganic ion transport and metabolism have been identified as important survival determinants (Gruzdev et al, 2012; Finn et al, 2013; Mandal and Kwon, 2017). *Salmonella* mutants lacking genes encoding Fep*CDG* (an iron-enterobactin transporter), Pst (a cytoplasmic ATPase), PhoU (orthophosphate uptake regulator), CorA (a magnesium/nickel/cobalt transporter), and NhaA (a sodium/proton antiporter), KdpA (the potassium transport channel protein) were impaired for survival on plastic surfaces (Mandal and Kwon, 2017). Moreover, genes involved in Fe-S clusters formation (*iscA*, *iscS*, and *fdx*), phosphate transport (*pstACS*), potassium transport (*kdpFABC*), zinc/cadmium exporter (*zntA*) were upregulated in *Salmonella* desiccated on plastic surfaces and stainless steel (Gruzdev et al., 2012; Finn et al., 2013).

1.6.6 Alternative sigma factors and DNA damage repair systems

Alternative sigma factors, such as RpoS and RpoE, are important for survival of *Salmonella* in low-moisture environments (Finn et al, 2013). Alternative sigma factors regulate the expression of several stress-induced genes, including genes encoding proteins that are required for survival (Finn et al, 2013). The expression of *rpoE* and *rpoH* was upregulated in *Salmonella* desiccated on plastic and stainless-steel surfaces (Gruzdev et al., 2012; Finn et al., 2013), and the deletion of *rpoE rpoS*, and *rpoN* significantly impaired the survival of *Salmonella* on those abiotic surfaces (Gruzdev et al., 2012; Finn et al., 2013; Mandal and Kwon, 2017). Genes that are involved in DNA repair and recombination are important for the desiccation tolerance of *Salmonella* (Mandal and Kwon, 2017; Maserati et al, 2018). *Salmonella* under desiccation on microglass beads contained significant abundance of DnaJ and UvrD, proteins involved in DNA repair, compared to cells in a non-desiccated suspension (Maserati et al, 2018). Moreover, *Salmonella* mutants lacking *dam* (DNA adenine methylase) and *xerCD* (encoding site-specific tyrosine recombinase genes) were significantly impaired for survival on plastic surfaces (Mandal and Kwon, 2017).

These findings suggest that DNA damage occur in *Salmonella* under desiccation, but the extent and significance of the damage has not been characterized. Additionally, it is unclear if certain genomic regions are more susceptible to damage or if desiccation-induced DNA repair causes adaptive mutations in *Salmonella* that confer higher tolerance to desiccation.

1.7 Impact of desiccation on stress-tolerance of *Salmonella*

The heat resistance of *Salmonella* increases considerably in low-moisture environments (Podolak et al, 2010). Thermal resistance of bacteria is often measured in terms of the decimal reduction time (*D*- value) which is the time required to kill 90% (or achieve 1-log reduction) of

the bacteria at a given temperature (Finn et al, 2013). The *D*-value of *Salmonella* in sucrose solution with Aw 0.98 and 0.83 at 65.6 ^{o}C was 0.29 and 40.2 min, respectively (Sumner et al, 1991). Further, *Salmonella* inoculated into peanut butter (A_w = 0.35) was dramatically resistant to heat, with only about 1.4, 2.2, and 2.5-log reduction when heated at 70, 80 and 90 ^{o}C for 5 min, respectively (Shachar and Yaron, 2006). The heat resistance of *Salmonella* in wheat flour increased drastically as the A_w decreased, with the *D*-value at 75 ^{o}C increasing from 3.25 to 14.5 min as the A_w decreased from 0.7 to 0.3 (Smith et al, 2016). The higher resistance of *Salmonella* in LMFs has been attributed to various factors including low thermal diffusivity in low-moisture conditions, protective effect of food matrices, and increased stress tolerance of the bacteria (Smith et al, 2016; Podolak et al, 2010; Finn et al, 2013).

Desiccation tends to increase the resistance of *Salmonella* to several additional other stressors (Gruzdev et al, 2011). *Salmonella* desiccated on plastic surfaces was significantly more resistant to ethanol (10 to 30%, 5 min), sodium hypochlorite (10 to 100 ppm, 10 min), didecyl dimethyl ammonium chloride (0.05 to 0.25%, 5 min), hydrogen peroxide (0.5 to 2.0%, 30 min), NaCl (0.1 to 1 M, 2 h), bile salts (1 to 10%, 2 h), dry heat (100°C, 1 h), and UV irradiation (125 $\mu W/cm^2$, 25 min) compared to non-desiccated suspensions (Gruzdev et al, 2011). However, desiccated *Salmonella* cells became susceptible to stressors when were resuspended in water, suggesting that the acquisition of increased stress tolerance was not permanent (Gruzdev et al, 2011; Mattick et al., 2001). These findings suggest that inactivation strategies against *Salmonella* in high-moisture foods may be ineffective in low-moisture foods.

1.8 CONCLUSION

LMFs are important vehicles for *Salmonella* outbreaks due to the ability of *Salmonella* to survive on these commodities for a long period of time. LMFs may become contaminated with

Salmonella before, during and after processing and most LMF-associated outbreaks occurred due to failures in the execution of various control measures designed to mitigate contamination of food products. Although some genes and molecular mechanisms have been identified, further investigations are needed to fill critical knowledge gaps in our understanding of the mechanisms that mediate the survival of *Salmonella* in low-moisture conditions. The majority of studies that have investigated the mechanisms for survival involved transcriptome analysis. However, transcriptional response of genes may not necessarily correlate with importance for survival and some genes that may be important for survival may not be differentially expressed. Therefore, a combination of mutagenesis approaches and transcriptome analysis may prove vital for the identification of novel genes and mechanisms that are important for survival. In addition, research efforts with focus on survival mechanisms on LMFs are needed in order to identify genes that may be specific for survival of LMFs, since the majority of previous studies assessed survival mechanisms on abiotic surfaces.

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CHAPTER 2

Induction of the Viable but Non-Culturable State in *Salmonella* Contaminating Dried Fruit

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Key words: *Salmonella*, low-moisture foods, dried fruit, viable but non-culturable, survival, confocal microscopy.

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2.1 ABSTRACT

Salmonella can become viable but non-culturable (VBNC) in response to environmental stressors but the induction of the VBNC state in *Salmonella* contaminating ready-to-eat dried fruit is poorly characterized. Dried apples, strawberries and raisins were inoculated with approx. 9 log CFU/g of a five-strain mixture of *Salmonella*, dried until the water activity (A_w) approximated that of uninoculated LMFs and stored at 4 and 25°C in the dark. For dried fruit inoculated in bulk, *Salmonella* could not be recovered after drying on selective or non-selective media, even after enrichment. On spot-inoculated dried apples, the population of *Salmonella* decreased progressively to approx. 2.6 log CFU/g at 40 days and *Salmonella* could not be recovered after about 46 days of storage at 25°C, even following enrichment. *Salmonella* survived longer on dried apples incubated at 4°C, with with a 2-log reduction at 40 days and failed to be recovered after about 82 days. However, fluorescence microscopy and laser scanning confocal microscopy after staining with the LIVE-DEAD *Bac*Light Bacterial Viability kit showed that a large proportion (56-85%) of *Salmonella* cells present on dried fruit were viable at the times when *Salmonella* could not be recovered on growth media. Findings from this study suggest that *Salmonella* can enter the VBNC state on dried fruit. Therefore, microscopic and molecular methods should be used in addition to culture-based methods for efficient detection and enumeration of *Salmonella* contaminating dried fruit.

2.2 INTRODUCTION

Bacteria in the viable but non-culturable (VBNC) state fail to grow under laboratory conditions which would normally permit growth, although they remain alive and metabolically active (Oliver, 2010). Since the pioneering studies on the induction of the VBNC state in *Escherichia coli* and *Vibrio cholerae* several decades ago (Xu et al, 1982, Colwell RR, 2019), many bacteria including foodborne pathogens have been reported to enter into the VBNC state in response to stressful environmental conditions (Oliver et al 2005; Patrone et al, 2013; Wei and Zhao, 2018). Various stressors such as desiccation, low pH, low humidity, UV-radiation and thermosonication have been shown to induce the VBNC state in diverse serotypes of *Salmonella* (Cunningham et al, 2009, Gruzdev et al, 2012; Morishige et al, 2014; Liao et al, 2018). Stressors in foods or food processing environments can also induce the VBNC state (Zhao et al, 2017). For instance, a large percentage of the population of *Salmonella enterica* serovar Thompson contaminating spinach leaves transitioned into the VBNC state when exposed to chlorine (Highmore et al, 2018). Treatment of chicken breasts with lactic acid and peracetic acid induced the VBNC state in *Salmonella* contaminating the product (Purevdorj-Gage et al, 2018). Moreover, *Salmonella* inoculated into grape juice became non-culturable but remained viable after storage at 4^{o}C for 48h (Nicolo et al, 2011). However, there are major knowledge gaps regarding the occurrence or induction of the VBNC state in *Salmonella* contaminating low-moisture foods (LMFs), defined as those with water activity (A_w) below 0.6 (Beuchat and Mann, 2014).

LMFs are important vehicles for human salmonellosis and have been implicated in several salmonellosis outbreaks (Isaacs et al, 2005; Russo et al, 2013; CDC 2018; Sheth, et al, 2011). Several studies have shown that *Salmonella* is able to survive low-moisture conditions in

LMFs and on dry abiotic surfaces for an extended period of time (Beuchat et al, 2013; Finn et al, 2013). It is possible that VBNC *Salmonella* was involved in LMF-associated outbreaks where low numbers of *Salmonella* were recovered from the contaminated products (Gill et al, 1983; Danyluk et al, 2007; Gruzdev et al, 2012, Asakura et al, 2002). However, experimental evidence for the induction of the VBNC state in *Salmonella* contaminating LMFs is lacking.

Unlike many other LMFs, dried fruits are characterized by a unique combination of traits including not only low moisture content (18-26), but also high sugar content (38-66%), low pH (< 4.5), and antimicrobial phenolic compounds (USDA, 2018; Dauthy ME, 1995; Beuchat and Mann, 2014). Although the incidence of foodborne outbreaks associated with dried fruit is low compared to fresh fruit, detection of *Salmonella* and other foodborne pathogens on dried fruit raises public health concerns (Witthuhn et al, 2005; Beuchat et al, 2013). The survival of *Salmonella* on dried fruit is considerably lower than on other LMFs (Beuchat et al, 2013; Finn et al, 2013; Beuchat and Mann, 2015). The population of *Salmonella* inoculated on dried fruit (cranberries, raisins, strawberries) was reduced by approx. 2 log CFU/g after drying for 1h under atmospheric conditions (Beuchat and Mann, 2014). Upon storage of the inoculated dried fruit at 25^{o}C, *Salmonella* failed to be recovered (< 1 cfu/25g) after 21-42 days, depending on the type of fruit (Beuchat and Mann, 2014).

In the course of an investigation of the transcriptional responses of *Salmonella* to prolonged storage on various types of LMFs, presented in Chapter three , we noted that transcripts could be readily obtained from *Salmonella* inoculated on dried apples at time points that failed to yield *Salmonella* growth on selective or non-selective media. Since bacterial RNA is short-lived, with half-life ranging from seconds to a few hours (Laalami et al, 2014; Selinger et al, 2003), we hypothesized that *Salmonella* cells contaminating the dried apples may have

transitioned into the VBNC state and hence became unculturable on growth media. Therefore, the objective of the current study was to assess the induction of the VBNC state in *Salmonella* contaminating a panel of dried fruit. The survival of *Salmonella* on the dried fruit, as affected by inoculation methods and storage temperature was determined using culture-based microbiological methods. Furthermore, the viability of *Salmonella* inoculated on the dried fruit at various sampling times was determined using fluorescence and confocal microscopy.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial cultures and inoculum preparation

The strains used in this study are shown in Table 1. All the strains, except for *S.* Typhimurium D05, were provided by Dr. Nathan Anderson, U.S Food and Drug Administration, Institute for Food Safety and Health, Bedford Park, Illinois. *Salmonella* Typhimurium D05 was provided by Dr. Michael McClelland, University of California Irvine, Irvine, California. They were preserved in trypticase soy broth (TSB; Becton, Dickinson & Co. Sparks, MD) containing 0.6% yeast extract (TSB-YE, Becton, Dickinson & Co.), and 20% glycerol (Fisher Scientific, Fair Lawn, NJ) at - 80^{o}C. To prepare the inoculum suspension, frozen stock cultures of individual strains were streaked on TSA-YE plates and incubated at 37^{o}C for 24 h. A single colony of each strain was then inoculated in 3-ml TSB-YE in 15-ml conical centrifuge tubes (Becton, Dickinson & Co.) and incubated at 37^{o}C overnight. From the overnight cultures of individual strains, 100 µl was spread-plated on TSB-YE with 1.2% agar (TSA-YE, Becton, Dickinson & Co.), followed by incubation at 37^{o}C for 24 h to achieve a bacterial lawn. For each strain, the bacterial lawn from two agar plates was harvested using sterile plastic loops (Fisher Scientific), resuspended in 10 ml sterile deionized water (dH_2O), washed twice and then combined in equal volumes to achieve a 5-strain cocktail (50 ml) used to inoculate the LMFs.

2.3.2 Characteristics of dried fruit and measurement of water activity (A_w), pH, and Brix

Dried apples, dried strawberries and raisins were provided by industry sources and stored at 25°C until usage. Dried apples were of unknown variety and were provided as dried-fruit fragments of average dimensions 9 × 9 × 2 mm and average weight 0.2g / fragment; whole dried strawberries and raisins were provided as entire dried fruit, with average weight of 3 and 0.7g per individual fruit, respectively.

The A_w of dried fruit before and after inoculation, after drying, and periodically during storage was determined using a benchtop water activity meter (AquaLab model 3TE, Decagon Devices, Pullman, WA). The A_w of *Salmonella*-inoculated samples could not be measured due to pathogen concerns. Therefore, dried fruit that were inoculated with heat-killed nonpathogenic *Escherichia coli* K12 at the same volume/weight ratio as LMFs inoculated with *Salmonella* were used instead. Dried strawberries were sliced into smaller pieces in order to fit the loading chamber of the water activity meter, while dried apple fragments and raisins were analyzed without size reduction. To determine the pH of dried fruit homogenates, the dried fruit (10g) were macerated in 10 ml dH_2O in a 50-ml conical centrifuge tubes (VWR Int.) using a flame-sterilized spatula and then vortexed for 60 sec. The pH of the homogenate was determined using a benchtop pH meter (Orion model 410Aplus, Thermo Scientific, Waltham, MA). The amount of sugars and other dissolved solids in rinsates of dried fruit was measured as Brix (°Bx). Dried fruit (5g) were transferred into 10ml dH_2O in a 50-ml conical centrifuge tubes (VWR Int.), the mixtures were vortexed at high speed for 2 min and the Brix of the rinsates was determine using a pocket refractometer PAL-3 (Atago, Minato-ku, Tokyo, Japan). The A_w, pH and Brix were determined in triplicate for each type of dried fruit.

2.3.3 Inoculation, storage, and microbiological analysis of dried fruit

Dried fruit was weighed (200g) into separate sterile Whirl-Pak stomacher bags (750ml, Nasco, Fort Atkinson, WI) and inoculated at 10% volume per weight by transferring 20 ml of cell suspension into the bags in a series of four additions of 5ml each, with each addition followed by vigorous shaking by hand for 30 sec. After inoculation, the bags were shaken again for 30 sec to further mix the content and the inoculated dried fruit was spread on perforated ceramic plates in a Nalgene polypropylene desiccator (Thermo Scientific, Waltham, MA) containing drierite (W.A. Hammond drierite Co. Ltd., Xenia, OH) as desiccant. The desiccator was kept inside an incubator at 22°C and 33-48% RH for 7 days when the A_w of the inoculated dried fruit approximated that of the uninoculated product. To enumerate the population of *Salmonella* on dried fruit immediately after inoculation and after drying, 5g of dried fruit was transferred into 10 ml of dH_2O in 50-ml conical centrifuge tubes (VWR Int.). The mixtures were vigorously shaken at high speed for 60 sec, serially diluted in dH_2O and appropriate dilutions were plated on TSA-YE and XLD agar plates, followed by incubation at 37°C for 24h. The experiment was performed in three independent trials, with duplicate samples analyzed at each time point.

Further assessment of survival of *Salmonella* on dried apples utilized *Salmonella* preadapted to low pH, as described in a previous study (Beuchat and Mann, 2014). Briefly, individual strains of *S.* Agona, *S.* Tennessee, *S* Montevideo, *S* Mbandaka and *S.* Reading (described above) were grown in 1-ml TSB-YE at 37°C for 24h. From the overnight bacterial cultures, 100µl cell suspension of each strain was individually spread-plated on TSAYE supplemented with 1% glucose (TSA-YEG, Acros Organics, Fair Lawn, NJ, USA), followed by incubation at 37°C for 24h. Harvesting of the cells from the lawns and preparation of the 5-strain

cocktail as inoculum were done as described above. Dried apples were inoculated with acid-preadapted cells or non-preadapted cells and for 7 days as described above. Enumerations were done as described above on TSA-YE and XLD agar plates which were incubated at 37ºC for 24h under aerobic, microaerobic and anaerobic conditions.

In addition to commercially-provided dried apples, some experiments utilized Red Delicious and Granny Smith apples were purchased from local stores. The fruit were peeled, sliced into fragments (10 × 10 x 2 mm) and dehydrated for 12 h using a 6-tray Cabela household dehydrator (Model 54-1646, Sidney Nebraska, US). They were then stored in Whirl-Pak stomacher bags (750ml, Nasco, Fort Atkinson, WI) until use. Before inoculation, 5g of each type of dried fruit was transferred into 10 ml of dH_2O in 15-ml centrifuge tubes (VWR Int., Radnor, PA). The mixtures were vortexed at high speed, serially diluted in dH_2O and appropriate dilutions were plated on TSA-YE and Xylose Lysine Deoxycholate agar (XLD, Becton, Dickinson & Co.), followed by incubation at 37ºC for 24h to enumerate total aerobic bacteria and *Salmonella*, respectively. Each inoculation was performed in triplicate.

2.3.4 Enrichment for *Salmonella* from inoculated dried fruit

When *Salmonella* inoculated on the dried fruit was below the limit of detection (20 CFU/g), samples were enriched for *Salmonella* according to the FDA Bacteriological Analytical Manual (Andrews et al, 2016) protocol with modifications. Briefly, 5g of commercially-supplied dried apples, dried strawberries and raisins were transferred into 15 ml lactose broth (Becton, Dickinson & Co.) in 50-ml centrifuge tubes (VWR Int.) and incubated at 37ºC for 24 h. After 24h, 100µl of the lactose broth was transferred into 9 ml Rappaport-Vassiliadis broth (Oxoid, Basingstoke, UK) and incubated at 42ºC for 48 h. Suspensions (100 µl) from the lactose and

Rappaport-Vassiliadis broth were plated on XLD agar plates which were incubated at 37°C for 24h.

2.3.5 Spot-inoculation and cold storage of dried apples

Fragments of the industry-supplied dried apples were placed in sterile Petri dishes and spot-inoculated with 20 μl per fragment (approx. 10^9 CFUs) of the 5-strain *Salmonella* strain cocktail described above. The inoculated fragments were dried in a biosafety cabinet for 3 h with the Petri dish lid off, and then the Petri dishes were covered, sealed with parafilm, and stored in the dark at 22 °C or 4 °C. For enumerations, individual fragment were transferred into 1 ml of dH_2O in 1.5-ml Eppendorf tubes, and the population of *Salmonella* was enumerated on TSA-YE as described above. Dilutions of rinsates were also plated on TSA-YE with 0.1% sodium pyruvate (TSA-YEP, Acros Organics, Fair Lawn, NJ, USA) and incubated at 37°C for 24h. The experiment was conducted in three independent trials, analyzing duplicate samples at each time point.

2.3.6 Assessment of the survival of *Salmonella* in apple juice

Organic pasteurized apple juice was purchased from a local store and stored at 4 °C until usage. The pH, Brix, and background microbiota of the juice was determined as described above. Fructose (9 g, Sigma-Aldrich, St. Louis, MO) was added to 30 ml of apple juice at 30 % w/v to increase the Brix of the juice to that of dried apple rinsate described above. To test the effect of high solute concentration on the survival of *Salmonella*, 30 ml of apple juice with or without fructose in 50-ml conical centrifuge tubes (VWR Int.) was inoculated with the *Salmonella* cocktail (prepared as described above) to obtain 10^8 CFU/ml and stored in the dark at 25°C. For *Salmonella* enumerations immediately after inoculation and periodically during storage, 1 ml of inoculated apple juice with or without fructose was serially diluted in sterile dH_2O, and

appropriate dilutions were spread-plated on TSA-YE and TSA-YEP. The experiment was repeated in three independent trials.

2.3.7 Viability staining and visualization of *Salmonella* on dried fruit

Rinsates of *Salmonella*-inoculated dried fruit after drying for 7 days were stained with the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Eugene, Oregon) according to the manufacturer's instructions. Briefly, the green (SYTO 9) and red (propidium iodide) stains were mixed in equal volumes, 1µl of the mixture was added to 300 µl of rinsates from inoculated dried fruit and the dye-rinsate suspensions were incubated in the dark (25 ^{o}C) for 10 min. The suspensions were then spotted (3 µl) on glass slides, dried under ambient conditions for 2 min to fix the cells onto the slides and visualized with a fluorescence microscope (Olympus BX63, Waltham, MA). Green fluorescence (live bacteria) was captured with FITC filters while TR filters was used to detect red fluorescence (dead bacteria). The cell suspension used as inoculum and rinsates of uninoculated dried fruit were similarly stained and visualized. Visualization of *Salmonella* in rinsates of dried fruit was performed in two independent trials with duplicate samples processed per trial. Images were acquired from at least three different microscopic fields per replicate of each trial using Olympus cellSens image analysis software (Olympus, Waltham, MA). Fluorescing (live and dead) bacterial cells were counted with ImageJ (Schneider et al, 2012) to compute the percentage of viable cells as the number of green fluorescing cells over the total number of cells.

Salmonella was also visualized directly on the surfaces of the spot-inoculated dried apples using laser scanning confocal microscopy. Briefly, an equal-volume mixture of SYTO 9 and PI was diluted 1:4 with dH_20, and 5µl of the resulting mixture was added onto the *Salmonella*-inoculated spot, followed by incubation in the dark at 25^{o}C for 15 min. A small (3× 3

mm) fragment with as flat a surface as possible was excised from the stained region using a flame- sterilized razor blade and placed in 300µl dH_2O in a glass cell culture dish (P35G-1.5-20-C, MatTech, MA) with the stained surface immersed in the water. Image acquisition was performed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Inc. Thornwood, NY, USA) at the Cellular and Molecular Imaging Facility at North Carolina State University. A multiline argon laser light source and an inverted LD C-Apochromat 40×/1.1 W Korr M27 water immersion objective lens were used for all experiments. Simultaneous acquisition with two main dichroic beam splitters (MBS) at 488 and 561 nm were used to image the double marker line with the pinhole maintained at 1 AU (1.1 µm). SYTO 9 was excited at 488 nm and emission was collected at 499–561 nm while PI was excited at 561 nm with emission collected at 578 - 669nm. Images were processed using the Zeiss Zen Blue software (Zeiss, Obercohen, Germany). The experiment was conducted in three independent trials with duplicate samples in each trial, and image acquisition from at least three different microscopic fields per replicate of each trial. Fluorescing bacterial cells were enumerated as described above. VBNC *Salmonella* in inoculated apple juice with or without fructose was detected by staining 300 µl of apple juice. Visualization was performed using 2 µl of stained apple juice and viable cells was enumerated as described. The experiment was performed in three independent trials.

2.3.8 Bacterial DNA extraction and real-time PCR assay

Individual fragments of spot-inoculated dried apples after drying for 3h, and at 50 and 110 days of storage at 25 and 4°C, respectively, were transferred into 1 ml of dH_2O in1.5-ml micro-centrifuge tubes (VWR Int.). The mixtures were vortexed at high speed for 60 sec, the apple fragments were removed, and the remaining suspensions were centrifuged at 15,000 × g, 25°C for 2 min. The genomic DNA of the bacterial cells in the resulting pellet was extracted with

the Qiagen DNeasy Blood & Tissue kit (Qiagen Sciences Inc, Germantown, MD) according to the manufacturer's instructions. Genomic DNA was extracted in duplicate in two independent trials and DNA concentration was determined with NanoDrop spectrophotometer (Model ND2000C, Thermo Scientific). The quantity of the *invA* target in each DNA sample was determined using the StepOneplus Real-Time PCR systems (Applied Biosystems, Foster City, CA). The real-time PCR mix consisted of 1x iTaq Universal SYBR green supermix (Bio-Rad Laboratories Inc, Des Plaines, IL), 600nM each of forward (*invA*Forward, 5' TCGCCAGTACGATATTCAGTG 3') and reverse (*invA*Reverse, 5' AGGCCTTCGGGTTGTAAAGT 3') primers, and the DNA template in a 20-µl reaction. The thermocycler conditions used included 95^{o}C for 10 min followed by 40 cycles of 95^{o}C for 15 sec and 60^{o} C for 1 min. For dried apples after 3h of drying, five reactions containing 0.01, 0.1, 1, 10, and 100ng of template DNA were run. DNA samples from 50 and 110 days of storage at 25 and 4^{o}C, respectively, were used as template in separate reactions. The threshold cycle (C_T) values and concentrations were exported into Microsoft Excel 365 (Redmond, WA) and a plot of average threshold cycle (C_T) values against DNA concentration was computed. Since *invA* is a single-copy gene in *Salmonella* (Galan et al, 1992), detected copies of *invA* would correspond to the population of *Salmonella* (live and dead) in the sample. Total population of *Salmonella* / fragment was calculated from the standard curve generated with gDNA from dried apples after 3h of drying. Number of viable cells (cells / fragment) was estimated based on $\frac{N_v}{N_T} \times T_c$, where N_v and N_T are fluorescent microscopy-based counts of viable (green) cells and total (green or red) cells, respectively, determined as described above, while T_c is the total population of *Salmonella* (live and dead) calculated from qPCR data.

2.3.9 Assessment of the virulence and resuscitation of *Salmonella* on dried apples

The virulence of *Salmonella* on spot-inoculated commercially-supplied dried apples that were stored at 4 and 25 ^{o}C for 50 days was assessed using the *Galleria mellonella* infection model. Live fourth-instar larvae of *Galleria mellonella* (the honeycomb moth) were purchased (Vanderhorst Wholesale, Inc., Saint Mary's, OH, USA), kept at 15 ^{o}C in the original shipping containers and used within 7 days of receipt. Individual fragments of dried apples were transferred into 1ml of dH_2O inside 1.5-ml micro-centrifuge tubes (VWR Int, LLC. Radnor, PA, USA). The mixtures were vortexed at high speed for 60 s, serially diluted in dH_2O and 10µl of appropriately diluted rinsates was injected into the last left proleg of larva using a sterile 1-ml syringe and 30-G needle (Becton, Dickinson & Co.). Ten larvae were inoculated per dilution of rinsates of contaminated dried apples and another set of 10 larvae were each injected with 10 µl of appropriate dilutions of a 5-strain *Salmonella* mixtures that was prepared as described. In addition, 10 larvae were each inoculated with 10 µl of rinsate from uninoculated dried apples. All procedures were performed in a biosafety cabinet. Inoculated larvae were placed in sterile petri plates with the lids covered and incubated at 37 °C for 5 days. Larval death was monitored daily and larvae that showed no response to touch were considered dead and promptly removed. Virulence assessments were conducted in three independent trials with duplicate samples per trial.

To determine if non-culturable *Salmonella* in rinsates of contaminated dried apples would regain culturability after inoculation into larvae, commercially-supplied dried apples were spot-inoculated with cell suspension (prepared as described) of *S.* Typhimurium strain D05 and stored at 25 ^{o}C as described. Rinsate of inoculated dried apples at 65 days of storage when *Salmonella* on dried apples failed to grow on agar plates was serially diluted and appropriate dilutions were

inoculated into *G. mellonella* larvae. Another set of 10 larvae were each injected with 10µl of appropriate dilutions of the inoculum cell suspension. Inoculated larvae were incubated at 37 ^{o}C and monitored daily for mortality as described. After 24 h of incubation, three dead and three live larvae were removed, surface-disinfected by swabbing with 75 % ethanol, and transferred into 3-ml dH_2O inside a 15-ml centrifuge tubes (three dead larvae in one tube and three live larvae in another tube). The larvae were crushed with flame-sterilized glass rods, vortexed at high speed for 60 sec, and the resulting mixtures were serially diluted in dH_2O. Appropriate dilutions of the mixture were spread-plated on XLD supplemented with 50 µg/ml kanamycin (XLD+Kan, Fisher Scientific), followed by incubation at 37 ^{o}C for 24 h. The experiment was conducted in duplicates of two independent trials

2.3.10 Assessment of the capacity of *Salmonella* on dried apples to invade mammalian cells

Rinsates of commercially-sourced spot-inoculated dried apples that were stored at 25 ^{o}C for 50 days (prepared as described) were centrifuged at 15,000 × g, 25 ^{o}C for 2 min. The supernatant was removed, and the resulting pellet resuspended in 60 µl of dH_2O to be used to infect mammalian cell lines. Human intestinal Caco-2 epithelial cells was provided Dr. Jun Ninomiya-Tsuji at North Carolina State University. The mammalian cells were cultured in 500 µl of Dulbecco's modified Eagle's medium (DMEM, Fisher Scientific) supplemented with 10% bovine growth serum inside 24-well plates at 37°C in 5% CO_2 for 72 hours. The cells were then seeded in fresh 500 µl DMEM 24-well plates at a concentration of 5×10^5 cells per well on the day of infection. The mammalian cell lines were infected with 5-stain cocktail of *Salmonella* at approx. 10^5 CFU/ml to achieve a multiplicity of infection (MOI) of 1 : 1. To another sets of mammalian cell lines, 50 µl of suspensions from inoculated dried apples (described above) was added followed by incubation at 37 ^{o}C in 5 % CO_2 for 30 min. To kill unattached bacterial cells,

1 ml of gentamicin solution (100 µg/ml, Fisher Scientific) was added to the wells and the mixtures were incubated at 37 ^{o}C in 5 % CO_2 for 48 h. After the incubation, the wells were washed with sterile 500 µl PBS and then lysed in 500 µl PBS containing 0.2% Triton X-100 (Fisher Scientific). The lysates were serially diluted in PBS and appropriate dilutions were spotted (10 µl/spot) on TSA-YE agar plates which were incubated at 37 ^{o}C for 24 h. The experiment was performed in two independent trials with triplicate samples per trial.

2.4 RESULTS

The total aerobic bacterial count of commercially-supplied dried apples and dried strawberries was below the limit of detection of 20 CFU/g. Commercially-supplied raisins and laboratory-dried Red Delicious and Granny Smith apples contained approx. 2-3 log CFU/g aerobic bacteria (data not shown). *Salmonella* was not detected in any of the dried fruit before inoculation. The average A_w of dried fruit before and after inoculation and after drying is shown in Table 2. The average A_w of uninoculated dried fruit ranged from 0.41 (commercially-supplied dried apples) to 0.64 (laboratory-dried Red Delicious apples) (Table 2) and the average A_w of the inoculated dried fruit did not change significantly during storage (data not shown). The average pH of dried fruit homogenates ranged from 3.1 (Granny Smith) to 4.6 (raisins) (Table 3). The average Brix of rinsates from the commercially-supplied dried apples, strawberries and raisins was 29.4, 24.1 and 21.4^{o} Bx, respectively, while rinsates of the laboratory-dried Granny Smith and Red Delicious had average brix of 17 and 14 ^{o} Bx.

2.4.1 The viable but von-culturable state was induced in *Salmonella* contaminating dried fruit

The population of *Salmonella* on bulk-inoculated dried strawberries, commercially-supplied dried apples and raisins was 9 ± 0.2 log CFU/g immediately after inoculation. However,

Salmonella could not be recovered by culture (limit of detection, 20 CFU/g) after drying for 7 days to the original A_w (Fig 1), even following enrichment. Survival assessments were also performed using bulk-inoculated laboratory-dried Red Delicious and Granny Smith apples dried in-house, in order to determine if the rapid loss in culturability was peculiar to the supplied dried fruit, possibly due to addition of sulfites. After drying for 7 days, the population of *Salmonella* on dried Red Delicious apples decreased to 5.7 and 4.3 log CFU/g on TSA-YE and XLD, respectively (Fig. 1). Even more pronounced reductions seen with Granny Smith, where *Salmonella* could not be detected in one trial and populations were reduced to 1.6 and 1.2 log CFU/g on TSA-YE and XLD, respectively, in the two other trials (Fig. 1).

To test the potential impact of preadaptation to low pH on survival of *Salmonella* on dried apples, inoculum (9.4 log CFU/g) was prepared with cultures grown (pH 4.4) on TSA-YEG (37 ^{o}C, 24 h). However, the pre-adapted *Salmonella* cells also became undetectable after drying for 7 days (< 20 CFU/g); no colonies could be obtained on XLD or TSA-YE. Agar plates (XLD and TSA-YE) were also incubated under anaerobic and microaerobic conditions (37 ^{o}C, 24 h) to assess the potential impact of oxidative stress on the culturability of *Salmonella*. However, *Salmonella* failed to yield colonies under these conditions. In comparison to the inoculum where 99.5% of the cells fluoresced green (Fig. 2A), a significant fraction, 85± 9 and 67.3±6% in rinsates of bulk-inoculated commercially-supplied apples and strawberries, respectively (Fig. 2B and C), appeared to remain viable upon completion of the 7-day drying period. Since no fluorescing bacteria were detected on uninoculated dried apples (Fig. 2H) or strawberries (data not shown), the fluorescing bacterial cells on the inoculated products were considered to be *Salmonella.* Uninoculated raisins had significant background fluorescence from

bacterial cells which were indistinguishable from *Salmonella* (data not shown), and therefore were not pursued further with fluorescence microscopy

2.4.2 *Salmonella* survived longer on spot-inoculated dried apples stored at 25 and 4 °C

The population of *Salmonella* spot-inoculated on dried apples was 9 log CFU/g. The A_w of spot-inoculated dried apples immediately after inoculation and after drying was 0.75 and 0.54, respectively. In contrast to the rapid population reductions observed on bulk-inoculated dried apples described above where no *Salmonella* could be recovered upon drying to the original A_w, there was no significant decline on the population of *Salmonella* on the spot-inoculated after drying, and the populations on spot-inoculated apples stored at 25°C decreased progressively until *Salmonella* was undetectable (< 10 CFU/g) on agar plates on day 46 ± 2 (Fig. 3). Growth was assayed on pyruvate-supplemented media to test if protection against oxidative stress would resuscitate VBNC *Salmonella* in the current study. Assessments of the effect of storage temperature and growth on pyruvate-supplemented growth medium was performed with spot-inoculated dried apples due to the progressive reduction of *Salmonella* population. Significantly longer survival ($P < 0.0001$) was noted when the spot-inoculated apples were stored at 4°C, where *Salmonella* could be detected for as long as 82 ± 3 days (Fig. 3). At both storage 25 and 4°C, recovery of *Salmonella* was significantly higher ($P < 0.001$) on media supplemented with pyruvate (Fig. 3), in agreement with a previous study that showed improved recovery of VBNC *Salmonella* induced by H_2O_2-treatment when grown on media supplemented with sodium pyruvate (Morishige et al, 2017).

Despite *Salmonella* on spot-inoculated dried apples stored at 4 and 25 °C failing to form colonies on agar plates at 110 and 50 days, respectively, confocal microscopy showed that a high

percentage of *Salmonella* were viable at these times. Findings suggested that 68±6% of the population of *Salmonella* on spot-inoculated dried apples stored at 25ºC were viable after 50 days (Fig. 2E), and the percentage significantly ($P < 0.05$) decreased to 54±4% at 110 days of storage (Fig. 2F). Similarly, spot-inoculated dried apples stored at 4ºC contained 56±9% of viable *Salmonella* at 110 days of storage, despite not yielding colonies on growth media (Fig. 2G).

The total populations (dead and live) of *Salmonella* on spot-inoculated commercially-supplied dried apple fragments after *Salmonella* failed to be recovered by culture (50 and 110 days for apples stored at 25 and 4 ºC, respectively) were determined using qPCR. The concentration of genomic DNA isolated per fragment of inoculated commercially-supplied dried apples at 3h of drying, 50 days at 25ºC or 110 days at 4ºC was approx. 12.4 ± 1.2, 11.3 ± 0.8 and 10.6 ± 1.1µg, respectively. The average *invA* C_T value s of 100, 10, 1, 0.1, and 0.01ng DNA was approx.11.6, 15.3, 18.7, 22.3, and 25.4, respectively (Fig. 4). The *invA* C_T values of DNA samples from commercially-supplied apples stored at 25ºC for 50 days and 4ºC for 110 days were 12.12 and 12.88, respectively (Fig. 4). Based on the percentage of viable (green-fluorescing) cells in the total population, the population of viable *Salmonella* on dried apples stored at 25ºC for 50 days and 4ºC for110 days was estimated to be 8.7 and 8.6 log cells/fragment, respectively.

2.4.3 Assessing the role of high sugar concentration on the survival of *Salmonella*

The A_w and pH of pasteurized apple juice with was 0.99 and 3.9, respectively, and addition of fructose reduced the A_w to 0.83 but the pH was unchanged. Aerobic bacteria were not detected before inoculation (<10 CFU/ml). In apple juice without fructose, the population of

Salmonella was reduced from 8 log CFU/ml upon inoculation to 6.5 log CFU/ml after 24 hours, and further reduced to 5.2 log CFU/ml after 5 days of storage at 25ºC (Fig 5) Population reductions were significantly ($P < 0.001$) higher in apple juice supplemented with 30% fructose, as the population decreased to 5.3 log CFU/ml after 24 hours and was undetectable at 5 days (< 10 CFU/ml) (Fig. 5). However, despite the apparent impact of fructose content on recovery on growth media, fluorescence-based viability was not noticeably impacted as most (92 ± 4%) of the *Salmonella* cells in apple juice at 5 days of storage fluoresced green with or without fructose (Fig. 2I and J).

2.4.4 Rinsates of contaminated dried apples were lethal to *Galleria mellonella* larvae

Larvae inoculated with inoculum suspension at 10^4 CFU/ larvae showed 100% mortality after 24 h (data not shown) while inoculations at 10^3 CFU/larvae achieved 55 and 25% survival after 24 h and 5 days of incubation at 37 ºC, respectively (Fig. 6). The larvae seemed not to be affected by rinsates of uninoculated dried apples as they showed 100 % survival after 24 hours and only 20 % mortality at 5 days. However, despite not forming colonies on agar plates, rinsates of commercially-supplied, spot-inoculated dried apples after stored at 25ºC for 50 days seemed to be virulent as undiluted rinsates caused 75 and 95 % mortality in *G. mellonella* larvae after 24 h and 5 days, respectively (Fig. 6). The virulence tended to decrease with dilutions of the rinsates, as 10-fold dilutions of the rinstaes of dried apples stored at 25 ºC caused 47 and 80 % mortality in inoculated larvae at 24 and 5 days, respectively (Fig. 6).

Similarly, the survival of inoculated *G. mellonella* larvae correlated with rinsate concentration was observed in dried apples stored at 4 ºC. *G. mellonella* larvae injected with undiluted rinstates of dried apples stored at 4 ºC for 50 days showed 100% mortality after 24 h (data not shown). Larvae inoculated with 10- and 100-fold dilutions of the rinsates showed 5 and

45% survival after 24 h, respectively (Fig. 6). Furthermore, 100 % of the larvae inoculated 10-fold dilutions died after 48 hours while 20 % of larvae inoculated with 100-fold dilutions of the rinstates survived after 5 days. Since the population of *Salmonella* on dried apples stored at 4 ^{o}C for 50 days was approx.10^{6} CFU/ml (Fig. 3), 10- and 100-fold dilutions of the rinsate would correspond to 10^{5} and 10^{4} CFU/ml, respectively. Therefore, larvae inoculations with 10- and 100-fold dilutions would achieve 10^{3} and 10^{2} CFU/ larvae, respectively.

Recovery of *Salmonella* from larvae (dead or alive) proved difficult as microbiota from the larvae grew on XLD agar plates, which made identification and enumeration of *Salmonella* impossible, even at higher dilutions. Therefore, kanamycin-resistant *S.* Typhimurium KD05 was spotted on commercially-supplied dried apples so that enumerations could be made on XLD+Kan. The population of *S.* Typhimurium KD05 spot-inoculated on commercially-supplied dried apple was 10^{8} CFU/fragment at inoculation. However, *S.* Typhimurium KD05 on dried apples stored at 25 ^{o}C entered the VBNC state at 60 days of storage, and therefore failed to grow on agar plates. The survival of larvae inoculated with inoculum suspension of *S.* Typhimurium KD05 at 10^{3} CFU/ml was comparable to that of the 5-strain cocktail described above (data not shown). Further, the virulence of undiluted rinsates of dried apples inoculated *S.* Typhimurium KD05 and stored at 25 ^{o}C for 60 days was approx. 95%, as that of dried apples inoculated with 5-strain cocktail described above (data not shown). The population of *Salmonella* recovered from larvae inoculated at 10^{3} CFU/ml after incubation at 37 ^{o}C for 24 h was 10^{5} CFU/ larvae, regardless of whether the larvae was dead or alive. However, VBNC *S.* Typhimurium KD05 in the rinstaes of inoculated dried apples was not resuscitated on agar plates.

The invasion of Caco-2 cells by VBNC *Salmonella* on dried apples inoculated with 5-strain *Salmonella* cocktail was assessed to determine the virulence potential of VBNC

Salmonella. Approximately 10^4 CFU/ml of *Salmonella* was recovered from Caco-2 cells infected with inoculum suspension, indicating that the *Salmonella* strains used in the current study were able to invade Caco-2 cells. However, Caco-2 cells infected with rinsates of inoculated dried apples failed to yield *Salmonella* on agar plates.

2.5 DISCUSSION

The exact routes and levels of contamination of low-moisture foods with *Salmonella* remain largely unknown, but contamination from sources with high water activity is one of several possible scenarios. A high inoculum level was used here to inoculate the products in order to better monitor population reductions over relatively long periods of time, since *Salmonella* tend to survive longer in low-moisture environments at high inoculum level (Blessington et al, 2012; Beuchat and Mann, 2015). In addition, we wished to compare the survival data from the current study to transcriptional analyses of inoculated dried fruit and other low-moisture foods, which will be presented separately and where similarly high inoculum levels were employed.

The rapid decline in the culturabilty of *Salmonella* on dried fruit even at high inoculation level used in this current study emphasized the level of stress imposed by dried fruit. Moreover, preadaptation of *Salmonella* to acidic pH 4.4 before inoculating dried apples did not improve survival, as preadapted and non-preadapted *Salmonella* became similarly undetectable on dried apples after 7 days. Since rapid reductions in the population of *Salmonella* were also noted with apples dried in-house, especially Granny Smith, it is unlikely that the rapid reduction on the supplied apples was due to any added antimicrobial agents.

A previous study also reported rapid reduction in the population of *Salmonella* inoculated on dried fruit (dried cranberries, raisins, dried strawberries, date paste), but the observed

population reductions were lower compared to the current study (Beuchat and Mann, 2014). In that study, the inoculum was a suspension of a 5-strain *Salmonella* cocktail, preadapted to pH 4.7 and mist-inoculated on the dried fruit and date paste at approx. 7 log CFU/g followed by drying for 1 hour and storage at 4 and 25°C (Beuchat and Mann, 2014). The investigators reported reductions by 1 and 2 log CFU/g on raisins and dried strawberries, respectively, after the one-hour drying period, and by 3.5 and 4.3 log CFU/g, respectively, after storage at 25°C for 6 days (Beuchat and Mann, 2014). Differences with the current study may be due to the different inoculation methods and drying times used. Different sources of dried fruit could also result in variations in the survival of *Salmonella*. Of the five strains used in the cocktails, only *S.* Tennessee strain K4643 was used in both studies (Beuchat and Mann, 2014).

Our data suggest that the failure of *Salmonella* contaminating dried fruit to be recovered on growth media may not be due to cell death but results from the induction of the VBNC state. The ability of *Salmonella* to enter the VBNC state on dried fruit raises potential food safety concerns. Such VBNC *Salmonella* may not be detected with standard culture-based methods, resulting in false-negative results and potentially allowing contaminated foods to be released to the public. The potential health risk is particularly accentuated by the fact that dried fruit are considered a ready-to-eat product.

The method of inoculation noticeably impacted the rate at which the VBNC state was induced in *Salmonella* on dried apples. *Salmonella* on spot-inoculated dried apples stored at 25°C were culturable for much longer periods of time compared to bulk-inoculated dried apples. The faster reduction in the culturability of *Salmonella* on bulk-inoculated dried apples may be due to the extended drying time (7 days) required to restore the A_w to that of uninoculated dried apples. Additionally, drying of bulk-inoculated apples in a desiccator, in contrast to air-dried spot-

inoculated apples, may have constituted additional stress, thereby inducing the VBNC state earlier than in spot-inoculated fragments.

Salmonella survived significantly longer ($P < 0.0001$) on commercially-supplied, spot-inoculated dried apples stored at 4^{o}C than at 25^{o}C. Although the effect of temperature on survival was only tested on spot-inoculated dried apples in the current study, the protective effect of low temperature is in agreement with previous studies which used bulk-inoculation (Kimber et al, 2012; Blessignton et al, 2012) and mist-inoculation methods (Beuchat and Mann, 2014). Therefore, the ability of *Salmonella* to survive longer on LMFs at refrigeration temperature seems to be independent on the inoculation methods. However, our findings suggested that the induction of the VBNC state is possible under low temperature storage as well. Entry into the VBNC state is induced in *Salmonella* when exposed to stresses, but the exact triggering factors in dried fruits are unknown. Several stressors, including low pH, high sugar content, desiccation, and naturally-occurring antimicrobials are imposed by most dried fruit (Beuchat and Mann, 2014). We tested the survival of *Salmonella* in apple juice with or without added fructose in order to assess the impact of high sugar concentration on the induction of VBNC state in *Salmonella.* The recovery of *Salmonella* was significantly impacted by sugar concentration, with high sugar concentration resulting in more rapid loss of culturability, although viability of *Salmonella* was not noticeably impacted. Therefore, the induction of the VBNC state in *Salmonella* contaminating dried fruit may a combinatorial and potentially synergistic effect of high solute concentration, acidic pH and other stressors on dried fruit, and not solely low water activity or acid stress.

Several tools have been used to detect bacteria in the VBNC state (Ramamurthy et al, 2014). A criterion distinguishing live and dead bacteria cells is membrane integrity, which is

assumed to be intact in viable cells but disrupted in those that are dead (Berney et al, 2007). The LIVE/DEAD *Bac*Light bacterial viability stain has been widely used to determine viability of *Salmonella* (Gruzdev et al, 2012; Purevdorj-Gage, 2018). The viability stains (SYTO 9 and propidium iodide, PI) bind to nucleic acid, with propidium iodide being able to penetrate only cells with damaged cytoplasmic membranes while SYTO 9 penetrates both intact and damaged membranes cells (Stiefel et al, 2015). However, the nucleic acid binding affinity of PI is 30-fold higher than that of SYTO 9 and thus PI outcompetes SYTO 9 in dead cells (Stiefel et al, 2015). The proportion of viable *Salmonella* cells on spot-inoculated commercially-supplied dried apples stored at 25^{o}C decreased significantly ($P < 0.05$) from 68 ± 6% at 50 days to 54 ± 4% at 110 days, suggesting that a fraction of VBNC *Salmonella* died upon prolonged exposure to dried apples. The potential of VBNC *Salmonella* to cause illness is poorly understood, but a growing body of evidence suggests that VBNC cells can become resuscitated under favorable conditions (Zhao et al, 2017).

Supplementation of growth media with pyruvate and catalase, and co-culturing with eukaryotic cells are some of the methods that have been employed to resuscitate VBNC *Salmonella* (Gupte et al, 2003; Senoh et al, 2012; Morishige et al, 2017). However, the resuscitation strategies that we employed, including incubation under anaerobic and microaerobic conditions and supplementation of growth medium with sodium pyruvate, failed to resuscitate VBNC *Salmonella* in the current study. Although the recovery of *Salmonella* from the dried apples improved in the presence of pyruvate, *Salmonella* cells on spot-inoculated commercially-supplied dried apples stored at 4 and 25^{o}C for 110 and 50 days, respectively, could not be recovered on growth media in the presence of absence of pyruvate, in spite of the high fraction of live cells based on the LIVE/DEAD stain (Fig. 3).

It is worth noting that in previous studies that reported resuscitation of VBNC *Salmonella*, only a fraction of the VBNC population were resuscitated while a significant proportion remained unculturable regardless of the methods for resuscitation (Gruzdev et al, 2012; Gupte et al, 2003; Senoh et al, 2012; Morishige et al, 2017). The mechanisms involved in the resuscitation of VBNC cells remain poorly understood, but the bacterial agent involved, the factors that triggered the VBNC state, the age of the VBNC cells and method for resuscitation play important roles in resuscitation (Zhao et al, 2017; Ayrapetyan and Oliver, 2016). Therefore, although procedures employed in the current study to resuscitate VBNC *Salmonella* were not successful, other conditions that may be encountered in the environment, food processing plants, or in the human gastrointestinal tract may allow resuscitation.

The *G. mellonella* infection model has been used widely to assess the virulence of foodborne pathogens, including *Salmonella* (reference). Findings from *G. mellonella* infection assay suggested that VBNC *Salmonella* from dried apples were virulent in the larvae. However, considering that 10^8 viable cells/fragment were putatively present on inoculated dried apples stored at 25 ^{o}C for 50 days (described in Results section 2.4.2), the potential virulence of VBNC *Salmonella* seemed attenuated compared to inoculum suspension. On the other hand, rinsates of dried apples stored at 4 ^{o}C for 50 days appeared to be more lethal in *G. mellonella* larvae than the inoculum suspension inoculated at the same population (10^3 CFU/larvae). Since approx. 10^8 viable cells/fragment was potentially present on dried apples stored at 4 ^{o}C at 110 days (described in Results section 2.4.2), it is possible a fraction of the population of *Salmonella* on dried apples stored 4 ^{o}C at 50 days had entered the VBNC state, and thus not detected on agar plates. In that case, the fraction of *Salmonella* in the VBNC state may have contributed to the lethality of the rinsates in inoculated larvae.

Although rinsates of inoculated dried apples appeared to be lethal in *G. mellonella* larvae when *Salmonella* was undetectable, it is unclear if the lethality was due to potential virulence of VBNC *Salmonella* or due to the presence of lipopolysaccharides (LPS) in the membrane of both dead and live *Salmonella* cells. The LPS is an important virulence determinant of Gram-Negative bacteria and the LPS of heat-killed *Salmonella* activated TLR4 (Chessa et al, 2014), which plays an important role an in the development of septic shock during *Salmonella* infection (Wilson et al, 2008). Furthermore, the failure to recover *Salmonella* from Caco-2 cells infected with VBNC *Salmonella* could have resulted from the inability of VBNC *Salmonella* to invade Caco-2 cells. However, it is also possible that VBNC *Salmonella* invaded Caco-2 cells but remained non-culturable, and hence not detectable on agar plates. Therefore, assessments of the virulence of VBNC *Salmonella* would require further investigation using diverse infection assays, and ideally an avirulent *Salmonella* surrogate as an avirulent control.

2.6 CONCLUSIONS

The present findings provide evidence of the induction of the VBNC state in *Salmonella* on dried fruit. Although cold storage tended to prolong the culturability of *Salmonella* spot-inoculated on dried apples, it did not prevent the formation of VBNC cells. The combination of diverse stressors (such as acidic pH, high solute concentration, low $A_{w,}$ and antimicrobials) on dried fruit may be responsible for the induction of the VBNC state in *Salmonella* inoculated on these commodities. Given the potential food safety concerns associated with ready-to-eat dried fruit contaminated with VBNC *Salmonella*, molecular and microscopic methods may be valuable in supplementing culture-based approaches for accurate detection and enumeration of *Salmonella* contaminating dried fruit.

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Table 1: Bacterial strains used in this study

Strain	Description
Salmonella Agona strain 447867	Isolate from the 2008 rice and wheat puff cereal outbreak (Russo et al, 2013)
Salmonella Tennessee strain K4643	Clinical isolate from the 2006-2007 peanut butter outbreak (CDC, 2007)
Salmonella Montevideo strain 488275	Isolate from the 2009/2010 black pepper outbreak (Gieraltowski et al, 2013),
Salmonella Mbandaka 698538	Isolated from the 2013 sesame tahini paste outbreak (CDC, 2013),
Salmonella Reading	(unknown source)
Salmonella Typhimurium strain D05	A kanamycin-resistant mutant of *S*. Typhimurium 14028s containing deletions in 5 contiguous genes (*STM14_1359 to STM14_1363*).

Table 2: Water activity (A_w) measurements of low-moisture foods. In-shell pistachios, cornflakes and chocolate chunks were ground into smaller particulates while dried strawberries were sliced into smaller pieces as described in Materials and Methods. A_w measurements were taken in triplicates using a benchtop water activity meter described in Materials and Methods.

Dried fruit	Uninoculated	After inoculation	After Drying
Apple	0.56	0.65	0.53
Raisin	0.42	0.77	0.44
Strawberry	0.41	0.63	0.49
Red delicious apple	0.64	0.72	0.65
Granny Smith apple	0.51	0.64	0.51

Table 3: pH measurements of low-moisture foods employed in this study. Ground in-shell pistachios, cornflakes and chocolate chunks were each transferred into 10 ml of dH_2O while dried apples, dried raisins and dried strawberries were macerated in 10 ml of dH_2O, as described in Materials and Methods. The pH of the homogenates was determined in triplicate as described in Materials and Methods.

Dried fruit	pH
Apple	4.5
Raisins	4.6
Strawberry	4.4
Red Delicious apple	4.1
Granny Smith	3.2

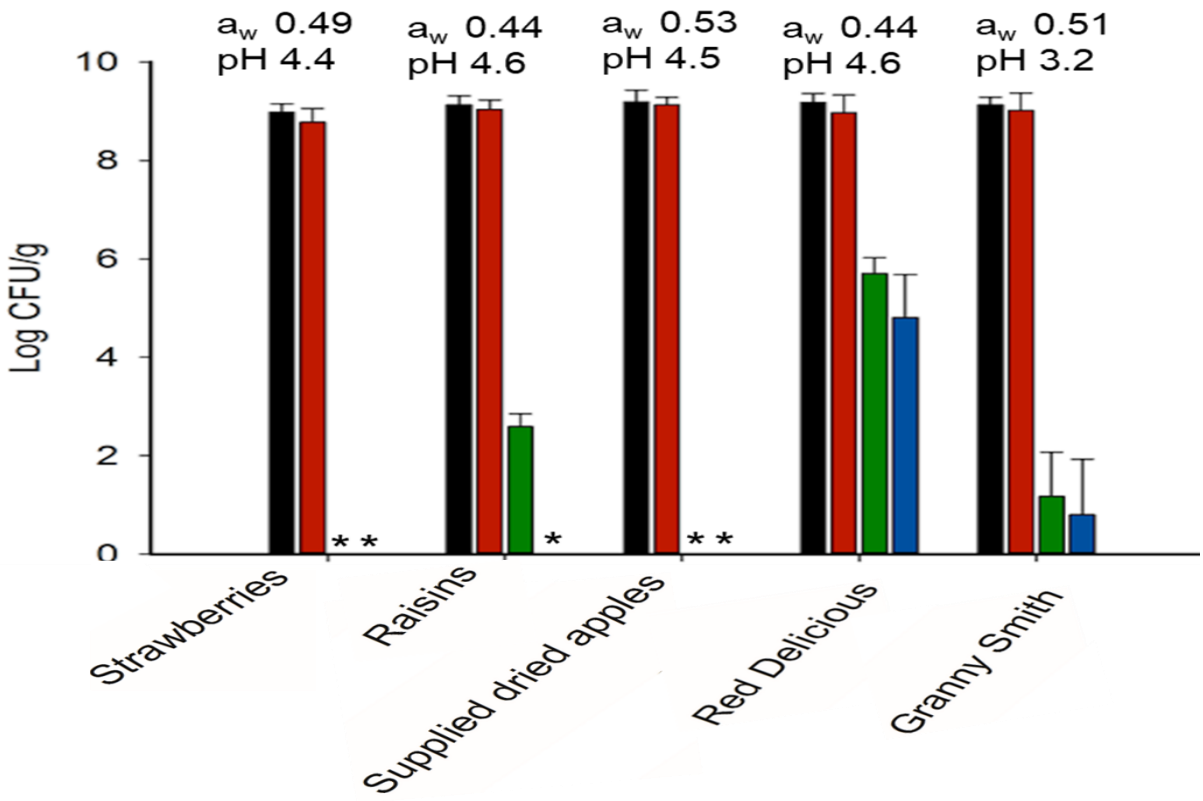
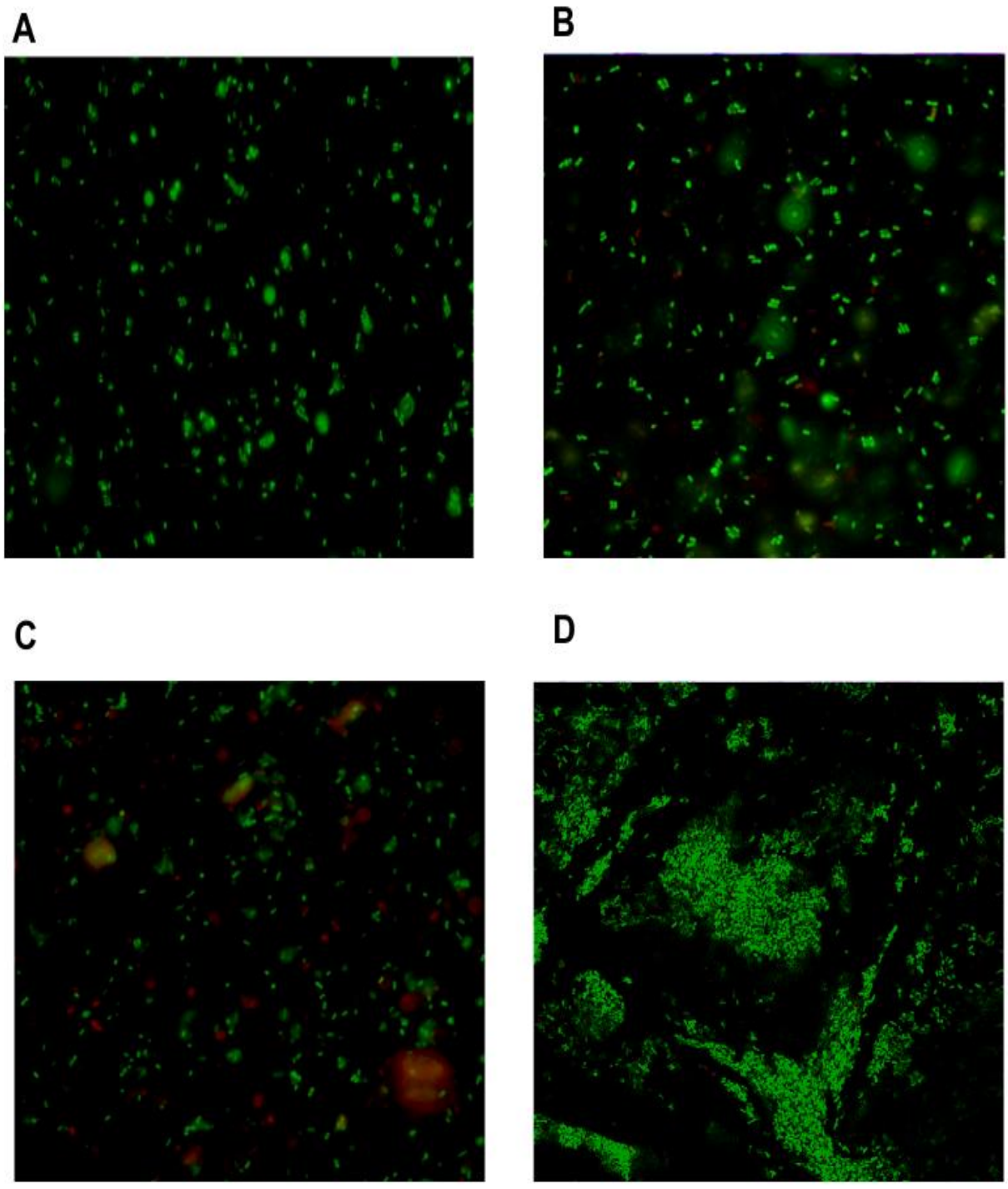


Figure 1: Survival of *Salmonella* on dried on strawberries, raisins, Red Delicious dried apples, and Granny Smith dried apples. The dried fruits were inoculated, dried and stored as described in Materials and Methods (subsection 2.1.4). Population of *Salmonella* contaminating the LMFs was enumerated as described in Materials and Methods (subsection 2.1.4) immediately after inoculation and drying. Data represent averages from two independent trials with three samples processed per trial and error bars indicate standard deviation. (*) indicates that *Salmonella* was below the limit of detection (20 cells/g). *Salmonella* was undetected on dried Granny Smith apples in the first trial; thus, survival data represent average of the second and third trials.

Figure 2: Visualization of viable and dead *Salmonella* cells contaminating low-moisture foods by a fluorescence (A-C) or a laser scanning confocal microscopy (D-J). Live (green) and dead (red) cells were visualized after staining with LIVE/DEAD BacLight bacterial viability kit as described in Materials and Methods (A); inoculum suspensions, (B); rinsate of *Salmonella*-contaminated commercially-supplied, spot-inoculated dried apples after drying for 7 days, (C); rinsate of *Salmonella*-contaminated dried strawberries after drying for 7 (D); surface of *Salmonella*-contaminated dried apples after drying for 3h, (E); surface of *Salmonella*-contaminated dried apples after storage for 50 days at 25^{o}C, (F); surface of *Salmonella*-contaminated dried apples after storage for 110 days at 25^{o}C, (G); surface of *Salmonella*-contaminated dried apples after storage for 110 days at 4^{o}C (H); surface of uninoculated dried apples, (I); apple juice with 30% fructose stored at 25^{o}C for 5 days, (J): apple juice without fructose stored at 25^{o}C for 5 days. Representative fields are shown.


A
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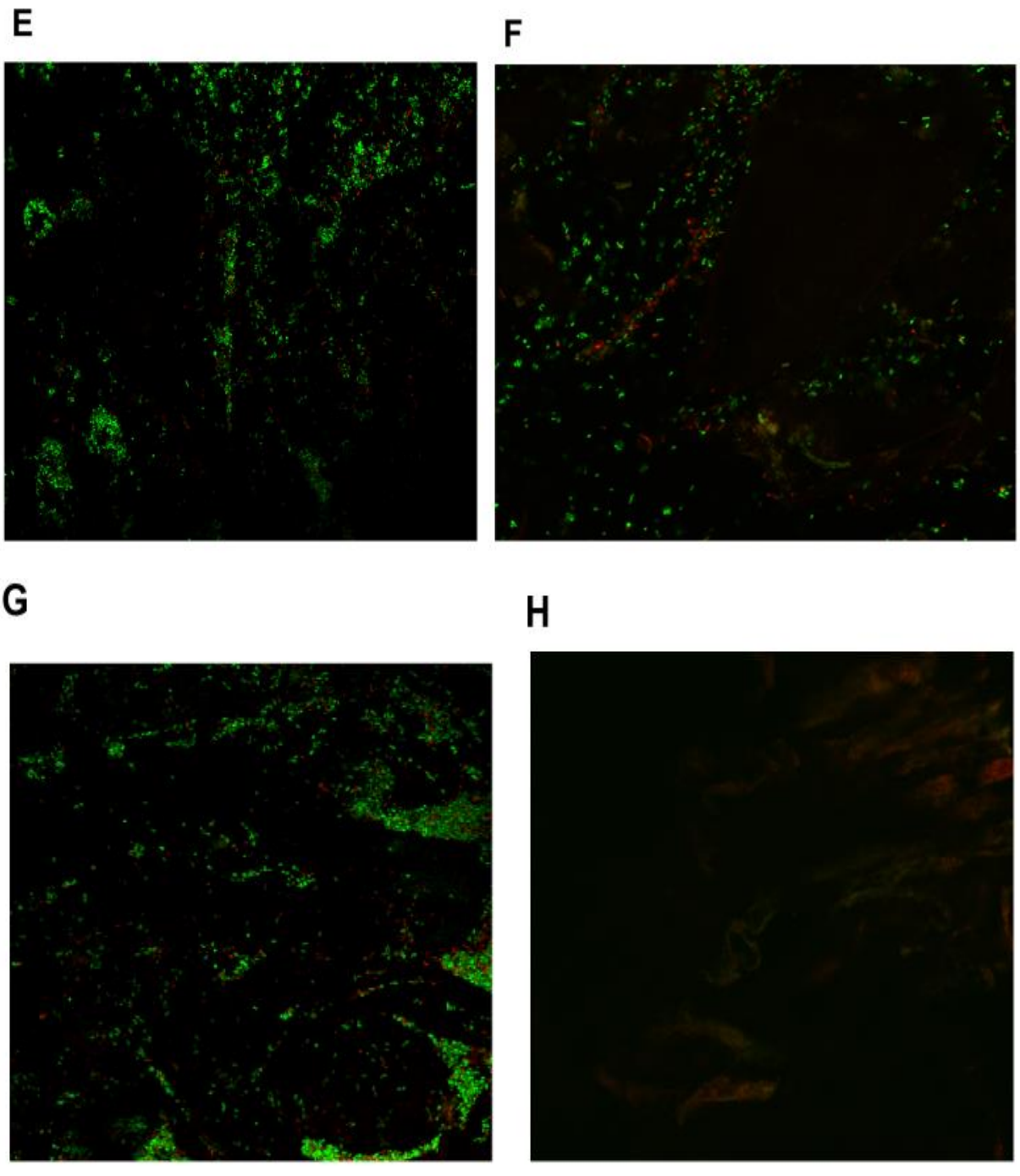
E
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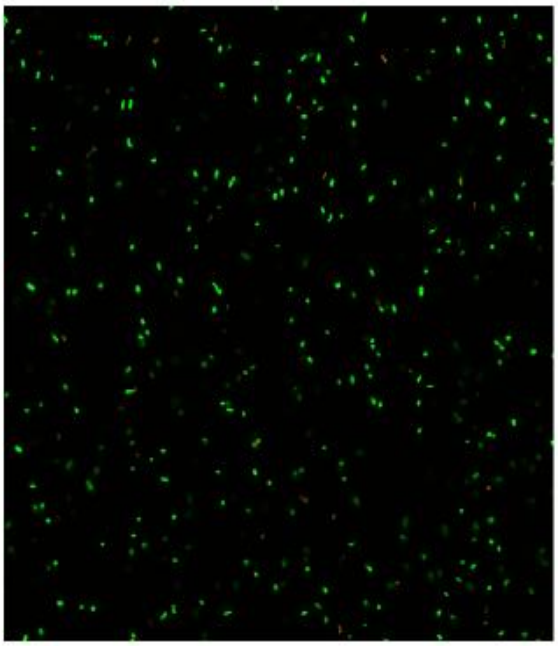
I



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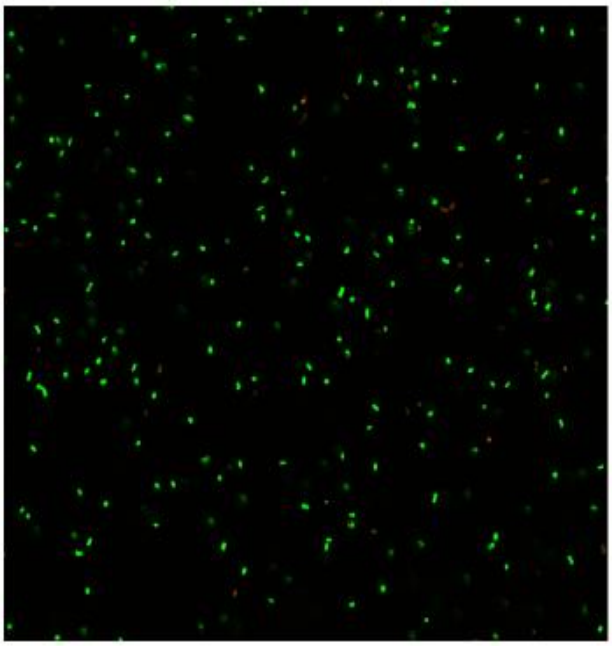

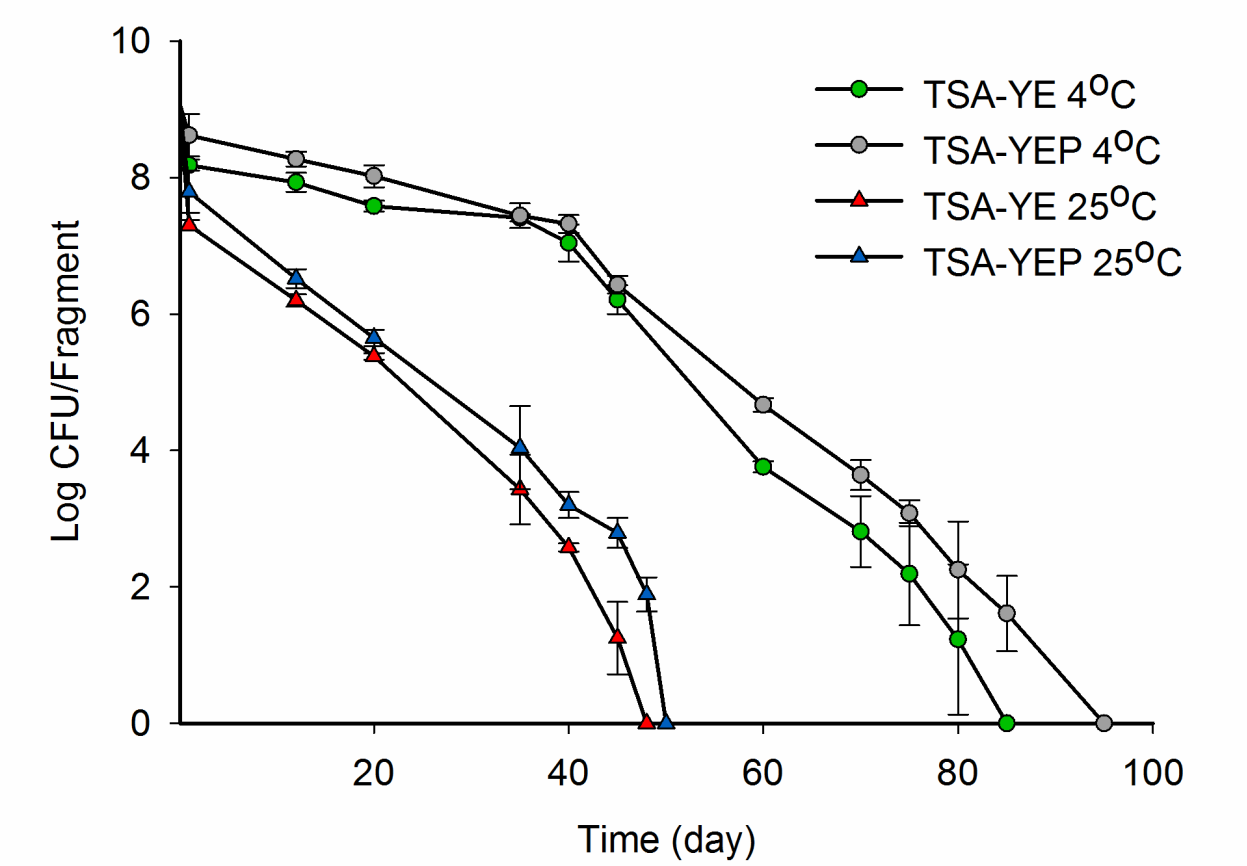

Figure 3: Survival of *Salmonella* on commercially-supplied, spot-inoculated dried apples. Individual fragments of dried apples were inoculated, dried and stored at 4 and 25°C as described in Materials and Methods. Population of *Salmonella* contaminating the LMFs was enumerated as described in Materials and Methods immediately after inoculation, after drying, and periodically during storage. Data represent averages from two independent trials with three samples processed per trial and error bars indicate standard deviation.

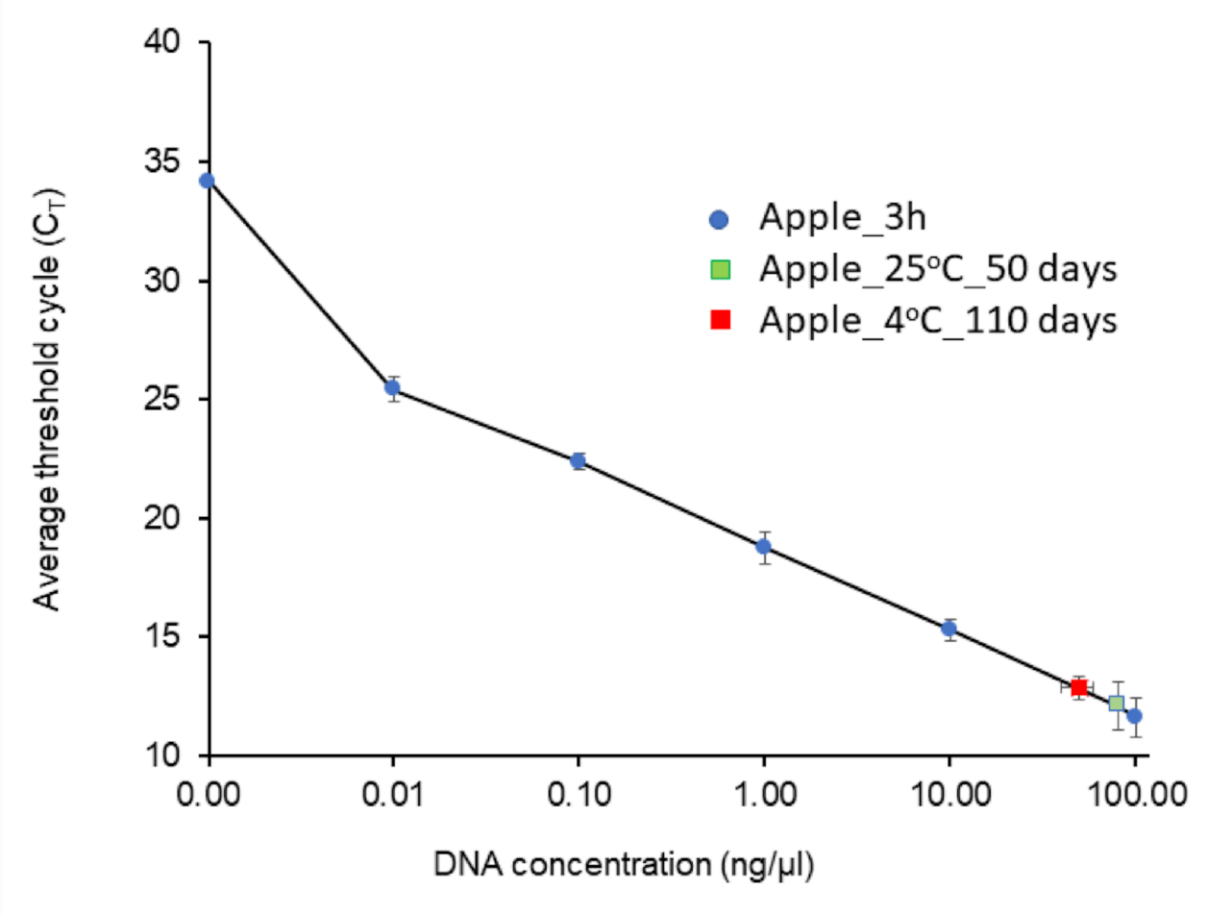

Figure 4: Standard curve for quantitative detection of total (live and dead) *Salmonella* contaminating commercially-supplied, spot-inoculated dried apples at 3h of drying (blue) 50 days of incubation at 25°C (green), and 110 days of incubation at 4°C (red). Bacterial Genomic DNA was extracted from contaminated dried apples and subjected to quantitative PCR using *invA* as target genes as described in Materials and Methods (subsection 2.1.8). The *invA* C_T values obtained by RT-qPCR were plotted against quantity of DNA. Data represent averages from two independent trials with duplicate fragments processed per trial. Vertical and horizontal error bars indicate standard deviations of C_T values and DNA concentration, respectively.

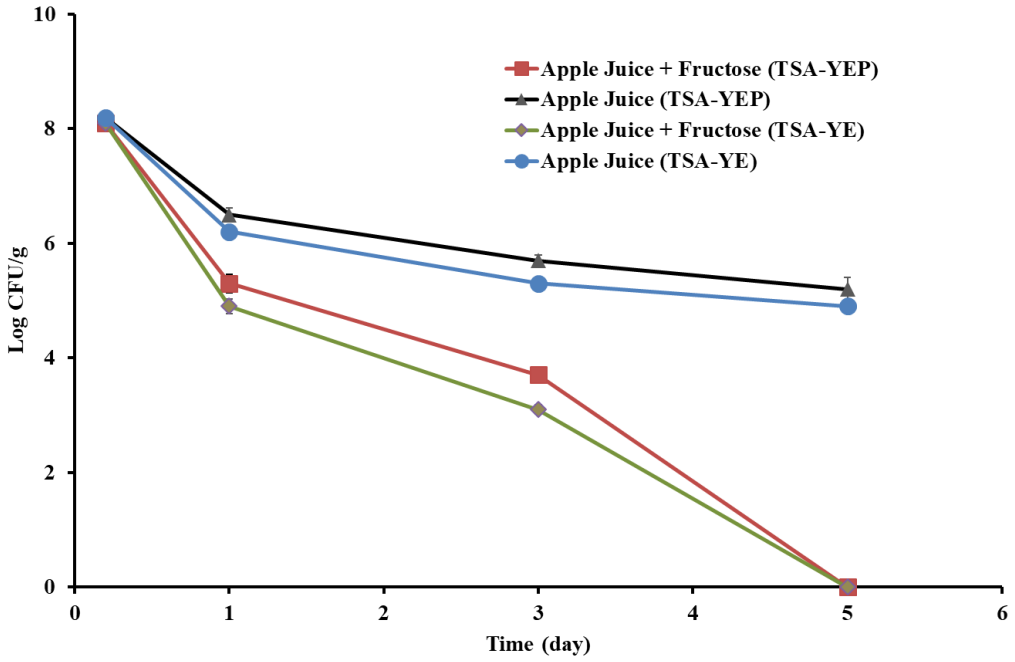

Figure 5: Survival of *Salmonella* in apple juice with (red and green lines) or without 30% fructose (black and blue lines). Thirty ml of apple juice with or without fructose was inoculated with *Salmonella* as described in Materials and Methods and stored in the dark at 25°C. The population of *Salmonella* immediately after inoculation and periodically during storage was enumerated on TSA-YE (green and blue lines) and TSA-YEP (red and black lines) as described in Materials and Methods. Dashed horizontal lines denotes the limit of detection of 10 CFU/ml. The experiment was repeated in three independent trials and error bars represent standard deviations.

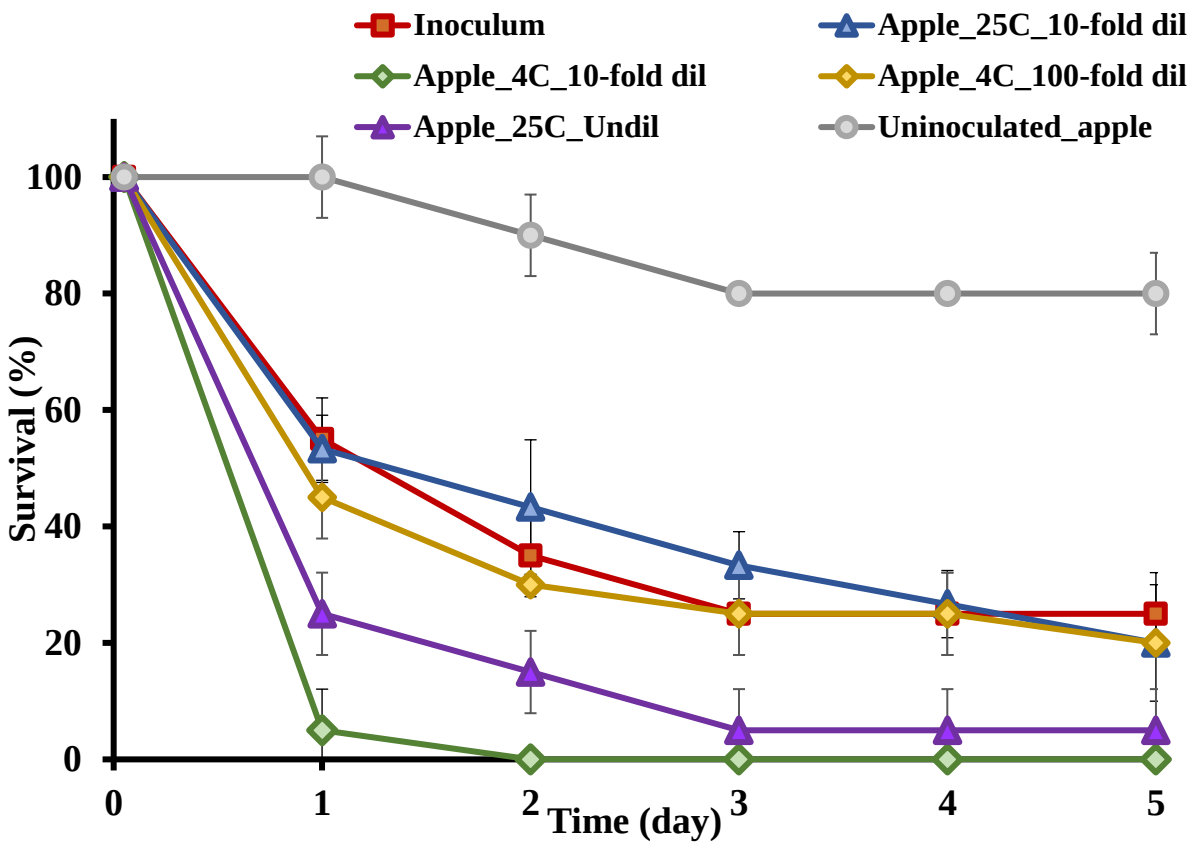

Figure 6: Survival of *Galleria mellonella* larvae injected with *Salmonella* on commercially-supplied, spot-inoculated dried apples at 50 days of storage at 4 and 25 ^{o}C. *G. mellonella* larvae were inoculated 10µl of inoculum cell suspension (red) at 10^3 CFU/ larvae; undiluted rinsate of uninoculated dried apples (grey); undiluted (purple) and 10-fold dilutions (blue) of rinsates of inoculated apples stored at 25 ^{o}C for 50 days; 10-fold dilutions (10^3 CFU/larvae, green) and 100-fold dilutions (10^2 CFU/larvae, yellow) of rinsates of dried apples stored at 4 ^{o}C for 50 days as described in Materials and Methods. The experiment was repeated in two independent trials with duplicate fragments processed per trial and 10 larvae inoculated per dilution.

CHAPTER 3

Limitations of surrogate *Salmonella enterica* serovar Typhimurium strain MMH112 as an avirulent control in the *Galleria mellonella* infection model

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Key words: *Salmonella*, avirulent, *Galleria mellonella*, virulence

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3.1 ABSTRACT

The *Galleria mellonella* infection model has been widely used to assess virulence of pathogens, including *Salmonella enterica*. However, the unavailability of naturally-occurring avirulent *Salmonella* strains that can be used as controls can complicate the interpretation of *Salmonella* virulence in *G. mellonella* larvae. *S.* Typhimurium strain MMH112 is a mutant of *S.* Typhimurium 14028s developed in a previous study as an avirulent surrogate for applications in food safety studies. In the current study, we assessed the suitability of this strain as avirulent controls in the *G. mellonella* model. Live fourth-instar larvae of *G. mellonella* were inoculated at 10^3, 10^4, and 10^5 CFU/larvae of *S.* Typhimurium 14028s and *S.* Typhimurium MMH112, followed by incubation at 37 ^oC for 5 days, and mortality of the larvae was monitored daily. A dose-dependent mortality of larvae was observed as inoculations at 10^5 CFU/larvae showed 100% mortality in larvae inoculated with *S.* Typhimurium 14028s and *S.* Typhimurium strain MMH112 after 24 h. Inoculations at 10^4 CFU/larvae caused 80 and 75% mortality in larvae inoculated with Typhimurium 14028s and *S.* Typhimurium strain MMH112, respectively after 24 h. At a lower inoculation level of 10^3 CFU/larvae, 55 and 40% of larvae inoculated with Typhimurium 14028s and *S.* Typhimurium strain MMH112, respectively, died after 24h. Overall, the virulence of *S.* Typhimurium strain MMH112 was comparable to that of *S.* Typhimurium 14028s, thus indicating the limitations of *S.* Typhimurium strain MMH112.

3.2 INTRODUCTION

The *Galleria mellonella* infection model has been widely applied to assess the virulence of various pathogens such as *Listeria monocytogenes, Staphylococcus aureus*, *Escherichia coli*, *Salmonella* and numerous microorganisms (Martinez et al, 2017; Jønsson et al, 2017; Tsai et al, 2016). For most virulence assessment applications, cell suspensions are injected into the haemocoel of last-instar *Galleria* larvae through a proleg, typically the last proleg, followed by incubation of the inoculated larvae contained at 37 ^{o}C. Mortality of the larvae is monitored daily and larvae that fail to respond to touch are considered dead (Ramarao et al, 2012). The advantages of using the *G. mellonella* larvae as an infection model are compelling. There are some structural and functional similarities between the insect immune response to bacterial infection and the innate immune response of mammals (Bergin et al, 2005). Moreover, there are positive correlations between infection outcomes in *Galleria* and other well-established models using murine, *Drosophila*, and *Caenorhabditis elegans* (Tsai et al, 2016). Lastly, the inoculated larvae can be incubated at 37° C, a temperature required for optimal growth and expression of virulence factors by many human pathogens (Ramarao et al, 2012). In addition, the *Galleria* model is easy to handle and cost-effective, and its use is not subject to the strict animal use and welfare guidelines associated with vertebrate models.

The virulence of *Salmonella* in *Galleria mellonella* larvae was demonstrated several decades ago with S. Typhimurium strains LT2 and 7 Suc LL, with the latter showing higher virulence (Kurstak et al, 1969). The study revealed that *Salmonella* caused the lysis of the hemocytes of the inoculated larvae, thereby resulting in death (Kurstak et al, 1969). Since then, several studies employed the *G. mellonella* model to assess differences in virulence among various strains of *Salmonella* (Card et al, 2016; Viegas et al, 2013; Deblais et al, 2018). Since

there is no naturally avirulent *Salmonella enterica* strain that can be used as avirulent control, previous virulence assessments used sterile diluents (sterile PBS or water) or the nonpathogenic *E. coli* strain K12 as negative control (Card et al, 2016; Viegas et al, 2013; Deblais et al, 2018). However, using the above-listed reagents as avirulent controls precludes assessments of baseline virulence of *Salmonella* in *G. mellonella* larvae.

In the current study, we assessed the suitability of *S.* Typhimurium strain MMH112 as a negative control in the *G. mellonella* model. *S.* Typhimurium strain MMH112 was derived from *S.* Typhimurium 14028s by the deletion of the major *Salmonella* pathogenicity islands SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, and *phoN* (encoding alkaline phosphatase) as well as the virulence plasmid pSLT (de Moraes et al, 2016). *S.* Typhimurium strain MMH112 was completely avirulent in the mice as female BALB/c mice that were orally-infected at 10^8 CFU did not show disease symptoms (de Moraes et al, 2016). Moreover, no cells of the avirulent strain were detected in the spleen, liver, Peyer's patches, or large intestine of the animals (de Moraes et al, 2016). In contrast, mice infected with the *S.* Typhimurium 14028s showed disease symptoms and the organs were positive for the wild-type strain (de Moraes et al, 2016). Further, the persistence of this avirulent strain on tomatoes and leafy greens as well as its biofilm formation and stress tolerance were comparable to that of the wild-type (de Moraes et al, 2016). Thus, the avirulent strain has potential as surrogate in food safety studies. Therefore, the objective of the current study is to assess the virulence of *S.* Typhimurium strain MMH112 in *G. mellonella* larvae.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains and Inoculum preparation

Salmonella enterica sv Typhimurium strain 14028s, isolated from 4-week-old chickens in 1960 (Jarvik et al, 2010), was kindly provided by Dr. Michael McClelland (University of California, Irvine, CA). *S.* Typhimurium strain MMH112 (de Moraes et al, 2016) was provided by Dr. Elliot Ryser (Michigan State University, East Lansing, MI). The strains were preserved at -80 ^{o}C in Luria-Bertani broth (LB; Becton, Dickinson & Co. Sparks, MD) containing 20% glycerol (Fisher Scientific, Fair Lawn, NJ). Bacterial cultures were grown in LB or on LB agar containing 1.2% agar (Becton, Dickinson & Co.) at 37 ^{o}C for 24 h. To prepare the inoculum, single colonies were grown in 3 ml of trypticase soy broth (TSB; Becton, Dickinson & Co. Sparks, MD) in 15-ml centrifuge tubes (VWR Int. Radnor, PA) at 37 ^{o}C for 24 h. The bacterial cultures were centrifuged at 15,000 ×g, 25 ^{o}C for 1 min and the pellets were washed twice with sterile deionized water (dH_2O).

3.3.2 Inoculation of *Galleria mellonella* larvae

Fourth-instar larvae of *G. mellonella* were purchased (Vanderhorst Wholesale, Inc., Saint Mary's, OH, USA), kept in their original shipping containers at 15 ^{o}C, and used within 5 to 7 days. For inoculations, cell suspensions were serially diluted in sterile dH_2O and 10 µl injected into the last left proleg of each larva at 10^3, 10^4, and 10^5 CFU/larva using a sterile 1-ml syringe and 30-G needles (Becton, Dickinson & Co. Sparks, MD). Ten larvae were inoculated per dilution per strain and control larvae included 10 that were injected with the equivalent volume of sterile dH_2O, as well as 10 that were not inoculated. The larvae were placed in 150-mm plastic Petri plates (10 larvae per plate) and incubated at 37 °C for up to 5 days. Larval death was monitored daily based on melanization as well as a lack of movement of the larva in response to

touch, and dead larvae were promptly removed. Virulence assessments were conducted in four independent trials.

3.3.4 RESULTS AND DISCUSSION

Larvae injected with sterile dH_2O or non-injected larvae showed no mortality at 3 days and only 10% or lower mortality at 5 days. Dose-dependent mortality of *G. mellonella* larvae was noted upon inoculated with *S.* Typhimurium 14028s and *S.* Typhimurium MHM112 as inoculations with 10^5 CFU resulted in 100% mortality after 24 h for both strains (data not shown). Inoculation with 10^4 CFU *S.* Typhimurium 14028s caused 80 and 97% mortality at 24 h and 5 days, respectively (Fig. 1A). Similarly, larvae inoculated with *S.* Typhimurium MHM112 at 10^4 CFU / larva showed 70 and 87.5 % mortality at 24 h and 5 days, respectively. Lower mortality was noted with 10^3 CFU, with approx. 50% mortality at 24 h for both strains respectively (Fig. 1B). Overall, the virulence of *S.* Typhimurium MMH112 was comparable to that of the wild-type with no significant difference ($P > 0.05$) in mortality at infection dose 10^3 CFU/larva (Fig. 1B). At dose 10^4 CFU/larva, the mortality of larvae inoculated with *S.* Typhimurium 14028s was significantly higher than that of *S.* Typhimurium MHM112 at 3 and 4 days ($P < 0.01$), but not at other sampling points (Fig 1A).

The virulence of *Salmonella* observed in the current study corroborates findings from previous studies that reported 100% mortality in larvae injected with 10^5 CFU, and 80 to 90% mortality with 10^4 CFU within 24 hours (Bender et al, 2013; Card et al, 2016). Further, melanization (systemic formation of the brown-black pigment melanin) is part of the immune response of Arthropoda to infection and can be used to evaluate insect health (Bender et al, 2013). In the current study, melanin production by *G. mellonella* larvae inoculated with *S.* Typhimurium 14028s and *S.* Typhimurium MMH112 was similar (Fig. 2B and C). The virulence

of *S.* Typhimurium MHM112 was unexpected as previous studies have shown that certain *Salmonella* mutants that were avirulent in mice were also avirulent in *G. mellonella* larvae (Viegas et al, 2013; Mil-Homens et al, 2018; Wang-Kan et al, 2017).

However, the genes (*arcB*, *bolA*, RNase E and RNase III) deleted in these previous studies (Viegas et al, 2013; Mil-Homens et al, 2018; Wang-Kan et al, 2017) were different from the genes (SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, and *phoN*) deleted in the current study. Moreover, *Salmonella* mutants harboring deletions in SPI-1 and SPI-2 showed strong invasion impairment in hemocytes isolated from *G. mellonella* hemolymph but showed insignificant attenuation of virulence in *G. mellonella* larvae (Viegas et al, 2013). Although SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5 deleted in *S.* Typhimurium MMH112 are known virulence determinants, other virulence factors that are still functional in *S.* Typhimurium MMH112 may be responsible for its lethality in *G. mellonella* larvae. Therefore, *S.* Typhimurium MMH112 may not be suitable as an avirulent control in the *G. mellonella* infection model. The potential of *Salmonella* mutants lacking other virulence determinants, such as *hfq* and LPS genes as avirulent determinants in the *Galleria mellonella* should be investigated.

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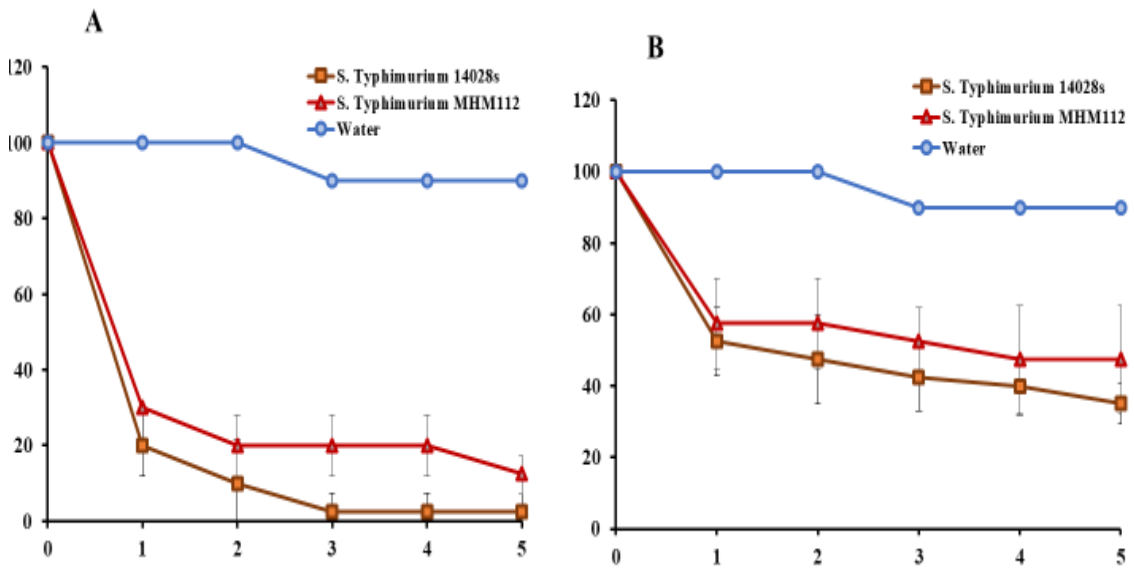
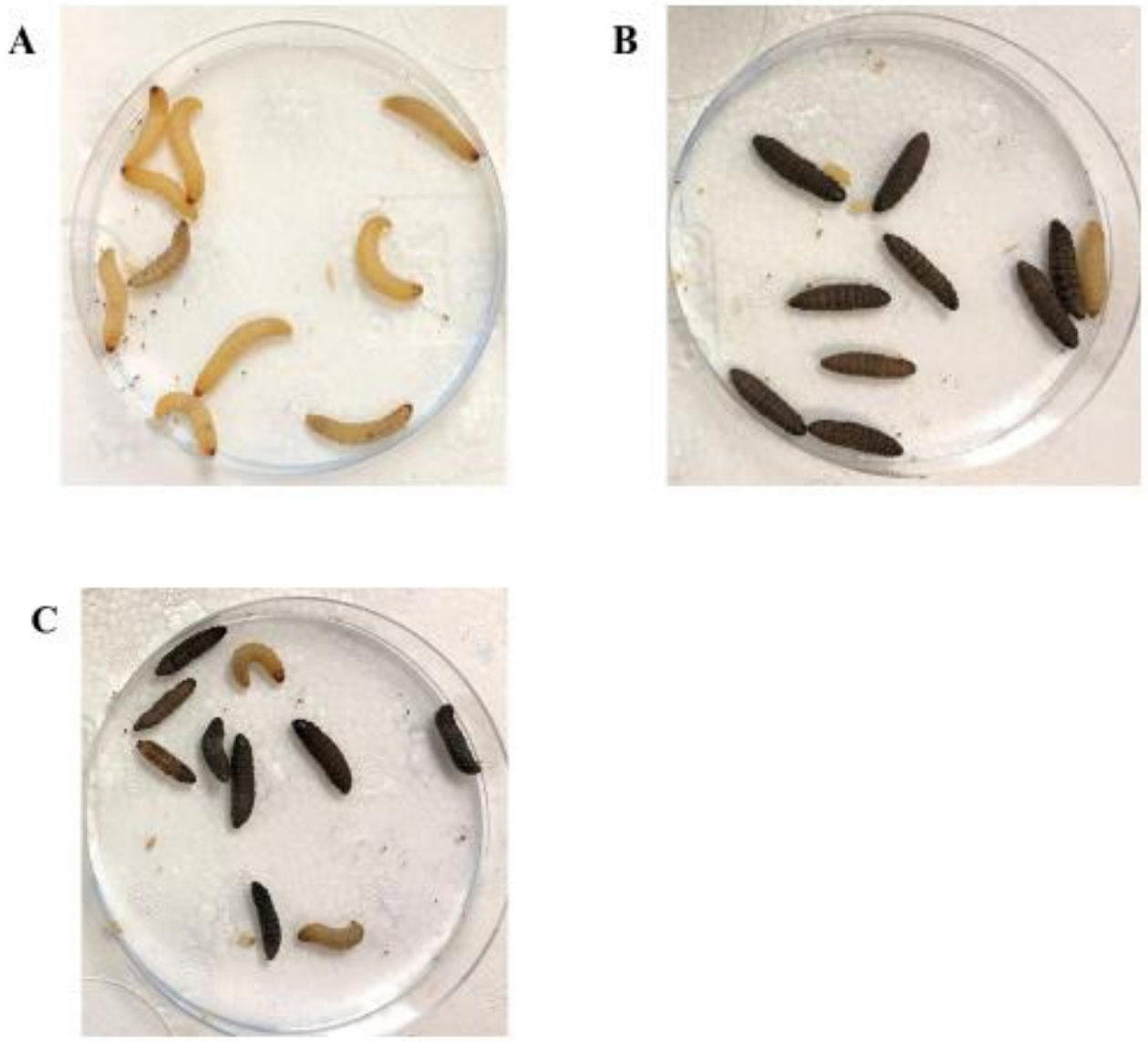


Figure 1. Dose-dependent killing of *G. mellonella* by S. Typhimurium 14028s and *S.* Typhimurium MMH112. *G. mellonella* larvae were inoculated with, (A): 10^4 CFU or cells/larva and (B): 10^3 CFU or cells/larva, incubated at 37 ^{o}C, and monitored for daily for 5 days as described at Materials and Methods. Data represent average from four independent trials with 10 larvae inoculated per dilution per trial. Error bars represent standard deviation



Figures 2: *Galleria mellonella* larvae injected with (A): sterile water, (B): 10^4 CFU/ larva of *S.* Typhimurium 14028s, and (C): 10^4 CFU/ larva of *S.* Typhimurium MMH112 after incubation at 37^{o}C for 24 h.

CHAPTER 4

Global transcriptomic analysis of *Salmonella enterica* serovar Enteritidis (PT30) on low-moisture foods

4.1 ABSTRACT

Salmonella is the leading foodborne pathogen associated with low-moisture foods (LMF) but the mechanisms underlying its survival in LMF are not fully understood. In the current study, the global transcriptional response of *Salmonella* in selected low-moisture foods was assessed. Individual fragments of in-shell pistachios, cornflakes and dried apples were spot-inoculated (20 µl /fragment) with suspensions of *Salmonella* enterica serovar Enteritidis PT30. The inoculated LMFs were placed in petri dishes, air-dried for 3 h and stored at 25 ^{o}C in the dark. The population of *Salmonella* on the LMFs immediately after inoculation, after drying for 3h and periodically during storage was enumerated on TSA-YE and on XLD agar. Total RNA of *Salmonella* in the inoculum and on LMFs at 3h, 1, 5 and 12 weeks was isolated, ribo-depleted, sequenced and analyzed. The population of *Salmonella* on all tested LMFs was approx. 10^9 CFU /fragment after inoculation with no significant reduction after drying for 3 h. The population of *Salmonella* on cornflakes and pistachios gradually decreased to approx. 7 log CFU/fragment at 12 weeks of storage. *Salmonella* was undetectable in dried apples after 42 d even with enrichment, although *Salmonella* transcripts were detected from dried apples at 12 weeks. Several genes were differentially expressed on the LMFs as soon as after 3 h of drying compared to the inoculum suspension. For all products, the transcriptome of *Salmonella* after drying at 3h was noticeably different from transcriptome at 1, 5 and 12 weeks of storage, with no major changes in transcriptome thereafter. Several differentially-expressed genes were identified in all tested LMFs at all tested times, while some were upregulated only in specific LMF-time combinations. Some of the differentially expressed genes included those involved in fatty acid biosynthesis, osmoprotectant biosynthesis and transport, DNA repair and RNA degradation, biosynthesis of aromatic compounds and stringent response factors.

4.2 INTRODUCTION

Salmonella can survive on low-moisture foods and dry abiotic surfaces for a long period of time, but the molecular mechanisms employed for survival are not fully characterized. Few studies have identified some determinants for survival of *Salmonella* under low-moisture conditions. Transcriptome analysis of *Salmonella* desiccated on stainless steel revealed significant upregulation of genes involved in phosphate transport (*pstACS*) and osmoprotectant transport (*proP, proU* and *osmU*) and the deletion of *proP* negatively impacted the survival of *Salmonella* on stainless steel (Finn et al, 2013). However, phosphate transport and osmoprotectant transport genes were not differentially expressed in *Salmonella* desiccated on plastic surfaces (Gruzdev et al, 2012). Instead, the *kdpFABC* operon, encoding a potassium transport channel, were among the most highly-induced genes in *Salmonella* desiccated on plastic, and a *kdpA* deletion mutant was impaired for long-term survival (Gruzdev et al, 2012). In another study, genes involved in fatty acid metabolism were the most significantly upregulated genes in *Salmonella* on filter paper discs (Li et al, 2012). Potential involvement of some virulence factors in the desiccation tolerance of *Salmonella* has been shown as virulence factors (*sseA, sseD, sscA, sopD, e.t.c*) were significantly upregulated in *Salmonella* desiccated on polycarbonate filters, and *ΔsseD* and *ΔsopD Salmonella* deletion mutants were impaired for survival on glass beads (Maserati et al, 2017).

Overall, these studies suggested that genes involved in various molecular functions, such as amino acid metabolism, biosynthesis of trehalose (osmoprotectant), inorganic ion transport, diverse transcriptional regulators and transporters, and several stress-induced genes were differentially expressed in *Salmonella* desiccated on various abiotic surfaces (Li et al, 2012; Finn et al, 2013a; Gruzdev et al, 2012; Maserati et al, 2017). However, there were variations in

the transcriptional response depending on the type of surface and the environmental conditions (Li et al, 2012; Finn et al, 2013a; Gruzdev et al, 2012; Maserati et al, 2017). Therefore, transcriptional response and mechanisms that may be unique to survival on LMFs may not have been detected in the studies that analyzed the transcriptome of *Salmonella* on abiotic surfaces.

A recent study analyzed the transcriptome of *Salmonella* on chocolate, black pepper, skim milk and dried pet food and identified some genes that were differentially expressed on these products but not on abiotic surfaces (Crucello et al, 2019). Various functional groups, including propionate metabolism, citric acid cycle, lipopolysaccharide biosynthesis, glyoxylate and dicarboxylate metabolism were enriched in *Salmonella* inoculated on the LMFs after 72 hours (Crucello et al, 2019). However, the study involved a single trial and focused on the transcriptional response of *Salmonella* to only short-term (72 hours) survival on LMFs (Crucello et al, 2019) which may not have captured the mechanisms employed for long-term survival.

The objective of the current study was to analyze the transcriptional response of *Salmonella* on three model LMFs (pistachios, dried apples, and cornflakes) after drying and during a short, medium and long-term storage. The *Salmonella* transcripts on each product were sequenced using RNA-Seq and analyzed to identify genes that were differentially expressed on the products relative to the inoculum cell suspension. The transcriptome of *Salmonella* was compared among the products and across the sampling time points to determine the effects of product type and storage time on the transcriptional response of *Salmonella*.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strain and growth conditions

The *Salmonella enterica* serovar Enteritidis strain ATCC BAA-1045 (PT30) was isolated from contaminated almonds implicated in the 2005 international *Salmonella* outbreak (Isaacs et

al, 2005) and kindly provided by Dr. Wei Zhang (Institute for Food Safety and Health, Illinois Institute of Technology, Bedford Park, IL). The strain was preserved at -80 ^{o}C in Luria-Bertani broth (Becton, Dickinson & Co. Sparks, MD) and 20% glycerol (Fishr Scientific, Fair Lawn, NJ). Unless otherwise stated, *Salmonella* was grown statically in trypticase soy broth (TSB; Becton, Dickinson & Co. Sparks, MD) containing 0.6% yeast extract (TSB-YE, Becton, Dickinson & Co.), TSB-YE with 1.2% agar (TSA-YE, Becton, Dickinson & Co.), and Xylose Lysine Deoxycholate agar (XLD, Becton, Dickinson & Co.) at 37 ^{o}C for 24 h.

4.3.2 Characteristics of low-moisture foods and measurement of water activity (Aw) and pH.

The model low-moisture foods (LMFs) used in this study include in-shell pistachios, cornflakes, and dried apples. These LMFs were supplied by industry sources and stored at 25 ^{o}C until usage. Dried apples were provided as dried-fruit fragments of average dimensions (9 × 9 × 2 mm) and average weight 0.2 g/fragment. In-shell pistachios and cornflakes had average weight of 0.9 and 0.1g /fragment, respectively. Before inoculation, 5g of each LMF was transferred into 10 ml of dH_2O in 50-ml centrifuge tubes (VWR Int., Radnor, PA). The mixtures were vortexed at high speed, serially diluted in dH_2O and appropriate dilutions were plated on TSA-YE and XLD followed by incubation at 37 ^{o}C for 24 h to enumerate total aerobic bacteria and *Salmonella*, respectively. Each inoculation was performed in triplicate.

The A_w of LMFs before and after inoculation, after drying, and periodically during storage was determined using a benchtop water activity meter (AquaLab model 3TE, Decagon Devices, Pullman, WA). The A_w of *Salmonella*-inoculated samples could not be measured due to pathogen concerns. Therefore, LMFs that were inoculated with heat-killed nonpathogenic *Escherichia coli* K12 at the same volume/weight ratio as LMFs inoculated with *Salmonella* were

used instead. Pistachios and cornflakes were ground into smaller particulates a coffee grinder (Mr. Coffee, Cleveland, OH) for 30 s for A_w measurement, while the A_w of dried apples was measured directly without size reduction. To determine the pH of LMFs, dried apples (10g) were macerated in 10ml dH_2O in a 50-ml conical centrifuge tubes (VWR Int.) using a flame-sterilized spatula, while ground pistachios and cornflakes (10g) were transferred into separate 10ml dH_2O in a 50-ml conical centrifuge tubes and then vortexed for 60 sec. The pH of the homogenate was determined using a benchtop pH meter (Orion model 410Aplus, Thermo Scientific, Waltham, MA).

4.3.3 Inoculation, storage and microbiological analysis of low-moisture foods.

To prepare the inoculum suspension, frozen stock cultures of *S.* Enteritidis PT30 was streaked on TSA-YE plates and incubated at 37ºC for 24 h. After 24 h, a single colony was grown in 3-ml TSB-YE in 15-ml conical centrifuge tubes (Becton, Dickinson & Co.) at 37 ºC overnight. From the overnight cultures, 100 µl of bacteria was spread-plated on TSA-YE, followed by incubation at 37 ºC for 24 h to achieve a bacterial lawn. The bacterial lawn from one plate was harvested using sterile plastic loops (Fisher Scientific, Fair Lawn, NJ), resuspended in 10-ml sterile deionized water (dH_2O), washed twice and used to inoculate the LMFs. Individual fragments of each LMF was spot-inoculated with 20 µl of cell suspensions per fragment (approx. 10^9 CFUs). The inoculated fragments were dried inside a biosafety cabinet for 3 h with the Petri dish lid off, and then the Petri dishes were covered, sealed with parafilm, and stored in the dark at room temperature (approx. 22 ºC). For enumerations, individual fragments were each transferred into 2ml of dH_2O in 15-ml conical centrifuge tubes, and the population of *Salmonella* was enumerated on TSA-YE and XLD as described above. The experiment was conducted in three independent trials with duplicate fragment analyzed per trial.

4.3.4 Isolation of total bacterial RNA

Total bacterial RNA in the inoculum cell suspension, and on LMFs after drying for 3 h and at 1, 5 and 12 weeks of storage was isolated using a combination of chloroform extraction and the Promega SV Total RNA Isolation System. In brief, two fragments of each LMF were transferred into 3 ml RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) in 15-ml conical centrifuge tubes (Becton, Dickinson & Co.). The mixtures were incubated at room temperature or 5 min to ensure that the bacterial RNA was stabilized and then vortexed at high speed for 2 min. The rinsates were transferred into two 1.5-ml centrifuge tubes (VWR Inc.), centrifuged at 12,000 ×g, 4 ^{o}C for 5 min, and the cell pellets were washed twice in 1ml cold Tris-EDTA buffer (TE; 10 mM Tris, 1 mM EDTA [pH 8.0]; Fisher Scientific), followed by centrifugation (as described) and the supernatants were discarded. The resulting cell pellets were lysed with 100 µl of TE containing 6 mg/ml lysozyme (Sigma-Aldrich, Darmstadt, Germany) at room temperature for 5 min, followed by the addition of 75-µl cold RNA lysis buffer (Promega SV system). The mixture was mixed by pipetting, 300 µl of RNA dilution buffer (Promega SV system) was added, and the resulting mixtures were mixed thoroughly by inversion. Chloroform (200µl, Sigma-Aldrich) was added to the mixtures, followed by incubation at room temperature for 5 min with the mixtures thoroughly mixed by inversion every 1 min during incubation. The mixtures were then centrifuged at 15,000 ×g, 4 ^{o}C for 10 min and the clear liquid layer containing RNA was transferred into fresh 1.5-ml centrifuge tubes. To precipitate RNA, an equal volume of 95% ethanol (Fisher Scientific) was added to the liquid, the mixture was vortexed at high speed for 1 min and transferred into the Spin columns included in the Promega SV total RNA isolation system. RNA purification and DNA removal were performed with the Promega

SV total RNA isolation system according to the manufacturer's instructions and purified RNA samples were preserved at -80 ^{o}C until sequencing.

4.3.5 RNA-seq and data analysis

RNA samples were processed and sequenced at the Genomic Sciences Laboratory at North Carolina State University. In brief, total RNA integrity was assessed using capillary electrophoresis with 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) and ribosomal RNA were depleted with the Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, CA, USA) according to the manufacturer's instructions. The resulting mRNA samples were converted to Illumina sequencing libraries using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England Biolabs Inc., Ipswich, MA) and sequenced with Illumina HiSeq 2500 (150 b, single-read, Illumina Inc., San Diego, CA) to yield 9-10 million reads per sample. Reads were quality-filtered and demultiplexed with CASAVA (Consensus Assessment of Sequence and Variance) version 1.8, pipeline (Illumina, Inc.).

Demultiplexed sequences were analyzed using the CLC Genomics Workbench version 12 (Qiagen Bioinformatics, Redwood City, CA). Sequence reads were mapped to *Salmonella* Enteritidis strain P125109 (GenBank accession number AM933172.1) allowing maximum of two mismatches. Transcripts in each sample were counted, normalized as transcript per million (TPM) and reads per kilobase of transcript, per million mapped reads (RPKM), and gene expression levels in *Salmonella* recovered from LMFs were compared to inoculum cell suspension to determine changes in gene expression on LMFs, Log_2 foldchange (Log_2FC). Genes with at least $Log_2FC \geq | 2 |$ change in expressed with an adjusted *P* value (False discovery rate, FDR) < 0.05 were considered differentially expressed. Gene ontology analysis and functional

annotation were performed using the eggNOG-Mapper (Huerta-Cepas et al, 2017) and Gene Ontology Resource (Mi et al, 2019), respectively.

4.4 RESULTS AND DISCUSSION

4.4.1 Survival of *Salmonella* on low-moisture foods

The population of *Salmonella* on pistachios, cornflakes, and dried apples immediately after inoculation was approx. 10^9 CFU /fragment with no significant reduction in population after drying for 3 hours to the original A_w. The products were inoculated at a high inoculum level (10^9 CFU /fragment) to ensure that enough quantity of bacterial RNA could be isolated from the samples during long-term storage. The population of *Salmonella* on the products decreased progressively during storage to about 10^6 and 10^7 CFU /fragment on pistachios and cornflakes, respectively, after 12 weeks of storage (**Fig. 1**). However, on dried apples the reduction in the population of *Salmonella* was rapid and *Salmonella* was undetectable at 60 days (**Fig. 1**). Further analysis of the survival of *Salmonella* on dried apples (and other dried fruit) are described in Chapter 2.

4.4.2 Transcriptional response of *Salmonella* to desiccation on low-moisture foods

The concentration of total RNA isolated from the inoculated LMFs at various sampling times ranged between 15 to 24 µg while the RNA integrity number (RIN), a quality index, ranged between 7.6 and 10. RNA yield and quality were independent on product type and storage time. RNA-seq yielded an average of 9.5 million sequence reads per sample with 92-94% of the reads mapped to the reference *S.* Enteritidis P125109 genome. The high RNA quality and percentage of mapped sequence reads in the current study could be attributed to the removal of *Salmonella* cells from the LMFs before RNA isolation. Moreover, the LMFs used in the current

study had no detectable background microbiota, thus minimizing possible interference of transcripts from background microbiota.

4.4.3 Differentially expressed genes (DEGs) in *Salmonella* in low-moisture foods

Transcriptional response of *Salmonella* to the low-moisture environment in the LMFs was rapid as several genes were differentially expressed after drying for 3 hours, with 93, 372, and 341 genes upregulated after drying on dried apples, cornflakes and pistachios, respectively, while 34, 40 and 90 genes were downregulated after drying on dried apples, cornflakes and pistachios, respectively (Table 1). All differentially expressed genes (DEGs) in *Salmonella* on each LMF at all sampling points are shown in Supplementary materials. Several genes were differentially expressed on the LMFs, suggesting that *Salmonella* remained metabolically active during long-term storage of the inoculated LMFs, even on dried apples after 12 weeks of storage, where *Salmonella* where could not be recovered (Fig. 1). The number of DEGs was noticeably lower on dried apples than on pistachios and cornflakes during drying and storage (Table 1), although RNA yields from dried apples were comparable to yields from pistachios and cornflakes at all sampling time points. It should be noted that the number of detected but not differentially expressed genes was higher on dried apples than on pistachios and cornflakes (Table 1), thus the low number of DEGs on apples was not due to the absence of transcripts of the non-DEGs.

4.4.5 Transcriptome response of *Salmonella* on low-moisture foods varied with time

The current study assessed the impact of storage time on the transcriptional response of *Salmonella*. Hierarchical clustering analysis, visualized with heat maps (Fig. 2), was used to characterize the similarities among the transcriptome of *Salmonella* on each product at various sampling points. Findings showed changes in the transcriptome of *Salmonella* based on time, as

transcriptome after 3 hours of drying, and at 1, 5 and 12 weeks of storage clustered differently for all LMFs (Fig. 2). The most noticeable difference was between the transcriptome after 3 hours of drying compared to the storage times (Fig. 2).

On dried apples, only 8 DEGs (4 upregulated and 4 downregulated) were shared at drying and all storage sampling time points. Four genes that were upregulated on apples during drying and all subsequent storage sampling points include *mdoG* (glucan biosynthesis protein G), *SEN_RS13015* (a putative membrane protein), *SEN_RS16195* (DEAD/DEAH family ATP-dependent RNA helicase), and *SEN_RS19715* (serine/threonine protein kinase). The downregulated genes shared during drying and storage are; *pagC* (a virulence membrane protein), *SEN_RS15475* (polysaccharide deacetylase), *SEN_RS14755* (amino-acid N-acetyltransferase), and *SEN_RS17560* (glycerophosphodiester phosphodiesterase). Notable among the genes that were upregulated after drying but not during storage are *carAB* operon encoding the carbamoyl-phosphate synthase, respectively, and *SEN_RS07750* (malto-oligosyltrehalose trehalohydrolase) and *SEN_RS07755* (malto-oligosyltrehalose synthase). Carbamoylphosphate is a precursor to the biosynthesis of arginine and pyrimidine nucleotides (Deyroede et al, 2006) while malto-oligosyltrehalose is a compatible solute that accumulates in the cytoplasm of bacteria under osmotic stress (Chen et al, 2019).

Moreover, 58 DEGs (25 upregulated and 33 downregulated) in *Salmonella* on dried apples were shared among all storage sampling time points but not with drying. Examples of genes that were upregulated at all storage times but not at drying include *znuC* (Mn2+/Zn2+ABC transporter ATP-binding protein), *SEN_RS06400* (K+/H+ antiporter NhaP2), *lrhA* (transcriptional regulator LrhA), *lpxP* (lipid A biosynthesis palmitoleoyl acyltransferase), *recN* (DNA repair protein), *srkA* (stress response kinase A), and *plsB* (glycerol-3-phosphate 1-O-

acyltransferase). Some down regulated genes shared among all storage sampling points include genes involved in 2-methylcitrate cycle (*prpB, prpC, SEN_RS01810*, and *prpE*), flagellar biosynthesis (*flgL*, *flgK*, and *flgM*), and NADH-mediated electron transfer (*nouN*, *nouL*, and *nouJ*). Possible implications of these DEGs are discussed later in the chapter. The considerable differences in the transcriptome of *Salmonella* on dried apples during drying compared to storage suggests that different processes mediated survival of *Salmonella* on dried apples during drying versus storage.

Salmonella on cornflakes shared more DEGs (mostly upregulated) at drying and storage compared to dried apples, with 305 (287 upregulated and 18 downregulated) DEGs shared. A few examples of these shared DEGs are discussed below (section 4.4.7). Very few genes (*e.g SEN_RS22340* encoding methyl-accepting chemotaxis protein and *SEN_RS08120* encoding serine protease) were highly induced at drying but not during storage, although these genes were not repressed at storage. A few genes (*e.g SEN_RS19295* encoding XRE family transcriptional regulator and *SEN_RS13710* encoding carbon starvation induced protein CsiD) were strongly repressed only at 3h. Further, some genes (*e.g SEN_RS12965* encoding IMP dehydrogenase and *SEN_RS13970* encoding nitric oxide reductase transcriptional regulator) were strongly induced, while few repressed genes (*e.g SEN_RS13705* encoding tripartite tricarboxylate transporter permease and *SEN_RS15415* encoding CoA ester lyase) at all sampling time points during storage but not after drying.

Interestingly, more genes were strongly induced in *Salmonella* on pistachios compared to dried apples and cornflakes (Fig. 2), and several (151) of these induced genes remained upregulated throughout storage. Some of these strongly induced genes, which were also induced on cornflakes and dried apples (to a lesser extent) are discussed below (section 4.4.7). Five

strongly downregulated genes were shared during drying and storage, and these include: *SEN_RS15475* encoding polysaccharide deacetylase, *SEN_RS13575* encoding biofilm-associated protein BapA, *SEN_RS03815* encoding histidine ammonia-lyase, *SEN_RS09020* encoding arginine N-succinyltransferase, and *SEN_RS03810* urocanate hydratase. However, the importance of these genes to the survival of *Salmonella* on LMFs have not been characterized.

For all LMFs, the majority of the DEGs were shared at 1, 5 and 12 weeks, with only a few time-specific DEGS, suggesting that the transcriptome of *Salmonella* remained relatively unchanged on these LMFs during storage. These similarities could be because the shared DEGs were continuously differentially expressed during storage, or transcripts of the shared DEGs were protected from degradation, and thus remained stable during storage. Several factors, such as stalled ribosomes, certain small non-coding RNAs, and RNA chaperone proteins (for example, Hfq) can protect bacterial mRNA from degradation by ribonucleases (McCullen et al, 2010; Hui et al, 2014). Regardless of the factors responsible for the stability of *Salmonella* transcriptome on the LMFs during storage, the maintenance of the transcript levels of shared DEGs suggests their importance for survival on LMFs.

4.4.6 Transcriptome of *Salmonella* was dependent of the types of low-moisture foods.

The physicochemical characteristics of the different low-moisture foods tested impacted the transcriptional response of *Salmonella*. In the current study, we identified LMF-specific transcriptome response as well as transcriptome responses that were shared by at least two LMFs, at different sampling time points. There were 51, 79, 52 and 42 DEGs that were common to all three LMFs after drying for 3 hours, and at 1, 5 and 12 weeks of storage, respectively (Fig. 4). Some of these shared DEGs are described in the section below. Overall, more DEGs were shared between *Salmonella* on pistachios and cornflakes than either of these two products shared

with dried apples (Fig. 4). It should be noted that survival of *Salmonella* was comparable on pistachios and cornflakes. Findings corroborate other findings that have identified variations in the transcriptional response of *Salmonella* to different low-moisture abiotic surfaces such as stainless steel and plastic surfaces as well as to different LMFs such as chocolate, (Crucello et al, 2019; Finn et al, 2013; Gruzdev et al, 2012; Li et al, 2012; Maseratti et al, 2017). The differences in the transcriptional response of *Salmonella* on the different LMFs suggest that different mechanisms may be employed by *Salmonella* to survive depending on other factors present in the food.

4.4.7 Biological functions of selected differentially-expressed genes that were shared on at least two different low-moisture foods.

As described above, several product- and time-specific DEGs were identified. we observed. However, we hypothesized that genes that are important for desiccation tolerance of *Salmonella* on LMFs would be differentially expressed on majority of LMFs. Therefore, DEGs that were shared among at least two of the three LMF products tested were considered top candidates with respect to their importance for survival on LMFs and are further discussed below. These commonly shared DEGs are involved in diverse biological processes and pathways. Examples of biological processes that were enriched on all products during storage include *de novo* purine biosynthesis, aromatic compound, amino acid phospholipid biosynthetic processes, mRNA and ncRNA metabolic processes (Table 2). Some processes were also noticeably downregulated, and these include the 2-methylcitrate cycle (a propionate catabolic process), oxidation of short-chain fatty acids, motility, ATP synthesis coupled to electron transport, and the 2Fe-2S cluster assembly (Table 2).

4.4.8 Upregulated genes

Fatty acids and aromatic compound biosynthesis

Several of the genes involved in fatty acids biosynthesis, such as *plsB, plsX, fabZ, lpxD, lpxL*, and *lpxM* were upregulated. Fatty acid synthesis in bacteria is achieved by a highly conserved type II fatty acid biosynthetic pathway, in which *fabZ* catalyzes the dehydration of hydroxyacyl-acyl carrier proteins while *plsB* and *plsX* catalyze the transfer of long-chain fatty acids to glycerol-3-phosphate component of membrane phospholipids (Zhang and Rock, 2008). The genes *lpxL*, *lpxM*, and *lpxD* encode proteins that catalyze the addition of laurate (and palmitoleate), myrisate, and R-3-hydroxymyristoyl to Kdo_2-lipid IV_A component of the lipid A, which is an important component of the lipopolysaccharides (Six et al, 2008). These findings suggest that *Salmonella* on LMFs modifies the fatty acid composition of the lipid bilayer of membrane cell membrane for survival. Previous studies have shown that the exposure of *Salmonella* to antimicrobials and magnetic fields resulted in significant changes in membrane fatty acid composition with resultant effects on membrane fluidity and permeability (Mouhoub et al, 2017; Dubois-Brissonnet et. al, 2011). However, the importance of membrane fatty acid composition of the membrane lipid bilayer on the desiccation tolerance of *Salmonella* remains to be elucidated.

A previous transcriptome study reported upregulation of *fadA, fadE, fadH, fadI* genes involved in fatty acid degradation as well as genes associated with short-chain fatty acid biosynthesis, propionic (*prpEBCD*) and butyric acid (*gabTBD*) in *Salmonella* Tennessee and Typhimurium LT2 desiccated on filter paper discs for 2 hours (Li *et al*, 2012). Interestingly, some of the upregulated genes in that study, i.e., *fadA, fadE, fadH, fadI*, and *prpEBCD* were downregulated in the current study (Table 2). In the current study, it seems that *Salmonella* on

LMFs downregulates the degradation of fatty acids and biosynthesis of short-chain fatty acids in favor of biosynthesis of aromatic compounds, as several genes that are involved in the biosynthesis of aromatic compounds were upregulated. These included *aroB, aroE, aroG, aroK*, and *aroM* involved in the synthesis of shikimate, a precursor for many aromatic compounds, including aromatic amino acids, and ubiquinone (Zucko et al, 2010). Although findings from the current study suggest possible synthesis and accumulation of aromatic compounds in *Salmonella* on LMFs, further investigation is necessary to confirm this observation and determine the type and functions of such compounds. Such investigations may identify unique compounds that can be used as biomarkers for rapid detection of *Salmonella* on LMFs.

Compatible solute synthesis and ion transport

Intracellular accumulation of low molecular weight, highly soluble compatible solutes (osmoprotectants) is one of the mechanisms of osmoregulation in bacteria (Sleator and Hill, 2002). In the current study, *otsA* and *otsB* involved in the synthesis of a compatible solute, trehalose from glucose are upregulated (Table 2). The trehalose-6-phosphate synthase OtsA catalyzes the condensation of UDP-glucose and glucose-6-phosphate to form trehalose-6-phosphate and the trehalose-phosphatase OtsB then dephosphorylates trehalose-6-phosphate to yield trehalose (Elbein et al, 2003). These findings corroborate reports from previous studies that reported upregulation of *otsA* and *otsB* in *Salmonella* desiccated on stainless-steel and paper discs (Li et al, 2012; Finn et al, 2013). Interestingly, *treF* which catalyzes the breaking down of trehalose to form glucose (Horlacher et al, 1996), was also upregulated in *Salmonella* on the LMFs. A previous study also reported upregulation of *treF* in addition to *otsA* and *otsB* in *Salmonella* under desiccation on stainless steel (Finn et al, 2013). Upregulation of genes that

mediate biosynthesis and catabolism of trehalose at the same time suggest that *Salmonella* maintain balance between trehalose and glucose concentrations in the cytoplasm.

Additional transport-related genes upregulated in *Salmonella* on LMFs were *kdpA* and *kdpB*, encoding two of the four subunits that make up a potassium transport channel protein. Potassium (K^+) is essential for various physiological functions in bacteria including regulation of intracellular pH and maintenance of turgor pressure (Loukin et al, 2005). Loss of water in *Salmonella* in low-moisture environment causes loss of turgor which serves as a signal that trigger the induction of the *kdpFABC* operon (Potts, 1994). Previous studies have reported upregulation of *kdpA* in *Salmonella* in response to desiccation on plastic surfaces and black pepper, and the deletion of *kdpA* severely impacted long-term survival of *Salmonella* on plastic surfaces (Gruzdev et al, 2012, Crucello et al, 2019). However, the none of the *kdp* genes were differentially expressed in other studies that analyzed the transcriptome of *Salmonella* on stainless steel surfaces and filter paper (Finn et al, 2013; Li et al, 2012; Maserati et al, 2017). Thus, it is possible that the importance of K^+ transport in the survival of *Salmonella* on LMFs depends on other factors in the environment.

DNA repair and RNA degradation

Genes that are involved in DNA repair systems, including *uvrB*, *uvrC*, *recN*, *recR*, and *recQ* were upregulated in *Salmonella* on all the LMFs (Table 2). UvrB and UvrC are central components of the highly-conserved nucleotide excision repair (NER) systems in prokaryotes, while RecN, RecR and recQ are involved in recombinational repair of DNA damage that often involves double-stranded DNA breaks (Kisker et al, 2013; Kuzminov et al, 2011). The *uvrB* and *recN* genes were also upregulated in *Salmonella* on chocolate bars and black pepper after 72 hours of storage (Crucello et al, 2019). However, other previous studies have identified genes

involved in the DNA repair system other than those identified in the current study (Mandal and Kwon, 2017; Maserati et al, 2018, Crucello et al, 2019). Specifically, *uvrD*, *dam*, and *xerCD* were reported to the induced and required for desiccation tolerance of *Salmonella* on plastic surfaces (Mandal and Kwon, 2017; Maserati et al, 2018). Therefore, observations from the current study provide additional evidence that *Salmonella* under desiccation undergoes DNA damage and thus require a functional DNA repair system for survival. However, the extent of DNA damage in *Salmonella* on LMFs and the significance of the repair needs to be investigated.

Moreover, we found evidence of RNA turnover in *Salmonella* on the LMFs as several genes that are involved in RNA degradation were upregulated, such as *rhlB*, *deaD*, *SEN_RS02995*, *SEN_RS06900*, *SEN_RS09690*, and *SEN_RS16655*). *SEN_RS02995*, *SEN_RS06900*, *SEN_RS09690*, and *SEN_RS16655* encode ribonuclease I, exoribonuclease II, ribonuclease E and ribonuclease G, respectively, and have been shown to mediate the degradation of mRNA, rRNA, tRNA and sRNA in *Salmonella* (Chao et al, 2017; Viegas et al, 2007). However, the target RNA molecules and the relevance of their degradation to the survival of *Salmonella* on LMFs require further elucidation. Several other genes that are involved in the stringent response, transcriptional regulation, transporters and other processes were upregulated in *Salmonella* on LMFs (Table 2).

Downregulated genes

Several processes were downregulated in *Salmonella* on the LMFs and some are listed in Table 2. Genes encoding various subunits of NADH-ubiquinone oxidoreductase and F0-F1 ATP synthase complex were among the downregulated genes. These complexes mediate the electron transport chain that is coupled to ATP synthesis under normal growth conditions (Kracke et al, 2015; Guo et al, 2019). The NADH-ubiquinone oxidoreductase and F0-F1 ATP synthase genes

were also downregulated in *Salmonella* on chocolate, powdered milk, black pepper and dry petfood (Crucello et al, 2019). However, previous studies that analyzed the transcriptome of *Salmonella* on abiotic surfaces did not detect differential expression of the genes involved in these complexes (Li et al, 2012; Finn et al, 2013, Gruzdev et al, 2012; Maserati et al, 2017). Such findings suggest that these complexes may be specific for survival on LMFs. The downregulation of NADH-Ubiquinone oxidoreductase genes could be due to possible low abundance of NADH in *Salmonella* on LMFs. NADH is a product of the glycolysis pathway (Sebastiaan et al, 2015), which would be expected to the downregulated in *Salmonella* under desiccation as glucose becomes unavailable. Bacterial dispersion by means of motility is generally repressed in low-moisture environments (Tecon and Or, 2016). Therefore, it was not unexpected that genes involved in flagellar biosynthesis (*fliC*, *flgE*, *flgK*, and *flgL*) were downregulated.

4.5 CONCLUSIONS

Findings from the current study revealed that *Salmonella* possesses a large repertoire of genetic determinants for survival in low-moisture environments for an extended period. The relative stability of transcriptome responses of *Salmonella* on the LMFs during storage suggests that the mechanisms required for long-term survival were expressed early and remained relatively unchanged. Several LMF-time specific DEGs were identified, indicating that the mechanisms of survival may be depend on other factors present on the foods. Although the transcript levels of a gene do not necessarily correlate to the gene's importance for survival, genes that were significantly differentially expressed on all products in the current study may be good candidates for further characterization with mutagenesis methods.

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Table 1: Number of differentially expressed genes of *Salmonella* on low-moisture foods. Gene expression levels in *Salmonella* on the LMFs were compared to those of the inoculum suspension and expressed as Log_2Fold Change. Genes with at least $Log_2FC \geq | 2 |$ with an adjusted *P* value (False discovery rate, FDR) < 0.05 were considered differentially expressed.

Sample	Upregulated	Downregulated	No change in expression
Apple 3h	93	34	1146
Apple 1wk	75	62	918
Apple 5wk	42	53	950
Apple 12wk	35	42	1008
Cornflakes 3h	372	40	1016
Cornflakes 1wk	439	146	869
Cornflakes 5wk	474	147	800
Cornflakes 12wk	433	118	858
Pistachios 3h	341	90	1056
Pistachios 1wk	470	195	770
Pistachios 5wk	401	129	816
Pistachios 12wk	347	119	932

Table 2: Selected genes that were differentially expressed in *Salmonella* on at least two low-moisture foods during drying and storage.

Biological process	Gene	Molecular function
Upregulated		
Transporters/ permeases	*mdlA*	SmdA family multidrug ABC transporter
	mdlB	SmdB family multidrug efflux ABC transporter
	znuA	zinc ABC transporter substrate-binding protein
	znuB	zinc ABC transporter permease subunit
	znuC	zinc ABC transporter ATP-binding protein
	pitA	inorganic phosphate transporter
	pstS	phosphate ABC transporter substrate-binding protein
	ptsP	phosphoenolpyruvate--protein phosphotransferase
	dtpA	dipeptide/tripeptide permease
	dtpD	MFS transporter
	ybhR	ABC-2 family transporter protein
	ybhS	ABC-2 family transporter protein
Fatty acid biosynthesis	*plsB*	glycerol-3-phosphate 1-O-acyltransferase
	plsX	phosphate acyltransferase
	fabZ	3-hydroxyacyl-ACP dehydratase
	lpxD	UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase
	lpxL	lauroyl/palmitoleoyl acyltransferasee
	lpxM	lauroyl-Kdo(2)-lipid IV(A) myristoyl transferase

Table 2 Continued

Biological process	Gene	Molecular function
Upregulated		
Osmoprotectants and ion import	*otsA*	alpha,alpha-trehalose-phosphate synthase
	otsB	trehalose-phosphatase
	treF	alpha,alpha-trehalase TreF
	kdpA	potassium-transporting ATPase subunit KdpA
	kdpB	potassium-transporting ATPase subunit KdpB
DNA repair	*uvrB*	excinuclease ABC subunit B
	uvrC	excinuclease ABC subunit C
	recN	DNA repair protein
	recR	recombination protein
	recQ	ATP-dependent DNA helicase
Aromatic compounds	*aroB*	3-dehydroquinate synthase
	aroE	shikimate dehydrogenase
	aroG	3-deoxy-7-phosphoheptulonate synthase
	aroK	shikimate kinase
	aroM	AroM family protein
RNA degradation	*rhlB*	ATP-dependent RNA helicase RhlB
	deaD	DEAD/DEAH family ATP-dependent RNA helicase
	SEN_RS02995	ribonuclease I
	SEN_RS06900	exoribonuclease II
	SEN_RS09690	ribonuclease E
	SEN_RS16655	ribonuclease G
Stringent response factors	*sspA*	stringent starvation protein A
	sspB	ClpXP protease specificity-enhancing factor

Table 2 Continued

Biological process	Gene	Molecular function
Upregulated		
Transcriptional regulators	*lrhA*	transcriptional regulator LrhA
	gcvA	transcriptional regulator GcvA
	zur	zinc uptake transcriptional repressor Zur
	SEN_RS09250	MerR family transcriptional regulator
	SEN_RS17465	IclR family transcriptional regulator
	phoB	phosphate response regulator transcription factor
Downregulated		
ATP synthesis coupled to electron transport	*nouN*	NADH-quinone oxidoreductase subunit NuoN
	nouJ	NADH-ubiquinone/plastoquinone oxidoreductase chain 6
	nuoL	NADH-Ubiquinone oxidoreductase (complex I), chain 5 N-terminus
	nuoE	NADH-quinone oxidoreductase subunit NuoE
	nuoG	NADH-quinone oxidoreductase subunit NuoG
	nuoH	NADH-quinone oxidoreductase subunit NuoH
	pntB	Catalyses transhydrogenation between NADH and NADP which is coupled to ATP hydrolysis
	gndA	NADP-dependent phosphogluconate dehydrogenase
	atpC	F0-F1 ATP synthase subunit epsilon
	atpD	F0-F1 ATP synthase subunit beta
	atpG	F0-F1 ATP synthase subunit gamma

Table 2 Continued

2-Methylcitrate cycle	*prpB*	Cleaves2-methylisocitrate to yield pyruvate and succinate
	prpC	2-methylcitrate synthase
	prpD	2-methylcitrate dehydratase
	prpE	propionate--CoA ligase
Motility	*fliC*	Polymerizes to form the filaments of bacterial flagella
	flgE	Flagellar basal body protein FlaE
	flgK	Flagellar basal body rod FlgEFG protein C-terminal
	flgL	Bacterial flagellin N-terminal helical region
Short-chain fatty acids oxidation	*fadA*	acetyl-CoA C-acyltransferase
	fadB	fatty acid oxidation complex subunit alpha
	fadE	acyl-CoA dehydrogenase
	fadH	NADPH-dependent 2,4-dienoyl-CoA reductase
	fadL	long-chain fatty acid transporter

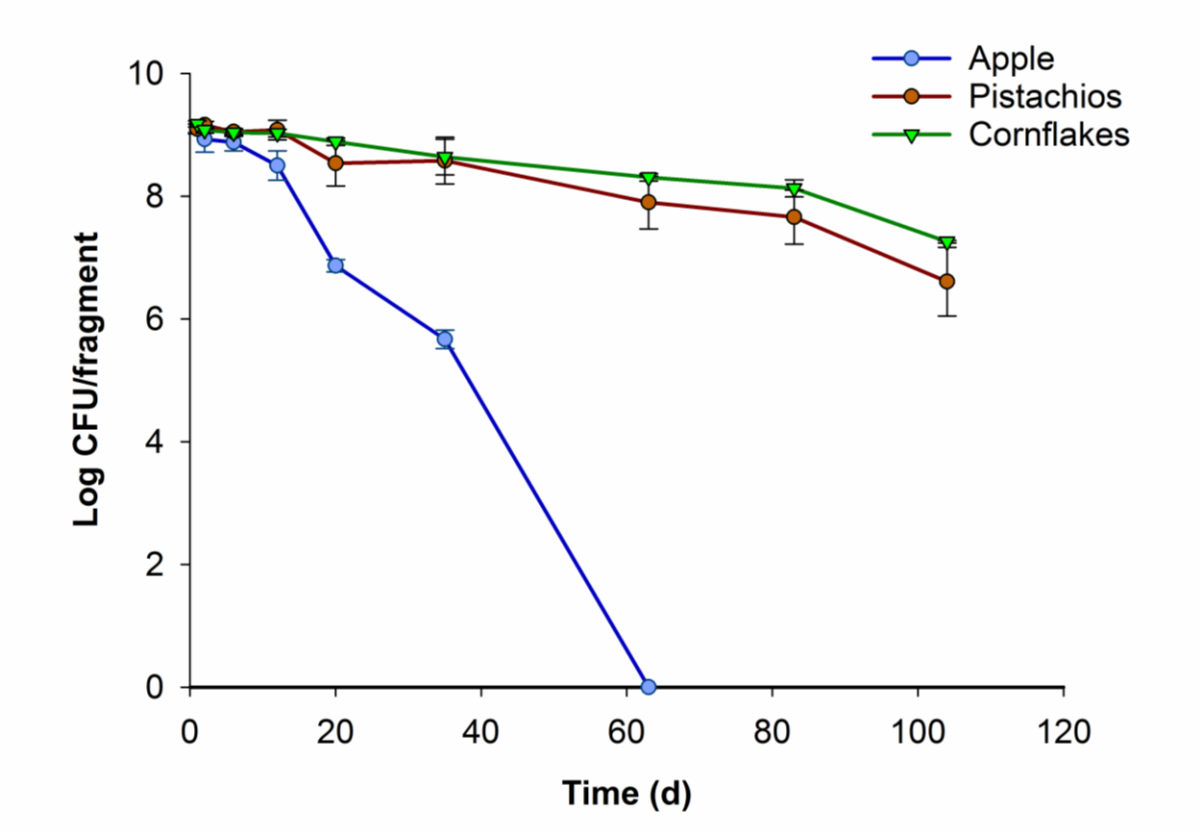
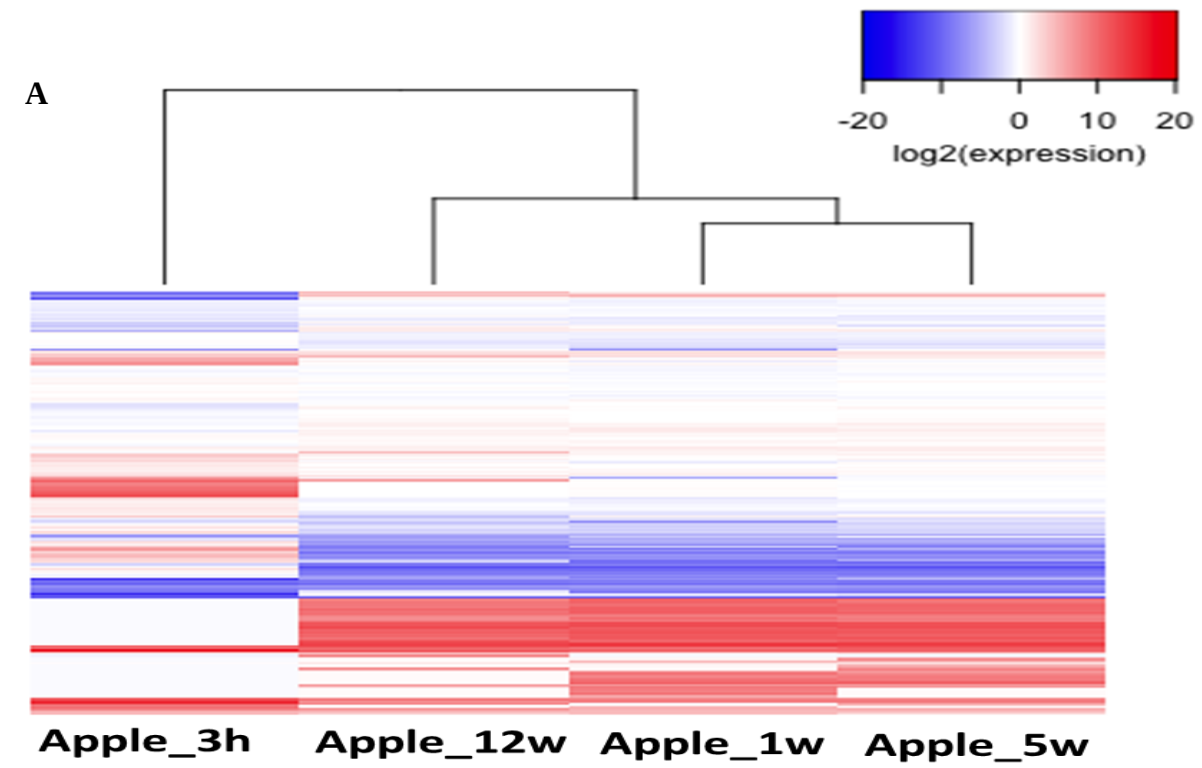
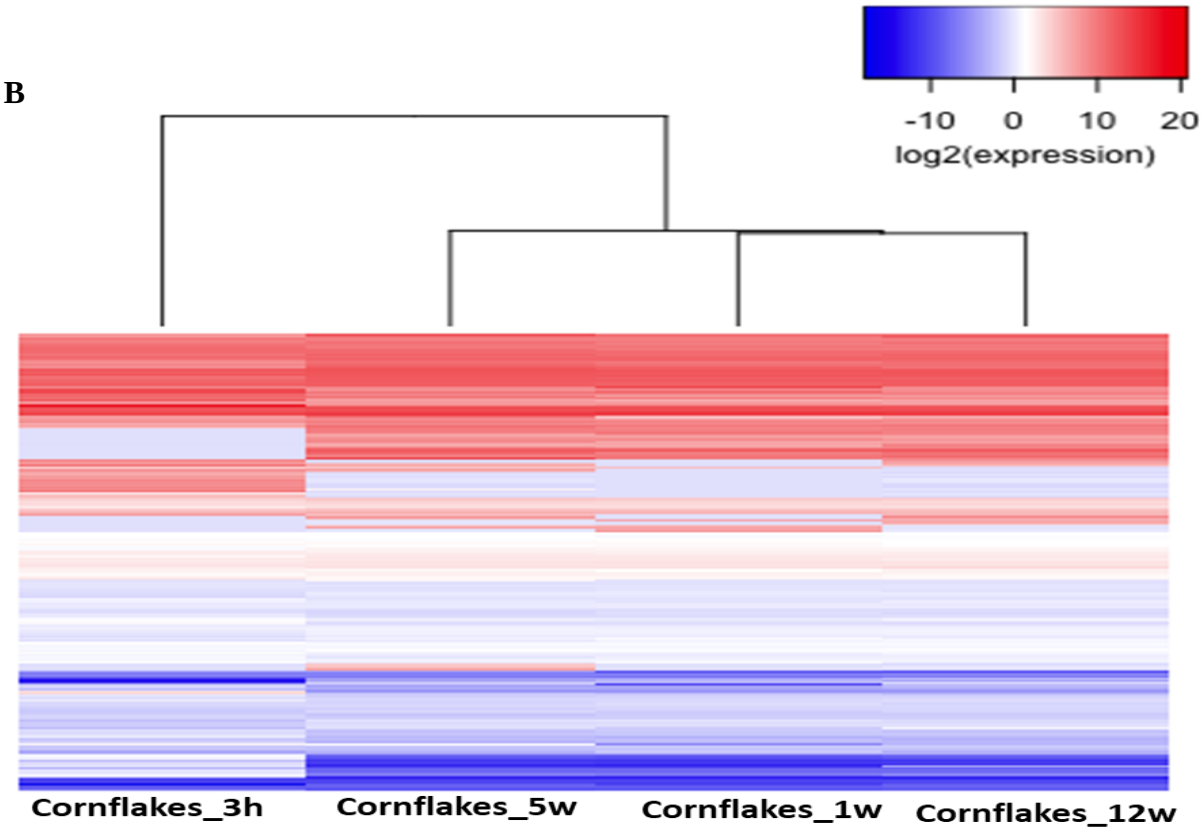


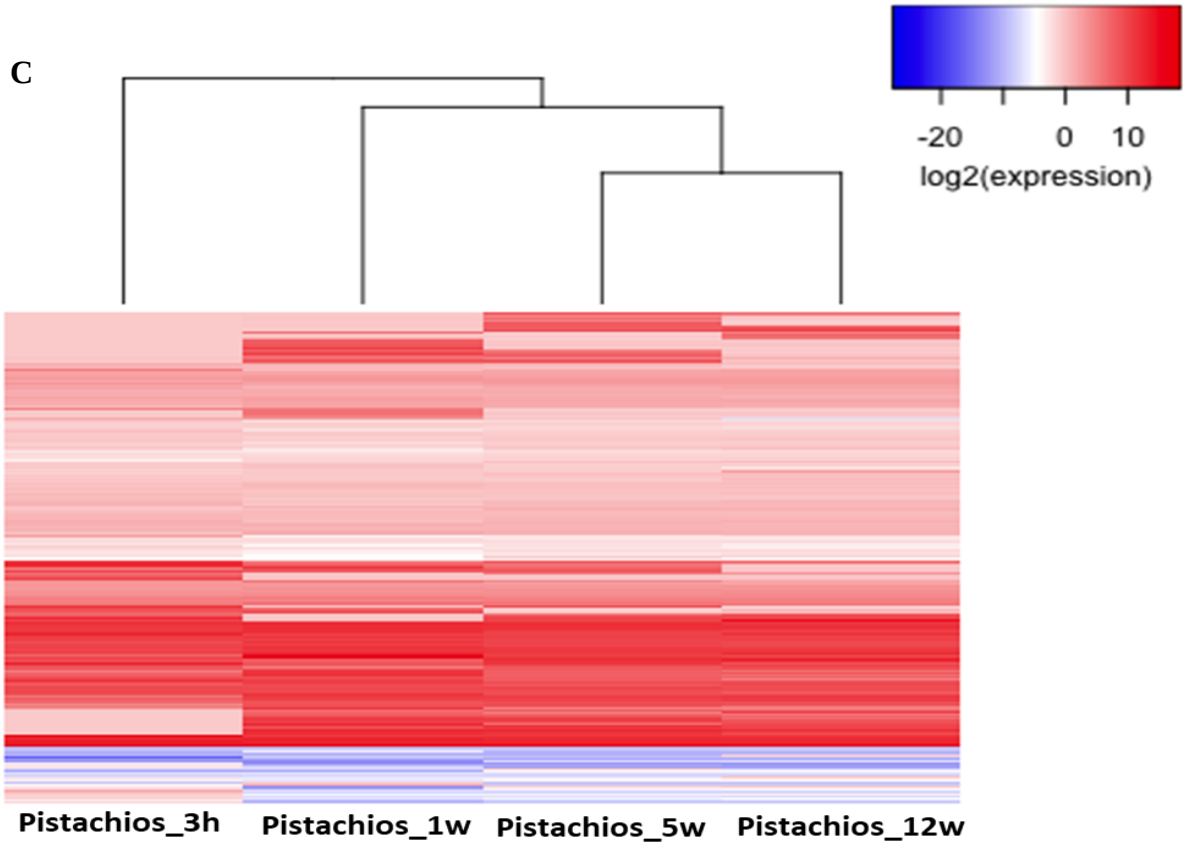
Figure 1: Survival of *Salmonella enterica* serovar Enteritidis strains on dried apples, pistachios and cornflakes. The LMFs were inoculated, dried and stored, and populations of *Salmonella* were enumerated as described in Materials and Methods immediately after inoculation, after drying and periodically during storage. Data represent averages from three independent trials with duplicate samples processed per trial. Error bars indicate standard deviation (n = 6).

Figure 2: Hierarchical clustering of the transcriptome of *Salmonella* on (A) dried apples, (B) cornflakes, (C) pistachios after drying for 3 hours and at 1, 5 and 12 weeks of storage. Total transcriptional changes on LMFs relative to the inoculum suspension (either differentially expressed or not) were used to compare expression similarities using the Euclidean distance.


A
-20
0
10
20
log2(expression)
Apple_3h
Apple_12w
Apple_1w
Apple_5w


B
-10
0
10
20
log2(expression)
Cornflakes_3h
Cornflakes_5w
Cornflakes_1w
Cornflakes_12w

C


-20
0
10
log2(expression)
Pistachios_3h
Pistachios_1w
Pistachios_5w
Pistachios_12w

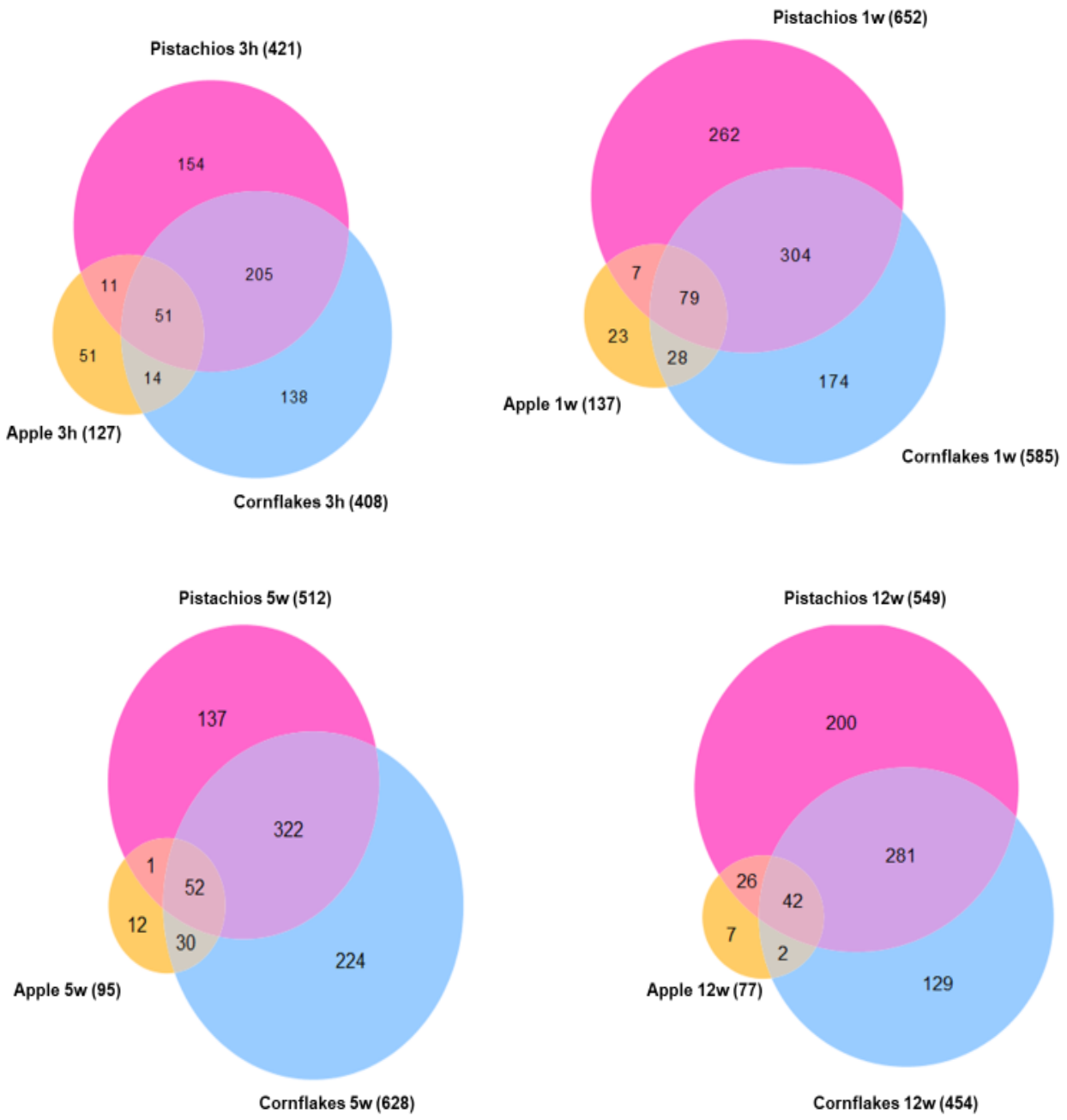


Figure 3: Distribution of differentially expressed genes in *Salmonella* on dried apples, cornflakes and pistachios at 3 hours of drying and after 1, 5 and 12 weeks of storage.

CHAPTER 5

Identification of novel genes mediating survival of *Salmonella* on low-moisture foods via transposon sequencing analysis

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Running title: Genes mediating *Salmonella* survival on low-moisture foods

5.1 Abstract

Salmonella enterica is the leading foodborne pathogen associated with outbreaks involving low-moisture foods (LMFs). However, the genes involved in *Salmonella's* long-term survival on LMFs remain poorly characterized. In this study, in-shell pistachios were inoculated with Tn*5*-based mutant libraries of *S.* Enteritidis P125109, *S.* Typhimurium 14028s, and *S.* Newport C4.2 at approx. 10^8 CFU/g and stored at 25ºC. Transposon sequencing analysis (Tn-seq) was then employed to determine the distribution of distinct Tn*5* insertion sites immediately after inoculation (T_0), after drying (T_1) and at 120 days (T_{120}). In *S.* Enteritidis, *S.* Typhimurium and *S.* Newport mutant libraries, the relative abundance of 51, 80, and 101 Tn*5* insertion sites, respectively, was significantly lower at T_1 compared to T_0, while in libraries of *S.* Enteritidis and *S.* Typhimurium the relative abundance of 42 and 68 Tn*5* insertion sites, respectively, was significantly lower at T_{120} compared to T_1. Tn*5* insertion sites with reduced relative abundance were localized in DNA repair, lipopolysaccharide biosynthesis and stringent response genes. Testing of 14 single-gene deletion mutants revealed that mutants in *sspA, barA, uvrB, damX, rfbD, uvrY, lrhA, yifE, rbsR* and *ompR* were impaired for survival on pistachios. The findings highlight the value of the combined mutagenesis and sequencing strategy to identify novel genes important for the survival of *Salmonella* in low-moisture foods.

5.2 INTRODUCTION

Low-moisture foods (LMFs) have low water activity (A_w), generally below 0.6, and thus are not conducive to microbial growth (Beuchat et al, 2012). However, the ability of certain foodborne pathogens to persist on LMFs for long periods has rendered these commodities important vehicles for outbreaks of foodborne illness (Beuchat et al, 2013; Finn et al, 2013b). LMFs have been associated with numerous outbreaks, having led to 7,315 cases of foodborne illnesses and 63 deaths worldwide between 2007 and 2012 (Farakos and Frank, 2014). In the United States, an estimated 83% of LMF-associated multistate foodborne outbreaks between 2007 and 2018 have involved *Salmonella* (CDC, 2019). Examples of extensively-investigated foodborne salmonellosis outbreaks involving LMFs include the 2008-2009 peanut butter outbreak that resulted in 714 cases, 171 hospitalizations and 9 deaths (CDC, 2009), and a pistachio-associated salmonellosis outbreak in 2016 (CDC, 2016).

The food safety concerns of *Salmonella*-contaminated LMFs are accentuated by the fact that most LMFs are considered ready-to-eat foods and have a relatively long shelf life. Moreover, *Salmonella* contaminating LMFs exhibits unusually high tolerance to other stressors including heat and exposure to bile salts and sanitizers (Gruzdev et al, 2011; Smith et al, 2016). Thus, preventive control and inactivation strategies that may be effective against *Salmonella* contaminating high-moisture foods may be less effective on LMFs. Furthermore, salmonellosis outbreaks associated with LMFs can span several months because *Salmonella* can survive and remain infectious on LMFs for long periods of time (Isaacs et al, 2005; CDC, 2009). Indeed, several studies have demonstrated *Salmonella*'s capacity to persist in the absence of growth on a diverse array of LMFs (Kimber et al, 2012; Hokunan et al, 2016; Beuchat and Mann, 2015).

However, the molecular mechanisms and genetic determinants mediating long-term survival of *Salmonella* on LMFs are still poorly understood.

Findings from previous studies have suggested that the immediate response of *Salmonella* to desiccation involves accumulation of compatible solutes such as glycine betaine and trehalose (Csonka et al, 1991; Li et al, 2012; Finn et al, 2013b). Transcriptome analyses of *Salmonella* under desiccation on abiotic surfaces suggested that long-term survival involved complex metabolic and cellular processes (Li et al, 2012; Finn et al, 2013a; Gruzdev et al, 2012; Maserati et al, 2017). Processes and traits found to be upregulated under desiccation included potassium influx, the stress-induced sigma factors (*rpoE* and *rpoS*), Fe-S cluster, fatty acid and amino acid metabolism, stress response and virulence (Finn et al, 2013b; Gruzdev et al, 2012; Maserati et al, 2017, 2018). In addition, genes involved in propanoate metabolism, citrate cycle, and lipopolysaccharide biosynthesis were downregated in *Salmonella* on milk chocolate, powdered milk, black pepper, and dried pet foods (Crucello et al, 2019). Formation of thin aggregative fimbriae and cell filamentation were implicated in desiccation tolerance of *Salmonella* (Mattick et al, 2000; White et al, 2006; Maserati et al, 2017). It must be kept in mind, however, that most of these these studies tested desiccation tolerance of *Salmonella* over relatively short periods of time on dry abiotic surfaces such as plastic, stainless steel and paper which may not be representative of the mechanisms employed by *Salmonella* to survive on LMFs.

The objective of the current study was to identify genetic determinants required for survival of *Salmonella* on LMFs. Mutant libraries created by random Tn5 insertion in the genomes of three *Salmonella* serovars were selected for both short and long-term survival on in-shell pistachios, followed by transposon-sequencing analysis to identify genetic determinants

required for survival. The role of a subset of 14 genes identified via this combined mutagenesis and sequencing approach was then directly investigated by assessing the capacity of single-gene deletion mutants to survive on pistachios.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial strains and growth conditions

Salmonella strains used in this study included *Salmonella enterica* sv Typhimurium strain 14028s, isolated from 4-week-old chickens in 1960 (Jarvik et al, 2010); *Salmonella* Enteritidis PT4 str. P125109, a clinical strain associated with a poultry outbreak in the United Kingdom in (Toro et al, 2016); and *Salmonella* Newport C4.2, isolated from a tomato field in Virginia, USA (de Moraes et al, 2018). Single-gene deletion (SGD) mutants of *S.* Typhimurium strain 14028s harbor a kanamycin (SGD-Kan^R) or chloramphenicol (SGD-Cm^R) resistance gene, oriented in the antisense and sense direction, respectively, in regard to the deleted gene (Porwollik et al, 2014). The strains were preserved at -80 ^oC in Luria-Bertani broth (LB; Becton, Dickinson & Co. Sparks, MD) containing 20% glycerol (Fisher Scientific, Fair Lawn, NJ). Bacterial cultures were grown in LB or on LB agar containing 1.2% agar (Becton, Dickinson & Co.) at 37 ^oC for 24 h unless indicated otherwise. When necessary, growth media were supplemented with 60 µg/ml kanamycin (LB+Kan; Fisher Scientific) or 20 µg/ml chloramphenicol (LB-Cm, Acros Organics, NJ).

5.3.2 Construction of Tn*5* mutant libraries

The methods for construction of the barcoded Tn*5* libraries were previously described (de Moraes et al, 2017; de Moraes et al, 2018). In brief, a library of Tn*5* insertion mutants was constructed with each strain with a mini-Tn*5* derivative into which we inserted an N_{18} random barcode using PCR. This derivative was integrated into the genome using the EZ-Tn5 <

T7/KAN-2 > promoter insertion kit (Epicentre Biotechnologies, Madison, WI, United States). Mapping of barcoded transposons to specific locations in the genome was performed as described (de Moraes et al., 2017).

5.3.3 Negative selection of mutant libraries for survival on pistachios

Frozen stocks of mutant libraries were thawed on ice and 1 ml was transferred into two separate 30ml LB+Kan in 50-ml centrifuge tubes (Becton, Dickinson & Co.) and incubated at 37 ^{o}C with shaking for 12 h. The cultures were washed twice in 5-ml sterile deionized water (dH_2O), and combined to yield 10 ml of cell suspension, to be used as inoculum. In-shell pistachios were obtained from a commercial source and analyzed for total aerobic counts and *Salmonella* before use. For inoculation, 200 g of pistachios were weighed into sterile Whirl-Pak stomacher bags (5441 ml, Nasco, Fort Atkinson, WI) and inoculated at 4% volume per weight by transferring 8 ml of the cell suspension in a series of four additions of 2 ml into the bag, with each addition followed by vigorous shaking by hand for 30 sec. The inoculated pistachios were dried in a Nalgene polypropylene desiccator (Thermo Scientific, Waltham, MA) containing drierite desiccant (W.A. Hammond drierite Co. Ltd., Xenia, OH) for 24 h, at which time the A_w approximated that of uninoculated nuts. After drying, the nuts were placed into several 50-ml centrifuge tubes (VWR Int., Radnor, PA), capped tightly, and stored at 25 ^{o}C in the dark.

To recover *Salmonella* from the inoculated pistachios, 10 g of nuts (approx. 10 pistachios) were transferred into 20 ml LB+Kan in 532-ml Whirl-Pak bags (Nasco). The bag was vigorously hand-massaged for 1 min and incubated at room temperature for 10 min. For enumerations, 100 µl of the rinsate was serially diluted in dH_2O and appropriate dilutions were plated on LB+Kan agar plates, followed by incubation at 37 ^{o}C for 24 h. The remainder of the mixtures of pistachios and LB+Kan were incubated statically at 37 ^{o}C for 12 h to amplify the

pistachio-associated *Salmonella* populations, shaken manually for 30 sec, and 1 ml of the resulting suspension was transferred into a 1.5-ml microcentrifuge tube (Fisher Scientific) and centrifuged at 15,000 ×g, 25 ^{o}C for 2 min. The pellets were then preserved at -80 ^{o}C. The population of *Salmonella* in the enriched mixture was enumerated as described. The experiment was conducted in three independent trials with duplicate samples processed per trial.

5.3.4 DNA library preparation, sequencing and data analysis

The methods for DNA library preparation, sequencing and data analysis were previously described and (de Moraes et al, 2017; de Moraes et al, 2018). In brief, bacteria were recovered and grown in LB+60 μg/ml kanamycin. Bacteria were pelleted, lysed and the lysate used as template for PCR using primers directly flanking the N_{18} barcode. The frequency of each barcode was determined by Illumina sequencing of 20 bases. The aggregated abundances for the input and output libraries were statistically analyzed using edgeR (Robinson et al, 2010), and the log_2-fold changes and FDRs were reported.

5.3.5 Assessment of the survival of single-gene deletion mutants on pistachios

Single-gene deletion mutants of *S.* Typhimurium 14028s were constructed previously (Porwollik et al, 2014). The mutants were grown in 30 ml of LB+Kan in 50-ml conical tubes (VWR Int.) at 37 ^{o}C for 24 h with shaking. The cultures were pelleted, washed twice in dH_2O, and used to inoculate 50 g of in-shell pistachios at 4% volume per weight as described above. The inoculated nuts were dried and stored as described above. For *Salmonella* enumerations from the inoculated pistachios, 2 g of nuts (approx. 2 pistachios) were transferred into 5-ml dH_2O in a 15-ml conical tube (Becton, Dickinson & Co). The mixtures were vortexed at high speed for 60 sec, and appropriate dilutions of the liquid were plated on LB+Kan agar, followed by incubation at 37 ^{o}C for 24 h. The experiment was conducted in two independent trials with

duplicate samples per trial. For mutants that showed significantly higher reduction compared to the wild-type strain, counterpart mutants with the same gene deletion harboring a chloramphenicol resistance gene oriented in the antisense direction (Porwolli et al, 2014) were also tested for survival on pistachios, with enumerations done on LB-Cm.

5.3.6 Statistical analysis

Colony forming units (CFU) from the survival assays were entered into an Excel 2013 spreadsheet (Microsoft, Redmond, WA), log transformed, exported to SigmaPlot 14.0 (Systat Software Inc., San Jose, CA) and plotted against time to generate survival curves. Comparisons of log-reductions between the populations of the wild-type and the single-gene deletion mutants on pistachios at different time points during storage were analyzed using one-way analysis of variance, with Tukey's test used to compare multiple means (α, 0.05).

5.4 RESULTS AND DISCUSSION

5.4.1 Survival of *Salmonella* wild-type strains and random mutant libraries on pistachios

In the current study, the population of *Salmonella* (WT strains and mutant libraries) on pistachios immediately after inoculation was approx. 8.5 log CFU/g. The A_w of pistachios before inoculation, immediately after inoculation and after drying was 0.25, 0.43 and 0.32, respectively, with no significant changes in A_w during storage (data not shown). We inoculated pistachios with higher levels of *Salmonella* in this model than are likely to contaminate LMFs in real life, in order to be able to adequately detect changes during survival and identify relevant mutants. We noted differences in survival among the three strains that were employed. The population of *S.* Typhimurium 14028s and *S.* Enteritidis P125109 wild-type strains on pistachios was reduced by 1 log CFU/g after drying, with further reductions by 1.5 log CFU/g after storage for 120 days

(Fig. 1). A higher reduction of 2 log CFU/g was observed in the population of S. Newport CD4.2 after drying, and the population was below 6 log CFU/g after 30 days of storage (Fig. 1).

The observed survival of *S.* Typhimurium 14028s and *S.* Enteritidis P125109 on pistachios in this study corroborates findings from a previous study that reported reductions in the population of *Salmonella* on pistachios by approx. 1 and 1.4 log after drying and during storage at 24 ^{o}C for 120 days, respectively (Kimber et al, 2012). The noticeably lower survival of *S.* Newport CD4.2 on pistachios is quite unusual for *Salmonella. S.* Newport strains have been implicated in LMF-associated *Salmonella* outbreaks and their desiccation tolerance was found to be comparable to *S.* Typhimurium and Enteritidis (Kirk et al, 2004; Gruzdev et al, 2012; CDC, 2014). However, salmonellosis outbreaks involving strains of serovar Newport tend to be associated with fruit and vegetables (de Moraes et al, 2018). Indeed, functional analysis of the *S.* Newport CD4.2 genome revealed unique adaptations to persistence in plants that are not shared by strains of other serotypes, including *S.* Typhimurium 14028s (de Moraes et al, 2018). Therefore, the poor survival of *S.* Newport strain CD4.2 in low-moisture foods may reflect trade-offs in this particular strain for adaptations related to plant colonization.

Overall, the survival of wild-type strains was slightly (0.3-0.5 log CFU/g) higher than observed with their respective mutant libraries strains, but this difference was not significant. The observed differences may have been due to the small fraction of mutants with reduced survival. (Fig. 1). As also observed and discussed above with the parental strain, the mutant library of *S.* Newport CD4.2 declined markedly relative to the others, with population levels declining to < 6 logCFU/g after 30 days of storage (Fig. 1). Because the mutant libraries needed to consist of more than 10^6 distinct mutants in order for the complexity of the library to be

maintained and for profiles of mutant fitness to be determined, survival assessments of *S.* Newport CD4.2 and its mutant library were discontinued after 30 days.

Mutant library populations from the inoculated pistachios immediately after inoculation (T_0), after drying for 1 day (T_1) and at 120 days of storage (T_{120}) were amplified during growth in LB+Kan at 37°C for 12 h. This was done to facilitate detachment of strongly-adhering cells and resuscitation of injured cells, and to ensure extraction of genomic DNA at sufficient concentration for sequencing. The population of all mutant libraries after enrichment was approx. 10^{10} CFU/g at all sampling time points.

5.4.2 Distribution of the relative abundance of Tn*5* insertion sites in *Salmonella* mutant libraries

The plots of the relative abundance of Tn*5* insertion sites (TIS) in mutant libraries that were selected on pistachios are shown in Fig. 2. TIS with significant change ($P < 0.05$) in abundance at T_1 relative to T_0, or T_{120} relative to T_1, and with at least 2-fold change (that is, $Log_2FC \geq | 1 |$) were considered to be selected, and thus the corresponding unmutated locus was considered to have a putative role in survival. In principle, TIS in mutants impaired for survival would be negatively selected ($Log_2FC \leq -1$, $P < 0.05$) as the mutants would have lower relative abundance on pistachios after drying or storage compared to the input mutant pools. The corresponding gene would thus have a role in enhancing survival under the experimental conditions. Conversely, TIS in mutants with enhanced survival would be positively selected and have higher relative abundance ($Log_2FC \geq 1$, $P < 0.05$). The corresponding gene would thus have a role in reducing survival under the experimental conditions or might not be required at all. In the latter case, a mutant might be more fit than the wild type if it does not waste resources producing determinants not needed for survival under the tested conditions.

The most extensive selection occurred in the *S*. Newport C4.2 mutant library after drying (T_0_T_1) with selection of 135 TIS, of which 101 and 34 were negatively and positively selected, respectively (Fig3A). This was particularly interesting in light of the above-discussed finding that this strain was markedly more impaired in survival on the dry pistachios than the other two strains (Fig. 1). In mutant libraries of *S*. Enteritidis P125109 and *S*. Typhimurium 14028s, 63 and 84 TIS were selected, respectively with the majority (51 and 80 TIS, respectively) being negatively selected (Fig. 3A). Further storage of inoculated pistachios at 25ºC for 120 days resulted in selection of 45 and 71 TIS in the mutant libraries of *S*. Enteritidis P125109 and *S*. Typhimurium 14028s, respectively (Fig. 3B). Overall, higher selection occurred during drying than storage for all mutant libraries, suggesting that drying imposed the highest desiccation-related selection pressure.

It should be noted that each library contains different abundance distributions for insertions of Tn5, partly because some genes may not be shared by all three *Salmonella* strains used in this study. With this in mind, the TIS (genes and intergenic regions) that were selected in at least two libraries during drying and storage are listed in Table 1. Although the majority of the selected TIS were strain-specific, several (n = 53) were selected in at least two mutant libraries while 12 were selected (10 negatively and 2 positively) after drying in all three mutant libraries (Fig. 3A). The TIS that were selected in two mutant libraries but not in the third one were either absent in a specific library (6 in *S*. Newport CD4.2 library, 2 in *S*. Enteritidis P125109 library, and 1 in *S*. Typhimurium 14028s library) or were present but did their abundance did not change significantly (Table 1).

The TIS that were negatively selected in all three mutant libraries during drying were localized in several genes, including *uvrA*, *uvrB*, *uvrY*, *rpoS*, *hupA*, *sspA*, *yifE*, *rbsR*, *damX* and

STM14_2365, while *phoP* and *flhD* insertions were positively selected in all three libraries (Table 1). Similarly, 21 TIS were negatively selected during storage in both *S.* Enteritidis P125109 and *S.* Typhimurium 14028s mutant libraries (Fig. 3B). With the sole exception of the insertion in *hupA*, TIS that were negatively selected in all three mutant libraries during drying were also negatively selected during dry storage (Table 1).

5.4.3 Genes putatively important for survival of *Salmonella* on pistachios

Several genes, including those listed above, and intergenic regions appeared to be important for the survival of *Salmonella* on pistachios. Some of the genes encoded hypothetical proteins while others were implicated in DNA repair, transcriptional regulation, and osmolarity responses. These genes are categorized based on their molecular functions and discussed below, starting from the category that has the highest number of genes that are putatively important for survival of *Salmonella*.

DNA recombination and repair mechanisms

Tn*5* localization in several genes involved in DNA recombination and repair, including *uvrA, uvrB, uvrD, recN*, *recG, dam,* and *dcm* was significantly underrepresented in the *Salmonella* mutant populations from the dried or stored pistachios, suggesting that the corresponding mutants were impaired for survival on this dry product (Table 1). This TIS distribution suggested that *Salmonella* undergoes DNA damage in low-moisture conditions, with survival requiring functional DNA repair systems. UvrA, UvrB and UvrD proteins are central components of the highly-conserved nucleotide excision repair (NER) systems in prokaryotes (Kisker et al, 2013). The UvrA-UvrB complex identifies conformational changes induced by DNA lesions, a prerequisite for subsequent steps in NER, and facilitates the recruitment of UvrC, UvrD, DNA polymerase I and DNA ligase for DNA base repair (Kisker et al, 2013; Truglio et al,

2004). On the other hand, RecG, RecN and UvrD are involved in recombinational repair of DNA damage that often involves double-stranded DNA breaks (Kuzminov et al, 2011). DNA adenine methyltransferase (Dam) and DNA cytosine methyltransferase (Dcm) methylate the majority of methylated DNA bases in *E. coli* and *Salmonella* (Marinus et al, 2014). The importance of Dam in DNA mismatch repair, induction of the SOS response, and oxidative stress tolerance has been extensively documented and discussed (Marinus et al, 2014; Stephenson and Brown, 2016; Chatti et al, 2012).

Interestingly, *uvrA*, *uvrB* and *uvrY* were selected during both drying and storage, while *dam* and *dcm* were only selected during drying, and *uvrD*, *recN* and *recG* were only selected during storage (Table 1). This differential selection of DNA repair genes suggests that the NER pathway and methylation-directed DNA repair systems were especially important for repair of DNA damage during drying, when viability was also most markedly impacted. The findings agree with data from proteomic analysis of *Salmonella* under desiccation on glass beads which revealed significant abundance of DnaJ and UvrD, proteins involved in DNA repair, compared to cells in the non-desiccated inoculum suspension (Maserati et al, 2018). In addition, a *Salmonella dam* mutant exhibited significantly-impaired survival on plastic petri plates (Mandal and Kwon, 2017). On the other hand, none of the DNA recombination and repair genes that appear to be important for survival in the current study were differentially expressed in transcriptome analyses of *Salmonella* under low-moisture conditions (Li et al, 2012; Gruzdev et al, 2012; Finn et al, 2013a; Maserati et al, 2017). It is worthy of note that these transcriptome studies employed *Salmonella* on desiccated abiotic surfaces such a glass beads, plastics and stainless steel, in contrast to desiccation on pistachios in the current study. Moreover, some genes that are

important for desiccation tolerance of *Salmonella* may not be differentially expressed under desiccation.

The induction of DNA damage in response to desiccation has been reported in other bacteria, including *Deinococcus radiodurans*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* and spores of *Bacillus subtilis* (Tanaka et al, 2004; Cytryn et al, 2007; Humann et al, 2009; Dose et al, 1992). The exact causes of DNA damage under desiccation remain poorly understood but may include covalent modifications and intracellular accumulation of reactive oxygen species (ROS) (Humann et al, 2009). Moreover, it is not clear if desiccation-induced DNA repair causes adaptive mutations in *Salmonella* that confer higher tolerance to desiccation. For example, exposure of *Listeria monocytogenes* to sublethal acidic pH selected for point mutations in *rpsU*, encoding ribosomal protein S21, that conferred significantly higher tolerance to lethal acidic pH and other stressors (Metselaar et al, 2015). Therefore, the potential of desiccation-induced DNA damage as an adaptive desiccation survival strategy needs to be investigated.

Lipopolysaccharide biogenesis

Several genes involved in LPS biogenesis including *rfbP*, *rfbN*, *rfbC*, *rfbD*, *rfaL*, *rfaJ* and *rfaH* were found to be important for survival of *Salmonella* on pistachios (Table 1). RfbP, RfbN, RfbC, and RfbD are involved in O-antigen synthesis while RfaL, RfaJ and RfaH mediate LPS core synthesis (Schnaitman et al, 1993). Previous studies reported that mutations in LPS biosynthesis genes significantly impaired survival of *Salmonella* on polystyrene petri dishes and formica melamine resin laminate under ambient conditions. (Garmiri et al, 2008; Mandal and Kwon, 2017). Furthermore, LPS is a known virulence determinant of *Salmonella* and plays

important roles in swarming motility, host invasion and oxidative stress tolerance (Toguchi et al, 2003; Thomsen et al, 2003; Choi and Groisman et al, 2013; Bogomolnaya et al, 2014).

Transcriptional regulators and stringent response

Tn*5* insertions in several transcriptional regulators including *rpoS, lrhA, nsrR, hupA*, and *emrR* appeared to result in impaired survival of *Salmonella* on pistachios, by approx. 2- to 8-fold during drying, storage, or both drying and storage (Table 1). Localization in *rpoS* had the greatest impact on survival with approx. 8-fold (i.e, $\log_2$FC = - 3) reduction in relative abundance in all the libraries during drying, and further reduction by 8-fold during storage (Table 1). The *rpoS* gene encodes the alternative sigma factor RpoS which is a global regulator of stress-induced genes, virulence determinants and several metabolic processes in Gram-negative bacteria (Ibanez-Ruiz et al, 2000; Levi-Meyrueis et al, 2014). The impaired survival on pistachios of *Salmonella* mutants with Tn*5* localization in *rpoS* suggests that certain genes important for survival may be under RpoS control.

Regulation of RpoS intracellular levels is essential for homeostasis in Gram-negative bacteria (Hengge-Aronis, 2002). LrhA, a LysR homolog, is an important regulator of RpoS and other cellular functions such as flagellar synthesis, chemotaxis and NADH-dependent electron transport (Peterson et al, 2006; Lehnen et al, 2002; Tran et al, 1997). Deletion of *lrhA* in *E. coli* resulted in pleiotropic stationary-phase defects due to decreased RpoS levels (Gibson et al, 1999). Mutants with Tn*5* insertions in *lrhA* showed reduced survival during drying and storage in at least two mutant libraries (Table 1). Interestingly, RpoS regulation by LrhA is dependent on the small RNA (sRNA) chaperone Hfq (Peterson et al, 2006), and in the current study *Salmonella* mutants with Tn*5* insertions in *hfq* showed reduced survival during storage (Table 1). These findings suggest that RpoS and its regulation are important for survival of *Salmonella* on

low-moisture foods. Interestingly, previous studies did not identify *rpoS, lrhA, hfq* and the other transcriptional regulators identified in the current study as important survival determinants in *Salmonella* under desiccation (Li et al, 2012; Gruzdev et al, 2012; Finn et al, 2013a; Maserati et al, 2017).

Localization of Tn*5* in *rpoC* and *rpoZ* impaired survival of *Salmonella* on pistachios (Table 1). RpoC and RpoZ make up the β′ and ω subunits of the RNA polymerase and are both involved in the stringent response in *E. coli* (Igarashi et al, 1989; Yamamoto et al, 2018). Mutational studies have shown that the RpoC and RpoZ subunits bind to the alarmone ppGpp which signals the induction of the stringent response in *E. coli*, and the RNA polymerase without these subunits failed to bind to ppGpp (Igarashi et al, 1989; Ross et al, 2013; Zuo et al, 2013). In the current study, insertion of Tn*5* in ppGpp synthase, *relA* and the stringent starvation protein A (*sspA*) impaired survival of *Salmonella* on pistachios (Table 1). These observations suggest that *Salmonella* contaminating LMFs are starved and require stringent response genes for survival.

Osmolarity response

Salmonella must be able to detect osmolarity changes in the environment in order to survive under desiccation. The EnvZ/OmpR two-component system is a well-characterized phosphorelay signal transduction system that mediates osmotic stress responses in Gram-negative bacteria (Cai et al, 2002). In the current study, *Salmonella* mutants with Tn*5* insertions in *envZ* and *ompR* were impaired for long-term survival on pistachios (Table 1). Under osmotic stress, EnvZ (inner cytoplasmic membrane-bound histidine kinase) phosphorylates OmpR in the cytoplasm (Cai et al, 2002). Phosphorylated OmpR serves as a pleiotropic transcriptional regulator of outer membrane porin genes such as *ompC* and *ompF* and several other genes, including *ssrA* and *ssrB* that form a two-component regulator of *Salmonella* pathogenicity island

2 (SPI-2) genes and *hilC* which is a known SPI-1 regulator (Lee et al, 2000; Oropeza and Calva, 2009; Cameron et al, 2012). OmpC, OmpF and other membrane porins modulate membrane permeability in Gram-negative bacteria in response to environmental stress (van der Heijden et al, 2016). Therefore, failure to to activate the EnvZ/OmpR two-component system in *Salmonella* mutants with Tn*5* insertions in *envZ* and *ompR* was likely responsible for impaired for survival on pistachios.

In *S.* Newport C4.2 and *S.* Typhimurium 14028s mutant libraries, *mdoH* and *mdoG*, encoding osmoregulated periplasmic glucans (Opgs) OpgH and OpgG, respectively (Bohin, 2000), were positively selected during drying but not during storage of inoculated pistachios (Table 1). OpgH and OpgG are believed to catalyze the addition of glucosyl branches to a glucose backbone to form highly-branched oligosaccharides that accumulate in the periplasmic space of most Proteobacteria, in response to low-osmolarity environments (Bohin, 2000; Lequette et al, 2004). However, the effect of Opgs on desiccation tolerance has remained elusive. In the current study, inactivation of *mdoH* and *mdoG* appears to be advantageous to survival of *Salmonella* in the low-moisture food model that was investigated.

Other genes and intergenic regions

Impaired survival of *Salmonella* on pistachios was also associated with localization of Tn*5* in several other genes (*nlpD, rnfD, rbsR, rbsK, barA, gpmA, aroA, fabF* and *rnb*) encoding proteins with various known functions as well as several genes (*yhcB, yifE, STM14_4207, STM14_4197.J, STM14_1261* and *STM14_2365.RJ*) encoding putative or hypothetical proteins (Table 1). Of these, only *nlpD* has been previously reported to be putatively involved in *Salmonella* desiccation tolerance (Gruzdev et al, 2012). Moreover, localization of Tn*5* in six intergenic regions in *Salmonella* was associated with impaired survival on pistachios (Table 2).

BLAST analysis suggested that these regions are conserved in diverse serotypes of *Salmonella*. Several of these intergenic regions were flanked by genes in which Tn*5* insertions were also correlated with either impaired (e.g., *rpoS, nlpD, dam,* and *damX*) or enhanced (*flhD*) survival (Table 2). Mutants with Tn*5* localized in *aroK* and *hofQ,* flanking one of these intergenic regions, were present in the mutant libraries but were not selected in this model system. However, mutants with Tn*5* localized in *hdfR*, found only in the *S*. Typhimurium 14028s mutant library, showed significantly ($P < 0.001$) reduced survival by $\log_2$ -2.29 and -3.30 after drying and storage, respectively. Mutants with Tn*5* insertions in *maop,* flanking one of the selected sequences (Intergen_3365, Table 2) were not detected in any of the three mutant libraries.

5.4.4 Survival of single-gene deletion (SGD) mutants on pistachios

Phenotype evaluation on pistachios was done using mutants that were selected based on significant ($P < 0.05$) 2-fold reduction in relative abundance after drying for 1 day (T_1) and at 120 days of storage (T_{120}) in at least one strain, or with significant 2-fold reduction at either T_1 or T_{120} in at least two strains. Both the kanamycin and chloramphenicol-resistant variants of these mutants were tested for survival in order to rule out the effect of polarity. Mutants with reduced relative abundance at both T_1 and T_{120} included *ΔrbsR*, *ΔdamX*, *ΔrfbD*, *ΔlrhA*, *ΔuvrB*, *ΔhupA*, *ΔyhcB*, *ΔOmpR*, *ΔbarA*, *ΔuvrY*, *ΔyifE*, and *ΔsspA*, and mutants in six of these (*uvrB, uvrY, sspA, damX, rbsR,* and *yifE*) were negatively selected in all three libraries (Table 1). Two mutants (*ΔmdoH* and *ΔmdoG*) with significant ($P < 0.05$) increase in relative abundance at either T_1 or T_{120} in at least two strains were also tested for survival on pistachios.

The population of *Salmonella* mutants and parental strains inoculated on pistachios ranged from 8.23 to 8.79 log CFU/g, with reductions by approx. 1 log CFU/g after drying for 1 day. Although Tn*5* localization in these genes (except *mdoH* and *mdoG*) was associated with

impaired survival during drying (Table 1), survival of the SGD mutants on pistachios was not significantly impaired during drying (Fig 4 A and B). However, upon storage of inoculated pistachios at 25^{o}C for 110 days, 10 of the 14 SGD mutants showed significantly (P < 0 .05) impaired survival (Fig. 4A and B) with population reductions exceeding those of the parental strain by more than 100-fold for mutants *ΔrfbD*, *ΔlrhA*, *ΔuvrB*, and *ΔsspA* (Fig. 4A and B). Survival of two SGD mutants, *ΔhupA* and *ΔyhcB*, was similar to that of the parental strain (Fig. 4B). The negative selection of these two mutants could have resulted from impaired fitness during competition with other mutants in the mutant libraries. In agreement with the findings from mutant library selection, the deletion of *mdoH* and *mdoG* did not impair survival of *Salmonella* during drying and storage (Fig. 4A and B). Overall, the survival phenotypes of most (12/14) SGD mutants corroborated the findings from negative selection and transposon sequencing analysis of the mutant libraries. Similar data were obtained with the chloramphenicol-resistant derivatives of all tested SGD mutants.

As indicated earlier, most of the genes identified by mutagenesis to be important for survival in the current study were not identified in previous studies that used differentially expressed RNAs or proteins as an assay to investigate the molecular basis of survival of *Salmonella* (Li et al, 2012; Finn et al, 2013a; Gruzdev et al, 2012; Maserati et al, 2017). One explanation for some of these differences may be that the levels of RNA or protein product of a gene do not necessarily correlate with the importance of the gene for survival (Price et al, 2013). This is supported by the finding thatdeletion of some genes that were differentially expressed in *Salmonella* under low-moisture conditions did not impact survival (Finn et al, 2013a; Gruzdev et al, 2012). Another explanation may be that the previous studies primarily tested survival of

Salmonella on abiotic surfaces and for shorter period of time. Thus, *Salmonella* may deploy different desiccation survival strategies depending on other factors in the environment.

5.5 CONCLUSIONS

In the current study, we have integrated transposon mutagenesis and sequence analysis to identify novel determinants important for survival of *Salmonella* on pistachios, employed as model LMF. Our findings suggest that DNA recombination and repair, osmolarity regulation, lipopolysaccharide biogenesis, stringent response and stress-induced sigma factors are some of the mechanisms employed by *Salmonella* to survive on LMFs. Elucidation of the molecular basis of the survival of *Salmonella* on LMFs may aid the development of improved detection and inactivation strategies, thereby reducing the food safety burden associated with contamination of low-moisture foods.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

MM, VJ and SK conceived the study; VJ, MM, SP, WC performed experiments and analyzed the data; VJ drafted the manuscript; SK, MM, and SP revised the manuscript; JF and SK secured funding.

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Table 1: Transposon insertion sites with significant ($P < 0.05$) change by 2-fold or more in relative abundance in output compared to input mutant pools of at least two *Salmonella* Tn*5* mutant libraries. NC denotes genes or intergens that were present in the corresponding mutant library but did not change significantly ($Log_2FC \geq | 1 |$ and $P < 0.05$) while ND denotes genes or intergens that were absent in the corresponding mutant library and thus not assayed. * denotes that the genes were impacted in all the three libraries.

	Log_2Fold Change T_{input}_T_{output}				
	***S.* Newport T_0_T_{1d}**	***S.* Enteritidis T_0_T_{1d}**	***S.* Typhimurium T_0_T_{1d}**	***S.* Enteritidis T_{1d}_T_{120d}**	***S.* Typhimurium T_{1d}_T_{120d}**
DNA repair mechanism					
STM14_5112 (*uvrA*)*	**-2.56**	**-1.18**	**-1.41**	**-2.97**	**-3.16**
STM14_0926 (*uvrB*)*	**-2.57**	**-1.29**	**-1.48**	**-3.68**	**-4.70**
STM14_4752 (*uvrD*)	NC	NC	NC	-1.37	-1.33
STM14_2365 (*uvrY*)*	**-2.32**	**-2.61**	**-2.22**	**-3.88**	**-3.52**
STM14_3289 (*recN*)	NC	NC	NC	-1.03	-1.51
STM14_4509 (*recG*)	-1.5	NC	NC	-1.39	-1.25
STM14_4196 (*Dam*)	-3.11	NC	-2.35	NC	NC
Osmolarity response					
STM14_1317 (*mdoG*)	3.45	NC	1.08	NC	NC
STM14_1318 (*mdoH*)	3.35	NC	1.01	NC	NC
STM14_4217 (*ompR*)	NC	NC	NC	-1.81	-3.84
STM14_4216 (*envZ*)	NC	NC	NC	-2.14	-3.73

Table 1 Continued

	Log$_2$Fold Change T_{input}_T_{output}				
	***S.* Newport T_0_T_{1d}**	***S.* Enteritidis T_0_T_{1d}**	***S.* Typhimurium T_0_T_{1d}**	***S.* Enteritidis T_{1d}_T_{120d}**	***S.* Typhimurium T_{1d}_T_{120d}**
Lipopolysaccharide biogenesis					
STM14_2576 (*rfbP*)	NC	-2.25	-2.95	NC	NC
STM14_2579 (*rfbN*)	ND	-2.00	-3.03	-1.24	-1.18
STM14_2588 (*rfbC*)	NC	-1.37	-2.41	NC	NC
STM14_2590 (*rfbD*)	NC	-1.82	-2.28	NC	NC
STM14_4479 (*rfaL*)	NC	-1.67	-2.25	NC	NC
STM14_4478 (*rfaJ*)	NC	-1.38	-2.35	NC	NC
STM14_4783	-1.55	-2.14	NC	NC	NC
Stringent response					
STM14_4991 (*rpoC*)	-1.29	NC	-1.32	NC	NC
STM14_4506 (*rpoZ*)	NC	-1.19	-1.08	NC	-1.72
STM14_3564 (*relA*)	-1.13	NC	-1.17	NC	-1.28
STM14_4033 (*sspA*)*	**-2.18**	**-1.59**	**-1.86**	**-1.08**	**-3.77**

Table 1 Continued

	Log$_2$Fold Change T_{input}_T_{output}				
	***S.* Newport T_{0}_T_{1d}**	***S.* Enteritidis T_{0}_T_{1d}**	***S.* Typhimurium T_{0}_T_{1d}**	***S.* Enteritidis T_{1d}_T_{120d}**	***S.* Typhimurium T_{1d}_T_{120d}**
Transcriptional regulators					
STM14_1409 (*phoP*)	1.35	1.31	1.01	NC	NC
STM14_3397 (*emrR*)	NC	NC	NC	-1.54	-2.52
STM14_3526 (*rpoS*)*	**-3.29**	**-2.64**	**-3.66**	**-3.33**	**-2.63**
STM14_5011 (*hupA*)	-2.37	-1.24	-1.66	NC	NC
STM14_5249 (*nsrR*)	-1.17	NC	-1.42	NC	-1.65
STM14_2873 (*lrhA*)	-1.03	NC	-1.25	-1.29	-1.28
STM14_5242 (*hfq*)	-2.25	NC	NC	-1.36	-2.42
STM14_4694 (*yifE*)*	**-2.02**	**-1.73**	**-2.22**	**-2.05**	**-3.46**
STM14_1755 (*rnfD*)	-1.71	NC	-1.2	NC	NC
STM14_4683 (*rbsR*)*	**-1.55**	**-1.48**	**-1.80**	**-1.13**	**-1.92**
STM14_4682 (*rbsK*)	-1.34	NC	-1.36	NC	-1.47
STM14_3566 (*barA*)	NC	-1.95	-1.08	-3.06	-2.56
STM14_4197 (*damX*)*	**-1.24**	**-1.94**	**-2.24**	**-3.00**	**-1.74**
Hypothetical proteins					
STM14_4207	2.03	1.66	NC	NC	NC
STM14_4197.J	ND	ND	-2.48	ND	-3.85
STM14_1261	ND	NC	NC	-2.57	-2.94
STM14_2365.RJ*	**-2.92**	**-2.34**	**-1.91**	**-3.61**	**-3.46**

Table 1 Continued

	Log$_2$Fold Change T_{input}_T_{output}				
	***S.* Newport T_{0}_T_{1d}**	***S.* Enteritidis T_{0}_T_{1d}**	***S.* Typhimurium T_{0}_T_{1d}**	***S.* Enteritidis T_{1d}_T_{120d}**	***S.* Typhimurium T_{1d}_T_{120d}**
Intergenic regions					
Intergen_1718 (2033472…2033683)	1.05	2.27	NC	1.07	NC
Intergen_2515 (3086724…3086785)	ND	-2.43	-3.03	-3.87	NC
Intergen_3017 (3653422…3653601)	-1.84	NC	-1.98	NC	NC
Intergen_3018 (3654880…3654976)	ND	-2.34	-2.32	-3.69	-3.04
Intergen_3020 (3656644…3657107)	-4.48	NC	-1.3	NC	NC
Intergen_3365 (4120085…4120190)	-1.86	ND	-2.08	ND	2.73

Table 2: Genes flanking the intergenic regions on the 5 and 3' ends

Intergenic regions	**Gene flanking at 5'**	**Gene flanking at 3'**
Intergen_1718	flagella transcriptional regulator (*flhD*)	hypothetical protein (*STM14_2343*)
Intergen_2215	alternative sigma factor (*rpoS*)	murein hydrolase activator (*nlpD*)
Intergen_3017	DNA adenine methyltransferase (*dam*)	putative cell division protein (*damX*)
Intergen_3018	putative cell division protein (*damX*)	3-dehydroquinate synthase (*STM14_4198*)
Intergen_3020	shikimate kinase gene (*aroK*)	DNA uptake porin gene (*hofQ*)
Intergen_3365	HTH-type transcriptional regulator gene (*hdfR*)	macrodomain ori organization protein encoding gene (*maop*).

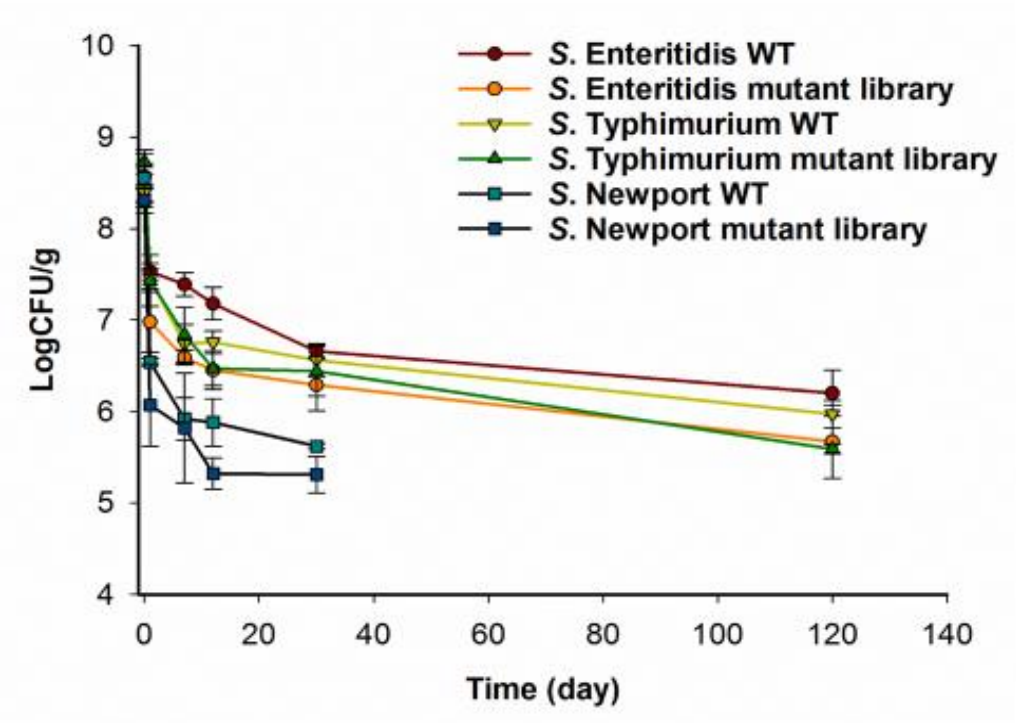
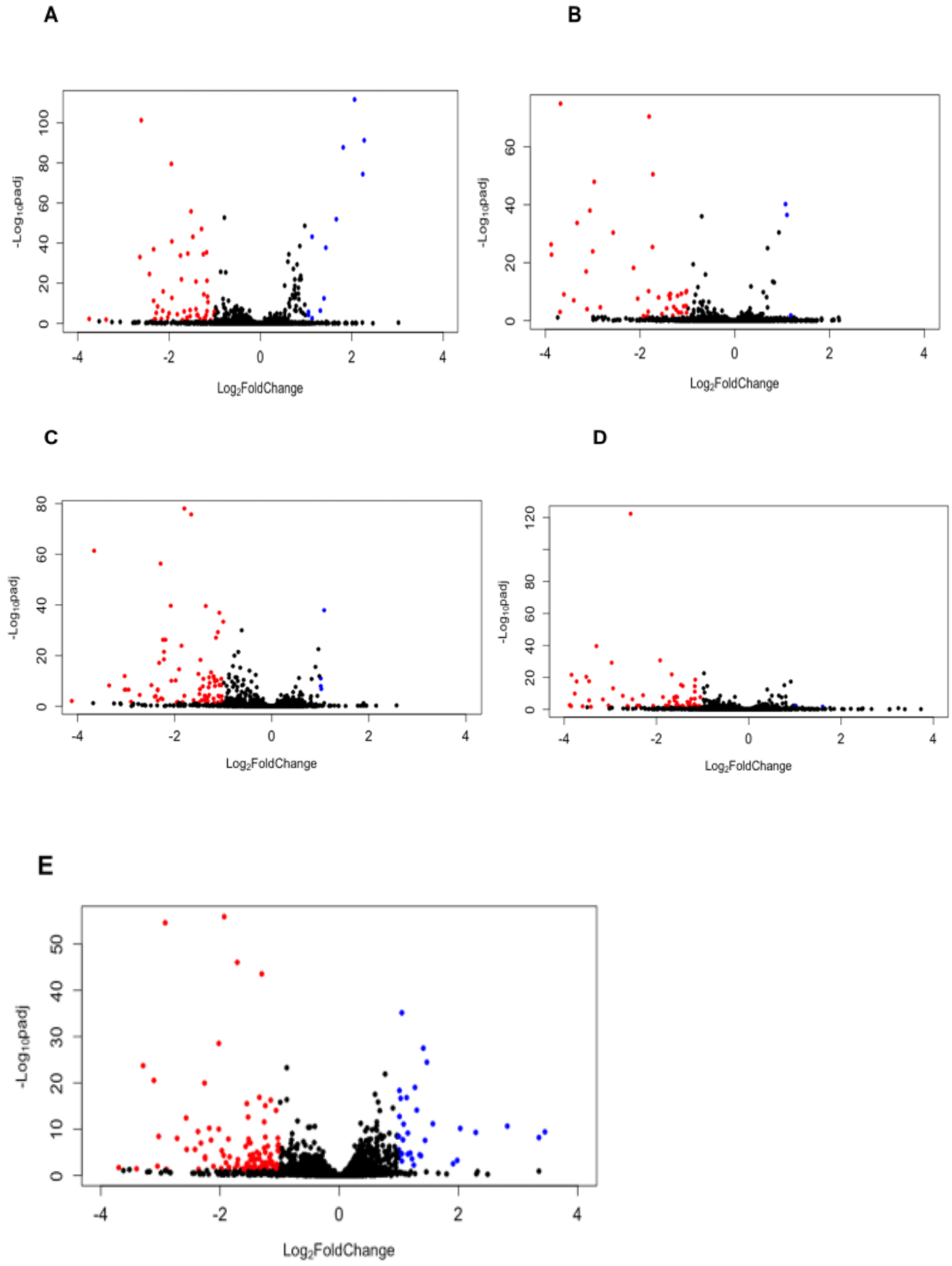


Figure 1: Survival of *Salmonella* wild-type (WT) strains and Tn*5* mutant libraries on in-shell pistachios. Pistachios were inoculated, dried and stored, and populations of *Salmonella* were enumerated as described in Materials and Methods immediately after inoculation, after drying and periodically during storage. Data represent averages from three independent trials with duplicate samples per trial. Error bars indicate standard deviation (n = 6). Enumerations of *S.* Newport C4.2 on pistachios were discontinued when the population was less than 6 log CFU/g.

Figure 2: The relative abundance of distinct Tn*5* insertion sites in the mutant libraries. (A), *S.* Enteritidis P125109 mutant library on pistachios after drying for 1 day (T_{1d}) relative to immediately after inoculation (T_0); (B), *S.* Enteritidis P125109 mutant library on pistachios at 120 days of storage (T_{120d}) relative to T_{1d}; (C), *S.* Typhimurium 14028s mutant library on pistachios at T_{1d} relative to T_0; (D), *S.* Typhimurium 14028s mutant library on pistachios at T_{120d} relative to T_{1d}; (E), *S.* Newport C4.2 mutant library on pistachios at T_{1d} relative to T_0. Red dots indicate significant ($P < 0.05$) reduction by at least 2-fold, blue dots indicate significant ($P < 0.05$) increase by at least 2-fold, and black dots indicate non-significant or less than 2-fold change in abundance. Data represent average of three independent trials and duplicate samples per trial (n = 6).


A
B
C
D
E
-Log10padj
Log2FoldChange

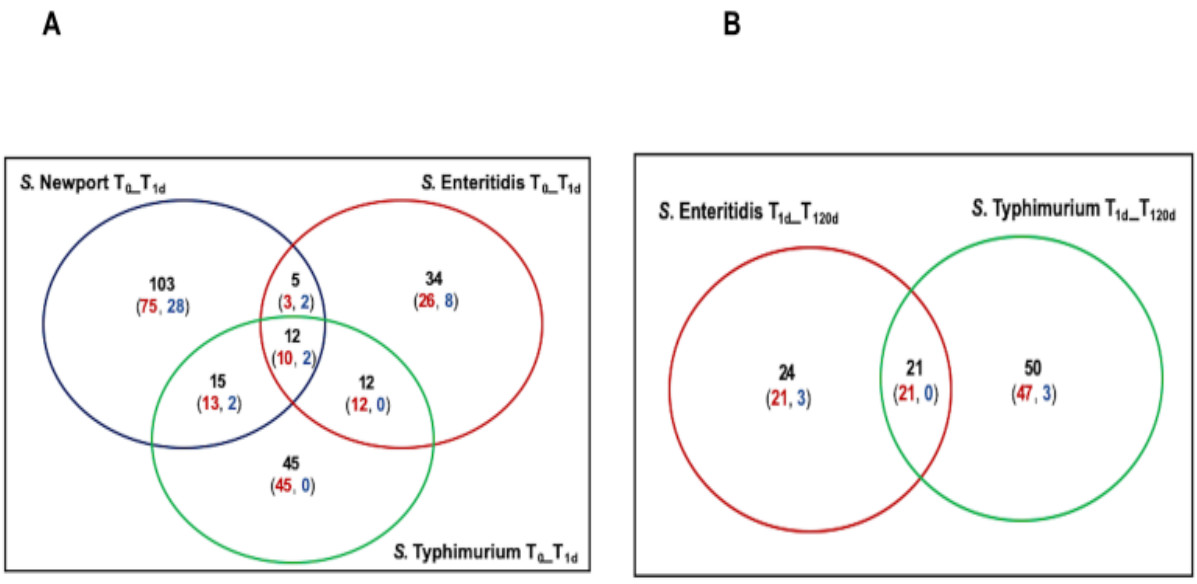


Figure 3: Distribution of Tn*5* insertion sites among tested *Salmonella* mutant libraries. Insertion sites with significant ($P < 0.05$) difference by at least 2-fold in relative abundance are shown in (**A**), after drying (T_{1d}) compared to immediately after inoculation (T_0), and (**B**), at 120 days of storage (T_{120d}) compared to T_{1d}. Black, red, and blue font indicate numbers of distinct Tn*5* insertion sites with significant ($P < 0.05$) change by at least 2-fold, at least 2-fold reduction, and at least 2-fold increase in relative abundance, respectively.

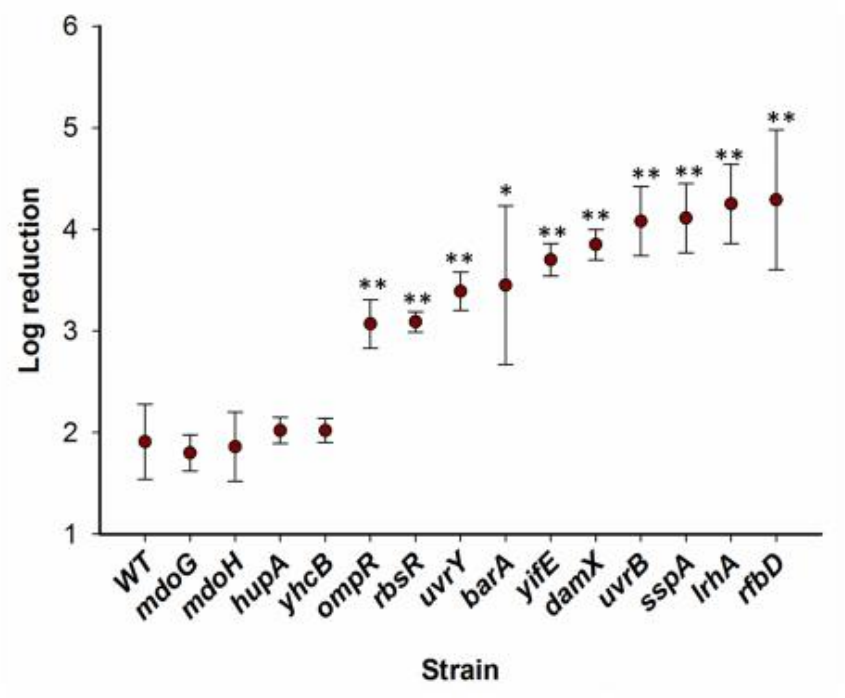


Figure 4: Survival of *Salmonella* Typhimurium 14028s and its single-gene deletion mutants on dry in-shell pistachios after 110 days. Pistachios were inoculated, dried and stored and populations of *Salmonella* were enumerated as described in Materials and Methods. Data represent the negative log10 difference in CFU at day 110 versus day zero (immediately after inoculation). The mean and standard deviation of two independent trials with duplicate samples are plotted. * and ** indicate P <0.05 and P<0.005, respectively, based on two tailed Student's t-test.

CHAPTER 6

Validating a method for multiplex screening of *Salmonella* mutants for survival on dry surfaces

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Keywords: *Salmonella,* abiotic surfaces, low-moisture foods, mutants, pistachios, nitrocellulose, survival

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6.1 Abstract

Salmonella is a leading foodborne pathogen that can survive on dry abiotic surfaces and low-moisture foods (LMFs) for long periods, but underlying survival mechanisms remain poorly understood. In the current study, a nitrocellulose-based screening protocol was used to identify *Salmonella* mutants with impaired desiccation tolerance. Multi-gene deletion (MGD) mutant libraries of *S.* Typhimurium strain 14028s were grown in 96-well plates and spotted both on regular media and on sterile nitrocellulose (NC). The NC was dried under ambient conditions, stored at 25^{o}C for 16 days, and then overlaid on agar plates which were incubated at 37 ^{o}C for 24 h. Mutants that grew on regular media but did not form colonies on the overlaid NC were considered to be putatively impaired for survival, and the survival of several such mutants was quantitatively assessed. Of 448 MGD mutants tested, 83 were putatively impaired for survival, and quantitative survival assessments of 29 of these revealed 14 that were significantly impaired. Three of the latter were assessed for survival on pistachios revealing impairment in survival on pistachios in two of the three mutants. Assessment of the survival of single-gene deletion mutants on NC revealed that genes mediating the biosynthesis of thin aggregative fimbriae (*csgD* and *csgA*), fimbriae (*fimY* and *fimA*), and *recA* may be required for survival of *Salmonella* on NC while fimbriae biosynthesis may not be important for survival on pistachios.

6.2 INTRODUCTION

Salmonella is a leading cause of foodborne illnesses globally (Hoffmann et al, 2017). In the United States alone, *Salmonella* is estimated to cause 1 million cases of foodborne illnesses annually, accounting for 28% of all hospitalizations and 35% of all deaths attributed to known foodborne bacterial pathogens (Mead et al, 1999; Scallan et al, 2011). Low-moisture foods (LMFs), i.e., foods with water activity (A_w) typically below 0.6, are important vehicles for *Salmonella* infections and several LMFs have been involved in multistate outbreaks of *Salmonella* (Podolak et al, 2010; Beuchat et al, 2013). LMFs accounted for 16 (21%) of 76 selected *Salmonella* outbreaks reported by the Centers for Disease Control and Prevention (CDC) from 2007 to 2018 (CDC, 2019). *Salmonella* can survive low-moisture conditions in foods and food processing environments for long periods, but the underlying mechanisms remain poorly understood. (Margas et al, 2014; Kimber et al, 2012; Beuchat and Mann, 2015).

Transcriptome analysis of *Salmonella* under low-moisture conditions revealed that several genes such as those involved in osmoprotectant biosynthesis and transport, inorganic ion transport, DNA repair, stress responses, virulence, amino acid metabolism, carbohydrate and lipid transport and metabolism, were differentially expressed under desiccation (Finn et al, 2013a; Gruzdev et al, 2012; Deng et al, 2012; Maserati et al, 2017; Li et al, 2012). However, genes induced under desiccation may not be always required for desiccation tolerance of *Salmonella*, as knock-out mutants lacking these genes were not impaired for survival (Finn et al, 2013a; Gruzdev et al, 2012). Moreover, transcriptome-based analysis would fail to identify genes that may be important for desiccation tolerance but not differentially expressed.

High-throughput phenotypic screening methods such as transposon-sequencing that combine random mutagenesis and next-generation sequencing (NGS) constitute powerful

approaches that can be employed to overcome some of the limitations of transcriptome-based methods (Mandal and Kwon, 2017). Although the cost of NGS has become substantially reduced, costs of reagents, DNA library preparation and sequencing are still considerably high for routine applications in many labs. In this study, we tested the capacity of a non-NGS-based, multiplex screening assay to identify mutants of *Salmonella* that are impaired for survival in low-moisture conditions. The construction of multi-gene deletion (MGD) mutants of *Salmonella* Typhimurium 14028s (Porwollik et al, 2014) has provided the opportunity for hierarchical screening of multiple genes for various phenotypes, including survival in low-moisture environments.

The objective of the current study was to test the capacity of a multiplex screening protocol to identify *Salmonella* MGD mutants that were impaired for survival in low-moisture environments and to identify the genes required for survival. A panel of MGD mutants of *S.* Typhimurium 14028s were tested for survival on a model abiotic surface, i.e., dry nitrocellulose, and several MGD mutants that were identified to be survival-impaired were assessed for survival on a low-moisture food, pistachios. To identify the genes involved in the desiccation tolerance of *Salmonella*, relevant single-gene deletion mutants of *S.* Typhimurium 14028s were also assessed for survival on dry nitrocellulose.

6.3 MATERIALS AND METHODS

6.3.1 Bacterial strains and culture preparation

Salmonella enterica sv Typhimurium 14028s (wild-type parental strain), two collections of multi-gene deletion (MGD) mutants (each mutant containing deletions of two or more contiguous genes), and a panel of single-gene deletion (SGD) mutants of *S.* Typhimurium 14028s were utilized in this study. *S.* Typhimurium 14028s was originally isolated from chickens

by the Centers of Disease Control and Prevention in 1960 (Jarvik et al, 2010). The construction and characterization of the MGD and SGD mutants were previously described (Porwollik *et al,* 2014). One of the two MGD mutant collections, MGD-Cam^R, was comprised of 251 mutants in three libraries, MGD_Cm_01 (n=92), MGD_Cm_02 (n=88), and MGD_Cm_03 (n=71), in which the deleted genes were replaced with a chloramphenicol resistance gene oriented in the antisense direction. The second MGD mutant collection, MGD-Kan^R, was comprised of 197 mutants in five libraries, MGD_Kan_069/070 (n=7), MGD_Kan_071/072 (n=46), MGD_Kan_073/074 (n=40), MGD_Kan_075/076 (n=60), and MGD_Kan_077/078 (n=44) in which the deleted genes were replaced with a kanamycin resistance gene oriented in the sense direction. Some genes were deleted in more than one MGD mutant, although none of the mutants in each MGD collection harbored exactly the same gene deletions. Overall, 140 MGD-Kan^R mutants harbored the same gene deletions as 140 MGD-Cam^R mutants. Hereafter, MGD mutants in the Kan^R and Cam^R collections with the same gene deletions will be referred to as the kan^R and Cam^R derivatives, respectively. In the SGD mutants, individual genes have been replaced with a kanamycin (SGD-Kan^R) or chloramphenicol (SGD-Cam^R) resistance gene, oriented in the antisense and sense direction of the gene, respectively. The MGD collections were preserved in 96-well plates in Luria-Bertani broth (LB, Becton, Dickinson & Co., Sparks, MD) while the SGD mutants were preserved in cryovials containing LB, both at -80 ^{o}C.

6.3.2 Multiplex screening of multi-gene deletion mutant libraries of *Salmonella* for survival on nitrocellulose

Frozen cultures of MGD mutants in 96-well plates were thawed in a laminar flow biosafety cabinet at 25^{o}C for 10 min. Using a multichannel pipette, 20 µl of bacterial cell suspension of each mutant was transferred into new 96-well plates (Greiner Bio-One, Monroe,

NC) containing 180 µl of trypticase soy broth (TSB, Becton, Dickinson & Co.) and 0.6 % yeast extract (TSB-YE, Becton, Dickinson & Co.), and incubated statically at 37 ^{o}C for 24 h. The optical density (OD_{560}) of the cultures was determined using the Infinite ® 200 PRO NanoQuant spectrophotometer (Tecan Group Ltd., Mannedorf, Switzerland). The cultures were spotted using a flame-sterilized metal stamp on autoclaved pieces of nitrocellulose membranes (0.2 µm, 7 × 7 cm^2, Pall Gelman Laboratory, Ann Arbor, MI) placed in sterile polystyrene Petri dishes (100 mm × 15 mm, Fisher Scientific, Fair Lawn, NJ). The spotted NC was dried (with the Petri dishes covered with lids) in a laminar flow biosafety cabinet at 25 ^{o}C for 5 min, and the Petri dishes were then covered and stored in the dark at 25 ^{o}C for 16 days. The bacterial cultures were also spotted on TSB-YE with 1.2% agar (TSA-YE, Becton, Dickinson & Co.) and 60µl ml^{-1} kanamycin (Fisher Scientific, Fair Lawn, NJ; TSA-YE+Kan) or 20µl ml^{-1} chloramphenicol (Acros Organics, NJ; TSA-YE+Cam) followed by incubation at 37 ^{o}C for 24 h. At 16 days of storage, each spotted NC was overlaid on TSA-YE plates with the inoculated side facing up and incubated at 37^{o}C for 24 h. Mutants that yielded confluent growth on TSA-YE+Kan or TSA-YE+Cam but formed few or no colonies on the overlaid NC were considered to be putatively survival-impaired. The experiment was conducted in three independent trials with duplicate NC membranes in each trial.

6.3.3 Quantitative assessment of the survival of multi-gene deletion mutants on NC

To confirm the impaired survival of MGD mutants that formed few or no colonies on NC individual MGD mutant and wildtype strain (WT) were grown in 1 ml TSB-YE in1.5-ml Eppendorf tubes (VWR Int. Radnor, PA) at 37^{o}C for 24 h. The cultures were washed twice in sterile deionized water (dH_2O) and serially diluted in dH_2O. Cell suspensions of the WT and mutants were spotted in a series of 10^2 - 10^7 CFU/spot on autoclaved NC (7 × 7 cm^2) that were

placed in sterile polystyrene petri dishes. For each mutant, the 10^2- 10^4 dilutions were spotted in duplicate on one NC and the 10^5-10^7 dilutions on another, with the same dilutions of the WT spotted on each NC. The spotted NC was dried as described above in a laminar flow biosafety cabinet at 25 ^oC for 5 min, and the Petri dishes containing the spotted NC were covered and stored in the dark at 25 ^oC for up to 7 days. Populations of *Salmonella* on the spotted NC were enumerated at 24 h and 7 days by overlaying the NC on TSA-YE as described above. CFUs on the NC were counted and logarithmic reductions in the populations were calculated. The experiment was conducted in two independent trials, with two NC membranes spotted in duplicate per mutant for each trial.

6.3.4 Confirmation of deletions in *Salmonella* multi-gene deletion mutants

The expected gene deletions in MGD mutants of interest were confirmed with polymerase chain reaction (PCR). The primers for PCR amplifications were purchased from Eton Bioscience Inc. (Durham, NC) and are listed in **Table 1**. The forward primers were designed to bind to 400-600 bp upstream of the expected deleted regions while the reverse primers were complementary to nucleotide sequences within the deleted regions. Therefore, mutants harboring the expected deletions would fail to yield DNA amplicons, while the parental strain would yield DNA amplicons with the expected size. The PCR reaction consisted of 1 µl of bacterial cell suspension (MGD mutant or WT) as template, 1x GoTaq Green Master Mix (Promega, Madison, WI), 1µM each of the forward and reverse primers, and nuclease-free water in a 20-µl reaction volume. The thermocycler conditions for the PCR were set at 95^oC for 5 min; 34 cycles of 95^oC for 1 min, 55^o C for 30 s, and 72^oC for 1.30 s; 72^oC for 5 min for all mutants except MGD_Cm_01_D01/ MGD_Kan_71/72_D01, for which the annealing temperature was

set at 50°C for 30 s. PCR amplicons were separated on 0.8% agarose by gel electrophoresis (100 V, 60 min) and stained with ethidium bromide for visualization.

6.3.5 Single-gene deletion mutant characterization

Individual SGD mutants and survival-impaired MGD-Cam^R were grown in 1 ml TSB-YE at 37 °C for 24 h, washed in dH_2O, spotted in a series at 10^2- 10^7 cells /spot on NC, dried and stored for 15 days as described above. The populations of *Salmonella* at 1, 7, 10, 12 and 15 days of storage were enumerated as described above. The experiment was conducted in two independent trials, with two NC membranes spotted in duplicate for each trial.

6.3.6 Survival assessment of multi-gene deletion mutants on in-shell pistachios

In-shell pistachios were obtained from a commercial source and kept under ambient conditions. The total aerobic plate count of in-shell pistachios was determined by transferring 10 g into 10 ml dH_2O in 50-ml conical centrifuge tubes (VWR Int.), followed by vortexing at high speed for 1 min. The liquid was serially diluted and appropriate dilutions were plated on TSA-YE, followed by incubation at 37 °C for 24 h. To determine the A_w of pistachios before and after inoculation, in-shell pistachios (10 g) were ground into smaller particulates using a coffee grinder (Mr. Coffee, Cleveland, OH) for 30 s, and the A_w was measured using a benchtop water activity meter (AquaLab model 3TE, Decagon Devices, Pullman, WA).

For inoculum preparation, a single colony of each mutant and WT was individually grown in 1-ml TSB-YE at 37 °C for 24 h, and 100 µl was then spread-plated on TSA-YE and incubated at 37 °C for 24 h. The resulting bacterial lawn was transferred using sterile loops (Fisher Scientific) into 5 ml dH_2O in 15-ml conical centrifuge tubes (VWR Int.), washed twice in 5 ml dH_2O and then added to in-shell pistachios (100 g) at 4% v/w. Inoculated pistachios were dried inside Nalgene polypropylene desiccators (Thermo Scientific, Waltham, MA)

containing drierite (W.A. Hammond drierite Co. Ltd., Xenia, OH) as desiccant for 24 h, when the A_w of the inoculated pistachios approximated that of uninoculated pistachios. The dried inoculated pistachios were placed into several 50-ml centrifuge tubes (VWR Int.) and stored in the dark at 25°C and 35-48% RH. The populations of *Salmonella* immediately after inoculation of the pistachios, after drying and periodically during storage were enumerated on TSA-YE and Xylose Lysine Deoxycholate agar (XLD, Becton, Dickinson & Co.) that were incubated at 37°C for 24 h. The experiment was conducted in two independent trials with duplicate samples analyzed per trial.

6.3.7 Statistical analysis

Bacterial counts from each replicate were entered into Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA) and $\log_{10}$- transformed to compute the log CFU per ml, g, or NC spot as appropriate. Significance of differences between mutants and wild type strain at different times was determined with one-way analysis of variance (ANOVA) with Tukey's test used to compare multiple means (α, 0.05), using SPSS (SPSS version 22, IBM Corporation Software Group, Somers, NY).

6.4 RESULTS

6.4.1 Multiplex screening on NC identified survival-impaired multi-gene deletion mutants

The optical density (OD_{560}) of cultures of MGD mutants ranged from 0.4 to 1.2, with the majority (70 and 76% of MGD_Cam^R and MGD_Kan^R, respectively) having wild-type OD_{560} of 1.2 (data not shown). However, all mutants grew on the spotted TSA-YE with no apparent differences in growth capacity. MGD mutants that grew on TSA-YE agar plates but formed few or no colonies on NC at 16 days (Fig. 1) were considered to be putatively impaired for survival. Out of 250 MGD-Cam^R and 197 MGD-Kan^R , mutants, 53 (21.5%) and 30 (15%), respectively,

failed to be recovered from NC at 16 days, with survival-impaired mutants being identified in each of the Cam^R and Kan^R libraries (Supplementary Material). A subset of the MGDs were accompanied with impaired survival on NC in both the Cam^R and Kan^R versions. Of the 53 MGD_Cam^R mutants, 37 had Kan^R derivatives with the same gene deletions, and for 8 of these survival was impaired in both the Cam^R and Kan^R derivatives. In addition to these 8 MGD-Kan^R (that also showed impairment in the Cam^R derivatives), 1 MGD-Kan^R mutant which did not have a Cam^R derivative was also impaired for survival on NC, while the remainder 16 of 25 MGD-Kan^R mutants that were tested survived on NC. Thus, of 140 mutants with the same deletions in both the Cam^R and Kan^R MGD systems, 8 were impaired in survival in both the Cam^R and Kan^R versions.

The survival of 29 (20 MGD-Cam^R and 9 MGD-Kan^R) of the 83 putative desiccation-intolerant MGD mutants was quantitatively assessed on NC. The mutants selected for further testing included the 8 MGD-Cam^R and 8 MGD-Kan^R mutants that were impaired in survival in both the Cam^R and Kan^R versions (described above), 3 MGD-Cam^R mutants with no Kan^R derivatives, 9 MGD-Cam^R mutants for which the Kan^R derivatives were found to be not impaired, and 1 MGD-Kan^R mutant with no Cam^R derivative. The growth of all 29 MGD mutants in TSB-YE was comparable to that of the WT (10^9 CFU /ml). The population of the WT strain was reduced on NC by 2 and 4 log CFU/spot at 1 and 7 days of storage, respectively (Table 2). Of the 29 tested mutants, 12 (8 MGD-Cam^R and 4 MGD-Kan^R) showed significantly ($P < 0.05$) higher reductions on NC in comparison to the WT, with population reductions ranging from 2.9 and 5.9 log CFU/spot after 1 day and 4.3 to 6.3 log CFU/spot after 7 days (**Table 2 and 3**). All 4 of the survival-impaired MGD-Kan^R mutants had counterparts among the survival-impaired MGD-Cam^R mutants (**Table 2**). These were MGD_Cm_01_D01 and

MGD_Kan_071/072_D01; MGD_Cm_01_B03, and MGD_Kan_071/072 _B03; and MGD_Cm_02_A09 and MGD_Kan _073/074_A09, and MGD_Cm_01_C03 and MGD_Kan_71/72_C03 (**Table 2**). Population reductions of the remaining 16 mutants were not significantly different from the WT (data not shown). Four MGD-Cam^R mutants (MGD_Cm_02_D08, MGD_Cm_03_G10, MGD_Cm_03 _C02, and MGD_Cm_03_ C06) showed significantly-impaired survival in comparison to the WT strain (**Table 3**) but their corresponding MGD-Kan^R derivatives were not impaired (**data not shown**). One MGD-Cam^R mutant (MGD_Cm_03_H06) showed significantly higher reductions on NC than the WT (**Table 3**) but lacked a Kan^R counterpart.

6.4.2 Identification of relevant genetic determinants in survival-impaired MGD mutants

To determine which gene deletion conferred the survival-impaired phenotype of MGD mutants that were impaired for survival in both Cam^R and Kan^R derivatives, we assessed NC survival of SGD mutants harboring deletions in single genes. SGD mutants were available for genes lacking in MGD_Cm_01_D01 and MGD_Kan_071/072_D01; MGD_Cm_01_B03, and MGD_Kan_071/072_B03; and MGD_Cm_02_A09 and MGD_Kan_ 073/074_A09. SGD mutants in genes deleted in MGD_Cm_01_C03 and MGD_Kan_71/72_C03 (genes deleted are listed in Table 2) were not available. All genes deleted in MGD_Cm_01_B03 constituted the *Salmonella fim* operon (*fimAICDHFZYW*), and SGD mutants in two of these genes, *fimA,* encoding the major fimbrial protein subunit and *fimY*, encoding a transcription regulator for fimbriae biosynthesis, were selected for survival assessment. The SGD *fimA* mutants were *ΔfimA*-Kan^R and *ΔfimA*-Cam^R, while SGDs in *fimY* were *ΔfimY*-Kan^R and *ΔfimY*-Cam^R.

Of the 18 genes deleted in MGD_Cm_01_D01, 9 were part of the cell surface glycoprotein (*csgDEFG/BAC,* also annotated as *agfDEFG/BAC*), which had been previously

linked to desiccation tolerance and persistence in *Salmonella* (White et al, 2006). Therefore, SGD mutants lacking a transcription regulatory gene (*csgD*), *ΔcsgD*-Kan^R and a curli major subunit gene (*csgA*), *ΔcsgA*-Kan^R and *ΔcsgA*-Cam^R, were selected for survival assessments; the *ΔcsgD*-Can^R construct was not available.

In MGD_Cm_02_A09, 15 of the 20 deleted genes were also deleted in MGD_03_Cm_H10 which was not impaired for survival, and thus not considered relevant for further testing. Of the remaining 5 genes, mutants *ΔrelA*-Kan^R and *ΔrelA*-Cam^R with deletion of *relA,* encoding an enzyme that mediates production of the alarmone guanosine tetraphosphate, a well- characterized stringent response factor (Potrykus and Cashel 2008; Mechold et al, 2013) were selected for survival assessments. Additional SGD mutants that were further assessed were *ΔygaD*-Kan^R and *ΔygaD*-Cam^R, harboring deletion of *ygaD,* encoding a hypothetical protein.

After 7 days of storage at 25^{o}C, the population of the WT strain on NC was reduced by approx. 3 log CFU/spot, with further reductions by approx. 2 log CFU/spot after 15 days of storage (**Fig. 2A, B, C**). The populations of MGD_01_Cm D01, MGD_01_Cm_B03, and MGD_02_Cm A09 were reduced by approx. 6 log CFU after 7 days of storage and the mutants were undetectable on the NC after 10 days (Fig. 2A, B and C). At 7 days, all tested SGD mutants showed population reductions of approx. 4-5 log CFU/spot on NC which were significantly ($P < 0.05$) higher than reductions of the WT strain but also significantly ($P < 0.05$) lower than observed with the corresponding MGD mutants (**Fig. 2A, B, C**). The populations of the tested SGD mutants decreased progressively, and no recovery was observed after 15 days on the NC, except for *ΔygaD*-Kan^R and *ΔygaD*-Cam^R with approx. 1.4 log CFU **(Fig 2. A, B, C).**

6.4.3 Survival of desiccation-intolerant multi-gene deletion mutants on in-shell pistachios

Three of the survival-impaired MGD-Cam^R, whose Kan^R derivatives were also impaired in survival on NC (MGD_01_Cm_B03, MGD_01_Cm D01 and MGD_02_Cm_A09) were selected for survival assessment on pistachios. The population of the WT and mutants on pistachios immediately after inoculation was approx. 10 log CFU/g. After drying for 1 day, the population of WT, MGD_01_Cm_B03, MGD_01_Cm D01 and MGD_02_Cm_A09 were reduced by approx. 1.2, 1.6, 3.5 and 2.5 log CFU/g, respectively (**Fig. 3A**). The populations decreased progressively during storage, with MGD_01_Cm_D01 and MGD_02_Cm_A09 showing significant higher reductions compared to the WT strain (**Fig. 3B**). However, the decline of MGD_01_Cm_B03 did not differ significantly from that of the WT at any of the sampling time-points (**Fig. 3B**).

6.5 DISCUSSION

The ability of *Salmonella* to survive on LMFs is associated with significant food safety concerns. In the current study, we assessed the capacity of a simple, low-cost multiplex screening protocol to identify genes mediating the survival of *Salmonella* in low-moisture conditions on nitrocellulose (NC) membranes used as a model dry surface. NC membranes are permeable to the growth medium and can be spot-inoculated with multiple strains at the same time, rendering them suitable for multiplex growth assays. In contrast to other abiotic surfaces (e.g., plastic, paper, or stainless steel) that were used in previous studies, enumeration of *Salmonella* surviving on NC in the current study did not require removal of cells from the surface, which tends to be problematic for strongly-adherent cells (Hokunan et al, 2016, Finn et al, 2013a; Hiramatsu et al, 2005). The survival of *Salmonella* on nitrocellulose was assessed for up to 16 days because the WT strain showed approx. 5 log reduction after storage on dry NC for

15 days (**Fig. 2**). Therefore, desiccation for 16 days was deemed sufficient to identify mutants impaired for survival. However, there is a possibility that longer desiccation periods may be required to identify mutants impaired for long-term survival.

Nonetheless, some mutants that were impaired in survival were identified using the NC-based screening assay. While the survival phenotype (including impaired and non-impaired mutants) of majority of mutants with deletions in both Cam^R and Kan^R derivatives were consistent, variations in survival between the two versions were observed. The discrepancy between the survival phenotype of the Kan^R and Cm^R derivatives of these mutants may be due to polarity effects (Powolik et al, 2014), or may reflect other, unidentified genetic changes in one of the derivatives. Quantitative survival assessments revealed that the survival of *Salmonella* WT on nitrocellulose in this current study was comparable to survival on stainless steel reported previously (approx. 4 log reduction after 7 days) but considerably less than survival on plastic (Margas et al, 2014; Finn et al, 2013a; Hokunan et al, 2016). The quantitative assessments confirmed that 48% (14 of 29) of the mutants identified via the multiple screening protocol were significantly impaired in survival. Therefore, quantitative survival assessments should be included to confirm the phenotype of mutants found to be survival-impaired with the multiplex screening protocol.

Seven of the genes deleted in MGD_01_Cm_D01 and its Kan^R derivative form a cell surface glycoprotein operon (*csgDEFG/BAC* or *agfDEFG/BAC*) which is involved in the biosynthesis and assembly of thin aggregative fimbriae (Tafi or curli) in *Salmonella* and is conserved in several other enterobacterial species (White et al, 2006; Zogaj et al., 2003). Tafi and cellulose production are positively regulated by *csgD*, and a *ΔcsgD Salmonella* mutant was impaired for long-term survival on plastic surfaces (White et al, 2006). Similarly, in the current

study, a *ΔcsgD*-Kan^R mutant was impaired for survival on NC. Moreover, *Salmonella* mutants *ΔcsgA*-Kan^R and *ΔcsgA*-Cm^R, lacking the curli major subunit gene *csgA* were also impaired for survival on NC. Tafi and cellulose production are important for the formation of a recalcitrant, protective multicellular matrix, conferring the RDAR (red, dry and rough) morphotype, which has been speculated to aid the persistence of *Salmonella* in the environment (Collins et al, 1991; White and Surette, 2006). The findings from the current study showed that mutants lacking either the curli major subunit gene (*csgA*) or the transcriptional regulator (*csgD*) were impaired for survival. The regulator *csgD* was also reported in a previous study to be important for desiccation tolerance of *Salmonella* on dry plastic (White et al, 2006).

The fimbrial gene cluster *fimAICDHFZYW* is involved in the biosynthesis of type I fimbriae in *Salmonella* (Zeiner et al, 2012). Within this gene cluster, *fimAICDHF* constitutes a single operon transcribed form the *fimA* promoter region (P_{fimA}) while transcriptional regulators *fimZ*, fim*Y* and fim*W* are independently transcribed (Kolenda et al, 2019). Fimbriae are adhesive organelles that facilitate adherence of *Salmonella* to surfaces, and *Salmonella* mutants lacking the major fimbrial protein subunit fimA were afimbriated (Zeiner et al, 2012; Hansmeier et al, 2017). Although the roles of fimbriae as an intestinal colonization factor (Humphries et al, 2001) and in biofilm formation (Boddicker et al, 2002) of *Salmonella* have been reported, their relevance in desiccation tolerance of *Salmonella* has not been characterized. In the current study, MGD mutants with deletions in the *fim* genes and SGD mutants (*ΔfimA*-Kan^R , *ΔfimA*-Cam^R , *ΔfimY*-Kan^R , and *ΔfimY*-Cam^R) were all impaired for survival on NC, thus underlining the importance of the type I fimbriae system in the desiccation tolerance of *Salmonella*. Collectively, the findings from the current study suggests that the ability of *Salmonella* to adhere to surfaces via curli or fimbriae is important for survival in low-moisture conditions.

Our findings also showed that the *recA* gene was important for the survival of *Salmonella* on NC. The RecA protein is a well characterized member of the SOS network and is involved in DNA recombination and repair (Anderson et al, 1998; Benson et al, 2000). The impairment of the *recA* deletion mutant of *Salmonella* for survival on NC suggest the importance of DNA damage repair for survival. Several other genes that encode proteins involved in DNA damage repair, such as *uvrA*, *uvrB*, *uvrC*, *recN*, and *recX* were not deleted in the MGD mutants, and thus not assessed for their roles in survival in the current study. Interestingly, MGD mutants that contained deletions in some genes, such as *proP*, *sopD*, *sseD*, *kdpA*, and *aceA* that have been described to be important for survival of *Salmonella* on abiotic surfaces in previous studies (Finn et al, 2013; Maserati et al, 2017; and Gruzdev et al, 2012), were not impaired for survival under the conditions tested in the current study. It is noteworthy that the conditions, such as the drying methods and storage times, employed in those previous studies were different from the current one. Therefore, such differences, in addition to inherent variations in the sensitivity of different methods may cause variations in the determinants identified as relevant for the survival of *Salmonella* under desiccation stress.

Three MGD mutants that were impaired for survival on NC were tested for survival on pistachios in order to determine if the determinants for the survival on abiotic surfaces were also required for survival on LMFs. *Salmonella* (both mutants and WT) survived longer on pistachios than on nitrocellulose membrane in agreement with previous studies that reported that *Salmonella* survive longer on LMFs (e.g., chocolate and almonds) than on plastic and paper surfaces (Hokunan et al, 2016, Hiramatsu et al, 2005). Our findings suggest that cell surface glycoprotein (genes deleted in MGD_01_Cm D01) and RecA (gene deleted in MGD_02 _Cm_A09) were important for survival of *Salmonella* on both abiotic surface and LMFs, while

fimbriae biosynthesis (genes deleted in MGD_01_Cm_B03) was required for survival on NC but not on pistachios. We hypothesize that fimbriae biosynthesis was important for survival on NC because fimbriae were needed to attach to relatively smooth surfaces, e.g., NC membranes, but dispensable for attachment to rough surfaces such as those of in-shell pistachios.

6.6 CONCLUSIONS

The nitrocellulose-based multiplex screening protocol with MGD mutants was able to identify several *Salmonella* mutants that are impaired for survival on dry abiotic surfaces. Testing mutants with deletions in one of the multiple genes deleted in the MGD mutants revealed genes that were important for the desiccation tolerance of *Salmonella*. While certain determinants such as thin aggregative fimbriae (*csgD* and *csgA*) and *recA* were required for survival on model abiotic surfaces (NC) and LMFs (pistachios), others such as fimbriae (*fimY* and *fimA*), that were important for survival on abiotic surfaces may not be required for survival on LMFs. Therefore, research efforts aimed to characterize molecular determinants for survival on LMFs should assess the survival of *Salmonella* on LMFs.

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Table 1: Primers used in this study

MGD mutant	Primers	Sequence (5' to 3')
MGD_CM_01_D01	D01_Forward	AGATCAGTCTGCCGAAGATG
MGD_KAN_071/072_D01	D01_Reverse	TAGCGGGATGATAGCGTCCGTT
MGD_CM_01_B03	B03_Forward	CATTCGCCGGGAATAAAGCC
MGD_KAN_071/072_B03	B03_Reverse	CATGATAAAGGTGGCGTCGG
MGD_CM_03_C01		
MGD_CM_01_D04	D04_Forward	GGTACGATCGTCGCCTTCAA
MGD_KAN_071/072_D04	D04_Reverse	AGAGGCTTCGCGAATACTGTCG
MGD_CM_02_A09	A09_Forward	CTGGCGATAAAGCGCATCAC
MGD_KAN_073/074_A09	A09_Reverse	CGAACCCGTAGTGGTCGAAA
MGD_CM_02_D08	D08_Forward	TCAACACCACGAGTGAGAGC
MGD_KAN_077/078_G06	D08_Reverse	CTCGCGCAGGAAAGTGTTGC
MGD_CM_03_C02	C02_Forward	GCCAGAAGGGACGCTATGTC
MGD_KAN_075/076_C02	C02_Reverse	CGGTCAATGTGAATCGCCAC
MGD_CM_03_ H06	H06_Forward	TTAGCGGGTATAACGGCAGC
	H06_Reverse	GCGCTGGCATAGTCCAGATT

Table 2: Reductions in the population of multi-gene deletion mutants of *Salmonella* Typhimurium 14028s that were impaired for survival on nitrocellulose membrane in both the Kan^R and Cam^R derivatives. N_0, N_1, and N_7 represent bacterial population at inoculation, at 1 and 7 days of storage, respectively. Data represent average ± standard deviation of two independent trials, with duplicate NC each spotted in duplicate per mutant for each trial. Genes highlighted in bold denotes genes that were also deleted in other MGD mutants that were not impaired for survival.

Strain	**N_0**	**N_0-N_1**	**N_0-N_7**	**Genes deleted**
S. Typhimurium 14028s	7.1 ± 0.1	2.0±0.2	4.1±0.1	Wild type
MGD_Cm_01_D01	7 ± 0.3	5.8 ± 0.4	6.1 ± 0.1	*msyB, yceK, mdoH, mdoG, STM14_1316, mdoC, ymdC, STM14_1313, ymdA, csgC, csgA, csgB, STM14_1308, STM14_1307, csgD, csgE, csgF, csgG*
MGD_Kan_71/72_D01	6.6 ± 0.7	4.5 ± 0.7	5 ± 0.2	
MGD_Cm_01_B03	6.8 ± 0.6	5.4 ± 1.2	5.7 ± 0.7	*fimA, fimI, fimC, fimD, fimH, fimF, fimZ, fimY, STM14_0643, fimW*
MGD_Kan_71/72_B03	6.7 ± 0.6	5.2 ± 0.8	5.1 ± 1.2	
MGD_Cm_03_C01	7.3 ± 0.1	5.1 ± 0.1	5.9 ± 0.1	
MGD_Cm_02_A09	7.1 ± 0.2	4.9 ± 0.3	5.7 ± 0.4	*recA, ygaD, STM14_3419, mltB, STM14_3421*, ***srlA, srlE, slrB, srlD, gutM, STM14_3427, srlR, gutQ, ygaA, STM14_3431, ygbD, STM14_3433, hypF, STM14_3435, hydN***
MGD_Kan_73/74_A09	7.1 ± 0.3	4.8 ± 0.4	5.3 ± 0.9	

Table 2 Continued

Strain	N_0	N_0-N_1	N_0-N_7	Genes deleted
MGD_Cm_01_C03	7.0 ±0.1	4.3 ± 0.2	4.8 ± 0.3	STM14_1013_to_STM14_1016
MGD_Kan_71/72_C03	7.1 ±0.1	4.3 ± 0.05	4.3 ± 0.2	

Table 3: Reductions in the population of multi-gene deletion mutants of *Salmonella* Typhimurium 14028s that were impaired in survival on nitrocellulose membrane in only one version (either Cam^R or Kan^R derivatives). N_0, N_1, and N_7 represent bacterial population at inoculation, at 1 and 7 days of storage, respectively. Data represent average ± standard deviation of two independent trials, with duplicate NC each spotted in duplicate per mutant for each trial. Genes highlighted in bold denotes genes that were also deleted in other MGD mutants that were not impaired for survival. * denotes that the MGD-Cam^R mutant did not have a Kan^R version.

Strain	**N_0**	**N_0-N_1**	**N_0-N_7**	**Genes deleted**
MGD_Cm_02_D08	6.9 ± 0.03	5.9 ± 0.01	5.9 ± 0.01	*STM14_4042, oadB, oadA, oadG, STM14_4046, STM14_4047, STM14_4048*
MGD_Cm_03_G10	7.3 ± 0.03	4.8 ± 0.02	6.3 ± 0.07	*pgtE, pgtA, pgtB, pgtC,* ***pgtP, STM14_2951, STM14_2952, ddg, STM14_2954, STM14_2955, glk, STM14_2957, STM14_2958, STM14_2959, STM14_2960****, STM14_2961, mntH, nupC, STM14_2964*
MGD_Cm_03_C02	7.2 ± 0.04	4.7 ± 0.01	5.8 ± 0.05	***STM14_0648, STM14_0649, STM14_0650, STM14_0651, gtrA, STM14_0653, STM14_0654, STM14_0655****, STM14_0656 STM14_0657, STM14_0658, STM14_0659, STM14_0660, STM14_0661, STM14_0662 pheP, STM14_0664, STM14_0665, ape, STM14_0667*

Table 3 Continued

Strain	N_0	N_0-N_1	N_0-N_7	Genes deleted
MGD_Cm_03_C06	7.3 ± 0.1	4.9 ± 0.1	5.8 ± 0.4	*ybdL, ybdM, ybdN, ybdO, dsbG, ahpC, ahpF, STM14_0709, STM14_0710, STM14_0711, STM14_0712, ybdQ, STM14_0714, STM14_0715, ybdR, rnk*
MGD_Cm_03_H06*	7.1 ± 0.05	2.9 ± 0.08	5 ± 0.2	*STM14_3298, STM14_3299, STM14_3300, STM14_3301, STM14_3302, STM14_3303 STM14_3304, STM14_3305, STM14_3306*

Supplementary Table: Multigene deletion mutants of *Salmonella* Typhimurium 14028s that were putatively impaired for survival on nitrocellulose membrane. Mutants were grown in 96-well plates, spotted on TSA-YE agar plates and NC, followed by storage of spotted NC in the dark at 25^{o}C for 16 days as described (Materials and Methods). Mutants that formed colonies on TSA-YE but formed small or no colonies on NC at 16 days of storage were considered to be putatively impaired for survival on NC. The MGD mutants that were quantitatively assessed for survival on NC are highlighted in bold. * indicates that either the Cam^{R} or Kan^{R} derivative (not shown) of the mutants survived on NC while ** means the mutant lacks either a Cam^{R} or a Kan^{R} derivative. The experiment was conducted in three independent trials with duplicate NC membranes spotted per trial.

	Chloramphenicol-resistant MGD	**Kanamycin-resistant MGD**	**Genes deleted**
1	**MGD_Cm_01_B03**	**MGD_Kan_71/72_B03**	**STM14_0635_to_STM14_0644**
2	**MGD_Cm_01_C03**	**MGD_Kan_71/72_C03**	**STM14_1013_to_STM14_1016**
3	**MGD_Cm_01_D01**	**MGD_Kan_71/72_D01**	**STM14_1320_to_STM14_1303**
4	**MGD_Cm_01_F02**	**MGD_Kan_71/72_F02**	**STM14_2020_to_STM14_1998**
5	**MGD_Cm_02_A09**	**MGD_Kan_73/74_A09**	**STM14_3436_to_STM14_3417**
6	**MGD_Cm_03_A08**	**MGD_Kan_75/76_A08**	**STM14_0169_to_STM14_0180**
7	**MGD_Cm_03_B03**	**MGD_Kan_75/76_B03**	**STM14_0438_to_STM14_0456**
8	**MGD_Cm_03_D12**	**MGD_Kan_75/76_D12**	**STM14_1567_to_STM14_1580**
9	**MGD_Cm_01_D04 ***		**STM14_1359_to_STM14_1363**
10	**MGD_Cm_01_D05***	`	**STM14_1375_to_STM14_1383**
11	**MGD_Cm_02_D08***		**STM14_4042_to_STM14_4048**
12	**MGD_Cm_03_B08 ***		**STM14_0534_to_STM14_0545**
13	**MGD_Cm_03_B09 ***		**STM14_0547_to_STM14_0550**
14	**MGD_Cm_03_C02 ***		**STM14_0648_to_STM14_0667**
15	**MGD_Cm_03_C06 ***		**STM14_0702_to_STM14_0717**
16	**MGD_Cm_03_E11 ****		**STM14_2620_to_STM14_2633**
17	**MGD_Cm_03_F02 ****		**STM14_2258_to_STM14_2274**

Supplementary Table Continued

18	**MGD_Cm_03_G10 ***		**STM14_2946_to_STM14_2964**
19	**MGD_Cm_03_H03 ***		**STM14_3100_to_STM14_3112**
20	**MGD_Cm_03_H06 ****		**STM14_3298_to_STM14_3306**
21	MGD_Cm_01_A06*		STM14_0214_to_STM14_0204
22	MGD_Cm_01_C06**		STM14_1098_to_STM14_1107
23	MGD_Cm_01_C07**		STM14_1130_to_STM14_1125
24	MGD_Cm_01_ C10**		STM14_1230_to_STM14_1224
25	MGD_Cm_01_G05**		STM14_2489_to_STM14_2498
26	MGD_Cm_02_C06**		STM14_3761_to_STM14_3782
27	MGD_Cm_02_ D10*		STM14_4853_to_STM14_4861
28	MGD_Cm_02_E09*		STM14_4078_to_STM14_4092
29	MGD_Cm_02_F03**		STM14_0169_to_STM14_0176
30	MGD_Cm_03_A04*		STM14_0067_to_STM14_0074
31	MGD_Cm_03_A09*		STM14_0192_to_STM14_0201
32	MGD_Cm_03_A10**		STM14_0219_to_STM14_0239
33	MGD_Cm_03_B02*		STM14_0367_to_STM14_0376
34	MGD_Cm_03_C05*		STM14_0693_to_STM14_0700
35	MGD_Cm_03_C08*		STM14_0802_to_STM14_0807
36	MGD_Cm_03_C09*		STM14_0816_to_STM14_0837
37	MGD_Cm_03_C10*		STM14_0904_to_STM14_0923
38	MGD_Cm_03_C11*		STM14_0938_to_STM14_0957
39	MGD_Cm_03_C12**		STM14_0959_to_STM14_0985
40	MGD_Cm_03_D02*		STM14_1029_to_STM14_1062
41	MGD_Cm_03_D04**		STM14_1150_to_STM14_1184
42	MGD_Cm_03_D05*		STM14_1191_to_STM14_1208
43	MGD_Cm_03_D08*		STM14_1295_to_STM14_1321
44	MGD_Cm_03_D09*		STM14_1375_to_STM14_1391
45	MGD_Cm_03_D11**		STM14_1490_to_STM14_1561
46	MGD_Cm_03_E02*		STM14_1627_to_STM14_1634
47	MGD_Cm_03_E03**		STM14_1640_to_STM14_1662
48	MGD_Cm_03_E04**		STM14_1770_to_STM14_1871
49	MGD_Cm_03_E07*		STM14_4494_to_STM14_4503
50	MGD_Cm_03_E12*		STM14_2235_to_STM14_2244
51	MGD_Cm_03_F04*		STM14_2368_to_STM14_2416
52	MGD_Cm_03_F07**		STM14_2533_to_STM14_2572

Supplementary Table Continued

53	MGD_Cm_03_F08*		STM14_2587_to_STM14_2598
54		**MGD_Kan_77/78_E08****	**STM14_4513_to_STM14_4529**
55		MGD_Kan_71/72_D02*	STM14_1334_to_STM14_1324
56		MGD_Kan_71/72_D03*	STM14_1354_to_STM14_1341
57		MGD_Kan_71/72_D06*	STM14_1385_to_STM14_1391
58		MGD_Kan_71/72_D07*	STM14_1501_to_STM14_1490
59		MGD_Kan_71/72_E02*	STM14_1611_to_STM14_1616
60		MGD_Kan_71/72_E06*	STM14_1748_to_STM14_1765
61		MGD_Kan_71/72_E10*	STM14_1813_to_STM14_1835
62		MGD_Kan_71/72_F03*	STM14_2022_to_STM14_2027
63		MGD_Kan_71/72_F04*	STM14_2040_to_STM14_2033
64		MGD_Kan_71/72_F05**	STM14_2053_to_STM14_2062
65		MGD_Kan_71/72_ F12*	STM14_2280_to_STM14_2225
66		MGD_Kan_71/72_G03*	STM14_2388_to_STM14_2388
67		MGD_Kan_71/72_G04*	STM14_2397_to_STM14_2403
68		MGD_Kan_71/72_G06*	STM14_2522_to_STM14_2534
69		MGD_Kan_71/72_G08*	STM14_2616_to_STM14_2603
70		MGD_Kan_71/72_H04*	STM14_2833_to_STM14_2829
71		MGD_Kan_75/76_A01**	STM14_0002_to_STM14_0011
72		MGD_Kan_75/76_A02*	STM14_0016_to_STM14_0036
73		MGD_Kan_75/76_A07**	STM14_0128_to_STM14_0140
74		MGD_Kan_77/78_D05**	STM14_4823_to_STM14_4861

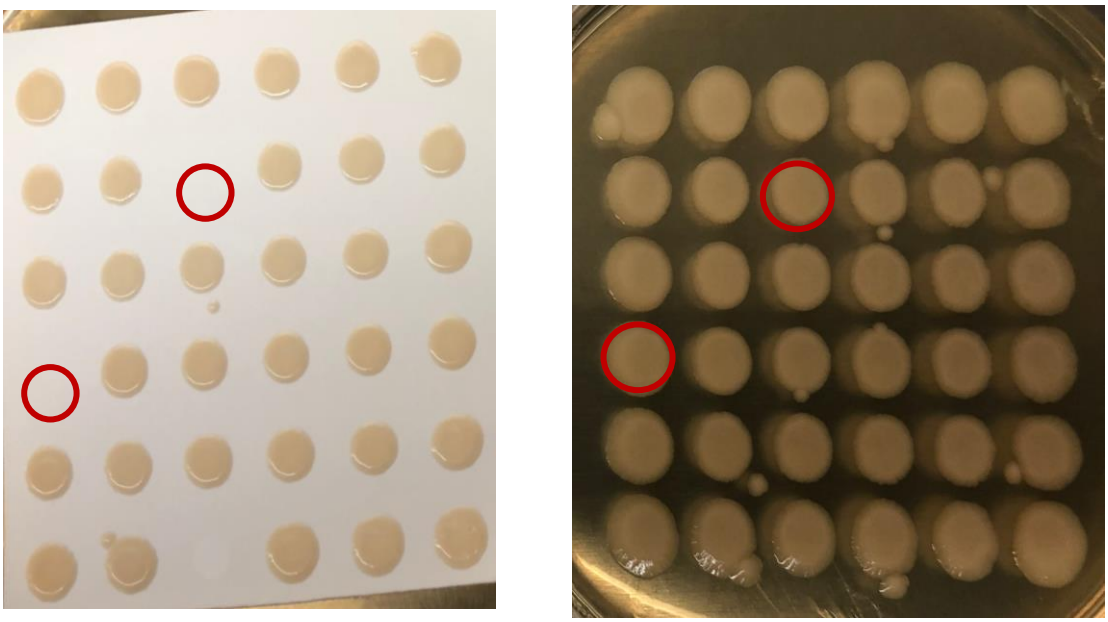
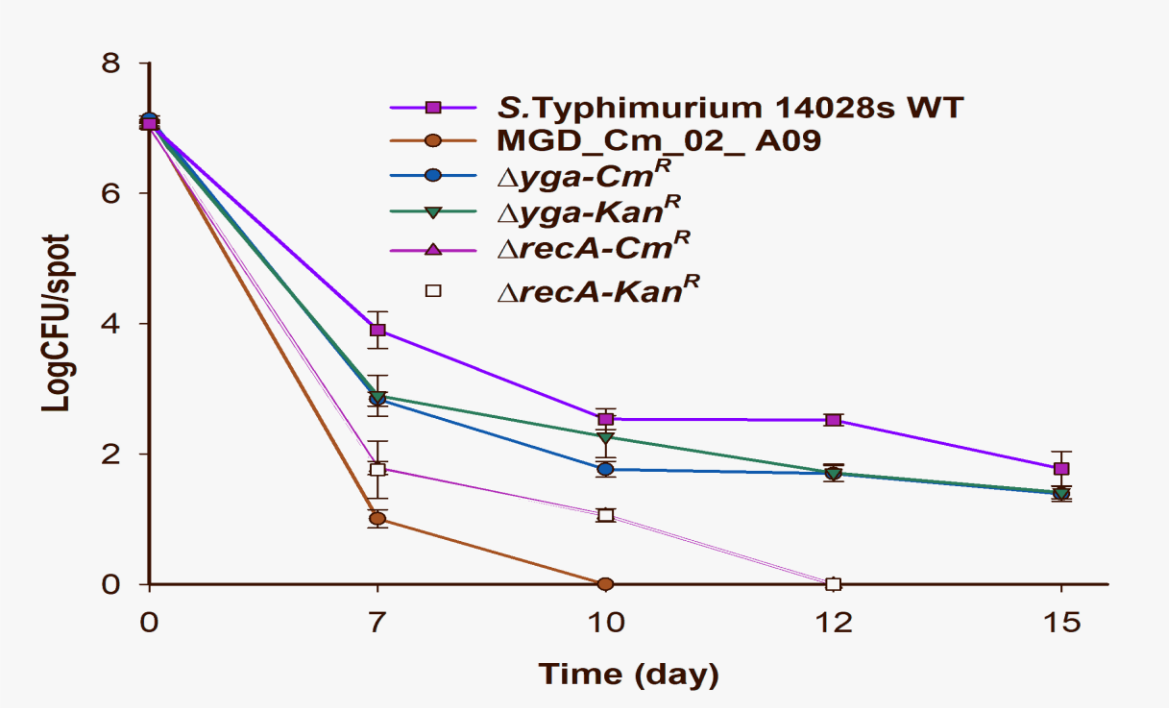


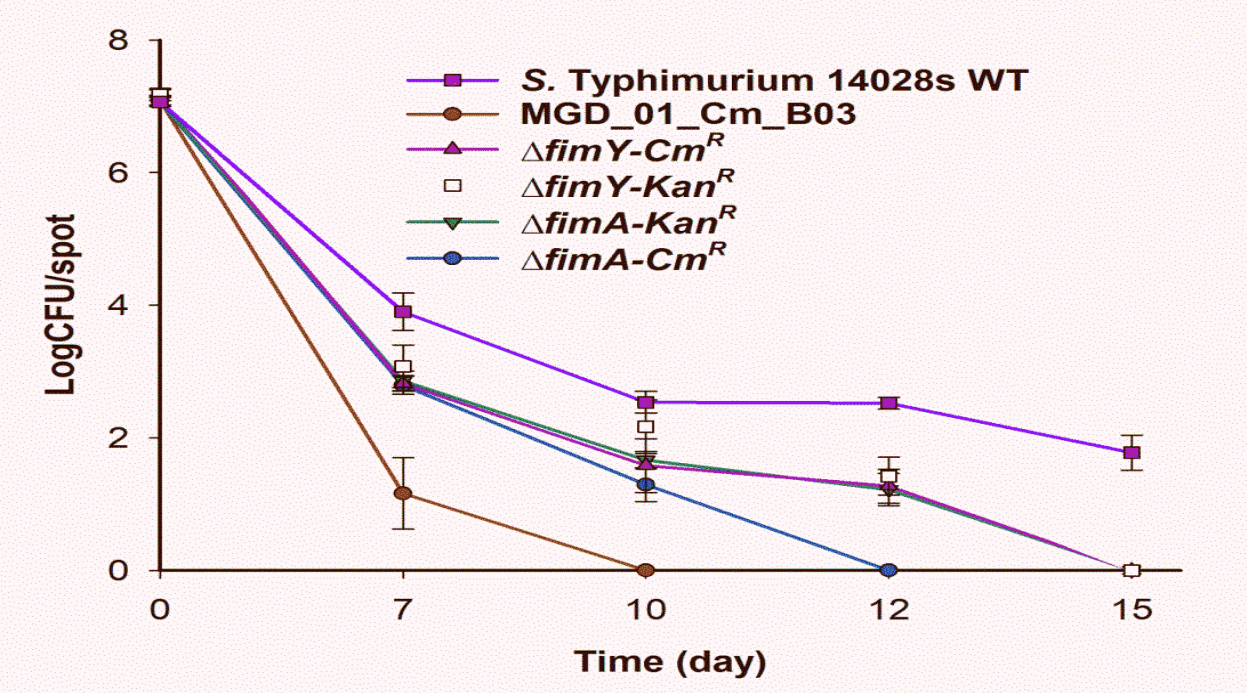
Figure 1: Growth of multi-gene deletions (MDG) mutants on TSA-YE agar plate and on spotted nitrocellulose membrane overlaid on TSA-YE. MGD mutants were grown in TSB-YE inside 96-well plates at 37^{o}C for 24h, spotted on TSA-YE agar plates (37^{o}C, 24h) and on autoclave-sterilized nitrocellulose membrane (NC) as described in **Materials and Methods**. Spotted NC were dried, stored in the dark at room temperature for 16 days, overlaid on TSA-YE as described in Materials and Methods, and incubated at 37^{o}C for 24h. Mutants, such MGD_Cm_01_D01 and MGD_Cm_01_B03 highlighted in red circles, that grew on agar plates but not on NC were considered tentatively impaired for survival.

Figure 2: The survival of multi-gene and single-gene deletion mutants of *Salmonella* Typhimurium 14028s on nitrocellulose membrane. Bacterial cell suspensions of the WT strain, (A): MGD_Cm_02_A09 and its corresponding SGD mutants, (B): MGD_Cm_01_B03 and its corresponding SGD mutants, and (C): MGD_Cm_01_D01 and its corresponding SGD mutants were spotted an dried on NC as described in **Materials and Methods.** The population of *Salmonella* as enumerated by overlaying inoculated NC on TSA-YE agar plates as described in **Materials and Methods.** Data represent average ± standard deviation of two independent trials, with duplicate NC each spotted in duplicate per mutant for each trial.

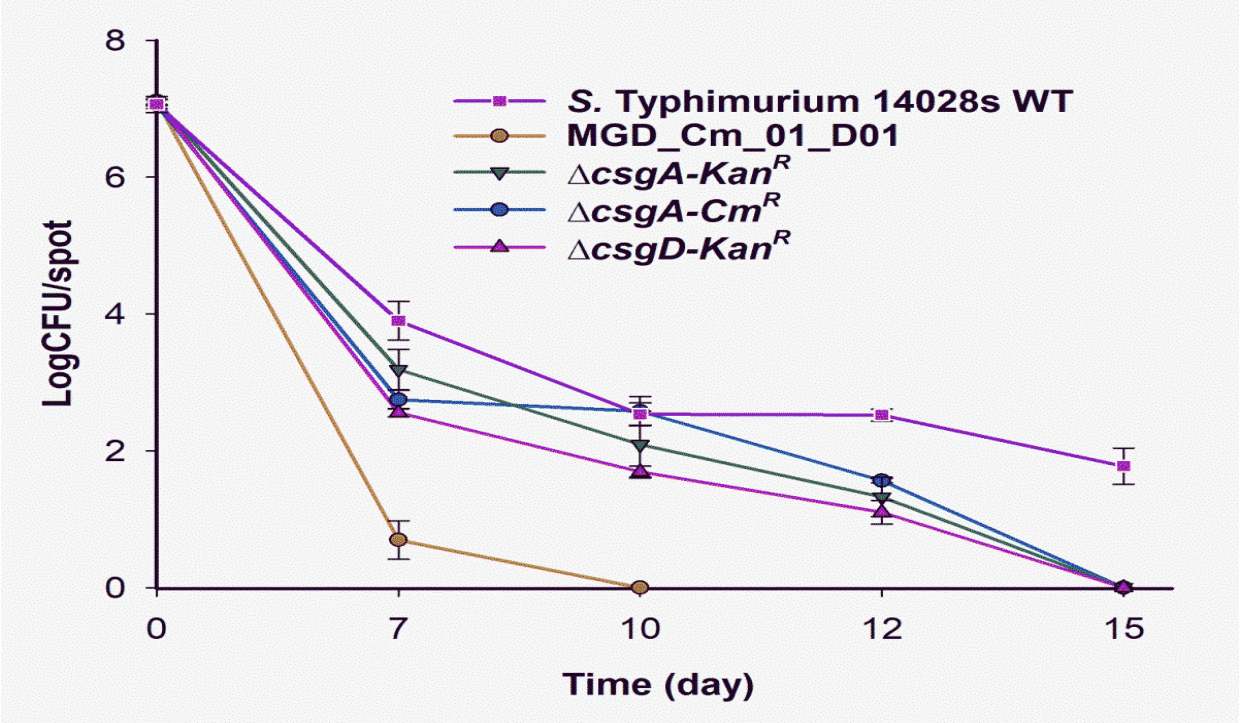
A


S.Typhimurium 14028s WT
MGD_Cm_02_ A09
ΔygaCmR
ΔygaKanR
ΔrecACmR
ΔrecAKanR
LogCFU/spot
Time (day)
0
2
4
6
8
0
7
10
12
15

B


S. Typhimurium 14028s WT
MGD_01_Cm_B03
ΔfimYCmR
ΔfimYKanR
ΔfimAKanR
ΔfimACmR
LogCFU/spot
Time (day)
0
2
4
6
8
0
7
10
12
15

C


S. Typhimurium 14028s WT
MGD_Cm_01_D01
ΔcsgA-Kan^R
ΔcsgA-Cm^R
ΔcsgD-Kan^R
LogCFU/spot
Time (day)
0
2
4
6
8
0
7
10
12
15

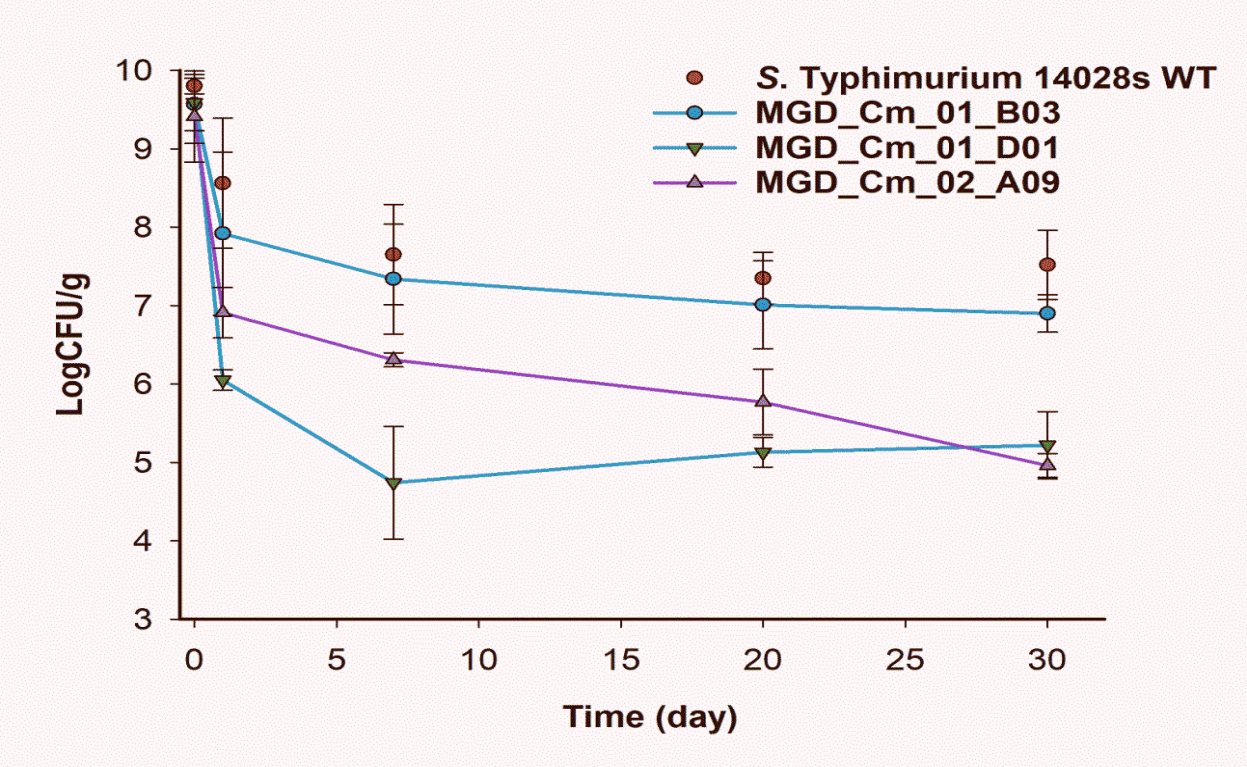


Figure 3: The survival of multi-gene deletion mutants of *Salmonella* Typhimurium and the wild-type strain on in-shell pistachios. Pistachios were inoculated, dried and stored, and populations of *Salmonella* were enumerated as described in **Materials and Methods** immediately after inoculation, after drying and periodically during storage. Data represent average ± standard deviation from two independent trials with duplicate samples processed per trial.

CHAPTER 7

Validation of an ampicillin selection protocol to enrich for mutants of *Listeria monocytogenes* unable to replicate on fresh produce

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Key words: *Listeria monocytogenes*, growth, mutants, genes, produce, cantaloupe, ampicillin selection.

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7.1 ABSTRACT

Several outbreaks of listeriosis have implicated fresh produce but genetic factors required for growth of *Listeria monocytogenes* on produce remain poorly characterized. Since β-lactam antibiotics specifically kill growing bacterial cells, we hypothesized that ampicillin selection can enrich for *L. monocytogenes* mutants unable to grow on produce. For validation, we examined relative recovery of *L. monocytogenes* strain 2011L-2858 and its cold-sensitive mutant L1E4 following inoculation of cantaloupe rind with 1:1 mixture of the strains and incubation at 4°C with or without ampicillin. *L. monocytogenes* from fragments inoculated with the mixed cultures and incubated in the presence of ampicillin were used to inoculate fresh rinds for a second round of enrichment. In the presence of ampicillin, the proportion of L1E4 increased from 55% on day 0 to 78% on day 14, with higher recovery (85% after 14 days) in the second round of enrichment. These data suggested that L1E4 was enriched on cantaloupe rind while growing cells of the wildtype were killed by ampicillin. Application of this protocol to transposon mutant libraries from three *L. monocytogenes* strains yielded several mutants unable to grow on cantaloupe. Thus, ampicillin selection can facilitate discovery of genes essential for growth of *L. monocytogenes* on fresh produce.

7.2 INTRODUCTION

Listeria monocytogenes is responsible for potentially severe foodborne disease (listeriosis) with symptoms that include meningitis, bacteremia and abortions or stillbirths (Drevets and Bronze, 2008; Buchanan et al. 2017; Maury et al. 2016). In the United States alone, *L. monocytogenes* is estimated to cause 1600 cases of foodborne illness annually resulting in 1500 hospitalizations and 260 deaths, and listeriosis is a major cause of severe foodborne disease worldwide (Scallan et al. 2011; Lomonaco et al. 2015; de Noordhout et al. 2014). Several recent listeriosis outbreaks have been linked to fresh fruits and vegetables including celery, cantaloupe, caramel apples, stone fruit and salad greens (Gaul et al. 2013; McCollum et al. 2013; Jackson et al. 2015; CDC, 2015; Gardner and Kathariou, 2016).

Although the capacity of *L. monocytogenes* to adhere and grow on the surface of various types of fresh produce has been reported (Martinez et al. 2016; Kaminski et al. 2014; Salazar et al. 2016, 2017), the underlying molecular mechanisms remain poorly characterized. Previous studies suggested the involvement of motility-related genes on the ability of *L. monocytogenes* to attach and colonize fresh-cut radish tissue, broccoli, alfalfa seeds, and cabbage (Gorski et al. 2003, 2009; Palumbo et al. 2005). A putative transcription factor (*lmo0753*) and a putative cellulose binding protein (*lmof2365_0859*) were demonstrated to be important for attachment of *L. monocytogenes* on lettuce leaves, cantaloupe rinds and baby spinach, as deletion mutants of these genes showed reduced attachment relative to the parental strains (Salazar et al. 2013; Bae et al. 2013). Transcriptome analysis of *L. monocytogenes* grown on cantaloupe slices at 10^{o}C revealed significant increase in the expression of genes involved in flagellar assembly, *prfA*-regulated virulence (*plcA, hly, actA*, and *plcB*), iron uptake and biosynthesis of branched chain amino acids such as valine, leucine and isoleucine (Kang et al. 2019). However, information on

the genetic determinants required for growth of *L. monocytogenes* on fresh produce remains limited.

In principle it is possible to identify genes required for growth of *L. monocytogenes* on produce by enriching a transposon mutant library for mutants unable to grow on produce. The mutants can then be further characterized to identify the relevant genes. In the current study, we tested the capacity of ampicillin selection to enrich for cells that were not replicating on the rind of fresh cantaloupe. Selective enrichment of non-replicating mutants by treating the mutant pool with β-lactam antibiotics has long been described (Davis 1948). The selection protocol is based on the principle that β-lactam antibiotics only kill replicating bacterial cells by inhibiting peptidoglycan synthesis and cell wall assembly in growing cells (Bayles 2007). Methicillin, a β-lactam antibiotic, has been used to selectively enrich *L. monocytogenes* mutants unable to replicate in a macrophage cell line (Camilli et al. 1989). The objective of the current study was to validate the capacity of an ampicillin selection protocol to selectively enrich for *L. monocytogenes* that were unable to grow on cantaloupe rind. In addition, we tested the suitability of the protocol to enrich for growth-impaired mutants from mutant libraries from three different produce-associated *L. monocytogenes* strains.

7.3 MATERIALS AND METHODS

7.3.1 Bacterial strains and growth conditions

L. monocytogenes strains used in this study are listed in Table 1 and were obtained from the *Listeria* collection at our laboratory at North Carolina State University. *L. monocytogenes* serotype 1/2b strain 2011L-2858, hereafter designated as strain 2858, was isolated from cantaloupe implicated in the 2011 multistate cantaloupe outbreak that involved whole cantaloupe (McCollum et al. 2013). *L. monocytogenes* strain L1E4 is a cold-sensitive mutant of strain 2858 with a *mariner*-based transposon insertion in the *lmo0866* homolog, encoding a DEAD box RNA

helicase (Price et al. 2018). *L. monocytogenes* strain F8027 (serotype 4b) was isolated from celery (Lang et al. 2004) while strain 2010L-1723 (serotype1/2a) was implicated in a celery-associated outbreak of listeriosis in Texas in 2010 (Gaul et al. 2013). All strains were susceptible to ampicillin, being unable to grow at ampicillin levels of 0.25 µg ml^{-1}. Mutant libraries of *L. monocytogenes* strains 2011L-2858, F8027 and 2010L-1723 were constructed using pMC-38 (Cao et al. 2007). The number of transposon copies inserted in the chromosome of mutants of interest were determined by Southern blots and the transposon insertions were localized by two-step arbitrary PCR as previously described (Cao et al. 2007, Parsons et al. 2017).

L. monocytogenes was grown in trypticase soy broth (TSB; Becton, Dickinson & Co. Sparks, MD) containing 0.6% yeast extract (TSB-YE, Becton, Dickinson & Co.) or in brain heart infusion (BHI) broth (Becton, Dickinson & Co.). Agar cultures were in TSB-YE supplemented with 1.2% agar (TSA-YE, Becton, Dickinson & Co.). Modified oxford agar (MOX; EMD Chemicals Inc., Gibbstown, NJ) supplemented with MOX antimicrobial supplement (MOX; Becton, Dickinson & Co.) was used as *Listeria* selective medium. When required, the growth media were supplemented with erythromycin (Em; MP Biomedicals, Solon, Ohio) at 6µg ml^{-1}. *L. monocytogenes* strains 2858 and L1E4 were grown at 37^{o}C in standing cultures overnight and preserved at -80^{o}C in BHI with 20% glycerol (Fisher Scientific, Fair Lawn, NJ). Mutant libraries were preserved at -80^{o}C in BHI.

7.3.2 Inoculation of cantaloupe with *L. monocytogenes* and ampicillin selection

Synchronized cultures of *L. monocytogenes* strains 2858 and L1E4 used for inoculation were prepared by growing each strain in 5ml TSB-YE in 15-ml conical centrifuge tubes (Becton, Dickinson & Co.) at 37^{o}C overnight, followed by transferring 50µl of the overnight culture into 5ml of fresh TSB-YE in 15-ml conical centrifuge tubes which were then incubated at 37^{o}C for

12h. The cultures were centrifuged at 6500×g (Rotor JA-14, Beckman, Palo Alto, CA) for 5 min at 25^{o}C, and the pellet was washed twice in sterile deionized water (dH_2O) and then resuspended in 5ml dH_2O. Inoculum for individual mutant libraries was prepared as described (Parsons et al. 2017), by transferring 10µl of the frozen mutant libraries into 5ml BHI-Em and incubating at 40^{o}C overnight. The cultures were then washed twice and resuspended in 5ml dH_2O. The washed cultures were serially diluted in dH_2O and appropriate dilutions were used to inoculate cantaloupe fragments.

Conventionally grown cantaloupe were purchased from retail store and held at 4^{o}C for 24h before use. Fragments of cantaloupe (2 × 2 × 1cm, 4cm^2 rind surface area) were prepared as described (Martinez et al. 2016) and aseptically placed in sterile petri dishes. The rind was inoculated with approx. 10^5 CFU per fragment of strain 2858, L1E4 or a 1:1 mixture of both strains by spotting 100µl of inoculum as a series of 10-µl drops on the rind. The fragments were air-dried in a laminar flow biosafety cabinet at room temperature for 1h. The inoculated fragments were then placed rind-surface down in sterile plastic 15 ×100-mm petri dishes (two fragments/petri dish) with sterile water (10ml) with or without ampicillin (100µg ml^{-1}) (Sigma, St. Louis, MO) so that the inoculated rind surface was completely immersed in the liquid. The petri dishes were covered with their lids and incubated at 4^{o}C for up to 14 days.

The population of *L. monocytogenes* on the rind was determined immediately after drying, and again at 7 and 14 days by transferring each fragment into 10ml of sterile dH_2O in sterile 50-ml conical centrifuge tubes (Becton, Dickinson & Co.) which were then vortexed at high speed for one minute. Two fragments were tested at each time point, and the experiments were done in three independent trials. The resulting rinsates were serially diluted and plated on the selective medium MOX to enumerate listeriae after 48h at 37^{o}C. For the second round of

enrichment, 14 days after inoculation of the fragments with a 1:1 mixture of strains 2858 and L1E4, typical *Listeria* spp. colonies (approx. 1,600 CFU) were swabbed off the MOX plates using sterile cotton-tipped swabs (Medline, IL), washed twice and resuspended in 5ml dH_2O. The suspension was then used to inoculate the rind of new cantaloupe fragments as described above. The newly-inoculated fragments were placed in petri dishes with 10ml dH_2O with or without ampicillin (100µg ml^{-1}) and incubated at 4°C for up to 14 days. Rinsates from fragments inoculated with the mixture of 2858 and L1E4 were plated on MOX and MOX-Em to enumerate total listeriae and L1E4, respectively. In a preliminary experiment, plating dilutions of L1E4 on MOX and MOX-Em yielded the same CFUs (data not shown).

7.3.3 Enrichment of *L. monocytogenes* mutant libraries for growth-deficient mutants

Fragments inoculated with mutant libraries (approx. 10^5 CFU per fragment) were air-dried, incubated in 10ml dH_2O with or without ampicillin (100µg ml^{-1}) and incubated at 4°C for up to 14 days, and total listerial populations were enumerated on MOX as described above. A subset of randomly-chosen mutants from 7 and 14 days of the primary enrichments of each library were tested for growth on cantaloupe at 25 and 4°C over 14 days in two independent trials with duplicates fragments per trial, as described.

7.3.4 Determination of cold sensitivity

Colonies from the mutant libraries were randomly selected from MOX plates on days 7 and 14 and tested for cold sensitivity at 4°C. Individual colonies were inoculated into 200µl TSB-YE in a 96-well plate (Greiner Bio-One, Monroe, NC) and incubated at 37°C for 24h. Bacterial cultures in the 96-well plates were spotted on duplicate TSA-YE plates using a flame-sterilized metal stamping device and the agar plates were incubated at 4°C for 21days or 37°C for 24h. Mutants that grew on TSA-YE plates at 37°C but not at 4°C by 21 days were considered

cold-sensitive. Growth of such mutants was also assessed in TSB-YE at 4°C over 21 days. Each mutant was inoculated into 5ml TSB-YE in 15-ml conical centrifuge tubes (BD) to a final concentration of 10^3CFU/ml in duplicate, followed by incubation at 4°C for 21 days. The population of each mutant was enumerated periodically on TSA-YE.

7.3.5 Statistical analysis

Bacterial counts from each replicate were $\log_{10}$-transformed to compute the log CFU per fragment or ml as appropriate. Significant changes in the listerial populations at different times was tested with one-way analysis of variance (ANOVA) with Tukey's test at $P < 0.05$, using SPSS version 22 (IBM Corporation Software Group, Somers, NY). Differences in the percentage of strain 2858 and L1E4 recovered from fragments inoculated with 1:1 mixture of the two strains were similarly analyzed using ANOVA with Tukey's test at $P < 0.05$.

7.4 RESULTS AND DISCUSSION

7.4.1 Ampicillin selection at 4°C results in enrichment of the cold-sensitive mutant L1E4

At 22°C, the cold-sensitive mutant L1E4 was previously shown to grow on cantaloupe rind similarly to its wildtype parental counterpart, strain 2858 (Price et al. 2018). Therefore, the selection capacity of the ampicillin-based enrichment protocol was evaluated at 4°C, a temperature that permits the growth of 2858 but not L1E4. As expected, *L. monocytogenes* 2858 grew on cantaloupe rind in the absence of ampicillin at 4°C, increasing by approx. 0.5 and 1.4 log CFU/fragment by 7 and 14 days, respectively, while the population of strain L1E4 remained unchanged (Fig. 1). However, there was a significant reduction ($P = 0.001$) by approx. 0.4 log CFU/fragment in the population of *L. monocytogenes* 2858 at 7 days on fragments immersed in water with ampicillin (Fig. 1), as would be expected by the bactericidal effect of the antibiotic against the cells that were replicating on the rind. There was no significant difference in the

population of L1E4 on fragments with or without ampicillin at either 7 or 14 days at 4^{o}C (Fig. 1**)**, in agreement with the fact that this strain did not grow on the cantaloupe rind at 4^{o}C, and thus would not be susceptible to ampicillin.

At 4^{o}C, the population of the mixed cultures on cantaloupe rind without ampicillin increased by approx. 0.4 and 0.9 log CFU/Fragment at 7 and 14 days, respectively (Fig. 2A), in the first round and by ~ 0.2 and 0.5 log CFU/fragment during the second round of enrichment (Fig. 2B). Lower growth of the mixture in the secondary enrichment may be due to lower relative abundance of strain 2858 at time 0, compared to time 0 in the primary enrichment (Fig. 3). Since L1E4 is incapable of growth at 4^{o}C (Fig. 1), the population increase of the mixed culture without ampicillin treatment can be attributed solely to 2858. In the absence of ampicillin, the percentage of strain 2858 recovered from the rind fragments inoculated with the mixed cultures increased from 45% on day 0 to 75 and 89% on day 7 and 14, respectively, in the first round, and from 34% on day 0 to 66 and 84% on day 7 and 14, respectively, for the second round (Fig. 3). In the presence of ampicillin, the population of the mixture on cantaloupe rind did not increase for either round of enrichment (Fig. 2). However, the relative abundance of L1E4 increased from 55% on day 0 to 69 and 78% on day 7 and 14, respectively, in the first round of enrichment, and from 66% on day 0 to 77 and 85% on day 7 and 14, respectively, in the second round (Fig. 3). The relative increase in abundance of L1E4 reflects the expected die-off of the growing wildtype cells in the presence of ampicillin. Therefore, these data suggest that the ampicillin selection protocol can effectively enrich for *L. monocytogenes* unable to grow on the cantaloupe rind.

7.4.2 Ampicillin selection of mutant libraries identified novel mutants impaired for growth on cantaloupe rind

To demonstrate the effectiveness of the ampicillin selection protocol, ampicillin selection was used to enrich mutant libraries derived from *L. monocytogenes* strains 2858, F8027 and 2010L-1723 (hereafter designated as strain 1723) for mutants unable to replicate on the rind at 4°C. In the absence of ampicillin, the populations of the mutant libraries increased progressively during incubation at 4°C, with 1.6, 1.8 and 2.2 log increase on day 7 and an additional 0.5, 0.4 and 0.5 log increase on day 14 for libraries from strain 2858, 1723 and F8027, respectively (data not shown). However, in the presence of ampicillin, the populations decreased by 1.3, 0.9 and 1.5 log on day 7 and an additional 0.3, 1.1 and 0.3 log on day 14 for 2858, 1723 and F8027 libraries, respectively (data not shown). Since the original inoculum was 10^5 CFU/fragment on day 0, such decreases often resulted in low numbers of CFUs on the MOX plates, especially after 14 days.

Panels of mutants from 7 and 14 days of the primary enrichments of the mutant libraries were randomly chosen for further characterization. A significant portion (23/81, 28%) of the randomly-selected colonies from the mutant library of strain 2858 were found to be cold-sensitive, with comparable prevalence of such cold-sensitive mutants on day 7 (16/52, 30%) and day 14 (7/29, 24%). Of the remainder, 57 of the 81 (70%), including 21 of the 29 (72.4%) on day 14 were not cold-sensitive on laboratory media and also grew on cantaloupe at 4°C. However, of the 29 mutants tested from day 14, one (28C3) was unable to grow on cantaloupe at 4°C (Fig. 4A) even though it grew normally on laboratory media at 4°C (Fig. 5A). Thus, promising mutants specifically impaired for growth on the cantaloupe rind at 4°C represented a minority

(1/29, 3.4%) of the population at day 14, but were still markedly enriched, from none detected among the 52 tested mutants on day 7 (<0.02%) to 3.4% on day 14.

Similar findings were obtained with the mutant libraries of the other two strains. Of 79 colonies randomly selected from the F8027 mutant library, a total of 27 including 13/52 (25%) on day 7 and 14/27 (52%) on day 14 were cold-sensitive. Of the remainder, 51 (65%) were not cold-sensitive and also grew on cantaloupe, while one (mutant F8C2) from day 14 was impaired for growth on cantaloupe (Fig. 4B) while capable of growth at 4^{o}C on laboratory media (Fig. 5B). For the library of strain 1723, of 41 tested mutants (27 and 14 from day 7 and 14, respectively) 14 (34%) were cold-sensitive, 23 (56%) grew both on laboratory media and on cantaloupe at 4^{o}C while three (mutants 17C1, 17C2, and 17C, all from day 14) were unable to grow on cantaloupe (Fig. 4C). These three mutants grew on TSA-YE at 4^{o}C over 21d, but their growth was slower compared to the parental strain (Fig. 5C). Thus, the protocol was able to enrich for promising mutants from the libraries of each of the tested strains after 14 days of ampicillin selection.

As indicated above, the majority (56 to 70%) of the mutants appeared to be false positive, since they grew at 4^{o}C both on laboratory media and on cantaloupe. These may have survived the ampicillin selection by being in a non-replicating state during the exposure to the antibiotic. Such a non-replicating state may be especially common at the low temperature used for these experiments. Even though *L. monocytogenes* is a psychrotroph, its growth rate is low at 4^{o}C (Lado and Yousef, 2007). Therefore, a secondary round of enrichment, as described above for enrichment of the cold-sensitive mutant L1E4, may be necessary to increase the relative abundance of desired mutants in an enriched population.

The five mutants which were found to be impaired for growth on cantaloupe rind at 4ºC but grew on laboratory media at this temperature were further characterized. All mutants were susceptible to ampicillin, being unable to grow at ampicillin levels of 0.25 µg ml^{-1}, similarly to their parental counterparts. All but one (mutant F8C2) harbored single transposon insertions, which were localized in diverse genes (Table 1). Mutant F8C2 yielded evidence of more than two transposon copies via southern blots, but PCR-based identification of the insertion site identified only one genomic location, even after repeated trials (Table 1). Interestingly, none of the genes disrupted in the identified mutants was previously implicated in growth of *L. monocytogenes* on produce. The transposon-inactivated genes in 28C3 (*lmo1079* homolog) and 17C2 (*lmo0231* homolog) are hypothetical proteins with unknown functions, while the gene disrupted in 17C3 (*lmo2690* homolog) is a TetR family transcriptional regulator which is poorly characterized in *L. monocytogenes.* Mutant 17C1, derived from strain 1723, was noteworthy in harboring a transposon insertion in the homolog of *lmo0582* (*iap*), encoding the invasion-associated protein P60, implicated in peptidoglycan hydrolysis (Kuhn and Goebel, 1989, Rigden et al. 2003). In addition to its inability to grow on cantaloupe rind and impaired growth at 4ºC, 17C1 had filamentous cells, as also previously reported for *L. monocytogenes iap* mutants (Gutekunst et al. 1992; Pilgrim et al. 2003). Thus, the ampicillin selection protocol was successful in identifying mutants which are impaired in growth on cantaloupe even though they able to growth at 4ºC. In addition, the protocol appears suitable for produce-relevant strains of diverse serotypes and genotypes.

7.5 CONCLUSIONS AND PERSPECTIVES

The collective data from the mutant libraries of three different strains indicate that the ampicillin selection protocol can indeed successfully enrich for mutants unable to grow on cantaloupe at 4^{o}C. The ampicillin selection protocol has previously been used successfully to isolate *L. monocytogenes* mutants of strain 10403S (serotype 1/2a) that were not replicating in a macrophage cell line, leading to the discovery of genetic determinants required for intracellular growth of *L. monocytogenes* (Camilli et al. 1989). However, our data clearly indicate the ampicillin selection protocol also has potential for food microbiology applications, including identification of mutants unable to grow on fresh produce, or impaired in their capacity to grow at refrigeration temperature. In the current study, we have provided evidence supporting the capacity of ampicillin selection to enrich for cells unable to replicate on the rind of cantaloupe. The ampicillin selection protocol was optimized and validated at 4^{o}C using a specific strain pair, 2858 and L1E4, but we show that the protocol can be adapted for use with diverse produce-relevant strains.

Using the ampicillin selection protocol, we were able to recover the desired mutants after 14 days, from each of the tested mutant libraries, where they constituted 3.4 to 21.4% of the tested mutants. A large fraction of mutants enriched by the ampicillin selection method were cold sensitive, thus confirming the effectiveness of this method to enrich for non-growing bacterial cells, as these cold-sensitive mutants would be expected to be unable to grow on the produce at the 4^{o}C temperature that was employed in the experiments. On the other hand, the protocol also enriched for a second group of mutants which were not cold-sensitive and also grew on cantaloupe, thus appearing to be false-positives that may have resulted from special physiological states that rendered them resistant to the antibiotic. Therefore, further screening of

enriched mutant populations is necessary to identify mutants that are impaired for growth. Overall, the ampicillin selection protocol coupled to an effective screening method can constitute a powerful tool to identify genetic determinants mediating growth of *L. monocytogenes* on fresh produce.

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Table 1: *Listeria monocytogenes* strains used in this study

***L. monocytogenes* Strains**	**Genotype and features**
2011L-2858	Serotype 1/2b strain from 2011 cantaloupe outbreak (Martinez et al, 2016)
2010L-1723	Serotype 1/2a strain from 2010 celery outbreak (Gaul et al, 2013)
F8027	Serotype 4b strain isolated from celery (Parsons et al, 2016)
2011L-2858 mutant library	Mariner-based transposon mutant library of 2011L- 2858 (this study)
F8027 mutant library	Mariner-based transposon mutant library of strain F8027 (this study)
2010L -1723 mutant library	Mariner-based transposon mutant library of 2010L-1723 (this study)
L1E4	Cold-sensitive *mariner*-based transposon mutant of 2858 with transposon insertion in homolog of *lmo0866* (Price et al 2018)
17C1	Mutant of 2010L-1723 with a *mariner*-transposon insertion in *lmo0582 homolog* (this study)
17C2	Mutant of 2010L-1723 with a *mariner*-transposon insertion in *lmo*0231 homolog (this study)
17C3	Mutant of 2010L-1723 with a *mariner*-transposon insertion in *lmo*2690 homolog (this study)
28C3	Mutant of 2011L-2858 with a *mariner*-transposon insertion in *lmo1079* homolog (this study)
F8C2	Mutant of F8027 with a *mariner*-transposon insertion in the intergenic region between *lmo2453* and *lmo2454* homologs (this study). F8C2 may contain more than one copies of *Tn.*

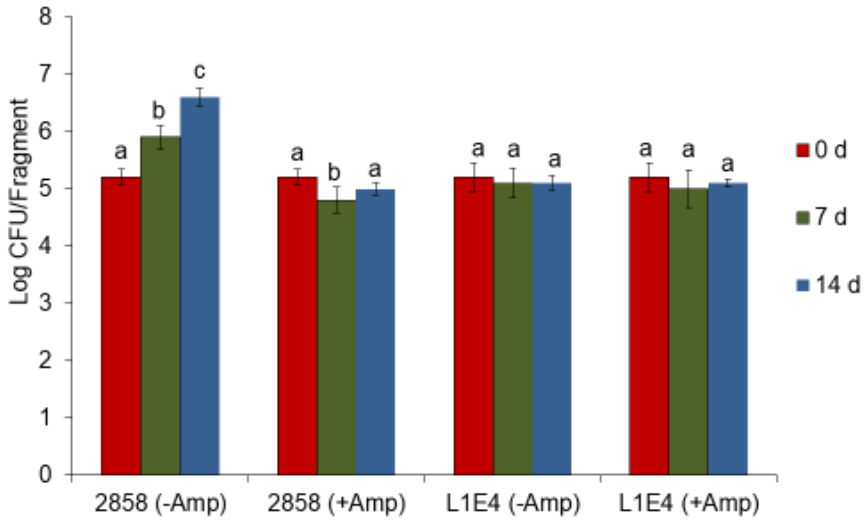

Figure 1: Growth of *L. monocytogenes* strain 2858 and its cold-sensitive mutant L1E4 on cantaloupe rind incubated in 10-ml sterile water in the presence or absence of ampicillin. Rind fragments were inoculated as described in Materials and Methods and incubated at 4°C with (+Amp) or without (-Amp) ampicillin (100µg ml^{-1}) over 14 days. Populations were enumerated as described in Materials and Methods immediately after drying for 1h (day 0) and on day 7 and 14 days. Data represent averages from three independent trials with two fragments per trial and error bars indicate standard deviation. Within each category, different letters on bars indicate significant difference ($P < 0.05$).

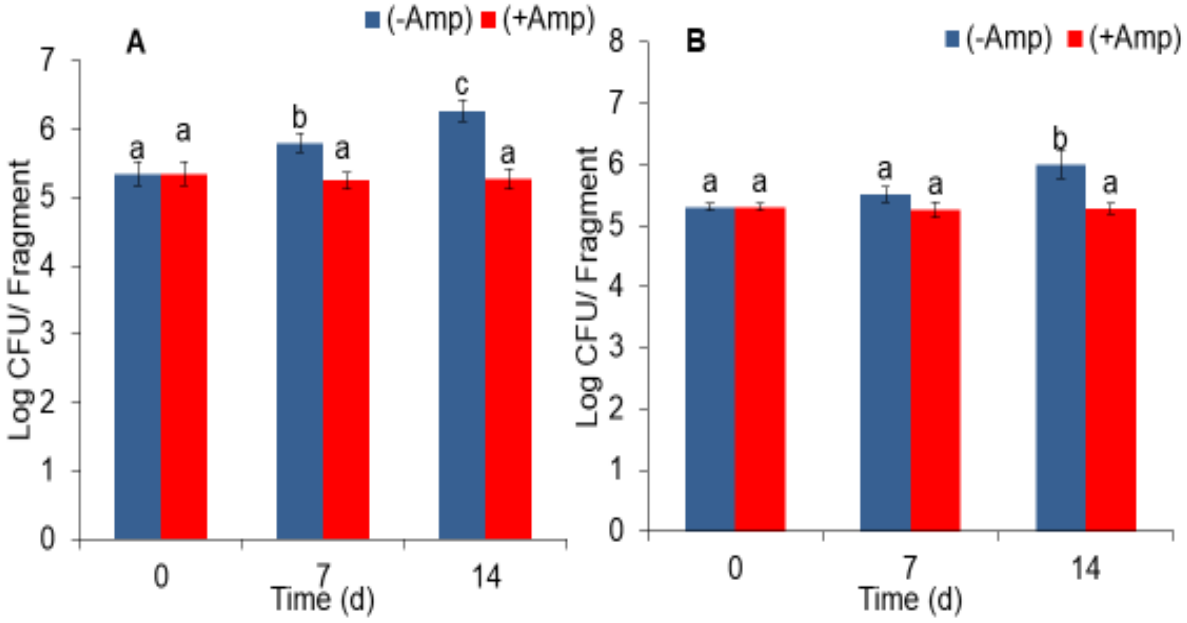

Figure 2: Growth of the mixture of *L. monocytogenes* 2858 and its cold-sensitive mutant L1E4 (1:1 mixture) on cantaloupe rind. Strain mixture (1:1) was inoculated in 10ml sterile water with (+Amp) or without (-Amp) ampicillin (100µg ml^{-1}) in (A) the first and (B) the second round of enrichment at 4°C, as described in Materials and Methods. Populations were enumerated as described in Materials and Methods immediately after drying for 1 h (day 0) and on day 7 and 14. Data represent averages of three independent trials with two fragments per trial and error bars indicate standard deviation. Different letters on bars indicate significant difference ($P < 0.05$).

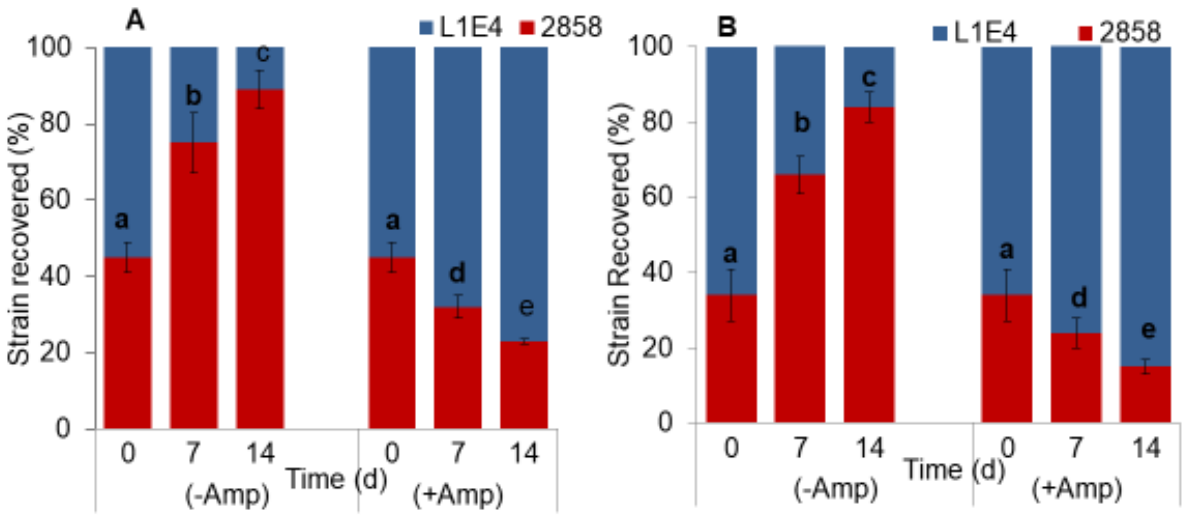

Figure 3: Relative abundance of *L. monocytogenes* strain 2858 and its cold-sensitive mutant L1E4 on cantaloupe rind in the (A) first and (B) second round of enrichment at 4°C. Procedures were as described in the legend for Fig. 2. Populations of each strain were enumerated as described in Materials and Methods immediately after drying for 1 h (day 0) and on day 7 and 14. Data represent averages from two independent trials with two fragments per trial and error bars indicate standard deviations. Within each category, different letters on bars indicate significant difference ($P < 0.05$).

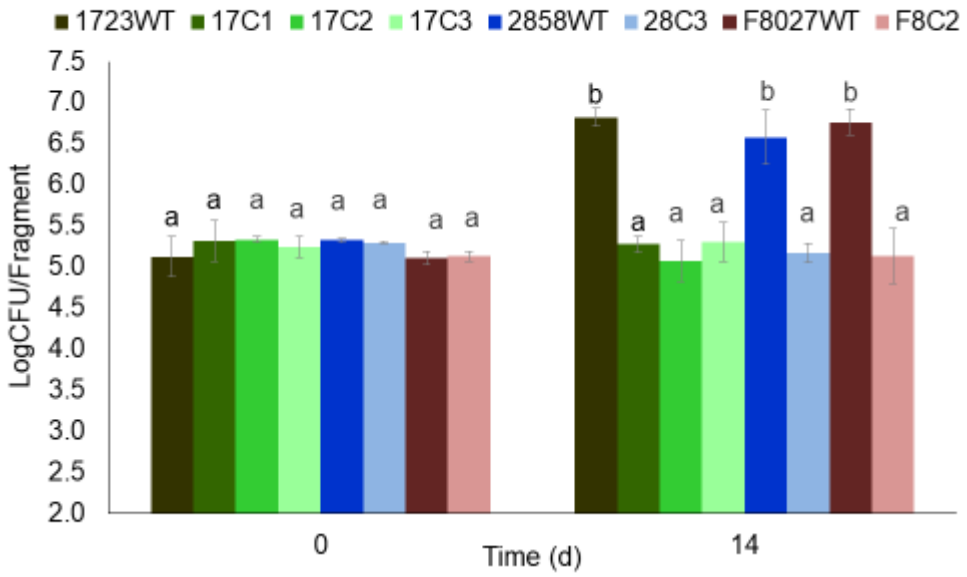

Figure 4: Growth of selected mutants and their parental strain counterparts (WT) on cantaloupe rind at 4^{o}C by 14 days. The selected mutants include (A) mutant 28C3 and its parental strain 2858; (B) mutant F8C2 and its parental strain F8027; (C) and mutants 17C1, 17C2, 17C3 and their parental strain 2010-1723. Rinds were inoculated and incubated at 4^{o}C over 14 days described in Materials and Methods. Populations of each strain were enumerated immediately after drying for 1 h (day 0) and on day 7 and 14 days as described in Materials and Methods. Data represent averages of two independent trials with duplicates fragments per trial, and error bars represent standard deviations. Different letters on the bars indicate significant differences ($P < 0.05$).

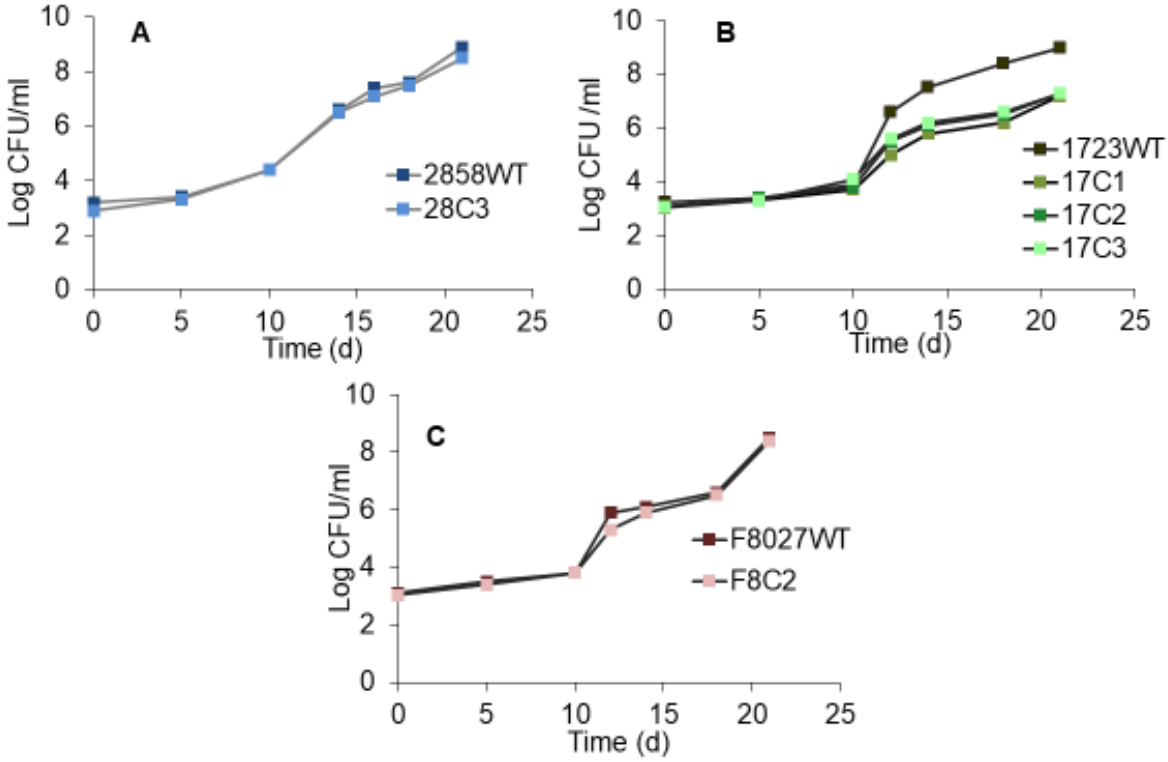

Figure 5: Capacity of mutants obtained via the ampicillin selection protocol to grow in the cold (4ºC) on laboratory media. Strains included (A) Strain 2858 and its mutant 28C3; (B) strain F8027 and mutant F8C2; (C) strain 2010L-1723 and its mutants 17C1, 17C2 and 17C3. Growth was assessed over 21 days at 4ºC as described in Materials and Methods. Data represent averages of two replicates from one representative experiment.