

ABSTRACT

Title of Dissertation: EVALUATION OF THE COPPER HOMEOSTASIS AND SILVER RESISTANCE ISLAND AND ITS ROLE IN PERSISTENCE OF *SALMONELLA ENTERICA*

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Salmonella enterica is one of the leading bacterial cause of foodborne illness in the United States. Although there are many serovars, only a small subset causes human illness. Since *Salmonella* is ubiquitous in the environment, the Food and Drug Administration has established regulations for food processors to ensure that the products are free of contamination. Low-moisture foods are commonly ready-to-eat, and due to the low water activity do not promote growth of bacteria. However, *Salmonella* has been shown to persist in these foods. There have been two outbreaks and multiple recalls in the United States due to contaminated pistachios. Based on a retrospective study, results show that there is evidence of a contamination in the growing orchard and a significant number of *Salmonella* isolates from the environments contain the Copper Homeostasis and Silver Resistance Island (CHASRI) cassette. This raises several questions: what is the prevalence of CHASRI among different *Salmonella* isolates from food and environmental sources? Does presence of CHASRI enable *Salmonella* to survive better against copper stress? And whether presence of CHASRI provide cross-protection against other stresses such as desiccation and thermal treatment? This dissertation attempts to answer those questions.

The prevalence of the CHASRI in *Salmonella* was determined by the use of publicly available whole genome sequencing data. The CHASRI was found in 61 different serovars and types of sources. The presence of the CHASRI in isolates from low-moisture foods that have caused previous outbreaks (peanut butter, nuts, spices) was interesting to note, and leads to future studies on correlations between this island and virulence. Based on results of phylogenetic analysis of CHASRI sequences from closed genomes, we determined there were four types of CHASRI found in *Salmonella*. Traditionally, the *Salmonella* Genomic Island-4 (SGI-4) is found but in addition the CHASRI can incorporate by itself, within a variant of SGI-4, or via a rare plasmid. Interestingly, the sequence of the CHASRI from SGI-4 and the variant SGI-4 were highly different. The high SNP differences in sequence along with the difference in the arsenic operon led to the conclusion that these variants arose independently.

A *Salmonella* Senftenberg strain (CFSAN047523), isolated from pistachios, was used to create three knockouts (Δcus , Δpco , and $\Delta CHASRI$). Previous studies have looked at the minimum inhibitory concentration (MIC) of strains with and without the CHASRI but have omitted the minimum bactericidal concentration (MBC). In this study, we used the knockouts to test for both MIC and MBC. While the MIC was similar for the strains, the MBC was greater in the wild type and partial CHASRI knockouts. Growth and inactivation kinetics were measured in different concentrations of copper sulfate. At higher levels of copper sulfate, the presence of the CHASRI made cells more resilient to inactivation by copper sulfate.

Evidence shows that the stress response in *Salmonella* has the ability to crosstalk and provide protection against multiple stresses. To investigate this phenomenon further, our isolates were

tested against a multitude of stresses to evaluate for cross-protection that may be due to the CHASRI. Cells undergoing copper stress were better equipped to survive lethal copper concentrations and desiccation if the CHASRI was present. The presence of *Salmonella* in final pistachio products that have been fully processed identifies that some adaptation and stress response is occurring in the processing facility. Inoculated pistachios with the wild type and Δ CHASRI strain were thermally processed to test for survivors. This study showed that the presence of the CHASRI gave the isolate an advantage to survive thermal processing after desiccation.

Overall, this study presents the prevalence of the CHASRI in *Salmonella enterica* as well as the important role it plays in copper tolerance. The evidence of cross-protection and tolerance to copper leads to future research regarding gene expression and virulence assessment.

EVALUATION OF THE COPPER HOMEOSTASIS AND SILVER RESISTANCE ISLAND
AND ITS ROLE IN PERSISTENCE OF *SALMONELLA ENTERICA*

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DEDICATION

I would like to dedicate my work to my family who have supported me for this entire journey.

My grandparents were always strong supporters of my education and always pushed me to go to the next level. My mom and dad taught me how to work hard to get what you want and gave me the genetics to be able to be a mom, have a career, and pursue higher education all at the same time. My brother, who has always been my biggest cheerleader. My in-laws, who were always there for anything I may need be it a place to write, dinner, or teaching me about front matter.

Ultimately, I dedicate this work to my husband and my boys. Rob, you have always had my back and told me to stress less and were willing to do anything to help. Thank you for bringing me coffee or treats when you know I secretly needed them. Jackson and Connor, being your mom is my favorite job. I hope I can be a good role model and show you that you can do whatever you dream of.

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LIST OF ABBREVIATIONS

AMP: Ampicillin

AMR: Antimicrobial Resistance

a_w : Water activity

BHIB: Brain Heart Infusion Broth

BPW: Buffered Peptone Water

BSAAO: Biological Soil Amendments of Animal Origin

CDC: Centers for Disease Control

CHASRI: Copper Homeostasis and Silver Resistance Island

CM: Chloramphenicol

EPA: Environmental Protection Agency

FDA: Food and Drug Administration

GI: Genomic Island

GMP: Good Manufacturing Practices

LB: Luria-Bertani

LMF: Low moisture food

MBC: Minimum Bactericidal Concentration

MIC: Minimum Inhibitory Concentration

MHA: Mueller-Hinton Agar

MHB: Mueller-Hinton Broth

MPPST: Most Prevalent Poultry-Associated *Salmonella* Serotypes

mTSAYE: Modified Trypticase Soy Agar with Yeast Extract

NCBI: National Center of Biotechnology Information

NGS: Next-Generation Sequencing

PBS: Peptone Buffered Solution

PFGE: Pulsed Field Gel Electrophoresis

RH: Relative Humidity

RT-qPCR: Reverse Transcription Quantitative PCR

SNP: Single Nucleotide Polymorphism

SPI: *Salmonella* Pathogenicity Island

SRA: Sequence Read Archive

ST: Sequence Type

T3SS: Type-3 Secretion System

TCS: Two-component signal transduction system

TSA: Trypticase Soy Agar

TSB: Trypticase Soy Broth

USDA: United States Department of Agriculture

WHO: World Health Organization

wgMLST: Whole Genome Multilocus Sequence Typing

WGS: Whole Genome Sequencing

CHAPTER 1: LITERATURE REVIEW AND STATEMENT OF PROBLEM

Salmonella enterica, Food Safety, and Preventive Controls

Salmonella enterica subspecies *enterica* is a gram-negative, facultative anaerobe, rod-shaped bacterium. There are over 1,500 known serovars that are classified based on the combination of O, H, and Vi antigens (2). *Salmonella* has relatively low nutrition requirements and can grow at a wide range of temperatures and pH (3). The temperature which *Salmonella* can grow ranges from 5.2 – 46.2 °C with an optimal temperature for growth between 35 – 43 °C. Similar to the broad range of temperature, *Salmonella* can also grow in a broad range of pH (3.8 – 9.5) with the optimal pH at 7.0 – 7.5 (3). The ability for *Salmonella* to grow is based on a combination of temperature, pH, salts, and the type of acid present. A minimum water activity (a_w) of 0.93 is required for growth although many studies have shown *Salmonella* to survive for long periods at lower a_w (3).

Salmonella is the leading cause of bacterial foodborne illness and has shown to cause around 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year (<https://www.cdc.gov/Salmonella/index.html>). Although there are many serovars of *Salmonella enterica*, not all frequently cause human illness and only a small subset of serovars are commonly recovered from clinical patients (Table 1-1). The two most common serovars in the United States in 2022 are Enteritidis and Typhimurium which account for 41.5% of the outbreaks of domestic Salmonellosis and 60% worldwide (4). Historically, serovars Enteritidis, Heidelberg, and Hadar have been attributed to 80% of outbreaks attributed to eggs or poultry (5). More than 50% of the outbreaks caused by serovars Javiana, Litchfield, Mbandaka, Muenchen,

Poona, and Senftenberg have been attributed to plant commodities (5). *Salmonella* was placed on the global priority list of antimicrobial-resistant bacteria that needs more emphasis on research and development of new antibiotics by WHO in 2017 (6).

Salmonella depends on a wide range of pathogenicity islands and virulence factors that allow it to survive in multiple conditions in the journey from the environment to a host, to cause infection. Pathogenicity islands are acquired by horizontal gene transfer, are typically large genomic regions, and are absent non-pathogenic bacteria (7). Some characteristics of pathogenicity islands are they contain one or more virulence genes, are commonly adjacent to tRNA genes, and are frequently associated with mobile elements. There are 24 *Salmonella* Pathogenicity Islands (SPIs) known presently and the presence and organization of the SPIs may provide insights to the evolution and pathogenicity of *Salmonella* (8, 9). *Salmonella* pathogenicity island 1 (SPI-1) encodes a Type-3 Secretion system (T3SS) and several other effector genes that are secreted in the cells of the intestines, which allows for internalization of the bacteria (10). SPI-2 also encodes a T3SS which differs from the one present in SPI-1 (11). When *Salmonella* invades M cells, the SPI-1 T3SS needs to be expressed, but to replicate inside the macrophages the SPI-2 T3SS needs to be turned on (12). Table 1-2 provides information and highlights on select SPIs that are found in all *Salmonella*. Additional SPIs are found among different serotypes and may account for virulence differences among these serotypes (8).

Table 1-1: List of Serovars associated with Culture-confirmed Clinical Illnesses of Salmonellosis, United States, 2016 (13)

Rank	Serotype	Number Reported	Percent	Incidence (per 100,000)
1	Enteritidis	7,830	16.8	2.44
2	Newport	4,728	10.1	1.47
3	Typhimurium	4,581	9.8	1.43
4	Javiana	2,719	5.8	0.85
5	I 4,[5],12:i:-	2,179	4.7	0.68
6	Infantis	1,281	2.7	0.4
7	Muenchen	1,216	2.6	0.38
8	Montevideo	1,018	2.2	0.32
9	Braenderup	1,001	2.1	0.31
10	Thompson	792	1.7	0.25
11	Saintpaul	778	1.7	0.24
12	Heidelberg	754	1.6	0.23
13	Oranienburg	692	1.5	0.22
14	Mississippi	536	1.1	0.17
15	Typhi	423	0.9	0.13
16	Bareilly	412	0.9	0.13
17	Berta	369	0.8	0.11
18	Agona	362	0.8	0.11
19	Paratyphi B var. L(+) tartrate+	343	0.7	0.11
20	Anatum	257	0.6	0.08
	Subtotal	32,271	69.2	
	Other Serotyped*	7,709	19.3	2.4
	Unknown Serotype	5,625	12.1	1.75
	Partially Serotyped	801	1.7	0.25
	Rough, mucoid, and/or nonmotile	217	0.5	0.07
	Subtotal	14,352	30.8	
	Total	46,623	100	14.51

Table 1-2: A subset of Salmonella Pathogenicity Islands (12, 14, 15)

Pathogenicity Island	Genes of Interest	Notes:
SPI-1	<i>invA</i> <i>sipA</i> <i>sipB</i> <i>sipC</i> <i>sopE</i>	<ul style="list-style-type: none"> - Best characterized of the SPIs - Type 3 secretion system - - Virulence phenotype is dependent on secretion of effector proteins into host cell cytosol
SPI-2	<i>ssaJ</i> <i>spiC</i>	<ul style="list-style-type: none"> - Type 3 secretion system - essential for growth of bacteria; - SPI2 mutants fail to replicate similar to wild type strains. - Necessary for spread to lymph nodes, liver, or spleen
SPI-3	<i>mgtCB</i>	<ul style="list-style-type: none"> - Intramacrophage survival and virulence in mice - Transport of Magnesium at low Mg²⁺ conditions
SPI-4	<i>siiA-F</i>	<ul style="list-style-type: none"> - Type I secretion - toxin secretion similar to <i>E.coli</i> hemolysin secretion - Highly conserved among different serovars
SPI-5	<i>sopB/sigD</i> <i>pipB</i>	<ul style="list-style-type: none"> - Encodes the effector proteins for both SPI-1 and SPI-2 T3SS
SPI-6	<i>sag</i> <i>pagN</i>	<ul style="list-style-type: none"> - Recognized in serovars Typhi and Typhimurium - Encodes fimbriae
SPI-9		<ul style="list-style-type: none"> - Adhesion Factors - Type 1 Secretion System - Induced under low pH and high osmolarity
SPI-11	<i>pagC</i> <i>pagD</i>	<ul style="list-style-type: none"> - Virulence factors but not fully characterized

Along with SPIs, *Salmonella* can also horizontally acquire Genomic Islands (GIs). These elements have similar characteristics of SPIs but do not contain virulence genes and tend to include antimicrobial resistance (AMR) genes or other resistance genes (16). SGI-1 was initially reported in serovar Typhimurium and encoded resistance to the antimicrobials ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (17, 18). There have been many variants of SGI-1 identified, which contain the same backbone but contain different assortments of AMR genes (17). SGI-2 is very similar to SGI-1 but has been determined to have evolved independently (19). SGI-3 and SGI-4 were once considered different but have the same genomic backbone and are found in the same chromosomal location REF ARAI 2019 Branchu 2019 (8). This SGI differs from SGI-1 and SGI-2 in that it contains heavy metal tolerance genes and not AMR genes. The advancement of genomics is enhancing the detection and identification of genomic islands. However, an updated scheme for nomenclature and the how to discriminate between novel SGIs and variants needs to be better established to reduce issues based on a previous instance with SGI-3 and SGI-4. One suggestion is to use the genomic location of the islands to determine nomenclature similar to methodology employed by pathogenicity islands. (8).

Outbreaks of salmonellosis have been linked to various food commodities such as produce, spices, nuts, meats, and seafood which represents a wide range of water activity, pH, and processing events (20, 21). This illustrates the ability *Salmonella* has to survive in a wide range of environments found in natural and food industry settings (22) which make it particularly challenging for food safety. Preventive controls are defined as risk-based, procedures, practices, and processes that when employed significantly minimize or prevent the hazards identified. The

preventive controls employed to tackle *Salmonella* in a low-moisture processing setting are process controls, sanitation controls, supply-chain controls, and recall plans (23). Some process controls typically used to reduce pathogens are the application of heat, dehydration, and holding temperature. Studies have shown that *Salmonella* can adapt to stress and possibly become better equipped to survive these process controls (22, 24, 25). *Salmonella* can also form extensive biofilms that consist of curli fimbriae, cellulose, and other proteins which can provide protection against desiccation, biocides, and the host immune system (26). Identification of these adaptations may provide answers on how these outbreaks are occurring, better risk management practices, and how to prevent them in the future. One area of importance for research is studying how *Salmonella* can survive in a low-moisture environment and contribute to outbreaks of salmonellosis.

Low Moisture Foods (LMF) and Salmonella

Low-moisture foods (LMF) are naturally low in moisture or are produced from higher moisture foods where the water activity is reduced through drying or a dehydration processes (27). LMFs are shelf-stable due to the low moisture content, commonly ready-to-eat, and are perceived as safe by consumers. LMFs include cereals, chocolate, dried fruits and vegetables, flour, spices, nuts, peanut butter, seeds, and pasta. LMFs have a water activity (a_w) < 0.85, which is below the approximate level for bacteria to grow ($a_w = 0.87$) although many pathogenic bacteria are capable of surviving the drying process (27). There have been numerous, large outbreaks associated with LMFs (Table 1-3) which has added to the importance of research in the ability of pathogens to persist in this environment and on these foods (28). The route of contamination of these products can occur in numerous stages of the processing operations such as drying, hulling,

milling, packaging, and storage (29). Cross-contamination between the facility environment is a common factor among outbreaks, due to the lack of wet cleaning, and the extended duration between full facility cleanings (29). There are numerous factors related to low-moisture foods that increase survivability of foodborne pathogens. It has been shown that bacteria survive better in a lower a_w of 0.37 as compared to 0.57 in tree nuts (30). Lower a_w can also provide secondary protection against heat as seen in a study comparing survival of *Salmonella* inoculated in low-moisture foods with varying degrees of a_w (31). The composition of the food also alters how pathogens respond to the low-moisture environment. Fats, proteins, and sugar content all play large roles in desiccation resistance. Nuts, nut products, and seeds accounted for the majority of outbreaks and recalls in low moisture foods from 2012-2020 (28). Specifically, pistachios have accounted for three outbreaks and at least nine recalls with a very limited number of serovars recovered.

Table 1-3: Multistate outbreaks of Salmonellosis attributed to Low-Moisture foods from 2000-2023 (32, 33, 34, 35) Salmonella isolated from product donated by an *

LMF Product	Salmonella Serovar	Country	Year	Cases
Almonds	Enteritidis*	USA Canada	2000 - 2001	168
	Enteritidis	USA Canada	2003 - 2004	47
	Typhimurium*	Australia	2012	43
Toasted Oat Cereal	Agona*	USA	1998	400+
Puffed Rice or Wheat Cereal	Agona*	USA	2013	33
	Mbandaka*	USA	2018	136
Cake Mix, raw – in ice cream	Typhimurium*	USA	2009	26
Cake Mix	Agbeni*	USA	2018	7
Flour	Infantis*	USA	2023	14
Peanut Butter	Tennessee*	USA	2006 - 2007	715
	Typhimurium*	USA Canada	2008 - 2009	714
	Bredeney	USA	2012	42
	Braenderup	USA	2014	6
	Senftenberg	USA	2022	21
Pine Nut	Enteritidis*	USA	2011	53
Pistachio	Montevideo* Newport* Senftenberg*	USA	2008 - 2009	83
	Senftenberg*	USA	2013	8
	Senftenberg* Montevideo*	USA	2015 - 2016	11
	Montevideo* Mbandaka*	USA	2013	16
Tahini	Concord*	USA	2018	8
	Montevideo*	USA	2009 - 2010	272

Stress Response in *Salmonella enterica*

Many pathogens, including foodborne pathogens, contain a genetic defense system against the many different potential environmental conditions that may be encountered. These systems must be able to protect against temperature fluctuations, availability of nutrients and oxygen, and enhance competition among other microbes. *Salmonella* has a complex regulatory system, including transduction systems and sigma factors, which can turn on a stress response cascade.

Two-component signal transduction systems (TCS) are composed of sensors and transcriptional regulators and can be used to respond to changes in the environment. The sensor is typically membrane-bound protein kinase. A signal substrate binds to the sensor which will cause an autophosphorylation event that will trigger the response regulator to bind to DNA to induce transcription. SoxRS, a TCS, senses and responds to oxidative stress and nitrosative stress, by activating a diverse set of antioxidant defense genes (36).

Sigma factors can assist with recruitment of RNA polymerase for specific gene translation. *Salmonella* has six sigma factors: σ^D (RpoD) is responsible for expression of housekeeping genes, whereas σ^S (RpoS), σ^E (RpoE), σ^H (RpoH), σ^F (σ^{28}), and σ^N (RpoN) are activated during stress or changes in growth conditions (36). RpoS, is one of the most studied sigma factors, and is primarily involved in the general stress response by activating up to 500 genes (37, 38, 39). RpoS accumulates during stationary phase and is degraded during exponential growth periods. Bacteria tend to be more resilient to environmental stresses during the stationary period, which may be a result of RpoS accumulation. RpoE responds to extracytoplasmic stress, such as extreme heat and is essential for membrane and periplasmic homeostasis (36, 40). RpoS and

RpoE have been linked with survival during starvation and osmotic stress conditions, and RpoE is important for desiccation tolerance (24). RpoH is responsible for regulating genes during a heat shock events, oxidative stress, and during stationary phase survival. σ^F (σ^{28}) is used for flagellar biogenesis and motility (41). The RpoN regulon is diverse and controls genes response to nitrogen limitation, nitric oxide stress, alternative carbon sources and many others (42).

Expression studies are a valuable tool to identify the genes responsible for survival and persistence in the presence of various stressors. These studies can be gene specific using Real-time Reverse Transcription Quantitative PCR (RT-qPCR) or capture all gene expression using RNA-Sequencing (RNA-seq). Direct expression studies using RT-qPCR target a selection of genes and compare expression of those genes to a known housekeeping gene. However, creating the panel for qPCR can be onerous and may not be efficient for studies if many genes are involved. Therefore, global expression studies utilizing RNA-seq whole genome sequencing technology to sequence all the transcripts present is a better assay. Using bioinformatic tools, the ability to pinpoint any number of genes that are being expressed is achieved. A combination of the two techniques provides the most accurate results, where qPCR is used as a confirmatory tool.

Exposure of *Salmonella* to an initial stress increases the probability of developing cross-protection to multiple stresses and leading to more virulent cells. Table 1-4 provides a summary of previous studies looking at stress response and cross-protection. A shift in research has occurred where focus on the environment and the source of the *Salmonella* plays a more important role in virulence and persistence. The study by Furtado et al (43) highlights the

different phenotypes *Salmonella* can display depending on which part of the processing chain it is derived from. Future studies should focus on testing persistence of a strain from all locations in the farm to fork continuum to identify components leading to the bacteria adaptations to different niches.

Table 1-4: Previous Research on Stress Response in *Salmonella enterica*

Author	Year	Stress Response	Findings
Leyer G.J., Johnson E.A. (44)	1993	Acid → Multiple Stresses	Acid adapted cells were able to survive thermal treatments, osmotic stress, H ₂ O ₂ , surface active compounds.
Greenacre E.J., Brocklehurst T.F. (45)	2006	Acid → Salt	Cells were adapted with acetic acid and challenged with osmotic stress. These preadapted cells were protected against exposure to 2.5M NaCl. The protection was not induced by KCl adaptation.
Xu H., Lee H.Y., Ahn J. (46)	2008	Acid → Acid and Cold	Acid adaptation induced cross-protection and viable nonculturable (VBNC) state against low acid and cold stress.
Alvarez-Ordóñez A., Lopez M. (47)	2010	Acid	The results were affected by the type of acid used. Acid resistance was dependent on growth temperature. Acid adapted cells showed improved survival in BHI pH 3.0 compared with non-acid adapted.
Gruzdev N., Sela S. (48)	2011	Desiccation → Multiple Stresses	UV irradiation showed a 2-log reduction on desiccated cells at 25 minutes vs. an 8-log reduction of non-desiccated cells. Desiccated cells were more tolerance of ethanol, bile salts, hydrogen peroxide, and NaCl.
Kang IB., Seo K. (49)	2018	Acid-Salt → Heat	Acid adaptation increased heat tolerance, which may be due to outer cell membrane protein regulation. The combination of Acid, Salt, and refrigeration were able to reduce the tolerance to heat.
Kim C., Wynn C. (50)	2019	Heat → Heat	Bacteria grown at lower temperatures display decreased thermal resistance.
Jayeola V., Kathariou S. (51)	2020	Desiccation	Used Tn5 mutagenesis to determine genes necessary for persistence in LMF. Genes important included those for DNA repair, transcriptional regulation, and osmolarity response
Furtado M.M, Sant'Ana A.S. (43)	2023	Desiccation → Rehydration, Heat	This study showed differences of desiccation survival both intra-serovar and inter-serovar. Temperature of drying and storage time see to affect survival. This study also identified differences in desiccation resistance depending on where in the processing chain the isolate was derived.

Copper use in agriculture and bacterial toxicity

Copper is one of eight essential plant micronutrients, being required for many plant enzymatic activities and the production of chlorophyll and seeds (52). Copper has been used in agriculture for centuries as a fungicide and as a deficiency corrector. Copper may be essential for food sustainability and research has been funded by USDA's National Institute of Food and Agriculture to evaluate how copper effects crop fertility and grain yield (53, 54, 55). The usage of copper has been restricted in Europe over the last few decades due to the expansive dissemination of the *Salmonella* ST34 clone, with a final objective to totally phase out its use. This has a strong possibility to lead to crop loss due to plant disease and nutrition deficiencies (56). There are other avenues of heavy metal accumulation in soil such as anthropogenic activities, animal manure applications, pesticide usage, and sewage sludge (57). The concentrations of copper in present-day soil reflect past usage, illustrating how the accumulation can persist for long periods of time. Copper is an essential micronutrient for bacteria as both an electron carrier and a redox catalyst, however excess copper is cytotoxic (58). Copper exists in an equilibrium between two forms, Cu^+ and Cu^{2+} . Cu^{2+} is more biologically inert and can be found in the bacterial periplasm whereas Cu^+ is more toxic since it can perform Fenton-like reactions and produce hydroxy radicals. Bacteria rely on oxidases to convert copper to the less toxic form (Cu^{2+}). Bacterial toxicity due to copper is greater in an anaerobic environment due to reduced activity of cellular oxidases and therefore, the bacteria can accumulate more toxic copper under these conditions (58). Eukaryotes have exploited the bactericidal effects of copper by increased expression of copper transporters in macrophages as a primary defense against bacterial such as *Salmonella enterica* (59). Copper was recognized as the first antimicrobial metal by the United States Environmental Protection Agency (US EPA) in 2008 (60). The

bactericidal effect of copper is attributed to copper ions affecting the cell membranes and generating intracellular oxidative stress. Copper Sulfate (CuSO_4) has been used in agriculture as a herbicide, fungicide, insecticide, and nematicide (61). Copper sulfate is typically mixed with water and sprayed onto plants as a fertilizer and to guard against plant diseases.

Generalized Copper Response in Salmonella

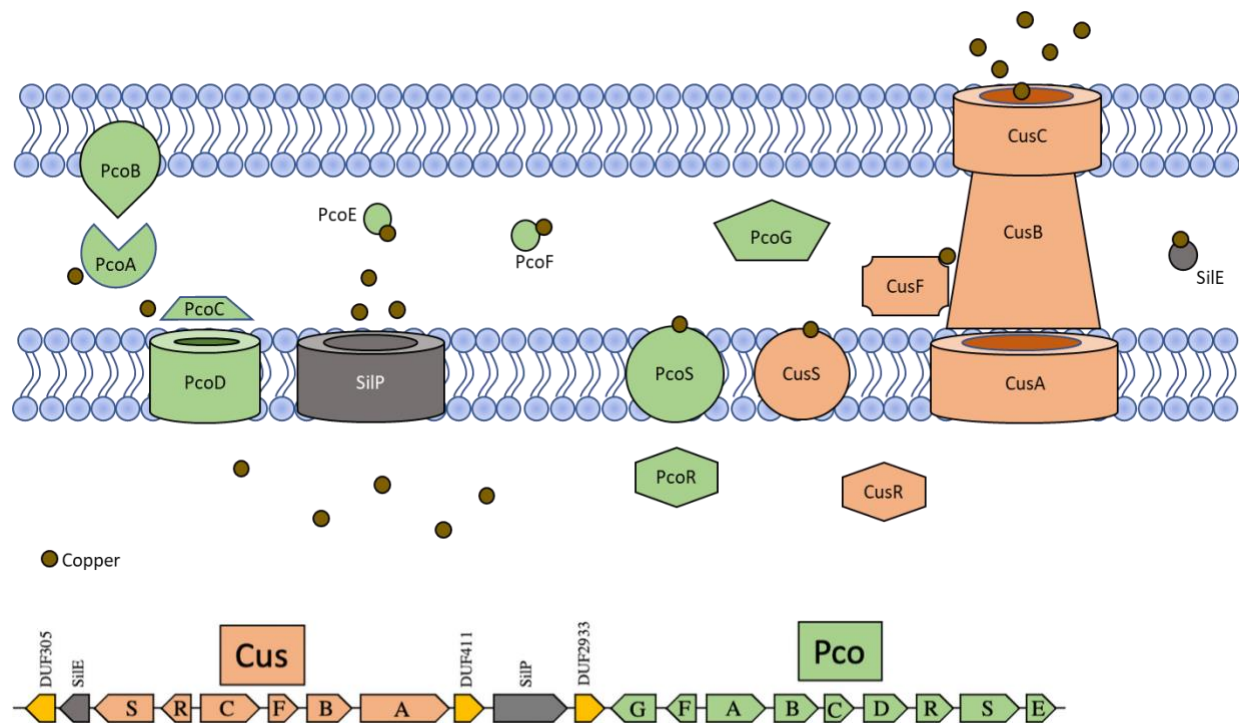
Mediation of intracellular copper stress in *Salmonella* is controlled by core regulons such as the *cue* regulon and the *gol* regulon. Regulons are defined as a group of genes that are regulated as a single unit, typically under the same repressor and activator. In the *cue* regulon, CueR turns on transcription of *copA* (a P-type ATPase) and *cueO* (a multicopper oxidase). CopA is thought to move Cu^+ from the cytoplasm to the periplasm where it is then oxidized by CueO into Cu^{2+} (62). This regulon is functionally conserved in bacteria although additional adaptations have been seen in different species, such as in *Salmonella* with the addition of an additional periplasmic chaperone (CueP) (58). The *gol* regulon is homologous to the *cue* regulon. GolS regulates the expression of an additional P-type ATPase (GolT) and a cuprochaperone (GolB) with the addition of a cytosolic copper storage protein (Csp3). In addition to the previous systems, other stress responses such as CpxR/CpxA and SoxR/SoxS are induced by copper. Copper can prevent outer membrane maturation, therefore the *cpx* response inhibits cell growth, cell division, and promotes biofilm formation (58).

Copper Homeostasis and Silver Resistance Island (CHASRI)

The addition of extrachromosomal tolerance loci allows some bacteria to persist in environments containing high levels of copper ions that would normally become toxic. Gram-negative bacteria

(*E. coli*, *P. syringae*, and monophasic *Salmonella*) have been isolated from agricultural areas contaminated with high levels of copper by repeated usage of copper salts as feed additives, bactericides, and fungicides (63). An extrachromosomal cassette, the Copper Homeostasis and Silver Resistance Island (CHASRI) (Figure 1), has been previously identified in different Enterobacteriaceae including *Escherichia coli*, *Enterobacter cloacae*, and *Salmonella enterica* (64). This island combines two different systems of copper homeostasis the *cus* system and the *pco* system and most likely derived from a single linkage event in a close relative of *Enterobacter cloacae* (64).

Figure 1-1: Diagram of the Copper and Silver Resistance Island (CHASRI) regulons and a schematic of the proteins encoded. (64)



The *pco* (plasmid-borne copper resistance) system was initially identified on a plasmid (pRJ1004) in an *E. coli* strain isolated from an Australian piggery where copper supplementation

was occurring (65). The *pco* cluster contains up to 9 genes, *pcoGFABCDRSE*, which are under control of three promoters (*PpcoA*, *PpcoE*, and *PpcoF*) (64, 66, 67). PcoRS is a two-component regulatory system with PcoS, the sensor-kinase, located in the periplasmic membrane. PcoA (multicopper oxidase) and PcoC (periplasmic metal binding) have been hypothesized to be crucial to this system (64, 66). PcoD is an inner membrane copper pump and PcoB is an outer membrane protein, that may inhibit the uptake of copper (68). PcoE is homologous to another CHASRI gene, *silE*. The role of PcoE is thought to be a primary defense mechanism to sequester copper in the periplasm before the rest of the system is induced and has been named a “copper sponge” (66, 69). PcoE also has the advantage of being induced by CusRS if PcoRS is lost. Additionally, PcoF and PcoG which encode for a putative periplasmic metal binding and M23 metallopeptidase, respectively make up the remainder of the operon.

The *cus* (Cu-sensing) system, *cusSRCFBA*, was first identified in *E. coli*, and comprises of an efflux system which removes both copper and silver from the periplasm and cytoplasm (69). The regulators CusRS form a two-component regulatory system, where CusS is the sensor kinase located in the cytoplasmic membrane and CusR, the response regulator, activates transcription of *cusCFBA* (70). The *cus* cluster is induced by the presence of copper and to a lesser extent by silver. CusA, CusB, and CusC form an efflux pump that can remove copper from the cytoplasm and periplasm. CusA is located on the inner membrane, CusC forms the outer membrane channel, and CusB provides the periplasmic portion of the pump. CusF, a periplasmic protein, can bind to copper and helps direct it to the CusCBA efflux pump for detoxification of the periplasm (71). Cus-mediated copper detoxification is more integral under anaerobic conditions where oxidases are not active but can also participate in copper removal in aerobic conditions.

The three major mechanisms of copper detoxification provided by this island include: 1) the action of the periplasmic detoxification *pco* system, 2) the *cus* system tricomponent efflux pump and transporters, and 3) the cytoplasmic detoxification system (SilP). Copper tolerance from this cassette is provided in aerobic, anaerobic and during the transition between the two environments which is beneficial for facultative anaerobes. Dispersal of the CHASRI among Enterobacteriaceae via horizontal transfer has been identified in environments under both natural and anthropogenic copper stress. This island is present in *Salmonella* genomic island 4 (SGI-4) along with additional heavy metal resistance genes including those for arsenic (72). SGI-4 is a characteristic of the monophasic *Salmonella* Typhimurium ST34 clade which is primarily associated with pigs. The selection of this clone in Europe may have been driven by overuse of copper as a growth promoter in the swine industry (73).

Previous Minimum Inhibitory Concentration (MIC) studies have looked at the ability for bacteria to survive challenge with copper sulfate (CuSO_4) in *E. coli* and with a limited scope in *Salmonella*. The core copper homeostasis genes provide better tolerance in the presence of oxygen (MIC = 5.5 mM) than when in an anaerobic environment (MIC = 0.70 mM) (62), which is due to the activity of the oxidases. The addition of the CHASRI allows for an increase of MIC to ~12–15 mM and results in more similar MIC values in both the aerobic and anaerobic conditions (74). These studies in *Salmonella* have been done using agar dilution methods and are focused on inhibition. Thus, there are gaps in the research and analysis of different methods for MIC with follow-up testing of Minimum Bactericidal Concentration (MBC) should be conducted in *Salmonella*. An additional focus of this study will be to determine if the expression of the

CHASRI in *Salmonella* also provides cross-protection against secondary stress such as heat or desiccation.

Statement of problem

Based on the extensive literature review performed, it is evident that:

- 1) LMFs are resulting in an increasing number of outbreaks and recalls due to *Salmonella* contamination. Due to the inherent nature of these foods, controls that are used for microbial reduction in foods with higher water activity are less effective. Although, the dry environment does not allow for microbial proliferation, foodborne pathogens have been shown to persist for extended periods of time. The low a_w of these foods also can contribute to the increased thermal tolerance of *Salmonella*.
- 2) There have been numerous storage studies of *Salmonella* in nuts but very little research on the actual source of the contamination and if there are genetic components that are behind the continued isolation of *Salmonella*.
- 3) The CHASRI has been previously identified in a subset of *Salmonella* serovars. There have been previous studies utilizing different MIC assays to determine protective effects of the CHASRI in bacteria when exposed to copper. These studies have varying results which may be due to methodology (agar vs. broth) and conditions (aerobic vs. anaerobic). In my opinion, these studies have not shown a strong enough advantage for *Salmonella* to acquire this genomic island.
- 4) The stress response cascade has been shown to provide cross-protection against additional stresses in bacteria. There are gaps in research evaluating the persistence of *Salmonella* undergoing multiple stresses that are inherent to the food processing pathway.

Specific objectives

- 1) Investigate repeated outbreaks of *Salmonella* in pistachios to identify potential genomic targets that are contributing to persistence.

Hypothesis: The outbreaks of *Salmonella enterica* from 2009 and 2016 in pistachios are due to an environmental contamination that occurred prior to 2009 and contain genetic elements leading to its persistence in the pistachio environment.

- 2) Evaluate the distribution of the CHASRI genomic element across *Salmonella enterica* and determine the benefits of the inclusion of CHASRI to the bacteria.

Hypothesis: Presence of CHASRI is widespread throughout numerous serovars of *Salmonella enterica* and enhances survival in environments with increased copper concentrations.

- 3) Determine if the CHASRI has a protective effect in *Salmonella* desiccated on pistachios and then heat treated.

Hypothesis: Induction of the primary stress response will provide increased protection against a secondary stress in the strains containing the CHASRI in comparison to the knockout.

CHAPTER 2: IDENTIFICATION OF THE POTENTIAL SOURCE
AND GENETIC MARKERS THAT MAY BE CONTRIBUTING TO
PERSISTENCE OF TWO SEROVARS OF *SALMONELLA* IN THE
PISTACHIO SUPPLY-CHAIN

Publication Status:

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Abstract

Pistachios have been implicated in two salmonellosis outbreaks and multiple recalls in the U.S. This study performed an in-depth retrospective data analysis of *Salmonella* associated with pistachios as well as a storage study to evaluate the survivability of *Salmonella* on inoculated inshell pistachios to further understand the genetics and microbiological dynamics of this commodity-pathogen pair. The retrospective data analysis on isolates associated with pistachios was performed utilizing short-read and long-read sequencing technologies. The sequence data were analyzed using two methods: the FDA's Center for Food Safety and Applied Nutrition Single Nucleotide Polymorphism (SNP) analysis and Whole Genome Multilocus Sequence Typing (wgMLST). The year-long storage study evaluated the survival of five strains of *Salmonella* on pistachios stored at 25 °C at 35% and 54% relative humidity (RH). Our results demonstrate: i.) evidence of persistent *Salmonella* Senftenberg and *Salmonella* Montevideo strains in pistachio environments, some of which may be due to clonal resident strains and some of which may be due to preharvest contamination; ii.) presence of the Copper Homeostasis and Silver Resistance Island (CHASRI) in *Salmonella* Senftenberg and Montevideo strains in the pistachio supply chain; and iii.) the use of metagenomic analysis is a novel tool for determining the composition of serovar survival in a cocktail inoculated storage study.

Introduction

Salmonella has been shown to persist in low-moisture environments and represents a potential hazard for a wide range of low-moisture foods and ready-to-eat products such as nuts, peanut butter, spices, and flour (75). The ability for *Salmonella* to survive for extended periods of time in these low-moisture products and cause clinical infections is a notable public health concern (27). Contamination of these products with *Salmonella* can occur pre-harvest, during harvest, during storage, or post-harvest (e.g., after a heat-treatment step) (76).

Salmonella enterica subsp. *enterica* serovar Montevideo (*Salmonella* Montevideo) and *Salmonella enterica* subsp. *enterica* serovar Senftenberg (*Salmonella* Senftenberg) have been repeatedly isolated from pistachios dating back to 2008. These isolates were identified during multi-state salmonellosis outbreaks linked to pistachio consumption in 2013 and 2016 (77, 78, 79, 80, 81), as well as multiple product recalls across multiple time periods (e.g., 2009, 2010, 2012, 2013, 2016, and 2018) (82, 83). A previous study noted that as many as six different *Salmonella* serovars (e.g., Agona, Liverpool, Montevideo, Tennessee, Senftenberg, and Worthington) have been isolated from pistachios, and a comparison of the limited number of serovars to the numbers identified in surveys of other tree nuts may suggest a narrow and persistent contamination source (84, 85). Multiple potential sources of contamination with foodborne pathogens should be considered regarding the production of produce, including pistachios. Previous land use should be considered in regard to total risk, for example, if the current farmland was known to have previously been used as an animal feeding operation (86). Adjacent land use may also be a source of potential biological hazards to consider since there is evidence that dust and microorganisms can move a short distance into adjacent almond orchards

from an upwind animal operation (87). Moreover, pathogen-contaminated runoff can occur, and wild animals can leave waste containing pathogens. Although many of these potential sources would concentrate on the orchard floor where there may not be any direct contact with the pistachios due to the use of catch frames during harvest, various farming activities such as cultivation, spraying foliar treatments, and harvest have the potential to aerosolize pathogens and deposit them on pistachios (86).

The United States is the leading producer and exporter of pistachios in the world (88). Domestically, approximately 99% of the pistachios consumed in the United States are grown in California (89). In 2018, 264,000 bearing acres of pistachios in California resulted in a total production of 967 million pounds (90). The harvesting, transportation, wet abrasion (hulling), float tank submersion, drying, and storage of pistachios all include inherent risks for *Salmonella* contamination according to a risk assessment published in 2018 (91). The researchers were able to model atypical situations and calculate the mean risk of illness from pistachios. In particular, this study showed an increased risk of contamination due to certain pistachio production steps, including use of a float tank and a delay in drying, both of which had the greatest impact on consumer risk (92).

Multistate outbreaks of salmonellosis in 2013 and 2016 linked to contaminated pistachios were investigated by the U.S. Food and Drug Administration (FDA), the Centers for Disease Control and Prevention (CDC), as well as state public health labs (79, 80). The 2013 outbreak included 8 clinical cases from six states infected with the same strain of *Salmonella* Senftenberg and isolates recovered from pistachio and environmental swabs. The onset of infection was over a 5-month

span (January to May 2013), likely owing in part to the long shelf-life of these products. The clinical strains and those recovered from the implicated facility were identified as related by PFGE, producing an identical PFGE pattern. At the time of this outbreak WGS was not routinely performed but in retrospect three clinical isolates, four pistachio isolates, and 17 environmental swabs were sequenced, and the data was uploaded to NCBI. The outbreak in 2016 had a total of eleven documented clinical cases identified by PulseNet (nine cases of *Salmonella* Montevideo and two cases of *Salmonella* Senftenberg), with two hospitalizations in nine states. Onset dates of illnesses from patients covered a 3-month span (January to March 2016). Clinical and food isolates were characterized by Pulsed Field Electrophoresis (PFGE), as well as whole genome sequencing (WGS). FDA reported raw pistachio samples collected at firms supplied by the implicated facility yielded *Salmonella* Montevideo and Senftenberg, while samples of raw pistachios collected during the investigation of the implicated facility yielded *Salmonella* Senftenberg. These *Salmonella* Senftenberg isolates were nearly indistinguishable from the clinical isolates based on WGS results (81).

Next-Generation sequencing (NGS) now regularly allows for further investigation into the genomes of *Salmonella enterica* that are responsible for illnesses, outbreaks, (93, 94, 95) and other contamination events, including a determination of whether a resident strain may be causing contamination (96). Additionally, NGS techniques provide an abundance of data regarding virulence genes, phages, and antimicrobial resistance gene patterns. The ability to capture all this data in one assay that can be used for multiple downstream analyses makes it very appealing. FDA and CDC utilize SNP and cgMLST/wgMLST analysis in the determination of outbreaks of foodborne pathogens (97). Although these are different methods, the results are

very comparable. WGS data are essential to fully characterize a bacterial pathogen of interest and can often assist in finding ways to prevent future contamination (93, 94, 95).

In this study, sequence data from *Salmonella* Senftenberg and *Salmonella* Montevideo isolates associated with pistachio outbreaks, recalls, and investigations over a nine-year period (2009-2018) were analyzed to better understand evolutionary relationships among these isolates and, subsequently, to better evaluate their persistence in the pistachio growing and processing environment. In addition to analyzing WGS data, in parallel, we also conducted a storage experiment over a year-long time frame that included the 2016 outbreak strains of *Salmonella* Montevideo and Senftenberg. This latter study focused on the survival rate of these strains on inshell pistachios stored at two humidity levels typical of low moisture foods ($a_w < 0.7$) and further underscored persistence phenotypes among these adapted strains. The novel application of metagenomic sequencing of the cocktail-inoculated pistachios allowed for simultaneous detection of the five different serovars to determine survivability of the strains over the storage period.

Materials and Methods

Bacterial isolates used for WGS analyses

The isolates used in this study were identified by querying the Pathogen Detection tool (<https://www.ncbi.nlm.nih.gov/pathogens/>), a publicly available database curated by the National Center of Biotechnology Information (NCBI) (98), that routinely updates genomic sequences from food, environmental sources, and patients. SNP clusters for further analysis were identified based on the following criteria: serovar “Montevideo” or “Senftenberg”, source “pistachio”, and location “United States”. A total of four SNP clusters (two for Senftenberg: PDS000031814,

PDS000031739 and two for Montevideo: PDS000027237, PDS000032600) were identified based on our parameters. All isolates found in the SNP cluster associated with pistachios, as well as any other isolates that were closely related within the clade of the NCBI phylogenetic tree, were downloaded for analysis. The metadata (date of collection, source, and location) were also downloaded from NCBI for use in the analysis. The final data set derived from the four SNP clusters consisted of 204 isolates (109 Senftenberg and 95 Montevideo) obtained from clinical, food, and environmental sources; these were sequenced using Illumina technology. The sources of the 109 Senftenberg isolates consisted of almonds (1), chicken (2), clinical (10), environmental swab (43), fertilizer (1), pistachios (51), and tahini (1). These isolates were collected from 1987 to 2018. The sources of the 95 Montevideo isolates used for phylogenetic analysis consisted of almonds (1), raw intact beef (1), cattle (1), chicken (2), clinical (30), environmental swabs (9), blend trail mix (4), missing source (2), mixed nuts (9), pistachios (35), and swine (1). These isolates were collected from 2008 to 2018. Geographically, there were 107 Senftenberg and 89 Montevideo isolates from the United States and 2 Senftenberg and 6 Montevideo from other countries (including Canada, Mexico, and the United Kingdom). The 2013 outbreak investigation isolates (3 clinical, 4 pistachios, and 17 environmental) were found in SNP cluster PDS000031739 (Senftenberg). The 2016 outbreak investigation isolates were contained in SNP clusters PDS000031814 (Senftenberg) and PDS000027237 (Montevideo). There was a total of thirteen pistachio isolates (8 Senftenberg and 5 Montevideo) and 10 clinical isolates (2 Senftenberg and 8 Montevideo) associated with the 2016 outbreak investigation. The complete list of isolates and accompanying metadata can be found in S1 Table. The isolates were sequenced using the Illumina sequencing chemistry by participating GenomeTrakr laboratories (99), CDC PulseNet laboratories, or private labs. Raw reads for all isolates were downloaded

from the Sequence Read Archive (SRA) (<https://www.ncbi.nlm.nih.gov/sra>) using command line tools from the SRA toolkit available on NCBI. *De novo* assemblies were generated with SPAdes v3.13.0 (100) using k-mer lengths of [21, 33, 55, 77, 99, 127], and the options “--careful” and “--only-assembler”.

In silico multi-locus sequence typing (MLST)

Initial sequence analysis of the isolates was performed using an *in silico* MLST approach, based on information available at the *Salmonella enterica* MLST database (101). Seven loci (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) were used for MLST analysis to generate a sequence type (ST).

Genome closure

Five *Salmonella* Senftenberg genomes and five *Salmonella* Montevideo genomes isolated from pistachios were completely closed using long read sequencing technology for this study (Table 2-1). These specific isolates were selected as they were sourced from pistachios, readily available in our laboratory, and had different collection dates. Six of the *Salmonella* isolates were sequenced with the PacBio Sequel system (Pacific Biosciences, Menlo Park, CA, USA) and four isolates were sequenced with the Oxford Nanopore GridION (Oxford Nanopore Technologies, Oxford, UK). The ten closed genomes were submitted in DDBJ/EMBL/GenBank and annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (102). Eight of the complete genomes were reported with a detailed method description in two separate genome announcements (1, 103).

Gene-by-gene comparison approach

To provide gene-by-gene comparison, we aligned all our closed genomes based on serovar using MAUVE aligner version 20150226 using the progressive algorithm with default settings, and phages were identified using PHASTER (104). Any unique genomic regions, phages, and plasmids identified were imported into Ridom SeqSphere⁺ v. 6.0.2 (Ridom GmbH, Munster, Germany) (105) and used as a reference to interrogate the 201 short read isolate assemblies used in this study for presence or absence.

Phylogenetic analyses (wgMLST and SNP)

Targeted core genome MLST (cgMLST) and accessory genome analysis of the isolates was performed using Ridom SeqSphere⁺ software v 6.0.2 (105) by creating *ad hoc* wgMLST databases derived from the closed genomes previously described. Using the cgMLST target definer tool and accessing our genomes from NCBI, an *ad hoc* wgMLST scheme for *Salmonella* Senftenberg was developed. The scheme was based on the closed reference genome CFSAN047866 (CP029040), comprised of 4,768 genes, and query genomes (CP029036, CP029038, CP037892, and CP037894) found in Table 1 using the thresholds previously published (106). The resulting scheme consists of 4,548 loci (3,908 core targets and 640 accessory targets). The 109 *Salmonella* Senftenberg assemblies (54 nuts/seeds, 44 environmental, 10 clinical and 1 poultry) were typed using this scheme.

The same process was repeated to create an *ad hoc* wgMLST scheme for *Salmonella* Montevideo using the closed genome CFSAN051296 (CP029336), comprised of 4,664 genes, as the reference and the four other closed genomes from this study (CP029035, CP029039, CP040379,

and CP040380) as query genomes using the same settings to define the core genome loci. The resulting scheme consists of 4,447 loci (3,876 core targets and 571 accessory targets). The 95 *Salmonella* Montevideo assemblies (49 nuts/seeds, 30 clinical, 9 environmental, 2 poultry, 2 missing source, 1 beef, 1 swine, and 1 cattle) were typed using this scheme.

For each serovar, the alleles identified were used to establish a matrix disregarding missing allele values. A minimum spanning tree of all common alleles was then constructed to visualize the results using the Ridom SeqSphere⁺ software. Clusters were automatically assigned using the default distant threshold for the software of ≤ 10 alleles.

Due to the large allele differences identified in the wgMLST analysis, phylogenetic SNP analysis was performed separately on the four different sequence types of *Salmonella* Senftenberg and Montevideo using the CFSAN SNP Pipeline (107). In short, WGS reads were mapped to the reference genome using Bowtie2 and the resulting files were processed using SAMtools. The variant sites were identified using VarScan, and a SNP matrix was produced using custom scripts. Four SNP matrices were generated using the closed genomes CFSAN000878 (ST14), FSW0104 (ST185), CFSAN000258 (ST316), and CFSAN005645 (ST138) as references. The SNP matrices can be found in S2-S5 Tables. Phylogenetic trees were constructed using the RAxML (Randomized Accelerated Maximum Likelihood) program with 500 bootstrap replicates (108). All RAxML analyses were performed with the default parameter settings and the GTRCAT nucleotide substitution model. The trees were visualized using Figtree software (version 1.4.4).

Pistachio storage study

A storage study was conducted to evaluate the ability for *Salmonella* (including *Salmonella* Senftenberg and Montevideo isolates associated with the 2016 outbreak) to persist on raw, inshell pistachios in conditions simulating a range of typical storage conditions. A cocktail containing four different *Salmonella enterica* serovars (Anatum, Montevideo, Oranienberg, and Senftenberg) associated with low moisture food commodities and one strain of *Salmonella* Newport isolated from tomatoes was used in this experiment (Table 2). The genomes of all five isolates were completely closed using Pacbio long read technology (1, 109). For this study, organic, raw inshell pistachios were purchased and tested for background microbial populations using the Biomerieux Tempo AC kit (Biomerieux, Durham, NC, USA), and then inoculated with the 5-strain cocktail. Inoculum was prepared from stock culture as described in Keller et al. (110) with slight modifications. Briefly, a single colony from each working stock was transferred to Trypticase Soy Broth (TSB, BD, Franklin Lakes, NJ, USA) and incubated overnight at 37°C. After incubation, 100 µl of inoculum was spread plated on Trypticase Soy Agar (TSA, BD) plates. The plates were incubated overnight at 37°C and then bacteria were harvested by adding 1.0 ml of Buffered Peptone Water (BPW, BD) to the surface of the plate and gently scraped using an L-shaped plate spreader. Each plate yielded ~0.5 ml of harvested culture at approximately 10 log CFU/ ml. The stock cocktail was formed by combining equal volumes of all five harvested cultures and then a high-population cell suspension was prepared by combining 18 ml of the five-serotype *Salmonella* stock cocktail with 1,800 ml of sterile deionized water. A total of 900 g of pistachios were soaked in the high-population cell suspension bath for 1 minute, and then placed on trays lined with absorbent towels in a biosafety cabinet to air-dry overnight at room temperature. After 24 h, pistachios were stored in desiccator cabinets equilibrated to either

35% relative humidity (RH) or 54% RH at 25°C, which were selected to represent a low and high water activity (a_w) within the range of water activity levels typical of low moisture foods (30). Uninoculated control pistachios were held under the same conditions. To determine the surviving populations, triplicate (10g) samples were removed for analysis at 0, 1, 2, 4, 6, 13, 27, 55, 83, 180, 270, and 365 days. The water activity was recorded for the inoculated and control pistachios from the different relative humidity conditions using an Aqualab model 4TE water meter (Decagon Devices, Pullman, WA). Two random 10 g samples from the uninoculated control group were tested using the Biomerieux Tempo AC kit to determine background microbe counts. The triplicate samples of inoculated pistachios were diluted 1:9 in BPW and appropriate dilutions were plated in duplicate on the differential media m-TSAYE [Trypticase Soy Agar with Yeast Extract supplemented with 0.05% (wt/vol) ammonium iron citrate and 0.03% sodium thiosulfate]. After overnight incubation at 37°C, colonies were counted, and log CFU/g was calculated. A repeated measure analysis of variance (ANOVA) was performed in R v3.6.1 to determine the differences in log CFU/g over time between the high and low humidity conditions. The differences in the slopes (or rate of reduction in log CFU/g) between the two humidity conditions were also investigated using R.

Two methods were used to determine composition of surviving serovars at different time points, the Luminex xMAP® *Salmonella* Serotyping Assay (Luminex, Madison, WI, USA) and metagenomic analysis of shotgun sequencing using the Illumina MiSeq benchtop sequencer (Illumina, San Diego, CA, USA). For the Luminex assay, 12 – 16 individual colonies from each condition were selected from the m-TSAYE plates and DNA was extracted using Bio-Rad InstaGene™ matrix (Bio-Rad, Hercules, CA) following the manufacturer's protocol for DNA

extraction from bacterial cells. DNA was analyzed for molecular determination of serotype in *Salmonella* following the protocol published by the CDC (111, 112). For the sequencing assay, the initial 1:9 dilution of pistachio:BPW was incubated at 37 °C overnight. A 2 ml aliquot of the overnight enrichment was centrifuged at 6000 RPM for 10 minutes and then DNA was extracted using the Maxwell RSC Cultured Cells DNA Kit (Promega, Madison, WI, USA) according to manufacturer's recommendations with the addition of RNase A after lysis. Sequencing libraries were prepared using Nextera DNA Prep Library kit (Illumina, San Diego, CA) following the manufacturer's protocols. The finished libraries were sequenced on the Illumina MiSeq using v3 600-cycle sequencing chemistry with 2×250 paired end reads. Determination of serovar composition from shotgun sequencing raw reads was conducted using custom C++ programs developed to compile a k-mer signature database containing multiple unique 30 bp sequences per species and then identification of each read in the input file using the 30 bp probes (113). For each bacterial species or subspecies, each non-duplicated 30-mer from a reference whole genome sequence was placed into a database. We removed any k-mers not found in at least 2/3 of a set of additional genome sequences of the same species and we removed any k-mer found in genomes of other species. The resulting k-mer database used in this work contains 5,900 target entries, each consisting of approximately 40,000 (range 44 to 80,000) unique k-mers. The database includes 1,100 different bacterial genera, and 3,500 species. Normalization is performed to correct for bias due to differing number of k-mers used per database entry and the results are tabulated as percent of identified reads (contribution to the microbial population of identified species) for each database entry.

Results and Discussion

In silico MLST

In silico seven locus MLST was performed on the 109 *Salmonella* Senftenberg and 95 Montevideo isolates identified from the Pathogen Detection browser. MLST results for the *Salmonella* Senftenberg isolates identified two different STs. The 54 isolates from NCBI SNP cluster PDS000031814 belong to ST14, while the 55 isolates from SNP cluster PDS000031739 belong to ST185. The ST14 group includes seven of the clinical isolates and 39 isolates from pistachios with a date of collection ranging from 2009 to 2016. The ST185 group contains three clinical isolates and 12 isolates from pistachios collected between 2013 and 2018.

The MLST results for *Salmonella* Montevideo isolates also detected two STs. SNP cluster PDS000027237 is a member of ST316, while SNP cluster PDS000032600 belongs to ST138. The ST316 group contains a total of 82 isolates, of which 24 were clinical isolates and 33 were isolates from pistachio sources ranging in collection date from 2009 to 2018. ST316 has previously been described as the “outbreak” clade due to its linkage with numerous outbreak-associated plant products including tahini, sprouts, spices, as well as pistachios (114). The ST138 group of 13 isolates contains 6 clinical isolates and 3 isolates from pistachios. The ST results can be found in Appendix 1 and clearly indicate that pistachios have been contaminated by multiple strains of *Salmonella* Senftenberg and Montevideo.

Comparative analysis of closed genomes

Long Read Sequencing technology was employed to generate 10 closed genomes for 2-3 representatives of each ST to use as a reference for SNP analysis, as well as to identify the positions of mobile elements and assess genetic differences between STs. The *Salmonella*

Senftenberg isolates ranged from 4.7 – 4.8 mb in size while the *Salmonella* Montevideo isolates were ~ 4.6 mb. One plasmid, over 300 kb in size, was found associated with *Salmonella* Senftenberg CFSAN000878. The genome statistics for these ten isolates can be found in Table 2-1.

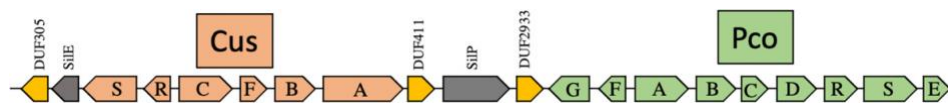
The content of the ten closed genomes were compared to each other to find and identify genomic elements that a clonal strain may acquire or dispose of over time. The closed genomes were evaluated for conserved genes shared within the same sequence type, as well as between both serovars (Senftenberg and Montevideo). As expected, the isolates within the same sequence type showed higher similarity. Interestingly, one unique region was identified to be shared between the ST14 and ST316 closed genomes but missing in the ST185 and ST138 genomes. This region was determined to be the Copper Homeostasis and Silver Resistance Island (CHASRI) (Figure 2-1) and is ~ 21 kb in size (64). Based on analysis using Ridom SeqSphere⁺, this island was confirmed in all the short-read sequence data of isolates associated with ST14 and ST316 and was not present in the ST185 or ST138 isolates. The island is located on the chromosome in both serovars and contains the genes *pco* operon, *cus* operon, *silE*, and *silP*. The sequence of the CHASRI had a 100% identity intra-serovar and a 99.95% identity inter-serovar when the region was analyzed with BLAST (115). The CHASRI has been identified previously in *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Salmonella enterica* (64). This island has previously been shown to increase the ability of a bacterium to survive in an environment with higher levels of copper and silver, as well as the ability to provide tolerance during the transition between aerobic and anaerobic environments, affording a fitness advantage for facultative

Table 2-1: Closed genome statistics from this study.

Sample ID	Serovar	MLST ST	Collection Year	Chromosome/ Plasmids Size (bp)	Sequencing Technology	Coverage	GenBank Accessions
CFSAN000258	Montevideo	ST316	2009	4,694,397	PacBio	500×	CP029035 ⁽¹⁾
CFSAN045764	Montevideo	ST316	2016	4,694,399	PacBio	375×	CP029039 ⁽¹⁾
CFSAN051296	Montevideo	ST316	2016	4,694,373	PacBio	204×	CP029336 ⁽¹⁾
CFSAN005645	Montevideo	ST138	2009	4,615,193	ONT	103×	CP040380 ⁽⁵⁸⁾
CFSAN010209	Montevideo	ST138	2009	4,619,529	ONT	73×	CP040379 ⁽⁵⁸⁾
CFSAN000878	Senftenberg	ST14	2009	4,794,849	PacBio	479×	CP029036 ⁽¹⁾
pCFSAN000878				319,930			CP029037 ⁽¹⁾
CFSAN045763	Senftenberg	ST14	2016	4,766,139	PacBio	275×	CP029038 ⁽¹⁾
CFSAN047866	Senftenberg	ST14	2016	4,807,052	PacBio	477×	CP029040 ⁽¹⁾
FSW0104	Senftenberg	ST185	2013	4,820,913	ONT	77×	CP037894 ⁽⁵⁸⁾
CFSAN087304	Senftenberg	ST185	2018	4,819,673	ONT	25×	CP037892 ⁽⁵⁸⁾

anaerobes (64, 72). The use of copper as a growth promoter in swine and poultry has led to the selection of this island in *Salmonella* associated with these sources in other countries (116, 117). The use of copper as a fungicide and bactericide in agriculture has been reported for many years, and it is generally considered safe for both conventional and organic produce use (118, 119). Copper deficiency is reportedly common in pistachios and the use of copper in foliar and soil treatments has been recommended to the industry (120). Copper-containing foliar sprays may be applied at various times while pistachios are developing in the trees (121). The presence of the CHASRI in the pistachio-associated isolates may be an acquired adaptation by these specific *Salmonellae* in response to copper stress in the growing environment.

Figure 2-1: Diagram of the CHASRI from the closed genomes in the study



The IncH12/IncH12A plasmid (CP029037) identified in *Salmonella* Senftenberg CFSAN000878, with a length of 319,930 bp, contains multiple heavy metal resistance genes which confer arsenic resistance (*arsB*, *arsC*, *arsH*), mercury resistance (*merA*, *merC*, *merD*, *merP*, *merR*, *merT*), and tellurium resistance (*terABCDEF*), as well as a copy of *silE*, *cusA*, and *cusS*. The plasmid also contains one antimicrobial resistance gene, MCR-9.1, which confers Colistin resistance and is of significant public health importance (122, 123). The plasmid sequences for the duplicated genes, *silE*, *cusA/czcA*, and *cusS*, differ from the same genes found in the chromosome with 91.2%, 93.7%, and 95.1% identity, respectively. This plasmid was only

identified in one other isolate (CFSAN016599) from pistachio, also collected in 2009. Since the plasmid has not been identified in any isolates after 2009, it is plausible that it was lost over time due to its large size and the genes on this plasmid not being necessary but rather redundant for increased fitness due to the incorporated CHASRI already in the chromosome.

Phages can encode factors that increase the virulence of *Salmonella* by enhancing adhesion, intracellular survival, and host entry (124). Phages were identified using PHASTER, and those sequences were then used as references in Ridom SeqSphere⁺ to interrogate the short-read assemblies. PHASTER identified a unique intact phage, Salmon_SPN3UB_NC019545, which was found in one of the closed genomes from ST14. Specifically, this phage was found in the closed genome of CFSAN047866 (CP029040) as well as in the short-read data from 7 of 54 isolates by analysis with Ridom SeqSphere⁺. Interestingly, these isolates were not associated with a single group on the SNP tree. Phages identified in the other sequence types were found in 98-100% of all isolates, and the phages identified can be found in Appendix 2.

Phylogenetic analyses using wgMLST and SNPs

Whole genome multi-locus sequence typing (wgMLST)-based methods have been previously employed for analysis of *Salmonella enterica* outbreaks and clusters (106, 125) and are less computationally expensive in comparison with SNP analysis. An advantage of using an *ad hoc* wgMLST approach is the ability to analyze isolates without the need for a reference sequence. In this study, two *ad hoc* wgMLST schemes were created for analysis of the *Salmonella* Senftenberg and *Salmonella* Montevideo isolates. In addition to the wgMLST analysis, the CFSAN SNP pipeline (107) was utilized to perform a reference-based variant detection analysis

which is compatible for isolates that are closely related and allows for higher resolution of these isolates. This application should only be used on isolates that belong to the same NCBI SNP cluster (126), therefore the pipeline was utilized here to produce four individual SNP matrices for the different STs.

Salmonella Senftenberg

A minimum spanning tree produced by analysis of all common allele calls from both the core genome and accessory genome shows that *Salmonella* Senftenberg isolates in ST14 are distinct from isolates in ST185 by a difference of 3,534 alleles (Figure 2-2). This allele difference between ST14 and ST185 is meaningful and illustrates that the primary contamination sources for these isolates are not the same. The analysis separated the isolates belonging to ST14 into three clusters (Clusters 1, 2, and 3) and the ST185 isolates into one cluster (Cluster 4). Cluster 1 contains the majority of the ST14 isolates, including those isolates associated with the 2016 outbreak. The pistachio isolates from ST14 have more branching and allele difference (range of 0-11) over an eight-year period (2009-2017). All the ST185 isolates associated with pistachios including the 2013 outbreak isolates are located in Cluster 4 and have low allele differences (0-7) over a five-year period (2013-2018), illustrating genome conservation and clonality.

The reference-based SNP analysis of the 55 *Salmonella* Senftenberg isolates that are typed as ST14, using CFSAN000878 as the reference, identified 146 variant positions and a maximum likelihood tree was constructed using RAxML (Figure 2-3A). Anonymized facility identifiers were used for those isolates where the source facility was known. The pistachio isolates from 2009 – 2017 share a common ancestor, and do not form distinct lineages based on the year of

isolation. The isolates, both clinical and those associated with pistachios from the 2016 outbreak investigation, have a median of 4 SNPs and a range of 0 – 9 SNPs. The outbreak investigation associated isolates appear throughout the tree, suggesting that multiple subpopulations of *Salmonella* strains are potentially contaminating pistachios. Akin to this observation, five other clinical isolates, that are not associated with the 2016 outbreak, have relatively close relationships to pistachio isolates. For instance, PNUSA008582 is 1 SNP away from both CFSAN048429 and CFSAN048427, while the other four clinicals have a minimum SNP distance of 5 – 13 SNPs from the nearest 2016 pistachio outbreak strain. The main clade has a total diversity range of 0 – 31 SNPs with a median of 7 SNPs. Previous research has shown SNP differences in *Salmonella* outbreaks to be <21 SNPs (94, 99, 127, 128, 129) with an outlier in serovar Agona with 30 SNP differences (130). From our study, a 2015 fertilizer (bone meal) isolate, OSF067866, was noted to be 9 SNPs distant from a 2009 pistachio isolate, CFSAN015119. While a definitive linkage of these two isolates is unattainable, it is notable that the use of pistachio and almond hulls as a soil amendment has been explored (131, 132, 133, 134, 135). Additional examination of soil amendments and fertilizer blends containing tree nut hulls may shed additional light on potential reservoirs and persistence of *Salmonella* in orchard soil. The results from both wgMLST and SNP analysis were comparable and are indicative of *Salmonella* residence in the pistachio primary production environment (i.e., orchard) for isolates belonging to ST14. Further evidence supporting this hypothesis of *Salmonella* residence in the orchard environment is that there are isolates collected from five additional facilities (including pistachio and environmental sources), located in California, and not linked to the 2016 outbreak, over multiple years are found throughout the SNP tree. Finally, the finding that all the ST14 isolates contain the CHASRI island – coinciding with the known use of copper in pistachio

orchards which may be providing selective pressure for this adaptation, as well as previous studies showing poultry isolates containing this island – adds support that these strains have established residence in or around the production environment. Although, the initial source of the environmental contamination (e.g., adjacent animal operations, use of biological soil amendments of animal origin (BSAAO), water, or wildlife) of this strain cannot be determined, our analysis shows that it was able to establish residence.

For the second Senftenberg SNP tree, closed genome FSW0104 was used as the reference genome to analyze the 55 isolates identified by MLST to belong to ST185 (Figure 2-3B). The SNP analysis identified 109 variant positions within these isolates. The isolate FSE0085, tahini from Mexico, was the closest related isolate (75 – 79 SNPs) to the rest of the isolates (n=54) which grouped together to form a single monophyletic clade. The 51 pistachio and environmental swab isolates in this clade were collected from two different facilities, Facility C and Facility D, both located in California. The 2013 outbreak isolates (clinical, pistachio, and environmental swabs) have a median 1 SNP distance and a range of 0 - 3 SNPs from each other. This outbreak investigation also includes two isolates of roasted pistachios (FSW0103 and FSW0104) from Facility C that were shipped to another facility (Facility C*). This outbreak investigation resulted in identification of deficiencies in Good Manufacturing Practices (GMPs) at Facility C, which may have contributed to the outbreak (80). This strain has not only been associated with the 2013 outbreak but repeated isolation from pistachios has resulted in additional product recalls. For example, the pistachio isolates collected in the 2018 recall form a group that is a median of 4 SNPs distant (range 3 – 8 SNPs) from those collected in 2013, 2014, and 2015. Both phylogenetic analyses indicate genome conservation and clonality with very little

variability between the isolates from these two facilities (C and D). The SNP analysis yields a median of 1 SNPs distance and a range of 0 – 8 SNPs over a five-year period and the wgMLST results illustrates a distinct central node within the ST185 isolates with low allele differences (0-7). The minimal genetic differences of these isolates is indicative of a persistent strain that is not under stress and experiencing slower than normal evolutionary events (136). The 2013 outbreak investigation illustrated how the same strain can move between facilities (C and C*). Therefore, some theoretical explanations for the presence of *Salmonella* isolates from Facility C and D with 0 SNP and allele distances between them include: a) *Salmonella* establishing residence in the facilities after both receiving pistachios contaminated by a possible shared grower, harvester, or transporter in or before 2013 or b) a business relationship that allows for pistachios or equipment to be shared or exchanged between the two facilities. It seems unlikely that two strains with no SNP/allele differences would occur in disparate facilities without some type of indirect or direct relationship between them. Rather, the small number of genetic differences over an extended period would suggest the transmission of the same strain between the two facilities and therefore a clonal strain taking harborage (96, 137). Furthermore, the lack of any measurable SNP distance between the *Salmonella* isolates from these two facilities suggests that such residence would be in a setting with relatively low environmental stress, such as an environment devoid of implementation of more robust sanitation controls.

Figure 2-2: Minimum spanning tree of the wgMLST allelic profiles for *Salmonella* Senftenberg isolates (n=106) using Ridom SeqSphere+

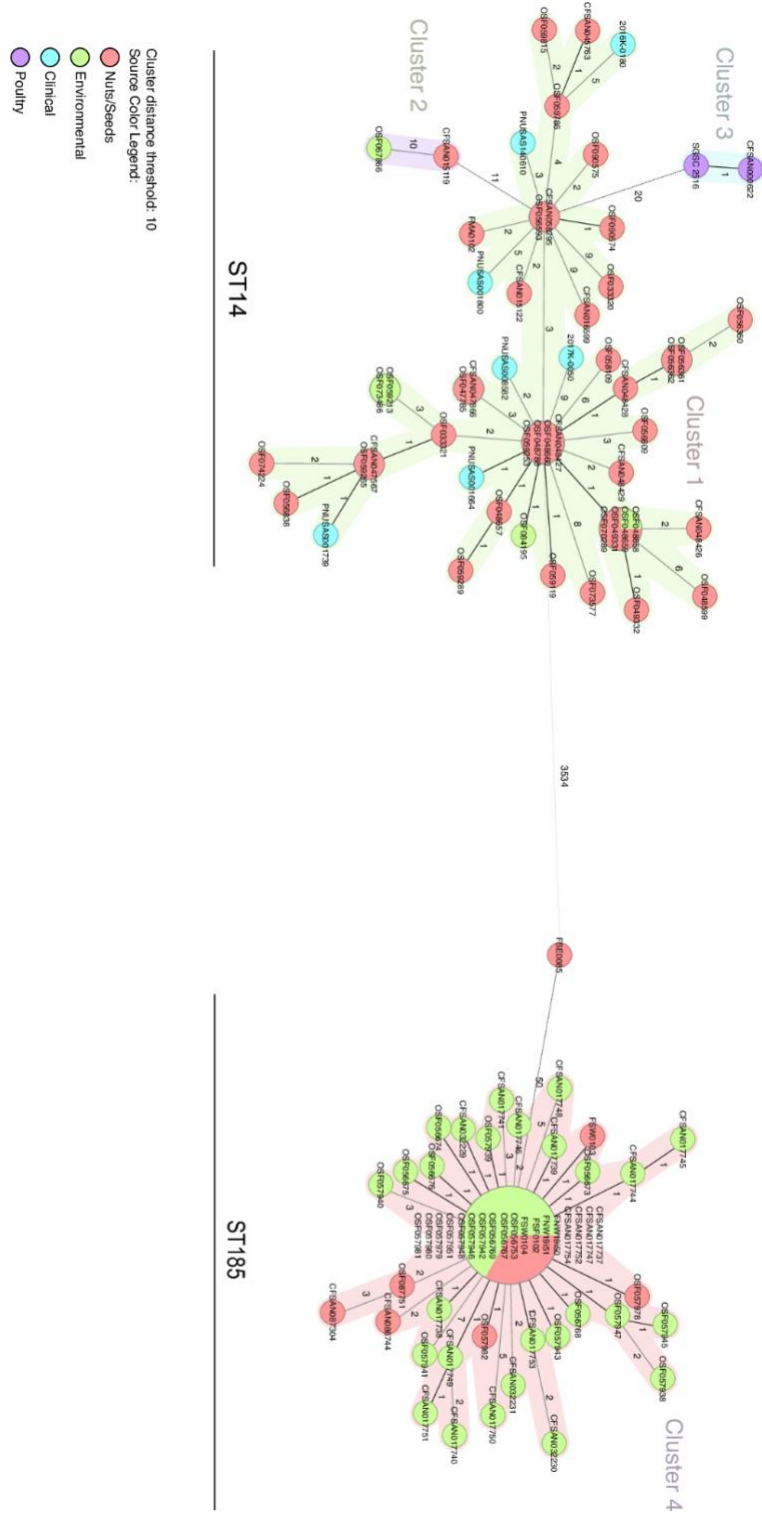
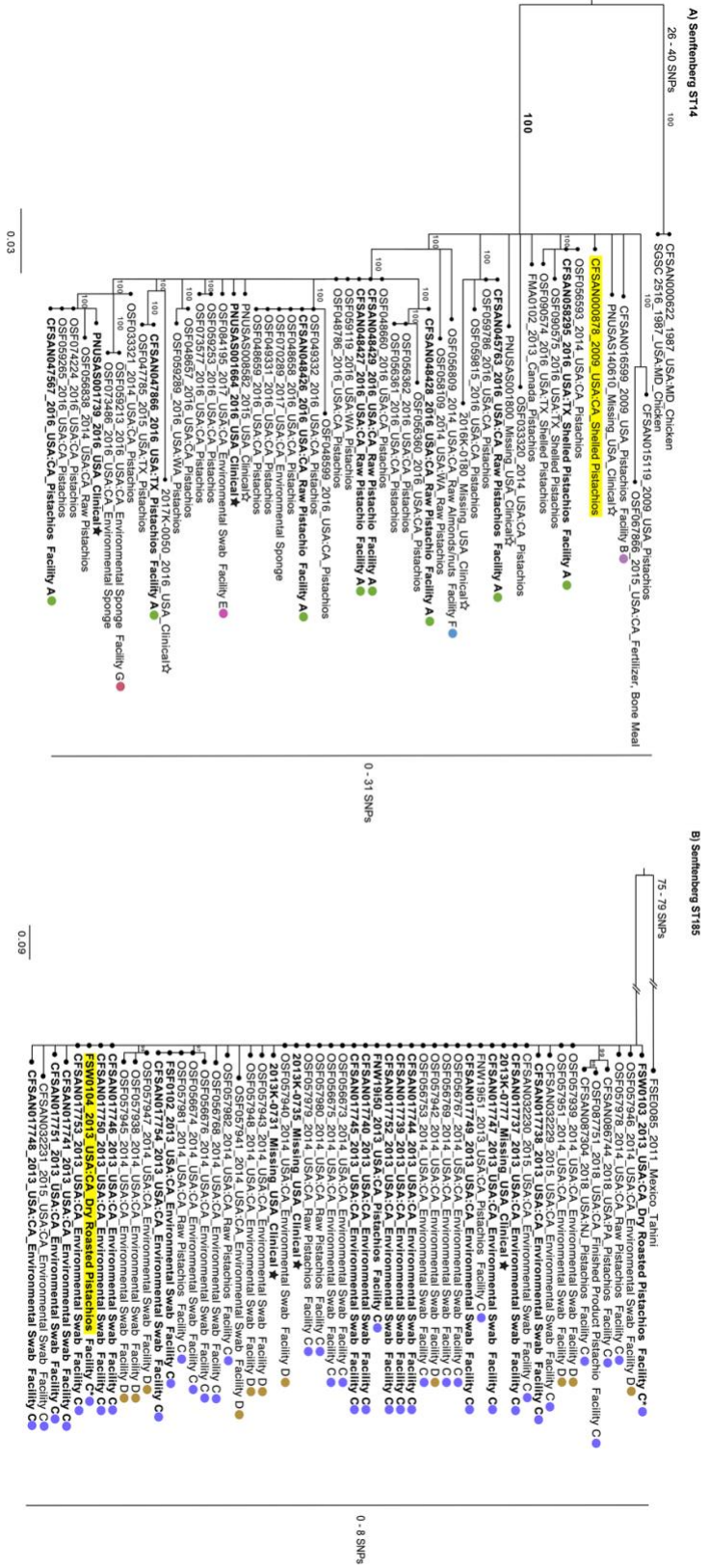


Figure 2-3: Phylogenetic analysis based on SNPs found in the *Salmonella* Senftenberg strains of this study (A) ST14, (B)ST185



Salmonella Montevideo

A minimum spanning tree produced by distance calculation for all common allele calls from both the core genome and accessory genome shows a distinct difference of 2,428 alleles between the *Salmonella* Montevideo isolates from ST316 and ST138 (Figure 2-4). Similar to Senftenberg isolates, these isolates also possess a sizeable allele disparity between the sequence types, which would be indicative of different sources of the contamination. Four well-defined clusters were identified, two for each ST (ST316 – Cluster 1 and 2, ST138 – Cluster 3 and 4). A majority of the isolates from pistachios are in a single cluster for each ST (Cluster 1 and Cluster 3), whereas those isolates associated with the 2016 outbreak are in Cluster 1. A single isolate from trail mix (CFSAN015114 – 19 alleles) collected in 2008, as well as two clinical isolates (PNUSAS034916 – 47 alleles and PNUSAS051029 – 14 alleles) were found to fall outside of Cluster 1.

Interestingly, five clinical isolates from the United Kingdom were found in proximity to the outbreak Cluster 1 of pistachio isolates (0 – 4 alleles). One of these isolates was collected prior to the outbreak in 2014, and four were collected after the outbreak (2017-2019). There are two clinicals, PNUSAS041766 and PNUSAS045784, that are a match to each other and are one allele from the large node of outbreak-associated isolates. The ST138 pistachio isolates are found in Cluster 3. Three clinical isolates with 3-4 allele differences from an ST138 pistachio isolate are in Cluster 3 with one of these isolates, 12-1128, located in Canada.

Reference-based SNP analysis identified a total of 107 distinct SNP variants in the isolates of ST316 using CFSAN000258 as the reference genome. A resultant phylogenetic tree, capturing the relationship among the 82 isolates belonging to ST316, yielded several important findings (Figure 2-5A). Isolates related to the 2016 outbreak are not associated with one specific lineage

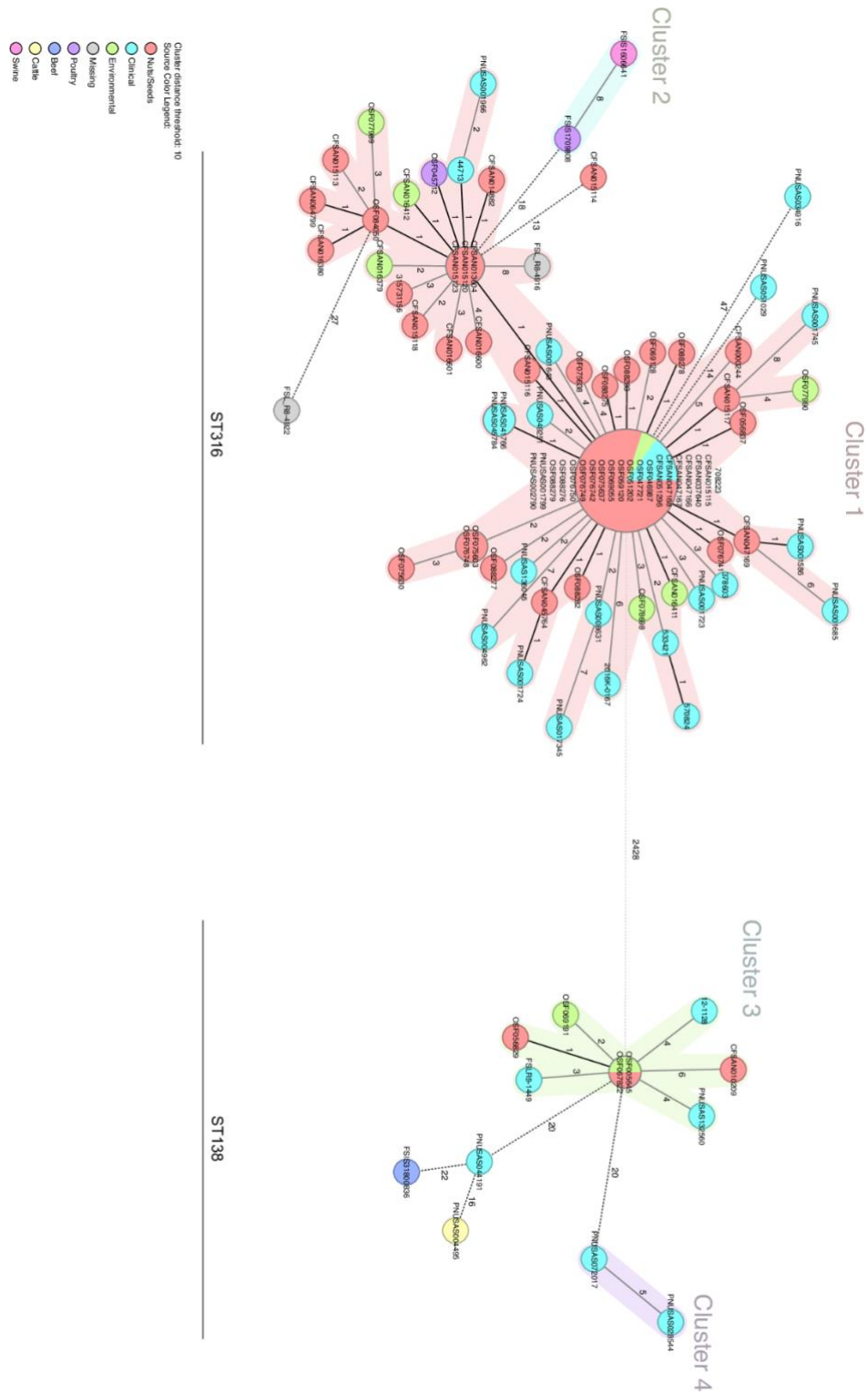
on the tree. Isolates from seven different facilities can be found throughout the tree. The paraphyletic grouping of the clade breaks down into smaller subclades, with a total SNP distance ranging from 0 – 16 SNPs with a median of 3 SNPs. The 2016 outbreak strains have a median distance of 2 SNPs (range 0 – 10 SNPs). As observed in the wgMLST analysis, other non-epidemiologically linked clinical isolates from the United States and the United Kingdom were found to be closely related to pistachio isolates, with a minimum SNP distance range of 0 –11 SNPs and 0 – 4 SNPs, respectively. With the continued rise in imported/exported goods, the observation of the large number of clinical isolates that are clustered near pistachio isolates from both the United States and the United Kingdom further highlights the benefit of global partnerships in the area of food safety and public health and the need for increased metadata inclusion and transparency (138).

The presence of a chicken isolate (OSF045712) from Facility H located within the California nut clade can possibly be explained by the use of a tree nut supplemented animal feed (139, 140, 141). This scenario is supported by domestic pistachio firms advertising the distribution of their hulls for animal feed to promote sustainability (142, 143). The chicken isolate is 2 – 5 SNPs away from other tree nut isolates in the clade. This potential connection between a contaminated component of animal feed and livestock that eventually enters the food supply for humans illustrates the interconnectedness of many food production systems and the importance of a “One Health Approach” (144) for food safety. Akin to the Senftenberg ST14 group, the CHASRI is identified in each of these isolates. The combined findings of CHASRI presence, the increased changes occurring over eight years, and that the isolates are derived from seven different

facilities are consistent with the hypothesis of a persistent strain in the pistachio production environment that is most likely co-residing with *Salmonella* Senftenberg.

A phylogenetic tree was constructed for the 13 Montevideo isolates from ST138 (Figure 2-5B) based on a total of 48 distinct SNP variants identified by reference-based SNP analysis using CFSAN010508 as the reference genome. The three clinical isolates are within 1 – 8 SNPs (median 3 SNPs) from the pistachio isolates from three different facilities (Facility A, Facility G, and Facility I), however, without epidemiological data we are unable to positively link these illnesses with contaminated pistachio exposure. Three isolates from Facility G, two environmental swabs and one pistachio, also belong to this clade. The SNP distance between the strains from Facility G (isolated in 2009 (OSF005645), 2015 (OSF069191), and 2017 (OSF067822)) is only 2 SNPs. The wgMLST shows this same relationship with 0 allele differences between OSF005645 and OSF067822 and 1 allele difference to OSF056829. This observation points to substantial conservation within the genome over a 5-year period. The pistachio and swab isolates from three unrelated facilities (Facility A, Facility G and Facility I), that were collected over a wide timespan (8 years) with low diversity, suggests a single contamination event with a persistent *Salmonella* strain. These strains are likely to reside in a lower-stress environment than in the orchard environment, due to the low SNP/allele distances suggest a strain that is not undergoing selective pressure and may also have a slower generation time similar to a resident pathogen (96). Possible low-stress environments could include shared farm equipment, transport vehicles, or an intermediate space such as silos.

Figure 2-4: Minimum spanning tree of the wgMLST allelic profiles for *Salmonella* Montevideo isolates (n=95) using Ridom SeqSphere+



Storage study to assess persistence in pistachios

To further explore the nature and likelihood of long-term environmental persistence of these *Salmonella*, we tested the survival of *Salmonella* strains in a cocktail after inoculation on raw inshell pistachios. The strains used in this study were associated with low-moisture food products (peanuts (Anatum), pecans (Oranienburg), pistachios (Montevideo and Senftenberg)) and a strain isolated from tomato (Newport) (Table 2-2). The Senftenberg and Montevideo strains used contain the CHASRI and are from ST14 and ST316, respectively. The basis behind using the Newport strain was to determine if there were differences in survivability on a low-moisture food based on the initial source of the isolate (i.e., a high-moisture food). Pistachios were inoculated with a five-serovar cocktail of *Salmonella enterica* and stored at one of two different relative humidity levels (35% RH and 54% RH) at 25°C.

Table 2-2: Strains used in storage study to assess persistence in pistachios.

Strain	Serovar	Source	MLST (ST)
CFSAN076215	Anatum	Peanuts	64
CFSAN051296	Montevideo	Pistachio	316
CFSAN000836	Newport	Tomato	118
CFSAN076211	Oranienburg	Pecans	3613
CFSAN045763	Senftenberg	Pistachio	14

Salmonella in the five-strain cocktail was able to persist in pistachios for up to one year (the length of the study) (Figure 2-6). There was a ~1 log CFU/g reduction in *Salmonella* during the period of desiccation (24 hour drying time) prior to storage. The *Salmonella* in the inoculated pistachios stored at 35% relative humidity (mean $a_w = 0.3398$, stdev = 0.0228) were able to persist for a year with only an additional ~1 log CFU/g reduction after desiccation (a total of ~2 log CFU/g reduction). The *Salmonella* in the pistachios stored at 54% relative humidity (mean a_w

= 0.5337, stdev = 0.0370) showed an additional ~3 log CFU/g reduction over a year. A repeated measures ANOVA analysis showed the impact of humidity condition (high vs low) over time. The rate of reduction was higher for the high humidity condition based on the observed difference in the slope (low and high humidity was -0.0026 and -0.0085 per day, respectively) between the two conditions ($p < .0001$) (storage data shown in Appendix 3). Differences in log CFU/g between low and high humidity conditions was most pronounced after 6 months (Fig. 2-6). These findings are consistent with those reported in literature (30, 110, 145). One main objective of the study was to determine if there was a difference in which serovars were surviving within the cocktail inoculum. A previous storage study by Kimber et al. (146) evaluated the surviving serovars from the cocktail at the end of the storage study, hence one goal of our study was to analyze the composition of surviving serovars throughout storage, which allows for discrete timepoints of possible serovar elimination. Two methods were used to determine the serovars present, the Luminex xMAP® *Salmonella* serotyping assay and metagenomic shotgun sequencing with data analysis using a custom kmers database. The Luminex assay identified each serovar except for one timepoint at 35% RH and two timepoints at 54% RH (Appendix 4). However, the results from the shotgun metagenomic analysis identified all five serotypes present in each of the triplicate samples at every time point for both storage conditions (Figure 2-7).

Sequencing was conducted post-enrichment, therefore the serovars identified from the raw reads are from viable bacteria. The limitation of the molecular serotyping assay is the number of colonies that are selected and tested. Unintentional bias could be introduced into the results based on colony selection by either over- or under-selection, whereas there is a higher

probability of sequencing all strains that are present above a certain threshold with the sequencing method. The Kimber et al. 2012 study used the same Oranienburg strain as in our experiments, but the researchers did not identify this strain in the one hundred colonies they tested at the end of their study (14.5 months) (146). In contrast, we were able to still recover the Oranienburg strain both in sequencing and by colony picks at 12 months. Two possibilities for the absence of Oranienburg in the Kimber et al. 2012 study could be that Oranienburg was missed due to the bias that can occur when picking colonies for serovar determination or Oranienburg simply did not survive. This could be determined by using metagenomic sequencing (removes colony pick bias) or by picking colonies at different time points (identify survival cutoff). The use of the metagenomic/bioinformatic pathway allows for a robust determination of the cocktail composition while reducing the bias and time of traditional molecular serotyping. Another interesting finding was that the primary source of the *Salmonella* (low-moisture vs. high-moisture environment) may not necessarily determine the ability to survive in a different environment, as shown with the survivability of the Newport strain on the pistachios.

This storage study reinforces the capability for *Salmonella* to survive for extended times on this low-moisture commodity. The results from our study conclude that the five-strain cocktail of *Salmonella* can survive in pistachios and be detected by direct plating methods when stored at different humidity conditions for at least one year. The extended shelf-life of pistachios combined with our evidence of *Salmonella* survival for up to a year highlights the importance of surveillance of this pathogen.

Figure 2-6: Survival curve of inoculated pistachios with a 5-strain Salmonella cocktail stored at different relative humidity levels.

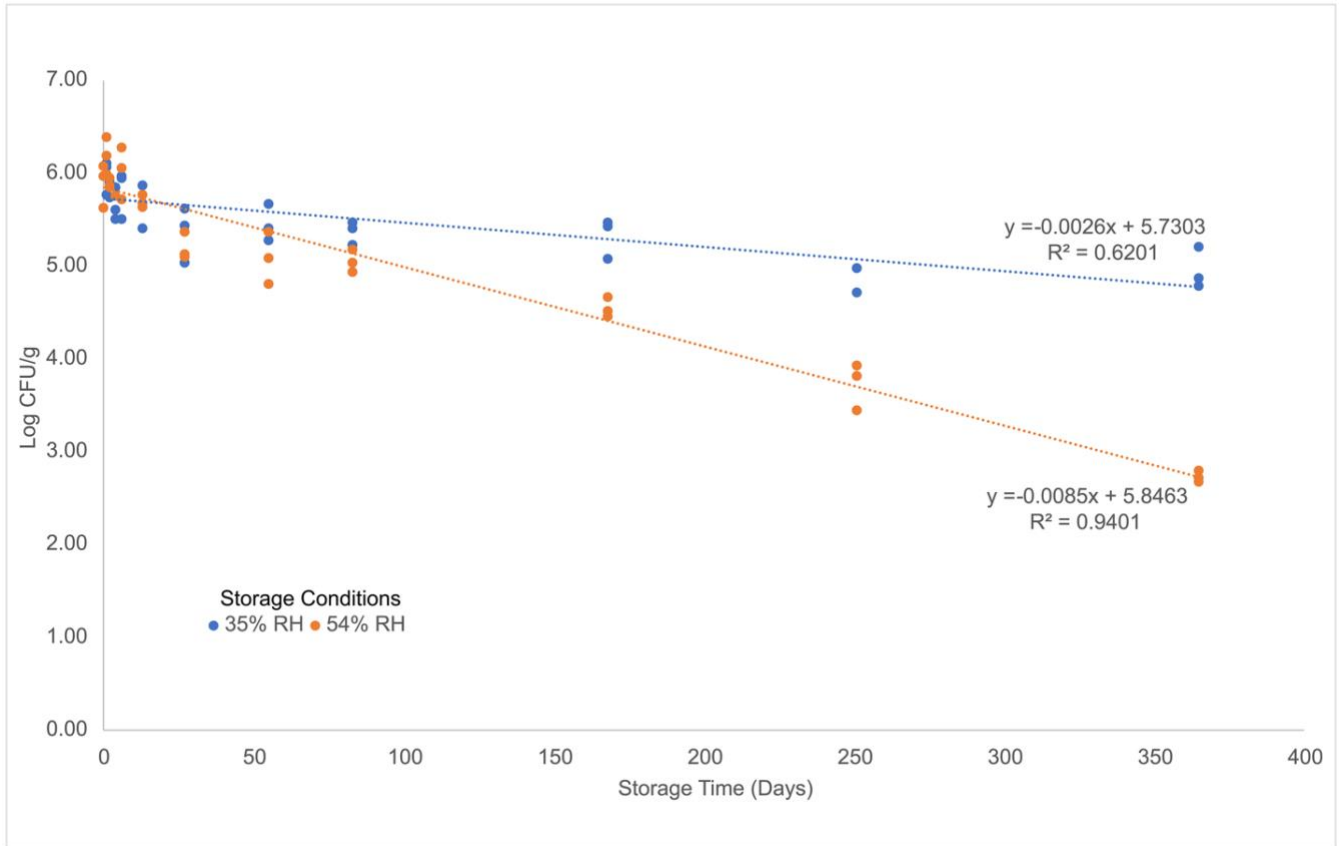
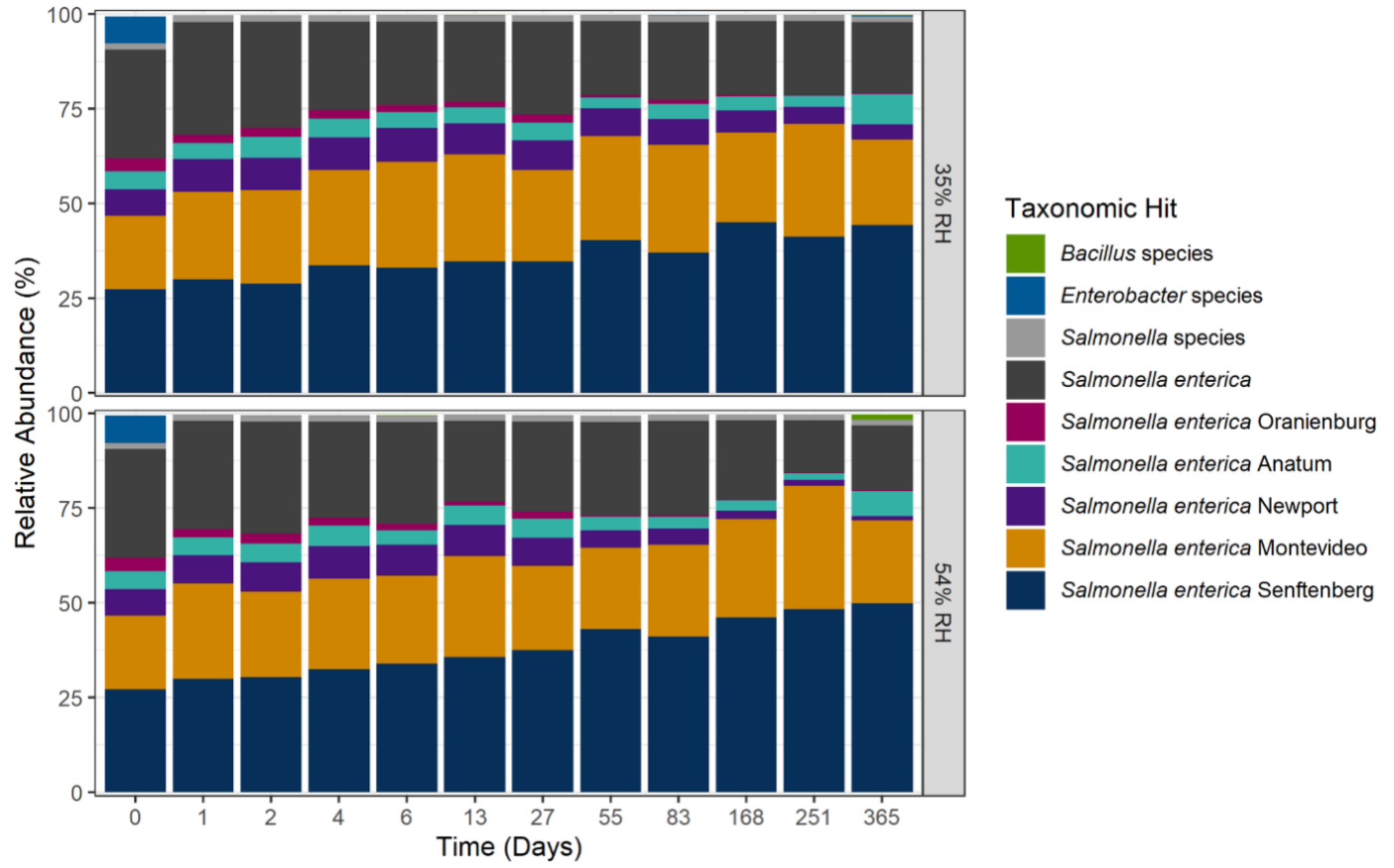


Figure 2-7: Results from Shotgun Metagenomic Sequencing



Conclusions

Pistachios may be contaminated with pathogenic bacteria in the growing, production, and processing environments and present a hazard due to the ability for *Salmonella* to persist in low-moisture environments and foods. During outbreak investigations, epidemiological, traceback, and laboratory information are used to link illnesses to food sources. Diagnostic capabilities have been greatly enhanced due to the development and increasing applicability of WGS, which is now fully deployed as a molecular epidemiological tool to assist in foodborne outbreak investigations (93, 94, 95). We were able to match clinical and food *Salmonella* Senftenberg and Montevideo isolates and define SNP differences between closely related strains by using WGS in two different phylogenetic analyses. Further, by implementing long read sequence technology, we identified the presence of the heavy metal tolerance island (CHASRI) in strains likely associated with preharvest contamination, possibly indicative of their adaptation to the presence of copper in this niche. Presence of the CHASRI in *Salmonella* Senftenberg (ST14) and *Salmonella* Montevideo (ST316) may explain the increased level of recovery of these strains from pistachios or pistachio environments due to selective pressure from copper usage, even though previous data has shown a greater diversity of serovars present (84). Finally, this analysis points to at least three possible pistachio-associated environments where closely related *Salmonella* Senftenberg and Montevideo strains could have been residing as recently as 2018 including 1) multiple environmental adapted strains in the pre-harvest or orchard environment (ST14 and ST316); 2) a clonal strain at the pistachio handling facilities which could include storage silos (ST185) and 3) a clonal strain in the intermediate post-harvest environment (e.g., shared farm equipment or transport equipment) (ST138) .

Our storage study demonstrates that *Salmonella* will survive in pistachios for a minimum of one year at both high and low relative humidity. The use of a non-nut associated strain, *Salmonella* Newport, in our cocktail and its ability to be recovered at the 12-month time point, showed that the original source may not necessarily have a significant impact of survival of *Salmonella* on a low-moisture food. Pistachios may be stored untreated for 12 months or longer by pistachio handlers and then consumers may store them up to an additional 12 months (147). The ability of *Salmonella* to survive long-term in a dry environment highlights the need for adequate preventive controls in the processing of pistachios. Harris et al. (10) report that majority of the pistachios sold in the U.S. market are roasted inshell. Identification and surveillance of the possible sources of contamination, (e.g., the growing environment, shared equipment, or processing environment) would likely augment implementation of specific production changes that could further reduce the risk of contaminated nut products.

Taken together, this study demonstrates the following: i.) evidence of persistent *Salmonella* Senftenberg and Montevideo strains in pistachio environments as recently as 2018, ii.) presence of the Copper Homeostasis and Silver Resistance Island (CHASRI) in *Salmonella* Senftenberg and Montevideo strains, suggesting an adaption in response to extrinsic pressures in the pistachio supply chain, and iii.) use of metagenomic sequencing in conjunction with storage studies using a cocktail of *Salmonella* strains can identify strains that are surviving over time. This study further emphasizes the power of WGS in conjunction with metadata, which helps to define outbreaks and resident strains and possible routes of contamination, as well as give insights into genetic factors that could lead to fitness in a given niche. Moreover, these findings underscore the importance of industry in adhering to food safety standards established for this commodity.

CHAPTER 3: GENOMIC AND FUNCTIONAL CHARACTERIZATION OF THE COPPER HOMEOSTASIS AND SILVER RESISTANCE ISLAND

Publication Status:

Manuscript ready for submission

Abstract

Copper is essential for all living organisms, but in excess can become toxic. Biological systems have evolved mechanisms to keep organisms in copper homeostasis. *Salmonella enterica* has core copper homeostasis genes integrated into the genome that are an important virulence factor to combat the host immune response. Studies have shown that *Salmonella* can acquire mobile genetic elements to provide enhanced tolerance to stressed environments. One such cassette, the Copper Homeostasis and Silver Resistance Island (CHASRI), has become more prevalent in certain serovars that have enhanced copper exposure. In this study, whole genome sequence data was used to determine how prevalent the CHASRI is in *Salmonella* from foodborne and environmental sources. The results show the CHASRI is in more serovars than previously thought and can be isolated from many different food sources. SGI-4 was previously described as the mobile element in which the CHASRI was transferred to *Salmonella*, however the results from a comparative study using sequences from closed genomes find that the CHASRI can also be found alone, or as a component of an SGI-4 variant. Knockouts were developed to analyze the kinetics of copper tolerance in a strain that was previously identified as a pistachio orchard strain. The results from the kinetic assays verified that the CHASRI and its components enhance tolerance to increased levels of copper.

Introduction

Copper is an essential micronutrient for bacteria, although excessive copper is cytotoxic, and must be controlled by different cellular copper pathways (58). Bacterial pathways to control copper toxicity are effective in aerobic environments and copper toxicity has increased prevalence in anaerobic environments (58). The majority of *Salmonella enterica* strains contain the Cue and Gol regulon to assist with copper homeostasis (58, 62).

The use of copper in agriculture and manufacturing has exposed bacteria to copper stress and has led to the evolution of cellular systems to survive in a copper rich environment. The Copper Homeostasis and Silver Resistance Island (CHASRI) is a mobile element that can be found both on plasmids and chromosomes in Enterobacteriaceae and contains two operons: *pco* and *cus* (64). The combination of these two operons provide protection in both aerobic and anaerobic conditions and during the transition of these two states which makes it highly suitable for facultative anaerobes. Previous studies have looked at the presence of *Salmonella* Genomic Island-4 (SGI-4) in *Salmonella* 4,[5],12:i:- strains in Europe (72, 74). SGI-4 contains both the CHASRI, an arsenic tolerance operon, and the necessary genes for conjugative transfer and replication, which defines a mobile genetic element. The presence of the copper and zinc in livestock feed may have driven the selection pressure for the *Salmonella* strains (72). The CHASRI is also associated with certain STs of *Salmonella* Montevideo and Senftenberg, which have been linked to previous outbreaks (114, 148). However, the overall prevalence of this mobile element in *Salmonella* and its role in copper tolerance is not fully defined.

NCBI has expanded the AMRfinder plus tool to include a variety of heavy metal genes including those found in the CHASRI (149). There are over 500,000 *Salmonella enterica* sequences deposited to NCBI and this data is a valuable source to investigate the prevalence of the CHASRI.

The CHASRI has been previously documented in both certain serovars of *Salmonella enterica* and in isolates from certain sources. We have found this genomic island in previous studies and believe that the CHASRI can be identified in isolates associated with agriculture or anthropogenic contamination of copper in the environment other than the previously identified niches in swine and poultry environments (74, 148, 150). The purpose of this study was to find the prevalence of the CHASRI in *Salmonella* from food and environmental sources and to characterize the different variations of the CHASRI from published *Salmonella enterica* closed genomes. Additionally, this study generated scarless knockouts to assess any differences between the wild type and the knockouts regarding the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), growth kinetics, and inactivation kinetics in the presence of copper sulfate.

Materials and Methods

Analysis of short read sequence data of isolates containing the CHASRI

The NCBI Pathogen Detection browser was used to find all *Salmonella* isolates that contain the *silA* gene (component of the CHASRI) and subsequently the raw sequence data of 6,195 isolates was downloaded from the Sequence Read Archives using fastq-dump. The metadata for these

isolates was downloaded, and any isolates that were lacking geographical, isolation source, or date of collection were removed from analysis. The raw reads were *de novo* assembled using SPAdes v3.13.0 (100) and assemblies were quality checked with Quast v5.0 (151). Those assemblies with >300 contigs or genome size >6 Mb were removed from further analysis. The serovar of the isolates was determined *in silico* using Seqsero2 v1.2.1 (152). Those isolates where a serovar was unable to be determined were removed from analysis. The remaining assemblies were annotated with Prokka using CFSAN051296 (NZ_CP029336.1) as reference for gene naming convention (153). For this analysis, a quality check requirement was to have the entire CHASRI present on a single contig. The multifasta function of Roary was utilized to pull the amino acid sequences for the individual genes in the CHASRI (154). These sequences were aligned to determine similarity. The geographical location was amended to the country level of identification. The isolation source was categorized in 22 categories (Table 3-1).

Table 3-1: Modified isolation sources used in this study.

Modified Source	Examples of Isolation Sources
Animal Feed	Raw Hide Bone, Pig Ears, Meat and Bone Meal
Beef	Ground Beef, Comminuted Beef, Raw-Intact-Beef
Companion Animal	Cat, Dog
Composite Commodity	RTE Corn Dog, Salted Nut Crackers
Dairy	Cheese, Milk
Eggs	Egg Yolks, Egg Whites, Whole Eggs
Environmental Sample	Sponges, Swabs
Equine	Equine Feces, Equine, Equine Transtracheal Wash
Fruits	Papayas, Avocados
Fertilizer	Finished Fertilizer, Raw Fertilizer
Goat/Sheep	Goat, Lamb, Caprine feces
Grains/Beans	Rice, Soy
Leafy Greens	Romain Lettuce, Spinach
Nuts/Seeds	Pistachios, Almonds, Peanut Butter
Pork	Pork Chop, Ground Pork, Raw-Intact-Pork, Pork Meat
Poultry Products	Ground Turkey, Chicken Breast, Finished Ground Chicken
Seafood	Crab Cakes, Tilapia, Tuna Steak, Shrimp
Spices	Celery Seed, Red Pepper Flakes
Sprouts	Sprouts, Alfalfa Sprouts
Vine Stalk Produce	Cucumber, Tomatoes, Peppers
Water/Soil	Creek Water, Irrigation Water, Soil, Sewage
Wild Game	Quail, Rabbit, Moose

Bioinformatic analysis of Closed Genomes containing CHASRI

The use of closed genomes was necessary to determine the location of the CHASRI (chromosome vs. plasmid) and to determine the presence of additional tolerance or antimicrobial genes. Closed genomes were identified on NCBI and the genbank files were downloaded. Sequences from *Citrobacter freundii* (NZ_LS992183), *Escherichia coli* (KU248945), *Citrobacter portucalensis* (NZ_CP046347), and the CHASRI from *Salmonella enterica* (NZ_CP061046) were downloaded and used for analysis. The nucleotide sequence of the region spanning the CHASRI were aligned using MUSCLE (155) and a minimum spanning tree was produced using RAxML (108). The genomes were queried for the presence of SGI-4 by using a

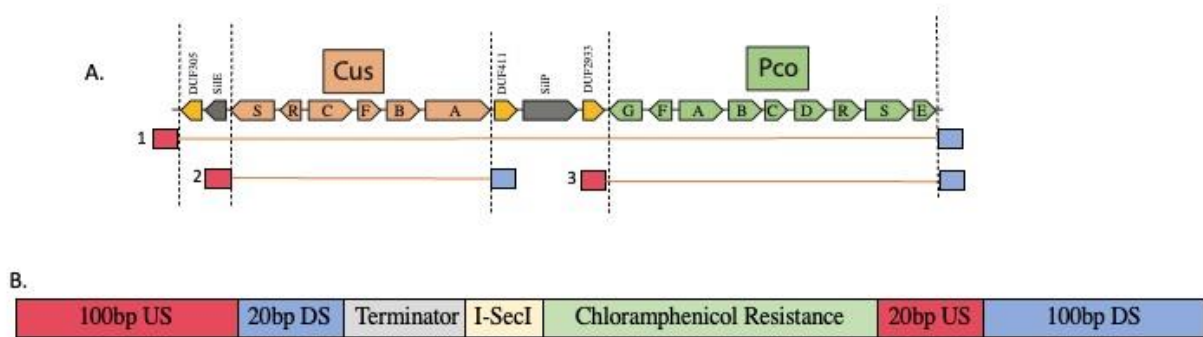
blast query against the SGI-4 reference (MN730129.1) and the variant (OK209934.1) with 85% coverage and 70% homology cutoffs. PlasmidFinder was used to identify plasmid replicon sequences in those isolates where the CHASRI was located on a plasmid.

Creation of Knockouts using Red Recombinase Scarless Deletion

The *Salmonella enterica* serovar Senftenberg isolate, CFSAN045763 (NZ_CP029038.1), isolated from pistachios was previously identified to contain the CHASRI inserted into the chromosome (148). The CHASRI in this isolate is 21,782 base pairs in length and does not occur within SGI-4. A scarless red-recombinase deletion method that has been previously described was performed with minor modifications (156).

Three gBlocks were designed to individually delete the *Cus* operon, the *Pco* operon, and the entire CHASRI cassette using Geneious Prime. Each gBlock consisted of sequences upstream and downstream of the desired deletion site, a terminator sequence, the gene for chloramphenicol (CM) resistance, and the cutsite for *SceI* (Figure 3-1). These gBlocks were ordered from IDT (Coralville, Iowa) and the lyophilized products were reconstituted to a concentration of 20 ng/ μ l with molecular grade water. The sequences for the gBlocks can be found in Appendix 5.

Figure 3-1: (A) The location of the upstream (US) and downstream (DS) sequences used for the gBlock design as seen in (B).



One change to the published method was an additional overnight incubation at 30 °C on Luria-Bertani (LB) agar supplemented with 100 mg/ml ampicillin (AMP) and 20 mg/ml chloramphenicol (CM) (FisherScientific, Pittsburgh, PA) to confirm integration of the gBlock and to help aid in recovery. After confirmation of the scarless recombination to remove chloramphenicol resistance, the protocol was modified to increase the efficiency of plasmid removal. Although the pSLTS plasmid is heat sensitive, for efficient removal of the plasmid from our *Salmonella* knockout strains, the cells need to be incubated in a nutrient deficient media to facilitate expulsion of the plasmid. The cells were incubated overnight at 42 °C in a 1:4 dilution of LB broth:Water. The overnights were streaked to isolation on LB. Isolates were then patched on LB and LB+AMP to identify those colonies that had ejected the plasmid. These isolates were then used to create freezer stocks of our knockout strains (Table 3-2).

Table 3-2: Strains used in this study.

Strain	CHASRI genes present	Source
CFSAN045763	<i>pcoESRDCBAFG</i> <i>silP, silE</i> <i>cusABFCRS</i>	This work
CFSAN045763 Δ <i>cus</i>	<i>pcoESRDCBAFG</i> <i>silP, silE</i>	This work
CFSAN045763 Δ <i>pco</i>	<i>silP, silE</i> <i>cusABFCRS</i>	This work
CFSAN045763 Δ CHASRI	None	This work

The knockout and wild type strains were sequenced on the PacBio Sequel IIe to determine effective knockout of the individual operons and the entire cassette. The libraries were prepared using the SMRTbell Template Prep Kit 3.0 (Pacific Biosystems, Menlo Park, CA) according to published protocols (157). The final library was sequenced on the PacBio Sequel IIe with 30 hours collection time. Raw data was demultiplexed and *de novo* assembly was performed using the package Microbial Assembly in SMRTLink v11 (Pacific Biosystems, Menlo Park, CA). The resulting assemblies were checked for full closure and oriented to the *oriC* gene.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The strains (CFSAN045763, CFSAN045763 Δ *cus*, CFSAN045763 Δ *pco*, CFSAN045763 Δ CHASRI) were grown overnight in TSA at 37 °C. A stock of 100 mg/ml CuSO₄ (Sigma-Aldrich, St. Louis, MO) was made in molecular grade water. The solution was then filter-sterilized and different concentrations of CuSO₄ were mixed with Mueller Hinton Cation II broth (MHB) (FisherScientific, Pittsburgh, PA). A single colony from each isolate was resuspended to get a 0.1 OD₆₀₀. The samples were further diluted 1:100 in MHB. The range of copper sulfate concentrations was 1.2 mg/ml to 2.1 mg/ml in 0.1 mg/ml increments. The

different concentrations of copper sulfate and the diluted samples were added to a microtiter plate and incubated overnight at 37 °C. After overnight incubation, presence or absence of turbidity was recorded for the wells. The MIC was determined by the concentration of the first concentration that did not show growth. To determine MBC, 5 µl was spot inoculated on Mueller Hinton agar (MHA) (FisherScientific, Pittsburgh, PA) and incubated overnight. The MBC was the concentration that did not have growth on the agar plate. Samples were tested in biological triplicates and technical duplicates.

Growth Kinetics

The strains (CFSAN045763, CFSAN045763 Δ *cus*, CFSAN045763 Δ *pco*, CFSAN045763 Δ *CHASRI*) were grown overnight in TSB at 37 °C. A stock of 100 mg/ml CuSO₄ was made in molecular grade water. The CuSO₄ stock solution was then filter-sterilized and diluted to 1.0 mg/ml, 2.0 mg/ml, and 3.0 mg/ml in MHB. The overnight cultures were diluted to 0.1 OD₆₀₀ in Peptone Buffered Solution (PBS) and then further diluted 1:25 in MHB. The diluted cultures were mixed with the MHB+CuSO₄ in a 1:1 ratio in the Bioscreen Honeycomb 2 plate. The final concentrations of CuSO₄ tested for growth were 0 mg/ml, 0.5 mg/ml, 1 mg/ml, and 1.5 mg/ml. All samples were tested in biological triplicate and with technical triplicates on the Bioscreen C° Pro instrument (Oy Growth Curves, Finland). The instrument was programmed to incubate at 37 °C and take measurements every 10 minutes for 48 hours. Specific growth rates and maximum growth capacity was determined by analysis using an online program by S. Castillo and D. Barth (<https://scsandravtt.shinyapps.io/SGRapp/>), based on the Grofit package for R (158). The smoothing factor of 0.7 was used to analyze all data (159). Analysis of variance was used to compare the specific growth rates and maximum OD values for the different strains

growing in the same levels of copper sulfate. Secondary statistical analysis was performed using unpaired t-test on the averages of the three replicates with $\alpha = 0.05$ in Prism9 GraphPad.

Copper Inactivation Kinetics

The strains (CFSAN045763, CFSAN045763 Δ *cus*, CFSAN045763 Δ *pco*, CFSAN045763 Δ *CHASRI*) were grown overnight in TSB at 37 °C. A stock of 100 mg/ml CuSO₄ was made in molecular grade water and filter sterilized. The copper sulfate was diluted to a concentration of 4 mg/ml, 6 mg/ml, and 8 mg/ml in MHB. The 5 ml overnight cultures were centrifuge at 5,000 RPM for 5 minutes and then resuspended in fresh MHB. Equal parts of cells and MHB+CuSO₄ were added and incubated at 37 °C. The final testing concentration of CuSO₄ were 2 mg/ml, 3 mg/ml, and 4 mg/ml. The samples were plated on TSA at predetermined timepoints (0, 2, 4, 6, and 24 hours) to determine log CFU/ml reduction. Data for biological triplicate samples was collected. An unpaired t-test was performed on the data to test for significant log reduction at different time points.

Results and Discussion

Distribution of the CHASRI in *Salmonella enterica*

The NCBI Pathogen Detection browser was used to identify those *Salmonella enterica* isolates which contain the *silA* gene. This query returned over 28,000 results. After removal of clinical isolates, those with missing metadata, and those from livestock, a total of 6,195 isolates remained. The short reads for these isolates were downloaded, *de novo* assembled, and the *Salmonella* serotype was determined. The isolates that failed to serotype were removed from further analysis. The serotyped assemblies were annotated and those isolates that had a complete

CHASRI on a single contig were used for data analysis. A total of 4,954 *Salmonella* isolates passed the quality control criteria of the study.

The date of collection ranged from 1972 – 2020 and the isolates were collected in 49 different countries. A total of 61 different serovars were identified with the predominant ones being Senftenberg, Kentucky and I 4,[5],12:i:- . It is noted that Senftenberg sequence type 14 (ST14), the predominate ST for this serovar (160), contains the CHASRI similar to Montevideo ST316 (161). Similarly, ST34 (I 4,[5],12:i:-) has been previously described to contain SGI-4, which includes both the CHASRI as well as other heavy metal genes (74, 150, 162). The metadata for environmental swabs and sponges, (18% of the total isolates) is ambiguous and since the swabbing location was unknown it was difficult to distinguish the primary source of the bacteria. Therefore, these isolates were removed from further analysis.

The top two sources of the isolates are animal products (58%) and low moisture foods (12%) (Figure 3-2). The majority of the animal-derived product isolates were from poultry (38%) and pork (15%). The increased recovery of isolates from these sources that contain the CHASRI further illustrates the connection between copper supplementation and the selective pressure for bacteria to acquire heavy metal tolerance genes (150). Figure 3-3 illustrates the relationship between the serovars containing the CHASRI and their source. It is of note that Senftenberg isolates containing the CHASRI are widely distributed among the three different source groupings (Figure 3-3). There are several serovars that are predominately associated with animal-derived product sources. I 4,5[12]:i:- was the main serovar associated with the pork-derived products (40.2%). Serovars Kentucky, Schwarzengrund, and Heidelberg are considered

an MPPST (most prevalent poultry-associated *Salmonella* serotypes) (163) and were associated with 28.7%, 21.1%, and 12.6% of the poultry-derived products containing a CHASRI respectively. The serovars Senftenberg, Tennessee, Montevideo, and Mbandaka were large contributors to *Salmonella* containing the CHASRI and were associated with spices and nuts/seeds, all of which have been previously associated with outbreaks in these low moisture commodities (95, 137, 148, 164). The serovars, Kentucky, Schwarzengrund, and I 4,5[12]:I:-, which were associated with animal-derived products were also associated with animal feed isolates which may be of importance too.

Figure 3-2: Breakdown of Source Attribution of the 4,954 isolates

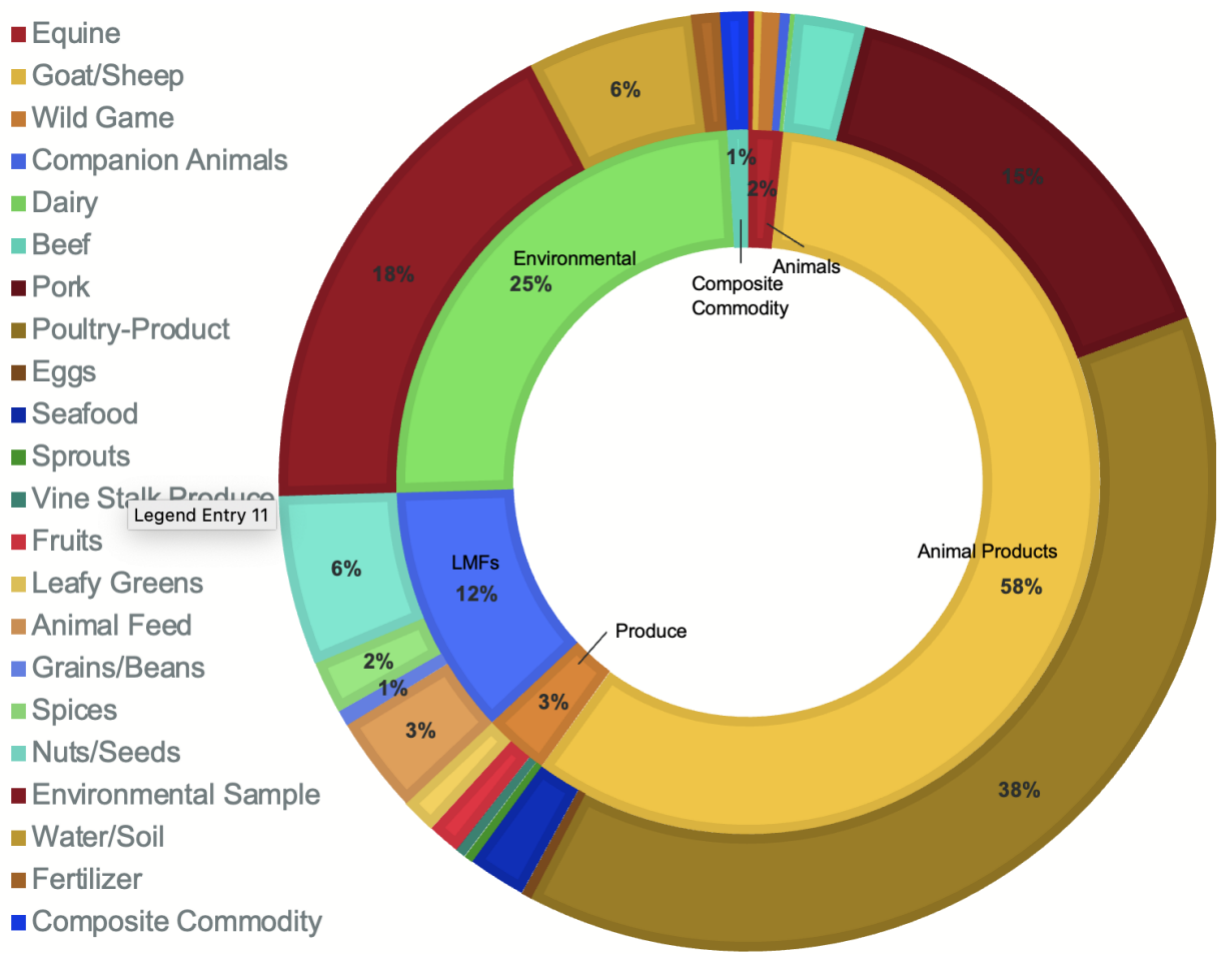
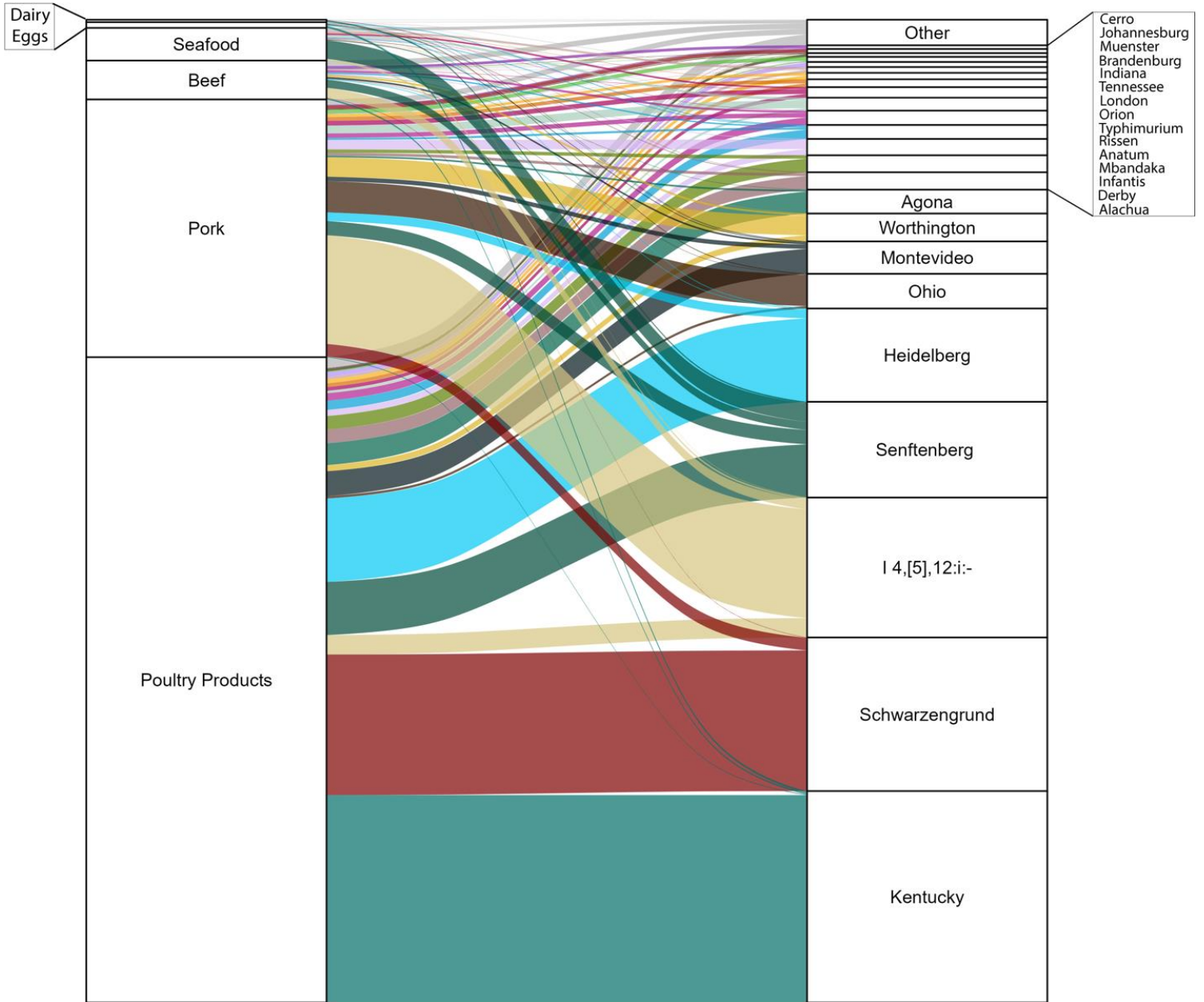
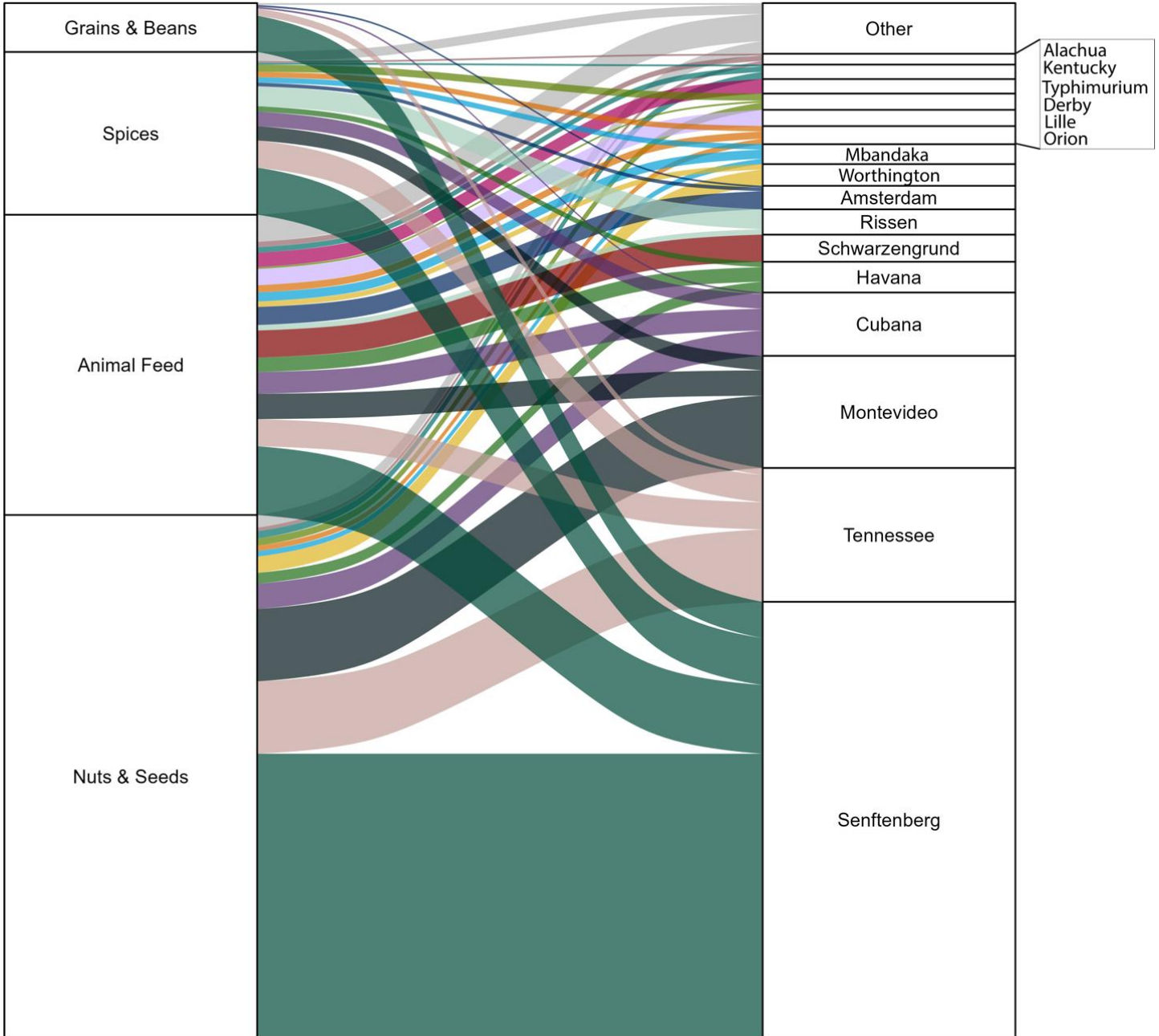


Figure 3-3: Serovar-Source Group Attribution. A. Animal Derived Products, B. Low-moisture foods, C. Produce and Environment Associated

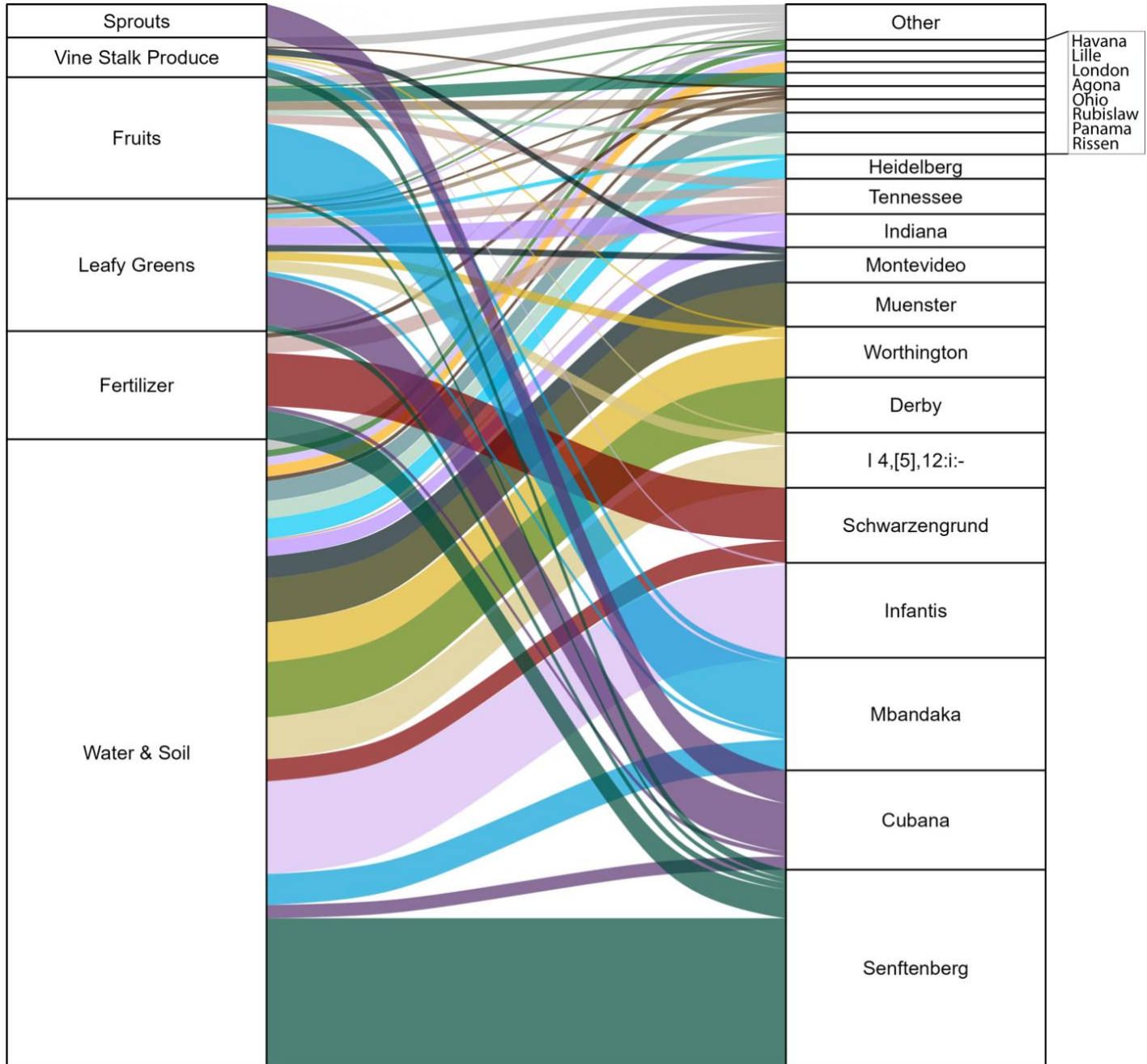
A. Animal Derived Products



B. Low-Moisture Foods



C. Produce and Environment Associated



Analysis of the CHASRI in closed genomes

A total of 165 nucleotide sequences from closed genomes and plasmids as well as 11 nucleotide sequences from short read sequencing of the CHASRI were used for the maximum likelihood tree in Figure 3-4. The CHASRI was located on the chromosome in 131 isolates, and on the plasmid in 45 isolates. There was also one instance where the CHASRI was found on both the chromosome and plasmid in the same isolate which is denoted by the (**) on the tree. There are three main clades found within this tree and will be referred to as Clade 1, 2, and 3.

Clade 1 was identified by an analysis of the amino acid sequence of the CHASRI of the previously defined 4,954 isolates which identified a clade that had high genetic diversity that contained 33 isolates (Appendix 6). These isolates were from 8 different serovars and originated from poultry-derived products, wild animals, fertilizer, leafy greens, and fruits. BLAST analysis in NCBI of the CHASRI sequence from these strains identified a close match with a CHASRI, located on a plasmid, from a strain of *Citrobacter portucalensis* (NZ_CP046347). The CHASRI of *C. portucalensis* was found on an Inc-FII plasmid and PlasmidFinder identified Inc-FII (10 of 11) and Inc-C (1 of 11) which are both associated with multi-drug resistant plasmids (165). Although closed genomes were primarily used for construction of the maximum likelihood tree, to ensure a complete analysis of all variations of the CHASRI located within *Salmonella* the short read data from a subset (n=11) of these isolates was used. The sequences are genetically similar to each other (0 – 5 SNPS) within Clade 1 but far more distant from Clade 2 and Clade 3, with a median of 3,817 SNP differences (range of 3,765 – 5,745 SNPs). The significance of this plasmid is unknown currently, it is noted, however, that the 33 isolates are resided in 11 different SNP clusters on the Pathogen Detection database. Although, the instance of this CHASRI

sequence is rare, it is interesting to see it in sources that all share an agricultural niche, and that the plasmid is being transferred horizontally to the other serovars.

Clade 2 contained 43 *Salmonella* isolates, comprised of 13 different serovars. The CHASRI sequence from this clade was similar to the CHASRI from *Citrobacter freundii* (NZ_LS992183) and mainly found incorporated into the chromosome (78%) while the others were located on IncHI2 type plasmids. Notably, this variation of the CHASRI was found on strains associated with previous outbreaks of Salmonellosis in low moisture foods including pistachios (Montevideo, Senftenberg, Worthington), spices (Montevideo), and peanut butter (Tennessee) (1, 166, 167, 168).

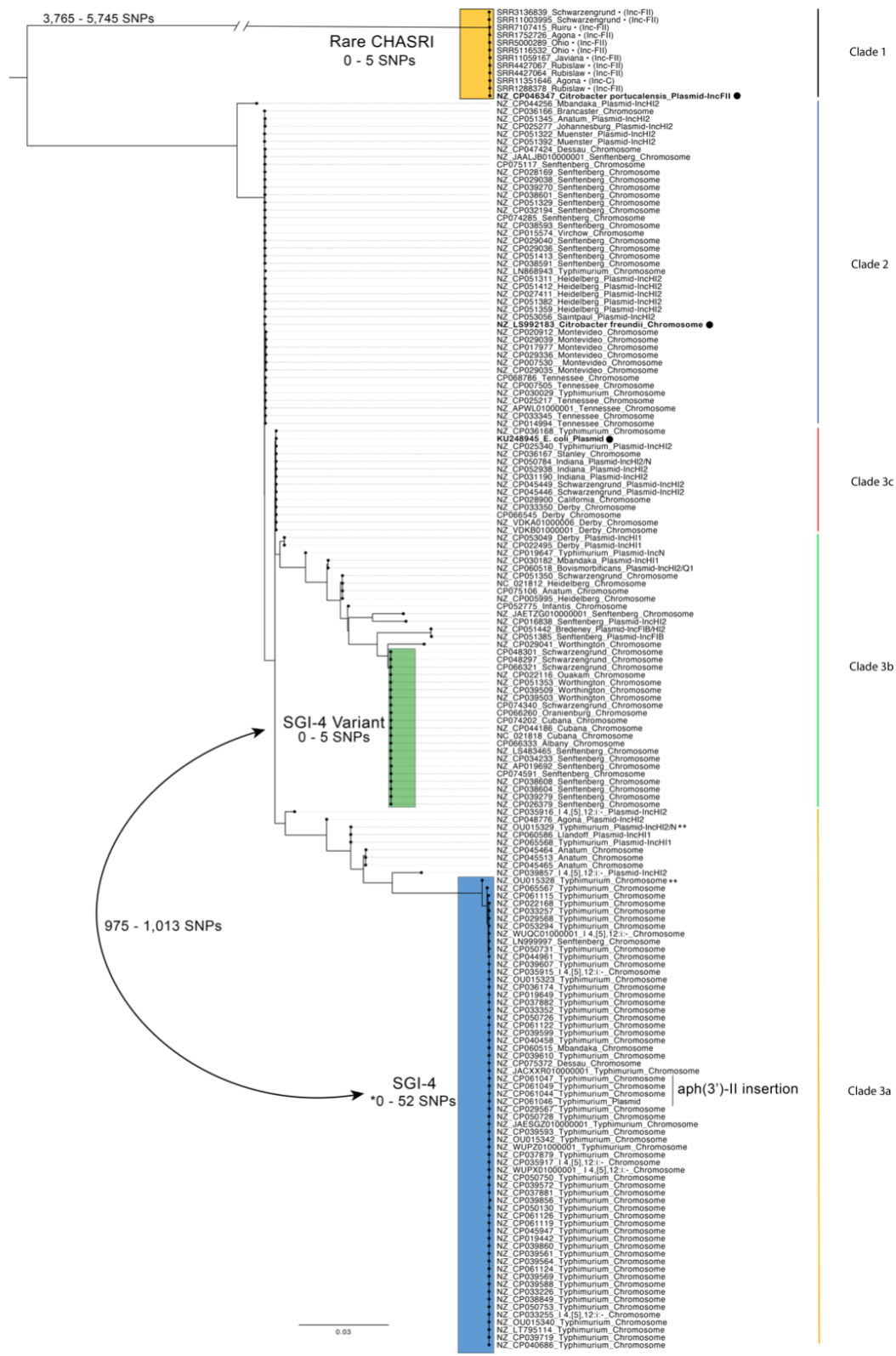
Clade 3a contains the sequences where the CHASRI is found as part of *Salmonella* Genomic Island-4 (SGI-4). Interestingly, less than half of the isolates (37.1%) contained the CHASRI as part of the SGI-4 (n =62) marked in blue on Figure 3-4. The 62 sequences that contained the SGI-4 cassette were predominately serovar Typhimurium (n = 59), but there was also one isolate each for serovar Senftenberg, Dessau, and Mbandaka, respectively. An interesting insertion of the gene for aph(3')-II occurred in the region between DUF2933 and *pcoG* in 4 of the isolates (1 plasmid, 3 chromosome), due to this insertion these sequences were removed from determination of SNP distances in this clade. The rest of the sequences had a median SNP distance of 0, where the largest difference in the sequences was found in the *pcoB* gene of NZ_OU15328. The *pcoB* gene encodes an outer membrane protein that may inhibit the uptake of copper, it is unknown if these changes may alter the function of this protein (68). Previously published data referenced the SGI-4 to be primarily associated with serovar Typhimurium and I 4,5[12]:i:- (ST34) but this

study shows other serovars also incorporate the island into their genome. There were eight isolates that were below the BLAST cutoff for 85% coverage, but these isolates were inspected visually and did contain the genomic island.

The SGI-4 variant, highlighted in green, is found in Clade 3b. SGI-4 (MN730129.1) and SGI-4 variant (OK209934.1) are not closely related (64.6% pairwise similarity) and this difference is also seen in the CHASRI sequence (median 1,008 SNPs). The main difference between the SGI-4 and the variant is within the arsenic operon. The variant SGI-4 contains a different combination of arsenical genes than the typical SGI-4. The impact of these different genes was not investigated during this study. The CHASRI sequence for the 21 isolates that contained the SGI-4 variant was highly similar with a median 0 SNPs (range 0 – 5). Due to the large number of SNP differences between the CHASRI sequence and the presence of a different *ars* operon, it is possible that this genomic island has a very distant ancestor or more likely, evolved separately.

Clade 3c contains sequences not associated with the SGI-4 or the variant SGI-4. These sequences are closely related to a CHASRI from an *E. coli* plasmid (KU248945). These sequences have a median of 3 SNPS and a range of 0 – 6. The phylogeny gives support to the hypothesis that these *Salmonella* isolates obtained this CHASRI from horizontal transfer with an *E. coli* plasmid.

Figure 3-4: Minimum spanning tree for the nucleotide sequences of the CHASRI based on the 176 sequences of the CHASRI found in a subset of Salmonella genomes



Enhanced tolerance to bactericidal effects of copper in vitro

The scarless deletions were confirmed by sequencing the strains using the PacBio Sequel II. The long-read data was *de novo* assembled, and annotated with Prokka for comparison with the previously closed genome of CFSAN045763 (1). The deletion was visualized using Geneious as shown in Figure 3-5. The method was effective at deleting the entire operons in a single effort and were indeed scarless by not producing any indels.

Figure 3-5: Geneious visualization of the wild type and knockout strains from the closed genomes.

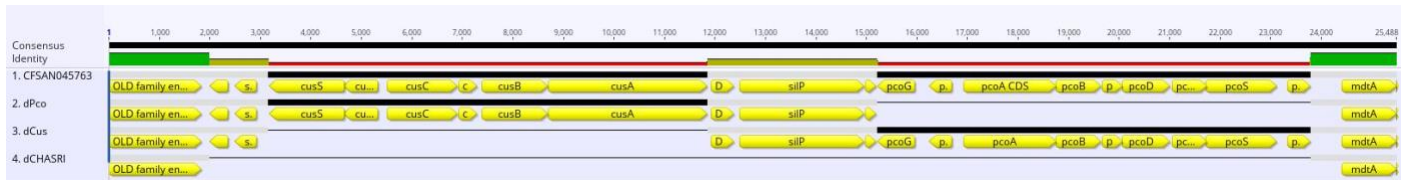


Table 3-3 shows the MIC and MBC measurements for the wild type and knockout strains. The MIC is reported as the range of the six values (three biological and two technical replicates). This variation is most likely due to assay-to-assay variation between individual cultures. The MBC was the same for each measurement, therefore is reported as a single number. Although, each sample has similar MIC values, the MBC was slightly higher for the strains containing the entire or partial CHASRI in comparison to the complete knockout. These results are comparable with previously published MIC assays of *Salmonella* in copper (64, 74, 169). Previous studies show that the CHASRI gives a more significant difference in MIC under anaerobic conditions which were not assessed in this study (64, 74, 169). However, the MBC illustrates that copper has a bacteriostatic effect on *Salmonella* effect at certain concentrations which is important information where copper is used as an agent to control microbial activity such as copper plating in processing plants, or as agricultural additives.

Table 3-3: MIC and MBC Values of the wild type and knockout strains from this study

Strain	MIC Range	MBC
CFSAN045763	1.5 mg/ml – 1.7 mg/ml	2.0 mg/ml
CFSAN045763 Δcus	1.4 mg/ml – 1.6 mg/ml	2.0 mg/ml
CFSAN045763 Δpco	1.5 mg/ml – 1.6 mg/ml	2.0 mg/ml
CFSAN045763 $\Delta CHASRI$	1.4 mg/ml – 1.5 mg/ml	1.8 mg/ml

Effects of Copper Sulfate on Growth Kinetics

The wild type strain and the three knockouts assessed for differences in growth kinetics in the presence of copper. All strains were able to grow in the presence of copper sulfate up to the maximum tested concentration of 1.5 mg/ml (Figure 3-6). Overall, the results from the Bioscreen C° was reproducible between the wells which may be in part due to the ability to run two 100-well plates concurrently. Table 3-4 shows the average and standard deviation of the specific growth rate (Table 3-4A) and the maximum OD (Table 3-4B) of the nine measurements. The average of the triplicates for each biological replicate was used for statistical analysis.

Statistically there was no difference in the maximum OD that was reached for any of the isolates grown in the different concentrations of copper sulfate. However, there were statistical differences in the specific growth rate at 0.5 mg/ml between the wild type vs. Δcus and $\Delta CHASRI$ ($p = 0.0232$ and 0.0310 respectively) as well as Δpco vs. Δcus ($p = 0.0402$). The difference between the growth rate of Δpco and $\Delta CHASRI$ was close to significantly different ($p = 0.0535$). Although overall they eventually reached similar maximum OD in 0.5 mg/ml of copper sulfate, there is significant difference between the area under the curve measurements for the Δcus and $\Delta CHASRI$ which may be due to a slower adaptation to the environment. At 1.5 mg/ml of copper sulfate the $\Delta CHASRI$ isolate also showed a similar max OD but the time it took to achieve this was longer, and the area under the curve was not significantly different from the

others. The specific growth rate, maximum OD, and area under the curve declined with the increase in copper concentrations, which is to be expected. There is no visual difference in lag time until the concentration went to 1.5 mg/ml as seen in Figure 3-6.

Table 3-4 A. Specific growth rate (h^{-1}), B. Maximum OD, C. Area under the Curve Data is shown as average of the 3 biological replicates \pm standard deviation. Subscript letters in the same row denote significant difference ($p < 0.05$) by unpaired t test.

A. Specific Growth Rate (h^{-1})

[CuSO ₄]	CFSAN045763	Δ_{cus}	Δ_{pco}	Δ_{CHASRI}
0 mg/ml	2.20 \pm 0.25	2.33 \pm 0.29	2.14 \pm 0.26	2.20 \pm 0.24
0.5 mg/ml	1.55 \pm 0.24 ^{a,b}	0.94 \pm 0.17 ^{a,c}	1.79 \pm 0.46 ^{c,d}	1.04 \pm 0.12 ^{b,d}
1 mg/ml	0.48 \pm 0.04	0.39 \pm 0.02	0.54 \pm 0.13	0.47 \pm 0.10
1.5 mg/ml	0.17 \pm 0.03	0.14 \pm 0.02	0.13 \pm 0.03	0.13 \pm 0.03

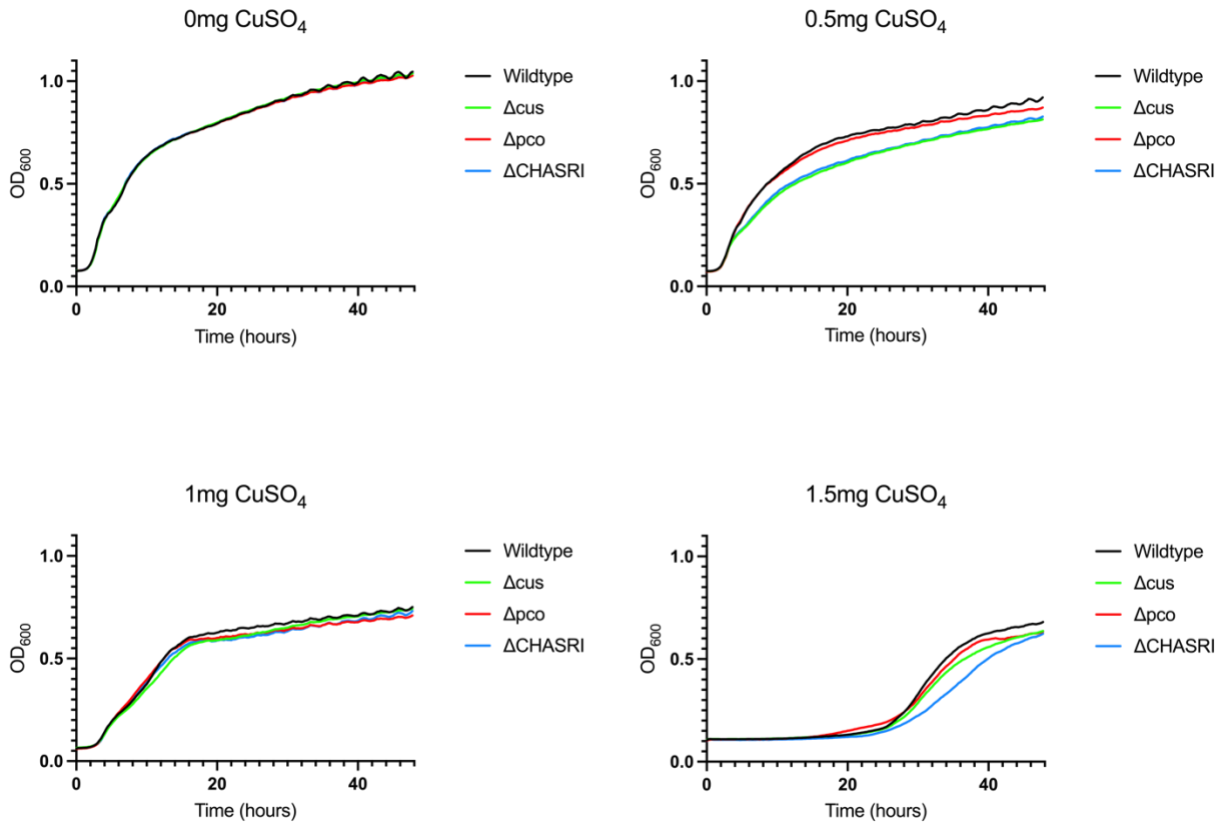
B. Maximum OD

[CuSO ₄]	CFSAN045763	Δ_{cus}	Δ_{pco}	Δ_{CHASRI}
0 mg/ml	1.04 \pm 0.03	1.03 \pm 0.04	1.02 \pm 0.01	1.03 \pm 0.01
0.5 mg/ml	0.91 \pm 0.07	0.81 \pm 0.07	0.87 \pm 0.02	0.82 \pm 0.04
1 mg/ml	0.75 \pm 0.04	0.74 \pm 0.09	0.71 \pm 0.01	0.72 \pm 0.04
1.5 mg/ml	0.68 \pm 0.04	0.64 \pm 0.04	0.63 \pm 0.03	0.62 \pm 0.01

C. Area under the Curve

[CuSO ₄]	CFSAN045763	Δ_{cus}	Δ_{pco}	Δ_{CHASRI}
0 mg/ml	33.3 \pm 0.75	33.3 \pm 0.96	33.0 \pm 0.40	33.1 \pm 0.40
0.5 mg/ml	28.9 \pm 1.63 ^{a,b}	24.4 \pm 1.96 ^{a,c}	28.2 \pm 1.06 ^{c,d}	24.9 \pm 0.90 ^{b,d}
1 mg/ml	23.6 \pm 1.23	22.5 \pm 2.03	22.8 \pm 1.05	22.5 \pm 0.74
1.5 mg/ml	9.4 \pm 1.46	8.1 \pm 0.85	9.0 \pm 1.71	6.9 \pm 1.22

Figure 3-6: Growth curves generated using the Bioscreen Co instrument. The average of the technical replicates and biological replicates were graphed after normalizing for background.



Inactivation of *Salmonella enterica* by Elevated Copper Sulfate Concentrations

The wild type and knockout overnight cultures were subjected to incubation at varying concentrations of copper sulfate during the stationary phase and sampled to determine the effects of an elevated copper environment. At a concentration of 2 mg/ml, the log reduction was not significantly different at each time point for all four strains (Figure 3-7A). There was ~ 2 log CFU/ml reduction (range 2.13 – 2.69 log CFU/ml) after 24 hours incubation in 2 mg/ml copper sulfate for all four strains. There was also no significant difference in log reduction between the samples taken up to 6 hours when incubated with 3 mg/ml of CuSO₄. However, there was significant difference between the log reduction at 3 mg/ml CuSO₄ incubation for the WT vs. ΔCHASRI ($p = 0.028$) and Δpco vs. ΔCHASRI ($p = 0.011$) strains at 24 hours based on unpaired

t-test. The WT and partial knockouts also showed increase survival in comparison to the complete deletion when incubated with 4 mg/ml CuSO₄ (Figure 3-8). There was a significant difference in log reduction between the wild type and partial knockouts vs. Δ CHASRI at both 1 hour (p value < 0.34) and 1.5 hours (p value < 0.02) of incubation. There was more variability in survival at time points greater than 2 hours at this higher copper concentration which could suggest the presence of a persistent subpopulation. The Δ pcp knockout appears to be better equipped at handling short term copper stress (up to 6 hours) which may be due to the presence of the efflux pump system in the *cus* operon. Overall, the results illustrate the benefit of the inclusion of the CHASRI, be it in partial or in entirety, when *Salmonella* is exposed to higher concentrations of copper sulfate.

Figure 3-7: Log Reduction (CFU/ml) of the strains grown in A. 2 mg/ml copper sulfate and B. 3 mg/ml copper sulfate sampled at different timepoints up to 24 hours.

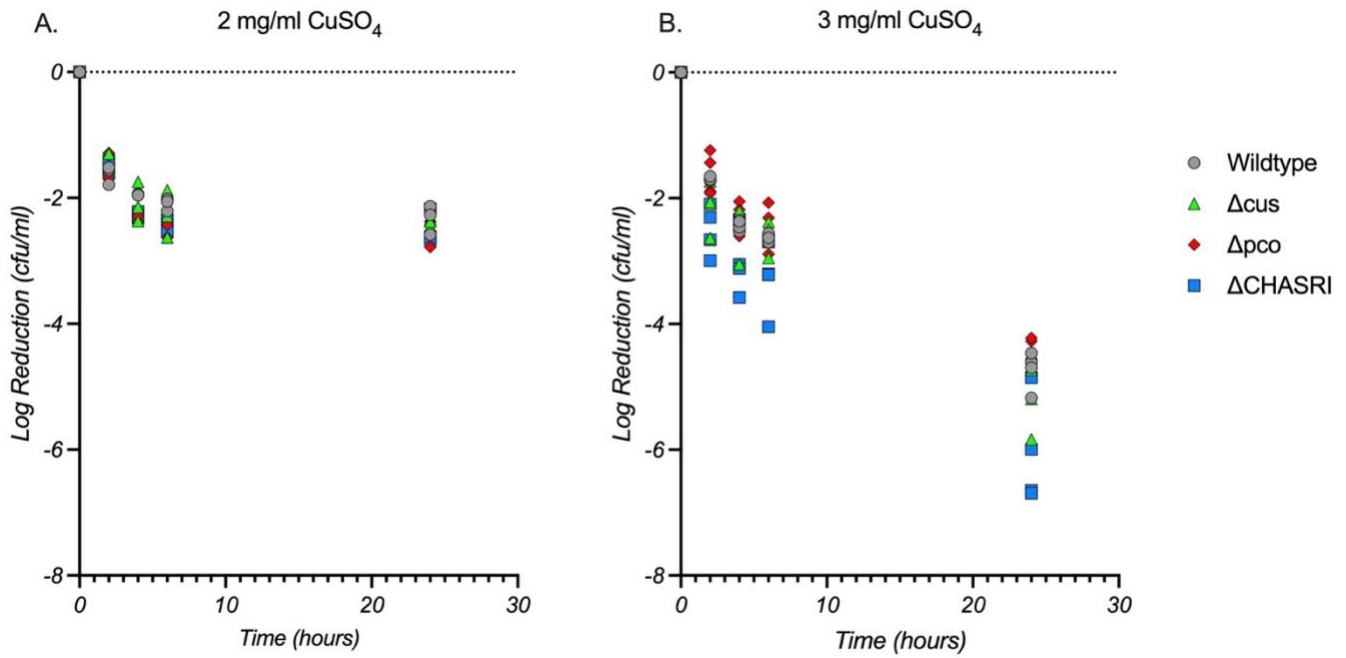
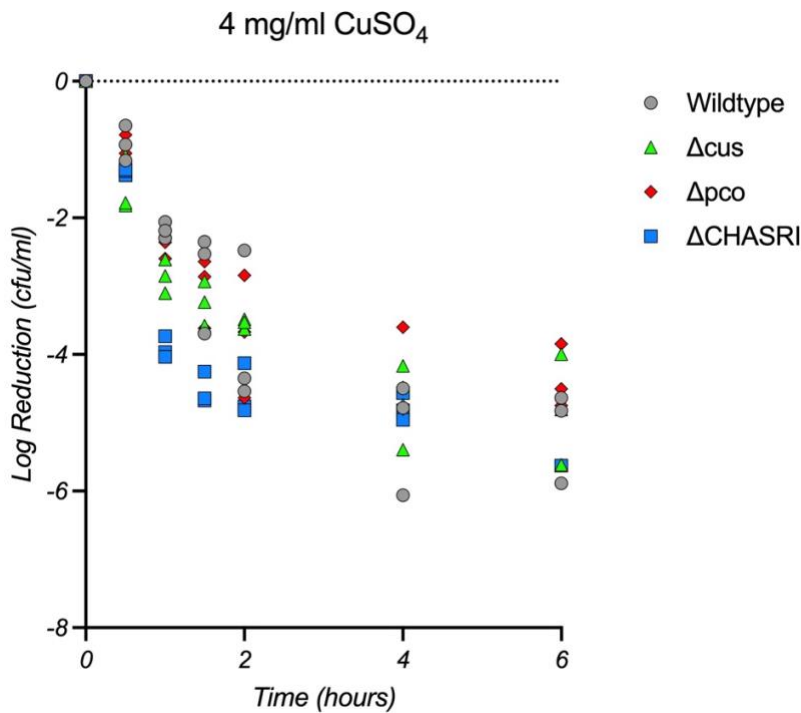


Figure 3-8: Log reduction (CFU/ml) of the strains grown in 4 mg/ml copper sulfate and sampled up to 6 hours.



Conclusions

Salmonella enterica has been isolated from a large variety of sources and can survive in many different environments. The CHASRI is a genomic island that has become integrated in *Salmonella* due to outside pressures from extensive copper usage. This study shows that the CHASRI is prevalent in other serovars, for example Senftenberg, Tennessee, and Montevideo beyond the previously reported serovar Typhimurium. The CHASRI also is found in sources not typically associated with poultry or pork such as low moisture foods, fruits and leafy greens. This study determines that the CHASRI is multifaceted and acquired in *Salmonella* through multiple pathways. There is evidence of the CHASRI in isolates derived from low-moisture sources that are not associated with SGI-4 or the variant. Future studies should be performed to see if the CHASRI gives any advantage to *Salmonella* in a low moisture environment. This analysis also identifies that the SGI-4 and the variant are not similar, and more studies on the phenotypic differences of these islands should be addressed. It is also very interesting to find a CHASRI in a small subset of the isolates that is highly related to a plasmid from *Citrobacter* which seems to be a novel introduction of the CHASRI into *Salmonella*.

The knockouts created allowed for phenotypic assays to evaluate the advantages of acquisition of the CHASRI for growth in a copper environment as well as survival under copper stress. The growth rate was significantly different when grown at 0.5 mg/ml of copper sulfate as well as the area under the curve was lower for the Δcus and $\Delta CHASRI$ strains. Lag time was increased at 1.5 mg/ml copper sulfate and area under the curve was lower for the $\Delta CHASRI$ strain. There was not a significant difference between the MIC and MBC under aerobic conditions for the wild type

and knockout strains. However, the results from the inactivation assay show an increase tolerance to higher levels of copper for short periods of time.

CHAPTER 4: THE PROTECTIVE EFFECTS OF THE CHASRI AND
THE IMPACT ON *SALMONELLA* SURVIVAL TO MULTIPLE
STRESSES

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Abstract

The stress response of bacteria to different environments is an important area of research and has been the focus of many functional genomic studies. Studies have shown that genes are able to turn on a cascade of protective measure for bacteria and sometimes provide cross-protection. The prevalence and properties of the CHASRI were a focus of the previous chapter. The goal of this study was to investigate if the CHASRI can provide any cross-protection against successive stresses. In this study, the strains from Chapter 3 were challenged with lethal copper concentrations, desiccation, and desiccation followed by heat with and without pretreatment with of 1 mg/ml of copper sulfate. Surviving bacteria was detected by direct plating on mTSAYE and log CFU/g was determined. The results of the study show that conditioning the strains with copper will turn on the CHASRI and provide immediate protection against lethal copper. Cells that are copper stressed can tolerate desiccation if the CHASRI is present. Interestingly, the study in pistachios showed that the CHASRI has some contribution to providing protection against desiccation and thermal treatment regardless of the presence of excess copper.

Introduction

Salmonella has been isolated from many different types of foods, animals, and environments. Bacteria are known to adapt to different environments and may acquire genes to increase tolerance. It has been previously hypothesized that monophasic *Salmonella* Typhimurium (ST34) has acquired the CHASRI as a response to selective pressure from copper supplementation in swine (72, 73). We have shown that the CHASRI is more widespread in serovars other than Typhimurium and sources outside of livestock. Our previous study showed that the CHASRI aids in survival at elevated copper environments (up to 4 mg/ml), but any other benefits are still unknown. Copper sulfate is used in the pre-harvest environment as an organic fungicide and as a deficiency corrector. The levels used in the field are low, and below the bactericidal concentration we identified in Chapter 3 (170). There have been multiple outbreaks of *Salmonella* in pistachios that have been linked to the orchard environment as defined in Chapter 2. The outbreak strain from the orchard environment has been isolated from finished product that underwent processing including thermal treatment. It is understood that when bacteria become stressed, sigma factors are turned on and a cascade of events can provide several protective effects. These protective effects can be beneficial when a secondary stress is introduced. One example of cross-protective effects is *Salmonella* grown in high acid can be protected against heat and cold stress (46, 49). Similarly, desiccated cells are more protective against thermal processing and acids (48).

In this chapter, the main objective is to determine whether the CHASRI has cross-protective effects that may benefit *Salmonella* in a stressed environment. This will be performed by pre-conditioning with copper prior to a treatment with a lethal concentration of copper or

desiccation. Additionally, since this isolate is a pistachio orchard strain that has been found in final product, the strains will be tested in pistachios undergoing heat treatment.

Materials and Methods

Conditioning of strains to increase copper tolerance

Preconditioning Cultures in Subinhibitory Concentrations of Copper Sulfate

The effects of preconditioning bacteria with low levels of copper were tested by the following method. The wild type strain, CFSAN045763, was grown overnight on TSA from freezer stocks. Single colonies were inoculated in 22 ml of MHB and incubated at 37 °C for 20 hours. A 100 mg/ml stock of copper sulfate was prepared, and filter sterilized. This stock was then diluted to 3.8 mg/ml and 3.4 mg/ml in MHB. The overnight culture of bacteria was further diluted by adding 20 ml of the overnight culture to 160 ml of MHB. The diluted bacteria were combined with the MHB + CuSO₄ in a 1:2 fashion, giving final concentrations of 1.7 mg/ml and 1.9 mg/ml CuSO₄+MHB as previous determined to be subinhibitory concentrations for this strain. This concentration was below the inhibitory concentration previously identified in Chapter 3. The samples were incubated at 37 °C and samples were taken at the following timepoints: 0, 5, 10, 15, 30 minutes and 24 hours for RNA extraction. At these timepoints 10 ml samples were taken and pelleted by centrifugation at 8,586 g for 5 minutes. The supernatant was removed, and the pellets were immediately put into the -80 °C freezer until RNA extraction was performed.

Incubation at bactericidal concentration of 2 mg/ml CuSO₄

After 24 hours of pre-conditioning, 10 ml of the cultures were pelleted by centrifugation at 8,586 g for 5 minutes and then resuspended in 40 ml of MHB. These strains were then combined with 4.0 mg/ml of CuSO₄ in a 1:2 dilution to create a final concentration of 2.0 mg/ml of CuSO₄. This

concentration was found to be bactericidal in Chapter 3. The inocula were incubated at 37 °C. Plate counts were performed at 0h, 4h, and 24h, log CFU/ml calculations were determined, and ANOVA was performed on these log reduction values. Samples for RNA extraction were taken at 0, 5, 10, 15, 30 minutes and 24 hours. This experiment was conducted in biological triplicate.

RNA extraction and Reverse Transcriptase Quantitative PCR (RT-qPCR)

RNA was extracted using the Total RNA Purification Plus Kit (Norgen Biotek, ON, Canada) following the manufacturer’s suggested protocol. RT-qPCR was performed on all the RNA samples in duplicate along with a no template control (NTC) using the Luna® Universal One-Step RT-qPCR kit (New England Biolabs, Ipswich, MA) on the ABI7500 Fast instrument (Applied Biosystems, Waltham, MA) following manufacturer’s protocols. The primers used were designed for this experiment and amplified regions of genes associated with copper homeostasis (Table 4-1). The real-time RT-qPCR results were analyzed using the $\Delta\Delta C_T$ method to calculate the relative fold gene expression of the samples (171). The $\Delta\Delta C_T$ is calculated by the following equations:

$$\Delta C_T = C_{T \text{ Target Gene}} - C_{T \text{ Housekeeping Gene}}$$

$$\Delta\Delta C_T = \Delta C_{T \text{ Treatment}} - C_{T \text{ Control}}$$

$$-\Delta\Delta C_T = \text{Log Fold Change in Expression}$$

Upregulated genes will have a $-\Delta\Delta C_T > 1$ and downregulated genes will have a $\Delta\Delta C_T < 1$. The C_T results from all biological and technical replicates was used for the above calculations. Any outlier C_T (difference of $> 2 C_T$ from average) was excluded from calculations.

Table 4-1: Primers used in the Real-time RT-qPCR assay.

gene		Forward Primer	Reverse Primer
<i>golT</i>	Core Homeostasis	AAGTGACGCTGTGGTTTGTGTC	TGAGTAGCTGTAACGCCTCC
<i>copA</i>	Core Homeostasis	TAGTATTGCCGCGCTTGAAC	AGACGTTTAATCGCATCGGC
<i>cueO</i>	Core Homeostasis	CACAGCGGGATGAATCATGG	GAACTGCGTGCCGTGAATAT
<i>cusA</i>	CHASRI	AGAGTTTATCGTGGTGGCCA	CCCTCCCAGCGACATGATAT
<i>pcoB</i>	CHASRI	TCACAGCTTTCTATTGCGGC	TCGCATGCCCTGTAAATTG
<i>rpoD</i>	Housekeeping gene	CAATGACCATCTGCCGGAAG	CATCTTCATCGGTGCTGGTG

Cross-protection of pre-conditioning with copper exposure against desiccation stress

Preparation of Inocula

The wild type (CFSAN045763) and knockout strains (CFSAN045763 Δ *cus*, CFSAN045763 Δ *pco*, CFSAN045763 Δ *CHASRI*) produced in Chapter 3, were grown on TSA from the freezer stocks. Colonies were transferred to TSB and grown overnight at 37 °C. The overnight cultures were used to inoculate MHB or MHB supplemented with 1 mg/ml of CuSO₄ at a 1:20 ratio, and then incubated overnight at 37 °C. The 20 ml cultures were centrifuged at 8,586 g for 5 minutes and the supernatant was removed. The cell pellet was resuspended with 2.2 ml of PBS.

Desiccation stress

Desiccated cells were prepared using similar methods to Mandal et al. (25). Briefly, ten 100 μ l spots were aliquoted onto disposable polystyrene petri dishes and were air-dried inside the biosafety cabinet with the blower on for 4 hours at room temperature. Afterwards, the plates were covered with the lids and incubated at room temperature (~30% relative humidity) for 24 hours. At 24 hours, the desiccated cells were recovered by adding 10 ml of Brain Heart Infusion

Broth (BHIB), and gently pipetting to resuspend the cells from the petri dish. The 10 ml of recovered cells were centrifuged for 5 minutes at 8,586 g and the supernatant was removed. The cell pellet was resuspended in 1 ml of PBS. Direct plating was performed in duplicate for log CFU determination. The four strains were tested in biological triplicate. The log CFU reduction for all strains, both control and pre-conditioned with copper were analyzed for statistical significance using Student's t-test.

Cross-protection study in pistachios to assess overall tolerance in a simulated processing environment

Preparation of Inoculum

The wild type and Δ CHASRI strains were grown on TSA from the freezer stocks. A single colony was transferred into 10 ml of TSB and incubated overnight at 37 °C. Three lawn plates were prepared by spread plating 100 µl of inoculum on to TSA and then incubating overnight. Bacteria was harvested by adding 10 ml of Modified Buffered Peptone Water (mBPW) (172) to the surface of the plate and gently scraping using an L-shaped spreader. The harvested culture was approximately 10 log CFU/ml.

Preparation of Pistachios

Raw, unsalted pistachios were purchased and analyzed for the presence of *Salmonella* before inoculation. Subsets of pistachios were mixed with mBPW in a 1:2 dilution, and hand massaged. Direct plating was conducted by spread-plating 100 µl of the enrichment on to mTSAYE in duplicate and incubated overnight at 37 °C. The presence of black colonies were counted, and the total log CFU/g of *Salmonella* was calculated. There were two categories of pistachios used

for inoculation – a control group and a pretreated group. The pistachios from the pretreated group were weighed into 400 g samples and treated using 25 ml of a 1 mg/ml of CuSO₄ solution. These nuts were allowed to dry in the biosafety cabinet for 3 days and then stored in a sealed bag until inoculated.

Inoculation Procedure

The methods were adapted from the previous study done by Casulli et al. (173). The pretreated or control pistachios were weighed out in to 400-g batches and were inoculated with 25 ml of bacterial suspension in a whirl-pak bag. The bag was gently massaged to evenly distribute the inoculum. The pistachios were then spread out on autoclaved trays and placed into the biosafety cabinet to air-dry overnight at room temperature. Homogeneity testing was conducted by sampling 6 subsamples of 5-g each, to ensure evenly distributed bacteria. After 24 hours, the nuts were transferred to a humidity-controlled glove box and stored at 45% RH for at least 3 days before thermal treatment. Water activity was measured using an Aqualab model 4TE water meter (Decagon Devices, Pullman, WA).

Thermal Treatment

Thermal treatment was conducted in an Heratherm™ General Protocol Oven (ThermoFisher, Waltham, MA) set to 118 °C. The come-up time was determined using a probe that was inserted between the pistachio shell and the meat of the nut. Duplicate 20-g samples were placed on sterile glass petri dishes. The oven temperature was monitored throughout the testing time, and the samples were heated for a duration up to 3 hours. At each time point, the pistachios were immediately diluted 1:2 with mBPW that was at 4 °C. Each sample was hand massaged and then

serial dilutions were plated on mTSAYE. After overnight incubation at 37 °C, the black colonies were counted, and plate counts were converted to log CFU/g. Each category of pistachios was tested in biological triplicate, and each time point in technical duplicate.

Weibull Modelling and Statistical Analysis

Duplicate plate counts were collected for each time point of thermal processing. The log reduction was calculated for each time point by $\log N_t - \log N_0$. The log reduction values were fitted by the Weibull Model (174) with the nonlinear regression function of JMP Pro 15 software (Cary, NC, USA) to determine the microbial inactivation kinetics using the equation:

$$\log\left(\frac{N_t}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^\beta$$

In this equation, α represents the time for a log reduction and β is the shape parameter of the inactivation curve. The time to reach a 2-log and 5-log reduction (t_{D2} , t_{D5}) was calculated based on the model parameters and in this equation:

$$t_{Dn} = \alpha(2.303n)^{\frac{1}{\beta}}$$

GraphPad Prism 9 software (Boston, MA, USA) was used to perform the unpaired t-test to determine statistical significance of these reduction times.

Results and Discussion

Conditioning to increase copper tolerance

The wild type strain was incubated with subinhibitory concentrations (1.7 and 1.9 mg/ml) of CuSO₄ and Real-time RT-qPCR was conducted at different time points on five different copper genes. These primed bacteria were then subjected to incubation in 2.0 mg/ml CuSO₄ + MHB.

The log fold expression changes for the core copper homeostasis genes (*golT*, *copA*, and *cueO*) can be found in Figure 4-1. The genes *golT* and *copA* showed upregulated gene expression throughout the incubation period with both subinhibitory and lethal copper concentrations. The expression for *golT* was higher during the first 5 minutes of incubation with 2 mg/ml for the pre-conditioned strains but became the same regardless of conditioning. The *golT* expression at 30 minutes was similar to the expression after 24 hours for both low and high concentrations of copper. Expression for *copA* was very similar to *golT*. *CopA* expression levels for the strains incubated in 2 mg/ml were higher than those at incubated at 1.7 and 1.9 mg/ml. These two genes are P-type ATPases that transport copper from the periplasm to the cytoplasm. These genes belong to operons that are homologous to each other, therefore it can be expected that their expression profiles be similar (58). The fold expression changes for *cueO*, another core homeostasis gene that is a copper oxidizer was upregulated at all timepoints except for at 15 minutes in samples incubated in 1.9 mg/ml of copper sulfate. The log fold change was higher in the 1.9 mg/ml preconditioned cells compared to the unconditioned cells when subsequently incubated at 2 mg/ml. Eventually after 24 hours of incubation at this high copper concentration, the expression was similar regardless of the preconditioning ($p > 0.05$).

Figure 4-2 shows the log fold change over time for the two CHASRI genes that were assayed. The expression for *cusA*, a component of the efflux pump, was upregulated for all timepoints. The expression for the samples incubated at 2 mg/ml was higher than when at lower concentrations of copper. The expression for *cusA* peaked in the first 5 minutes regardless of conditioning when incubated with 2 mg/ml CuSO₄. This operon is important for copper tolerance in anaerobic environments since it does not require oxygen like oxidases (175). The expression

for *pcoB* was upregulated in all samples at the higher concentration of copper but varied when incubated at lower levels. *PcoB*, is an outer membrane protein that may inhibit the uptake of copper and may be more important in high level copper environments (68). Expression of *pcoB* was highest for the 1.9 mg/ml preconditioned cells during the first 5 minutes of incubation with 2 mg/ml CuSO₄.

Overall, the expression levels for the copper genes were more elevated when incubated with 2 mg/ml of CuSO₄. However, when the preconditioned cells were subjected to a higher concentration of copper sulfate, the expression of the CHASRI genes was at their highest during the earlier timepoints. This elevation in gene expression was also identified in the core copper homeostasis genes.

The plate counts for 4 and 24 hours (Table 4-2) showed less reduction of bacteria if the cells were preconditioned prior to incubation with 2 mg/ml. The results from the ANOVA of the log reduction values at 4 hours ($p = 0.001$) and 24 hours ($p = 0.002$) showed a significant difference between the treatments. The upregulated expression of the CHASRI genes that can be seen early in the incubation period with 2.0 mg/ml must have a protective effect on the preconditioned cells. Future studies on acclimating the bacteria to slowly increasing levels of copper would be beneficial since we know that copper accumulates in the environment and can persist long-term (176).

Table 4-2: Log reduction values (log CFU/ml)

	Pre-Conditioning		
	No Conditioning	1.7 mg/ml	1.9 mg/ml
4 hours	1.06 ± 0.16	0.09 ± 0.18	0 ± 0.04
24 hours	1.82 ± 0.19	0.21 ± 0.21	0.80 ± 0.42

Figure 4-1: Log Fold Change of the Core Copper Homeostasis genes. Pre-conditioning time frame (0:05 – 24:00), and elevated copper concentration time frame 24:05 – 48:00

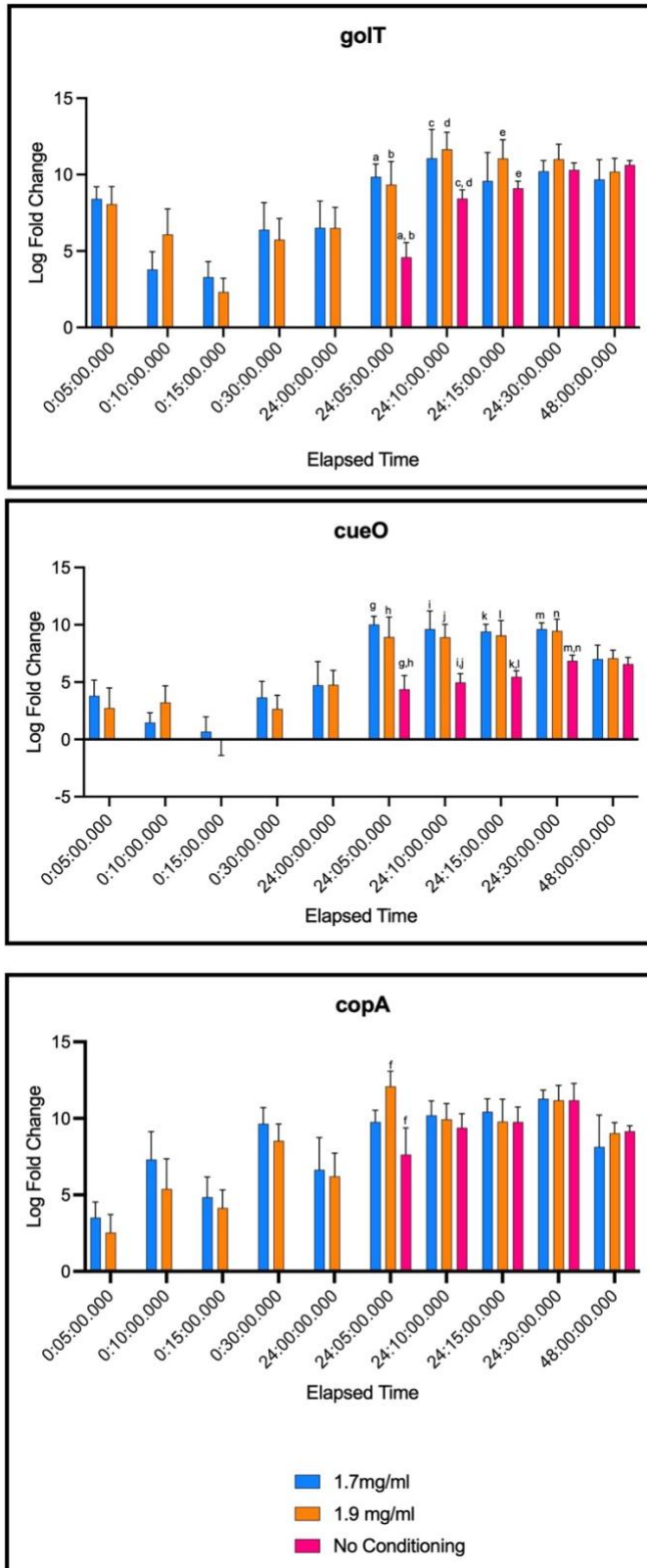
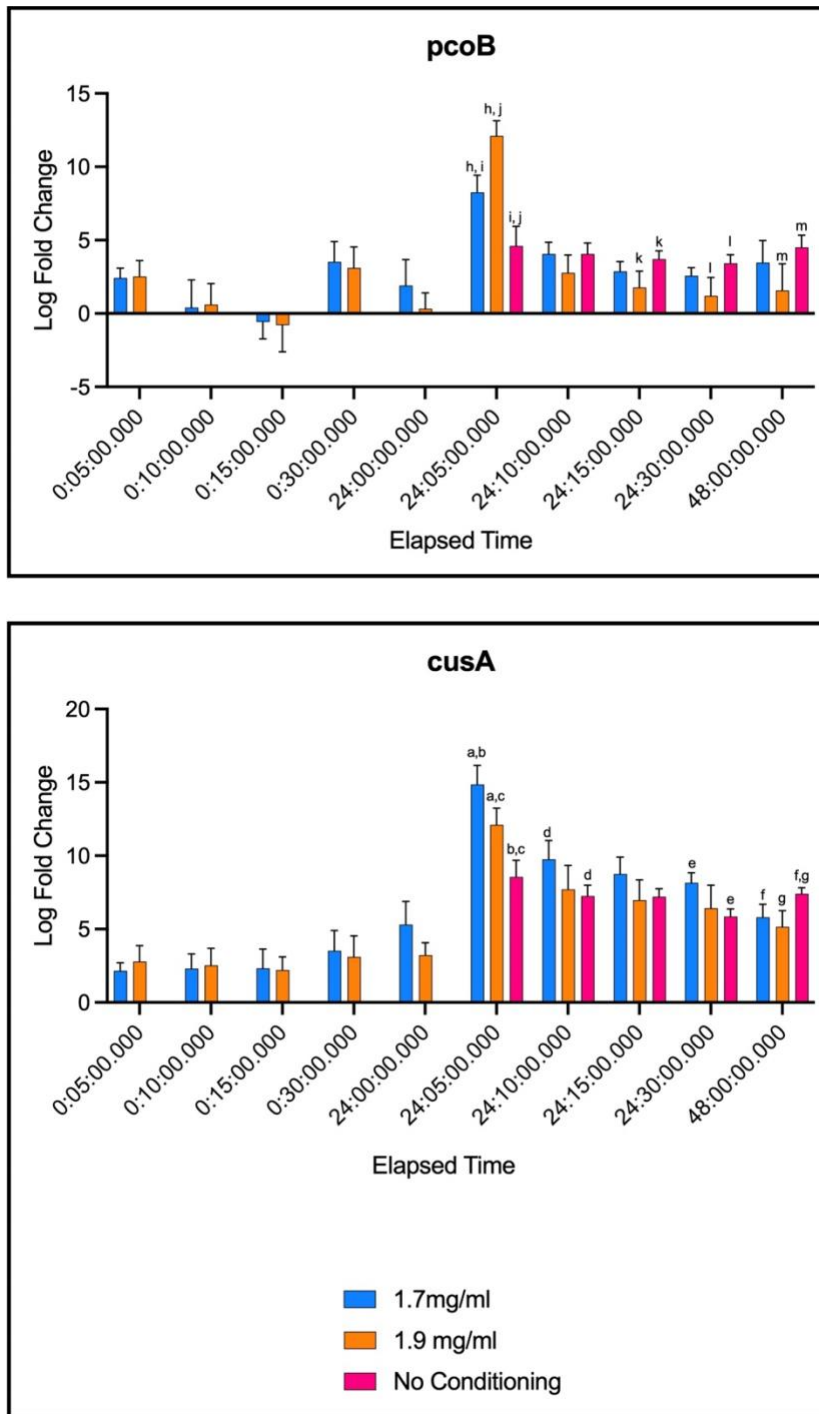


Figure 4-2: Log Fold Change of CHASRI genes. Pre-conditioning time frame (0:05 – 24:00), and elevated copper concentration time frame 24:05 – 48:00



Copper preconditioning and cross-protection during desiccation

All four strains (WT and three KOs) were grown overnight in the presence or absence of copper and then desiccated on petri dishes. *Salmonella* was recoverable from all four strains from both growth conditions after 24 hours of desiccation. The results of the log reduction can be found in Table 4-3. The strains that were grown normally in MHB alone prior to desiccation did not have a significant difference in log reduction after 24 hours with a range of reduction from 1.2 to 1.6 log CFU/ml. Interestingly, the cells that were pre-conditioned in CuSO₄ prior to desiccation were more stressed and had significantly higher loss during desiccation (range 2.8 to 3.7 log CFU/ml) in comparison to the control ($p < 0.0001$). The concentration of 1 mg/ml CuSO₄ was previously (Chapter 3) found to not inhibit growth but must have some implications in overall health and resilience of the *Salmonella* strains. However, although the reduction was greater, the wild type strain survived desiccation significantly better ($p = 0.0123$) than the Δ CHASRI knockout. The partial knockout, Δ cus, also had significantly less reduction ($p = 0.0137$) than the Δ CHASRI knockout. The CHASRI does not have any benefit on desiccation stress itself but provides some protection against desiccation as a secondary stress. This illustrates the benefit and possible cross-protection interactions between the CHASRI and a secondary desiccation stress. It is known that copper sulfate is used in the pre-harvest environment has fungicides and growth promoters, and we have shown that the strains recovered from the pistachio orchard contain the CHASRI. It is possible that the strains are being primed by copper sulfate in the orchard and that is then improving the ability to survive desiccation on the food surface. Copper causes a phenotypic change in the cell wall of *Salmonella* and turns on the filamentous production (177), perhaps the CHASRI enhances this response to better aid in desiccation tolerance.

Table 4-3: Log Reduction at 24 hours post-desiccation. Matching superscript initials represent statistically significant difference between the means based on t-test .

	Control	Pre-Conditioned
WT	1.299 ± 0.266	2.867 ± 0.220 ^a
Δcus	1.523 ± 0.234	3.048 ± 0.090 ^b
Δpco	1.498 ± 0.066	3.579 ± 0.398
$\Delta CHASRI$	1.553 ± 0.053	3.738 ± 0.270 ^{a,b}

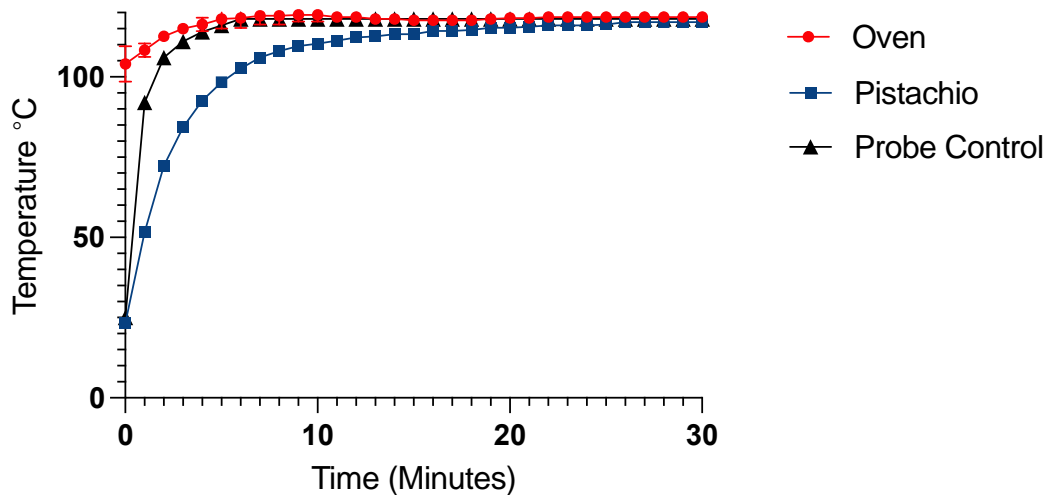
Cross-protection of cells against sequential desiccation and thermal stress in pistachios

The strain CFSAN045763 originated in pistachios and was determined to be due to a contamination in the orchard in Chapter 2. A purpose of this study was to determine if the CHASRI has additional benefits to overall survival of *Salmonella* in pistachios in a postharvest environment. To test this hypothesis, pistachios were inoculated with the wild type and $\Delta CHASRI$ strain, then were subjected to the stress of desiccation followed by thermal processing. Pistachios can be treated in the orchard with copper sulfate as a foliar spray. A subset of pistachios was pretreated with copper prior to inoculation to mimic that increased concentration of copper on the surface.

The pistachios were inoculated with ~8.5 log CFU/g of *Salmonella* and were found to be homogeneous (average 8.72 ± 0.18 log CFU/g). There was ~ 1 log reduction after desiccation which is comparable to many previous studies (146, 173) and our study from Chapter 2 (148). The a_w was 0.45 to 0.55 prior to thermal treatment. The time to temperature for the inside of the pistachio shell increased exponentially and was at 90% of the processing temperature at 7 minutes, and eventually reached the target temperature at 15 minutes (Figure 4-3). The external

temperature of the pistachio was unable to be measured, but it is reasonable to state that the external temperature reached processing temperature within the first 7 minutes.

Figure 4-3: Time-Temperature Graph for Oven, Pistachio, and Probe



Salmonella was recovered after thermal treatment of 118 °C for up to 3 hours as shown in Figure 4-4. There was no significant difference in the log reduction of the strains based on the conditioning of the pistachios. However, the wild type strain inoculated on the control pistachios had an average log reduction of 3.5 log CFU/g which was significantly less than the knockout with a reduction of 5.2 log CFU/g ($p = 0.019$). The same trend was identified in the copper pretreated pistachios with the average reduction of 3.8 (wild type) and 4.8 (knockout) log CFU/g ($p = 0.0006$).

The Weibull model was used to determine the thermal inactivation kinetics from the observed data. The α and β parameters were used to calculate the time to a 2 log and 5 log reduction which are shown in Figure 4-5. Interestingly, there was not a significant difference in the 2D-

value but there was for the 5D (control $p = 0.034$, pretreated $p = 0.023$). This would fit the theory of a secondary population becoming more tolerant to the thermal stress. The US Food and Drug Administration recommends a minimum 5-log reduction for peanuts and pistachios (178). The log reduction results from this study were different than those by Casulli et al. The two main differences were in strain selection and the use of a moist-air convection oven. These differences highlight the need to improve microbial inactivation models and ensure the results are consistent with the processing workflow. The current study shows the inverse relationship of dry air on D-values. Although the overall reduction is similar, the slope of the models is visibly different between the control and pretreated pistachios. It may be of interest to evaluate this further. I hypothesize that either the copper is causing a difference in where the majority of the *Salmonella* population is persisting (outside shell vs. inside shell) therefore causing a thermal inactivity change, or that the increased copper concentration is having additional cross-protection effects similar to the desiccation study.

Figure 4-4: Log Reduction of Salmonella in inoculated pistachios. Data shown as the average of the replicates with standard deviation. The solid lines represent the Weibull Model.

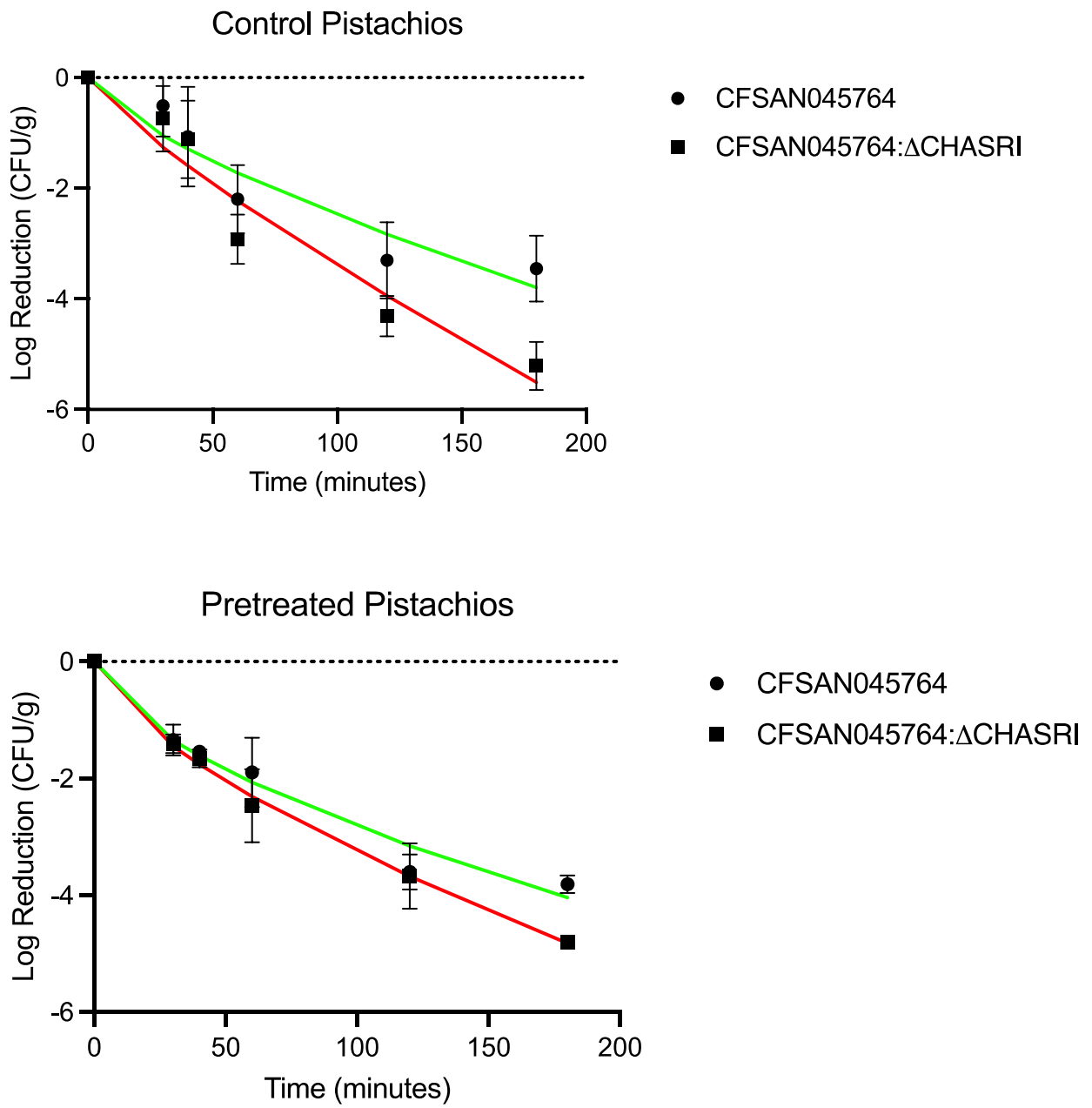
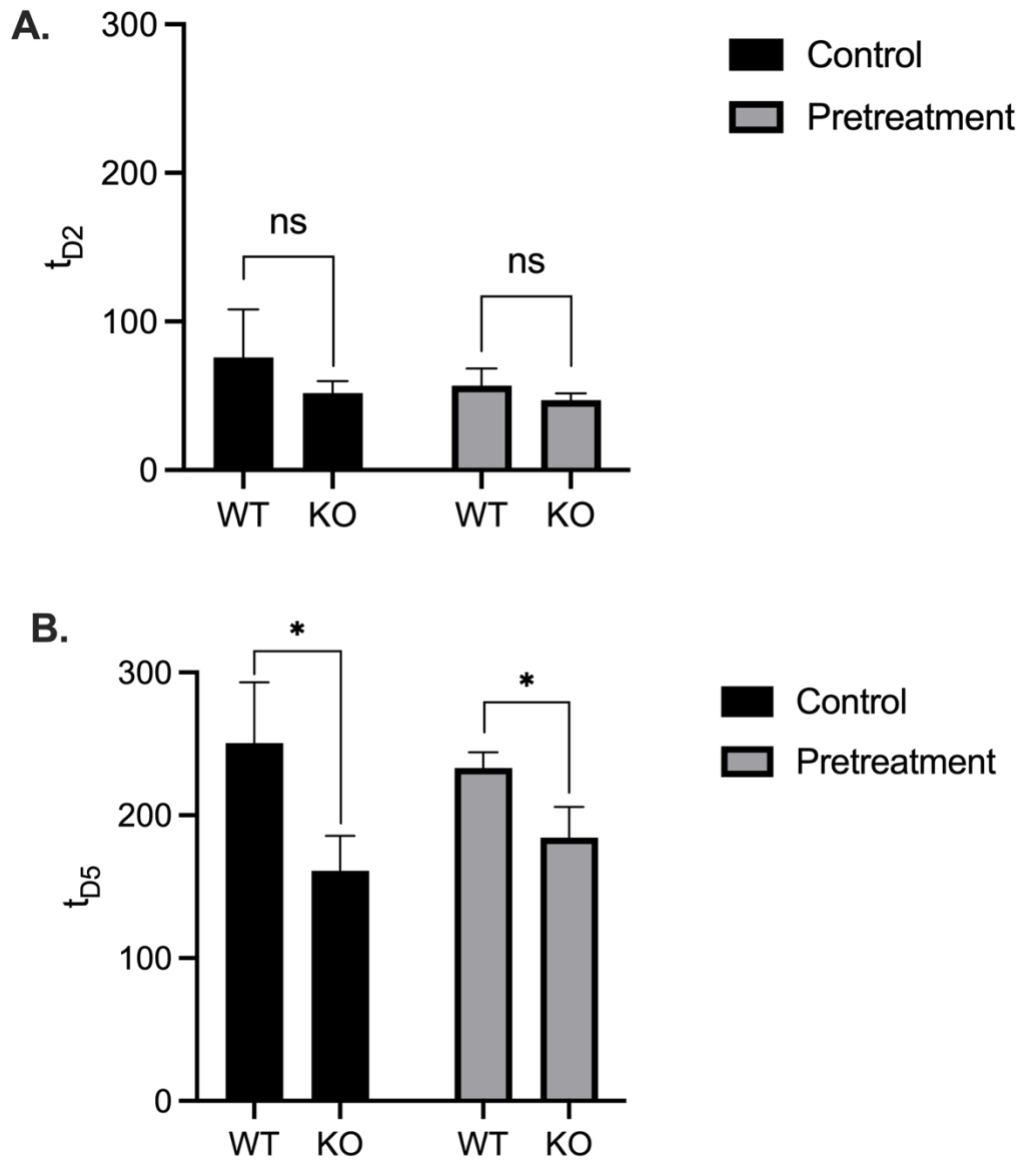


Figure 4-5: Time to an (A) 2-log reduction and a (B) 5-log reduction. Graph is based on average of three replicates and standard deviation. * represents a p value < 0.05



Conclusion

The stress response of bacteria is still a largely unknown phenomenon, and very complex studies are ongoing using vast mutagenesis libraries to determine the cascade of genes that can provide protection. This study was able to evaluate the impact of the CHASRI, a mobile cassette, by utilizing a knockout that was genetically identical. The CHASRI allows preconditioned *Salmonella* to adapt to handle a lethal level of copper sulfate and reduce overall log reduction. This is explained by the upregulation of copper homeostasis genes earlier in the incubation period. The CHASRI not only seems to provide protection against copper, but also provides downstream protection against a secondary stress of desiccation. The wild type and the Δcus strain showed reduced cell death after desiccation when preceded by copper stress. Copper sprays are used in the preharvest environment, which may lead to *Salmonella* being better equipped to desiccate and persist on produce and nuts if the CHASRI is present. The results of our pistachio study identify that the CHASRI provides tolerance to long-term heat exposures. Surrogate bacteria are routinely used in process validation and this study shows that strain selection is very critical to ensure the process will provide the necessary results. Presence of a strain in a facility that has certain genetic markers should be used when validating methods for preventive controls.

CHAPTER 5: OVERALL CONCLUSIONS AND FUTURE WORK

Overall Conclusions

Salmonella enterica is a significant contributor to cases of foodborne illness worldwide, being mainly attributed to consumption of animal products or fresh produce. We now know that *Salmonella* can also be found in many other sources and environments such as soil, water, and low-moisture environments. While the characteristics of low-moisture foods do not allow for the proliferation of *Salmonella*, they do not prevent persistence once contaminated. Many low-moisture foods are also ready-to-eat or previously deemed safe due to this nature but have been implicated in many foodborne outbreaks. It is critically important to understand the mechanisms behind this persistence.

Outbreaks of salmonellosis caused by the consumption of pistachios have occurred multiple times with the same serovars (Senftenberg and Montevideo). In Chapter 2, an in-depth comparison study was performed to determine the source of these outbreaks and recalls. A total of 204 isolates were identified by using the NCBI Pathogen Detection Browser. MLST identified four sequence types among these isolates: ST14, ST185, ST316, and ST138. Utilizing the advancements in sequencing technology, high quality reference genomes were generated for ten isolates. These closed genomes allowed for in-depth analysis of gene content. This analysis identified a unique genomic island only in one ST from each serovar, the Copper Homeostasis and Silver Resistance Island (CHASRI). Additionally, determinations of contamination points for the isolates were determined by phylogenetic analysis. The isolates belong to ST14 and ST316, carried the CHASRI and were isolated from multiple facilities and over multiple years. The higher number of SNPs and the location of related isolates spread throughout the trees, leads

to these strains being from an orchard contamination. The ST185 and ST138 isolates were unusual in the fact that they had limited number of SNPs. The ST185 isolates were from two facilities and show harborage within that facility where as the ST138 isolates may be from shared equipment used by different firms.

A storage study was also conducted in pistachios with a cocktail of *Salmonella*. The pistachios stored at a lower relative humidity were able to persist for up to a year with very little log reduction. Metagenomic sequencing was able to determine that each strain of the cocktail was able to survive the whole storage time. Interestingly, one of the strains was not originally from a low-moisture source. This illustrates that the persistence of these strains may not be impacted by their origination but that there may be other genetic or gene expression factors at action.

The identification of the CHASRI in Chapter 2 directed the research to investigate this genomic island further and to identify the prevalence in *Salmonella enterica* in chapter 3. A total of 4,954 isolates that passed quality measures were determined to contain the CHASRI as a single cassette in their genome. These isolates were derived from 22 source categories and 61 serovars. These isolates came from 49 different countries and spanned a 48-year period. Animal derived products accounted for many of these isolates (58%) but 12% were from LMFs. Interestingly, serovar Senftenberg was found to be from a wide range of sources.

Further analysis of the CHASRI was conducted using closed reference-based genomes found on NCBI. This analysis identified four variations of the CHASRI in *Salmonella*. A rare form was identified in a very small subset of isolates that was identical to the CHASRI from a *C.*

portucalensis plasmid. The CHASRI is a component of SGI-4, a genomic island that also contains other heavy metal tolerance genes. The SGI-4 CHASRI was primarily associated with serovar Typhimurium (95%). Interestingly, an SGI-4 variant was identified in 21 isolates, which had a CHASRI sequence that was highly different than the one in SGI-4. This variant was found in multiple serovars excluding Typhimurium. In addition to the CHASRI on the genomic island, the CHASRI was found in a subset of isolates on its own. There was no correlation of the sequence and if the CHASRI was found on the chromosome or plasmid.

A *Salmonella* Senftenberg strain that we identified from Chapter 2 to be from the pistachio orchard was used to create CHASRI knockouts in Chapter 3. The Red-Recombinase Scarless Deletion method was able to produce genetically identical strains except for the region deleted. The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration assay showed no difference in the MIC of the wild type strain and the knockouts but the MBC for the Δ CHASRI isolate was lower than the others. The growth kinetics in 0.5 mg/ml of CuSO₄ were significantly different between the wild type and the Δ *cus* and Δ CHASRI isolates. This was also identified between the Δ *pco* and the Δ *cus* and Δ CHASRI isolates. The inactivation of the strains in 2 mg/ml CuSO₄ was not significantly different but differences were identified in higher concentrations (3 mg/ml and 4 mg/ml). The full knockout was not as effective in surviving at these higher concentrations compared to the wild type and the partial knockouts.

The stress response in *Salmonella* is complicated and previous studies have identified cross-protection benefits of genes. Based on this knowledge, it is interesting to know if the CHASRI can also induce similar effects. In Chapter 4, pre-conditioning the wild type strain with sub-

inhibitory concentrations of copper sulfate provided protection against a subsequent lethal concentration. The results of real-time RT-qPCR verified that the copper genes were able to upregulate faster and at a higher magnitude to try to protect the cell. The strains that contained the CHASRI, intact or partial, were able to survive a desiccation stress preceded by copper incubation significantly better than the complete knockout.

Successive stresses were also tested using inoculated pistachios. Pistachios inoculated with the wild type and the Δ CHASRI strains were subjected to desiccation and thermal treatment. There was no significant difference in the reduction after desiccation between these strains. However, there was significant difference in the log reduction after thermal treatment. This same trend was found in pistachios that were pretreated with copper sulfate prior to *Salmonella* inoculation. The Weibull model was employed for inactivation kinetics. Interestingly, the *Salmonella* strains inoculated in both the control pistachios and pretreated pistachios exhibited similar time to a 2-log kill. However, for increased levels of inactivation (5-log), the time was significantly higher for the wild type strain.

Future Work

This is just the beginning for the next steps in fully understanding the impact of the CHASRI and the stress response of *Salmonella*. The entire cascade of stress response genes needs to be evaluated during the stress exposure experiments. Utilizing RNA-seq will allow us to see what other genes are being upregulated in response. Using the knockout strains, we can see how the CHASRI is interacting with other genes to provide enhanced tolerance. The use of copper chelators is also another area to research. We saw that cells grown in a low level of copper were

able to survive desiccation if the CHASRI was present. Is it possible to stop the expression of some stress response genes using a chelator in the pre-harvest environment? Also of interest, is the timeframe that these protections may have effect. If the time between stresses is increased or decreased, does that change effectiveness of treatment? Does the time of copper addition to produce influence the protective effect. The CHASRI has also been identified in *Pseudomonas syringae* isolated from tomato fields in California where copper is applied as an antifungal agent. On the eastern shore of Maryland and Virginia, there has been an endemic issue with *Salmonella* Newport in tomatoes. Is it possible that there is competition for resources on the west coast that the *P. syringae* is able to outcompete due to the CHASRI. In the same thought, is the reason we are seeing outbreaks of Montevideo and Senftenberg in pistachios due to the presence of the CHASRI? Lastly, is the copper exposure to these strains with CHASRI priming the bacteria to be more virulent and able to protect against the host immune response, is this a contributing factor to these strains causing outbreaks?

CHAPTER 6: APPENDICES

Appendix 1: Isolates and metadata for comparative genomics study in Chapter 2

Strain	Location	Isolation Source	SNP cluster	ST	BioSample	SRA	Collection Date	Facility
2016K-0180	USA	Clinical	PDS000031814	14	SAMN04592565	SRR3315923	Missing	
2017K-0050	USA	Clinical	PDS000031814	14	SAMN06312264	SRR5238274	2016	
CFSAN000622	USA: MD	Chicken	PDS000031814	14	SAMN02367754	SRR1060630	1987	
CFSAN015119	USA	Pistachios	PDS000031814	14	SAMN02844939	SRR2585751	2009	
CFSAN015122	USA	Shelled Pistachios	PDS000031814	14	SAMN02844942	SRR2585428	2009	
CFSAN016599	USA	Pistachios	PDS000031814	14	SAMN02846418	SRR1816865	2009	B
CFSAN045763	USA:CA	Raw Pistachios	PDS000031814	14	SAMN04451263	SRR3168967	2016	A
CFSAN047567	USA:CA	Pistachios	PDS000031814	14	SAMN04531980	SRR3309442	2016	A
CFSAN047866	USA:TX	Pistachios	PDS000031814	14	SAMN04549535	SRR3272258	2016	A
CFSAN048426	USA:CA	Raw Pistachio	PDS000031814	14	SAMN04607364	SRR3340981	2016	A
CFSAN048427	USA:CA	Raw Pistachio	PDS000031814	14	SAMN04607365	SRR3340982	2016	A
CFSAN048428	USA:CA	Raw Pistachio	PDS000031814	14	SAMN04607366	SRR3340985	2016	A
CFSAN048429	USA:CA	Raw Pistachio	PDS000031814	14	SAMN04607367	SRR3340986	2016	A
CFSAN058295	USA:TX	Shelled Pistachios	PDS000031814	14	SAMN06125464	SRR5120752	2016	A
FMA0102	Canada	Pistachios	PDS000031814	14	SAMN02698166	SRR1258573	2013	
OSF033320	USA:CA	Pistachios	PDS000031814	14	SAMN03702143	SRR2890018	2014	
OSF033321	USA:CA	Pistachios	PDS000031814	14	SAMN03702144	SRR2890019	2014	
OSF047785	USA:TX	Pistachios	PDS000031814	14	SAMN04535551	SRR3286882	2015	
OSF048599	USA:CA	Pistachios	PDS000031814	14	SAMN04607380	SRR3372311	2016	
OSF048657	USA:CA	Pistachios	PDS000031814	14	SAMN04632053	SRR3391910	2016	
OSF048658	USA:CA	Pistachios	PDS000031814	14	SAMN04632057	SRR3391911	2016	
OSF048659	USA:CA	Pistachios	PDS000031814	14	SAMN04632058	SRR3391913	2016	
OSF048660	USA:CA	Pistachios	PDS000031814	14	SAMN04632059	SRR3657446	2016	
OSF048786	USA:CA	Pistachios	PDS000031814	14	SAMN04858522	SRR3437480	2016	
OSF049331	USA:CA	Pistachios	PDS000031814	14	SAMN04934443	SRR3490039	2016	
OSF049332	USA:CA	Pistachios	PDS000031814	14	SAMN04934444	SRR3490040	2016	
OSF056360	USA:CA	Pistachios	PDS000031814	14	SAMN05968031	SRR5057162	2016	
OSF056361	USA:CA	Pistachios	PDS000031814	14	SAMN05968029	SRR5057161	2016	
OSF056362	USA:CA	Pistachios	PDS000031814	14	SAMN05968028	SRR5057160	2016	
OSF056593	USA:CA	Pistachios	PDS000031814	14	SAMN06033741	SRR5070635	2014	

Strain	Location	Isolation Source	SNP cluster	ST	BioSample	SRA	Collection Date	Facility
OSF056809	USA:CA	Raw Almonds/nuts	PDS000031814	14	SAMN06034002	SRR5116313	2014	F
OSF056838	USA:CA	Raw Pistachios	PDS000031814	14	SAMN06033973	SRR5120068	2014	
OSF058109	USA:W A	Raw Pistachios	PDS000031814	14	SAMN06114141	SRR5152278	2014	
OSF059119	USA:W A	Pistachios	PDS000031814	14	SAMN06213829	SRR5182252	2016	
OSF059213	USA:CA	Environmental Sponge	PDS000031814	14	SAMN06213743	SRR5217564	2016	G
OSF059253	USA:CA	Pistachios	PDS000031814	14	SAMN06213719	SRR5217444	2016	
OSF059265	USA:CA	Pistachios	PDS000031814	14	SAMN06213707	SRR5217482	2016	
OSF059289	USA:W A	Pistachios	PDS000031814	14	SAMN06213683	SRR5229127	2016	
OSF059786	USA:CA	Pistachios	PDS000031814	14	SAMN06270093	SRR5279048	2016	
OSF059815	USA:CA	Pistachios	PDS000031814	14	SAMN06270175	SRR5251448	2016	
OSF067866	USA:CA	Fertilizer, Bone Meal	PDS000031814	14	SAMN07525954	SRR5990714	2015	
OSF070289	USA:CA	Environmental Sponge	PDS000031814	14	SAMN07792017	SRR6179039	2017	
OSF073486	USA:CA	Environmental Sponge	PDS000031814	14	SAMN08200061	SRR6386672	2016	
OSF073577	USA:CA	Pistachios	PDS000031814	14	SAMN08245531	SRR6424913	2016	
OSF074224	USA:CA	Pistachios	PDS000031814	14	SAMN08294372	SRR6487131	2016	
OSF084195	USA:CA	Environmental Swab	PDS000031814	14	SAMN09765131	SRR7643277	2017	E
OSF090574	USA:TX	Shelled Pistachios	PDS000031814	14	SAMN10719997	SRR8428217	2016	
OSF090575	USA:TX	Shelled Pistachios	PDS000031814	14	SAMN10720013	SRR8428324	2016	
PNUSAS001664	USA	Clinical	PDS000031814	14	SAMN04535330	SRR3240307	2016	
PNUSAS001739	USA	Clinical	PDS000031814	14	SAMN04570735	SRR3289842	2016	
PNUSAS001800	USA	Clinical	PDS000031814	14	SAMN04595059	SRR3392769	Missing	
PNUSAS008582	USA	Clinical	PDS000031814	14	SAMN06328296	SRR5260040	2015	
PNUSAS140610	USA	Clinical	PDS000031814	14	SAMN14517896	SRR1146236 5	Missing	
SGSC 2516	USA: MD	Chicken	PDS000031814	14	SAMN01933164	SRR5598938	1987	
CFSAN017737	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847614	SRR3151938	2013	C
CFSAN017738	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847615	SRR3151939	2013	C
CFSAN017739	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847616	SRR3151940	2013	C
CFSAN017740	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847617	SRR3151941	2013	C
CFSAN017741	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847618	SRR3151942	2013	C
CFSAN017744	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847621	SRR3151943	2013	C

Strain	Location	Isolation Source	SNP cluster	ST	BioSample	SRA	Collection Date	Facility
CFSAN017745	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847622	SRR3151944	2013	C
CFSAN017746	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847623	SRR3242178	2013	C
CFSAN017747	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847624	SRR3242179	2013	C
CFSAN017748	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847625	SRR3242180	2013	C
CFSAN017749	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847626	SRR3242181	2013	C
CFSAN017750	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847627	SRR3242182	2013	C
CFSAN017751	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847628	SRR3242184	2013	C
CFSAN017752	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847629	SRR3242185	2013	C
CFSAN017753	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847630	SRR3340442	2013	C
CFSAN017754	USA:CA	Environmental Swab	PDS000031739	185	SAMN02847631	SRR3340443	2013	C
CFSAN032229	USA:CA	Environmental Swab	PDS000031739	185	SAMN03473632	SRR1979272	2015	C
CFSAN032230	USA:CA	Environmental Swab	PDS000031739	185	SAMN03473633	SRR1982146	2015	C
CFSAN032231	USA:CA	Environmental Swab	PDS000031739	185	SAMN03473634	SRR1979316	2015	C
CFSAN086744	USA:PA	Pistachios	PDS000031739	185	SAMN10251117	SRR8113236	2018	C
CFSAN087304	USA:NJ	Pistachios	PDS000031739	185	SAMN10498876	SRR8261878	2018	C
FNW19i50	USA:CA	Pistachios	PDS000031739	185	SAMN02678810	SRR1614998	2013	C
FNW19i51	USA:CA	Pistachios	PDS000031739	185	SAMN02678811	SRR1646543	2013	C
FSE0085	Mexico	Tahini	PDS000031739	185	SAMN02344969	SRR1212317	2011	
FSF0102	USA:CA	Environmental Swab	PDS000031739	185	SAMN02698064	SRR1535718	2013	C
FSW0103	USA:CA	Dry Roasted Pistachios	PDS000031739	185	SAMN02678830	SRR1257291	2013	C
FSW0104	USA:CA	Dry Roasted Pistachios	PDS000031739	185	SAMN02678831	SRR1257282	2013	C
OSF056673	USA:CA	Environmental Swab	PDS000031739	185	SAMN06033882	SRR5099892	2014	C
OSF056674	USA:CA	Environmental Swab	PDS000031739	185	SAMN06033881	SRR5099891	2014	C
OSF056675	USA:CA	Environmental Swab	PDS000031739	185	SAMN06033880	SRR5099888	2014	C
OSF056676	USA:CA	Environmental Swab	PDS000031739	185	SAMN06033879	SRR5099890	2014	C
OSF056753	USA:CA	Environmental Swab	PDS000031739	185	SAMN06033912	SRR5105418	2014	C
OSF056767	USA:CA	Environmental Swab	PDS000031739	185	SAMN06033898	SRR5116302	2014	C
OSF056768	USA:CA	Environmental Swab	PDS000031739	185	SAMN06033897	SRR5116309	2014	C
OSF056769	USA:CA	Environmental Swab	PDS000031739	185	SAMN06033896	SRR5116457	2014	C

Strain	Location	Isolation Source	SNP cluster	ST	BioSample	SRA	Collection Date	Facility
OSF057938	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110528	SRR5120074	2014	D
OSF057939	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110527	SRR5120108	2014	D
OSF057940	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110526	SRR5120103	2014	D
OSF057941	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110525	SRR5120104	2014	D
OSF057942	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110524	SRR5120105	2014	D
OSF057943	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110523	SRR5120107	2014	D
OSF057945	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110521	SRR5120102	2014	D
OSF057946	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110520	SRR5230332	2014	D
OSF057947	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110519	SRR5230170	2014	D
OSF057948	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110518	SRR5230325	2014	D
OSF057951	USA:CA	Environmental Swab	PDS000031739	185	SAMN06110515	SRR5230171	2014	D
OSF057978	USA:CA	Raw Pistachios	PDS000031739	185	SAMN06110542	SRR5230465	2014	C
OSF057979	USA:CA	Raw Pistachios	PDS000031739	185	SAMN06110541	SRR5230320	2014	C
OSF057980	USA:CA	Raw Pistachios	PDS000031739	185	SAMN06110540	SRR5230330	2014	C
OSF057981	USA:CA	Raw Pistachios	PDS000031739	185	SAMN06110539	SRR5230168	2014	C
OSF057982	USA:CA	Raw Pistachios	PDS000031739	185	SAMN06110538	SRR5230385	2014	C
OSF087751	USA:CA	Finished Product Pistachios	PDS000031739	185	SAMN10395404	SRR8176592	2018	C
44713	United Kingdom	Clinical	PDS000027237	316	SAMN03465686	SRR1957791	2014	
378603	United Kingdom	Clinical	PDS000027237	316	SAMN10922314	SRR8568765	2017	
533421	United Kingdom	Clinical	PDS000027237	316	SAMN10855875	SRR8515722	2018	
570824	United Kingdom	Clinical	PDS000027237	316	SAMN10816097	SRR8484198	2018	
708223	United Kingdom	Clinical	PDS000027237	316	SAMN11244187	SRR8774349	2019	
315731156	Missing	Pistachios	PDS000027237	316	SAMN00710616	SRR500745	2009	B
2016K-0167	USA	Clinical	PDS000027237	316	SAMN04572175	SRR3278065	Missing	
CFSAN000244	Missing	Pistachios	PDS000027237	316	SAMN00710595	SRR500472	2009	B
CFSAN013604	USA	Pistachios	PDS000027237	316	SAMN02843424	SRR2559381	2008	
CFSAN014882	USA	Pistachios	PDS000027237	316	SAMN02844702	SRR1973753	2009	
CFSAN015113	USA	Nantucket Blend Trail Mix	PDS000027237	316	SAMN02844933	SRR2559383	2008	B
CFSAN015114	USA	Nantucket Blend Trail Mix	PDS000027237	316	SAMN02844934	SRR2586832	2008	B
CFSAN015115	USA	Pistachios	PDS000027237	316	SAMN02844935	SRR2585772	2009	B

Strain	Location	Isolation Source	SNP cluster	ST	BioSample	SRA	Collection Date	Facility
CFSAN015116	USA	Nantucket Blend Trail Mix	PDS000027237	316	SAMN02844936	SRR2586844	2009	B
CFSAN015117	USA	cashew/almond/pistachio blend	PDS000027237	316	SAMN02844937	SRR2585430	2009	B
CFSAN015118	USA	Pistachios	PDS000027237	316	SAMN02844938	SRR2585429	2009	B
CFSAN015120	USA	Pistachios	PDS000027237	316	SAMN02844940	SRR2585750	2009	B
CFSAN015123	USA	Nantucket Blend Trail Mix	PDS000027237	316	SAMN02844943	SRR2559382	2008	B
CFSAN016379	USA:CA	Environmental Swab	PDS000027237	316	SAMN02846198	SRR1816812	2009	B
CFSAN016380	USA:CA	roasted & salted garlic/onion pistachios	PDS000027237	316	SAMN02846199	SRR1816860	2009	B
CFSAN016411	USA:CA	Environmental Swab	PDS000027237	316	SAMN02846230	SRR1816817	2009	B
CFSAN016412	USA:CA	Environmental Swab	PDS000027237	316	SAMN02846231	SRR1816831	2009	B
CFSAN016600	USA	Pistachios	PDS000027237	316	SAMN02846419	SRR1816838	2009	B
CFSAN016601	USA	Pistachios	PDS000027237	316	SAMN02846456	SRR1816839	2009	B
CFSAN037640	USA:IL	Shell Pistachios	PDS000027237	316	SAMN04348626	SRR3038647	2015	A
CFSAN045764	USA:CA	Raw Pistachios	PDS000027237	316	SAMN04451264	SRR3168966	2016	A
CFSAN047166	USA:CA	Pistachios	PDS000027237	316	SAMN04523269	SRR3242198	2016	A
CFSAN047167	USA:CA	Pistachios	PDS000027237	316	SAMN04523270	SRR3242197	2016	A
CFSAN047168	USA:CA	Pistachios	PDS000027237	316	SAMN04523271	SRR3242196	2016	A
CFSAN047169	USA:CA	Pistachios	PDS000027237	316	SAMN04523272	SRR3242195	2016	A
CFSAN051296	USA	Raw Pistachios	PDS000027237	316	SAMN05181544	SRR3707433	2016	
CFSAN064799	USA	Shelled Pistachios	PDS000027237	316	SAMN07205601	SRR5758423	2017	K
FSIS1606641	USA:MI	Swine	PDS000027237	316	SAMN05150610	SRR3555186	2016	
FSIS1709808	USA:GA	Raw Intact Chicken	PDS000027237	316	SAMN06250977	SRR5196071	2016	
FSL_R8-4916	Missing	Missing	PDS000027237	316	SAMN00990693	SRR493332	Missing	
FSL_R8-4922	Missing	Missing	PDS000027237	316	SAMN00990695	SRR493336	Missing	
OSF045712	USA:IN	Chicken	PDS000027237	316	SAMN04451189	SRR3191464	2015	H
OSF046987	USA:CA	Nuts	PDS000027237	316	SAMN04516950	SRR3225384	2015	F
OSF047721	USA:CA	Pistachios	PDS000027237	316	SAMN04535485	SRR3294561	2015	
OSF051202	USA:W A	Pistachios	PDS000027237	316	SAMN05176310	SRR3606932	2016	
OSF056837	USA:CA	Raw Pistachios	PDS000027237	316	SAMN06033974	SRR5120069	2014	
OSF059120	USA:W A	Pistachios	PDS000027237	316	SAMN06213828	SRR5182248	2016	
OSF069055	USA:CA	Environmental sponge	PDS000027237	316	SAMN07682574	SRR6108679	2015	
OSF069128	USA:CA	Raw Pistachios	PDS000027237	316	SAMN07714156	SRR6109685	2015	

Strain	Location	Isolation Source	SNP cluster	ST	BioSample	SRA	Collection Date	Facility
OSF075603	USA:CA	Pistachios	PDS000027237	316	SAMN08452546	SRR6848181	2016	
OSF075630	USA:W A	Pistachios	PDS000027237	316	SAMN08452491	SRR6782992	2016	
OSF075637	USA:CA	Pistachios	PDS000027237	316	SAMN08452454	SRR6848879	2016	
OSF075638	USA:CA	Pistachios	PDS000027237	316	SAMN08452472	SRR6848843	2016	
OSF076741	USA:CA	Pistachios	PDS000027237	316	SAMN08640413	SRR6848188	2016	
OSF076742	USA:CA	Pistachios	PDS000027237	316	SAMN08640405	SRR6848319	2016	
OSF076748	USA:CA	Pistachios	PDS000027237	316	SAMN08640428	SRR6849216	2016	
OSF076749	USA:CA	Pistachios	PDS000027237	316	SAMN08640429	SRR6849236	2016	
OSF076750	USA:CA	Pistachios	PDS000027237	316	SAMN08640444	SRR6849235	2016	G
OSF077989	USA:CA	Environmental sponge	PDS000027237	316	SAMN08719745	SRR6971655	2016	
OSF077990	USA:CA	Environmental sponge	PDS000027237	316	SAMN08719747	SRR6967965	2016	
OSF078698	USA:CA	Environmental sponge	PDS000027237	316	SAMN08923813	SRR7064464	2016	
OSF084050	USA:CA	Finished Almonds	PDS000027237	316	SAMN09756424	SRR7638853	2017	J
OSF088275	USA:CA	Nuts	PDS000027237	316	SAMN10461370	SRR8272032	2018	G
OSF088276	USA:CA	Nuts	PDS000027237	316	SAMN10461356	SRR8272067	2018	G
OSF088277	USA:CA	Nuts	PDS000027237	316	SAMN10461355	SRR8274725	2018	G
OSF088278	USA:CA	Nuts	PDS000027237	316	SAMN10461349	SRR8274723	2018	G
OSF088279	USA:CA	Nuts	PDS000027237	316	SAMN10461371	SRR8272153	2018	G
OSF088282	USA:CA	Nuts	PDS000027237	316	SAMN10461374	SRR8272155	2018	G
OSF088283	USA:CA	Nuts	PDS000027237	316	SAMN10461359	SRR8272614	2018	G
PNUSAS001586	USA	Clinical	PDS000027237	316	SAMN04522164	SRR3277620	Missing	
PNUSAS001648	USA	Clinical	PDS000027237	316	SAMN04532523	SRR3277288	2016	
PNUSAS001685	USA	Clinical	PDS000027237	316	SAMN04526253	SRR3270925	2016	
PNUSAS001723	USA	Clinical	PDS000027237	316	SAMN04568469	SRR3289850	2016	
PNUSAS001724	USA	Clinical	PDS000027237	316	SAMN04550286	SRR3270998	2016	
PNUSAS001745	USA	Clinical	PDS000027237	316	SAMN04867243	SRR3495148	Missing	
PNUSAS001799	USA	Clinical	PDS000027237	316	SAMN04571760	SRR3289840	2016	
PNUSAS001966	USA	Clinical	PDS000027237	316	SAMN04893760	SRR3473884	2016	
PNUSAS002790	USA	Clinical	PDS000027237	316	SAMN05357709	SRR3897960	2016	
PNUSAS004962	USA	Clinical	PDS000027237	316	SAMN06009836	SRR5031400	2016	
PNUSAS008631	USA	Clinical	PDS000027237	316	SAMN06446431	SRR5336282	Missing	
PNUSAS017345	USA	Clinical	PDS000027237	316	SAMN07359245	SRR5864604	Missing	
PNUSAS034916	USA	Clinical	PDS000027237	316	SAMN08743521	SRR6870271	2018	

Strain	Location	Isolation Source	SNP cluster	ST	BioSample	SRA	Collection Date	Facility
PNUSAS041766	USA	Clinical	PDS000027237	316	SAMN09283774	SRR7235831	Missing	
PNUSAS045784	USA	Clinical	PDS000027237	316	SAMN09754681	SRR7633156	Missing	
PNUSAS049251	USA	Clinical	PDS000027237	316	SAMN09813368	SRR7688270	Missing	
PNUSAS051029	USA	Clinical	PDS000027237	316	SAMN09881758	SRR7739852	Missing	
PNUSAS136046	USA	Clinical	PDS000027237	316	SAMN14176056	SRR1117434 7	Missing	
12-1128	Canada	Clinical	PDS000032600	138	SAMN06030184	SRR5055291	Missing	
CFSAN010209	USA:CA	Pistachio	PDS000032600	138	SAMN02678532	SRR5384655	2009	A
FSIS31800836	USA:UT	Beef	PDS000032600	138	SAMN09779928	SRR7657490	2018	
FSLR9-1449	USA:NY	Clinical	PDS000032600	138	SAMN09756961	SRR8502795	2013	
OSF005645	USA:CA	Pistachio	PDS000032600	138	SAMN02265272	SRR958039	2009	G
OSF056829	USA:CA	Raw Pistachios	PDS000032600	138	SAMN06033982	SRR5120042	2014	I
OSF067822	USA:CA	Environmental sponge	PDS000032600	138	SAMN07526058	SRR5991129	2017	G
OSF069191	USA:CA	Environmental sponge	PDS000032600	138	SAMN07713973	SRR6109466	2015	G
PNUSAS004495	USA:WY	Cattle	PDS000032600	138	SAMN05879184	SRR4418296	Missing	
PNUSAS028544	USA	Clinical	PDS000032600	138	SAMN08044050	SRR6317156	Missing	
PNUSAS044191	USA	Clinical	PDS000032600	138	SAMN09487752	SRR7429448	Missing	
PNUSAS072017	USA:MD	Clinical	PDS000032600	138	SAMN12617948	SRR9972052	Missing	
PNUSAS132560	USA	Clinical	PDS000032600	138	SAMN13975837	SRR1100527 0	Missing	

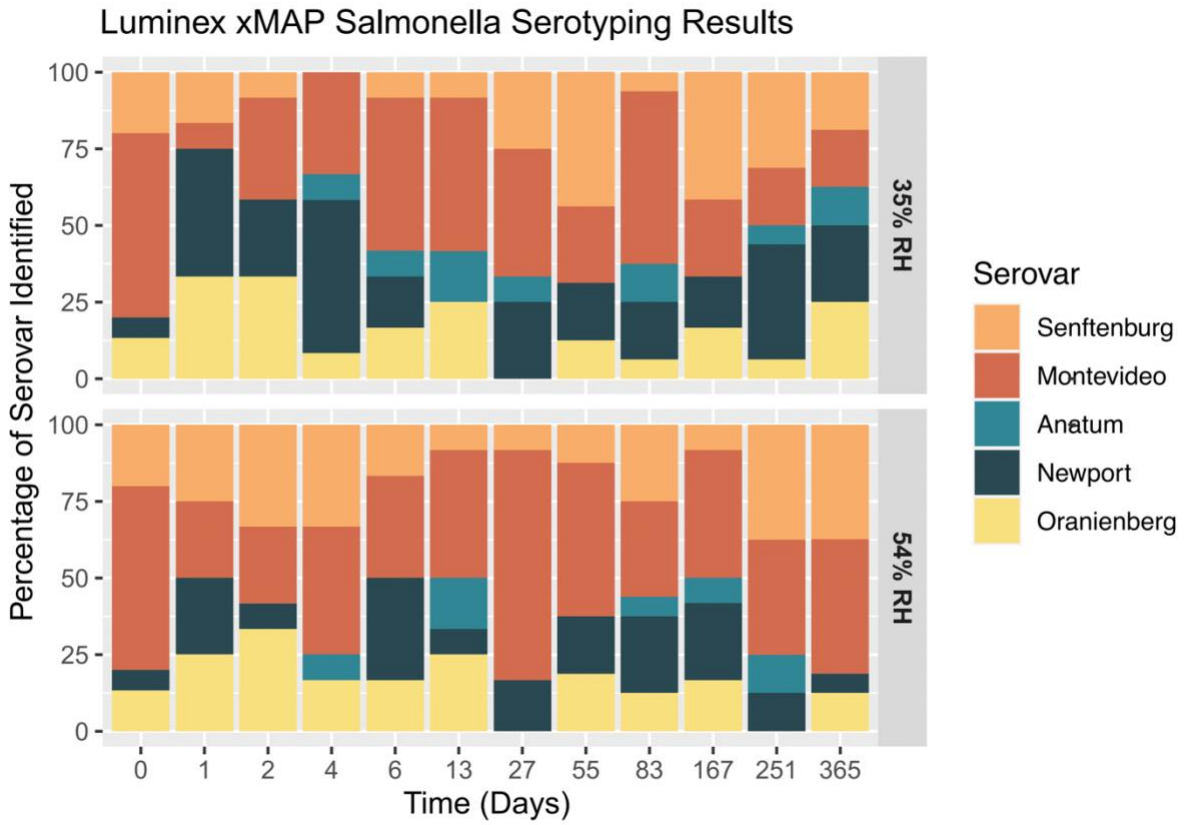
Appendix 2: Phages identified in study isolates

Closed Genome(s)	Sequence Type	Phage(s) Identified
FSW0104 CFSAN087304	ST185	Aeromo_phiO18P_NC009542 Salmon_118970_sal3_NC031940
CFSAN000258 CFSAN045764 CFSAN051296	ST316	Salmon_Fels2_NC010463 Salmon_vB_SosS_Oslo_NC_018279
CFSAN005645 CFSAN010209	ST138	Salmon_Fels2_NC010463 Escher_D108_NC_013594

Appendix 3: Data from storage study of inoculated pistachios stored at 35% RH and 54% RH

Day	Log CFU/g				Water Activity	
	Inoculation	Post-Desiccation	35% RH	54% RH	35% RH	54% RH
-1	7.05 7.10 7.08					
0		5.63 5.97 6.08	5.63 5.97 6.08	5.63 5.97 6.08	0.4601	0.4601
1			5.77 6.07 6.11	6.39 5.99 6.19	0.4183	0.5663
2			5.91 5.90 5.74	5.95 5.87 5.85	0.3697	0.5723
4			5.51 5.61 5.85	5.76 5.76	0.3466	0.5613
6			5.97 5.95 5.51	6.28 5.72 6.06	0.3438	0.5622
13			5.87 5.41 5.68	5.64 5.77 5.68	0.443	0.5504
27			5.04 5.62 5.44	5.10 5.13 5.37	0.3372	0.5559
55			5.67 5.28 5.41	4.81 5.37 5.09	0.3274	0.5495
83			5.47 5.41 5.23	4.94 5.18 5.04	0.3327	0.5492
168			5.08 5.47 5.43	4.67 4.46 4.52	0.3127	0.5355
251			4.72 4.98 4.98	3.82 3.45 3.93	0.2853	0.5031
365			4.79 4.87 5.21	2.80 2.72 2.68	0.3258	0.5406

Appendix 4: Results of the Luminex Serotyping of colonies picked at different timepoints from inoculated pistachios stored at 35% RH and 54% RH.



Appendix 5: gBlocks used in the Scarless Red Recombinase Deletion Protocol

>delta_cus

TTGATAAAGCAATCCAGCCTTCCTGATAAGTTGCACTAATTATATCGAATGGCTTCT
GTTTGCTGCATGACAGGCTAATGACATCTTTGTCATTTAAATC TACCTGAAAGGGCA
CCCCCTGATCTCAAGAGTGGCAGCGGTTCTGTTAAGTAACTGAACCCAATGTCGTTA
GTGACGCTTACCCGCAAAAAACCCCGCTTCGGCGGGGTTTTTTTCGCTCTTAAGAGGT
CACTGACCTAACAAAAAAAACCCCGCCCTGACAGGGCGGGGTTTTTTTTGGTCT
TGAGTGGCAGAGTCAGTTATCGCGAGCAGTATGTAAGTAGATCCTCAGTGTCCAGCTA
GGGATAACAGGGTAAT CCTGGTGTCCCTGTTGATACCGGGAAGCCCTGGGCCAACTT
TTGGCGAAAATGAGACGTTGATCGGCACGTAAGAGGTTCCAACCTTTCACCATAATGA
AATAAGATCACTACCGGGCGTATTTTTTGTAGTTATCGAGATTTTCAGGAGCTAAGGA
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ATCGTAAAGAACATTTTGTAGGCATTTTCAGTCAGTTGCTCAATGTACCTATAACCAGA
CCGTTTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGT
TTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGAATTCCG
TATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTACCCCTTGTTACAC
CGTTTTCCATGAGCAAACCTGAAACGTTTTTCATCGCTCTGGAGTGAATACCACGACGA
TTCCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCT
GGCCTATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGG
GTGAGTTTACCAGTTTTGATTTAAACGTGGCCAATATGGACAACCTTCTTCGCCCC
GTTTTACCATGGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGCCGCTGGCG
ATTCAGGTTTCATCATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGAA
TTACAACAGTACTGCGATGAGTG CATCTTTGTCATTTAAATC TACCTGAAAGGGCAC
CCCCTGTGGGTGTCCTTCTTACTGATTCACCCTGACGTCAGGGTTTAAATCGATAAT
ATACAGAGGTGAGTATGAAAAAAGTGGT

>delta_pco

GACTGATGCTCAGTGAGTTACTACGCAGTCACTCA TTTCCACGGCCACGGAGGGCATGG
AGATCATCAACATCACGGAAGTGAAAACGATAAAAAAATTAATCCGGCAGACGGG
GCCGCGTCGCGGTCCCGTTA CCCGCAGCTCCACTTGTTAGATCGATCGATCATTAGT
GACGCTTACCCGCAAAACCCCGCTTCGGCGGGGTTTTTCGCTCTTAAGAGGTCCTAG
CGCATGGATGGACCCGTAGGGATAACAGGGTAAT CCTGGTGTCCCTGTTGATACCGG
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CCAACCTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGTAGTTATCGA
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AATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGA
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TGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTG
GCGTGTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAGAATATGTTT
TTCGTCTCAGCCAATCCCTGGGTGAGTTTACCAGTTTTGATTTAAACGTGGCCAATA
TGGACAACCTTCTTCGCCCCCGTTTTACCATGGGCAAATATTATACGCAAGGCGACA
AGGTGCTGATGCCGCTGGCGATTCAGGTTTCATCATGCCGTTTGTGATGGCTTCCATG

TCGGCAGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCCGCGTCGCGGTC
CCGTTACCCGCAGCTCCACTTGTAGACCCTCATTGACGCCGAAGTCACTGGCTTA
CGCTCCCGTCCGGGAGCGTTTTTTTTTCCCATATATCAAACTTAACTCTATCTGGTGA
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>delta_CHASRI

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GTTTTTCGCTCTTAAGAGGTCAGTACTTGTAGTGGCAGAGTCAAGTATCGCG
AGCAGTATGTAAGTAGATCCTCAGTGTGAGCTAGGGATAACAGGGTAATCCTGGTGT
CCCTGTTGATACCGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACGTTGATCG
GCACGTAAGAGGTTCCAACCTTTCACCATAATGAAATAAGATCACTACCGGGCGTATT
TTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAATCA
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TTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCCT
TTTTAAAGACCGTAAAGAAAAATAAGCACAAAGTTTTATCCGGCCTTTATTACATTC
TTGCCCGCCTGATGAATGCTCATCCGGAATTCGATGGAATGAAAGACGGTGAGC
TGGTGATATGGGATAGTGTTCACCCTTGTACACCGTTTTCCATGAGCAAACCTGAAA
CGTTTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATAT
ATTCGCAAGATGTGGCGTGTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTA
TTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTACCAGTTTTGATTT
AAACGTGGCCAATATGGACAACCTTCTTCGCCCCCGTTTTTCACCATGGGCAAATATTA
TACGCAAGGCGACAAGGTGCTGATGCCGCTGGCGATTTCAGGTTTCATCATGCCGTTTG
TGATGGCTTCCATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGCGATGAGTG
TCGTGACATTTCTACAATGTCCCGCAGCTCCACTTGTAGACCCTCATTGACGCCGA
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ACTC

Key:

IDT Adapter

Upstream Sequence

Downstream Sequence

Terminator

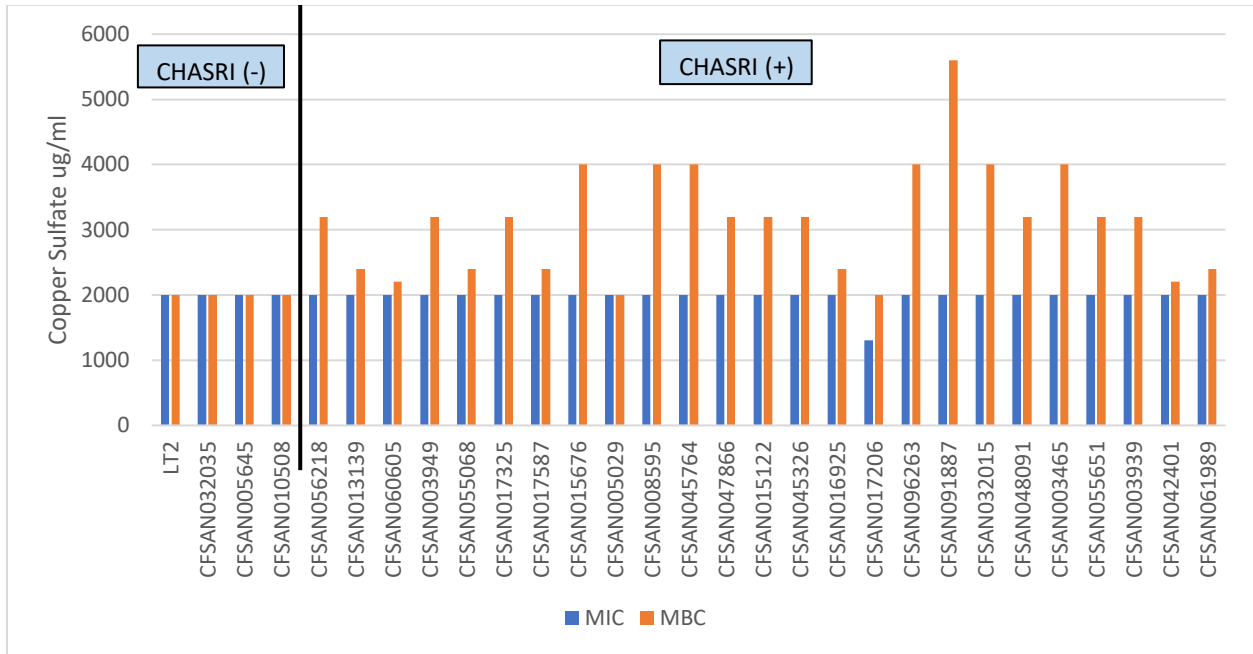
Restriction Site

Chloramphenicol Sequence

Appendix 6: 33 Strains that have the plasmid similar to Citrobacter portucalensis

SRA Accession	Serovar	Source	Location
SRR4427064	Rubislaw	Avocado	USA
SRR5058957	IV 48:z4,z32:-	Environmental swab	USA
SRR4427067	Rubislaw	Avocado	USA
SRR5058958	IV 48:z4,z32:-	Environmental swab	USA
SRR4427068	Rubislaw	Avocado	USA
SRR4427066	Rubislaw	Avocado	USA
SRR1288378	Rubislaw	Cilantro	USA
SRR1656995	IV 48:z4,z32:-	Avocado	USA
SRR11510983	Agona	Pork	USA
SRR11351646	Agona	Pork	USA
SRR11059176	Javiana	Mongoose	Grenada
SRR11059167	Javiana	Mongoose	Grenada
SRR1288385	Rubislaw	Cilantro	USA
SRR3136839	Schwarzengrund	Poultry	USA
SRR11003995	Schwarzengrund	Poultry	USA
SRR5201512	Schwarzengrund	Poultry	USA
SRR11284266	Schwarzengrund	Poultry	USA
SRR3185089	Schwarzengrund	Poultry	USA
SRR5000289	Ohio	Spinach	USA
SRR5116532	Ohio	Animal Feed	USA
SRR6366587	Ohio	Fertilizer	USA
SRR5172936	Ohio	Environmental swab	USA
SRR6109521	Ohio	Environmental swab	USA
SRR5172916	Ohio	Fertilizer	USA
SRR5076861	Ohio	Animal Feed	USA
SRR8863430	Bere	Pork	USA
SRR1752726	Agona	Poultry	USA
SRR3660557	Ohio	Wild Animal	USA
SRR7974097	Ohio	Pork	USA
SRR6261935	Agona	Poultry	USA
SRR7107415	Ruiru	Pork	USA
SRR3286810	Ruiru	Poultry	USA
SRR1752879	Agona	Poultry	USA

Appendix 7: MIC and MBC from different strains of Salmonella enterica to copper sulfate



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